THE ACTION OF TRYPSIN ON NORMAL SERUM INHIBITORS OF INFLUENZA VIRUS AGGLUTINATION.

A. A. DE C. SAMPAIO* AND A. ISAACS.

From the World Influenza Centre, National Institute for Medical Research, Mill Hill, London, N.W. 7.

Received for publication November 21, 1952.

AT least two types of inhibitors of influenza virus agglutination have been described in normal animal sera. The first type (Francis, 1947) is heat stable (100° for 30 minutes), much more active against heated than unheated influenza B viruses, readily inactivated by the receptor destroying enzyme (RDE) of *V. cholerae* (Anderson, 1948) and is thought to be a mucopolysaccharide ; it is often known as " Francis inhibitor." A second type, present in normal rabbit serum, was described by McCrea (1946) ; it is heat labile (65° for 30 minutes), active against freshly isolated but not laboratory adapted strains of influenza virus A and is precipitated by ammonium sulphate along with gamma globulin. Heat labile inhibitors in guinea-pig, rabbit and mouse sera were later described (Ginsberg and Horsfall, 1949), and these may be similar in nature to the rabbit serum inhibitor of McCrea. However, it was Chu (1951) who first pointed out the characteristic feature of this second type of inhibitor, that it was highly active against unadapted strains of influenza virus A, but only weakly active or inactive against mouse adapted lines of the same strain ; this inhibitor may be conveniently referred to as " Chu inhibitor." Sampaio (1952) has recently studied the presence of Francis and Chu inhibitors in a number of normal animal sera. The α and β inhibitors described by Smith, Westwood and Belyavin (1951) in rabbit serum presumably correspond to Francis and Chu inhibitors.

Van der Veen and Mulder (1950) have shown that crude extracts of *V. cholerae* inactivate the inhibitor in normal ferret serum. Isaacs and Bozzo (1951) found that normal ferret serum contained mainly inhibitor of the Francis type which could be readily inactivated by purified RDE of *V. cholerae*. However, purified RDE was without effect on the Chu inhibitor present in normal rabbit serum. Chu (1951) pointed out that crude *V. cholerae* extracts, but not purified RDE, inactivate the inhibitor (Chu inhibitor) present in normal mouse serum, and Magill and Jotz (1952) and Mulder and Brans (personal communication) have found that crude *V. cholerae* extracts inactivate normal rabbit serum inhibitor.

The present investigation was begun to define more closely the action of crude *V. cholerae* extracts on Chu inhibitor. The results suggested that a trypsin-like enzyme known to be present (Stone, 1949) might be the active factor in crude *V. cholerae* extracts. It was subsequently found that crystalline trypsin was highly effective in inactivating both Francis and Chu inhibitors in all the sera examined ; the present report describes these results.

* W.H.O. Visiting Fellow.

MATERIALS AND METHODS.

Viruses.—A/England/1/51 virus was used for measuring Chu inhibitor, and Crawley (1946, England B) strain after heating at 56° for 30 min., for measuring Francis inhibitor.

Trypsin.—The preparation of trypsin used in most of these experiments was obtained from Messrs. Armour & Sons, Chicago, U.S.A. The trypsin is best dissolved in a small volume of 0·01 N HCl and then made up to the required strength by dilution in phosphate buffer, pH 8·0. Once made up in phosphate buffer the trypsin gradually loses its activity on storage at 4°. It was therefore found advisable to weigh out only small quantities at a time or to keep it in 0·01 N HCl.

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 suspension of fowl cells added. The same volume of virus was at once added in a dilution which gave 8 partial agglutinating doses per 0·25 ml. and the cells were allowed to settle at room temperature (20–25°). Tests were carried out in plastic plates and readings were made by the pattern method. The end-point was taken as partial (50 per cent) agglutination, and the titres in the tables are expressed as the reciprocal of the initial dilution of serum present at the end-point.

Technique of trypsin treatment.—Four parts of trypsin, made up in phosphate buffer pH 8·0 were mixed with 1 part of serum and heated at 56° for 30 min. The amounts of trypsin used are expressed in the tables as weight of crystalline trypsin per ml. of serum.

RESULTS.

Preliminary experiments were carried out to investigate the effect of crude *V. cholerae* extracts on Chu inhibitor, using normal rabbit serum as a convenient source. An extract prepared from the 4Z strain of *V. cholerae* by the method of Burnet and Stone (1947) showed only weak activity on normal rabbit serum inhibitor. Through the kindness of Professor J. Mulder and Dr. L. M. Brans, Leiden, however, a sample of crude *V. cholerae* extract prepared in their laboratory was made available to us. This preparation completely inactivated the inhibitor in normal rabbit serum and was used in the following experiments.

Action of crude V. cholerae *extracts on normal rabbit serum inhibitor.*

The technique which Mulder and Brans (personal communication) recommended for demonstrating the action of crude cholera extract was to mix four parts of extract with one of serum, incubate them together overnight at 37° and then heat for 1 hr. at 56°. In the present experiments it was found that the overnight incubation at 37° was unnecessary. Table I shows an experiment in which four parts of cholera extract were mixed with one part of serum, which was then heated at 56° for different times and titrated for inhibitory activity.

TABLE I.—*Action of Crude* V. cholerae *and Heat on Normal Rabbit Serum Inhibitor.*

Material tested.	Heated at 56° for—	Inhibitory titre.
V. cholerae extract + rabbit serum	0 min.	384
" " + " "	5 "	60
" " + " "	15 "	<12
" " + " "	30 "	<12
" " + " "	60 "	<12
Saline + rabbit serum	0 "	384
" + " "	60 "	192

Inhibitory activity was assayed against 8 agglutinating doses of A/England/1/51 virus.

Inhibitory activity was abolished by heating with cholera extract at 56° for 15 min. Controls showed that in the absence of heating the inhibitory activity was unaltered, while heating serum at 56° for 1 hr. in the presence of saline, phosphate buffer pH 8·0 or normal agar extract caused a two- to four-fold reduction in the inhibitory titre of rabbit serum. It appeared therefore that the combined action of a factor in crude cholera extract and the effect of heating were required for complete inactivation of inhibitor. In further experiments, therefore, the technique was modified so that the cholera extract and serum were simply mixed, heated for 30 min. at 56° and tested for inhibitory activity.

Properties of active factor in crude V. cholerae *extracts.*

Some of the properties of the active factor in crude *V. cholerae* extracts were next investigated. It was found that the active factor was adsorbed on Seitz filtration through a Ford's sterimat SB filter pad and on a 0·72 μ A.P.D. gradocol membrane. It was heat labile (Table II), and was precipitated by 50 per cent ammonium sulphate. These results suggested that the active factor might be

TABLE II.—*Effect of Heat on the Active Factor in Crude* V. cholerae *Extract.*

Material tested.	Preliminary treatment of *V. cholerae* extract.	Inhibitory titre.
Heated *V. cholerae* extract + rabbit serum .	Heated 56° for 30 min. .	20
,, ,, ,, + ,, ,, .	,, 60° ,, .	80
,, ,, ,, + ,, ,, .	,, 62·5° ,, .	140
,, ,, ,, + ,, ,, .	,, 65·5° ,, .	160
V. cholerae extract + rabbit serum . . .	*nil* .	<10
Saline + ,, ,, . . .	,, .	280

Inhibitory activity was assayed against 8 agglutinating doses of A/England/1/51 virus.

macro-molecular, possibly a protein, and to test this possibility further the crude *V. cholerae* extract was incubated for 24 hr. at 37° with crystalline trypsin in a concentration of 8 mg./ml. of extract. The trypsin had apparently no effect on the cholera factor. However, controls showed that trypsin itself completely abolished the inhibitory action of normal rabbit serum. As with the cholera extract, it was simply necessary to mix trypsin and normal rabbit serum and heat at 56° for 30 min. in order to remove all inhibitory activity. An experiment which illustrates these results is shown in Table III.

TABLE III.—*Effect of Trypsin on Crude* V. cholerae *Extract.*

Material.	Treatment.	Inhibitory titre of serum.
Saline + rabbit serum	*nil* .	384
,, + ,, ,, ...	Heated 56° for 1 hour .	192
V. cholerae extract + rabbit serum	,, ,, ,, .	<12
Trypsin treated *V. cholerae* extract + rabbit serum .	,, ,, ,, .	<12
Trypsin + rabbit serum	,, ,, ,, .	<12

Inhibitory activity was assayed against 8 agglutinating doses of A/England/1/51 virus.

The active factor in *V. cholerae* extract shares a number of properties with trypsin. Thus, when trypsin and normal rabbit serum were mixed, heated at 56° for different times and then tested for inhibitory activity, the results, which

are shown in Table IV, were very similar to those which are shown in Table I for crude cholera extract. Controls showed that trypsin heated with serum did not agglutinate red cells. Crystalline trypsin made up in phosphate buffer

TABLE IV.—*Action of Trypsin and Heat on Normal Rabbit Serum Inhibitor.*

Material tested.	Heated at 56° for—	Inhibitory titre of serum.
Trypsin + rabbit serum	0 min.	640
„ + „ „	5 „	60
„ + „ „	15 „	15
„ + „ „	30 „	15
„ + „ „	60 „	15
Buffer + „ „	0 „	640
„ + „ „	60 „	320

Inhibitory activity was assayed against 8 agglutinating doses of A/England/1/51 virus.

pH 8·0 was inactivated after heating at 56° for 30 min. However, its heat stability was increased when it was made up in an extract of normal agar, as used in the preparation of the cholera extract. One property which appeared at first to differentiate the two was that the active factor in crude *V. cholerae* extract was not so effective as trypsin when mixed with normal rabbit serum and incubated overnight at 37° without subsequent heating at 56°. However, in these conditions *V. cholerae* extract contains RDE, which causes " slipping " of red cells, an effect which might simulate the action of a serum inhibitor. It was found that when dilutions of the reagents were carried out in 2 per cent citrate-saline, which greatly reduces RDE action, this " prozone " was abolished ; citrate-saline alone caused only a slight reduction in the prozone. In addition, the prozone was produced by *V. cholerae* extract, red cells and virus in the absence of serum. It is concluded, therefore, that *V. cholerae* extract is active on rabbit serum inhibitor after incubation at 37° alone (*cf.* Chu, 1951), and that the properties of the active factor in *V. cholerae* extract are similar in a number of respects to those of trypsin.

Action of trypsin on normal serum inhibitors.

The action of trypsin on Francis and Chu inhibitors in different animal sera was next investigated. Preliminary experiments showed that trypsin mixed with normal serum and heated at 56° for 30 min. reduced greatly, or abolished, both Francis and Chu inhibitors. An experiment was therefore carried out with different amounts of trypsin in order to determine its action on Francis and Chu inhibitors in different sera. Falling dilutions of trypsin were mixed with a constant amount of normal serum and heated at 56° for 30 min. ; controls consisted of serum plus the diluent phosphate buffer, and serum plus saline. The sera were then tested for inhibitor against A/England/1/51 and heated Crawley viruses ; these viruses virtually measure Chu and Francis inhibitors respectively. The results are shown in Table V.

Table V shows that the inhibitors in different sera vary in their sensitivity to trypsin. The control titres of Chu inhibitor were usually lower than the Francis inhibitor titres, and the former appeared to be more sensitive to trypsin treatment than the latter. The Francis inhibitor in rabbit serum was rather resistant, and 10 per cent of the control titre was present after treatment with 32 mg.

trypsin/ml. serum. With this exception trypsin treatment reduced inhibitory activity of these sera to an insignificant level.

TABLE V.—*Effect of Trypsin on Chu and Francis Inhibitors in Different Sera.*

Trypsin.	Serum.									
	Ferret.		Fowl.		Mouse.		Rabbit.		Guinea-pig	
	E.	H.C.	E.	H.C.	E.	H.C.	E.	H.C.	E.	H.C.
32 mg./ml. serum	<10	<10	<10	<10	<10	<10	<10	60	<10	10
16 ,, ,,	<10	<10	<10	<10	<10	<10	<10	80	<10	20
8 ,, ,,	<10	20	<10	10	<10	<10	<10	120	<10	320
4 ,, ,,	<10	960	<10	30	<10	<10	10	320	15	320
2 ,, ,,	<10	1280	<10	40	10	10	40	640	60	320
nil-phosphate control	30	1920	<10	240	20	15	60	320	60	480
nil-saline control	30	1920	<10	320	40	20	120	640	120	480

Table gives the inhibitory titres of different animal sera after treatment with trypsin and assayed against—

E. = 8 agglutinating doses of A/England/1/51.
H.C. = 8 agglutinating doses of Crawley virus heated at 56° for 30 min.

Action of trypsin on specific antibody.

In a number of experiments it was found that trypsin treatment caused a sharp drop in the normal inhibitor content of immune animal sera without any apparent action on specific antibody. Table VI shows the effect of treatment with 8 mg. trypsin/ml. serum on the normal inhibitor and specific antibody contents of immune ferret and fowl sera (to PR8 virus).

TABLE VI.—*Absence of Effect of Trypsin on Specific Antibody Titre.*

Serum.	Heated at 56° with—	Virus.		
		PR8.	A/England/1/51.	Heated Crawley.
PR8 immune ferret	Trypsin	5120	<10	10
	Buffer—control	5120	35	1280
PR8 immune fowl	Trypsin	960	<10	<10
	Buffer—control	960	<10	160

Table gives inhibitory titres of untreated and treated sera against 8 agglutinating doses of the viruses shown.

In other experiments it was found that following similar treatment, fowl, ferret, rabbit, guinea-pig, mouse and human sera showed no significant drop in antibody titre immediately or after 2 weeks' storage at 2°. Van der Veen and Mulder (1950) found no destructive effect of crude *V. cholerae* extract on specific antibody in human and ferret serum. We observed that after heating trypsin with normal serum at 56° for 30 min., tryptic activity was abolished, since on addition of a second volume of serum and heating again no further destruction of inhibitor took place.

Action of different preparations of trypsin.

Chu (1951) found that a semi-purified preparation of trypsin (Fairchild) caused a reduction in the inhibitory titre of normal mouse serum ; a crystalline preparation was without effect. Chu suggested that the activity was due to an

unidentified agent in the crude enzyme preparation. We have tested three different batches of crystalline trypsin prepared by Armour & Co., and one sample which was prepared from ox pancreas and kindly made available to us by our colleague Dr. J. Humphrey; all four preparations were highly active. The active factor was found to be heat-labile, to be effective after 30 min. incubation with serum at 37° or 56°, but not at 0°, and to show a greater activity after prolonged incubation (24 hr.) at 37°.

DISCUSSION.

These experiments were commenced in an attempt to identify the factor in crude *V. cholerae* extracts which inactivated the Chu inhibitor in normal sera. This factor was found to show similar properties to an active agent in a crystalline preparation of trypsin, and it is known that a trypsin-like enzyme is present in crude *V. cholerae* filtrates (Stone, 1949). A final identification of these factors has not yet been made, but it seems likely that the trypsin-like enzyme is the active factor in crude *V. cholerae* extract. There is as yet no evidence to suggest that the activity of the crystalline trypsin preparations is due to any impurity.

An obvious practical application of these results is in the removal of normal inhibitors from sera used for antigenic analysis of influenza viruses. Previously, workers have used either partially purified receptor-destroying enzyme of *V. cholerae* which acts on Francis inhibitor but not, apparently, on Chu inhibitor, or crude *V. cholerae* extract which acts on both Francis and Chu inhibitors. The former preparation is effective in treating ferret and fowl sera, which contain mainly Francis inhibitor, and it has the advantage that it can be readily standardised. The crude preparation was formerly the preparation of choice in treating rabbit, guinea-pig and mouse sera, but it was extremely difficult to standardise, and in addition, different batches of extract showed unpredictable variations in potency. The use of trypsin presents many advantages. It is a stable product which is active against Francis and Chu inhibitors under conditions where it does not affect the titre of specific antibody, and since it is available commercially it presents no difficulties in preparation or standardisation. It would be a further advantage if trypsin were equally effective in the treatment of human serum. However, non-specific and specific inhibitory activities are not so readily distinguished in human as in animal sera, and further investigation will be required before trypsin treatment can be safely applied to them.

SUMMARY.

A study was made of the action of crude *V. cholerae* extracts on the substance (Chu inhibitor) in normal rabbit serum which inhibits haemagglutination by influenza virus. The results suggested that a trypsin-like enzyme known to be present in crude *V. cholerae* extracts was responsible for the inactivation of Chu inhibitor. Crystalline trypsin was found to be highly active in destroying Chu and Francis inhibitors in normal ferret, fowl, rabbit, guinea-pig and mouse sera in conditions where no significant effect on specific antibody was observed.

We should like to thank Drs. C. H. Andrewes and R. Porter for their advice and criticism, and Messrs. E. Owen and D. Eade for their most valuable technical help.

REFERENCES.

ANDERSON, S. G.—(1948) *Aust. J. exp. Biol. med. Sci.*, **26**, 347.
BURNET, F. M., AND STONE, JOYCE D.—(1947) *Ibid.*, **25**, 227.
CHU, C. M.—(1951) *J. gen. Microbiol.*, **5**, 739.
FRANCIS, T., JR.—(1947) *J. exp. Med.*, **85**, 1.
GINSBERG, H. S., AND HORSFALL, F. L., JR.—(1949) *Ibid.*, **90**, 475.
ISAACS, A., AND BOZZO, A.—(1951) *Brit. J. exp. Path.*, **32**, 325.
MCCREA, J. F.—(1946) *Aust. J. exp. Biol. med. Sci.*, **24**, 283.
MAGILL, T. P., AND JOTZ, A. C.—(1952) *J. Bact.*, **64**, 619.
SAMPAIO, A. A. DE C.—(1952) *Bull. World Hlth. Org.*, **6**, 467.
SMITH, W., WESTWOOD, J. C. N., AND BELYAVIN, G.—(1951) *Lancet*, ii, 1189.
STONE, JOYCE D.—(1949) *Aust. J. exp. Biol. med. Sci.*, **27**, 229.
VAN DER VEEN, J., AND MULDER, J.—(1950) 'Studies on the Antigenic Composition of Human Influenza A Virus Strain.' Leiden (Stenfert Kroese).