INFLUENZA VIRUS HAEMAGGLUTINATION. THE MECHANISM OF THE FRANCIS PHENOMENON.

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THE phenomenon described by Francis (1947) served to focus attention on serum constituents, other than specific antibodies, which can inhibit influenza virus haemagglutination, more especially if the virus has been degraded by heat treatment. Susceptibility of Francis inhibitor to the destructive action of the virus enzyme, which attacks red blood cell receptors, seemed to offer an adequate explanation of the phenomenon (Hirst, 1948*b*), and more recently Stone (1949) has demonstrated in a number of strains of virus a close correlation between their susceptibility to inhibition by Francis inhibitor and the degree of inactivation of their enzymes. According to this theory, living virus is able to free itself from, and destroy, attached inhibitor, while heated virus, being without enzymic activity, is unable to do so with the result that the haemagglutinin receptors remain blocked.

This explanation is, however, untenable, since the phenomenon can be as readily demonstrated at 0° C. as at room temperature (Anderson, Burnet, Fazekas de St.Groth, McCrea and Stone, 1948; Stone, 1949). Nevertheless, it is still widely accepted, and used as a basis for further hypotheses relating to virus infectivity. In this laboratory we have recently produced by ether treatment a virus preparation which, while retaining its full enzymic activity as measured by its capacity to elute from red blood cells, is as efficient an indicator of Francis inhibitor as its heat-treated counterpart. There are numerous minor observations which conflict with the enzyme hypothesis, but these two in themselves appear to us to be conclusive.

An alternative hypothesis has been advanced by Burnet and his co-workers, that there exists a dynamic equilibrium in the competition between inhibitor and red cell for the virus receptors, the degree of inhibition reflecting the relative avidities of the virus particle for the two competing substrates (Anderson *et al.*, 1948). Burnet suggests that heat treatment so modifies the virus surface as to increase its affinity for inhibitor at the expense of the red cell.

The importance of understanding the mechanism of inhibitor action is obvious and it seemed to us that Burnet's hypothesis could be put to experimental test. It will be shown that it, likewise, is untenable, and an explanation based on the experimental findings will be presented.

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Definitions.

So much confusion exists in the literature of virus haemagglutination that the precise meaning which we intend to attach to certain terms must be made clear.

The Francis phenomenon or Francis effect was originally described in relation to Influenza B (Lee) (Frances, 1947), and a number of sera, both " normal " and immune. Exactly the same phenomenon occurs with strains of Influenza A, namely, an increase in the inhibitory action of many normal sera against a standard agglutinating dose of virus when the virus is heated, usually at 56° C. for 30 minutes. We use the terms, therefore, in respect of Type A virus and the sera of normal rabbits.

The terms " Francis inhibitor " and " non-specific inhibitor " have been the cause of much confusion. By virtue of its original connection with Type B virus, Francis inhibitor is sometimes used to denote inhibitor to B virus only. Sometimes it covers inhibitors to both A and B viruses, and again it is used to denote inhibitor to heated as opposed to living virus, the term " non-specific inhibitor " being used to denote inhibitor to the latter. We, therefore, intend to avoid use of these terms, and will use instead the term " serum inhibitor " or simply " inhibitor," without prejudice to the question as to whether inhibitors to A and B strains are qualitatively distinct.

The question of whether inhibitor to heated virus is qualitatively different from that effective against living virus is fundamental to the mechanism of the Francis response and requires more detailed consideration. Briody (1950) gives a concise review of the present position. Two qualitatively different inhibitors are held to be involved. The inhibitor of living virus, called non-specific inhibitor, acts on living A and B viruses; the inhibitor of heated virus called Francis inhibitor acts on heated A and B viruses. The demonstration of these two inhibitors, as qualitatively distinct entities is attributed to McCrea (1946, 1948) and to Burnet and McCrea (1946). The main distinction claimed to exist between the two is briefly as follows : Non-specific inhibitor of living virus is a γ-globulin, completely recoverable at 33 per cent saturation with ammonium sulphate, rapidly destroyed at 62° C. and relatively unaffected by periodate. Francis inhibitor of heated virus, on the other hand, is a mucoprotein, not completely recovered at 33 per cent saturation with ammonium sulphate, stable to 100° C. and rapidly inactivated both by periodate and the receptor destroying enzyme (R.D.E.) of *V. cholerae*.

In contradistinction to these findings Hirst (1948*a*, 1948*b*) found that non-specific inhibitor was stable to 100° C. for 15 minutes, while at 62° C. a complex reaction took place, probably involving other serum constituents. Furthermore it was destroyed by periodate and by living virus. Van der Veen and Mulder (1950) found that non-specific inhibitor was absorbed out by heated Lee B virus, was destroyed by incubation with living Lee B virus, and was destroyed by periodate and R.D.E.

A partial explanation of these contradictions may lie in the fact that McCrea's work on non-specific inhibitor was done in 1946 before the Francis phenomenon had been described, and there is no record that purified non-specific inhibitor was ever tested against heated virus. In his work on Francis inhibitor in 1948, a living A virus (BELD) and a heated B (Lee B) were used to measure non-specific and Francis inhibitor activity respectively. Living B and heated A were not used.

Thus the comparison made was not between inhibitors to living and heated virus, but between inhibitors to living A and heated B. At the same time it is stated that "even purified mucoid . . . may still show moderate inhibitory activity against haemagglutination by some active viruses" (McCrea, 1948). Moreover, since the majority of Francis inhibitor activity was recovered at 33 per cent saturation with ammonium sulphate, it follows that the fraction tested as non-specific inhibitor must also have contained the majority of the Francis inhibitor activity. There is thus no evidence that the purified mucoid is not a portion broken off, by the drastic technique of its preparation, from a larger molecule incorporating both activities.

To summarize, we do not feel that the evidence is convincing that the activities against living and heated virus are represented by qualitatively distinct inhibitors. It seems probable that McCrea's concept of a protein-mucoprotein complex is nearer the truth. The work reported in this paper has been restricted to the serum of normal rabbits and a P.R.8 strain of Influenza A virus. By "serum inhibitor" we, therefore, imply a substance or complex present in the serum of normal rabbits which is capable of inhibiting the haemagglutinating activity of the P.R.8 strain used. To what extent generalizations can be made from the mechanism of the interaction studied will be discussed in a later section.

MATERIALS AND METHODS.

Virus.

The virus used in these investigations designated P.R.8/Stock (P.R.8/S), was an egg-adapted P.R.8 strain of Influenza A obtained from the National Institute for Medical Research, Mill Hill, in 1947. It has undergone 169 allantoic passages in this laboratory. For all absorption experiments the virus was purified by adsorption on to red cells and subsequent elution into saline. The strain P.R.8/X used in preliminary experiments was a mutant strain derived from P.R.8/S, and characterized by its poor Francis response after heating.

Serum.

Normal rabbit sera were used throughout. In the early experiments sera from two individual rabbits were used. For later work two pools of sera, Pool 1 from eleven rabbits and Pool 2 from nine rabbits, were used. Both these serum pools were inactivated at 56° C., Pool 1 for 45 minutes and Pool 2 for 15 minutes. Both were filtered through 0·74μ Gradocol membranes and stored at $-$ 20° C.

Red blood cells.

Fowl cells from nine fowls were used usually in pools obtained from two to four fowls. They were stored at 4° C. as packed cells and washed before use. Cells older than seven days were not used. All titrations were carried out using fowl cells, but human Group O cells were used for most absorption experiments.

Filtration.

Gradocol membranes 0·74μ A.P.D. were used for preliminary filtration of sera and for clarification of virus-serum mixtures before their filtration through finer membranes.

Membranes of 0·2 to 0·17 μ A.P.D. were used for removal of virus from mixtures. In all cases they were found to be effective in removing all traces of virus detectable by haemagglutination. They were, however, freely permeable to serum inhibitor. Filtration was by negative pressure up to 40 cm. Hg.

Cholera enzyme.

Receptor destroying enzyme (R.D.E.), was prepared according to the method of Burnet and Stone (1947), and freeze-dried. It was used in a menstruum of borate buffer pH 6·8 with 0·1 per cent calcium chloride. Virus titrations in the presence of R.D.E. were carried out in borate buffer pH 6·8 with 2·5 per cent sodium citrate.

Haemagglutination technique.

Titrations were performed by the method of Hirst and Pickels (1942), using a photoelectric densitometer. All red cell suspensions were standardized, so that invariably a 1 in 4 dilution gave the same galvanometer reading, which was accepted as the 50 per cent agglutination end-point; the approximate concentration was 1 per cent packed cells. For virus titrations the dilutions were made in 1 ml. volumes and 1 ml. of R.B.C. suspension added. For serum inhibition titrations serum dilutions were made in 0·5 ml. volumes, 4 A.Ds. of virus in 0·5 ml. were then added and finally 1 ml. of R.B.C. suspension. Tubes were mixed by inversion, and read 75 minutes from the time of mixing.

Interpolated titres were calculated from the readings above and below the 50 per cent end-point. Extrapolation from the graph of the titration was occasionally used when the curve justified the procedure. Titres are expressed as the reciprocal of the calculated final dilution of virus or serum at the 50 per cent agglutination end-point.

EXPERIMENTAL.

Preliminary Investigations.

Effect of heat inactivation of virus.

The relationship between duration of heating virus at 56° C., and its susceptibility to inhibition by normal rabbit serum was studied. Fig. 1 shows typical response curves for both the P.R.8/S and P.R.8/X strains. The change in the virus which gives rise to the Francis effect is progressive, the serum titre continuing to rise with increased time of heating as long as the virus retains sufficient haemagglutination activity to make possible the employment of a standard dose of 4 A.D. This effect was obtained regularly in all experiments. Some irregularity in the response is frequently observed during the first hour of heating, but this does not affect the general trend of the curves. Since the power of elution from red cells of both the virus strains is consistently and completely destroyed by heating at 56° C., for 30 minutes, progressive destruction of the virus enzyme would not appear to be an adequate explanation of the phenomenon.

Francis effect at 2° C.

Comparative titrations of rabbit serum inhibitor against living and heated viruses were carried out at room temperature and at 2° C. In each case the virus

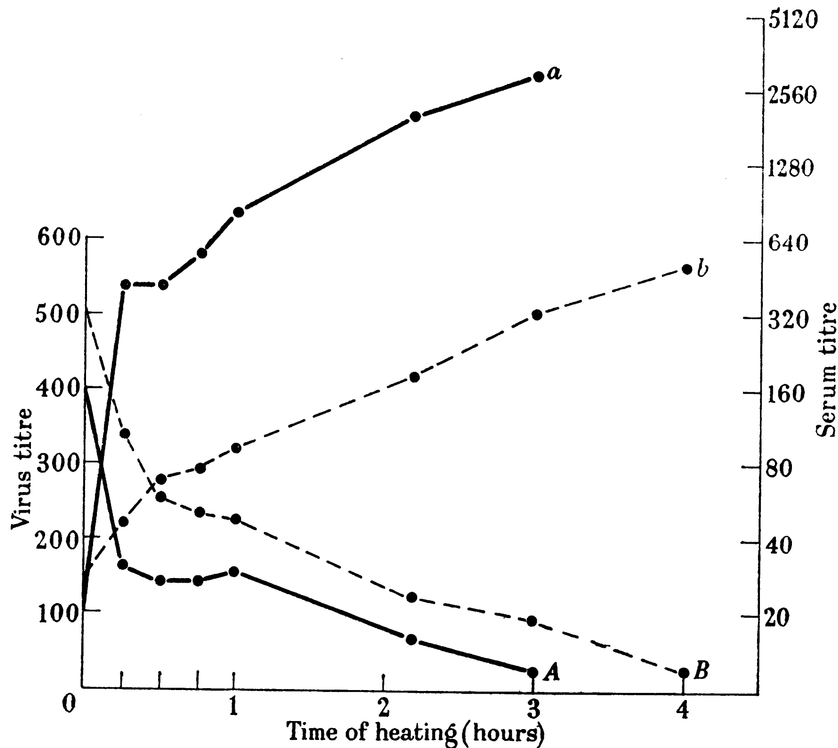

FIG. 1.—Effect of duration of heat treatment of virus on the Francis phenomenon. Curves A and B show virus titres ; curves a and b show serum inhibitor titres ; unbroken lines, virus strain P.R.8/S ; broken lines, virus strain P.R.8/X.

dose (4 A.D.) was calculated from a preliminary virus titration at the respective temperature. The results summarized in Table I fully confirm previous reports that the Francis effect occurs equally well at either temperature. Exact correspondence between the H/L ratios would, of course, not be expected, as temperature affects both the speed of reactions between the reagents and the rate of cell sedimentation.

TABLE I.—*Comparison of Francis Effects at Room Temperature and at 2° C.*

Expt. No.	Serum.	Virus strain.	Serum inhibitor titres v. 4 A.D. virus. Room temperature. L.	H.	H/L.	2° C. L.	H.	H/L.
1	Rabbit 1	P.R.8/S	49	245	5·0	95	1280	13·5
		P.R.8/X	30	57	1·9	70	121	1·7
2	Rabbit 2	P.R.8/S	50	148	3·0	160	471	2·9
		P.R.8/X	20	77	3·8	40	238	6·0
3	Pool No. 2	P.R.8/S	40	214	5·3	140	349	2·5

L = living virus ; H = heated virus.

Filterability of virus and serum inhibitor.

As a preliminary to absorption experiments with virus serum mixtures it was necessary to devise a method for the separation of free and absorbed inhibitor. This was achieved by filtration through Gradocol membranes of known porosity. Normal rabbit serum, 40 ml. of Pool 2, was filtered through a membrane of average pore diameter 0·74μ. Aliquots were then passed through membranes of A.P.D. 0·31μ, 0·24μ and 0·19μ and the four filtrates titrated against both living and heated virus. It is evident from the results shown in Table II that serum inhibitor will pass without detectable loss through membranes of A.P.Ds. down to 0·19μ. Tighter membranes were not tested, as it was found that virus was completely held back at 0·2μ, and that only about 2 per cent, as tested by haemagglutinin titres, passed at 0·24μ. Membranes of A.P.D. 0·2μ or less were accordingly used for recovery of free inhibitor in the absorption experiments.

TABLE II.—*Passage of Serum Inhibitor through Gradocol Membrane.*

Virus (4 A.D.).	Titres of serum filtrates.			
	0·74μ.	0·31μ.	0·24μ.	0·19μ.
P.R.8/S living	40	36	37	31
,, heated	238	234	261	266

Absorption Experiments.

Inhibitor absorption in absence of cells.

Burnet's hypothesis of a dynamic equilibrium demands that heated virus should possess greater affinity for inhibitor than living virus relative to their affinities for red cell receptors. In view of the extraordinary and progressive exaltation of the Francis effect on continued heating, it seemed possible that the heat treatment might increase the absolute affinity of virus for inhibitor quite irrespective of the presence of red cells in the reaction mixture. In such case the relative avidities of living and heated virus could readily be compared by titration of the filtrates of absorption mixtures for their residual inhibitory activity.

Three experiments designed to this end are summarized in Table III. In order to facilitate filtration the mixtures were passed in each case first through a coarse membrane, and then immediately thereafter through one of 0·17μ A.P.D. Filtrates were titrated against 4 A.D. heated virus. Experiment 1 shows that virus subjected to the usual heat treatment for demonstration of the Francis effect does in fact form a firm complex with inhibitor which is removable by filtration, whilst allowing free inhibitor to pass through. Experiment 2 shows that virus, even when completely inactivated so that it is no longer capable of causing any sign of red cell agglutination, can still absorb inhibitor. Experiment 3 compares the absorptive capacities of equal volumes of living and heated virus as a direct test of the thesis that heating may increase absorptive power. In this case the absorptions and filtrations were carried out at 2° C. to avoid enzymic destruction of inhibitor by the living virus. Clearly the living virus showed not less but greater absorptive power than the heated virus. Indeed, it must have absorbed nearly all the inhibitor present, for at the lowest dilution of filtrate

tested, 1 in 80, there was 71 per cent red cell agglutination compared with 79 per cent given by the 4 A.D. virus-saline control.

TABLE III.—*Absorption of Inhibitor by Living and Heated Viruses.*

	Absorbing virus P.R.8/S.				Serum absorbed.		
Expt. No.	Treatment.	Titre.	Dose. ml.	Dose. (A.D.)	Vol. ml.	Initial titre.	Residual titre.
1	Heated 56° C./30 min.	146	9	657	0·1	996	<400
2	Totally inactivated 56° C./ 7 hr.	0	5	0	0·5	387	178
3	Living untreated.	293	5	685	0·5	188	<80
	Heated 56° C./30 min.	89	5	160	0·5		97

The employment of equal volumes of living and heated virus for comparison of their absorptive powers is only justifiable on the assumption that with heat treatment all the virus elements present undergo a similar degree of degradation. An alternative possibility is that some virus may be totally destroyed and some partially degraded, in which case reduced absorptive power of a given volume would be inevitable. The Francis effect, however, is obtained when sera are titrated against standard 4 A.D. doses of both living and heated virus. Absorption tests were therefore made with the amounts of absorbing viruses equated both on the basis of their haemagglutinating activities and by volume.

Selection of dosage.—Because comparative absorption tests in which living virus is used must be done at a low temperature in order to obviate inhibitor destruction by enzyme action, the virus agglutination titres used for the calculation of absorption doses should ideally be those exhibited at the same low temperature. It was found, however, that the H/L virus titre ratios from titrations carried out at room temperature, and at 2° C. were almost identical. This was considered to justify the equating of absorbing doses on the basis of titrations at room temperature so as to reduce to a minimum the amount of work in the cold room.

TABLE IV.—*Ratios Heated/Living Virus Titres when Estimated at R.T. and at 2° C.*

Expt. No.	Virus strain.	H/L ratios. R.T.	H/L ratios. 2° C.
1	P.R.8/S	0·45	0·39
2	,,	0·64	0·61
3	,,	0·41	0·45
4	P.R.8/X	0·5	0·5
5	,,	0·39	0·46

An absorption experiment was carried out, using the following virus doses :

1. Living virus 1·7 ml. = 1600 A.D.
2. ,, ,, 0·43 ml. = 400 A.D.
3. Heated virus 1·7 ml. = 400 A.D.

Each of these was mixed with 0·5 ml. serum in a total reacting volume of 10 ml. and the respective filtrates obtained. The titration curves for residual inhibitor are shown in Fig. 2. At 400 A.D. dosage the curves of living and heated virus are practically superimposed, whereas the curves for equal volumes are widely separated. Thus A.D. for A.D. living and heated virus have exactly the same absorbing power for serum inhibitor, although the latter gives a pronounced Francis effect.

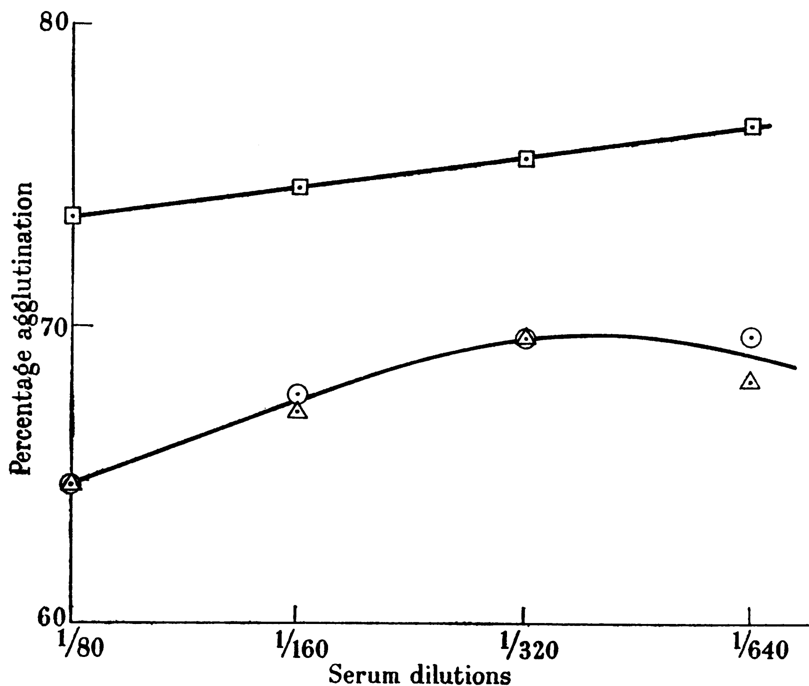

Fig. 2.—Titration curves of a serum absorbed with living and heated virus. ⊡ Living virus; absorption dose 1·7 ml. = 1600 A.D. ⊙ Living virus; absorption dose 0·43 ml. = 400 A.D. △ Heated virus; absorption dose 1·7 ml. = 400 A.D.

Inhibitor absorption in presence of red cells.

In spite of the equal absorbing potencies of living and heated virus in simple virus-serum mixtures, there remained the possibility that the two might differ in their relative avidities for red cell receptors, so that the dynamic equilibrium in serum inhibition titration mixtures would stabilize at different levels. The point was investigated by absorptions in the presence of agglutinable red blood cells. To weight the conditions in favour of the Burnet hypothesis the red cells were added to a concentration double that employed by us in all titration work. Absorption mixtures in the majority of experiments consisted of 0·5 ml. serum, 200 A.D. virus and 1 ml. packed R.B.Cs. in a total volume of 10 ml., the reagents being suitably diluted before mixing. All absorptions were done with chilled materials in the cold room at approximately 2° C. The reagents were mixed in the same order as in a normal Francis inhibitor titration, serum and virus being allowed 5 minutes' contact before the introduction of red cells. For the first

experiment pooled fowl cells were used; thereafter human group O cells were substituted as the large amounts required imposed too great a strain on the fowls available. After 15 minutes' further contact the cells were spun off and the supernatant fluids filtered through membranes of 0·2μ or 0·19μ A.P.D. in order to remove virus. At this point work was transferred from the cold room to the laboratory, where titrations of residual inhibitor were done against 4 A.D. of a suitable indicator virus, usually the same heated virus as was used for absorption. Fowl cells were used for all the titrations.

The results of four experiments, including a control experiment without admixture of red cells, are shown in Table V. The "units of inhibitor" are calculated on the basis that one unit neutralizes one A.D. of virus. At the 50 per cent titration end-point three out of four A.D. of the virus are neutralized, so that 2 ml. of the serum dilution at this end point contain 3 units of inhibitor. In all these experiments 0·5 ml. serum was absorbed, so that inhibitor content is given by the formula $0{\cdot}5 \times \text{titre} \times 3/2$. This unit is purely arbitrary, and the apparent potency of a serum will vary with the virus used for the titration; nevertheless it serves as a useful basis for the comparison of the absorption powers of viruses in any single experiment.

TABLE V.—*Absorption of Normal Rabbit Serum in Presence and Absence of R.B.C.*

		Absorbing virus.			Serum.			
Expt. No.	R.B.C.	Batch.	Nature.	Dose A.D.	Titre.	Units of inhibitor present.	Units of inhibitor absorbed.	Units absorbed per A.D. of virus.
1	Human	E/150	*Nil* (control)	—	640	480	—	—
			Living	200	88	66	414	2·1
			Heated	200	136	102	378	1·9
2	,,	E/150	*Nil* (control)	—	560	420	—	—
			Living	200	160	120	300	3·0
			Heated	200	139	105	315	3·2
3	Fowl	E/166	*Nil* (control)	—	2308	1731	—	—
			Living	151	30	23	1708	11·3
			Heated	135	86	65	1666	12·3
4	Nil	E/158	*Nil* (control)	—	533	400	—	—
			Living	100	270	178	222	2·2
			Heated	100	270	178	222	2·2

For explanation of "Unit of Inhibitor" see text.

The close agreement between units of inhibitor absorbed by living and heated viruses shown in the last column of Table V is remarkable in view of the complexity of the experiments. The conclusion appears inescapable that, in the presence of red cells under conditions comparable with those obtaining in the mixtures of a Francis titration, equal haemagglutinating doses of living and heated virus exhibit not only equal avidity for serum inhibitor, but also equal relative avidities for inhibitor and the red cell receptors. As a final check Francis titrations were carried out in parallel with an absorption experiment, with the same serum and virus preparations, both at room temperature and at 2° C. Pronounced Francis effects were obtained in each case.

Further experiments were carried out in which aliquots of serum were absorbed with a given dose of living virus, with and without the presence of red cells.

The amounts of inhibitor absorbed were identical in one experiment and not significantly different in another. This indicates that the virus-inhibitor complex is not dissociated by the subsequent addition of red cells—a reaction which would be necessary in order to establish a dynamic equilibrium in Francis titrations. Of course, if the virus and cells were mixed first and the serum added subsequently, much less inhibitor would almost certainly be absorbed, for the order of adding reagents profoundly affects the results of inhibitor titrations. As, however, the addition of cells to previously mixed virus and serum dilutions is universally practised for serum titrations this point was not directly tested.

The time factor in inhibitor absorption.

The period of contact of virus and serum prior to the addition of red cells may, or may not, affect the magnitude of the Francis effect. This is because such contact always increases the serum titre against heated virus, sometimes to quite fantastic heights, whereas it may or may not increase the titre against living virus. This discrepancy in behaviour of different batches of living virus of the same strain has not been elucidated, but in any case cannot be the basis of the Francis effect; firstly because equivalent Francis effects may often be obtained with and without prior contact of virus and serum, secondly because reduction of combining speed from heat treatment would tend to give exactly the reverse effect, namely a lower inhibitor titre with heated virus than with living virus.

These time effects are illustrated by the experiments recorded in Tables VI and VII and Fig. 3. The two experiments in Table VI were comparative Francis titrations done at 2° C. Contact periods of 6 hours and 4 hours raised the H/L serum titre ratios to a remarkable degree, for in each case the L titres remained unaffected. Fig. 3 graphically records the result of an experiment of like nature also at 2° C. in which the effect of time of contact was followed over a period of $3\frac{1}{2}$ hours by setting up the virus-serum dilutions in quadruplicate and adding red cells to the sets at intervals. Here the living and heated virus curves run roughly parallel, giving approximately equal H/L serum titre ratios throughout. Table VII gives the result of an absorption test carried out at 2° C. in parallel with the Francis titration experiments and with the same materials. The absorption mixtures consisted of 0·5 ml. serum and 100 A.D. virus in 10 ml. total volume, red cells not being present. Aliquots were filtered immediately and after two hours' contact, period of contact being taken as the time elapsing between mixing reagents and completion of filtration. Filtrates were titrated against two different batches of heated indicator virus. The differences between the titres of serum absorbed by living and heated virus at corresponding times are not significant.

TABLE VI.—*Effect of Time of Contact of Virus and Serum on the Francis Effect.*

Expt. No.	R.B.C. added to serum-virus mixtures.	Serum titres v 4 A.D. virus.		Ratio H/L.
		L.	H.	
1	Immediately	95	960	10·1
	After 6 hours	80	>2560	>32·0
2	Immediately	61	640	10·5
	After 4 hours	65	>10,240	>158·0

TABLE VII.—*Influence of Time of Contact on Absorption of Inhibitor by Living and Heated Virus.*

Absorbing virus.			Titres of absorbed serum against indicator viruses.	
	Dose A.D.	Time of contact (minutes).	H1. 4 A.D.	H2. 4 A.D.
Nil (control)	—	—	533	1002
Living	100	3	270	463
,,	100	120	143	385
Heated	100	5	270	501
,,	100	120	181	349

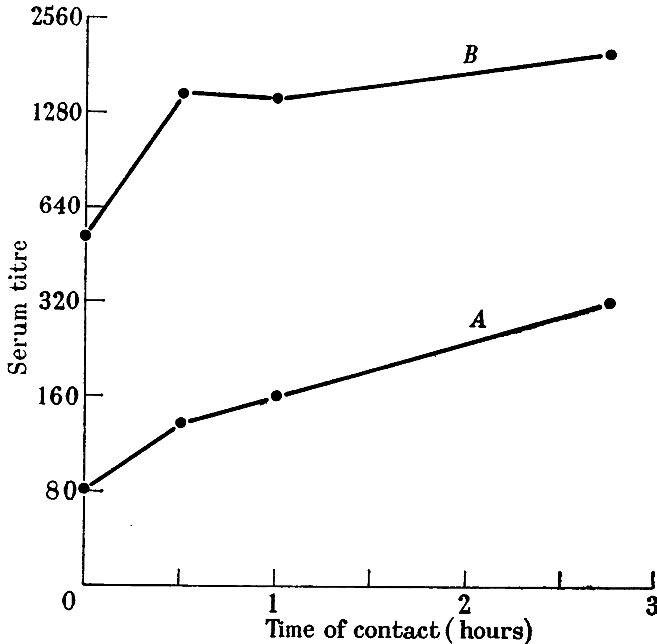

FIG. 3.—Effect of time of contact of virus and serum on the Francis effect at 2° C. A, Serum titres against 4 A.D. living virus ; B, serum titres against 4 A.D. heated virus.

DISCUSSION.

In the experiments reported living and heated virus, A.D. for A.D. behaved identically, both in respect of combining capacity for serum inhibitor and also rate of combination. It must be emphasized, however, that all absorptions were done within the range of virus-serum proportions required to relate the occurrences to the mechanism of the Francis phenomenon. In order to elucidate completely the dynamics of virus-inhibitor reactions it would be necessary to carry out multiple absorptions on the chess-board pattern, testing each of a large range of virus quantities against each of a similarly extended range of serum quantities, and furthermore making in each test replicate titrations of the free

inhibitor at various time intervals. Our resources hitherto have not been adequate for a task of such magnitude. However, from the present limited investigation it is clear that neither of the two hypotheses which have been previously advanced to account for the Francis effect is valid.

It is impossible that enzymic destruction of inhibitor by living virus, if it occurs at all at 2° C., should be as great as or greater than that occurring at room temperature. Yet the Francis effect is often more pronounced at the lower temperature. Also, not all virus strains possessing demonstrable enzyme activity show the increased sensitivity to inhibitor on heating. Again, the inhibitor sensitivity continues to increase with prolonged heating long after all demonstrable enzymic activity has been lost.

Burnet's hypothesis of a dynamic equilibrium rests upon the assumption that the degree of haemagglutination inhibition in a serum titration mixture is a measure of the relative absorptive affinities of the virus for inhibitor and red cell receptors. He suggests that on heating a virus " changes in its surface configuration are produced which increase its absorptive affinity for various mucoids " (Anderson *et al.*, 1948). We believe that heat treatment does in fact alter surface configuration, but that this is without appreciable effect upon its relative absorptive affinities. Hence the equivalent behaviour of living and heated virus with the same number of agglutinating doses of each, contrasted with the reduced combining power of the heated virus when equal amounts of the virus material are used.

The retention of absorbing power by totally inactivated virus suggests the possibility of there being two types of virus receptors with different grades of heat lability and different combining affinities. Such a dual receptor hypothesis, however, also runs counter to our experimental findings, because whatever affinity differences are postulated for the two receptor types, it follows logically that the living virus should combine with more inhibitor and at greater speed than should the heated virus. The phenomenon is probably based upon the well-established fact that in all serological reactions certain threshold doses of the reagents are required in order to produce visible changes, and that in different reactions with the same reagents the thresholds are not necessarily the same. " Total inactivation " may imply merely that the number of virus receptors has fallen below the required threshold, or that their distribution has been changed, so that they can no longer clump red cells, but are still capable of combining with inhibitor.

A hypothesis which we consider adequate to explain all the facts so far elicited is one which might be termed " the hypothesis of steric hindrance." We suggest that heating alters the surface configuration of the virus so that a given amount of inhibitor will blanket the combining groups to an extent sufficient for haemagglutination neutralization, whilst the same amount of inhibitor is insufficient to blanket living virus to the same extent. It is inconceivable that living virus, treated with such a critical dose of serum and still capable of causing haemagglutination should be unable to absorb more inhibitor ; obviously it can do so because it is possible with sufficient serum to neutralize its haemagglutination activity completely. What our experiments have shown is that heated virus continues to absorb inhibitor at approximately the same rate long after the neutralization end-point has been reached. This presents no serious intellectual difficulty for, even assuming complete chemical identity of red cell receptor substrate and inhibitor, the former exists as part of the complex surface structure

of the relatively enormous red cell, whilst the latter is a serum constituent, probably of molecular size. The siting of the red cell receptors over the cell surface requires a complementary spatial arrangement of recepors on the surface of the virus for haemagglutination to occur. On distortion of this spatial arrangement any combined inhibitor may well prevent the apposition of adjacent free receptors to reactive cell sites, as well as neutralizing the virus receptors to which it is directly united. The distortion, however, may be insufficient to interfere with the approach and union of further molecules of inhibitor.

This hypothesis conforms to what is known to occur during the earlier stages of heat denaturation of many proteins. According to Astbury (1939) there is an unfolding of the polypeptide chains and the work of Pauling and Campbell (1942) on the artificial production of antibodies was based on this particular effect. Influenza virus consists largely of nucleoprotein, and it seems likely that the virus receptors form a part of protein complexes at the surfaces of the elementary bodies, especially in view of the closely associated enzymic activity. Unfolding of protein molecules at the surface would inevitably cause structural distortion, but would not necessarily destroy combining power for certain substrates.

We do not claim that the maximum absorptive powers of living and heated virus at the saturation point are equal ; in fact we have reason to believe from the results of further experiments that this is not so. Any such differences at or near the saturation point, however, can have nothing to do with the mechanism of the Francis phenomenon.

It should be explicitly stated that the work reported in this paper has been confined to the interaction of a single strain of Influenza A virus and normal rabbit serum. Other strains may behave differently. Also species differences in the content and behaviour of serum inhibitory substances have been clearly demonstrated (Burnet and McCrea, 1946 ; Smith and Westwood, 1949). Nevertheless, it seems improbable that a multiplicity of mechanisms exist for a phenomenon so widely distributed amongst the influenza viruses as is the Francis phenomenon, and we suggest that the explanation offered is probably applicable both to other strains and other sera.

SUMMARY.

Prolonged heating of Influenza A virus causes a progressive degradation, which results in a progressive increase of the Francis effect as long as any haemagglutination activity remains.

The Francis effect is demonstrable equally at room temperature and at 2° C. The H/L titre ratios of a virus are the same at the two temperatures.

Heat treatment of virus, sufficient to convert it to a good indicator of the Francis effect, does not appreciably affect the power of a given number of agglutinating doses to absorb serum inhibitor. Over a wide range of dosage both total combining capacity and rate of combination remain unchanged. The situation is not affected by the presence of agglutinable red blood cells in the reaction mixtures.

The significance of these findings is discussed, and a new hypothesis is presented to explain the mechanism of the Francis phenomenon.

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