THE EFFECT OF ENZYME INHIBITORS ON THE MULTIPLICATION OF INFLUENZA A AND B VIRUSES IN CHICK EMBRYOS

by

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INTRODUCTION

Although the viral diseases have plagued man and animals through the ages, the etiologic agents were not clearly identified until the beginning of the 20th century. Historically a number of virus infections were easily recognized by the symptoms which they produced and physicians of the day prescribed their favorite remedies. Among these were pine, honey, and garlic for the common cold. The bark of the green willow, salicylic acid plasters, and the prickly poppy were recommended for the treatment of warts. Yellow plants were prescribed for certain types of jaundice. Rabies yielded to garlic, river crabs and the liver of the mad dog. Herpes zoster responded to the application of cats blood. Even Shakespeare criticized the chicanery of the medical profession when he said, "The most sovereign prescription in Galen is but empiricutic". (1)

With the establishment of bacteriology as a science, attempts were made to find chemotherapeutic substances which would inhibit bacterial growth in the host. As late as 1920 hope was all but abandoned in this quest.

Then in rapid succession came the sulphonamides and the antibiotics. As late as 1930 the inhibition of viral multiplication was thought useless because of the intra-cellular position of the disease agent. It was held that the metabolic activities of host and virus was so closely interwoven that what would kill one would also destroy the other. The development of such antibiotics as aureomycin and chloramphenicol have shown that inhibition of viral multiplication is feasible.

The information contained in this thesis focuses attention on viruses as related to attempts at chemotherapy. Wherever possible the mechanism of action of a drug is given prominence. The experimental material is concerned with attempts to prevent multiplication of influenza A and B viruses by means of enzyme inhibitors.

SULPHONAMIDES

Mechanism of Action

The first real attempts at a chemotherapeutic approach to bacterial infections were made by Stamp [2] who isolated inhibitory substances from Group A and C streptococci. Green [3] also obtained a similar component from Brucella abortus. These observations were overshadowed by the discovery of the sulphonamides which stimulated a great deal of investigation into the action of the drugs against susceptible organisms. The demonstration by Woods [4] that p-aminobenzoic acid (PABA) would negate the effect of sulphanilamide was an outstanding contribution later confirmed by many workers. Woods [4] and Fildes [5] postulated that the sulphonamides blocked an essential metabolic system in susceptible organisms which was reversed by PABA. Woods [4] conceived the relationship between PABA and the sulphonamides to be a competitive one. He concluded that any organism which would form enough PABA to neutralize the sulphonamides would remain immune; or, that organisms which utilized PABA in the form of pteroylglutamic acid (PGA) would be resistant to sulphonamide action. In the field of bacterial metabolism the application of this concept of

antagonists has led to striking successes by Fildes [6], McIlwain [7], Woolley and White [8].

In his review, Henry [9] has objected to the Woods-Fildes theory on the grounds that the respiratory and growth preventing effects of the sulphonamide drugs have not been clearly separated. The inhibitory qualities of the sulphonamides are greater on cells in active multiplication than upon resting cells. Furthermore PABA, itself, in high enough concentration actually inhibits respiration. This fact led Sevag [10,11,12] to the conclusion that a sulphonamide-sensitive respiration exists, inhibition of which causes cessation of growth. He considers that PABA is not an essential metabolite but rather a less toxic chemical analogue of the sulphonamides with affinity for the same enzyme system. Thus he showed that coenzyme I and II and the carboxylases of pyruvic acid are inhibited by sulphonamide drugs. In addition, sulfathiazole, which exerts the greatest effect, is most nearly related to co-carboxylase (thiamine pyrophosphate). From this Sevag reasoned that PABA has a non-specific ability to protect the enzyme from sulphonamide action.

Work [13] defends the Woods-Fildes theory and points out that the critical aspect of Sevag's postulate rests on two premises: the observation that PABA itself may be inhibitory and that the action is non-specific. He feels that the first premise is invalid since an excess of any metabolite frequently exhibits inhibitory rather than stimulating properties. Secondly Work believes that PABA is an essential metabolite for certain microorganisms and that Sevag's "non-specific" effect is based on the use of a less sensitive bacterial strain. The exact enzyme systems which are involved have not been established as yet, and many problems remain to be settled. Woods [14] raises the question, "How does the nature of the products of the reaction correlate with PABA?" In addition, folic acid, purines, pyrimidines, thymines and certain amino acids as serine, lysine, and methionine have been shown to be concerned with the action of PABA, since many of these will reverse sulphonamide inhibition. Methionine has received special consideration because it does not act in a competitive manner. Other amino acids as glycine, serine, and allothreonine do not antagonize sulphonamides by themselves but each enhances methionine.

Kohn [15], therefore, has suggested the following extension of Wood's theory to account for the role of PABA in the synthesis of purines, certain amino acids and possibly pyrimidines. The primary reaction in which PABA takes part may be inhibited by the sulphonamides. This in turn causes growth suppression because of failure of the cell to produce essential amino acids. The block is circumvented and growth is restored by the addition of methionine, xanthine, glycine and guanine to the media.

Disclosure of the formula of folic acid indicates that PABA might be displaced by the sulphonamides in the pteroyl glutamic acid molecule. Sulphonamide sensitive strains of bacteria were found to grow in the presence of PGA. These results suggest that Kohn's scheme could be modified. Although PGA antagonizes the action of sulphonamides in several organisms, it cannot replace PABA in Lactobacillus arabinosus or certain mutants of E. coli.

In addition, using certain folic acid inhibitors and staphylococcus, it was found that their effect was nullified by PABA, PGA, pteroic acid and even by sulfathiazole, but not by glutamic or p-aminobenzoylglutamic acid. The inhibitory action of sulphonamides, however,

against this strain of staphylococcus was antagonized by PABA, by pteroic, but not by PGA. This suggested to Martin et al [16,17] that pteroic acid and not PGA is involved in staphylococcus metabolism.

O'Meara and coworkers [18] believe that reductone, CHO.C(OH).CHOH, is the strongly reducing non-sulfhydryl substances found in the log phase of bacterial growth, and it may well be that reductone forms the three carbon unit in the formula below, in which the three carbons are marked with asterisks, and that the sulphonamides interfere with the synthesis of pteroic acid from PABA by competing with the latter for reductone, resulting in an inert analogue of pteroic acid.

N N
C C CH*
H
N C C*CH$_2$* - NH⟨ ⟩CO -NH- C - CH$_2$ -CH$_2$ -COOH
N N COOH

Pterin moiety PABA moiety Glutamic moiety
Pteroyl Radical

<u>Folic Acid (PGA)</u>

CHEMOTHERAPY OF VIRUS DISEASES WITH SULPHONAMIDE DRUGS

The observation that sulphonamide therapy is somewhat effective in certain virus diseases has stimulated extensive investigation of the mechanism involved. Findlay [19] observed that if some strains of the virus of lymphogranuloma venereum are mixed with sulphonamide infectivity is not destroyed, but virulence is reduced. Since, however, other strains became non-viable when placed in contact with the drug, he proposed an extension of the PABA theory. Those viruses which require PABA are susceptible to the action of sulphonamides, and those which prove to be insusceptible synthesize enough PABA to neutralize the sulphonamide inhibition or they utilize PABA at a higher level, namely PGA. Henry [8] believes that cellular depression by sulphonamide therapy might affect multiplication of the virus by slowing down cell growth. This concept seems reasonable because the sulphonamide drugs are known to increase the severity of most rickettsial diseases [20]. This observation has been correlated with the fact that the rickettsia grow better in cells with a lower metabolic rate, as is produced by dinitrophenol [21] or other

depressants [20]. Henry further postulates that if a virus is a self-propagating particle, the sulphonamides might hamper its autocatalytic properties. Until such a method of virus propagation is proven, this concept must remain merely a collection of words. Henry [9] gives as his final alternative the thought that interference may be due to an adsorption of the sulphonamide to the virus particle.

It might be well at this point to call attention to a well-defined family of viruses, the lymphogranuloma-psittacosis group, in which the sulphonamides have proven to be particularly effective. There is doubt, at this time, as to whether the lymphogranuloma-psittacosis group should be classed with the viruses at all. In size they range from 200 to 400 mu in diameter, the larger particles are not filterable and may be seen with the ordinary microscope, they contain thymonucleic acid, they progress through a definite morphological cycle within the cell and are antigenically similar. The 6th edition of Bergey's Manual of Determinative Bacteriology has separated them from the viruses. This is probably premature, for there is by no means a unanimity of opinion as to their ultimate classification.

In a recent review [1] and from the work of individuals, general agreement has been reached on the effectiveness of sulphonamide therapy in lymphogranuloma venereum. It has been shown that there is a beneficial in vivo therapeutic response in man. In the experimental animal the protection obtained by the sulphonamide drugs is also reversible by PABA [19]. In the treatment of psittacosis the evidence is conflicting, but in man poor results have been noted [22] and strain differences in susceptibility to sulfadiazine have been observed [23]. In experimental studies Early and Morgan [24] have shown that the inhibitory effect of sulfadiazine on strain 6BC is reversed by PABA and PGA[25]. Subsequently Morgan[26] found that PABA and pteroic acid will inhibit the growth of psittacosis virus in a competitive manner while PGA exhibits a non-competitive sulphonamide antagonism. From these data they reasoned that pteroic acid was an intermediate in the synthesis of PGA by the psittacosis virus. A sulphonamide-resistant strain has been developed from the original 6BC strain [27]. In mice, sulfamerazine was effective in the treatment of mouse pneumonitis [28] and PABA reversed its action [1]. Sulphonamide failed in the treatment of feline pneumonitis, a related virus[29].

The treatment of trachoma has been hampered by many variables such as the presence of strains of low and high virulence, secondary infection, inadequate dosage and faulty diagnosis. Two clinical reviews on this subject lead one to believe that sulphonamide treatment is efficacious [30]. Inclusion conjunctivitis is cured by the use of sulfadiazine, especially in young children [31] and since this virus is rarely associated with secondary infection, the action must be directly on the virus. One should bear in mind that purely clinical evaluations of a drug are usually not reliable.

PENICILLIN

Mechanism of Action

With the introduction of penicillin great hopes were raised that it would prove effective against virus diseases. This has been found true only to a limited extent. Despite the extensive study of penicillin, carried out in the last five years, relatively little is known concerning its mechanism of action. The pertinent literature is summarized below.

Gros and Macheboeuf [32] have shown that the utilization of the nucleic acids is inhibited by penicillin. Similarly, Krampitz [33], using Staphylococcus aureus, demonstrated that normally ribosenucleic acid (RNA) is enzymically decomposed, liberating ribose for energy. The addition of penicillin resulted in failure of the organism to decompose RNA but they were still able to utilize ribose. He also found that the presence of penicillin inhibited RNA synthesis as well. Gale and Taylor [34] and Gale et al [35,36,37] have shown that penicillin interferes with glutamic acid metabolism. This amino acid is synthesized by gram negative organisms but must be supplied to gram positive ones.

It is transported across the cell wall by an energy-requiring metabolic reaction which is absent in gram negative organisms. On the basis of his data, Gale feels that penicillin exerts its toxic effect on gram positive organisms by preventing the transmembrane passage of glutamic acid.

Isolated observations concerning the possible mechanism of action of penicillin are recorded below. Interference with SH groups, particularly glutathione, has been suggested by Pratt and Dufrenoy [38]. A comparison of the effects on gram positive and negative organisms indicates that catabolism of the mononucleotides is blocked [38]. The proper use of cobalt decreases the penicillin requirement in vivo and in vitro [38], suggesting some effect on coenzymes. Simmonds and Fruton [39] found that glycine was incorporated into a peptide before being metabolized and that penicillin prevented this bacterial peptide synthesis. An inositol-utilizing strain of yeast has been described which, unlike most yeasts, was sensitive to penicillin. D-glucose-1-phosphate and fructose-1-6-phosphate neutralized the effect of the drug. Similar results were

obtained with Lactobacillus arabinosus and Staphylococcus aureus [40].

Organisms trained to dispense with certain amino acids became more resistant to penicillin without any contact with the drug. Furthermore, penicillinase was not produced by all resistant strains [41]. In experiments using radioactive penicillin it was found that resting sensitive organisms could take up small amounts. When large amounts were used, the penicillin uptake remained the same. The authors postulated, therefore, that there is a direct chemical combination with a cellular component [42].

Hotchkiss [43] demonstrated that penicillin-treated staphylococci did not differ markedly from untreated organisms in their rate of oxygen uptake, phosphate, glutamic acid or amino acid utilization. However, the normal cells metabolized the amino acids to form protein, whereas the treated cells did not. In addition, the treated staphylococci produced extracellular non-amino acid nitrogenous substances in quantities approximately equal to the amino acid nitrogen used.

From the data presented it is evident that penicillin probably acts by SH inhibition and interference with nucleic acid formation. Peptide and polypeptide synthesis disorganization has been shown but the intermediate steps in all the above processes are, as yet, unknown.

Experimental and Clinical Effects of Penicillin

Experimentally, penicillin has not inhibited the growth of lymphogranuloma venereum virus except when huge doses were used [44], although it seems to be effective in the treatment of the clinical disease[45]. The curative action of penicillin on ornithosis and psittacosis in mice was first demonstrated by Heilman and Herrell [46] but the action is virustatic rather than virucidal. Penicillin has inhibited psittacosis and ornithosis infections in man [47]. The meningopneumonitis virus is also susceptible to penicillin both in chick embryos and in mice [48]. Treatment of inclusion conjunctivitis has given remarkable results[49].

Its use in the pox viruses is without effect -- so much so that purified penicillin is added to vaccinia

virus to inhibit pathogenic organisms which might cause infection [21]. However, when vaccinia and crude penicillin are mixed and injected into rabbits the viral lesions are abolished [50]. Investigation has shown that the substance producing this inhibition is probably an impurity of penicillin which resists boiling for 15 minutes [50]. It has been suggested that the active substance is O-hydroxyphenylacetic acid [51].

STREPTOMYCIN

While penicillin and streptomycin overlap in their antibacterial spectra, they probably exert their inhibitory effects in entirely different manners. Streptomycin is more active against gram negative and acid fast organisms which reproduce at a slower rate than the gram positive ones. If penicillin-treated assay plates with E. coli as test organisms are placed in contact with a carbolic solution of trypan blue the dye is retained in the cells in the outermost zone of inhibition. By contrast streptomycin-treated cells, when stained, show no signs of such dye absorption and furthermore the bacteriostatic zone occupies a much larger area of the inhibition segment [52].

Den Doonen de Jong [53] has suggested that streptomycin may form a "bridge" between two molecules of nucleic acid. This hypothesis is further supported by the demonstration that nucleic acid is precipitated by streptomycin [54]. It might be well to comment here that the relative inertness of nucleic acid-rich viruses to streptomycin makes this concept seem improbable as a major mechanism of action. Inhibition of oxidation also has its proponents. Hirsch and Dosdogru [55]

believe that streptomycin interferes with respiratory enzymes while Fitzgerald et al [56] have shown a decrease in benzoic acid oxidation by certain strains of mycobacteria in the presence of streptomycin.

In the most convincing work, Umbreit and associates [57,58,59,60] have observed that streptomycin is probably effective in the region of the pyruvate-oxalacetic cycle. Their experimental data suggest that it is the oxalacetic-pyruvate condensation which prevents "a variety of substances from entering the terminal-respiration system that resembles the citric acid cycle". Furthermore, dependent and resistant strains of E. coli have the ability to bypass and dispense with this condensation. Streptomycin is prevented from affecting the oxalacetic-pyruvate cycle of the normal cell by two barriers of either a chemical or physical nature. The first is present in the external cell membrane and the second is found at the level of the mitochondria. If streptomycin was able to bypass either or both of these barriers it then suppressed the oxalacetic-pyruvate condensation of the cell. Such barriers to the action of the drug were absent in E. coli and present in tissue

cells, a fact which explained the differential effect of the drug. Dihydrostreptomycin penetrated these cell barriers more slowly than streptomycin, with consequent fewer toxic reactions to the animal.

Streptomycin and Gamexane, an insecticide, are inositol derivatives and it has been suggested that both compounds are effective because they are incorporated into the cell in place of inositol (61,13,14). Further proof of this concept is the inactivation of over 3000 parts of streptomycin by one part of lipositol, a phospholipid isolated from brain and soy bean. Chemical examination has characterized lipositol as being an inositol derivative with a galactose residue. The presence of lipositol as a constituent of nerves is again interesting because of the toxic effects of streptomycin on the eighth nerve.

Effect against Viruses

Streptomycin has been found to be without any demonstrable effect against most of the viruses and, in fact, is commonly used in isolations from material contaminated with bacteria.

CHLORAMPHENICOL

Mode of Action

Smith et al[62] tested the effect of chloramphenicol on 45 isolated enzyme systems and failed to demonstrate any inhibition of cellular respiration, transamination, or breakdown of proteins. The only action of the drug was against purified bacterial and liver esterases. In doses up to 1 microgram no change was noted, 1 to 3 micrograms gave a definite inhibition of activity but 3 to 50, however, resulted in a marked stimulation of esterase activity (50-80%) while doses of 50 micrograms or more gave complete suppression of esterase activity. The antiesterase effects on mitochondria and liver homogenates were noticeably different from the above. When mitochondria or animal cells were used the action was incomplete in that the drug inhibited only 40 to 50% of the esterase activity exhibited by the mitochondria. At concentrations of 100 mg or more no significant inhibition could be demonstrated. These observations on normal tissue cell barriers to chloramphenicol correlate with those of Umbreit on streptomycin [60].

Effect on Viruses

Remarkable rickettsiostatic activity was described in the earliest laboratory reports on chloramphenicol [63] and, experimentally, strain 6BC of psittacosis was shown to be susceptible to chloramphenicol treatment while the viruses of Japanese encephalitis and influenza were resistant. Chloramphenicol possesses considerable therapeutic activity in embryonated eggs and mice infected with the viruses of psittacosis or lymphogranuloma venereum and this activity is comparable in amount to that demonstrated by others for sulfadiazine and penicillin tested under similar conditions [64]. However, infection was not prevented in mice treated prophylactically nor were tissues freed of virus when the drug was used for chemotherapy. Synthetic chloramphenicol has been shown to produce the same virustatic effect [65]. Clinically the larger viruses have responded rather well to chloramphenicol therapy, including the psittacosis-lymphogranuloma group, infectious mononucleosis, atypical pneumonia, herpes zoster, and certain ocular viruses, but the smaller viruses still remain resistant [66,67,68].

AUREOMYCIN

Mode of Action

There has been no important contribution to the mechanism of action of aureomycin in the literature to date.

Effect on Viruses

Eaton [69] has demonstrated in chick embryos and mice that aureomycin has a therapeutic effect in atypical pneumonia while with chloramphenicol under the same conditions there was an irregular result. Wagner [70] has shown inhibitory properties of aureomycin against ten strains of the psittacosis-lymphogranuloma group which was characterized as virustatic rather than virucidal. The drug is ineffective against herpes simplex growing in chick embryos [71].

Clinically the use of aureomycin would appear to be indicated in all cases of atypical pneumonia [72], psittacosis-lymphogranuloma infections, trachoma [73] and certain ocular virus diseases [74]. Preliminary clinical reports, in contradistinction to experimental ones, suggest that aureomycin may be of use against herpes simplex and herpes zoster [75]. The reported effectiveness of aureomycin in the treatment of

influenza by Finland et al [76] is far from convincing. Thus like chloromycetin, aureomycin has no certain or definite suppressive ability against the smaller viruses.

Figure I

EFFECT OF ANTIBIOTICS ON RICKETTSIAE AND VIRUSES (78)

Disease Agents	Size mμ	Sulfonamides		Penicillin		Streptomycin		Chloram-phenicol		Aureomycin		Terramycin	
RICKETTSIAE	475	–	–	–	–	+	–	+	+	+	+	+	+
PSITTACOSIS	450	+	+	+	+	–	–	+	±	+	+	+	0
LYMPHOGRANULOMA	440	+	+	+	–	–	0	+	+	+	+	+	0
TRACHOMA		0	+	0	±	0	–	0	+	0	±	0	0
ATYPICAL PNEU-MONIA		0	–	0	–	0	–	0	+	0	+	0	±
MUMPS	340	–	–	–	–	–	–	–	+	–	–	0	0
VACCINIA	225	–	–	–	–	–	–	–	0	–	0	0	0
VARICELLA	200	0	–	0	–	0	–	0	+	0	–	0	0
HERPES ZOSTER	200	0	–	0	–	0	–	0	+	0	+	0	+
HERPES SIMPLEX	150	–	–	–	–	–	–	0	+	+	±	0	0
INFLUENZA	115	–	–	–	–	–	–	–	–	–	–	±	–
KERATOCONJUNC-TIVITIS	85	0	–	0	–	0	–	0	+	0	+	0	0
EQUINE ENCEPHALO-MYELITIS	50	–	–	–	–	–	–	–	–	–	–	0	0
POLIOMYELITIS	25	–	–	–	–	–	–	–	±	–	–	0	0

Legend: First Row = Experimental Second Row = Clinical
0 = Not Done – = Ineffective + = Effective ± = Doubtful

TERRAMYCIN

Mode of Action

There has been no description of mechanism of action of this new antibiotic at the present time. This drug is probably closely related to aureomycin.

Effect on Viruses

A recent publication by Finlay et al [77] has described the antibiotic as effective against certain of the large viruses and preliminary publications stated that it was also effective against influenza A.

* * * * *

In general, it is clear from Figure 1 and also from the discussion that certain of the antibiotic agents, namely, aureomycin, chloramphenicol and terramycin are effective therapeutic agents against the psittacosis-lymphogranuloma group of viruses. This finding represents an outstanding achievement since it demonstrates clearly that it is possible to attack and limit the growth of some of the intracellular agents of disease. Of particular interest are the claims for efficacy of aureomycin and chloramphenicol against some of the smaller viruses, especially

herpes zoster and simplex as well as keratoconjunctivitis, the latter being only 85 millimicrons in diameter. The establishment of virustatic rather than virucidal properties should also be noted. These observations provide hope that it may be possible, soon, to modify the development of extremely small viruses such as poliomyelitis.

CHEMICAL COMPOSITION OF VIRUSES

All studies to date show that viruses have one component in common, nucleic acids, and these in turn may be separated into desoxyribosenucleic (DNA) and ribosenucleic (RNA) types. The plant viruses, which will not be reviewed here, have been obtained in crystalline form and consist entirely of pure RNA[79]. This finding has created the impression that all viruses are, therefore, nucleoproteins. The animal viruses, however, contain varying quantities of nucleoprotein of the DNA type with the exception of poliomyelitis [82], western equine encephalitis (WEE) and eastern equine encephalitis (EEE). The amount of nucleic acids present in different viruses ranges from a low of 4% (EEE) to 45% in bacteriophage T_2 [81,82]. DNA and RNA have been found in bacteriophage [83,96] as well as in influenza by Knight [85] and Taylor [86]. Knight [87], however, has been unable to verify Taylor's work in all respects. Papilloma, the most highly purified of animal viruses, consists of DNA, no RNA being found by existing chemical methods [88]. In addition to nucleoprotein, most animal viruses consist of lipid

Figure II

GENERAL CHEMICAL COMPOSITION OF SOME VIRUSES [82]

Virus	Nucleoprotein	Lipid
TOBACCO MOSAIC	+	–
SHOPE PAPILLOMA	+	1.5%
T2 BACTERIOPHAGE	+	2 %
EQUINE ENCEPHALOMYELITIS	+	48 %
NEWCASTLE	+	27 %
INFLUENZA	+	23 %
VACCINIA	+ also copper, biotin & flavins	4 %

Figure III

PHOSPHOROUS AND NUCLEIC ACID CONTENT OF VIRUSES (83)

Virus	Nucleic Acid Percent	Type	Percent of Total
TOBACCO MOSAIC	40	RNA	Probably 100%
TOMATO BUSHY STUNT	17	RNA	Probably 100%
TOBACCO NECROSIS	18	RNA	Probably 100%
E. COLI BACTERIOPHAGE T2	45	DNA	99 - 100%
RABBIT PAPILLOMA	9	DNA	90%
VACCINIA	5.6	DNA	95%
EPIDEMIC RICKETTSIA	-	DNA	No ribose found
EQUINE ENCEPHALOMYELITIS	4.4	RNA	
INFLUENZA	5	RNA & DNA	

and carbohydrate [79,82,85], among them being WEE and EEE which have a high lipid fraction consisting of phospholipid, cholesterol, and fatty acid [89]. More carbohydrate is present in influenza virus than can be accounted for by nucleic acid, and it consists of either mannose or a glucose-galactose complex [87]. Newcastle virus, which resembles influenza, has glucose as its carbohydrate [79]. A summary of the chemical composition of various viruses is presented in Figures 2 and 3.

With vaccinia virus DNA was shown to be the only nucleic acid present [90] along with a carbohydrate complex other than nucleic acid. Although RNA has been reported in vaccinia, Hoagland et al [91,92] found that infectivity remained constant despite treatment of the elementary bodies with ribonucleotidase. They also found nucleic acid intimately associated with purified elementary bodies and that adenine and guanine were present. Thymine was not isolated as such, but a positive test for its presence was obtained with purified material [93]. Lipid, neutral fat and cholesterol were present, the latter being removable

without changing infectivity [94,95]. Biotin was shown to be present by biological tests [96]. Copper and flavine adenine dinucleotide were also demonstrated [92,97,98,99].

ENZYMES IN VIRUSES

McFarlane and Dolby [95] found no zymohexase, enolase, glucosidase or nucleosidase and Hoagland et al no dehydrogenase [97,98]. The latter group did show phosphomonoesterase, phosphodiesterase as well as ribonuclease and desoxyribonuclease activity [90]. Bauer [100] is critical of the method for determining the latter two because of the extended incubation time of 99 hours. He maintains that a 30 minute incubation period should be sufficient to demonstrate activity if present. The phosphodiesterase activity is criticized because of the possibility of bacterial contamination. The detection of large amounts of biotin is conducive of acceptance [98] but riboflavin may well be a contaminant [100]. Sigurdsson [101] reported that many viruses are inhibited by high acid concentration, indicating the presence of acid-sensitive catalase. Since influenza virus will attach to the surface of a variety of red blood cells, elute therefrom and again agglutinate red cells with no appreciable dimunition of activity, it is concluded that the virus contains a mucinase [100]. With a clearer conception of the nature of the substrate this conclusion might

be justified. The demonstration that vaccinia elementary bodies readily adsorb phosphatase, catalase and lipase [97] makes it seem improbable that enzymes claimed to be associated with elementary bodies are genuine constituents [100]. Contaminating substances from host tissue have made the enzyme study of animal viruses highly complex and unreliable. To date no incontestable evidence has been brought forward to show that enzyme systems, including those of respiration, are normal constituents of viruses. The data have been summarized in Figure 4.

Metabolic Activities of Viruses

The neurotropic strain of yellow fever, lymphocytic choriomeningitis and lymphogranuloma venereum cause an increase in xanthine oxidase activity in mouse brain [102, 103] and in chick embryos [104]. Pearson and Winzler using Theilers GD VII virus, in mouse brain tissue cultures, found no increase in oxygen or glucose and lactic acid production over their controls [105]. Further, in similar cultures using radioactive phosphorous (P), they found the activity concentrated in the RNA fraction and an increase in the total RNA at the expense of DNA [106,107]

Figure IV

ENZYME SYSTEMS CONCERNED IN VIRUS GROWTH (100)

Virus	Absent	Present
VACCINIA	Zymohexase Enolase Phosphoglucomutase Adenosine nucleosidase Peptidase Triosephosphate dehy-drogenase Cytochrome oxidase Cytochrome C Hyaluronidase	Phosphodiesterase Ribonuclease Desoxyribonuclease Phosphomonoesterase Riboflavin Copper Biotin
INFLUENZA	Mucinase Desoxyribonuclease Phosphatase Xanthine oxidase Adenosine triphospha-tase Succinic dehydrogenase	Mucinase
LANSING	Hyaluronidase	
MM	Hyaluronidase	

The presence of the virus also increased incorporation of glucose fragments into the protein fraction and decreased incorporation into the lipid fraction. Racker and Krimsky [108] reported studies showing the inhibition of anaerobic glycolysis by homogenates of mouse brain infected with the Lansing strain of poliomyelitis virus.

If, three hours after infecting embyros with influenza, radioactive phosphorous is injected allantoically and then allowed to incubate 48 hours and assayed, most of the phosphorous (P) is found in the pentose nucleic acid fraction [109]. In herpes infection of chick embryos, Ackerman and Francis observed a 34 to 42% increase in weights of liver and heart, which development was at the expense of other organs. There was an increase in the total nucleic acids but the DNA/RNA ratio remained the same. Infected heart muscle showed increased succinicoxidase activity by 27% and decreased α-ketoglutaric activity around 20%. Despite an increased weight, the liver metabolism remained normal. The total loss of metabolic ability was compensated for by the increase in size of the organs so that the actual decrease of succinicoxidase activity was not apparent (110).

Knight found about 7% aspartic and glutamic acid in influenza viruses and about 5% arginine and isoleucine [111]. Hoagland also has shown that different strains of virus have significant differences in amino acid content [84] and Stanley has made the same observation with plant viruses [112].

IN VITRO INACTIVATION OF VIRUSES

The problem of in vitro inactivation of viruses is beyond the scope of this thesis and only pertinent observations from the extensive literature are included below. Among the in vitro experiments of interest are those of Adams [113] who demonstrated that viruses could be inactivated by violent shaking or bubbling of gas through the solution and that the addition of protein had a sparing effect. He feels that the spreading of a protein at a gas-liquid interface results in denaturation of protein and that shaking or bubbling enormously increases the area of this gas-liquid interface. If, then, protein is added, there is competition for the interface with a resulting sparing action on the virus. He further states, "in denaturation the proteins are probably unfolded from a highly specific globular substance into a relatively unspecific polypeptide which probably exposes hidden SH groups and phenol groups to the action of chemicals etc." Burnet and Lush [114] have also commented on the effect of surface-acting agents on viruses.

It has long been known that viruses are extremely susceptible to certain oxidizing agents, and Wagner and Stacy [115] have recently shown that p-benzoquinone, potassium permanganate and periodate are effective against influenza A. Goebel et al [116] have suppressed ribonuclease and type III pneumococcus activity as well as WEE by incubation with periodic acid. It has been held that periodic acid is chiefly concerned with carbohydrates and the destruction of fowl red blood cell receptors for certain viruses by periodic acid was indirect evidence for the carbohydrate nature of such receptors. They present data, however, that periodic acid may act on proteins by severing the carbon linkages between adjacent hydroxyl or hydroxyl and amino groups. Ascorbic acid destruction of viruses was shown to be due to hydrogen peroxide [117]. Other animal viruses have been inactivated by incubation with small amounts of salicylates [118]. Certain lipid extracts of normal sera from various animals are able to suppress psittacosis virus in mice [114]. Phospholipid-free and sphyngomyelin-cerebroside fractions exerted a 10 to 100 fold inactivating effect while lecithin and cephalin decreased the titer 10,000 to 100,000 fold, with lecithin ten times

more effective than cephalin. Kaiser destroyed vaccinia and myxoma virus by 30 second exposure to iodine vapors and the virus was not reactivated by thiosulphate [119]. Comments about the arsenicals and mercury are reserved for the discussion on SH inhibitors.

CHEMOTHERAPEUTIC ATTEMPTS

Over 400 compounds other than the sulphonamides and various antibiotics have been tested against a variety of viruses. Unfortunately the effect of these substances against enzymes is not known since the investigators were more concerned with chemotherapeutic results rather than the metabolic pathways concerned in viral multiplication. It seems reasonable then to divide the compounds into two general groups: those which show therapeutic promise and those which did not. This information is further summarized in Figures 5, 6, and 7.

Therapeutically Ineffective Compounds

In 1942 Coggeshall and Maier [120] tested, in influenza and poliomyelitis infected mice, some 67 different compounds, among them being sulphonamide derivatives, sulfones, quinolines and miscellaneous chemicals. The following year Krueger [121] studied a large number including sulphonamides, acridines, neoarsphenamine and several antibiotics including Subtilin. There was no demonstrable protection for the mice. Coincident with

Figure V

ADDITIONAL CHEMOTHERAPEUTIC ATTEMPTS AT VIRAL INHIBITION

Virus	Inhibitor Used	Author Reference Number
WEE	Anesthetics	124
NEUROTROPIC	Anesthetics	125
	Trypan Red	126
LANSING	Thyroid	127
	Arsenicals	128
	Hormones	129
INFLUENZA	Synthetic detergents	130
	Quinine	131
	Atropine sulfate	132
	Isocyanates	133
	Atabrine and quinacrine	134
	Pyridoxine, inositol, and Biotin deficiency	135
FOWLPOX	Penicillin & Patulin	136
VIRUS DISEASE OF CHICKENS	Sulphonamides	137
LYMPHOCYTIC CHORIOMENINGITIS	Prontosil & Neoprontosil	138
MURINE & FELINE PNEUMONITIS	Sulfamerazine	139

Figure VI

COMPOUNDS SHOWING SLIGHT EFFECT ON VIRAL MULTIPLICATION

Virus	Compound Used	Author Reference Number
VACCINIA	Choline & similar compounds	140
PSITTACOSIS	Nitroakridin (mice)	141
LANSING	Substituted pyrimidines	142
THEILERS	Potassium & phosphorus def.	143

Figure VII

INHIBITORS OF VIRUS MULTIPLICATION USED IN TISSUE CULTURE

Virus	Compounds Effective	Compounds Ineffective	Author Reference Number
VACCINIA	Chloro- & bromacylamides.		144
	Dinitrophenol, cyanide, Azide, atabrine, proflavine, iodoacetic acid and substituted amino acids.	Analogues of pantothenic & nicotinic acid.	145
	B-2-thienylalanine		146
	O-iodosobenzoate and hydroquinone		147

this work was the attempt of Andrewes et al [21] who investigated more than 125 compounds known to possess some bacteriostatic action against bacteria. Influenza lung consolidations in mice were not reduced by any of these. Cutting and associates [122] examined 150 compounds without effect against herpes simplex, neurovaccinia, and influenza in mice and chick embryos. Among the substances tested were acridines, metallic compounds, pyridines, surface tension reducers, compounds with labile methyl groups, amino acid derivatives, intermediates in fat and carbohydrate metabolism, vitamins and vitamin analogues and nucleic and related acids. The same investigators [123] unsuccessfully attempted in 1948 to inhibit vaccinia virus in eggs and mice, as well as staphylococcus bacteriophage with pentoses, including arabinose and inositol, seven pyrimidine drugs, antihistamines, antifolic compounds, cyclohexanes and uracils. Benzoxazole exerted a slight but irregular therapeutic effect with vaccinia virus in mice [123].

In 1949 Schaffer et al [148] tried several anesthetics, morphine, magnesium sulphate, cytochrome C (because of value in combating anoxia), dithiobiuret (possible enzyme inhibitor of nerve cell) and

dithiocarbamate against certain strains of poliomyelitis, encephalitis and influenza PR 8 strain. Certain carbamates were shown to give a minimal depression of herpes simplex multiplication[149].

Compounds with Therapeutic Promise

Acridine compounds have been tested by several investigators [150,151,152,122,141]. Green et al [151] reported that nitroakridine 3582 completely prevented hemagglutination by influenza B virus provided that the dosage did not exceed 10 infecting units. An increase in the virus inoculum beyond 10 units resulted in a lower hemagglutination titer of the allantoic fluid than was obtained in the control embryos. Rasmussen et al [152] in a follow-up paper noted that nitroakridine was less effective against influenza A virus but believed that they had demonstrated an in vivo inhibition of both strains. Their results may be criticized on the grounds that their method of viral assay depended upon hemagglutination titers which are 1000 times less sensitive than actual infectivity titers. One must, therefore, suspect that their successful results were, at the most, very minimal ones indeed.

Other investigators have been unable to confirm the above findings [122,141]. In mice Hirst did not demonstrate inhibition of EEE, louping-ill, St. Louis encephalitis, rabies and influenza A viruses with nitroakridine 3582 [141].

Of much more significance are the results of Green [153] using neutralized tannic acid as an inhibitor of influenza A virus in chick embryos. With suitable doses of tannic acid (1 mg) the actual multiplication of influenza as measured by infectivity is prevented. The drug is effective when given 6 hours before or one hour after the virus. These observations have been confirmed in part by the writer. Unfortunately, while tannic acid combines with proteins and the resulting combination is resistant to enzyme action[154] relatively little is known about metabolic effects of the compound.

With the larger viruses Eaton et al [155] suppressed cat pneumonitis in chick embryos with acriflavine and several related compounds. Proflavine, atabrine and others were not effective. Various nitro compounds have been tested against other viruses of the psittacosis-lymphogranuloma group by Eaton and Jackson [156]. These compounds are of interest in view of their relationship

to chloramphenicol. In eggs chloramphenicol was effective against cat pneumonitis, lymphogranuloma venereum and meningo-pneumonitis; p-nitrobenzamide and a sulphonamide derivative also were inhibitory. Eaton et al [157] were able to secure good inhibition of cat pneumonitis virus with sodium arsenamide using chick embryos, but the results in mice were not as clear or as pronounced. If arsenamide is given one hour after inoculation of chick embryos with cat pneumonitis virus, almost complete inhibition of multiplication occurs. If given 24 to 49 hours after the virus, partial inhibition occurs. A related compound was less active and the results were not as striking with all chemicals against meningo-pneumonitis virus.

Robbins in 1942 [158] delayed the development of a non-lethal pox on chickens by the use of quinine -- an observation not confirmed, although earlier Manwell and Goldstein [159] had shown that mercurochrome would prevent lesions from developing on the chorio allantoic membrane. While podophyllin was shown to be effective against venereal warts by Cult et al [160,161] it probably acts on the tissue rather than the virus itself, by preventing cell division in the late prophase.

Szanto and Felsenfeld observed prolongation of life in more than one half of the mice infected with low concentrations (10^{-7}) of the Lansing virus by means of malononitrile (162). The dilutions of virus used were so high, however, that it is doubtful that they were working with the Lansing strain, and casts doubt upon their conclusions. Subsequently Melzer and Adelman (163) used a confirmed strain of Lansing virus (10^{-3}, 10^{-4} dilution) and found that malononitrile had no effect whatsoever.

Salle (164) has described the inactivation of influenza A and Newcastle disease viruses by Subtilin (a polypeptide antibiotic). Using chick embryos, 3 mgs of Subtilin reduced the hemagglutination titer four-fold when a 10^{-3} dose was used, while a 10^{-4} infecting dose of influenza resulted in complete suppression of hemagglutination. As with the nitroakridine experiments of Green (151) no infectivity titers were done, however. The inactivation would appear to be an important contribution to the study of viral multiplication were it not for Krueger (121) who previously found Subtilin ineffective in the treatment of influenza infected mice.

It is claimed that anesthetics reduce mortality in mice infected with certain neurotropic viruses [124,125] and similar results have been observed in man [165] but the mechanism of action has not been determined. Mice infected with neurotropic viruses show prolonged survival by the administration of thyroid [127] and hormones [129]. Poor general nutrition [165,166,167,168], especially thiamine deficiency [169,170,171], and panthothenic acid [172] have apparently decreased susceptibility to infection in experimental animals. The discovery that certain polysaccharides would prevent multiplication of mumps and influenza viruses, as well as the nature of the inhibition, will be discussed in the following section.

BACTERIOPHAGE

A study of bacteriophage constitutes an ideal system for determining essential virus-host relations for the following reasons:

1. Pure strains of phage are antigenically distinct and specific in their action.

2. It is possible to purify phage sufficiently for chemical analysis and to examine the purified product for biological activity.

3. The invasion of the bacterial cell may be visualized by means of the electron microscope.

4. The chemical constituents of the media for growing bacteria can be controlled.

5. Resistant variants which grow up following the action of phage can be studied.

6. The system is particularly well adapted for enzyme study in the Warburg apparatus.

Despite all the above advantages, the use of bacteriophage has not, as yet, furnished crucial information on the mechanism of multiplication of the bacterial viruses because of the complexity of the problem. The use of varied strains and media, lack of adequate controls in many published experiments and the presentation of

conflicting data, even when the same strains are used, all prevent any clear, concise summation of bacteriophage growth and multiplication. It is also becoming increasingly evident that information obtained with bacteriophage and bacterial systems cannot be applied without considerable reservations to the animal viruses where the host-parasite relationships may be even more complex.

Amino Acid Relationships

Cohen and Fowler [173], studying the amino acid requirements for phage, found that the omission of phenylalanine, tryptophane, leucine, valine, glutamic acid and methionine and histidine were detrimental. Phage will not grow in distilled water but will in a dilute solution of glycine [174]. The addition of asparagine, glucose, arginine, nucleic acids and alanine are without effect while combinations of asparagine and glucose were inhibitory [174]. Anderson [175] was able to enhance the growth of T4 and T6 viruses by the addition of amino acids in the following order: tryptophane, phenylalanine, diodotyrosine and tyrosin; tryptophane was found necessary as an absorption factor or coenzyme. Leucine and cysteine are able to inhibit phage growth [176].

Chemical Effects on Phage

SH groups have been shown to be present in phage by inactivation and reactivation with appropriate SH destroying or furnishing groups [177]. Inactivation of bacteriophage by tannin, mercuric chloride and heat is a chain reaction and particles that are inactivated appear to infect particles and accelerate their denaturation [178]. Citrate and oxalate ions [179,180] have the ability to decrease virus multiplication, although under certain conditions phage may be trained to dispense with their presence [181]. Among the ions, sodium [182,183], potassium [183], calcium [181], and magnesium have been shown to have a relationship to phage multiplication.

Chemical Examination of Bacteriophage

Cohen and Fowler [176] observed that purified T_2 phage contains DNA. They further demonstrated that with certain phage "the sole nucleic acid produced is of the DNA type and that the nucleic acid constitutes the sole phosphorous-containing constituent of the virus". Price [184] in contradistinction to Cohen found no difference between normal and infected cells in amounts of inorganic phosphorus, adenosine triphosphate (ATP), RNA or DNA. It is important, however, to keep in mind that

Price used penicillin to suppress bacterial growth and this antibiotic may have interfered with nucleic acid metabolism as well. All phage strains which have been examined chemically have shown lipid fractions which consist only of fat in contradistinction to many animal viruses [185]. Cohen [83] also found that the sole source of phosphorus in phage came from the external media, and this was confirmed by Putnam et al [186].

However Goldwasser [187], using a non-lysing strain of bacteriophage and a biological method of purification as well as chemical, confirmed the suspicion that chemical methods are not sensitive enough. He found that chemical procedures removed 95% of the phosphorus in crude lysates which had been assumed to represent that present in phage. Removal of 90% of phage by adsorption with bacteria, on the other hand, left the phosphorus content of the medium unchanged. Goldwasser concluded, therefore, that the amount of phosphorus present in centrifuged lysates could not be used as an index of the amount of phage present. He also showed, in contrast to Cohen, that there was a rapid turnover of RNA. Each investigator worked with different bacteriophages and this may explain the discrepant results. The data do, however, emphasize the danger of placing too much reliance

on chemical methods alone. Final evaluation must await further chemical and biological studies.

Respiratory Quotient of Viruses

The RQ of infected staphylococci fluctuates periodically, which indicates an explosive liberation of carbon dioxide. Dehydrogenases are among the first enzymes to disappear from bacteria undergoing lysis. Before lysis takes place the phage-infected bacteria appear to be asphyxiated [188].

Henry and Henry [189] observed that phage-resistant strains of bacteria manifested slower rates of growth, decreased aerobic oxidation of sugars and dissimilation of pyruvate, but aerobic glycolysis of fructose and mannose was greater than with susceptible strains. By replacing the oxygen supply with nitrogen or by the use of cyanide or iodoacetate, Cohen [190] demonstrated immediate lysis of E. coli infected with bacteriophage. He believed this phenomenon was due to " a rapid reorganization of the cell substance, which in the absence of the host's energy supply is uncontrollable, leading to autolysis and disrupting of the structures essential to phage synthesis".

The observations [191] that the RQ remains the same in infected and uninfected cells and that adsorbed

inactive ultra-violet irradiated phage also stops bacterial multiplication with no change in RQ, points to the fact that despite crucial damage to the reproductive mechanism of the cell, the energy systems are not modified but merely diverted to new synthesis. The mechanism of such a synthesis is obscure since Monod and Wollman [192] found that adaptive enzyme formation is suppressed by bacteriophage.

The Effect of Vitamins and Analogues on Bacteriophage

Woolley and Murphy [193] observed that desoxypyridoxine inhibited the multiplication of T_2 bacteriophage and this effect was relieved by pyridoxine, formic, acetic, butyric and valeric acids, and glucose-6-phosphate and pyruvic acids. Lactic, malic, fumaric and succinic acids were somewhat less effective. Roberts et al [194] have shown that vitamin B-12 increases the rate of DNA formation and in turn stimulates the rate of phage formation [195]. Price [184] found that if nicotinic acid, a co-factor from broth and phage were added simultaneously to a synthetic medium containing organisms, multiplication of phage occured. If the co-factor and nicotinic acid were placed in the medium prior to the phage they were rapidly absorbed by the bacterial cells and phage synthesis was prevented. He concluded that his results

were an argument against the Precursor theory [177].

Metabolic Antagonists

Addition of methionine sulfoxide, the analogue of glutamic acid, as well as indole-3-acetic acid in varying dilutions have suppressed phage growth [176]. Cohen and Fowler [196] found that certain concentrations of 3-indoleacetic acid inhibits phage formation while a 20-fold dilution stimulates virus growth. Iodoacetate, fluoride, azide and gramicidin were able to prevent the synthesis of phage [184]. The first three are known to block ATP formation in the carbohydrate cycle while gramicidin prevents the uptake of inorganic phosphorous from the medium. Arsenite was used by Spizizen [197] as an inhibitor of phage. When 2-4-dinitrophenol (DNP) is added to certain cultures of E.coli, growth ceases but respiration continues [198]. If phage is added to this system, respiration is decreased, lysis is increased and multiplication of phage does not occur [192,199]. Trypaflavine and acridine, as well as malachite green, p-aminophenol, thiamine and DNP will inhibit phage formation while sodium fluoride is less effective [200]. Fitzgerald and Lee, and Fitzgerald and Babbit [201.202] have demonstrated that several acridines including "phosphime GRN" are capable of suppressing phage.

In perhaps the most interesting work, Foster [203] has shown that proflavine concentrations which do not affect bacterial growth inhibit completely the multiplication of T4 and T6 phage. The concentration of inhibitor required is inversely related to the length of the latent period, which represents the time necessary from the addition of phage to the formation of new particles. Of importance is the finding that when proflavine is introduced during the latent period it effectively prevents phage formation. Inhibition of the bacteriophages may be obtained if the drug is added as late as 12-15 minutes after infection, a period about equal to one half the latent period. If proflavine is added during the period of multiplication, decreased yields of virus are obtained; the extent of inhibition is an inverse function of the time of addition of the drug. Apparently proflavine blocks some late step in the process of multiplication of phage but does not interfere with earlier steps since the removal of the drug during the first part of the latent period permits normal multiplication. Cohen and Anderson [204] have inhibited T2 phage with 5-methyl tryptophane and reversed it with tryptophane [205]. Like

proflavine, complete inhibition is obtained only if the drug is added during the first half of the latent period. These findings, along with Fowler's, suggest that inactive virus is present during the first half of the latent period and that phage formation is, therefore, a synthetic process in which the virus is built up stepwise until maturity is reached.

Theories of Multiplication

Cohen [83] postulates, "one readily apparent mechanism for virus synthesis is the time-worn template hypothesis; since virus does not seem to be a self-duplicating unit but rather a duplicated unit" -- which is synthesized from the model it presents. Because virus components, like phosphorus, may be obtained from an external source the theory that precursors exist in the cell which are autocatalytically transformed to virus by the infecting agent is negated. Cohen, therefore, concludes that , "a virus is a parasite which organizes a specific enzymatic environment for its own multiplication".

Wyckoff [206], by means of electron microscope pictures of T2 bacteriophage, sees particles developing in ruptured bacterial protoplasm until they have used up most of it. The particles are small and suggest forms

which resemble the multiplication of cocci. He assumes that most of the growth takes place in liberated protoplasm rather than in the intact bacteria. The facts that phage has a complex organization, that amino acid differences exist between host and phage, and that discrepancies are found between diffusion rates and particle size are presented by Wyckoff as proof of "an independence of particle movement that presumably would sustain some kind of metabolic activity". Such a concept postulates "living" self-reproducing particles much smaller than some protein molecules, as the hemocyanins. Merlind [207] also thinks that phage forms intracellular colonies by binary fission.

More recently Luria and Human [208] have used chromatin staining methods on infected bacteria and concluded:

1. that phage is a more "spatially organized process" than ordinary bacterial growth,
2. that bacterial genes are supplanted by virus, which directs a new synthesis, and
3. that inactivated phage carries the process to a certain point before disappearing.

They found specific changes due to phage, such as an alteration of the chromatinic bodies in bacteria, then a swelling of the cells with the formation of granular

chromatin ("phage nucleoprotein"). If inactivated phage was used the granular chromatin developed and then gradually faded away.

POLYSACCHARIDES

In 1932 Levine and Frisch (209) demonstrated that extracts of Salmonella, probably polysaccharide in nature, combined specifically with their homologous bacteriophages and inhibited multiplication. The polysaccharide nature of the inhibitor was further established by Gough and Burnet in 1934 (210). Subsequently Ellis and Spizizen (182) demonstrated that phage also could be inactivated by large concentrations of starch, inulin and gum arabic. Maurer and Woolley (211) observed that pectin did not prevent multiplication of phage but it did inhibit bacterial lysis. The first successful inhibition of a plant virus (tobacco mosaic) was obtained by Takahashi (212), who utilized a polysaccharide from yeast for this purpose. With the animal viruses Maurer and Woolley (213) reported that the growth of the agent of influenza was arrested by polysaccharides derived from flaxseed, myrrh, gum acacia, apple and citrus pectin. In addition some polygalacturonides were also found to be effective. During the same year Horsfall and McCarty (214) reported that a number of bacterial polysaccharides were capable of preventing the multiplication of pneumonia virus in mice (PVM). Outstanding among these was the capsular substance

of the Friedlander's bacillus where as little as 2 micrograms per mouse proved to be sufficient. These observations were extended to the mumps virus by Ginsberg et al [215] who observed that as little as 5 micrograms of the Friedlander's polysaccharide (FP) inhibited multiplication in the chorioallantoic sac of the embryonated egg.

A detailed study of these phenomenon revealed the following [216,217]:

1. The polysaccharide, itself, was non toxic to the chick embryos both grossly and microscopically as far as could be determined.

2. The mumps virus was not inactivated as result of contact for 30 minutes at 4 degrees C. with the polysaccharide as measured by infectivity titers of serially diluted allantoic fluids.

3. A significant degree of inhibition was obtained as long as 96 hours after infection with the virus.

4. Formation of new virus was abruptly prevented by the introduction of polysaccharide into the infected egg but at no time did a decrease in the amount of virus already formed occur.

5. A fact not stressed by the authors is that massive doses of polysaccharide did not completely abolish viral multiplication.

6. The viral inhibiting portion of the polysaccharide molecule was independent of the serologic part of the molecule as shown by treatment with hydroxyl ions and periodic acid.

7. Polysaccharide did not destroy the virus receptors of the host cell. This was shown by experiments in which the polysaccharide was introduced into the allantoic sac and subsequently washed out with saline. The introduction of virus into the sac following this procedure resulted in maximal multiplication.

On the basis of the above facts the authors conclude that the Friedlander's polysaccharide does not inhibit mouse pneumonia and mumps virus per se but rather that it blocks "a metabolic step . . . which is required for the multiplication of both viruses".

Woolley et al [213] had originally concluded from their observations with apple pectin and influenza A virus that the inhibitor had combined with or destroyed the cell receptor. He later modified this view and suggested that the inhibition was due to a competition between virus and apple pectin for a specific cell substrate [218]. It is of interest here to note that Friedlander's polysaccharide does not inhibit multiplication of the influenza A or the Newcastles viruses[216].

The data presented above may require reinterpretation in the light of a recent study by Heilbrunn and Wilson [219]. These authors investigated a polysaccharide obtained from S. marcescens which was capable of inhibiting cell mitosis. Their previous studies convinced them that just prior to division the protoplasm of the cell undergoes gelation and that mitosis was dependent upon this phenomenon. Heparin, a polysaccharide,

prevented mitosis by interfering with gelation. They reasoned, therefore, that because the marcescens polysaccharide was able to inhibit certain mouse cancers, its action was probably due to a heparin-like effect. This hypothesis was substantiated by experimental evidence to show that the two substances were closely related, if not identical chemically.

Ginsberg et al [216] did not consider an effect on mitosis as a mechanism for the inhibition by polysaccharide of the mumps and PVM viruses. Other observations, previously mentioned, such as suppression of phage multiplication by citrate and oxalate ions or the absence of calcium ions are in agreement with the Heilbrunn-Wilson concept.

It is of interest that the nuclear apparatus is rather intrinsically associated with animal viruses [220] and also with bacteriophage multiplication [208]. The predominance of DNA in animal viruses again emphasizes this relationship. The intimate association between viral multiplication and cell division suggests that nuclear activity may be a key to the entire problem. Certainly it would seem important to determine if Friedlander's polysaccharide and heparin are also related.

INTERFERENCE PHENOMENA

Of growing interest is the phenomenon of interference between two animal viruses in a given host. For several reasons this interest is justified: first, it may possibly lead to a system of classification or separation, and secondly, it may throw some light on the possible method of enzyme synthesis of certain viruses. Several investigators [221,222], including Delbruck and Price [223], have worked with this problem and have reviewed the subject.

Jenner in 1804 observed that herpetic lesions would prevent formation of vaccinia lesions and even today some physicians utilize this observation in reverse, that is, give smallpox vaccination in an effort to relieve herpetic infection [224]. In other reports it was noted that vaccinia would not take on the cornea of rabbits recently recovering from herpes simplex infection [224]; also the antagonistic effect of vaccinia on foot-and-mouth disease and neurovaccinia on rabies has been demonstrated.

Interference may occur between immunologically related viruses, between antigenically unrelated viruses, between activated and inactivated viruses and

finally dual infections of a host may take place. Examples of the above, in order, would be interference of a neurotropic strain in the chick embryo [225], foot-and-mouth disease interfering with rabies [226], inactivated influenza A against active influenza A[227] and finally the simultaneous growth of influenza A and B viruses [228].

The injection of certain viruses in concentrated amounts may lead to a phenomenon called "auto-interference" [229]. It has been postulated that in undiluted viruses inhibiting substances may be present which are lost on dilution. In this connection it has been shown that the injection of undiluted influenza virus leads to the formation of two well-defined particles of different sizes, as determined by physical means. The larger one possesses all the properties associated with influenza virus and the smaller one (precursor) is non-infectious, causes hemagglutination and also interference. The precursor concept has been supported in a different manner by the finding that injection of undiluted virus material is followed by the development of hemagglutinins within 3 to 4 hours, whereas infectivity remains stationary for 5 to 6 hours [230]. Hoyle [231] has also made similar observations.

When one attempts to explain the phenomenon of interference the theories are numerous and conflicting. The use of inactivated influenza virus to suppress active influenza would tend to negate the theory of exhaustion of cellular metabolite [229]. The theory of interference by metabolic substances of another virus still requires the demonstration of such products. It has been thought that the attachment of one phage particle to an organism would not allow further infection; this has been shown to be untrue [232,233] and therefore the penetration theory thus falls with the others. The well known phenomenon of the attachment of hemagglutinating viruses to red cells with a subsequent elution of unchanged virus which results in a permanent alteration of the cell, has led to the concept of cell receptors being present on the susceptible cells which combine to attach the virus. Many of the mucoid substances, cholera enzymes, etc., probably owe their effectiveness to the combination with or destruction of cell receptors [234][235]. These receptors, then, if neutralized would prevent infection. The receptor blockade theory, therefore, remains a possibility but has not been indisputably substantiated [224].

The blockade or key enzyme theory remains as the most plausible explanation for interference [222,236] and fits most of the observed facts after ruling out other factors [230,237,238]. Thus, an inactivated virus particle could combine with the specific enzyme and block out the active particle [191]. If virus production is stepwise then interference could occur at any point in the process of virus formation. The addition of precursors to such a concept will require expansion of the theory and must be considered in future experiments dealing with this phenomenon. The theory must also explain such observations as those of Syvertan and Berry [239] who presented evidence of the simultaneous infection of a single cell with three viruses.

EXPERIMENTAL

Introduction

The plan of the following experiments was based on the hypothesis that the synthesis of virus could be blocked by the introduction of key enzyme inhibitors into the cell environment. Once this had been accomplished it would then be necessary to identify the particular enzyme involved and thereby elucidate the mechanics of multiplication.

The influenza group of viruses were selected because of the ease of cultivation and the clear-cut nature of the end-point, namely, red blood cell agglutination. The embryonated egg was utilized as a culture medium because it represented an easily standardized medium free of contaminating viruses. The inhibitors and viruses could be easily introduced into such an intact system and samples removed at will. The preliminary experiments recorded below represent attempts at standardization of procedures. The final experiments may be taken as examples of the application of the technique to the problem originally posed.

Methods

Influenza A (PR 8) and B (Lee) viruses were derived from Dr. T. Francis, Ann Arbor ,Michigan. They were maintained by allantoic passage in 9-10 day old chick embryos. Stock virus was lyophilized. Passage was accomplished by the allantoic injection of 10^{-4} dilution of 0.1 ml amounts and the eggs incubated for 48 hours. In these studies the viruses were kept at high infectivity by repeated passage and storage, for very brief periods, in the carbon dioxide box.

The inhibitors used were obtained from various commercial scources by purchase with the exception of mono sodium fluoroacetate and Mapharsen, which were given by the Monsoto Chemical Company and Parke-Davis Company, respectively.

Fertile hen eggs were obtained from a local hatchery; 8 to 10 day old embryos were used and the same scource of supply was employed throughout all experiments.

The following general plan was carried throughout all experiments. It consisted of first establishing the 50% lethal dose (LD 50) of the inhibitor by injecting, allantoically, previously candled eggs. The embryos, except where noted, were candled at 24 and 48 hours and the deaths recorded. The virus, in measured

doses, was mixed with various concentrations of inhibitor and both injected allantoically, although in early experiments the inhibitor was usually given first followed by the virus. Usually the total volume of both inhibitor and virus never exceeded 0.2 ml but this varied with particular experiments. The eggs were then sealed with paraffin and incubated. At the end of incubation they were candled and placed in the refrigerator for at least 2 hours. The top of the shell was then broken by a small forceps , exposing the embryo membranes. A 10 ml Luer syringe was used as an aspirator and 1 ml quantities were withdrawn and pooled in equal amounts. Usually hemagglutination tests were performed immediately.

Two methods were employed for hemagglutination determinations. When chick cells were used, 0.2 ml of undiluted allantoic fluid was placed in 0.8 ml of saline in Kahn tubes. Serial two-fold dilutions were then made in tubes containing 0.5 ml of saline. The last tube served as a control. Then 0.5 ml of a 0.25% chicken cells was added to each tube, shaken and allowed to stand at room temperature until button formation in the tube was clearly defined, usually in 1 to 1.5 hours. When possible, agglutination patterns were checked independently by two observers. This method was used in all preliminary studies but the following hemagglutination technique was used for the larger and final type experiments. It

consisted of making serial dilutions of virus as above and to each 0.5 ml amount adding 0.5 ml of inactivated rabbit serum. To each tube 0.1 ml of 0.75% human type O cells was pipetted, the mixtures were shaken, and they were allowed to stand at room temperature for about 2.5 hours or until the control had sufficiently sedimented. In some cases the tubes were resuspended and placed in the refrigerator; final readings were made the following morning.

The control tubes without virus, by either method, usually showed a small packed mass of cells with sharply defined edges in the bottom. In tubes which contained virus the cells seemed to spread over the bottom in a single layer or show a definite ring of cells, with scattered cells both outside and inside the ring. No attempt was made to estimate the intensity of agglutination of each tube but the results were simply recorded as negative or positive. It might be added here, however, that in later experiments while hemagglutination tests were done, reliance was placed on infectivity tests as the final criterion of virus multiplication.

Infectivity titers were done by serially diluting allantoic fluids in sterile saline in units of ten. A clean pipette was used with each transfer and the contents

of each tube shaken for thorough mixing before passage to the next tube. In the early experiments using relatively large amounts of virus (10^{-2}), few infectivity tests were done. Later influenza A was passed at a 10^{-7} dilution in 0.1 ml amounts. Influenza B was passed in 10^{-6} dilution. At the conclusion of the experiment the virus, rediluted to original passage amount, was inoculated into no less than three fertile eggs. All were incubated 48 hours at 37 C. and hemagglutination titers performed on pooled fluids.

In preparing the inhibitors for inoculation aminopterin, 3-acetylpyridine and nitrogen mustard were obtained in sterile ampules and were diluted with sterile saline to appropriate concentration. The diluent used for nitrogen mustard was kept ice-cold in all dilutions. All other chemicals were dissolved in saline and heated to 60 C. for one hour with the exception of the following: Mapharsen was made up in sterile saline and allowed to stand for several hours in the ice box; p-chloromercuricbenzoate was dissolved in weak alkali, neutralized and sterilized while tannic acid was neutralized with sodium hydroxide and autoclaved for 15 minutes.

INITIAL EXPERIMENTS

Potassium Cyanide

Because of the large amount of work done on potassium cyanide and, in particular, its effect on respiratory systems, it was thought that this chemical would be a useful inhibitor with which to begin this investigation.

In experiment 1 a constant quantity of influenza A virus (10^{-2}), ten million ID, was used with varying amounts of potassium cyanide. The cyanide was first injected into the allantoic cavity of 9-10 day old chick embryos. Immediately following this procedure the virus was introduced. Embryos dead prior to 48 hours incubation at 37 C. were removed and refrigerated until the end of the experiment. In these and all subsequent experiments the living embryos were placed in the refrigerator for two hours before the allantoic fluids were withdrawn. The fluids from comparable eggs were pooled and tested. It can be seen in Table 1 that the LD 50 of KCN, in 48 hours, was in the region of 0.05 M. With increasing doses of cyanide the embryos expired rapidly and there was little opportunity for viral multiplication. In the living embryos, however, the red cell agglutination (RCA) titer of allantoic fluids was comparable with those of the controls.

TABLE 1

The Effect of Varying Quantities of KCN on the

Multiplication of Influenza Virus

KCN Molarity	ml	Embryos Number	Embryos Status	Incubation in Hours	Hemagglutination Titer
0.1	0.2	6	D	8	0
	0.1	6	D	8	0
0.075	0.1	5	D	8	0
		1	L	8	nt*
0.05	0.1	3	D	30	0
		2	L	48	1280
0.025	0.1	6	L	48	2560
0.001	0.1	6	L	48	2560
none	none	6	L	48	2560

Dose: 0.1 ml 10^{-2} virus

*not tested D = Dead L = Living

Table 2 presents a similar experiment with influenza A virus in which intermediate concentrations of KCN were employed. Embryos found dead after 12, 20, and 40 hours at 37 C. were placed in the refrigerator. After 48 hours incubation, pools of allantoic fluid were prepared from the surviving eggs. It can be seen that fluids from all living embryos, with one exception (0.07 M KCN) showed RCA titers equal to or exceeding the controls. Embryos which had expired previous to 40 hours contained varying quantities of virus or none at all. The data are not consistent but they show that even embryos dying of cyanide intoxication within 12 hours after infection often contained considerable quantities of virus as measured by hemagglutination. Subsequent studies using infectivity titers would indicate that in this experiment virus was probably present in dilutions of 10^{-5} to 10^{-7} despite early death of the embryos and the short period of incubation.

In the next experiment, varying quantities of KCN were given, followed by 10^{-2} influenza A virus. A total of 36 eggs were injected for each dilution of cyanide together with 12 controls, which received saline. At

TABLE 2

The Effect of Varying Quantities of Potassium Cyanide and Incubation Time on the Multiplication of Influenza A Virus

KCN M 0.1 ml	Embryos Number	Embryos Status	Incubation in Hours	Hemagglutination Titer
0.07	10	D	12	1250
	1	D	20	320
	1	L	48	0
0.06	2	D	12	1-240
	3	D	20	80
	3	D	40	0
	3	L	48	10240
0.05	5	D	20	640
	6	D	40	1280
	1	L	48	10240
0.04	1	D	20	0
	4	D	27	10240
	4	D	40	10240
	3	L	48	2560
0.03	12	L	48	10240
none	12	L	48	5120

D = Dead   Dose: 0.1 ml 10^{-2} virus   L = Living

6, 12, 24, 36, and 48 hours after inoculation, three living and three dead (when possible) as well as three control eggs were removed and placed in the refrigerator. The data are presented in Table 3, where it can be seen that the rate of formation of virus in the living embryos was not appreciably influenced by the presence of KCN. In the dead eggs there was a suggestive but not conclusive retardation of viral growth. These results again indicate that lethal and sub-lethal doses of cyanide do not appreciably alter the ability of cells to regenerate influenza virus.

The effect of prolonged contact between equal parts of influenza A virus and 0.05 M KCN was determined in experiment 5. Virus and inhibitor were mixed and held in the refrigerator for varying periods prior to inoculation into chick embryos. A total dose of 0.2 ml of the mixture was then injected and the eggs incubated for 48 hours at 37 C. Hemagglutination titers of pooled fluids, as shown in Table 4, were the same for both experimental and control eggs.

In experiment 6 the dose of influenza virus was varied and the amount of cyanide (0.1 ml of 0.045 M) kept constant. Six control eggs were used for each virus

TABLE 3

The Effect of Incubation Time and Different Doses of Potassium Cyanide on the Multiplication of Influenza Virus

KCN M 0.1 ml	Embryo Status	Hemagglutination Titer of Allantoic Fluid Pools Hours of Incubation				
		6	12	24	36	48
0.06	L	0	0	280	1280	2560
	D	0	0	80	320	2560
0.04	L	0	20	5120	1280	2560
	D	0	0	5120	1280	2560
none	L	0	320	2560	640	5120

D = Dead Dose: 0.1 ml 10^{-2} virus L = Living

TABLE 4

The Effect on Influenza Virus Multiplication of Incubation with Potassium Cyanide for Varying Periods of Time

KCN 0.05 M	Embryos Number	Embryos Status	Hemagglutination Titer
½ hr.	6	L	2560
1 hr.	6	L	2560
1½ hrs.	6	L	2560
2 hrs.	6	L	2560
Virus alone	6	L	2560

0.1 ml virus cyanide mixture injected
Incubation time—48 hrs.

dilution. The data represents the results of HCA titers from pooled allantoic fluids. It can be seen that maximal titers were obtained in all instances. In order to determine if the KCN treated virus was viable the pooled fluids were then diluted to the degree used in the original inoculum and injected intra-allantoically into chick embryos. In all cases infectivity remained constant, showing (Table 5) that both hemagglutination and infectivity were unchanged by exposure of the virus to cyanide.

In all previous experiments large doses of influenza virus (one to ten million ID) had been used to determine if a growth retarding effect of KCN could be obtained; with the following, more dilute inocula of virus were used. Table 6 presents data in which 24 eggs were injected with a 10^{-2} influenza virus and 24 eggs with a 10^{-6} dilution of virus following the introduction of 0.05 M KCN. 12 eggs were used as controls for each dilution of virus respectively. There was no appreciable difference in the experimental or control HCA titers. Again the allantoic fluids from dead embryos also agglutinated red cells to a degree equal to or slightly less than the controls.

The above observations were extended in experiment 7, in which 42 eggs were injected with 0.1 ml of 0.048 M KCN. 12 of these were inoculated with 0.1 ml of a 10^{-2}

TABLE 5

The Effect on Hemagglutination and Infectivity in Potassium Cyanide Treated Eggs Infected with Varying Quantities of Influenza A Virus

Number Eggs	ml KCN 0.045M	0.1 ml Virus Diluted to	Hemagglutination Titer	Allantoic Fluid Pools* Dilution	Infectivity
12	0.1	1×10^{-2}	10240	1×10^{-2}	+
6	-	1×10^{-2}	10240	1×10^{-2}	+
11	0.1	1×10^{-3}	10240	1×10^{-3}	+
6	-	1×10^{-3}	10240	1×10^{-3}	+
11	0.1	2.5×10^{-3}	10240	2.5×10^{-3}	+
6	-	2.5×10^{-3}	10240	2.5×10^{-3}	+
11	0.1	5×10^{-3}	10240	5×10^{-3}	+
6	-	5×10^{-3}	10240	5×10^{-3}	+

*Cyanide given first followed by virus

TABLE 6

Multiplication in Living and Dead Embryos Infected with Varying Quantities of Influenza A Virus and Constant Amounts of Potassium Cyanide

ml KCN 0.05 M	Inoculum 1 x 10⁻	Embryos Number	Embryos Status	Hemagglutination Titers
0.1	2	22	D	5120
		2	L	2560
0.1	6	21	D	2560
		3	L	2560
none	2	12	L	2560
none	2	12	L	5120

D = Dead L = Living Incubation time 48 hours

TABLE 7

Multiplication in Embryos Treated with 0.045 M Potassium Cyanide

and Varying Amounts of Influenza A Virus

ml KCN 0.045 M	Inoculum $1 \times 10^{-}$	Embryos Number	Embryos Status	Hemagglutination Titer
0.1	2	5	D	nt
		7	L	2560
none	2	6	L	2560
0.1	6	4	D	nt
		8	L	2560
none	6	6	L	5120
0.1	7	6	L	5120
0.1	8	2	D	nt
		4	L	0
0.1	9	4	D	nt
		2	L	0

nt = not tested D = Dead L = Living

influenza A virus and 12 with a 10^{-6} dilution. Six eggs each received 0.1 ml of a 10^{-7}, 10^{-8}, and 10^{-9} dilution of virus. For the 10^{-2} and 10^{-6} dilutions 6 eggs were used for each as controls. With larger numbers of eggs than in the previous experiments the results were practically the same: neither concentrated or dilute virus multiplication was inhibited. The absence of hemagglutination in the 10^{-8} dilution was suggestive of viral inhibition, bu the result was probably due to the fact that the end-point of the titration had been reached at 10^{-7}; see Table 8 for example.

Summary

The experiments included in this section show that sub-lethal doses of KCN do not, in any way, retard the in vivo multiplication of influenza A virus in chick embryos. This fact holds true irrespective of the concentration of both KCN and virus. Furthermore, contact between KCN and virus from ½ to 2 hours did not result in any in vitro inactivation by the chemical.

TABLE 8

Multiplication of Influenza A Virus in Living and Dead Cyanide Treated Eggs

ml KCN	Inoculum	Embryos		Hemagglutination
0.05 M	$1 \times 10^{-}$	Number	Status	Titer
0.1	2	14	D	0
		10	L	5120
0.1	6	15	D	1280
		9	L	5120
none	2	12	L	2560
none	6	12	L	2560
none	7	12	L	80
none	8	12	L	0

D = Dead L = Living Incubation time 48 hours

Nitrogen Mustards

Observations that nitrogen mustard (methyl bis b-chloroethyl amine HCL) prevented viral multiplication by virtue of its ability to combine with nucleic acids prompted the use of this inhibitor in the following series of experiments.

Table 9 represents a composite of three separate experiments; nitrogen mustard was injected first, followed by 0.1 ml of a 10^{-2} influenza virus. Adequate controls were also included using various virus saline mixtures alone. The quantity of nitrogen mustard was varied in amount and it may be seen from the Table that between 0.2 and 0.3 mgs was close to the 50% LD for chick embryos. No hemagglutination titers were performed on the allantoic fluids of embryos dying before 48 hours, at which time the experiment was terminated. The RCA titers of treated eggs was equal to, or occasionally higher than, the controls.

A summary of the data shows that the final hemagglutination titer of pooled allantoic fluids was in no way decreased by the presence of sub-lethal concentrations of nitrogen mustard, provided the embryo was able to survive. Additional experiments with the mustards will be presented subsequently and interpretation of findings is reserved for the discussion.

TABLE 9

Results of Pooled Experiments with Varying Quantities of Nitrogen Mustard in Influenza A Infected Embryos

Number Eggs	mg Nitrogen Mustard	Embryos Number	Embryos Status	Hemagglutination Titer
6	0.4	6	D	nt
8	0.3	5	L	5120
16	0.3	13	D	nt
		3	L	5120
8	0.2	8	L	5120
14	0.2	8	D	nt
		6	L	2560
6	none	6	L	5120
10	none	10	L	1280
8	none	8	L	2560

D = Dead Dose: 0.1 ml 10^{-2} virus L = Living

Fluoroacetate

To determine the effect of mono sodium fluoroacetate on influenza A virus multiplication, a series of preliminary experiments were run. Table 10 presents 3 separate experiments which have been combined. The fluoroacetate was injected first in varying doses followed by a 10-2 influenza A virus dilution in 0.1 ml amounts. Controls are included for each of the three experiments.

In general there was no suppression of viral multiplication demonstrated, as measured by hemagglutination titers of pooled allantoic fluids. It can be seen that when small numbers of eggs were used, the final titrations varied widely and point to the necessity of adequate sized pools. Furthermore, embryos which survived 15 hours (not included in Table 10) also contained considerable quantities of virus.

Similar results were obtained when 0.1 mg amounts of fluoroacetate were tested against virus diluted to 10-6. It may also be noted in Table 11 that even in dead eggs viral multiplication took place almost equal to that of the control embryos. The fluctuations in HCA titer may be due to chance oe else to the small number of allantoic fluids per pool. Additional studies using fluoroacetate will be presented in the section dealing with final experiments.

TABLE 10

Composite of Three Experiments Using Varying Doses of Fluoroacetate Against Influenza A Virus

Number Eggs	Fluoroacetate mg	Embryos Number	Embryos Status	Hemagglutination Titer
24	0.5	16	L	2560
2	0.5	1	L	1280
2	0.4	1	L	1280
2	0.3	1	L	1280
2	0.2	2	L	1280
3	0.2	3	L	2560
2	0.1	2	L	1280
3	0.1	3	L	2560
2	0.05	2	L	2560
3	0.02	3	L	2560
3	0.01	3	L	2560
3	-	3	L	640
2	-	2	L	640
8	-	8	L	2560

Dose : 0.1 ml 10^{-2} virus L = Living

TABLE IX

The Effect of Fluoroacetate in Living and Dead Embryos on the Multiplication of Influenza A Virus Using Dilute Inocula

Fluoroacetate mg	Embryos Tested Number	Status	Hemagglutination Titer
0.4	3	D	320
	7	L	640
0.5	3	L	320
	9	L	2560
none	12	L	640

D = Dead L = Living Dose: 0.1 ml 10^{-6} virus

Sodium Azide

While the exact mechanism of sodium azide inhibition on certain enzymes has been incompletely elucidated it has, nevertheless, been used extensively by many investigators of viral multiplication. Its effect on influenza A virus multiplication was evaluated in the following series of experiments.

In a preliminary experiment sodium azide was inoculated allantoically into four different groups of embryos, using 6 eggs for each dilution. 0.1 ml of a 10^{-2} influenza virus was then injected. Six eggs were included for the controls. As shown in Table 12, in no case was there evidence of suppression of viral multiplication in living embryos. Furthermore, virus was present in the dead embryos which were tested.

In Table 13 is presented an experiment in which larger numbers of embryos were used together with more dilute virus. 24 eggs were inoculated with 0.26 mg of sodium azide and 12 each with 0.13 and 0.0013 mgs respectively. This was followed by a 10^{-7} dilution of influenza virus (0.1 ml) into each egg and to 12 control embryos. In addition 4 control eggs each were injected with 10^{-8} and 10^{-9} dilutions of virus.

TABLE 12

The Effect of Incubation Time and Varying Amounts of Sodium Azide on Influenza A Multiplication in Chick Embryos

Na Azide mg	Embryos Number	Embryos Status	Incubation in Hours	Hemagglutination Titer
0.65	5	D	20	0
	1	D	27	nt
0.48	3	D	27	640
	3	D	45	1280
0.32	5	D	45	2560
	1	L	45	nt
0.16	5	L	45	2560
none	6	L	45	5120

L = Living   D = Dead   nt = not tested

TABLE 13

Effect of Sodium Azide in Varying Doses on the Multiplication of Influenza A Virus in Dilute Inocula in Infected Embryos

Na Azide mg	Inoculum $1 \times 10^{-}$	Embryo Number	Embryo Status	Hemagglutination Titer	Infectivity $1x10^{-}$
0.26	7	12	L	5120	7
0.13	7	12	L	1280	7
0.0013	7	12	L	1280	7
none	7	12	L	1280	nt
none	8	4	L	2560	nt
none	9	4	L	640	nt

D = Dead L = Living nt = not tested

Surprisingly, as shown in Table 13, the embryos containing the largest amount of azide had the highest titer as determined by red cell agglutination. Furthermore all experimental eggs gave positive infectivity tests at 10^{-7} dilution. Of passing interest was the apparent multiplication of the 10^{-9} control, a dilution at which infectivity is usually lost.

Experiment 14 was a semi-duplication of the previous protocol. In this case, however, the virus and inhibitor were mixed together and immediately injected in 0.2 ml amounts (0.1 ml of 10^{-7} virus and 0.1 ml of azide in appropriate strength). 61 eggs received 0.265 mgs of virus-azide mixture and 33 living and 5 dead were removed at the end of 18 hours incubation. 24 eggs each were inoculated with azide-virus mixtures containing 0.12 and 0.0013 mgs respectively. As with the larger dose, 12 eggs of each were removed at the end of 18 hours and the remaining embryos at the end of 36 hours. 12 eggs each received 0.1 ml of 10^{-7}, 10^{-8} and 10^{-9} dilutions of influenza virus and were incubated at 37 C for the entire 36 hours. Table 14 presents the above data and it is apparent that in embryos living over 18 hours, virus multiplication took place and that at the end of 36 hours

TABLE 14

The Effect of Sodium Azide and Incubation Time on the Multiplication of Influenza Virus as Measured by Hemagglutination and Infectivity Tests

Na Azide mg	Inoculum 1 x 10⁻	Embryo Number	Embryo Status	Incubation in Hours	RCA Titer	Infectivity 1 x 10⁻
0.265	7	5	D	18	0	3
		33	L	18	2560	7
		2	D	36	5120	7
		21	L	36	10240	7
0.13	7	12	L	18	2560	7
		12	L	36	10240	7
0.0013	7	12	L	18	2560	7
		12	L	36	10240	7
none	7	12	L	36	10240	7
none	8	12	L	36	10240	7
none	9	12	L	36	10240	nt

D = Dead L = Living nt = not tested

virus was present in both living and dead eggs. Although no hemagglutination was demonstrable in the 5 eggs dead at the end of 18 hours incubation from 0.265 mgs of azide, the virus, nevertheless, was present at a dilution of 10^{-3}. This discrepancy corroborates observations made by the investigators that infectivity is more sensitive indicator of the presence of virus than hemagglutination. The remaining embryos contained virus to the maximal titer of 10^{-7}.

Summary

In doses of sodium azide which permitted embryo survival no inhibition of viral multiplication was noted and, in addition, embryos which died within 18 hours did show evidence of virus multiplication by infectivity tests even though hemagglutination tests were negative.

Aminopterin

The relationship of folic acid to sulphonamide action, as mentioned in previous discussions, led to the examination of aminopterin as a possible inhibitor of influenza multiplication by virtue of its ability to antagonize the action of folic acid.

As shown in Table 15, large numbers of embryonated eggs were used with amounts of aminopterin ranging from 0.5 mg to 0.01 mg. Embryo death was variable as the protocol shows, but no inhibition of a 10^{-7} virus inoculum was demonstrated. Further aminopterin studies will be presented in the section dealing with final experiments as well as in the discussion.

TABLE 15

The Effect of Aminopterin on the Multiplication of Influenza A Virus in Living Chick Embryos

No. Eggs	Aminopterin mg	Embryo Number	Embryo Status	Hemagglutination Titer
18	0.5	15	L	640
18	0.1	15	L	640
12	0.05	12	L	640
12	0.01	11	L	1280
12	none	11	L	640

Dose: 1×10^{-7} virus

Miscellaneous Inhibitors

In the following composite of six different preliminary types of experiments, influenza A and B viruses were used as shown in Table 16. Influenza A was diluted to 10^{-2} while influenza B was diluted 10^{-6} prior to inoculation. The inhibitor, in each case, was added first followed by the virus and the eggs were incubated only 24 hours. Pooled allantoic fluids were tested for RCA but no infectivity tests were done. With sodium thioglycollate, mercuric chloride and p-chloromercuribenzoate there was no demonstrable inhibition of viral multiplication. On the other hand, dithiobiuret and thioflavine treated embryos showed lower RCA titers generally than the controls, but since some influenza B virus was found it was felt there had been no suppression of growth. Similarly, oxine was shown to be ineffective by spot agglutination tests for the presence of the virus, only 1 tube being used for each separate pool of allantoic fluids.

In summary, as with other previously mentioned compounds, the above chemicals showed no evidence of inhibitory action against influenza A or B viruses as measured by preliminary testing.

TABLE 16

The Effect of Miscellaneous Inhibitors Against Influenza Viruses in Living Chick Embryos

Inhibitor	Dose mg	Virus	Dilution	Living Embryos	HCA Titer
Sodium thio-glycollate	5	A	10^{-2}	2	2560
	4			2	2560
	3			2	2560
	none			2	2560
Mercuric chloride	0.1	A	10^{-2}	3	320
	0.01			3	1280
	0.001			4	640
	0.0001			4	160
	none			8	640
p-chloro-mercuri-benzoate	1	A	10^{-2}	12	2560
	0.5			12	2560
	none			6	2560
Dithio-biuret	0.02	B	10^{-6}	4	160
	0.04			4	320
	none			4	640
Thio-flavine	0.0005	B	10^{-6}	10	40
	none			6	640
Oxine	0.5	B	10^{-6}	3	+
	0.1			3	+
	0.05			3	+
	0.01			3	+
	0.005			3	+
	none			4	+

Inhibitor added first   + = 1 tube test

Terramycin

With the announcement of the release of Terramycin, a new antibiotic, it was claimed in preliminary reports that the compound gave promise as a therapeutic agent for the treatment of influenza virus infections. Terramycin was obtained and tested in two separate experiments with influenza B. Propylene glycol (20%) was used as the diluent, it being established previously that the amount used was not toxic to chick embryos. Table 17 shows the results of two separate experiments with influenza B virus. In the first experiment RCA titers were done with the usual number of tubes, but in the second protocol only 1-tube spot agglutination tests were carried out. Gradually descending doses from 0.5 to 0.0015 mgs of Terramycin were dissolved in 20% propylene glycol and 0.1 ml injected allantoically into fertile eggs just previously inoculated with 0.1 ml of a 10^{-6} influenza B virus, which also was diluted in glycol solution. The eggs were incubated 24 hours. In the pools of allantoic fluid from the living eggs RCA titers were positive. Any suppression of virus multiplication which took place was, therefore, minimal (Table 17) and certainly not worthy of further trial at this time.

TABLE 17

The Effect of Terramycin on the Multiplication of Influenza B Virus

No. Eggs	Terramycin mg	Embryo Number	Embryo Status	Hemagglutination Titer
4	0.5	3	L	160
4	0.25	2	L	80
7	0.125	6	L	+
2	0.062	2	L	80
7	0.062	7	L	+
2	0.031	2	L	80
7	0.031	7	L	+
7	0.015	7	L	+
7	0.0015	7	L	+
6	none	6	L	+
2	none	2	L	+

Terramycin dissolved in Prop. Glycol 20%
Influenza B control diluted in 20% Prop. Glycol

FINAL EXPERIMENTS

A critical review of the data which had been obtained to this point led to the formation of the following concepts for testing inhibitors against the influenza viruses:

1. Freshly passed virus (24 hours previous to each experiment) was used since deterioration occurs on storage in the CO_2 box.
2. The use of dilute virus was desirable and from 100 to 1000 ID 50 was selected as the standard inoculum. This amount could be obtained by diluting influenza A to 10^{-7}, influenza B to 10^{-6}, and injecting 0.1 ml.
3. An LD 50 of inhibitor was chosen in order to be certain that a maximal metabolic effect was produced. To rule out zone effects, decreasing quantities of inhibitor were included in each experiment.
4. Virus and inhibitor were mixed together simultaneously to insure that both reached the same cells. Dilutions and injections were made as quickly as possible to obviate any in vitro effect.

5. An incubation of 24 hours was selected for influenza A because almost maximal infectivity titers were obtainable at that time. Longer periods of incubation might allow the embryo to detoxify the added inhibitor and would, therefore, hide any apparent or real effect. As influenza B has been shown to grow less readily than A, it was necessary to increase the incubation time to 36 hours.
6. A minimum pool of at least 8 chorioallantoic fluids was desirable for final testing since a smaller amount might give variable results which would lead to erroneous interpretation.
7. For the infectivity titers the dilutions used were dependent upon the height of the RCA. Usually a screening type of test with 0.1 ml of a 10^{-6} B or 10^{-7} A virus was sufficient to prove that virus had regenerated to maximum. Minor differences in end titer were avoided since major response was being searched for (an all or none phenomenon) and there were too many variables in titrations to assay significance of 10-1000 fold differences.

8. Once a significant result was obtained then detailed types of experiments would have to be performed to elicit the mechanism of action.

In order to test the validity of the above concepts tannic acid was utilized. This agent had been reported by Green [153] as showing both in vitro and in vivo inhibition of influenza A virus. The tannic acid was neutralized to a pH of 7.2 and sterilized by autoclaving. 0.1 ml of solution, containing 1 mg of sodium tannate was mixed with dilute influenza A virus and injected allantoically immediately thereafter. In addition, 12 eggs were given tannate first, followed by virus, and 12 embryos were given virus first and then tannate. The same procedure was followed for influenza B. Six eggs were included for each control.

Table 18 shows that irrespective of the order in which the inhibitor and virus were inoculated into the embryos, hemagglutination was absent in the allantoic pools containing the tannic acid. This observation was confirmed in experiment 19. Inhibition of influenza A and B viruses by tannic acid was complete as measured by both hemagglutination and infectivity titers (see Table 19). In fact, no virus at all could be found by

TABLE 18

The Effect on the Multiplication of Influenza A and B Viruses by the Injection of Tannic Acid in Living Embryos

No. Eggs	Na Tannate mg	Virus and Order Given	Embryo No.	Embryo Status	HCA Titer
12	1	A and T mixed	8	L	0
12	1	A, first	1	L	0
12	1	T, first	9	L	0
6	none	A alone	5	L	2560
12	1	B and T mixed	6	L	0
12	1	B, first	11	L	0
12	1	T, first	11	L	0
6	none	B alone	6	L	320

A = Influenza A virus
B = Influenza B virus
T = Sodium Tannate
24 hour incubation

TABLE 19

The Effect of Tannic Acid on the Multiplication of Influenza A and B Viruses as Measured by Hemagglutination and Infectivity Titers

No. Eggs	Na Tannate mg	Virus	HCA Titer	Infectivity 1 x 10⁻
8	1	A	0	0
8	none	A	5120	7
8	1	B	0	0
8	none	B	160	6

Virus and inhibitor mixed before injecting
Total dose 0.2 ml. Inc. time 48 hours

sub-inoculation of undiluted allantoic fluid despite an extension of the incubation period to 48 hours. These data served to confirm Green's findings regarding tannic acid and also established the validity of the experimental technic and means of measuring inhibition of viral multiplication. In all subsequent studies a similar (final type) technic was the one utilized.

With the above concepts in mind, experiments with influenza A and B viruses were designed with physostigmine, aminopterin, sodium azide, nitrogen mustard, sodium citrate, fluoroacetate, 3-acetylpyridine and Mapharsen as inhibitors. Equal parts of 10^{-7} influenza A or 10^{-6} B were mixed with inhibitor and injected allantoically, in 0.2 ml amounts, as quickly as possible to minimize in vitro action. Influenza A was incubated 24 hours and influenza B 36 hours. Only pooled allantoic fluids from the living embryos were used for RCA titers and infectivity tests were performed the same day, when possible. Previous to this time chicken cells had been used for RCA titers; however, in this series human type O cells were used (see methods).

Table 20 presents the data in which physostigmine was tested against both viruses. In Table 21 the effect

TABLE 20

The Effect of Physostigmine, in Varying Amounts, on the Multiplication of Influenza A and B Viruses

No. Eggs	Virus	Physostigmine mg	Embryos Tested	HCA Titer	Infectivity Titer
24	A	0.5	22	640	10^{-7}
12		0.25	8	320	10^{-7}
12		0.0025	12	640	10^{-7}
12		none	12	320	10^{-7}
24	B	0.5	21	80	10^{-6}
12		0.25	8	80	10^{-6}
12		0.0025	11	80	10^{-6}
12		none	12	80	10^{-6}

of nitrogen mustard was determined while in Table 22 is listed the results with sodium azide. Tables 23,24, 25 and 26 present the data obtained with 3-acetyl-pyridine, sodium citrate, aminopterin, fluoroacetate respectively. It is apparent through Tables 20-26 that while HCA titers varied with either or both viruses, maximum multiplication occurred as measured by infectivity titers. It was concluded that the formation of influenza A and B viruses was in no way influenced by the presence of varying concentrations of the above enzyme inhibitors.

The experimental results with Mapharsen, however, proved entirely different in that inhibition of viral multiplication did occur. Three doses were used, 0.2, 0.1 and 0.001 mgs respectively for both influenza A and B viruses. As can be seen in Table 27, hemagglutination was absent in embryos given 0.2 and 0.1 mg doses of the drug. Further, when these pooled allantoic fluids were tested for infectivity following dilution to 10^{-7} or 10^{-6} virus was not demonstrable. The 0.001 mg dose of Mapharsen, on the other hand, had no such effect. A similar result was obtained in a duplicate experiment not recorded herein.

TABLE 21

The Effect of Nitrogen Mustard on the Multiplication of Influenza A and B Viruses

No. Eggs	Virus	Nitrogen Mustard mg	Embryos Tested	RCA Titer	Infectivity Titer
24	A	0.2	14	5120	10^{-7}
12		0.1	12	5120	10^{-7}
12		0.001	12	5120	10^{-7}
12		none	12	5120	10^{-7}
24	B	0.2	19	320	10^{-6}
12		0.1	11	160	10^{-6}
12		0.001	11	160	10^{-6}
12		none	11	160	10^{-6}

TABLE 22

The Effect of Sodium Azide on the Multiplication of Influenza A and B Viruses

No. Eggs	Influenza Virus	Sodium Azide mg	Embryos Tested	RCA Titer	Infectivity Titer
24	A	0.26	19	2560	10^{-7}
12		0.13	12	1280	10^{-7}
12		0.0013	12	2560	10^{-7}
12		none	12	2560	10^{-7}
24	B	0.26	18	80	10^{-6}
12		0.13	8	160	10^{-6}
12		0.0013	10	80	10^{-6}
12		none	7	40	10^{-6}

TABLE 23

The Effect of 3-Acetylpyridine on the Multiplication of Influenza A and B Viruses

No. Eggs	Influenza Virus	3-acetylpyridine micrograms	Embryos Tested	HCA Titer	Infectivity Titer
24	A	600	24	640	10^{-7}
12		300	12	640	10^{-7}
12		3	12	640	10^{-7}
12		none	12	640	10^{-7}
24	B	600	24	640	10^{-6}
12		300	12	2560	10^{-6}
12		3	12	2560	10^{-6}
12		none	12	2560	10^{-6}

TABLE 24

The Effect of Sodium Citrate on the Multiplication of Influenza A and B Viruses

No. Eggs	Influenza Virus	Sodium Citrate mg	Embryos Tested	RCA Titer	Infectivity Titer
24	A	16.6	24	80	10^{-7}
12		8.3	12	80	10^{-7}
12		0.083	12	80	10^{-7}
12		none	12	40	10^{-7}
24	B	16.6	24	640	10^{-6}
12		8.3	9	640	10^{-6}
12		0.083	12	640	10^{-6}
12		none	7	640	10^{-6}

TABLE 25

The Effect of Aminopterin on the Multiplication of Influenza A and B Viruses

No. Eggs	Influenza Virus	Aminopterin mg	Embryos Tested	HCA Titer	Infectivity Titer
24	A	0.25	16	640	10^{-7}
12		0.125	12	320	10^{-7}
12		0.00125	12	640	10^{-7}
12		none	12	320	10^{-7}
24	B	0.25	20	40	10^{-6}
12		0.125	12	80	10^{-6}
12		0.00125	12	80	10^{-6}
12		none	12	160	10^{-6}

TABLE 26

The Effect of Fluoroacetate on the Multiplication

of Influenza A and B Viruses

No. Eggs	Influenza Virus	Fluoroacetate mg	Embryos Tested	HCA Titer	Infectivity Titer
24	A	5	17	640	10^{-7}
12		2.5	10	640	10^{-7}
12		0.025	12	1280	10^{-7}
12		none	12	1280	10^{-7}
24	B	5	16	1280	10^{-6}
12		2.5	10	2560	10^{-6}
12		0.025	12	2560	10^{-6}
12		none	12	2560	10^{-6}

TABLE 27

The Effect of Mapharsen on the Multiplication

of Influenza A and B Viruses

No. Eggs	Influenza Virus	Mapharsen mg	Embryos Tested	HCA Titer	Infectivity Titer
24	A	0.2	16	0	10^{-7} 0
12		0.1	10	0	10^{-7} 0
12		0.001	12	2560	10^{-7} +
12		none	12	2560	10^{-7} +
24	B	0.2	16	0	10^{-6} 0
12		0.1	11	0	10^{-6} 0
12		0.001	11	640	10^{-6} +
12		none	12	640	10^{-6} +

Having established in a preliminary manner that Mapharsen would inhibit the influenza viruses, it was thought of interest to determine the smallest amount of the compound which would give consistent results. Table 28 shows results in which varying doses of Mapharsen, from 0.05 to 0.015 mgs, were used. Influenza B virus RCA was completely inhibited in all of the above doses, as was influenza A with the exception of the 0.02 mg dose in which virus was present

In an attempt to narrow the dosage still more, 6 eggs each were injected with amounts of Mapharsen varying from 0.05 to 0.0025 mgs for both viruses. RCA titers were again used as a rough index of the presence of virus. Table 29 presents the results of the above experiment. Influenza B virus RCA titer was effectively inhibited down to and including the 0.015 mg dosage. However influenza A was inhibited only down to and including the 0.02 mg dosage. For a time it was thought that influenza B was, therefore, slightly more susceptible to Mapharsen than A virus. Subsequent experimentation showed that this was merely a variation which could be expected when working in narrowed ranges of dosage.

TABLE 28

The Effect of Varying Amounts of Mapharsen on the Multiplication of Influenza A and B Viruses

No. Eggs	Mapharsen mg	Influenza Virus	Hemagglutination Titer
6	0.05	A	0
6	0.025		0
6	0.02		+
6	0.015		0
6	none		+
6	0.05	B	0
6	0.025		0
6	0.02		0
6	0.015		0
6	none		+

TABLE 29

The Effect of Mapharsen on the Multiplication of Influenza A and B Viruses

No. Eggs	Mapharsen mg	Influenza Virus	Hemagglutination Titer
6	0.05	A	-
6	0.025		-
6	0.02		-
6	0.015		+
6	0.01		+
6	0.005		+
6	0.0025		+
6	0.05	B	-
6	0.025		-
6	0.02		-
6	0.015		-
6	0.01		+
6	0.005		+
6	0.0025		+
6	none	A	+
6	none	B	+

Presented in Table 30 is a similar type experiment to the above to which, in addition, infectivity tests were done. Dosages of Mapharsen as low as 0.25 effectively suppressed hemagglutination with both influenza A and B virus. Inhibition of influenza B was confirmed by infectivity tests, but influenza A, despite absence of agglutination, in effective dose was still infective up to 0.75 mg doses of Mapharsen, at which point infectivity correlated with the absence of red cell agglutination.

As shown in the above experiments, the viruses were always used in 10^{-6} or 10^{-7} dilutions. Now a constant amount of Mapharsen (0.03 mg) was mixed with varying dilutions of influenza B virus and injected allantoically; only undiluted pooled allantoic fluids were used in infectivity tests. Table 31 shows that RCA titers were inhibited down to and including 10^{-4} dilutions, as was infectivity. A similar experiment, not included, was done with influenza A with the same results.

In all previous experiments the virus had been mixed with the Mapharsen and then injected. In the following studies attempts were made to demonstrate an in vivo effect by injecting virus first and Mapharsen later.

TABLE 30

The Effect of Varying Amounts of Mapharsen on Influenza A and B Virus Multiplication as Measured by Hemagglutination and Infectivity Tests

No. Eggs	Influenza Virus 0.1 ml	Mapharsen mg	Embryos Tested	HCA Titer	Infectivity Titer
24	A	0.2	17	0	10^{-0} -
12		0.1	11	0	10^{-0} -
8		0.075	7	0	10^{-0} -
8		0.050	6	0	10^{-0} +
8		0.025	7	0	10^{-0} +
8		0.001	6	10240	10^{-7} +
8		none	6	10240	10^{-7} +
24	B	0.2	18	0	10^{-0} -
12		0.1	12	0	10^{-0} -
8		0.075	8	0	10^{-0} -
8		0.050	8	0	10^{-0} -
8		0.025	8	0	10^{-0} -
8		0.001	8	320	10^{-6} +
8		none	7	640	10^{-6} +

TABLE 31

The Effect of Constant Amounts of Mapharsen on Varying Dilutions of Influenza A Virus

No. Eggs	Mapharsen mg	Inoculum 1 x 10-	Hemagglutination Titer	Infectivity Titer
6	0.03	7	0	0
6	0.03	6	0	0
6	0.03	5	0	0
6	0.03	4	0	0
6	0.03	3	2560	+
6	0.03	2	2560	nt
6	none	7	2560	nt

nt = not tested

The data in Table 33 show that introduction of the virus prior to the Mapharsen results in a complete loss of the inhibitory effect of the drug, thus indicating that the inactivation was purely an in vitro one. This experiment was repeated using both cysteine and BAL. It is apparent from the data that no in vivo activity could be shown and in addition that SH groups were involved in vitro by the ability of cysteine to reverse such inhibition. When slightly greater amounts of Mapharsen (0.05 mgm) were used, no in vivo inhibition could be demonstrated either as shown in Tables 33 and 34. Furthermore, since Mapharsen supposedly inactivates SH groups the attempt was made to reactivate the virus by adding cysteine. This was accomplished as shown in Table 33 in which a positive spot hemagglutination test was obtained.

At this time the results were irregular and unreliable probably due to differentiation of the Mapharsen which had been used without a stabilizer. Time did not permit further evaluation of these findings but their significance will be reserved for the discussion.

Table 35 gives data on the attempt to inhibit in vivo the multiplication of influenza viruses by the use of another arsenical, sodium arsenamide. If arsenamide was given before or after virus no difference could be

TABLE 32

Reversal of Mapharsen Effect on Influenza A and B Viruses by Cysteine

No. Eggs	Mapharsen mg	Virus and Order Given	Hemagglutination Titer
6	0.03	A first	+
6	0.03	A mixed*	+
6	0.03	A mixed	0
6	none	A	+
6	0.03	B first	+
6	0.03	B mixed*	+
6	0.03	B mixed	0
6	none	B	+

* = cysteine added

TABLE 33

The In Vivo Effect of Mapharsen on Influenza A and B Viruses

No. Eggs	Mapharsen 0.05 mg	Influenza Virus	Hemagglutination Titer
12	Before virus	A	5120
12	After virus	A	5120
6	Mixed	A	0
6	none	A	5120
6	Before virus	B	160
6	After virus	B	640
6	Mixed	B	0
6	none	B	640

TABLE 34

The In Vivo Effect of Mapharsen on Influenza A Virus Multiplication

No. Eggs	Mapharsen mg	Time After Virus	Hemagglutination Titer
6	0.05	1 hour	+
6	0.05	2 hours	+
6	0.05	24 hours	+
6	0.05	mixed	-
7	none	———	+

TABLE 35

The In Vivo Effect of Arsenamide on the Multiplication of Influenza A and B Viruses

No. Eggs	Arsenamide 0.1 mg	Influenza Virus	Hemagglutination Titer
12	Given first	A	5120
12	Given after	A	5120
6	Control	A	5120
6	Given first	B	320
6	Given after	B	320
6	Control	B	320

noted and titers of red cell agglutination were as high as the controls in both A and B viruses.

Discussion

The hypothesis which stimulated this study is based upon the prevalent notion that viruses are synthesized within the cytoplasm by a process which involves the utilization of extra and intracellular enzymes. Therefore, interference with an essential reaction would be reflected by a corresponding decrease in virus content of the chorio-allantoic fluid. This hypothesis has been subjected to experimental tests by means of the methods previously outlined.

Chick embryo techniques have been selected for in vivo studies because of the following factors:

1. The chick embryo presents an integrated physiologic unit which under certain conditions may detoxify harmful substances, neutralize excessively acid or alkaline compounds, or bypass certain substances.

2. The ease of manipulation, ready access to material and relative immunity to bacterial and viral contamination make the embryo particularly desirable as a test system.

The ability of the intact egg to detoxify certain compounds does not negate the use of this method for there is a physiological limit to adaptability and since compounds are introduced in excess in a closed system they must remain there for the duration of the experiment. This does not infer that the above method

is ideal or that a chemical which may be active in the chick embryo will remain so in other animals, as, for instance, in mice. But if a certain chain of enzyme reactions is found responsible for the multiplication of viruses in the chick embryo, the data may be logically extended to studies using other hosts.

The use of tissue culture, as a laboratory tool, has been deliberately avoided since it represents an artificial system which is not necessarily physiological. Furthermore, slight changes in pH or temperature and bacterial contamination might produce non-specific inhibition of viral multiplication. However, in future experimentation, the method could be employed as a supplementary one for focussing attention on certain enzyme systems.

It is apparent that diffusion of compounds through cell membranes represents one of the major assumptions of the experimental procedure which was adopted. By its very nature this problem remains somewhat unresolvable. One must assume that, under certain conditions, inhibitors are able to diffuse through living membranes and further that they will diffuse equally with the virus to susceptible cells. Certainly the ability of any compound

to produce death in the embryo attests to its effect on vital cells. Whether this involves major areas of virus multiplication cannot be elucidated at the moment, but would be subject to experimental approach.

It is also assumed that the mode of action of a particular inhibitor which has been elucidated by experiments with bacteria, muscle-strips, liver, etc. may, by analogy, be transferred to intact chick embryo as well. Such an assumption is logical since single-celled organisms have much in common with multi-cellular ones and size is not an index of non-susceptibility to known metabolic inhibitors. The apparent ability of some cells to bypass certain metabolic requirements, under stress, is likewise appreciated.

The ideal control of inhibitor action, finally, should be purely chemical in nature. If membranes subjected to drastic treatment were analyzed by Warburg techniques, the percentage of inhibition of any enzyme could be determined. But even such highly refined methods are not without inherent errors. A biological approach at this stage is desirable but will require eventual confirmation by means of Warburg techniques.

The data obtained thus far will be interpreted very broadly in an effort to portray their general significance to the problem of viral multiplication and also to stimulate interest in a purely chemical approach.

Potassium cyanide has long been used as an enzyme inhibitor since Warburg's publications in 1923. His work enforced the belief that cellular respiration was due entirely to the reaction between molecular oxygen and a complex intracellular compound, and he concluded that all these respiratory enzymes were cyanide sensitive [240,241]. Experiments by other workers [242] have shown that inhibition by cyanide is not complete and that roughly two thirds of the respiratory activity is managed by cyanide sensitive enzymes, the remaining ones not being effected. The flavoproteins, which contain riboflavin as an essential component, are the cyanide resistant enzymes of the respiratory system. Keilin[243] has shown that the intracellular iron porphyrin proteins known as cytochrome a, b, and c are characterized by their ability to undergo reversible oxidation and reduction. It was further demonstrated that a fourth component existed, called cytochrome oxidase[244] which catalyzes the oxidation

of the cytochromes by molecular oxygen. Grieff [23] comments that the latter enzyme is probably identical with Warburg's cyanide sensitive system. There is a rough parallelism between the respiratory activity of aerobic cells and the concentration of cytochrome and cytochrome oxidase. Cyanide inhibits the action of cytochrome oxidase, but there is good evidence that the cytochromes themselves are cyanide resistant [245].

Among others Grieff and Pinkerton [20] have observed that cyanide favors the growth of rickettsiae because it decreases the respiratory rate of the embryo. Unlike viruses, the rickettsiae reproduce best in slowly growing tissues. Maitland and Lang [246] working with vaccinia found that incubation of minced tissue at 37 C for 5 days destroyed its ability to support growth of vaccinia virus. They could show no correlation between the amount of tissue respiration and this labile factor even though potassium cyanide in M/100 concentrations abolished the growth-initiating property of the unincubated media. The authors did not establish definitely, but suggested that the cyanide sensitive system and the labile factor might be identical. If this result is substantiated, it would indicate that cytochrome oxidase is an essential enzyme

for the multiplication of vaccinia virus. The failure of KCN, on the other hand, to inhibit influenza virus multiplication points to two alternatives: (1) cyanide is either not able to penetrate all cells in inhibiting amounts or (2) the needed energy is supplied by cyanide insensitive systems.

Concerning the first alternative, there is a direct relationship between pK and diffusibility of membranes and, in general, substances having the properties of weak acids penetrate only in the undissociated form [247]. Cyanide has a pK of 9.14 and is 93% undissociated. A recent publication by Ronkin [248] has shown that phosphate uptake is markedly inhibited in the excised mussel gill when treated with cyanide. Thus, there is presented evidence that cyanide does effectively penetrate cells and inhibit phosphate uptake. If, by analogy, the chorioallantoic cells are affected similarly then cytochrome oxidase cannot be considered as essential for influenza virus multiplication.

The overall production picture of energy in the Kreb's cycle is shown by the formula: Coenzyme 1 - flavoprotein-cytochrome c - cytochrome oxidase - O_2 = H_2O + high energy phosphate. As the experiments in this study show, even

embryos dying of cyanide intoxication contained high concentrations of virus which is further evidence that cytochrome oxidase is not a key enzyme. Whether or not the flavoproteins can furnish enough energy for influenza virus multiplication has not, as yet, been determined.

Barron et al [249] in an extensive study of the effect of nitrogen mustard (methyl bis B-chloroethyl amine hydrochloride) on tissues and enzymes have noted the striking resemblance between one of the nitrogen mustards and choline. Nitrogen mustard was shown to inhibit choline activity and thiosulfate was able to reverse such inhibition. Tryptophane, tyrosine, cysteine and histidine had no preventive effect. Acetylcholine esterase is also inhibited by nitrogen mustard. It has also been stated that in alkaline solution the mustards react strongly with SH groups but Cori does not support this contention. A partial inhibition in the metabolism of inorganic pyrophosphate, phosphocreatine and the enzymes phosphokinase and hexokinase was also demonstrated by Cori. In tissue metabolism studies [249] pyruvate oxidation has been shown to be inhibited by nitrogen mustard. Two SH enzymes were also affected, hexokinase and phosphokinase. These enzymes are very susceptible to nitrogen mustard but in vivo confirmation of this effect has not been obtained.

Finally, amounts of inhibitor too small to inactivate any systems have been found to produce fundamental changes in mitotic activity (250). The mustard gases combine readily with all substances rich in nucleic acid (251). Among these are included the nucleoproteins, the pneumococcus transforming principle (relatively pure DNA), and a number of viruses including bacteriophages. The following viruses are susceptible to in vitro inactivation by nitrogen mustards: EEE, rabies, hog cholera, Newcastle, feline pneumonitis, rabbit papilloma, tobacco mosaic and several phage species.

Despite the fact that nitrogen mustard was quite toxic for the developing chick embryo, it had no suppressive effect on the multiplication of influenza A and B viruses as measured in both living and dead embryos. It is, therefore, believed that choline, acetylcholine esterase and pyruvate are not essential in the metabolism of influenza virus under the conditions of these experiments.

The finding that PABA is an essential constituent of folic acid suggested the possibility of using 4-amino pteroyl glutamic acid as a viral inhibitor. While folic acid seems to be concerned with the synthesis of certain pyrimidines and related compounds its status is incompletely

wprked out. Karnofsky et al [252] have noted the use of analogues of folic acid as producing a stunting in chick embryos together with a decrease in the vascular network of the chorioallantoic membrane. They tend "to favor the more general interpretation that the 4-amino folic acids interfere with the growth of tissues -- those growing the most rapidly being the most effected". The thesis that it interferes with the growth of certain tissues, in a highly specific manner, remains to be proved.

Dinning and coworkers have shown [254] that the livers of monkeys given aminopterin were devoid of choline oxidase activity while catalase activity was normal. Williams and Elvehjem [253] have inhibited guanine metabolism in rat livers with folic acid and the oxidation of xanthine oxidase is prevented by folic acid, but if folic acid and xanthine oxidase are together some oxidation of the folic acid appears to occur.

Studies on the metabolism of folic acid have shown that rat liver slices form from PGA a substance utilized for growth by Leuconostoc citrovorum, often referred to as the citrovorum factor (CF). Further, it was determined that reducing substances, such as ascorbic acid, stimulated the formation of CF, both from synthetic PGA and from compounds present in normal rat liver. Aminopterin is one of the most potent analogues of PGA known

but the inhibition is not competitive and the further addition of PGA reverses such inhibition inefficiently. In contradistinction, the transformation of PGA to CF not only prevents the toxicity of aminopterin but is itself a biologically active derivative of PGA. Presumably aminopterin competes with CF for combination with an apoenzyme or enzyme with which CF normally reacts. It has also been demonstrated that aminopterin competes directly with CF [255]. If aminopterin treated chick embryos are injected with thymine, hypoxanthine, folic acid, or vitamin B 12 the antagonism of aminopterin is not relieved. However, thymidine partially counteracts aminopterin antagonism but a combination with hypoxanthine-desoxyriboside is more effective [256]. The above data again indicate that the anti-folic acid activity of aminopterin is not competitive but rather represents non-dissociated combination with an enzyme or apoenzyme. It is clear from the above that the role of PGA in metabolism is poorly understood except for the fact that it may be considered an essential growth factor. Apparently PGA, or some component thereof, may not be essential for the multiplication of influenza viruses since its antagonist aminopterin failed to inhibit viral growth in chick embryos.

Sodium fluoroacetate is a highly specific protoplasmic poison, its extreme toxicity being further attested to by the fact that between 5 - 7.5 mgs per kilogram of body weight represents an LD_{50} for the M. rhesus monkey. Chick embryos were relatively more resistant, requiring 5 mgs to produce an approximate LD_{50}.

Bartlett and Barrow [257], in 1947, had shown interference in vitro and in vivo with the oxidation of acetic and pyruvic acids. Buffa and Peters [258], in later studies, have observed that fluoroacetate's particular action is centered specifically around the utilization of citrate in the citric acid cycle. Citric acid accumulates in the tissues of fluoroacetate treated animals. They concluded that either aconitase or isocitric dehydrogenase was inhibited. Further, they were able to show that the toxicity of fluoroacetate was not due to a binding of calcium ions by the accumulated citric acid.

One molecule of the energy producing compound adenosinetriphosphate (ATP) is formed in the degradation of citric to α-ketoglutaric acid. In turn -ketoglutaric acid when decarboxylated to form succinic goes through a smaller cycle ending as oxalacetic acid, in which three molecules of ATP are formed. Theoretically,

certain amino acids may be transformed into glutamic acid which in turn is changed to α-ketoglutaric acid by a process of transamination. It is apparent then that in theory those amino acids such as arginine, proline, etc. are able to bypass citric acid and yield less ATP than had they gone through the complete cycle.

When fluoroacetate is used aconitase is blocked, which allows citrate to accumulate because of failure of successive steps in metabolism to function and which normally is changed to α-ketoglutaric acid. Were this the only means of energy being formed the organism would immediately die, but, as shown above, amino acids may take up this "slack" by bypassing the block with a corresponding reduction in the ATP formed.

Fluoroacetate was completely ineffective in inhibiting influenza virus multiplication in either living or embryos dying 18 hours after injection. A priori, this allows the interpretation that aconitase does not function in influenza virus multiplication. The previously reported facts that cyanide and azide, which are able to inhibit the whole Kreb's cycle, fail to completely inhibit influenza virus multiplication intimates that the amount of energy needed

for viral multiplication is, indeed, small. The results with fluoroacetate further support this thesis in a less dramatic manner, since only limited interference in the energy cycle occurs.

Like cyanide, sodium azide has been shown to inhibit oxidation of cytochrome oxidase [259]. Azide is also a powerful inhibitor of aerobic and anaerobic metabolism and has been shown to prevent enzyme synthesis [260]. It is able to prevent the accumulation and formation of organic phosphate bonds in carbohydrate metabolism. Finally, the incorporation of C14 labeled glycine into intestinal tissue protein is prevented by azide [261] which would presuppose, among other mechanisms, interference with diffusability of amino acids or inactivation of enzymes necessary in peptide synthesis.

It is conceivable, then, that azide exerts its effect either at the cell surface or by diffusing through the membrane and disrupting enzyme synthesis, or by destroying cytochrome oxidase together with certain anaerobic pathways of metabolism. With regard to diffusion Ronkin [248] has shown that azide exists in a highly dissociated form and, unlike cyanide, penetrates the membranes of mussels very poorly.

In the experiments presented, sodium azide was totally ineffective in preventing the multiplication of influenza A and B viruses despite the fact that the compound exerts a specific action on cytochrome oxidase, as well as other metabolic processes of vital significance to the cell. The data support the cyanide experiments in excluding cytochrome oxidase as essential for the multiplication of influenza viruses. The fact that sodium azide is only poorly diffusable through the cell wall may be offered as evidence that the experimental data were completely invalid . Such an objection could be countered by the demonstration that virus was capable of maximal multiplication even in embryos dying soon after receiving the drug. Since death was attributable to the sodium azide, it must be assumed that the compound did penetrate cells in toxic quantities.

If the structure of nicotinic acid is changed by replacing the carboxyl group with a methyl ketone, an analogue of nicotinic acid is formed, 3-acetylpyridine. This compound is highly effective in producing nicotinic acid deficiencies in mice and dogs. Although its antinicotinic action is demonstrable against bacteria, certain strains are completely resistant[262]. The fact

that 3-acetylpyridine may be recovered from non-susceptible treated microorganisms rules out any theory of destruction of the compound in vivo. There seems little doubt that the antimetabolite penetrates the cells. It is possible that "animals have functions for nicotinic acid which microorganisms lack and with which 3-acetylpyridine interferes or that specific proteins with which nicotinic acid reacts in animals differ sufficiently from similarly reactive proteins in bacteria, so that the analogue will no longer combine with them " [262].

The current idea for some time, that vitamins were only accessory substances, is no longer tenable with the demonstration that certain enzymes form with these vitamins essential compounds necessary for metabolism. Nicotinic acid is one of these vitamins. It has been shown to be an essential part of coenzymes I and II. It has also been demonstrated that the injection of 600 micrograms of 3-acetylpyridine into the yolk sac of 4 day old embryonated eggs is lethal in 24 hours[263]. The same dosage was used in the influenza experiments, but no deaths were produced in the embryos. It is reasonable to suppose that, even in these older embryos, deaths could be produced upon prolonged incubation.

There was no demonstrable effect on influenza virus multiplication with either the A or B strains. Though indirectly related, any effect the analogue had would be referable to the energy cycle again through the mediation of coenzymes I and II. This is considered as confirmation of previous observations that the energy cycle plays no direct role in influenza virus multiplication.

The following miscellaneous inhibitors were employed for the reasons stated below:

The fact that chloramphenicol has been shown to inhibit liver esterase [62] and to possess experimental and clinical effectiveness against some of the larger viruses led to the use of physostigmine which is one of many of the esterase inhibitors [264]. Since chloramphenicol is devoid of any activity against influenza viruses, it was not expected to find that physostigmine was any more effective. It is also hoped that this phase of the problem will be investigated with other viruses.

Among others Cutting et al [122] have used excessive amounts of metabolite in an attempt to inhibit chain reactions in the metabolic stream. Sodium citrate, also in excessive amounts, was used for this purpose in

the hope of demonstrating some inhibitory action against influenza. The compound was also included because it is said to combine with calcium and magnesium ions. No deaths were recorded in the treated embryos although slightly larger doses did produce 100% mortality. Nevertheless, doses of citrate as high as 16 mgs resulted in no visible inhibition of the influenza viruses.

In preliminary experiments mercuric chloride and p-mercuribenzoate, both well known SH inhibitors, were shown to possess no in vitro action against influenza viruses and reference to the significance of this will be reserved for later discussion. In addition thioflavine and oxine (8-hydroxyquinoline) were shown ineffective in suppressing viral multiplication. The former compound was used because of reported successes with other flavine compounds in virus inhibition, and the latter drug was chosen because of its ability to remove certain metals from solution by a process of chelation. Thioglycollate may be used as a reducing agent and also inhibits cytochrome c, but was not toxic for the embryos in the amounts used, nor was any viral suppression noted.

Arsenoxide or Mapharsen was originally investigated by Ehrlich but pronounced too toxic for clinical use, the high therapeutic efficiency of the drug not being appreciated. Like most arsenicals its clinical efficiency is correlated with its ability to neutralize SH groups (265). Gordon and Quastel (266) have called attention to the work of other investigators who have shown: that the toxic action of arsenoxide is due to condensation with thiol groups present in cytoplasm, leading to a dysfunction of the system regulating cell respiration; that arsenic compounds are capable of combining with compounds containing thiol groups (such as thioglycollate); and, lastly, that the toxicity of organic arsenic compounds can be reduced by adding thiol compounds such as cysteine, glutathione, etc.. Quastel et al (266) have demonstrated that succinic dehydrogenase, choline dehydrogenase and pyruvic oxidase are greatly inhibited by Mapharsen. Glucose and lactate oxidation are depressed probably due to blocking of the respiratory chain at the pyruvic enzyme stage. Liver esterases were inhibited but the results were not definite while invertase, cytochrome oxidase and catalase were unaffected.

Perez and Kline [267] were able to inactivate influenza virus by incubating the virus with bichloride of mercury for ½ hour. Upon injection of this mixture in animals or chick embryos, with previously or simultaneously administered BAL (a highly efficient SH compound), the virus was reactivated.

The properties of pneumonia virus of mice have been shown to be effected by the inactivation of SH groups by Volkert and Horsfall [268]. Burney and Golub [269] using various SH inhibitors have been able to inactivate psittacosis virus (by one hour incubation with drug) and reactivate by adding glutathione to the incubated mixture and injecting it into chick embryos. However, virus was not inhibited by the same procedures in tissue cultures. If chick embryos are inoculated with psittacosis virus four hours after the addition of SH-inhibiting compounds no effect was demonstrable. The authors are critical of this method, believing that uniform distribution of inoculum is uncertain and that it is difficult to employ an effective virustatic concentration and at the same time avoid toxic effect to the host.

Using the same techniques, as discussed previously

Mapharsen was shown to inactivate both influenza A and B viruses and that this inactivation was practically instantaneous. Further study established the effective range of Mapharsen as related to the strength of the inoculum. There was an occasional variability as shown in Table 29, but generally results were duplicated at will. Further examination demonstrated that this was not an in vivo inactivation but rather an in vitro one which was reversible by treatment with adequate amounts of cysteine, pointing, then, to SH groups as being involved. The irregular results with BAL, in experiments not included, were explained by the deterioration of the Mapharsen which was supplied without buffer or preservative. Unfortunately, a fresh supply of Mapharsen was unobtainable at the time, and clarification of this discrepancy must await further study. Additional problems include more intricate experiments designed to rule out a non-specific effect due to acidity alone, and experiments allowing more intimate contact of the drug with virus for ½ hour prior to injection and incubation.

The in vivo failure of Mapharsen to inactivate influenza virus is perplexing. Uneven distribution of inoculum, as pointed out by Burney et al [269], poor

diffusion through cell membranes, the presence of easily accessible SH groups other than the virus are all factors which may explain the result. Until future study can rule out these factors one by one, it is assumed that Mapharsen is inactivated by cell protein SH groups before it has had an opportunity to combine with influenza virus.

Eaton et al (157) were able to secure good inhibition of the psittacosis-lymphogranuloma group with sodium arsenamide in chick embryos, but with mice the results were not as clear or pronounced. When arsenamide, an SH inhibitor, was used against influenza virus in chick embryos no in vivo effect could be demonstrated which correlates with the data using Mapharsen, mercuric chloride and p-mercuribenzoate. While the in vitro inhibition by Mapharsen is of interest, the data as a whole indicate that in vivo destruction of SH inhibitors does not interfere with influenza virus multiplication.

Terramycin, like other antibiotics, was shown to be without effect on influenza virus in contradistinction to earlier claims of other investigators. Up to this time no further studies have been published substantiating their earlier preliminary claims of effectiveness.

Experimental work has borne out Green's [153] findings on the efficacy of tannic acid. Unfortunately, the specific mode of action of tannic acid is unknown although Green states it is classed as a general enzyme inhibitor and protein precipitating agent. Its actual inhibiting focus is a matter for further investigation. Nevertheless, tannic acid is able not only to inhibit further influenza multiplication but is virucidal, as found in the experimental work. This is, indeed, interesting as previously discussed antibiotics have never been shown to kill viruses, but only to possess a virustatic action. Further study of tannic acid is definitely indicated.

An attempt has been made in this discussion to indicate the direction of one phase of research in the field of viral multiplication. One might inquire, then, just how do the data fit with present theories of virus formation as summarized below:

1. Viruses possess all the enzymes necessary for metabolic purposes and also for the synthesis of new protein within the host cell. This concept, which would class them, like bacteria, as autonomous parasites, is generally rejected.

2. It is believed by some that viruses contain only those enzymes necessary for reproductive purposes and that the host cell contributes the metabolic environment.

3. A few individuals consider that viruses do not possess enzymes at all but rather that they reproduce by directing the formation of new protein from enzymes already present in the host.

All of these concepts imply that in some manner, through enzymes, new virus protein is formed in a step-wise synthetic process involving the construction of larger and larger units which finally emerge as mature virus. Some of the data which have been presented may be interpreted as favoring the synthesis idea. Thus, chloramphenicol, by interfering with esterase activity, appears to inhibit the reproductive capacity of viruses, rickettsiae, and a wide variety of bacteria as well. Such a finding, if true, would imply a step-wise reproductive proces common to all three groups of parasites. Selective inhibition plays a role too, as in the case of rickettsial agents where the substitution of a sulfonamide group in place of a carboxy radicle in the same position on the benzene ring reverses the action of the drug. Data of this type suggest a complex and variable

reproductive system or else a secondary effect on the primary pathway. The results using enzyme inhibitors and metabolic antagonists are not in agreement with the concept of step-wise synthesis of virus protein from smaller units within the cell environment. It is difficult to visualize a complete immunity of the infected cell toward such drastic interference with metabolic processes as was imposed by the experimental conditions adopted. The results could be criticized on the grounds that all cells were not equally affected or that the chemicals did not penetrate into the region where the virus was being formed. Even if such reasoning were correct, at least a partial decrease in virus titer should have occurred and this was not evident from the data which were obtained. In the case of influenza viruses one is tempted to reject the unit synthesis theory as unlikely and to consider another alternative; namely, that influenza virus protein is formed by conversion from mature cell protein, a process which would involve minimal expenditure of energy and relatively few enzymes. Some evidence in favor of such a hypothesis can be obtained from the electron microscope, from studies of chemical similarities and differences between virus and cell protein, as well as from the use of experiments of the type described herein.

In these studies particular attention has been focused on inhibitors affecting the energy yielding processes of the cell such as cyanide, azide, fluoroacetate, 3-acetylpyridine and sodium citrate. All of the data suggest that the energy cycle is probably not directly concerned with influenza virus formation except as the yield is influenced by cell viability. It remains to be seen if other viruses are similarly unaffected before any definite conclusions can be drawn. In any event, an elucidation of the mechanism of viral multiplication will require a chemical approach to the problem and an intimate knowledge of the mechanism of protein synthesis.

SUMMARY

1. A group of metabolic inhibitors was selected which had been shown by other investigators to inactivate certain enzyme systems.

2. The approximate LD_{50} dose of inhibitors for chick embryos has been established.

3. Their effect on the multiplication of influenza A and B viruses was tested. The following were totally ineffective: cyanide, sodium azide, fluoroacetate, 3-acetylpyridine, aminopterin, nitrogen mustard, physostigmine, sodium citrate, mercuric chloride, thioflavine, p-mercuribenzoate, oxine, thioglycollate, arsenamide and Terramycin.

4. As demonstrated by Green, the ability of tannic acid to inactivate influenza virus was confirmed.

5. Mapharsen was shown to possess an in vitro activity against influenza A and B viruses.

6. Comments on the possible significance of these observations in relation to viral metabolism have been discussed.

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