THE ANTIGENIC COMPLEX OF STREPTOCOCCUS HÆMOLYTICUS.

IV. ANAPHYLAXIS WITH TWO NON-TYPE-SPECIFIC FRACTIONS.

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Anaphylactic experiments with bacterial proteins have presented more difficulties to investigators in this field than similar experiments with proteins from other sources. This has probably been due, to a certain extent, to the primary toxicity of many bacterial proteins and, in part, to the difficulty of obtaining a sufficient quantity of concentrated material.

Zinsser and Parker (1), working with a slightly alkaline saline extract of typhoid bacilli, noted its primary toxicity and the quantitative rather than the qualitative differences between passively sensitized and normal guinea pigs when relatively large amounts of antigen were injected; whereas with smaller amounts, they did not obtain acute anaphylactic death. Using the isolated uterus method of Dale (2), they were able to eliminate the error due to toxicity of the antigen, since normal uteri did not react with typhoid antigen. They showed, in this way, that the reactions obtained with bacterial antigens were essentially similar to those obtained with such antigens as horse serum. A critical review of the previous literature on bacterial anaphylaxis is given by these authors.

The experiments reported in this and in the succeeding paper were undertaken primarily to throw some light on the question of the antigenicity of the various fractions isolated from the hemolytic streptococcus. It had already been shown that certainly two and possibly three distinct reactive substances, all non-type-specific, were present in extracts of hemolytic streptococci in addition to the type-specific protein, and that these could be separated chemically and serologically (3). Certain points of interest soon arose with regard to the anaphylactic reactions caused by these fractions, and these observations are recorded. The results presented in this paper were
obtained with the group-reactive nucleoprotein (P) and with the species-specific substance (C) which is probably a carbohydrate; while the results with the type-specific protein (M) and the non-type-specific protein (Y) sometimes associated with it are given in the succeeding paper.

Methods.

The preparation of the extracts used in these experiments has been previously described (3). Briefly, the nucleoprotein (P) was the fraction of NaOH extracts precipitable in the cold with dilute acetic acid, while the probable carbohydrate (C) was obtained from the supernatant fluids after precipitation of the other reactive fractions from their respective extracts. It was freed of protein as far as the small yields permitted. The type-specific (M) protein was obtained from HCl extracts.

The preparation of antibacterial sera was described in detail in a preceding paper and the most satisfactory method of preparing anti-P sera, also described previously (3), is summarized here for convenience. Rabbits were immunized with P by intravenous injections of 1 per cent solutions. Four daily injections of 10 mg. each were made, followed by a 3-day rest, then four more injections of 20 mg. each followed by 3 days of rest as before. A similar third series of injections of 30 mg. doses was given, then a fourth series of 40 mg. doses. If the titer of the test bleeding, taken 7 days later, was unsatisfactory, further series of 40 mg. doses were made until a satisfactory titer was obtained. The animal was then bled and the serum stored in the ice box without preservative.

Guinea pigs were sensitized passively by the intraperitoneal injection of 0.5 cc. of immune serum. Occasionally larger amounts of serum were employed and are thus recorded in the tables. Since it was impossible to obtain enough guinea pigs of exactly the same weight for use in all experiments, animals of approximately the same weight were selected for each experiment. The weights, which usually ranged between 150 gm. and 200 gm., are omitted from the tables. The sensitized animals were tested by intravenous injections of the appropriate extracts at varying intervals after sensitization, as shown in the tables. The smallest amount of extract which regularly caused acute anaphylactic death was called the minimal anaphylactic dose (M. A. D.), following the terminology of Weil (4). Since the concentration of active substances in the extracts was sometimes unknown, the dose could not always be expressed in mg. and was, therefore, in these instances given as M. A. D. for guinea pigs sensitized with the homologous serum. Sometimes both methods of recording the dosage were employed. In those instances in which it was desirable to test the anaphylactic reactions of a sensitized guinea pig with more than one substance, it was found necessary to wait 24 hours between injections in order to give the animal time to recover from the non-specific reduction of reactivity due to the first shock. The necessity for this interval has also been noted by Wells and Osborne in their study of the
relationships of certain plant proteins by means of the anaphylactic reaction (5). Control animals were always injected with doses at least as large as those used for the sensitized guinea pigs, sometimes following an intraperitoneal injection of normal rabbit serum and sometimes without this preliminary injection. They never showed symptoms of shock with the doses employed and survived indefinitely, most of them being kept under observation for several weeks.

A convenient method for making intravenous injections into guinea pigs was brought to our attention by Dr. C. H. Hitchcock. The hair was shaved from the back of the leg and a superficial longitudinal vein was easily laid bare by a small incision in the skin just above the ankle. This vein was small but was satisfactory for intravenous injections with a 25 gauge Yale needle. As many as five separate injections have been made into the same vein, although usually the veins in both legs were used for repeated injections, and occasionally similarly located veins in the forelegs were employed. All intravenous injections reported here were made in this manner.

The results of anaphylactic tests of each reactive fraction injected into guinea pigs sensitized with anti-P and with antibacterial sera are considered in order. The group-reactive P antigen is discussed first since a pure anti-P serum was available for its study. Since Zinsser and Parker reported optimal sensitization after an interval of 5 to 8 days rather than earlier, the following experiment was performed to determine the optimal time for the shocking injection after passive sensitization with this serum.

Experiment 1.—A series of guinea pigs was sensitized with anti-P serum. The M. A. D. of the homologous P was determined at 24-hour intervals throughout a 4-day period. The results are tabulated in Table I.

The experiment showed that the optimal time for passive sensitization with this anti-P serum was not reached until the 2nd or 3rd day, as judged by the size of the M. A. D., and that guinea pigs became less sensitive after the 3rd day.

The next experiment was devised to show the relationships among nucleoproteins from different types of hemolytic streptococci as well as from Streptococcus viridans and pneumococcus.

Experiment 2.—Another series of guinea pigs was sensitized with anti-P serum. The M. A. D. of nucleoprotein from strains representing three different types of

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1 In each instance the details of preparation of immune serum are given in the tables; hence they will not be discussed in detail in the text.
hemolytic streptococcus was determined on the day after sensitization by the intravenous injection of varying amounts of a 1 per cent solution. Sensitized

**TABLE I.**

**Anti-P Reactions.**

**Time Required for Passive Sensitization with an Anti-P Serum.**

Guinea pigs sensitized with anti-P serum, R500, from a rabbit immunized with P from hemolytic streptococcus, Strain S39, Type S23.

| Guinea pig | Days after sensitization | Dose | Result   |
|------------|--------------------------|------|----------|
| A-1        | 1                        | 5.0  | +++      |
| A-2        | 1                        | 7.5  | 15 min.  |
| A-3        | 2                        | 0.5  | +        |
| A-4        | 2                        | 1.0  | 4 min.   |
| A-5        | 3                        | 0.5  | ±        |
| A-6        | 3                        | 1.0  | ++       |
| A-7        | 3                        | 2.0  | 5.5 min. |
| A-8        | 4                        | 2.0  | +        |
| A-9        | 4                        | 5.0  | 3.5 min. |

20 mg. of P did not shock unsensitized control guinea pigs.

In all tables the following symbols are employed:

- indicates no shock.

±? " trace of shock.

± " slight shock.

± " mild shock.

+++ " moderate shock.

++++ " moderately severe shock.

++++ " severe shock.

++++ " very severe shock.

† " animal died.

Hom. = homologous.

Het. = heterologous.

M. A. D. = minimal anaphylactic dose.

Guinea pigs were also tested with P from a strain of green streptococcus and with P from pneumococcus, Type III. This protein was an oxidized extract kindly supplied by Dr. Julianelle.
reinjected on the following day with a dose of hemolytic streptococcus P several times the M. A. D. in order to determine whether they had become desensitized. Control animals were not shocked by 20 mg. of P.

**TABLE II.**

Anti-P Reactions.

**Minimal Anaphylactic Dose of Nucleoprotein, P, from Different Strains.**

Guinea pigs passively sensitized with anti-P serum, R500, from a rabbit immunized with P from hemolytic streptococcus, Strain S39, Type S23.

| Guinea pig | 1st test 1 day after sensitization | 2nd test 2 days after sensitization |
|------------|-----------------------------------|-----------------------------------|
|            | Antigen: P from strain of *S. hemolyticus* | dose | result | Antigen: P from strain of *S. hemolyticus* | dose | result |
| B-1        | S39 (hom.)                          | 5.0  | +       | S39 (hom.)                          | 10*  | ±      |
| B-2        | " "                                | 7.5  | 13 min. | " "                                | " "  | 10     | ±?     |
| B-3        | S43 (het.)                          | 5.0  | +       | " "                                | 7.5  | 14.5 min. |
| B-4        | " "                                | 7.5  | " "    | 10                                  | ±?     |
| B-5        | S3                                  | 5.0  | ++**   | S3                                  | 10    | ++     |
| B-6        | " "                                | 7.5  | 14.5 min. |
| B-7        | V92 (S. viridans)                   | 10.0 | +      | S39 (hom.)                          | 10    | 14 min. |
| B-8        | " "                                | 15.0 | +++    | " "                                | 20    | +++    |
| B-9        | " "                                | 20.0 | +++    | S39 (hom.)                          | 10    | +++    |
| B-10       | Pneumococcus, Type III              | 2cc. | ++     |

* * 10 M. A. D. for 48 hours. See Table I.

** Found dead next day. Small Gram-negative bacillus in heart's blood culture.

*** Concentration of P unknown. Test made 48 hours after sensitization. A control animal was unaffected by the same dose.

Table II shows the results of this experiment. Although the three hemolytic streptococcus proteins represented three distinct serological types, the M.A.D. for guinea pigs sensitized with Serum R500 was the same; while shock, but not death, was produced by considerably
larger doses of green streptococcus protein. A pneumococcus protein injected 48 hours after sensitization resulted in delayed death. Animals surviving shock from the hemolytic streptococcus proteins were completely protected against shock on the following day with 10 mg. of homologous P, although with this interval after sensitization 1 mg. was the M.A.D.; and those surviving shock from the green streptococcus protein were partly protected against the homologous P.

The nucleoproteins were also tested in guinea pigs sensitized with antibacterial sera. Since these sera contained antibodies for all the reactive substances but in different amounts depending on the method of immunization or the individual response of the rabbit, the tests with them were partly incidental to other experiments and were collected as Experiment 3.

Experiment 3.—Guinea pigs sensitized with 0.5 cc. to 1.0 cc. of antibacterial sera were tested 1 to 6 days later by intravenous injections of nucleoprotein P in doses ranging from 4 mg. to 10 mg. The M. A. D. was usually determined. The results are recorded in Table III.

The table is self-explanatory and shows that guinea pigs passively sensitized with antibacterial sera died in acute anaphylactic shock when injected intravenously with 7.5 mg. to 10 mg. of homologous or of heterologous hemolytic streptococcus nucleoprotein. Proteins from the different strains, except possibly from the homologous strain, were approximately equally effective in producing shock. Since homologous P solutions always contained some type-specific protein M, a smaller M.A.D. was to be expected. Precipitin tests, not tabulated here, also showed the presence of P antibodies in all these sera.

Active sensitization was obtained with nucleoprotein antigens in a few instances. After numerous doubtful results, unsuccessful apparently on account of dosage or timing between injections, the following satisfactory experiment in active anaphylaxis was performed.

Experiment 4.—Three guinea pigs were given intravenous injections of P from hemolytic or from green streptococci, as shown in Table IV. 22 days later each guinea pig was reinjected with 20 mg. of hemolytic streptococcus P. Table IV summarizes the experiment.

All three guinea pigs suffered typical anaphylactic shock on reinjection with hemolytic streptococcus proteins. Although the intensity
of the shock varied with different sensitizing antigens and was most marked with the most distantly related P, still the postmortem findings

TABLE III.

Anti-P Reactions.

Effect of Nucleoprotein, P, on Guinea Pigs Sensitized with Antibacterial Sera.

| Guinea pig | No. | Ct. | Days after sensitization | Antigen: P from *S. hemolyticus* strain | Dose | Result |
|------------|-----|-----|--------------------------|----------------------------------------|------|--------|
| C-1        | R323 | 1.0 | 6                        | S39 (het.)                             | 7.5  | +      |
| C-2        |     | 1.0 | 6                        | " " " "                               | 10.0 | ++++   |
| C-3        |     | 1.0 | 6                        | S60 (hom.)                             | 10.0 | 4 min. |
| C-4        | R321 | 0.5 | 3                        | S3 (het.)                              | 10.0 | ¶       |
| C-5**      | R261 | 0.5 | 1                        | " " " "                               | 4.0  | -      |
| K-1        |     | 0.5 | 1                        | " " " "                               | 10.0 | ¶11 min.|
| C-6        |     | 1.0 | 6                        | S60 " "                               | 5.0  | ++     |
| C-7        |     | 1.0 | 6                        | " " " "                               | 7.5  | ¶3.5 min.
| C-8        |     | 1.0 | 6                        | " " " "                               | 7.5  | ¶45 "   |
| C-9        |     | 1.0 | 6                        | " " " "                               | 10.0 | ¶3.5 "  |
| M-2        | Q308 | 1.0 | 3                        | S3 " "                                | 10.0 | ¶3.5 "  |
| M-3        |     | 1.0 | 2                        | " " " "                               | 10.0 | ¶50 "   |
| M-6        |     | 1.0 | 3                        | " " " "                               | 10.0 | ++      |
| C-10       | R446 | 0.5 | 1                        | S43 " "                               | 5.0  | ++      |
| C-11       |     | 0.5 | 1                        | " " " "                               | 7.5  | +++++   |
| C-12       |     | 0.5 | 1                        | " " " "                               | 10.0 | ¶3.5 min.|

* Serum R323 was against Strain S43, Type S60.

** Three other guinea pigs in this group received the same dose with the same result.

were typical of anaphylactic death in all instances. This experiment offered sufficient evidence that nucleoprotein antigens from streptococci could sensitize guinea pigs actively.
These experiments confirm the conclusions previously reached as a result of precipitin tests and absorption experiments that the nucleoprotein fraction P is common to hemolytic streptococci and that it is related to similar protein fractions of green streptococci and of pneumococci.

The anaphylactic reactions of the second non-type-specific substance were next investigated. This is referred to as the C substance and is probably a complex carbohydrate chemically similar to the type-specific soluble substances of certain other bacteria. It will be recalled however, that this fraction, while it is species-specific for hemolytic streptococci, shows no type specificity whatever in its precipitin reactions. Thus, with antibacterial serum from Rabbit R446 typical disc precipitates were formed in high dilutions of C preparations from all strains of hemolytic streptococcus tested. Consequently, it was important to find out whether this antigen-antibody system could cause anaphylaxis, and the following experiment was performed to determine this point.

**Experiment 5.—** A series of guinea pigs was sensitized with Serum R446. On the following day the animals were tested with intravenous injections of varying amounts of “purified” C from two heterologous strains of hemolytic streptococcus. The results are given in Table V.

### TABLE IV.
**Anti-P Reactions.**
**Active Sensitization with Nucleoprotein Antigens.**

| Guinea pig | Sensitizing injection* | Shocking injection* 22 days later |
|------------|------------------------|----------------------------------|
|            | Antigen P from strain** | Dose | Result | Antigen P from strain | Dose | Result |
| D-1 S43 (S. hemolyticus) | 20 | 135 | S43 (S. hemolyticus) | 20 | ++++ (lethality)*** |
| D-2 S3 " " | 20 | 180 | " " "  "  "  " | 20 | 150 min. |
| D-3 S92 (" viridans) | 20 | 173 | " " "  "  "  " | 20 | " |

* Intravenous.
** Strain S43 represents Type S60; Strain S3 represents Type S3.
*** Lungs distended as in acute anaphylactic death.
In this experiment typical anaphylactic shock was produced with a substance which is probably a carbohydrate. Titration of C from Strain S43 showed that 0.04 mg. was the M.A.D. for guinea pigs sensitized on the preceding day with Serum R446. Similarly, the M.A.D. of C from Strain S23 was 0.03 mg. The results exactly parallel the precipitin tests recorded in Table I of the preceding paper (3). A

| Guinea pig | Shocked by intravenous injections 1 day after sensitization |
|------------|----------------------------------------------------------|
|            | "Purified" C from strain | Dose | Result |
| E-1        | S43 (het.) | 0.02 | ++++ |
| E-2        | " " | 0.04 | 3.5 min. |
| E-3        | " " | 0.2 | 3.5 " |
| E-4        | " " | 2.0 | " |
| E-5        | S23 " | 0.01 | ± |
| E-6        | " " | 0.03 | 4 min. |
| E-7        | " " | 0.1 | 5 " |

The three strains used in this experiment represent three distinct types of S. haemolyticus:

- S3 represents Type S3.
- S43 " " S60.
- S23 " " S23.

Crude C extracted with antiformin from another strain of Type S23 also produced typical anaphylactic death in guinea pigs sensitized with this serum. Precipitin tests with this extract were negative for M and for P substances, and paralleled the anaphylaxis results in being positive for the C substance. The results with this extract are not included in the table.

Since the C substance produced undoubted anaphylactic shock, the question of its chemical nature became of even greater interest and
theoretical importance than before. As already pointed out, it was supposed that this fraction was probably a carbohydrate, and the facts supporting this supposition have been considered previously (3), as well as the fact that the chemical analyses do not exclude the possibility that the active material is combined with protein or is protein itself. Exposure of C to active trypsin, however, did not alter its activity in the precipitin test, and the effect of such digestion on the anaphylactic reaction is shown in the following experiment.

**TABLE VI.**

*Anti-C Reactions.*

**Exposure of C Substance to Trypsin: Effect on Anaphylactic Shock.**

Guinea pigs sensitized with Serum R446 from a rabbit immunized with Strain S3, Type S3.

| Guinea pig | Shocked by intravenous injections 1 day after sensitization | Dose* | Result |
|------------|----------------------------------------------------------|-------|--------|
|            | Purified C from Strain S3, Type S3, treated with:        | mg.   |        |
| F-1        | Inactive trypsin                                          | 0.05  | ++++   |
| F-2        | " " "                                                    | 0.075 | 13 min. |
| F-3        | Active "                                                   | 0.05  | ++++   |
| F-4        | " " "                                                    | 0.075 | 13.5 min. |

* These figures may be somewhat inaccurate on account of the small volumes with which the experiment was performed.

Experiment 6.—"Purified" C substance was subjected to the action of 0.5 per cent trypsin for 50 minutes. The technique of the experiment was the same as that employed in similar experiments described in previous papers (3). The trypsin was shown to be active by a simultaneous experiment in which the type-specific protein M was completely digested under the same conditions. (See Experiment 3 in the following paper.) Titrations of the C substance exposed to inactivated trypsin (the control) and of that exposed to active trypsin were made in guinea pigs sensitized with Serum R446.

Table VI shows that trypsin did not digest the C substance or change its titer in the anaphylactic reaction under conditions which completely destroyed other reactive fractions of the hemolytic streptococcus.
A few additional experiments were made to test the reactivity of C in guinea pigs sensitized with an anti-P serum and with antibacterial sera which showed only a low titer of C antibodies in the precipitin test. Experiment 7 gives some of these results.

Experiment 7.—Two guinea pigs, sensitized 2 days earlier with an anti-P serum, were given intravenous injections of C in doses which represented 3.3 and 20 M. A. D. respectively for animals sensitized with serum potent in C antibody.

TABLE VII.

Anti-C Reactions.

Effect of C Substances on Guinea Pigs Sensitized with:

(1) Anti-P Sera with No C Precipitins.

(2) Antibacterial Sera with Low Titer of C Precipitins.

| Guinea pig | Seraized with serum | Kind of serum* | Days after sensitization | Purified C extract from strain | Dose | Result |
|------------|---------------------|----------------|--------------------------|-------------------------------|------|--------|
| G-1        | R500                | Anti-P         | 2                        | S23 (hom.)                    | 0.1  | 3.3    |
| G-2        | R322                | "              | 2                        | "                             | 0.6  | 20.0   |
| G-3        | R322                | "              | 1                        | S43 (het.)                    | 0.2  | 5.0    |
| G-4        | 264                 | "              | 1                        | "                             | 0.2  | 5.0    |
| G-5        | "                   | "              | 1                        | S23 (hom.)                    | 0.1  | 3.3    |

* R500 was a rabbit immunized with P from Strain S39, Type S23.
R322 was a rabbit immunized with Strain S43, Type S60.
R264 = " " " " " " S39, " S23.

** M. A. D. in terms of Serum R446. See Table V.

*** Intravenous injections of suitable homologous P or M extracts on the following day produced typical anaphylactic death in these animals.

(See Experiment 5.) Since they were not shocked, the sensitiveness of one of these animals, G-2, was tested on the following day by an intravenous injection of nucleoprotein P.

Guinea pigs sensitized with antibacterial sera which had a low titer of C precipitins were also tested with C substances but showed no symptoms of shock, whether the C was obtained from homologous or from heterologous strains. Two of these animals, G-3 and G-5, subsequently received homologous M extracts to prove that they had been really sensitized.
This experiment emphasized the fact that this non-type-specific substance was not the same as the non-type-specific nucleoprotein P since it did not shock Guinea Pigs G-1 and G-2, sensitized with a potent anti-P serum. It also showed that C would shock guinea pigs sensitized with antibacterial sera, only if the serum had a relatively high content of C precipitins. Thus, G-3, G-4, and G-5 showed no shock when given 3 to 5 M.A.D. of C, although subsequent injections of homologous type-specific M extracts proved that this group of animals had been properly sensitized. These results parallel the precipitin reactions. This experiment will be referred to in another connection to show that the C substance is different from another non-type-specific substance (designated Y) which gave cross-reactions with certain antibacterial sera (notably, with Serum R264).

No attempts to sensitize actively with C could be made on account of the small amounts of material available.

DISCUSSION.

The anaphylactic reactions of two of the cell derivatives of *Streptococcus hemolyticus* paralleled in every instance the precipitin reactions previously reported (3). The similarity of nucleoproteins (P) from different types as well as from different strains of hemolytic streptococci was again evident; while the partial relationships of P from related species of bacteria, such as *Streptococcus viridans* and pneumococcus, were also apparent. These conclusions are based on passive anaphylactic experiments with anti-P and with antibacterial sera and on active sensitization with P which was also accomplished.

The species-specific C substance was also effective in producing anaphylactic death in passively sensitized guinea pigs, but only if the serum showed a high titer of the non-type-specific C precipitins. Amounts as small as 0.03 mg. to 0.04 mg. were sufficient to cause death in such animals. This is an instance in which a substance, probably carbohydrate in nature, causes passive anaphylactic shock, an interesting finding in view of its chemical nature and the failure to immunize with it. It seems to be a hapten capable of reacting with antibody but not capable of stimulating antibody formation. Insufficient quantities have been available to determine its capacity to produce active anaphylaxis. A similar result has been reported since the conclusion of these experiments by Tomcsik (6) who obtained from *E. lactis*
*aerogenes* 5 gm. of a specific substance which was largely carbohydrate, although he could not free it of 0.9 per cent nitrogen. 0.03 mg. of this product was the minimal anaphylactic dose for guinea pigs passively sensitized with antibacterial serum, a figure strikingly similar to that obtained with the probable carbohydrate of the hemolytic streptococcus. Tomcsik’s material was non-antigenic in that it did not produce active anaphylaxis in guinea pigs nor antibodies in rabbits into which it was injected. His work is analogous to that reported here with the C substance; and in both instances the final proof that the material is pure carbohydrate is still lacking, although the amount of nitrogen present in one minimal anaphylactic dose is certainly small. In the case of the streptococcus C, which contained approximately 4 per cent nitrogen, the minimal anaphylactic dose, therefore, contained only about 0.000001 gm. of nitrogen. In the present anaphylactic experiments, additional evidence that C is not a protein is the failure to digest it with trypsin; and its failure to shock guinea pigs passively sensitized with anti-P serum is further evidence that it is distinct from the non-type-specific nucleoprotein, P.

SUMMARY.

The anaphylactic reactions of two non-type-specific fractions of hemolytic streptococcus extracts parallel the precipitin reactions. The nucleoprotein, P, is a true antigen, in that it stimulates antibody production in rabbits, as shown before, and produces anaphylactic shock in guinea pigs actively as well as passively. The probable carbohydrate, C, on the other hand, does not induce antibody formation in rabbits, so far as known at present, but does produce typical anaphylactic shock in guinea pigs passively sensitized with antibacterial serum provided the serum shows a high titer of C precipitins. This is an instance of a hapten, probably carbohydrate in nature, causing anaphylactic shock in passively sensitized guinea pigs.

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