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The role of D-benzylpenicillenic acid in the sensitization of erythrocytes

The discovery by LEY *et al.*[1] that erythrocytes that had been incubated with penicillin were agglutinated by the sera of some patients with penicillin allergy provided a valuable method for the study of this condition. This procedure has been used by several workers as a diagnostic test[2,3], and some characteristics of the antibody have been established[2,4]. However, there is still little known about the mechanism of attachment of the penicillin molecule to the erythrocytes, a matter of importance in any attempt to describe the penicillin-antibody interaction on a molecular level.

Recent work by DE WECK AND EISEN[5] and LEVINE[6] has suggested that penicillenic acid, a spontaneously formed, unstable breakdown product which can combine irreversibly with protein, is responsible for the antigenicity of penicillin. In order to investigate the possible role of this and other breakdown products in the agglutination phenomenon we have attempted to sensitize erythrocytes with five penicillins now in common use, G, V, O, α-phenoxyethylpenicillin and 2,6-dimethoxyphenylpenicillin; 6-aminopenicillanic acid; *dl*-penicillamine; and the following breakdown products of penicillin G: D-Benzylpenicillenic acid, penillic acid, penilloic acid and penicilloic acid*. The last three compounds were prepared by well-known methods, were recrystallized from appropriate solvents and had melting points in agreement with published values[7]. D-Benzylpenicillenic acid was prepared according to the method of LEVINE[8]. The D isomer of this compound has not been crystallized alone, but the above procedure gives preparations with purities as high as 90–95% when assayed by the ultraviolet absorption at 322 mμ.

The general procedure for sensitizing cells is as follows: Equal volumes of a 40% suspension of erythrocytes previously washed in 0.15 M phosphate–saline buffer (pH 7.2) and a 0.1 M solution of the sensitizing agent in the same buffer are mixed and incubated for 1 h at 37°. The cells are then washed with the buffer and tested by addition to serum prepared by immunization of rabbits with penicillin G following the method of JOSEPHSON[9].

Cells prepared in this way with penicillins G, V, O, α-phenoxyethylpenicillin and 2,6-dimethoxyphenylpenicillin gave positive agglutination tests, while penillic acid, penilloic acid, penicilloic acid and penicillamine gave negative results. 6-Aminopenicillamic acid gave anomalous results in that when it was dissolved in an $NaHCO_3$ solution before addition to the erythrocytes, as was done by CHISHOLM *et al.*[10], the results were positive, while they were negative if the cells were treated the same way but without the bicarbonate.

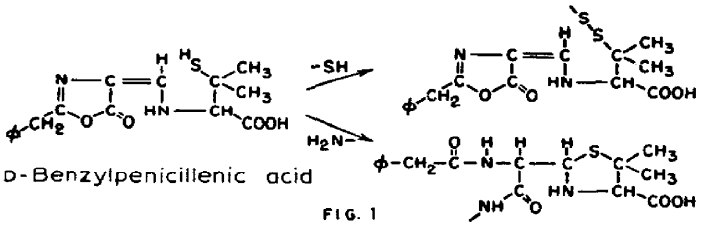
When erythrocyte ghosts are substituted for whole cells in the above procedure, those incubated with penicillin G have the ability to adsorb the agglutinating factor from active sera, while control ghosts handled in the same way but without the penicillin do not do this, thus showing that the penicillin is attached to the cell wall, and that integrity of the cell structure is not necessary for the attachment to occur.

For D-benzylpenicillenic acid which is unstable and only slightly soluble in aqueous solution, a modified procedure was used. 1 ml of a 40% suspension of washed

* We gratefully acknowledge the following sources of the compounds used here: Penicillin G, Squibb Institute, New Brunswick, N.J.; Penicillin V, Eli Lilly and Company, Indianapolis, Ind.; Penicillin O, The Upjohn Company, Kalamazoo, Mich.; 2,6-dimethylphenylpenicillin, α-phenoxyethylpenicillin and 6-aminopenicillamic acid, Bristol Laboratories, Inc., Syracuse, N.Y. (U.S.A.).

erythrocytes was allowed to stand at room temperature for 1 h. At times 0, 20 and 40 min a 0.01-ml aliquot of a solution of the agent to be tested was added, D-benzylpenicillenic acid in ethanol, the others in phosphate–saline buffer. The cells were then washed and tested as before. Penicillic acid, penilloic acid and penicilloic acid did not sensitize cells at any concentration tried. Penicillin G sensitized at a minimum concentration of 0.25 M in the added aliquots, while D-benzylpenicillenic acid required a concentration of only 0.025 M. Since the two major breakdown products of D-benzylpenicillenic acid under these conditions are penillic acid and penicilloic acid, neither of which sensitize cells at even much higher concentrations, it seems likely that D-benzylpenicillenic acid itself is responsible for sensitization. That the penicillenic acid form is present in solutions of the 5 penicillins that do give positive reactions is shown by the increasing absorption at 322 mμ of solutions of these compounds incubated at 37° in the phosphate–saline buffer. Absorption at this wavelength is specific for penicillenic acid among the penicillin breakdown products.

LEVINE[8] has demonstrated two reactions which proceed under conditions similar to those used here by which D-benzylpenicillenic acid could combine with protein: by forming mixed disulfides with protein sulfhydryl groups and by forming α-amides of penicilloic acids with free amino groups (Fig. 1). Either of these methods would provide a strong covalent bond between the penicillin molecule and the cell wall, which would explain the common finding that the cells remain sensitized for as long as two weeks despite frequent washings. The weak, reversible binding known to occur between penicillin and plasma proteins[11,12] could not explain such behavior.



D-Benzylpenicillenic acid

FIG. 1

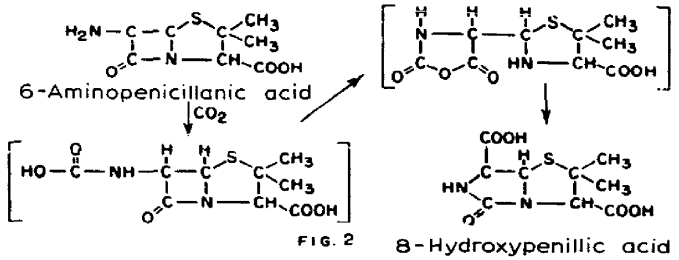


FIG. 2

8-Hydroxypenillic acid

At present it is not possible to make a definite choice between the sulfhydryl and the α-amide mechanism of attachment, but we tend to favor the latter for two reasons. First, the sulfhydryl attachment leaves exposed to the antibody a molecular configuration that bears little resemblance to penicillin itself, while the α-amide reaction provides a penicilloic acid moiety. Since free penicillin and penicilloic acid are known to inhibit the agglutination reaction, it seems likely that the antibody is designed to fit a structure similar to them. If the antigenicity of penicillin is due to its combining with tissue protein through the α-amide reaction as has been suggested

by LEVINE, the antibody may indeed be designed to fit penicilloic acid rather than penicillin itself. This would explain our finding that penicilloic acid and penilloic acid are actually better inhibitors than any of the complete penicillins. Also this model explains the apparent paradox that penicilloic acid while inhibiting agglutination does not itself cause sensitization, for penicilloic acid itself would not be expected to form the α-amide bond under the conditions used here.

The second reason arises from the CO_2 dependence of sensitization with 6-aminopenicillanic acid. JOHNSON AND HARDCASTLE[13] have recently described a reaction of 6-aminopenicillanic acid with CO_2 to produce a substance which they have named 8-hydroxypenillic acid (Fig. 2). The proposed intermediate contains an oxazole ring similar to D-benzylpenicillenic acid which should likewise be susceptible to nucleophilic attack to form the α-amide of a penicilloic acid. This reaction would not be possible in the absence of CO_2. In this case the possibility of binding through the sulfhydryl group would not be available.

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