only, using the Wilcoxson matched-pairs signed-ranks test, there was no significant difference for maxima after phenoxybenzamine at 10^{-8}M and maxima after phenoxybenzamine in the presence of cocaine at 4×10^{-6}M.

In the other two cases, the maxima were significantly smaller ($0{\cdot}01 > P > 0{\cdot}005$ and $P = 0{\cdot}01$ respectively). This indicates that, in this preparation, cocaine does not increase the apparent efficacy or intrinsic activity of the noradrenaline α-adrenoceptor interaction. In view of the specialized architecture of the rat anococcygeus muscle (Nash, Gillespie & Robertson, 1974), we would suggest that the leftwards shift is due to increases in the local concentration of noradrenaline, a result in keeping with Trendelenburg's prediction. The post-synaptic potentiation found by Nakatsu & Reiffenstein (1968) in the relatively densely innervated rat vas deferens should, perhaps, be considered as an exception to the general rule proposed by Trendelenberg (1973).

March 3, 1976

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Chemical aspects of penicillin allergy: mechanism of imidazole-catalysed penicilloylation

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The principal antigenic determinant in penicillin allergy is the penicilloyl group, which is thought to be formed by reactions of penicillin with nucleophilic groups of protein such as ε-amino-groups of lysine residues (for reviews, see Schwartz, 1969; Schneider, 1970). Since penicilloylation of primary amino-groups at physiological pH and temperature only proceeds slowly (Tsuji, Yamana & others, 1975; Bundgaard, 1975), catalysis is likely to be important in the immunochemical binding of penicillins to serum proteins or other tissue macromolecules. Aminolysis of penicillins by imidazole is an efficient process at neutral pH (Bundgaard, 1971, 1972a, b) and imidazole-catalysed penicilloylation could be a pathway involved in the formation of penicilloyl-protein conjugates *in vivo* (Bundgaard, 1972c; Yamana, Tsuji & others, 1975). This possibility is supported by studies involving the blocking of imidazole-groups in proteins (Wagner, Truex & Hall, 1973).

The imidazole-catalysed penicilloylation of amino- or hydroxyl-groups has been suggested to proceed either *via* the initially formed product in the reaction of penicillin with imidazole, *N*-penicilloylimidazole, or *via* its intramolecularly isomerized product, penicillenic acid (Bundgaard, 1972a). Since penicillins which are structurally incapable of undergoing rearrangement into penicillenic acids have been demonstrated to be as immunogenic as e.g. benzylpenicillin (Schneider & de Weck, 1966; Schneider, 1970), it is important to know whether the highly reactive *N*-penicilloylimidazole formed from all types of penicillins is able to transfer its penicilloyl group to various functional groups of proteins with the formation of more stable penicilloyl compounds. Experiments with 6-ethoxycarbonylaminopenicillanic acid (ethoxypenicillin) have led to the conclusion that *N*-penicilloylimidazole is capable of penicilloylating amino- and hydroxy-groups in intermolecular reactions (Bundgaard, 1972c). This conclusion has recently been contested by Yamana & others (1975) for the following reasons: (1) ethoxypenicillin itself as well as *N*-ethoxypenicilloylimidazole are not, as concluded by Bundgaard (1972c), unable to rearrange into ethoxypenicillenic acid; (2) 6-(α-toluensulphonamido)-penicillanic acid which cannot form a penicillenic acid, did not give rise to any significant penicilloylamide formation through reaction with imidazole in the presence of ε-aminocaproic acid, conditions under which benzylpenicillin as well as ethoxypenicillin readily reacted to produce penicilloylated ε-aminocaproic acid. Therefore the authors concluded that the intermolecular imidazole-catalysed penicilloylation of amino-groups by benzylpenicillin does not proceed through the intermediary *N*-benzylpenicilloylimidazole, but exclusively through its isomerized product, benzylpenicillenic acid.

I now wish to present results that support the original statement of the reactivity of *N*-penicilloylimidazole (Bundgaard, 1972c) and that also differ from the conclusions drawn by Yamana & others (1975) about the mechanism of the intermolecular

imidazole-catalysed reactions of benzylpenicillin.

The penicillins used were ethoxypenicillin sodium (Bundgaard, 1972c), benzylpenicillin sodium, and sodium 6-(α-benzenesulphonamido)-penicillanate. The latter was prepared as follows. A solution of benzenesulphonyl chloride (Fluka AG) (5·3 g; 0·030 mol) in acetone (180 ml) was added dropwise with stirring to a solution of 6-aminopenicillanic acid (5·4 g; 0·025 mol) in 250 ml of 3% aqueous sodium bicarbonate and 75 ml of acetone at 0–5°. The 6-aminopenicillanic acid used had been recrystallized by titration of a neutral aqueous solution with hydrochloric acid to its isoelectric point (about pH 4·8). Stirring was continued for a further 2 h at room temperature. The reaction mixture was cooled to 5° and washed with 2 × 300 ml of ether. The aqueous layer was covered with ether (200 ml) and acidified to pH 2 with 5N hydrochloric acid. The layers were separated, and the aqueous portion was extracted with a further 100 ml of ether. The combined ethereal extracts were washed with 100 ml of water in two portions, dried over anhydrous sodium sulphate, diluted to 800 ml with anhydrous ether, and cooled in ice. A 30% solution (20 ml) of sodium 2-ethylhexanoate (0·025 mol) in n-butanol was added dropwise with stirring to produce a white precipitate which was filtered, washed with ether, and dried before recrystallization (without heating) from water–n-butanol (1:20). Yield 4·2 g (45%). The infrared spectrum (in KBr) showed characteristic bands at 1775 (β-lactam carbonyl), 1605 (carboxylate), 1330 and 1160 cm^{-1} (sulphonamide). Found: C, 44·3; H, 4·2; N, 7·4. Calc. for $C_{14}H_{15}N_2O_5S_2Na$: C, 44·4; H, 4·0; N, 7·4%. In thin-layer chromatography (silica gel G; iso-amyl acetate– methanol– conc. formic acid– water, 13:4:2:1) the compound (40 μg) moved as a single spot (R_F 0·53), and was visualized with iodine vapour.

Reactions of the penicillins with imidazole in the presence of varying amounts of different primary amines and methanol were carried out under conditions similar to those already described (Bundgaard, 1972c). Completed reaction solutions were analysed for penicilloylamide or ester and penicilloate by the specific penamaldate assay (Schwartz & Delduce, 1969). As shown in Fig. 1 the percentage yield of the formed penicilloylamides or penicilloyl methyl ester is exactly the same for all three penicillins. In the concentrations used the rate of the penicilloylation is solely determined by the rate of reaction of imidazole with penicillin, cf. Bundgaard (1972c). The results obtained with 6-(α-benzenesulphonamido)-penicillanate differ from those reported by Yamana & others (1975) with a similar penicillin, 6-(α-toluensulphonamido)-penicillanic acid. Yamana and co-workers found no formation of penicilloylamides by reaction of this penicillin under similar experimental conditions (ε-aminocaproic acid was used as penicilloyl acceptor amine). It should be noted that the penicilloyl derivatives of 6-(α-benzenesulphonamido)-penicillanic acid had only about half

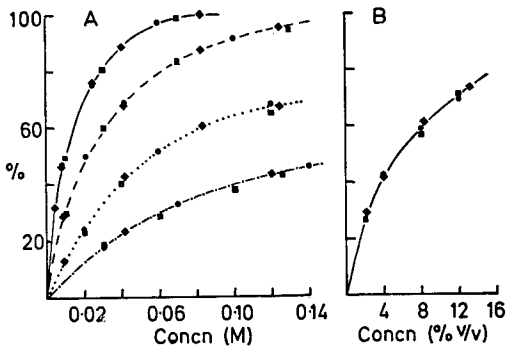


FIG. 1. Effect of amine concentration (M) and methanol concentration (% v/v) on the percentage yield of penicilloylamides (A) and penicillolyl methyl ester (B) formed by reaction of various types of penicillins (10^{-3}M) with imidazole (0·25M) and glycine ethyl ester (——), glycylglycine (- - -), glycine (. . . .), ε-aminocaproic acid (— · —), and methanol (Fig. B). All reactions were carried out at pH 7·40 and 37° (μ = 0·5). ● Benzylpenicillin sodium; ◆ ethoxypenicillin sodium; ■ sodium 6-(α-benzenesulphonamido)-penicillanate.

the molar absorptivity of corresponding benzylpenicilloyl derivatives in the penamaldate assay. Since no penicillenic acid formation is possible with the sulphonamidopenicillin the results can only be interpreted as showing a capacity of *N*-penicilloylimidazole to transfer its penicilloyl group to the acceptor amines and methanol.

The claim by Yamana and co-workers that ethoxypenicillin can isomerize to the corresponding penicillenic acid seems untenable. They observed the appearance of an absorption maximum at 320 nm in the reaction solutions of ethoxypenicillin and imidazole in the presence of mercuric chloride. In the present study an absorption maximum at 317 nm was observed, but in contrast to benzylpenicillenic acid mercuric mercaptide this less intense absorption band was not stable, but disappeared slowly. On treatment with an excess of 2M sodium hydroxide the absorption band disappeared and was not replaced by a new band with a maximum around 300 nm. A similar treatment of benzylpenicillenic acid mercuric mercaptide leads to quantitative formation of the strongly absorbing (λmax 298 nm) 2-benzyl-4-hydroxymethyleneoxazol-5 (4*H*)-one (Bundgaard, 1971; 1972a). Furthermore, when benzylpenicillin and ethoxypenicillin (5 × 10^{-3}M) were allowed to react with imidazole (0·25M) in the absence of mercuric chloride (pH 7·4, 37°) an increase of absorption at 310–325 nm was only observed in the benzylpenicillin solution. Finally, no oxazolone has ever been reported to be formed from an alkoxycarbonylamino-acid or from any of its activated derivatives such as anhydrides, nitrophenylesters or imidazolides (Mazur, 1963; Vajda, Kuziel & others, 1965; Determann, Heuer & others, 1966; Detar, Silverstein & Rogers, 1966; Young, 1972). Also in the reaction of 6-(α-benzenesul-

phonamido)-penicillanate with imidazole in the presence of mercuric chloride an unstable absorption band with λmax at 315 nm was produced. The compounds responsible for this absorption may be *N*-penamaldyl-imidazole mercuric mercaptides formed by reaction of the *N*-penicilloylimidazoles with mercuric chloride.

To determine whether the imidazole-catalysed penicilloylation of amines by benzylpenicillin proceeds through the initially formed *N*-benzylpenicilloyl-imidazole or through its isomerized product, benzyl-penicillenic acid, rate experiments have been made with benzylpenicillenic acid (Sigma, St. Louis). Pseudo-first-order rate constants (kobs) have been determined for the disappearance of benzylpenicillenic acid in aqueous solutions (pH 7·4, 37°) containing imidazole (0·25M) and varying concentrations of glycylglycine and also in solutions containing only imidazole or only glycylglycine. The results (Fig. 2) show that at concentrations of glycylglycine of 0·02–0·08M, the reaction of benzylpenicillenic acid (4×10^{-5}M) with imidazole (0·25M) is preferred to the reaction with glycylglycine. The pure imidazole reaction was a nucleophilic catalysis of hydrolysis *via* *N*-benzylpenicilloylimidazole. Other oxazolones react with imidazole in a similar way (de Jersey, Willadsen & Zerner, 1969). Fig. 2 also shows that the rates of disappearance of penicillenic acid in solutions containing both imidazole and glycylglycine are markedly higher than the rates calculated on the basis of separate reactions with imidazole and glycyl-glycine (illustrated by the dotted line). The aminolysis of benzylpenicillenic acid by either imidazole or glycylglycine was not found to be subject to general acid-base catalysis by a second molecule of amine therefore suggesting that the penicilloylating agent in the experimental conditions employed is *N*-benzyl-penicilloylimidazole and not, benzylpenicillenic acid. The non-linear dependence of kobs on the concentration of glycylglycine (Fig. 2) shows that the rate constant becomes less dependent with higher glycylglycine concentrations. This behaviour is consistent with *N*-benzylpenicilloylimidazole being a metastable intermediate through which the penicilloylation by benzyl-penicillenic acid proceeds.

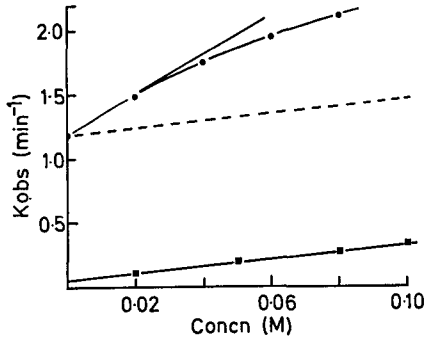
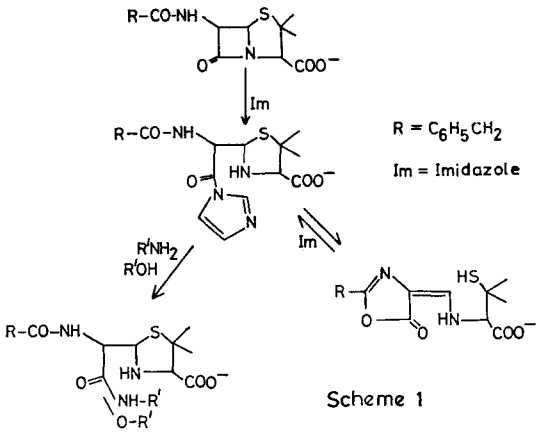


FIG. 2. Effect of glycylglycine concentration (M) on rate of disappearance of benzylpenicillenic acid (4×10^{-5}M) in aqueous solution containing imidazole (0·25M) (●) and in solutions without content of imidazole (■). All reactions were carried out in thermostated quartz cuvettes at pH 7·40 and 37° ($\mu = 0{\cdot}5$). Reaction rates were measured by recording the decrease in absorbance at 322 nm.

Further evidence that *N*-penicilloylimidazole is the predominant penicilloylating agent in imidazole-catalysed penicilloylations is provided by the following experiments. In the absence of the penicillenic acid-stabilizing mercuric chloride the formation of benzyl-penicillenic acid from benzylpenicillin sodium ($5{\cdot}6 \times 10^{-3}$M) in solutions of imidazole (0·25M) with or without methanol (1·67% v/v) or glycylglycine (0·021M) proceeds in a short time to an almost constant concentration of benzylpenicillenic acid in all solutions. The equilibrium concentrations are, however, smaller in the imidazole solutions containing methanol ($1{\cdot}32 \times 10^{-5}$M) and glycylglycine ($0{\cdot}85 \times 10^{-5}$M) than in the pure imidazole solutions ($1{\cdot}69 \times 10^{-5}$M). This difference in equilibrium concentrations can be explained either by an enhanced rate of degradation of penicillenic acid due to reaction with the methanol or glycylglycine or by a smaller equilibrium concentration of the penicillenic acid precursor, *N*-benzylpenicilloylimidazole, caused by its reaction with the two penicilloyl acceptor compounds. However, the rate of degradation of benzylpenicillenic acid in a 0·25M imidazole solution is only slightly enhanced by the addition of glycylglycine (0·021M) (see Fig. 1), and was found to be totally unaffected by the addition of 1·67% v/v methanol. Therefore the lower equilibrium concentrations of penicillenic acid must be due to reaction of the added compounds with the intermediary *N*-benzylpenicilloylimidazole (see Scheme 1).



Scheme 1

In summary, it has been shown that intermolecular imidazole-catalysed penicilloylation of amino- or hydroxyl-groups by benzylpenicillin as well as by penicillins that are structurally incapable of undergoing rearrangement into penicillenic acids proceeds predominantly through the highly reactive *N*-peni-

cilloylimidazole. This mechanism may also be involved in intramolecular imidazole-catalysed penicilloylations (Yamana & others, 1975).

February 2, 1976

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LETTERS TO THE EDITOR

The enolization of 4, 5-dehydromuscarone

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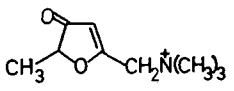
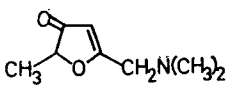
It has been stated that 4,5-dehydromuscarone (I), 4,5-dehydronormuscarone (II) and similar 3(2H)-furanones show no tendency to enolize (Rosenkranz, Allner & others, 1963; Bollinger & Eugster, 1971). However, we have found that 4,5-dehydromuscarone iodide (I) (m.p. 137–8°, prepared by the method of Meister, 1967) in aqueous (D_2O) solution at 40°, undergoes spontaneous enolization at an appreciable rate in solutions of pH greater than 5; this enolization could readily be followed with the aid of nmr spectra.

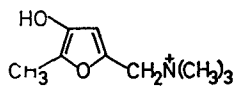


I



II

At pH 5, the proportion of the enolic form (III) present reached a maximum of 34% after 200 min and remained at this value until decomposition of the dehydromuscarone produced an acidic fragment which lowered the pH of the solution and caused a reduction in the proportion of the enolic form present (11% after 20 h). At higher pH values, enolization was rapid and more extensive.



III

Nmr spectra (Varian A60A spectrometer) of freshly prepared solutions of I showed peaks having shifts and integrals in good agreement with those expected for the keto form (δ(ppm) = 1·55, d, $-CH_3$; 3·37, s, $-N(CH_3)_3$; 4·62, s, CH_2N; 5·00, q, 2–H; 6·22, s, 4–H).

Spectra of stored solutions of I showed additional peaks with shifts and integrals (δ(ppm) = 2·26, s, CH_3; 3·12, s, $N(CH_3)_3$; 4·45, s, $-CH_2N$; 6·66, s, 4–H), in good agreement with those expected for the enolic form, the peaks for the ring methyl and proton being

† Correspondence.