Studies on the Stability of Trimelamol, a Carbinolamine-Containing Antitumor Drug

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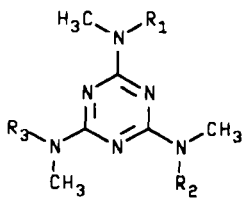
Abstract □ The stability of trimelamol (N^2,N^4,N^6-trimethylol-N^2,N^4,N^6-trimethylmelamine) a synthetic carbinolamine-containing antitumor drug, has been studied. Two major degradation pathways have been characterized and a unified mechanism proposed to rationalize the chemistry involved. One degradation pathway involves the consecutive loss of hydroxymethylene units by elimination of formaldehyde until the parent trimethylmelamine (**4**) results. An HPLC method was used to obtain kinetic data for the loss of trimelamol and to monitor the order of appearance of three degradation products. This pathway was shown to follow first-order kinetics at all pH values studied at both 18 and 37 °C. The second pathway involves the coupling of two trimelamol molecules via a methylene bridge to form bis(trimelamol) (**6**) which had been previously referred to in the literature as a "polymer". This reaction is acid catalyzed and temperature dependent. Bis(trimelamol) is virtually water insoluble and adheres strongly to glass surfaces. Finally, $t_{1/2}$ values have been determined for trimelamol in aqueous solution at different temperatures, and the kinetics of formation of degradation products has been studied over a period of 30 h under a variety of conditions of pH and temperature. The data reported here are relevant to both the formulation and clinical administration of trimelamol, and may contribute to an understanding of mechanism of action and future analogue development studies.

Trimelamol (N^2,N^4,N^6-trimethylol-N^2,N^4,N^6-trimethylmelamine, TMM; **1**) is a potent, synthetic,[1] carbinolamine-containing *S*-triazine antitumor agent similar in structure to hexamethylmelamine (HMM, **7**) and pentamethylmelamine (PMM, **8**), but differing in that it does not require bioactivation by oxidative *N*-demethylation for in vitro antitumor activity[2] and it is more water soluble. Hexamethylmelamine is extensively *N*-demethylated in vivo,[3-6] presumably through the loss of formaldehyde units from *N*-hydroxymethyl intermediates.[7] Both HMM and PMM are only cytotoxic in vivo after prolonged exposure or after activation by liver microsomal preparations.[2,8] The inhibitory effect of nonactivated HMM and PMM is reversed by drug removal, but the cytotoxic effects of *N*-hydroxymethylmelamines such as TMM are not.[9] *N*-Hydroxymethylmelamines are significantly more toxic in vitro than HMM or PMM.[2] Trimelamol exhibits antitumor activity towards mouse PC6 plasmacytomas and human lung tumor xenografts,[8,10] although its toxicity is similar to PMM in rodents, with negligible neurotoxicity.[11] In addition, the known species differences in the metabolic activation of PMM (*N*-demethylation of PMM is less efficient in rats and humans than in mice), suggest that *N*-hydroxymethyltriazines may be useful for the treatment of human tumors.[11,12] Trimelamol has already shown promise in the clinic.[13]



1 : $R_1 = R_2 = R_3 = CH_2OH$ (Trimelamol)
7 : $R_1 = R_2 = R_3 = CH_3$ (HMM)
8 : $R_1 = R_2 = CH_3$; $R_3 = H$ (PMM)

The development of iv formulations of TMM has proved problematic due to its poor aqueous solubility, its chemical instability, and its tendency to form an insoluble precipitate.[11] One formulation developed for clinical use is stored as a lyophylized mixture of TMM and mannitol which is reconstituted with 5% dextrose at pH 9.0 prior to administration.[13] After reconstitution, a white solid, previously thought to be a polymer,[11] precipitates at a rate dependent on the conditions of the solution. This is undesirable for an injectable formulation.

Prior to this investigation, initial studies on the chemical stability of TMM had been reported by Rutty and co-workers[11,13] and by Judson.[14] Using reversed-phase HPLC (Spherisorb 5 μm octyl packing; methanol:0.05 M ammonium bicarbonate, 30:70, pH 8.1) they were able to estimate the purity of samples of TMM. Using a different mobile phase (acetonitrile:0.05 M ammonium bicarbonate, 9:91), separation of trimethylmelamine (**4**) and N^2-monomethylol-N^2,N^4,N^6-trimethylmelamine (**3**) was reported. The effects of organic solvents and lyophylization on solubility and dissolution rate were also studied, and the destabilizing effect of low pH and high ionic strength and temperature described. Consecutive loss of hydroxymethylene units was postulated (e.g., **1** → **2** → **3** → **4**; Scheme I), and a "polymerization" reaction involving the formation of methylene bridges was suggested to account for the white precipitate. This reaction was thought to be enhanced by the presence of undissolved drug particles, low pH, high TMM concentration, and increased temperature. More recently, Judson and co-workers reported an investigation of the pharmacokinetics of TMM in humans.[15]

The present studies[16] were undertaken to extend this work and to investigate the mechanism of degradation of TMM with the objective of developing more stable formulations. In addition, an understanding of the mechanism of degradation could relate to the mode of action of compounds of this type, ultimately leading to the design of more effective analogues. Through these investigations, the sequential degradation pathway proposed by Rutty and co-workers[11,13] has been confirmed. The kinetics of degradation has been measured in aqueous solution to the point of total loss of TMM, and rate constants and $t_{1/2}$ values have been determined for a range of

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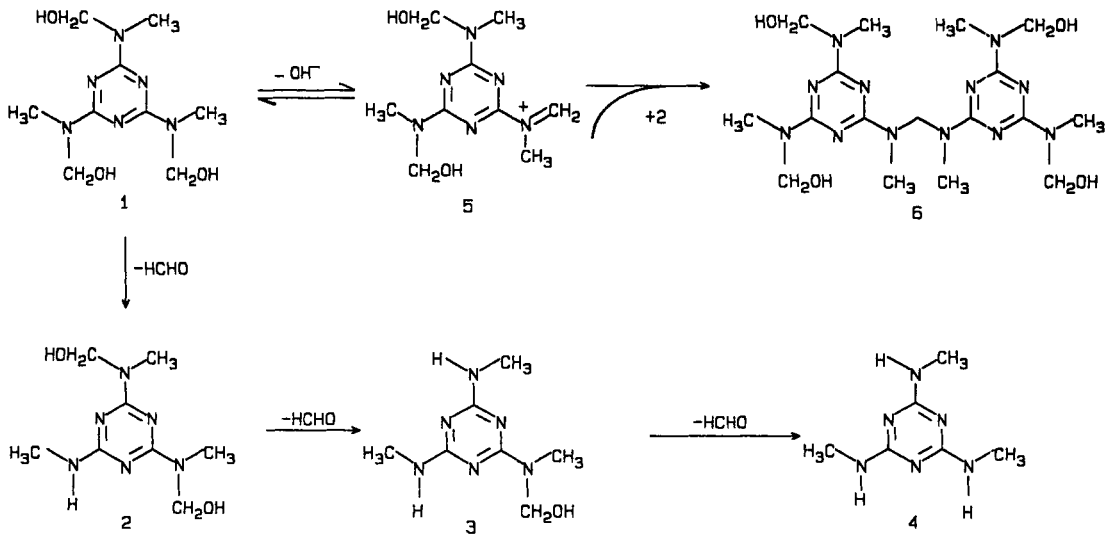


Scheme I—Degradation pathways of trimelamol (1) in aqueous solution.

pH values (Table I). The polymerization process referred to by Rutty and co-workers[11,13] has also been investigated. Rather than a polymer, the white, electrostatic, water-insoluble solid formed via the second degradation pathway has been identified as bis(trimelamol) (6). This process does not appear to proceed further to higher molecular weight polymers as suggested by Rutty and co-workers,[11] probably due to the low aqueous solubility (<0.02 mg/mL) of 6.

Thermogravimetric studies on the solid-state degradation of TMM indicate that at higher temperatures (>125 °C) a true "polymerization" process may occur, rather than a stepwise loss of formaldehyde units.

Experimental Section

Materials and Reagents—Acetonitrile (HPLC grade), ammonium bicarbonate (GPR), resorcinol (GPR), NaOH (GPR), and HCl (GPR) were purchased from BDH Chemicals Ltd., Poole, U.K., and DMSO (HPLC grade) was purchased from Aldrich Chemical Company, Ltd. Water was glass distilled and filtered prior to use. Trimelamol (TMM; 1) was synthesized by Cyanamid UK Ltd. (Batch No.: RM.150).

High-Performance Liquid Chromatography (HPLC)—The system was comprised of a Pye-Unicam LC3-X3 pump, a Kratos Spectroflow 773 variable-wavelength UV detector set at 225 nm and 0.002 AUFS, and a model 7125 syringe-loading injection valve (Rheodyne; Cotati, CA) fitted with a 20-μL loop. A μ-Bondapak C_{18} 10-μm column (3.9 mm × 30 cm, Waters P/N 27324) was used for all analytical determinations. The mobile phase, acetonitrile:0.05 M ammonium bicarbonate (12:88) at pH 8.1, was filtered (0.45 μm Nylon-66 membrane, Rainin Instruments Company, Woburn, MA) and deaerated prior to use. A flow rate of 0.8 mL/min with a column inlet pressure of 1700 psi gave a retention time of 22 min for TMM (Figure 1). Peak areas were measured with a Spectraphysics SP4100 computing integrator. A pH meter (Electronics Institute Ltd., model EIL7055) fitted with combination electrodes (Kent Industrial Measurements Ltd., Stonehouse, Gloucester, U.K.) was used for all pH measurements.

Internal Standard Preparation—Resorcinol was used as an internal standard to initially assess the assay procedure. A sample of resorcinol was accurately weighed (~100 mg), transferred to a volumetric flask (250 mL), and made up to volume with water. An aliquot (5 mL) was pipetted into a second volumetric flask (50 mL) and made up to volume with mobile phase. The solution was stored in the dark at 4 °C.

Sample Preparation—Accurately weighed TMM (~500 mg) was transferred to a volumetric flask (500 mL) with water, sonicated until completely dissolved, and then made up to volume with water. An aliquot of this solution (50 mL) was transferred to a 1000-mL three-necked round-bottomed flask containing water (450 mL) that had been previously deaerated with nitrogen. The center neck of the flask was fitted with a pH electrode and nitrogen was passed through the outer necks. The solution was initially sparged with nitrogen to purge carbon dioxide and a head of nitrogen was maintained above the solution throughout the study. The entire flask assembly was submerged in a thermostated waterbath and the temperature was allowed to equilibrate prior to adding the TMM solution. This procedure was repeated at pHs 3.0, 4.0, 7.4, 9.0, and 10.0 at 18 °C, and pH 7.4 at 37 °C. The pH adjustments were made by the addition of

Table I—First-Order Rate Constants, Correlation Coefficients, and Half-life ($t_{1/2}$) Values for the Degradation of Trimelamol in Aqueous Solution at Various pH Values and Temperatures

pH	First-Order Rate Constant ($\times 10^{-4}$), min^{-1}	Correlation Coefficient (r)	$t_{1/2}$, h
3.0[a]	5.92	0.996	19.7
4.0[a]	2.18	0.994	53.2
7.4[a]	1.41	0.989	82.2
9.0[a]	1.56	0.937	74.5
10.0[a]	2.23	0.972	52.0
7.4[b]	13.18	0.995	8.8

[a] 18 °C. [b] 37 °C.

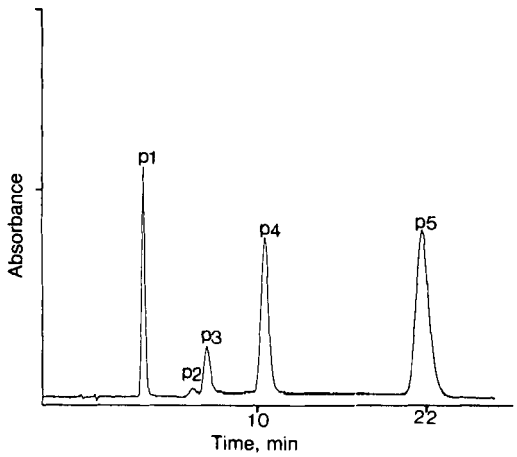


Figure 1—Typical HPLC chromatogram of trimelamol (1) undergoing degradation in aqueous solution. Peaks are numbered in order of elution: P1 = resorcinol (internal standard); P2 = N^2,N^4,N^6-trimethylmelamine (4); P3 = N^2-monomethylol-N^2,N^4,N^6-trimethylmelamine (3); P4 = N^2,N^4-dimethylol-N^2,N^4,N^6-trimethylmelamine (2); and P5 = trimelamol (1).

either HCl (10%) or NaOH (20%) solutions.

Assay Method—At each predetermined time point, a 0.5-mL aliquot (Finnpippette, Jencons Scientific, Leighton Buzzard, U.K.) was removed from the flask and internal standard (0.5 mL) was added, followed by vigorous mixing (1 min) using a rotary mixer (Rotamixer; Hook and Tucker Instruments, U.K.). The 20-μL loop was completely filled (100-μL syringe, Hamilton) and the contents were injected onto the analytical column. Log areas of TMM peaks were plotted against time (min) for each pH and temperature (Figures 2A and 2B). For each condition, the three degradation products were plotted as peak areas versus time (min). One example (pH 7.4 at 37 °C) is given in Figure 3 and the rest of the data are summarized in Table II. The precision and long-term reproducibility of the assay procedure was assessed by monitoring the area of the internal standard peak.

Preparative High-Performance Liquid Chromatography—The analytical system described above was fitted with a 5-mL injection loop and a Dynamax-60A C_{18} column (8 μm, 21.4 mm × 25 cm semi-preparative, fitted with a 21.4 mm × 5 cm C_{18}, 8 μm guard column). A mobile phase consisting of acetonitrile:water (15:85) was filtered, deaerated, and delivered at a flow rate of 8 mL/min. The TMM (500 mg) was dissolved in acetonitrile and made up to 500 mL in a volumetric flask. An aliquot (25 mL) was accurately diluted with water to 250 mL in a volumetric flask, and the solution was left to stand overnight at 18 °C. An aliquot (5 mL) of this partially degraded TMM solution was injected onto the loop and loaded onto the column, and the effluent was monitored at 233 nm with an attenuation of 0.16 AUFS. Fractions containing discrete peaks were collected, lyophylized, combined using methanol as solvent, and then re-lyophylized. The integrity and relative retention time of individual components were established by re-injection onto the analytical HPLC system.

Preparation of Pure Bis(trimelamol)—Trimelamol (2.16 g) was dissolved in dimethylsulfoxide (10 mL) and then diluted with distilled water (30 mL). After stirring mechanically for 20 min, a thick white precipitate formed. Collection by vacuum filtration afforded a white amorphous solid (1.01 g, 52%, mp 144–146 °C) which was purified by recrystallization from chloroform to afford colorless prisms (194 mg, mp 157–158 °C).

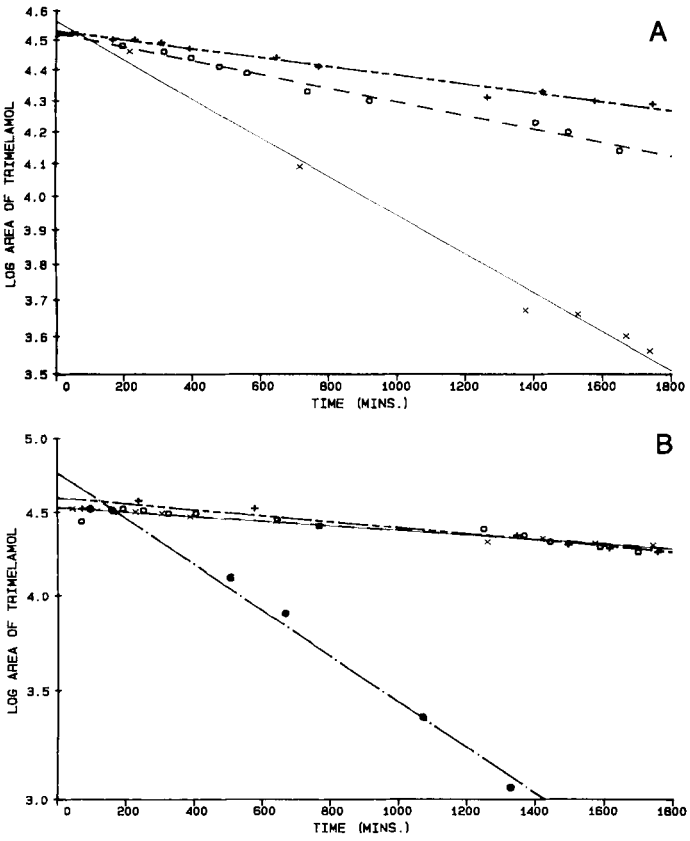


Figure 2—First-order rate plots of loss of trimelamol at pH (—X—) 3.0, (- -○- -) 4.0, and (– - - + - - –) 7.4 (18 °C) (Figure 2A), and at pH (–X–) 7.4, (- -○- -) 9.0, and (– - - + - - –) 10.0 (18 °C) and (-●-) 7.4 (37 °C) (Figure 2B).

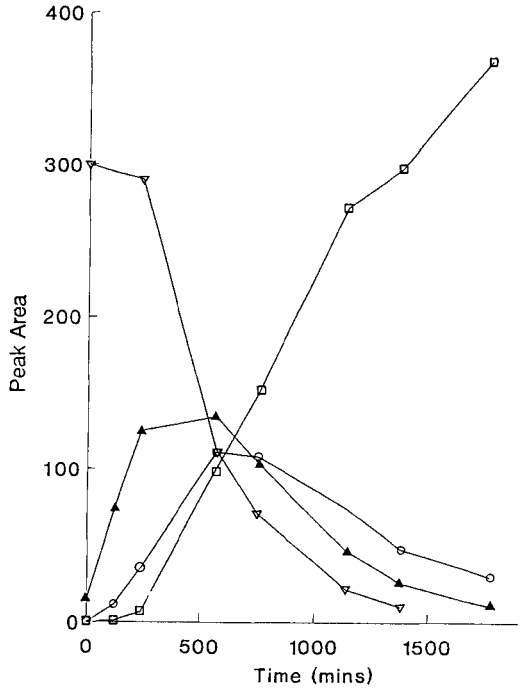


Figure 3—Plot of relative concentrations of trimelamol (**1**) and all degradation products versus time at pH 7.4 and 37 °C. Key: (▽) **1**; (▲) **2**; (○) **3**; (□) **4**.

Combustion analysis: calc. for C, 43.59; H, 6.89; N, 35.92 ($C_{17}H_{32}N_{12}O_4$). Found: C, 43.21; H, 6.87; N, 35.60.

Gravimetric Assay—The TMM samples (5, 75, 150, 200, and 1000 mg) were weighed into Nessler tubes (50 mL), dissolved in DMSO (2 mL for samples <20 mg; 5 mL for 20-mg sample), and then diluted to volume with water of the appropriate pH (3.0, 7.4, and 10.0, prepared by the addition of either 10% HCl or 20% NaOH) to give the concentrations shown in Table III. After thorough mixing, followed by incubation at 18 or 37 °C for 24 h, the resultant solutions or suspensions were filtered through pre-dried and weighed acid-washed sintered glass filters (Pyrex G3, Scientific Furnishings, Chichester, Hants., U.K.). The collected filtrates were dried to constant weight and the dry yields were calculated as a percentage of the weight of TMM present at time zero (Table III).

Nuclear Magnetic Resonance (NMR) Spectrometry—Lyophylized samples (~30 mg) were dissolved in DMSO-d_6 (Goss Scientific Instruments, Ingatestone, Essex, U.K.), and one- and two-dimensional ^1H and ^{13}C NMR spectra were measured at 270 MHz using a JEOL GSX 270 FT NMR spectrometer coupled to a JEOL DA 5000 data system (Table IV).

Mass Spectrometry—Mass spectra were measured using a JEOL JMS DX 303 GC mass spectrometer coupled to a JEOL DA 5000 data system. Trimelamol (TMM; **1**): EI: 258 ($M^{+\cdot}$, 37), 241 (M^+, –OH, 14), 239 (15), 228 (M^+, –HCHO, 37), 222 (29), 211 (M^+, –HCHO, –OH, 19), 209 (26), 198 ($M^{+\cdot}$ –2 × HCHO, 36), 181 ($M^{+\cdot}$ –2 × HCHO, –OH, 64), 179 (20), 168 ($M^{+\cdot}$ –3 × HCHO, 81), 151 (23), 139 (64), 124 (17), 113 (10), 110 (6), 96 (46), 82 (80), 69 (14), 57 (100) [a weak ion at 348 corresponding to **6** was usually observed in TMM samples at higher temperatures, probably representing dimerization in the probe]; CI (ACE isobutane): 259 ($MH^{+\cdot}$, 2), 241 (2), 229 (16), 211 (11), 199 (36), 181 (13), 169 (49), 57 (100) [weak ions corresponding to **6** were observed at 469 ($MH^{+\cdot}$), 439 (MH^+, –HCHO), 409 ($MH^{+\cdot}$ –2 × HCHO), 379 ($MH^{+\cdot}$ – 3 × HCHO), and 349 ($MH^{+\cdot}$ – 4 × HCHO). Bis(trimelamol) (**6**): CI (isobutane): 349 ($MH^{+\cdot}$ – 4 × HCHO, 100), 181 (43), 169 (100); FAB (glycerol): 469 ($MH^{+\cdot}$). N^2,N^4-Dimethylol-N^2,N^4,N^6-trimethylmelamine (**2**) and N^2-monomethylol-N^2,N^4,N^6-trimethylmelamine (**3**) (isolate from preparative HPLC, isobutane): 229 (2, MH^+, 10), 211 (11), 199 (3, MH^+, 51), 181 (30), 169 (100), 85 (53). Trimethylmelamine (**4**): EI: 168 (M^+, 59), 139 (34), 124 (11), 96 (5), 83 (100), 68 (5), 57 (29).

Table II—Time of First Appearance and Maximum Concentration of Degradation Products of Trimelamol at Various pH Values and Temperatures

pH	Temperature, °C	Degradation Products					
		2 (P4)[a]		3 (P3)[a]		4 (P2)[a]	
		TFA[b]	MAX[c]	TFA[b]	MAX[c]	TFA[b]	MAX[c]
3.0	18	0	12–22	2–6	28+	22	28+
4.0	18	0	27	3–10	40–53	10–13	125+
7.4	18	0	23–50	3–6	50	10–13	106+
9.0	18	0	20–33	3–6	33–50	6–13	120+
10.0	18	0	21–30	2–4	28	18–20	28+
7.4	37	0	2–6	0	6–10	1–2	28+

[a] See Figure 1 for explanation of P2–P4. [b] Time of first appearance of degradation products in hours. [c] Time of maximum concentration of degradation products in hours.

Table III—Yield of Bis(trimelamol) Formed from a Range of Concentrations of Trimelamol in DMSO:H_2O at Various pH Values

Trimelamol, mg/mL	Temperature, °C	Yield, %		
		pH 3[a]	pH 7.4[a]	pH 10[a]
0.1	18	NP[b]	NP	NP
1.5	18	NP	NP	NP
3.0	18	4.1 ± 1.1[c]	NP	NP
4.0	18	35.6 ± 1.5	NP	NP
20.0	18	66.4 ± 0.9	NP	NP
20.0	37	73.2 ± 1.3	49.9 ± 6.7	NP

[a] Yield of precipitate (% w/w) based on amount of trimelamol at the start. [b] No precipitate formed. [c] Data derived from three replicate experiments.

Table IV—Proton and Carbon-13 Nuclear Magnetic Resonance Data (δ) for Trimelamol and Degradation Products in DMSO-d_6

Signal	Compound				
	TMM (1)	2	3	4	6
$N(CH_2OH)CH_3$	3.05 (s, 9H)	2.93 (s, 6H)	3.04 (s, 3H)	—	2.98[a], 3.04 (s, 18H)[b]
$N(H)CH_3$	—	2.75 (s, 3H)[c]	2.72 (s, 6H)[c]	2.71 (s, 9H)	—
$N\text{-}CH_2\text{-}O$	4.97 (d, 6H)[d]	4.93 (bs, 4H)[e]	4.93 (bs, 2H)[e]	—	5.00 (d, 8H)[f]
$N\text{-}CH_2\text{-}N$	—	—	—	—	5.60 (s, 2H)
OH	5.44 (bs, 3H)[g]	5.43 (bs, 2H)[g]	5.43 (bs, 1H)[g]	—	5.50 (bs, 4H)[g]
$NHCH_3$	—	6.65 (bs, 1H)[g,h]	6.65 (bs, 2H)[g,h]	6.36 (bs, 3H)[g,i]	—
$N(CH_2OH)CH_3$	32.6	26.9, 32.3		27.2	32.3[a], 32.6
$N\text{-}CH_2O$	70.8	70.3		—	70.8
$N\text{-}CH_2\text{-}N$	—	—		—	58.1
Ring C	165.3	165.0, 166.0		166.2	165.1, 166.1

[a] Bridge methyl [triazine-$N(CH_3)CH_2$-]. [b] 1:2 integral for 2.98 and 3.04 signals. [c,e] Assignment of these signals could be reversed. [d] J = 7.5 Hz. [f] J = 6.5 Hz. [g] Exchangeable with D_2O. [h] Small broad D_2O-exchangeable signal at 6.45. [i] Signal covers 6.10–6.62.

Thermogravimetric Analysis (TGA)—Measurements were made on a Perkin-Elmer Series 7 thermogravimetric analyzer. Trimelamol (18.97 mg, Batch RM.150) and 6 (5.30 mg) were separately heated in a platinum crucible in a stream of nitrogen gas at 18 psi and a ramp rate of 5 °C/min (Figures 4 and 5, respectively). The major mass losses were observed between ~100 and 325 °C. The characteristic odor of formaldehyde was detected in the exhaust gases from both samples. In both cases, heating to 325 °C afforded clear transparent rigid solids which were insoluble in water, acetone, and acetonitrile. The residue from heating TMM was further analyzed by HPLC.

Results and Discussion

To carry out these studies, an HPLC system was required that would allow qualitative and quantitative evaluation of the degradation of TMM. Rutty and co-workers[11] had previously reported two reversed-phase (C_{18}) HPLC methods, both of which used ammonium bicarbonate in the mobile phase. Initial efforts in the present study were directed towards developing a mobile phase for preparative HPLC devoid of buffer salts that might interfere with subsequent NMR and MS determinations. Also, degradation of TMM is accelerated in the presence of buffer salts, and "on-column" degradation may occur when buffered mobile phases are used.[17] However,

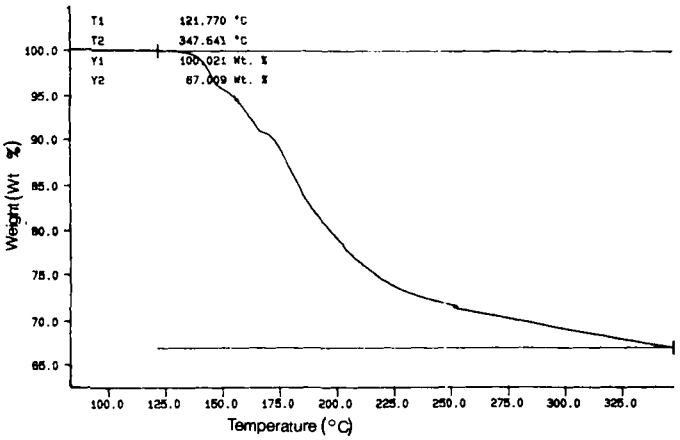


Figure 4—Thermogravimetric analysis of trimelamol (1) at a rate of 5.0 °C/min.

a separate trial, with a Dynamax-60A, C_{18}, 8 μm, 4.6 mm × 25 cm column and mobile phases ranging from 8% to 30%

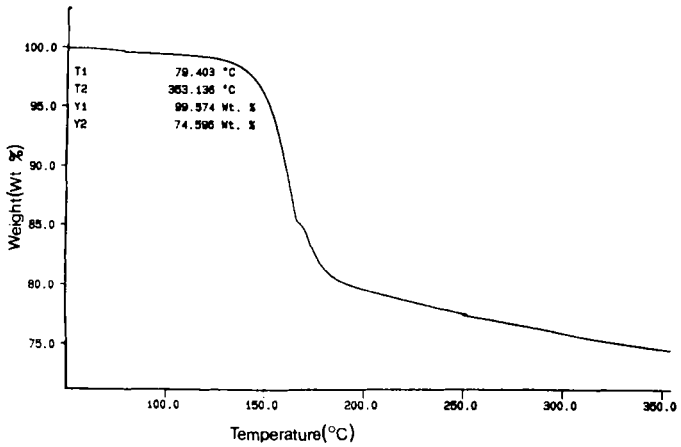


Figure 5—Thermogravimetric analysis of bis(trimelamol) (**6**) at a rate of 5.0 °C/min.

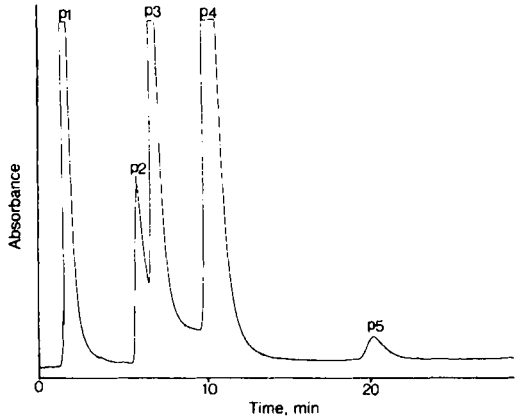


Figure 6—Analytical HPLC trace of the lyophylizate from preparative HPLC. Peaks are numbered in order of elution: P1 = DMSO (solvent); P2 = N^2,N^4,N^6-trimethylmelamine (**4**); P3 = N^2-monomethylol-N^2,N^4,N^6-trimethylmelamine (**3**); P4 = N^2,N^4-dimethylol-N^2,N^4,N^6-trimethylmelamine (**2**); and P5 = trimelamol (**1**).

acetonitrile in H_2O, resolved only two peaks (15 and 26 min for 15% acetonitrile) for a partially degraded sample of TMM together with a broad baseline band encompassing both peaks. This broad band was identified as 4, and the 26-min peak as TMM, implying that the first and second degradation products were co-eluting at 15 min. The separation was improved by using a mobile phase consisting of acetonitrile:0.05 M ammonium bicarbonate (12:88, pH 8.1).[11] For a partially degraded sample of TMM, three peaks at 30, 16, and 11 min were observed and assigned to TMM, 2, and a mixture of 3 and 4, respectively. Complete resolution was obtained with the same mobile phase and a Waters μ-Bondapak C_{18} (10 μm, 3.9 mm × 30 cm, P/N 27324) column. Peaks were observed at ~7, 8, 12, and 22 min, as shown in Figure 1.

Degradation Pathway I—The HPLC assay described above was used to investigate the consecutive loss of formaldehyde from TMM. Resorcinol was chosen as an internal standard (t_r = 5 min). It was shown to be stable in the presence of TMM by allowing a solution of a mixture of both to stand at room temperature. This led to only 0.6% degradation of resorcinol after 2 days. Trimelamol (TMM; 1) and 4 were identified by co-injection with standards and the first degradation product (2) was identified by MS and NMR, after isolation by preparative HPLC. Fractions corresponding to the peak assumed to be the first degradation product were collected, combined, immediately frozen, and then lyophylized over a period of 3 days to afford a white electrostatic solid.

Analysis by NMR in DMSO-d_6 (Table IV) indicated the presence of more than one species, and further degradation was assumed to have occurred. Two $N(H)CH_3$ signals were observed at 2.75 and 2.72δ, and two $N(CH_2OH)CH_3$ signals at 2.93 and 3.04δ, respectively. The $N\text{-}CH_2OH$ (4.93δ), -OH (5.43δ), and $NHCH_3$ (6.65 and 6.45δ) signals were also visible. Re-injection of this DMSO-d_6 solution onto the analytical HPLC system (Figure 6) showed that it was predominantly the first degradation product (2, P4, 11.8 min, 63%), contaminated with the second (3, P3, 8.2 min, 27%) and third (4, P2, 6.9 min, 8%) products, along with residual TMM (1, P5, 22 min, 2%). Further degradation had apparently occurred during the period of lyophylization (62 h). Trimethylmelamine (4) gave a small signal at 6.36δ, consistent with a spectrum of pure sample, and a contribution to the $N\text{-}CH_3$ signals at 2.72δ (2.71δ in pure 4).

According to HPLC, the first and second degradation products (2 and 3, respectively) were present in a 2:1 ratio. As the ratio of $N(H)CH_3$ to $N(CH_2OH)CH_3$ protons for 2 and 3 should be 1:2 and 2:1, respectively, the tallest signal in the $N(CH_2OH)CH_3$ area at 2.93δ could be tentatively assigned to 2. Due to contamination with 4, similar assignments could not be made for the $NHCH_3$ signals at 2.72 and 2.75δ. The $N\text{-}CH_2\text{-}O$ signals for both 2 and 3 resonated at 4.93δ, with hydroxyl signals at 5.43δ.

The ^{13}C NMR (Table IV) and MS data were also consistent with the above assignments. The EI (and CI) mass spectrum of TMM is characteristic, with a molecular ion at 258 and fragment ions at 228, 198, and 168 corresponding to the consecutive loss of three molecules of formaldehyde. In addition, ions at *m/z* 241, 211, and 181 correspond to the consecutive loss of −OH groups. Mass spectrometry (CI) of the lyophylizate from preparative HPLC indicated the presence of the first (2) and second (3) degradation products with MH^+ ions at 229 and 199, respectively.

In order to study the kinetics of degradation of TMM under various conditions, an aqueous solution at a concentration of 0.1 mg/mL was adjusted to a specific pH and maintained at 18 or 37 °C. Degradation was monitored using the HPLC system described above for pH values of 3.0, 4.0, 7.4, 9.0, and 10.0 at 18 °C and also for pH 7.4 at 37 °C. Plots of log (peak area) for TMM concentration versus time (Figures 2A and 2B) are linear, implying that loss of TMM is first order over this range of pH values and temperatures. The rate constants and $t_{1/2}$ values for these conditions are given in Table I. The resolution of all three degradation products of this pathway (2, 3, and 4) by HPLC enabled a complete concentration profile of TMM and all its degradation products to be obtained for each set of conditions. One example for clinically relevant conditions (pH 7.4 at 37 °C) is shown in Figure 3. Table II summarizes the times of first appearance and maximum concentration of degradation products for all sets of conditions. At 37 °C, degradation is so rapid that the second degradation product (3) is already present at the first sampling (>1 min). The maximum concentrations of the first and second degradation products are reached in just 2–6 and 6–10 h, respectively, and the third degradation product appears in just over 1 h, reaching a maximum at ~28 h. As the precise number and structure of biologically active species resulting from a dose of TMM in animals or humans have not yet been determined, this data may be of significance for future studies in this area. As degradation products 2 and 3 still possess carbinolamine functionalities, they are likely to be biologically active and may contribute to the overall clinical effectiveness of TMM.

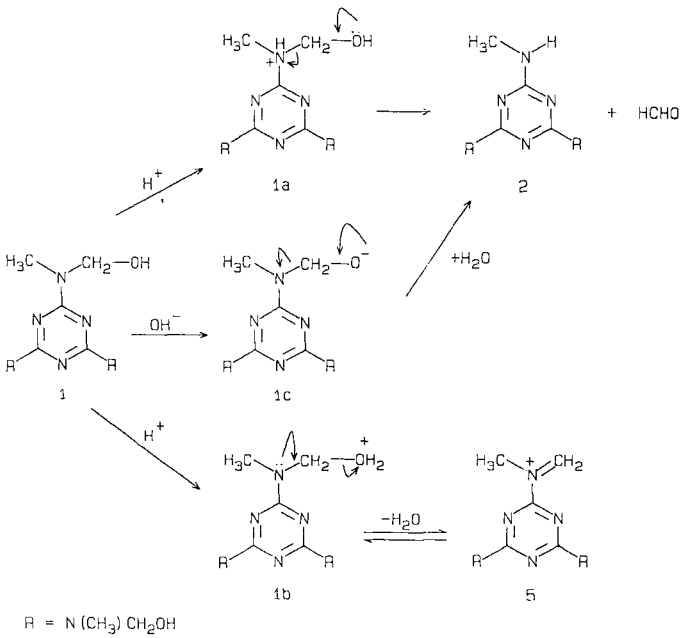
Significant features of the data for 18 °C include the relatively delayed appearance of the final degradation product (4) at the extreme pH values of 3.0 (22 h) and 10.0 (18–20 h). Conversely, the maximum concentration of the first

degradation product is reached significantly more rapidly (12–22 h) at pH 3.0 than at any of the other pH values. Maximum concentration of the second and third degradation products are reached more rapidly at the extreme pH values of 3.0 and 10.0.

The acid catalysis of this degradation pathway may be rationalized by a requirement for *N*-protonation prior to the loss of a hydroxymethylene unit as shown in Scheme II (**1** → **1a** → **2**). As successive hydroxymethylene units are lost, the system appears to become less stable (e.g., Scheme I: stability = **1** > **2** > **3**). This may result from further protonation of the —$NHCH_3$ groups formed, causing the triazine ring to become progressively more electron deficient and thus encouraging the loss of formaldehyde according to the type of mechanism shown for **1a** → **2**. The base catalysis may result from a change of mechanism whereby the hydroxyl of the hydroxymethylene group is initially deprotonated (e.g., **1** → **1c**, Scheme II), followed by the elimination of formaldehyde (**1c** → **2**).

Degradation Pathway II—The second degradation pathway affords a white, electrostatic, water-insoluble solid, previously described in the literature as a "polymer."[11] This material is problematic in iv formulations since it precipitates either upon storage or shortly after reconstitution. It has an apparent affinity for glass and plastic surfaces and, once precipitated, does not appear to react further. In addition, during HPLC analysis it may block analytical columns, causing unacceptably high back pressures. Rutty and coworkers[11] found that it eluted with methanol from their reversed-phase HPLC column as a complex series of peaks, suggesting the existence of a number of polymeric species of different molecular weights. However, the results of this study demonstrate that the material is essentially homogenous. Elution from an HPLC column as a series of bands occurs only because of the relative positions of "plugs" of **6** on the column following multiple injections.

Initial studies were aimed at elucidating the structure of this degradation product. A crude sample was produced by filtration of a concentrated solution of TMM in DMSO:H_2O, and a pure sample was obtained as colorless prisms by recrystallization from chloroform. The structure was unambiguously assigned as **6** from ^1H and ^{13}C NMR, mass spectrometry, elemental analysis, and thermogravimetric data.



Scheme II—Possible mechanisms of degradation.

Unlike TMM, there are two different chemical environments for both the methyl and methylene protons of **6**. Separate ^1H NMR signals are observed for the outer $N(CH_2OH)CH_3$ (3.04δ) and inner bridge $N\text{-}CH_3$ (2.98δ) methyls in the expected ratio of 2:1. Comparison with TMM [$N(CH_2OH)CH_3$, 3.05δ] allows the lower field signal to be assigned to the central bridge $N\text{-}CH_3$ groups. Similarly, a doublet at 5.00δ could be assigned to the four external $N\text{-}CH_2\text{-}OH$ groups (cf. 4.97δ in TMM), and a new two-proton singlet at lower field (5.60δ), not present in the TMM spectrum, could be assigned to the central methylene bridge based on literature values for similar nitrogen-disubstituted methylenes.[18] The appropriate integral of 4:1 was observed for these methylene signals and the overall ratio of methyl-to-methylene signals was the expected 10:18. Similarly, two different $N\text{-}CH_3$ signals were observed in the ^{13}C spectrum, one of which (32.3δ) could be assigned to the new bridge $N\text{-}CH_3$ groups by comparison with TMM. A new signal was also observed at 58.1δ, corresponding to the bridge $N\text{-}CH_2\text{-}N$.[18] Inequivalency of the triazine ring carbons was now evident, with signals appearing at both 165.1 and 166.1δ. Mass spectral data were also consistent with this structure. Bis(trimelamol) affords an ion (CI) at *m/z* 349, corresponding to the loss of four HCHO units. Two other fragments at 181 and 169 correspond to asymmetric cleavage on one side of the central methylene bridge (EI of 4 gives $M^{+\cdot}$ of 168). Interestingly, CI mass spectra (isobutane) of pure samples of TMM typically show low intensity ions at 469, 439, 409, 379, and 349, corresponding to the $MH^{+\cdot}$ of **6** and the consecutive loss of four units of HCHO. These ions are only observed at higher probe temperatures and when all of the TMM has left the probe. One explanation is that TMM samples always contain a small amount of **6** which tends to leave the probe only at higher temperatures due to its low volatility. Alternatively, **6** may form in the probe.

The amount of **6** formed from solutions of TMM was found to depend on conditions of pH and temperature. In order to investigate this, attempts were made to develop an HPLC system to assay **6**. However, it proved too insoluble to assay with either a normal- or reversed-phase column using a variety of solvent systems. Gravimetric analysis was a viable alternative. Table III shows the results of an experiment carried out under conditions of differing concentration, pH, and temperature. A TMM solution was allowed to degrade and **6** was collected by filtration, dried, and weighed. The data indicate that at 18 °C, **6** is only produced at pH 3.0 and the amount formed is related to the concentration of TMM, with no detectable amount formed at concentrations of 1.5 mg/mL or below. Elevating the temperature to 37 °C enhances the formation of **6** from a 20 mg/mL TMM solution at pH 3.0. At the same concentration, **6** was formed at pH 7.4 only when the temperature was elevated to 37 °C. No bis material was formed at pH 10.

The fact that the production of **6** is acid catalyzed suggests a likely mechanism for its formation from the first degradation product (**2**) and the iminium species **5** (Scheme I). The iminium ion (**5**), formed by loss of hydroxyl from TMM, is likely to be highly reactive and not disposed to accumulate. Since TMM is non-nucleophilic, it cannot react with the imine, but the first degradation product (**2**) reacts immediately upon formation. As with the first degradation pathway, imine formation is likely to be acid rather than base catalyzed, which explains the observed result. Protonation of the hydroxyl group (**1** → **1b**, Scheme II) is required prior to its expulsion as a water molecule to generate the iminium ion (**1b** → **5**).

It may be concluded from the above studies that the first degradation pathway, involving the loss of formaldehyde

units, is the more predominant route for TMM loss under the conditions described. For example, according to both the gravimetric and kinetic studies, no 6 was produced in a 0.1 mg/mL solution at pH 7.4 and 18 °C after 24 h. However, based on the $t_{1/2}$ (82.2 h) measured under identical conditions, an 18.3% loss of TMM via pathway I would occur during the same time period.

Solid-State Degradation—Further evidence for the degradation of TMM via consecutive loss of hydroxymethylene units was obtained through thermogravimetric (TGA) studies. Figure 4 is a plot of the percent weight loss of a sample of TMM as a function of increasing temperature. Three steps are visible in the TGA trace, representing the net consecutive loss of three formaldehyde units. The first step occurs at 140 °C, corresponding to the loss of 3.5% by mass. Similarly, from 140 °C to the next step at 169 °C corresponds to the loss of an additional 5.75%. In some experiments, the heating was stopped at this point, leaving a residue of a transparent, non-rigid solid. Analysis of this residue by HPLC and ^{1}H NMR indicated a complex mixture. According to analysis of HPLC peak areas, the residue comprised ~43% unchanged TMM, 37% 2 (loss of one formaldehyde unit), 18% 3 (loss of two formaldehyde units), and 2% trimethylmelamine (4) loss of three formaldehyde units). The formation of 6 could not be assayed by HPLC, although its presence was detected by NMR.

The fact that the steps in the TGA curve are reproducible suggests that, in the solid state, the sample is not degrading homogeneously. A possible explanation is that the elimination and local accumulation of formaldehyde may prevent other TMM molecules from similarly degrading (Le Chatelier's principle), thereby stabilizing the remainder of the sample. Since the formaldehyde is ultimately lost as vapor on the outside of the sample, the degradation must proceed more rapidly on the outside than in the center of the sample, at any given temperature.

In this model of degradation in the solid state, the position of the observed steps in the TGA curve should be partly a function of sample size and shape since this would define the rate of loss of formaldehyde. The subsequent degradation up to 325 °C and the nature of the residue obtained suggest that polymerization to a rigid cross-linked melamine:formaldehyde polymer may eventually occur:

$$\underset{(258)}{\text{Trimelamol}} = \underset{\text{-(186)-}}{\text{Polymer}} + \underset{(72)}{1.5CH_2(OH)_2}$$

The formation of such a polymer would require a 27.9% mass loss, which approximates to the plateau in the TGA curve in Figure 4 at ~240 °C.

Similarly, Figure 5 shows a TGA curve for 6 which suggests that similar reactions are occurring to form initially a mixture of degradation products. By the time 325 °C is reached, most of the hydroxymethylene groups should have degraded and the formaldehyde should be lost, resulting in a similar cross-linked melamine:formaldehyde polymer:

$$\underset{(468)}{\text{Dimer}} = \underset{\text{-(372)-}}{\text{Polymer}} + \underset{(36)}{2H_2O} + \underset{(60)}{2HCHO}$$

Again, the theoretical mass loss of 20.5% appears to correspond to the plateau in the TGA curve at ~180 °C.

Conclusions

These studies confirm the existence of two pathways for the degradation of TMM. One pathway involves the temperature-dependent acid- and base-catalyzed consecutive loss of formaldehyde units as observed by both HPLC and solid-state TGA. The second degradation pathway involves temperature-dependent acid-catalyzed attack of 2 on a TMM iminium ion intermediate of type 5. At the concentrations investigated, the first pathway appears to predominate for a given pH and temperature. A mechanistic scheme has been constructed to rationalize the chemical reactions observed in both pathways, and this may be used to explain the acid-catalyzed nature of both these routes of degradation. Similarly, base catalysis of the first pathway may be explained by assuming a change of mechanism from initial protonation of the nitrogen (**1** → **1a**; Scheme II) to deprotonation of the hydroxyl instead (**1** → **1c**). The fact that the second degradation pathway is acid and not base catalyzed is significant as, according to the proposed mechanism, protonation of the hydroxyl (e.g., **1** → **1b**) is a prerequisite for iminium ion formation (**1b** → **5**), a process that should not occur at higher pH values.

Of particular interest from a clinical standpoint is the relatively short $t_{1/2}$ (8.8 h) for TMM at physiological pH and temperature, and the consecutive appearance of degradation products 2, 3, and 4, at least two of which are likely to possess antitumor activity of their own. Furthermore, this degradation is likely to be more rapid in biological fluids of significant ionic strength.[11] Conversely, the $t_{1/2}$ at room temperature (18 °C) was found to be much longer (82.2 h). The information reported here should help in the elucidation of the mechanism of action of TMM, and in the design of more effective analogues.

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