STUDIES IN IMMUNIZATION BY A SPECIES ANTIGEN

I. PREPARATION OF SPECIES ANTIGEN FROM PNEUMOCOCCI

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That it is possible to obtain a species as opposed to a type-specific immunity to an organism, such as the *Pneumococcus*, by injection of antigenic preparations, is generally admitted. But there is little agreement on the methods to be used, the nature of the antigen responsible, and the comparative value of the protection secured. The following summary of the results recorded by different observers shows the need of further study of the problem.

Previous work

Tillett (1927) obtained immunity in a rabbit against a rabbit-virulent strain of type 3 *Pneumococcus* by repeated injections of a vaccine of another type *Pneumococcus* and also of R avirulent strains, but not by injections of pneumococcal nucleo-protein.

Day (1933) found that species protection of mice against pneumococci of different types could be obtained by a single injection of type pneumococci that had undergone autolysis. The sera of rabbits injected with these preparations conferred passive immunity on mice. Day suggested that the antigen responsible might be formed by a dissociation of the type-specific antigen, and called it 'species antigen'.*

Harley (1935) confirmed these results and showed that species antigen could be obtained from type pneumococci by simple chemical means, excluding autolytic processes. In his final method he prepared species antigen by heating pneumococci in dilute HCl and treating the extract with weak alkali at 37° C.

Felton (1934) dissolved type pneumococci to a large extent by the action of cold alkali ($N/10$ NaOH) on dried organisms. This preparation was treated with acid till it turned cloudy, kept cold and centrifuged next day. The acid-soluble fraction contained the type and species C carbohydrate; it gave type protection on injection in mice. The acid-insoluble residue gave much less type protection, but it afforded some species immunity.

Enders, Wu & Shaffer (1936) were able to immunize mice and to obtain a protective antiserum from rabbits against type 3 *Pneumococcus* by repeated injections of an avirulent strain derived from type 1. They found by exhaustion experiments that C carbohydrate appeared responsible for this immunity, since it neutralized the antibody.

Downie (1938) was unable to obtain species immunity by the methods of Harley and Day. He confirmed the findings of other observers that rabbits, unlike mice, do not react to the injection of type-specific antigen in solution, and stressed the importance of filtration before testing alleged solutions.

Dubos (1938) prepared a filtrate from autolysed type 1 pneumococci, employing both virulent (S) and avirulent (R) strains. On injection in rabbits these provoked the formation of antibodies that rendered the sera protective against different types of pneumococci. His results from active immunization of mice with these filtrates were not so good.

* For the sake of convenience this short title, though imperfect, will be used in this paper.

Present study; methods

In the present investigation a uniform method adopted in previous work was followed, to reduce the number of variables. Types 1 and 2 pneumococci of full virulence to mice were employed for most experiments, since these two types are specially sensitive to the action of both type-specific and species antibodies.

The pneumococci were grown in trypsinized broth containing 4% of serum for 18 hr. at 37° C., then for a further period of 1½ hr. after the culture medium had been enriched by the addition of half its volume of glucose broth. On an average this addition resulted in a tenfold increase in numbers. The organisms were then collected by centrifuge and subjected to various treatments to be described later (p. 534); the final product was made up to the same volume as that of the enriched culture. A single dose of 0·3 c.c. of each preparation was given by intraperitoneal injection to mice, and the animals tested a week later for immunity. When the degree of type-specific immunity was investigated, certain preparations were used in 10- to 1000-fold dilutions, the inoculum remaining 0·3 c.c.

The content of type-specific material in the preparation was roughly estimated by the amount of the precipitate given by a unit volume when mixed with an equal volume of a 1 in 10 dilution of the type antiserum. The mixture was drawn up in a pipette, sealed and set vertically. The size of the precipitate was read after standing 48 hr. at room temperature.

For the test doses of living pneumococci, serial tenfold dilutions were made from 0·1 or 0·2 c.c. of an 18 hr. serum-broth culture. Equal parts of broth and saline, warmed beforehand, were used as diluent, and the dilutions kept warm until actually injected. To secure accuracy, a fresh or cleaned pipette must be used for making each dilution. The number of pneumococci present was determined by plate culture of 0·1 c.c. of the 10^{-6} dilution on blood agar. The smallest test dose employed was 0·2 of the 10^{-7} dilution.

For each preparation and control a single series of mice was employed, and the animals observed for 1 week. Significant results were checked by repeating the experiment several times with fresh preparations. Experience showed that variable results depended far more on slight modifications in the technique of preparations than on individual differences in the response of the mice injected.

Filtration of preparations through ordinary filters absorbs so much of the active antigens that it is necessary to employ large amounts of material, the first portion of which can be discarded. To obviate this drawback, gradocol membranes of 0·25 mμ pore size were used for filtering.

Chemical process (Harley)

The method devised by Harley for the preparation of species antigen was chosen as the starting-point of the present investigation. It has the advantage of employing simple chemical means in place of less controllable autolytic processes. Harley found that a hot acid extract of pneumococci, which produced pure type immunity on injection in mice, after treatment with alkali afforded species protection. This result suggested that alkali treatment of type-specific antigen converted it into a less differentiated form which acted as a species antigen. While this process usually gave satisfactory results, partial failures were not uncommon in the experience of its originator (personal communication).

On repeated trials I also failed to obtain consistent results; occasionally the preparation afforded no species protection whatever, as shown below:

Type 1 pneumococci Average test dose	Controls	Type 2 pneumococci. Harley's process Five separate preparations				
6 cocci	D	S	S	D	D	S
60	D	S	S	D	D	D
600	D	S	D	D	D	D
6,000	—	S	D	D	D	D
60,000	—	D	S	D	D	D

S, survived; D, died.

Similar variations in results were obtained in mice injected with type 1 preparations and tested against type 2 pneumococci. Although this inconsistency apparently depended on some error in technique, it appeared that slight modifications should make little difference if the conversion of type antigen was the objective. Accordingly, the successive stages of the process were analysed in a series of experiments to determine the effect of one variation at a time, as compared with the original process.

The stages of the chemical method are as follows:

(1) Type pneumococci, collected as described in the previous section, are resuspended in saline of half the culture volume. To this suspension, half its volume of $N/10$ HCl is added, giving a strength of $N/30$ HCl, and the preparation heated to 60° C. for 1 hr.

(2) The suspension is cooled and centrifuged clear of the cocci.

(3) The acid extract is neutralized with $N/10$ NaOH. An excess of the alkali is added to bring the *p*H to 9, and the preparation incubated at 37° C. for 3 hr.

(4) The preparation is neutralized, made slightly acid and heated to 60° C. for 25 min. to secure sterility. An acid reaction during heating is necessary to prevent destruction of antigen.

Variations tried

(1) *Acid extract.* The strength of the acid and duration of the heating could be varied to a considerable extent without affecting the final result. A longer period of heating in $N/30$ HCl at 60° C., as for 3 hr., was somewhat superior.

The object of heating in acid was to secure a solution of type-specific antigen. This was invariably obtained; the extract (cleared by centrifuge) produced excellent type immunity in mice. The usual dose, described in the section on methods, was needlessly excessive; one-hundredth of this dose was sufficient in the case of types 1 and 2 pneumococci.

While this acid extract on injection in rabbits provoked the formation of type-specific agg "tinin, it lost this power after filtration. As Downie has shown, the extract though apparently clear contains cocci which hold undissolved type-specific antigen; only these induce agglutinin formation in rabbits. In mice, however, the type-specific immunity produced by injection of an acid extract was diminished but not abolished by filtration; this is best brought out when the effect of equal dilutions of ordinary and filtered extracts are compared.

Saline extract. The use of hot acid to procure a solution of type-specific antigen was open to the objection that it might cause chemical changes in the material present in the extract. To obtain a simple solution of this antigen, fresh pneumococci were suspended in saline at *p*H 7 and heated for an hour at 60° C., as in the preparation of

vaccines. After standing, the cocci were removed by centrifuge and the clear saline extract investigated. Three doses of a filtrate given to a rabbit failed to induce the formation of type-specific agglutinin. On injection in mice the saline extract gave as good type immunity as that afforded by an acid extract. The centrifuged extract contained pneumococci and filtration reduced its immunizing action in mice. But the saline extract differed from an acid extract in one important particular. Treatment of the saline extract with alkali or with hot acid followed by alkaline incubation did not affect its action. It still gave only type-specific immunity without species protection.

Microscopical examination of pneumococci, heated in acid and in saline respectively, revealed that acid heating profoundly affected a proportion of the cocci, reducing them to feebly staining particles, whereas saline had no such effect.

(2) *Centrifugalization.* The foregoing experiments indicated that: (*a*) Species antigen was not formed from type-specific antigen. (*b*) The chief source of species antigen obtainable from an acid extract lay in the reduced remnants of cocci left in suspension. Further tests showed that the variable results given by Harley's method depended on the clearing of the acid extract by the centrifuge. If the extract were rendered water-clear or filtered, a very poor or negative yield of species antigen was obtained on alkaline treatment. On the other hand, if the extract was not cleared to a sufficient degree, subsequent treatment with alkali gave a poor result. This variation is illustrated by the following experiment:

		Type 2 pneumococci. Heated in *N*/30 HCl 1 hr.		
Type 1 pneumococci Test doses	Controls	Acid extract largely cleared by centrifuge*	Clearer extract after second centrifuging*	Residue from second centrifuging*
5 cocci	D	S	S	D
50	D	S	S	D
500	D	D	S	D
5,000	—	D.	S	D
50,000	—	D	D	D

All three preparations incubated at *p*H 9 for 3 hr.

It will be seen that the almost clear extract gave a much better result. The residual cocci, separated by a second spinning from almost invisible remnants, appeared inert in protective value, and their presence in the first preparation reduced its effect.

To vary the process, pneumococci were heated in acid, then the suspension was made alkaline and incubated, and finally centrifuged clear. This preparation failed to give species protection.

Tests with type antiserum showed that hot acid extracts a proportion only of the type-specific carbohydrate; alkaline incubation dissolves out a larger amount. If many cocci be left in an acid extract, subsequent alkaline treatment liberates more type-specific antigen together with some protein and species antigen. The failure of such preparations to excite species protection, except to a slight degree, could not be explained satisfactorily.

(3) *Alkaline incubation.* The main effect of alkali on the acid extract is to secure solution of the coccal remnants. By the use of stronger alkali (*N*/60 NaOH) the action can be expedited. Whether alkali was necessary for the formation or activity of species antigen remained for further investigation.

(4) *Final heating.* A short period of final heat at 60° C., keeping the preparation just acid to avoid destruction of antigen, was introduced to ensure sterility. But control

experiments demonstrated that this acid heating increased the immunizing value of the preparation, sometimes to a considerable extent. The increase appeared proportional to the strength of alkali used previously. The following experiment proved the value of final heating:

Type 2 pneumococci Test doses	Controls	Type 1 pneumococci Acid extract, incubated alkaline	
		(*a*) Unheated	(*b*) Final acid heated
3 cocci	D	S	S
30	D	S	S
300	D	S	S
3,000	—	D	S
30,000	—	D	S

Autolytic process (Day, Harley)

For autolysis, type pneumococci were collected by centrifuge from a reinforced culture and suspended in a small volume of saline. An equal volume of alcohol was added down the sides of the centrifuge tubes and then well mixed. The pneumococci thus killed were at once centrifuged down and resuspended in slightly acid saline, in volume equal to or half that of the culture fluid. The object of the alcohol treatment was to secure more uniform autolysis than occurs in suspensions of living organisms.

Autolysis was allowed to proceed for a day or two at room temperature or for longer in the refrigerator (4° C.). The latter method was slower but minimized the risk of contamination. Tests for sterility were made before further treatment of the suspension. In the original method autolysis was more prolonged, and concluded by keeping the suspension alkaline for a few hours to secure more solution. This can be done after heating the autolysate (Harley). It is somewhat more efficacious to employ a preliminary heating in $N/30$ HCl, as shown by the following experiment:

Type 1 pneumococci Test doses	Controls	Type 2 pneumococci Suspension kept 11 days at 4° C.			
		(*a*) Heated		(*b*) Heated in HCl	
		No more treatment	Incubated alkaline	No more treatment	Incubated alkaline
8 cocci	D	S	S	S	S
80	D	D	S	S	S
800	D	D	D	S	S
8,000	—	D	D	D	S
80,000	—	D	D	D	D

Other experiments confirmed this result that heating in acid followed by alkaline incubation yielded the best results. The alkaline preparations were neutralized before injection; this caused some cloudiness from precipitation of bacterial protein. A final heating of the preparation (at acid *p*H) tended to diminish its efficacy. These autolysates contained very large amounts of type-specific carbohydrate.

Stages of the process

The initial treatment with alcohol and resuspension in saline at once removed most of the Gram-positive material from the pneumococci. In 24–36 hr. at room temperature almost all the type-specific carbohydrate from type 1 organisms had passed into solution. Filtrates gave excellent type-specific immunity, even in high dilution (1 in 1000) when injected in mice, but no species protection. The coccal bodies, collected by centrifuge and treated with hot acid followed by alkaline incubation, gave a preparation which

usually afforded a slight or irregular degree of species immunity. The supernatant fluid, turbid from incomplete separation of the most reduced cocci, in spite of its high content of type-specific antigen, conferred species protection on similar treatment with HCl and alkali, usually equal to that obtained from preparations of the coccal deposit. The following table shows the inconstant results of a single injection of twelve separate preparations made from partially autolysed pneumococci, types 1 and 2, at different times:

Type 2 pneumococci Test doses Average no.	Controls	Autolysed type 1 pneumococci. HCl and alkali preparations Prep. no. 1	2	3	4	5	6	7	8	9	10	11	12
7 cocci	D	S	D	S	S	S	S	D	D	S	S	D	S
70	D	S	S	D	S	S	S	D	S	S	D	D	S
700	D	D	S	D	S	D	D	D	D	S	D	D	D
7,000	—	D	D	D	D	D	S	D	D	S	D	S	D
70,000	—	D	D	D	D	D	D	D	D	D	D	D	D

Type 1 pneumococci Test doses Average no.	Controls	Autolysed type 2 pneumococci. HCl and alkali preparations Prep. no. 1	2	3	4	5	6	7	8	9	10	11	12
8 cocci	D	S	S	D	S	D	D	D	D	S	S	S	D
80	D	S	D	D	S	D	S	D	D	S	S	D	D
800	D	S	D	S	D	D	S	S	D	D	D	D	D
8,000	—	D	D	D	S	D	D	D	S	D	S	D	S
80,000	—	D	D	D	D	D	D	D	D	D	D	D	D

No better results were obtained from autolysis of other type pneumococci.

From pneumococcal autolysates, as from acid extracts in Harley's process, a potent preparation of species antigen was best secured by solution of the smallest coccal remnants that tend to remain in suspension after centrifuging. This was demonstrated by experiment. An autolysate was centrifuged twice, and the first and second lots of reduced cocci so collected were dissolved by hot HCl followed by alkali. The apparently clear supernatant fluid was subjected to the same chemical process. Only this last preparation conferred species immunity on injection in mice.

Different types of pneumococci vary in the comparative rate of solution of their constituents during autolysis. With type 1 almost all the type-specific material passes into solution before any of the species component is dissolved. With type 2 the separation is less distinct, and from type 3 species material dissolves out more readily than from type 1.

Collection. Since experience with Harley's method had shown that greatly reduced pneumococci were the best source of species antigen, some way of recovering these remnants from autolysates was much to be desired. Satisfactory results were obtained by acid agglutination, which was facilitated by brief previous treatment with a drop of weak alkali, or by heating (keeping the suspension just acid). When autolysates so treated were centrifuged a clear supernatant fluid was seen, with a deposit adherent to the sides as well as collected at the bottom of the tubes. From the cocci collected by these means it is possible to get better preparations of species antigen, as in the experiment below:

Type 1 pneumococci Test doses	Controls	Type 2 pneumococci, partially autolysed. Cocci separated by simple centrifuge, special collection (each treated with HCl, then alkali)	
7 cocci	D	S	S
70	D	S	S
700	D	D	S
7,000	—	D	S
70,000	—	D	D

Other methods of reduction. In place of simple autolysis, various solvents, antiseptics and protein coagulants were employed in weak solutions to obtain reduction of the pneumococci and removal of type-specific material. Of these agents, carbolic and weak acid gave satisfactory final preparations.

Carbolic acid. Fresh pneumococci were treated with 1 in 20 carbolic acid for half an hour; then 7 volumes of saline were added to reduce the strength to 1 in 160. In this medium the cocci were kept for a week in the refrigerator, or for 5 days with an 18 hr. period at 37° C. The cocci reduced by this process could be collected fairly well by centrifuge. They were resuspended in saline and kept 3 more days in the refrigerator and again collected; this intermediate period allowed more type-specific material to dissolve away. The cocci were then heated in $N/30$ HCl and kept alkaline (pH 9) for 3 hr.

In the following table the results of twelve experiments with separate preparations by this process from type 1 pneumococci are collated:

Type 2 pneumococci Test doses Average no.	Controls		Inoculated mice	
	Survived	Died	Survived	Died
4 cocci	0	12	10	2
40	0	12	9	3
400	0	12	7	5
4,000	—	—	4	8
40,000	—	—	3	9
400,000	—	—	0	12

Two of the preparations were unsatisfactory. A final heating of the preparation (at acid pH) slightly increased its efficacy.

Dilute acid. If pneumococci were first treated with 50% alcohol and subsequently kept in $N/40$ or $N/400$ HCl (made by diluting $N/10$ HCl with saline), the type-specific material slowly dissolved out leaving the species material in the reduced bodies of the cocci. At room temperature this process took about 20 days for type 1 pneumococci, about 9 days for type 2. From this acid suspension the cocci were easily collected by centrifuge, but had been made more resistant to solvents.

The following experiment gives the results obtained from different periods of treatment with cold dilute acid:

Type 2 pneumococci Test doses	Controls	Preparation of type 1 pneumococci after keeping in $N/40$ HCl for	
		9 days	20 days
4 cocci	D	S	S
40	D	D	S
400	D	D	S
4,000	—	D	S
40,000	—	D	D

Type 1 pneumococci Test doses	Controls	Preparation of type 2 pneumococci after keeping in $N/40$ HCl for	
		9 days	20 days
5 cocci	D	S	S
50	D	S	D
500	D	S	D
5,000	—	S	D
50,000	—	D	D

Solvents. Other solvents were tested in place of the usual hot acid followed by alkali. NaOH alone (in $N/40$ or $N/60$ strength) dissolved reduced pneumococci readily, but

these solutions seldom gave species protection when injected, even when treated by hot acid and alkali. This failure was not due to a destructive effect on species antigen, since alkali solutions of alcohol precipitates (described in the section below) afforded species immunity.

Taurocholate (bile salt) readily dissolves fresh or kept pneumococci, but has less solvent action on heated or otherwise hardened organisms. When simple taurocholate (5%) failed to secure a satisfactory degree of solution, its action could be increased by the addition of HCl, adding some alcohol to keep most of the taurocholate acid in solution. For this purpose a mixture of 4 parts 5% taurocholate, 1 part of $N/10$ HCl and 1 part of alcohol was employed, first at 37° C. for 1–2 hr., then at 60° C. for 45 min. Good results were obtained without complete solution of the cocci. From taurocholate preparations the species antigen with protein was precipitated by the addition of 2 volumes of alcohol. This precipitate was taken up in saline (without drying) made alkaline (pH 9) and incubated for 2 hr.

The following experiments illustrate the results obtained from preparations made from carbolized pneumococci by (*a*) the HCl and alkali process, (*b*) the taurocholate method:

Type 2 pneumococci Test doses	Controls	Type 1 pneumococci treated with carbolic. Cocci separated and treated: (*a*) HCl and alkali	(*b*) Taurocholate process
8 cocci	D	S	S
80	D	S	S
800	D	S	S
8,000	—	S	S
80,000	—	S	D

Type 1 pneumococci Test doses	Controls	Type 2 pneumococci treated with carbolic. Cocci separated and treated: (*a*) HCl and alkali	(*b*) Taurocholate process
6 cocci	D	S	S
60	D	S	S
600	D	D	S
6,000	—	D	S
60,000	—	S	D

Formamide, the bacterial solvent introduced by Fuller (1938), dissolved fresh pneumococci at a temperature of 150° C. An acetone precipitate, taken up in acid saline and filtered, contained active type-specific antigen, as proved by the immunization of mice:

Type 1 pneumococci Test doses	Controls	Type 1 pneumococci Formamide preparation Diluted 1 in 50
11 cocci	D	S
110	D	S
1,100	—	S
11,000	—	S
110,000	—	D
1,100,000	—	D

Reduced pneumococci were dissolved in a few minutes at a lower temperature, 100–

amide,

much occurring in the cold. Acid alcohol, as used by Fuller to precipitate protein from formamide solutions, brought down most of the species antigen with the protein. Hence 2 volumes

reduced

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pneumococci. This precipitate was treated (wet) with alkaline saline (*p*H 9) to dissolve out the species antigen.

Formamide was very useful to secure solutions of reduced pneumococci that had been hardened by previous treatment and were resistant to other solvents. The following experiment illustrates its efficiency:

Type 1 pneumococci Test doses	Controls	Type 2 pneumococci. Alcoholized and kept in *N*/400 HCl for 11 days at 4° C. Cocci collected	
		(*a*) HCl 60° C. then alkali, centrifuged clear	(*b*) Residue from (*a*) formamide preparation
15 cocci	D	S	S
150	D	D	S
1,500	—	S	S
15,000	—	D	S
150,000	—	D	S

Origin of species antigen

In previous experiments species antigen had been obtained by chemical and autolytic solution of pneumococci. To determine whether species antigen was a natural component of the cocci or was a product of some chemical change, the effect of simple mechanical disintegration of the organisms was tried.

Type 2 pneumococci, obtained from a young culture, were heated in saline to 80° C. for 20 min. to destroy enzymes. The cocci were then dried and ground lightly in saline. The resulting suspension was centrifuged and the supernatant fluid, which contained much type-specific material, discarded. The residue was reground in slightly alkaline saline, the suspension just over-neutralized and centrifuged clear. This extract was injected in mice which were tested a week later and proved immune to 2000 fatal doses of virulent type 1 pneumococci.

Though chemical action might be unnecessary for the formation of species antigen, yet the use of acid appeared necessary for its action, when the antigen was dissolved in slightly alkaline saline.

In the following experiment type 2 pneumococci were ground as in the preceding protocol and then the suspension filtered at *p*H 8. This filtrate was divided in two parts; one was kept as control, the other acidified and heated to 60° C.:

Type 1 pneumococci Test doses	Controls	Type 2 pneumococci. Heated. Ground Residue reground: filtrate	
		Unheated solution	Heated acid
14 cocci	D	D	S
140	D	D	S
1,400	—	D	S
14,000	—	D	D

A further experiment showed that species antigen could be obtained in a solution apparently free from protein. Partially autolysed type 1 pneumococci were collected and extracted in saline acidified with acetic acid. This extract was centrifuged clear and treated with an equal volume of picric acid in saturated watery solution. After 2 days the preparation was again centrifuged and treated with 2 volumes of alcohol, which gave a small precipitate in 3 days. This precipitate was washed in alcohol and dissolved in saline. It gave no precipitate with salicyl-sulphonic acid nor with Millon's reagent.

It gave no precipitin reaction with type 1 antiserum, but a weak Molisch reaction. On injection in mice it afforded definite species immunity.

To find the solubility of species antigen in alcohol, preparations were made from partially autolysed type 2 pneumococci by Harley's process. The acid extract was made well alkaline and kept at 37° C. for 1½ hr., then neutralized. This extract was treated with different amounts of alcohol, 1, 2 and 3 volumes respectively. The resultant precipitates were dissolved in saline and injected in mice, which were afterwards tested for type-specific and for species immunity. Most of the species antigen was precipitated by 1 volume of alcohol, all by 2 volumes. Type 2 specific antigen required 2–3 volumes of alcohol for precipitation. For the success of this separation it is necessary to get an extract almost or quite free from bacterial protein. If much protein be present, the antigens are brought down as an adsorbate with the protein precipitated by alcohol or other agent.

INTERFERENCE WITH IMMUNIZATION

The conclusion that species antigen was a natural constituent of pneumococci, and could be extracted by simple means from fresh cocci, demanded a new interpretation of the effects of alkaline solution and of acid heating which greatly influenced the potency of preparations. Experimental results had revealed two peculiarities of species immunization which were of special significance in this inquiry:

(*a*) Pneumococci reduced by autolysis (and largely deprived of type-specific carbohydrate), when heated and injected undissolved, induced only a minimum degree of species protection. Injection of a complete solution of these cocci gave no better results. Yet fractional solution of the cocci proved that they contained an ample amount of species antigen. This anomaly is in strong contrast with the efficiency of other bacterial antigens contained in heated intact bacteria (ordinary vaccines) which readily induce immunization.

(*b*) The highest degree of species immunity was afforded by preparations of remnants of pneumococci from which constituents other than species antigen had been largely removed. The inclusion of less reduced cocci in the material greatly reduced the efficiency of the preparations, and appeared chiefly responsible for the variations in the results observed.

These considerations suggested that the failure of intact pneumococci and of many pneumococcal preparations to induce species immunity was due to an excess of some constituent. Removal of this interfering material would then allow of better immunization.

The following experiment proved the value of this procedure:

Type 1 pneumococci, treated with 50% alcohol and resuspended in saline, had undergone autolysis for 1 day at room temperature and 2 days more in the refrigerator. The suspension was then heated at 80° C. to destroy enzymes, the reduced cocci collected by acid agglutination and centrifuge, resuspended in neutral saline and kept 10 days in the refrigerator till wanted. The suspension was then heated in *N*/30 HCl for an hour at 60° C., neutralized, made well alkaline (*p*H 9·5–10) and kept at 37° C. for an hour, when solution appeared complete. On over-neutralization to *p*H 6·5 some cloudiness from precipitation of protein was seen; this was more evident after standing overnight. The following day the slightly acid preparation was divided in two parts. One portion

was kept as control; the other centrifuged clear, its supernatant fluid removed (prep. 2) and the precipitate largely dissolved in bile salt. This last preparation was treated with 2 volumes of alcohol and the precipitate collected after an hour. This was taken up in alkaline saline, kept at 37° C. for an hour (imperfect solution) and finally heated at *p*H 6 for half an hour. The original cloudy preparation, the cleared portion and the preparation made from the precipitate were injected in three sets of mice, which were tested a week later for cross immunity to type 2 *Pneumococcus*, with the following result:

Type 2 pneumococci Test doses	Controls	Type 1 pneumococci. Reduced by autolysis HCl and alkali. Over-neutralized		
		(1) Not clear	(2) Cleared	(3) Prep. of ppt.
5 cocci	*D*	S	S	S
50	D	D	S	S
500	D	D	S	S
5,000	—	D	S	S
50,000	—	D	S	S

From this experiment it seemed evident that the precipitated matter in the original uncleared preparation prevented proper immunization by the species antigen present. The precipitate consisted of protein to which much species antigen had been adsorbed, but by partial solution this antigen could be largely recovered.

No particular attention had been paid to the clarity of the preparations on neutralization before injection in former experiments, since it was known that undissolved particles of pneumococci contained species antigen, and that precipitated protein carried down more or less of the species antigen by adsorption. But this last experiment demonstrated the presence of a factor in the precipitate which could almost nullify the immunizing effect of species antigen.

The improvement in species immunization obtained by the removal of precipitated material from pneumococcal preparations was confirmed by further experiments and found to apply also to undissolved matter.

In the following example, autolysed type 1 pneumococci were heated in dilute HCl and then dissolved in hot formamide. The precipitate obtained on the addition of 2 volumes of acetone to this solution was collected and taken up in neutral saline. Next day this preparation was divided in two parts, one of which was centrifuged clear. Both parts were injected in mice which were tested for species immunity a week later:

Type 2 pneumococci Test doses	Controls	Type 1 pneumococci. Autolysed cocci, formamide preparation	
		Uncleared	Cleared
6 cocci	D	D	S
60	D	D	S
600	D	D	D
6,000	—	D	D
60,000	—	D	S

It is noticeable that the cleared solution had not been freed from protein in solution and did not give a solid immunity.

The addition of acid to neutral or alkaline solutions containing protein is not always effective in precipitating all the interfering material. But if the acidified preparation be first cleared of precipitated material by centrifuging, and then heated to 60° C., any remaining protein is coagulated and interference with immunization minimized,

even when no visible precipitate is seen. This result is shown in the following experiment:

Type 1 pneumococci Test doses	Controls	Type 2 pneumococci. Reduced by autolysis Formamide solution-acetone ppt. Left in alkaline saline 18 hr.	
		Acidified and centrifuged clear	Same preparation final heating
11 cocci	D	D	S
110	D	D	S
1,100	—	S	S
11,000	—	D	S
110,000	—	D	D

Here the acidified preparation, simply cleared by centrifuge, still contained enough interfering material to prevent proper protection. The paradoxical type of immunity conferred appears characteristic of such preparations; other instances can be seen in the tables of results obtained by the treatment of autolysed pneumococci with hot acid and alkali, when no further treatment was adopted. The solid immunity afforded by the heated preparation is similar to that obtained by Harley's process after the final acid heating.

Nature of substance interfering with species immunization

The constituents in pneumococcal preparations which might be responsible for this interference include type-specific material, an excess of species antigen itself, lipoids, material derived from the culture medium and the bacterial protein.

Type-specific material. An excess of this substance might well explain the failure of a preparation made by Harley's method when the acid extract contained a considerable number of unreduced cocci. But the successful species immunization obtained from whole autolysates or their fluid portion, which contained an enormous amount of type-specific material, refuted this possibility. Complete solutions of avirulent rough pneumococci, which contain no type-specific substance, were found to give no species protection when injected. Lastly, the addition of a good type-specific immunizing extract to a preparation which induced species immunity did not reduce the efficacy of the latter.

Species antigen. The possibility that an excess of species antigen itself might prevent efficient immunization was excluded by special experiments. Preparations made by dissolving reduced pneumococci, if not purified, conferred only a minimum degree of species immunity, although containing much species antigen. Injected in dilutions of 1 in 5, 1 in 10 and 1 in 50, such preparations failed to induce species immunity, in spite of the progressively reduced amount of the antigen administered.

Lipoids. Since the interfering material was precipitated along with species antigen by alcohol and acetone, it was unlikely to be of lipoid nature. Extraction of reduced pneumococci with warm ether and with chloroform did not remove the interfering substance.

Culture medium. It was conceivable that material such as serum protein in the culture medium might be carried on to preparations of pneumococci and render mice susceptible to infection by living pneumococci grown in a similar medium. To test this hypothesis, experiments were made with preparations made from pneumococci that had been well washed with saline before treatment; also a different animal serum was used for test

cultures. Neither of these modifications affected the presence of interfering material in the preparations.

Bacterial protein. The exclusion of other constituents by experiment, and the fact that measures which removed or coagulated protein enhanced the species' protective power of pneumococcal preparations, led to the conclusion that bacterial protein or a closely related substance was the factor that opposed immunization. This factor was not a product confined to autolysates, for it was found in preparations made from fresh heated pneumococci. It was unaffected by acid and alkali in moderate strength and was not destroyed by hot formamide.

Previous results explained

The presence of inconstant amounts of an antagonistic factor explained the variable degrees of species immunity induced by injection of pneumococcal preparations made by the same method. Thus in Harley's process, failure to remove insufficiently reduced cocci from the acid extract resulted in the solution of enough opposition factor by alkali to nullify the immunizing effect of the species antigen liberated. Heating the final solution (at acid *p*H) precipitated and coagulated dissolved protein containing opposition factor, thus reducing its activity and enhancing the immunizing value of the preparation.

In the autolytic process, the incomplete solution of reduced cocci or of alcohol precipitates of solutions, gave a fair measure of species protection, since species antigen was partly dissolved out, leaving most of the opposition factor in coagulated and relatively insoluble protein. Complete solutions of reduced pneumococci contain too much opposition factor to afford species immunity on injection. The slow reduction of pneumococci by carbolic or dilute mineral acid rendered the bacterial protein less soluble, with the result that the usual solvent methods procured some selective extraction of species antigen and gave fairly reliable preparations.

Purification of species antigen

The separation of species antigen from the protein, with which the opposition factor is associated, is not a simple matter. Pneumococci, even when considerably reduced by autolysis, yield extremely little species antigen to extractives which do not dissolve the bacterial protein. The addition of a protein coagulant to solutions containing species antigen and much protein results in the adsorption of nearly all the species antigen to the protein precipitated. A converse method (Felton's) for precipitation of polysaccharide with calcium phosphate failed to separate the antagonistic components.

The following methods of purification on a small scale have been used with success:

(*a*) Harley's process applied to partially autolysed pneumococci. The remnants of cocci left in the acid extract contain so little protein that final acid heating should cause no visible turbidity. After this heating the preparation should be centrifuged.

(*b*) Fractional disintegration by successive solvent processes of pneumococci reduced by autolysis. By this means the bulk of the protein can be removed, especially from type 1 pneumococci, leaving much species antigen in the remnants. Bile salt can be used first, then the undissolved remnants collected and dissolved by formamide as described previously.

(*c*) The removal of protein from solutions of reduced pneumococci under conditions which minimize adsorptions of species antigen. The cocci are heated in *N*/30 HCl at

60° C. for 1 hr., neutralized with $N/10$ NaOH and solution obtained by the addition of extra alkali to bring the pH to 9 or 10, maintained for 1–2 hr. at 37° C. If solution is then incomplete, the preparation is cleared of undissolved material by the centrifuge.

(1) The alkaline solution is made just acid (pH 6·5) to precipitate protein, then kept almost neutral overnight to allow of some solution of adsorbed antigen. Next day it is centrifuged clear, made acid (pH 6) and heated to 60° C. for 20 min. If a further precipitate forms, this is removed by centrifuge.

(2) If alkaline incubation does not give a clear solution, the residue is collected by centrifuge and dissolved by formamide at 120° C. This solution is precipitated by 2 volumes of alcohol and the precipitate taken up in the first extract (at pH 9–9·5). After standing an hour the preparation is neutralized and kept overnight, just acid (pH 6·5). Next day it is centrifuged clear, and finally heated at acid pH.

The following experiment shows the extent of species immunity secured by injection of a single dose of (*a*) reduced pneumococci, simply heated, (*b*) treated by a combined solvent process (HCl, alkali, then formamide) to exclude protein:

Type 1 pneumococci Test doses	Controls	Type 2 pneumococci. Reduced by autolysis	
		(*a*) Heated only	(*b*) Solvent process
12 cocci	D	S	S
120	D	D	S
1,200	—	S	S
12,000	—	D	S
120,000	—	D	S
1,200,000	—	D	D

Discussion of these results is best deferred until some further aspects and details of species immunity are described, as it is hoped, in future communications.

SUMMARY

From pneumococci a species antigen can be obtained which on injection gives protection against pneumococci of different types.

Species antigen is a somatic constituent of pneumococci and can be obtained in solution free of protein. It may be of carbohydrate nature, but is not derived from the type-specific material.

The bodies of pneumococci contain another substance which on injection opposes species immunization. This opposition factor is closely associated with the protein content.

Inconstant results from injection of preparations containing species antigen are due to the presence of opposition factor in varying amount.

A high degree of species immunity can be secured by the use of species antigen freed from opposition factor.

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