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REVIEW ARTICLE

Chemical Aspects of Penicillin Allergy

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While allergic reactions have been noted with many drugs, they are observed more often with the penicillins than any other group of compounds and penicillin allergy has been and remains a major clinical problem. With the advent of the semisynthetic penicillins following the isolation of 6-aminopenicillanic acid in 1959 (1) it was hoped that a nonallergenic penicillin could be found. Despite the fact that this has not been achieved, the intensive basic research effort resulting from the revived interest in the penicillins has yielded a great deal of new information concerning the chemical and immunologic mechanisms underlying allergic reactions to these drugs.

It is safe to say that without a knowledge of the chemical and biochemical behavior of the penicillins, success in studying their allergenicity could not have been achieved. This review is devoted to a consideration of those chemical reactions of penicillins, both *in vitro* and *in vivo*, which have been, or may be, implicated in allergy. While these topics cannot be entirely divorced from the immunologic and clinical aspects of the problem, the limitations of space preclude consideration of the types of antibodies involved, the clinical manifestations of allergy, or the immunologic and clinical test methods. These topics have been well documented in a number of reviews to which the reader is referred (2–7).

Biochemical Basis of Drug Allergy—Allergy is mediated by antigen-antibody reactions, the antibody having been formed as a response to exposure of the individual to an immunogenic substance. It is a generally accepted hypothesis that drug molecules of low molecular weight are nonimmunogenic themselves, but must first combine irreversibly with a tissue macromolecule in order to elicit antibody formation (8–10). The macromolecule is usually a protein but may be a high molecular weight carbohydrate or lipid. Antibodies formed in response to exposure to the hapten-protein conjugate have some degree of specificity for the hapten group *and* usually for portions of the protein carrier.

The requirement of combination of small molecule to a macromolecular carrier has been established empirically from the work of a number of investigators which showed that only those compounds capable of reaction with proteins were able to induce an immune response.

Once antibody is formed, it can react with an antigen, presumably formed on subsequent exposure to drug, and initiate the allergic reaction. The antibody may also react with an antigen resembling, in chemical structure, the hapten to which it is specific. The extent of this *cross-reactivity* will often depend upon not only how closely the two molecules resemble each other, but also how the hapten is linked to the carrier protein, the amino acid residue to which it is conjugated, and perhaps residues surrounding the one to which the hapten is linked.

Determination of antibody specificity, that is, the structure of the antigenic determinants which elicited

antibody formation, is usually carried out by hapten-inhibition tests. Whole antigens, which can elicit antigen-antibody reactions measurable by a number of immunologic techniques, must be "multivalent." That is, they must contain more than one antigenic determinant per molecule of carrier. Antigen-antibody reactions are usually inhibited by "monovalent" haptens, *i.e.*, small molecules which consist of only a single haptenic group attached to a carrier. The carrier in the latter case might be a single amino acid, for example, and not necessarily a large molecule. The antibody usually has a certain affinity for the monovalent hapten, thus preventing combination with a multivalent antigen. The degree of inhibition will, of course, depend on the concentration of monovalent hapten and its structural similarity to the haptenic group to which the antibody is specific. By measurement of degree of inhibition of a particular antigen-antibody reaction, with monovalent haptens of known structure, the specificity of the antibody may be determined.

A careful distinction must be drawn between those substances which are immunogenic, *i.e.*, capable of eliciting antibody formation, and those which may act as antigen by reaction with existing antibody. The latter substances may or may not be immunogenic while an immunogenic substance will almost certainly react with antibody formed in response to its prior administration.

The objectives and importance of chemical studies in drug allergy thus become clear. First, the specificity of the antibodies to particular chemical structures must be elucidated. This information provides the basis for development of specific test reagents by which a particular patient's potential for allergic reaction may be accurately assessed. Such substances would be multivalent conjugates of the haptenic group to a carrier and could elicit a mild response, thus detecting the presence of antibody, but ideally should be nonimmunogenic themselves.

From a single drug a number of different antigenic determinants may form *via* degradation or metabolism. Thus, in a population of individuals allergic to the same drug, a variety of antibody specificities may be prevalent. Also, single individuals may develop antibodies to more than one antigenic determinant. A foolproof test for allergy will often require a number of specific reagents. As will be seen, this is the case with the penicillins.

With knowledge of the structure of antigenic determinants of a particular drug it may be possible to prepare monovalent derivatives which could be used to "desensitize" patients prior to administration of drug. Presumably, these derivatives could block antibody-combining sites and thus allow the drug to be utilized safely. Needless to say, such a technique would be particularly useful with penicillins which are often needed for life-threatening infection.

A third objective of studies of the chemical reactions involved in allergy to a particular drug would be the preparation of chemical modifications of the drug which would be nonallergenic, while retaining the therapeutic properties of the original compound. Since chemical modifications of penicillins and cephalosporins are made relatively easily by varying the side chains, the possibility of discovery of a nonallergenic derivative should not be overlooked.

ANTIGENIC DETERMINANTS OF PENICILLIN ALLERGY

In this section consideration will be given to the present state of knowledge of the chemical nature of the haptenic groups involved in penicillin allergy. It should be noted at the outset that only the penicilloamide group has been definitely established as a specific antigenic determinant although it is known that there are others. Examination of the chemistry of the penicillins may allow some speculation as to the nature of these other determinants.

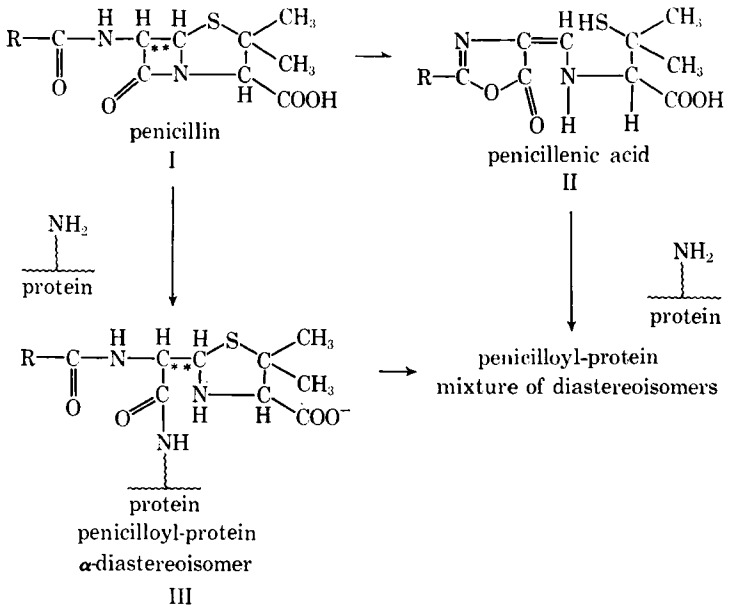
A complete treatment of the known or potential antigenic determinants of penicillins and cephalosporins[1] must include the following: (*a*) The possibility of direct reaction of drug with protein *in vivo* and the structure of the conjugate thus formed. (*b*) Degradation products of the drug which can react with protein. These may result from instability of drug or reaction with a pharmaceutical adjuvant in the dosage form. (*c*) Impurities which may be present in the drug prior to formulation into dosage forms. (*d*) Metabolites of drug, formed *in vivo* subsequent to administration, and capable of conjugating to protein.

Penicilloamide Group—Several groups (11–14) independently found that the principal antigenic determinant of penicillin allergy was the penicilloyl moiety bound to ε-amino groups of lysine residues of proteins. This was accomplished through hapten-inhibition tests in which it was found that *N*-penicilloyl-ε-aminocaproic acid (15) was the most efficient inhibitor. This substance, of course, markedly resembles ε-*N*-penicilloyl-lysine.

Two possible routes for formation of penicilloyl derivatives are apparent and are shown in Scheme I. One pathway is through rearrangement of penicillin to penicillenic acid (14, 16) which is known to be able to react quite rapidly with amines (17) and could easily form the penicilloyl conjugate. An alternate pathway is the *direct* aminolysis of penicillin by the protein. The latter route produces the α-diastereoisomer, whereas the penicillenic acid route must yield a mixture of diastereoisomers. The D-α-conjugate can, however, slowly isomerize to produce also a mixture of diastereoisomers (18).

A considerable controversy has developed as to which route is more important *in vivo*. The early evidence in favor of the penicillenic acid pathway may be summarized as follows (7): (*a*) Penicillenic acid has been detected in solutions of benzylpenicillin kept at neutral pH and even in dry crystalline material (see Table I) (19, 20). (*b*) Penicillenic acid is a very reactive compound being easily attacked by nucleophiles, *e.g.*, water and amines, to form pencilloyl derivatives (17). (*c*) Direct reaction of penicillin with amines was known to be very slow at neutral pH although the half-life of aminolysis at pH 11.5 was of the order of minutes (7).

[1] The cephalosporins are included in this review because of their close relationship to the penicillins in chemical structure, biological activity and, as will be seen, allergy problem.



Scheme I—Formation of penicilloyl-protein.

(*d*) Specificity of antibody in sera of patients known to be allergic to penicillin was found, in some cases, to be directed to a mixture of diastereoisomers of penicilloyl rather than the D-α-isomer (13, 21–24). (*e*) Penicillenic acid and benzylpenicillin were found to exhibit "allergenic equivalence" in contact sensitivity in guinea pigs (21, 22). That is, they both introduced the same antigenic determinant into epidermal proteins.

Except for *d*, this evidence is of a circumstantial nature and does not, of itself, prove that penicillenic acid is a necessary intermediate. The question of stereospecificity is also not yet resolved. Other workers have found antibodies with greater specificity for the α-diastereoisomer than a mixture (25). Furthermore, specificity toward a mixture of diastereoisomers does not necessarily require penicillenic acid mediation of conjugation to protein, since the conjugate itself may epimerize after formation by the direct route. There is now a considerable body of evidence supporting the view that direct aminolysis of penicillins is a more probable route to penicilloyl-protein *in vivo*.

It has been shown (20) that the rate of formation of penicillenic acid from penicillins is dependent upon the concentration of undissociated penicillin acid in solution and is given by Eq. 1:

$$\text{Rate} = \frac{k(\text{H}^+)\,(\text{Penicillin})}{K_a + (\text{H}^+)} \qquad \text{(Eq. 1)}$$

where k is the specific first-order rate constant and K_a is the acid dissociation constant of the drug. Since the pKa for most of the penicillins is around 2.7, the concentration of undissociated penicillin at neutral pH would be extremely low. The half-life for this reaction for methicillin at pH 7.4 at 35° would be about 1,000 days, and methicillin rearranges faster than any of the other known penicillins in clinical use.

It should be noted that if penicillenic acid is present in a solution prior to administration, it can react very rapidly with nucleophiles (17) and therefore, has the capability to be a potent sensitizer. From Eq. 1 it can be seen that formation of penicillenic acid is favored at lower pH. Thus, it would seem to be very important

Table I—Penicillenic Acid Formation from Penicillin

Penicillin	Side Chain	Penicillenic Acid Formation Rate[a]	Penicillenic Acid Found in Dry Penicillin, %[b]	Alkaline Hydrolysis Rate[c]	Rate of Aminolysis by Glycine[d]	Penicilloyl Groups Bound to Protein[e]	Penicilloyl Groups Bound to Poly-L-lysine[f] (μmole)
6-APA	—	—	—	(4)[g]	0.44[h]	0.5	—
Benzylpenicillin	$C_6H_5CH_2$—	4.4	0.0–0.35	12.5	2.3	28.6	19.3
Ampicillin	α-Aminobenzyl	—		12.3	2.7	12.0	15.1
Methicillin	2,6-Dimethoxyphenyl-	4.8	2.15	6.5	1.6	21.0	12.3
Phenoximethyl penicillin	$C_6H_5OCH_2$—	0.13	0.12	16.5	3.7	41.7	21.1
Phenethicillin	α-Phenoxyethyl	0.16	0.20	—	—	46.5	22.6
Propicillin	α-Phenoxypropyl-	—	—	—	—	42.0	—
Oxacillin	3-Phenyl-5-methyl-4-isoxazolyl	—	—	13.0	2.1	—	16.7
Cloxacillin	3-(*o*-Chlorophenyl)-5-methyl-4-isoxazolyl	—	—	20.0	—	71.0	—
Penicillin O	Allylthiomethyl-	1.1	0.36	—	—	—	22.7

[a] Micromoles/mole penicillin/min. (pH 7.4 phosphate buffer, 37°) (8). [b] Calculated from absorbance at 320–330 mμ in freshly dissolved material (pH 7.4 phosphate buffer, 4°) (8). [c] Specific (OH^-) rate constant (M^{-1} min.) at 31.5° (28). [d] Specific rate constant for glycine anion at 50° in M^{-2} min.$^{-1}$ (26). [e] Micrograms/ml. bound to human serum (29). [f] After incubation at pH 7.4, 4 hr. at 37° (27). [g] Estimated from data at 50°. [h] Units are M^{-1} min.$^{-1}$.

to buffer penicillin preparations at pH's as high as possible to minimize penicillenic acid formation.

There is considerable variation in rate of penicillenic acid formation (26, 27) (see Table I) among the various penicillins, which correlates well with their known rates of acid instability. On the other hand, the aminolysis and alkaline hydrolysis rates of penicillins do not vary widely. In the aminolysis of penicillins by glycine (26), for example (Table I), only about a two-fold variation in rate was noted between the fastest (penicillin V) and the slowest (methicillin) reacting penicillins. These rates correspond well with the alkaline hydrolysis rates of the penicillins. The variation in acid stability, on the other hand, is over 300-fold (30). Therefore, it seems probable that the penicillenic acid route is not the only mechanism by which the penicilloyl determinant can be formed under physiologic conditions.

It has been suggested that formation of penicillenic acid *in vivo* may be more rapid than *in vitro* due to enzymatic or metal-ion catalysis (7). While an enzyme catalysis cannot be entirely ruled out, no direct evidence for such a reaction has been found. In catalysis by cupric ion of penicillin degradation it has been shown that cupric ion is consumed in the reaction and *no* penicillenic acid was observed in the pH range 4–7 (31).

Aminolysis of Penicillins—While it is true that penicillins generally react relatively slowly with primary amines at neutral pH, several recent studies have shown that under certain circumstances aminolysis of penicillins can be quite rapid under conditions which could prevail *in vivo*.

First of all, studies on the mechanism of penicillin aminolysis have shown that general base or general acid catalysis seems to be required (26, 32). In the reaction with glycine (26), for example, the rate law included only dependence upon the square of glycine anion ($NH_2CH_2COO^-$) concentration. It would be expected, therefore, that compounds containing both the nucleophile (amine) and general acid-base groups could react more rapidly with penicillin. This was suggested by data obtained for reaction of penicillin with a series of aliphatic diamines $NH_2(CH_2)_nNH_2$ (n = 2–6), where both inter- and intramolecular general base catalysis of aminolysis was noted (32). An excellent example of a rapid aminolysis of penicillin G was also seen with compounds containing the grouping NH_2—$CH(R)$—CH_2—SH (33). These data suggested that the nucleophile was the thiolate ion which formed a penicilloyl ester and transferred the penicilloyl group to the amine forming the more stable amide. This reaction was fairly rapid at pH 7–8; the half-life at pH 7.4 for a 0.01 M solution of aminoethanethiol at 23° was only about 35 min.

When one considers that these combinations of functional groups on protein molecules should be readily accessible to penicillins, then it is not at all surprising that these drugs may readily conjugate to the protein *in vivo* by direct aminolysis.

Another possible means by which aminolysis of penicillin may occur more rapidly has recently been reported (34). Poly-L-lysine has been shown to catalyze the aminolysis of benzylpenicillin by tris(hydroxymethyl)aminomethane (tris). Although this reaction showed a maximum rate at pH 8.8, the rate at pH 7.4 was substantial. The mechanism apparently involves "activation" of penicillin by the polymer for nucleophilic attack by tris. On the basis of this mechanism, one might speculate that a similar type of catalysis could occur *in vivo*.

The most reliable evidence favoring the direct reaction of protein with penicillin to produce conjugates comes from studies of this reaction under conditions approximating those found *in vivo*. In one study (29) in which a number of penicillins and 6-APA were incubated for 48 hr. at 37° with human serum diluted 1:10 with pH 7.4 phosphate buffer and small molecular weight products removed by dialysis, it was found that conjugation of the drugs to protein had occurred. The results, expressed in terms of amount bound as penicilloyl group, are given in the next to last column in Table I. Cloxacillin and phenethicillin, which conjugated to the greatest extent, form their respective penicillenic acids much more slowly than benzylpenicillin or methicillin. It thus appears that direct aminolysis of the penicillins did take place. It is interesting to note that the extent of conjugation was in the same order as the extent of reversible binding of these penicillins to human albumin. Perhaps the high local concentration of drug in the vicinity of protein as a result of reversible binding accelerated the rate of aminolysis. This seems most likely in the case of cloxacillin, which is known to be over 90% bound to serum proteins, when administered in normal doses. The low values obtained with 6-APA were attributed by the author to an assay problem. While this may be true it is apparent that 6-APA is less susceptible to nucleophilic attack than the penicillins based on the relative alkaline hydrolysis rates (26), and should conjugate to a lesser extent to protein on this basis also.

In the other work (27), the extent of conjugation of penicillins to polylysine after incubation for 4 hr. at 37° at pH 7.4 was measured and results are shown in Table I, last column. Again, there is a better correlation of these data with susceptibility of the penicillin to nucleophilic attack rather than to rate of penicillenic acid formation. The same authors also showed that 2,4-dinitrophenyl-6-APA, a compound which cannot rearrange to penicillenic acid, could form a conjugate with aminocaproic acid at about the same rate as benzylpenicillin.

Thus, the direct penicilloylation of proteins appears to be a general route by which the penicilloamide antigenic determinant is formed *in vivo*, although the penicillenic acid pathway cannot be excluded.

The finding that the penicilloamide group is a major antigenic determinant of penicillin allergy has been of great significance. Probably the most important result has been the development of a test reagent, penicilloyl-poly-L-lysine which is specific for the penicilloamide moiety. This multivalent conjugate may be prepared by treating poly-L-lysine (PLL) with penicillenic acid (35) or by direct reaction with penicillin at high pH (36, 37). The former method gives a derivative containing significant amounts of other determinants (penicillenate and penamaldate) while the direct method yields a material virtually free of these

substituents. Levine (37) used varying ratios of penicillin to PLL in the preparations and found that a maximum of 60% of the amino groups of PLL could be penicilloated probably because of steric interference from the bulky penicilloyl groups. He further treated the conjugates with succinic anhydride, which blocked at least 97% of the remaining free amino groups. Some succinylation of the nitrogen of the thiazolidine ring of the penicilloyl groups also took place.

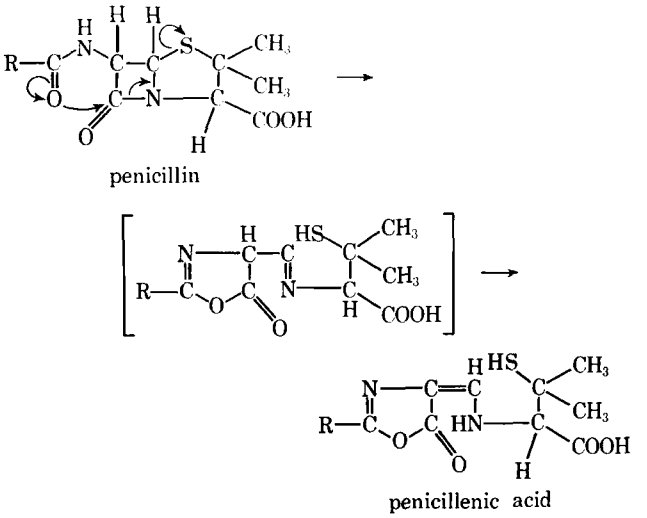
The direct penicilloation of PLL yields the α-diastereoisomeric penicilloyl while the penicillenic acid route gives a mixture of diastereoisomers (37). The direct route is quite advantageous in that PLL derivatives of virtually any penicillin may easily be prepared without the necessity of first preparing the corresponding penicillenic acid. The latter preparation is often difficult, tedious, and gives low yields of material which is relatively impure.

As was mentioned earlier, the ideal test reagent should be able to elicit a reaction but should be nonimmunogenic itself. Levine has found that exhaustive succinylation of penicilloyl-PPL resulted in complete loss of immunogenicity without affecting its ability to react with antibodies to the penicilloamide group (38). In the same study he reported that a heavily coupled conjugate was also nonimmunogenic, whereas lightly coupled penicilloyl-PLL did stimulate formation of antibodies in guinea pigs. There has been a report of a systemic reaction following a test with penicilloyl-PLL (39). Levine also found that penicilloyl conjugates of poly-D-lysine were nonimmunogenic in guinea pigs (40). Others have obtained essentially the same results (41, 42).

There have been a number of reports of the relative effectiveness of penicilloyl-PLL in detecting propensity for allergy to penicillin in patients with histories of such reaction. These have been summarized by DeWeck and Blum (6). While it is true that a good percentage of patients with a history of penicillin allergy do respond positively to penicilloyl-PLL, a great many do not (43–47) and presumably these individuals have developed antibody specificity for other determinant groups. Many of these patients have shown positive reactions when tested with penicillin itself (7, 44, 46, 48, 49), with penicilloic acid (50), or other degradation products (25). These results indicate that there are probably a variety of structures which may be antigenic determinants in penicillin allergy. The next sections will explore the chemical reactions by which these groups may be formed.

Penicillenic Acid—As mentioned earlier, this very reactive degradation product of penicillins is formed by a rearrangement (Scheme II) initiated by attack of the side-chain carbonyl upon the β-lactam (51). Benzylpenicillenic acid is characterized by a very high molar absorptivity (ϵ = 26,600) in the UV at 322 mμ which facilitates its detection at very low concentrations.

While a great deal has been learned about the formation of penicillenic acid, relatively little is known about its fate. Aminolysis to form penicilloamides has already been discussed (see Scheme III, depicting this and other reactions discussed below). The rate of hydrolysis of benzylpenicillenic acid in pH 7.5, 0.1 *M* phosphate

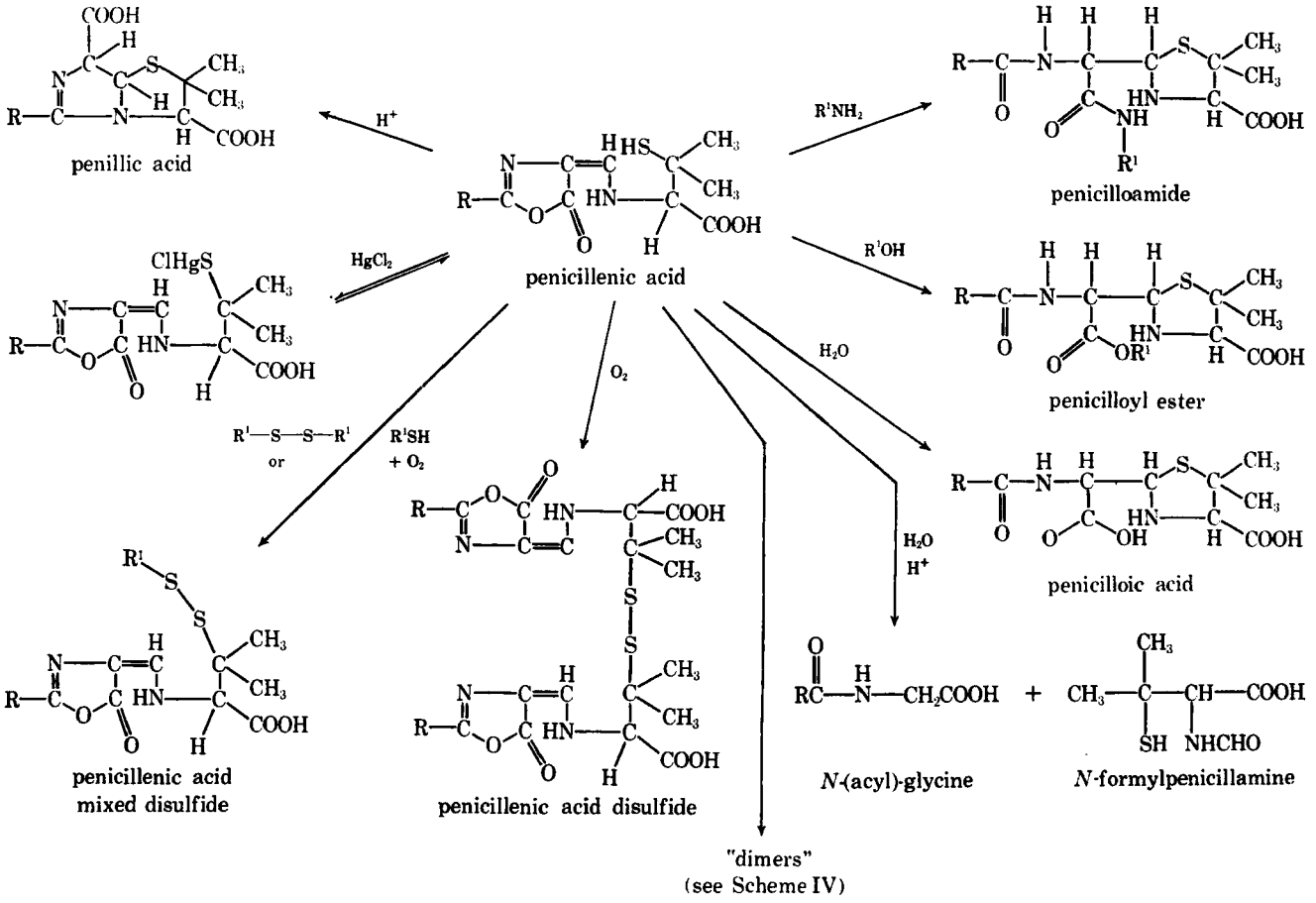


Scheme II

buffer was measured by following the decrease in absorbance at 322 mμ (17). Half-lives at 25 and 37° were 21.5 and 6.5 min., respectively. Similar measurements made over a wide range of pH in the present author's laboratory (52) showed the rate to be minimal at about pH 6 where the half-life was about 15 min. at 35°. At both lower and higher pH the rates were much faster. It was also noted that phosphate buffers exerted a catalytic effect as did acetate and carbonate. The rapid degradation rate of penicillenic acid would make the measurement of its rate of formation very difficult were it not for the fact that it forms a relatively stable mercaptide with mercuric chloride. The measurements of rate of penicillenic acid formation, referred to earlier, were made in the presence of equimolar amounts of mercuric chloride (26). It was found that mercuric chloride had no effect on rate of penicillenic acid formation in concentrations up to 16 times that of the penicillin. Incidentally, the formation of penicillenic acid from penicillins in acid solution is the basis for a very sensitive assay of penicillin (53).

The hydrolysis of penicillenic acid in neutral and alkaline solution leads to formation of penicilloic acid (17), which can react further as will be discussed in a subsequent section. Presumably, penicillenic acid will react with compounds containing hydroxyl groups to form α-penicilloyl esters. These are not as stable as the amides and hydrolyze to penicilloic acid (54).

In acid solution benzylpenicillenic acid is probably converted in part to benzylpenicillic acid (55). This conclusion was based on the fact that the fraction of penicillin G converted to benzylpenicillic acid was maximal at about pH 3. It can be shown that if benzylpenicillic acid formation depends upon concentration of undissociated penicillin in solution (as does penicillenic acid formation) that the fraction would be a maximum at pH about 3. An alternative explanation is that both penillic acid and penicillenic acid come from the same intermediate (51) (see Scheme II). It should be noted that *no* penillic acid has been found among the degradation products of any of the newer semisynthetic penicillins although reports of thorough investigations for most of these are absent from the literature.



Scheme III—Reactions of penicillenic acid.

It is known that penicillenic acid can react with disulfide linkages to form mixed disulfides, as exemplified by its reaction with oxidized glutathione (17). This is one possible route of conjugation to protein which would preserve the oxazolinone structure as part of the antigenic determinant. The same type of linkage would be formed with cysteine sulfhydryl groups in the presence of oxygen. The penicillenic acid determinant has been shown to be immunogenic in animal studies (14, 35). Antibodies to this same determinant have been detected in a small number of penicillin-allergic patients on the basis of inhibition of immunologic tests with the monovalent hapten *S*-(*N*-ethylsuccinimidyl)penicillenate (14). It is known that skin proteins contain more sulfhydryl groups than serum proteins (56) and one might speculate that there is more likelihood therefore, of the penicillenate determinant being involved in the contact hypersensitivity developed in some individuals who handle the drug. The skin has a lower pH than serum and this would promote formation of penicillenic acid from penicillin which penetrated into the skin lipids. An aqueous medium is not a requisite for the penicillenic acid rearrangement (57).

One contaminant of penicillenic acid is usually its disulfide. In following the hydrolysis of penicillenic acid, a residual absorbance at 322 mμ of about 11% of the original value was attributed to the presence of this compound (17). Thus, this material may be present as a contaminant in preparations of penicillin and may, being divalent, elicit a reaction with antibody specific for the penicillenate determinant (58).

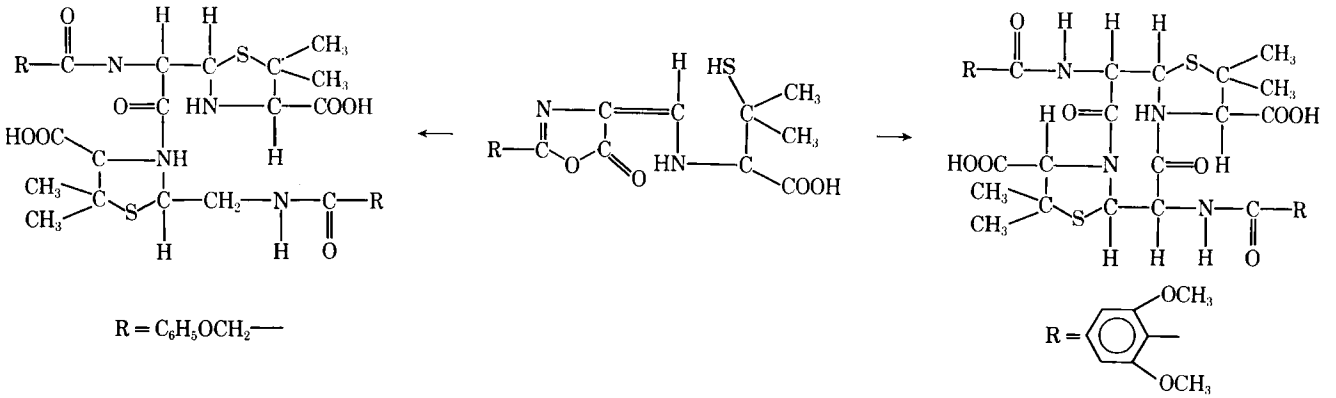
One group of investigators (35) conjugated penicillenic acid to poly-L-lysine and found significant absorbance at 320 mμ and in the 280–290 mμ region indicating the presence of both penicillenate and penamaldate groups. Treatment of the conjugate with 2-mercaptoethanol, which cleaves disulfide bonds, reduced the absorption above 300 mμ. It was inferred from these results that, while in penicilloyl-protein the presence of penicillenate groups could be ascribed to mixed disulfide formation with cysteinyl residues, an alternative explanation was required for the polylysine conjugate. Since conditions of preparation and purification precluded the presence of penicillenic acid disulfide, the inference was drawn that penicillenate groups were substituents on the polymer through mixed disulfide linkage with penamaldate groups. These could arise by direct acylation of amino groups on the polymer by penicillenic acid disulfide or by rearrangement of penicilloyl groups to penamaldate which could then form mixed disulfides with penicillenic acid. The feasibility of the direct acylation by penicillenic acid disulfide was demonstrated by showing a reduction in absorbance at 322 mμ when the disulfide was incubated with ε-aminocaproic acid for 24 hr.

Some doubt concerning the ability of penicillenic acid to conjugate directly to thiol groups *in vivo* through disulfide bonds has been raised by some recent work

by Wagner (59) who found that benzylpenicillenic acid reacts with ethanethiol in neutral medium to produce a thiol ester of penicilloic acid. No evidence was found for any residual penicillenate in the reaction mixture (absorbance at 320 mμ decreased to zero).

It would seem then, that penicillenic acid, whether formed *in vivo* or present as an impurity in a penicillin preparation, is more likely to produce conjugates of the penicilloyl group than to retain the penicillenate structure as an antigenic determinant. One way in which penicillenate can be a determinant in allergy is if a stable conjugate develops in a penicillin or one of its dosage forms prior to administration. This possibility will be discussed further later.

In Scheme IV are shown two of the "dimers" that have been found as products of acid decomposition of phenoxymethylpenicillin (60) and methicillin (57). These could result from attack by the amine of penicillenic acid on the oxazolinone carbonyl of a second molecule of penicillenic acid or of the penicillin itself. This seems a likely route by which polymers of some penicillin could form (see section on *Impurities*).



Scheme IV—"Dimers" from penicillenic acid.

Johnson and Panetta found (57), as products of methicillin degradation, both 2,6-dimethoxyhippuric acid and *N*-formylpenicillamine. These presumably arise from cleavage of the penicillenic acid. While the hippuric acid derivative is relatively inert, *N*-formylpenicillamine could combine with protein through Schiff-base formation between the aldehyde and a lysine amino group, or *via* disulfide linkage. *N*-Formylpenicillamine has not been isolated as a degradation product from any other penicillins, however, and its potential as an antigenic determinant, while not highly probable, is mentioned here only as a possibility.

Penicilloic Acid—Penicilloic acid is the principal hydrolysis product of penicillin and can act as a monovalent inhibitor of antibody to penicilloamide although it is not as efficient as *N*-penicilloyl-ε-aminocaproic acid (61). Batchelor and Dewdney (62) have found no evidence that penicilloic acid plays any other role in penicillin allergy. On the other hand, others have found that some patients give positive immunologic reactions when tested with penicilloic acid (50), although negative to penicilloyl-polylysine and benzylpenicillin. For this reason, Levine has included it in his "minor determinant mixture" (63).

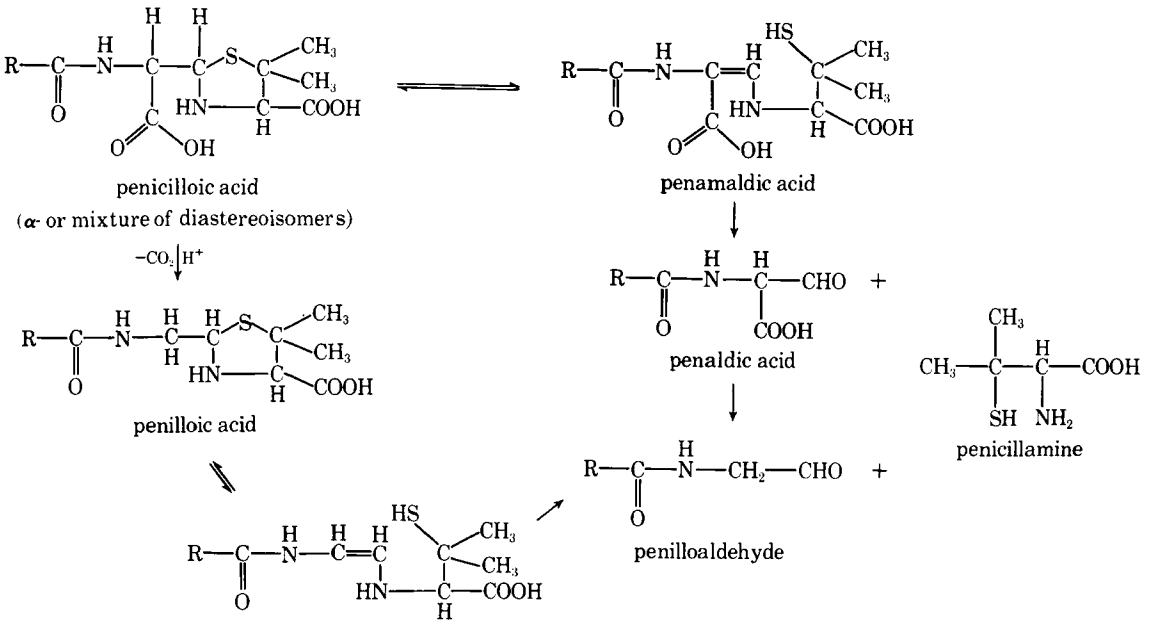
While penicilloic acid cannot conjugate directly to protein as the penicilloyl moiety, one can visualize a number of ways in which other structures capable of combining with protein could be formed from penicilloic acid *in vivo*. Some of these are shown in Scheme V.

Like the penicilloamides, isomerization of penicilloic acid can take place, probably through penamaldic acid (18, 65). The penicilloic → penamaldate transformation is an example of a reverse Michael addition reaction which is generally reversible (64). Thus, there is probably always some penamaldic acid coexisting in equilibrium with penicilloic acid. In the formation of the mixture of diastereoisomers of penicilloic acid and penicilloamides, it has been assumed that all four possible diastereoisomers would be present in the product. It seems likely, however, that only two products will predominate. The usual course of the Michael reaction is *trans* addition of the proton following attack by the nucleophile. Since the sulfhydryl may attack from either of two directions, *trans* addition of proton would yield only two products.

The isomerization apparently is catalyzed by copper in neutral solution and is more rapid in acidic medium (13). It has been found, however, to be independent of pH throughout the range pH 7.3–12.5 (18). The rate probably depends upon concentration of the anion (pKa about 5.3) and is not hydroxyl-ion catalyzed. Usually the Michael reaction is base-catalyzed.

The mutarotation reaction (like the hydrolysis of penicillin to penicilloic acid) may be followed by monitoring the optical rotation of the reacting solution. The α-diastereoisomer has only about half the specific rotation of penicillin while the mixture of diastereoisomers has even lower specific rotation (13). It is worth noting that penicilloamides have been found to mutarotate in alkaline medium fairly rapidly, but hardly at all at pH 7.4 (18). It was concluded that the amide derivatives isomerize by a different mechanism than penicilloic acid, *i.e.*, not *via* penamaldate. The amides could undergo mutarotation *via* a carbanion resulting from deprotonation by base of the asymmetric carbon alpha to the carboxamide. Since protein conjugates of penicillins have been prepared in highly alkaline media for use in immunologic studies, care must be taken to minimize the isomerization of these penicilloamides.

In acidic solution penicilloic acid decarboxylates to



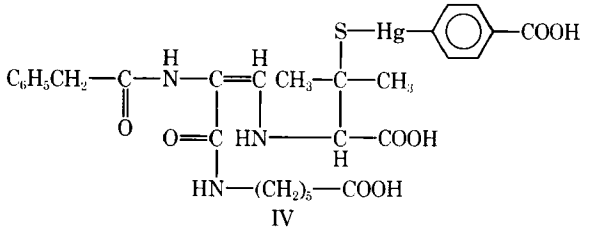
Scheme V—Reactions of penicilloic acid.

penilloic acid, which may be in equilibrium with penaldic acid. The decarboxylation mechanism has not been thoroughly studied but apparently the rate depends upon undissociated carboxyl concentration since penicilloate is relatively stable at higher pH. The pKa of the acid group formed upon hydrolysis of penicillin is about 5.3 (66). Thus, above pH 7.3, less than 1% of the free acid will exist. There should also be two diastereoisomers of penilloic acid.

Penaldic acid can further degrade to penilloaldehyde with release of penicillamine. These are the same products formed by treatment of penicilloic acid or penilloic acid with mercuric chloride in acid solution. The reaction of penicilloic acid with $HgCl_2$ buffered in alkaline medium has been utilized as an assay for penicilloic acid, since in alkaline solution penamaldate is formed and has an absorbance maximum at 282 mμ in the UV with molar absorptivity about 8,000. This absorbance is lost rather rapidly, however, in contrast to the penamaldate produced by treating penicilloamides or penicilloyl esters with $HgCl_2$ where the penamaldate is more stable (67). This difference has been utilized as a means of assay for combinations of penicilloic acid and penicilloyl derivatives in solution. More recently a somewhat improved method based on the kinetics of the reactions was developed (68). The instability of the reaction product of $HgCl_2$ with penicilloic acid is probably due to decarboxylation to penaldate.

It can be seen in Scheme V that several of the products formed from penicilloic acid contain functional groups capable of conjugation to protein, *i.e.*, penamaldate, penicillamine, and penilloaldehyde.

Penamaldate—There is some evidence that the penamaldate moiety is a specific antigenic determinant in penicillin allergy. Thiel *et al.* (25) found one allergic patient with whom the best monovalent inhibitor was a penamaldate derivative formed by treating D-α-benzylpenicilloyl-ε-aminocaproic acid with *p*-hydroxymercuribenzoic acid in alkaline solution (IV).

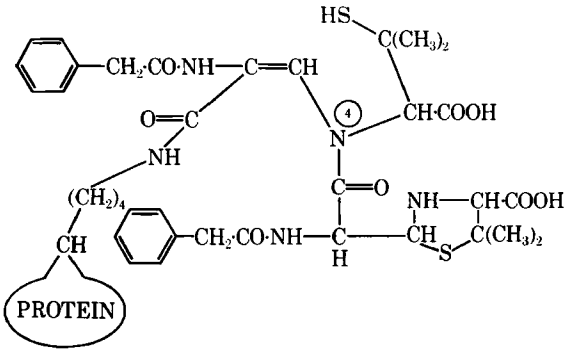


IV

The penamaldate-protein conjugate could be formed *in vivo* from penicilloic acid through a mixed disulfide interchange with cystine residues or by hydrolysis of a penicillenic mixed disulfide conjugate. The latter route seems less likely, since none of the patients in this and other studies (24) had antibody specific for the penicillenate determinant which would be expected if the formation of penamaldate were mediated by penicillenate. Also, penicillenate disulfide conjugates are known to be fairly stable at pH 7.5 (17).

Penamaldate groups may also be formed from penicilloamides. As mentioned previously, it was found (13) that penicilloyl derivatives of proteins and of polylysine, prepared by reaction of penicillenic acid with the protein in alkaline solution, contained penamaldate as evidenced by the UV absorbance at 280 mμ. The authors suggested this was due to acylation of the N^4 of a penamaldoyl protein by penicillenic acid (V). Presumably, such a derivative would not form if the penicilloyl-protein conjugate were prepared directly from penicillin in alkaline solution where no penicillenic acid could be formed.

Isomerization of penicilloamide to penamaldate *in vivo* catalyzed by copper may be one means by which the penamaldate moiety comes to be an antigenic determinant in penicillin allergy. The mean concentration of copper in serum of female patients in one report (69) was 142 mcg./100 ml. (2.2×10^{-5} *M*) which is

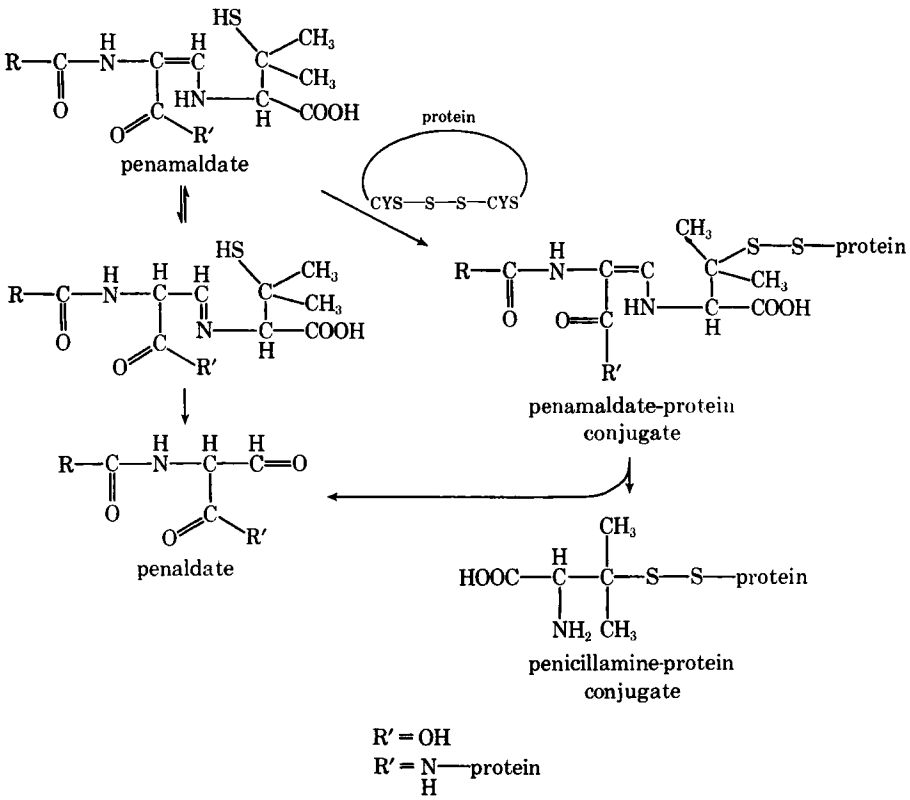


V

probably more than enough to react with the penicilloamide present on a mole-for-mole basis.

Penicillamine—As shown in Scheme VI, penicillamine is one of the products of penamaldate degradation. Penamaldate is in equilibrium with a Schiff base formed from penicillamine and penaldic acid (70). There seems to be little doubt that the penicillamine moiety is an antigenic determinant as is penicillin allergy. Levine and Price (24) found that a small percentage of humans allergic to penicillin reacted positively to a multivalent penicillamine conjugate prepared by treating human serum albumin and penicillamine with potassium ferricyanide as oxidizing agent. Specificity for the penicillamine moiety was demonstrated by hapten inhibition using penicillamine-cysteine mixed disulfide as the monovalent hapten. D-Penicillamine itself was unable to elicit a reaction in these patients, a fact which the authors attribute possibly to hapten inhibition by excess penicillamine. However, it may also be due to inability of penicillamine to form *in vitro* the multivalent conjugate necessary for reaction. It may be that such conjugates are more readily formed by reaction of penamaldate (from penicilloic acid) with skin proteins through disulfide interchange, and subsequent breakdown to leave penicillamine conjugate. Further support for this hypothesis comes from the fact that the same patients sensitive to the multivalent penicillamine conjugates also reacted to benzylpenicillin and benzylpenicilloic acid. It should be noted here that penicillinase, which has been used as treatment for penicillin allergy, produces large amounts of penicilloic acid, and thus, may aggravate an allergy due to penicillamine. Also, penicilloic acid and penicillin in milk products may provoke penicillamine reactions.

Using guinea pig contact dermatitis as the test system, it was found (21, 22) that penicillamine cross-reacted with both benzylpenicillin and benzylpenicillenic acid. Both of these can, *via* benzylpenicilloic acid, introduce the penicillamine group into proteins. Benzylpenicilloic acid was shown to react with cystine *in vitro* to yield penicillamine-cysteine mixed disulfide (71).



Scheme VI—Reactions of penamaldates.

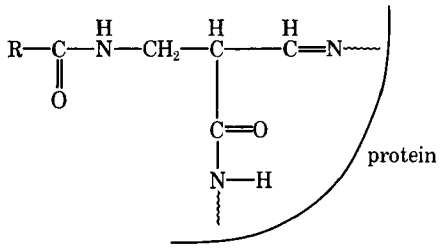
In the guinea pig contact dermatitis system cross-reaction was also noted between penicillin G and penicillin O (allylthiomethyl penicillin) (21). This was probably due to formation of the penicillamine antigen from both penicillins. Since all the penicillins have the same nucleus, the penicillamine determinant could be an antigenic determinant common to all of them. (See discussion of cross-reaction below.)

Penilloaldehyde—While this substance does form from penamaldate and is capable of reaction with amino groups to form a Schiff-base-type conjugate,

it has not been demonstrated to be an antigenic determinant in penicillin allergy. In the guinea pig contact dermatitis system, for example, penilloaldehyde did not cross-react with penicillin G (21).

It has been suggested (25) that penilloaldehyde formed from penilloate might react with amino group of protein and the conjugate might be stabilized by reduction of the double bond. Such a transformation might explain the strong reactions sometimes observed with penilloic acid (25).

One group of workers (25) point out an interesting possibility, whereby a penicilloamide group on a protein might be converted to penilloaldehyde *in vivo* and a Schiff base formed with an adjoining amino group. They found that penilloaldehyde reacts with ε-aminocaproic acid in 0.1 *M* phosphate pH 7.4 yielding a derivative with absorption maximum at 280 mμ. The postulated derivative *in vivo* might have Structure VI.



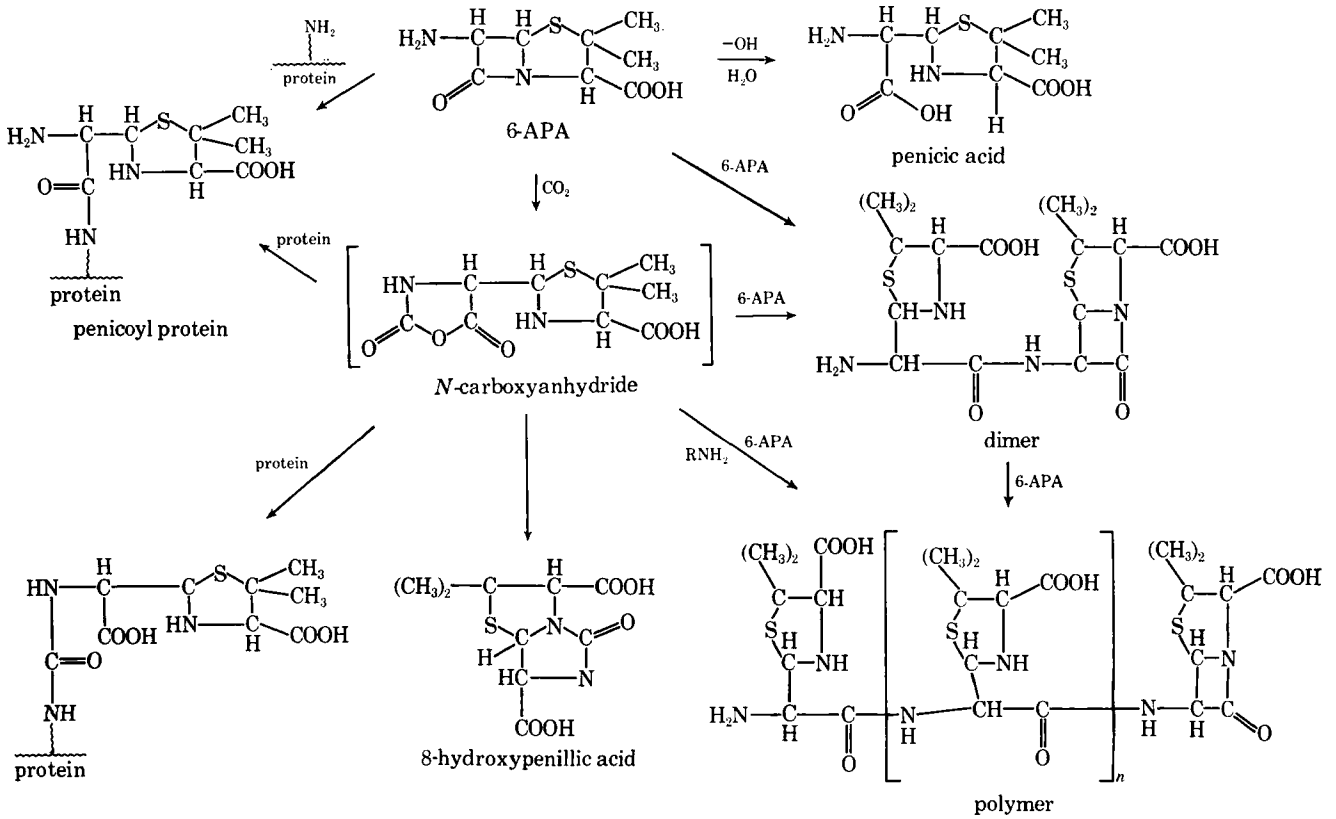
VI

It would probably be difficult to prepare a monovalent hapten which would correspond to such a derivative, since the two protein amino groups might be quite far apart. One suggestion might be to utilize an aliphatic diamine, form the monopenicilloamide, and treat with $HgCl_2$ in alkaline medium to yield the aldehyde which could combine with the other free amino group.

6-Aminopenicillanic Acid (6-APA)—This key intermediate is present in penicillin fermentation broths and is used in the preparation of all the semisynthetic penicillins. As such, it may appear in small amounts as a contaminant in penicillin preparations and should be suspect as a potential source of allergy. It has also been found in the urine of persons ingesting penicillin G (72) and may be a metabolite of penicillins. It is known that certain enteric bacteria produced an enzyme (penicillin amidase) which catalyzes the removal by hydrolysis of the side chain of penicillins G and V leaving the β-lactam intact and this intestinal hydrolysis may be a source of 6-APA *in vivo*.

It has been found that small amounts of 6-APA are produced as a result of alkaline hydrolysis of penicillins *in vitro* (73). Of the compounds tested, penicillin V produced the most 6-APA, but even there, only very small amounts were formed. The experiments were conducted at pH 8.5 at 70° in 0.05 *M* phosphate buffer, conditions far different from those under which penicillins are usually handled during processing. It seems unlikely therefore, that alkaline hydrolysis is a significant source of 6-APA.

The known reactions of 6-APA pertinent to the present discussion are shown in Scheme VII. It has been found that 6-APA can react with poly-L-lysine (64, 65),



Scheme VII—Reactions of 6-APA.

ε-aminocaproic acid (29, 74), and proteins (29) at pH 7.4 to produce the penicoyl derivative which is, of course, analogous to the penicilloyl determinant of penicillins. This may be one route by which 6-APA may be conjugated to protein *in vivo*.

6-APA, having no side chain, cannot form a penicillenic acid directly. It has been shown, however, to react with carbon doxide to form 8-hydroxypenillic acid (76) probably through the *N*-carboxyanhydride. The latter would have the potential for reaction with protein amino groups to form penicoylprotein or a ureide derivative. One would not expect the latter to cross-react immunologically with penicillins as well as the penicoyl derivative.

6-APA is known to polymerize in neutral solution to form a 7–8 unit linear polymer (77). This reaction was observed in fairly concentrated solutions and apparently proceeds *via* nucleophilic attack by the amino group on the β-lactam carbonyl of a neighboring molecule. The reaction could also proceed in the presence of CO_2 *via* the *N*-carboxyanhydride. Dennen studied the kinetics of 6-APA degradation in solution in the pH range 5–10 and found both hydrolysis and dimerization taking place (78). Below pH 7.5, the hydrolysis reaction predominated while above this value the dimerization was more pronounced as evidenced by observation of second-order kinetics. The pH of minimum rate was about 8.0. The work is subject to criticism, however, since ionic strength was not controlled and no account was taken of the effects of buffers. Tris buffers were used in the pH 8–9 region, for example, and almost certainly tris acts as a nucleophile toward 6-APA as it does with penicillins (68). One interesting finding was the development of a new absorption band in the UV at 304 mμ after heating 6-APA solution at pH 6.3, at 75° for 2.5 hr., while no such band was formed with penicic acid. This new band could be due to formation of a penicillenic acid following formation of dimer. The dimer, being an acyl-6-APA like the penicillins, could rearrange to a penicillenic acid. The latter should be capable of facile reaction with protein or with another 6-APA to produce higher polymers.

The UV absorption band at 304 mμ is somewhat lower than usually observed with penicillenic acids, but does not seem unreasonably low in the absence of side chain. It may be that the absence of a side chain influences the wavelength of maximum absorbance of the penamaldate formed from 6-APA protein conjugate upon addition of $HgCl_2$. If so, lower values would be obtained in the assay for penicilloyl groups if the readings were taken at 282 mμ as with penicillins, and this may explain the low result observed by Batchelor *et al.* for 6-APA (29).

The polymer of 6-APA is likely the Factor 1 found by Batchelor *et al.* in preparations of 6-APA (79). This material was not found in the penicillin from which the 6-APA was prepared, showed greater antibacterial activity than 6-APA, and its appearance coincided with decrease in β-lactam as noted by hydroxylamine assay. Two other materials, designated Factors 2 and 3, were also found and the greatest yields were obtained at pH 7–9. This result correlates well with a polymerization proceedng *via* nucleophilic attack of free amine (pKa's of 6-APA are about 2.3 and 4.9) (66) upon the β-lactam of a second molecule. The materials found by Batchelor *et al.* may be polymers of varying size.

Earlier studies on 6-APA showed it to be a potent immunogen in rabbits (80) and to cross-react with antibodies to the benzylpenicilloyl group (81, 82). It is now felt that this allergenicity of 6-APA is primarily due to penicilloyl-protein impurities carried through from fermentation since 6-APA is made from benzylpenicillin. Some aspects of the role of 6-APA in allergy will, therefore, be discussed in the section on impurities below.

Metabolism of Penicillins—It is indeed surprising that so little work has been done in identifying the metabolites of the penicillins when one considers the extensive research effort expended on these antibiotics. While it is generally known that the major portion of an intravenous dose of most penicillins is excreted unchanged in the urine (83), some fraction of the dose is usually metabolized. Several studies have demonstrated the presence of biologically active metabolites of some penicillins. Vanderhaeghe *et al.* (84) identified *p*-hydroxyphenoxymethylpenicillin as the principal metabolite of phenoxymethyl penicillin in humans and also found another active metabolite, possibly a dihydroxy derivative. Hydroxylation of benzene rings is a well-known metabolic pathway (85) and probably other penicillins are metabolized in this way. From the point of view of allergy to penicillins, the reactivity of these hydroxylated compounds should be investigated.

The only biologically inactive metabolite to be identified thus far is penicilloic acid following administration of benzylpenicillin (86). Since it is clear that penicilloic acid is probably an important intermediate in formation of certain antigenic determinants *in vivo*, its formation as a metabolite of penicillins may be a key step in the pathway to hypersensitization. It should be noted in this connection that phenaceturic acid has also been found as a metabolite (86), showing the potential for further reactivity of penicilloic acid.

A study of metabolism of nafcillin (2-ethoxy-1-naphthoyl penicillin) using ^{14}C-labeled drug, revealed the presence of three biologically inactive metabolites (87). None of these was positively identified, but 2-ethoxynaphthoic acid was excluded.

The presence of 6-APA in urine of humans and experimental animals fed benzylpenicillin has already been mentioned. No other studies have appeared which confirmed these findings.

The need for identification of penicillin metabolites has not been felt in the past, probably because of the low order of toxicity of the drugs and the fact that the greater portion of a dose is usually excreted unchanged. It seems quite obvious, however, that much greater effort must be devoted to this area in the future. With the variety of penicillins now available (and more to come) differences in both metabolism rates and products may be expected. The potential role of these substances as allergens is unknown but, since only small amounts could cause problems, they should be identified and an assessment made of their immunogenicity.

Impurities—Impurities in penicillins, even if present in only small quantities, represent a potential source of allergen. Since benzylpenicillin is made by fermenta-

tion, thus exposing the drug to protein, the possibility of penicilloyl-protein formation during processing and its subsequent carry-over through extraction into the final product cannot be overlooked. Furthermore, the principal source of 6-APA is benzylpenicillin treated with an amidase from an *E. coli* strain to remove side chain. Therefore, any protein impurity could either remain from the benzylpenicillin or be picked up in the deacylation process, or both, and be carried over into 6-APA and subsequently, the semisynthetic penicillins. Such impurities were found in commercial benzylpenicillin and 6-APA (88–90). Fractionation of sodium 6-APA on synthetic polysaccharide (Sephadex) yielded a high molecular weight component which elicited an immune reaction in guinea pigs sensitized to "crude" 6-APA in the manner used by Chisholm *et al.* (80). The purified 6-APA from the fractionation did not elicit any reaction. Thus, the previously recognized potent immunogenicity of 6-APA was probably due to impurity.

The fraction containing impurity was found to contain the penicilloyl moiety by penamaldate analysis and a protein by amino acid analysis. It was immunogenic in guinea pigs and rabbits while purified 6-APA was not. It was concluded that the impurity arose from the *E. coli* amidase preparation which had been penicilloylated by the original benzylpenicillin, from which the 6-APA was prepared.

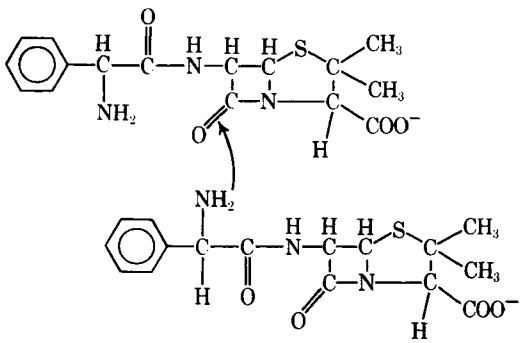
By dialysis of large volumes of solution of benzylpenicillin, several commercial batches were shown to contain a high molecular weight impurity containing both proteinaceous material and bound penicilloyl groups (89). This impurity was also found to be immunogenic in animals, producing antibodies with penicilloyl specificity. After removal of this protein contaminant another polymeric material developed in solution upon standing and amino acid analysis indicated this material was derived from the penicillin itself. This polymeric substance probably forms *via* penicillenic acid and could combine with the proteinaceous residue in the crude penicillin to form a potent allergen.

A polymeric fraction also developed in purified 6-APA and contained several components similar to the material found by Grant *et al.* (77).

Comparisons were made in sensitivity tests on human volunteers with history of penicillin allergy using commercial penicillin and impurity-free material (90). While a number of these patients reacted to the crude material but not to the purified, one patient reacted only to the pure penicillin and many reacted to both preparations. Thus, the protein contaminants in benzylpenicillin are certainly not the only source of immunogen.

Contrasted with the findings detailed above are those of Dursch (91), who found small quantities of retentate after exhaustive dialysis of aqueous solutions of commercial benzylpenicillin but found no evidence for the presence of proteinaceous material. It is interesting to note that dialysis was performed at low temperature (3°) in order to obtain reproducible results. At higher temperatures, the amounts of retentate were quite variable. This might have been due to polymerization of the penicillin which would be faster at the higher temperature. These results indicate that the extraction and other techniques used in processing penicillin may influence the degree of contamination in the drug and this can vary among manufacturers.

A further study by Stewart (92) considered the role of macromolecular impurities in other penicillins on their allergenicity. Concentrated solutions of benzylpenicillin, ampicillin, and methicillin were fractionated on synthetic polysaccharide. With benzylpenicillin and ampicillin both high and intermediate molecular weight fractions were found, while with methicillin there was only one high molecular weight fraction. All three also gave a low molecular weight dialyzable fraction containing the penicillin itself with some degradation products. Only with benzylpenicillin was a proteinaceous material found in the high molecular weight fraction. The polymer found in methicillin and both macromolecular fractions from ampicillin contained amino acids derived from the penicillin itself and presumably are formed by some self-polymerization mechanism. The author does not suggest a chemical mechanism other than that it "appears to depend primarily upon penicillenic acid acting as a starter." This, however, does not seem likely for ampicillin which is one of the most acid-stable penicillins (30) and does not readily form a penicillenic acid. A more likely mode of polymerization would be similar to that proposed for 6-APA, *i.e.*, nucleophilic attack of the side-chain free amine upon the β-lactam carbonyl of a second molecule (VII). The pKa of the amino group in



VII

ampicillin is about 7.1 and, therefore, at neutral pH there would be a considerable amount of unprotonated amine available for such a reaction. This reaction may account in part for the rapid degradation of sodium ampicillin in solution where the pH is above 8.0. It would be less likely to occur at pH about 5.0 where the zwitterion form of the drug predominates. Further study of the products of ampicillin degradation is certainly warranted.

Methicillin, on the other hand, is readily degraded to penicillenic acid from which the macromolecular nonproteinaceous fraction probably is derived. It is not known whether the "dimer" of Johnson and Panetta (57), found upon degradation of methicillin, is dialyzable. If not, it may be one of the principal components in this fraction. Stewart (92) did not report a molecular weight for this material.

The nondialyzable fractions from methicillin and ampicillin showed only very low immunogenicity and ability to elicit reactions in hypersensitive patients.

While they may conjugate *in vivo* to proteins and play a role in delayed reactions, they probably have little if anything to do with the more important immediate reactions to these penicillins.

The fact that no proteinaceous impurity was found in methicillin and ampicillin is significant. These compounds are prepared by acylation of 6-APA which itself is prepared from benzylpenicillin. Evidently, the processing (extraction, *etc.*) is sufficient to eliminate any protein residue which might have been present in the original benzylpenicillin. It seems, therefore, that a protein-free benzylpenicillin could be prepared by the same method used for methicillin, *i.e.*, acylation of 6-APA with phenylacetyl chloride. While this may be more expensive, it would certainly be worthwhile in view of the high immunogenic potency exhibited by the impurity.

A more detailed study of the role of protein impurities and polymers in penicillin allergy has been reported by DeWeck *et al.* (58), including in the discussion a critical appraisal of the previous findings. It is pointed out that the small amounts of benzylpenicilloyl-protein (0.02–0.2%) found as an impurity could hardly be an elicitor of allergic reactions of benzylpenicilloyl specificity in practice, since the benzylpenicillin with which it is administered has sufficient affinity for the antibodies to cause complete inhibition as a monovalent inhibitor. This situation, termed "inbuilt inhibition," may explain why patients hypersensitive to the penicilloyl determinant often do not react to a dose of benzylpenicillin. DeWeck *et al.* also found no formation of large polymers in penicillin solutions kept at controlled pH around neutrality. In the prior study, the solutions were not buffered and, since acid is formed upon hydrolysis of penicillin, the pH could drop considerably, increasing the rate of penicillenic acid formation through which these polymers probably form.

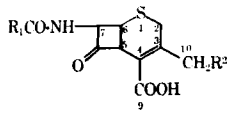
From the results of immunologic tests on penicillin-hypersensitive patients with various chromatographic fractions from penicillin solutions which had been kept at neutral pH for 1 week at room temperature, DeWeck *et al.* concluded that a number of low molecular weight substances were capable of eliciting reactions. Furthermore, it seems clear from the responses observed that several different antigenic determinants are involved. None of the substances were identified chemically, however, and this most important step must await future work.

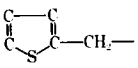
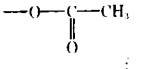
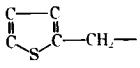
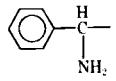
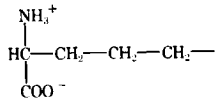
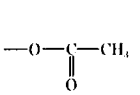
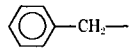
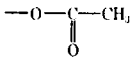
Another relevant question concerns the immunogenicity of the "crude" *versus* "purified" benzylpenicillin. DeWeck *et al.* (58) found that the purified material was as immunogenic for rabbits as "crude" benzylpenicillin and that alkaline hydrolysis destroyed most of the immunogenic potential of both materials. It would not be expected that mild hydrolysis conditions would sufficiently alter any penicilloyl-protein impurity in crude penicillin so as to remove its immunogenicity. It was concluded, therefore, that any protein impurity present in the crude penicillin is nonimmunogenic in the low concentrations found.

As mentioned earlier, it is now felt that the early observed immunogenicity of 6-APA was probably due to a penicilloyl-protein impurity. The amount of this material probably varied considerably between lots and among the several manufacturers, thus explaining the fact that some investigators found no reactions with 6-APA even in patients highly sensitive to benzylpenicillin (93). Since 6-APA has no affinity for antibody to the benzylpenicilloyl group, even small quantities of impurity should elicit reactions in patients sensitive to the penicilloyl determinants, *i.e.*, no inbuilt inhibition (58).

In their study of antibody specificity, Thiel *et al.* (25) found three patients with greater specificity toward 6-APA derivatives than other potential determinants. They used both penicoyl-aminocaproic acid and poly-6-APA as inhibitors and found that the latter was generally the more effective of the two. It may be that these patients were sensitized by a conjugate of the polymer to protein if the polymer had been carried through the processing of one of the semisynthetic penicillins.

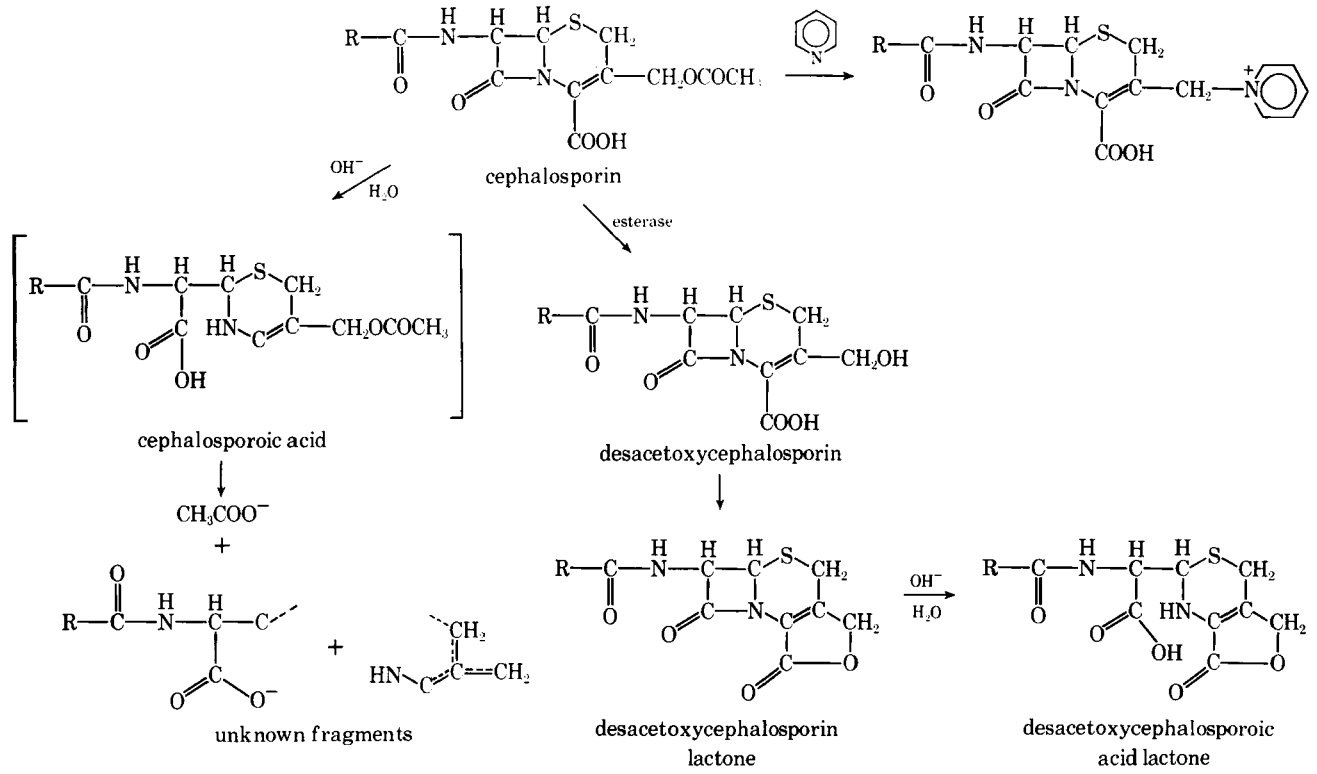
Table II—Cephalosporins



Name	R_1	R_2
Cephalothin		
Cephaloridine		
Cephalexin		—H
Cephalosporin C		
Cephaloram		

Cephalosporins—The cephalosporins (see Table II) are acyl derivatives of 7-aminocephalosporanic acid and are similar to the penicillins in that they have the common β-lactam structure and many similar biological properties. Table II lists some of the derivatives commercially available or being tested and cephalosporin C. Because of the structural differences between the penicillins and cephalosporins, some hope was offered that the latter might prove to be nonallergenic or at least not cross-reactive with the penicillins. As will be noted below, these hopes have not yet been realized. It is worthwhile, however, to explore the chemistry of the cephalosporins as it relates to their potential allergenicity and to their possible mode of cross-reaction with penicillins.

In contrast to the well-known chemistry of penicillin, relatively little work has been done on those chemical reactions of cephalosporins which may be related to allergy. Several recent reviews, however, provide a good portion of the known chemistry and some of the



Scheme VIII—Reactions of cephalosporins.

biological properties of these drugs (94–97). Scheme VIII depicts some pertinent reactions of the cephalosporins.

Alkaline hydrolysis of the β-lactam takes place almost as readily with cephalothin as with benzylpenicillin, and with cephaloridine the rate is even faster (98), probably because the repulsion of the attacking hydroxyl ion by carboxylate is reduced in the presence of the positive charge on the latter. The expected hydrolysis product, cephalosporoic acid, is not stable however, and probably has only a transitory existence (94). It is further fragmented while expelling the acetoxy or other substituent at the 3-position but the products have not yet been characterized (95). While only one equivalent of acid is produced upon hydrolysis of a penicillin, two equivalents of acid result from cephalosporin hydrolysis. In addition, a characteristic absorption band in the UV at 260 mμ is lost with the latter group. Studies on metabolism of cephalothin and cephaloridine in the rat (99) yielded thienylacetamidoethanol as a metabolite along with thienylacetylglycine, and another unidentified polar substance. A common precursor of the identified metabolites was postulated to be thienylacetamidoacetaldehyde. This is the equivalent of the penilloaldehyde which results from penicillin degradation. The cephalosporins in this study had been administered orally to the animals and the authors concluded that these derivatives undergo extensive degradation in the gastrointestinal tract, with some of the products being absorbed. It seems equally likely, however, that metabolism could have occurred following oral absorption of the drug, and the aldehyde formed as one of the fragments following hydrolysis. By analogy, it is known that benzylpenicillin metabolism (86) does produce the corresponding penicilloic acid which can degrade further to penicilloaldehyde. The aldehyde should be able to combine with ε-amino groups of lysine residues forming a hapten-protein conjugate.

The cephalosporins do not appear to form compounds analogous to penicillenic acid and are relatively stable in acid solutions (97). Furthermore, lacking the gem-dimethyl group in the penicillins, the cephalosporins cannot break down to yield an amino acid analogous to penicillamine. Thus, several routes of potential hapten formation, noted with penicillins, are not available to the cephalosporins.

The acetoxy group of cephalothin can be removed by esterases and the resulting desacetoxy compound, which is biologically active (100), can form a lactone. The lactones upon hydrolysis yield compounds analogous to penicilloates which are relatively stable. The desacetoxy compound of cephalothin has been found as a metabolite (99) and it seems not unlikely that this material may form lactone which could further react with tissue proteins to form cephalosporoyl-protein. This could be antigenic itself or perhaps cross-react with antibodies to penicilloyl-protein if the side chain was of similar structure to that on the sensitizing penicillin.

Direct aminolysis of cephalosporins by protein amino groups apparently does take place *in vivo*, as evidenced by the data of Shibata *et al.* (101) who found that protein incubated with cephalosporins at pH 8.5 contained 20–35 fewer ε-amino groups than the native proteins measured by formal titration. The product of aminolysis of cephalosporins is probably not a cephalosporoyl protein, however. It has been reported (97) that reaction of cephalosporin C with ε-aminocaproate at pH 10 leads to slow formation, from an intermediate, of a product which has an absorption maximum at 274 mμ

and is highly labile in dilute acid. This product may be a penamaldate-type structure.

One potential means of conjugation of cephalosporins, but not penicillins, to proteins, is by reaction at the 3-position. For example, cephaloridine is prepared from cephalothin by reaction with pyridine at neutral pH (102). Other nucleophiles may also be substituted (103). It may be possible for nucleophilic groups on proteins to participate in such reactions also yielding conjugates which may be immunogenic. This possibility has not been explored. Another possible consequence of such a reaction is to bring the reactive β-lactam into close proximity to other nucleophilic groups on the protein and thus facilitate attack at that site resulting in cephalosporoyl conjugate.

Although early clinical studies (104–110) indicated that cephalosporins might not cross-react with penicillins, it has now been well established that such cross-reactions do indeed occur in experimental animals and humans (112–119). It is of interest (82, 101, 111) to speculate on the chemical nature of the eliciting antigens which might be formed from cephalosporins *in vivo* and their relationship to penicillin conjugates. The principal antigenic determinant of penicillin, the penicilloyl group, is sufficiently close in structure and mode of conjugation to protein to the cephalosporoyl group so that cross-reaction might be expected. However, the known fragmentation of the cephalosporin nucleus following conjugation would argue against this, except if the lactone derivative of the cephalosporin were involved. It should be noted that the probable products of fragmentation of a cephalosporoyl conjugate would markedly resemble similar fragmentation products of penicilloyl conjugates, *i.e.*, penilloaldehyde or Schiff-base derivatives. Perhaps studies with such compounds as monovalent inhibitors would reveal the nature of the haptens involved.

Of interest was the fact that Batchelor *et al.* found no evidence of extensive cross-reaction between 6-APA and cephalosporins (111). Evidently, the side chain is an important factor in determining cross-reactivity of these molecules.

Until more is known concerning the specific structure of penicillin antigenic determinants other than penicilloyl, it will be extremely difficult to ascertain the chemical nature of the cross-reacting cephalosporin determinants.

In addition to being cross-reactive with penicillins, the cephalosporins have been shown to be immunogenic in animals through the use of protein conjugates prepared *in vitro* (82, 110, 111). There has also been a report of a reaction to cephaloridine in a patient who had received penicillin with no ill effects (120). It thus seems likely that it will eventually be found that cephalosporins produce allergy *via* chemical reactions leading to at least two types of antigenic determinants, one resembling a penicillin determinant and another unlike any penicillin product.

Cross-Reactions and Role of the Penicillin Side Chain —There are a number of penicillins currently available and more may be expected in the future. It is pertinent therefore, to attempt to answer a number of questions concerning the role of the side chain in penicillin allergy.

Are there differences in immunogenicity among the various penicillins? Are the same antigenic determinants formed *in vivo* from these different drugs? Do they cross-react with previously available penicillins? Can any predictions be made, on the basis of present knowledge, of side chains which will be nonallergenic or at least less allergenic than the penicillins now used?

The preponderance of work being reviewed in this report has been done with benzylpenicillin with much less effort having been devoted to other penicillins. It may be stated at the outset that none of these presently known is nonallergenic. Clinical reports have appeared detailing allergic reactions to oxacillin (121), phenethicillin (122), ampicillin (119), methicillin (123), and others, but the mechanisms responsible for these reactions have not been defined. They may be due to the fact that the patients were previously sensitized to benzylpenicillin and the antibodies cross-react with eliciting antigens formed by the new penicillin, or the new derivative itself may be immunogenic.

There have been a number of experimental studies which demonstrate cross-reactivity among various penicillins. Stewart (104) for example, found that five subjects sensitized to benzylpenicillin reacted to ampicillin, methicillin, and *p*-aminobenzyl penicillin. Parker and Thiel (42) have shown, using polylysine conjugates of various penicillins, that the extent of cross-reaction is quite variable among individuals, and others have made similar observations. Since antibodies to the benzylpenicilloyl group are directed not only against the particular side chain and nucleus, but also the lysine residue and adjoining groups on the protein chain (61) one might conclude that the side chain of a particular penicillin might not be an important factor. Considerable differences would be expected, however, in response of antibenzylpenicilloyl antibody to some eliciting determinant other than penicilloyl produced by the new penicillin. The relative ability of the new penicillins to combine with proteins *in vivo* to form penicilloyl-protein conjugate may reflect their cross-reactivity with benzylpenicillin. As already noted, there are only very small differences in rate of reaction of the various penicillins with nucleophiles (Table I) and it is not surprising, therefore, to find cross-reactions with most of the semisynthetics.

In patients with antibodies not specific for the penicilloyl determinant the situation may be quite different. Presumably the new penicillin would be required to form *in vivo* an eliciting antigen of similar structure to the original benzylpenicillin immunogen. If penicillenic acid formation were prerequisite in the process, for example, then those penicillins which form penicillenic acid only very slowly would not be expected to cross-react. Unfortunately, the precise structure of the "minor" antigenic determinants is not known and insufficient attention has been paid to the relative rates of many of the reactions which may be involved among the newer penicillins. The penicillamine determinant group, for example, can be formed from all penicillins but the rates of formation may differ considerably and indeed, some of the newer penicillins may prove to be safe for administration to patients with proven penicillamine specific antibody. Until detailed information is available

on the ability of the semisynthetics to form antigen *in vivo*, all of the penicillins must be considered unsafe for use in persons known to be hypersensitive.

Another problem to be faced with the newer penicillins is their immunogenicity, *i.e.*, their ability to form antigen *in vivo* which will elicit formation of antibody specific for their particular structures. In their review, DeWeck and Blum (6) reported quantitative differences observed in immunogenicity of a number of penicillins by measuring antibody titer after immunization of rabbits with equimolar amounts of penicillins. They classified as "highly antigenic," benzylpenicillin, allylthiomethyl penicillin, and phenoxymethyl penicillin. "Somewhat lower antigenicity" was noted for phenethicillin, methicillin, propicillin, oxacillin, cloxacillin, and α-phenoxybenzylpenicillin. Ampicillin appeared "definitely less antigenic." These results could not be correlated with *in vitro* rates of penicillenic acid formation, nor with aminolysis rates. Ampicillin seems to be a special case, as it has been found to be a particular offender when used in treating infectious mononucleosis (124). It has not been confirmed, however, that the rashes noted in these cases are allergic in origin, and they may be a manifestation of some other toxicity.

Bunn *et al.* (125) studied the ability of six penicillins to stimulate antibody production in rabbits, and found that all (penicillin G, phenoxymethyl penicillin, phenethicillin, methicillin, oxacillin, and ampicillin) did so but with varying titers. Of this group, oxacillin was least immunogenic, whereas methicillin and ampicillin gave the greatest response. In discussing their results, the authors state that if there were common degradation product(s) *in vivo* from any and all of the penicillins studied, the resulting reactive substances should be substantially the same for each penicillin. They ignore, however, the possibility that the *rates* of the reactions may vary and produce thereby different amounts of the same products in a given time, leading to quantitative differences in response. In this work there was considerable cross-reactivity noted among the penicillins.

In contrast to the results cited above, one study has shown a correlation between immunogenicity and rate of penicillenic acid formation. Feinberg (126) measured penicillenic acid formation from benzylpenicillin, methicillin, phenethicillin, and propicillin. The latter showed no penicillenic acid formation, even at pH 5.0, in 2 weeks while phenethicillin showed only a slow rate. The other two penicillins both produced considerable amounts of penicillenic acid at pH 5.0 as would be expected. The four penicillins were tested for immunogenicity by injecting into rabbits in a water-in-oil emulsion based on a silicone oil and containing lanolin and a nonionic emulsifier.[2] In one set of experiments, penicillins prepared from specially purified 6-APA were used. The antibody titers indicated that while benzylpenicillin and methicillin were highly immunogenic, a much lower order of activity was seen with phenethicillin and propicillin. The purified preparations gave somewhat higher titers than commercial batches though the differences were not large.

[2] Arlacel A, Atlas Chemical Industries, Wilmington, Del.

It is difficult to correlate these observations with those of previous studies. It may be that the pH in the emulsions was not controlled and more penicillenic acid was formed in the two penicillins found to be immunogenic prior to administration. The author does not comment upon this aspect of the study.

CONCLUSIONS

From the results of testing a number of patients in several laboratories, it is quite clear that a variety of antigenic determinants are involved in penicillin allergy. This is not completely surprising in view of the number of ways in which penicillin can degrade and react with protein. The elucidation of the structure of most of the antigenic determinants has been complicated by the relative instability of the derivatives used as inhibitors and cross-reactions between closely related structures. The preparation of stable monovalent derivatives of those structures under suspicion as allergens is a prime requisite. Also needed are multivalent conjugates, to a suitable carrier, of the same determinant groups, for use as diagnostic reagents.

There are probably one or more determinants of penicillin allergy which are now neither known nor even suspected. This is apparent from the fact that some patients react to penicillin, but not to penicilloic acid or penicilloyl-PLL. There is a definite need, therefore, for further study on how penicillins may be transformed either *in vivo* or *in vitro* into presently unknown intermediates capable of reaction with macromolecules. The previously neglected metabolism of these drugs requires more attention, particularly with regard to characterization of products rather than rates. Stability studies on penicillins, *e.g.*, benzylpenicillin (127, 128), phenethicillin (129), and methicillin (130), which have in the past been primarily directed toward determination of rate of loss from solution, should focus more attention in the future upon products of degradation, their formation rates, and their ability to react with protein. This information would be of value in assessing the potential antigenicity of new penicillins and in formulating products so that rates of degradation to reactive intermediates might be minimized. For example, proper control of pH in a penicillin product should reduce penicillenic acid and subsequent "polymer" formation to a minimum (58).

Similarly, it has been seen that the conditions under which a penicillin is manufactured may have an influence on the presence in the final product of certain impurities which might be allergenic. A case in point is penicillenic acid, which can form in nonaqueous solution of penicillin acid. Extraction into nonaqueous medium is usually one step in the purification of penicillins. If the drug is kept too long in that state then it can be seen that considerable quantities of penicillenic acid may form, especially from methicillin and benzylpenicillin where the rate is fairly high. This may explain the presence of material absorbing at 320 mμ in crystalline penicillins (8).

The formulator of penicillin products must be aware of potential reactivity of the pharmaceutical adjuvants with the active drug. Penicillins are known to react with

certain carbohydrates (54, 131) and the conjugates of benzylpenicillin found to be immunogenic in animals (54). Pharmaceutical dosage forms often contain starch, sugars, and other materials capable of reaction with penicillin.

One of the most fascinating questions is whether or not a nonallergenic penicillin can be made. In essence, this is really two questions: (*a*) can a penicillin be prepared which will be nonimmunogenic itself, and (*b*) given a nonimmunogenic penicillin, will it cross-react with antibody formed in response to a previous exposure to a penicillin?

Most allergic individuals have been sensitized by benzylpenicillin and the major antigenic determinant is the penicilloyl group. The rates of aminolysis of penicillins, it has been seen, are not affected to a great extent by the side chain. It would be expected, therefore, that new penicillins would form penicilloyl derivatives at comparable rates and the degree of cross-reactivity would depend on the structural similarity of the conjugates. Since the side chain seems to have only a moderate influence on degree of cross-reaction, it should be expected that those individuals sensitized to the penicilloyl determinant will react to new penicillins. On the other hand, individuals sensitized to one or more of the minor determinants may not react to a new penicillin if the latter does not form the required structure *in vivo*. This further emphasizes the need for suitable test reagents which will be able to pinpoint the antibody specificity of an individual.

The question of immunogenicity of penicillins is still open to conjecture. The possibility that immunogenicity is related to rate of penicillenic acid formation has again been put forth by Feinberg (126) in contrast to results of others (6, 27, 29). Pertinent to this discussion is the mode of action of penicillins, which exert their antibacterial action by inhibition of certain enzymes involved in cell-wall synthesis. This inhibition is a result of penicilloation of the enzymes to form a stable derivative unable to react further. The inhibition of these enzymes then is the same reaction by which the penicilloyl-protein antigenic determinant is formed. It might be expected, therefore, that a penicillin which might react only very slowly with a protein because of some property of its side chain, might also have a relatively low order of antibacterial activity.

One possibility for producing a nonimmunogenic penicillin lies in chemical modification of the nucleus. The cephalosporins, while cross-reacting with penicillins, appear to be less immunogenic themselves, although they have not been used clinically for a sufficient time in which to make a good estimate of their potential allergenicity. Perhaps further nucleus modification would be a step in the right direction.

Another approach to the penicillin allergy problem would be the development of compounds which could specifically inhibit the antigen-antibody reaction. In addition to the monovalent specific inhibitors which were mentioned earlier in this regard, it is also conceivable that other compounds not related to penicillin may have sufficient affinity for the antibody active site to block interaction with antigen. It has been reported (132) that chlorophenesin selectively suppressed certain immunologic reactions elicited by penicillin-protein conjugates in guinea pigs. The drug did not destroy penicillin, did not affect its antibiotic action, was devoid of antihistamine and anti-inflammatory activity, and did not act by generalized depression of reactivity of the organism. Here then is one potential technique for safe "desensitization" of patients, *i.e.*, without using a penicillin.

It seems clear that the further investigation of penicillin allergy will require the collaboration of the pharmaceutical chemist, immunologist, and clinician if success is to be achieved. Furthermore, these studies should provide a model for the investigation of other drug-hypersensitivity problems.

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RESEARCH ARTICLES

Solvency and Hydrogen Bonding Interactions in Nonaqueous Systems

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Abstract □ A study of the importance of hydrogen-bonding interactions in the formation of complexes is useful in providing knowledge of the physical and chemical properties of drug molecules and hopefully it will eventually lead to rational formulation of drugs into suitable dosage forms. The present report deals with a study of some of the methods available for the experimental measurement of these interactions and the means of determining the association (equilibrium) constants from the experimental results. An attempt is made to develop suitable methods for the quantitative analysis of hydrogen-bonding data so that useful estimates of association constants can be made *a priori*. The effect of the nonaqueous solvent on the value of the association constant is also discussed and a method is given whereby an estimate of solvent interaction can be calculated.

Keyphrases □ Solvency-hydrogen bonding interactions—nonaqueous systems □ Complex formation—hydrogen bonding interaction □ Solubility method—association constants □ Liquid-liquid partition method—association constants □ Optical rotatory dispersion method—association constants □ UV spectrophotometry method—association constants □ Linear free energy relationship—hydrogen bonding □ Solvent effects—stability constants, complexes

An attempt has been made in this paper to organize and bring together much of the current information available on the overwhelming role of hydrogen-bond formation on the properties of pharmaceutical and related solutions. A substantial portion of the material presented has been drawn from the literature, the remainder has been based on various published and unpublished studies carried out in the authors' laboratories at The University of Wisconsin and The University of Kansas. The present treatment has been limited to the interactions of organic species in essentially nonaqueous systems.

DISCUSSION

Hydrogen-bond formation plays an extremely important role in controlling various physical processes of prime interest to the pharmaceutical chemist. Solubility, rate of dissolution, rate of zone migration in GLC, TLC, and paper chromatography, partition coefficient, rate of drug release, differential volatility, activity coefficients, *etc.*, are usually controlled by and predictable on the basis of hydrogen-bond formation.

Although, for example, various theories and hypotheses have been proposed in the area of solubility behavior of nonelectrolytes the most pharmaceutically useful approach appears to be that based on the concept that such solutions represent summation of effects arising from interactions of a large number of equilibrium systems. In nearly every instance the more important interactions in these solutions are due to rapid formation and breaking of hydrogen bonds. Thus, if we were to consider solvency of substance A in solvent B it is evident that

$$\text{total } A \text{ in solution} = [A] + 2[A_2] + 3[A_3] + \ldots + [AB] + 2[A_2B] + 3[A_3B] + \ldots + [AB_2] + 2[A_2B_2] + 3[A_3B_2] + \ldots + \ldots$$

where the various terms in the right part of the equation represent the various species present in solutions which contain one or more molecules of A per unit. The concentration of each species can be related to the monomer concentrations of A and B if the stability constant for the particular species was known. Thus, for example,

$$[A] = K_{1:0}$$

and

$$[AB] = K_{1:1}[A][B]$$

and

$$[AB_2] = K_{1:2}[A][B]^2$$

and

$$[AB_n] = K_{m:n}[A]^m[B]^n$$

It must, however, be kept in mind that $[B]$ is not always equal to the reciprocal of the molar volume of B even for the pure solvent,