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EFFECTS OF pH ON THE ACTIVITY OF NICOTINE AND NICOTINE MONOMETHIODIDE ON THE RAT DIAPHRAGM PREPARATION

BY

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Results have been obtained which substantiate the view that it is the univalent nicotinium ion rather than the un-ionized base which acts at the neuromuscular junction.

Nicotine is dibasic, containing a strongly basic pyrrolidine nitrogen atom and a weakly basic pyridine nitrogen atom. Taylor (1951) deduced that from its dissociation constant, determined by Vickery & Pucher (1929), nicotine was mostly in the form of the univalent (pyrrolidinium) cation at physiological *p*H, and, by analogy with acetylcholine and other onium compounds, it might be expected that it is in this form that the substance acts at ganglia and at the neuromuscular junction. The amounts of divalent cation present at this *p*H are so small (less than 1 in 100,000) that it is unlikely that this species is active. However, although the predominant species is the univalent cation, considerable amounts of the completely un-ionized base are also present (over 40% at *p*H 7.6 and 37° C, see below) and there exists the possibility that this may have some effect. To investigate this we have studied the effects of *p*H on the activity of nicotine.

In studies on the effects of *p*H on the activity of local anaesthetics (Trevan & Boock, 1927; Skou, 1954) the concentrations of drug which blocked conduction were determined in buffer solutions of different *p*H. Changes in *p*H alone, however, may affect the sensitivity of tissues to drugs, so changes in activity with *p*H may not simply be due to changes in the degree of ionization. We have therefore studied the effects of *p*H on the relative activities of nicotine and nicotine monomethiodide (monomethylnicotinium iodide). These substances are extremely similar in structure, differing only in that one has a tertiary pyrrolidine nitrogen atom where the other has a quaternary pyrrolidine nitrogen atom. It seemed reasonable to expect that changes in the sensitivity of the tissues brought about by altering the *p*H should affect both substances to a similar extent. Consequently changes in the relative activity of the two compounds should be related to the degree of ionization of nicotine, if it is as the monovalent ion that this substance is active. A similar technique has been employed by Kalow (1954), who studied the effects of *p*H

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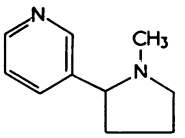
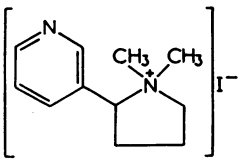


Nicotine



Nicotine monomethiodide

change on the relative activity on the frog rectus of tubocurarine chloride and dimethyltubocurarine chloride in order to assess the effects of ionization on the activity of the former.

Our observations were confined to the rat diaphragm preparation (Bülbring, 1946). We had hoped also to use the chick biventer-cervicis preparation (Ginsborg & Warriner, 1960), but found that this would not tolerate the changes in *p*H we wished to make.

METHODS

Chemical

Compounds. Nicotine was used as the hydrogen tartrate (B.D.H.) and as the monohydrochloride (K. & K. Labs. Inc., Jamaica, N.Y.). The latter was recrystallized before use.

Nicotine monomethiodide was prepared by the method of Barlow & Dobson (1955) but crystallized in an anhydrous form, m.p. 137–138.5° C; found, C, 43.1; H, 5.65; I^-, 42.1; calculated for $C_{11}H_{17}N_2I$, C, 43.4; H, 5.65; I^-, 41.8%. The ultra-violet absorption spectrum in de-ionized water was identical with that of the hydrated material, $\lambda_{max.}$ 225 and 260 mμ, log ϵ (molar) 4.17 and 3.57; Barlow & Dobson recorded $\lambda_{max.}$ 225 and 260 mμ, log ϵ (molar) 4.14 (the value 4.41 is a misprint) and 3.55. The absorption at 225 mμ is due to the iodide ion; for sodium iodide $\lambda_{max.}$ is 225 mμ with log ϵ (molar) 4.13.

Monomethylnicotinium dipicrate, recrystallized from aqueous ethanol, had m.p. 163.5–164.5° C; found C, 43.4; H, 3.54; $C_{23}H_{22}N_8O_{14}$ requires C, 43.5; H, 3.51%.

Dissociation constants. Values of pK_a were determined by electrometric titration as described in a previous paper (Barlow & Hamilton, 1962).

Biological

Preparations. The rat phrenic nerve-diaphragm preparation was set up as described by Bülbring (1946) and stimulated with square-wave shocks of 0.75 msec duration at a rate of 5/min. The bath volume was 25 ml. and the temperature 37° C.

Assay. The two compounds were tested for their ability to block neuromuscular transmission in the rat diaphragm. Their activities were compared on a molar basis by a standard 4-point assay procedure. In one set of experiments the preparation was set up in Krebs bicarbonate Ringer solution gassed with pure oxygen (solution A), the assay performed, the aerating gas changed to 95% oxygen plus 5% carbon dioxide (solution B), and the assay repeated. In a second set of experiments the preparation was set up in a modified Krebs bicarbonate Ringer solution containing a higher concentration of bicarbonate than usual (solution C), and after the assay this was replaced by a modified Krebs bicarbonate Ringer solution containing a lower bicarbonate concentration than usual (solution D) and the assay repeated. In a third set of experiments the assays were performed in solution A and then in solution D. At least 40 min was allowed between the assays for the preparations to become adjusted to the changes in *p*H.

The composition of the solutions is shown in Table 1.

TABLE 1

VALUES OF *p*H IN THE ORGAN BATH USING VARIOUS SALT SOLUTIONS

To ensure stable conditions the aerating gas was bubbled through the solution in the reservoir as well as the solution in the organ bath itself. This was particularly necessary when, as with solution A, pure oxygen was used as the aerating gas. There was a gradual drift towards alkalinity as the carbon dioxide was displaced. The *p*H approached a limiting value which in the organ bath was found, electrometrically, to be 8.05. Values in parenthesis indicate the number of determinations

(mM/l.)	A	B	C	D
NaCl	118	118	103·5	139·5
KCl	4·71	4·71	4·71	4·71
$CaCl_2$	2·54	2·54	2·54	2·54
$MgSO_4$	1·19	1·19	1·19	1·19
$NaHCO_3$	25	25	40	4
KH_2PO_4	1·19	1·19	1·19	1·19
Glucose	11·1	11·1	11·1	11·1
Gas	100% O_2	95% O_2 + 5% CO_2	95% O_2 + 5% CO_2	95% O_2 + 5% CO_2
*p*H in organ bath (mean±standard error)	8·05 ±0·02 (6)	7·51 ±0·02 (7)	7·69 ±0·01 (7)	6·73 ±0·01 (5)

RESULTS

The pK_a *values* for nicotine at 25° C were 3.10 and 8.01. The value for nicotine monomethiodide was 3.15. Each value was the mean of two estimations and the results agree with those of Vickery & Pucher (1929), who obtained pK_a values of 3.22 and 8.11 for nicotine at 20° C. Using the temperature correction suggested by Albert (1952), the pK_a for the pyrrolidine nitrogen atom of nicotine at 37° C should be $8.01 - 12 \times 0.022 = 7.75$; Vickery & Pucher's results give a value of 7.74. At a *p*H of about 7.6, therefore, there will be less than one molecule in 100,000 in the form of the pyridinium ion, but 42% of the nicotine is present as the un-ionized base.

Assays. The effects of *p*H on the equipotent molar ratio are summarized in Table 2.

TABLE 2

EFFECT OF *p*H ON THE ACTIVITY OF NICOTINE RELATIVE TO THAT OF NICOTINE MONOMETHIODIDE ON THE RAT DIAPHRAGM PREPARATION AT 37° C.

The equipotent molar ratio indicates the number of molecules of nicotine needed to produce the same effect as one molecule of the methiodide

Solution	*p*H	Number of estimations	Equipotent molar ratio		Percentage univalent ion present
			Mean±s.e.	Logarithm	
A	8·05	8	10·10±0·72	1·004 (0·972–1·033)	33
C	7·69	6	9·14±0·56	0·961 (0·933–0·987)	54
B	7·51	6	6·53±0·56	0·815 (0·776–0·851)	64
D	6·73	8	5·59±0·58	0·747 (0·700–0·790)	92

If only the univalent nicotinium ion is the active species, the equipotent molar ratio (M) should be related to the *p*H by the expression,

$$M \times \frac{10^{pKa-pH}}{1+10^{pKa-pH}} = \text{constant},$$

M being the (total) molar concentration of nicotine divided by the concentration of nicotine monomethiodide producing the same effect (this latter concentration being

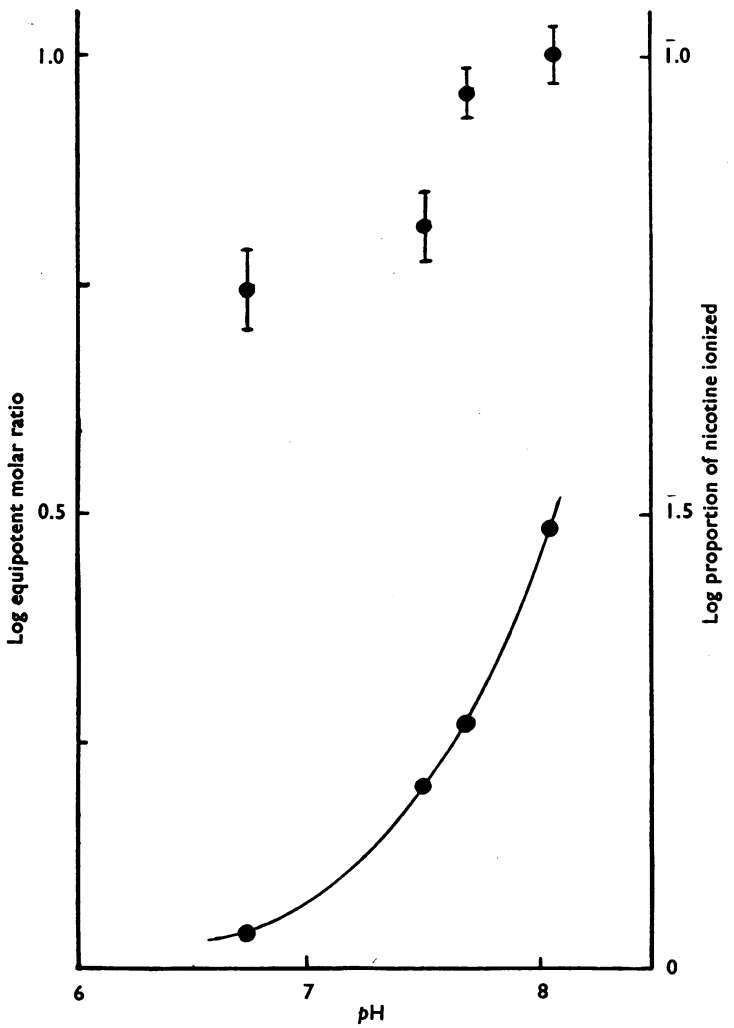


Fig. 1. Effect of *p*H on the activity of nicotine in blocking the rat diaphragm preparation. Ordinate on the left side (for the upper points), the logarithm of the number of molecules of nicotine producing the same effect as one molecule of nicotine monomethiodide; abscissa, *p*H. Values are taken from Table 2: the range corresponds to the standard error. Ordinate on the right side (for the lower and continuous curve), the logarithm of the proportion of nicotine which is ionized, the scale being inverted so that high values indicate a low degree of ionization and should be related to high values of the left-hand ordinate which indicate low activity.

assumed to be constant). In acid solutions this should approach a limiting value; ionization will be virtually complete, 10^{pK_a-pH} being much greater than 1 (this is neglecting possible effects of ionization of the pyridine nitrogen atom). In alkaline solutions, when 10^{pK_a-pH} is less than 1, the relationship becomes

$$M \times 10^{pK_a-pH} = \text{constant}$$

and log M should vary linearly with *p*H, the slope being unity.

The graph of *p*H against the logarithm of the equipotent molar ratio is shown in Fig. 1, together with the graph of the logarithm of the fraction of the nicotine present as the univalent ion. These show a qualitative similarity, but no more than this.

A quantitative test of the hypothesis can be made by measuring the equipotent molar ratio at two *p*H values and comparing the change in activity with the corresponding change in the concentration of the univalent nicotinium ion. The results are set out to show this in Table 3.

TABLE 3

EFFECT OF *p*H CHANGE ON ACTIVITY AND ON DEGREE OF IONIZATION

*p*H change	Equipotent molar ratio at alkaline *p*H / Equipotent molar ratio at acid *p*H	Mean	Nicotinium ion conc. at acid *p*H / Nicotinium ion conc. at alkaline *p*H
8·05 to 7·51 (solutions A to B)	1·66, 1·61, 1·55, 1·59, 1·58, 1·59	1·60	1·91
7·69 to 6·73 (solutions C to D)	1·35, 1·20, 1·80, 1·64, 1·53, 1·68	1·53	1·71
8·05 to 6·73 (solutions A to D)	2·34, 2·24	2·29	2·75

DISCUSSION

The quantitative agreement between the observed and expected results is not as good as was hoped. The experimentally determined ratio in Table 3 is invariably lower than the theoretical value. This might be taken to indicate that the un-ionized base itself has some effect, but the present experiments do not justify a definite conclusion about this. The rat diaphragm, unlike the frog rectus used by Kalow (1954), is quite sensitive to changes in *p*H. If, for example, the actual doses of nicotine monomethiodide added to the bath in these experiments (and producing between 25 and 50% inhibition of the contractions) are plotted against the *p*H (Fig. 2a), it will be seen that the sensitivity to the blocking action of the drug varies considerably, being greatest at the more extreme values of *p*H. The differences between this curve and that for nicotine (Fig. 2b) should be ascribable only to the effects of *p*H on the ionization of the nicotine. These differences, however, are not so very much greater, proportionately, than those brought about by *p*H changes alone on the activity of nicotine monomethiodide. The values in Table 3, therefore, may be taken to do no more than substantiate what has hitherto been

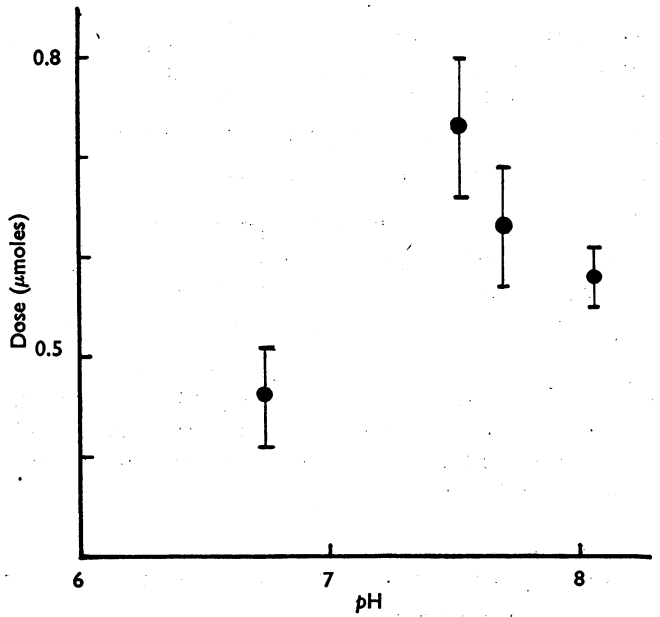


Fig. 2a. Effect of *p*H on the sensitivity of the rat diaphragm to nicotine monomethiodide. Ordinate dose in μmoles producing between 25 and 50% inhibition when added to a 25-ml. bath; abscissa, *p*H. The values at the highest and lowest *p*H are the mean of 8 experiments, the others of 6 experiments. The standard error is indicated.

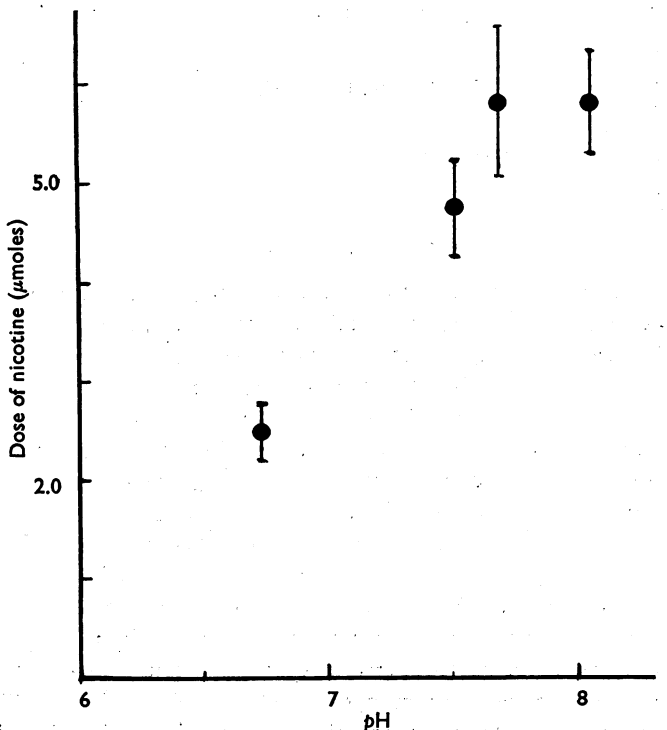


Fig. 2b. Effect of *p*H on the sensitivity of the rat diaphragm to nicotine, plotted as in Fig. 2a but with the scale of the ordinate multiplied by ten (nicotine being less active than the monomethiodide). The number of experiments is the same as in Fig. 2a.

assumed, that the univalent nicotinium ion is the active species to the neuromuscular junction. The possibility of investigating further whether the un-ionized base is partly active appears to depend upon finding a test preparation whose sensitivity to blocking drugs is less affected by changes of *p*H than is the rat diaphragm. The chick biventer-cervicis is certainly not such a preparation.

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