THE USE OF *V. CHOLERAE* FILTRATES IN THE DESTRUCTION OF NON-SPECIFIC INHIBITOR IN FERRET SERA.

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SINCE the haemagglutination-inhibition reaction was first employed in the serological diagnosis of influenza (Hirst, 1942) the presence of inhibitors of haemagglutination in normal sera has been recognized as a disadvantage in the use of the test. Three types of inhibitor have been found :

1. McCrea (1946) described an inhibitor in normal rabbit sera which inhibited agglutination by unheated influenza A viruses, was precipitated by 33 per cent saturation with ammonium sulphate along with gamma globulin, and was inactivated by heating at 62° for 30 minutes.

2. Francis (1947) noted that many normal sera inhibited heated influenza viruses to high titre. The so-called Francis inhibitor has been extensively investigated and is thought to be a mucopolysaccharide. It is heat stable and resists 100° for 30 minutes (McCrea, 1948), but is inactivated by the receptor-destroying enzyme (RDE) of *V. cholerae* filtrates and by prolonged incubation with influenza viruses (Anderson, 1948). Francis inhibitor has been recognized by Anderson (1950) in the sera and tissues of a number of different species.

3. Ginsberg and Horsfall (1949) described an inhibitor in human, guinea-pig, mouse and rabbit sera which inhibits haemagglutination and prevents infection by influenza viruses. The inhibitor is inactivated by heating at 56° for 30 minutes, and acts only in the presence of calcium. Chu (1951) has investigated an inhibitor present in normal mouse sera, which is active against unadapted but not against mouse-adapted influenza strains. This inhibitor is also inactivated by heating at 56° for 30 minutes and may be identical with that described by Ginsberg and Horsfall.

Hirst (1948) investigated the inhibitor in normal rabbit serum and his conclusions differ from those of McCrea. He found the inhibitor of agglutination by unheated and heated influenza virus to be heat stable (100° for 15 minutes), and to resemble Francis inhibitor in its properties. Recently, Smith and Westwood (1950) have discussed the problems raised, and suggest that the evidence is not convincing that the activities against unheated and heated influenza virus are represented by qualitatively distinct inhibitors. They favour McCrea's concept of the inhibitor as a protein-mucoprotein complex, and agree that the purified mucoid which he studied may have been broken off from a larger molecule incorporating both activities.

The extensive use of ferret sera in investigations of the antigenic characters of influenza virus strains makes the inhibitory action of normal ferret sera of

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particular interest. Burnet and McCrea (1946) have described an inhibitor in normal ferret sera which resembled specific antibody in its properties, being inactivated by heating at 65° for 30 minutes, and Anderson (1950) mentions the presence of Francis inhibitor in normal ferret sera. In order to overcome non-specific inhibition of haemagglutination by normal ferret sera, Mulder and van der Veen (1948) recommended the use of filtrates of *V. cholerae.* These authors noted that crude *V. cholerae* filtrates inactivated the non-specific inhibitor in normal ferret serum with only slight destruction of specific antibody. In a later paper (Van der Veen and Mulder, 1950) they have specifically recommended the use of crude *V. cholerae* filtrates, and have noted a lack of correspondence between the RDE activity and ability to destroy non-specific inhibitor in these filtrates. Also, Chu, Andrewes and Gledhill (1950) and Appleby and Stuart-Harris (1950) have reported that purified preparations of the receptor-destroying enzyme, RDE, did not inactivate the non-specific inhibitor in normal ferret sera. However, crude *V. cholerae* filtrates contain a number of enzymes which might influence the results of haemagglutination-inhibition tests, e.g., the receptor-destroying enzyme, RDE (Burnet and Stone, 1947), a mucinase (Burnet, 1948) and a trypsin-like enzyme (Stone, 1949), and it seemed desirable, therefore, to investigate the nature of the inhibitory activity in normal ferret serum and of the factors in *V. cholerae* filtrates responsible for its destruction. The present paper reports the results of this investigation. We have used the term " non-specific inhibitor" to describe the inhibitor in normal serum for unheated influenza virus, and " Francis inhibitor " for the inhibitor for heated influenza virus.

MATERIALS AND METHODS.

Virus.—The following strains were used :—

Influenza A : WS, PR8, MEL, Shope 15 strain of Swine influenza, FM1, A/Sweden/3/50, A/Belfast/2/51, A/Madrid/2/51.

Influenza B : Lee, Crawley (England, 1946), Warner (Australia, 1948), B/Budapest/1/49, B/Johannesburg/1/50.

Allantoic fluids were used from embryos inoculated at 10 days and incubated for a further 2 or 3 days at 35°.

Red cells.—Fowl cells were washed thrice in normal saline and made up in a 0·5 per cent suspension (v/v) in normal saline. The concentration of cells was calibrated by a photo-electric densitometer. As far as possible, vaccinia-insensitive, inhibitor-sensitive fowl cells were used and they were not kept for longer than 5 days.

Sera.—Ferret sera were stored at 2° with the addition of 1/10,000 merthiolate. They were heated at 56° for 30 minutes immediately before use.

V. cholerae *filtrates.*—The 4Z strain of *V. cholerae* was obtained through the courtesy of Sir Macfarlane Burnet. The preparation of filtrates was as described by Burnet and Stone (1947).

Titration of non-specific inhibitor.—Serial two-fold dilutions of serum (0·25 ml.) were prepared in saline and an equal volume of 0·5 per cent fowl cells added. Finally a third volume of virus diluted in saline to give 4–8 partial agglutinating doses (A.D.) was added and the cells allowed to settle at room temperature (20–25°). Readings were made by the pattern method, the end-point being

taken as partial agglutination. Titres in the tables are expressed as the reciprocal of the initial dilution of serum present at the end-point.

Titration of Francis inhibitor.—Serial two-fold dilutions of serum (0·25 ml.) were prepared in saline and an equal volume of heated Lee virus (heated at 56° for 30 minutes) containing 5 A.D. was added; this was allowed to stand at room temperature for 30 minutes. A third volume of red cells was added and readings were made in the same manner as above.

RDE titration.—The titration was carried out after the method of Burnet and Stone (1947). Serial two-fold dilutions (0·25 ml.) of RDE were prepared in calcium acetate buffer, pH 6·0, and an equal volume of fowl cells added. After incubation for one hour at 37° one drop of saturated solution of sodium citrate was added, followed by 0·25 ml. of MEL virus diluted in saline to give 5 A.D. The end-point of the reaction was taken as partial agglutination, and the titre of RDE is expressed as the reciprocal of the initial dilution of RDE at the end-point.

EXPERIMENTAL.

In a study of haemagglutination-inhibition by normal ferret sera there were three variables to consider—serum, red cells and virus. The normal ferret sera which we studied came from an inbred stock of ferrets bred in the National Institute for Medical Research Farm at Mill Hill, and their sera were surprisingly uniform in inhibitor content. Much more variable were the results obtained with red cells from different fowls and with different strains of influenza virus.

Haemagglutination-inhibition titres with different fowl red cells.

Stuart-Harris (1943) drew attention to the wide variation of specific anti-haemagglutinin titres with influenza B viruses when different fowl cells were used. Table I shows the same phenomenon.

TABLE I.—*Haemagglutination-Inhibition Titres with Different Fowl Cells.*

Serum.	Red cells.	
	Fowl 11.	Fowl 12.
Normal ferret serum . . .	120	<10
Immune ferret serum . .	480	20

5 A.D. of B/Johannesburg/1/50 virus were used.

Cells of fowl 12 give very low inhibitory titres in tests with B/Johannesburg/1/50 virus and normal and immune ferret sera. However, fowl 11 and 12 cells gave identical inhibition titres in tests with A/Sweden/3/50 virus. Cells such as those of fowl 12 are described by Anderson, Burnet and Stone (1946) as "inhibitor-insensitive" and they were not used in these experiments. The other fowl cells which we used were all "inhibitor-sensitive," and gave titres not greatly different from those of fowl 11.

It was also important to avoid the use of vaccinia-positive fowl cells, since crude filtrates of *V. cholerae* were found to agglutinate vaccinia-positive fowl cells (*vide infra*).

Haemagglutination-inhibition titres with different virus strains.

Large variations in inhibitory titres were encountered in tests with different virus strains. The highest inhibitory titres, of roughly 5000 when inhibitor-sensitive cells were used, were found in titrations of normal ferret sera with heated influenza B virus (Francis, 1947). Next, a freshly isolated strain of influenza A virus (A/Belfast/2/51) showed a non-specific inhibitory titre of 1280 when first egg passage amniotic or allantoic fluid in the " intermediate phase " (Burnet and Stone, 1945) was used. The same virus after one further allantoic passage was now in the " D phase " (Burnet and Bull, 1943), and was inhibited by normal ferret sera to a titre of 30. The influenza B viruses which we tested generally gave inhibitory titres of 100–400, and a number of A and "A-prime " strains gave inhibitory titres of the order of 20–80. Swine virus was not inhibited by normal ferret serum (cf. Smith and Westwood, 1949). From the strains examined, B/Johannesburg/1/50 and Crawley viruses were selected and used in the majority of experiments. It was found that there was an inverse linear relationship between the amount of virus and the inhibitory titre of serum. When it was necessary to magnify the inhibitory titre of a serum, small amounts of virus, 3–4 partial agglutinating doses, were used.

Destruction of non-specific inhibitor in normal ferret serum by filtrates of V. cholerae.

Mulder and van der Veen's (1948) observation on the destruction of non-specific inhibitor in normal ferret sera by crude filtrates of *V. cholerae* was readily confirmed. As an aid in identifying the active factor in the *V. cholerae* filtrates an investigation of its properties was carried out.

1. *Effect of pH.*—A series of two-fold dilutions (0·2 ml.) of crude *V. cholerae* filtrate was prepared in phosphate buffer and to each dilution was added 0·05 ml. of normal ferret serum. After overnight incubation (16 hours) at 37° the mixtures were inactivated by heating at 56° for one hour and then titrated for inhibitor against Crawley virus. The results are shown in Table II ; destruction of non-specific inhibitor was favoured by a slightly acid pH.

TABLE II.—*pH of Action of* V. cholerae *Filtrate on Non-Specific Inhibitor.*

Dilution of *V. cholerae* filtrate.	Inhibitory titre at pH		
	6·3.	7·0.	8·0.
1:2	<10	<10	15
1:4	<10	30	60
1:8	60	60	120
1:16	80	100	140
1:32	120	120	140
∞	120	160	160

3 A.D. of Crawley virus were used.

2. *Heat stability.*—It was found that the active factor in *V. cholerae* filtrates was completely destroyed by heating at 56° for 30 minutes. When the filtrate was diluted in calcium acetate buffer, pH 6·0, and then heated at 56° for 30 minutes, no destruction of activity occurred.

3. *Calcium requirement.*—Dilutions of *V. cholerae* filtrate were prepared in (1) acetate buffer, pH 6·0, (2) acetate buffer plus 0·1 per cent calcium chloride,

and (3) acetate buffer plus 2 per cent sodium citrate. Titrations of the activity of the *V. cholerae* factor in these diluents were then carried out as above. The results are shown in Table III.

TABLE III.—*Effect of Calcium and Sodium Citrate on* V. cholerae *Factor.*

Dilution of *V. cholerae* filtrate.	Diluent for *V. cholerae* filtrate.		
	Acetate.	Acetate plus calcium.	Acetate plus citrate.
1:2	<10	<10	160
1:4	<10	25	160
1:8	15	30	160
1:16	40	40	160
∞	160	160	160

The table shows the non-specific inhibitory titres against 3 A.D. of Crawley virus.

Table III shows that citrate completely inhibited the action of the *V. cholerae* factor, but the addition of 0·1 per cent calcium chloride did not increase the action.

4. *Adsorption to and elution from fowl red cells.*—It is known that the receptor-destroying enzyme of *V. cholerae*, RDE, is adsorbed to fowl red cells and readily elutes following enzymic inactivation of the influenza red cell receptors (Burnet and Stone, 1947). Experiments showed that the factor responsible for destruction of non-specific inhibitor in normal ferret serum was similarly rapidly adsorbed on to fowl cells at 2°, and could be eluted into saline on incubation at 37°. A comparison was then made of the rate of adsorption and elution of the active factor and RDE.

Washed, packed fowl cells in a final concentration of 5 per cent (v/v) were added to filtrates of *V. cholerae* at 2°. After varying periods the cells were centrifuged at 2° and the supernatants removed. Saline was added to the same volume and elution was carried out for varying periods at 37°. The supernatants and eluates were then titrated for (1) ability to destroy non-specific inhibitor in normal ferret serum, (2) RDE activity. It was found that the two activities showed a corresponding drop in titre on absorption with red cells and a corresponding rise in titre on elution. The results of a representative experiment are shown in Table IV.

TABLE IV.—*Effect of Absorption on Red Cells and Elution on* V. cholerae *Factor.*

Material tested.	Non-specific inhibitory titre. *V. cholerae* dilution.						RDE titre.
	1:1.	1:2.	1:4.	1:8.	1:16.	∞	
V. cholerae filtrate .	<10	<10	<10	20	40	120	. 96
Filtrate absorbed with fowl cells . . .	40	40	60	80	120	..	. 8
Eluate	<10	<10	<10	30	60	..	. 64

Table shows titration of untreated *V. cholerae* filtrate, filtrate absorbed with fowl cells for 1 min. at 2° and then eluted for 30 min. at 37°. The materials were titrated for their action on non-specific inhibitor in normal ferret serum and for their RDE titre.

5. *Presence of active factor in purified preparations of RDE.*—A purified preparation of RDE was available through the courtesy of Mr. G. L. Ada of the Walter and Eliza Hall Institute, Melbourne. The RDE was supplied in dried form, in ampoules containing 40,000 units each, and the material was made up in 2 ml. of calcium acetate buffer, pH 6·0.

Dilutions of RDE were prepared in acetate buffer, pH 6·0, and were tested for their action on the non-specific inhibitor in normal ferret serum. The results of an experiment are shown in Table V.

TABLE V.—*Action of Purified RDE on Non-Specific Inhibitor in Normal Ferret Serum.*

RDE dilution.	Inhibitory titre.
1:1	120
1:2	30
1:4	<10
∞	80

8 A.D. of Crawley virus were used.

The undiluted, purified RDE had no action on non-specific inhibitor, but the same material was fully active when diluted 1 in 4. This inhibitory zone has not been investigated fully, owing to difficulty in reproducing the effect. Thus, in experiments with other batches of purified RDE, inhibitor destruction occurred with undiluted and diluted RDE (Table VII).

Calcium requirement of inhibitor-destroying factor of V. cholerae.

These results show that the factor in *V. cholerae* filtrates which destroys non-specific inhibitor in normal ferret sera shows a striking resemblance in properties to the receptor-destroying enzyme, RDE (Burnet and Stone, 1947). The only apparent difference encountered was in calcium requirement. Both reactions were inhibited by 2 per cent sodium citrate, but the action of RDE was increased by the addition of 0·1 per cent calcium chloride, whereas the action of the inhibitor-destroying factor was, if anything, slightly diminished. However, the difference would be no more than an apparent one if the serum supplied the calcium necessary for the reaction. This was tested by estimating the RDE titre of a *V. cholerae* eluate in the following diluents :

1. Acetate buffer, pH 6·0.
2. Acetate buffer plus 0·1 per cent calcium chloride.
3. Acetate buffer plus 0·1 per cent calcium chloride plus 2 per cent sodium citrate.
4. Acetate buffer plus normal ferret serum 1/5.
5. Acetate buffer plus normal ferret serum 1/5 plus 2 per cent sodium citrate.

However, it was first necessary to remove non-specific inhibitor from the normal ferret serum ; this was carried out by previous overnight incubation of the serum with *V. cholerae* eluate at 37°, and the RDE was then destroyed by heating at 56° for one hour. The results of the RDE titrations are shown in Table VI.

TABLE VI.—*Receptor-destroying (RDE) Titre of a Red Cell Eluate of* V. cholerae *in Various Diluents.*

Diluent.	RDE titre.
Acetate pH 6·0	2
Acetate + 0·1 per cent $CaCl_2$	80
Acetate + 0·1 per cent $CaCl_2$ + 2 per cent sodium citrate	16
Acetate + 1/5 inactivated serum	80
Acetate + 1/5 inactivated serum + 2 per cent sodium citrate	10

It can be seen that the titre of RDE is greatly increased in the presence of either 0·1 per cent calcium chloride or 1/5 normal ferret serum. Citrate has an inhibitory effect on both reagents, suggesting that the effect of the serum is due to its calcium content.

Properties of the inhibitor in normal ferret serum.

These results suggested that the active factor in *V. cholerae* filtrates was RDE, and it might be expected, therefore, that the inhibitory property of normal ferret sera was due to Francis inhibitor. One method of investigating this was as follows:

A series of dilutions of purified RDE in acetate buffer was prepared, and to each was added a constant amount of normal ferret serum. After overnight incubation at 37° the sera were heated for one hour at 56°, and each dilution was titrated for (*a*) non-specific inhibitor against Crawley virus and (*b*) Francis inhibitor against heated Lee. The results are shown in Table VII, where it is seen that the highest dilution of RDE which destroys the inhibitors is in each case 1/128. This suggests that the Francis inhibitor is responsible for both the high titre inhibition of heated virus and the lower titre inhibition of unheated virus.

TABLE VII.—*Action of Purified RDE on Non-specific Serum Inhibitor and Francis Inhibitor.*

Titration.	RDE dilution.										
	1:1.	1:2.	1:4.	1:8.	1:16.	1:32.	1:64.	1:128.	1:256.	1:512.	∞
Non-specific inhibitor .	<10	<10	<10	<10	<10	<10	<10	<10	15	60	80
Francis inhibitor .	<50	<50	<50	<50	<50	<50	<50	<50	100	1400	4800

Non-specific inhibitor was titrated against 6 A.D. of Crawley virus. Francis inhibitor was titrated against 6 A.D. of heated Lee.

Heat stability of inhibitor in normal ferret serum.—Normal ferret serum was diluted 1 in 10 in normal saline, and aliquots were heated at 65° and 56° for 30 minutes, and then titrated for non-specific inhibitor and Francis inhibitor. The results of such an experiment are shown in Table VIII.

A two-fold reduction in both inhibitory titres occurred on heating at 65°. Altogether 13 normal sera from our ferret stock have been tested, and in all cases

TABLE VIII.—*Heat Stability of Inhibitors in Normal Ferret Serum.*

Titration.	Serum heated 30 minutes at— 56°.	65°.
Non-specific inhibitor	640	320
Francis inhibitor	6400	3200

Non-specific inhibitor was titrated against 3 A.D. of Crawley virus. Francis inhibitor was titrated against 6 A.D. of heated Lee.

the non-specific inhibitory titre was unaffected or only slightly reduced following heating at 65° for 30 minutes. In addition, a batch of pooled normal ferret serum obtained through the courtesy of Dr. P. von Magnus of the State Serum Institute, Copenhagen, and two normal sera obtained from bought ferrets through the courtesy of Professor C. H. Stuart-Harris, were examined, and the non-specific inhibitor found to be resistant to heating at 65° for 30 minutes. The results were the same whether the non-specific inhibitor was tested against A or B strains or against a newly isolated A strain in the first egg passage.

Action of influenza virus on non-specific inhibitor in normal ferret serum.

Incubation of normal ferret serum with influenza virus (strains A/Madrid/2/51 and Lee), followed by heating at 65° for 30 minutes to destroy all remaining haemagglutinin, produced a drop in non-specific inhibitory titre and a corresponding drop in Francis inhibitor, although complete destruction of these inhibitors did not occur. The results of an experiment of this kind are shown in Table IX.

TABLE IX.—*Effect of Influenza Virus on Non-specific Serum Inhibitor and Francis Inhibitor.*

Serum incubated with—	Titration. Non-specific inhibitor.	Francis inhibitor.
A virus	10	75
B virus	30	100
Saline control	60	300

Serum was incubated overnight at 37° with 100 A.D. of A/Madrid/2/51 and 50 A.D. of Lee. Inactivation—30 minutes at 65°. Non-specific inhibitor was titrated against 5 A.D. of Crawley virus. Francis inhibitor was titrated against 4 A.D. of heated Lee.

Action of trypsin on non-specific inhibitor in normal ferret serum.

A preparation of crystalline trypsin was kindly supplied by Dr. W. J. Elford. When diluted in phosphate buffer, pH 8·0, in a concentration of 0·1 per cent and incubated for 2 hours at 37° with normal ferret serum it was found to destroy both the non-specific inhibitory titre and the Francis inhibitor (Burnet, McCrea and Anderson, 1947).

Use of crude V. cholerae *filtrates in destroying non-specific serum inhibitor.*

The use of crude *V. cholerae* filtrates for the destruction of non-specific serum inhibitor is undesirable, since they may contain a number of complicating factors.

The following factors, which are known to occur in crude *V. cholerae* filtrates, may interfere with the results of haemagglutination-inhibition tests.

1. A proteinase is present which may be identical with the trypsin-like agent described by Stone (1949). The proteinase destroys specific antibody. Most workers who have used crude *V. cholerae* filtrates have noted destruction of specific antibody, and we have repeatedly noticed this in our experiments. It can be shown that crude *V. cholerae* filtrates produce liquefaction of gelatin. When RDE was absorbed by fowl red cells all detectable proteinase activity remained in the supernatant fluid; eluates did not produce liquefaction of gelatin.

2. One *V. cholerae* filtrate when incubated with normal ferret serum for short periods (1 hour) at 37°, followed by heating for one hour at 56°, has shown the property of increasing the inhibitory titre ten-fold. The responsible factor is not inactivated by heating at 60° for 30 minutes, but is destroyed by boiling for 5 minutes.

3. Crude *V. cholerae* filtrates contain a lipoid which agglutinates vaccinia-sensitive cells (Stone, 1946) to a titre of approximately 32. This was previously observed by Chu (1948). It was also found that a lipoid haemagglutinin was present to a titre of 640 in a preparation of purified RDE supplied by Mr. G. L. Ada. Our results do not suggest that the lipoid and RDE are intimately associated; thus the lipoid agglutinin was absorbed from *V. cholerae* filtrates by vaccinia-sensitive and insensitive fowl cells (*cf.* Burnet and Boake, 1946) but did not elute along with the RDE.

Use of V. cholerae *red cell eluates in destroying non-specific inhibitor.*

It seems clear from the results that the non-specific inhibitor in normal ferret serum corresponds to the Francis inhibitor and that it can be inactivated by RDE. Red cell eluates of RDE are easily prepared, and afford a relatively simple method of obtaining the enzyme free from significant amounts of proteinase. Eluates have been prepared by absorption with 5 per cent fowl cells for 1 minute at 2°, followed by elution into normal saline for 30 minutes at 37°; the eluates were then diluted in acetate buffer pH 6·0 and titrated for their ability to destroy non-specific inhibitor. They have been used at the highest effective dilution. A number of experiments have been carried out with RDE eluates in studies of the antigenic structure of recently isolated strains of influenza A and B viruses; sera treated in this way have shown complete destruction of non-specific inhibitor with no reduction in the titre of specific antibody.

DISCUSSION.

The conclusions which these results suggest are that the active factor in *V. cholerae* filtrates is the receptor-destroying enzyme, RDE, and that the non-specific inhibitor in the ferret sera under investigation is largely, if not wholly, Francis inhibitor. The evidence for this can be summarized briefly:

1. A slightly acid pH (6·0) favours the action of the active factor and of RDE.
2. The active factor and RDE are both inactivated by heating at 56° for 30 minutes; both are protected from the destructive effect of heat by diluting in calcium acetate buffer, pH 6·0.

3. Both factors require calcium for their action and both are inhibited by sodium citrate.

4. Both factors are adsorbed to and can be eluted from fowl red cells; absorption and elution of the active factor and RDE occur at the same rate.

5. Purified preparations of RDE destroy non-specific inhibitor; the effective dilution is commensurate with the RDE titre of the preparation.

6. RDE inactivates the non-specific inhibitor and the Francis inhibitor in normal ferret serum at the same rate.

7. The non-specific inhibitor in normal ferret serum is not inactivated by heating at 65° for 30 minutes.

8. The non-specific inhibitor is inactivated on incubation with influenza virus.

The difference between the present results on the activity of purified preparations of RDE and those of previous workers may be due to the pro-zone effect described above; some purified preparations of RDE were found to be ineffective in destroying non-specific inhibitor when tested undiluted but were active when diluted. The ferret sera which we have examined have not contained significant amounts of the heat labile (65°) globulin inhibitor described by Burnet and McCrea (1946). It may be that sera from some ferret stocks differ in their inhibitor content from those which we have examined. In general, our results with ferret sera are in agreement with the conclusions reached by Smith and Westwood (1950) in their study of the inhibitor in normal rabbit sera.

The use of red cell eluates of *V. cholerae* is clearly preferable to that of the crude filtrates. By this means it may be possible to obtain a more accurate picture of the antigenic structure of influenza viruses, uncomplicated by non-specific inhibitory effects. How important this is can be seen from present studies of influenza B viruses (Bozzo, in preparation), in which the titre of non-specific inhibitor in an immune ferret serum may be greater than the titre of specific antibody for a particular virus under test. The use of RDE-treated ferret sera has been invaluable in the study of the large number of A-prime strains received at the World Influenza Centre following the recent epidemic in Europe. It has been possible to use antisera in the confident knowledge that treatment with RDE eluate destroys all non-specific inhibitor without in any way affecting the titre of specific antibody. It may be worth while emphasizing, however, that this method will not be applicable to sera from other species unless it is found that their non-specific inhibitory titre is due predominantly to Francis inhibitor.

SUMMARY.

The factor in *V. cholerae* filtrates responsible for the destruction of non-specific inhibitor in normal ferret serum has been identified as the receptor-destroying enzyme, RDE.

The non-specific inhibitor in 16 normal ferret sera has been found to be due to the activity of Francis inhibitor.

The use of *V. cholerae* eluates is recommended for the destruction of non-specific inhibitor in ferret sera.

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REFERENCES.

ANDERSON, S. G.—(1948) *Australian J. exp. Biol.*, **26**, 347.—(1950) *Australian J. Sci.*, **12**, 147.
Idem, BURNET, F. M., AND STONE, J. D.—(1946) *Australian J. exp. Biol.*, **24**, 269.
APPLEBY, J. C., AND STUART-HARRIS, C. H.—(1950) *Brit. J. exp. Path.*, **31**, 797.
BURNET, F. M.—(1948) *Australian J. exp. Biol.*, **26**, 71.
Idem AND BOAKE, W. C.—(1946) *J. Immunol.*, **53**, 1.
Idem AND BULL, D. R.—(1943) *Australian J. exp. Biol.*, **21**, 55.
Idem AND McCREA, J. F.—(1946) *Ibid.*, **24**, 277.
Iidem AND ANDERSON, S. G.—(1947) *Nature*, **160**, 404.
Idem AND STONE, J. D.—(1945) *Australian J. exp, Biol.*, **23**, 151.—(1947) *Ibid.*, **25**, 227.
CHU, C. M.—(1948) *J. Hyg.*, **46**, 42.—(1951) *J. gen. Microbiol.*, in press.
Idem, ANDREWES, C. H., AND GLEDHILL, A. W.—(1950) *Bull. World Hlth, Org.*, **3**, 187.
FRANCIS, T. JR.—(1947) *J. exp. Med.*, **85**, 1.
GINSBERG, H. S., AND HORSFALL, F. L., JR.—(1949) *Ibid.*, **90**, 475.
HIRST, G. K.—(1942) *Ibid.*, **75**, 49.—(1948) *Ibid.*, **87**, 301.
McCREA, J. F.—(1946) *Australian J. exp. Biol.*, **24**, 283.—(1948) *Ibid.*, **26**, 355.
MULDER, J., AND VEEN, J. VAN DER.—(1948) *Acta med. brev. Neerl.*, **16**, 57.
SMITH, W., AND WESTWOOD, M. A.—(1949) *Brit. J. exp. Path.*, **30**, 48.
Idem AND WESTWOOD, J. C. N.—(1950) *Ibid.*, **31**, 725.
STONE, J. D.—(1946) *Australian J. exp. Biol.*, **24**, 197.—(1949) *Ibid.*, **27**, 229.
STUART-HARRIS, C. H.—(1943) *Brit. J. exp. Path.*, **24**, 33.
VEEN, J. VAN DER, AND MULDER, J.—(1950) 'Studies on the Antigenic Composition of Human Influenza Virus A Strains.' Leiden (Stenfert Kroese).