

## The nature of the toxic reaction of influenza virus towards lung tissue

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### SUMMARY

The inoculation of large doses of unadapted influenza virus intranasally into mice results in the production of severe lung lesions. This toxic effect is a result of the entry of virus particles into the lung cells followed by uncoating of the virus ribonucleic acid.

The toxic property of the virus is destroyed by procedures which destroy or modify the nucleic acid such as exposure to monochromatic UV light of wavelength 2537 Å, or treatment with hydroxylamine or Bayer A139. Reagents acting on amino groups are particularly effective as they react with the nucleic acid and probably also interfere with penetration of virus into the cell.

Toxicity is also destroyed by mercurials which probably prevent uncoating of the nucleic acid by union with disulphide bonds, and by oxidizing agents such as iodine, permanganate, osmic acid and hydrogen peroxide under conditions which suggest possible action on some constituent of the virus containing methionine.

The toxic effect produced by the inoculation of large doses of unadapted virus intranasally in mice is associated with the occurrence of an incomplete growth cycle in which there is full production of RNP antigen but no production of haemagglutinin or infective virus.

### INTRODUCTION

Inoculation of large doses of influenza virus into animals may cause severe pathological change and even death in the absence of detectable virus multiplication, apparently as a result of the toxic properties of the virus. The effects produced depend on the route of inoculation and toxicity towards lung tissue is of special interest as it is probably one of the major causes of death in human influenza.

Intranasal inoculation of large doses of unadapted virus in mice produces severe lung lesions (Sugg, 1949; Ginsberg, 1954). This paper describes a study of this phenomenon with an attempt to determine the particular virus component responsible for the toxic effect. Egg-adapted virus was treated with chemical reagents reacting with particular virus components or chemical groupings and the resultant effects on the toxicity towards mouse lung determined.

## METHODS

*Viruses*

The DSP (1943) and the A2/Hong Kong (1968) strains of influenza virus were used in these studies. These were originally isolated from man by egg inoculation and had been propagated by egg passage. Virus was precipitated from infected allantoic fluid by 7.5% polyethylene glycol, eluted into phosphate-buffered saline pH 6.5, and purified by adsorption-elution from guinea-pig red blood cells followed by differential centrifugation, the final deposited virus being resuspended in phosphate-buffered saline at pH 6.5 to a concentration of approximately 1% by volume. This virus concentrate was suitably diluted in appropriate phosphate-buffered saline immediately before use.

*Chemical treatment*

This was similar to the methods used in previous work on the chemical reactions of influenza virus proteins (Hoyle & Hana, 1966). The virus concentrate was diluted 1/20 in phosphate-buffered saline and mixed with an equal volume of reagent in similar buffer. The conditions of reagent concentration, pH, temperature and period of exposure were chosen for maximum possible specificity.

Excess reagent was removed by adsorbing the virus to guinea-pig red blood cells, washing with cold saline and eluting into phosphate-buffered saline at pH 6.5, unless the reagent reacted very rapidly or lysed red blood cells, in which case it was chemically neutralized.

Reagents insoluble in water were added in solution in ether and mixed by rapid shaking to produce an emulsion. Provided the final concentration of ether was less than 5% no destruction of virus properties was caused by the solvent.

In all cases controls were set up in which the virus was exposed to identical procedures and conditions of temperature and pH, but in the absence of reagent or in the presence of previously neutralized reagent.

The choice of suitable chemical reagents was limited to those active between pH 5 and 10, and at a temperature of 37° or less, owing to the instability of the virus outside these limits.

*Measurements of virus properties**Haemagglutinin titre*

HA titre was measured by titration by the Salk method against a 0.5% (v/v) suspension of guinea-pig red blood cells in saline.

*Neuraminidase activity*

Neuraminidase activity was determined by measuring the amount of *N*-acetyl neuraminic acid released from an ovomucin substrate under standard conditions by the Aminoff (1961) method and was expressed as the optical density reading at 549 nm.

*Complement-fixing antigen*

Complement-fixing antigen was measured by the long fixation method, using appropriate human convalescent sera containing antibody predominantly to either the strain specific antigen or to the group antigen.

*Toxicity*

An intranasal dose of 0.05 ml. of each preparation was given to each of four mice. The mice were killed with chloroform on the third day and their lungs examined for areas of consolidation visible to the naked eye, and lesions recorded as follows.

- (i) Mice dying on 2nd or 3rd day with severe lung lesions recorded as D<sub>2</sub>, D<sub>3</sub>,
- (ii) Severe lung lesions affecting all lobes recorded as + +.
- (iii) Moderately severe lung lesions recorded as +.
- (iv) Small lesions recorded as +/2.
- (v) No lesions recorded as 0.

In control experiments with untreated virus it was found that the inoculation of virus preparations with a haemagglutinin titre of 100 or less produced no lesions. With titres ranging from 200 to 2000, lesions of increasing severity were produced, but with inoculum titres from 4000–32,000 a maximal response was attained, the lesions produced by inocula of titre 32,000 being no more severe than with 4000. With these doses variations in the result with individual mice appeared to reflect differences in the degree of inhalation of the inoculum. In most experiments the virus concentrate was diluted to give an inoculum titre of 10,000 but because of differences in purification of the virus samples, and especially in the efficiency of the red cell adsorption-elution used in clearing the treated virus from chemical reagent, titres of actual inocula ranged from 4000 to 32,000.

*Infectivity tests*

These were carried out by a modification of the 6 hr. soluble antigen production test of Finter & Beale (1956) which although less sensitive than the orthodox infectivity test is more suitable for preparations containing large amounts of virus.

Two eggs were inoculated to the allantoic sac with 0.1 ml. of the virus preparation and after 6 hr. incubation the chorioallantoic membranes were removed, suspended in 1 ml. of phosphate-buffered saline of pH 6.5 containing 1/1000 sodium azide, frozen and thawed three times, centrifuged, and the content of S antigen in the supernatant fluid measured by complement fixation with a human convalescent serum containing antibody to the group antigen.

## RESULTS

First experiments were directed to determine if any component of the virus particle was directly toxic or if the toxic effect depended on union of virus with cells. Virus preparations were disintegrated into their component subunits by shaking with ether or dichloromethane, or by alternate freezing in CO<sub>2</sub> acetone and

Table 1. *Effect of virus disintegration on toxicity*

Material	Haemagglutinin titre	Neuraminidase activity	S Antigen titre	Toxicity Test	
				Dilution	Result
Hong Kong virus control	32,000	0.40	4	1/1	D <sub>3</sub> , ++, ++, 0
				1/5	D <sub>2</sub> , D <sub>3</sub> , ++, ++
				1/25	++, ++, 0, 0
				1/125	0, 0, 0, 0
Hong Kong virus treated with dichloromethane (aqueous phase)	16,000	0.34	96	1/1	0, 0, 0, 0
				1/5	0, 0, 0, 0
D.S.P. virus control	32,000	0.35	6	1/1	D <sub>2</sub> , D <sub>2</sub> , +, +
Ether treated D.S.P. virus (aqueous phase)	16,000	0.30	128	1/1	0, 0, 0, 0
Hong Kong virus control	8,000	0.375	4	1/1	++, ++, ++, +
Hong Kong virus frozen and thawed 5 times	8,000	0.375	3	1/1	D <sub>3</sub> , D <sub>3</sub> , ++, +/2
Hong Kong virus frozen and thawed 50 times	16,000	0.37	14	1/1	0, 0, 0, 0

Table 2. *The effect of diazotized sulphanilic acid on haemagglutinin and toxicity of influenza viruses*

	D.S.P. Virus		Hong Kong virus	
	Control	Virus + M/400 diazo reagent	Control	Virus + M/400 diazo reagent
Haemagglutinin titre	8,000	< 32	32,000	< 32
Toxicity test result	D <sub>2</sub> , D <sub>2</sub> , ++, +	0, 0, 0, 0	++, ++, +/2, 0	+/2, 0, 0, 0

thawing in water at 20° C. These procedures resulted in release of internal RNP antigen and total loss of toxicity (Table 1), although the haemagglutinating power and neuraminidase activity were not destroyed. Chemical reagents which destroy haemagglutinin do, however, also destroy toxicity. One of the more specific of these reagents is diazotized sulphanilic acid which reacts with histidine and tyrosine, one or other of which appears to be present in the active centre of the haemagglutinin (Hoyle & Hana, 1966). Virus concentrates were diluted 1/10 with borate-buffered saline pH 9.0 and mixed with an equal volume of M/200 diazotized sulphanilic acid and held at 0° C. for 16 hr. The reagent was then neutralized by addition of 2% iminazole. Controls were treated with diazotized sulphanilic acid previously neutralized with iminazole. Toxicity of the DSP virus was completely and that of Hong Kong virus almost completely destroyed (Table 2). Destruction of haemagglutinating activity therefore destroys toxicity, probably by preventing union of virus and cells. There are, however, many chemical reagents which react

with the virus components without destroying haemagglutinin and experiments were set up to determine if any of them would inactivate the toxic property.

#### *Reagents reacting with sulphhydryl or amino groups*

Results with these reagents are shown in Table 3. Under the condition used dichromate, iodacetamide, *N*-ethyl maleimide and nitroprusside react specifically with the SH group; acrylonitrile reacts strongly with SH and feebly with the amino group; fluorodinitrobenzene and phenyl isothiocyanate react with both groups; while formaldehyde, glyoxal and  $\beta$ -propiolactone are specific for the amino group. The results show clearly that toxicity is unaffected by reaction with SH groups but is destroyed by chemical action on the amino group.

#### *Reagents acting on the disulphide bond*

Allison (1962) found that the infectivity of influenza viruses was reversibly inactivated by mercurials and attributed this effect to action on disulphide bonds preventing uncoating of the virus nucleic acid. The combination of urea and dithiothreitol produces an irreversible rupture of the bond. The effect of these reagents on the toxicity of the Hong Kong strain is shown in Table 4. (Reagents acting on the disulphide bond destroy the neuraminidase activity of DSP virus which cannot be recovered after adsorption to red cells.) Toxicity is destroyed by the mercurials but not by urea + dithiothreitol.

#### *Oxidizing agents*

Oxidizing agents are very viricidal towards influenza virus and the toxic property can be inactivated without effect on the haemagglutinin. The possible lines of attack of these chemicals include the SH group of cysteine, the  $\text{CH}_2\text{S}$  group of methionine, the unsaturated fatty acids of the lipid and the aromatic rings of the nucleic acid and amino acids.

The action of iodine, permanganate, and osmic acid at concentrations of 1/10,000 at pH 6.0 and 0° C., and of hydrogen peroxide at 1/100, pH 7.0 and room temperature, were investigated. Under these conditions, 1/10,000 iodine is decolorized instantly by a 10-fold excess of cysteine or methionine, within a few seconds by tryptophane and slowly (30–60 min.) by tyrosine and uracil. Histidine and cytosine react in 24 hr. Water soluble agents containing the C=C double bond, such as maleic acid, do not react but iodine dissolves in oils containing unsaturated fatty acids such as arachis oil, and is slowly decolorized (Hoyle, 1964).

Under similar conditions permanganate is decolorized instantly by cysteine and maleic acid and within seconds by methionine and tyrosine. Tryptophane, histidine and uracil react in 10 min. and shaking with arachis oil causes rapid decolorization.

Osmic acid reacts instantly with cysteine and more slowly (2–10 min.) with methionine. There is no apparent reaction with maleic acid but on shaking with arachis oil a brown colour develops fairly rapidly. Osmic acid does not react with the aromatic rings in 1 hr.

Results are shown in Table 5. It is possible that the different oxidizing agents do not act on the same virus component but if they are acting in the same way the

Table 3. Action of amino and sulphydryl group reagents on the toxicity of DSP and Hong Kong viruses

(Composite table embodying the results of several experiments.)

Reagent and conditions of reaction	Probable reacting group	Hong Kong virus		DSP virus	
		HA titre	Toxicity test result	HA titre	Toxicity test result
Formaldehyde 1/1000, pH 7, 1 hr. 37° C	NH <sub>2</sub>	4,000	0, 0, 0, 0	16,000	0, 0, 0, 0
Glyoxal 1/200, pH 8, 1 hr. 37° C	NH <sub>2</sub>	8,000	0, 0, 0	16,000	0, 0, 0, 0
$\beta$ -propiolactone 1/800, pH 8, 1 hr. 37° C	NH <sub>2</sub>	32,000	0, 0, 0	16,000	0, 0, 0, 0
Fluorodinitrobenzene 1/1000, pH 7, 1 hr. 37° C	NH <sub>2</sub> SH	6,000	0, 0, 0, 0	8,000	0, 0, 0
Phenyl isothiocyanate 1/1000, pH 9, 18 hr. 4° C	NH <sub>2</sub> SH	24,000	0, 0, 0, 0	130,000*	0, 0, 0, 0
Acrylonitrile 1/200, pH 7, 1 hr. 37° C	(NH <sub>2</sub> ) SH	12,000	++ , + , +	16,000	D <sub>3</sub> , D <sub>3</sub> , D <sub>3</sub> , +/2
Sodium dichromate 1/10,000, pH 6, 2 hr. 37° C	SH	4,000	D <sub>3</sub> , D <sub>3</sub> , D <sub>3</sub> , +	16,000	++ , ++ , ++ , +
Iodacetamide 1/1000, pH 6, 2 hr. 37° C	SH	8,000	D <sub>3</sub> , ++ , + , +	16,000	D <sub>2</sub> , D <sub>3</sub> , + , 0
N-ethyl maleimide 1/500, pH 6, 2 hr. 37° C	SH	32,000	D <sub>3</sub> , D <sub>3</sub> , ++ , +	24,000	D <sub>3</sub> , ++ , ++ , ++
Sodium nitroprusside 1/2000, pH 7, 1 hr. 37° C	SH	24,000	D <sub>3</sub> , D <sub>3</sub> , ++	8,000	D <sub>3</sub> , ++ , ++ , +
Controls	—	4,000	D <sub>3</sub> , D <sub>3</sub> , D <sub>3</sub> , +	8,000	D <sub>3</sub> , D <sub>3</sub> , + , +/2
Conditions as in tests but without reagent		8,000	D <sub>3</sub> , D <sub>3</sub> , ++ , +	16,000	D <sub>2</sub> , D <sub>3</sub> , D <sub>3</sub> , +
		16,000	D <sub>2</sub> , D <sub>2</sub> , ++ , +	32,000	D <sub>2</sub> , D <sub>2</sub> , D <sub>3</sub> , ++
		32,000	D <sub>3</sub> , ++ , + , +	130,000*	D <sub>3</sub> , D <sub>3</sub> , D <sub>3</sub> , ++

\* High HA titres due to partial disruption of virus at pH 9.

Table 4. *Effect of reagents acting on the disulphide bond on toxicity of Hong Kong virus*

Reagent		Haemagglutinin titre	Toxicity test result
Mercuric chloride	Control	32,000	D <sub>3</sub> , +, +, +, +
	Virus + 1/5,000 HgCl <sub>2</sub> 1 hr. 37° C pH 6	32,000	0, 0, 0, 0
Parachlormercuribenzoate	Control	50,000*	D <sub>3</sub> , D <sub>3</sub> , D <sub>3</sub> , + +
	Virus + 1/1000 PCMB 1 hr. 37° C pH 8	130,000*	0, 0, 0, 0
Urea + dithiothreitol	Control	32,000	D <sub>2</sub> , D <sub>2</sub> , D <sub>3</sub> , D <sub>3</sub>
	Virus + 4M urea + 1/600 DTT 30 min. 20° C. pH 8	50,000	D <sub>3</sub> , D <sub>3</sub> , D <sub>3</sub> , + +

\* High HA titres due to partial disruption of virus at alkaline pH.

results suggest action on methionine. Action on SH groups can be excluded because of the results described above and only methionine and tryptophane are attacked by iodine sufficiently rapidly to account for the destruction of toxicity in 10 sec., while the rapid action of osmic acid would appear to exclude action on tryptophane. Hydrogen peroxide destroyed toxicity under conditions in which it was shown to oxidize methionine but other possible actions of peroxide were not studied. The CH<sub>3</sub>S group also reacts with alkyl halides to produce sulphonium salts. Exposure of virus preparations to ethyl chloride or ethyl bromide in sealed tubes resulted in loss of toxicity but it was found that the virus particles were disrupted with release of internal S antigen so that loss of toxicity could have been due to virus disintegration.

#### *Agents acting on nucleic acid*

All the procedures described above which destroy toxicity also render the virus preparations non-infective. The destruction of toxicity by reagents acting on the amino group may well be due to action on nucleic acid. Agents acting more specifically on nucleic acids include hydroxylamine which attacks the pyrimidines and the ethylene iminoquinone Bayer A 139 which attacks the phosphate sugar backbone (Scholtissek & Rott, 1963).

Virus preparations diluted in phosphate buffer pH 6.5 were mixed with an equal volume of 1/1000 hydroxylamine hydrochloride in buffer pH 6.5. At intervals samples were adsorbed with red cells, the cells washed, the virus eluted and tested for HA titre, toxicity and infectivity by the 6 hr. test. Similar experiments were done with 1/50 Bayer A 139 but in this case TRIS buffer was used in place of phosphate. Results are shown in Table 6. Haemagglutinin titres were unaffected but infectivity and toxicity were destroyed, infectivity as measured by the 6 hr. test being almost as sensitive as toxicity.

Virus preparations diluted in buffer pH 6.5 were exposed in shallow layers to monochromatic UV light of wavelength 2537 Å. At intervals preparations were

Table 5. *Effect of oxidizing agents on toxicity of DSP and Hong Kong viruses*

Reagent and conditions of reaction	Probable reacting groups	Hong Kong virus		DSP virus	
		HA titre	Toxicity test results	HA titre	Toxicity test result
Iodine 1/10,000, pH 6, 10 sec., 0° C. Neutralized with thiosulphate Control (virus + neutralized iodine)	SH SCH <sub>3</sub> Tryptophan —	16,000 16,000	0, 0, 0, 0 D <sub>3</sub> , D <sub>3</sub> , D <sub>3</sub> , +	6,000 6,000	0, 0, 0, 0 D <sub>3</sub> , D <sub>3</sub> , +, +
Potassium permanganate 1/10,000, pH 6, 0° C. A 1 min. B 10 min.	SH C=C (SCH <sub>3</sub> , Tyr) SH C=C SCH <sub>3</sub> , Tyr Trypt. Hist. Uracil —	16,000 6,000 16,000	D <sub>2</sub> , D <sub>3</sub> , + + 0, 0, 0 D <sub>3</sub> , +/2, 0	— — —	— — —
Neutralized with cysteine Control (virus + neutralized reagent)	—	16,000	D <sub>3</sub> , +/2, 0	—	—
Osmic acid 1/10,000, pH 6, 2 min. 0° C. Neutralized with cysteine Control (virus + neutralized reagent)	SH SCH <sub>3</sub> C=C —	4,000 3,000	0, 0, 0, 0 D <sub>3</sub> , D <sub>3</sub> , +/2, 0	3,000 4,000	0, 0, 0, 0 D <sub>3</sub> , D <sub>3</sub> , + +, + +
Hydrogen peroxide 1/100, pH 7, 20° C 30 min. Control	SCH <sub>3</sub> Others not tested —	12,000 8,000	+ +, 0, 0, 0 D <sub>3</sub> , + +, +, +	6,000 8,000	0, 0, 0, 0 D <sub>2</sub> , D <sub>3</sub> , D <sub>3</sub> , +



Table 6. Destruction of infectivity and toxicity of DSP and HK viruses by 1/2000 hydroxylamine hydrochloride and 1/100 Bayer A 139

Experiment	Time of exposure to reagent (hr.)	Haemagglutinin titre	Infectivity test (6 hr.)		Toxicity test result
			Egg 1	Egg 2	
DSP virus + 1/2000 hydroxylamine hydrochloride	0	2,000	192	256	D <sub>3</sub> , +, +
	½	2,000	14	12	+, +, +, +
	1	2,000	2	2	+, +/2, 0
	2	2,000	0	2	+/2, +/2, 0
	4	2,000	0	0	0, 0, 0
HK virus + 1/2000 hydroxylamine hydrochloride	0	25,000	112	80	+, +, +, +/2
	½	12,000	24	3	+, +, +/2
	1	8,000	4	0	+/2, 0, 0
	2	16,000	0	0	0, 0, 0
	4	16,000	0	0	0, 0, 0
DSP virus + 1/100 Bayer A 139	0	8,000	12	12	D <sub>3</sub> , D <sub>3</sub> , D <sub>3</sub> , +, +
	1	8,000	6	12	D <sub>2</sub> , +, +, +, +
	2	8,000	12	24	+, +, +, +/2, 0
	4	8,000	0	1	+/2, +/2, +/2, 0
	6	8,000	0	0	0, 0, 0, 0
HK virus + 1/100 Bayer A 139	0	32,000	24	14	D <sub>2</sub> , D <sub>3</sub> , 0
	1	32,000	24	24	D <sub>3</sub> , +, +, +/2
	2	32,000	20	28	D <sub>3</sub> , +, 0
	4	32,000	4	2	+, +, +, 0
	6	32,000	0	0	0, 0, 0

removed and tested for HA titre, infectivity and toxicity. Results in Table 7 show that infectivity as measured by the 6 hr. test was slightly more sensitive to UV light than toxicity.

*The relation between toxicity and intracellular synthesis of virus components*

*Relation between toxicity and production of RNP antigen by adapted and unadapted virus*

Mice were inoculated intranasally either with a purified non mouse-adapted Hong Kong virus with a haemagglutinin titre of 32,000 or with a mouse-adapted Hong Kong virus in the form of 1% lung extract.

At intervals three mice from each batch were killed, the lung lesions recorded and a pooled extract of the mouse lungs tested for RNP antigen content by complement fixation with an anti-S serum (Table 8). The unadapted virus produced a full yield of RNP antigen within 8 hr. but consolidated lung lesions did not appear until 48 hr. The amount of RNP antigen detectable in the lungs at 8 hr. was about 20 times the amount present in the inoculated virus.

The mice inoculated with adapted virus did not develop a full yield of RNP antigen until 24 hr. and consolidated lung lesions did not appear until 72 hr.

Table 7. *Effect of exposure to UV light of wavelength 2537 Å on toxicity and infectivity of DSP and HK viruses*

Virus	Time of exposure (sec.)	Haemagglutinin titre	Infectivity test (6 hr.)		Toxicity test result
			Egg 1	Egg 2	
			DSP	0	
	5	8,000	20	20	D <sub>3</sub> , +, +, +/2
	25	8,000	4	4	+, +/2, 0, 0
	125	16,000	0	0	+/2, 0, 0, 0
	625	8,000	0	0	0, 0, 0, 0
	3125	16,000	0	0	0, 0, 0, 0
HK	0	16,000	128	128	D <sub>2</sub> , D <sub>3</sub> , D <sub>3</sub> , +, +
	5	16,000	128	64	D <sub>3</sub> , +, +, +, +
	25	16,000	128	64	D <sub>3</sub> , +, +/2, 0
	125	16,000	0	0	+, +, 0, 0
	625	16,000	0	0	0, 0, 0, 0
	3125	16,000	0	0	0, 0, 0, 0

Table 8. *Production of ribonucleoprotein antigen and development of lung lesions in mice inoculated with adapted and unadapted virus*

Time after inoculation (hr.)	Mice inoculated with unadapted virus of HA titre 30,000		Mice inoculated with adapted virus of HA titre 32	
	S antigen titre in lung extract	Lesions (3 mice)	S antigen titre in lung extract	Lesions (3 mice)
8	192	0, 0, 0	2	0, 0, 0
16	256	0, 0, 0	48	0, 0, ?+
24	112	0, 0, 0	112	0, 0, 0
48	256	D <sub>2</sub> , D <sub>2</sub> , D <sub>2</sub>	128	0, 0, 0
72	64	D <sub>3</sub> , D <sub>3</sub> , +	128	+, +, +

*Relation between toxicity and haemagglutinin production by adapted and unadapted virus*

Mice were inoculated intranasally with unadapted virus with a haemagglutinin titre of 8000. At intervals three mice were killed, the lung lesions recorded and the haemagglutinin titre of pooled saline tracheal washings measured. No haemagglutinin was detected at any stage but severe lesions were found in mice examined at 48 and 72 hr.

When mice were similarly inoculated with adapted virus of haemagglutinin titre 8, haemagglutinin became detectable at 24 hr. and washings attained a titre of 512 at 40 hr.

*Relation between virulence of adapted and unadapted virus*

The mouse-adapted Hong Kong virus was maintained by serial passage in mice, harvesting the lungs on the 4th day. Of 60 mice inoculated in this way with fully adapted virus 2 died on the 3rd day, 22 on the 4th and 19 had lesions on the 4th day of such severity that death would have resulted on the 5th day.

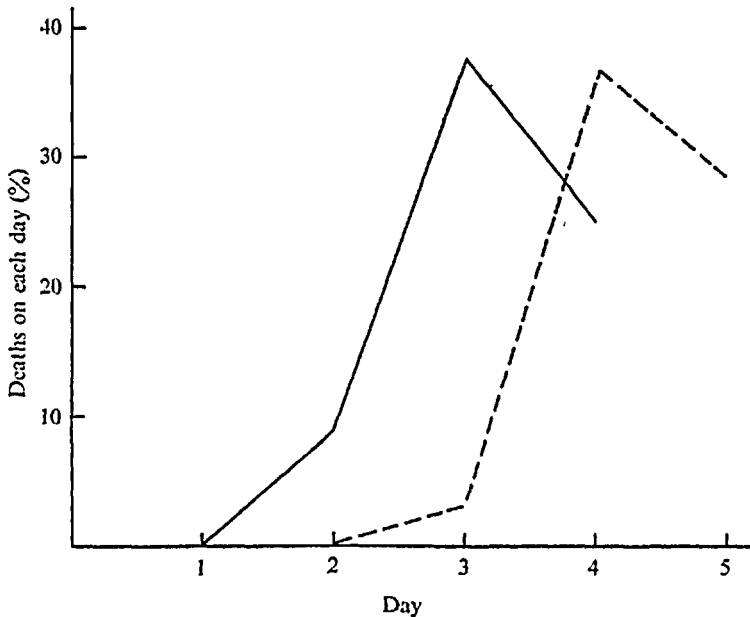


Fig. 1. Mortality of mice inoculated intranasally with adapted and unadapted virus. Mice inoculated with a large dose of unadapted virus (HA titre of inoculum  $> 4000$ ) —, Mice inoculated with a small dose of adapted virus (HA titre of inoculum  $< 4$ ) ----.

In tests of the effects of chemical reagents on toxicity, control mice were inoculated with large doses of unadapted virus and examined on the 3rd day. Of 88 such mice eight died on the 2nd day, 33 on the 3rd day and 22 had lesions on the 3rd day of a severity which would cause death on the 4th day (Fig. 1). The lung lesions produced by adapted and unadapted virus were macroscopically indistinguishable.

Figure 1 shows that the mortality curves obtained with the two types of virus were identical in shape and the overall death rate was almost the same, the only difference being that with a large dose of unadapted virus the peak mortality occurred on the 3rd day while with a small dose of adapted virus it occurred on the 4th day. This time difference probably represents the time taken for the adapted virus to multiply to a concentration in the lung equivalent to that produced by the large dose of unadapted virus. If virulence means ability to kill the two forms of virus are equally virulent.

#### DISCUSSION

The results described in this paper indicate that the toxic effect produced by intranasal inoculation of unadapted virus in mice depends on the penetration of virus into the lung cells and on the release of the virus nucleic acid.

Disruption of the virus particle destroys toxicity as the released nucleoprotein is unable to enter the cell. Destruction of the haemagglutinin prevents the initial union of virus and cell.

Ammonium salts and aliphatic amines are known to interfere with the penetration stage in influenza virus reproduction (Fletcher, Hirschfield & Forbes, 1965);

they possibly act by blocking a cell receptor for the virus amino group, and chemical reagents acting on the amino group are very viricidal and efficient in destroying toxicity. These agents probably have a dual action, reacting with the virus nucleic acid and also interfering with penetration.

Allison (1962) described a reversible inactivation of influenza virus by mercurials which he attributed to union with disulphide bonds preventing uncoating of the virus nucleic acid. The effects on toxicity of agents acting on the disulphide bond may well be explained in this way. Mercuric chloride which prevents uncoupling of the disulphide bond destroys toxicity while toxicity is unaffected by urea + dithiothreitol which uncouples the disulphide bond and would presumably not prevent uncoating of the nucleic acid.

Ackermann & Maassab (1955) showed that influenza virus reproduction was inhibited by the methionine analogue methoxinine which acted at an early stage of the growth cycle immediately after penetration. The effect was not due to interference with incorporation of methionine in protein synthesis but to interference with some other action of methionine. The possibility that the methionine involved was a virus component was not considered. The destruction of virus toxicity by iodine, permanganate and osmic acid may possibly be due to action on the  $\text{CH}_3\text{S}$  group of methionine. The group also reacts with mercuric chloride, hydrogen peroxide and alkyl halides all of which destroy toxicity.

Agents acting on the nucleic acid are particularly effective in destroying toxicity, and hydroxylamine, Bayer A 139 and exposure to UV light destroy toxicity under conditions in which there is no demonstrable action on other virus components. At one stage in the work the possibility was considered that the toxic effect might be due to damage to the cell walls produced during the penetration of the cell by large doses of virus, but the fact that toxicity can be destroyed by action on the nucleic acid shows that the toxic effects are produced at a later stage than penetration. The virus nucleic acid may itself be toxic but more probably the toxic effects are due to synthesis of some protein under the control of the virus RNA. The toxic effect would appear to be associated with the occurrence of some form of incomplete growth cycle (Schlesinger, 1953; Ginsberg, 1954).

When unadapted virus is inoculated intranasally to mice there occurs no demonstrable production of haemagglutinin or infective virus, but a full yield of RNP antigen is obtained after 8 hr. Severe lung lesions follow after an interval of some 40 hr.

With adapted virus also severe lung lesions occur at a similar interval after the production of RNP antigen has reached its peak. It appears that cell damage is induced at an early stage in the growth cycle but lung consolidation takes some time to develop.

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