THE ISOLATION AND PROPERTIES OF A MODIFIED STRAIN OF NEUROTROPIC INFLUENZA A VIRUS.

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In recent months the question of whether recombination occurs with influenza viruses, in the manner described for bacteriophage, has occupied a considerable amount of attention. That two different virus particles, either alive or inactivated, should be able to combine within the host cell so as to produce stable " offspring " with a more or less haphazard reassortment of hereditary characters is a question of more than academic interest, with far-reaching implications. It has clearly been shown (Sugg and Magill, 1948 ; Liu and Henle, 1951*a*) that two different strains of influenza virus may, under suitable conditions, be propagated together through several transfers, each maintaining its own characteristics. But Burnet and Lind (1951*a* and *b*) have found that under certain conditions, under which the full growth of both components of an influenza virus mixture is suppressed, viruses can quite readily be isolated, which carry some of the properties of both parents yet do not behave like a mixture. By inoculating mice intracerebrally with a mixture of one of various non-neurotropic A strains, and in each case neurotropic W.S., in such proportions that the growth of the latter was nearly but not quite prevented by interference, neurotropic virus was isolated which exhibited the serological properties of the non-neurotropic component of the mixture. In other properties these strains varied, and they were not always the same as either parent. Burnet and Lind concluded that these strains were not mixtures, but that a recombination of characters had occurred. The use of passage at high dilution (Isaacs and Edney, 1950*a*) formed an essential part of the technique, to show that simple mixtures were not responsible for the various properties of the strains.

In the case of bacterial viruses Luria and Dulbecco (1949) have demonstrated upon a numerical basis the production of active particles from ultra-violet irradiated and inactivated *Bact. coli* phage. This occurred when the number of phage particles was sufficient to produce multiple infections of a bacterial cell, and was attributed to a reassortment of irradiated units which had not undergone lethal mutations. Liu and Henle (1951*b*) have reported that such multiplicity reactivation also takes place with influenza viruses irradiated with ultra-violet light, when the dose of irradiation is such that the majority of particles receive single, or few, hits.

Because of the findings of Luria and Dulbecco (1949) the first experimental work here was planned to determine whether recombination could be demonstrated, with influenza virus, by a comparable method. If this were so, from an inactivated inoculum seeded allantoically in eggs, viable virus should only be isolated where the inoculum is sufficiently large to produce multiple infections of host cells, but

not from a similar inoculum distributed in small amounts amongst a number of eggs. It was found, however, that increase in haemagglutinins occurred sometimes with inocula of any size, from 0·01 ml. to 0·5 ml. of irradiated allantoic fluid, of PR8 or of Kunz virus. Results were difficult to interpret, largely because of two unknown factors—the extent of distribution of an inoculum in the allantoic cavity before the virus particles become intracellular, and the possible presence of a few viable particles in the inoculum which had survived irradiation. It was decided therefore to investigate the possibility of producing " recombinants " of influenza virus from two partially inactivated strains of originally different properties.

MATERIALS AND METHODS.

Viruses.

The two viruses chosen for this work were (1) Kunz, an egg-adapted A prime virus, isolated in Sheffield in 1947 (Stuart-Harris and Miller, 1947), and (2) Neuro W.S. (N.W.S.), a neurotropic variant derived from the classical W.S. strain (Stuart-Harris, 1939). These two viruses show no antigenic relationship in haemagglutination cross-titrations with ferret immune sera, and in addition they may be differentiated by intracerebral mouse inoculation. N.W.S. inoculated intracerebrally causes paralysis and death ; Kunz does not usually survive more than one generation.

Irradiation technique.

Allantoic fluids of Kunz and N.W.S. were irradiated separately, in films of about 2 mm. depth in flat glass dishes, for 5 and 3 minutes respectively, at a distance of 7 in. from an ultra-violet light source of wave-length 2537 Ä.u. Previous work had shown that this amount of irradiation did not alter the haemagglutinin titre, though reducing considerably the infectivity titre—in fact around this dose of irradiation fluids frequently appeared to be sterile upon egg passage in any dilution tested. Absolute sterility may be difficult to establish because of the interfering effects of large amounts of inactive virus inevitably present in an irradiated inoculum which may block the growth of the few particles remaining active, and also because, as Liu and Henle (1951*b*) have shown, new viable virus may under some conditions be regenerated from the inactive virus of the inoculum. That such recombination occurred with the dose of irradiation used here was difficult to establish upon a numerical basis. The effects of a decreased amount of irradiation were not investigated.

It remained to be found whether the dose of ultra-violet light used, though not detrimental to the haemagglutinin titre, would still leave unimpaired the more easily destroyed capacity (Henle and Henle, 1947) to enter the host cells and initiate the first stage of infection. It has been shown by Isaacs and Edney (1950*b*) that interference by one virus with the growth of a second virus is essentially an intracellular process. The capacity of irradiated virus to cause interference was therefore used as an indication of its power of entering the cells of the allantoic membrane. In the case of Kunz, this was determined as follows. Irradiated allantoic fluid was diluted 1 in 100, and 0·5 ml inoculated allantoically in four chick embryos. This was followed one hour later by 100 $I.D._{50}$ of unirradiated N.W.S: The second virus was prevented from growing in any of the eggs. The converse effect of interference by N.W.S. with the growth of Kunz in eggs is more difficult

to establish, and the irradiated fluid was not tested in this way. As, however, it received little more than half the dose of irradiation used for Kunz, it seemed likely that the capacity of N.W.S. to enter the host cell would not be impaired.

Recovery of modified virus.

After irradiation the haemagglutinin titres of the fluids were found. A mixture of undiluted fluids was prepared in which the respective haemagglutinin content of the two viruses was the same and the ratio of Kunz to N.W.S. virus was 1 : 1·6. Varying amounts of this mixture (from 0·1 ml. to 0·01 ml.) were then injected into the allantoic cavity of 11-day chick embryos. Virus-containing fluids were harvested from 11 of 12 eggs thus inoculated, after 48 hours' incubation at 37°. The haemagglutinating property of these fluids was tested at a dilution of 1/10 in the presence of immune ferret sera previously treated with cholera filtrate (Appleby and Stuart-Harris, 1950). In every case Kunz antiserum inhibited, but N.W.S. serum failed to inhibit, haemagglutination.

Six of these fluids taken at random were passed intracerebrally in mice, where four readily gave rise to signs of cerebral involvement. Brain emulsions from these mice harvested 72 hours after inoculation were then passed in eggs allantoically, both with and without N.W.S. antiserum. In most cases, no growth occurred in the presence of serum, and brain emulsion propagated without serum, when titrated in haemagglutination tests in the presence of immune sera, behaved serologically like N.W.S. These were not investigated further. One mouse brain emulsion, however, gave rise to a haemagglutinating fluid which was not neutralised by N.W.S. serum *in ovo*, but was neutralised by Kunz serum *in vitro*. This strain was passaged further.

It was found that this virus when propagated in eggs in the presence of N.W.S. antiserum for one generation maintained its pathogenicity for mice, intracerebrally, through six consecutive generations, each passage causing paralysis and death, while showing the serological characters of Kunz.

The series of steps by which the new strain (N–K) was established are shown as follows :

Irradiated { Kunz + N.W.S. } in eggs ——→ allantoic fluid intracerebrally in mice (encephalitic symptoms after 5 days) ——→ brain emulsion allantoically in eggs with N.W.S. antiserum (haemagglutinins neutralised by Kunz serum)

——→ allantoic fluid intracerebrally in mice—6 generations (paralysis and death) ——→ brain emulsion allantoically in eggs—4 generations (haemagglutinins neutralised by Kunz serum)

Passage at limiting dilution.—In an investigation of recombination it is of prime importance to rule out the possibility that a " recombinant " may in fact be a mixture of both parents. For purification of strain N–K use was therefore made of the limiting dilution technique in eggs, recommended by Isaacs and Edney (1950*a*) as follows :

1st passage, with fluid diluted 10^{-8}, 2/8 egg fluids positive.
2nd passage, with fluid diluted 10^{-8}, 2/4 egg fluids positive.
3rd passage, with fluid diluted 10^{-8}, 2/7 egg fluids positive.

The two virus-containing fluids from the last passage were examined and found to be the same in neurotropic and serological properties as the original strain. One of these was then passed at limiting dilution at the National Institute for Medical Research by Dr. Isaacs, in 16 eggs, of which 5 showed growth of virus. These fluids were all alike in their outstanding properties and resembled the original virus.

It was thus established that this strain was stable through many mouse and egg transfers, and two components could not be separated by this means.

Characters of Neuro-Kunz Strain.

Methods which may be reliably employed to characterise an influenza virus are limited. Strain N–K was compared with the two parent viruses in the following properties : antigenic relationships, growth in mouse brain and lung, and behaviour toward a haemagglutinin inhibitor from sputum.

Virus preparations were used in the form of egg allantoic fluids. In every experiment the three fluids to be compared were treated in the same way both before and during the experiment and the conditions were made as nearly as possible the same for each virus.

1. *Cross titrations of immune sera.*

Convalescent ferret sera were prepared from ferrets inoculated intranasally with allantoic fluids. They were titrated by cross-haemagglutinin inhibition tests, using a modification of Salk's method. In order to minimise non-specific effects sera were treated with cholera filtrate before titration. To one part of serum was added two parts of cholera filtrate with two parts of normal saline, and the mixture incubated at 37° overnight and then heated at 58° for 50 minutes. It was found that this treatment removed non-specific inhibitors of haemagglutination from normal ferret sera, but it did not appear to reduce the antibody level for homologous viruses (Appleby and Stuart-Harris, 1950). In each case pre-infection sera from the ferrets when treated with cholera filtrate failed to inhibit agglutination of the homologous virus used for infection. Antigens (allantoic fluids) were adjusted to contain 4 M.H.D.

TABLE I.—*Comparative Titres of Ferret Antisera with N–K Strain and Parent Viruses.*

Antiserum.	Virus.		
	Kunz.	N.W.S.	N–K.
Kunz	1536	<24	1536
N.W.S.	<24	768	<24
N–K	1536	48	1536

The results recorded in Table I showed that the new virus was similar in antigenic structure to Kunz, but had some apparent relationship to N.W.S.

2. In vivo *neutralisation tests.*

Neutralisation by immune ferret sera *in vivo* revealed a rather closer relationship of N–K to N.W.S.

(a) *In eggs.*—One in five serial dilutions of freshly harvested allantoic fluids of N–K were mixed in each case with equal volumes of either Kunz or N.W.S. undiluted immune ferret serum. Of the mixtures 0·1 ml. was injected into the allantoic cavity of 11-day embryos. After 3 days' incubation the growth of virus was determined by haemagglutination.

Under these conditions no growth occurred in eggs receiving 0·1 ml. of a 1/10 dilution of virus in the presence of Kunz immune serum, while N.W.S. serum reduced the 50 per cent infectivity titre from 10^{-8} to 10^{-3} (Table II). The possibility that this neutralisation might have been in some part due to non-specific serum substances was not investigated.

TABLE II.—*Neutralisation of N–K Strain by "Parent" Immune Sera in Eggs.*

Ferret antiserum.	Dilutions of virus.							
	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.
None	—	—	—	—	—	—	12/12	4/11
Kunz	0/6	0/5	—	—	—	—	—	—
N.W.S.	—	4/6	3/8	0/6	—	—	—	—

The denominator represents the number of eggs inoculated; the numerator the numbers from which virus was recovered.

(b) *In mice.*—Similar effects were obtained upon intracerebral inoculation of the virus in mice. Inocula were prepared as for egg neutralisation tests. As before, the neutralising effect of Kunz serum was greater than that of N.W.S., as it accounted for rather more than 100 $L.D._{50}$ of virus, while N.W.S. serum neutralised about 10 $L.D._{50}$ (Table III).

TABLE III.—*Neutralisation of N–K Virus by Parent Immune Sera in Mouse Brain.*

Ferret antiserum.	Dilutions of virus (allantoic fluid).				
	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.
None	4/4	4/4	4/4	3/4	0/4
Kunz	4/4	3/4	0/4	0/4	0/4
N.W.S.	4/4	4/4	3/4	0/4	—

The denominator represents the number of mice inoculated; the numerator the number of deaths.

3. *Tissue tropism.*

(a) *Mouse brain inoculation.*—No detectable difference was found in the symptoms produced by N–K and N.W.S. strains when given, in dilution, intracerebrally in mice. Both viruses caused paralysis, followed by death after (usually) 5–8 days.

The titre of infective virus in mouse brains 3 days after inoculation, determined by passage of mouse brain emulsion in serial dilutions in eggs, was approximately the same for both viruses. The 50 per cent infectivity dose for each virus lay between 10^{-5} and 10^{-6}.

Kunz virus, even in large doses, did not produce neurotropic symptoms, and was not recoverable from mouse brains after the second transfer.

(b) *Mouse lung inoculation.*—Intranasal inoculation showed N–K virus to be intermediate in its affinity for mouse lung tissue between the two parent strains, but perhaps rather closer to Kunz.

Groups of 6 mice were inoculated with undiluted allantoic fluid under light ether anaesthesia. Of these 6 mice, 2 were killed after 2 days, and their lungs emulsified to provide passage material; 4 were killed after 7 days and the lungs examined.

N.W.S. was lethal for mice in the second mouse lung generation, producing almost complete consolidation. Mice inoculated with Kunz virus had in the third generation few symptoms but showed scattered small lung lesions. N–K caused extensive areas of consolidation in the third generation, with 1 death in 4 mice after 6 days.

4. *Behaviour toward inhibitor from sputum.*

A solution of haemagglutinin inhibitor was prepared from human sputum by thorough maceration with powdered glass, centrifugation, and filtration through tightly packed cotton-wool. Sufficient penicillin and streptomycin were added to ensure bacteriostasis, but the preparation was not heated. Such a fluid regularly exhibits powerful inhibitory properties in the haemagglutination test with influenza viruses.

Virus-infected egg allantoic fluids were either freshly harvested, or recently stored at −72° with no more than one freezing and thawing. Red cells of different fowls, while giving somewhat different inhibitory titres, did not alter the relative titres or the significance of the results.

To serial 1 in 2 dilutions in normal saline an equal volume of virus dilution containing 4 M.H.D. was added. A 0·25 per cent suspension in normal saline of fowl red cells was added (*a*) simultaneously with the virus, and (*b*) after varying periods of incubation of inhibitor-virus mixture at 37°.

As an example, the results of one experiment, in which the contact period at 37° of virus and inhibitor was 45 minutes, are given in Table IV.

TABLE IV.—*Titres of Sputum Inhibitor with N–K and Parent Strains.*

Virus.	Inhibitor titres.	
	No contact.	45 min. contact.
Kunz	128	64
N.W.S.	1,024	4,056
N–K	4,056	16,224
Lee*	128	2,048

* Allantoic fluid heated at 56° for 30 minutes.

The inhibitor titres obtained upon adding red cells and virus simultaneously, reflecting the relative affinity of each virus for the inhibitor and for the red cell receptors, show a difference between the three strains. N.W.S. has a markedly greater affinity for this inhibitor than has Kunz. N–K was inhibited to a significantly greater degree even than N.W.S.

During the 45 minutes' contact of virus with the inhibitor two events may occur. Burnet (1948) has shown that purified mucoid inhibitor of the Francis type

requires some period of contact with inactivated virus before the maximum inhibitor titre of the preparation is reached. This fact is illustrated in Table IV, with the inhibitor preparation used here, where the titre to heated Lee virus is 16-fold higher after 45 minutes' contact. It seems reasonable to expect that a similar progressive change may occur with live virus particles, and Smith and Westwood (1950) have shown that this may sometimes occur with live PR8 virus and rabbit serum inhibitor during three hours at 2°. At the same time virus strains possessing enzymic activity are capable of bringing about a progressive destruction of inhibitor, thus reducing the latter's titre. Enzymic activity is a property which varies very much with different strains of influenza virus, and it is a relatively constant characteristic for a strain. The final titre of inhibitor after the contact period would therefore depend upon the balance between these two processes.

It might be deduced from Table IV that N.W.S. and N–K possess little enzymic activity, since the inhibitor titre increased on contact, though to a less extent than with heated Lee virus. Burnet's work (1951) suggests that N.W.S. is relatively feeble in content of enzyme of this nature. In the case of Kunz, however, the increase in titre of inhibitor expected upon contact of a virus devoid of enzyme was not found, and in fact there was a decrease. It appears difficult to reconcile the behaviour of N–K in this respect with the possession of any Kunz component.

DISCUSSION.

The new strain of influenza virus which has been isolated appears to have a combination of the characters of both parents, so distributed that it is difficult to interpret its behaviour as a mixture of any fixed proportions of the parent viruses. If it were such a mixture, while intracerebral passage in mice shows it to be predominantly N.W.S., the same fluid by neutralisation tests *in vitro* is predominantly Kunz antigenically.

It is stable upon repeated mouse and egg passages, and in environments which would suppress one or other component of a mixture. Thus, virus recovered from the 6th consecutive mouse brain generation is neutralised by Kunz antiserum in spite of the fact that such intracerebral passage would favour the N.W.S. neurotropic virus at the expense of the Kunz component originally employed.

In behaviour toward sputum inhibitor, where there is a marked difference between the two parents, the new strain possesses the N.W.S. character of weak enzymic activity.

There remains the possibility that the altered properties of the virus were a direct result of mutation induced by U–V irradiation of either Kunz or N.W.S. From a survey of the discovered properties of the strain it would seem very unlikely that a single irradiation could have produced these combined effects.

The new virus appears therefore to be a stable strain possessing genetic components of both parents, whose origin could be explained by the process of recombination. It will be recalled that the strain was only recovered from one of the egg fluids obtained from the batch of eggs originally inoculated with the two partially inactivated parent viruses. Five other eggs yielded viruses of the Kunz or N.W.S. strains of which one virus only survived passage in the mouse brain. It is likely, therefore, that the egg fluids contained mixtures of the two viruses, and that recombination occurred in the mouse brain inoculated with the mixture.

The technique employed would render survival of a neurotropic virus possessing an antigen of the Kunz type theoretically possible, and would tend to suppress growth equally of a non-neurotropic agent or of a virus with the W.S. antigen. Final proof of the mode of origin of the new strain was not however obtained.

SUMMARY.

By the simultaneous inoculation into embryonated eggs of two ultra-violet irradiated influenza A strains of different antigenic properties and tissue tropism, a new strain of influenza virus has been isolated. This possesses some characters of both parents, but so distributed that it appears improbable that this virus could in fact be an unpurified mixture of both parents. Its origin could be attributed to a process of recombination.

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REFERENCES.

APPLEBY, J. C., AND STUART-HARRIS, C. H.—(1950) *Brit. J. exp. Path.*, **31**, 797.
BURNET, F. M.—(1948) *Aust. J. exp. Biol. med. Sci.*, **26**, 389.—(1951) *J. gen. Microbiol,*. **5**, 46.
Idem AND LIND, P. E.—(1951*a*) *Ibid.*, **5**, 59.—(1951*b*) *Ibid.*, **5**, 67.
HENLE, W., AND HENLE, G.—(1947) *J. exp. Med.*, **85**, 347.
ISAACS, A., AND EDNEY, M.—(1950*a*) *Brit. J. exp. Path.*, **31**, 209.—(1950*b*) *Aust. J. exp. Biol. med. Sci.*, **28**, 231.
LIU, O. C., AND HENLE, W.—(1951*a*) *J. exp. Med.*, **94**, 291.—(1951*b*) *Ibid.*, **94**, 305.
LURIA, S. E., AND DULBECCO, R.—(1949) *Genetics*, **34**, 93.
SMITH, W., AND WESTWOOD, J. C. N.—(1950) *Brit. J. exp. Path.*, **31**, 725.
STUART-HARRIS, C. H.—(1939) *Lancet*, i, 497.
Idem AND MILLER, H. M.—(1947) *Brit. J. exp. Path.*, **28**, 394.
SUGG, I. Y., AND MAGILL, T. P.—(1948) *J. Bact.*, **56**, 201.