THE INFLUENCE OF HEATING THE IMMUNIZATION MATERIAL UPON THE ANTIBODY-INVOKING EFFECTIVENESS OF THE TYPE-SPECIFIC AND SPECIES-SPECIFIC ANTIGENS OF TYPE II PNEUMOCOCCUS CELLS.

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(Received for publication, October 3, 1927.)

INTRODUCTION.

Avery and Heidelberger and their associates (1–4) have presented convincing evidence that the virulent pneumococcus cell contains two different types of antigens: (1) a type-specific antigen (SP) which gives rise to a type-specific antibody; (2) a species-specific antigen (P)[1] which gives rise to a species-specific antibody. The type-specific antigenic complex is contained in the intact cell of strains possessing the soluble specific substance; immunization of a suitable animal with suspensions of pneumococcal cells invokes a type-specific antibody which reacts specifically with the soluble specific substance whether contained in solution (S-precipitation) or disposed on the periphery of the bacterial cell (agglutination); the same antibody is also apparently the one responsible for the passive protection of mice, at least against Type II pneumococci (5). The species-specific antigen, in contrast to the type-specific one, gives rise to an antibody

* Mr. Gaspari's cooperation in this work was made possible by a grant from The Henry Strong Denison Medical Foundation.

[1] The term species-specific "antigen" is used in the inclusive sense of Avery and Heidelberger (1, 2) to refer to the protein (mainly nucleoprotein and mucoid) which reacts with the species-specific precipitins that are invoked by immunization with protein derived from all types of pneumococci. It is not important in this paper that the protein may include a number of separate but unrecognized antigens, provided they are sufficiently alike in chemical and physical properties to be affected in similar manner by heat.

reactive with the protein derived from all types of pneumococci. This antibody is especially prominent in the immune serum obtained by the injection of solutions of the pneumococcus nucleoprotein (3) or of filtered solutions of the endocellular substances which are liberated when pneumococci are dissolved by bile or disintegrated by freezing and thawing (4). The prominence of these species-specific antiprotein precipitins in the immune serum invoked by solutions of pneumococci is due to the absence of the type-specific antibody which is dominant in the usual antibacterial serum obtained by injection of the intact cells. However, certain amounts of the species-specific antibody are also present although seldom so prominent, in the type-specific immune serum.

Although both anti-S and anti-P occur together in the usual antibacterial serum, sufficient evidence (1–4, 6) has been presented to prove that each of the antibodies is distinct and separate and that each is invoked by a distinct and separate antigen. The present investigation proceeds upon that basis, and consists of a comparison of the effect of heating of the immunization material (suspensions of pneumococci) upon the antibody-invoking property of the type-specific (SP) and species-specific (P) antigens of Type II pneumococcus cells.

Any comparison of the influence of heat upon the antibody-invoking effectiveness of antigens should take into account the size of the doses injected into the animals, the total amount of antigenic material, number of injections or stimuli, and the length of time of the immunization. Although these factors are frequently ignored, it is obvious that comparisons of the effectiveness of unheated antigen with heated antigen are of little value if the dosages of total antigen are greatly in excess of that required for the maximum immunity response of the animal (7). All these factors were carefully controlled in our experiments; the animals were immunized with two different dosages and the sera tested at different stages of the immunization. While individual differences are to be expected, and were encountered, in the degree of responses of the individual rabbits, a sufficient number of rabbits (41) were included to furnish a valid basis for a comparison of the influence of heat upon the effectiveness of the antibody-invoking property of the type-specific and species-specific antigenic constituents of Type II pneumococci.

In one sense, the species-specific antigen (P) can be considered an endocellular antigen contained inside of the type-specific pneumococcal cell. Since these bacteria autolyze readily, the suspensions of pneumococci employed in routine immunizations contain significant amounts of free intracellular substances in solution; and, as pointed out by Avery and Heidelberger (2), animals injected with such material receive, in fact, a mixture of varying proportions of free or dissociated P together with the undissociated type-specific antigenic complex. This free P can be expected to give rise to species-specific antibody and thus the amount of autolysis which has occurred *in vitro* before injection of the material is certainly a factor in determining the species-specific antibody response. Precautions to avoid the presence of autolytic products were especially necessary with the unheated bacteria which autolyze more rapidly *in vitro* than do the heated cells, and for this reason fresh bacterial suspensions were prepared at least every 2nd day. However, in spite of all precautions, it is doubtful whether it is possible to obtain a suspension of pneumococcus cells, either heated or unheated, in which no cellular changes (freeing of the P) have occurred. Since dissolution of pneumococci takes place in the animal body, *in vivo* lysis should also be considered a possible source of the species-specific antigen when animals are immunized with suspensions of pneumococcus cells (2). In the present investigation, both possible sources of the species-specific antigen are considered since both filtered solutions of intracellular substances and vaccines with the minimum of autolysis were employed as immunization material.

EXPERIMETAL.

Tests of the Immune Sera.—The immune sera were tested for type-specific and species-specific antibodies by the same general methods as those employed at the Hospital of The Rockefeller Institute (6). The type-specific antibody (anti-S) was recognized by three methods: (1) precipitation of the soluble substance from solution; (2) type-specific agglutination of broth cultures of the homologous strain; (3) passive protection of mice (8). The precipitation of the soluble substance (S) was tested with filtrates of young, unautolyzed broth cultures of pneumococci; the substitution of these fluids for solutions of the purified S substance being justifiable since they were devoid of the species-specific protein and contained no other detectable serologically reactive substance other than the type-specific S which is liberated in the early growth of pneumococci. The tests for the species-specific antiprotein precipitin were made with a filtered solution of Type I pneumococcus cells in which the species-specific antigen is the only substance reactive with Type II immune serum.

Experiment 1. Influence of Heat upon the Antibody-Invoking Properties of the Protein Contained in Solutions of Pneumococcus Cells.

Solutions of pneumococcus cells lack entirely the effective antigenic complex which is responsible for the production of the type-specific antibody invoked

by suspensions of intact pneumococcus cells and hence the dominant antigen contained in the bacterial solutions is the species-specific protein (P) which gives rise to anti-P precipitins. Although our primary interest was in a study of the conditions determining the relative proportion of the two types of antibodies (anti-S and anti-P) invoked by the intact bacterial cells (vaccine), the first step in the investigation consisted of a study of the influence of heat upon the antibody-invoking property of the protein as contained in the cellular solutions. Previous experiments (9) had shown that the anti-protein precipitin response was slightly diminished by heating pneumococcus solutions for 10 minutes at 55°C. and greatly decreased by boiling. In the following experiment, the bacterial solutions were heated for 30 minutes at 55°, 75°, 100°, and 120°C., and the antibody-invoking activity of the heated solutions was compared with that of unheated pneumococcus solutions. In order to make the comparison more valid, two test doses were employed, one of which was 20 times as great as the other; the use of the two dosages furnishing a basis to compare the antigenic effectiveness of small amounts of the unheated solution with the effectiveness of large doses of the heated material. Test bleedings were made after two and after five courses of injections; the examination of the sera after two different periods of immunization furnishing a basis for comparison of the effectiveness of unheated and heated antigen from the standpoint of the relations of the total amount of antigen and of the number of injections required to invoke comparable immunity responses. The results are summarized in Table I.

The results of this experiment (Table I) showed that heating for 30 minutes at any temperature between 55° and 120°C. reduces the antibody-invoking activity of the species-specific protein contained in solutions of pneumococcus cells. Two courses of injections with a small dose (0.1 cc.) of the unheated solution invoked a more effective response than was obtained by immunization with the heated material, not only when the doses of unheated and heated material were equal but also when the dosage of heated material was 20 times as great as that of the unheated antigen solution. The same effect was evident when the immunization was continued to a total of five courses of injections: although the prolonged immunization with heated solution caused some increase in the anti-P precipitin content of the sera, the antibody responses to the heated antigen were always smaller and approached that invoked by the unheated material only in animals which had received doses 20 times as large as those required when the unheated solution was used. Thus, a comparison of the response to the unheated pneumococcus solution with the response to the heated solutions, shows that 30 minutes exposure to temperatures from 55°

to 120°C. diminished the effectiveness of the species-specific protein antigen, both from the standpoint of the degree of antibody response to equal doses of unheated and of heated solution, and also from the standpoint of the amount of total antigen (dosage) and number of injections required for an effective response.

TABLE I.

Influence of Heat upon the Antibody-Invoking Properties of Solutions of Pneumococcus Cells.

Antigen used in immunization: Pneumococcus cell solution		Species-specific protein precipitins (anti-P)				Type-specific antibodies (anti-S)
		Serum after 2 courses of injection		Serum after 5 courses of injection		Serum after 5 courses of injections
Treatment of antigen	Size of dose injected	Protein solution				Agglutinins Anti-S precipitins Passive protection
		Undiluted	1/10 diluted	Undiluted	1/10 diluted	
	cc.					
Unheated	0.1	++	+	+++	++	0
Heated 30 min. at 55°C.	0.1	±	0	+	±	0
	2.0	+	±	++	+	0
Heated 30 min. at 75°C.	0.1	0	0	±	0	0
	2.0	+	+	++	+	0
Heated 30 min. at 100°C.	0.1	0	0	±	0	0
	2.0	+	±	+	±	0
Heated 30 min. at 120°C.	0.1	0	0	0	0	0
	2.0	±	0	±	0	0

0 = no detectable reaction; ± = faint cloudiness without sedimentation; + = cloudiness with slight sedimentation; ++ = marked cloudiness with moderate sedimentation; +++ = marked cloudiness with large amounts of sediment.

A comparison of the responses to equal doses of the solutions which had been heated at different temperatures shows a more or less gradual diminution of the antibody-invoking property with increase in the temperature. The solution heated for 30 minutes at 55° or at 75°C. induced somewhat better responses than did the solution heated at

100°C. but the difference is less than that between the unheated solution and the solution heated at any of the test temperatures. While heating at 120°C. almost completely destroyed the antigenicity, prolonged immunization with the larger dose (2.0 cc.) invoked a weak anti-protein response.

The retention of some degree of antigenicity by the P substance of Pneumococcus after 30 minutes exposure to 120°C. is especially interesting in view of the relatively large loss in activity which follows heating at only 55°C. The antigenic effectiveness of coagulated proteins (9) involves fundamental questions which are encountered in the study of enzymes as well as in immunology, *i.e.*, whether the diminished but not destroyed response is due to a partial but common inactivation of all of the protein molecules, or to traces of active substance which remain uncoagulated at the final end-point of the coagulation reaction, or perhaps to a gradual *in vitro* or *in vivo* reversal of the coagulated protein to its original form.

Experiment 2. Influence of Heating at Different Temperatures upon the Antibody-Invoking Properties of Suspensions of Pneumococcus Cells.

The following experiment dealt with a comparison of the influence of the temperature of heating upon the antibody-invoking properties of suspensions of pneumococcus cells. In contrast to the solutions of bacterial substances studied in the preceding experiment, the suspensions of the bacterial cells contain two effective antigens: the antigenic complex (SP) which gives rise to the type-specific (anti-S) antibodies, as well as the species-specific antigen (P) which gives rise to the species-specific (anti-P) antibodies. Hence, this experiment consisted essentially in a comparison of the influence of the different temperatures upon two types of antigens (type-specific and species-specific) both of which are contained in the same pneumococcus cell.

The bacterial sediment of a centrifuged 12 hour broth culture was resuspended in one-tenth volume of salt solution, so that 1 cc. of the suspension was equivalent to 10 cc. of culture. The suspension was divided into four equal portions; the separate lots were then heated for 30 minutes at one of the following temperatures: 55°, 75°, 100°, and 120°C. Eight rabbits of approximately the same age and weight were selected and divided into two series. The individual rabbits in the first series received doses of vaccine equivalent to 0.5 cc. of culture and the individuals in the second series received doses equivalent to 5.0 cc. of culture; one animal in each series being injected with vaccine which had been heated at one of the four test temperatures. The immunization consisted of three courses of six daily

intravenous injections, with 1 week's rest between each course. 10 days after the last injection, the animals were bled, and the immune serum tested for type-specific and species-specific antibodies by the methods described.

The immune serum obtained with suspensions of pneumococci which had been heated at different temperatures (55° to 120°C.) contained the usual type-specific antibodies, regardless of the temperature at which the vaccine had been heated. However, the response of the individual animals to the different amounts of the same vaccine did not show the definite relation between amount of antigen and degree of antibody response which was evidenced in the previous experiment with the pneumococcus solutions. The serum from the rabbit injected with the smaller dose frequently contained as great an amount of type-specific antibody as did the serum obtained from animals which had received doses 10 times as large of the same material. Due to the irregularity of response and to the lack of a definite difference between the responses to the two test doses of vaccine heated at the same temperature, it was impossible to show from this experiment any general distinction between the influence of the different temperatures upon the effectiveness of the type-specific and species-specific antigens. The results as a whole, however, indicated that heating at any temperature had less effect upon the effectiveness of the type-specific antigen than upon the effectiveness of the species-specific antigen.

Experiment 3. Comparison of the Antibody (Type-Specific and Species-Specific) Response to Unheated Suspensions of Pneumococci with the Response to Heated Suspensions of Pneumococci.

The results of the preceding experiment had shown no definite difference between the responses to the pneumococcus suspensions which had been heated at different temperatures. It seemed probable that a more clean-cut distinction between the type-specific and species-specific antigens would be evidenced by limiting the comparison to the differences in the antigenic effectiveness of unheated and heated suspensions, rather than by attempting to show differences in the effect of heating at different temperatures.

In the following experiment, one series of rabbits was injected with unheated or live suspensions, and two other series were injected with suspensions heated at 55° and at 100°C. Since it was desired, if possible, to base our comparisons upon test doses which showed some direct relation between dosage and degree of response to the same lot of vaccine, it was decided to increase the difference between the test doses employed in Experiment 2, and to use for the larger dose in each series, an amount of vaccine 15 times as great as that represented by the smaller dose. The immunization was continued for four courses of injections with bleedings after the second and fourth courses. The use of two test doses and the examination of the immune serum after two and four courses of injections furnished a more valid basis for a distinction between the effect of heat upon the antigenic

effectiveness of the type-specific and species-specific antigens of the pneumococcus cell.

Twelve male rabbits of approximately the same age and weight were divided into three series. The first series of four animals received one course of injections of pneumococcus suspension which had been heated 8 minutes at 55°C. and three courses of unheated suspension. The second and third series of rabbits received four courses of the same suspension of pneumococci differing only in that the suspension had been heated for 30 minutes at 55° and at 100°C., respectively. Two rabbits in each series received daily doses equivalent to 0.4 cc. of broth culture, and the other two rabbits in the series received doses 15 times as large (equivalent to 6.0 cc. of culture). Each course of injections consisted of six daily doses with the usual period of 1 week between the first and second, and between the third and fourth courses. Bleedings were made 10 days after the last injection of the second and fourth courses, which caused an 11 day rest period between the second and third courses. Two rabbits (Nos. 25 and 29) died during the third course of injections.

The results of tests of the immune sera are collected in Table II.

The results in Table II can be analyzed as follows: After both the second and fourth courses of injections, the sera of all of the animals which had received the larger amount of vaccine contained a greater amount of type-specific antibody than did any of the animals which had received the smaller test dose. This is important, for the fact that the smaller test dose was less than that required for the maximum antigenic response furnishes a more desirable basis for a comparison of the effect of heat upon the effectiveness of the two types of pneumococcus antigens than if both test doses had been above that required for the maximum response.

If a comparison is made of the type-specific antibody responses to the larger test dose of vaccine, it is evident that no distinction can be made between the sera obtained by immunization with unheated pneumococcus cells and that obtained by immunization with suspensions which had been heated either at 55° or at 100°C. The same general relation holds true in the sera obtained by immunization with the smaller dose: although none of these sera contain as high a content of anti-S antibodies as do the sera obtained by use of the larger dose of vaccine, the use of the unheated bacterial cells did not cause a more effective response than did the pneumococci which had been heated. The results obtained with the animals immunized with the smaller doses are in one respect more convincing than that obtained

TABLE II.

Comparison of Antibody-Invoking Property of Unheated Suspensions of Pneumococcus Cells with That of Suspensions Which Had Been Heated at Different Temperatures.

Antigen used in immunization: Suspension of pneumococcus cells			Species-specific anti-protein precipitins		Type-specific antibodies							
			Serum after 2 courses of injections	Serum after 4 courses of injections	Serum after 2 courses of injections				Serum after 4 courses of injections			
					Type II agglutinins		Passive protection		Type II agglutinins		Passive protection	
Rabbit No.	Treatment of antigen	Dose in terms of broth culture	Precipitation with ¼ dilution of test solution	Precipitation with ¼ dilution of test solution	Dilution of serum		Dose of culture		Dilution of serum		Dose of culture	
					1/10	1/80	0.1 cc.	0.01 cc.	1/10	1/80	0.1 cc.	0.01 cc.
		cc.										
18	Unheated	6.0	+	+++	xxx	x	S	S	xxx	x	S	S
19	Unheated	6.0	+	+++	xxx	x	S	S	xxx	x	S	S
20	Heated 30 min. at 55°C.	6.0	±	+	xxx	x	S	S	xxx	x	S	S
21	Heated 30 min. at 55°C.	6.0	±	+	xxx	0	S	S	xxx	x	S	S
22	Heated 30 min. at 100°C.	6.0	±	+	xxx	x	S	S	xxx	x	S	S
23	Heated 30 min. at 100°C.	6.0	±	+	xxx	0	S	S	xxx	x	S	S
24	Unheated	0.4	±	+++	x	0	D	S	xx	0	S	S
25	Unheated	0.4	±	*	xx	0	S	S	*	*	*	*
26	Heated 30 min. at 55°C.	0.4	0	0	xx	0	S	S	xx	0	S	S
27	Heated 30 min. at 55°C.	0.4	0	0	x	0	D	S	x	0	D	S
28	Heated 30 min. at 100°C.	0.4	0	0	x	0	D	S	xx	0	S	S
29	Heated 30 min. at 100°C.	0.4	0	*	x	0	D	S	*	*	*	*

* Rabbit died during immunization.

In Tables II and III 0 = no detectable reaction; ± = faint cloudiness without sedimentation; + = cloudiness with slight sedimentation; ++ = marked cloudiness with moderate sedimentation; +++ = marked cloudiness with large amounts of sediment; x = definite granulation without formation of compact disc; xx = compact disc with faintly cloudy supernatant fluid; xxx = compact disc with clear supernatant fluid; D = mouse died in 18–60 hours; S = mouse survived 7 days.

with the larger test dose, for the fact that the test dose is known to be less than that required for the maximum response indicates that the lack of any essential difference in apparent antigenic effectiveness is not obscured by the injection of excessive amounts of total antigen.

Quite different relations are revealed in a comparison of the species-specific antibody (anti-P) response. In the case of the animals injected with the larger test dose, the sera obtained by immunization with the unheated pneumococci contain much more of the species-specific antibody than do those immunized with the heated bacterial cells. While the sera of the rabbits receiving the heated material increased in their anti-P precipitin content as the immunization was continued, it is evident that even after four courses of injections none of these animals produced more of the species-specific antibody than that invoked in other animals by two courses of injections of the unheated material. The same distinction is revealed in the results obtained in the animals which received the smaller test dose. Again, the animals which received unheated pneumococci produced more anti-P than did those which received the same amount of heated bacteria. The distinction between the anti-P response to the heated and the unheated bacterial cells is in striking contrast to the apparent lack of any difference in the anti-S response to equal doses of heated and unheated pneumococcus suspensions. Whether or not the bacterial cells have been heated is apparently the most important factor in determining the relative amount of the species-specific antibody in antipneumococcus sera, for although size of dose and length of the period of immunization also increased the anti-P response to the heated bacterial suspension, doses 15 times as great of the heated bacteria failed to invoke as effective an anti-P response as that obtained by small doses of unheated pneumococci.

These results are of additional interest as further evidence of the individuality of the type-specific and of the species-specific antigens of Pneumococcus, for the existence of two different antigens which are affected to a different extent by heat are required in order to obtain immune sera in which the relative proportion of the two antibodies is determined by the use of unheated or heated bacterial cells in the immunization.

Experiment 4. Comparison of the Antibody Response to Subsequent Injections of Unheated and of Heated Pneumococcus Cells by Rabbits Previously Immunized with Heated Pneumococci.

The following experiment differs from the preceding ones in that the animals received a preliminary immunization treatment with heated pneumococcus cells; and were then divided into two groups, one of which was given additional immunization with heated pneumococci, and the second was given additional immunization with unheated pneumococci.

It is a common practice in the preparation of antibacterial serum to employ heated bacteria in the first part of the immunization treatment and to inject live bacteria in the later stages of the immunization in the hope of increasing the protective value of the serum. Since passive protection of mice against Type II pneumococci is apparently a function of the type-specific antibody (5), the results of the preceding experiments furnished considerable ground for the belief that unheated pneumococci would be no more effective than heated bacterial cells in the production of a serum of high protective or high agglutinating value. However, in view of the possibility of differences in the responses of previously immunized animals, the following experiment was designed to compare the responses induced by subsequent immunization with unheated bacteria with the responses induced when the immunization was continued with heated bacteria alone. All of the animals selected for the final test immunization, had responded to previous immunization with the heated bacteria with the production of immune serum of high titres of type-specific antibody before the final test comparison of the effect of heat upon the bacterial cells injected in the subsequent immunization.

Eighteen rabbits were immunized with three courses of daily injections of heated vaccine, each dose being equivalent to 5.0 cc. of broth culture. The immune sera were then tested by the usual methods for type-specific and species-specific antibodies. From the original eighteen animals, twelve were selected for the final test comparison. Six rabbits which gave weak responses to the preliminary immunization were eliminated from the experiment. Each of the selected twelve rabbits had produced, in response to the preliminary immunization, sera which contained high and approximately equivalent amounts of the usual type-specific antibody (anti S precipitins, agglutinins, and passive protective action). Since the preliminary immunization consisted of eighteen injections of relatively large amounts of vaccine, it was assumed that at this stage the animals had had sufficient immunological stimulus to invoke an anti-S response approximately equivalent to the responding capacity of the individual animals. It is obvious, thus, that the following comparison of the effect of subsequent immunization with unheated and with heated bacterial cells, was limited to animals which had already given evidence of effective response to heated pneumococci and which had in fact already produced as high titres of type-specific antibody as is usually obtained in

Type II antipneumococcus rabbit serum. None of the animals had produced more than the small amount of the species-specific antibody commonly found in antipneumococcus serum of rabbits immunized with heated vaccine.

These twelve previously immunized rabbits were then divided into two series: Series *A* received heated pneumococci in the subsequent immunization; Series *B* received the same amount of unheated pneumococci; the subsequent immunization consisted of two courses of six daily injections of bacterial suspension equiva-

TABLE III.

Comparison of the Antibody Responses to Subsequent Injections of Unheated and Heated Pneumococci by Rabbits Previously Immunized with Heated Pneumococci.

Rabbit No.	Treatment of pneumococcus suspension injected in subsequent immunization	Type-specific antibody										Species-specific antibody			
		Agglutinins				Passive protection						Antiprotein precipitins			
		After 3 preliminary courses of heated pneumococcus		After subsequent immunization		After 3 preliminary courses of heated pneumococci			After subsequent immunization			After 3 preliminary courses of heated pneumococci		After subsequent immunization	
		Serum dilution				Dose of culture						Dilution of test solution			
		1/20	1/80	1/20	1/80	0.2 cc.	0.1 cc.	0.03 cc.	0.2 cc.	0.1 cc.	0.03 cc.	1/4	1/20	1/4	1/20
30	Heated	xx	x	xx	0	D	D	S	D	S	S	+	±	+	±
31	"	xx	0	xxx	0	D	S	S	S	S	S	±	0	+	±
32	"	x	0	xx	0	S	D	D	D	S	S	0	0	±	0
33	"	xx	x	xxx	x	D	S	S	D	D	S	±	0	±	0
34	"	xx	0	xxx	x	S	S	S	S	S	S	±	0	+	±
35	"	xxx	0	xxx	0	S	S	S	S	S	S	±	0	±	0
36	Unheated	x	0	xx	0	S	D	S	S	S	S	+	±	++	+
37	"	xx	0	xx	0	S	D	S	D	S	S	0	0	++	+
38	"	xx	0	xxx	x	D	S	S	S	S	S	0	0	++	+
39	"	x	0	xx	0	D	D	S	D	S	S	±	0	++	+
40	"	xx	0	xxx	x	D	D	S	S	D	S	±	0	++	+
41	"	xx	0	xxx	0	D	D	S	D	S	S	±	0	++	+

lent to 5.0 cc. of broth culture. After the end of this treatment, a second bleeding was made, and the immune serum obtained was tested by the same methods employed in the tests of the first bleeding.

The results are collected in Table III. Since the anti-S precipitins and the type-specific agglutinins proved approximately parallel in all the sera, the results of the anti-S precipitin tests are omitted in Table III.

The facts presented in Table III can be analyzed to advantage by considering first the type-specific antibody response. All of the animals in both Series *A* (heated) and *B* (unheated) had produced sera containing high titres of the type-specific antibody after the first three courses of injections. This response apparently represented the maximum response of the individual animals, since only slight increases and at times slight decreases in the content of type-specific antibody was obtained by continued immunization with either heated or with unheated pneumococci. Apparently, with the selected previously immunized rabbits used in this experiment, the slight differences that existed in the potency of the sera of the different animals was determined by the capacity of the animal to respond and was independent entirely of whether or not the bacterial cells had been heated.

This apparent total lack of any real difference between the type-specific antibody content of the sera obtained by subsequent injection of the previously immunized animals with unheated pneumococci in comparison to that obtained with heated bacterial cells was just as pronounced in passive protection experiments as in agglutination tests. All of the sera protected against about 0.1 cc. of culture and attempts to show differences by increasing the test doses failed due to the irregularities usually encountered in protection tests with doses of culture near the zone of mass infection. Other experiments were made in which a constant dose of culture (1×10^{-4} cc.) was tested against different amounts of serum. The results of these experiments showed some differences in the potency of the different sera but the differences were irregular and had no relation to the heating treatment of the immunization material.

An analysis of the species-specific antibody responses induced in the previously immunized rabbits reveals entirely different relations than those obtaining for the species-specific antibody. The sera of all the rabbits contained only small amounts of the anti-P precipitin at the end of the preliminary immunization which, as shown before, had yielded sera containing high contents of anti-S. When these previously immunized animals were subsequently injected with additional doses of heated pneumococci there was only a slight increase in the species-specific antibody yield. On the other hand, the animals which

received unheated pneumococci in the subsequent immunization treatment responded with large amounts of the species-specific antibody so that their sera after the final two test courses of injections contained anti-P precipitins in titres comparable to those obtained by immunization with large amounts of solutions of pneumococcus cells or of pneumococcus protein.

The most important facts shown by this experiment can be illustrated best by summarizing the results of Table III in the form given

TABLE IV.

Summary of the Antibody Responses to Subsequent Injections of Unheated and Heated Pneumococci by Rabbits Previously Immunized with Heated Pneumococci.

Immunization		Increase in antibody content of sera due to subsequent immunization of the previously immunized rabbits	
		Type-specific antibodies	Species-specific antibodies
Animals previously immunized with three courses of injections of heated pneumococcus cells	Animals which subsequently received two additional courses of heated pneumococci	No significant increase	No increase by 3 rabbits; slight increase by 3 rabbits
	Animals which subsequently received two additional courses of unheated pneumococci	No significant increase	Marked increase by all (6) rabbits

in Table IV. The relations evident there (Table IV) indicate that individual rabbits can produce only a certain amount of the type-specific antibody and that the maximum response can be obtained by the injection of heated pneumococcus cells; and that after this maximum responding capacity has been reached, the further injection of unheated pneumococci serves only to increase the species-specific antiprotein precipitin content without increasing significantly the type-specific antibody content of the serum.

Experiment 5. The Antihemotoxin Response of Rabbits Immunized with Heated and with Unheated Pneumococcus Cells.

Pneumococcus antihemotoxin, like the anti-P precipitin, is a species-specific and not type-specific antibody (11). The hemotoxin, the antigen which gives rise to the antihemotoxin antibody, is an endocellular substance and like the antigens which give rise to the anti-P precipitins is present in solutions of pneumococcus cells. In a previous paper (10), it was shown that the hemotoxin can be distinguished from the antigens related to the anti-P precipitins by the fact that the antibody-invoking property of the hemotoxin antigen is destroyed by heating treatment (55°C.) which diminishes but does not destroy the effectiveness of the precipitinogens. Since the hemotoxin like the P precipitinogens is contained as an endocellular constituent in the pneumococcus cell suspensions injected for the production of the usual antibacterial antipneumococcus serum, one would expect the relative proportion of the antihemotoxin contained in antipneumococcus serum to be influenced by the heating treatment of the bacterial suspensions employed in the immunization. In fact, from analogy with the results obtained in immunization with heated solutions of pneumococcus cells, one would expect the antihemotoxin content to be almost entirely dependent upon whether the bacterial cells were heated or unheated, for we failed entirely to detect any antihemotoxin in the immune serum of animals injected with heated hemotoxin solutions (10).

In order to determine the effect of the heating treatment of suspensions of pneumococcus cells upon the antihemotoxin content of antipneumococcus serum, antihemotoxin titrations were made with the sera of the forty-one animals employed in the preceding experiments. All of the sera obtained by immunization with unheated pneumococcus cells, like the sera obtained by immunization with unheated solutions of the bacterial substances, contained significant amounts of antihemotoxin. With only one exception, none of the sera obtained by immunization with heated pneumococcus cells contained any detectable amount of the antihemotoxin. The one exception consisted in the serum of a rabbit which had received prolonged immunization with large amounts of bacterial cells heated at 100°C. (Rabbit 23 in Table II). It is impossible to explain this apparent discrepancy.

In spite of the observed exception, it is logical to believe that the antihemotoxin content of antipneumococcus serum, like the anti-P precipitin content of the same sera, is determined largely by the heating treatment of the bacterial cells employed in the immunization. One would expect to find especially large amounts of this species-specific antibody in the antipneumococcus serum obtained from horses which had received large doses of live or unheated pneumococci during the later stages of the immunization.

DISCUSSION.

The preceding experiments have dealt with the influence of heat upon the antibody-invoking effectiveness of the type-specific and species-specific antigens of Type II pneumococcus cells. The results of the study of the effect of heat upon the species-specific antigen as contained in filtered solutions of the intracellular substances of pneumococci showed that 30 minutes exposure of the bacterial solutions to 55°C. or higher temperatures, caused a marked decrease in the antibody-invoking effectiveness of the species-specific antigen, not only from the standpoint of the degree of antibody response to equal doses of unheated or of heated solution, but also from the standpoint of the amount of total antigen and number of injections required for an effective response. The experiments with suspensions of pneumococcal cells were more complicated than those in which solutions were used as the immunization material, since the latter experiments included the type-specific as well as the species-specific antibodies. More differences existed in the responses of individual animals immunized with the same lot of bacterial suspension than were encountered among the rabbits immunized with the bacterial solutions. However, the large number of rabbits included, the use of different doses in the immunization, and the examination of the immune serum after different periods of immunization, furnished convincing evidence that the antibody-invoking property of the species-specific antigen as contained in the suspensions of bacterial cells was more greatly diminished by heat than was that of the type-specific antigen. For example, a series of rabbits injected with large but equal doses of heated (30 minutes at 55° or 100°C.) or of unheated suspensions of pneumococci, contained in common a high content of type-specific antibody independent of the heating treatment of the antigen: and in contrast to their common content of the type-specific antibody, the same sera contained large amounts of the species-specific antibody only where unheated bacteria had been employed in the immunization. Moreover, a comparison of the sera of another series of rabbits showed that the immune serum obtained by injection of small amounts of unheated pneumococcus suspension (below the dose required for the most effective response) contained less type-specific but more species-

specific antibody than did the serum of other rabbits which had received doses 15 times as great of the heated bacterial cells. The production of immune serum in which the relative proportion of type-specific to species-specific antibody seems to be directly dependent upon whether or not the immunization material had been heated or unheated, presents convincing evidence of differences in the effect of heat upon the antibody-invoking effectiveness of the type-specific and species-specific antigens of Pneumococcus. It is important to observe that these relations were established in experiments in which dosage and length of time of immunization were carefully controlled, for it would be theoretically possible to obtain similar responses to heated and unheated antigen if the total amount of antigen were greatly in excess of the dose required for the maximum immunity response.

The species-specific antigen of Pneumococcus is endocellular and it can be assumed that the bacterial cells must be dissolved, either before or after injection into the animals, in order to liberate the antigen and permit it to come into direct contact with the cells involved in antibody production. Since unheated pneumococci autolyze *in vitro* more readily than do the heated bacterial cells and are likewise more soluble in bile, the question might be raised whether a greater rate of lysis of the unheated cells *in vivo* may not be a factor in the greater effectiveness of the endocellular antigen. But *in vivo* and *in vitro* dissolution of pneumococci are distinctly different processes: one of them due in large part to the specific heat-labile bacteriolytic enzyme produced by the bacteria themselves (12) and the other to lytic action of the cells or fluids of the animal. However, the possibility of differences in the ease of lysis of the unheated and heated pneumococci, while not to be ignored, does not seem to be an important point for the fact that the antibody-invoking effectiveness of the protein as contained in the solutions employed in the first experiment was influenced as markedly by heat as was the protein contained within the undissolved cells in later experiments, indicated that the decrease in antigenic activity represents a real change in the bacterial protein which occurs whether heated inside or outside the bacterial cell.

The distinct difference in the relative effect of heat upon these two antigenic constituents of the pneumococcus cell is of interest from both theoretical and practical points of view. It is important to note that

our experiments have dealt with the antibody-invoking and not with the antibody-combining property of the two antigens. The type-specific S substance when free in solution, dissociated from the antigenic complex SP, does not possess the antibody-invoking property whether heated or unheated, but it retains its antibody-combining property after even the most drastic heating treatment (in approximately neutral solutions). The resistance of the antibody-combining property of the S substance to the autoclaving and boiling in the open water bath, both of which are preliminary steps in the preparation of the purified substance (1, 2), is an outstanding example of heat stability among immunologically reactive substances. However, even the antigenic complex itself (SP), undissociated and in the antibody-invoking effective form, is relatively heat-resistant in comparison to many antigens of bacterial origin and, from the antibody-invoking standpoint is decidedly more heat-stable than the species-specific protein antigens contained within the same pneumococcus cell. Possibly, combination with the carbohydrate or S substance endows pneumococcus protein with new physical properties (resistance to heat coagulation) as well as conferring upon it the new chemical or immunological properties which are responsible for type specificity.

The difference in the heat lability of the type-specific and species-specific antigens of Pneumococcus must be an important factor in determining the relative proportion of the type-specific and species-specific antibodies in all antipneumococcus serum produced by injection of pneumococcus suspensions. While the type-specific antibody (anti-S) is the predominant and important one, a certain amount of the species-specific antibody accompanies it in practically all type-specific sera. The species-specific antibody content and the ratio between type-specific and species-specific antibody vary in sera produced in different laboratories; one serum may contain relatively large amounts of the species-specific antibody and another may contain only difficultly detectable traces of the same antibody, even though the type-specific antibody content of the two sera is approximately identical. The factors which operate in determining the relative amount of species-specific antibody must include those which determine the degree of the response to any antigen: treatment of the immunization material, dosage, period of immunization, and the re-

sponding capacity of the individual animal. However, if the vaccine is prepared from virulent, S-producing pneumococci and reasonable doses are injected in a course of immunization not unnecessarily long, one could expect that heating of the pneumococcus suspension injected would greatly decrease the production of species-specific antibodies (anti-P) without an appreciable effect upon the production of type-specific antibodies (anti-S). This is shown to be true with rabbits in the preceding experiments.

The results of the experiments on subsequent immunization of rabbits previously immunized with heated pneumococci, indicated that the only thing gained by the subsequent immunization is an increase in the species-specific antibody content of the serum. Since this latter antibody is not involved in truly type-specific agglutination, the conclusion may be drawn that continued immunization of animals which already have responded with high titres of type-specific antibody adds nothing at all to the diagnostic value of the serum. The use of live or unheated bacteria is unnecessary in order to produce a highly potent type-specific sera, and, if the sera are to be used for typing, the increase in species-specific antibody without increase in the type-specifically reacting substances is undesirable.

SUMMARY.

This paper presents an experimental comparison of the effect of heating of the immunization material upon the antibody-invoking effectiveness of the type-specific (SP) and species-specific (P) antigens of Type II pneumococci. Heating of the pneumococcus suspension (vaccine) invariably decreased the production of species-specific antibodies (anti-P) without a comparable effect upon the production of type-specific antibodies (anti-S).

For diagnostic typing purposes, the ideal antipneumococcus serum should contain the maximum content of type-specific, and the minimum of species-specific antibody. Our results with forty-one rabbits indicate that the ideal serum from the type-specific standpoint would be obtained by immunization with the heated cells of virulent pneumococci over a comparatively short immunization period; and that the only thing gained by continued immunization or by the use of unheated bacteria at any stage of the immunization, is an increase in

the species-specific antibody which is undesireable in sera to be used for diagnostic purposes.

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