A NEW CHEMICAL ASPECT OF PENICILLIN ALLERGY: THE DIRECT REACTION OF PENICILLIN WITH ε-AMINO-GROUPS

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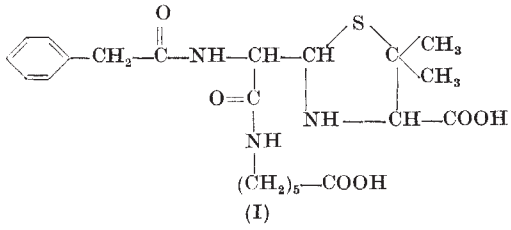
IT seems well established that the penicilloyl group bound to carrier protein structures *in vivo* is a main antigenic determinant in penicillin allergy[1–3]. On the other hand, the chemical basis for the formation of these penicilloyl protein conjugates has not yet been satisfactorily elucidated. Earlier views on the formation of the penicilloyl haptenic structure have held that a highly reactive degradation product of penicillin, penicillenic acid, might be the only form in which penicillin reacts irreversibly with proteins. Penicillenic acid is, in fact, spontaneously formed from penicillin in neutral solution and is able to react with free amino-groups, thereby forming a penicilloyl-amide[4]. The possibility that penicillins could react directly with ε-amino-groups by an opening of the β-lactam ring has been neglected mainly because an experimental demonstration of the reaction between penicillins and such amino-groups on proteins, peptides or low molecular weight amines in neutral aqueous solution was lacking[5].

Two facts have been known for some time to throw some doubt on the concept of penicillenic acid being a main intermediate in the formation of the penicilloyl haptenic structure: (*a*) There is no correlation between the *in vitro* rate of formation of penicillenic acid from various penicillins bearing different acyl side-chains and the immunogenicity of these penicillins; (*b*) 6-aminopenicillanic acid (6-APA), being structurally incapable of rearrangement to a penicillenic acid intermediate, was shown to be a strong inducer of the allergic state in rabbits[6].

In experiments initiated by these considerations, the incubation of penicillins with amines in neutral aqueous solution was examined. It was established that penicillins do react with amines under such conditions to yield penicilloyl-amides. In this article we report in a preliminary form some of our results together with findings obtained with dinitrophenyl-6-aminopenicillanic acid, demonstrating that the direct reaction of the β-lactam of penicillins with amines is an important general route to the penicilloyl-amide antigenic determinant. (A full account of this work will be published in *Helv. Chim. Acta.*)



(I)

Benzylpenicillin (0·5 M) was incubated at 37° with ε-aminocaproic acid (molar ratio, 1 : 4·25) in 0·5 M phosphate buffer *p*H 7·4, and also in 0·1 M sodium bicarbonate at *p*H 7·4. Additions of small amounts of sodium hydroxide kept the *p*H at the desired value. After one day a viscous gum was precipitated from the reaction mixture by acidification. From the precipitate, crystalline benzylpenicilloyl-ε-aminocaproic acid (I) in the form of its dibenzylammonium salt was obtained. The products obtained in the two runs agreed in properties (m.p. 110°–113° and 111·5°–113°. Corrected capillary melting points are reported; $[\alpha]_D^{24} = +69{\cdot}7°$ and $+69{\cdot}2°$ (c = 1, water); penamaldate assay[7]: Em = 24,000) with an authentical sample of benzylpenicilloyl-ε-aminocaproic acid dibenzylammonium salt prepared in alkaline solution. Mixed melting points were not depressed. The yields were 20–30 per cent. In addition, benzylpenicillin was similarly incubated at physiological *p*H with poly-L-lysine of mean molecular weight about 1,000, the molar ratio of penicillin to ε-amino-groups being 1 : 0·4. Passage of the incubation mixture through a 'Sephadex *G*-25' column neatly separated the polylysine material (peak I) from the residual material (peaks II and III) (Fig. 1). Penicilloyl groups attached to the polylysine were measured by a modified penamaldate assay[7]. In accordance with the behaviour expected for a benzylpenicilloyl polylysine[7], the material from the column (peak I) specifically precipitated antisera from rabbits which had been immunized with benzylpenicilloyl-bovine γ-globulin.

Other penicillins were incubated for 4 h at 37° with polylysine in 0·05 M phosphate buffer *p*H 7·4 at a concentration of 75 mM, the molar ratio of penicillin to ε-amino-groups being 1 : 1. Polylysine material was isolated from the incubation mixture through 'Sephadex *G*-25' gel-filtration and the amount of penicilloyl-amide on the polylysine was established by penamaldate analysis. That no other material developing under the conditions of the incubation and detectable by penamaldate assay, was carried with the polylysine peak, was verified in each case by a run in which polylysine was added only at the end of the incubation period. The polylysine peaks

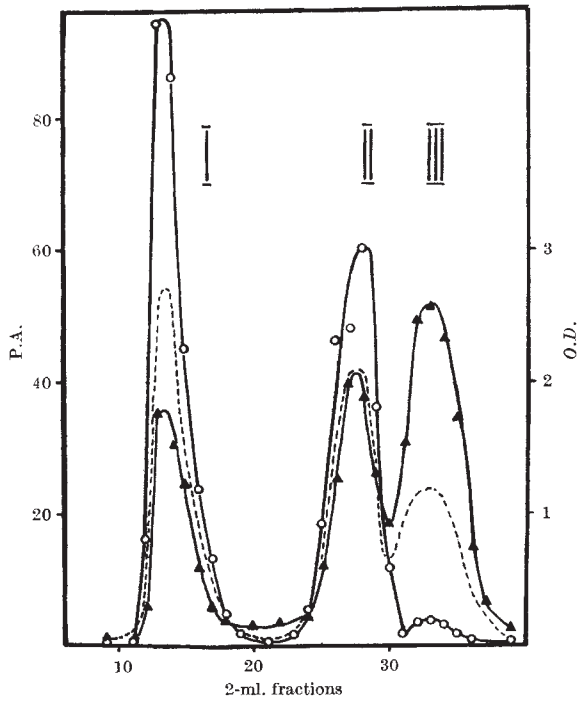


Fig. 1. Gel-filtration of 1 ml. of a solution of benzylpenicillin (250 mg) and polylysine (40 mg) in 4·0 ml. of McIlvaine's citrate-phosphate buffer incubated for 24 h at 37°, *p*H 7·4. The column (1·8 cm × 30 cm) containing 'Sephadex *G*-25' was equilibrated and eluted with McIlvaine's citrate-phosphate buffer. Analysis of eluate: ○, penamaldate assay (P.A.) as increase in optical density at 282 mμ after $HgCl_2$ treatment: ▲, optical density at 257 mμ; – – – Folin-Lowry colour (ref. 8) as optical density at 750 mμ. Optical measurements were made in 1 cm cells

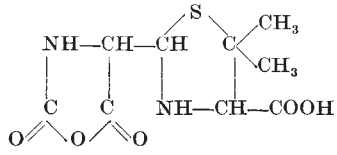
Table 1. Aminolysis of Penicillins and Degradation to Penicillenic Acid

Penicillins	Penicilloyl groups on polylysine after incubation of 300 μmoles of penicillins with excess polylysine for 4 h at *p*H 7·4, at 37° μmoles*	Degradation to penicillenic acid at 37°, from (ref. 6) Mole parts per thousand per min at *p*H 4·0	Mole parts per thousand per h at *p*H 7·4
Benzylpenicillin	19·3	2·0	0·26
Phenoxymethylpenicillin	21·1	0·06	0·008
Allylthiomethylpenicillin	22·7	0·5	0·06
Phenoxyethylpenicillin	22·6	0·08	0·01
Dimethoxyphenylpenicillin	12·3	2·5	0·29

* Calculated from the penamaldate assay with a mean molar extinction of 24,000.

obtained in these control runs did not contain significant amounts of material detectable by penamaldate analysis. The results are shown in Table 1.

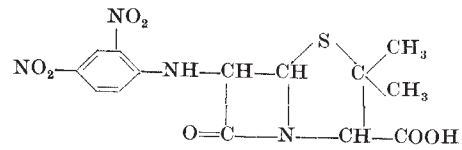
These experiments demonstrate the formation of the penicilloyl-amide bond in aqueous solutions at physiological *p*H. They make it unlikely that the route to penicilloyl-amides proceeds in general through penicillenic acid as a main intermediate, since no correlation between conversion to penicillenic acid of the various penicillins and the formation of penicilloyl polylysine is found (Table 1).



(II)

However, the use of a penicillin-like molecule unable to undergo the penicillenic acid rearrangement, but capable of acylating amines in neutral aqueous solution and of inducing the formation of specific antibodies *in vivo*, is required in order to demonstrate unambiguously that the direct acylation of amino-groups by the penicillin molecule through opening of the β-lactam ring, without involvement of a penicillenic acid type intermediate, occurs and constitutes an important route *in vivo* to the penicilloyl-amide antigenic determinant. 6-APA would seem to be a good choice in this respect. However, this compound is peculiar as it reacts easily with carbon dioxide, thereby yielding 8-hydroxypenillic acid[9–11]. The reaction probably involves a CO_2-6-APA adduct (II) (ref. 9) as an intermediate which conceivably could react with amines. Furthermore, 6-APA is capable of intermolecular acylation by reaction of the amino-group of one 6-APA molecule with the β-lactam ring of another. On repetition of this process, polymers of 6-APA are formed for which evidence has been reported[12,13]. Since the dimer penicillanyl-aminopenicillanic acid as well as the higher polymers are, in fact, penicillins, they are in principle capable of the penicillenic acid rearrangement. Therefore, the occurrence of a reaction between 6-APA and amines at neutrality could be considered as proof of a direct reaction of the β-lactam with amines only if the reaction products were identified as penicillanyl amides, or if additional information indicated that the contribution of the polymerization under the conditions used is negligible. On the other hand, the first step of the 6-APA polymerization, the *N*-acylation of a 6-APA molecule by a second one, obviously cannot proceed via a penicillenic acid intermediate and constitutes a demonstration of the direct attack of an amino-group on the β-lactam. However, the equivalence in this respect of the reacting amino-group of 6-APA and ε-amino-groups could not be taken for granted.

We have obtained preliminary evidence that 6-APA reacts with polylysine in neutral aqueous solution in a nitrogen atmosphere free of carbon dioxide to yield a conjugate which specifically precipitated a rabbit anti-benzylpenicilloyl antiserum. The reaction was followed by penamaldate assay. It seemed diminished in sodium bicarbonate solution. We have further observed that, in the absence of polylysine and carbon dioxide, a considerable increase of material showing a penamaldate assay results on incubation of 6-APA alone in neutral solution. This could be due to hydrolysis but in part also to polymerization of 6-APA.



(III)

In view of the complications connected with 6-APA work, we proceeded with dinitrophenyl-6-aminopenicillanic acid (DNP-APA) (III). This substituted 6-APA contains all the features of penicillins required for the demonstration of a direct aminolysis of the β-lactam since a penicillenic acid rearrangement cannot occur and potential side-reactions of 6-APA are avoided by the blocking of the 6-amino-group. The compound was prepared by reacting 6-APA with dinitrofluorobenzene in a mixture of acetone and sodium bicarbonate solution. The DNP-APA could be well crystallized as the free acid from ether and from acetone–water (m.p. 164°. Analysis, calc. for $C_{14}H_{14}O_7N_4S$: C, 43·98; H, 3·69; N, 14·65; found: C, 44·23; H, 3·90; N, 14·58; neutralization equivalent, calc. 382·3; found: 383).

This compound (2·5 g) was incubated with ε-aminocaproic acid (3·8 g) in 0·1 M phosphate buffer (8 ml.) at 37° for one day. The incubation solution was kept at *p*H 7·2 with the aid of a *p*H-stat, delivering 1 N sodium hydroxide to the reaction vessel. Dinitrophenyl-6-aminopenicillanyl-ε-aminocaproic acid was isolated in the form of its crystalline dibenzylammonium salt in nearly 40 per cent yield (m.p. 135°–137°. Analysis, calc. for $C_{34}H_{45}O_9N_7S$: C, 56·10; H, 6·23; N, 13·47; found: C, 55·84; H, 6·71; N, 12·93; penamaldate assay: E_m = 25,000). Manipulations were performed in subdued light, since the desired product as well as DNP-APA itself are light-sensitive in neutral solution.

The formation of dinitrophenyl-6-aminopenicillanyl-ε-aminocaproic acid from DNP-APA and ε-aminocaproic acid in 0·05 M phosphate buffer at *p*H 7·4 was compared to the formation of benzylpenicilloyl-ε-aminocaproic acid from benzylpenicillin and ε-aminocaproic acid under the same conditions. To this end, solutions (0·36 M) of DNP-APA and benzylpenicillin in 0·05 M phosphate buffer were incubated in a *p*H-stat at *p*H 7·4 at 37° alone and in the presence of 1·45 M ε-aminocaproic acid. The opening of the β-lactam ring was followed by penamaldate assay[14]. Figs. 2 and 3 show the very similar rates of

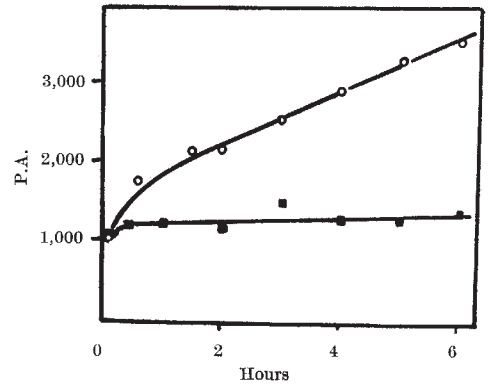


Fig. 2. Incubation of DNP-APA at *p*H 7·4, at 37°. ■, Alone; ○, in the presence of ε-aminocaproic acid. Penamaldate assay (P.A.) as increase in optical density at 282 mμ after $HgCl_2$ treatment (1 = 1 cm)

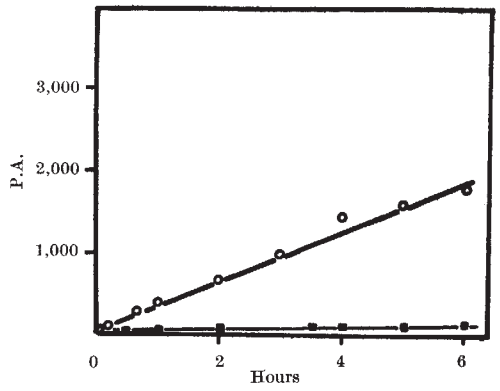


Fig. 3. Incubation of benzylpenicillin at *p*H 7·4 at 37°. ■, Alone; ○, in the presence of ε-aminocaproic acid. Penamaldate assay (P.A.) as increase in optical density at 282 mμ after $HgCl_2$ treatment (1 = 1 cm)

formation of the dinitrophenyl-6-aminopenicillanyl group and the benzylpenicilloyl group in the presence of ε-aminocaproic acid. The rate of hydrolysis of both DNP-APA and benzylpenicillin appears negligible under the conditions used. This result indicates similar rates of aminolysis for DNP-APA and benzylpenicillin and implies that the contribution of the penicillenic acid route to the aminolysis of benzylpenicillin could only be small under the *in vitro* conditions used.

The immunogenicity of DNP-APA was tested in rabbits. Animals were injected with DNP-APA and complete Freund's adjuvant (Difco) according to a procedure described previously[1]. Antibodies specific for the dinitrophenyl-6-aminopenicillanyl determinant were demonstrated in the rabbit sera by haemagglutination[1]. For the haemagglutination reaction, rabbit antisera absorbed with normal sheep erythrocytes were incubated with sheep erythrocytes previously treated with DNP-APA or benzylpenicillin in 0·1 M veronal buffer at *p*H 9·0. The haemagglutination titres of anti-DNP-APA sera with DNP-APA treated cells were comparable to those obtained with anti-benzylpenicillin rabbit sera and benzylpenicillin treated cells. The haemagglutination reaction was specifically inhibited by dinitrophenyl-6-aminopenicillanyl-ε-aminocaproic acid and by benzylpenicilloyl-ε-aminocaproic acid. The former compound was a more effective inhibitor. Only 1×10^{-4} M dinitrophenyl-6-aminopenicillanyl-ε-aminocaproic acid was required in order to achieve the same degree of haemagglutination inhibition as obtained with 6×10^{-3} M benzylpenicilloyl-ε-aminocaproic acid.

The experiments described here demonstrate the reaction of penicillins with ε-amino-groups at physiological *p*H. It is shown that the resulting penicilloylamides are formed by direct penicilloylation without necessarily involving a penicillenic acid intermediate. Even in the case of benzylpenicillin, the contribution of the penicillenic acid route appears small under certain *in vitro* conditions. DNP-APA, which has no possibility of reacting via a penicillenic acid intermediate, was shown to be immunogenic in rabbits. It is therefore suggested that the direct penicilloylation of carrier protein structures is a general route to the penicilloyl antigenic determinant *in vivo*.

While this article was being prepared, the paper by Batchelor, Dewdney and Gazzard[15] came to our notice. It is remarkable that some of the lines of thought were quite similar in the two laboratories.

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OSMOTROPOTAXIS IN THE HONEY-BEE

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OLFACTORY orientation in insects can follow two different paths: (*a*) Sampling of the scented area with movable antennae in temporal sequence makes it possible to perceive differences in the concentration of the scent and to make adjustments in a klinotactic orientation. This alone can be carried out with a single sensory organ on one side. (*b*) Simultaneous spatial perception of the scent gradient by both antennae: the animals succeed in orienting themselves tropotactically to the scent source. That two sense organs lie symmetrically on the body must be assumed (theoretically receptor can replace the word sense organ[1]).

Many authors believe that osmotropotactic orientation can be shown in one-antenna animals. One-antenna animals move in circles toward the antenna side in a scent current, in a different scent area, and also in a scent gradient, *Geotrupes silvaticus* Panz. and *Geotrupes vernalis* L.[2], *Calliphora erythrocephala*[3], *Leptinotarsa decemlineata* Say[4], meal-worm beetle[5], *Drosophila melanogaster*[6,9], *Habrobracon juglandis* Ashmead[7], *Ips curvidens*[8], and silkworm[10].

On the other hand, experiments have been performed with one-antenna animals as proof of a klinotactic olfactory orientation. The animals with one antenna still show a fully developed olfactory orientation; sometimes they show only a slight deviation towards the antenna side. Attaining the goal results from klinotaxis, and for this one antenna suffices for *Rhodnius prolixus*[11], *Nemeritis canescens*[12] and *Geotrupes silvaticus* Panz[13].

Other authors discuss olfactory orientation as quite a different mechanism. The scent acts only as a stimulant. Actual finding of the objective is only possible when an air current gives the animal the chance to fly rheotactically against the scent gradient[6,14–17].

For the first time we have succeeded in showing osmotropotactic orientation in a scent area for bees[18,19]. The way it works, its quantitative effect, and its interaction with osmoklinotaxis are described in this article.