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SHORT COMMUNICATION

A Nuclear Magnetic Resonance Study of Compound 48/80

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SUMMARY

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Compound 48/80, a potent liberator of histamine from mast cells, is a polymer prepared by heating 4-methoxy-N-methylphenethylamine with an equimolar amount of formaldehyde in acid solution. We have attempted to elucidate the structure of 48/80 with the aid of carbon-13 nuclear magnetic resonance (NMR). The NMR spectrum of 48/80 suggests that it is a polymer in which methylene groups link 4-methoxy-N-methylphenethylamine moieties via their 3 and 3′ positions. Some of the polymer chains of 48/80 are terminated by $-CH_2OH$ groups. In accord with the findings of Read and co-workers (J. Med. Chem. 15: 320, 1972; *ibid* 16: 1292, 1973), the most active high molecular weight polymers appear to be hexamers; however, contrary to the suggestion of these authors, there was no evidence for the presence of tetrahydro*iso*quinoline moieties in 48/80. Light scattering and fluorescence probe measurements with 1-anilino-naphthalene-8-sulfonic acid indicate that at concentrations above 2 mg/ml (0.01 M based on the monomer molecular weight) compound 48/80 formed micelles. This is reflected in the NMR spectrum of 48/80 by the sharpness of the $N-CH_3$ resonance.

INTRODUCTION

Compound 48/80 is probably the most important member of a large and diverse group of drugs and other agents which cause the rapid degranulation of mast cells. The reaction initiated by these drugs differs from reaginic antibody-antigen stimulated release in several ways. Compound 48/80-induced degranulation is more rapid (1) and efficacious (2), does not require extracellular calcium (3) and is not potentiated by phosphatidyl serine (4). However, the secretory process mediated by 48/80 and other low molecular weight histamine liberators is similar to the anaphylactic reaction in that all of these agents ultimately release histamine and other potent pharmacological substances after the noncytotoxic fusion of the mast cell plasma and perigranular membranes. Although reaginic antibody-antigen complex formation on the cell surface has been studied (5) and the IgE receptor partially characterized (6), the molecular events which are initiated by

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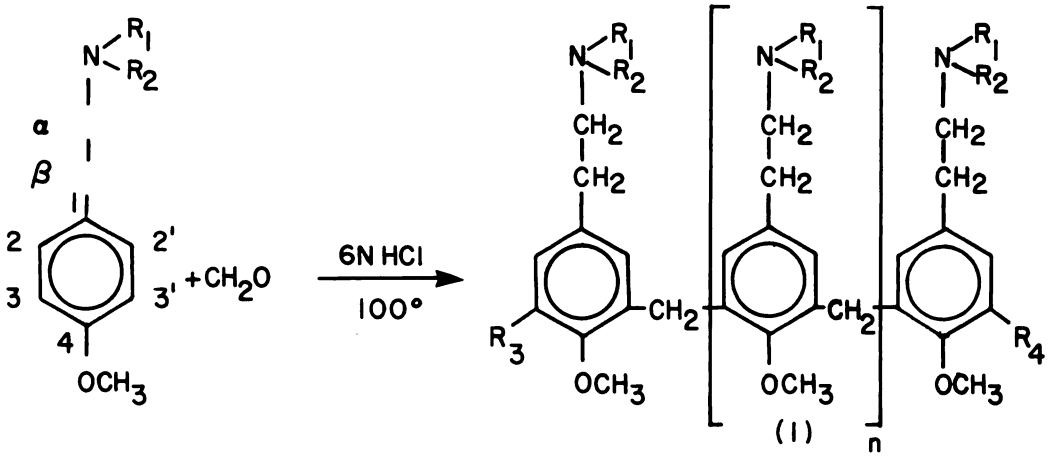
48/80 and similar agents are unknown. Furthermore, despite the widespread use of compound 48/80 as an investigative tool, the structure of this polymer has never been proven conclusively.

Compound 48/80 was first synthesized by Baltzly and co-workers (7) by heating equimolar quantities of 4-methoxy-N-methylphenethylamine and formaldehyde in acid solution. They postulated that the product consisted of a family of copolymers formed according to reaction scheme:



where R_1 = H and R_2 = CH_3 and R_3 = R_4 = H, CH_2OH, CH_2Cl. Baltzly *et al.* suggested that the trimer (I, R_1 = H, R_2 = CH_3, n = 1) was the active polymer and some later papers reported 48/80 concentrations on a molar basis using the trimer molecular weight. Subsequently, De Graw and co-workers synthesized the dimer (I, R_1 = R_2 = CH_3, R_3 = R_4 = H, n = 0) (8) and the trimer (I, R_1 = R_2 = CH_3, R_3 = R_4 = H, n = 1) (9) and found them both to be inactive. Gel permeation and dialysis studies by Read and Lenney (10) indicated that the degree of polymerization of active constituents of 48/80 ranged from tetramer to octamer with the average being the hexamer. Finally, Read and co-workers (11) synthesized a 48/80 analogue from 7-methoxytetrahydro*iso*quinoline which was more active than 48/80 itself. They suggested that 4-methoxy-N-methylphenethylamine may undergo partial cyclization during heating with formaldehyde to yield polymers containing some tetrahydro*iso*quinoline monomers. In this study, we have employed carbon-13 NMR[2] to study the structure of 48/80.

Compound 48/80 was prepared by heating 4-methoxy-N-methylphenethylamine (12) on a steambath with an equimolar amount of formaldehyde in 6 N HCl for 4 hr according to the procedure of Baltzly *et al.* (7). The reaction mixture was then cooled and evaporated under reduced pressure. The resultant syrup was dissolved in 50 ml of hot aqueous ethanol and 50 ml of hot ethyl acetate added. After standing overnight in the refrigerator, an equal volume of ether was added and the resultant precipitate filtered off and dried *in vacuo*.

Trifluoroacetyl 48/80 was prepared by suspending 48/80 (200 mg) in a mixture of ethyl acetate (5 ml) and pyridine (1 ml) and adding trifluoracetic anhydride (1.5 ml) dropwise. After the resultant yellow solution had been allowed to stand at room temperature overnight, the solvent was removed under reduced pressure and the residue was dissolved in chloroform (10 ml). The chloroform solution was washed with dilute HCl (3 × 10 ml) and then evaporated to dryness at reduced pressure. The residue was redissolved in spectral grade chloroform and the infrared spectrum of the solution recorded with a Perkin Elmer spectrometer.

For the preparation of the C^{13}-enriched

[2] Abbreviations used: NMR, nuclear magnetic resonance; ANS, 1-anilinonaphthalene-8-sulfonic acid; TMS, tetramethylsilane.

48/80, paraformaldehyde (0.25 g $HC^{13}HO$; 90% enriched, Batch A-583 purchased from Merck and Co.) was suspended in a mixture of water (2.2 ml) and concentrated HCl (3.2 ml) and heated on a steambath until dissolved. The solution was then cooled, 4-methoxy-N-methylphenethylamine hydrochloride (1.58 g) was added and the resultant solution heated on a steambath for 4 hr. The C^{13}-enriched 48/80 was isolated as described above for the unenriched polymer.

Gel permeation chromatography of 48/80 was carried out in a 100 cm × 4.5 cm column containing Sephadex G-25 (medium) suspended in 0.03 N acetic acid adjusted to pH 3 with HCl (10). Samples (350 mg) were applied to the column in 3 ml of 0.03 N acetic acid (pH 3.0) and eluted with the same solution. The effluent was continuously monitored at 280 nm and 11 ml fractions were collected and lyophilized.

The NMR spectra were recorded on a JEOL FX-60 spectrometer operated at 15.03 MHz for carbon-13 in a 10 mm variable temperature dual probe. 8 K data points were collected with a 1 sec recycle time and 2 μsec pulse width (90° = 8 μsec). A 1 Hz broadening factor was introduced through apodization of the free induction decay. Samples were prepared by dissolving 40 mg of material in approximately 2 ml of D_2O (Merck and Co.) to give a 0.1 M (based on the monomer molecular weight) solution. *p*-Dioxane (10–50 μl) was used as an internal standard. Reference materials which were not soluble in D_2O were dissolved in methanol-d_4 (Merck and Co.).

Fluorescence measurements were made in an Aminco-Bowman fluorometer. Solutions contained 1-anilinonaphthalene-8-sulfonic acid (10 μM), sodium phosphate buffer pH 7.4 (0.05 M) and compound 48/80 (2–20,000 μg/ml). The solutions (2 ml) were placed in a 1 cm^2 cuvette and fluorescence measured using activation and emission wavelengths of 392 nm and 465 nm respectively. For the light scattering experiments, the same solutions were employed but the activation and emission monochromators were set at the same wavelength (450 nm).

Histamine release was measured using unpurified rat peritoneal cells. The cells were obtained by injecting 20 ml of Locke's solution into the peritoneal cavity of decapitated male Sprague-Dawley rats. The abdomens were massaged for 1–2 min, and the solution was reclaimed by cutting the abdominal wall and allowing the fluid to drain while the animal was held over a funnel. The mast cells in this fluid contained virtually all of the histamine and constituted 5–10% of the total cell population.

The 48/80 from each column fraction was neutralized, lyophilized and redissolved in Locke's solution at equal concentrations (determined by UV absorption at 278 nm). The specific activity was determined by incubating approximately 10^5 mast cells in 1 ml of each fraction (containing 0.5 μg/ml 48/80) for 10 min at room temperature. The cells were then centrifuged for 10 min at 300 × *g*. The percent histamine released into the supernatant was determined on the basis of the total originally present in the cells. Histamine was measured using the fluorometric method of Shore (13).

The biological activity of the 48/80 used in this study was tested by measuring its ability to release histamine from rat peritoneal mast cells. The ED_{50} values (i.e., the concentration required to release 50% of the histamine) for our 48/80, and Burroughs Wellcome 48/80 (Lot No. 46663)

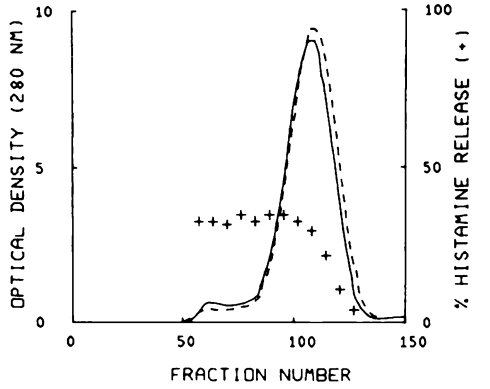
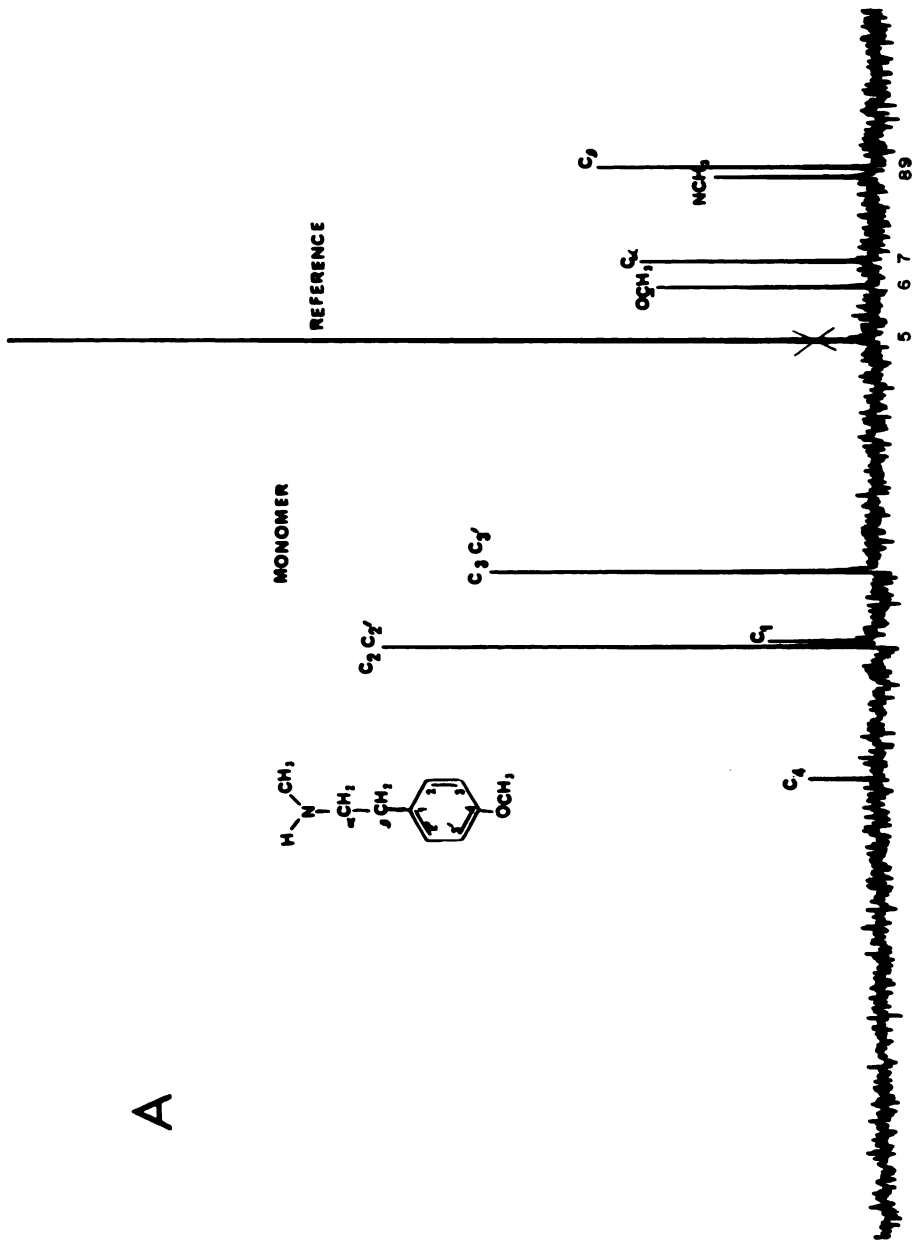


FIG. 1. *Gel chromatography of 48/80 over Sephadex G-25*

The column dimensions were 100 cm × 4.5 cm. ——, 48/80; - - -, 48/80 synthesized with carbon-13-enriched formaldehyde; ++, the release of histamine from rat peritoneal mast cells by solutions containing 0.5 μg/ml of 48/80.


A
REFERENCE
MONOMER
C5
NCH3
C4
OCH3
C3 C3'
C2 C2'
C1
C6
5
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8
9

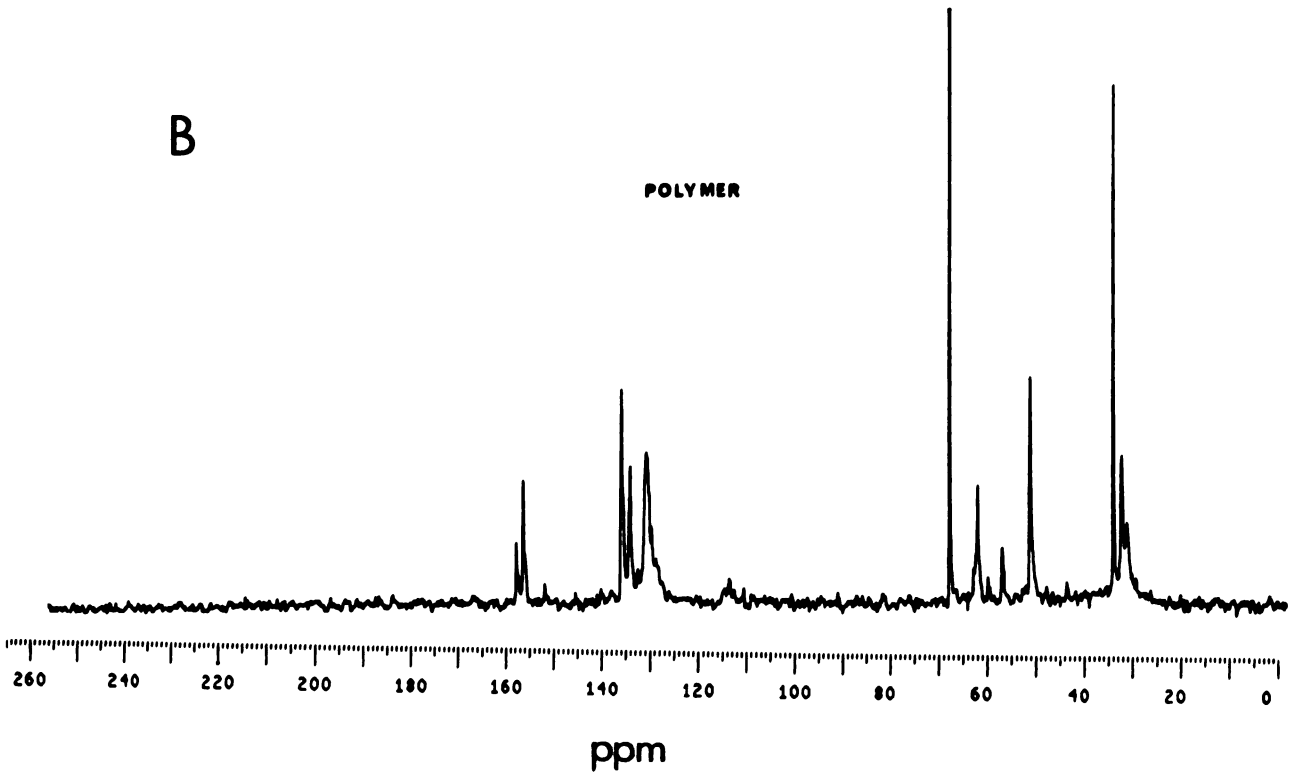
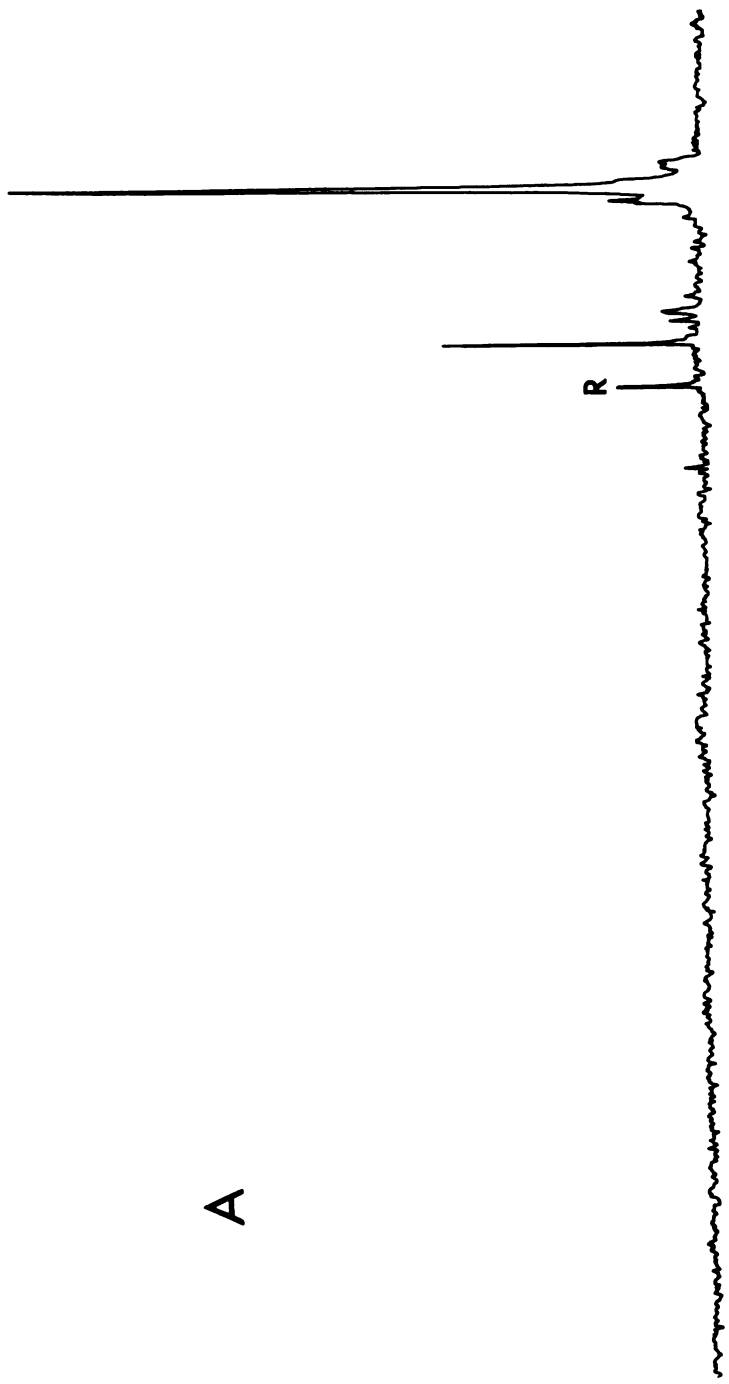


FIG. 2. *The carbon-13 NMR spectrum of 4-methoxy-N-methylphenethylamine and compound 48/80 in D_2O*

A, 4-methoxy-N-methylphenethylamine; B, compound 48/80. Solutions contained 20 mg/ml of the compounds as their respective hydrochloride salts. The 48/80 sample was obtained by combining fractions 51–77 (see Fig. 1) and freeze drying them. The reference compound was *p*-dioxane.



A

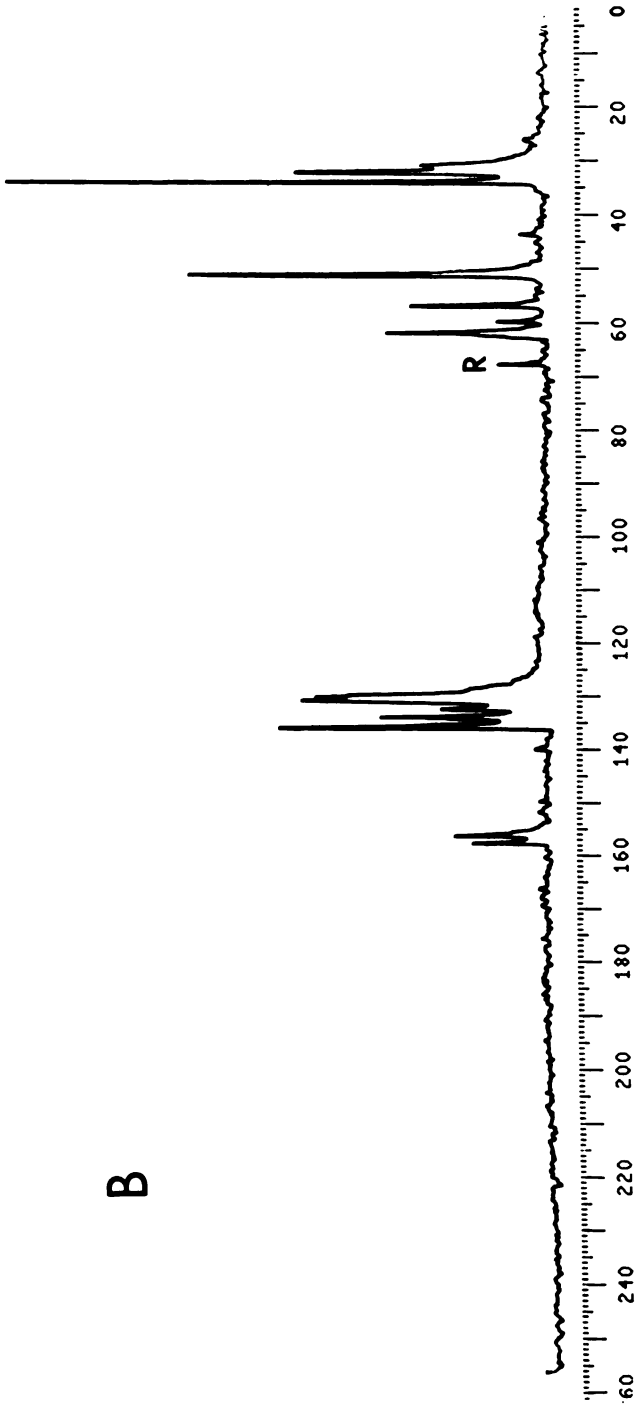


FIG. 3. *The carbon-13 NMR spectrum of compound 48/80 and compound 48/80, prepared from carbon-13 enriched formaldehyde, in D_2O*
A, carbon-13 enriched 48/80; B, unenriched 48/80. Solutions contained 20 mg/ml of the polymers as their respective hydrochloride salts. The polymers were not subjected to gel permeation chromatography. The reference compound (R) was *p*-dioxane.

were 0.54 μg/ml, and 0.40 μg/ml, respectively.

The elution profile of 48/80 separated over a Sephadex G-25 gel filtration column according to the procedure of Read and Lenney (10) is shown in Figure 1. While a small fraction of the 48/80 appeared as a shoulder, most of the material emerged as a single symmetrical peak. Selected fractions were diluted to a concentration of 0.5 μg/ml and then assayed for their histamine-releasing capacity. The results suggest (Fig. 1) that most of the biological activity resides in the leading edge of the peak, which undoubtedly contains the higher molecular weight species.

The NMR spectra of 4-methoxy-N-methylphenethylamine and 48/80 are shown in Figure 2. The resonances of 4-methoxy-N-methylphenethylamine (Table 1) were assigned with the aid of an off resonance experiment together with the known resonances of analogous compounds and partial structures. A comparison of the NMR spectrum of 48/80 with that of 4-methoxy-N-methylphenethylamine permitted the assignment of many of the major aliphatic resonances in the polymer (Table 1). With the exception of carbon atom 4, the aromatic resonances of compound 48/80 were combined into a broad band of resonance lines in the 129-137 ppm region. The 3 and 3′ resonances of 4-methoxy-N-methylphenethylamine monomer were shifted down field in the spectrum of 48/80 thus confirming that polymerization does indeed occur at these positions. When 48/80 was prepared from carbon-13 enriched formaldehyde, only the intensities of resonances at 30.62 ppm and 59.52 ppm were enhanced (Fig. 3, Table 2). While the peak at 30.62 ppm can be attributed to the linking methylene groups in 48/80, this resonance is at higher field than the corresponding carbon atom of diphenylmethane (δ_c = 37.0 ppm) (14). One possible explanation for this observation may be that steric crowding causes an upfield shift. This type of shift has been discussed by several authors (15, 16). The enriched resonance at 59.52 ppm is closer to an oxygen bearing carbon like the methylene resonance of benzyl alcohol (δ_c = 64.7 ppm) than it is to the chlorine bearing carbon of benzyl chloride (δ_c = 46.6 ppm). This would suggest the terminating groups of 48/80 (R_3, R_4 in I) are -CH_2OH.

Read *et al.* have suggested that, during the preparation of 48/80, some of the aliphatic side chains may cyclize to give tet-

TABLE 1

Chemical shifts[a] of the carbon atoms in 4-methoxy-N-methylphenethylamine and 48/80

Carbon	p-methoxy-N-methyl-phenethyla-mine[b] δ_c (ppm)	48/80[b] δ_c (ppm)
β	31.58 (T)	31.77 (T)
N-CH_3	33.59 (Q)	33.59 (Q)
α	51.00 (T)	50.74 (T)
OCH_3	56.27 (Q)	56.59 (Q), 61.50 (Q)
3,3′	115.39 (D)	—[c]
1	129.69 (S)	—[c]
2,2′	130.69 (D)	—[c]
4	158.86 (S)	155.68 (S), 157.18 (S)
CH_2OH	—	59.50 (T)
-CH_2-	—	30.79 (T)
HCHO[d]	82.65	

[a] Shift obtained with *p*-dioxane as an internal standard and converted to TMS using 67.4 ppm as shift for dioxane.

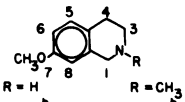
[b] Off resonance identified the peaks as singlet (S), doublet (D), triplet (T), quartet (Q).

[c] These peaks could not be assigned.

[d] Carbon-13 enriched.

TABLE 2

Chemical shifts[a] of the carbon atoms in 7-methoxytetrahydroisoquinoline, 7-methoxy-N-methyltetrahydroisoquinoline and carbon-13 enriched 48/80

Carbon	 R = H δ_c (ppm)[b]	R = CH_3 δ_c (ppm)[b]	C-13 enriched 48/80 δ_c (ppm)
1	45.60	55.7	—
N-CH_3	—	43.4	—
3	43.1	52.8	—
4	25.0	25.3	—
-CH_2OH	—	—	59.52 (T)
-CH_2-	—	—	30.62 (T)

[a] Shift obtained with *p*-dioxane as an internal standard and converted to TMS using 67.4 ppm as shift for *p*-dioxane.

[b] These chemical shifts were kindly supplied by Dr. J. F. Weber, Department of Chemistry, University of Hawaii.

rahydro*iso*quinoline moieties within the polymer. The chemical shifts of the aliphatic resonances of two model compounds, 7-methoxytetrahydro*iso*quinoline and 7-methoxy-N-methyltetrahydro*iso*-quinoline, are given in Table 2. If the postulated cyclization occurred via the Pictet-Spengler mechanism (17), then the product would be analogous to 7-methoxy-N-methyltetrahydro*iso*quinoline and C^{13}-enriched 48/80 should show a strong resonance from carbon atom 1 at or near 55.7 ppm. While C^{13}-enriched 48/80 does exhibit a resonance at 59.52 ppm several lines of evidence suggest that it is not due to a tetrahydro*iso*-quinoline moiety but that it can be safely assigned to a terminal $-CH_2OH$ group. Firstly, the chemical shift of the resonance is independent of the pD of the solution over the range 4–9. The chemical shift of carbon atom 1 of tetrahydro*iso*quinoline would be expected to shift under these conditions due to the ionization of the adjacent nitrogen atom. Secondly, the infrared spectrum of trifluoracetylated 48/80 exhibits carbonyl stretching vibrations at 1790 cm^{-1} and 1735 cm^{-1} which can be attributed to $-OCOCF_3$ and $-NCH_3COCF_3$ groupings, respectively. Finally, the C^{13} NMR spectrum of 7-methoxytetrahydro*iso*quinoline has a characteristic resonance at 25 ppm, due to carbon atom 4 (Table 2), which is absent in 48/80 (Fig. 2).

It is of interest that carbon atom 4 of 4-methoxy-N-methylphenethylamine (δ_c = 158.86 ppm) is replaced by two resonances (δ_c = 155.68 ppm and 157.18 ppm) in 48/80. Similarly, the OCH_3 resonance (δ_c = 56.27 ppm) of the monomer is also replaced by two resonances (δ_c = 56.59 ppm and 61.50 ppm) in the polymer. The two resonances in 48/80 could be due either to the same carbon atom in two different environments or to the presence of two different carbon atoms. Attempts to cause coalescence of the resonances at 155.68 ppm and 157.18 ppm by heating or changing solvent were unsuccessful, indicating that they probably result from two different carbon atoms. We suggest that these resonances may represent carbon atoms at the end of the polymer (δ_c = 157.18 ppm and 56.59 ppm) and in the middle of the polymer (δ_c = 155.68 ppm and 61.50 ppm). The ratio of integrated peak intensities for the resonances at 157.18 ppm and 155.68 ppm were 0.96 in unfractionated 48/80 (Fig. 3), 0.53 in combined fractions 51–77 (Fig. 2), 0.82 in fractions 78–84 and 1.15 in fractions 92–119. The expected values would be 2 in the trimer, 1 in the tetramer, 0.66 in the pentamer and 0.5 in the hexamer. This would suggest that the initial most active fractions contain mainly the hexamer which is in agreement with the findings of Read and Lenney (10).

It was also noted that the resonance due to the $N-CH_3$ group in the phenethylamine chain of 48/80 was much sharper than the remaining resonances (Fig. 2). Light scattering measurements of solutions of 48/80 showed a sharp increase in scatter above 100 μg/ml (Fig. 4). Similarly, when the fluorescent probe ANS was present, there was a dramatic increase in fluorescence intensity at concentrations of 48/80 above 100 μg/ml (Fig. 4). These findings strongly suggest that 48/80 forms micelles at higher concentrations. Since the NMR measurements were made at a concentration of 20 mg/ml, most of the 48/80 was probably present in the form of micelles. The sharper resonance of the $N-CH_3$ group can therefore probably be attributed to the greater mo-

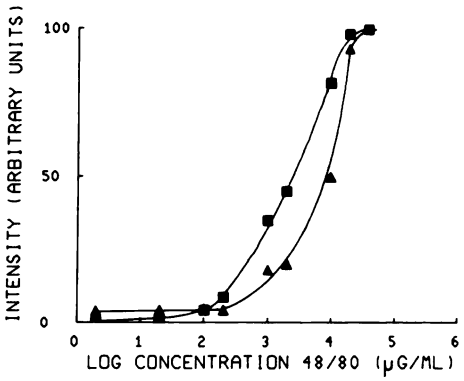


FIG. 4. *The effect of 48/80 concentration on light scattering and the fluorescence of ANS*

Solutions contained ANS, 10 μM, and 0.05 M sodium phosphate buffer, pH 7.4. ■——■, fluorescence intensity using activation and emission wavelengths of 392 nm and 465 nm, respectively; ▲——▲, light scattering measured using activation and emission wavelengths of 450 nm.

bility of carbon atoms which are located on the outside of the 48/80 micelles. It should be emphasized that micelle formation occurs at a 48/80 concentration that is almost three orders of magnitude higher than that required to degranulate mast cells, indicating that micelles are not necessary for the biological activity of the polymer.

These studies have shown that the reaction of 4-methoxy-N-methylphenethylamine with formaldehyde in strong acid solution probably proceeds in the manner proposed by Baltzly and co-workers (7) (Scheme I). The NMR spectrum of 48/80 prepared from carbon-13 enriched formaldehyde provides clear evidence for the presence of linking methylene groups and for the termination of the polymer chains by $-CH_2OH$ moieties. Finally, there was no evidence for the presence of tetrahydro*iso*quinoline moieties in 48/80.

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