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EFFECTS *IN VITRO* AND *IN VIVO* OF THIOUREAS ON ACETYLCHOLINE ESTERASE ACTIVITY OF RAT TISSUES

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Abstract—In view of data available that accumulation of acetylcholine in the lung leads to pulmonary edema formation, the effects of thiourea (TU), phenylthiourea (PTU) and α-naphthylthiourea (ANTU) on lung acetylcholine esterase (AchE) activity were examined *in vitro* and *in vivo*. ANTU and PTU produced a concentration-dependent inhibition of lung AchE activity *in vitro*. The ED_{50} values of ANTU and PTU were 2.8×10^{-4} and 5×10^{-4} M respectively. No significant inhibition in AchE activity of the lung occurred in response to any concentration of TU. The mechanisms of AchE inhibition due to ANTU appeared to be competitive. The apparent K_m for lung AchE averaged 7.23×10^{-4} M and the K_i for ANTU averaged 1.11×10^{-4} M. ANTU at 5×10^{-4} M inhibited the AchE activity of all examined tissues *in vitro*. An edema-producing dose of ANTU had no effect on AchE activity of any tissue except the brain. A failure to demonstrate the inhibitory effect *in vivo* of ANTU on AchE activity has been discussed in this paper. Pretreatment with atropine offered no protection against the pulmonary edema-producing ability of ANTU. The data collected in the present report indicate that inhibition of lung AchE is probably not the mechanism of ANTU-induced pulmonary edema. The significance of brain AchE inhibition in response to an edema-producing dose of ANTU is not known.

Pulmonary edema is a pathological state of abnormal extravasation of plasma fluid in lung spaces. The edema may be of cardiogenic or noncardiogenic origin. In the latter category, the etiological factors may include environmental pollutants (O_3, NO_2, H_2S and phosgene gases) or a variety of chemicals such as thioureas, alloxan, oleic acid and ammonium chloride [1]. Thiourea (TU) and its derivatives, phenylthiourea (PTU) and α-napthylthiourea (ANTU), have been employed in animal models as experimental tools to study characteristics of pulmonary edema common to a variety of drugs and toxic substances. These are: (1) production of tolerance [2,3]; (2) the innate resistance of young animals; and (3) species differences [4,5].

The acute toxic signs associated with the administration of thiourea-like compounds are massive pulmonary edema, capillary hemorrhage, pleural effusion and inflammation of the tracheobronchial tree [4,6]. The mechanism whereby TU, PTU and ANTU produce acute pulmonary edema is not clearly understood. A causative relationship between bradycardia and pulmonary edema has been demonstrated in the genesis of pulmonary edema. For example, stimulation of the peripheral end of the cut vagus nerve produces lung edema in the dog [7]. Intravenous administration of acetylcholine (Ach) produces lung edema [8], while inhibitors of acetylcholine esterase (AchE) have been reported to produce fatal acute pulmonary edema in response to an excessive buildup of Ach [9,10]. This study was undertaken to test the hypothesis that inhibition of AchE by thiourea-like compounds is related to their edema-producing effect. We have examined the effects *in vitro* of TU, PTU and ANTU on AchE activity of the lungs. The effects of ANTU on AchE activity of different tissues were studied both *in vitro* and *in vivo*. Also studied were the kinetic properties of lung AchE.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 350–400 g were purchased from Simenson Laboratory Inc., Gilroy, Calif. They were maintained, two per cage, on Purina rat chow and water *ad lib*. Acetylthiocholine iodide and dithiobisnitrobenzoic acid (DTNB) were obtained from Sigma Chemical Co. TU, PTU and ANTU were purchased from J. T. Baker Chemical Co., Phillipsbury, N.J. The first two compounds were dissolved in distilled deionized water and the last was suspended in propylene glycol.

Study in vivo. Rats were first anesthetized by ether and then transferred to a cold room where blood was collected in heparinized syringes via the inferior vena cava. Lungs and heart were removed *en bloc*. In order to wash out blood from the pulmonary capillary bed [11], lungs were perfused with cold isotonic saline at 4–5° through the right side of the heart with a slit on the left. A total volume of 50 ml saline was passed through the pulmonary vasculature over a period of 5 min. Liver, kidney and brain were repeatedly washed with cold isotonic saline to remove blood. All tissues were first minced into small pieces, then homogenized in phosphate buffer (0.1 M, pH 8) using a motor-driven Teflon pestle (Tri-R) to yield a concentration of 30 mg/ml. Homogenate and blood were centrifuged in a refrigerated centrifuge (Sorval RC-2B, SS-34 rotor) at 600 *g* for 10 min at 4°. The supernatant and plasma were carefully aspirated and used to measure AchE activity. Protein content of

the supernatant and plasma was measured according to the method of Lowry *et al.* [12].

The supernatant and plasma were incubated with different concentrations of ANTU, PTU or TU or appropriate vehicle for 10 min at 37° in a Dubnoff metabolic shaker before running the enzyme assay according to the colorimetric method of Ellman *et al.* [13]. Acetylthiocholine iodide was used as the substrate. The rate of enzymatic hydrolysis of the substrate was monitored by an increase in absorbancy at 416 nm at 37° by a Gilford spectrophotometer. The enzymatic reaction was linear for a period of at least 10 min. The change in absorbancy for 5 min was read on the linear part of the curve. Tissue and reagent blanks were run concurrently. The changes in absorbancy resulting from these blanks were taken into account when calculating the final enzyme activity. Enzyme activity was expressed as moles product formed/hr/mg of protein.

Study in vivo. In order to correlate the findings *in vitro* with effect *in vivo*, an edema-producing dose of ANTU, 20 mg/kg in propylene glycol, was injected i.p. Control rats received an equivalent volume of propylene glycol. Two and a half hr later, rats were anesthetized with ether. Blood and different tissues were obtained and processed for determination of AchE activity in the manner described above. This dose and the time interval after ANTU injection were chosen because rats always develop acute pulmonary edema with this regimen of treatment (see Table 5). The AchE activity of the lung for the study *in vivo* was expressed in two ways: (1) moles of the product formed/hr/mg of lung protein and (2) moles of the product formed/hr/lung.

Additional experiments *in vivo* were also carried out to find out whether or not a buildup of Ach secondarily to AchE inhibition of the lung is the mechanism for ANTU-induced pulmonary edema. Rats were first injected intraperitoneally either with saline or with 1 mg/kg of atropine 30 min prior to an edema-producing dose (20 mg/kg) of ANTU in propylene glycol. The rats in the control group received saline prior to receiving an equivalent volume of vehicle (propylene glycol). Two and a half hr later, rats were etherized. The abdominal cavity was opened and the descending aorta severed to allow free bleeding. For quantitation of pulmonary edema, the trachea was exposed and ligated. The thoracic cavity was then opened to remove lungs and heart *en bloc*. The lungs were separated from the heart, blotted gently and weighed after cutting the trachea at the point of bifurcation in a tared container. The dry weight of the lung was determined by heating in an oven at 60° until a constant weight was attained (72 hr). Both wet lung weight (WLW) and dry lung weight (DLW) expressed in g/kg body weight were used to evaluate the extent of pulmonary edema [14].

All data are reported as the mean ± standard error (S.E.). Student's *t*-test was employed to determine the degree of statistical significance between the means.

RESULTS

The effects of different concentrations of ANTU, PTU and TU on rat lung AchE activity *in vitro* are summarized in Table 1. ANTU had a potent inhibitory effect on lung AchE activity. The magnitude of inhibition was clearly related to the concentrations. At 10^{-3} M, AchE activity was inhibited by 75 per cent; at 5×10^{-4} M, inhibition was 60 per cent; and at 10^{-4} M the activity was inhibited by 25 per cent. There was no inhibition of AchE activity at the 10^{-5} M concentration of ANTU. The vehicle, propylene glycol, had no effect on AchE activity. A dose-related inhibition of AchE activity was also noticed in response to PTU treatment. A statistically significant inhibition of AchE activity, however, occurred only at a concentration of 10^{-4} M. It was interesting to note that TU produced no significant inhibition in AchE activity at any concentration. At 10^{-3} M, the highest concentration of TU employed, AchE activity was reduced only by 16 per cent.

The ED_{50} values (the concentration that inhibits the enzyme activity by 50 per cent) of ANTU and PTU were obtained by plotting the enzyme activity as per cent of control against their concentrations. The ED_{50} for ANTU was 2.8×10^{-4} M as compared to 5×10^{-4} M for PTU.

Table 1. Effects of different concentrations of α-naphthylthiourea (ANTU), phenylthiourea (PTU) and thiourea (TU) on rat lung AchE activity *in vitro**

Concn of inhibitor (M)	Enzyme activity† (moles × 10^{-4} product released/hr/mg protein)					
	ANTU	P values‡	PTU	P values‡	TU	P values‡
PO_4 buffer (control)§	4.17 ± 0.21 (5)		7.26 ± 0.36 (5)		7.26 ± 0.21 (5)	
Propylene glycol (control)	5.34 ± 0.45 (8)					
Inhibitor (10^{-3})	1.23 ± 0.27 (6)	<0.001			6.12 ± 0.36 (5)	<0.10
Inhibitor (5×10^{-4})	2.04 ± 1.80 (3)	<0.001				
Inhibitor (10^{-4})	4.04 ± 0.24 (8)	<0.05	4.71 ± 0.57 (5)	<0.02	6.60 ± 0.24 (5)	NS‖
Inhibitor (10^{-5})	4.56 ± 0.21 (7)	NS	6.33 ± 0.36 (5)	NS	7.20 ± 0.45 (5)	NS
Inhibitor (10^{-6})			6.84 ± 0.42 (5)	NS		

* Rats were sacrificed by ether anesthesia. Lungs and heart were removed *en bloc*. Lungs were perfused with cold isotonic saline through the right side of the heart, then minced and homogenized in PO_4 buffer (0.1 M, pH 8). The homogenate was centrifuged at 600 *g* for 10 min at 4°. The supernatant was incubated with and without different concentrations of thioureas for 10 min at 37° before assaying AchE activity. The tissue and reagent blanks were run simultaneously and were taken into consideration in calculating enzyme activity.

† Mean ± S.E. (N).

‡ All comparisons involving ANTU are made against the propylene glycol control, since this was the solvent for ANTU. PTU and TU were compared against their own PO_4 buffer controls run simultaneously.

§ P value between P_{O_4} buffer and propylene glycol is not significant at the 0.05 level.

‖ Not significant.

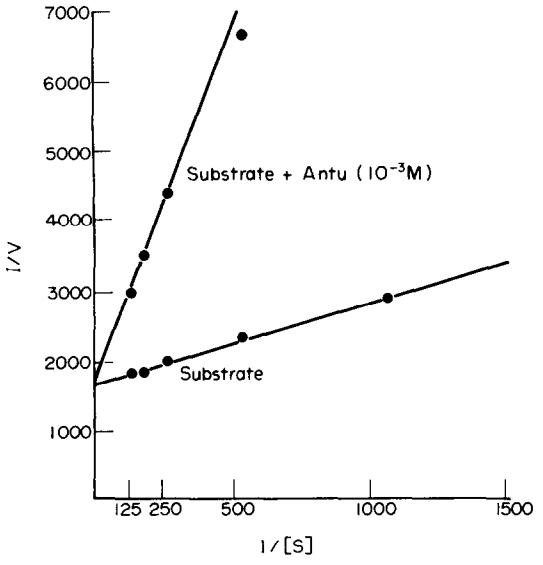

Fig. 1. Diagram, $1/V$ vs $1/[S]$ of acetylthiocholine iodide using lung-AchE with and without the presence of ANTU (10^{-3} M). Velocities are given as moles $\times 10^{-4}$ of product formed/hr/mg of protein; substrate concentrations are in moles/liter. The mean K_m and K_i values of several such studies are shown in Table 3.

The Michaelis constant (K_m) of lung AchE using acetylthiocholine iodide as substrate and the dissociation constant (K_i) of the inhibitor (ANTU at 10^{-3} M) were determined by the double reciprocal plot of Lineweaver and Burk [15] and Dixon [16]. An example of one such study is graphically shown in Fig. 1. It appears from the graph that ANTU acts as a competitive inhibitor of AchE. The K_m value for four separate determinations averaged 7.23×10^{-4} M, and K_i for three determinations averaged 1.11×10^{-4} M (Table 2).

Table 2. K_m and K_i of lung AchE using acetylthiocholine as substrate and α-naphthylthiourea as inhibitor*

	Mean ± S. E. (N)
K_m	$7.23 \times 10^{-4} \pm 0.53$ (4)
K_i	$1.11 \times 10^{-4} \pm 0.18$ (3)

* Double reciprocal plot of Lineweaver and Burk and Dixon was employed to determine the K_m and K_i.

Table 3. Effects of ANTU on AchE activity of different tissues of rat *in vitro**

Tissue	Enzyme activity† (moles $\times 10^{-4}$ of product released/hr/mg protein) Propylene glycol (control)	ANTU (5×10^{-4} M)	P value between control and ANTU
Kidney	0.95 ± 0.16	0.00 ± 0.00	
Brain	106.96 ± 5.10	76.60 ± 4.20	<0.05
Liver	2.64 ± 0.24	1.60 ± 0.26	<0.05
Lung	6.79 ± 0.51	2.99 ± 0.61	<0.01
Plasma	4.32 ± 0.54	1.38 ± 0.19	<0.01

* Procedures for preparing and assaying the enzyme activity are the same as described in the legend of Table 1.
† Mean ± S. E. of four animals.

The AchE inhibitor effect of ANTU on different tissues *in vitro* is summarized in Table 3. In this study, AchE activity of different tissues was examined after treatment with 5×10^{-4} M ANTU. It is obvious that ANTU had a generalized inhibitory effect on the AchE activity of all tissues examined in this study. A great deal of variation was found in sensitivity to the inhibitory effect of ANTU on AchE activity of varying tissues.

Studies *in vivo* were carried out in an attempt to correlate the inhibitory effect *in vitro* of ANTU on AchE activity with its pathophysiological effect. An edema-producing dose (20 mg/kg) of ANTU was injected (i.p.) 2.5 hr prior to sacrifice. AchE activity of different tissues and plasma was then measured. The results of this study are summarized in Table 4. The dose of ANTU which produced acute pulmonary edema failed to inhibit AchE activity on the lung, liver, kidney and plasma. A significant inhibition ($P < 0.05$) was noticed only in brain AchE activity.

A failure to demonstrate the inhibition of lung AchE activity in response to an edema-producing dose of ANTU could be attributed to two factors; first, an extensive perfusion *in vitro* of the lung possibly washes out the inhibitor; and second, the unwashed amount of ANTU in lung undergoes an extensive dilution after homogenization as well as in the reaction mixture *in vitro*. Attempts were made, therefore, to circumvent these possibilities and find

Table 4. Effects of α-naphthylthiourea (ANTU) on AchE activity of different rat tissue *in vivo**

Tissue	Control† (P/hr/mg protein)	Control† (P/hr/lung)	ANTU† (P/hr/mg protein)	ANTU† (P/hr/lung)	P values between control and ANTU
Lung	5.35 ± 0.42	5.89 ± 0.57	6.15 ± 0.45	6.09 ± 0.42	NS, NS‡
Liver	2.76 ± 0.36		3.09 ± 0.66		NS
Kidney	0.90 ± 0.09		0.91 ± 0.03		NS
Plasma	3.42 ± 0.18		3.21 ± 0.42		NS
Brain	102 ± 5.85		85.44 ± 1.20		<0.05

* Rats were injected with ANTU (20 mg/kg, i.p.) 2.5 hr prior to sacrifice. Lungs were perfused with cold isotonic saline through the right side of the heart and the remaining organs washed repeatedly before determining AchE activity.
† Enzyme activity is expressed in moles $\times 10^{-4}$ product (P) released/hr/mg of protein and moles $\times 10^{-2}$ of P/hr/lung; mean ± S. E. of five animals.
‡ Not significant.

Table 5. Effects of atropine pretreatment on α-naphthylthiourea (ANTU)-induced pulmonary edema as measured by wet lung weight (WLW) and dry lung weight (DLW) in g/kg body weight

Group	Pretreatment*	Treatment	Mean ± S. E. of five animals		P values between groups		
			WLW/kg	DLW/kg	Groups	WLW	DLW
1	Saline	Propylene glycol	3.89 ± 0.14	0.85 ± 0.02	1,2	<0.001	<0.01
2	Saline	ANTU	6.02 ± 0.28	1.14 ± 0.06	1,3	<0.001	<0.01
3	Atropine	ANTU	6.35 ± 0.31	1.07 ± 0.05	2,3	NS†	NS†

* Rats were pretreated with saline or atropine (1 mg/kg) 30 min prior to i.p. injection of ANTU in propylene glycol. Rats in the control group received an equivalent volume of propylene glycol. They were sacrificed 2.5 hr later using ether and exsanguination. The index of pulmonary edema in each case was determined by wet and dry lung weight.

† Not significant.

out if a buildup of Ach secondary to inhibition of AchE is the mechanism for ANTU-induced pulmonary edema. Rats were treated either with saline or atropine 30 min prior to an edema-producing dose of ANTU. Two and a half hr later, the extent of pulmonary edema was determined. The results of this study are summarized in Table 5.

An edema-producing dose of ANTU produced the same degree of pulmonary edema in both saline- and atropine-pretreated rats. This was reflected by a significant increase in WLW and DLW in rats of these two groups over the group injected with vehicle. There was, however, no difference in WLW or in DLW between the two groups of rats injected with ANTU.

DISCUSSION

TU, PTU and ANTU are known to produce acute pulmonary edema in animal models. The mechanism by which these compounds produce pulmonary edema is not understood. There is evidence that thiourea-like compounds affect the pulmonary vascular endothelium directly and are thus responsible for ultrastructural changes of the capillary endothelium leading to lung edema [1]. Other investigators have suggested that thiourea compounds may be metabolized in the lung or liver to form active but short-lived toxic intermediates that may then affect the integrity of the pulmonary endothelial cells [17,18]. We suggested earlier, in view of our findings, that thiourea or a metabolite covalently interacts with a polypeptide of lung, and this interaction may directly or indirectly lead to increased permeability of pulmonary capillaries and alveolar epithelium [19].

The interesting finding borne out of this study was that ANTU had a generalized inhibitory effect on AchE activity of all examined tissues *in vitro*. At 5×10^{-4} M, it inhibited AchE activity of kidney, plasma, lung, liver and brain to varying degrees. The relative potency of TU, PTU and ANTU as inhibitors of lung AchE activity was examined *in vitro*. In this regard, both PTU and ANTU caused a concentration-related inhibition of AchE activity. TU, on the other hand, had little or no effect. At 10^{-3} M, the highest concentration, it inhibited AchE activity only by 16 per cent.

It is conceivable that the ability of thioureas to inhibit AchE activity *in vitro* might be directly related to their lipophilic property. For example, ANTU and PTU being more lipophilic [20, 21] might somehow introduce adequate conformational changes in the AchE molecule so as to inhibit its activity. On the other hand, TU being relatively lipid insoluble [22] fails to introduce the conformational change and, therefore, enzyme activity.

Since there is no structural similarity between Ach and ANTU, it is unlikely that both compete for the same binding site on AchE. The alternative mechanisms involved in ANTU's competitive inhibition of AchE might be attributed to either steric hindrance or allosteric inhibition [23]. In either case, an increasing concentration of the substrate would tend to remove ANTU from the enzyme and thus help to restore AchE activity to a normal level. This would explain how ANTU acts as a competitive inhibitor of AchE.

A failure to demonstrate an inhibitory effect of ANTU on AchE activity of lung and other tissues after administration *in vivo* of ANTU could be attributed to the extensive perfusion of the lung which washes the drug from the tissue and also to the extensive dilution *in vitro* of the unwashed ANTU in the reaction mixture. Because of these difficulties, it is not possible to draw any definite conclusion whether or not the inhibition *in vivo* of lung AchE is the mechanism of ANTU-induced pulmonary edema. Two lines of evidence provided in this report, however, suggest that pulmonary edema due to ANTU is probably not being mediated through the inhibition of lung AchE. First, there was an obvious lack of even an inhibitory effect *in vitro* of a similar compound (thiourea) at 10^{-3} M. This is a much higher concentration than that needed *in vivo* to produce acute pulmonary edema. Second, if the inhibition of lung AchE by administration *in vivo* of ANTU allows the buildup of Ach, then atropine pretreatment, which is known to exert protection against pulmonary edema induced by Ach-like drugs [24], should minimize or abolish the pulmonary edema caused by ANTU. In the present study, rats pretreated with atropine developed pulmonary edema in response to ANTU as much as those pretreated with saline. These lines of evidence tend to negate the involvement of lung AchE as one of the possible mechanisms of ANTU-induced pulmonary edema.

Our results are in agreement with the results of other investigators who have studied the effects of thiourea-like compounds on other enzymes. For example, DuBois and Erway [25] had reported that none of the thiourea derivatives inhibited the cytochrome oxidase or succinic dehydrogenase of lung or

liver tissues of rats given lethal doses of these compounds. High concentrations of these compounds were necessary to inhibit the activity of these enzymes *in vitro*. A significant inhibition, however, occurred in brain AchE activity after the administration of an edema-producing dose of ANTU. Whether the inhibition of brain AchE activity is the cause or the effect of pulmonary edema is not presently known.

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REFERENCES

1. M. B. Visscher, F. J. Haddy and G. Stephens, *Pharmac. Rev.* **8,** 389 (1956).
2. K. K. Carroll and R. L. Noble, *J. Pharmac.* **97,** 478 (1949).
3. H. E. Stokinger and W. D. Wagner, *Archs ind. Hlth* **14,** 158 (1956).
4. S. H. Diekes and C. P. Richter, *Proc. Soc. exp. Biol. Med.* **62,** 22 (1946).
5. A. R. Gregory and C. P. Hine, *Proc. Soc. exp. Biol. Med.* **128,** 693 (1968).
6. C. P. Richter, *Recent Prog. Horm. Res.* **2,** 255 (1948).
7. L. Fores, *Dt. Arch. klin. Med.* **87,** 339 (1906).
8. J. Driessens and A. Clay, *C. r. Séanc. Soc. Biol.* **146,** 258 (1952).
9. E. Adelson and F. Brunn, *Ann. intern. Med.* **30,** 838 (1949).
10. R. Altschul and M. M. Laskin, *Archs Path.* **41,** 11 (1946).
11. S. R. Cronin and S. N. Giri, *Proc. Soc. exp. Biol. Med.* **156,** 120 (1974).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
13. G. L. Ellman, K. D. Courtney, V. Andres, Jr. and R. M. Featherstone, *Biochem. Pharmac.* **7,** 88 (1961).
14. K. A. Gaar and L. D. Seager, *Proc. Soc. exp. Biol. Med.* **118,** 287 (1965).
15. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56,** 658 (1934).
16. M. Dixon, *Biochem. J.* **55,** 170 (1953).
17. A. L. Cunningham and J. V. Hurley, *J. Path.* **106,** 25 (1972).
18. B. Meyrick, J. Miller and L. Reid, *Br. J. exp. Path.* **53,** 347 (1972).
19. M. A. Hollinger, S. N. Giri, M. Alley, E. R. Budd and F. Hwang, *Drug. Metab. Dispos.* **2,** 521 (1974).
20. P. G. Stecher, *The Merck Index*, 8th Ed, p. 719. Merck & Co., Rahway, N.J. (1968).
21. P. G. Stecher, *The Merck Index*, 8th Ed, p. 820. Merck & Co., Rahway, N.J. (1968).
22. A. Collander, *Physiologia Pl.* **2,** 300 (1949).
23. I. H. Segal, *Enzyme Kinetics*, Chap. 3. A. Wiley Interscience, New York (1975).
24. J. L. Miller and S. A. Matthews, *Archs intern. Med.* **4,** 356 (1909).
25. K. P. DuBois and W. F. Erway, *J. biol. Chem.* **165,** 711 (1946).