Some Factors Influencing the Production of Certain Biosynthetic Penicillins

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The work reported in this paper grew out of a study of factors influencing the production of penicillin X and was extended to include observations on the production of other types of penicillin namely, penicillins G, F, K and para-aminobenzyl penicillin.

Material and Methods

Basic media. (a) Natural. A medium containing 4 per cent corn steep solids, 4 per cent lactose, 1.5 per cent Cerelose1 (92 per cent glucose) and 0.0044 per cent ZnSO4·7H2O was prepared in 95 per cent of the final volume. After adjustment to pH 5.6 to 5.8, it was sterilized by autoclaving. A suspension (20 per cent) of calcium carbonate was autoclaved separately. The calcium carbonate suspension was added to the broth to a final concentration of 1 per cent. The addition was made at the time of inoculation.

As antifoam agent, soya bean oil or lard oil was added as indicated below.

(b) Chemically defined. A medium of known composition was prepared using the concentrations of ammonium acetate, ammonium lactate, and inorganic salts reported by Jarvis and Johnson (1947). To obtain maximum yields with the strains of Penicillium used, it was found necessary to increase the concentration of lactose to 4 per cent and of Cerelose to 1 per cent. Moreover, to maintain suitable pH levels during fermentation, 0.8 per cent calcium carbonate was added. The antifoam agent used will be indicated in the experiments described below.

Strains of Penicillium chrysogenum. A number of mutants of P. chrysogenum Q176 were developed in our Laboratories by Farrell (1953). Several of these were shown to possess marked penicillin X-producing properties. In the experiments to be reported, one of these, UV15, was used. It was added as a spore suspension prepared in Foster sporulation broth (Foster et al., 1945). The ratio of spore suspension was kept constant at 2.5 ml per 100 ml of fermentation medium.

Incubation and containers. The medium was distributed in 1-L Erlenmeyer flasks. These were incubated on a rotary shaker at 270 complete cycles per min. The temperature of the incubator was approximately 26 C.

Estimation of the various penicillins. Total penicillin was determined by the cup-plate method using Staphylococcus aureus (Micrococcus pyogenes var. aureus) strain 209P as the test organism.

A simple chromatographic procedure was used to estimate the relative concentrations of the various penicillins produced during fermentation. Filter paper strips, ¼ in. by 23 in., were cut from Schleicher and Schuell2 no. 589 filter paper. It was found that the zones of inhibition were of a more regular shape when the strips were cut across the water mark than when cut along the water mark. Immediately before use, the upper 13 in. of the strip were dipped in a procaine citrate buffer pH 5.3 (5 g citric acid plus 12.5 g procaine base per 100 ml buffer). Excess buffer was removed by laying the strip on a clean towel.

The developing solvent was water-saturated amyl acetate. After application of the sample, the solvent was allowed to pass down the strips from a reservoir for a period of 8 hr. The wet strip was then cut just above the spot of application and laid on a tray of agar seeded with S. aureus 209P. After incubation for 18 hr, clear,

1 Schleicher and Schuell Co., Keene, New Hampshire

Figure 1. The effect of variation of the volume of medium per flask on the amounts of penicillins X and G produced in shake flasks. The medium used was corn steep liquor without added precursor. The points shown are the average values from ten experiments.

1 Canada Starch Co., Cardinal, Ontario, Canada.
round to oblong zones of inhibition were obtained. Under these conditions, complete separation of penicillins X and G was obtained when a sample containing approximately 1 unit of total penicillin was applied to the strip.

Maximum width of the zones of inhibition was measured with dividers. An estimation of the proportion of the various penicillins was made according to Kluener's method (1949).

Sampling. Duplicate flasks were prepared for each experimental trial, along with suitable controls. Each flask was sampled on the 4th, 5th, 6th, and 7th days of fermentation and, occasionally, on the 8th day. Each sample from the paired flasks was tested for total penicillin. For the chromatographic separation, the duplicate samples were pooled and tested on at least two strips.

Precursors. In the experiments to be reported, two specific precursors have been used, \( p \)-hydroxyphenylacetic acid and \( p \)-amino phenylacetic acid. The latter was prepared as described by Gilman (1932) and the \( p \)-hydroxy compound derived from this.

RESULTS

The Effect of Volume of Medium per Flask on the Proportion of Penicillins

In corn steep medium. Paired flasks were set up containing 100, 200 and 300 ml corn steep medium in 1-L Erlenmeyers. The antifoam agent, soya bean oil, was considered as a simple ingredient of the medium and

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**Fig. 2.** The effect of variation of the volume of medium per flask on the amounts of penicillins X and G in corn steep medium containing 0.1% \( p \)-hydroxyphenylacetic acid. The points shown are the average values from seven experiments.
was varied in the same proportion as the medium per flask, at a level of 0.25 ml per 100 ml. The ratio of the inoculum to the volume of medium also was constant. A second group of flasks containing the same three volumes of medium to which 0.1 per cent p-hydroxyphenylacetic acid was added was also fermented. The effects of the six variations of volume and medium were examined on a number of occasions. The averages of all determinations on total penicillin and penicillins X and G are shown graphically in figures 1 and 2. The individual determinations of all penicillins on the six day samples from the flasks containing 100 and 300 ml medium (no added precursor) in 10 consecutive experiments are shown in table 1.

In chemically defined medium containing p-hydroxyphenylacetic acid. In the absence of added precursors, the modified Jarvis and Johnson medium gives very low yields of penicillin. A single experiment in which synthetic medium containing 0.1 per cent p-hydroxyphenylacetic acid, distributed in 100, 200, and 300 ml volumes was set up. The antifoam agent was lard oil. Results are shown in figure 3.

The results shown in figures 1, 2, and 3 indicate that the amounts of penicillins X and G are markedly influenced by the volume of medium contained in shaken flasks. In corn steep medium, both with and without added p-hydroxyphenylacetic acid and in a chemically-defined medium containing p-hydroxyphenylacetic acid, an increase in the volume of medium resulted in an increase in penicillin X and a decrease in penicillin G. A comparison of the individual determinations as shown in table 1 indicates that an increase in volume of medium was accompanied by an increase in penicillin X in all experiments. In 9 of the 10 trials, an increase in volume resulted in a decrease in penicillin G; in the one exception, total penicillin was abnormally low. The effect of volume on the F penicillins is not striking. Penicillin K appears to follow the same pattern as penicillin X, an increase in volume of medium resulting in an increase in K.

**The Effect of Antifoam Agents**

In the above experiments, the volumes of antifoam agent were varied in the same proportion as the volumes of medium per flask were varied. If one assumes that the shaking to which the flasks were subjected produced a coarser emulsion in the larger volumes of liquid, then the amount of oil per surface would be larger, the larger the volume. Such conditions could affect the degree of aeration. The effect of the volume of liquid on the relative production of penicillins G and X could depend on aeration and/or the antifoam agent. The possible effect of the antifoam agent was explored.

To this end, a highly unsaturated and a relatively saturated oil, namely soya bean and lard oil, were chosen. The iodine numbers for these two oils are 130 to 135 and 75, respectively.

**The Effect of Soya Bean Oil and Lard Oil on the Proportion of Penicillins Formed in Corn Steep Medium without Added Precursor**

To paired flasks containing 200 ml corn steep medium, 0.5 and 2.0 ml soya bean oil were added; and to

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**TABLE 1**

<table>
<thead>
<tr>
<th>Penicillins</th>
<th>X</th>
<th>G</th>
<th>F</th>
<th>Dihydro</th>
<th>K</th>
<th>Total</th>
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<td>300</td>
<td>100</td>
<td>300</td>
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<td>(ml)</td>
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<tr>
<td>Ten consecutive experiments</td>
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<td>105</td>
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<td>268</td>
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<td>137</td>
<td>261</td>
<td>167</td>
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<td>108</td>
<td>144</td>
<td>108</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td>82</td>
<td>205</td>
<td>164</td>
<td>121</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Avg</td>
<td>88</td>
<td>170</td>
<td>189</td>
<td>135</td>
<td>170</td>
<td>188</td>
</tr>
</tbody>
</table>

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**Figure 5.** The effect of variation of the volume of a chemically defined medium containing 0.1% p-hydroxyphenylacetic acid on the amounts of penicillins X and G produced in shake flasks.
other paired flasks, the same amounts of lard oil were added. Control flasks contained no added antifoam agent. The experiment was set up on two occasions.

The effect of the different antifoam agents is shown in figure 4.

(1) *Penicillin X*. In the absence of antifoam agent, penicillin X averaged 108 units per ml. Its level was increased by the addition of either oil, greater stimulation being found with lard oil.

(2) *Penicillin G*. On the whole, the level of penicillin G was depressed by the addition of either oil.

(3) *Penicillin F*. The concentration of penicillin F was increased by the presence of both oils, marked stimulation taking place with the more unsaturated oil, soya bean.

(4) *Penicillin dihydro F*. Like F, the concentration of dihydro F was increased by both oils. A striking increase was observed in the presence of 2.0 ml lard oil, the more saturated agent.

(5) *Penicillin K*. The effect of the two oils on the production of penicillin K closely resembles that found with dihydro F. The addition of 2.0 ml lard oil doubled the concentration found in the absence of antifoam agent.

*Figure 4.* The concentrations of various penicillins produced in shaken flasks in the presence of soya bean (S.B.O.) and of lard oil. For each penicillin the height of the bar represents the average of the concentrations on the 4th, 5th, 6th, and 7th days derived from two experiments.
The Effect of Variation of the Volume of Medium per Flask in the Presence of a Constant Amount of Oil

To determine whether the effect of increase in media volume was independent of the action of the antifoam agent, the following experiment was carried out in duplicate. Flasks were prepared containing 100, 200, and 300 ml corn steep medium. To two flasks of each volume, 1 ml lard oil was added. Two flasks of each volume contained no oil. The levels of penicillins X and G obtained are shown in figure 5.

The results shown in figure 5 (left-hand side of graph) indicate that an increase in volume of medium produces an increase in the concentration of penicillin X both in the presence and absence of lard oil. It would appear that the effect of increased volume is largely independent of the effect of oil, although, higher levels are attained when oil is present.

The right-hand side of the graph indicates that penicillin G levels are lower in the larger volumes of medium and that the effect of the volume variation is also largely independent of the effect of lard oil. Also lard oil appears to depress the formation of penicillin G.

The Effect of Volume of Medium per Flask on the Formation of p-Aminobenzyl Penicillin

In corn steep liquor medium. Corn steep liquor broth containing 0.1 per cent p-aminophenylacetic acid was prepared. This was distributed in 1-L Erlenmeyer flasks in 100, 200, and 300 ml amounts. Lard oil in the proportion of 0.5 ml per 100 ml medium was added.

The peak titers of total penicillin and the units per ml in the various chromatographic fractions are shown in table 2.

![Graph showing the effect of volume of medium per flask in the presence of a constant amount of oil](image)

In chemically defined medium. An experiment, similar to that described above was set up using chemically defined medium. The results are shown in table 3.

The data in tables 2 and 3 indicate that the production of a hydrophilic penicillin, presumed to be p-aminobenzyl penicillin, is depressed as the volume of medium is increased. The effect of increasing volume, in the presence of p-aminophenylacetic acid, on the formation of a hydrophilic penicillin is the direct opposite of the effect found when the medium contained p-hydroxyphenylacetic acid.

Acknowledgments

The author wishes to express thanks to Dr. P. J. Moloney for his suggestions and continued interest in this work.

TABLE 2

<table>
<thead>
<tr>
<th>Penicillin</th>
<th>Volume of Medium per Flask (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Hydrophilic</td>
<td>1040 (80.5%)</td>
</tr>
<tr>
<td>G</td>
<td>168</td>
</tr>
<tr>
<td>F</td>
<td>38</td>
</tr>
<tr>
<td>Dihydro F</td>
<td>30</td>
</tr>
<tr>
<td>K</td>
<td>17</td>
</tr>
</tbody>
</table>

Medium containing 0.1% p-aminophenylacetic acid was distributed in 100, 200 and 300 ml amounts. No attempt was made to identify the penicillin in the hydrophilic band as p-aminobenzyl penicillin so it is designated only as "hydrophilic." Its position on the chromatograms is approximately the same as that found by Brewer and Johnson (1953).

TABLE 3

<table>
<thead>
<tr>
<th>Penicillin</th>
<th>Medium Containing 0.1% p-aminophenylacetic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume per flask (ml)</td>
<td>100</td>
</tr>
<tr>
<td>Hydrophilic</td>
<td>1040 (80.5%)</td>
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<tr>
<td>G</td>
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<td>Dihydro F</td>
<td>30</td>
</tr>
<tr>
<td>K</td>
<td>17</td>
</tr>
</tbody>
</table>

Chemically defined medium containing 0.1% p-aminophenylacetic acid was distributed in 100, 200, and 300 ml amounts. A medium containing 0.1% p-hydroxyphenylacetic acid was used as a control.

Figure 5. The effect of variation of the volume of medium per flask in the presence and absence of lard oil. Flasks contained 100, 200, and 300 ml medium. To one group no oil was added. The point shown for the level of penicillin in each variation is an average of eight determinations: the level for the 4th, 5th, 6th, and 7th days of incubation from two experiments.
Summary

The results which have been presented indicate the following:

The proportions and amounts of penicillins X, G and p-aminobenzyl penicillin produced in fermentation liquors are markedly influenced by the ratio of volume of medium to volume of shake flask. An increase in the volume of medium was accompanied by an increase in penicillin X and a decrease in penicillin G under the conditions described. The highest yields of p-aminobenzyl penicillin were obtained in small volumes of medium, similar to those found optimum for penicillin G.

The presence and nature of certain oils can influence the amounts of particular penicillins produced. For example, the addition of a highly unsaturated oil gave marked stimulation to the production of the unsaturated penicillin F. A more saturated oil favored the production of the saturated straight-chain penicillins, dihydro F and K. The production of penicillin X was increased by the addition of both soya bean oil and lard oil, the greater stimulation being observed with the latter. The production of G was not markedly affected by the presence or absence of either oil.

The results suggest that the optimum conditions for the production of penicillins X and G differ markedly from each other. The degree of agitation in the small volumes was greater than in the larger volumes. This suggests that difference in aeration is one factor which accounts for the differences observed. Another important factor is the amount and kind of antifoam agent used.

Results presented indicate that in shake flask experiments, added precursors may be utilized in the formation of a specific penicillin without resulting in a significant increase in the total yield. Experiments designed to assess precursor activity should include variation in the amount and kind of antifoam agent used. Results of such experiments could be of practical value in determining the optimum conditions for large-scale fermentations.

REFERENCES


