Activity and Properties of \textit{para}-Aminobenzyl Penicillin

GLENN A. BREWER, JR., AND MARVIN J. JOHNSON

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin

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Eagle (1947a) has shown that \textit{p}-hydroxybenzyl penicillin (X) was more effective than benzyl penicillin (G) in curing mice infected with \textit{Streptococcus pyogenes} and \textit{Streptococcus pneumoniae}. Hewitt and Pittman (1946) have obtained similar results in animals infected with \textit{Hemophilus influenzae}. It was further shown that penicillin X remains in the blood of human subjects longer than penicillins G or K (Eagle, 1947b). It may be seen from these experiments that penicillins X and G differ in their therapeutic usefulness.

The introduction of the phenolic hydroxyl group in benzyl penicillin to give rise to penicillin X is accompanied by increased water solubility (Clarke et al., 1949a). This property is also reflected in the paper chromatographic behavior; that is, penicillin X has a relatively low Rf value compared to those for penicillins G, F, dihydro F, and K (Karnovsky and Johnson, 1949).

In this study a penicillin more hydrophilic than penicillin X was sought for a comparison with penicillin G. For this purpose a group of compounds were tested for their ability to act as precursors for such a penicillin. In order to facilitate the selection of the proper precursor a paper chromatographic method was employed. Of the several precursors tested, only five produced penicillins with a lower Rf value than that of penicillin X. Among these, \textit{p}-aminobenzyl penicillin was chosen for the work described in this paper. Although the formation of this penicillin has been reported previously (Corse et al., 1948; Clarke et al., 1949b), its properties or partial purification have not been described.

\textbf{Materials and Methods}

\textit{Production of the penicillins.} Spores of \textit{Penicillium chrysogenum} strain, W49-133, carried on a soil stock were transferred to the agar sporulation medium of Gailey \textit{et al.}, (1946). A spore suspension prepared from these plates was used as inoculum for the synthetic inoculum medium of Jarvis and Johnson (1947). After 40 hours of incubation at 25 C on a Gump rotary shaker (270 cycles per minute), the vegetative inoculum was transferred into the synthetic fermentation medium of Thorn and Johnson (1950) and was incubated under the same conditions. After an initial growth period of 24 hours the appropriate precursors were added twice daily as aqueous solutions of the sodium salts at a level of 0.01 per cent. The flasks were harvested at about 100 hours.

\textit{Preparation of precursors.} \textit{p}-Aminophenylacetic acid was prepared from \textit{p}-nitrobenzylcyanide according to the method described in \textit{Organic Synthesis} (Gilman and Blatt, 1948). The final product had a melting point of 197 C (reported 199 to 200 C). \textit{p}-Hydroxyphenylacetic acid was prepared by the decomposition of the diazotium salt of \textit{p}-aminophenylacetic acid (melting point observed 148 C, reported 148 C).

\textit{Paper chromatographic method.} The chromatographic method of Karnovsky and Johnson (1949) has been modified to give a better resolution of the polar penicillins. The original method suffered from the fact that the water phase frequently separated from the ether phase, thus causing waterlogging of the strips. In the present method the chromatogram is developed at 25 C with ether equilibrated against 28 per cent (wt/wt) ammonium sulfate solution.

The ether was purified according to the original method and equilibrated at 25 C against the ammonium sulfate solution prepared by dissolving 40 g of the salt in 100 g of water. Filter paper strips (0.5 inch wide, Eaton-Dikeman no. 613) were impregnated with 10 per cent (wt/v) potassium citrate buffers of pH 5.0, 5.5, 6.0, or 6.5. The pH of the buffer determined the rate of movement of the various penicillins on the developing strip. For example, penicillin G varied in Rf value from 0.20 at pH 6.5 to Rf 0.58 at pH 5.5. A pH of 6.5 was used to resolve the non-polar penicillins whereas a pH of 5.5 gave satisfactory Rf values for the polar penicillins encountered in this work. After development the strips were assayed by the method of squares described by Karnovsky and Johnson (1949).

\textit{Microbiological assay of penicillin.} Penicillin in broth samples was assayed by the Oxford cup plate method with \textit{Micrococcus pyogenes} var. \textit{aureus}, strain H, as the assay agent. The paper chromatograms were assayed by the method of squares either by plate or turbidimetric assay. \textit{M. pyogenes} var. \textit{aureus}, \textit{Bacillus subtilis}, Marburg type, and \textit{Sarcina lutea} were used for the plate assays, while \textit{M. pyogenes} var. \textit{aureus} and \textit{Streptococcus lactis}, strain Rogers, were used as assay agents in the turbidimetric assay. Except for the strain of \textit{Sarcina lutea}, which was obtained from E. R. Squibb and Sons, (New Brunswick, N. J.) the cultures used were supplied by Professor E. McCoy of the Department of Bacteriology, University of Wisconsin. The rough stage of \textit{Bacillus subtilis} was obtained by four successive 12-hour transfers in nutrient broth, followed by plating.
and selection of a colony of rough morphology. The culture was then held in the rough stage by 24-hour transfers.

The agar medium used for the plate assay was the same as that for the Oxford cup plate method. The turbidimetric assay medium differed for each organism. That for *M. pyogenes* var. *aureus* consisted of 1 per cent glucose, 0.3 per cent peptone, 0.3 per cent yeast extract and 0.5 per cent phosphate buffer adjusted to pH 7.0. The medium for *S. lactis* consisted of 0.5 per cent glucose, 0.5 per cent peptone, 0.5 per cent yeast extract and 0.5 per cent phosphate buffer adjusted to pH 7.0.

**Table 1. Differential assay values for several penicillins**

<table>
<thead>
<tr>
<th>Type of Penicillin</th>
<th>Chromatographic Rf at pH 5.5</th>
<th>Differential Assay Values*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacillus subtilis (smooth)</td>
<td>Bacillus subtilis (rough)</td>
</tr>
<tr>
<td>p-aminobenzyl</td>
<td>0.16</td>
<td>1.80</td>
</tr>
<tr>
<td>X</td>
<td>0.46</td>
<td>1.50</td>
</tr>
<tr>
<td>G</td>
<td>0.58</td>
<td>1.00</td>
</tr>
<tr>
<td>F</td>
<td>0.68</td>
<td>1.00</td>
</tr>
<tr>
<td>K</td>
<td>0.85</td>
<td>0.50</td>
</tr>
</tbody>
</table>

* Differential assay value = units of penicillin found with test organism units of penicillin found with *Micrococcus pyogenes* var. *aureus.*

Fig. 1. Comparison of blood levels of benzyl, *p*-aminobenzyl and benzyl penicillin containing the impurities present in the *p*-aminobenzyl penicillin preparation in a dog. Blood levels are expressed in units per ml blood per unit injected per gram of body weight.

were incubated for three hours, steamed for ten minutes, and read on a Lumetron colorimeter.

**Animal experiments.** A 10.4 kg dog was catheterized and injected intravenously with 22,500 units of partially purified *p*-aminobenzyl penicillin. Blood samples were collected periodically, diluted with 0.2 ml of 4 per cent ammonium oxalate and assayed by the *S. lutea* filter paper disc method. A preliminary experiment indicated that whole blood had no effect on the results of this assay and that as little as 0.1 unit per ml could be determined. Urine samples were taken at the same time intervals via the catheter tube. After collecting the urine the bladder was rinsed with sterile saline, the combined urine and washings were diluted with buffer and assayed by the Oxford cup plate method. Penicillin G (50,000 units) was injected into the same dog in a separate experiment. In another series of experiments penicillin G and penicillin G containing acid inactivated *p*-aminobenzyl penicillin were injected in the same manner.

**Experimental Results**

**Purification of *p*-aminobenzyl penicillin.** About 3 million units of *p*-aminobenzyl penicillin were produced by the shake flask fermentation method. The activity was adsorbed from the broth by charcoal and subsequently eluted with aqueous acetone buffered at pH 6.5. The eluted material was concentrated and the aqueous solution lyophilized. *p*-Aminobenzyl penicillin was highly water soluble and was not appreciably extracted by chloroform at pH 4.0, while practically all the penicillins F, dihydro F and K present in the crude lyophilizate could be extracted under these conditions. *p*-Aminobenzyl penicillin could, however, be extracted...
into butanol from a nearly saturated ammonium sulfate solution (5 g of ammonium sulfate per 10 ml of solution) adjusted to pH 6.5. Various further purification procedures were tried, but no appreciable purification could be obtained. This material was found to be satisfactory for animal injections and microbiological studies.

**Stability of p-aminobenzyl penicillin.** In the course of the purification studies the marked acid stability of p-aminobenzyl penicillin was noted. Further studies showed that p-aminobenzyl penicillin had a half-life of 26 minutes at pH 2.0 and 24 C as compared to that of 18 minutes for penicillin G. This latter value agrees well with that found by Benedict et al., (1946).

**Bacterial spectrum.** It was decided that the bacterial spectrum of p-aminobenzyl penicillin could be obtained by resolving a mixture of penicillins on a paper chromatogram and using the method of squares to assay the activity. The pencillins used in this study were G, F, K, X and p-aminobenzyl penicillin. A fermentation without precursor addition produced substantial amounts of penicillins F and K, p-hydroxyphenylacetic acid was used as a precursor of X, and p-aminophenylacetic acid served as a precursor of p-aminobenzyl penicillin. The various solutions of the penicillins were blended until a mixture was obtained which contained assayable amounts of all the constituents. The mixture (2 to 6 units) was applied to the buffered strips at pH 6.5 and 5.5 and the chromatogram developed as described previously. The developed strips were dried, cut in half lengthwise and each half assayed with the appropriate organism. The results presented in table 1 are ratios of the activity of each penicillin to the test organism as compared to the activity found towards *M. pyogenes* var. *aureus.*

**Animal injection.** The results of the injection of p-aminobenzyl penicillin into a dog are found in figures 1 and 2.

**Discussion**

As has already been mentioned, the relative activity of the various penicillins has been determined for a variety of organisms. In several cases penicillin X has been found to have greater activity than penicillin G, F, or K. Since penicillin X is more hydrophilic than the latter, it is of considerable interest to study the properties of a penicillin more polar than penicillin X. The results (table 1) indicate that p-aminobenzyl penicillin behaves in a similar manner to penicillin X.

The main purpose in studying p-aminobenzyl penicillin was to determine whether it would remain in the blood longer than penicillin G. It has been demonstrated several times that penicillin X gives higher blood levels than penicillin G. Figure 1A indicates that rate of decrease in concentration of p-aminobenzyl penicillin in the blood is much slower than that of penicillin G. A comparison of the blood levels in figure 1B indicates that the impurities present in the p-aminobenzyl penicillin preparation had only a slight effect on maintaining higher blood levels of penicillin G.

It may be seen from figure 2 that the rate of renal excretion of p-aminobenzyl penicillin is only about one-third as fast as that for penicillin G. It is also apparent from this figure that the renal excretion was affected very little by the impurities present in the p-aminobenzyl penicillin preparation. It appears from these experiments that p-aminobenzyl penicillin can maintain a higher concentration in the blood for a longer period than penicillin G. This behavior on injection has been previously demonstrated with penicillin X (Eagle, 1947b). It may be seen that both polar penicillins have the desirable property of remaining in the blood stream longer than does penicillin G.

**Acknowledgment**

The authors wish to thank Dr. Stanley Gershoff for his help in carrying out the animal experiments.

**Summary**

p-Aminobenzyl penicillin has been produced by using p-aminophenylacetic acid as a precursor in penicillin fermentations. This penicillin has been partially purified and its properties have been studied. The activity of this penicillin toward several microorganisms has been compared to the activities shown by penicillins X, G, F, and K. In animal experiments p-aminobenzyl penicillin has been shown to maintain higher blood levels than penicillin G.
A Modified Mueller Medium Without Native Protein for Tetanus Toxin Production

JOSEPH L. STONE

Massachusetts Department of Public Health,1 Boston, Mass.

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It is generally desirable when possible to eliminate proteins from media for cultures whose products may eventually be used for human injection. Some of the reasons for this are: (1) to minimize sensitization reactions to ingredients of the medium or to avoid inducing sensitivity to them; (2) to avoid the complication of separating the proteins of the medium from the desired protein during purification of a protein biological product; and (3) to attempt to reduce the cost of the medium since proteins are frequently an expensive source of nutrient.

This report is concerned with the production of tetanus toxin in the Mueller medium modified to eliminate veal infusion by adaptation of the culture to the modified medium.

Materials and Methods

The media used for toxin production were the most recent Mueller formulas (Mueller, 1951) with and without veal infusion. The cultures used were the Harvard strain of Clostridium tetani (Z-3A) and strain variants of this original culture. The seed cultures were grown in fluid thioglycollate broth (BBL) prior to inoculation of the toxin medium.

Method of Developing Strain Variants

The parent strain (Z-3A) was grown at 35°C for 1 or 2 days in the toxin medium containing a diminished amount of veal infusion. A transfer from this culture was then made to the fluid thioglycollate broth. This second generation was inoculated into the toxin medium with a lesser amount of veal infusion and the culture was again transferred to fluid thioglycollate broth. Successive transfers of this sort were accomplished until a toxin-producing culture could be isolated from the toxin medium which contained no veal infusion. The actual schedule of transfers leading to the modified strain can be seen in table 1.

Toxins were titrated according to procedures outlined in "Manual of Methods for Pure Culture Study of Bacteria" (1947). The Li is defined as the amount of toxin equivalent to 1 unit of antitoxin as established by flocculation.

Experimental Results

Preliminary trials with the original strain of Cl tetani (Z-3A) yielded very poor toxin titers in the absence of

REFERENCES


1 From the Biologie Laboratories, Institute of Laboratories, Massachusetts Department of Public Health, Forest Hills, Boston 30, Massachusetts.