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Synthetic Penicillin

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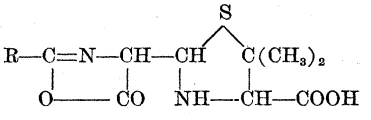
THIS REPORT IS A SUMMARY of the investigations that have led to the synthesis and isolation of G-penicillin (now termed *benzylpenicillin*). Most of the preliminary work, which is briefly described here, was performed by American and British chemists working under the joint auspices of the Committee on Medical Research, OSRD (Washington) and the Medical Research Council (London) (cf. *Science*, 1945, **102**, 627). The details of this preliminary work will be published in a monograph on penicillin chemistry, now in preparation.

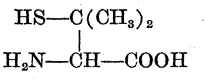
The isolation in crystalline form of the active synthetic product and the unequivocal proof of its identity with natural benzylpenicillin have been carried out in this Laboratory since the termination of the OSRD contracts. Complete details of this latter work will be reported following the publication of the monograph.

In the early stages of the investigation of the chemistry of penicillin, the oxazolone-thiazolidine structure (I) was favored by the majority of laboratories



(I)

as representing the structure of penicillin. Consequently, much effort was directed toward the synthesis of compounds of Type I. An approach toward the synthesis of such compounds, which involved the condensation of an appropriate oxazolone, possessing a free or potential aldehyde group, with *d*-penicillamine (II) (*1*), was explored by several laboratories both in this country and in England. Oxazolones of Type III which possessed a potential aldehyde group were soon synthesized (*2*).



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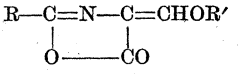


(III)

The formation, in such reactions, of products possessing antibiotic activity was demonstrated independently and almost simultaneously in the United States and in England and announced to the collaborating groups in the first months of 1944. The earliest record of this discovery is contained in a report from the laboratories of Merck and Company, Inc. (*3*), in which it was stated that "d(+)-penicillamine hydrochloride and the azlactone [(2-benzyl-4-methoxymethylene-5(4)-oxazolone) (III, R = $C_6H_5CH_2$–; R′ = CH_3–)] have been reacted to give a product showing

This article was submitted for publication at the request of and through the Editorial Board of the monograph on the Chemistry of Penicillin being prepared under the auspices of the National Academy of Sciences.

The nomenclature used by the authors conforms with that to be employed in the forthcoming monograph on the Chemistry of Penicillin. According to this nomenclature G-penicillin is designated *benzylpenicillin*; K-, F-, and X-penicillins are designated, respectively, *n-heptylpenicillin*, *Δ^2-pentenylpenicillin*, and *p-hydroxybenzylpenicillin*. This style of nomenclature is carried over into the naming of various degradation and rearrangement products of the penicillins. For example, G-penicilloic acid is designated *benzylpenicilloic* acid.

0.5 unit/mg. *in vitro* biological activity by the standard assay for penicillin G. This low order of activity has been obtained repeatedly, and is believed to be real. The active substance has properties similar to that of penicillin G." Shortly thereafter, the production of a similarly small amount of antibiotic activity from the condensation in cold glacial acetic acid containing sodium acetate, of 2-styryl-4-ethoxymethylene-5(4)-oxazolone with *dl*-penicillamine was described in detail by the Oxford group (*4*). It was later noted that in experiments where *l*- instead of *d*-penicillamine was used in the reaction no antibiotic activity was produced (*5*).

Although during the next year a considerable number of experiments by many laboratories were reported, in which the two compounds (*d*-penicillamine hydrochloride and 2-benzyl-4-methoxymethylene-5(4)-oxazolone) were allowed to react together under a variety of conditions, the best product that was obtained assayed only 3.6 units/mg. of material (the accepted value for sodium benzylpenicillin is 1,667 units/mg.). Because of the low order of activity of the material produced in this reaction, and also because it had become known that other compounds, not structurally related to penicillin, possessed detectable antibiotic activity when submitted to the routine assay methods, there was some doubt as to whether the activity produced in this reaction was due to the formation of a minute amount of penicillin or to the more extensive formation of substances of low intrinsic activity. During the next year further studies bearing on this point were reported.

In a study that was made of the relative stability of the antibiotic activity of the synthetic mixture and of benzylpenicillin, when treated with methanol, acid, or diazomethane, no difference in the gross rate of disappearance of the two activities was detected (*6*). A comparison was made of the relative antibiotic activities of the synthetic material and benzylpenicillin against seven bacteria. The two products gave the same relative responses (*6*).

This similarity of the synthetic material to benzylpenicillin in stability and "bacterial spectrum" offered strong evidence in support of the theory that penicillin was actually being synthesized in this reaction, but stronger evidence was soon obtained by the use of the isotope "tracer" technique (*7*). *dl*-Penicillamine containing radioactive sulfur was prepared and condensed with 2-benzyl-4-methoxymethylene-5(4)-oxazolone. Natural benzylpenicillin was added to the condensation product and isolated as the triethylammonium salt. This crystalline triethylammonium benzylpenicillin contained radioactive sulfur which was retained through a number of recrystallizations and through conversion to two derivatives, namely, sodium benzylpenicillin and *benzylpenillic* (G-penillic) acid. Within experimental error the content of radioactive sulfur of the two derivatives remained constant.

Further evidence for the presence of penicillin in the synthetic reaction mixture was obtained through the use of the enzyme penicillinase (*8*). This enzyme, which rapidly inactivates penicillins, destroyed the antibiotic activity present in the synthetic reaction mixture.

The above evidence for the identity of the active material in the condensation reaction stimulated extensive investigations on the conditions of the reaction and also led to experiments designed to concentrate the antibiotic activity in the reaction mixture. Although no significant improvement in the yield of antibiotic activity in the synthetic reaction was obtained, some success in the concentration of the antibiotic activity was realized.

Partition chromatography of the condensation mixture over silica at pH 7 resulted in the ultimate isolation in The Upjohn Company laboratories of material with an activity of 44 units/mg. (*9*). A procedure of fractionation of the condensation mixture, based on the "counter-current distribution" principle (*10*), led to the preparation in our Laboratory of concentrates with activities of 30–50 units/mg., and in one instance a fraction with an activity of 275 units/mg. was isolated (*11*).

It was found that concentrates of 30–50 units/mg., when examined in the infrared region, showed an absorption band at 5.63 μ.[1] This is the region of a characteristic absorption band in penicillin. A comparison was made of the *in vivo* excretion of concentrates of the synthetic activity and natural benzylpenicillin in rabbits. It was found that the ratio of excreted activity to that administered was the same within experimental error (*11*). Furthermore, the distribution coefficients for the synthetic and natural products were found to be the same in five different solvent pairs (*11*).

This overwhelming accumulation of evidence toward identity of the synthetic activity with benzylpenicillin warranted the conclusion that the condensation of *d*-penicillamine hydrochloride and 2-benzyl-4-methoxymethylene-5(4)-oxazolone represented a synthesis of benzylpenicillin, albeit in minute yield (*11*). Nevertheless, it was recognized that the only unequivocal confirmation of this conclusion lay in the actual isolation of the crystalline synthetic benzylpenicillin from the reaction mixture. This has now been

[1] The infrared measurements, carried out in the Physics Department of the University of Michigan, were performed on materials prepared in both the Upjohn and Cornell laboratories.

obtained through recent investigations in this Laboratory, which have culminated in the isolation of benzylpenicillin from the reaction mixture as the crystalline triethylammonium salt (*12*).

In the synthesis of benzylpenicillin for the isolation experiments a new procedure, which arose from studies on the mechanism of the synthetic reaction, was used. It had been found that when equimolar quantities of *d*-penicillamine hydrochloride and 2-benzyl-4-methoxymethylene-5(4)-oxazolone were condensed in pyridine containing triethylamine, a product was formed which was apparently free of starting material but possessed no antibiotic activity. When this product was then heated in pyridine containing pyridinium chloride, antibiotic activity was produced. These findings were utilized in modifying the original procedure. In the first step the *d*-penicillamine hydrochloride and the oxazolone were condensed to give a biologically inactive intermediate product, to be described later, which in the second step was activated by heating in pyridine containing pyridinium chloride. By this method a readily reproducible yield of activity was obtained in the synthesis, and the product so obtained appeared much more amenable to fractionation in our hands than products obtained by the one-stage synthesis.

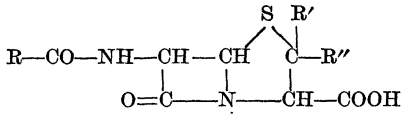
The above synthesis resulted in a reaction mixture which contained activity equivalent to about 0.1 per cent benzylpenicillin. The difficulty of isolation of the penicillin from the reaction mixture was enhanced by its innate instability. The method of fractionation that met with success in this particular case was based on the "counter-current distribution" principle (*10*). However, the isolation of the crystalline triethylammonium salt in amounts large enough for full characterization was realized only after careful standardization of each step of the procedure.

A preliminary concentration of the active material was effected by extraction of the penicillin from chloroform into 1.31 M phosphate buffer at pH 5.2. This was followed by an 8-plate "counter-current distribution" of the active material between ether and 2 M phosphate buffer at pH 4.88. The material obtained from the most active fractions of the 8-plate distribution was dissolved in chloroform, and a further concentration was obtained by extraction of the penicillin with 2 M phosphate buffer (pH 4.88). Finally, the last material was subjected to a 25-plate "counter-current distribution" between chloroform and 2 M phosphate buffer at pH 4.88. Crystals of the crude triethylammonium benzylpenicillin were prepared from the most active fractions of the 25-plate distribution. The salt was purified by crystallization from methylene dichloride by the addition of ether and finally recrystallized from acetone.

The melting point, ultraviolet and infrared absorption spectra, refractive indices, antibiotic activity, and specific rotation of the isolated triethylammonium salt of the synthetic material agreed, within the limits of experimental error, with the triethylammonium salt of natural benzylpenicillin. It is particularly noteworthy that the material isolated from this reaction mixture was the same optical isomer as the natural penicillin.

The isolation of benzylpenicillin from this reaction mixture proved conclusively that penicillin can be synthesized. Because of the obscurity of the reaction mechanism, the synthesis, at this stage of development, cannot be used as synthetic proof of structure of penicillin. However, as long as an unequivocal synthesis of penicillin by any other approach has not been demonstrated, the study of the mechanism of the above synthesis is extremely important. Such a study might lead either to a proof of structure or to an improved method of synthesis or both.

It should be pointed out that even at the present time it is possible to make, although in minute yields, new pencillins by the above reaction. The isolation of benzylpenicillin from the reaction of 2-benzyl-4-methoxymethylene-5(4)-oxazolone with *d*-penicillamine hydrochloride makes it clear that the antibiotic activity produced when oxazolones (*13*), substituted with other groups in the 2-position, were condensed with penicillamine was due to the synthesis of penicillins differing from benzylpenicillin in the nature of the group R, as illustrated on the basis of the β-lactam structure (IV). Moreover, the production of antibiotic activity by the condensation of 2-benzyl-4-methoxymethylene-5(4)-oxazolone with various α-amino-β-mercapto acids other than *d*-penicillamine, such as *d*-cysteine, the thiolthreonines, and β-mercaptoleucine (*14*), indicates the synthesis of analogues of penicillin differing from the known varieties of penicillin in the nature of the groups R′ and R″ (IV). All the penicillins so far produced by the mold have contained *d*-penicillamine; the above observation opens the way to the synthesis, from other α-amino-β-mercapto acids, of a series of entirely new and different penicillins which might possess desirable therapeutic properties.



(IV)

(*See page 450 for list of references.*)

Book Reviews

Mathematical methods of statistics. Harald Cramèr. Princeton, N. J.: Princeton Univ. Press, 1946. Pp. xvi + 575. (Illustrated.) $6.00.

The modern development of statistics has brought with it the use of mathematical techniques which have in the past been confined to the repertory of professional mathematicians. Unfortunately for nonmathematicians, the theory of probability, which is essential as a background to statistics, involves the use of advanced measure and integration theory, and the concomitant general apparatus of the theory of functions of real variables. Cramér's book is doubly welcome in that it contains both a remarkably complete treatment of the mathematical background to statistics and a treatment of statistical methodology itself, a combination not previously available.

The book is divided into three parts. The first part is devoted almost wholly to measure and integration. The second part gives the basic knowledge of probability required for statistics. The author's general point of view makes this part a study of random variables; games, permutations, combinations, etc., so dear to the hearts of probability textbook writers, are omitted, except as implicit in the binomial distribution. The development is carried through the central limit theorem (on the approximation to normality of the sum of a large number of independent random variables) and the law of large numbers. The problem of measure in infinitely many dimensions is avoided; probably for this reason the concept of convergence with probability 1 is omitted, and the function theoretic significance of the concept of convergence in probability (convergence in measure) is not explained. The third part of the book, comprising somewhat less than half, is devoted to statistical inference, subdivided into sampling distributions, tests of significance, and the theory of estimation. Periodogram analysis and the related theoretical problems of random processes are not treated because of lack of space.

The text presupposes a knowledge of calculus and familiarity with limiting processes. The writing, although almost always mathematically rigorous, does not sacrifice space to rigor. It is frankly a mathematics text, however, in spite of the numerous statistical examples, and as such is not for casual reading by outsiders. The full bibliography simplifies more extended study.

Both probability and statistics have been completely revolutionized in the 20th Century. The first has been put on a firm mathematical foundation, as rigorous as any other branch of analysis; the second, basing itself on probability, has been enabled to develop the delicate and elaborate techniques which give it its present importance. The author has succeeded admirably in his stated purpose of writing an exposition of the two fields, in their interrelations, from the modern point of view.

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(*Continued from page 433.*)

References

1. The synthesis of *d*-penicillamine hydrochloride was first described by E. P. Abraham, E. Chain, W. Baker, J. W. Cornforth, R. H. Cornforth, and R. Robinson in a report dated 4 October 1943.

2. The synthesis of several compounds of this type was announced by the investigators of Merck and Company, Inc., in a report dated 31 January 1944; the details of the preparation of 2-benzyl-4-methoxymethylene-5(4)-oxazolone were described in a report dated 17 March 1944. The synthesis of 2-phenyl-4-ethoxymethylene-5(4)-oxazolone was described by J. P. Wilson, J. B. Jepson, G. M. Robinson, E. P. Abraham, W. Baker, E. Chain, and R. Robinson in a reported dated 14 March 1944.

3. Merck and Company, Inc., report dated 31 January 1944. In a report dated 29 February 1944 the details of the synthesis of a product possessing antibiotic activity (1 unit/mg.) were described by the Merck group. In this procedure *d*-penicillamine and 2-benzyl-4-methoxymethylene-5(4)-oxazolone were heated in pyridine at 75° for 1½ hours.

4. G. M. Robinson, E. P. Abraham, W. Baker, E. Chain, and R. Robinson, report dated 27 March 1944. In consequence of the appreciable time lag in the exchange of technical information in the international collaboration, the Merck report dated 31 January 1944 (*3*) arrived in England after the submission of the report by the Oxford group.

5. Merck and Company, Inc., report dated 30 September 1944.

6. Department of Biochemistry, Cornell University Medical College, report dated 1 March 1945.

7. Department of Biochemistry, Cornell University Medical College, report dated 1 November 1945.

8. E. P. Abraham, W. Baker, E. Chain, and R. Robinson, report dated 26 November 1945.

9. The Upjohn Company, report dated 30 November 1945.

10. L. C. Craig. *J. biol. Chem.*, 1944, **155**, 519; L. C. Craig, C. Golumbic, H. Mighton, and E. Titus. *J. biol. Chem.*, 1945, **161**, 321.

11. Department of Biochemistry, Cornell University Medical College, report dated 15 December 1945.

12. The use of triethylamine to prepare a crystalline salt of natural benzylpenicillin was first described by the investigators of the Heyden Chemical Corporation in a report dated 22 May 1944.

13. Some other 5(4)-oxazolones which have been reported to give activity in this reaction are: 2-phenyl-4-ethoxymethylene (J. P. Wilson, J. B. Jepson, G. M. Robinson, E. P. Abraham, W. Baker, E. Chain, and R. Robinson, 14 March 1944); 2-propyl-4-hydroxymethylene and 2-amyl-4-hydroxymethylene (J. Cornforth, R. H. Cornforth, E. P. Abraham, W. Baker, E. Chain, and R. Robinson, 1–2 May 1944); 2-*p*-nitrostyryl-4-ethoxymethylene and 2-*p*-nitrophenyl-4-hydroxymethylene (F. C. Copp, W. M. Duffin, S. Smith, and S. Wilkinson, 29 November 1944); 2-*p*-nitrobenzyl-4-ethoxymethylene and 2-methyl-4-ethoxymethylene (Department of Biochemistry, Cornell University Medical College, 2 July 1945); 2-*n*-amyl-4-methoxymethylene (Merck and Company, Inc., 31 August 1945); 2-β-phenylethyl-4-methoxymethylene, 2-phenyl-4-thiolmethylene, and 2-phenyl-4-N-acetylanilinomethylene (G. M. Robinson, E. P. Abraham, W. Baker, E. Chain, and R. Robinson, 26 November 1945); 2-phenyl-5-chloro-oxazole-4-aldehyde also gives rise to penicillin-like activity when condensed with *d*-penicillamine (L. J. Goldsworthy, R. Robinson, E. P. Abraham, W. Baker, and E. Chain, 26 November 1945).

14. Department of Biochemistry, Cornell University Medical College, reports dated 1 March and 1 December 1945; Merck and Company, Inc., report dated 28 September 1945.