AMENDED SPECIFICATION

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PATENT SPECIFICATION

NO DRAWINGS

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The inventors of this invention in the sense of being the actual devisers thereof within the meaning of Section 16 of the Patents Act, 1949, are WILFRIED KAUFMANN, Wuppertal-Vohwinkel, Germany, Friedrich-Bayer-Strasse, 15, KLAUS BAUER, Wuppertal-Elberfeld, Germany, Briller, Höhe 22, both of German Nationality.

COMPLETE SPECIFICATION

6-Aminopenicillanic Acid

We, FARBENFABRIKEN BAYER AKTIENGESELLSCHAFT, a body corporate organised under the laws of Germany, of 22c Leverkusen-Bayerwerk, Germany, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention is concerned with a new biochemical process for the production of 6-aminopenicillanic acid.

The compound 6-aminopenicillanic acid forms a valuable starting material for the syntheses of new penicillins. The compound was first described by T. Kato (J. Antibiotics, Series A, *6*, 130, 184 1953), and subsequently by S. Murao (J. Agric. Chem.Soc.Japan, *29*, 400—07, June 1955). The latter author produced the compound by permitting penicillinamidase from *Penicillium chrysogenum* Q 176 to act upon the sodium salt of Penicillin G. In this reaction phenylacetic acid was split off. The strain of *Penicillium chrysogenum* Q 176 was caused to secrete penicillinamidase by pretreatment with sulphathiazole. This procedure is difficult to carry out, however, and does not make it possible to obtain relatively large amounts of 6-amino-penicillanic acid.

A further synthesis for the production of 6-aminopenicillanic acid is described in Specification No. 870,396 whereby *Penicillium chrysogenum* is grown in the usual media under conventional conditions, except that the precursor, such as phenylacetic acid or phenoxyacetic acid is omitted. It is, however, technically very difficult to separate and recover the desired product so that the process is hardly suitable for commercial operations.

The present invention is based upon our discovery that 6-aminopenicillanic acid can be obtained in very good yields and without great technical difficulties by permitting certain bacteria or extracts, enzymes or enriched enzyme extracts obtained from said bacteria to act upon penicillin G. For example, extracts of gram-negative bacteria such as *Escherichia coli*, *Bacterium proteus*, and *Aerobacter aerogenes*, have been found to be particularly suitable for use in our process. This is relatively surprising inasmuch as it is known that *Escherichia coli*, for example, is capable of forming penicillinase which splits the β-lactam ring in the penicillin molecule.

For best results, the process of the invention is carried out by causing suspensions of the bacteria to act upon penicillin G in the presence of toluene, chloroform or other preserving agents customarily employed in working with enzymes. Preferably, the penicillin G is employed in concentrations of from 5,000—10,000 units per cubic centimetre. Depending upon the specific bacteria employed, the process is effected at temperatures within the range of from 0—50° C., and preferably between 20 and 40° C., with 37° C. representing the optimum operating temperature under most circumstances. It is preferred to conduct the process at pH values within the range of from 5 to 9 and preferably at values between 6.5 and 8.0. The reaction time is dependent upon the amount of penicillin G and bacterium or extract thereof, employed and may range from

[*Price 4s. 6d.*]

15 minutes to 24 hours. Most desirably, the quantity of reactants is selected such as to provide a reaction time of about 5 hours. Under these conditions, it is found that the penicillin is transformed quantitatively into 6-aminopenicillanic acid.

With respect to the selection of suitable bacteria for use in the biochemical synthesis of our invention, it is interesting to note that the desired penicillin cleavage is generally effected efficiently in the presence of a Bacterium species or not at all. For this reason, it is not practical to define the operative strains of bacteria precisely by reference to genera, but rather, it is recommended that the selection be made by suitable testing of each species. In this connection, the following testing procedure serves admirably for purposes of determining the ability of bacteria to form 6-aminopenicillanic acid by reaction with penicillin G:

To approximately 10 cubic centimetres of a phosphate buffer solution (pH 7.0) containing 10,000 international units of Penicillin G per cubic centimetre, an equal volume of a test suspension of bacteria in a phosphate buffer solution (pH 7) is added and, after adding 0.2 percent toluene, the preparation is stored for 5 hours at approximately 37° C. The suspension of the bacteria is prepared by inoculating 100 cubic centimetres of a suitable nutrient broth, with a pure culture of the bacteria to be tested and cultivating the mixture on the shaking machine for about 18 hours at 28—32° C. Thereafter, the bacteria are separated by centrifuging, washed in 40 cubic centimetres of a phosphate buffer solution (pH 7) and resuspended in 10 cubic centimetres of the phosphate buffer solution at pH 7.

Following the 5 hour action of the cells of the bacterium on the Penicillin G, the residual penicillin content is microbiologically determined in the centrifuged solution free from cells. With various strains of bacteria, the following results are obtained in this test:

1. The solution may retain its full initial activity. In this case, it may be said that the cells of the bacterium under test did not develop any enzymatic action as against Penicillin G and, hence, the bacteria are unsuitable for use in the preparation of penicillin-splitting enzymes.

2. The solution may be found to be inactive or possessed of a greatly reduced activity. In this case, 500 milligrams of phenylacetyl chloride are then caused to act upon the solution with ice cooling and simultaneous addition of sodium bicarbonate. The solution is then tested again, with the following possible results being observed:

(a) no increase in activity as against the non-acylated solution, indicating that the penicillin was degraded irreversibly by penicillinase or similar enzymes developed during the reaction;

(b) a full reactivation of the penicillin action up to 10,000 international units per cubic centimetre of the solution indicating that the Penicillin G was cleaved by enzymatic action in such a way that 6-aminopenicillanic acid and phenylacetic acid are formed. The bacteria functioning in this manner are, of course, eminently suitable for use in the enzymatic formation of 6-aminopenicillanic acid by the process of the invention;

(c) a partial reactivation of the original penicillin activity of 10,000 international units per cubic centimetre of the solution occurs, indicating that penicillinase was present in the bacteria in addition to the desired enzyme by which phenylacetic acid is split-off. Bacteria exhibiting this type of activity can be employed for the preparation of 6-amino-penicillanic acid, provided the penicillinase inactivates only a negligible part of the penicillin, *or* if one succeeds in selectively eliminating the penicillinase activity.

The foregoing selection testing procedure can also be carried out in a modified form by investigating the enzymatic activity as against Penicillin G in cell-free culture filtrates. In our application of the selection test by the procedure described in detail hereinbefore, the following bacteria were examined as to their ability to form 6-aminopenicillanic acid from Penicillin G with splitting-off of phenylacetic acid: Bacillus subtilis, Sarcina lutea, Klebsiella pneumoniae, Streptomyces griseus, Staphylococcus aureus, Staphylococcus albus, Chromobacterium prodigiosum, Pseudomonas fluorescens, Pseudomonas pyocyaneum, Bacterium proteus (OX 19), Escherischia coli (ATCC 9637), Escherischia coli (ATCC 11105), Escherischia coli (N 14), Escherischia coli (W), Escherischia coli (CM 2), Escherishchia coli (CM 5A), Escherischia coli (CM 6) and Aerobacter aerogenes.

In the foregoing studies, it was found that Escherischia coli (ATCC 11105), Escherischia coli (ATCC 9637), and the laboratory strains of Escherischia coli (CM 2), (CM 5A) and (CM 6) and Aerobacter aerogenes were all well suited for use in the process of the invention. A distinct, although slightly weaker, cleavage capacity as against Penicillin G was exhibited by Bacterium proteus (OX 19). The remaining bacteria were unsuitable for use in the preparation of 6-aminopenicillanic acid.

In defining the operative bacteria for use in the process of the invention in terms of the foregoing test procedure, it may be said that any enzymes from bacteria may be employed which are capable of inactivating Penicillin G by at least 20 percent within 24 hours to yield a solution in which said inactivated Penicillin G can be at least partially reactivated by the addition of phenylacetyl chloride thereto.

It is believed that the invention may be best

understood by reference to the following specific Examples illustrating the application of the foregoing principles and procedures of the preparation of 6-aminopenicillanic acid from typical starting materials of the general class defined.

EXAMPLE 1.

A thick suspension of washed Bacterium coli (ATCC 9637) cells was suspended in a phosphate buffer solution of pH 6. To this suspension there was added sodium Penicillin G in a concentration of 5000 units per millilitre. Following the addition of 0.4 percent toluene, the solution was stored at 37° C. for 18 hours.

7.5 litres of the enzyme preparation were made up to 30 litres with acetone. The precipitate resulting from this addition was separated by centrifuging. The solution was then concentrated in a vacuum to 3.88 litres, as a result of which a precipitate formed. After adjusting the pH to 2.6, an extraction with 2 litres butyl acetate was effected, the extract was rejected, the aqueous phase adjusted to a pH of 4.3 by means of a sodium bicarbonate solution and, after clarifying it in a K—3—Seitz filter, the aqueous phase was concentrated under vacuum to 300 cubic centimetres, thus causing crystallisation to occur spontaneously. The crystals were recovered by centrifuging, washed portionwise with 300 cubic centimetres ice cold water and 150 cubic centimetres acetone. From the mother liquor, an additional, slightly less pure fraction of 6-aminopenicillanic acid can be recovered. The melting point of the product is 203° C. (decomposition). The ninhydrin test with a dilute aqueous solution shows orange to reddish-brown colouration.

The determination of 6-aminopenicillanic acid is best effected by testing the solution recovered after storage without further treatment against one of the conventional penicillin-sensitive organisms. In this experiment, 6-aminopenicillanic acid itself exhibits extremely low bacteriostatic activity. A second sample of 6-aminopenicillanic acid is treated carefully in the presence of ice cooling with an excess of phenylacetyl chloride and sodium bicarbonate. This causes phenylacetylation of the free amino group, and an amount of Penicillin G is obtained which corresponds to the quantity originally present.

EXAMPLE II.

A dense suspension of Escherischia coli (ATCC 11105) cells in a phosphate buffer solution of pH 7.5 was adjusted to a pH of 8.5 by means of a sodium carbonate solution. Upon the addition of 1 mg% lysozyme, 0.05 percent sodium desoxycholate, 0.01 mol of sodium ethylene diamine tetraacetate and 0.4 percent toluene, this suspension was held at 37° C. for 2 hours in order to bring about lysis of the bacterial cells. Thereupon, the pH of the preparation was brought back to pH 7.5 by the addition of hydrochloric acid, and the residual cells not affected by lysis and other insoluble residues were separated by centrifuging. The supernatant liquid was treated with potassium Penicillin G up to a concentration of 15000 units per cubic centimetres and, after the addition of 0.4 percent toluene, was held for 12 hours at 30° C.

In order to effect isolation of the 6-aminopenicillanic acid formed, an equal volume of acetone was added to the reaction mixture and it was stirred vigorously for a short period of time. The precipitate formed was separated. For extraction of the acetone, the liquid filtrate was shaken with an equal volume of butyl acetate. The separated, aqueous phase was next adjusted to pH 2.8 with hydrochloric acid at 0° C. for the removal of the non-split penicillin and shaken with an equal volume of cooled butyl acetate. The aqueous phase was separated, adjusted with potassium hydroxide to pH 4.3 and evaporated in a vacuum at 20—25° C. A white, crystalline precipitate of 6-aminopenicillanic acid was thus formed which was separated from the mother liquor, repeatedly washed with acetone and dried in a vacuum. The crystals of 6-aminopenicillanic acid thus obtained melt at 203—205° C. with decomposition. They exert practically no antibiotic action as against Bacillus subtilis. After its reaction with phenylacetyl chloride, there resulted an action of 2660 international units per milligram of 6-aminopenicillanic acid present. Ninhydrin test: orange to brownish-red reaction.

EXAMPLE III.

A dense suspension of Bacterium proteus (OX 19) cells in a phosphate buffer solution (pH 7.0) was acted upon with sodium Penicillin G up to a concentration of 5000 units per cubic centimetre. Following the addition of 0.2 percent chloroform, the solution was held for 15 hours at 37° C. From the resulting solution, 6-aminopenicillanic acid was isolated by the procedure described in Example II.

EXAMPLE IV.

To a dense suspension of the washed cells of the strain Escherischia coli (CM 2) in a phosphate buffer solution of pH 7.5, sodium Penicillin G was added in a concentration up to 10,000 international units per cubic centimetre. Following the addition of 0.0005 percent 2,4 dinitrophenol serving as a preservative, the solution was stored at 9 hours for 37° C. From the resulting solution, 6-aminopenicillanic acid was isolated by the technique described in Example I.

EXAMPLE V.

To a dense suspension of the washed cells of the strain Escherischia coli (CM 5A) in a phosphate buffer solution of pH 7.0, sodium Penicillin G was added at a concentration up to 10,000 international units per cubic centimetre. Following the addition of 0.2 percent toluene, the solution was held for 15 hours at 37° C. From the resulting solution, 6-amino-

penicillanic acid was isolated by the technique described in Example I.

EXAMPLE VI.

To a dense suspension of the washed cells of the strain Escherischia coli (CM 6) in a phosphate buffer solution of pH 7.5, sodium Penicillin G was added at a concentration of 10,000 international units per cubic centimetre. Following the addition of 0.2 percent chloroform, the solution was kept for 15 hours at 37° C. From the resulting solution, 6-aminopenicillanic acid was isolated by the technique described in Example I.

In our Specification No. 30,081/60 (Serial No. 897,618) there is described and claimed a process for the production of 6-acylaminopenicillanic acids, wherein 6-aminopenicillanic acid is reacted with a carboxylic acid, or a salt or derivative thereof, at a pH of not more than 6.5, in the presence of bacteria or extracts, enzymes or enzyme concentrates obtained from these bacteria which are capable of linking phenylacetic acid in an amide-like manner with the 6-positioned amino group of 6-aminopenicillanic acid with the liberation of water. It is to be explained that there exists an equilibrium between 6-acylamino-penicillanic acid and 6-aminopenicillanic acid but that a more alkaline pH value favours the formation of 6-aminopenicillanic acid.

WHAT WE CLAIM IS:—

1. Process for the production of 6-aminopenicillanic acid which comprises contacting penicillin G in an aqueous medium at a temperature within the range of from 0—50° C. and at a pH value within the range of from 5—9 for at least 15 minutes with a bacterium capable of inactivating penicillin G by at least twenty percent within 24 hours, or with an extract of such a bacterium or with enzymes, enzyme concentrations or enzyme extracts produced from such a bacterium, to yield a solution in which said inactivated penicillin G can be at least partially reactivated by the addition thereto of phenylacetyl chloride, and recovering said 6-aminopenicillanic acid from said aqueous medium.

2. Process according to claim 1, wherein the reaction is carried out at a temperature of 20—40° C.

3. Process according to claim 2, wherein the reaction is carried out at a temperature of 37° C.

4. Process according to any of the preceding claims, wherein the reaction is carried out at a pH within the range of 6.5 to 8.0.

5. Process according to any of the preceding claims, wherein the reaction is continued for a period of 15 minutes to 24 hours.

6. Process according to claim 5, wherein the reaction is carried out for a period of about 5 hours.

7. Process according to any of the preceding claims, wherein the bacterium used is Escherichia coli ATCC 9637, ATCC 11105, CM 2, CM 6 or CM5A or Bacterium proteus OX 19.

8. Process for the production of 6-aminopenicillanic acid according to claim 1, substantially as hereinbefore described and with reference to any of the specific Examples.

9. 6-aminopenicillanic acid, whenever prepared by the process according to any of claims 1—8.

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Carpmael Ransford

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EXAMPLE VI.

To a dense suspension of the washed cells of the strain Escherischia coli (CM 6) in a phosphate buffer solution of pH 7.5, sodium Penicillin G was added at a concentration of 10,000 international units per cubic centimetre. Following the addition of 0.2 percent chloroform, the solution was kept for 15 hours at 37° C. From the resulting solution, 6-aminopenicillanic acid was isolated by the technique described in Example I.

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WHAT WE CLAIM IS:—

1. Process for the production of 6-aminopenicillanic acid which comprises contacting penicillin G in an aqueous medium at a temperature within the range of from 0—50° C. and at a pH value within the range of from 5—9 for at least 15 minutes with a bacterium capable of inactivating penicillin G by at least twenty percent within 24 hours, or with an extract of such a bacterium or with enzymes, enzyme concentrations or enzyme extracts produced from such a bacterium, to yield a solution in which said inactivated penicillin G can be at least partially reactivated by the addition thereto of phenylacetyl chloride, and recovering said 6-aminopenicillanic acid from said aqueous medium.

2. Process according to claim 1, wherein the reaction is carried out at a temperature of 20—40° C.

3. Process according to claim 2, wherein the reaction is carried out at a temperature of 37° C.

4. Process according to any of the preceding claims, wherein the reaction is carried out at a pH within the range of 6.5 to 8.0.

5. Process according to any of the preceding claims, wherein the reaction is continued for a period of 15 minutes to 24 hours.

6. Process according to claim 5, wherein the reaction is carried out for a period of about 5 hours.

7. Process according to any of the preceding claims, wherein the bacterium used is Escherichia coli ATCC 9637, ATCC 11105, CM 2, CM 6 or CM5A or Bacterium proteus OX 19.

8. Process for the production of 6-aminopenicillanic acid according to claim 1, substantially as hereinbefore described and with reference to any of the specific Examples.

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