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Physicochemical and in-vitro biological studies on the possible association between theophylline and ethylenediamine in solution

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The possible association in solution between the components of aminophylline, theophylline and ethylenediamine, has been studied using physicochemical and biochemical techniques. Addition of ethylenediamine to solutions of theophylline caused characteristic changes in its u.v. and ^{13}C-n.m.r. spectra, which were due solely to the pH change, and not to any molecular association between the molecules in solution. Both compounds crossed the erythrocyte membrane extensively from plasma or 0·9% NaCl and had no influence on each other's behaviour. Theophylline partitioned approximately equally between octanol and pH 7·4 phosphate buffer, while ethylenediamine remained in the aqueous phase. Again, neither compound affected the properties of the other. Theophylline was reversibly bound to plasma protein, the extent of which was dependent upon the temperature used for equilibrium dialysis. However, ethylenediamine was not bound to plasma protein, and did not alter the binding of theophylline. These data show that the components of aminophylline behave independently in solution, and suggest that there is no association between theophylline and ethylenediamine in biological systems.

The naturally occurring methylxanthine theophylline (1,3-dimethylxanthine) is poorly soluble in water. A wide variety of agents have been used to facilitate its dissolution, the most important of which is ethylenediamine (1,2-diaminoethane). The combination of theophylline and ethylenediamine is known as aminophylline, and was introduced into therapy by Dessauer (1908) as a diuretic. It is now a drug of choice in the treatment of reversible airways obstructive disease, being given by the intravenous, oral and rectal routes.

The exact chemical nature of aminophylline in solid form or solution is uncertain. It has variously been described as a salt (American Hospital Formulary Service 1978), a stable mixture (Martindale 1982), or a combination of two moles of theophylline and one mole of ethylenediamine accompanied by variable amounts of water (British Pharmacopoeia 1980).

In the solid state, aminophylline has been described as a solid complex of theophylline with ethylenediamine (Nishijo et al 1982). The solubility of theophylline in an aqueous solution of ethylenediamine is greater than would be expected from the pH change due to ethylenediamine, and this has been attributed to the formation of a soluble complex between the two molecules (Okano et al 1967; Nishijo et al 1982). However, these studies involved the use of saturated solutions of theophylline in non-biological media, and thus may not be relevant to the behaviour of aminophylline in the body.

For pharmacological and therapeutic purposes, aminophylline and theophylline are generally assumed to be equivalent on a molar basis. However, we (Monks et al 1981) have recently shown that both the rate and extent of metabolism of theophylline in man are enhanced when it is given in combination with ethylenediamine. Plasma concentrations of theophylline in man following oral administration of theophylline were nevertheless identical with those seen after aminophylline ingestion (Aslaksen et al 1981). The pharmacological and clinical literature also contains accounts of differences between aminophylline and theophylline in animals (Van Heerswynghels 1937; Schmid et al 1956) and man (Marais & McMichael 1937; Arkinstall et al 1983), in particular suggesting that ethylenediamine contributes to the respiratory and cardiovascular effects of aminophylline.

It therefore becomes important to understand reasons underlying the interaction between theophylline and ethylenediamine. Possible mechanisms include displacement of theophylline from binding sites on plasma proteins, alteration of membrane passage of theophylline or the existence of a molecular complex between theophylline and ethylenediamine in biological milieux. This paper

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describes studies on these aspects of the disposition of aminophylline, using a variety of in-vitro methods. Part of these results have been communicated to the British Pharmacological Society (Caldwell & Cotgreave 1981).

MATERIALS AND METHODS

Compounds

[8-^{14}C]Theophylline (sp. act. 38 mCi mmol^{-1}, radiochemical purity >99%) and [U-^{14}C]ethylenediamine (sp. act. 25 mCi mmol^{-1}, radiochemical purity >99%) were purchased from Amersham International, Amersham, U.K. Theophylline and ethylenediamine (free base) were purchased from Sigma.

Liquid scintillation spectrometry. ^{14}C was assayed by liquid scintillation spectrometry with a Packard TriCarb instrument model 3385, using a Triton X-100-toluene based scintillant. Quench correction was by reference to an external standard.

Haematocrit determination. This used a Hawkesley MicroHaematocrit centrifuge and reference frame.

Spectroscopy. Ultraviolet (uv) spectra were obtained with a Cecil CE595 double beam instrument. ^{13}C-Nuclear magnetic resonance (n.m.r.) spectra were obtained with the Bruker HX90E instrument of the University of London NMR service, at 26·5 Hz using a 10 mm ^{13}C-dedicated probe at 35 °C and an external TMS reference.

Plasma-erythrocyte partitioning

Solutions of [^{14}C]theophylline, [^{14}C]ethylenediamine and [^{14}C]aminophylline, with the label in either the theophylline or ethylenediamine moieties, were prepared in 0·9% NaCl (saline). These were added to 5 ml aliquots of whole blood so as to produce concentrations in the range 0–50 μg ml^{-1} theophylline and 0–15 μg ml^{-1} ethylenediamine, and the blood incubated at 37 °C for 30 min with shaking. Plasma was separated by centrifuging at 900*g* for 15 min at 37 °C, and 50 μl aliquots counted for ^{14}C. Control experiments showed that equilibrium was achieved after 5 min incubation, and that the cells and plasma separated according to the haematocrit. Haemolysis was negligible, as shown by the absence of haemoglobin from the plasma (assay with Sigma kit no. 525).

Saline-erythrocyte partitioning

Erythrocytes were separated from whole blood by repeated centrifugation and suspension in saline until the saline was protein-free. The final suspension was adjusted to a haematocrit of 40%. The experiments described above were repeated using erythrocytes in saline with the exceptions that 1 ml of resuspended erythrocytes was used, and the centrifugation was for 4 min at 37 °C at 900*g*.

In some experiments, the supernatant saline was removed and replaced by the same volume of fresh saline. The cells were resuspended and incubated and centrifuged as before, the supernatant being counted for ^{14}C.

Plasma protein binding

Drug solutions in saline were added to 1 ml aliquots of plasma to give final concentrations of 1–30 μg ml^{-1} [^{14}C]theophylline containing 0–20% ethylenediamine and 1–15 μg ml^{-1} [^{14}C]ethylenediamine with and without 85% w/w theophylline and the plasma incubated at 37 °C for 30 min with shaking. The plasma was placed in Amicon CF-25A ultrafiltration membrane cones (Amicon Ltd., High Wycombe, U.K.) and these centrifuged at 900*g* at 37 °C for 25 min. The filtrate was counted for ^{14}C. Controls indicated no binding of theophylline to the ultrafiltration membranes, but there was a non-specific binding of 5–8% total ethylenediamine added.

Octanol-buffer partitioning

Solutions of [^{14}C]theophylline, [^{14}C]ethylenediamine and either theophylline- or ethylenediamine-labelled [^{14}C]aminophylline were prepared in 66 mM phosphate buffer pH 7·4. Small aliquots of these were made up to 5 ml with buffer saturated with octan-1-ol, 5 ml buffer-saturated octan-1-ol added, and the whole shaken for 1 h at 22 °C. The layers were allowed to settle for 30 min, separated and individually centrifuged for 1 h at 1000*g* at 22 °C. Aliquots of the aqueous and organic phases were counted for ^{14}C.

RESULTS

Ultraviolet spectroscopy

Table 1 summarizes the effects of variation of theophylline concentration, pH and ethylenediamine concentration upon the uv spectrum of theophylline. Theophylline exhibits two principal absorptions, at 210 and 271 nm, the latter showing a slight bathochromic shift of up to 3 nm with increasing concentration. The addition of up to 15% w/w ethylenediamine to a 30 μg ml^{-1} solution of theophylline also produced bathochromic shifts on both absorption maxima, to 218 and 274 nm. The pH

Table 1. Influence of concentration, ethylenediamine addition and pH change upon the ultraviolet absorption maxima of theophylline.

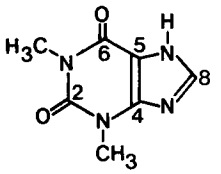
Drug solution	pH	λ_{max} (nm) 1	2
Theophylline 30 μg ml^{-1}	6·4	210	271
50 μg ml^{-1}	6·2	210	272
100 μg ml^{-1}	6·0	210	274
Theophylline 30 μg ml^{-1}			
+3·5% w/w ethylenediamine	10·4	216	272
+5% w/w ethylenediamine	10·7	217	272
+10% w/w ethylenediamine	11·0	218	273
+15% w/w ethylenediamine	11·1	218	274
Theophylline 30 μg ml^{-1}			
+NaOH	7·0	211	271
	8·0	213	271
	9·0	215	272
	10·0	216	272
	11·0	218	273
	12·0	220	274

of the solutions containing ethylenediamine rises 4–5 units, and so the pH of a 30 μg ml^{-1} theophylline solution was progressively increased by the addition of 2 M NaOH, which also gave pH-related bathochromic shifts in both absorptions. The uv spectrum of a theophylline solution at pH 11 was identical whether ethylenediamine or NaOH was used to increase the pH.

^{13}C-Nuclear magnetic resonance spectroscopy

^{13}C-N.m.r. spectra of solutions of theophylline and aminophylline were recorded at pH 5 and pH 9 and the results are summarized in Table 2. Signal assignment was by comparison with known ^{13}C resonances of purines (Jones et al 1970). The resonances of all carbon atoms in theophylline shift upfield upon increase in pH, the sizes of the shifts being in the order C8 > C5 > C6 > C4 > C2 > N-CH_3. The carbons of ethylenediamine similarly shift upfield by 3·1 ppm with change from pH 5 to pH 9. In comparison with the pH-related shifts in the resonances, the addition of ethylenediamine caused only very small shifts (less than 0·2 ppm) in the resonances of the theophylline carbons, all being downfield shifts with the exception of C2.

Table 2. Influence of addition of ethylenediamine and pH change upon the ^{13}C-n.m.r. resonances of theophylline.



Carbon atom	δ ppm*	Δ ppm in resonance upon addition of EDA† pH 5‡	EDA pH 9	NaOH pH 9
C2	152·58	+0·17	+0·17	+0·11
C4	147·71	−0·07	+1·12	+1·18
C5	107·19	−0·08	+4·16	+4·07
C6	155·97	−0·04	+1·65	+1·72
C8	141·24	−0·10	+3·56	+3·76
N-CH_3	30·30	−0·08	+0·03	+0·05
	28·30	−0·08	−0·16	−0·16
EDA	36·69	—	—	+3·11

* Relative to external TMS reference.
† EDA = ethylenediamine.
‡ Adjusted to pH 5 by addition of M HCl.

Plasma-erythrocyte and saline-erythrocyte partitioning

Fig. 1 shows the distribution of [^{14}C]theophylline and [^{14}C]ethylenediamine alone and in the form of aminophylline in whole blood. The extracellular concentration of theophylline and ethylenediamine was essentially independent of concentration and was slightly higher than plasma volume. The plasma/erythrocyte distribution of both compounds was unaffected when they were present as aminophylline.

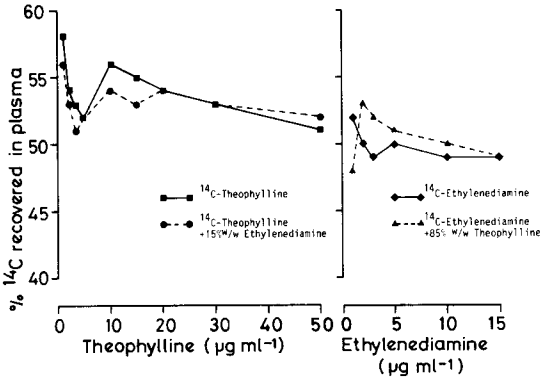


FIG. 1. Plasma/erythrocyte distribution of [^{14}C]theophylline, [^{14}C]ethylenediamine and [^{14}C]aminophylline (labelled in the theophylline or ethylenediamine moieties) at various concentrations. Details of the experiment are in the text. The haematocrit in each case was 48%. Points represent the means of 3 experiments. Results never varied by more than 5% and error bars are omitted for the sake of clarity.

The distribution of the same drug combinations between saline solution and erythrocytes is illustrated in Fig. 2. In all cases, the extracellular concentration was slightly lower than the supernatant volume, and was unaffected when the compounds were present as aminophylline. The reversibility of erythrocyte uptake is also illustrated in Fig. 2. Re-equilibration of the erythrocytes loaded with the drugs with fresh saline solution gave saline/

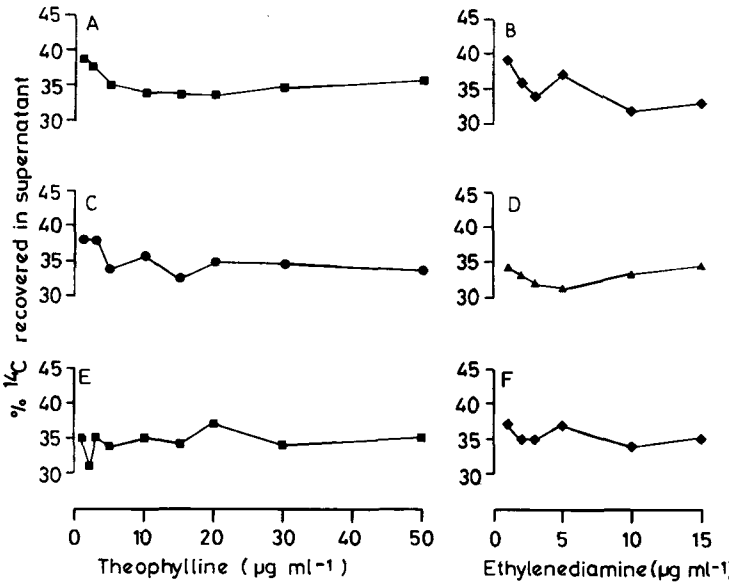


FIG. 2. Distribution of ^{14}C between 0·9% NaCl and washed erythrocytes of various concentrations of [^{14}C]theophylline (A), [^{14}C]ethylenediamine (B) and [^{14}C]aminophylline (labelled in the theophylline (C) or ethylenediamine (D) moieties) and of ^{14}C following re-equilibration of erythrocytes loaded with [^{14}C]theophylline (E) or [^{14}C]ethylenediamine (F) with 0·9% NaCl. Details of the experiment are given in the text. The haematocrit in each case was 40%. Points represent the means of 3 experiments. Results never varied by more than 5% and error bars are omitted for the sake of clarity.

erythrocyte distributions identical to those seen in the original experiments.

Octanol-buffer partitioning

Results of this study are presented in Table 3. It is seen that theophylline partitions between octanol and pH 7·4 buffer, with a slight preference for the aqueous phase. However, ethylenediamine is not soluble in the organic phase. The addition of 15% w/w ethylenediamine to [^{14}C]theophylline or of 85% w/w theophylline to [^{14}C]ethylenediamine did not influence the above results.

Plasma protein binding

Plasma protein binding studies were performed with [^{14}C]theophylline over the concentration range 1–30 μg ml^{-1} with 0–20% w/w ethylenediamine added, and with [^{14}C]ethylenediamine over the concentration range 1–15 μg ml^{-1}, in the presence and absence of 85% w/w theophylline. The results with [^{14}C]theophylline at 1, 10 and 30 μg ml^{-1} are shown in Fig. 3 and are representative of data at intermediate concentrations (2, 3, 5, 15 and 20 μg ml^{-1}). The plasma protein binding of theophylline was independent of concentration over the range studied, and of ethylenediamine concentration up to 20% w/w.

The dependence of the plasma protein binding of theophylline upon the temperature used for the ultrafiltration is shown in Fig. 4. It is remarkable that the % theophylline bound increases with decrease in temperature, even though equilibration was always performed at 37 °C.

Ethylenediamine was not bound to plasma protein over the concentration range tested, and this was unaltered when 85% w/w theophylline was added (data not shown).

Table 3. Octan-1-ol; 66 mM phosphate buffer pH 7·4 partition coefficients of [^{14}C]theophylline, [^{14}C]ethylenediamine and [^{14}C]aminophylline labelled in the theophylline or ethylenediamine moieties.

Drug solution	Partition coefficient of ^{14}C label
[^{14}C]Theophylline (10 mg ml^{-1})	0·755 ± 0·01
[^{14}C]Theophylline (10 mg ml^{-1}) + 15% w/w ethylenediamine	0·735 ± 0·02
[^{14}C]Ethylenediamine (1·75 mg ml^{-1})	0·00 ± 0·00
[^{14}C]Ethylenediamine (1·75 mg ml^{-1}) + 85% w/w theophylline	0·00 ± 0·00

DISCUSSION

The results of the various experiments described here all show that the two components of aminophylline behave essentially independently in these test systems, and are neither associated as a complex nor able to influence each other's properties.

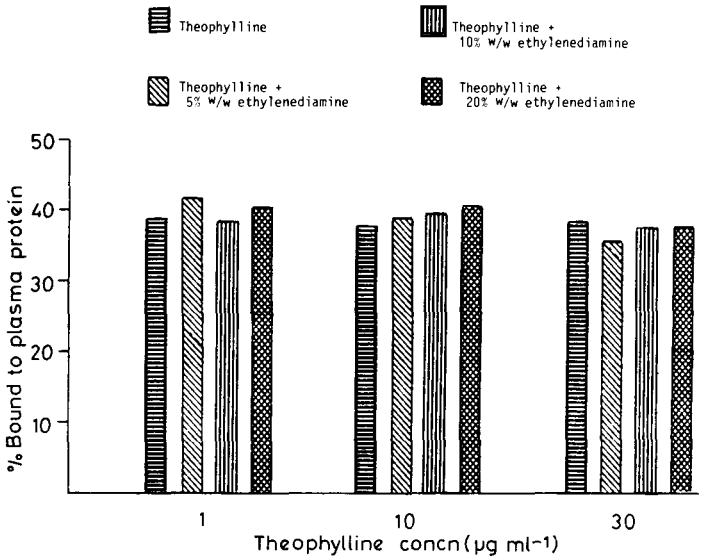


FIG. 3. Plasma protein binding of theophylline in the presence of various concentrations of ethylenediamine, assayed by ultrafiltration. Data are presented for 1, 10 and 30 μg ml^{-1} theophylline, and are representative of similar experiments at 2, 3, 5, 15 and 20 μg ml^{-1}. Details of the experiment are given in the text.

The uv and ^{13}C-n.m.r. spectroscopic studies show that the only perturbations of the theophylline moiety arising from the addition of the ethylenediamine are accountable for by the pH change of the solution. Solutions of theophylline show the same spectral changes upon moving from pH 5 to pH 9, regardless of whether ethylenediamine or NaOH is used to change the pH. Although not conclusive in themselves, the results of these spectral studies show that it is very unlikely that theophylline and ethylenediamine are physicochemically associated when aminophylline is dissolved in aqueous media.

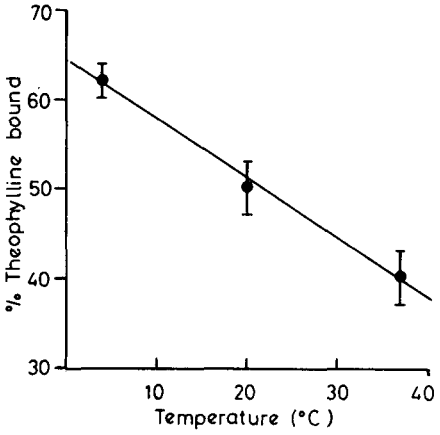


FIG. 4. Dependence of the plasma protein binding of theophylline upon the temperature used for ultrafiltration. 20 μg ml^{-1} theophylline was added to plasma at 37 °C and equilibrated for 30 min, followed by ultrafiltration at various temperatures. Points are the means of 4 experiments, and the bars represent ± s.d. The equation describing the line is $y = 64{\cdot}2 - 0{\cdot}666x$, $r = 0{\cdot}998$.

The possibility that theophylline and ethylenediamine influence each other's distribution in biological systems has been examined with the widely-used octanol/buffer system and with erythrocytes, in blood and in saline. The octanol/buffer distribution of theophylline was unaffected by the presence of ethylenediamine, while ethylenediamine did not distribute into the organic phase, either alone or in combination with theophylline.

Both theophylline and ethylenediamine entered erythrocytes in blood to approximately the same extent, which did not vary systematically with concentration, and had no influence upon each other's behaviour. When washed erythrocytes were used, again both compounds entered the cells to approximately the same extent and had no influence upon each other's distribution. When erythrocytes loaded with either theophylline or ethylenediamine were re-equilibrated with fresh saline, the distribution of the compound was unaltered, showing that there was no irreversible binding to red blood cells. The entry of both compounds into erythrocytes was greater from saline than from plasma, suggesting that plasma exerted a 'retarding' effect. However, it is unlikely that this was due to plasma protein binding in the case of ethylenediamine (see below).

Plasma protein binding of theophylline was ca

40% which agrees well with the recent report of Shaw et al (1982) who have drawn attention to the fact that differences in literature values for this figure probably arise from the use of different temperatures, between 18 and 37 °C, for the separation of free and bound drug by equilibrium dialysis or ultrafiltration. The data summarized in Fig. 4 confirm that the plasma protein binding of theophylline rises markedly with decreased temperature of ultrafiltration. The plasma protein binding of theophylline is independent of concentration up to 30 $\mu g\ ml^{-1}$ and not influenced by the addition of ethylenediamine up to 20% w/w. Ethylenediamine was not bound to plasma protein at any concentration tested in the presence or absence of theophylline.

Taken with our previous reports (Caldwell & Cotgreave 1982, 1983; Cotgreave & Caldwell 1983) that the plasma pharmacokinetics of theophylline and ethylenediamine following oral and i.v. administration of aminophylline to volunteers are very different, these data suggest very strongly that aminophylline behaves in solution in biological media as a simple mixture of theophylline and ethylenediamine, which behave entirely independently.

It is therefore necessary to find other reasons for the differences in metabolic (Monks et al 1981) and pharmacological (Marais & McMichael 1937; Arkinstall et al 1983) responses between theophylline and aminophylline, which might lie in the influence of ethylenediamine upon the drug metabolizing enzymes or upon some aspect of the pharmacodynamics of theophylline. These possibilities are the subject of continuing investigations.

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REFERENCES

American Hospital Formulary Service (1978) Vol. 2, Section 86.00. Washington: Am. Soc. Hosp. Pharm

Arkinstall, W. W., Anderton, R. C., Goughnour, B., Stewart, J. H., Uitvluct, M. (1983) Br. J. Clin. Pract. suppl. 23 8–14

Aslaksen, A., Bakke, O. M., Vigander, T. (1981) Br. J. Clin. Pharmacol. 11: 269–273

British Pharmacopoeia (1980) pp 24–25

Caldwell, J., Cotgreave, I. A. (1981) Br. J. Pharmacol. 74: 876P

Caldwell, J., Cotgreave, I. A. (1982) Br. J. Clin. Pharmacol. 14: 610P

Caldwell, J., Cotgreave, I. A. (1983) Br. J. Clin. Pract. Suppl. 23: 22–25

Cotgreave, I. A., Caldwell, J. (1983) J. Pharm. Pharmacol. 35: 378–382

Dessauer, P. (1908) Ther. Monatschr. (Berlin) 22: 401–407

Jones, A. J., Grant, D. M., Winkley, M. W., Robins, R. K. (1970) J. Am. Chem. Soc. 92: 4079–4087

Marais, O. A. S., McMichael, J. (1937) Lancet ii 437–439

Martindale's Extra Pharmacopoeia 28th Edn. (1982) Pharmaceutical Press, London p 342

Monks, T. J., Smith, R. L., Caldwell, J. (1981) J. Pharm. Pharmacol. 33:93–97

Nishijo, J., Ohno, K., Nishimura, K., Hukuda, M., Ishimaru, H., Yonetani, I. (1982) Chem. Pharm. Bull. 30: 771–776

Okano, T., Aita, K., Ikeda, K. (1967) Ibid. 15: 1621–1628

Schmid, E., Bartels, M., Taugner, R. (1956) Z. Aerosol-Forsch. Ther. 2: 149–152

Shaw, L. M., Fields, L., Mayock, R. (1982) Pharmacol. Ther. 32: 490–496

Van Heerswynghels, J. (1937) Arch. Int. Pharmacodyn. 56: 288–298