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END OF SYMPOSIUM ON THE CHEMICAL ASPECTS OF NUTRITION NEEDS

Additional papers given at Symposium are:
"Aspects of Vitamin 'A' Nutrition in Man," by J. E. Canham, E. M. Miller, D. L. Wallace, N. Raica, H. E. Sauberlich, and R. E. Hodges

"The Protective Action of Vitamin E Against O_2 Toxicity," by S. L. Kinzey, C. L. Fischer, and C. A. Mengel

Acetylcholinesterase Inhibition by Substituted Phenyl *N*-Alkyl Carbamates

Ching-Chieh Yu,*[1] Clyde W. Kearns, and Robert L. Metcalf

Acetylcholinesterase (AChE, EC 3.1.1.7) inhibition constants (binding, carbamylation, reactivation, and overall bimolecular rate constants) were studied for selected substituted phenyl *N*-alkyl carbamates. *N*-Methyl carbamates were better inhibitors than larger *N*-alkyl carbamates. AChE from different species of animals showed remarkable species specificity toward these inhibitors. *N*-Ethyl carbamates were the least potent inhibitors toward bovine erythrocyte AChE, while *N*-*n*-butyl or *N*-*n*-hexyl carbamates were the least potent inhibitors toward AChE from housefly (*Musca domestica* L.), honeybee (*Apis mellifera* L.), and house cricket (*Acheta domestica* L.). Thus, short chain *N*-alkyl (*N*-methyl to *N*-*n*-propyl) carbamates favored the inhibition of insect AChE, while longer *N*-alkyl (*N*-*n*-butyl and up) carbamates favored the inhibition of bovine erythrocyte AChE. AChE inhibited by short-chain *N*-alkyl carbamates recovered its activity faster than the long-chain *N*-alkyl carbamates. Bovine erythrocyte AChE inhibited by carbamates recovered its activity much faster than insect AChE, especially when the inhibition is made by long-chain *N*-alkyl carbamates. Insecticidal activity was decreased when *N*-alkyl chain length was increased.

Acetylcholinesterase (AChE, EC 3.1.1.7) inhibition by carbamates is analogous to acetylcholine hydrolysis which may be expressed as

$$\underline{EH + CX} \overset{Ka}{\rightleftharpoons} EH\cdots CX \xrightarrow{k_2} \underline{E{-}C} \xrightarrow{k_3} EH$$

$$\underline{\qquad\qquad\qquad\qquad\qquad\qquad}$$
$$k_1$$

in which EH is free enzyme; CX, the carbamate; EH · · · CX, the enzyme-inhibitor complex; E-C, the carbamylated enzyme. Main (1964) has described a procedure to measure the binding constant *Ka*, carbamylation constant k_2, and overall bimolecular rate constant k_1. The decarbamylation rate constant k_3 can be determined by the method of Wilson *et al.* (1960).

Almost all insecticidal carbamate inhibitors studied are *N*-methyl or *N*,*N*-dimethyl. It is generally realized that *N*-ethyl carbamates and *N*-phenyl carbamates are poorer inhibitors than *N*-methyl carbamates (Kolbezen *et al.*, 1954). However, no good explanation has appeared to explain this phenomenon. Therefore, several series of substituted phenyl *N*-alkyl carbamates were prepared. Main's analysis for inhibition constants (binding, carbamylation, and overall bimolecular rate constants) for AChE from housefly, honeybee, and bovine erythrocyte were studied in order to analyze the factors which cause the decrease of the inhibitory potency due to the increase of *N*-alkyl chain length.

The *in vitro* decarbamylation rate constants k_3 of AChE have been studied by Hellenbrand (1967), Kunkee and Zweig (1965), and Wilson *et al.* (1961). It is generally regarded that *N*-methyl or *N*,*N*-dimethyl carbamates behave as reversible inhibitors due to their fast decarbamylation rates. However, there are some variations in decarbamylation rates in regard to the *N*-methyl or *N*,*N*-dimethyl groups for AChE from different species of animals. Thus, k_3 of AChE from housefly and bovine erythrocyte are greater for *N*-methyl than for *N*,*N*-dimethyl carbamates. The opposite is true for honeybee AChE. Wilson *et al.* (1961) reported that k_3 of electric eel AChE decrease in the order of $-NH_2 > -N-Me_2 > -NH-Me$. There is no study extended to longer *N*-alkyl carbamates. Since bis-carbamates are known as irreversible

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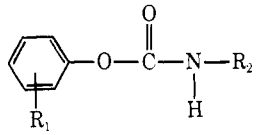
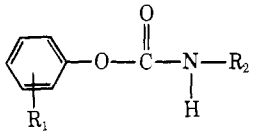
Table I. Properties of New Substituted Phenyl *N*-Alkyl Carbamates

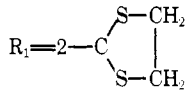


R_1	R_2	mp, °C	% Carbon		% Hydrogen	
			Calcd	Found	Calcd	Found
2-(1,3-dithiolane)	*n*-Propyl	110–111	55.09	55.72	6.05	6.32
	n-Butyl	83–84	56.53	57.40	6.44	6.68
	n-Hexyl	91–92	59.04	59.24	7.12	7.14
3-C-Me_3	*n*-Propyl	61	71.46	71.58	8.99	9.23
	n-Butyl	*a*				
	n-Hexyl	48–50	73.61	73.68	9.81	9.95
3-N-Me_2	*n*-Propyl	43–44[b]	64.84	65.09	8.16	8.75
	n-Butyl	69[b]	66.07	66.02	8.53	8.79
	n-Hexyl	38[b]	68.15	68.39	9.15	9.45
	n-Dodecyl	68–69[b]	72.37	72.55	10.41	10.64
3-N^+-Me_3I^-	*n*-Propyl	137–138	42.87	42.92	5.81	6.07
	n-Butyl	125–127	44.46	44.83	6.13	6.39
	n-Hexyl	116–118	47.30	47.48	6.70	6.90
	n-Octyl	114	49.77	49.79	7.19	7.42
	n-Dodecyl	108	53.88	54.30	8.01	8.21

[a] In liquid state. [b] Probably the samples were not completely dry.

Table II. Binding Constants *Ka*, Carbamylation Constants k_2, and Bimolecular Rate Constants k_i for the Inhibition of AChE from Bovine Erythrocyte, Honeybee, and Housefly by Substituted Phenyl *N*-Alkyl Carbamates and Their Toxicity to Housefly





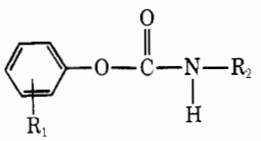
		R_1 = 2-(1,3-dithiolane)				R_1 = 3-N^+-Me_3I^-			
		R_2				R_2			
		Methyl	Ethyl	*n*-Propyl	*n*-Butyl	Methyl	Ethyl	*n*-Propyl	*n*-Butyl
Bovine erythrocyte	*Ka* (*M*)	1.35×10^{-5}	2.03×10^{-5}	4.00×10^{-5}	1.58×10^{-5}	3.50×10^{-6}	3.17×10^{-6}	4.58×10^{-6}	1.64×10^{-6}
	k_2 (min^{-1})	2.72	0.08	0.62	0.36	44.20	1.41	2.63	1.72
	k_i (M^{-1} min^{-1})	2.02×10^{5}	3.92×10^{3}	1.55×10^{4}	2.29×10^{4}	1.26×10^{7}	4.46×10^{5}	5.75×10^{5}	1.05×10^{6}
	i range (*M*)	2×10^{-6}	1×10^{-5}	2×10^{-6}	2×10^{-6}	4×10^{-8}	2×10^{-8}	8×10^{-7}	4×10^{-7}
		6×10^{-6}	1×10^{-4}	2×10^{-5}	8×10^{-6}	2×10^{-7}	1×10^{-5}	4×10^{-6}	1.5×10^{-6}
Honeybee	*Ka* (*M*)	2.82×10^{-6}	4.82×10^{-6}	9.85×10^{-6}	3.40×10^{-5}	7.86×10^{-8}	5.51×10^{-7}	5.62×10^{-7}	8.90×10^{-6}
	k_2 (min^{-1})	20.28	2.67	1.91	0.16	13.85	3.01	2.67	1.83
	k_i (M^{-1} min^{-1})	7.18×10^{6}	5.55×10^{5}	1.94×10^{5}	4.75×10^{3}	1.76×10^{8}	5.46×10^{6}	4.75×10^{6}	2.06×10^{5}
	i range (*M*)	1×10^{-7}	1×10^{-6}	4×10^{-6}	5×10^{-6}	4×10^{-9}	4×10^{-8}	1×10^{-7}	1×10^{-6}
		4×10^{-7}	4×10^{-8}	8×10^{-6}	3×10^{-5}	2×10^{-8}	4×10^{-7}	4×10^{-7}	4×10^{-6}
Housefly	*Ka* (*M*)	9.03×10^{-6}	3.19×10^{-4}	4.60×10^{-3}	2.86×10^{-4}				
	k_2 (min^{-1})	14.42	26.47	30.95	0.64				
	k_i (M^{-1} min^{-1})	1.60×10^{6}	8.31×10^{4}	6.73×10^{3}	2.24×10^{3}				
	i range (*M*)	1×10^{-7}	4×10^{-6}	2×10^{-5}	2×10^{-5}				
		2×10^{-6}	4×10^{-5}	3×10^{-4}	2×10^{-4}				
	LD_{50} (μg/g) (with piperonyl butoxide)	8.0	28.5	80.0	110.0				

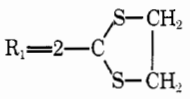
inhibitors (Wilson *et al.*, 1961; Davies *et al.*, 1970; Yu *et al.*, 1971), we were prompted to investigate the effect of the chain length of *N*-alkyl groups on the decarbamylation rate constant k_3 for AChE from bovine erythrocyte, housefly, honeybee, and cricket.

MATERIALS AND METHODS

Inhibitors. Eserine salicylate and neostigmine were obtained from Mann Research Laboratory. Other carbamates were prepared in a conventional manner (Kolbezen *et al.*, 1954). Most of the substituted phenols are commercially available. 2-Dithiolane phenol was prepared according to Durden and Weiden (1969). *3-N,N*-Dimethylaminophenyl *N*-alkyl carbamates were recrystallized from petroleum ether. They were then quaternized with iodomethane in acetone and recrystallized from acetone or ether. 2-Dithiolane phenyl *N*-alkyl carbamates were recrystallized from ethyl acetate. The properties of the new compounds are given in Table I.

Table III. Log k_i (M^{-1} min^{-1}) of Substituted Phenyl *N*-Alkyl Carbamates Against AChE from Honeybee, House Cricket, and Bovine Erythrocyte



R_1	R_2	Methyl	Ethyl	*n*-Propyl	*n*-Butyl	*n*-Hexyl	*n*-Octyl	*n*-Dodecyl
3-N^+-Me_3I^-	Honeybee	8.0	6.56	6.53	4.92	5.23	5.73	5.42
	Cricket	7.84	5.38	5.81	5.46	5.36	4.72	4.51
	Bovine erythrocyte	6.89	5.28	5.53	5.65	6.04	6.00	5.38
3-N-Me_2	Honeybee	5.56	3.76	3.87	2.40	2.64	...	...
	Cricket	4.26	2.11	2.36	1.69	1.57	...	...
	Bovine erythrocyte	4.23	1.94	2.49	2.49	3.04	...	...
3-C-Me_3	Honeybee	6.73	5.00	5.08	3.18	3.00	...	...
	Cricket	5.83	3.20	3.53	2.83	2.79	...	...
	Bovine erythrocyte	5.45	3.26	3.99	4.40	4.49	...	...
2- 	Honeybee	6.78	5.53	5.11	3.48	3.40	...	...
	Cricket	6.42	4.51	4.26	3.40	2.90	...	...
	Bovine erythrocyte	5.18	2.81	3.80	4.11	4.34	...	...

Enzymes. Bovine erythrocyte AChE was purchased from Nutritional Biochemicals Co. Insect AChE were prepared from housefly (*Musca domestica* L.), honeybee (*Apis mellifera* L.), and house cricket (*Acheta domestica* L.) head using the method of Yu *et al.* (1971). The specific activity of the enzyme is defined as number of micromoles of acetylthiocholine hydrolyzed per minute per mg of protein. The specific activities of the enzymes used in this study were bovine erythrocyte 1.0, housefly 9.0, honeybee 0.5, and cricket 0.3. The enzyme activities were inhibited more than 97% when the enzymes were preincubated with 1×10^{-6} *M* of eserine prior to the addition of acetylthiocholine as substrate. Therefore, they were considered to be true cholinesterases (acetylcholinesterases).

Inhibition. A quantity of 0.05 ml of the inhibitor in acetone (except the quaternized compounds which were dissolved in distilled water), was added to a test tube which contained 0.45 ml of AChE (with an activity which will hydrolyze about 0.1 μmol of acetylthiocholine per min in 0.1 *M* potassium phosphate buffer, pH 7.8). At various incubation times (15 sec to 30 min, depending on carbamylation rate) an aliquot of 0.05 ml of the reaction mixture was withdrawn and added to an assay solution which contained 2 ml of 0.1 *M* potassium phosphate buffer, pH 7.8, 0.1 ml of 0.01 *M* 5,5′-dithiobis-2-nitrobenzoic acid, and 0.02 ml of 0.075 *M* acetylthiocholine iodide to measure the residual enzyme activity at 412 nm colorimetrically. Syringes were used to facilitate rapid mixing. Inhibition constants (binding, carbamylation, and overall bimolecular rate constants) were obtained using Main's analysis (Main and Iverson, 1966) with some modifications. In some instances, the inhibitor concentrations used did not approach the *Ka* value (see Table II, inhibitor range) due to great carbamylation rate or the solubility of the inhibitor. Therefore, their inhibition constants were the estimated values. Least square method was used to obtain the best fit of log v against t and $i\Delta t/2.303\Delta \log v$ against i plots (in which v is enzyme activity, t is incubation time, and i is inhibitor concentration). Another independent measurement of bimolecular rate constants was obtained using Aldridge's method (Aldridge, 1950). The inhibitions were performed at 23 ± 0.5°C.

Reactivation. The method to determine the reactivation rate of the inhibited enzyme was essentially similar to that of Wilson *et al.* (1960) and was described by Yu *et al.* (1971).

RESULTS AND DISCUSSION

The inhibition constants (binding, carbamylation, and overall bimolecular rate constants) for 2-dithiolane phenyl *N*-alkyl carbamates and *2-N,N*-dimethylaminophenyl *N*-alkyl carbamate methiodides with AChE from bovine erythrocyte, honeybee, and housefly are summarized in Table II. The results showed that the effects of *N*-alkyl groups on binding and carbamylation were rather complicated. For *N*-alkyl groups changed from *N*-methyl to *N*-ethyl and *N*-*n*-propyl, the binding of enzyme-inhibitor complex tended to decrease. However, the effects on bovine erythrocyte AChE seemed to be less significant than on honeybee and housefly AChE. When *N*-alkyl groups were changed from *N*-*n*-propyl to *N*-*n*-butyl, the binding energy was increased for bovine erythrocyte and housefly AChE. However, the binding energy was decreased further for honeybee AChE. Carbamylation constants were greatly decreased from *N*-methyl to *N*-ethyl for bovine erythrocyte and honeybee AChE. But a slight increase was observed for housefly AChE. Further increase of *N*-alkyl chain length resulted in a great variation in carbamylation rates in different species of AChE. It is worthwhile to notice that carbamylation rates of uncharged 2-dithiolane phenyl *N*-*n*-butyl carbamate were dropped greatly for honeybee and housefly AChE.

It seemed that the variation of substituents on phenyl ring and the chain length of *N*-alkyl group had subtle effects on binding and carbamylation for AChE from different species. Iverson and Main (1969) also reported the orientation of the ring substitution in phenyl *N*-methyl carbamates was important in carbamylation rate. More study is needed to understand how it operates in different species in order to find the solid ground for the design of selective compounds for pest control.

Table IV. Reactivation Half-Lives of Honeybee, Housefly, Cricket, and Bovine Erythrocyte AChE Inhibited by 3-Dimethylaminophenyl *N*-Alkyl Carbamate Methiodides[a]

Carbamates	Honeybee	Housefly	Cricket	Bovine erythrocyte
N-Methyl[b]	1.33 (hr)	1.30	0.90	0.62
N,*N*-Dimethyl[c]	1.17	1.70	0.55	0.95
N-Ethyl	2.58	1.80	7.25	4.00
N-*n*-Propyl	14.00	11.30	93.00	2.03
N-*n*-Butyl	175.00	30.50	243.00	2.60
N-*n*-Hexyl	565.00	No recovery	400.00	2.60
N-*n*-Octyl	425.00	No recovery	700.00	8.70
N-*n*-Dodecyl	95.00	No recovery	47.00	3.10

[a] Two replicates were performed. [b] Eserine was used. [c] Neostigmine was used.

The overall inhibitory potency k_i ($= k_2/Ka$) is the reflection of the combination of binding and carbamylation constants. The results of independent measurement for k_i using Aldridge's method (Aldridge, 1950) are shown in Table III. Change of *N*-alkyl groups from *N*-methyl to *N*-ethyl resulted in a great decrease in overall bimolecular rate constants for all AChE studied. The causes of the decrease in inhibitory potency were different for AChE from different animals. For bovine erythrocyte and honeybee AChE, it is mainly due to the decrease in carbamylation rate. However, it is mainly due to the decrease in binding energy for housefly AChE.

When k_i values of four different substituted phenyl *N*-alkyl carbamates for AChE from three animal species are compared (see Table III), one can find that *N*-ethyl carbamates showed the least inhibitory potency toward bovine erythrocyte AChE. However, the inhibitory potencies were steadily increased starting from *N*-*n*-propyl until *N*-*n*-hexyl, which showed maximum activity. For insect AChE, it is *N*-*n*-butyl or *N*-*n*-hexyl which showed the least inhibitory potency. As discussed above, an improved binding for bovine erythrocyte AChE was obtained when *N*-alkyl groups were changed from *N*-*n*-propyl to *N*-*n*-butyl and no drastic decrease in carbamylation rate was obtained. However, decreased binding energy and carbamylation rate occurred for honeybee AChE. Therefore, increasing the *N*-alkyl chain length resulted in the decrease in the overall inhibitory potency for honeybee AChE.

AChE inhibited by short chain *N*-alkyl carbamates recovered its activity progressively (pseudo first-order). The half-lives (time required for 50% of the inhibited enzyme to recover) for AChE from honeybee, housefly, cricket, and bovine erythrocyte are summarized in Table IV. All four species of AChE inhibited by *N*-methyl carbamate recovered their activities much faster than those inhibited by longer *N*-alkyl carbamates. A remarkable species variation was seen in the decarbamylation rates when AChE were inhibited by *N*-ethyl and longer *N*-alkyl carbamates. Increasing the chain length of *N*-alkyl groups exponentially increased the half-lives of inhibited insect AChE. Maximum stability was reached on *N*-*n*-hexyl carbamate. Further increase in the alkyl chain length resulted in increased decarbamylation rates. No such pattern was observed for bovine erythrocyte AChE (in which *N*-ethyl and *N*-*n*-octyl showed maximum stability).

In general, bovine erythrocyte AChE recovered its activity much faster than that of insect AChE, especially when longer *N*-alkyl carbamates were used as inhibitors. It also has been shown that bovine erythrocyte AChE inhibited by biscarbamates recovered its activity much faster than that of insect AChE (Yu *et al.*, 1971). This remarkable difference in reactivation rates between insect and mammalian AChE may reflect the difference in enzyme active site. Increasing the *N*-alkyl groups may sterically interfere with the proper conformational shift during reactivation process. Thus, insect AChE were more sensitive to steric hindrance.

Insecticidal Activity. 2-Dithiolane phenyl *N*-alkyl carbamates were evaluated for toxicity to the housefly by topical application in a 5:1 ratio of piperonyl butoxide and carbamate in standard (w/v) acetone solutions. LD_{50} values are shown in Table II. One may see the toxicity correlated well with k_i values. Increasing the chain length of *N*-alkyl groups resulted in decreased toxicity.

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