EFFECTS OF ENZYME PREPARATIONS UPON PENICILLIN

II. AGENTS RESPONSIBLE FOR PENICILLIN INACTIVATION

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In a previous communication (Lawrence, 1945) were presented the relative effects of various enzyme systems or preparations upon the antibacterial action of penicillin. Of the several proteolytic and amylolytic ferments studied two products, taka-diastase and clarase, both derived from the fungus Aspergillus oryzae, were found to neutralize or inactivate completely the antibiotic substance. Bacterial amylase, a preparation obtained from Bacillus mesentericus, also proved to be antagonistic to the antibacterial properties of penicillin.

Other diastatic ferments, including several also derived from Aspergillus oryzae, failed completely to show any evidence of an antipenicillin effect. On the basis of these findings, it seemed worthwhile to extend the studies on the active preparations in an attempt to determine the agent present in these products which is responsible for the action.

Although the manufacturing procedures in preparing the diastatic enzymes, clarase and taka-diastase, are not known to the writer, it appeared not entirely in order for fungal amylase and mylase P, apparently derived from the same fungus, Aspergillus oryzae, to be devoid of a similar neutralizing action upon penicillin. As indicated in the previous paper, it was of interest to note that unlike the ineffectiveness of a malt diastase preparation and particularly of fungal amylase upon penicillin activity, the bacterial amylase used in the study compared in activity with taka-diastase. Upon inquiry concerning the source and possible nature of these two enzyme systems, the following information was obtained from Mr. Philip P. Gray of the Wallerstein Laboratories:

"The fungal amylase preparation is a highly active enzyme preparation obtained from a special strain of Aspergillus oryzae by extraction and purification and salt precipitation. This preparation is standardized using sodium sulfate as a diluent to a Lintner value of 1,000 and ordinarily may contain lactose as a carrier. Besides characteristic amylolytic enzymes, the preparation exhibits considerable activity of proteolytic enzymes, cytases, phosphatases, and maltase. Its amylolytic activity is exhibited best at pH range of 4.5 to 5.5 and at temperatures preferably not over 50 C." The mylase P product was described as "representing a specially fractionated mixture of enzymes from the above source (fungal amylase) which will be found to be especially rich in phosphatases, cytases, and hemicellulase, containing also some maltase."

The bacterial amylase preparation, obtained from the same laboratories, was described as "representing an enzyme derived from Bacillus mesentericus, a special preparation purified by precipitation and standardized to a definite
starch liquefying strength using sodium chloride, sodium sulfate, and phosphates as diluents. This preparation, while containing proteolytic and other enzymes, contains chiefly alpha amylase, exhibits powerful starch liquefying properties, highly active at high temperatures, and at pH values between 6.5 and 8.0."

The purpose of quoting the detailed description of the fungal and bacterial amylase preparations is to correlate the possible common enzyme systems contained in each and to suggest the presence of some enzyme fraction or substance in the bacterial product which may explain the effect of the latter upon penicillin. The active substance is evidently entirely lacking in the fungal amylase or is insufficient in quantity to be demonstrable in the test methods described in the previous communication.

The fact that certain bacterial extracts or enzymes derived from bacterial cells will inactivate penicillin has been known for some time. Fleming (1929) in his early studies on penicillin-containing filtrates observed that the colontyphoid-dysenteric group, as well as Pseudomonas pyocyaneus, Proteus vulgaris, and Vibrio cholera, was resistant or insensitive to penicillin. Abraham and Chain (1940) noted in their studies that an enzyme from Escherichia coli extracts destroyed penicillin activity. This enzyme was inactivated by heating at 90°C for 5 minutes and by incubation with papain (activated with potassium cyanide at pH 6.0) and was dialyzable through cellophane membranes. Hobby, Meyer, and Chaffee (1942) also found that supernatant filtrates from E. coli cultures inhibited the action of penicillin and noted, furthermore, that the latter agent was inactive against this organism. The author's studies confirm the findings mentioned and add to the list of penicillin-inactivating bacteria the following: Bacillus subtilis, B. mycoides, B. megatherium, B. mesentericus, B. panis, and B. adhaerens; Serratia marcescens; and Clostridium chauvei. The anaerobic organism mentioned was the only one of a series of pathogenic anaerobes studied which would completely inactivate penicillin. Among the several Clostridium cultures used in the investigation which failed to give evidence of a neutralizing effect on penicillin activity were C. tetani, C. novyi, C. welchii, C. botulinum, C. septique, C. sporogenes, and C. histolyticum. Bacillus brevis, the aerobic sporulating soil bacillus described by Dubos, which produces tyrothricin, will not inactivate the antibacterial effects of penicillin.

The method used in determining the effects of these and other organisms upon penicillin activity involved the addition of 1 ml of a 24-hour broth culture of organisms, or its sterile filtrate (Berkefeld), to 1 ml of a sodium penicillin solution containing approximately 40 to 48 Oxford units. Following incubation in a water bath at 40°C for two hours, the penicillin-bacterial test solutions were tested for penicillin activity by the Oxford cup-plate method (Abraham et al., 1941).

The information obtained on the neutralizing effects of various bacterial filtrates upon penicillin, particularly organisms of the aerobic, sporeforming B. subtilis and associated types, suggested the possibility that similar bacteria or their products might be encountered in the active enzyme preparations, namely, clarase, taka-diastase, and bacterial amylase, and would account for
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Bacterial counts were made, therefore, on the dry enzyme powders and the organisms present were identified by Mr. G. R. Goetchius, following the description and biochemical reactions given in Bergey's "Manual for Determinative Bacteriology" (5th edition, 1939). This was carried out as follows: Solutions or suspensions of the enzyme preparations were plated in beef extract agar and the colonies developing in the medium were counted at the end of 48 hours' incubation at 37°C. Following these counts, representative colony types were selected and a small inoculum from each was transferred to beef extract broth. At the end of 72 hours' incubation, 4-mm

<table>
<thead>
<tr>
<th>ENZYME PREPARATIONS</th>
<th>BEEF EXTRACT AGAR PLATE COUNTS ORGANISMS/Г</th>
<th>NUMBER COLONY TYPES</th>
<th>NUMBER IN-ACTIVATING PENICILLIN IN 2 HR</th>
<th>IDENTITY OF ORGANISMS RESPONSIBLE FOR PENICILLIN INACTIVATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Pepsin.............</td>
<td>240,000</td>
<td>2</td>
<td>2</td>
<td>Bacillus teres</td>
</tr>
<tr>
<td>2. Trypsin............</td>
<td>14,000</td>
<td>1</td>
<td>1</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>3. Epspin................</td>
<td>4,000</td>
<td>1</td>
<td>1</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>4. Pancreatin...........</td>
<td>23,000</td>
<td>1</td>
<td>1</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>5. Papain.............</td>
<td>21,000</td>
<td>1</td>
<td>none</td>
<td>-</td>
</tr>
<tr>
<td>6. Ficin..............</td>
<td>none</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7. Hurain.............</td>
<td>none</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8. Polidase...........</td>
<td>8,400,000</td>
<td>2</td>
<td>1</td>
<td>B. mesentericus</td>
</tr>
<tr>
<td>9. Invertase...............</td>
<td>5,000</td>
<td>1</td>
<td>1</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>10. Malt diastase........</td>
<td>170,000</td>
<td>3</td>
<td>1</td>
<td>Bacillus cohaerens</td>
</tr>
<tr>
<td>11. Mylase &quot;P&quot;............</td>
<td>99,000</td>
<td>1</td>
<td>1</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>12. Fungal amylase........</td>
<td>236,000</td>
<td>3</td>
<td>3</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>13. Bacterial amylase........</td>
<td>48,000,000</td>
<td>3</td>
<td>3</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>14. Clarase &quot;stand.&quot;.....</td>
<td>180,000,000</td>
<td>2</td>
<td>2</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>15. Clarase &quot;conc.&quot;.......</td>
<td>398,000,000</td>
<td>4</td>
<td>4</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>16. Taka-diastase........</td>
<td>12,000,000</td>
<td>2</td>
<td>2</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>17. Emulsin.............</td>
<td>1,000</td>
<td>1</td>
<td>1</td>
<td>B. subtilis group</td>
</tr>
<tr>
<td>18. Catalase.............</td>
<td>none</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19. Lysozyme...........</td>
<td>10,000,000*</td>
<td>2</td>
<td>1</td>
<td>B. subtilis group</td>
</tr>
</tbody>
</table>

- = no test made.
* = Aerobacter aerogenes predominant organism present.

A small sample of the broth culture was also tested at this point for antipenicillin effects by the Oxford cup-plate procedure. The estimated bacterial count per gram of enzyme powder and the colony types along with their identification are given in table 1.

It will be noted from the data that, in general, the total bacterial count varied considerably from one enzyme preparation to another. Furthermore, with but few exceptions, all the samples studied contained organisms which in pure culture were able to inactivate penicillin in less than two hours. Concentrated

loopfuls of the bacterial suspension were implanted in Durham tubes containing carbohydrate media as well as in tubes of Bacto-purple milk and nitrate broth. A small sample of the broth culture was also tested at this point for antipenicillin effects by the Oxford cup-plate procedure. The estimated bacterial count per gram of enzyme powder and the colony types along with their identification are given in table 1.

It will be noted from the data that, in general, the total bacterial count varied considerably from one enzyme preparation to another. Furthermore, with but few exceptions, all the samples studied contained organisms which in pure culture were able to inactivate penicillin in less than two hours. Concentrated
clarase gave the highest bacterial count of the products tested, 398,000,000 organisms per gram of dry preparation. Following this, in descending numbers of bacteria per gram of powder, were standardized clarase, bacterial amylase, and taka-diastase. The numbers of viable organisms estimated in these products were 180,000,000, 48,000,000, and 13,000,000 per gram, respectively. While certain morphological differences were observed in the colony types developing on agar plates, subsequent studies indicated all the strains present in the preparations mentioned could be classified as aerobic, sporeforming B. subtilis or related groups.

The predominant organism in the preparation of lysozyme, which contributed to the high count obtained in this preparation, was the gram-negative bacillus, Aerobacter aerogenes. This organism failed to show any evidence of an antipenicillin effect when incubated in the presence of the antibiotic agent and tested at the 2-hour period. A B. subtilis variant, however, which was capable of inactivating penicillin under the conditions mentioned, was isolated from this enzyme preparation. Poliadae was found to contain approximately 8,500,000 bacteria per gram of powder; however, two distinct colony types were isolated from the product, and only one was found to have an antipenicillin effect. The three colony types isolated from plates inoculated with fungal amylase were all identified as belonging to the B. subtilis group. The total bacterial count on this preparation, however, was relatively low, i.e., 236,000 bacteria per gram of dry powder.

On the basis of these studies it is evident that the mere presence of penicillin-inactivating organisms in a product does not necessarily indicate that the enzyme preparation will show evidence of an antipenicillin effect. Presence or absence of this action appears to be based upon the numbers of organisms (or possibly their metabolic substances) of the B. subtilis group in the products. This correlation in bacterial population with the resulting extent of penicillin inactivation has since been found to hold true for various samples of standardized clarase submitted for this test by the manufacturer. Certain preparations, which were found to be relatively free of bacteria of all types, evidenced little, if any, antipenicillin action.

Standardized clarase preparations were tested, moreover, which gave bacterial counts approximately that of the concentrated clarase sample (table 1, lot 1455) with a corresponding high titer antipenicillin action. The number of bacteria estimated to be present in several standardized clarase samples, and the extent to which freshly prepared sterile filtrates (Berkfeld) of the latter could be diluted and still show evidence of an antipenicillin effect, are given in table 2. These findings were compared with the bacterial counts and antipenicillin titers of the standardized clarase (lot 962) and concentrated clarase (lot 1455) preparations used throughout this and the previous study (Lawrence, 1945).

From the data presented in table 2, it is obvious that there is a correlation between the number of bacteria present in a clarase preparation and the extent to which the sample may be diluted and still show evidence of a neutralizing action against penicillin. Furthermore, the fact that this antipenicillin effect
is demonstrable in sterile Berkefeld filtrates of freshly prepared solutions or suspensions of the various clarase samples would indicate that the presence of viable organisms is not essential for the neutralizing action on the antibiotic agent. On the basis of these findings, therefore, it may be assumed that certain water-soluble, filterable bacterial end products in the sterile filtrates are directly responsible for the inactivation of penicillin noted throughout the studies reported previously (Lawrence, 1943, 1944, 1945).

Reference was previously to the fact that Abraham and Chain (1940) described an enzyme present in E. coli extracts which would destroy the antibacterial effects of penicillin. This enzyme was heat-labile to the extent that it was inactivated by heating at 90 C for 5 minutes. Therefore, the several enzyme systems showing antipenicillin effects in the present study were subjected to the temperature mentioned as well as to 70 C for 20 minutes and also were

heated in a boiling water bath. The preparations studied included sterile Berkefeld filtrates of standardized and concentrated clarase, taka-diastase, and bacterial amylase, and several filtrates of pure cultures of organisms known to show antipenicillin action. A test of the samples against penicillin by the Oxford cup-plate and the broth dilution methods revealed that the active agent present in all the preparations given was destroyed by the heat treatments mentioned.

In earlier articles on this subject (Lawrence, 1944, 1945) mention was made of the fact that in developing the sterility test for penicillin, solutions of the agent were sterilized by heating in a boiling water bath for 3 minutes and cooling rapidly to room temperature. This treatment had no apparent effect upon the potency of the antibiotic solutions.

In the studies presented in this communication, up to this point, the shortest time interval of testing the antibacterial effects of penicillin-enzyme solutions

<table>
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<th>TABLE 2</th>
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**Correlation of bacterial count with antipenicillin effect of clarase preparations**

<table>
<thead>
<tr>
<th>CLARASE PREPARATION</th>
<th>LOT NO.</th>
<th>ESTIMATED BACTERIA PER GRAM OF POWDER</th>
<th>COMPLETE INACTIVATION OF PENICILLIN AT TEST PERIODS (CUP-PLATE)</th>
<th>DILUTION IN FLUID MEDIUM SHOWING ANTIMICROBIAL EFFECT*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentrated clarase</td>
<td>1455</td>
<td>398,000,000</td>
<td>2 hr</td>
<td>1:500,000</td>
</tr>
<tr>
<td>Standardized clarase</td>
<td>962</td>
<td>138,000,000</td>
<td>2 hr</td>
<td>1:350,000</td>
</tr>
<tr>
<td>Standardized clarase</td>
<td>1339</td>
<td>30,000,000</td>
<td>2 hr</td>
<td>1:100,000</td>
</tr>
<tr>
<td>Standardized clarase</td>
<td>1351</td>
<td>135,000,000</td>
<td>2 hr</td>
<td>1:500,000</td>
</tr>
<tr>
<td>Standardized clarase</td>
<td>1426</td>
<td>120,000,000</td>
<td>2 hr</td>
<td>1:500,000</td>
</tr>
<tr>
<td>Standardized clarase</td>
<td>1466</td>
<td>740,000</td>
<td>18 hr</td>
<td>1:2,000</td>
</tr>
<tr>
<td>Standardized clarase</td>
<td>1500</td>
<td>172,000</td>
<td>none</td>
<td>&lt;1:1,000</td>
</tr>
<tr>
<td>Standardized clarase</td>
<td>1526</td>
<td>7,000,000</td>
<td>2 hr</td>
<td>1:20,000</td>
</tr>
</tbody>
</table>

* = Enzyme and penicillin (app. 42 to 48 Oxford units) contained in 10 ml Brewer's medium. One 4-mm loopful broth culture Staphylococcus aureus 209 added to each tube. Results tabulated at end of 7 days' incubation at 37 C. <1:1,000 = concentrations greater than 0.1 per cent not tested.
followed the initial two-hour incubation period at 40 C. With the information available that the antipenicillin agents were more sensitive than penicillin to heat, it appeared worth while to determine the effects of short time intervals of exposure of the antibiotic agent to the active enzyme preparations. This was carried out as follows: To a sample of penicillin solution (containing 42 to 48 Oxford units) was added a sterile filtrate of an active antipenicillin agent. The mixture was incubated in a 40 C water bath and small samples were removed at the end of 5, 10, 15, and 30 minutes and 1, 1½, and 2 hours. Immediately upon removal from the test mixture each sample was placed in a boiling water bath for 3 minutes, following which it was cooled rapidly to room temperature in an ice bath. These solutions, along with suitable controls, were tested for penicillin activity by the Oxford cup-plate procedure. The first sample removed (at the end of 5 minutes) proved to be devoid of any antibacterial effect. The mere contact of an active enzyme solution with penicillin was subsequently shown to be sufficient to suppress or neutralize completely the antibacterial agent. This was proved by adding the enzyme filtrate to a hot (100 C) solution of penicillin in a boiling water bath. Prolonged heating (boiling for 5 to 10 minutes) of an enzyme-penicillin solution, furthermore, did not result in the reappearance of an antibacterial action on the part of the antibiotic substance.

Although Abraham and Chain (1940) found that the antipenicillin enzyme present in E. coli filtrates dialyzes through cellophane membranes, the neutralizing substance present in highly active clarase preparations lacks this property completely. The dialytic studies were carried out in distilled water and in buffer solutions at pH 4.0, 6.0, and 8.0. Additional studies are in progress to determine the possible relationship of the antipenicillin agents present in various bacterial filtrates.

**SUMMARY**

Data are presented which indicate that the enzyme preparations that are active against penicillin contain certain water-soluble, Berkefeld-filterable substances of bacterial origin which are responsible for penicillin inactivation.

Broth filtrates of pure cultures of the organisms isolated from the active antipenicillin preparations neutralize the effects of the antibiotic substance. Similar penicillin-inactivating bacteria may be isolated from practically all the enzyme preparations studied.

The presence or absence of an antipenicillin effect in a preparation, including clarase, appears to be based upon the number of organisms (or their metabolic products) which the preparation contains.

The agent or agents present in the active enzyme products which neutralize penicillin activity are heat-labile. The action of active enzyme preparations upon penicillin appears to be almost instantaneous.

**REFERENCES**

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