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Isolation of Penicillin K by Continuous Countercurrent Solvent Fractionation

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During an investigation of the properties of the various natural penicillins, it became necessary to obtain appreciable quantities of pure penicillin K. It is the purpose of this paper to show how this was accomplished by continuous countercurrent solvent fractionation.

A knowledge of the distribution coefficients of the components of a mixture, under the conditions of operation, permit a reasonably close prediction of the results of a continuous countercurrent fractionation. Before this work was undertaken, the amounts of pure penicillins available were too small to permit their use in extensive distribution studies. For this reason, the Craig technique,[1,2] which does not require a foreknowledge of the distribution coefficients, was used with a series of eleven separatory funnels in order to establish an appropriate system of solvent and buffer for the separation of the various penicillins.

A system was developed which gave a satisfactory maximum difference of distribution coefficient between penicillin K and the other species; the system of choice was methyl amyl acetate[3] and 1 molar sodium phosphate buffer at *p*H 5.2. With this system a sample of amorphous penicillin from laboratory stock, when submitted to a 10-stage countercurrent distribution, gave the experimental bioactivity distribution curve shown in Fig. 1A.

When pure materials became available, the distribution coefficients of penicillins F, G and K were determined at 5° in the system 1 *M* sodium phosphate buffer–methyl amyl acetate at several *p*H's. The data thus obtained were correlated statistically and are shown in Fig. 2 plotted as log *D vs. p*H.

The experimental curve, Fig. 1A, is broken down in Fig. 1B into its G, F and K components. Subtracting the sum of these from the experimental curve gives the curve (D = 0.043) at the left of Fig. 1B, which is assumed to be penicillin X. The sum of the four individual curves is plotted in Fig. 1A as the theoretical curve.

Instead of going directly from batch runs in funnels to continous runs in an extraction train, a check run was carried out in separatory funnels using a modification of the procedure of Watanabe and Morikawa.[4]

Off-center feed, funnel 4 of a five funnel unit,

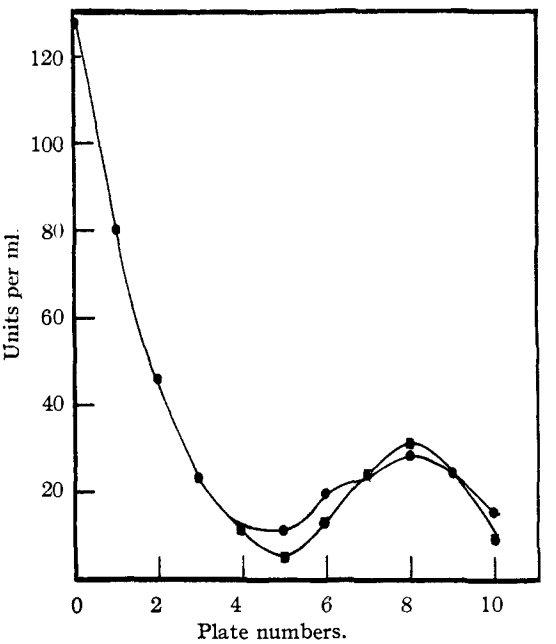

Fig. 1A.—Ten-plate countercurrent distribution curve of amorphous penicillin: ●—● experimental curve: ■—■ theoretical curve.

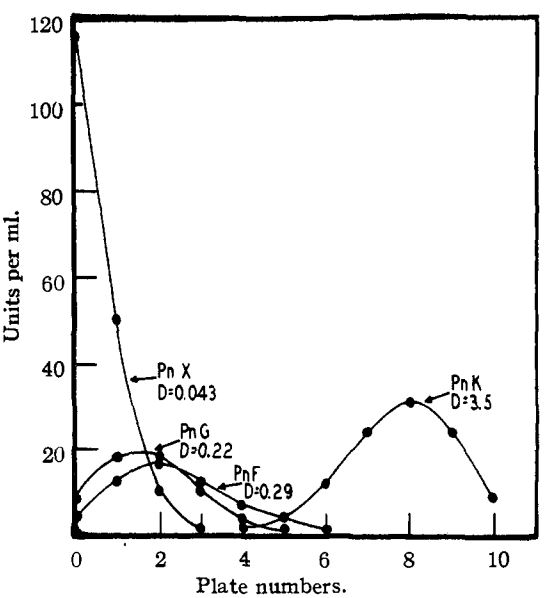

Fig. 1B.—Individual theoretical countercurrent distribution curves for the components of amorphous penicillin as calculated from Fig. 1A.

was employed which afforded one "back up" or scrubbing plate and three purifying plates. Details of the operation are described in the ex-

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(1) L. C. Craig, *J. Biol. Chem.*, **155**, 519 (1944).

(2) (a) L. C. Craig, C. Golumbic, H. Mighton and E. Titus, *J. Biol. Chem.*, **161**, 321 (1945); (b) Guy T. Barry, Yoshio Sato and Lyman C. Craig, *ibid.*, **174**, 221 (1948).

(3) 1,3-Dimethylbutyl acetate as obtained from Carbide and Carbon Chemicals Corp., New York, N. Y.

(4) Watanabe and Morikawa, *J. Soc. Chem. Ind. Japan*, **36**, 585B (1933).

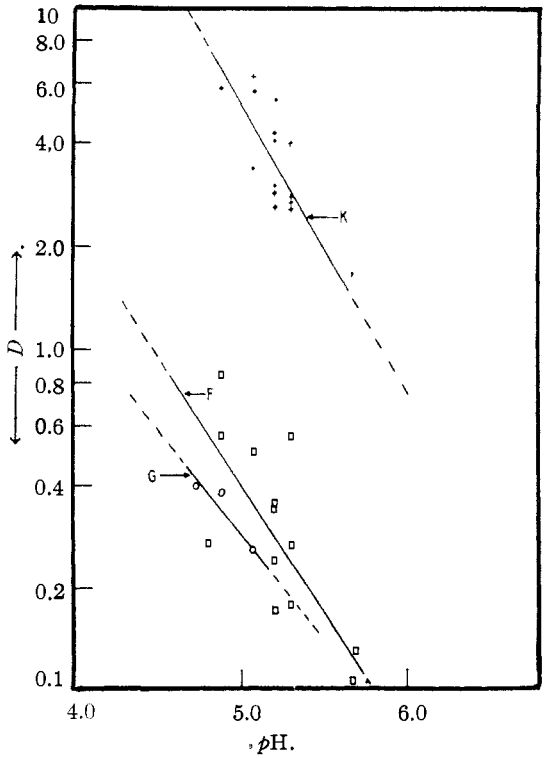

Fig. 2.—Log D *vs.* pH of experimental distribution coefficients of penicillins F, G and K.

perimental part. The penicillin K was recovered from the pooled solvent layers as the ammonium salt and twice crystallized from aqueous acetone to yield material analyzing well for pure ammonium penicillin K.

Having shown the system and mechanics for continuous countercurrent solvent fractionation to pure penicillin K to be operable, a large-scale run was carried out in a manner similar to the funnel run, but in centrifuges as described in the experimental part and depicted in Fig. 3.

The ammonium penicillin K isolated from this run was twice crystallized from aqueous acetone. The final product analyzed very well for penicillin K and was shown to be at least 95% pure with respect to other penicillins when analyzed by a 24-plate Craig distribution.

Experimental

Preparation of the Phosphate Buffer.—The phosphate buffer was prepared by mixing 1 M solutions of Na_2HPO_4 and NaH_2PO_4 to give a pH of 5.2 at 20°. Before each run the solvent and buffer were mixed to mutual saturation and separated. Under these conditions the buffer did not crystallize at 5° during the course of experimental work though this did happen occasionally on longer standing at temperatures a few degrees lower.

Determination of Distribution Coefficients.—Distribution coefficients were determined in separatory funnels at 5° at aqueous concentrations of 300–1500 u./cc. The buffer samples were assayed directly while the solvent samples were extracted into pH 6.7 sodium phosphate buffer. Distribution coefficients were calculated as D = concn. in solvent/concn. in aqueous.

Preliminary Batchwise Runs.—It was found convenient to use a funnel rack into which eleven one-liter separatory funnels were clamped, mounted so as to revolve on a horizontal axis. The distribution curve of a 10-plate Craig type run on a sample of amorphous penicillin is shown in Fig. 1A.

Continuous Countercurrent Runs in Funnels.—The same rotating funnel rack was used as in the batchwise runs with only five funnels in positions 1 to 5 from left to right. A position to the left of the 1 position was designated "E." A 500-milliliter erlenmeyer flask was placed beneath each funnel in positions numbered to correspond with those in the rack. A flask position "R" was marked to the right of the 5 position. To start the run, 500 cc. of methyl amyl acetate previously equilibrated with the phosphate buffer was placed in each funnel. To the funnels in position 1, 2, 3 and 4 were added 250-ml. portions of phosphate buffer. To the funnel in the 4 position was added 230,000 units of amorphous penicillin in 250 ml. of phosphate buffer. The funnels were closed, the contents mixed by rotation of the rack and allowed to separate. The buffer in each funnel was then dropped to the flask beneath. Each funnel was moved one position to the left and each flask, one position to the right. After the first step the funnel from the "E" position was moved to the number 5 position.

In the second step the contents of each flask was emptied into the funnel above. To the funnel in position 4 was added another portion of amorphous penicillin in pH 5.2 buffer. To the funnel in position 1 was added 250 ml. of fresh buffer. Again the funnels were closed, mixed, settled and the buffer phase separated into the flasks beneath. Again the funnels were moved one position to the left and the flasks one position to the right. Now the flask in the "R" position contained penicillin which was emptied into a larger container as raffinate pool. The cycle as outlined above was repeated for two more steps. After the fourth step, and thereafter, the funnel from position "E" was moved to an extraction stand and the 5 position filled by a funnel containing 500 ml. of fresh solvent.

The solvent in the funnels moved from the "E" position to the extraction stand contained the K penicillin which was extracted with two 125-ml. portions of pH 7.2 phosphate buffer. These successive portions of extract buffer were collected in a container as extract pool.

The material in the buffer extract was converted to the ammonium salt by extraction into ethyl ether and reextraction with 5% ammonium hydroxide to pH 7.0. This material when freeze dried gave a potency of 1620 u./mg. when tested against *Staphylococcus aureus* and a D. R.[5] of 0.28.

Crystallization of the Penicillin K Fraction.—10.8 grams of the freeze dried "K" fraction from two runs as described above was suspended in 200 ml. of 95% acetone and warmed on the steam-bath. Complete solution was effected by the addition of 25 cc. of 80% acetone. Crystallization was induced by the slow addition of 500 cc. of anhydrous acetone. Upon chilling, 4.2 g. of crystals was obtained which was recrystallized, yielding 2.65 g. of ammonium penicillin K.

Anal. Calcd. for $C_{16}H_{29}O_4N_3S$: C, 53.47; H, 8.13. Found: C, 53.70; H, 7.97; microbial assay, 2215 u./mg. D. R. 0.32.

Continuous Countercurrent Fractionation in an Extraction Train.—The extraction train used for this work consisted of five stainless steel centrifuges with disc type bowls (Fig. 3). Auxiliary equipment was arranged so that the solvent phase (methyl amyl acetate) passed continuously at a rate of 1 gal./min. through a line mixer into the 5 centrifuge, from the discharge spout of the 5 centrifuge through another line mixer into the 4 centrifuge, and

(5) D. R. denotes differential ratio, which is the ratio of the microbiological activity against *B. subtilis* to that against *Staph. aureus*. Pure sodium penicillin K is reported to have a potency of 2300 u./mg. against *Staph. aureus* and a D. R. of 0.31: "Chemistry of Penicillin," Princeton Univ. Press, 1949, p. 99.

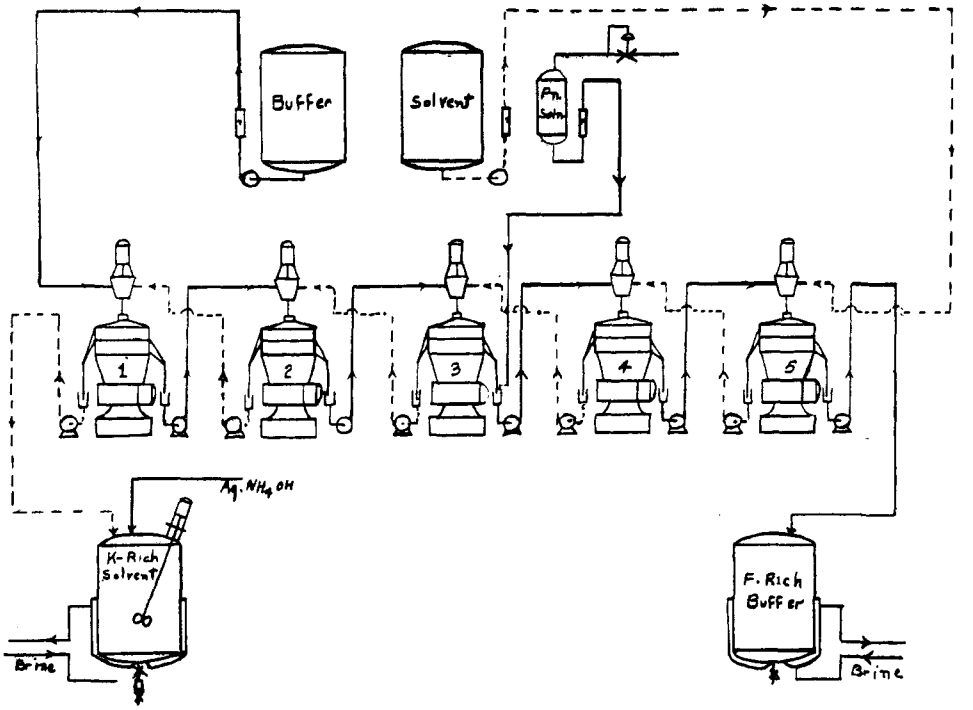

Fig. 3.—Flow diagram of large-scale extraction train.

in similar fashion through centrifuges 3, 2 and 1. From the discharge spout of the 1 centrifuge the rich solvent was pumped to a refrigerated holding tank. Fresh sodium phosphate buffer at *p*H 5.2 was fed at 0.85 gal./min. into the line mixer on the 1 centrifuge, from the discharge spout on the 1 centrifuge through a line mixer into the 2 centrifuge and in similar fashion through centrifuges 3, 4 and 5. From the discharge spout on the 5 centrifuge the raffinate buffer was pumped to a refrigerated holding tank. Before the run started the phases were cycled countercurrently through the train until all steps were in equilibrium. A solution of 820 million units of sodium penicillin in *p*H 5.2 buffer of a concentration of 49,200 cc./ml. was fed at about 0.12 gal./min. into the aqueous discharge of the 3 centrifuge from which it was pumped with the buffer stream into the line mixer on the 4 centrifuge.

The penicillin K rich solvent from the 1 centrifuge contained 242 million units and was, as noted above, accumulated in a 50-gal. stainless steel tank and extracted with 2% sodium bicarbonate solution. This extract was freeze dried and the solids obtained subjected to the crystallization procedure.

The buffer raffinate pool contained 550 million units. The total recovery of penicillin was 97% of the input.

Crystallization of the K Fraction.—The crude "K" fraction was converted to the ammonium salt by extraction from an aqueous solution at *p*H 2 into ethyl ether, then reextracted into water to *p*H 7 with 5% ammonium hydroxide. The final aqueous solution was freeze dried to a solid which was twice crystallized from aqueous acetone to yield 16 g. of crystals of ammonium penicillin K as a first crop.

Anal. Calcd. for $C_{16}H_{29}O_4N_3S$: C, 53.47; H, 8.13; N, 11.86. Found: C, 53.44; H, 8.07; N, 11.79; microbial assay, 2368 u./mg., D. R. 0.33.

Spectrographic examination failed to show the presence of a phenyl group while analysis of the Craig distribution curve showed the penicillin K content to be not less than 95% by weight.

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