NOTE ON THE BIOSYNTHESIS AND ISOLATION OF
³⁵S-LABELLED BENZYL PENICILLIN

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This appendix describes in the form of laboratory notes a simple and rapid method for producing and isolating ³⁵S-labelled benzyl-penicillin. The process yields 5–15 mg. of the compound virtually free from radioactive contaminants and with a specific activity up to *c.* 0.2 μ c./unit. Approximately 20 % of the ‘carrier-free’ ³⁵S added to the medium is converted into penicillin. This process was evolved as the result of work ancillary to other researches and is not claimed to be original. But as there appears to be no published account which describes in detail the preparation of ³⁵S-penicillin, the information given here may be useful to other workers in the field.

Organism and maintenance of the culture

Organism. *Penicillium chrysogenum* WIS 48-701. Under suitable conditions this strain synthesizes only benzylpenicillin. It is preserved as a freeze-dried preparation of spores.

Sporulation medium. Spores are produced on molasses agar medium of the following composition: glycerol, 0.75 %; molasses, 0.75 %; yeast extract, 0.5 %; NaCl, 4.0 %; MgSO₄.7H₂O, 0.005 %; KH₂PO₄, 0.006 %; ferric tartrate, 0.00016 %; CaSO₄, 0.025 %; CuSO₄ (1 % solution), 0.01 %; Bacto-agar, 3.0 %; pH value adjusted to 7.1. The medium is layered at least 0.5 cm. deep in plugged 20 oz. medical flats.

Method. Freeze-dried spores are taken up in a little Hartley beef broth and sown by spreading on the surface of the molasses agar. The bottles are incubated, medium uppermost, at 22° for 10 days. The cultures may then be stored in a cold-room (+2°) for at least 1 month.

Spores are separated from the mycelial mat by wetting the surface of the solid culture with 5 ml. 1 % aqueous ‘Teepol’ followed by washing with glass-distilled water and dilution to 250 ml. The resultant ‘standard spore suspension’ is used either as an inoculum for penicillin production (see below) or as a source of further freeze-dried preparations. For freeze-drying the spores are spun down, resuspended in about 10 ml. of a mixture made from equal volumes of inactivated horse serum and broth, and distributed in suitable ampoules.

Biosynthesis of penicillin

Medium. The organism is grown in a medium containing corn-steep solids, 2.0 %; lactose, 4.0 %; NaNO₃, 0.3 %; MgSO₄, 0.025 %; CaCO₃, 0.30 %; phenylacetic acid, 0.25 %; lard oil, 0.2 %; pH value adjusted to 5.6 before autoclaving; to this is added a 2 % (v/v) inoculum of ‘standard spore suspension’ and an aqueous solution of ‘carrier-free’ ³⁵S as ³⁵SO₄ in a volume not greater than 5 % of the total volume of medium.

Conditions of growth. A 250 ml. Erlenmeyer flask, capped with a 50 ml. beaker, is used as the growth vessel. To prevent rattling and permit free gas exchange the beaker is held clear of the neck of the flask by a collar made from three or four short pieces of pressure tubing threaded on wire. The flask, containing 40–50 ml. of medium, is swirled at 100 r.p.m. with a throw of 5 in. for 5–5½ days at 22° ($\pm 1^\circ$). No significant quantity of volatile radioactive compound is released during growth.

Yield. The concentration of penicillin in the harvested culture fluid is generally 750–950 units/ml. (by cup-plate assay). The proportion of added ^{35}S converted to labelled penicillin depends on the yield and the batch of corn-steep liquor used. At best the conversion rate is about 20 %.

Isolation of benzylpenicillin

The culture is brought to pH 7.0 with N-HCl , and filtered through a thin layer of 'Hyflo Supercel'. The penicillin is extracted from the filtrate by three successive liquid-liquid partition separations followed by chromatography on a column prepared and checked before the isolation is started.

Preliminary purification. (i) The filtrate is saturated with the minimum excess of 'Analar' $(\text{NH}_4)_2\text{SO}_4$ and the pH brought to 2–2.5 with 50 % phosphoric acid using thymol blue as external indicator. It is then extracted twice with one-fifth of its volume of 'Analar' amyl acetate and the aqueous phase rejected.

(ii) The combined amyl acetate phases are extracted twice with one-tenth of their volume of one-eighth saturated phosphate buffer, pH 6.5–7.0. The amyl acetate phase is rejected.

(iii) The combined aqueous phases are again acidified with 50 % phosphoric acid and extracted three times with one-half their volume of anaesthetic ether. The aqueous phase is rejected and the combined ether phases concentrated, if necessary, by evaporation with a stream of dry air. The final concentrate used for chromatography should contain about 5 mg. penicillin/ml. (assuming a 75 % yield so far).

Chromatography. The column described below operates satisfactorily with loads of up to 15 mg. of penicillin. Since optimal pH value and saturation of buffer increase with increasing column size, the dimensions given here are important.

7.5 g. of 'Hyflo Supercel' are thoroughly mixed with 3.75 ml. 25 %-saturated citrate buffer, pH 5.3. The material is packed 'dry', in about ten portions, into a tube of 1.1–1.2 cm. internal diameter, to give a column 18–20 cm. long.

The column is tested with a load of 15 mg. of pure benzylpenicillin in 3 ml. of ether, extracted from buffer by the process of (iii) above. The penicillin is washed through the column with anaesthetic ether saturated with the citrate buffer. Separation is unimpaired by operating at rates up to 3 ml./min. under a positive pressure of about 10 cm. Hg. Fractions of 3–4 ml. are collected in tubes containing about half the fraction volume of 0.0025 % bromthymol blue in neutral water. The fractions are titrated with vigorous agitation using 0.006 M-NaOH (1 ml. $\equiv$ 2 mg. benzylpenicillin) from a CO_2 -free automatic

burette. The plotted titration results show a single large peak, spread over about 25 ml. between the 45th and 80th ml. of effluent, which corresponds to benzylpenicillin. The exact position of this peak is characteristic of the particular column in use.

The tested column is now used for the partially purified sample of ³⁵S-penicillin. Load and operating conditions are unchanged, but phenylacetic acid and other non-penicillin compounds give an unimportant preliminary peak in the 12–18 ml. range. The fractions known to contain benzylpenicillin are collected in a vessel cooled in ice + salt freezing mixture. About 10 ml. of glass-distilled water are added to the vessel and the penicillin titrated, with vigorous shaking, using 0.006 M-NaHCO₃, until the pH value remains unchanged at 7.5 (bromthymol blue as external indicator). The aqueous phase is then separated from the ether, distributed in lightly plugged ampoules and freeze-dried. The product is sodium benzylpenicillinate contaminated mainly with sodium citrate and appears quite stable when stored under dry N₂ at atmospheric pressure. The final yield is 60–70 % of the total penicillin in the original culture.

Assays and tests for purity. The antibiotic activity contained in each ampoule is determined, when it is opened for use, by a cup-plate assay. Freedom of the product from radioactive contaminants is checked by the paper chromatography method of Glister & Grainger (1950) combined with radioactivity measurements (Lester Smith & Allison, 1952). Duplicate strips run against mixed penicillin standards show no antibiotic activity other than that due to the benzylpenicillin spot, which also exhibits 85–90 % of the total radioactivity of the strip. Almost all the remaining radioactivity is found at the origin, and is probably due to penicilloic acid produced by the decomposition of penicillin on the strip itself (Lester Smith & Allison, 1952).

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