IONISATION CONSTANTS OF SOME PENICILLINS AND OF THEIR ALKALINE AND PENICILLINASE HYDROLYSIS PRODUCTS

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Ionisation constants of several penicillins and of 6-aminopenicillanic acid in water at 25°, and approximate ionisation constants of the products of alkaline and penicillinase hydrolysis of some penicillins, are presented. The results suggest that mild alkaline hydrolysis of benzylpenicillin is not a simple reaction. The initial product (the penicilloic acid) has the same imino-group ionisation constant as the product of penicillinase hydrolysis, but this ionisation constant decreases after further treatment with alkali. The nature of this effect is discussed.

The acid-base properties of penicillins and their degradation products, including the penicilloic acids, were of considerable importance in the determination of the structure of the penicillins (Chain, 1949), and are of value in the study of antibacterial activity and drug absorption. The accuracy of the ionisation constants determined earlier (Neuberger, 1949) was limited by the availability and purity of the penicillins, and there do not appear to have been any subsequent publications of more accurate results. Approximate constants of some penicilloic acids were determined (Neuberger, 1949) by back titration of penicillin which had been decomposed by alkali, and more accurate values were obtained for benzylpenicilloic acid by titration of an apparently pure sample of the monosodium salt (Mozingo and Folkers, 1949a). The former procedure involves the assumption that mild alkaline hydrolysis of a penicillin gives only the penicilloic acid. It is shown below that this assumption is of doubtful validity.

Subsequently, titration of the penicillinase hydrolysis product of benzylpenicillin (Benedict, Schmidt and Coghill, 1945) provided some of the evidence for the now accepted belief that the product of this reaction is the penicilloic acid (Pollock, 1960), although the ionisation constants reported by these authors were considerably different from those reported for monosodium benzylpenicilloate.

In this paper we report the ionisation constants of 6-aminopenicillanic acid and a number of penicillins, as well as the constants of the alkaline and penicillinase hydrolysis products of some of the latter.

Experimental

Materials

6 *Aminopenicillanic acid.* A recrystallised preparation of this material was supplied by Mr. F. R. Batchelor.

Penicillins. The penicillins, except ampicillin, were available as the sodium or potassium salt. Samples of benzyl penicillin, phenoxymethyl

penicillin and methicillin were normal commercial material. The other penicillins, which were purified by recrystallisation, were supplied by Dr. M. J. Soulal.

The purity of the penicillins, which were stored in a desiccator at 6°, was determined by an alkalimetric titration method which is routinely used in our laboratories, viz. the determination of the equivalents of alkali consumed by mild hydrolysis of the penicillin.

Penicilloic acids. Solutions of penicilloic acids were prepared by reacting a solution of about one millimole of the penicillin in about 40 ml. of water with 1 ml. of penicillinase solution. The penicillinase solution, supplied by Mr. F. R. Batchelor, was the cell supernatant from a culture of *Bacillus cereus* in a casein hydrolysate medium (Pollock, 1957). A pH of 5 to 7 was maintained by gradual addition of sodium hydroxide until the pH remained constant, the pH was then adjusted to 8·7, because this is the approximate equivalence point for the disodium salt of a penicilloic acid. The solutions were made up to 100 ml.

The total amount of alkali added in all the reactions was very close to 1 equivilant per mole of penicillin, indicating complete hydrolysis of the penicillin. Some of the solutions were tested for residual penicillin by the hydroxamic acid method, with negative results. Consequently, the concentration of penicilloic acid was calculated by assuming 100 per cent hydrolysis of the penicillin to the penicilloic acid.

Some penicilloic acid solutions were also prepared by alkaline hydrolysis of the penicillins. Details of these are given in Table IV.

Procedure

All solutions were made with water that had been double distilled in glass apparatus, boiled for 10–15 min. to remove carbon dioxide and stored in an effectively carbon dioxide-free atmosphere.

Solutions of the penicillins and 6-aminopenicillanic acid in water were titrated with 0·4M hydrochloric acid to pH 2·25 or a higher pH if precipitation of the penicillin free acid occurred. With some penicillins, particularly cloxacillin, a low concentration had to be used so that the titration could be continued to a reasonably low pH, thus enabling enough pK_a values for averaging to be calculated. The random scatter of the pK_a values obtained suggests that supersaturation effects can be neglected. Ampicillin and 6-aminopenicillanic acid, which are amino-acids, were also titrated with 0·3M sodium hydroxide, until 1 equivalent of the latter had been added.

The initial concentrations of the penicillins, corrected for purity, are given in Table I.

The solutions of penicilloic acids, of initial concentration about 0·01M, were titrated with 0·4M hydrochloric acid to pH 2·25.

Titrations

75 ml. of sample solution was titrated in a glass vessel of 200 ml. capacity, kept in a water-bath at 25 ± 0·1°. Titrant was added in 0·1 ml. portions from a 10 ml. burette calibrated in 0·02 ml. divisions.

The solution, through which nitrogen was bubbled, was stirred by a glass paddle-stirrer driven by an electric motor. The pH values were measured by a glass and saturated calomel electrode system, and a Pye "Dynacap" pH meter calibrated in 0·02 pH unit divisions and run from a constant voltage transformer (230 V). The meter was standardised immediately before and immediately after all titrations with buffer solutions made from Soloid tablets and kept at 25°. If a change of more than 0·02 pH unit was observed in a particular titration the latter was rejected. Standardisation was effected at pH 4 and 7 for titrations with hydrochloric acid and at pH 7 and 9·15 for those with sodium hydroxide.

Calculations

Acid ionisation constants, defined by equation (1), were calculated at each pH value measured in the buffer region of the group's dissociation.

$$K_a = \frac{(H)\,[A^-]}{[HA]} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where (H) is the hydrogen ion activity. $[A^-]$ is the concentration of carboxylate ion or of un-ionised amino-compound, and [HA] is the concentration of un-ionised carboxylic acid or of the conjugate acid of the amino-compound, when K_a is the ionisation constant of a carboxy group or of an amino-(or imino-) group respectively. The constants so obtained are not thermodynamic constants since the definition of these involves the activities, instead of the concentrations, of all the species involved in the equilibrium. The constant defined by (1) has been called the "apparent" (Greenstein and Winity, 1961) or the "mixed" (Albert and Serjeant, 1962a) isonisation constant.

The constants were calculated from the relevant form of the Henderson (1908) equation; equation (2) for the titrations with HCl and equation (3) for the titrations with NaOH.

$$pK_a = pH - \log\left(\frac{c}{a - H} - 1\right) \quad .. \quad .. \quad (2)$$

$$pK_a = pH + \log\left(\frac{c}{a} - 1\right) \quad .. \quad .. \quad .. \quad (3)$$

where c is the total sample concentration, a is the titrant concentration and H is the hydrogen ion concentration. Increase in the total volume of solution caused by addition of titrant was allowed for when the concentrations were calculated.

The hydrogen ion concentration in (2) can be obtained either from the measured pH, or from the titration of a blank solution which contains all components of the sample solution except the pure substance under investigation (Glasstone, 1942). As a result of the definition of pH, the former method gives the hydrogen ion activity and not the concentration, but the use of activity in place of concentration here is not infrequently found in the literature (Albert and Serjeant, 1962b). When the latter method is used, (2) becomes:

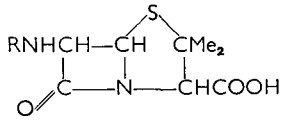
$$pK_a = pH - \left(\log \frac{c}{a - A_1} - 1\right) \quad .. \quad .. \quad (4)$$

where A_1 is the concentration of acid added to the blank titration at the relevant pH. This procedure involves two assumptions; (*a*) that hydrogen ion concentrations are identical when the sample and blank solutions are at the same pH, i.e., that the hydrogen ion activity coefficients are identical when the two solutions are at the same pH, and (*b*) that the titrant is 100 per cent dissociated in the blank solution. The validity of these assumptions is theoretically dubious and there seems to be no need to use this method unless the sample solution contains titratable substances other than the sample itself.

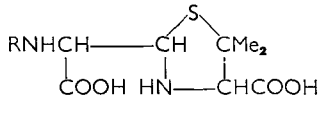
The ionisation constants of the penicillins were calculated by both these methods. The results of the two calculations agreed to within 0·02 pK_a unit for all the penicillins thus showing that the above assumptions are acceptable under the conditions used in this work. This is important because the blank titration method had to be used for the penicilloic acid calculations, there being some titratable material in the penicillinase preparation.

Results

The penicillins (I) and penicilloic acids (II) have structures as follows:



(I)



(II)

Penicillins. Ionisation constants of seven penicillins and of 6-aminopenicillanic acid are given in Table I. Each result is the average of a set of pK_a values calculated at each pH measured during the titration. In all cases results from two titrations are given. The lowest number of values in a set was 7 and most sets contained more than 10 values. The mean value and the maximum deviation of each set of values is quoted.

Penicilloic acids. The penicilloic acids have three ionisation constants, two attributable to carboxy groups and one to the imino-group. The two carboxy group constants are evidently of similar magnitude and it was not possible to evaluate them from the experimental data of the present work.

Imino-group ionisation constants of four penicilloic acids, obtained by penicillinase hydrolysis of the penicillins are given in Table II. Again the results are the averages of several values and results from two titrations are given. However, the pK_a values from each titration showed a tendency to decrease as the pH decreased. An example is shown in Table III. Possible reasons for this decrease are suggested in the Discussion. The results in Table II were taken from the earlier part of the titrations, where the values generally showed less consistent downward variations. Thus each result is the average of 11–13 values, obtained over the pH range 6·5–5·0, and in each case 3–5 values obtained

TABLE I

IONISATION CONSTANTS OF PENICILLINS AND 6-AMINOPENICILLANIC ACID IN WATER AT 25°

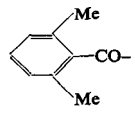
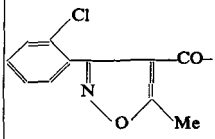
Sample	R	Purity per cent	Initial Conc. (M)	pKa –COOH	pKa $-NH_2$
6 APA	H–	100	0·0081	2·30 ± 0·05 2·29 ± 0·03	4·90 ± 0·05 4·92 ± 0·03
Benzylpenicillin ..	$PhCH_2CO-$	99·3	0·0099 0·0093	2·73 ± 0·03 2·71 ± 0·05	
Phenoxymethylpenicillin.. ..	$PhOCH_2CO-$	97·9	0·0098 0·0054	2·73 ± 0·05 2·74 ± 0·04	
Phenethicillin ..	PhOCHCO– | Me	97·5	0·0099 0·0082	2·72 ± 0·02 2·74 ± 0·03	
Propicillin.. ..	PhOCHCO– | Et	99·2	0·0048 0·0034	2·72 ± 0·03 2·72 ± 0·04	
Methicillin ..		97·5	0·0099 0·0096	2·76 ± 0·02 2·78 ± 0·03	
Cloxacillin ..		97·7	0·0025	2·73 ± 0·04 2·70 ± 0·03	
Ampicillin ..	PhCHCO– | NH_2	99·3	0·0079	2·53 ± 0·04 2·52 ± 0·02	7·24 ± 0·02 7·25 ± 0·03

TABLE II

IMINO-GROUP IONISATION CONSTANTS OF SOME PENICILLOIC ACIDS IN WATER AT 25°

Penicilloic acid	pKa
Benzyl	5·31 ± 0·05 5·33 ± 0·04
Phenoxymethyl	5·18 ± 0·03 5·12 ± 0·04
Phenoxyethyl	5·31 ± 0·02 5·28 ± 0·04
Phenoxypropyl	5·30 ± 0·05 5·31 ± 0·05

over the pH range 5·0–4·4 were ignored in obtaining the average because of the downward trend.

Table IV presents imino-group ionisation constants of benzylpenicilloic acid obtained by penicillinase and by alkaline hydrolysis of benzylpenicillin and titrated in 0·01M solution under various conditions. Here, because of the wide variations, the highest and lowest pK_a values are quoted from a set of about 15 values calculated from single titrations. The values in each set decreased with decreasing pH.

Discussion

The ionisation constants obtained for the penicillins examined here are consistent with the approximate results reported by Neuberger (1949) for benzylpenicillin and some other natural penicillins, and the results for 6-aminopenicillanic acid are in agreement with the approximate results reported by Batchelor, Chain, Hardy, Mansford and Rolinson (1961) for this compound. The side chain has very little effect on the ionisation constant of the penicillin, except when a free amino-group is present as in 6-aminopenicillanic acid or ampicillin. In these cases the presence of the positively charged amino-group slightly increases the acid strength of the carboxy group. The small effect of the side chain on the ionisation constant is to be expected because of the large distance between the two groups.

TABLE III

Variation of pK_a of benzylpenicilloic acid with pH

pH	6·46	6·22	6·04	5·88	5·76	5·64
pK_a	5·38	5·37	5·35	5·34	5·34	5·33
pH contd.	5·54	5·43	5·33	5·23	5·12	5·01
pK_a	5·33	5·32	5·31	5·31	5·29	5·29
pH contd.	4·89	4·75	4·61	4·43		
pK_a	5·27	5·25	5·24	5·21		

TABLE IV

Imino-group ionisation constants of the product of hydrolysis of benzylpenicillin under various conditions

Conditions of hydrolysis	Initial NaCl* conc. (M)	pK_a
Penicillinase	None	5·34–5·20†
Penicillinase	0·016	5·24–5·13‡
Penicillinase; then NaOH added to give pH 12; 21 hr. at 25°	0·016	4·89–4·78
NaOH; pH 12 at 25° overnight	0·016	4·98–4·82
NaOH; pH 12 at 23° 4 hr.§	0·004	5·34–5·15
Penicillinase	1·6	4·81–4·70
Penicillinase; 6M HCHO	None	4·2‖

* NaCl was either added as such or produced by neutralisation of excess NaOH.
† 15 values. 11 values averaged to 5·31 ± 0·05 quoted in Table II.
‡ 15 values. 11 values averaged to 5·22 ± 0·04.
§ Solution maintained at pH 12 by gradual addition of NaOH for 1½ hr. till pH stopped falling, then kept without addition of NaOH for further 2½ hr.
‖ pH at half-neutralisation.

The results presented for the penicilloic acids are incomplete because of the absence of carboxy group ionisation constants. There are no reliable values of these constants in the literature; the only results to be found are a value of pK_a 2·95 (Neuberger, 1949) and one of pK_a 2·16 (Benedict, Schmidt and Coghill, 1945), both for benzylpenicilloic acid. The former value was obtained from a sample of monosodium benzylpenicilloate, but it is not clear whether the presence of a second carboxy group was allowed for in the calculation. The latter value was obtained from a penicillinase hydrolysate of benzylpenicillin. However, Benedict and others do not seem to have realised that there are two overlapping

carboxy dissociations, and they attributed the second ionisation constant that they determined from their titration, one of pK_a 4·7, to an acid group. This phrase has been interpreted (Henry and Housewright, 1947; Hamilton-Miller, Smith and Knox, 1963) to mean that a carboxy group of pK_a 4·7 is present in penicilloic acids.

The evidence presented by Neuberger (1949), based on comparisons with thiazolidines of various structures, provides reasonably conclusive proof that the ionisation constant of about pK_a 5 in penicilloic acids is due to the imino-group. The fact that titration in the presence of formaldehyde decreases the ionisation constants of amino- and imino-groups, but has little effect on carboxy group constants, has been used (Harris, 1923–24; Harris and Birch, 1930) in the allocation of ionisation constants of amino-acids. The result in Table IV shows that titration of benzylpenicilloic acid in the presence of formaldehyde decreases the pK_a 5·3 value to about pK_a 4·2, thus confirming that this constant is not primarily due to a carboxy group.

The imino pK_a value of 5·32 found (Table II) for the product of penicillinase hydrolysis of benzylpenicillin is in good agreement with the value of 5·25 reported by Mozingo and Folkers (1949a) for monosodium benzylpenicilloate, and is considerably different from the value of 4·7 reported for a penicillinase hydrolysate by Benedict and others (1945). The results presented here thus provide confirmation of one point in the surprisingly small amount of evidence (Pollock, 1960) that shows the product of penicillinase hydrolysis to be the penicilloic acid.

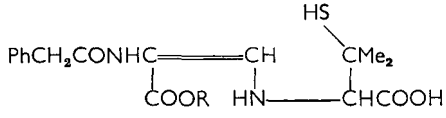
The only product of mild alkaline hydrolysis of a penicillin that has been reported (Mozingo and Folkers, 1949b) is the penicilloic acid. The results presented in Table IV show that the initial product obtained by hydrolysis at pH 12 for 4 hr. has approximately the same pK_a as penicilloic acid, but after 21 hr. at pH 12 a product with a lower pK_a is produced. Storage of a penicillinase hydrolysate at pH 12 for 21 hr. also gives a product of lower pK_a. The titrations done after storage at pH 12 for 21 hr. had higher initial sodium chloride contents than the other titrations, but this was shown not to be the cause of the lower pK_a's by titration of a fresh penicillinase hydrolysate in the presence of the relevant amount of sodium chloride (0·016M), which gave only a slight decrease of pK_a. Thus storage of benzylpenicilloic acid at pH 12 for 21 hr. has an effect which results in a considerable decrease of the pK_a of the imino-group. Since the basicity of the imino-group is unlikely to change as a result of conversion of the penicilloic acid to a different stereoisomer, the results imply that the acid is converted to some other compound. This conclusion is in agreement with observations of two spots on paper chromatograms of alkaline hydrolysates of some penicillins (Mansford, personal communication).

If this conversion occurs to some extent in penicillinase hydrolysates at pH 8·7, then the results in Table II–IV have been calculated from titrations of mixtures of penicilloic acid and the unknown compound. This could account for some of the decrease of pK_a observed in these titrations. However, an increase of 0·016M in initial sodium chloride

concentration decreases the pK_a by about 0·1 unit († and ‡, Table IV). The sodium chloride concentration increases by about 0·01M during titration over the pH range used for calculation of the pK_a values in Tables II–IV. Thus the salt effect associated with this increase probably accounts, at least in part, for the downward trend of pK_a values. Similarly, the low pK_a of 4·7 reported by Benedict and others (1945) could be attributed to a salt effect, because a pK_a of about 4·75 is obtained in the presence of 1·6M sodium chloride (Table IV).

The pK_a value of about 4·85 found after treatment with alkali for 21 hr. is close to the pK_a value of penilloic acids (Neuberger, 1949), which are the decarboxylation products of penicilloic acids. But decarboxylation at pH 12 would produce sodium carbonate, which would be titrated in the pH range 7·5–9. There was no evidence of this; thus titration of a penicillinase hydrolysate of benzylpenicillin in the presence of 0·5 mole sodium carbonate per mole penicillin required 0·5 ml. of titrant to change the pH from 9 to 6·7, while titration of the hydrolysate kept at pH 12 for 21 hr. required only 0·1 ml. of titrant for this pH change.

The formation of α-methyl penamaldate (III, R = Me) by the prolonged action of methanol on benzylpenicillin has been reported by Trenner (1949).

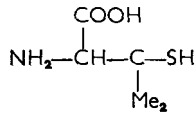


(III)

A tautomer of penamaldic acid (III, R = H) has been suggested (Goodall and Strafford, 1947) as a compound into which benzylpenicilloic acid might decompose before reacting with iodine. Its possible existence in equilibrium with benzylpenicilloic acid has been used (Cook, 1948) to explain the mutarotation of benzylpenicilloic acid in alkaline solution (Mozingo and Folkers, 1949b). The formation of penamaldic acid from benzylpenicilloic acid is consistent with the fact that thiazolidines generally exist in an equilibrium mixture of the ring form and an acyclic thiol form (Sprague and Land, 1957), although the presence of substituents generally stabilises the ring form. Some evidence for the existence of penamaldic acid in equilibrium with benzylpenicilloic acid was provided by the formation of penicillamine-cysteine disulphide on incubation of benzylpenicilloic acid with cystine at pH 7·5 and 37° (Levine, 1960).

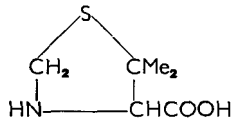
The imino pK_a of penamaldic acid will differ from that of benzylpenicilloic acid as a result of (*a*) the presence of a double bond, which will decrease the pK_a, and (*b*) opening the thiazolidine ring, which will increase the pK_a. The effect of a double bond α- to a secondary nitrogen was studied by Starr, Bulbrook and Hixon (1932), using pyrrolidines and Δ^2-pyrrolines. They found that introduction of the double bond decreased the pK_a by 2·5–3·2 units. The magnitude of (*b*) can be

determined by reference to penicillamine, IV, which has an amino pK_a of 7·7 (Mozingo and Folkers, 1949c).



(IV)

Introduction of an *N*-methyl group slightly increases the pK_a of primary amines [e.g., methylamine pK_a 10·62, dimethylamine pK_a 10·77 (Albert and Serjeant, 1962c)] so that *N*-methyl penicillamine will have pK_a about 7·9. Ring closure of *N*-methyl penicillamine will give 3-carboxy-4,4-dimethylthiazolidine (V), which has an imino pK_a of 5·98 (Neuberger, 1949).



(V)

Thus the net change of pK_a when the ring of a penicilloic acid is opened and a double bond introduced will be +1·9—(2·4 to 3·2), i.e.,—0·5 to 1·3. The observed effect when benzylpenicilloic acid is treated with alkali, a decrease of about 0·5 unit, is in reasonable agreement with the former value and penamaldic acid may well be a product of the action of alkali on benzylpenicilloic acid. Further work is in progress in an attempt to confirm this conclusion. If the conclusion is valid, it is clear from the results that the equilibrium between penamaldic acid and benzylpenicilloic acid is not rapidly established under the conditions of the titrations. This may be relevant to the large effect of experimental conditions on the iodine uptake of penicilloic acids (Weiss, 1959).

Less detailed experiments have indicated that alkali treatment of penicilloic acids other than benzyl has an effect similar to that discussed above.

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The paper was presented by DR. RAPSON.