standard error of 0·052. The method of calculating the correction for dye loss with time and the standard error of zero time estimate can be applied to other systems of dye determination times.

A test is described on six men of the existence of the effect, discovered in the cat, which leads to under-estimation of zero time concentration. Five of the six showed no sign of the effect, but the sixth gave evidence of its occurrence. Determinations of zero time concentrations by the customary method will therefore be valid generally, though subject to under-estimation occasionally.

The plasma volumes of the 53 students had a mean of 3·73 ± 0·079 litres, with a standard deviation of 0·575 litres. The lowest observed was 2·55 litres, and the highest, with one exception, 4·64 litres. The exception was 5·98 litres—a value which may be inflated by the occurrence of the cat effect. The standard error of a plasma volume determination varies with the volume itself and with the amount of dye injected, but averaged 0·2247 in the cases observed.

Plasma volume shows a correlation of 0·64 with height, 0·63 with surface area and 0·55 with weight in the 53 students. None of these measurements is a useful predictive of plasma volume.

REFERENCES.

CRUIKSHANK, E. W. H., AND WHITFIELD, I. C.—(1944) *Proc. physiol. Soc.*, **103**, 198.
MATHER, K.—(1946) 'Statistical Analysis in Biology.' London (Methuen, 2nd ed.).
MORRIS, C. J. O. R.—(1944) *Biochem. J.*, **38**, 203.

ANTICOLLAGENASE IN IMMUNITY TO *CL. WELCHII* TYPE A INFECTION.

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THE relative importance of α toxin, θ haemolysin and hyaluronidase in experimental *Cl. welchii* A infection, and of their respective antibodies in controlling the disease, has been the subject of a series of investigations during recent years (Evans, 1943*a* and *b*, 1945*a* and *b*). It was found that antisera containing α antitoxin were highly effective in protecting guinea-pigs against infection with a number of virulent strains of *Cl. welchii* A, whether the sera contained θ antihaemolysin and antihyaluronidase or not. On the other hand, antisera containing either θ antihaemolysin or antihyaluronidase but only a trace of α antitoxin had no influence on the course of the infection. Nor did they enhance the protective action of α antitoxin. It was also shown with 30 strains of *Cl. welchii* A from a variety of sources that the ability of a strain to produce fatal infection in guinea-pigs was related to the production of α toxin but not to the production either of θ haemolysin or hyaluronidase. These

results suggested that α toxin played the most important rôle in *Cl. welchii* A infection, and that α antitoxin was the significant antibody in the control of the disease.

Recently attention has been drawn to another antigenic enzyme produced by *Cl. welchii* A. Macfarlane and MacLennan (1945) observed that filtrates from broth cultures of *Cl. welchii* A were able to break down muscle by their action on collagen. They regarded the active substance as a collagenase, and suggested that it was responsible for the muscle destruction observed in gas gangrene. They further suggested that an antitoxin or toxoid designed to confer a high anticollagenase immunity might be more effective in the control of gas gangrene produced by *Cl. welchii* A than the present immunizing agents, which are judged by their α antitoxin effect. More recently Oakley, Warrack and van Heyningen (1946) found by *in vitro* tests that the collagenase was immunologically distinct from the other known antigens present in *Cl. welchii* A filtrates, and designed methods for determining the anticollagenase values of *Cl. welchii* antisera.

In view of this recent work, further tests have been made to investigate the protective action of anticollagenase in experimental gas gangrene. In the previous studies no account was taken of the anticollagenase activity of the antisera which were then employed, since the possible importance of this antibody was not appreciated at the time.

METHODS.

Two natural, unconcentrated *Cl. welchii* antisera prepared in horses at the Wellcome Laboratory have been employed. (1) R 6423 contained 75 units of α antitoxin per c.c. but no detectable anticollagenase, and (2) R 5434 contained 50 units of anticollagenase and only 0·2 units of α antitoxin per c.c. The arbitrary unit of anticollagenase is that chosen by Oakley and his colleagues. In the majority of the experiments antiserum was administered intramuscularly to guinea-pigs 20 hours *before* the infecting dose of organisms was given; in one experiment the antiserum was given intravenously 4 hours *after* infection. Guinea-pigs having an average weight of 450 g. were used, and kept under observation for at least 7 days after the onset of infection. Four virulent strains of *Cl. welchii* A were used, S.R. 9, S. 107, S.R. 12 and A. 118, all producing collagenase *in vitro*.

Three different methods of producing experimental gas gangrene were employed. In each a suspension of washed bacilli was prepared from a 3-hour liver broth culture which was centrifuged and the deposit of organisms washed once in saline, centrifuged, and resuspended in saline to give a concentration of about 250×10^6 organisms per c.c. From this suspension 10-fold dilutions in saline were made for injection.

In method 1 a dose of 0·2 c.c. of washed organisms was injected intramuscularly into the thigh of the animal 3 hours after an injection of $CaCl_2$ solution into the same site (Evans, 1943*a*).

Method 2 was based on the observation of Cooper (1946) that the infectivity of *Cl. welchii* was increased considerably when the organisms were injected with adrenaline; 0·2 c.c. of the bacterial suspension was injected about 10 minutes after an intramuscular injection of 0·5 c.c. of a 1 : 10,000 dilution of adrenaline

into the same site. This method produced fatal gas gangrene with dilute bacterial suspensions, and the course of the disease was similar to that observed when $CaCl_2$ was used as the adjuvant.

Method 3 was designed to produce a local skin infection. The bacterial suspension was mixed with equal parts of a 1 : 50,000 dilution of adrenaline and 0·2 c.c. of the mixture injected into the depilated skin of guinea-pigs. With small numbers of organisms a necrotic, oedematous infected lesion developed, which was often arrested after 24 hours, whereas with larger numbers the infection sometimes spread extensively and occasionally caused the death of the animal. With this method the protective effect of antisera was estimated on the smallest dose of organisms able to produce a skin infection.

RESULTS.

Experiments Using Calcium Chloride as Adjuvant.

Protective properties of anticollagenase.—The protective action of anticollagenase (R 5434) was tested as in Table I. Guinea-pigs were infected with *Cl. welchii* (S.R. 9) 20 hours after the intramuscular injection of serum. No protection was afforded by 50 units of anticollagenase. This result is confirmed by

TABLE I.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A* (*S.R.* 9) *and* $CaCl_2$ *after Receiving* (a) *No Serum, and* (b) *Anticollagenase.*

Dilution of *Cl. welchii* Suspension (0·2 c.c.).	Result with guinea-pigs (2 in each group) receiving—	
	No serum.	50 units of anticollagenase.
10^{-5}	D 4 ; D 1	D 1 ; D 2
10^{-6}	D 1 ; S +++	D 4 ; D 3
10^{-7}	D 2 ; D 5	D 4 ; S +++
10^{-8}	S — ; S —	S ++ ; S —

Key to Tables I–VI :
D 2 = death in 2 days :
S = survival ;
— = no reaction :
+ = small local swelling :
++ = large gangrenous lesion ;
+++ = extensive gangrene.

an observation from the previous investigations (Evans, 1943*a* and *b*) with an antiserum (R 5432) containing only a trace of α antitoxin, which has since been shown by Dr. Oakley to contain anticollagenase. The largest dose given (2 c.c.) now proves to have contained 80 units of anticollagenase, and was quite ineffective in protecting animals against infection by a number of strains of *Cl. welchii* A.

Protective properties of α antitoxin.—In a similar manner the protective properties of α antitoxic serum containing no anticollagenase (R 6423) was investigated with four strains of *Cl. welchii* A. Guinea-pigs receiving 50 units of α antitoxin were protected against at least 10,000 average lethal doses of *Cl. welchii* (S.R. 9) (Table II). One infecting dose only of strains S. 107, A. 118

TABLE II.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A (S.R. 9) and $CaCl_2$, after Receiving* (a) *No Serum, and* (b) *α Antitoxin.*

Dilution of *Cl. welchii* suspension (0·2 c.c.).	Result with guinea-pigs (2 in each group) receiving—	
	No serum.	50 units of α antitoxin.
10^{-3}	..	S — ; S —
10^{-4}	D 3 ; D 2	S — ; S —
10^{-5}	D 1 ; D 2	S — ; S —
10^{-6}	D 4 ; D 2	S — ; S —
10^{-7}	D 2 ; D 2	S — ; S —

TABLE III.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A (3 Strains) and $CaCl_2$, after Receiving,* (a) *No Serum, and* (b) *α Antitoxin.*

Strain.	Dilution of *Cl. welchii* Suspension (0·2 c.c.).	Result with guinea-pigs (3 in each group) receiving	
		No serum.	50 units of α antitoxin.
S.R. 12	10^{-3}	D 3 ; D 5 ; D 4	S — ; S — ; S —
S. 107	10^{-1}	D 3 ; D 4 ; D 4	S — ; S — ; S —
A. 118	10^{-3}	D 3 ; D 2 ; D 3	S — ; S — ; S —

and S.R. 12 was used, containing approximately 1000 average lethal doses, and again a dose of serum containing 50 units of α antitoxin and no anticollagenase protected the animals against fatal infection (Table III).

Protective properties of α antitoxin and anticollagenase combined.—An experiment was designed to observe whether anticollagenase would enhance the protective action of α antitoxin (Table IV). Guinea-pigs in one group were given graded doses of α antitoxin, and those in a second group the same doses of α antitoxin to which had been added 50 units of anticollagenase. Twenty

TABLE IV.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A (S.R. 9) and $CaCl_2$ after Receiving* (a) *α Antitoxin, and* (b) *α Antitoxin and Anticollagenase.*

Dose of α antitoxin (units).	Result with guinea-pigs (2 per dose) receiving approximately 100 lethal doses *Cl. welchii*.		Dose of α antitoxin and anticollagenase (K) (units).	Result with guinea-pigs (2 per dose) receiving approximately 100 lethal doses *Cl. welchii*.	
40 α	S — ;	S —	40 α + 50 K	S — ;	S —
20 α	S — ;	S +	20 α + 50 K	S + ;	S +
10 α	S + ;	D 3	10 α + 50 K	S + ;	D 4
5 α	S + + + ;	D 1	5 α + 50 K	D 4 ;	D 2
2·5 α	D 1 ;	D 4	2·5 α + 50 K	S + + + — ;	D 4
1·25 α	D 4 ;	D 2	1·25 α + 50 K	D 2 ;	D 4

hours later each guinea-pig was injected with 100 average lethal doses of *Cl. welchii* (S.R. 9) as determined by a simultaneous test on unprotected animals. Antiserum containing from 10 to 20 units of α antitoxin but no anticollagenase was protective, but no more so when given together with 50 units of anticollagenase.

Curative properties of α antitoxin.—A test was made, similar to those described previously (Evans, 1945*b*), of α antitoxin given intravenously to guinea-pigs *after* they had been infected with *Cl. welchii* A (Table V). Each of 18 guinea-

TABLE V.—*Treatment of Guinea-pigs with α Antitoxic Serum Containing No Anticollagenase 4 Hours after Infection with* Cl. welchii *A (S.R. 9) and* $CaCl_2$.

Dose of *Cl. welchii*.	Dose of intravenous α antitoxin (units).	Result (3 guinea-pigs per dose).
Approximately 100 lethal doses	2·5	D 4 ; D 3 ; D 5
	5	D 5 ; D 2 ; D 2
	10	S +++ ; S ++ ; S ++
	20	S +++ ; S ++ ; D 6
	40	S ++ ; S ++ ; S ++
	80	D 3 ; S + ; S +

pigs was infected with approximately 100 average lethal doses of S.R. 9, as determined by a simultaneous test on untreated animals. Four hours later varying doses of α antitoxin (R 6423) were given intravenously. It was evident that the α antitoxic serum containing no anticollagenase gave substantial protection against fatal gas gangrene when given after the onset of infection.

Experiments Using Adrenaline as Adjuvant.

Intramuscular infection.—A comparison was made of the protective action of anticollagenase and α antitoxin in fatal infection produced by *Cl. welchii* A activated by adrenaline (Table VI). Twelve animals each received 50 units

TABLE VI.—*Resistance of Guinea-pigs to Infection Produced by* Cl. welchii *A (S.R. 9) and Adrenaline after Receiving* (a) *No Serum*, (b) *α Antitoxin, and* (c) *Anticollagenase.*

Dilution of *Cl. welchii* suspension (0·2 c.c.).	Result with guinea-pigs (2 in each group) receiving—		
	No serum.	50 units of α antitoxin.	50 units of anticollagenase.
10^{-2}	D 2 ; D 2	S ++ ; S +	D 1 ; D 2
10^{-3}	D 2 ; D 2	S + ; S +	D 4 ; D 1
10^{-4}	D 2 ; D 3	S + ; S —	D 1 ; D 2
10^{-5}	D 4 ; D 3	S — ; S —	S ++ ; D 2
10^{-6}	S + ; D 2	S — ; S —	D 2 ; D 2
10^{-7}	S — ; S —	S — ; S —	S + ; S —

of α antitoxin (R 6423), 12 each received 50 units of anticollagenase (R 5434), and 12 were not given serum. Twenty hours later all 36 animals were each injected intramuscularly with 0·5 c.c. of a 1 : 10,000 dilution of adrenaline, and about 10 minutes later washed *Cl. welchii* organisms (S.R. 9) were injected in the same site. A dose of 50 units of α antitoxin protected against approximately 10,000 average lethal doses (the highest infecting dose used), but 50 units of anticollagenase gave no protection whatsoever against the smallest lethal dose.

Intradermal infection.—A similar result was obtained with intradermal infection by mixtures of *Cl. welchii* A and adrenaline. Groups of guinea-pigs were immunized as shown in Table VII. Twenty hours later each animal was

TABLE VII.—*Resistance of Guinea-pigs to Skin Infection Produced by* Cl. welchii *A (S.R. 9) and Adrenaline after Receiving* (a) *No Serum*, (b) α *Antitoxin, and* (c) *Anticollagenase.*

Dose of serum to each guinea-pig.	Skin lesions at 24 hours after receiving following dilutions of *Cl. welchii* suspension.						Number of guinea-pigs.	Number of deaths.
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}		
70 units α antitoxin	—	—	—	—	—	—	3	0
30 ,, ,,	—	—	—	—	—	—	3	0
10 ,, ,,	—	—	—	—	—	—	3	0
100 ,, anticollagenase	+ + +	+ + +	— — —	+ + +	+ + +	—	3	1
50 ,, ,,	+ + +	+ + +	— — —	+ + +	+ + +	—	3	2
None	— — —	— — —	— — —	+ + +	+ + +	—	3	2

Key to Table VII :

— = no reaction ;
+ = inflammatory reaction ;
+ + + = progressive infected lesion with necrosis.

given six intradermal injections of 10-fold dilutions (10^{-1} to 10^{-6}) of a washed *Cl. welchii* (S.R. 9) suspension (250×10^6 organisms per c.c.) mixed with equal parts of a 1 : 50,000 dilution of adrenaline. The minimal infecting dose was a dilution of 10^{-5}. All the animals receiving α antitoxin were protected against 1000 infecting doses of *Cl. welchii*, and even the injection of 10,000 infecting doses resulted only in a small indurated area which did not spread. None of the guinea-pigs receiving either 50 or 100 units of anticollagenase was protected against the smallest skin infecting dose (10^{-5} dilution), and all developed extensive infected lesions similar to those shown by the controls. Three of the animals receiving anticollagenase and two in the control group died in 5 to 6 days, but there were no deaths in the groups receiving α antitoxin.

CONCLUSIONS.

A number of antigens have been identified in *Cl. welchii* A filtrates : α toxin, θ haemolysin, hyaluronidase and collagenase. The relative importance of these antigens in *Cl. welchii* A gas gangrene, and of their respective antibodies in the control of the disease, is indicated by the fact that small doses of α antitoxin are effective in protecting guinea-pigs against fatal infection, whereas large doses of antisera containing either θ antihaemolysin, antihyaluronidase or anticollagenase, but no α antitoxin, are quite ineffective. Moreover, neither θ anti-

haemolysin, antihyaluronidase nor anticollagenase enhance the protective properties of α antitoxin.

It therefore appears that in the control of experimental gas gangrene in guinea-pigs α antitoxin is of prime importance. It is also reasonable to suppose that α toxin plays the most important part in the infectious process, and while it is recognized that the rapid spread of the disease may be associated with hyaluronidase production and that muscle destruction may be a result of the action of collagenase, there is so far no evidence to support the view that either of these enzymes plays any substantial part in the genesis of fatal gas gangrene.

SUMMARY.

Fatal gas gangrene was produced in guinea-pigs by injecting washed cultures of *Cl. welchii* A intramuscularly after an injection into the same site of (*a*) $CaCl_2$ solution or (*b*) adrenaline solution. A local skin infection was produced in guinea-pigs by the intradermal injection of a mixture of a washed culture of *Cl. welchii* A suspended in a dilute solution of adrenaline.

The protective action of α antitoxin and anticollagenase occurring in *Cl. welchii* A antisera was tested in these three types of infection. Antiserum containing α antitoxin and no anticollagenase was highly effective in protecting guinea-pigs against infection, whereas an antiserum containing anticollagenase but no α antitoxin was able neither to protect against infection nor enhance the protective properties of α antitoxin.

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REFERENCES.

COOPER, E. V.—(1946) *Lancet*, i. 459.

EVANS, D. G.—(1943*a*) *Brit. J. exp. Path.*, **24**, 81.—(1943*b*) *J. Path. Bact.*, **55**, 427.—(1945*a*) *Ibid.*, **57**, 75.—(1945*b*) *Brit. J. exp. Path.*, **26**, 104.

MACFARLANE, R. G., AND MACLENNAN, J. D.—(1945) *Lancet*, ii, 328.

OAKLEY, C. L., WARRACK, G. H., AND VAN HEYNINGEN, W. E.—(1946) *J. Path. Bact.*, **58**, 229.