THE EFFECT OF FORMALDEHYDE ON PNEUMOCOCCI

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Encapsulated pneumococci treated with sufficient concentrations of formaldehyde retain for some time their characteristic morphology, their positive reaction to the Gram stain, and their specific agglutinability in homologous antiserum. Moreover, rabbits and horses immunized by the intravenous route with formolized encapsulated pneumococci react with the production of the specific antibodies directed against the capsular polysaccharide of the bacterial cell used as antigen. It is generally considered, however, that pneumococcus antigens prepared by this technique are not very stable, and undergo with time a form of lysis accompanied by a loss of antigenicity (1).

It has been shown in a previous paper that the “capsular polysaccharide antigen” of Pneumococcus can be rendered ineffective by the action of an autolytic enzyme present in this bacterial species (2). Although this autolytic enzyme can be inactivated by a number of reagents, the inactivation is in many cases reversible (3-6). When, for instance, iodine is used in proper concentration to kill and “fix” pneumococci, the autolytic enzyme is inactivated and the cells retain their structure and their antigenicity; upon removal of the iodine, however, the enzyme may recover its activity and bring about lysis accompanied by loss of antigenicity (2, 5).

In the present study, an attempt has been made to analyze the action of formaldehyde on pneumococci in the light of the observations outlined above. Techniques to render stable the formolized pneumococcus antigens are also described.

EXPERIMENTAL

The bacteriological and immunological methods used in this study are the same as those described in a previous paper (2).
A commercial preparation of formalin (38 per cent formaldehyde) was used as source of formaldehyde.

The Effect of Different Concentrations of Formaldehyde on the Autolysis of Pneumococci.—A great many antiseptics have the apparently conflicting properties of activating the autolysis of different bacterial species when used in low concentrations, and of completely inhibiting the autolytic process when used in higher concentration (7, 8). The effect of different concentrations of formaldehyde on cultures of pneumococci is considered in Experiment 1.

**Experiment 1.**—A plain broth culture of Type III pneumococci 8 hours old was distributed in 5 cc. amounts into test tubes; the culture had reached a final pH of 7.1. Varying amounts of formaldehyde were added to the different samples to give final concentrations ranging from 0.005 to 0.5 per cent, and the formolized cultures were incubated at 37°C. and examined after different intervals of time. The presence of living cells was determined by streaking the cultures on blood agar plates, and the degree of autolysis was followed by microscopic examination of films stained by the Gram technique. The results are presented in Table I.

### TABLE I

<table>
<thead>
<tr>
<th>Final concentration of formaldehyde per cent</th>
<th>Growth on blood agar plates</th>
<th>Microscopic appearance of the cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 hrs.  24 hrs.  96 hrs.</td>
<td>24 hrs.  96 hrs.</td>
</tr>
<tr>
<td>0.5</td>
<td>−  −  −</td>
<td>Gram-positive</td>
</tr>
<tr>
<td>0.2</td>
<td>−  −  −</td>
<td>−</td>
</tr>
<tr>
<td>0.1</td>
<td>−  −  −</td>
<td>Mixture of Gram-positive and Gram-negative</td>
</tr>
<tr>
<td>0.05</td>
<td>−  −  −</td>
<td>Mixture of Gram-positive and Gram-negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gram-negative detritus</td>
</tr>
<tr>
<td>0.03</td>
<td>+  −  −</td>
<td>Gram-negative</td>
</tr>
<tr>
<td>0.02</td>
<td>+  −  −</td>
<td>−</td>
</tr>
<tr>
<td>0.01</td>
<td>+  +  −</td>
<td>Gram-positive</td>
</tr>
<tr>
<td>0.005</td>
<td>+  +  +</td>
<td>−</td>
</tr>
<tr>
<td>0</td>
<td>+  +  +</td>
<td>−</td>
</tr>
</tbody>
</table>

+ indicates growth of pneumococci on blood agar plates.
− " no growth of " " " " " " "

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The results of Experiment 1 show that an amount of formaldehyde corresponding to a final concentration of 0.05 per cent was sufficient to sterilize a culture of Type III pneumococci in 3 hours at 37°C. This amount, however, was not capable of preventing autolysis. In fact the cells treated with the smaller concentrations of formaldehyde (0.02 to 0.05 per cent) underwent autolysis more rapidly than the control cells. The cells treated with the largest concentrations of the antiseptic (0.2 per cent and 0.5 per cent) remained well formed and Gram-positive; in other words they were fixed by the reagent.

Partial Lysis of Formolized Pneumococci Washed Free of Formaldehyde.—The cells in Experiment 1 had remained in the presence of formaldehyde throughout the period of observation; the behavior of pneumococci killed with an amount of formaldehyde sufficient to inhibit the autolytic enzyme, then washed free of the antiseptic, is considered in Experiment 2.

Experiment 2.—500 cc. of plain broth culture of Type III pneumococcus was treated with enough formaldehyde to give a final concentration of 0.5 per cent. The formolized culture was kept at room temperature for 24 hours; the cells were then separated by centrifugalization, washed twice, and resuspended in 50 cc. physiological saline solution. The bacterial suspension consisting of well formed Gram-positive cells was then divided into two equal fractions, one of which received immediately 0.5 per cent formaldehyde. Both fractions were kept at room temperature and the microscopic appearance of the cells followed by the Gram stain.

The cells maintained in the presence of formaldehyde remained well formed and Gram-positive throughout the period of observation (4 weeks). Some of the cells in the suspension washed free of the antiseptic had on the contrary become Gram-negative within 24 hours, and hardly any Gram-positive cocci could be seen after 72 hours. The change from a Gram-positive to a Gram-negative state was accompanied by a reduction in size of the cocci, but no real disintegration of the cells could be observed. No further evidence of lysis appeared on prolonged incubation. The experiment was repeated at ice box temperature (5°C.) and at 37°C. The results were identical with the only difference that the rate of change was slower at 5°C. and faster at 37°C. It is therefore apparent that whereas pneumococci treated with an excess of formaldehyde retain their morphological
and staining characteristics as long as they are kept in the presence of this agent, they may undergo a limited form of lysis when washed free of the antiseptic.

Factors Affecting the Lysis of Formolized Pneumococci.—It has been shown elsewhere that the inactivation of some of the pneumococcus enzymes by iodine is a reversible process; these enzymes recover their activity when the iodine is removed by reducing agents (5, 6). It appeared possible that the lysis suffered by formolized pneumococci washed free of formaldehyde was due to the reactivation of some autolytic enzyme. To test this hypothesis, formolized pneumococci were kept under a variety of experimental conditions in order to control the action of the autolytic enzymes.

Experiment 3.—20 cc. of formalin were added to 1500 cc. of a young culture of Type III Pneumococcus. The formolized culture was kept at room temperature for 24 hours, then divided into six equal fractions and centrifugaled. The formolized cells were suspended in 5 cc. amounts of the following media.

(a) \textit{M}/20 phosphate buffer pH 7.0
(b) \textit{M}/20 phosphate buffer pH 7.0 + 0.1 cc. formalin
(c) \textit{M}/20 phosphate buffer pH 7.0; this suspension was immediately heated at 75°C. for 20 min.
(d) \textit{M}/20 acetic acid (final reaction pH 4.2)
(e) Lugol iodine solution

These cell suspensions were kept at 37°C. and stained by the Gram technique after different intervals of time.

After 24 hours, the cells in suspension (a) (neutral reaction, unheated and in the absence of additional formaldehyde or iodine) had become Gram-negative. In all the other preparations, the cells remained Gram-positive throughout the period of observation (2 weeks at 37°C.).

In other words, the change from a Gram-positive to a Gram-negative state is inhibited by the presence of formaldehyde or iodine, by heating the cell suspension, or by maintaining it at an acid or alkaline reaction. These findings may be interpreted as follows. The change from Gram-positive to Gram-negative is caused by an enzyme which is inactive in the presence of formaldehyde (b) or iodine (f) but which recovers its activity when the formaldehyde is removed and the prepa-
ration is incubated at neutral reaction (a); the enzyme is irreversibly inactivated by heating at 75°C. (c) and does not function at acid (e) or alkaline (d) reaction. To substantiate this hypothesis it was of interest to study the effect of the autolytic enzyme of Pneumococcus on formolized cells of this bacterial species.

The Effect of the Autolytic Enzyme of Pneumococcus on Formolized Pneumococci.—It is known that the autolytic enzyme of Pneumococcus can be obtained in an active form from autolysates and extracts of pneumococci (9, 10, 5). The effect of this enzyme preparation on formolized pneumococci is described in the following experiment.

Experiment 4.—3 cc. of formalin were added to 300 cc. of a young culture of Pneumococcus Type III in plain broth. The formolized culture was allowed to stand for 24 hours at room temperature. The cells were then separated by centrifugation, washed once in saline, and divided into two equal fractions. One fraction was resuspended in 15 cc. m/20 K_2HPO_4 (the final pH of the suspension was 7.8); the other was resuspended in 15 cc. m/20 phosphate buffer at pH 7.0 and this suspension immediately heated at 75°C. for 20 minutes. Both suspensions consisted of well formed Gram-positive cells.

The bacteriolytic enzyme was prepared according to a technique previously described (5). The cells from 150 cc. of a broth culture of R Pneumococcus, derived from Type II, were resuspended in 15 cc. of distilled water and allowed to autolyze in the presence of toluol for 48 hours at 37°C. The Gram-negative detritus was then removed by centrifugation and the active supernate used as enzyme.

To 1.0 cc. amounts of the two formolized bacterial suspensions (heated at pH 7.0, or resuspended in m/20 K_2HPO_4 and unheated) was added 1.0 cc. to 0.1 cc. of the solution of bacteriolytic enzyme. The mixtures were made up to 2.0 cc. volume with saline and incubated at 37°C. for 24 hours. At the end of this time films of the different mixtures were stained by the Gram technique to estimate the action of the enzyme on the two bacterial suspensions.

It appears from the results of Experiment 4 that the bacteriolytic enzyme of pneumococcus is capable of attacking pneumococci killed with formaldehyde. The enzymatic digestion however does not bring about a disintegration, a real “lysis” of the cell bodies; on the contrary the cells become Gram-negative, but retain their general morphology. In this respect, formolized cells differ from heat-killed cells which are completely disintegrated by sufficient amounts of the bacteriolytic enzyme. It is of special interest that the enzyme renders the formolized cells Gram-negative in a medium at pH 7.8,
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i.e. under conditions where the formaldehyde is not likely to dissociate from its combination with the cell structure. Since formolized pneumococci kept at pH 7.8 do not of themselves become Gram-negative, whereas they can be attacked by an active preparation of the enzyme, it seems logical to assume that fixation by formaldehyde is due not to its action on the cell substrate, but to the inactivation of the bacteriolytic enzyme. The results of Experiment 3 indicate that under proper conditions, the fixation of the cell, and therefore the inactivation of the enzyme, are reversible phenomena.

The Effect of Formaldehyde upon the Type Specific Antigenicity of Encapsulated Pneumococci.—Fresh preparations of formolized encapsulated pneumococci (Gram-positive) are capable of inciting the production of the type specific carbohydrate antibodies when injected into rabbits by the intravenous route. It has been shown elsewhere however, that the capsular polysaccharide antigen of pneumococci is inactivated by the action of an autolytic enzyme obtained from the same bacterial species (2). It appeared therefore possible that the partial lysis suffered by formolized pneumococci washed free of the antiseptic (Experiments 2 and 3) would be associated with a loss of type specific antigenicity. This is illustrated in the following experiment.

Experiment 5.—The same cell suspensions described in Experiment 3 were used for the immunization of rabbits by the intravenous route. The immunizing dose was the equivalent of 2 cc. of culture daily; three rabbits were used for each preparation. The animals were bled 1 week after the completion of the second course of immunization; their sera were tested for the presence of type specific agglutinins and for precipitins for the homologous capsular polysaccharide (Type III).
The sera of the three animals immunized with the formolized cells which had become Gram-negative (preparation (a)) failed to show any type specific agglutinin or precipitin in any dilution. On the contrary the sera of all the animals which had received the formolized cells maintained Gram-positive by destruction or inactivation of the autolytic enzyme exhibited high agglutinating titers and also precipitated the capsular polysaccharide of Type III Pneumococcus. Although there were, of course, marked individual variations between the animals, the best titers were obtained with preparations (c) (formolized cells heated at 75°C.) and (d) (formolized cells resuspended in dibasic potassium phosphate). Several other experiments were therefore instituted to confirm the antigenic efficacy of these two bacterial suspensions. Twenty-four rabbits were immunized with formolized-heated pneumococci Type III and 18 with formolized cells resuspended in m/20 dibasic potassium phosphate; the amounts of antigen used were the same as in Experiment 5. The sera of the forty-two animals showed type specific agglutinins in titers ranging from 1:40 to 1:640; the prozone phenomenon was observed with the sera of the highest titers. All but four sera were capable of precipitating the capsular polysaccharide of Type III Pneumococcus; the precipitin reaction was very intense in the best sera and was still detectable in a dilution of serum of 1/20. No appreciable difference could be detected between the two antigen preparations.

DISCUSSION

Formaldehyde, when added in sufficiently large concentration to cultures of pneumococci, acts as a fixing agent and preserves the Gram-positive character and the morphological integrity of the cells. When used in lower concentrations on the contrary, it activates markedly the autolytic disintegration of the cells of the same bacterial species. A similar phenomenon has been observed with different types of antiseptics acting on different microorganisms, namely that the same agent may activate, or on the contrary completely stop, the autolytic process according to the concentration in which it is used (7, 8). It has been suggested elsewhere (8) that large concentrations of the antiseptic (formaldehyde in this case) inactivate the autolytic enzymes, whereas smaller concentrations, by interfering with the normal physi-
ology of the cell, cause the autolytic enzymes to act on their specific cellular substrate.

When the cells fixed with sufficient concentrations of formaldehyde are washed free of the antiseptic and resuspended in a physiological medium at neutral reaction, they undergo a partial lysis. The lysis is not complete however, but leaves the cells in the form of small Gram-negative cocci; this "first phase of autolysis" had already been recognized by the use of other techniques in an earlier study (5).

What is the nature of the reaction which renders the formolized cells Gram-negative? One might assume that formaldehyde combines in a reversible manner with the substance or structure responsible for the Gram stain, and that the Gram-positive property is lost when the formaldehyde is allowed to dissociate from its complex with the cell. The following facts are in agreement with this hypothesis. Formaldehyde dissociates from its combinations more readily at pH 7.0 than at pH 8.0, and it has been shown in Experiment 3 that formolized cells may become Gram-negative at pH 7.0 but remain Gram-positive at pH 8.0. On the other hand however, formolized cells resuspended in acetic acid (pH 4.4) also remain Gram-positive although the formaldehyde complexes are least stable at acid reactions. It appears therefore that mere dissociation of formaldehyde from the Gram-positive structure cannot account for the change in staining reaction. The following theory seems to provide a satisfactory explanation of the facts described in Experiment 3.

Pneumococci are known to contain an enzyme capable of destroying the Gram-positive structure of the cells of this bacterial species (9,10,5). This enzyme is inactivated by a number of reagents, for example, iodine and formaldehyde. It has been shown that iodine inactivation is reversible (3, 4). We may suppose that formaldehyde inactivation also reverses when the bacterial cells are washed free of the antiseptic at neutral reaction; under these conditions, the enzyme recovers its activity and renders the cells Gram-negative. When, however, the formolized cells are rapidly heated at 75°C., the enzymes are destroyed and no autolysis can take place even at pH 7.0.

Both acid (pH 4.2) and alkaline (pH 7.8) reactions inhibit the action of the enzyme but through an entirely different mechanism. In the acid medium, the enzyme is freed from its combination with formalde-
hyde; but pH 4.2 is outside the range of enzymatic activity and therefore no lysis can take place. On the contrary it has been shown in Experiment 4 that an active preparation of the enzyme can attack formolized cells at pH 7.8. Since formolized pneumococci do not undergo any autolytic change when kept at this reaction, we are led to assume that the enzyme is maintained in an inactive form in the alkaline medium.

On the basis of these observations it is possible to prepare stable suspensions of formolized pneumococci by the following method. Enough formaldehyde is added to the culture to give a final concentration of 0.5 per cent; this stops immediately all autolytic process. The cells can then be separated at leisure from the medium and resuspended under such conditions that no further autolytic action can take place, for instance in a solution of m/20 dibasic phosphate or in an acetate buffer at acid reaction (pH 4.2), or in any physiological solution in which they are immediately heated at 75°C. for 20 minutes.

Cells of encapsulated pneumococci treated by any of these methods have proven to function as very effective type specific antigens even when used in small amounts; they incite rabbits to produce high titers of the type specific antibodies directed against the capsular polysaccharide of the bacterial cells. The results obtained with Type III Pneumococcus are the more striking when one considers that cells of this particular type are notoriously poor antigens. On the contrary, formolized pneumococci which have been allowed to become Gram-negative by removal of the antiseptic and incubation at neutral reaction, entirely fail to incite the production of the type specific carbohydrate antibodies in rabbits immunized by the intravenous route. This finding once more emphasizes the close correlation between the particular antigen concerned, and the structure responsible for the Gram-positive character of the cell (2).

The experimental results described in this paper deal with the effect of formaldehyde on Type III Pneumococcus. Identical results, however, have been obtained with pneumococci of Types I and II, and the methods of preparation of pneumococcus antigens outlined above have been used successfully for the production in rabbits of therapeutic antisera for the different pneumococcus types (11).
SUMMARY

When used in low concentration, formaldehyde increases the rate of autolytic disintegration of pneumococci whereas in large concentrations it completely inhibits autolysis and preserves both the morphological and staining characteristics of the cells.

Pneumococci treated with large concentrations of formaldehyde, then washed free of the antiseptic and resuspended in physiological solutions, rapidly undergo a change which renders them Gram-negative and smaller. The lysis is only partial, however, and is not accompanied by an actual disintegration of the cell. It is caused by the autolytic enzyme of the cell which remains inactive in the presence of an excess of formaldehyde but recovers its activity when the cells are resuspended in a neutral medium after removal of the antiseptic. If the autolytic enzyme is irreversibly inactivated by heating, or maintained inactive in acid or alkaline reaction, the formalized cells retain their staining characteristics and morphological integrity.

Formolized pneumococci which have become Gram-negative owing to the action of their autolytic enzyme, fail to elicit the type specific carbohydrate antibodies in rabbits. Formolized pneumococci in which the autolytic enzyme has been destroyed or maintained inactive, and which have retained their Gram-positive character, function as a very effective type specific antigen in the rabbit.

These observations emphasize once more the close relation between the Gram-positive structure of pneumococci and the capsular polysaccharide antigen of the cell. They can be used as a basis for the preparation of suspensions of formalized pneumococci which are stable and very effective as type specific antigens.

BIBLIOGRAPHY