[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Distribution of Pyridine Alkaloids in the System Buffer–*t*-Amyl Alcohol

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The partition coefficients of 14 N-heterocyclic compounds related to the tobacco alkaloids have been measured in the system *t*-amyl alcohol-buffer. When possible, the true partition coefficient and hydrolysis constant have been calculated. Suitable data are given for control of conditions so that countercurrent distribution can successfully separate these bases in admixture.

We report here the observed partition coefficients of model compounds, consisting of nicotine and its degradation products, between buffer and *t*-amyl alcohol. We are interested in these compounds because they are possible products resulting from the irradiation of nicotine in the presence of methylene blue and oxygen[2,3] or the fermentation of tobacco.[4]

Compounds closely related in structure may be separated if they are distributed between two immiscible solvents in successive countercurrent stages.[5,6] This technique is good for determining observed partition coefficients. The observed coefficient of these model compounds allows one to predict in which tubes the compounds will be present in an unknown mixture at a given *p*H. Golumbic, *et al.*,[7,8] showed that the observed partition coefficients of acids and bases depend upon the ionization constant, hydrogen ion concentration and true partition coefficient of the substance. It is desirable, therefore, to study partition coefficients, using buffers as the aqueous phase. By distributing an unknown complex mixture at several *p*H values and observing the movement of the peaks, thus allowing calculation of partition coefficients, it is possible to predict which of the model substances is in the mixture. This aids in the choice of derivatives for ultimate identification.

Given any known mixture of these model substances, a *p*H can be selected for distribution whereby separation can be made with the least number of transfers. The number of transfers required to separate two compounds with certain percentage impurities can be obtained simply from nomographs previously published.[9,10] For example, if one wanted to separate dihydronicotyrine and nicotine at *p*H 6 with 5% impurity, he would need approximately 18 to 20 transfers.

When a weak diacidic base such as nicotine is distributed between an organic solvent and an immiscible aqueous phase, hydrolysis occurs. Assuming there is no association in the organic phase, the observed partition coefficient k' is the result of the two equilibria: (a) the hydrolysis of the base in the aqueous phase and (b) the partition of the undissociated base between the immiscible phases. This true partition coefficient k is represented by the ratio of the undissociated base in the organic phase to the undissociated base in the aqueous phase. According to Golumbic's derivation, the observed partition coefficient may be expressed by

$$k' = \frac{[B]_0}{[B]_w + [BH^+]_w + [BH_2^{++}]_w} \qquad (1)$$

Substituting the first and second hydrolysis constants K_1 and K_2, resp., and rearranging terms

$$\frac{1}{k'} = \frac{1}{k} + \frac{K_1}{k[OH^-]} + \frac{K_1K_2}{k[OH^-]^2} \qquad (2)$$

It can be seen that equation (2) is that of a parabola of the form $y = a + bx + cx^2$, where $y = 1/k'$ and $x = 1/[OH^-]$, in which $(y - y_1)/(x - x_1)$ is linear with x. If three *p*H values are selected and if the degree of accuracy of the analytical and distribution measurements are commensurate with the hydrolysis constants, *i.e.*, 1×10^{-7}, rectification of equation (2) to a straight line plot or solution of three simultaneous equations would give values K_1, K_2 and k. This degree of accuracy is not required, however, to obtain good values for K_1 and k.

The third member of the right-hand side of equation (2) can often be omitted from the equation, since it has negligible effect except in the region where the hydroxyl ion concentration is a great deal less than the square-root of the product of the two hydrolysis constants. In dealing with the alkaloids having first hydrolysis constants less than the order 1×10^{-7}, selection of hydroxyl ion concentration greater then 1×10^{-9} permits deletion of this third term. The resulting equation then is that of a straight line.

Experimental

A 40-tube glass type Craig apparatus[11] was employed. Eight transfers were used for each determination of partition coefficient. Each tube had a capacity of 20 ml. of stationary phase.

The various heterocyclic bases used in this study were prepared and purified in this Laboratory according to techniques previously reported.[12-14] The *t*-amyl alcohol was purified by distillation.

Equal volumes of citrate or phosphate buffer, 0.05 molar, and *t*-amyl alcohol were mutually saturated and separated before use. The base to be distributed was dissolved in the buffer phase at a concentration of 0.5 to 1 mg./ml., and a 20-ml. aliquot was placed in the zero tube. Twenty ml. of

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t-amyl alcohol saturated with buffer was used for each transfer.

At the end of the distribution, the total contents of each tube was diluted with 95% ethanol to a standard volume of 100 ml. The citrate buffer of *p*H 6 required addition of 5 ml. of water prior to dilution with ethanol to ensure a homogeneous solution. Phosphate buffers at *p*H 7 and *p*H 8 required 7 and 9 ml. of water, respectively, to ensure homogeneity. The solutions were then read on a Beckman ultraviolet spectrophotometer using a 1-cm. cell, at a wave length of 259 mμ, to determine the concentration of base in each tube.

Discussion

The observed partition coefficient, $k' = C/C'$, where C = concentration of base in the upper phase and C' = concentration of base in the lower phase, was calculated from the relative concentrations of base in adjacent tubes according to Williamson and Craig.[15] When the peak was essentially in the zero tube, the coefficient was calculated from the equation $x = (1/1 + k')^8$ and when essentially in the eighth tube, it was calculated from the equation $x = (k'/1 + k')$,[8] where x is the fraction of base in the tube and k' is the partition coefficient.

The observed partition coefficients are given in Table I. All distributions were made at room temperature (24 ± 2°), and no attempt was made to control the temperature precisely, since this small change in temperature has negligible effect on the partition coefficient.[16] Figure 1 is a plot of $1/k'$ *vs.* $1/[OH^-]$. The best straight line was fitted to the experimental data by the method of least squares. The intercept gives a value for $1/k$, and the slope gives a value for K_1/k, from which, if the intercept is known, K_1 can be calculated.

Determination of the partition coefficient for N-methylmyosmine gave some difficulty. The distribution curves for this compound at *p*H 6.0, 7.0 and 8.0 are shown in Fig. 2. Although the compound was essentially pure as determined by ultraviolet spectra, its conventional distribution curve indicated that it was impure. The amount of impurity as calculated from the curves is dependent on the *p*H. Since the same preparation was used in each case, this cannot be true, unless a reaction is taking place. N-Methylmyosmine can exist in either an open-chain ketone structure or as the closed heterocyclic ring structure.[13] One possible explanation for this behavior is that the *p*H values employed were such as to shift the tautomeric equilibrium between the two forms. Myosmine, however, which is capable of existing in similar tautomeric forms,[17] did not show this anomaly at the *p*H values employed. The shape of the distribution curves would

TABLE I

OBSERVED AND TRUE PARTITION COEFFICIENTS OF PYRIDINE ALKALOIDS BETWEEN *t*-AMYL ALCOHOL AND BUFFER, TOGETHER WITH 1ST HYDROLYSIS CONSTANT

	Observed partition coefficient[a]				
	*p*H 6.0	*p*H 7.0	*p*H 8.0	k	K_1
N-Methylmyosmine	0.01	0.01_6	0.06	...	.....
4-Methylamino-1-(3-pyridyl)-1-butanol	.01	.02	$.04_5$	...	.....
Nornicotine	$.01_3$	.08	.46	...	.....
Anabasine	.03	.14	.86	...	.....
Metanicotine	.03	.07	.17	...	.....
Nicotine dioxide	.04	.04	.04	0.04	.....
Nicotine oxide	$.11_0$	$.11_7$	$.12_2$	0.12	9.4×10^{-10}
Nicotine	.29	2.05	5.59	6.5_9	2.2×10^{-7}[b]
Dihydronicotyrine	.53	2.99	5.89	6.3_5	1.1×10^{-7}
Myosmine	4.70	5.36	5.56	5.5_2	1.7×10^{-9}
2-Methyl-6-(3-pyridyl)-tetrahydro-1,2-oxazine	6.21	6.24	6.34	6.3_0	1.5×10^{-10}
Nicotyrine	12.8_5	13.4	13.6	13.5	3.4×10^{-10}
Nornicotyrine	14.0	14.1	14.4	14.3	2.1×10^{-10}
Pyridine	3.6	4.03	4.04	4.0_6	1.3×10^{-9}[c]

[a] All measurements made at room temperature, 24 ± 2°. [b] The following values are reported in the literature for nicotine: Kolthoff, *Ann. phys.*, **8**, 121 (1927), $K_1 = 7 \times 10^{-7}$; Craig and Hixon, THIS JOURNAL, **53**, 4367 (1931), $K_1 = 9 \times 10^{-7}$; Norton, *Ind. Eng. Chem.*, **32**, 241 (1940), $K_1 = 1 \times 10^{-6}$; Lowry and Lloyd, *J. Chem. Soc.*, 1626 (1932), $K_1 = 4.90 \times 10^{-7}$. [c] The following values are reported in the literature for pyridine: Goldschmidt and Salcher, *Z. physik. Chem.*, **29**, 89 (1899), $K = 2.4 \times 10^{-9}$; Barrow, *J. Biol. Chem.*, 12, 313 (1937), $K = 2.2 \times 10^{-9}$.

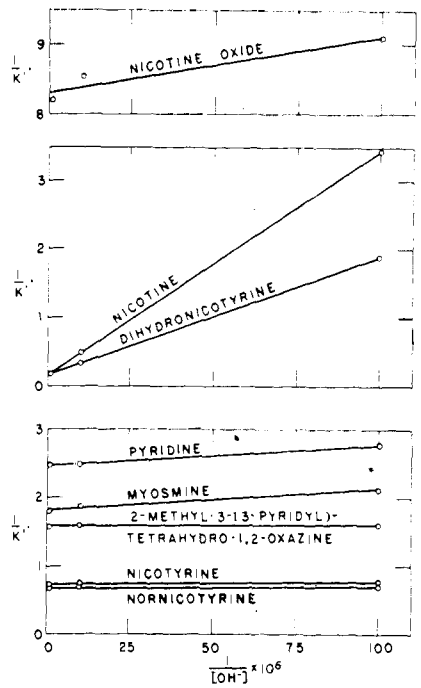

Fig. 1.—Effect of hydroxyl ion concentration on the observed partition coefficients.

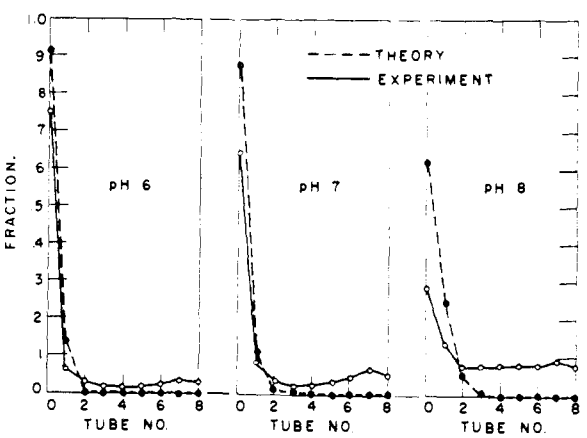

Fig. 2.—Countercurrent distribution of N-methylmyosmine at *p*H 6.0, 7.0 and 8.0.

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seem to suggest polymerization rather than a shift of tautomeric forms. Ready polymerization of N-methylmyosmine was encountered in the work previously cited.[13] This phenomenon was not studied further. The values of k' as reported were calculated from the major peak of these curves.

Values much greater than 10 or less than 0.1 cannot be determined accurately with an eight-tube transfer. For this reason, the values of the observed partition coefficients as reported in Table I for 4-methylamino-1-(3-pyridyl)-1-butanol, nornicotine, anabasine and metanicotine deviate from a straight line function sufficiently to make it impossible to calculate good values for the true partition coefficient or hydrolysis constant of these compounds. The observed values are in the correct order of magnitude and are reported here for use in separations of these compounds by countercurrent techniques.

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Relation between Precursor Structure and Biosynthesis of Penicillins[1]

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The addition of various carboxylic acids to synthetic-medium fermentation of *Penicillium chrysogenum* Q176 brought about the synthesis of the corresponding penicillin if the acid added was not substituted in the α-position. The paper chromatographic assay for penicillin types showed that in the presence of sorbic, cyclohexaneacetic and phenoxyacetic acids, the percentage of precursor-produced penicillin was as high as in the presence of phenylacetic acid. The over-all precursor efficiency of these acids was, however, much lower than that for phenylacetic acid. The rate of metabolism of certain precursor acids has been found to bear an inverse relation to precursor efficiency. Acids substituted in the α- or β- positions were not readily metabolized by the mold. If a rapidly metabolized acid such as sorbic was added to the fermentation at frequent intervals, its precursor efficiency became comparable with that for the slowly metabolized phenylacetic acid.

During the early coöperative investigations on penicillin[3] work was done in many laboratories on biosynthesis of penicillins. A large number of compounds were tested as possible precursors. It was found that when any one of a number of carboxylic acids was added to the fermentation, as such or as a suitable derivative, a penicillin was produced which was a substituted amide of the acid added, just as benzyl penicillin is a substituted amide of phenylacetic acid. Eleven new penicillins were crystallized. Later, Behrens and coworkers[4,5] reported tests on further possible precursors, and isolated 18 additional new penicillins. An additional five new penicillins were reported by Philip and others.[6] At the time most of this work was done, convenient criteria for production of new penicillins were not available. Stimulation of yield and variation of the penicillin activity ratio as measured on two test organisms were the methods used. After the advent of convenient paper chromatographic techniques, Thorn and Johnson[7] were able to show that the lower saturated fatty acids regularly produced biosynthetic penicillins, although such compounds had given no evidence of precursor activity in previous work.

The tentative conclusions regarding the relation of structure to precursor activity drawn from earlier work were[1] (a) that ring-substituted phenylacetic acids were excellent precursors, (b) that certain other ring systems could be substituted for the benzene ring, (c) that an "interrupting group" in the carbon chain, apparently to prevent β-oxidation, appeared advantageous, and (d) that substitution in α-position led to structures with no precursor activity.

The fact that acids which should be resistant to β-oxidation tended to be good precursors, together with the finding[5] that straight-chain aliphatic acids often acted as precursors for penicillins corresponding to acids containing two fewer carbon atoms, made it appear likely that precursor activity was inversely related to oxidizability. In the present investigation the chromatographic method for detection and determination of new penicillins has been applied to a number of possible precursors, many of which have been tested by other workers. A more accurate evaluation of their precursor activity has thus been gained, and the relation of precursor activity to oxidizability by the mold has been studied.

Results and Discussion

Table I lists the compounds tested as precursors, and the total penicillin yields obtained when they were used. The last column gives the percentage of "new" (*i.e.*, biosynthetic) penicillin formed, as determined by paper chromatography. Diagrams of representative paper chromatograms are given in Fig. 1. The data of Table I show that substitution of any one of a variety of groups at the α-carbon of the precursor acid made the resulting compound unavailable as precursor. An exception was α-ethylphenylacetic acid, which, in one of

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