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STRUCTURAL REQUIREMENTS FOR NICOTINE AND CYCLIC IMINE-INDUCED AMINE RELEASE FROM NEOPLASTIC MAST CELLS*

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Abstract—Nicotine, pyrrolidines and cyclic imines induced the release of biogenic amines from mouse neoplastic mast cells. Alkaline conditions were required to convert the releasing agents to the form of their free base thus making available the 2s-electron pair of the molecule's alicyclic nitrogen atom for interaction with the cells' trigger sites. The potency of the compounds as releasing agents was directly related to their basicity. The elevated pH was also needed to convert the cryptic or conformationally unreactive trigger site to an available or reactive form that permitted the interaction with the releasing agent to occur.

AMINE RELEASE from rat peritoneal mast cells can be induced by a wide variety of chemical agents[1] including alkaloids,[2–8] alkylamines,[2] toxins,[9–11] antibiotics,[12,13] antigens,[14] polypeptides,[15–17] adenosine-5′-triphosphate[18,19] and synthetic polymers such as compound 48/80.[20] Neoplastic mast cells of the mouse are unusually resistant to the effect of these agents. Nevertheless, we recently described conditions under which the release of histamine, 5-HT (serotonin, 5-hydroxytryptamine), heparin and protein could be induced by nicotine, other pyrrolidines, and by compound 48/80.[21] The liberation of these granule components from the tumor cells occurred only with alkaline pH conditions under which a cellular site became available to chemical agents that could initiate and maintain the release process.

Alkaline conditions were also required for the conversion of nicotine (and pyrrolidine) molecules to the form of their free base, which appeared to be the biologically active species needed for interaction with the mast cell trigger site. This contrasted with the ganglionic and neuromuscular junction sites[22,23] and the electroplax preparation,[24] where the pyrrolidinium ion form of the drug has been suggested as the active species. In addition, under optimum-releasing conditions, we observed that the pyrrolidine ring was the only portion of the nicotine molecule that was required for mast cell-releasing activity.[21]

In this paper, we have described the molecular structural requirements for the induction of the release of the mast cell granule components by nicotine, pyrrolidines and other cyclic imines. Further evidence is presented to suggest the involvement of a cryptic cellular trigger site. Data are presented in support of a proposed mechanism for the chemical initiation of the release process.

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MATERIALS AND METHODS

Neoplastic mast cells. The P-815Y mouse neoplastic mast cell line was routinely carried in ADK_2F_1 female mice and grown for the experiments in LAF_1 female mice (Jackson Labs., Bar Harbor, Me.). The origin and the propagation of this cell line have been described.[25] The cells were harvested 5–8 days after the injection of the tumor. Each tumor-bearing mouse received an intraperitoneal injection of 0·2 ml of a solution of 0·83 mg histamine dihydrochloride plus 1·15 mg 5-hydroxytryptamine (5-HT) creatinine sulfate/ml, 3 hr prior to harvesting of the cells. This was done to increase the intracellular amine content in order to raise the amine level for ease of assay. The cells contained 14·56 ±0·47 and 13·11 ±0·60 ng 5-HT and histamine/10^6 cells, respectively, at the time of harvest. The mice were sacrificed and the cells were obtained from their peritoneal cavities and centrifuged at 3000 *g* for 2 min in a Sorvall RC-2B, at 4°. The cells were washed twice with normal saline and centrifuged as above. They were resuspended in modified Tyrode-phosphate-albumin buffer at a concentration of 0·2 *g* ($\sim 2 \times 10^8$ cells) wet wt/ml. One ml of the cell suspension was used per incubation vessel containing a final volume of 5 ml.

Chemical assays. The intracellular and the extracellular amine levels were determined spectrofluorometrically with the Hitachi–Perkin Elmer MPF-2A, using the method of Shore *et al.*,[26] for histamine, and the modified method of Bogdanski *et al.*,[27] as described by Carlini *et al.*,[28] for 5-HT. 5-Hydroxytryptamine creatinine sulfate (CalBiochem) and histamine dihydrochloride (Eastman Organic Chemical) were used as standards.

Materials. L(−)Nicotine, piperazine (anhydrous), and piperidine were purchased from Matheson, Coleman & Bell (Norwood, Ohio). Pyrrolidine, 1-methyl-2-pyrrolidinone, 2-pyrrolidinone, pyrrole, 3-pyrroline, hexamethyleneimine, heptamethyleneimine, quinuclidine, 3-quinuclidone hydrochloride, 1-aminopyrrolidine hydrochloride, 1-(2-hydroxyethyl)pyrrolidine, and 2,5-dimethylpyrrolidine were purchased from Aldrich Chemical Co. (Milwaukee, Wisc.). 1-Methylpyrrolidine, 1-nitrosopyrrolidine, ethyleneimine, and 1-methylethyleneimine were obtained from K & K Labs (Plainview, N.Y.). 1,1-Dimethylpyrrolidinium iodide, 1-methylpyrrolidine-1-oxide, trimethyleneimine and tetrabromophenolphthalein ethyl ester were obtained from Eastman Organic Chemical (Rochester, N.Y.). 2,2,6,6-Tetramethylpiperidine and 1-formylpyrrolidine were purchased from Frinton Labs (S. Vineland, N.J.). Bovine serum albumin and imidazole were obtained from Sigma Chemical Co. (St. Louis, Mo.). 2,2,5,5-Tetramethylpyrrolidine hydrochloride, 1,2,2,5,5-pentamethylpyrrolidine hydrochloride and 1,2,2,6,6-pentamethylpiperidine tartrate (Pempidine) were a gift from May & Baker, Ltd. (Dagenham, Essex, United Kingdom). Cotinine was a gift from Dr. Herbert McKennis (Medical College of Virginia, Richmond, Va.). Nicotine-1′-oxide was synthesized by the method of Pinner and Wolffenstein[29] and characterized as described by Booth and Boyland.[30] p*K*a values were obtained from the literature[22,31,32] except for heptamethyleneimine and 3-quinuclidone which were determined by titration.[33] All values were corrected for a temperature of 37°.[22]

Incubation techniques. The incubation buffer consisted of NaCl, $1{\cdot}4 \times 10^{-1}$ M; KCl, $5{\cdot}4 \times 10^{-3}$ M; $CaCl_2$, $1{\cdot}8 \times 10^{-3}$ M; $MgCl_2$-6 H_2O, $1{\cdot}1 \times 10^{-3}$ M; $NaHCO_3$, $1{\cdot}2 \times 10^{-2}$ M; D-glucose, $5{\cdot}6 \times 10^{-3}$ M; NaH_2PO_4, $4{\cdot}2 \times 10^{-4}$ M; bovine serum albumin, 1·0 g/l. The pH of the buffer and of all solutions containing releasing agents

was adjusted with NaOH or HCl as required. The cells were resuspended in buffer at a concentration of 0·2 g wet wt/ml. All experiments were conducted at pH 8·9 unless otherwise stated. Incubations were carried out in 10-ml beakers, at 37°, and the vessels were gently agitated in a metabolic shaker. A final volume of 5 ml/incubation beaker was used. The incubations were terminated by centrifugation at 3000 *g* for 2 min, at 4°. The supernatants were divided into two equal parts and assayed for histamine and 5-HT, as described. Control release values were subtracted in all cases. Total amine concentrations/0·2 g wet wt of cells were determined prior to incubation for each experiment.

Partition coefficients. Partition coefficients were determined at room temperature, pH 8·9, with 5 or 6 ml of Tyrode-phosphate-albumin buffer as the aqueous phase and an equal volume of heptane (5 ml) or of chloroform (6 ml) as the lipid solvent organic phase. Samples were shaken for 15 min using an Eberbach shaker and then spun at 3300 rev/min for 10 min at 25° in an International centrifuge model K (No. 250 head) for chloroform and at 10,000 rev/min for 5 min at 4° in a Sorvall RC-2B (SM-24 head) for heptane. Four ml of the non-aqueous phase was removed and added to 1 ml of a chloroform solution of 0·2 mg/ml of tetrabromophenolphthalein ethyl ester.[34,35] Color intensities were determined with a Guildford 2400 spectrophotometer. The determinations of heptane–water partitions were made at 530 nm for 1-methylpyrrolidine; 570 nm for pyrrolidine, aziridine and azetidine; 575 nm for heptamethyleneimine; and 600 nm for 1-methylaziridine. Nicotine was measured directly (without dye) at 255 nm. The measurements of chloroform–water partition coefficients were made at 555 nm for 1-methylpyrrolidine; 565 nm for aziridine and heptamethyleneimine; 570 nm for pyrrolidine and azetidine; and 605 nm for 1-methylaziridine. Nicotine was measured directly at 260 nm (without dye). Buffered aqueous solutions of the releasing agents were prepared at concentrations sufficient to give measurable values in the organic solvent layer when compared with appropriate standards. The releasing agent concentrations tested were usually 10^{-2} M or less. The organic phases used were prepared as described by Hogben *et al.*,[36] and the calculation of the partition coefficient was made by dividing the concentration in the organic layer by the final concentration in the aqueous phase as determined by difference.[37]

RESULTS

Modification of the pyrrolidine ring by increasing the molecular aromaticity decreased the amine-releasing activity (Table 1). The introduction of a Δ^{3-4} double bond did not decrease biological activity but the introduction of a second unsaturation into a five-membered nitrogen-containing ring system, as in the case of imidazole, drastically lowered the biological activity. Imidazole, which is not as highly aromatic in character as pyrrole, has a nitrogen atom that shows electron-releasing properties.[38] In the pyrrole ring, however, the free electron pair on the single ring nitrogen atom participates in the resonance of the aromatic system. This compound showed no releasing activity, and it had a low p*K*a that required non-aqueous titration for its determination.[39]

Table 2 shows that the presence of electron-withdrawing substituents (formyl, nitroso) on the ring nitrogen atom of pyrrolidine substantially reduced or eliminated the amine-releasing ability of the compound. Pyrrolidines with alkyl (methyl) or other

TABLE 1. RING AROMATICITY AND 5-HT RELEASE*

Releasing agent	Per cent 5-HT release	
	10^{-3}M conc	10^{-2}M conc
Pyrrolidine	16·99 ±4·26	62·48 ±3·33
3-Pyrroline*	18·17 ±2·47	73·02 ±4·10
Imidazole	4·43 ±1·51	15·19 ±1·95
Pyrrole	1·58 ±0·69	4·29 ±1·30

* See incubation techniques and chemical assays under Materials and Methods. All values represent the mean of three to five experiments (± standard error of the mean).

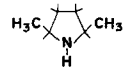
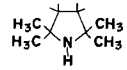
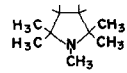
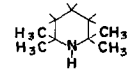
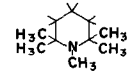
non-electron-withdrawing substituents (amino,2-hydroxyethyl) retained all or most of their activity. The importance of the ring nitrogen atom for releasing activity is further illustrated in Table 3. Increasing the number of methyl group substituents around the nitrogen atom in the pyrrolidine or the piperidine ring system had little effect upon amine release. The loss of releasing activity observed upon the introduction of a fifth methyl group (on the ring nitrogen) appeared to be greater than could be accounted for by the decrease in basicity that it produced. This was noted by comparing pyrrolidine-induced liberation of 5-HT (62 per cent)[21] with that of 1-methylpyrrolidine (55 per cent) which showed 7 per cent less releasing activity. 2,2,5,5-Tetramethylpyrrolidine produced 60 per cent release (2 per cent less than

TABLE 2. RING NITROGEN SUBSTITUENTS AND 5-HT RELEASE*

Releasing agent	Per cent 5-HT release	
	10^{-3}M conc	10^{-2}M conc
1-Methylpyrrolidine	12·95 ±2·52	55·02 ±5·12
1-(2-Hydroxyethyl) Pyrrolidine	17·28 ±2·35	69·50 ±2·61
1-Aminopyrrolidine	17·03 ±1·44	42·29 ±2·73
1-Formylpyrrolidine	0·00 ±0·00	0·64 ±0·30
1-Nitrosopyrrolidine	3·65 ±1·17	5·14 ±0·99

* See incubation techniques and chemical assays under Materials and Methods. All values represent the mean of three to thirteen experiments (± standard error of the mean).

TABLE 3. STEREOCHEMISTRY AND 5-HT RELEASE*

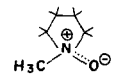
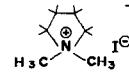
Releasing agent	Per cent 5-HT release	
	10^{-3}M conc	10^{-2}M conc
2,5-Dimethylpyrrolidine 	16·49 ±2·34	63·65 ±1·38
2,2,5,5-Tetramethylpyrrolidine 	19·99 ±3·00	60·39 ±4·79
1,2,2,5,5-Pentamethylpyrrolidine 	13·06 ±2·02	48·15 ±1·89
2,2,6,6-Tetramethylpiperidine 	23·23 ±2·90	70·08 ±3·86
1,2,2,6,6-Pentamethylpiperidine 	12·94 ±1·52	45·05 ±3·80

* See incubation techniques and chemical assays under Materials and Methods. All values represent the mean of three to six experiments (± standard error of the mean).

pyrrolidine) but 1,2,2,5,5-pentamethylpyrrolidine gave only 48 per cent release (14 per cent less than pyrrolidine) which was 7 per cent less than the value obtained with 1-methylpyrrolidine. This suggested the presence of a steric effect in addition to the decreased activity produced by the lower basicity (reflected by the lower p*K*a).

Table 4 presents additional evidence for the importance of the free electron pair (2s) of the ring nitrogen atom for releasing activity. 2-Pyrrolidinone and 1-methyl-2-pyrrolidinone are lactams or cyclic amides that are so weakly basic that their dissociation can only be measured in non-aqueous solution.[40,41] The free electron pair on the ring nitrogen is drawn toward the positively polarized carbon of the carbonyl group, making the electrons less available for interaction with the cells'

TABLE 4. ELECTRON DISTRIBUTION AND 5-HT RELEASE*

Releasing agent	Per cent 5-HT release	
	10^{-3}M conc	10^{-2}M conc
2-Pyrrolidinone 	2·56 ±0·85	3·16 ±1·22
1-Methyl-2-Pyrrolidinone 	0·00 ±0·00	2·78 ±2·78
1-Methylpyrrolidine-1-oxide 	1·84 ±1·84	1·78 ±0·88
1,1-Dimethylpyrrolidinium iodide 	1·63 ±0·69	1·58 ±1·40

* See incubation techniques and chemical assays under Materials and Methods. A.. values represent the mean of three to six experiments (± standard error of the mean).

TABLE 5. RING SIZE AND 5-HT RELEASE*

Ring size	Releasing agent	Per cent 5-HT release 10^{-3}M conc	Per cent 5-HT release 10^{-2}M conc
3	Ethyleneimine (aziridine)	7·66 ±2·30	50·10 ±2·11
4	Trimethyleneimine (azetidine)	9·96 ±2·62	59·28 ±4·13
5	Tetramethyleneimine (pyrrolidine)	16·99 ±4·26	62·48 ±3·33
6	Pentamethyleneimine (piperidine)	18·71 ±3·01	70·44 ±2·62
7	Hexamethyleneimine (homopiperidine)	22·67 ±4·97	76·20 ±6·35
8	Heptamethyleneimine	24·66 ±3·87	72·13 ±3·32

* See incubation techniques and chemical assays under Materials and Methods. All values represent the mean of three to six experiments (± standard error of the mean).

trigger sites. 1,1-Dimethylpyrrolidinium iodide and 1-methylpyrrolidinium-1-oxide do not possess a free electron pair due to the presence of the added substituent on the ring nitrogen atom, and they were inactive compounds.

In general, increasing imine ring size (Table 5) produced increasing releasing ability. Cyclic imines with a ring size of less than five or six members were slightly less potent on a molar basis.

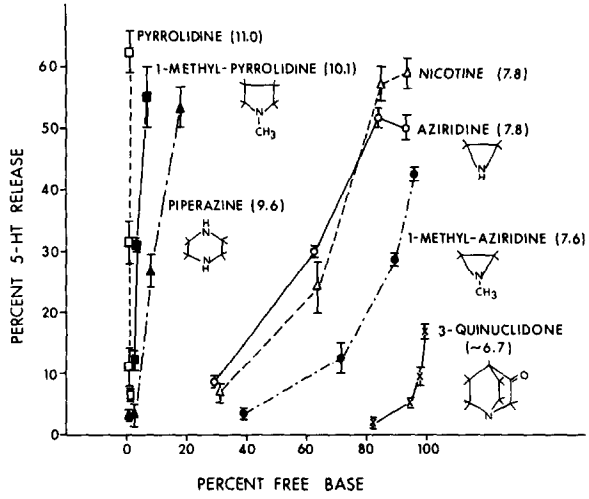


FIG. 1. Per cent free base and 5-HT release. All values represent the mean of three to twelve experiments (±S. E. of the mean).

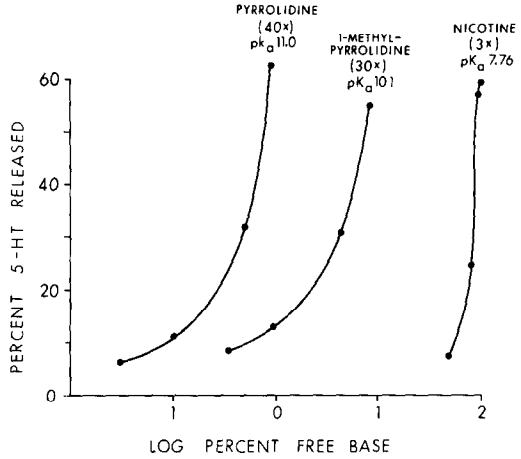


FIG. 2. Log per cent free base and 5-HT release.

The experimental results indicated that the free electron pair (2s) on the ring nitrogen atom was required for biological activity; therefore, the basicity of the compounds (or their ability to donate their free electron pair) was probably an important factor. Figure 1 shows that compounds with high p*K*a values (strong bases) produced optimum levels of amine release despite the fact that the per cent of the total number of molecules of the compound, that existed in the form of the free base, was low under optimum-releasing conditions. The lower the p*K*a of a compound, the greater the free base concentration of the compound required to produce a high level of cellular amine release. 3-Quinuclidone, a beta amino ketone with a p*K*a below 7·0, produced less than 20% 5-HT release at pH 8·9 even though its free base concentration at that pH approached 100 per cent. Quinuclidine, the analogous compound lacking the keto group, (p*K*a 10·6) was tested and found to be a potent-releasing agent (63·13 ±0·57) at pH 8·9.

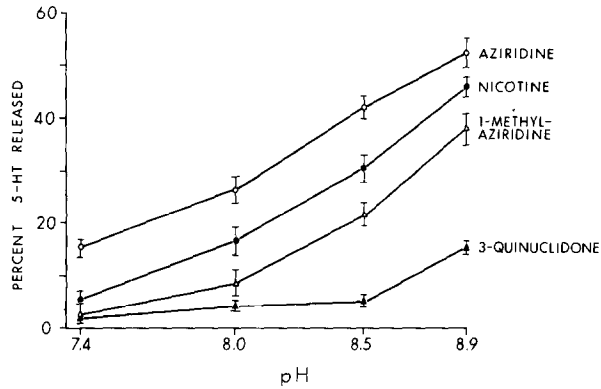


FIG. 3. 5-HT release at constant free base concentration with increasing pH. Free base levels of the compounds tested were aziridine (ethyleneimine) $9{\cdot}4 \times 10^{-3}$ M, p*K*a 7·78; nicotine $9{\cdot}5 \times 10^{-3}$ M, p*K*a 7·76; 1-methylaziridine (1-methylethyleneimine) $9{\cdot}6 \times 10^{-3}$ M, p*K*a 7·60; 3-quinuclidone 1×10^{-2} M, p*K*a 6·74. All values represent the mean of three to nine experiments (±S.E. of the mean).

TABLE 6. EFFECT OF INCREASED FREE BASE WITH CONSTANT pH ON 5-HT RELEASE*

Releasing agent	Molarity	Per cent free base (pH 7·4)	Concn. of free base (M)	Per cent 5-HT released
Nicotine	$1{\cdot}24\times10^{-2}$	30·58	$3{\cdot}80\times10^{-3}$	7·30±1·25
	$3{\cdot}73\times10^{-2}$	30·58	$1{\cdot}14\times10^{-2}$†	2·05±1·36
Aziridine	$1{\cdot}25\times10^{-2}$	29·61	$3{\cdot}69\times10^{-3}$	8·94±0·74
	$3{\cdot}74\times10^{-2}$	29·61	$1{\cdot}11\times10^{-2}$†	18·01±0·60
1-Methylaziridine	$1{\cdot}25\times10^{-2}$	38·91	$4{\cdot}85\times10^{-3}$	3·46±0·83
	$3{\cdot}12\times10^{-2}$	38·91	$1{\cdot}21\times10^{-2}$†	0·72±0·13

* See incubation techniques and chemical assays under Materials and Methods. All values represent the mean of three to nine experiments (± standard error of the mean).

† Molar concentration of the free base is equal to that obtained with $1{\cdot}2\times10^{-2}$ M releasing agent at pH 8·9.

Figure 2 presents data showing that a concentration of pyrrolidine (p*K*a 11·0) free base of $7{\cdot}8\times10^{-5}$ M produced amine release approximately equal to that produced by about 120 times as much nicotine free base ($9{\cdot}3\times10^{-3}$ M). 1-Methylpyrrolidine has a p*K*a one unit lower than that of pyrrolidine, and its base was about one-tenth as potent as pyrrolidine base as a releasing agent. The increase in free base concentration over the pH range tested varied from 3-fold for nicotine to 40-fold for pyrrolidine. The relationship between p*K*a and releasing ability was further strengthened by the results shown in Fig. 3. When the concentration of free base was kept constant, the per cent amine released at a given pH increased with increasing p*K*a of the releasing agents. These data supported the previous proposal that increasing the extracellular pH made a mast cell site available for the chemical initiation and the maintenance of the release process.[21] As shown in Fig. 3 and in Table 6, if the free base was kept constant over the entire pH range, then the releasing activity was a function of pH. Elevating the level of the free base under pH conditions at which the cellular site was unavailable did not produce amine release. For example (Table 6), increasing the free base of nicotine or 1-methylaziridine at pH 7·4 to the level that would be found at pH 8·9 did not produce the amount of amine release that occurred at the latter pH. Aziridine (ethyleneimine) did show some

TABLE 7. KINETICS OF RELEASE IN RELATION TO LIPID SOLVENT SOLUBILITY AND BASICITY

Releasing agent	p*K*a*	Time of optimum release (min)†	Partition coefficient‡	
			$K_{heptane}$	$K_{chloroform}$
Pyrrolidine	11·0	6	≪0·01	0·02
1-Methylpyrrolidine	10·1	9	0·01	2·01
Aziridine	7·8	~15	0·01	0·36
1-Methylaziridine	7·6	~15	0·01	0·27
Azetidine	11·0	6	≪0·01	0·02
Heptamethyleneimine	10·8	6	0·05	13·30
Nicotine	7·8	15	0·64	>100·00

* For p*K*a values, see Materials and Methods section.

† Amine release was determined as described in incubation techniques under Methods. All releasing agents were used at a concentration of 1×10^{-2} M.

‡ Partition coefficients were determined as described under Methods.

increase in amine release, but the level was far below the 50 per cent obtained at pH 8·9 using the same imine base concentration.

The rate of amine release produced by nicotine and the other compounds tested was primarily a reflection of their p*K*a. In some cases, lipid solubility was also a factor, as shown in Table 7. Pyrrolidine, which was strongly basic, had a low lipid solvent solubility, but it was a rapid-acting releasing agent (6 min). Heptamethyleneimine was less basic, but it had a much higher lipid solvent solubility and it, too, was a rapid releaser (6 min). Nicotine had relatively low basicity but high lipid solvent solubility; nevertheless, it was a slow releaser (15 min).

For the sake of brevity and consistency, all results have been presented as per cent 5-HT released. Data for histamine liberation were found to be qualitatively and quantitatively similar to those for 5-HT, in all cases, when both amines were measured (see Materials and Methods for histamine assays).

DISCUSSION

Basic monomeric substances, such as cyclic alkylamines (imines) and nicotine, were able to induce the release of low and high molecular weight components of mast cell granules.[21] In the case of mouse neoplastic mast cells, the release mechanism triggered by monomeric substances was temperature dependent, independent of oxygen and glucose, but the inducing agents did not need to contain more than one basic group as was previously reported for non-neoplastic rat peritoneal mast cells.[2] The simultaneous interaction of more than one molecule of the monomer with a single site or with adjacent sites may have compensated for the presence of only one basic group in the molecule. It was the free electron pair (2s) of the alicyclic nitrogen atom of nicotine and cyclic imine molecules' basic group that was able to interact with the cellular site. Molecular modifications, which decreased the availability of this electron pair for the interaction, produced a decrease or a complete loss of releasing activity. These chemical changes included: increased aromaticity of the molecule which resulted in electron delocalization (participation of the electrons in the aromatic system) (Table 1), the presence of electron-withdrawing substituents adjacent or attached to the ring nitrogen atom (Tables 2 and 4); the presence of nitrogen substituents that utilized all of that atom's electrons for chemical bond formation (Table 4); and the introduction into the molecule of groups that sterically hindered the nitrogen atom, thus decreasing its ability to donate the free electron pair (Table 3). Steric effects produced by changes in ring size were not critical unless they affected molecular basicity (Table 5).

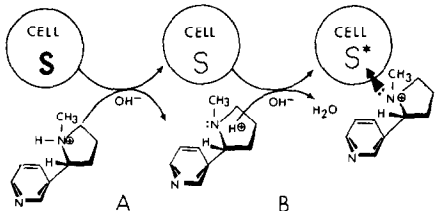
It was interesting to note that cotinine and nicotine-1′-oxide, which were "detoxication" products of nicotine produced by human metabolism,[42,43] involved the introduction of an electron-withdrawing substituent adjacent to the basic pyrrolidine ring nitrogen atom in the former, and directly attached to that nitrogen in the latter molecule. These compounds were tested and found to be inactive as releasing agents in the mouse mast cell system as were their pyrrolidine subunits (Table 4). It was also important to observe that ganglionic blocking activity did not require the retention of the basic strength of the compounds since quaternary derivatives were active in this regard[23] and as neurotransmitter releasers.[44,45] This lent additional support to the view that the pyrrolidinium or piperidinium ion forms were the active blocking agents.[22,23] In addition, piperidine derivatives must possess

at least three methyl substituents in the 2- and 6-positions and the pyrrolidines must have four methyl groups, two each in the 2- and 5-positions, in order to demonstrate blocking activity.[46] The smaller bond angles of the five-membered pyrrolidine ring caused a reduction in shielding of the nitrogen as compared with the larger angles of the piperidine ring system.[47] In the neoplastic mast cells' releasing process, the presence of three or four of these alkyl substituents did not affect activity; however, the addition of the fifth substituent (attached to the ring nitrogen) lowered the p*K*a and produced a steric effect which combined to decrease biological activity (Table 3).

The p*K*a of the releasing compounds was a measure of their basicity and their ability to donate the free electron pair of the nitrogen atom. Quinuclidine (p*K*a 10·6) was a potent releasing agent but 3-quinuclidone (Fig. 1) with a p*K*a below 7 displayed very weak activity. A similar situation was observed with pyrrolidine and 2-pyrrolidinone. These were electronic and not steric effects of the carbonyl group, since substitution of bulky methyl groups in the 2- and 5-positions of the pyrrolidine ring did not lower activity (Table 3). A steric effect was even less likely in the case of 3-quinuclidone, since the carbonyl group was beta to the ring nitrogen atom.

The lipid solubility of nicotine and the cyclic imines [as measured by their distribution between lipid solvents and water (partition coefficient)] did not appear to play a major role in determining the time of optimum release of the granule components. The drug partition between heptane and water reflected the level of the free base of each compound except for aziridine (ethyleneimine) and its 1-methyl derivative (Table 7). The aziridines also gave an unusual pattern of release that was characterized by the absence of a sharp maximum. Heptamethyleneimine displayed increased lipid solubility probably due to the large hydrocarbon portion of the molecule. The pyrrolidine molecules in the form of the free base at pH 8·9 were 0·78 per cent as compared with 1·15 per cent for heptamethyleneimine, yet the latter compound showed five to six times the lipid solvent solubility of the former, and it produced a comparable rate of release despite its slightly lower p*K*a value. In general, the partition coefficients obtained with the chloroform–water system followed the same over-all pattern as that obtained with heptane–water. The basic strength of the compounds was directly related to their rates of release and inversely related to their partition coefficients. The importance of basic strength was further emphasized by the data in Figs. 2 and 3, in which the release produced by nicotine can be seen to be related to its p*K*a. Approximately 15 times as much nicotine base was required to produce the release equivalent to that obtained with its 1-methylpyrrolidine subunit (Fig. 2). The p*K*a of nicotine was much lower than that of 1-methylpyrrolidine due to the "coulombic effect" of the electron-attracting pyridine ring in the nicotine molecule.[48].

The elevation of the extracellular pH, required to demonstrate the activity of the releasing compounds, caused a rise in the free base level of these agents. In the case of 3-quinuclidone, more than 80 per cent of the compound was in the form of the free base at pH 7·4 and greater than 99 per cent of the molecules was in the form of the base at pH 8·9. This compound showed no releasing activity at pH 7·4, but it did produce about 15 per cent release at pH 8·9. Raising the level of the compound that was present at pH 7·4 to give a free base concentration equal to the free base concentration that was present at pH 8·9 did not produce release (Table 6). Nicotine, a more potent base, gave identical results in such an experiment. Aziridine (ethyleneimine) showed some increase in release at pH 7·4, when its free base level was



S = CELL SITE IS CRYPTIC OR IN AN UNREACTIVE CONFORMATION
S = CELL SITE IS AVAILABLE OR IN A REACTIVE CONFORMATION
S* = CELL SITE + RELEASING AGENT INTERACTION

FIG. 4. Proposed mechanism for cell-releasing agent interaction.

elevated, for reasons that are not clear, but the release was far less than the 50 per cent liberation observed at pH 8·9 using the same free base concentration.

These data, plus the increased release observed with increased pH but a constant free base level, supplemented the evidence previously presented to suggest that elevation of the pH produces a change in the conformation or availability of a cell site that was required in order for the free base to trigger the release process. In Fig. 4, a mechanism has been suggested to explain these results. In part A of this figure, elevation of the pH by the addition of hydroxyl ion converts a nicotine molecule from the ionic form to that of the free base with the removal of a proton, thus freeing the 2s-electron pair on the pyrrolidine ring nitrogen atom. Simultaneously, cryptic or conformationally inadequate cell sites (**S**) are converted to available or conformationally adequate cell sites (S). The releasing agent molecules in the form of their free base can then interact with the cells' trigger sites by means of the free electron pair on the ring nitrogen atom. Such an interaction is indicated for the formation of S* and a quaternary or ionic ring nitrogen (of the releasing agent) bearing a positive charge. A complex series of reactions then leads to the liberation of low and high molecular weight granule components.

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