# NASAL MUCUS AND INFLUENZA VIRUSES

## I. THE HAEMAGGLUTININ INHIBITOR IN NASAL SECRETIONS

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(With 2 Figures in the Text)

In the course of studies on experimental influenza in mice it was observed that the haemagglutinin inhibitor, normally present in bronchial washings, disappeared in the early stages of infection (Fazekas de St Groth, 1950*a*). The absence of the inhibitor showed good correlation with the presence of active virus in the respiratory tract, and since the virus is capable *in vitro* of enzymically destroying the inhibitory capacity of bronchial mucus, it was assumed that the same reaction took place *in vivo*. Consequently, the drop in inhibitory titre can be regarded as a measure of virus activity in the air passages, and hence a diagnostic sign of influenza infection. Indeed, it has been shown (Fazekas de St Groth, 1950*b*) that the experimental disease of mice can be recognized as readily by this criterion as either by the recovery of virus from the lung tissue during the disease, or by the evaluation of macroscopic lesions seven days after infection, or, retrospectively, by the demonstration in the serum of specific antibodies in the stage of convalescence.

These findings prompted an attempt to develop a similar method for the diagnosis of human influenza, and the 1950 epidemic offered an opportunity to test the proposition. The work consisted of two, essentially independent, phases. First, the characterization of normal human respiratory mucus as regards its reactions with influenza viruses—the subject of the present communication. Secondly, observations made on established cases of influenza during the epidemic—to be reported in the accompanying paper. A preliminary note, containing a summary of the results, has appeared (Fazekas de St Groth, 1951).

### MATERIALS AND METHODS

*Normal saline* contained 0·9 % sodium chloride in distilled water.

*Red blood cells* were prepared from venous blood collected in 2 % sodium citrate. The erythrocytes were washed with three changes of normal saline, packed and stored at 4° C. Suspensions of required strength, specified below, were made up shortly before use. To avoid lipid agglutination (Stone, 1946), only 'lipid insensitive' fowl cells were used; in the case of human cells such a precaution is unnecessary, since they are uniformly insensitive to lipid agglutinins.

*Viruses* used in this study were grown allantoically or amniotically by the standard methods laid down by Beveridge & Burnet (1946). The history of the early strains has not been traced—it will suffice to say that all were isolated in ferrets and had numerous passages in mice and eggs. The more recent strains (from

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1942 onwards) were all isolated amniotically, and were not adapted in the laboratory to mammalian hosts.

The viruses used in form of allantoic fluids were:

*Type A strains*

| | |
|---|---|
| WSE | Substrain of WS, the prototype of influenza A virus (Smith, Andrewes & Laidlaw, 1933), adapted by extensive serial passage to grow and produce pocks on the chorio-allantois (Burnet, 1936) |
| PR8 | Francis (1934) |
| MEL | Burnet (1935) |
| MEL(m) | A mutant of MEL (Isaacs & Edney, 1950) |
| BEL | Burnet, Beveridge, Bull & Clark (1942) |

*Type A-prime strains*

| | |
|---|---|
| CAM | Anderson (1947) |
| FM1 | Prototype of A-prime virus (Rasmussen, Stokes & Smadel, 1948) |
| OcIs | Isaacs, Edney, Donnelley & Ingram (1950) |
| CHOM | A pre-epidemic strain in 1950 (Anderson, French & Kalra, 1952) |
| PHIL | One of the 'main wave' epidemic strains in 1950 (Anderson *et al.* 1952) |

*Type B strains*

| | |
|---|---|
| LEE | Prototype of influenza B virus (Francis, 1940) |
| MIL | Burnet, Stone & Anderson (1946) |

*Swine influenza*

| | |
|---|---|
| SW | The classical 'Strain 15' of Shope (1931) |

Besides these some A-prime strains were available in the O- or intermediate-phase, and were used in the form of amniotic fluid preparations.

*Receptor destroying enzyme* (*RDE*) of *Vibrio cholerae* was prepared by the method of Burnet & Stone (1947).

*Indicator-viruses* for the titration of inhibitors were obtained from all strains of active virus by a method which, in principle, is identical with that proposed by Stone (1949*a*, *b*). The details were slightly modified in order to obtain a treatment uniform for all viruses. 1·0 ml. of 20% sodium citrate in distilled water and 1·0 ml. of a 0·15 M-borate buffer at pH 8·5 were added to 10·0 ml. volumes of the respective virus preparations (preferably of high titre), and the mixtures were incubated in a water-bath for 30 min. at 56° C. This treatment is adequate for all strains, except WSE and SW. The latter two, having a lower heat resistance, were incubated for the same period (30 min.) at 52° C.

The indicator viruses will be designated by the name of the strain and the affix 'indicator-', or '-i' for short. Thus, for instance, WSE virus heated at 52° C. for 30 min. in citrate at pH 8·5 is *indicator-WSE* or *WSE-i*; or PR8 after the same treatment, but heated at 56° C., becomes *indicator-PR8* or *PR8-i*; and so on. This uniform notation is meant to simplify and replace the one used by Stone (1949*a–c*), and is being proposed with the consent of that author.

*Titration of haemagglutinin.* The small-volume-technique, a modification of the Hirst-Salk test, used extensively in previous work, has been adopted as standard technique.

In round-bottomed tubes of 10 mm. internal diameter serial twofold dilutions are set out in 0·25 ml. volumes of 0·5 % fowl cells, and the result is read by the pattern of settled cells after 30 min. standing at room temperature. Partial agglutination marks the end-point; the titre is the reciprocal of this dilution. One agglutinating dose (AD) in this test is defined as the amount of virus which causes partial agglutination of 0·25 ml. 0·5 % fowl red cells.

*Titration of haemagglutinin inhibitor.* It is common experience that 'weak' inhibitors, or inhibitors partially destroyed by viral enzymes, do not give clear-cut end-points, the range of partial and atypical agglutination extending over several tubes. The reason for this resides in the fact that the long sedimentation period required by the orthodox three-volume-test (0·25 ml. each of inhibitor, indicator-virus and red cells) allows for the dissociation of the complex of virus and inhibitor. Thus near the end-point dilution the cells which reach the bottom of the tube early in the 75 min. settling period form a central button (no agglutination = inhibition), while those arriving late are agglutinated by the virus which has become free during the long period available for equilibration. This shortcoming of the original technique (Burnet, 1948) became particularly manifest in the present studies, which were concerned specifically with the titration of partially degraded inhibitors.

A small-volume-technique has been devised therefore which eliminates this source of inaccuracy. The sample to be tested was diluted serially in 0·25 ml. volumes of normal saline and then one standard drop (0·025 ml. from an appropriate Pasteur pipette) containing 5 agglutinating doses of an indicator-virus was added. After an incubation of 30 min. at room temperature 0·025 ml. of a 5 % fowl red cell suspension was added (equivalent to 0·25 ml. 0·5 % cells) and the final reading made 30 min. later. Partial agglutination marked the end-point.

It can be seen that the absolute amounts of reagents were the same as in the test used by Burnet, and only the volume of diluent was reduced from 0·75 to 0·30 ml., thus reducing the interval between addition of red cells and the reading of the test by 45 min. (60 %). The use of standard drops for the addition of agglutinating doses and erythrocytes renders the test both simpler to perform and more accurate, inasmuch as drops from the same pipette are more uniform than volumes measured and delivered by hand.

As inhibitory titres of any mucoid vary, depending on the indicator-virus used in the test, when referring to titres it will be specified which indicator was used in the titration; thus WSE-titre, PR 8-titre, etc. By the same token, WSE-inhibitor, PR 8-inhibitor, ...; the use of this latter short-hand for 'inhibitory activity against WSE-i, PR 8-i, ...' does not imply, however, that the inhibitor is regarded as a mixture of separate entities, each of them possessing specific activity against one indicator. On the contrary, as demonstrated by Stone (1949*c*), titration of an inhibitor against several indicators measures but different aspects of a single component.

## EXPERIMENTS

### *Collection of samples*

As it is not feasible to collect routinely bronchial secretions from humans, the experimental material consisted of mucus taken on nasal swabs. The justification for this choice lies in the fact that the properties of nasal mucus and bronchial mucus (as tested on post-mortem material) were indistinguishable in their haem-agglutinin-inhibiting reactions, and that in the few instances when the isolation of virus was attempted from nasal material it was as successful as from throat washings, i.e. virus was present in the nose during the acute phase of influenza infection.

On the assumption that a standard-sized swab might collect a standard amount of mucus, initially 1 cm.$^2$ sized pieces of absorbent cotton cloth were introduced through the nares into the lower nasal passages and left there for 1 min. However, it became immediately evident that there was up to twenty-fold variation between samples collected from the two sides of the nose, or from the same side by taking hourly swabs. Consequently, as this tedious technique offered no advantage, the following simple method was adopted for all later tests.

Around one end of a wooden swab stick (20 cm. long, of 2 mm. diameter) was wound a small wad of surgical cotton-wool, making the swab proper 15 mm. long and of 4 mm. diameter. A large number of such swabs were put in screw-top jars and sterilized by autoclaving. To collect a sample of nasal mucus the swab was introduced horizontally into the lower nasal passage, pressed lightly against the septum, rotated once, and kept till further use in a sterile test-tube labelled with the patient's name. In the laboratory the end of the swab stick with the cotton-wool was inserted into a Wassermann-tube (70 × 10 mm.), broken off, and soaked in 2·0 ml. of normal saline. The extracting fluid, of which 1·75 ml. could be pipetted off easily, will be called '*undiluted nasal mucus*'.

### *Inhibitory titre of normal nasal mucus*

In the present study nasal mucus of nominally normal adults (Institute scientific staff) was used only, the work having been done entirely in an interepidemic period. The swabs were left soaking in saline for 1 hr., then the fluid was removed, and tested for inhibitory activity on the same day by the standard technique. Each sample was titrated against two types of indicator-virus, MEL-i and LEE-i. A representative set of results is tabulated below.

Table 1 contains the following points of interest: (1) the titre of any one sample depends on the indicator-virus used in its testing; (2) the titres with any one indicator vary widely, not only among different persons but between swabs from the same donor on different days, or on the same day but at hourly intervals, or simultaneously but from the right and left nasal passages; and (3) the ratio of the titres obtained with the two indicators is practically constant, and does not depend on the absolute titres of the sample.

These findings eliminate the possibility of using the absolute titres with any likelihood of reproducibility. On the other hand, they suggest another method of

evaluation. As the *ratio of titres* was found to be constant, both for tests on several samples from the same person and for different individuals, it appeared promising to continue with the investigation along this line.

Table 1. *Absolute inhibitory titres of nasal mucus*

| Time of washing (hr.) | Sample: Nasal passage | I | | II | | III | | IV | | V | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MEL-i | LEE-i | MEL-i | LEE-i | MEL-i | LEE-i | MEL-i | LEE-i | MEL-i | LEE-i |
| 0 | Right | 84 | 42 | 184 | 68 | 200 | 92 | 46 | 22 | 114 | 46 |
| | Left | 37 | 18 | 34 | 12 | 300 | 120 | 100 | 40 | 80 | 40 |
| 1 | Right | 70 | 20 | 75 | 45 | 45 | 23 | 140 | 75 | 80 | 37 |
| 2 | Right | 50 | 16 | 32 | 18 | 72 | 32 | 40 | 12 | 40 | 16 |
| 24 | Right | 160 | 50 | 40 | 20 | 280 | 80 | 460 | 220 | 80 | 33 |
| | Left | 120 | 50 | 40 | 20 | 210 | 120 | 210 | 100 | 320 | 115 |
| 48 | Right | 75 | 25 | 37 | 18 | 400 | 160 | 400 | 160 | 300 | 80 |

Each sample was titrated against ≡ 5 AD of the two indicators.

*The 'indicator spectrum' of nasal mucus*

As has been established by Stone (1949*b*) for several mucoids, the pattern of inhibitory titres obtained when testing against an extensive set of indicators is characteristic of the mucoid, and may be called its 'indicator spectrum'. It is independent of the absolute titres, and can be expressed therefore most conveniently as the ratio of titres.

To determine the indicator spectrum of nasal mucus, several samples were diluted in ten parallel rows and titrated against ten types of indicator by the standard method. The results are presented in Table 2.

Table 2. *The indicator spectrum of normal human nasal mucus*

| | Inhibitory titre against indicators | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample | WSE | PR8 | MEL | MEL(m) | BEL | CAM | FM1 | LEE | MIL | SW |
| I | 320 | 720 | 900 | 960 | 280 | 1200 | 960 | 320 | 240 | 1600 |
| II | 64 | 140 | 200 | 200 | 64 | 250 | 200 | 64 | 50 | 320 |
| III | 80 | 200 | 200 | 250 | 80 | 400 | 250 | 75 | 60 | 530 |
| IV | 230 | 520 | 600 | 800 | 200 | 800 | 600 | 160 | 160 | 1100 |
| V | 150 | 360 | 360 | 400 | 150 | 600 | 440 | 160 | 100 | 600 |
| Arithmetic mean | 169 | 388 | 452 | 522 | 155 | 650 | 490 | 156 | 122 | 830 |

Each sample was titrated against ≡ 5 AD of each indicator-virus.

Taking the lowest titre (against MIL-i) arbitrarily as unity, the ratios

MIL : BEL : LEE : WSE : PR8 : MEL : FM1 : MEL(m) : CAM : SW

are

1·0 : 1·3 : 1·3 : 1·4 : 3·2 : 3·7 : 4·0 : 4·3 : 5·3 : 6·8.

The fact that the indicator spectrum of any sample of normal nasal mucus does not differ significantly from the average, i.e. the variance of the individual ratios does not exceed the variance of replicate inhibitor titrations, is a valid confirmation

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of Stone's thesis. Moreover, it is a corollary of this finding that, if the inhibitors are acted upon by viral enzymes or the 'receptor destroying enzyme' (RDE) of *V. cholerae*, the pattern changes as the adsorptive power for different indicator viruses is reduced in a differential manner. It might be expected, then, that enzymic action on the nasal inhibitor could be detected from an alteration of the normal pattern.

*The 'inhibitor gradient' of nasal mucus*

In determining how the inhibitory pattern would change during enzymic destruction, use was made of the kinetic test designed by Stone (1949*c*) for similar purposes. In principle, the inhibitor is incubated with an enzyme preparation, at intervals samples are taken, and the remaining inhibitor titrated against a series of indicator-viruses. From the data the percentage of inhibitor destruction can be plotted against time, the slopes of the curves corresponding to the sensitivity of the mucoid to the various viruses. The viruses in order of their increasing action on the mucoid form the 'inhibitor gradient' (Stone, 1949*c*), which is just as characteristic for any inhibitor as is the 'receptor gradient' (Burnet, McCrea & Stone, 1946) for different types of erythrocytes (Stone, 1947).

Such an experiment is practicable, of course, only if the action of the enzymes can be stopped at will without impairing the inhibitor. This can be achieved in the simplest way by heating the virus-inhibitor mixture to a temperature that both inactivates the enzyme and destroys the viral haemagglutinin but leaves the inhibitor intact. It is known that influenza viruses do not stand up to heating above 60° C. for 30 min. in saline; however, the heat stability of the nasal inhibitor was not known, and had to be assessed in a preliminary test.

Three pools of nasal mucus, each from ten persons, were heated at temperatures from 56 to 100° C. for different periods of time. After quick cooling the samples were tested against five indicators derived from classical strains of influenza virus.

Table 3. *Heat resistance of human nasal inhibitor*

| | Reduction of inhibitory titre ($\log_2$ units) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 56° C. | | 65° C. | | | 78° C. | | | 100° C. | |
| Indicator | 30 min. | 60 min. | 15 min. | 30 min. | 60 min. | 5 min. | 15 min. | 30 min. | 5 min. | 15 min. |
| WSE-i | 0 | 0·1 | 0·3 | 1·3 | 1·9 | 1·7 | 3·3 | 4·2 | 2·9 | 4·9 |
| PR 8-i | 0 | 0 | 0 | 1·6 | 1·3 | 1·0 | 1·5 | 2·1 | 1·9 | 3·2 |
| MEL-i | 0 | 0 | 0·1 | 1·0 | 1·5 | 1·1 | 1·8 | 2·4 | 2·0 | 3·6 |
| LEE-i | 0 | 0 | 0 | 0·8 | 1·0 | 0·7 | 1·0 | 1·4 | 1·4 | 1·9 |
| SW-i | 0 | 0 | 0 | 0·9 | 1·0 | 0·7 | 1·0 | 1·6 | 1·4 | 2·1 |

Each value is the geometric mean from three pools of ten nasal swabs.

Dealing only with the practical aspects of Table 3, it will be seen that the inhibitor is completely stable at 56° C. for an hour; unfortunately, so are most of the viruses, rendering this temperature unsuitable for selective destruction. Even short treatments above 65° C. distort the indicator spectrum by causing a dis-

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proportionate drop in the inhibitory titres, e.g. the inhibitor of WSE is reduced by a factor higher than PR 8 or MEL, which in turn are less stable than LEE and SW. A compromise can be made at 65° C. for 30 min. Although the absolute titres are reduced somewhat, the distortion of the ratios is insignificant, and this treatment is known to destroy the influenza haemagglutinin; it was adopted therefore for the main tests.

For the determination of the inhibitor gradient a pool of nasal mucus was used, as in the previous experiment. Besides RDE three viruses of the influenza group were tested: MEL representing the A type, FM 1 as a typical A-prime virus, and LEE the classical B-strain.

25 ml. of inhibitor chilled to 0° C. was mixed with 3·0 ml. of an appropriate dilution of virus, in the cold. The mixture was placed in a water-bath at 37° C., and 5·0 ml. volumes were withdrawn at 0, 30, 60, 120 and 240 min. after the beginning of the experiment. The samples were heated at 65° C. for 30 min. to destroy the enzyme, chilled and titrated together at the end. Bulk dilutions in 2·50 ml. volumes of normal saline were made and then 0·25 ml. of each step distributed into ten tubes, thus giving ten sets of parallel dilutions. For each set a different indicator-virus was used. In Fig. 1 the titres are expressed as percentages of the initial titre, making direct comparison of the curves possible. Since, however, the absolute titres with the various indicators differed considerably (cf. Table 2), the range over which the test could be done was different for each indicator; yet, even the narrowest range extended over more than six $\log_2$ steps.

As regards the choice of the amount of active virus or RDE to be employed, the aim was to use an enzyme concentration which is just sufficient to bring the titres down within the experimental period of 4 hr. The dilutions required had titres of 27, 38, 2 and 0·4 for MEL, FM 1, LEE and RDE respectively.

Again leaving aside points of academic interest, the four families of curves of Fig. 1 show that, irrespective of the virus employed, it is the WSE-inhibitor which is most sensitive to enzymic destruction. The rest fall into four more or less clearly defined groups, giving the gradient WSE $\ll$ BEL < CAM, MEL(m), FM 1 < MEL, PR 8 < SW, MIL, LEE. Experiments like the above have been done also with the viruses WSE, PR 8, BEL, CAM, MIL and SW; the results were closely similar to those given in Fig. 1, the quantitative differences corresponding to the position of the respective virus in the inhibitor gradient of nasal mucus. It is worth mentioning that active WSE virus, while completely destroying (> 98·5 %) the inhibitor against WSE-i, did not alter the original titres with the other indicators, the only exception being the inhibitor against BEL-i which was reduced by 19 % in the same time. This is a further confirmation of the exceptional position of WSE-i at the top of the gradient.

### *Definition of the 'inhibitor index'*

Whenever nasal mucus comes into contact with an enzymically active virus its inhibitory titre will drop, and from knowledge of the inhibitor gradient it can be predicted that the reduction will be percentually the greatest when assessed by WSE-indicator. Such a destruction could be detected readily by the comparison

of two samples, viz. the inhibitor *before* and *after* having been acted upon. However, the same result may be obtained also by a more elegant method, by

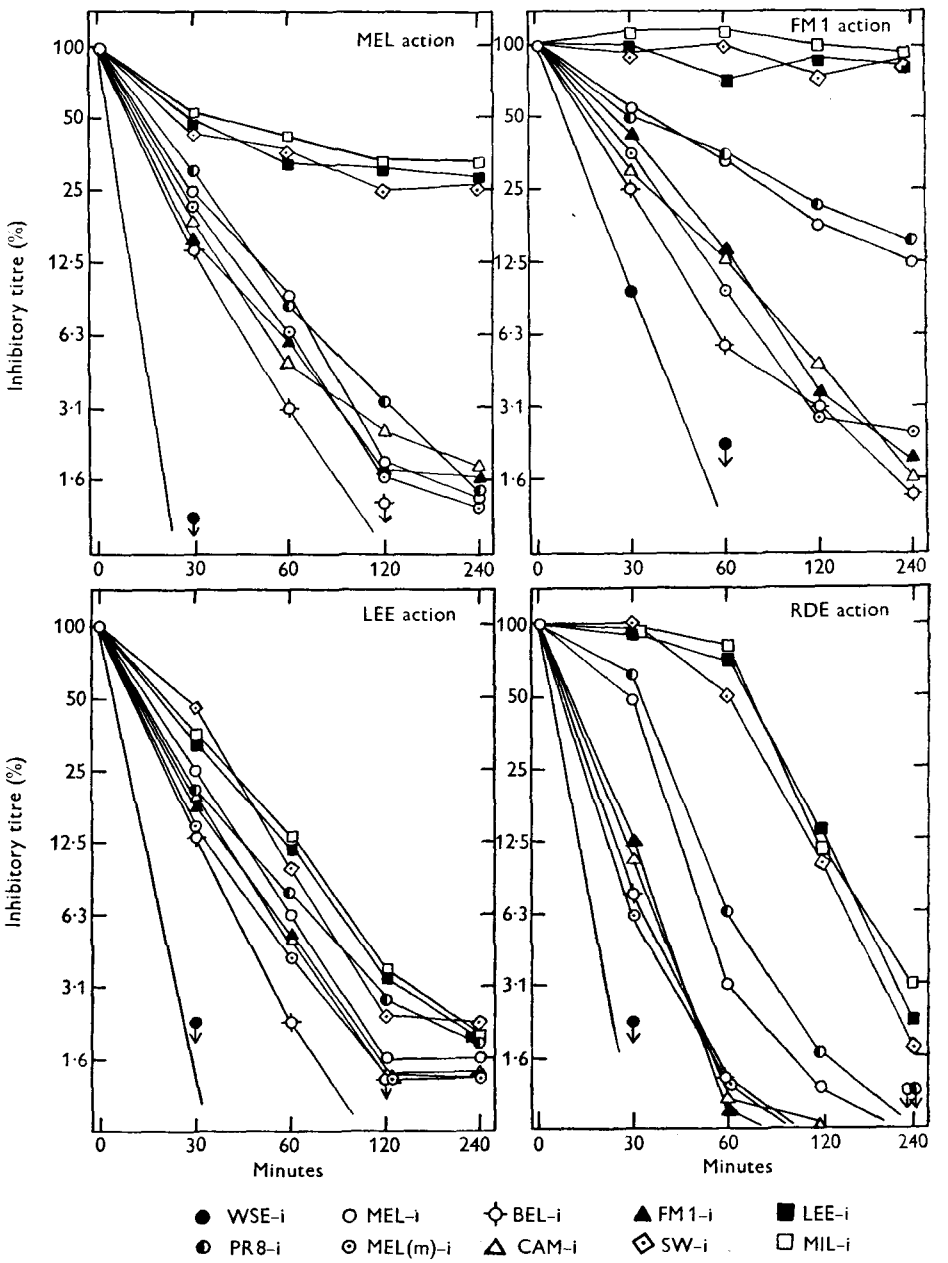


Fig. 1. Enzymic destruction of human nasal mucus. (For description see text.)

testing *only* the sample on which the enzyme had acted. If this sample is titrated in parallel against two indicators, one from the top and the other from the bottom of the gradient, the *ratio of the titres* will differ from the normal ratio given in the indicator spectrum, since the sensitive indicator will show relatively greater

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destruction. This is the principle on which rests the method devised for detecting changes in the nasal inhibitor.

As the sensitive indicator, WSE-i was the automatic choice. As regards the other, in whose terms the WSE-titre was to be expressed, two indicators were selected, MEL-i and LEE-i. The rationale of this arrangement was partly to provide a firmer basis for comparison, the variance of an average of two being lower than that of a single reading, and partly to obtain some information about the virus acting on the inhibitor, for enzymes above MEL in the gradient will widen the gap between WSE and both MEL and LEE, while those below MEL will show up by decreasing also the MEL : LEE ratio.

Accordingly, in all subsequent tests each sample was titrated in parallel against exactly five agglutinating doses of WSE-i, MEL-i and LEE-i, and the results are given in form of the *inhibitor index*, $10 \times \frac{\text{MEL-titre} + \text{LEE-titre}}{\text{WSE-titre}}$. Thus, for instance, the inhibitor index for sample I in Table 2 would be $10 \times \frac{900+320}{320} = 38$, of sample II $10 \times \frac{200+64}{64} = 41$, of sample III $10 \times \frac{200+75}{80} = 34$, and so on.

The rest of this paper will be concerned with the experimental definition of the 'normal' inhibitor index and its range of variation, and with the demonstration of changes brought about by the enzymic action of live viruses.

### *The normal inhibitor index*

(*a*) *Reproducibility of determinations.* Nasal swabs from twelve normal persons were soaked in saline for 60 min. at room temperature, and the extracting fluids titrated in four parallel tests, using the same reagents. From the titres against the three indicator-viruses (WSE-i, MEL-i, LEE-i) the inhibitor index was calculated by the method described in the previous sections. In reference to the average of each set of quadruplicate determinations the variance of the twelve samples was computed by the standard statistical procedure. In Table 4 the results are given as percentage of the respective mean, thus making direct comparison and summation possible.

The variance is of the order expected from such a test. Since the standard error of a single inhibitor titration is $\pm 16\%$ (calculated from triplicate tests on twenty-four samples, performed by four members of this Institute; unpublished), the standard deviation of the inhibitor index, which is based on the ratio of three inhibitor titrations, should be about 12%; indeed, it is $\pm 11{\cdot}4\%$. As will be shown in what follows, this value is less than the natural variance of 'normal' inhibitor indices, and thus the technique of determination will not be the limiting factor in the accuracy of the method.

(*b*) *Effect of storage.* Three swabs were taken from each of twelve normal persons. The first was soaked immediately in 2·0 ml. of saline for 60 min., after which the extracting fluid was removed from the swab and stored at 4° C. This sample was titrated on the day of collection and also on the following 2 days. The other two swabs were kept in separate tubes at 4° C. for 2 and 8 days respectively, and only

then extracted with saline and tested by the standard method. All results are expressed as inhibitor indices.

Table 4. *The normal inhibitor index*

I. Reproducibility of determinations.

| | Test | | | | |
|---|---|---|---|---|---|
| Sample | 1 | 2 | 3* | 4* | Variance (percentage) |
| I | 35 | 31 | 33 | 33 | 8·1 |
| II | 35 | 30 | 26 | 27 | 55·4 |
| III | 32 | 39 | 30 | 29 | 64·6 |
| IV | 31 | 35 | 29 | 33 | 20·8 |
| V | 39 | 43 | 37 | 40 | 17·5 |
| VI | 46 | 39 | 37 | 38 | 41·7 |
| VII | 39 | 31 | 36 | 33 | 35·8 |
| VIII | 21 | 28 | 31 | 26 | 66·7 |
| IX | 31 | 36 | 36 | 39 | 38·5 |
| X | 24 | 21 | 28 | 20 | 63·4 |
| XI | 37 | 40 | 33 | 35 | 25·0 |
| XII | 37 | 22 | 35 | 33 | 153·2 |
| | | | Av. | | 49·3 |

* Done with same reagents as 1 and 2, but 5 hr. later. Percentual variance $= \frac{\Sigma(M-x)^2}{n-1} \cdot \frac{100}{M}$, where $M$ is the mean of $n$ individual determinations ($x$).

Table 5. *The normal inhibitor index*

II. Effect of storage.

| | Length of storage (days) | | | | |
|---|---|---|---|---|---|
| | Dry swab* | | | Extract† | |
| Sample | 0 | 2 | 8 | 1 | 2 |
| I | 39 | 43 | 38 | 33 | 31 |
| II | 33 | 33 | 28 | 23 | 19 |
| III | 20 | 22 | 35 | 32 | 25 |
| IV | 27 | 45 | 33 | 41 | 45 |
| V | 45 | 40 | 24 | 36 | 30 |
| VI | 30 | 34 | 21 | 30 | 41 |
| VII | 31 | 45 | 48 | 33 | 34 |
| VIII | 33 | 32 | 34 | 40 | 30 |
| IX | 25 | 28 | 36 | 35 | 35 |
| X | 36 | 22 | 37 | 18 | 25 |
| XI | 38 | 38 | 45 | 27 | 46 |
| XII | 38 | 43 | 50 | 35 | 35 |
| Mean ± S.E. | 32·9 ± 2·0 | 35·4 ± 2·4 | 35·7 ± 2·6 | 31·9 ± 1·9 | 33·0 ± 2·4 |

* The swabs were kept at 4° C. in separate tubes for the period indicated, then soaked in 2·0 ml. saline for 60 min., and the extract tested immediately.

† The swabs were soaked in 2·0 ml. saline for 60 min., the extract removed and stored at 4° C. for the time shown.

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As shown in Table 5, neither 'dry' nor 'wet' storage alters the inhibitor index significantly. By averaging the five sets of results it also becomes clear that the readings are normally distributed around the arithmetic mean, and the areas covered by the standard errors overlap extensively. From the practical standpoint this means that swabs need not be tested on the day of collection; that several samples taken at intervals from the same person can be compared at any time chosen; and that any titration can be repeated and checked at a later date.

(*c*) *Effect of extraction time.* Swabs from normal donors were soaked in 2·0 ml. of normal saline, and the extracting fluid sampled at intervals ranging from 15 min. to 24 hr. All fluids were tested simultaneously after the conclusion of the experiment. The results are given in Table 6.

Table 6. *The normal inhibitor index*

III. Effect of length of extraction.

| | Length of extraction* | | | | |
|---|---|---|---|---|---|
| Sample | 15 min. | 30 min. | 60 min. | 120 min. | 24 hr. |
| I | 19 | 31 | 35 | 39 | 36 |
| II | 27 | 30 | 34 | 22 | 29 |
| III | 27 | 45 | 42 | 35 | 33 |
| IV | 48 | 38 | 34 | 38 | 52 |
| V | 38 | 48 | 37 | 36 | 41 |
| VI | 21 | 30 | 21 | 25 | 29 |
| VII | 27 | 24 | 18 | 27 | 33 |
| VIII | 28 | 21 | 24 | 21 | 32 |
| IX | 33 | 45 | 47 | 33 | 46 |
| Mean ± S.E. | 29·8 ± 3·0 | 34·7 ± 3·3 | 32·4 ± 3·2 | 30·7 ± 2·3 | 36·8 ± 2·7 |

* The swabs were soaked in 2·0 ml. saline at room temperature; at the time indicated samples of 0·25 ml. were withdrawn and stored at 4° C. till the end of the experiment. All titrations were done simultaneously.

The absolute inhibitory titres of each sample increased progressively during the first hour; after this there was no important further change. At the same time the ratio of titres, as expressed in the inhibitor index, remained fairly constant.

After this experiment 1 hr. was adopted as standard extraction time. Clearly, this choice is entirely optional: the only advantage it holds is to provide relatively high titres, but shorter or longer periods of extraction would be just as suitable as they do not affect materially the inhibitor index, only the absolute inhibitory titres.

(*d*) *Variation between swabs from the same person.* Under this heading three points were investigated: (1) comparison of daily samples, (2) difference between swabs from the two sides of the nose, and (3) the effect on the inhibitor index of repeated swabbing. The findings are presented in Table 7, 8 and 9.

The daily variation in inhibitor indices appears to be scattered normally about a mean. Table 7 also serves to show that the time of day has no effect on the inhibitor index of normal secretions, as the swabs were taken in the morning on days 0 and 8, at noon on day 1, and in the afternoon on day 2.

Table 7. *The normal inhibitor index*

IV. Difference between daily swabs from same donor.

| | Time of swabbing (day) | | | |
|---|---|---|---|---|
| Donor | 0 | 1 | 2 | 8 |
| I | 24 | 33 | 38 | 29 |
| II | 32 | 39 | 29 | 42 |
| III | 37 | 34 | 34 | 40 |
| IV | 30 | 45 | 42 | 38 |
| V | 30 | 28 | 34 | 32 |
| VI | 29 | 29 | 31 | 27 |
| VII | 27 | 35 | 33 | 31 |
| VIII | 31 | 40 | 42 | 30 |
| IX | 44 | 34 | 47 | 36 |
| X | 30 | 27 | 32 | 33 |
| XI | 35 | 29 | 27 | 32 |
| XII | 27 | 23 | 28 | 31 |
| Mean ± S.E. | 31·3 ± 1·5 | 33·0 ± 1·8 | 34·7 ± 1·8 | 33·4 ± 1·3 |

The right lower nasal passage of each donor was swabbed by the standard method.

Table 8. *The normal inhibitor index*

V. Difference between consecutive swabs from same donor.

| | Sample | | |
|---|---|---|---|
| Donor | 1st | 2nd | 3rd |
| I | 24 | 37 | 38 |
| II | 32 | 31 | 27 |
| III | 37 | 47 | 46 |
| IV | 30 | 35 | 33 |
| V | 30 | 31 | 34 |
| VI | 29 | 21 | — |
| VII | 27 | 31 | 42 |
| VIII | 31 | 26 | 38 |
| IX | 44 | 32 | 38 |
| X | 30 | 29 | 21 |
| XI | 35 | 26 | 31 |
| XII | 27 | 28 | 26 |
| Mean ± S.E. | 31·3 ± 1·5 | 31·2 ± 1·9 | 34·0 ± 2·2 |

The right lower nasal passage of each donor was swabbed thrice within a minute.

If several consecutive swabs are taken from the same nostril, there is a definite drop in absolute inhibitory titres in the later samples. This is due, presumably, to the decreasing amount of mucus that has been removed. In spite of these differences the inhibitor index does not change. For example, the average titre against the WSE-indicator was 114, 43 and 17 respectively for the three sets of swabs, while the corresponding indices were 31, 31 and 34.

Again, the same constancy of the inhibitor index in face of varying absolute inhibitory titres characterizes the results obtained by testing samples taken from the right and left nasal passages (Table 9).

Table 9. *The normal inhibitor index*

VI. Difference between the two sides of the nose.

| Donor | Nasal passage swabbed | |
|---|---|---|
| | Right | Left |
| I | 24 | 31 |
| II | 32 | 40 |
| III | 37 | 34 |
| IV | 30 | 39 |
| V | 30 | 23 |
| VI | 29 | 37 |
| VII | 27 | 34 |
| VIII | 31 | 39 |
| IX | 44 | 41 |
| X | 30 | 34 |
| XI | 35 | 31 |
| XII | 27 | 24 |
| Mean ± S.E. | 31·3 ± 1·5 | 33·9 ± 1·7 |

The two lower nasal passages were swabbed within 1 minute, by the standard technique.

(*e*) *Numerical value of the normal inhibitor index.* The 236 determinations on 141 samples from thirty-one individuals, as presented in Tables 4–9, provide sufficient material for definition of a valid average. Accordingly, the arithmetic mean is 33·18 with a standard deviation of ± 6·98. The values published in the preliminary note (Fazekas de St Groth, 1951), based on 137 samples from twenty-nine normal persons during the epidemic period was 33 ± 8 (or more precisely, even if not more accurately, 33·29 ± 7·83), almost identical with the inter-epidemic result. By pooling the two sets of observations the mean and standard deviation work out to 33·22 ± 7·38; this value is regarded as the normal inhibitor index of human nasal mucus, and the significance of any change brought about by the action of influenza viruses will be evaluated in terms of this norm and its variance. The frequency distribution of individual inhibitor indices and of cumulative group totals is presented in the histogram of Fig. 2.

*Effect of virus action on the inhibitor index of nasal mucus*

The final group of experiments served to assess the sensitivity of the method by determining the smallest amount of virus which is capable of significantly altering the inhibitor index of normal nasal mucus under standard conditions.

In order to avoid tedious testing by trial-and-error, extensive use was made of the Stone test for 'inhibitor destroying activity' of influenza viruses (Stone, 1949*c*). In this test serial dilutions of virus are mixed with a constant amount of inhibitor, the mixtures are incubated for a standard time at 37° C., then the virus is destroyed by heating and the samples tested for residual inhibitor by an appropriate indicator-virus. In those tubes where the active virus has reduced the inhibitory titre below a critical level the indicator will be able to agglutinate red cells added subsequently, in those tubes where enzymic destruction has been less complete there will be sufficient inhibitor left to prevent haemagglutination by the

dose of indicator virus. The end-point is that virus dilution which leaves exactly one unit of inhibitor undestroyed, i.e. the tube in which one observes partial (+) agglutination. If WSE-i is used as indicator, the end-point will show the smallest amount of virus needed to cause a significant reduction in the WSE-titre of the inhibitor. This amount of virus should be sufficient—by definition—to alter the normal inhibitor index. The residual inhibitor content of the end-point dilution and of the two neighbouring tubes was therefore titrated against the three indicators, and thus their inhibitor index determined. Such tests were performed with twelve strains of human influenza virus and with the virus of swine influenza, the final experiment being based in every instance on the results of a preliminary Stone test. To demonstrate the workings of the method, the findings with the first two viruses listed in Table 11 are given in full.

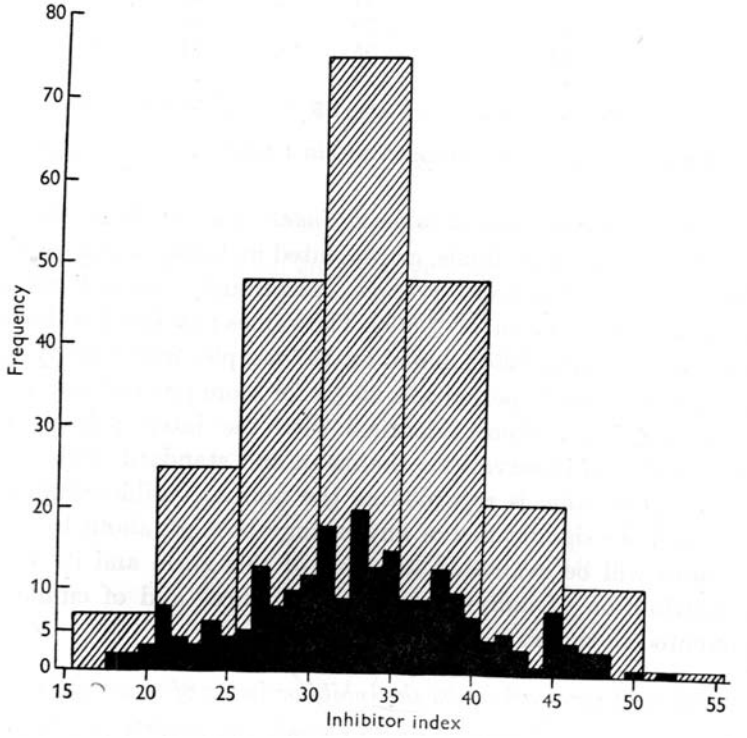


Fig. 2. Frequency distribution of inhibitor index values of normal persons. Black columns: individual readings; hatched columns: cumulative group totals.

Allantoic fluids harvested 2—48 hr. previously and containing the respective viruses were set out in doubling dilutions in 2·0 ml. volumes buffered (pH 6·2) saline, covering the range of 1 : 1 to 1 : 10,000. To each tube were added 32 units of nasal inhibitor in a 0·025 ml. drop, giving a concentration of four inhibitory doses for each 0·25 ml. This dilution was made from a stock of normal nasal secretions which had been titrated individually before pooling, and were stored in the frozen state at $-10°$ C. After thorough shaking the mixtures of virus and inhibitor were incubated in a water-bath at 37° C. for a period of 60 min. After this the racks were transferred into a 65° bath for 30 min. to destroy the active virus

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present. Following this 0·25 ml. volumes were removed into separate tubes, mixed with 5 AD of indicator-WSE, and kept for 30 min. at room temperature. Finally 0·025 ml. of 5% fowl cells were added to each tube, and the pattern of settled cells read after a further half hour. The end-point gives the 'inhibitor destroying titre' (ID-titre).

In the second part of the experiment the contents of the tube corresponding to the end-point dilution and of those on either side of it were titrated in parallel against the three indicators (WSE-i, MEL-i and LEE-i) used in the determination of the inhibitor index. If the ID-end-point has fallen between two tubes, then those two and the one following the higher dilution were tested. Since the expected maximum titre was only 4 against WSE-i, 10 against MEL-i and 4 against LEE-i, only five doubling dilutions were set out, starting at 1 : 1. From this test the

Table 10. *Assessment of enzymic efficacy against nasal mucus*

For description of technique see text.

(*a*) *Titration of inhibitor destroying activity*

| Strain | Haemagglutinin titre (HA-titre) | ID-titration 1 : 60 | 1 : 120 | 1 : 240 | 1 : 480 | 1 : 960 | 1 : 1920 | ID-titre | HA/ID |
|---|---|---|---|---|---|---|---|---|---|
| WSE | 160 | + + | + ± | tr. | — | — | — | 150 | 0·94 |
| PR8 | 360 | + + | + + | + + | ± ± | + | — | 960 | 0·38 |

Dilutions bracketed to be tested in (*b*).

(*b*) *Determination of the inhibitor index*

| Mucus pre-treated with: Strain | Dilution | Inhibitor titration: Indicator | Titre | Inhibitor index | Difference from normal inhibitor index | Efficacy (HA-titre / eff. titre) |
|---|---|---|---|---|---|---|
| WSE | 1 : 120 | WSE-i | 1·3 | 115 | Sig. | |
| | | MEL-i | 11·0 | | | |
| | | LEE-i | 4·0 | | | |
| | 1 : 240 | WSE-i | 3·0 | 57 | Sig. | $\frac{160}{240}=0.67$ |
| | | MEL-i | 12·0 | | | |
| | | LEE-i | 5·0 | | | |
| | 1 : 480 | WSE-i | 5·0 | 32 | Not. sig. | |
| | | MEL-i | 12·0 | | | |
| | | LEE-i | 4·0 | | | |
| PR8 | 1 : 480 | WSE-i | <1·0 | >160 | Sig. | |
| | | MEL-i | 11·0 | | | |
| | | LEE-i | 5·0 | | | |
| | 1 : 960 | WSE-i | 1·5 | 100 | Sig. | $\frac{360}{1500}=0.24$ |
| | | MEL-i | 11·0 | | | |
| | | LEE-i | 4·0 | | | |
| | 1 : 1920 | WSE-i | 4·0 | 43 | Not Sig. | |
| | | MEL-i | 12·0 | | | |
| | | LEE-i | 5·0 | | | |

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inhibitor index was calculated. Values exceeding the normal mean by three times its standard deviation ($33{\cdot}2 + 3 \times 7{\cdot}4 = 55{\cdot}4$) were registered as significant changes. The haemagglutinin titre of the active virus used was determined simultaneously by the standard method. The procedure can be followed from Table 10.

It was found quite regularly that the amount of virus which caused a significant rise in the inhibitor index by preferentially lowering the WSE-titre was half to one $\log_2$ unit higher than the ID-titre. Results of the complete experiment are given in Table 11.

Table 11. *Effect of viral enzyme action of the inhibitor index of nasal mucus*

I. Influenza virus strains adapted to allantoic growth.

| Strain | Type | HA-titre | ID-titre | HA/ID | Efficacy |
|---|---|---|---|---|---|
| WSE | A | 160 | 150 | 1·06 | 0·67 |
| PR 8 | A | 360 | 960 | 0·38 | 0·24 |
| MEL | A | 800 | 1100 | 0·73 | 0·40 |
| MEL (m) | A | 180 | 200 | 0·90 | 0·45 |
| BEL | A | 640 | 1100 | 0·58 | 0·37 |
| CAM | A-prime | 540 | 55 | 9·83 | 6·50 |
| FM 1 | A-prime | 400 | 320 | 1·25 | 1·00 |
| OcIs | A-prime | 420 | 150 | 2·78 | 2·10 |
| CHOM | A-prime | 160 | 400 | 0·40 | 0·25 |
| PHIL | A-prime | 240 | 720 | 0·33 | 0·20 |
| LEE | B | 400 | 1400 | 0·28 | 0·14 |
| MIL | B | 130 | 380 | 0·34 | 0·18 |
| SW | Swine | 640 | 1100 | 0·58 | 0·30 |

HA-titre = haemagglutinating titre against 0·5 % fowl cells. ID-titre = inhibitor destroying titre against WSE-i, as determined by the Stone test (1949*c*). HA/ID = smallest amount of virus (in agglutinating doses) capable of reducing the WSE-inhibitor by 80 % in 60 min. at 37° C. Efficacy = smallest amount of virus (in agglutinating doses) capable of causing significant distortion of the normal inhibitor index in 60 min. at 37° C.

All type A strains represented in Table 11 were grown allantoically, and were in the D-phase. As the human pathogen is the O-form (Burnet & Bull, 1943), a supplementary experiment was performed with the corresponding O-phase viruses available in this laboratory. From the CAM-O and OcIs-O stocks I was unable to grow a 'true O' virus (Burnet, Stone, Isaacs & Edney, 1949) which would not agglutinate fowl cells in the cold; these viruses were used therefore in the intermediate phase, showing high human and low fowl cell agglutinin titres. The technicalities of the test were the same as above.

Summarizing the results of Tables 11 and 12, it is evident that all of the strains tested show considerable enzymic action against the WSE-inhibitor of nasal secretions, and this fact is also reflected in the significant increase of the inhibitor index as compared to the normal mean. With the exception of CAM virus, which appears to have a rather sluggish enzyme both in the D and O phase, all strains are capable of destroying 75 % of the WSE-inhibitor in an hour at 37° C. when present in as small amounts as 1/15 to 1 agglutinating dose. If their action is tested by the alteration of the inhibitor index of the residual inhibitor, even smaller quantities are capable of bringing about significant changes. The higher efficiency

of the O-phase viruses might be more apparent than real: these fluids were harvested on the day of testing, while the others (Table 11) were previously stored at 4° C. for a period of 24–48 hr.

Table 12. *Effect of viral enzyme action on the inhibitor index of nasal mucus*
II. Influenza virus strains in the O and intermediate phases.

| Strain | Type | Phase | HA-titre (fowl/human) | ID-titre* | HA/ID† | Efficacy† |
|---|---|---|---|---|---|---|
| BEL | A | O | <10/96 | 1600 | 0·06 | 0·04 |
| CAM | A-prime | $\omega$ | 27/120 | 21 | 5·88 | 4·25 |
| OcIs | A-prime | $\delta$ | 60/320 | 640 | 0·50 | 0·38 |
| CHOM | A-prime | O | <10/200 | 1400 | 0·14 | 0·08 |
| PHIL | A-prime | O | <10/140 | 1200 | 0·12 | 0·06 |

* The Stone test for inhibitor destroying activity was performed with human cells instead of fowl cells.

† The titre against human cells was used in computing these ratios.

## DISCUSSION

The present study is essentially the ground-work required for the development of a potential diagnostic test based on the enzymic activity of influenza viruses. Were the conditions during the human epidemic disease comparable with those observed in experimental infection of mice, it should be expected that the large amount of active virus carried up along the respiratory tract would have some measurable effect on the mucus present, since the latter is known to contain a substrate for the viral enzyme and to act as haemagglutinin inhibitor (Fazekas de St Groth, 1948, 1950*a*). The way of detecting such a change, however, is bound to be fundamentally different in the case of mice and men. In experimental animals the respiratory mucus can be recovered quantitatively by washing the bronchial tract of excised lungs, and the inhibitor present can be expressed conveniently as a fraction of the normal, making a simple and direct comparison possible. In humans not only is this method inconceivable, but also—as has been shown above—there is no obvious means of collecting known volumes of respiratory secretions, even from the two lower nasal passages of the same person. Consequently one had to resort to some other principle on which to base a technique by which viral enzyme activity could be measured.

The most straightforward method, and one which is generally used both for qualitative and quantitative testing of enzyme action is the demonstration (and estimation) of split products. In the case of influenza virus this has been achieved with conspicuous success by Gottschalk and associates (1949, 1951). Promising as these first steps are, the chemical characterization of haemagglutinin inhibitors has not advanced far enough yet to provide means by which *minimal* action of viral enzymes could be detected. Although considerably more sensitive, the biological test based on haemagglutinin inhibitory properties of the mucus is similarly unsuitable for the present purpose. The usual practice of comparing two samples is impossible, not only because uniform swabs cannot be taken, but also because the first swab should be taken *before* infection had been initiated.

The method evolved to overcome these difficulties is based entirely on the principles defined by Stone (1949*b*, *c*, 1951) as governing the distribution of virus between red cells and mucoid inhibitors. Accordingly, each type of inhibitor has a characteristic '*indicator spectrum*': when titrated against a series of indicator viruses the titres will show a fixed ratio which is independent of the absolute amount of mucoid present. Further, if any enzymically active virus or RDE (the receptor destroying enzyme of *V. cholerae*) has been in contact with the inhibitor, its action will show up as a *differential reduction of titres* for the different indicators, that is, *the indicator spectrum will be distorted.* The order of sensitivity with which destruction can be detected by the different indicators gives the '*inhibitor gradient*' of the respective mucoid; this value, again, is characteristic of the inhibitor and independent of absolute titres.

Hence, the 'indicator spectrum' and 'inhibitor gradient' of nasal secretions were established first. Relevant results obtained in these experiments are: (1) the indicator spectrum of nasal secretions from several normal persons can be regarded as identical, and is normally distributed around a mean; (2) the WSE-indicator is the most sensitive detector of viral enzyme action;* and (3) all viruses and RDE reduce the inhibitory titre against WSE-i and distort the indicator spectrum at the same time. Based on these findings, a shortened version of the indicator spectrum was proposed for the characterization of the normal nasal inhibitor; it is termed *inhibitor index* and is given by the formula: $10 \times \frac{\text{MEL-titre} + \text{LEE-titre}}{\text{WSE-titre}}$. Since the WSE-inhibitor is the most readily destroyed, any enzyme action will reduce it disproportionately relative to the MEL and LEE titres, that is, the inhibitor index will rise.

The second part of the investigation was concerned with the statistical evaluation of the normal inhibitor index. In this section it was demonstrated that inhibitor index determinations by the standard techniques are reproducible with a standard deviation of $\pm 11{\cdot}4\,\%$, which is roughly half the normal variation of the inhibitor index in different normal specimens of nasal mucus. Thus the method is adequate—it is neither introducing a systematic error nor limiting the sensitivity of the test. The average value of the normal inhibitor index is 33·22 with a standard deviation of $\pm 7{\cdot}38$ (or $\pm 22\,\%$), the standard error of the mean, based on 373 determinations, being $\pm 0{\cdot}38$ (or $\pm 1\,\%$). In contrast to the absolute inhibitory titres which were found to vary widely, the inhibitor index is unaltered by storage of the swabs or their saline extracts; neither were any significant differences observed between consecutive or daily samples from the same person.

Incubation with active viruses, on the other hand, led to striking changes in the inhibitor index, even if only minute amounts of virus were used. From the data it would appear that with most strains of virus between 0·1 and 1·0 haemagglutinating doses are required to cause a significant increase of the normal inhibitor

* In view of the ready mutability of influenza virus strains this statement holds with certainty only for the local stock of WSE virus. The mouse-adapted variant of WS, for instance, falls in a different position in the gradient. The WSE strain used in these studies is obtainable from the Hall Institute, if requested.

index *in vitro* after an hour's treatment at 37° C. Since it is safe to assume that under natural conditions the virus will remain in contact with the inhibitor for periods longer than 1 hr., it follows with reasonable likelihood that its action will be detectable by the changed inhibitor index during the acute phase of influenza infection. However, not all strains are equally promising in this respect. CAM (A′), for one, is considerably less active than any other strain tested, and it is a most fortunate coincidence that the actual epidemic strains of 1950, whose effects were the test-case of the method, happened to be among those with the most active enzymes. As the practical evaluation of the test forms the subject of the accompanying paper, a fuller discussion of the quantitative relationship between virus and inhibitor is deferred till then.

## SUMMARY

A technique is developed by which minimal changes in haemagglutinin inhibitors can be detected.

It is shown that the *inhibitor index* $\left(\frac{10 \times \text{MEL-titre} + \text{LEE-titre}}{\text{WSE-titre}}\right)$ of normal human nasal secretions is 33·2 with a standard deviation of $\pm 7{\cdot}4$. This value is independent of the donor, of the absolute amount of mucus collected, of the time of extraction, and of the period of storage.

Minimal amounts of influenza viruses (less than one agglutinating dose) are capable of causing significant alteration in the inhibitor index upon interaction *in vitro* for 60 min. at 37° C.

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