THE EFFECTS OF ETHYL ETHER ON SOME BIOLOGICAL PROPERTIES OF INFLUENZA VIRUS.

WILSON SMITH, H. COHEN, G. BELYAVIN AND J. C. N. WESTWOOD.*

From the Department of Bacteriology, University College Hospital Medical School, London, W.C.1.

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THE biological properties of the influenza viruses have been studied intensively during recent years and some of them have been shown to be dependent upon specific reactive components of the virus surface. Practically nothing is known, however, about the virus architecture though it is probable that the spatial relationships of the various components concerned with biological activities like haemagglutination, complement fixation and enzyme action help to determine the efficiency of the virus unit as a whole. One obvious approach to an investigation of virus structure is the study of the effects of virus degradation by different chemical and physical agents. This paper reports the effects of ether treatment on the complement-fixing antigen, the haemagglutinin and the receptor-destroying enzyme of classical virus strains.

MATERIALS AND METHODS.

Virus preparations.

Influenza virus A (PR8) was used for most of the work but replicate experiments with Influenza B (Lee) established that results were applicable to both the major types of influenza virus. Both viruses were egg-adapted and were maintained by allantoic inoculation of 10-day embryonated eggs. Virus suspensions for experiment were obtained from infected allantoic fluid by adsorption on human group O red cells followed by elution into physiological saline.

Antisera.

Rabbits were inoculated subcutaneously with a single large dose of virus and bled at intervals thereafter. The sera were inactivated at 56° for 30 min. and stored in sealed ampoules at — 20°.

Haemagglutinin titrations.

These were done by the modification of the Hirst-Pickels photo-electric densitometer method described in a previous paper (Belyavin, Westwood, Please and Smith, 1951). In this method the densitometer readings are inversely proportional to the unagglutinated cell densities at the tube level traversed by the optical axis of the instrument. Except when specifically stated all red cell suspensions were prepared from the pooled blood of three fowls.

Complement fixation.

To measure the complement-fixing potency of an antigen preparation serial dilutions of both antigen and antiserum were mixed with a constant dose of complement. Both a "macro-" and a "micro-" technique were used. The former, employing a test volume of 0·5 ml. has previously been described (Smith, Westwood, Westwood and Belyavin, 1951).

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The micro-method (Belyavin, 1953) is identical in principle but permits the volume of each reagent to be reduced to 0·02 ml., the reagents being distributed by dropper pipettes on plastic sheets as described by Fulton and Dumbell (1949). Incubation is carried out in a humidity box and the fixation time is extended to 90 min. Thirty min. after the addition of standard volumes of 0·2 per cent sensitized sheep cells the plastic sheets are rocked on a centripetal rocker which brings any intact cells to the centres of the test drops as clearly visible dots or discs. The end-point, taken as complete haemolysis, is then easily detected by naked eye observation.

Antigen doses were adjusted from preliminary haemagglutinin titrations to commence at approximately 32 AD/ml. in the macro-technique and approximately 50 AD/ml. in the micro-technique.

When dilutions of both antigen and antiserum are used with a constant dose of complement to form a chessboard titration and the end-points for each row and column are plotted on a double logarithmic scale the area enclosed by the curves is proportional to the logarithm of the antigen concentration initiating the dilution series. This provides a reasonably accurate method for quantitative comparisons of the complement-fixing potencies of virus preparations before and after various types of treatment.

Ether treatment of virus.

" Aether puriss," B.D.H. was used. Five volumes of ether were added to one volume of virus suspension in a separating funnel. The mixture was shaken by a gentle to-and-fro motion for 2½ min. and the ether and aqueous phases then allowed to separate for a further 2½ min. This manipulation was repeated after which the ether and aqueous phases were either separated immediately or after being kept at 4° for various periods up to a maximum of 18 hr. The aqueous fraction was freed from residual ether by aeration. Any precipitate formed at this stage was removed by centrifugation and discarded as it was found to be completely inert in respect of both haemagglutination and complement fixation.

EXPERIMENTAL.

The Effect of Ether Treatment on Virus Haemagglutination.

Variability of effect.

The effect of ether treatment on the haemagglutinin titres of different PR8 virus preparations was extremely variable. In a number of experiments over 90 per cent of the initial haemagglutinating activity was destroyed but in others less than 30 per cent was lost. Many experiments were carried out to try to account for this variability ; they included the use of purified " Analar " and peroxide-free ether, extractions at different pH values ranging from pH 6·0 to 7·9, extractions in the presence of citrate ions, and extractions with ether acidified with acetic acid. The effect of variations in the ambient temperature was also investigated. Consistent results were not obtained by any of these modifications. It was noted that repeated extractions of single virus preparations usually yielded approximately constant results although there was one exception when the same preparation gave haemagglutinin recoveries ranging from 70 to 5 per cent in three successive extractions. These were done, however, on different days and we feel that the variable factor lies in the virus itself rather than in the technique of ether extraction. This possibility remains to be explored.

The qualitative change in the haemagglutination titration curve.

The haemagglutination curve obtained by plotting densitometer readings against an adequate range of virus dilutions always shows a marked optimum of virus concentration ; *i.e.*, all tubes containing either higher or lower con-

centrations than the optimal tube show less sedimentation of the fowl red cells at the standard reading time and hence give lower densitometer readings. Thus excess virus clearly causes a well-marked zone of haemagglutination inhibition analogous to the excess antigen inhibition zone of serum precipitation reactions. Provided that enough activity remains to allow demonstration of an inhibition zone any reduction of the haemagglutinin content of a virus preparation would be expected to shift the curve bodily to the left without any change in the densitometer reading at the optimal point. This is indeed the case when haemagglutinin activity is reduced by heat treatment. It is also the case with ether treatment provided that the treatment results in 90–95 per cent reduction. When however the reduction is relatively slight the curve assumes a different form. It is shifted downwards instead of to the left, with a very much lower densitometer reading at the optimal point. The results of a typical experiment with untreated, heated and ether-treated virus are illustrated in Fig. 1.

The variability of different fowl cells.

For quantitative determinations of this qualitative effect of ether treatment on virus haemagglutinin activity maximum densitometer readings could not be used as such, because densitometer readings are not linearly related to cell concentrations. When cell concentrations are low quite small changes of suspension density produce large changes in densitometer readings with the result that small effects tend to be spuriously magnified. To avoid this a simple calibration curve was constructed for the conversion of densitometer readings to percentage cell concentrations. Figures thus obtained actually represent cell concentrations at the optical level of the densitometer and are not strictly accurate assessments of the relative proportions of agglutinated and unagglutinated cells. They are however sufficiently accurate for the purpose. Throughout this paper the term " optimal reading " is used to designate the highest densitometer reading of a titration curve while the term " optimal concentration " refers to the concentration of unsedimented cells in the tube giving the optimal reading. The two optima are of course inversely related.

Four different virus batches were extracted with ether. Ether-treated and untreated samples of each batch were titrated in parallel against red cell suspensions obtained from 8 fowls. The optimal concentrations are tabulated in Table I.

TABLE I.—*Effect of Ether Treatment on Virus Haemagglutination.*

Fowl cells No.	Passages of PR8 virus. ES 154. Unt.	ES 154. Eth.	ES 165. Unt.	ES 165. Eth.	ES 169. Unt.	ES 169. Eth.	ES 171. Unt.	ES 171. Eth.
1531	0·11	0·155	—	—	—	—	—	—
1532	0·11	0·155	—	—	—	—	—	—
1533	0·08	0·155	0·085	0·19	0·08	0·15	0·085	0·135
1534	0·12	0·225	—	—	—	—	—	—
1536	0·11	0·20	0·09	0·225	0·08	0·225	0·075	0·18
1537	0·095	0·175	—	—	—	—	—	—
1538	0·09	0·145	—	—	—	—	—	—
1500	—	—	0·085	0·225	0·07	0·240	0·085	0·145

Figures give the " Optimal concentrations," for explanation of which term see text.

The downward shift of the titration curves produced by ether treatment is indicated by the appreciably higher optimal concentrations for the ether-treated samples of each virus batch as compared with those for the corresponding untreated samples. This happened with the cells of every fowl tested. The different cell suspensions however showed interesting differences of behaviour. It is well known that the cells of different fowls may vary in the readiness with

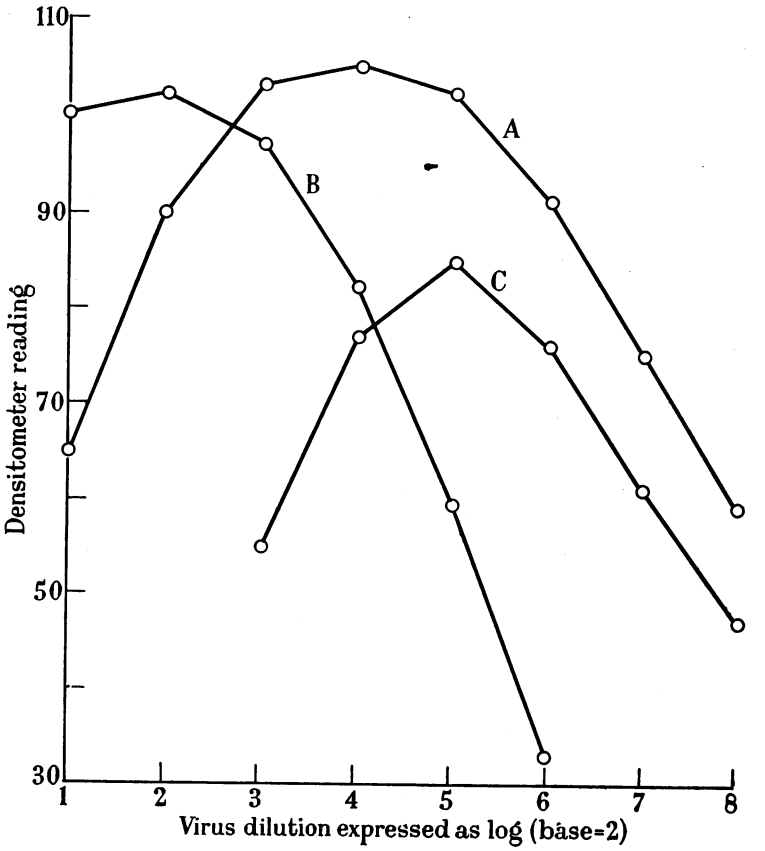

FIG. 1.—Haemagglutinin titration curves.
A. Untreated virus.
B. Heated virus.
C. Ether-treated virus.

which they are agglutinated by influenza viruses but those used for the experiment behaved with surprising uniformity against the four untreated viruses. In contrast the optimal concentrations against each ether-treated virus varied considerably and the relatively high or low susceptibility of each individual fowl's erythrocytes was maintained consistently in tests of the four different virus preparations. The relative susceptibilities of two cell suspensions against untreated and ether-treated virus may actually vary in opposite directions so that the suspension giving the lower optimal concentration with untreated virus

proves to be much less readily agglutinable than the other by the same virus after its ether extraction.

Three breeds of fowls were represented among the blood donors, White Leghorn, Brown Leghorn and Light Sussex, but so far as could be ascertained breed was not a factor in this variability of red cell susceptibility.

Consistent behaviour of cells of individual fowls.

In order to study the behaviour of cells obtained from individual fowls over a period of time a virus preparation was required which could be depended on to maintain haemagglutinin activity throughout the period. A PR8 virus eluate was distributed in 1 ml. quantities in ampoules, some of which were freeze-dried, some kept at 4° and some stored at — 20° after being rapidly frozen at — 76°. The freeze-dried virus lost considerable haemagglutinin activity in 3 days and much more in 7 days, and the rapidly frozen virus kept at — 20° lost over half its activity in 3 days. The virus at 4° showed no appreciable diminution of potency in 7 days and on ether treatment still gave a typical lowered titration curve. This batch was therefore used for the series of tests. At approximately weekly intervals samples were withdrawn in order to titrate the virus, both untreated and ether-treated, against the freshly bled red cells of three individual fowls.

The results are summarized in Table II. At each weekly test the three optimal concentrations for the untreated virus were almost identical. By itself this would apparently indicate that the cells of the three fowls were indistinguishable in haemagglutinin susceptibility. The parallel readings for the ether-treated samples however showed that this was not so. Not only did the cells differ widely in sensitivity at each test but the order of sensitivity was maintained throughout the series of five tests. Thus while all three cell suspensions regularly showed lowering of the titration curve against ether-treated virus as compared with the curve against untreated virus, the cells of two fowls consistently gave very high, and those of the third fowl consistently gave relatively low, optimal concentrations. Such differences as are shown by various bleedings from the same fowl are not statistically significant. It is thus evident that red cell sensitivity to ether-treated virus depends on some stable characteristic of the individual fowl donor. The uniform response of the virus eluate to ether treatment on five separate occasions is noteworthy and supports our contention that variability of the effect of ether treatment on different virus batches depends on some unknown variable in the virus itself.

TABLE II.—*Variable Susceptibility of Cells of Different Fowls to Ether-treated Virus.*

Virus.	Fowl cells.	Date of test. 17.vii.50.	21.vii.50.	27.vii.50.	11.viii.50.	18.viii.50.
Untreated	1500	0·09	0·085	0·09	0·08	0·065
	1533	0·085	0·09	0·085	0·085	0·07
	1536	—	0·09	0·085	0·08	0·075
Ether-treated	1500	0·205	0·255	0·255	0·24	0·205
	1533	0·115	0·190	0·195	0·175	0·180
	1536	0·25	0·225	0·260	0·24	0·255

Figures = optimal concentrations.

The significance of lowering of the virus titration curve.

It is generally assumed that any reduction of haemagglutination titre resulting from some treatment of the virus signifies that destruction of some of the haemagglutinin has occurred. This is not necessarily so. Any alteration of position of the titration curve in relation to either of its co-ordinates must affect the titre. If titre reduction is due to a reduction of the amount of haemagglutinin in the virus preparation, either by simple dilution or by partial haemagglutinin destruction, the shift of the curve will be to the left. This implies that titre reduction associated with downward displacement of the titration curve must depend upon some kind of haemagglutinin change distinct from quantitative reduction. It appears therefore that one effect of ether treatment may be a qualitative change in the virus receptors. Actual haemagglutinin destruction may also occur to a variable extent ; so that a reduction of titre following ether treatment may represent either a quantitative or qualitative change or a summation of both.

The validity of comparisons based on haemagglutination titrations rests on rigorous standardization of the method used so that experimental errors can be kept within small and defined limits. It is essential that the time allowed for cell sedimentation in each tube before taking the densitometer reading should be kept constant. In our method the time chosen is 75 minutes. Within any arbitrarily selected time any factor which slows the haemagglutination reaction will produce downward displacement of the titration curve. It seemed likely that the qualitative change in a virus produced by ether treatment might be such as to cause increase of haemagglutination reaction time. The temporal evolution of titration curves was therefore studied.

A series of replicate titrations was carried out on virus untreated and ether-treated. Dilutions were prepared in bulk and distributed to give several identical replicate dilution series. Each replicate was read after the lapse of a different time interval for cell sedimentation, ranging from 15 to 105 min. The series of titration curves thus obtained is illustrated in Fig. 2.

At 15 min. curves for untreated and ether-treated virus are almost identical and are flat because no appreciable cell sedimentation has yet occurred. At 45 min. they are parallel but widely separated. Subsequently both rise steadily so that both the untreated and ether-treated viruses give a series of ascending roughly parallel curves but at any give time the ether-treated virus curve is considerably lower than the corresponding untreated virus curve. It is clear therefore that the effect of ether treatment of virus on its haemagglutinating activity is operative in the early stages of the reaction and that in later stages the reaction proceeds normally. In the experiment illustrated the retardation time was approximately 30 min. so that ether curves for 75 and 105 min. are superimposed over the " untreated " curves for 45 and 75 min. respectively. In a second experiment using the cells of a different fowl the retardation time was about 15 min. and similar experiments with virus B (Lee) showed that its haemagglutinating activity was affected by ether treatment in the same way though usually with a shorter retardation time.

Given sufficient time all the cells of a suspension settle out leaving a clear supernatant fluid. The acceleration of sedimentation caused by virus is due to the formation of cell aggregates of density greater than that of discrete cells so that the major factors which determine the time relations of the reaction are

speed of aggregate formation and mean aggregate size. It seemed probable that both factors would be affected by the presence of virus-insensitive cells in a cell suspension and that the ether retardation phenomenon might be due to variability of sensitivity to ether-treated virus among fowl red cells. Experiments showed however that this is not the true explanation. The rates of development of titration curves were studied using an untreated virus against several

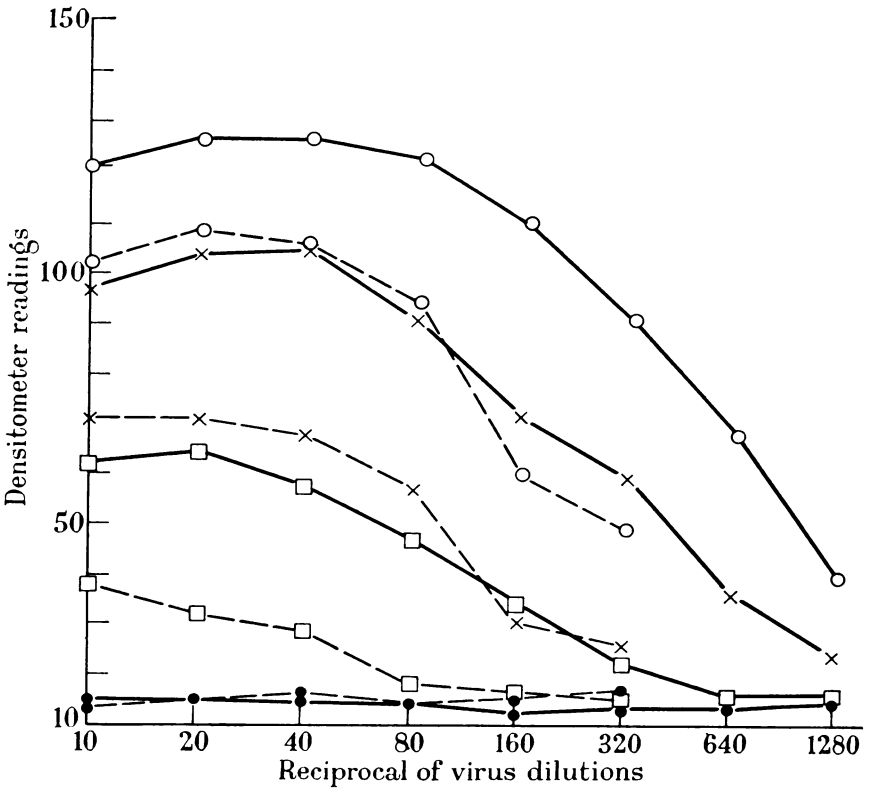

FIG. 2.—The temporal evolution of haemagglutinin titration curves with untreated and ether-treated virus.

●——● 15 minute readings.
⊡——⊡ 45 ,, ,,
×——× 75 ,, ,,
○——○ 105 ,, ,,

Unbroken lines = untreated virus.
Broken ,, = ether-treated virus.

cell suspensions containing various proportions of sensitive and unsensitive cells, the latter being obtained by means of purified *V. cholerae* receptor-destroying-enzyme prepared by the method of Burnet and Stone (1947). It was found that in such systems acceleration of sedimentation begins just as quickly as with normal cell suspensions due to the aggregation of the virus-sensitive cells which settle out to leave a slowly falling column of insensitive cells in suspension. This results in a deceleration of the reaction at a fairly early stage, a phenomenon never observed in ether-treated virus haemagglutination. In an ether-treated virus system acceleration of sedimentation is delayed but once having begun the

reaction proceeds throughout at the normal rate, indicating that the cell aggregates form more slowly than in an untreated virus system but that when formed they are of normal size. Possible reasons for this slower reactivity are discussed below.

The Effect of Ether Treatment on the Virus Complement Fixation Antigen.

While this work was in progress Hoyle (1950) reported that the complement-fixing potency of influenza virus was reduced by ether treatment. Our findings confirm this but their chief interest is that they show a dissociation of the effects of ether treatment on the virus complement-fixing potency and the haemagglutination activity in a manner directly contrary to that induced by heat inactivation. It should be emphasized that the complement-fixing antigens concerned are the strain-specific surface antigens of the virus elementary bodies, not the soluble antigens which are common to all strains within each of the major types A and B. The virus preparations used were purified eluates prepared from infected allantoic fluids ; such preparations are free from demonstrable soluble antigen.

Saline eluates of PR8 virus were extracted with ether by the method described above. The ether phase was evaporated at 30–35° under reduced pressure until the small fluid residue was devoid of any smell of ether ; this residue was then made up to the original volume with physiological saline. Some precipitate always formed in the aqueous phase ; this was removed by centrifugation and re-suspended in the original volume of saline. The clear supernatant fluid from the aqueous phase was considered to contain the degraded virus particles. The three fractions and a sample of the untreated virus eluate were tested for complement-fixing potency and were also titrated in parallel for haemagglutinin. The results of four experiments are summarized in Table III.

TABLE III.—*Effects of Ether treatment on C.F. Potency and Haemagglutinin Activity.*

		Complement fixing potency.*				Haemagglutinin titres.				
			After ether extr.					After ether extr.		
Virus preparation.	Time of ether contact.	Before ether extr.	Aqueous layer.	Ether layer.	Ppt.	Fowl cells used.	Before ether extr.	Aqueous layer.	Ether layer.	Ppt.
PR8/ES133	10 min.	30	20	0	0	pool	680	>256	6·6	<4
PR8/ES133	Overnight	22	6	0	0	pool	765	469	17	6
PR8/ES153	,,	26	13	nt	nt	1532	482	120	nt	nt
						1536	526	63	nt	nt
PR8/ES154	,,	28	4	nt	nt	1532	523	109	nt	nt
						1536	485	<40	nt	nt

* The units of potency are arbitrary and are expressed on a logarithmic scale.

0 = no activity.
nt = no test.

In view of the effects of ether treatment on the haemagglutination reaction it is obvious that direct quantitative comparisons between residual haemagglutinin and residual complement-fixing antigen are not valid. Nevertheless, even when haemagglutinin titrations were done with fowl cells least sensitive to ether-treated virus the reduction of complement-fixing potency was never less than the

reduction of haemagglutinin activity and was usually very much greater. Sometimes indeed preparations were obtained which were entirely devoid of complement-fixing activity while retaining appreciable haemagglutinin activity. The reverse effect never occurred. Yet this is precisely the effect which was always obtained by sufficient heat treatment of the virus. PR8 eluates were heated at 60° until all detectable haemagglutinin had been destroyed (titre < 2); they were then found to have retained about a quarter of their initial complement-fixing potencies. Eluates were heated at 56° for short periods so as to leave considerable residual haemagglutinin activity. Unheated and heated samples were then diluted so as to contain the same haemagglutinin concentration, namely 32 AD/ml., and compared directly in complement fixation tests. Two such experiments are summarized in Table IV. In both, the heat degradation caused an apparent increase of complement-fixing potency. Such apparent increase would of course result from a more rapid destruction of haemagglutinin than of complement-fixing antigen and this explanation was further supported by experiments in which virus was heated for various periods at different temperatures and complement fixation tests done in parallel on all the preparations after adjusting them to equivalent haemagglutinin content. The ratio of complement fixation units to AD showed a steady increase with increasing heat treatment.

TABLE IV.—*Relative Resistance of C.F. Antigen to Heat Inactivation.*

	Haemagglutination titres.		Complement fixing potency.*	
Virus preparation.	Before heating.	After heating.	Before heating.	After heating.
PR8	341	248	11	15
	408	147	10	12

* After adjustment of antigen to 32 AD/ml. using heated HA titre.

The Effect of Ether Treatment on the Receptor-Destroying Enzyme of the Virus.

Previous work had shown that ether-treated virus was more sensitive to normal serum inhibitor than untreated virus, although it retained considerable enzymic activity. This was advanced as conclusive evidence that the "indicator state" could not be dependent on destruction of virus enzyme, as was then generally believed (Smith *et al.*, 1951). Table V summarizes experiments in

TABLE V.—*Conversion of Virus to Indicator State by Ether-treatment.*

Exp. No.	Virus strain.	Inhibitory titres of normal rabbit sera against viruses		
		Untreated.	Heated.	Ether-treated.
1	PR8	<40	500	116
2	,,	<40	344	265
3	,,	32	377	1006
4	,,	<40	—	320
5	,,	<20	—	208
6	,,	<40	160	320
7	PR8/X	<40	<40	160
8	,,	<40	<40	320

PR8/X = a mutant of PR8 (Smith *et al.*, 1951). All the ether-treated preparations were shown to be enzymically active.

which ether-treated, heat-treated and untreated viruses were tested against normal rabbit serum. Without exception the ether treatment converted virus to the " indicator state " and in several instances produced a much more effective indicator virus than did heat treatment.

The function of the virus enzyme in the processes of infection has been the subject of much speculation but is still unknown. That it must play an important rôle is suggested by the fact that its destruction is invariably associated with loss of infectivity. Further study of its dissociation from haemagglutinin activity as a result of ether treatment was clearly indicated.

Quantitative assessments of the enzymic activity of virus preparations were made by measuring the proportion of virus which eluted after its adsorption on red cells. Packed washed fowl cells were added to equal volumes of virus, untreated and ether-treated, to give final cell concentrations of approximately 4 per cent. Adsorption was allowed to proceed for 30 min. at 4° ; then the cells were centrifuged out and re-suspended in saline to the original volume. The suspensions were incubated at 37° for 2 hr. after which the cells were removed by centrifugation. The supernatants were titrated in parallel for haemagglutinating activity.

The results of several such experiments on both PR8 and Lee viruses are presented in Table VI. They show that neither short (10 min.) nor prolonged (overnight) ether treatment markedly impaired the eluting powers of PR8 virus. This was in marked contrast to the behaviour of Lee virus, for which even 10 min. contact with ether caused at least 70–75 per cent reduction of eluting power and usually complete loss.

TABLE VI.—*Retention of Enzyme by Ether-treated Virus.*

Virus strain.	Passage No.	Period of ether contact.	Haemagglutination titres (ether-treated). Before absorption.	After elution.	Percentage yield on elution.
Type A	ES 157	10 min.	930	832	89
	ES 157	Overnight	452	395	88
	ES 161	10 min.	417	389	93
	ES 165	Overnight	125	94	75
	ES 169	10 min.	133	144	100
	ES 171	,,	217	77	36
PR8 mutant	EX 182	10 min.	996	862	86
Type B " Lee "	EB 9	10 min.	105	0	0
	EB 13	,,	160	30	18
	EB 14	,,	124	<20	—
	EB 15	,,	391	109	28

This method of estimating enzymic activity has a serious limitation. It depends on the retention of haemagglutinin activity by the eluted virus particles. Any virus which had suffered loss of its haemagglutinin receptors during the process of elution would be undetectable. Hence a dissociation of haemagglutinin and enzyme susceptibility to ether would tend to be obscured.

As an alternative the direct method of Lanni, Lanni and Beard (1951) for measuring enzymic activity was used. Virus preparations were mixed with egg

white and held at room temperature to allow progressive destruction of the egg white inhibitor. Aliquots were removed at various intervals and boiled for two min. to inactivate the virus. Finally all samples were titrated in parallel for residual inhibitor using heated PR8 virus as the indicator virus. It was found that within a certain range of virus dosage there was a linear relationship between inhibitor destruction and time so that a direct measurement of reaction velocity was possible. By this means it could be shown that ether-treated PR8 virus retained far more enzymic activity than could be accounted for by the residual haemagglutinin content. Clearly the virus haemagglutinin had been reduced preferentially in relation to the virus enzyme. Incidentally it was shown that the eluted ether-treated virus was completely non-infective ; an unknown infectivity factor must thus be more susceptible to destruction by ether than are haemagglutinin, complement-fixing antigen and virus receptor-destroying enzyme.

DISCUSSION.

Three important biological activities of the influenza viruses, specific complement fixation, haemagglutination and enzymic destruction of host cell receptors, are dependent on the integrity of surface components, but it is still unknown how far these components are independent or linked together in composite structures. It has been suggested that the enzyme may be an essential part of the virus haemagglutinin receptors which thus serve the dual purpose of cell-virus linkage and subsequent cell receptor destruction to facilitate cell penetration. Certainly separation of enzyme from the virus particle has not yet been accomplished. Our results, however, prove that haemagglutinin inactivation is not necessarily associated with loss of enzyme. Whereas virus treatment by heat or ultra-violet light invariably produces the inactivation sequence enzyme-haemagglutinin-C.F. antigen, exactly the reverse order of inactivation results from ether treatment. This suggests that the three types of biological activity may be functions of specific and distinct structures on the virus surface. Our results could equally well be due to the preferential destruction or distortion of subsidiary parts of a single complex surface entity by the different inactivating agents employed. If increased susceptibility of heated virus to non-specific inhibitors is a function of surface distortion as claimed by Smith and Westwood (1950) it follows that ether treatment must also induce such distortion because one of its effects is conversion of the virus to the indicator state. Sterically different surface distortions induced by different inactivating agents would adequately account for the observed phenomena.

A satisfactory interpretation of the qualitative change of haemagglutination behaviour induced by ether treatment demands a fuller elucidation of the mechanism of the optimal-reading phenomenon. This phenomenon is not merely an example of the immunological optimal proportions reaction dependent upon equivalence of antigen and antibody, for haemagglutination always appears first in the tube containing the highest concentration of virus. The rate of sedimentation in this tube, however, is outstripped by that occurring in the optimal tube. Neither can the inhibition zone be due to rapid enzyme action on red cell receptors when virus is present in excess, because such zones also occur in titrations carried out at 4°. As yet we have no satisfactory explanation to offer.

The evidence suggests that one effect of ether treatment of virus is to slow the speed of virus-red cell union. The most obvious way in which this could be

achieved in a mixture of fixed proportions of virus and cells would be by total inactivation of a proportion of the virus particles. That this is not the true explanation of the ether effect is clear from the absence of any shift to the left of the full haemagglutinin titration curve in many of our experiments. The speed of union and resulting aggregate formation however might equally be affected by the partial destruction of the receptors on each individual virus particle. If so, every particle would still be capable of linkage to red cells but enough linkages for aggregate formation would take considerably longer than with normal virus. It is possible that this intermediate stage always occurs in the inactivation of individual virus particles but that with heat treatment invariably, and with ether treatment sometimes, the phase of partial degradation is overshadowed by the rapidity with which it passes on to the phase of complete inactivation. An alternative explanation is the hypothesis of steric hindrance from surface distortion (Smith and Westwood, 1950). Extraction of ether-soluble lipids from the virus cannot fail to produce surface distortion and this may be of a type sterically different from that induced by heat. Quite small differences may well determine whether the receptors are unable to unite with the red cell receptor or are still able to do so though with difficulty. The highly variable effects of ether treatment would not be difficult to understand on this basis for the precise pattern of the induced distortion would depend on the original surface pattern of the treated virus. Our scanty knowledge of the phenomena associated with virus replication makes it extremely improbable that the surface pattern remains completely static throughout the reproduction cycle so that no matter how rigorously cultural conditions are standardized different batches of one and the same virus strain are unlikely to be absolutely identical. The complement fixation reaction, on the other hand, is not dependent on the pattern of the antigen in relation to other surface components and so is uniformly affected by ether treatment.

The anomalous haemagglutination behaviour of ether-treated virus is of some practical significance. With any method of haemagglutinin titration in which readings are taken before completion of cell sedimentation, the titre must be affected by the velocity of the reaction. The fact that reaction velocity may be affected by qualitative changes in a virus, independent of any quantitative reduction of its haemagglutinin, introduces a source of possible error into estimations by the photo-electric densitometer method. The precision of this method may therefore sometimes have to be sacrificed in favour of cruder methods in which titres are unaffected by reaction velocity.

SUMMARY.

The effects of ether treatment of influenza virus were variable in spite of rigorous standardization of experimental conditions. This is probably due to slight variation in different batches of a single virus strain.

The inactivation sequence with ether treatment is, complement fixation-haemagglutination-enzyme activity, which is the reverse of the sequence induced by heat or ultra-violet light inactivation.

Ether treatment causes a qualitative change of virus haemagglutination behaviour irrespective of any quantitative reduction of haemagglutinin. Speed of cell aggregate formation and hence of sedimentation is reduced. This affects

the position of the titration curve in relation to its co-ordinates in a manner different from that produced by quantitative reduction and hence also affects the apparent titre of the virus.

Some of the implications of the effects of ether treatment of virus are discussed.

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