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Inhibition of Complement by Carrageenin: Mode of Action, Effect on Allergic Reactions and on Complement of Various Species

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Summary. Carrageenin prevents the reaction of guinea-pig C′ with sensitized red blood cells, probably by a direct inactivation of the C'_1. The anti-complementary effect of carrageenin is antagonized by protamine sulphate. Marked reduction of circulating C′ activity in guinea-pigs resulting from intravenous injection of carrageenin, does not alter their susceptibility to acute or protracted anaphylactic shock. The C′ activities of human, guinea-pig, rabbit, rat and monkey sera are all inhibited *in vitro* by carrageenin.

INTRODUCTION

The haemolytic activity of guinea-pig complement is inhibited by very small amounts of the sulphated polysaccharide carrageenin, both when the carrageenin is added to the complement *in vitro* and when it is given intravenously to the animal before it is bled. Carrageenin appears to act at a stage prior to the fixation of the first component of complement (C'_1) to the sensitized red blood cell (EA) since cells in the forms EAC'_1, $EAC'_{1,4}$ or $EAC'_{1,4,2}$ still lyse in the presence of their appropriate reagents, irrespective of the presence of carrageenin (Davies, 1963). The present experiments were designed to elucidate further the mode of action of carrageenin, to observe the effect of carrageenin treatment on anaphylactic reactions in guinea-pigs and to test the effect of carrageenin on the complement activity of sera from species other than the guinea-pig.

MATERIALS AND METHODS

Buffer

Barbiturate buffer, pH 7·3, ionic strength 0·147, was prepared as described by Mayer (1961). Unless otherwise stated, it contains 0·00015 M Ca^{++} and 0·0005 M Mg^{++}. Ethylene diamine tetra-acetic acid, when required was used at a concentration of 0·01 M, in which case calcium and magnesium were omitted from the buffer.

Complement

Guinea-pigs, mice and rats were bled from the heart; monkeys and rabbits were bled from a vein, all under ether anaesthesia. Human blood was taken from a vein, without anaesthesia. Complement was estimated in terms of the number of 50 per cent haemolytic units ($C'H_{50}$) per ml., as described by Mayer (1961). Conditions optimal for the estimation of guinea-pig complement were also used for mouse, rabbit and monkey serum. Optimal conditions for the estimation of rat complement are slightly different (Osler, Hawrisiak,

Ovary, Siqueira and Bier, 1957). They involve the use of a higher concentration of haemolysin and the omission of calcium from the buffer. All estimations were done using a total volume of 7·5 ml., including 1 ml. of sensitized red blood cells (5×10^8). Optical densities were read at 541 mμ using a Unicam SP 600 spectrophotometer.

Sheep Erythrocytes

Sheep blood in Alsever's solution was purchased from Evans Medical Limited, Liverpool. Before use the cells were washed three times with barbiturate buffer and standardized spectrophotometrically to contain 10^9 cells/ml. Sensitization was effected by mixing these cells with an equal volume of haemolysin at its optimal concentration.

Haemolysin

Rabbits were immunized with boiled stromata from sheep red blood cells as described by Mayer (1961).

This haemolysin when added to a suspension of sheep erythrocytes, produced cells in the form EA. For the production of the intermediate complex EAC'_1, a sample of haemolysin was similarly prepared but was not heated before use.

Preparation of EAC'_1

Washed sheep erythrocytes (10^9 cells/ml.) were incubated for 20 minutes at 37° with an equal volume of unheated haemolysin, diluted 1/100 to 1/250 with buffer. These cells lysed in the presence of a reagent deficient in C'_1 (R1) (De Looze, Ransby and Leon, 1962). It should be noted that the C'_1 in this preparation is derived from the unheated rabbit haemolysin.

Reagent Deficient in C'_1 (R1)

This was prepared from guinea-pig serum according to Mayer (1961) and consisted of equal parts of euglobulin (prepared by dialysis and diluted 1/10) and guinea-pig serum heated at 56° for 20 minutes and diluted 1/10.

Carrageenin

Carrageenin, type 21, was kindly donated by Marine Colloids, Inc., 24 State Street, New York. Solutions were made by dissolving the carrageenin in boiling saline and allowing it to cool to room temperature.

Anaphylactic Shock in Guinea-Pigs

Guinea-pigs were sensitized by intraperitoneal injection of alum-precipitated, crystallized egg albumin (2·5 ml. of 2 per cent suspension) and used for experiments 3–4 weeks later. Acute anaphylactic shock was produced by the intravenous injection of crystallized egg albumin (0·2 ml. per 100 g. of a 0·2 per cent solution in saline). Protracted anaphylactic shock (Stone, 1959) was produced by subcutaneous injection of crystallized egg-albumin (0·4 ml. per 100 g. of a 10 per cent solution) in animals which had been dosed with mepyramine (2·5 mg. per 100 g. subcutaneously) 15 minutes before.

Permeability Factor

The permeability factor (PF/P) was prepared and tested by the method of Davies and Lowe (1960) by adding a pre-formed antigen–antibody complex (egg-albumin/rabbit

anti-egg albumin serum) to fresh guinea-pig serum and injecting the mixture into the skin of guinea-pigs previously injected intravenously with pontamine sky blue.

RESULTS

MODE OF ACTION OF CARRAGEENIN

Since cells in the form of the intermediate complex, EAC'_1, still progressed to lysis in the presence of C′ and a concentration of carrageenin which prevented lysis of EA by C′, it was suggested previously that carrageenin prevents the fixation of C'_1 to the sensitized cell (Davies, 1963).

The intermediate complex EAC'_1 can be prepared by allowing red blood cells to react with unheated haemolysin prepared in rabbits (De Looze *et al.*, 1962). Various concentrations (up to 100 μg./ml.) of carrageenin, in 1 ml. of buffer, were incubated with red blood cell suspension (0·5 ml.) and unheated haemolysin (0·5 ml. of 1/250) for 20 minutes at 37° before the addition of R1 (1 ml.) and buffer (4·5 ml.). A second set of tubes was prepared in which red blood cell suspension and unheated haemolysin were incubated together for 20 minutes before the addition of R1 and carrageenin. Both sets of tubes showed complete lysis during subsequent incubation at 37°. This result suggested the unlikely conclusion that carrageenin acted neither before nor after the fixation of C'_1. However, the haemolysin had been added to the cells a minute or so before the carrageenin and there was a possibility that the cells were already in the form EAC'_1 when the carrageenin was added. Unheated haemolysin (0·5 ml. of 1/250) was therefore incubated for 10 minutes with carrageenin (50 μg. in 1 ml.) before the addition of red blood cell suspension (0·5 ml.). Incubation was continued for a further 20 minutes and then R1 (1 ml.) and buffer (4·5 ml.) were added and incubation continued for a further 60 minutes. Similarly, carrageenin was incubated for 10 minutes with cells before the addition of haemolysin and, later, of R1 and buffer. There was no lysis in either of these tubes, indicating that carrageenin had prevented the formation of EAC'_1. This result also showed that, under these conditions, the formation of EAC'_1 was very rapid, as was the reaction between C′ and carrageenin. The following experiments supported these conclusions.

Sensitized cells were cooled to 0° and 1 ml. of the suspension added to 1 ml. of cooled C′ (1 : 100). The mixture was incubated at 0° for 10 minutes. Buffer (4·5 ml.) and carrageenin (5 μg. in 1 ml.) were then added. To a second tube, similarly prepared, buffer (5·5 ml.) alone was added. Both tubes were then incubated for 60 minutes at 37°. In a separate experiment, a series of tubes was prepared in which the various components of the reaction (EA, carrageenin, C′, and buffer) were mixed in different orders, at 30-second intervals at room temperature and then incubated for 60 minutes at 37°. The results showed (Table 1) that whereas cells incubated with C′ at 0° before the addition of carrageenin still progressed to lysis, there was little or no lysis when carrageenin was present before C′. Cells incubated at 0° with C'_1 will be in various forms (EA, EAC'_1, $EAC'_{1,4}$, etc.) but since the earlier experiments showed that the lysis of EAC'_1 is not inhibited by carrageenin the only possible explanations of the failure of cells to lyse is that the formation of EAC'_1 is prevented either by destruction of a C'_1 component or by a protective effect on the cell.

Müller-Eberhard and Kunkel (1961) and Müller-Eberhard (1961) have described a component of human complement, designated 11S component, which reacts with sensitized cells before C'_1 and in the absence of calcium. Its activity was lost upon heating at

56° for 45 minutes. Lepow, Naff, Todd, Pensky and Hinz (1963) identified this component with their C'_{1q}. Davies (1963) showed that the carrageenin-sensitive component in guinea-pig serum was not completely destroyed by heating at 56° for 20 minutes although haemolytic activity was destroyed by heating the serum for 10 minutes.

Because of this apparent similarity in heat-lability of C'_{1q} and the carrageenin-sensitive component and also because carrageenin appeared to act on a C'_1 component, it appeared that the two might be identical.

TABLE 1

INHIBITORY EFFECT OF CARRAGEENIN ON THE FORMATION OF EAC'_1

(a) EAC'_1 formation at 0°

	Lysis (%)
EA + C′ incubated at 0° before addition of carrageenin	83
EA + C′ incubated at 0° before addition of buffer	100

(b) Effect of mixing component in different orders

Order of mixing	*Lysis* (%)
EA; carrageenin; C′; buffer	0
EA; C′; buffer; carrageenin	50
EA; carrageenin; buffer, C′	5
EA; C′; buffer	100
EA; buffer; C′	100

On the assumption, as yet unproven, that the C'_1 complex of guinea-pig serum is similar to that of man, and taking advantage of the finding that C'_{1q} (11S) reacts with EA in the absence of calcium, an attempt was made to prepare the intermediate complex EAC'_{1q} from guinea-pig C′. It was argued that, if carrageenin inhibits C'_{1q}, then cells already in this form should lyse unhindered in the presence of C′ previously incubated with carrageenin.

At 0°, EA (4 ml.) were mixed with C′ (4 ml. of 1/10) and 22 ml. of buffer containing 0·01 M EDTA. After 10 minutes incubation at 0° the cells were washed once with EDTA buffer and three times with buffer containing calcium and magnesium and finally suspended in 4 ml. of this buffer. Another 4 ml. of EA were similarly treated, but the C′ was omitted and 4 ml. of EDTA buffer used in its place. The first lot of cells are referred to as 'postulated EAC'_{1q}'. To prepare the 'postulated $R1_q$', C′ (5 ml. of 1/10) was incubated with carrageenin (5 ml. of 25 μg./ml.) for 45 minutes at 37°. A 1 ml. sample of this mixture produced 9 per cent haemolysis when incubated with EA (1 ml.) and buffer (5·5 ml.). In order to neutralize excess carrageenin, protamine sulphate (500 μg. in 1 ml.) (see below) was added to the remainder and, after incubation for 5 minutes, 1 ml. of the mixture was added to EA (1 ml.) and buffer (5·5 ml.), to test for completeness of inactivation, and a further 1 ml. was added to EA (1 ml.), C′ (1 ml. of 1/200), and buffer (4·5 ml.) to test for anti-complementary activity. There was 26 per cent lysis in the first tube and complete lysis in the second, indicating almost complete destruction of complement activity during the preliminary incubation of C′ with carrageenin and little or no anti-complementary effect of the final reagent. One ml. amounts of 'postulated EAC'_{1q}', or control cells,

were then added to 1 ml. amounts of either 'postulated $R1_q$' or C′ (1/100). Buffer was added to a total volume of 7·5 ml. and the degree of haemolysis measured after incubation for 1 hour at 37°. Both kinds of cells showed complete lysis in the presence of C′ but only about 30 per cent lysis in the presence of 'postulated $R1_q$' (Table 2). It therefore seemed that if the cells were in fact in the form $EAC'1_q$ then their subsequent reaction with other components was blocked by carrageenin.

TABLE 2

EFFECT OF CARRAGEENIN ON 'POSTULATED C'_{1q}'

Reaction mixture	*Lysis* (%)
'EAC'_{1q}' + '$R1_q$'	32
'EAC'_{1q}' + C′ (1/100)	100
EA + '$R1_q$'	37
EA + C′ (1/100)	100

'EAC'_{1q}' = EA incubated with C′ in the presence of EDTA.
'$R1_q$' = C′ incubated with carrageenin and excess carrageenin neutralized by the addition of protamine sulphate.

REVERSAL OF THE ANTI-COMPLEMENTARY EFFECT OF CARRAGEENIN BY PROTAMINE SULPHATE

In order to study the reaction between complement and carrageenin it was necessary to find some means of neutralizing the effect of carrageenin.

Mutsaars and Lison (1948) showed that the anti-complementary effect of sulphated esters of polysaccharides was antagonized by basic dyes. Since the presence of basic dyes

TABLE 3

ANTAGONISM OF THE ANTI-COMPLEMENTARY EFFECT OF CARRAGEENIN BY PROTAMINE SULPHATE

Carrageenin (μg.)	*Protamine sulphate* (μg.)	*Lysis* (%)
10	25	100
10	12·5	14
10	0	7
0	100	100
0	0	100

1 ml. of guinea-pig C′ (1/100) in each tube.

was likely to interfere with the colorimetric estimation of haemoglobin, the basic protein protamine was tested for its ability to neutralize the effect of carrageenin. A concentration of 25 μg. per ml. of protamine sulphate antagonized the anti-complementary activity of 10 μg. per ml. of carrageenin (Table 3). The amount of protamine sulphate that could be used in experiments of this type was limited by the anti-complementary effect of protamine itself, and this, in turn, was antagonized by carrageenin (Table 4).

Antagonism of carrageenin activity by protamine enabled a study to be made of the rate at which complement activity was destroyed by carrageenin and, incidentally, to obtain evidence that carrageenin acted directly on some part of complement, rather than on the sensitized cell itself or on some intermediate complex of components of complement

with the sensitized cell. Complement (1 ml. of 1/100) and buffer (3·5 ml.) were pipetted into a series of tubes. At timed intervals, carrageenin solution (10 μg. in 1 ml.) was added and, at various intervals thereafter, protamine sulphate (50 μg. in 1 ml.) and EA suspension (1 ml.) were added and the tubes incubated for a further 60 minutes at 37°. The

TABLE 4

MUTUAL ANTAGONISM OF ANTI-COMPLEMENTARY EFFECTS BY CARRAGEENIN AND PROTAMINE SULPHATE

Carrageenin (μg.)	*Lysis* (%)					
	No protamine	12·5 μg. *protamine*	25 μg. *protamine*	50 μg. *protamine*	100 μg. *protamine*	200 μg. *protamine*
0	82	75	67	37	9	0
1	0·7	73	75	60	8	0
2	0·6	74	75		9	0
4	1·7	76	81	63	14	0
8	0	3	76	75	22	0
6	0	0	3	80	46	0

1 ml. of guinea-pig C′ (1/200) in each tube.

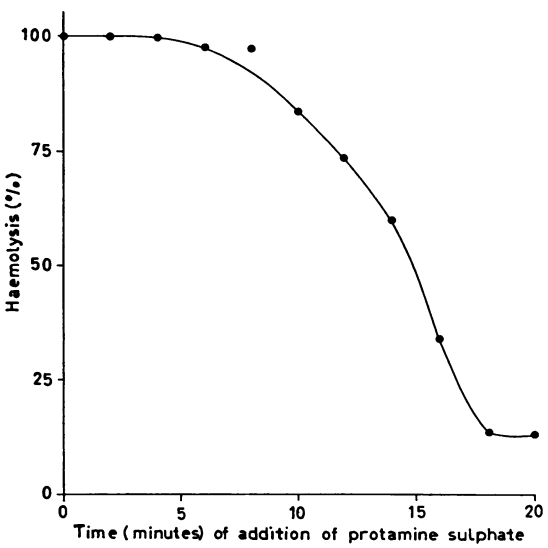

FIG. 1. The rate of destruction of C′ by carrageenin: Complement (1 ml. of 1/100) incubated with carrageenin (10 μg. in 1 ml.). Protamine sulphate (50 μg. in 1 ml.) and EA added at various intervals. Haemolysis measured after 1 hour at 37°.

amount of haemolysis in each tube was plotted against the period of contact between C′ and carrageenin. Under these conditions, complement activity was almost completely destroyed in about 20 minutes (Fig. 1).

EFFECT OF CARRAGEENIN ON ALLERGIC REACTIONS

Since intravenously-administered carrageenin inhibited the haemolytic complement activity in the serum of dosed guinea-pigs (Davies, 1963) it was of obvious interest to study the effect of such dosing on allergic reactions in this species.

Sensitized guinea-pigs were subjected to acute and protracted anaphylactic shock. Neither condition was affected by a dose of carrageenin (5 mg./kg.) given intravenously 10 minutes before the injection of antigen (Table 5). This amount of carrageenin reduced the C′ activity to less than 5 C′H_{50}/ml.

Davies and Lowe (1960) described a permeability factor formed by adding antigen–antibody complexes to guinea-pig serum. This factor, which they called PF/P appeared to require the presence of serum complement for its formation (Davies and Lowe, 1962). Its formation was not, however, inhibited by carrageenin.

TABLE 5

LACK OF EFFECT OF INTRAVENOUSLY ADMINISTERED CARRAGEENIN ON ANAPHYLAXIS IN GUINEA-PIGS

(a) Anaphylaxis		(b) Protracted anaphylaxis	
Survival time (minutes)		*Survival time (minutes)*	
Carrageenin (5 mg./kg. i.v.)	*Controls*	*Carrageenin (5 mg./kg. i.v.)*	*Controls*
3	4	42	45
4	3	57	29
2	2	31	Survivor
5	5	50	18
2	3		

EFFECT OF CARRAGEENIN ON THE COMPLEMENT OF SPECIES OTHER THAN THE GUINEA-PIG

Carrageenin inhibited the haemolytic activity of the complement of all five species tested. Its activity was not related to the C′ titre of the serum since it was equally active against human, monkey and guinea-pig C′. Both rat and rabbit C′ were very sensitive to the inhibitory effect when they were tested under conditions suitable for the assay of guinea-pig C′ (Table 6).

TABLE 6

INHIBITION OF COMPLEMENT BY CARRAGEENIN *in vitro*

Species	*C′H_{50} (ml.)*	*Haemolysis* (%)								
		200*	100	50	25	12·5	6·25	3·12	1·56	0·78
Guinea-pig	250	0	0	1·0	31	96	100	100	100	100
Human	65	0	0	12	37	81	88	91	100	100
Monkey	44	0	0	22	42	52	60	100	100	100
Rat	30	0	0	0	0	0	0	10	50	100
Rabbit	20	0	0	0	0	0	50	100	100	100

Serum (1 ml. of 1/10) incubated with carrageenin solution (1 ml.) for 30 minutes: EA (1 ml.) and buffer (4·5 ml.) added and incubation continued for a further 60 minutes. Both Ca^{++} and Mg^{++} added to buffer; red cells optimally sensitized for guinea-pig C′ activity.

* Concentration of carrageenin (µg./ml.).

Higher titres with rat C′ can be obtained if calcium is omitted from the buffer and a higher concentration of haemolysin used to sensitize the red blood cells (Osler *et al.*, 1957). We have confirmed this observation. The mean titre of thirty-one specimens of rat C′ estimated under these conditions was 84±2·9 C′H_{50}/ml. whereas under conditions suitable for the guinea-pig twenty-four samples gave titres of only 32±2·0 C′H_{50}/ml. When the

inhibitory activity of carrageenin was tested against rat C′ under these conditions of a higher level of sensitization and with added magnesium but no added calcium, a bi-phasic effect was observed, small amounts of carrageenin inhibited haemolysis whereas larger amounts permitted full haemolysis (Table 7). The reason for this anomaly is being investigated.

TABLE 7

INHIBITION OF RAT COMPLEMENT BY CARRAGEENIN

Concentration of carrageenin (μg./*ml.*)	*Haemolysis* (%)
400	100
200	93
100	68
50	27
25	21
12·5	21
6·25	34
3·125	77
1·56	93
0·78	100

Serum (1 ml. of 1/10) incubated with carrageenin solution (1 ml.) for 30 minutes: (EA 1 ml.) and buffer (4·5 ml.) added and incubation continued for a further 60 minutes. Mg^{++} but not Ca^{++} added to buffer: red cells optimally sensitized for rat C′ activity.

DISCUSSION

Lepow *et al.* (1963) isolated three fractions of the C'_1 complex of human complement. One of these fractions (C'_{1q}) appears to be identical with the 11S component isolated by Müller-Eberhard and Kunkel (1961). C'_{1r} could not be identified with any known complement activity and was presented as a new component. C'_{1s} was identified with C'_1 pro-esterase. All three fractions were necessary for the generation of C'_1 esterase activity.

Carrageenin, which allows cells in the form EAC'_1 to lyse in the presence of R1 ($C'_{4,2}$ and C'_3) has now been shown to prevent the formation of EAC'_1 by guinea-pig complement. This it could do either by inactivating directly some part of guinea-pig C'_1 or by preventing the sensitized cell from fixing C'_1 by an action on the cell itself. It is difficult at present to separate these two possibilities but the progressive inactivation of C′, illustrated in Fig. 1, suggests that carrageenin acts directly on complement rather than on the cells. Sub-fractionation of C'_1 has been achieved so far only with human complement. If guinea-pig C'_1 has an analogous composition then carrageenin does not prevent the fixation of the component analogous to C'_{1q}, if this fraction, as in human serum, is fixed to the sensitized cell in the absence of calcium (Müller-Eberhard, 1961). The effect of carrageenin on guinea-pig fractions analogous to C'_{1r} and C'_{1s} can only be determined when these fractions become available.

The antagonism of the anti-complementary effect of carrageenin by protamine is probably the result of an acidic molecule (carrageenin) reacting with a basic molecule (protamine) but it is tempting to speculate that the component of complement which is inhibited by carrageenin may also be a basic molecule.

The lack of effect of *in vivo* inhibition of complement by carrageenin on anaphylactic shock could have three possible explanations.

(a) The small residual C′ activity (less than 2 $C'H_{50}$/ml.) could still be sufficient to permit the subsequent stages to progress.

(b) The component of complement inhibited by carrageenin, although essential for haemolysis, is not required for the anaphylactic reaction, or

(c) C′ is not involved in anaphylaxis.

Similar arguments apply to the lack of effect of carrageenin on the formation of the permeability factor PF/P and further experiments on this point are in progress. The very complex problem of the role of complement in allergic reactions has been reviewed by Osler (1961) and it is apparent that a solution to this problem has not yet been reached.

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REFERENCES

DAVIES, G. E. (1963). 'Inhibition of guinea-pig complement *in vitro* and *in vivo* by carrageenin.' *Immunology*, **6**, 561.

DAVIES, G. E. and LOWE, J. S. (1960). 'A permeability factor released from guinea-pig serum by antigen-antibody precipitates'. *Brit. J. exp. Path.*, **41**, 335.

DAVIES, G. E. and LOWE, J. S. (1962). 'Further studies on a permeability factor released from guinea-pig serum by antigen-antibody precipitates: relationship to serum complement.' *Int. Arch. Allergy*, **20**, 235.

DE LOOZE, L. L., RANSBY, A. and LEON, M. A. (1962). 'Activity of C′1 in rabbit anti-sheep hemolysin.' *Proc. Soc. exp. Biol. (N.Y.)*, **109**, 393.

LEPOW, I. H., NAFF, G. B., TODD, E. W., PENSKY, J. and HINZ, C. F. (1963). 'Chromatographic resolution of the first component of human complement into three activities.' *J. exp. Med.*, **117**, 983.

MAYER, M. M. (1961). 'Complement and complement fixation.' *Experimental Immunochemistry* (By E. A. Kabat and M. M. Mayer), 2nd edn., pp. 133–240. Thomas, Springfield.

MÜLLER-EBERHARD, H. J. (1961). 'Isolation and description of proteins related to the human complement system.' *Acta Soc. Med. upsalien.*, **66**, 1.

MÜLLER-EBERHARD, H. J. and KUNKEL, H. G. (1961). 'Isolation of a thermolabile serum protein which precipitates γ-globulin aggregates and participates in immune hemolysis.' *Proc. Soc. exp. Biol. (N.Y.)*, **106**, 291.

MUTSAARS, W. and LISON, L. (1948). 'Recherches concernant l'alexine. III. Action des esters sulfuriques polysaccharidiques; effet des colorants basique.' *Ann. Inst. Pasteur*, **74**, 40.

OSLER, A. G. (1961). 'Functions of the complement system.' *Advanc. Immunol.*, **1**, 131.

OSLER, A. G., HAWRISIAK, M. M., OVARY, Z., SIQUEIRA, M. and BEIR, O. G. (1957). 'Studies on the mechanism of hypersensitivity phenomena. II. The participation of complement in passive cutaneous anaphylaxis of the albino rat.' *J. exp. Med.*, **106**, 118.

STONE, S. H. (1959). 'Acute and protracted anaphylactic shock in guinea-pigs after subcutaneous eliciting injection of antigen.' *J. Immunol.*, **82**, 138.