damaged in a similar manner. B_{12} enters the blood, thus resulting in elevation of vit. B_{12} and in a concomitant increase in urinary excretion. This injury provoked with the amounts of carbon tetrachloride used is apparently only temporary. The elevation of vit. B_{12} activity in serum is most likely due to an increase in vit. B_{12} *per se*, rather than alkali stable factors such as desoxyribosides.

Summary. (1) Administration of carbon tetrachloride increased the serum level of vit. B_{12} of rats fed a casein diet. No similar increase was observed in rats previously offered a low vit. B_{12} diet. (2) Administration of carbon tetrachloride likewise increased the urinary excretion of parenterally administered vit. B_{12} or of absorbed vit. B_{12} following oral administration. (3) Physiological significances of these findings are discussed.

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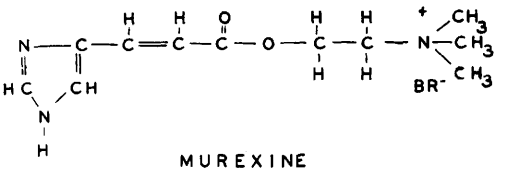
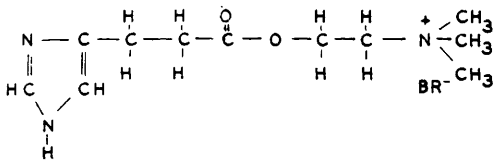
Interrelationship of Murexine, Dihydromurexine and Human Cholinesterases.* (22991)

FRANCIS F. FOLDES, ERVIN G. ERDÖS, NORA BAART AND SYDNEY P. SHANOR
Mercy Hospital and Department of Surgery, University of Pittsburgh School of Medicine, Pa.

It has been shown by Vincent and Julien (1) and later by Erspamer(2) that extracts from the hypobranchial glands of *Murex trunculus* contain several pharmacologically active agents. One of these, murexine (Mur) was identified as (β-(4-Imidazolyl)acrylyl) choline or the choline ester of urocanic acid (3).† The compound was first synthesized by Pasini *et al.*(4). Mur has a nicotinic effect on the autonomic ganglia and blocks myoneural transmission in laboratory animals(5) and man(6). Dihydromurexine (DhMur), (β-(4-Imidazolyl)propionyl) choline, the reduced derivative of Mur. (Fig. 1) is about 4 times as potent as Mur, at the autonomic ganglia and at the neuromuscular



MUREXINE



DIHYDROMUREXINE

FIG. 1. Structural formulae of murexine and dihydromurexine.

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† Murexine, dihydromurexine and urocanic acid (impurity less than 1%) were synthesized by Hoffmann-LaRoche, Nutley, N. J. and supplied by Dr. Leo A. Pirk.

junction; its duration of action, however, is considerably shorter than that of Mur. DhMur is less toxic in mice than Mur. In clinical trials(6), Mur proved to be a short acting depolarizing neuromuscular blocking agent. It was half as potent as succinylcholine in man.

Because of their pharmacological effect on physiological mechanisms associated with acetylcholine (ACh) metabolism, it seemed worthwhile to investigate the interrelationship of Mur and DhMur with human cholinesterases.

Material and methods. Stock solutions of Mur bromide, DhMur bromide and urocanic acid were made up with distilled water. The source of plasma cholinesterase was Cholase.‡ Cholase is the solution of a human plasma cholinesterase concentrate prepared similarly to Harvard fraction IV-6-3. The cholinesterase activity of 1 ml of Cholase with ACh substrate corresponds to 160 ml fresh, pooled, heparinized human plasma. "True" cholinesterase was prepared by hemolyzing washed heparinized human red blood cells. The hydrolysis of Mur by plasma cholinesterase was studied with an ultraviolet spectrophotometric method and with Ammon's manometric method(7). The hydrolysis of DhMur could only be assayed with the manometric technic since its u.v. absorption spectrum is unsuitable for analysis. The absorption spectra of Mur and DhMur are presented in Fig. 2. Since Mur is hydrolyzed by Cholase to urocanic acid and choline, the absorption spectrum of urocanic acid is also shown in Fig. 2. The rate of hydrolysis of Mur was followed by measuring decrease of absorption at a wave length of 317 mμ in Beckman model D.U. spectrophotometer. The system consisted of 5 x 10^{-5} M/lit. Mur and Cholase in 1:10,000 dilution in a pH 7.4 phosphate buffer. The quartz absorption cell had a 10 mm light path. Temperature of the absorption chamber was kept constant at 37°C. The blank contained the same dilution of Cholase in phosphate buffer. The enzyme and substrate were mixed at zero time and readings were taken at 30 second intervals until completion of hydrolysis. Hydrolysis of Mur and DhMur was also investigated with Ammon's technic(7), at 37°C and pH 7.4 using a bicarbonate-carbonate buffer system. The initial concentration of Mur in the experiments was 2.2 x 10^{-2} M/lit. and Cholase was diluted 1:800. In the DhMur experiments, the initial substrate concentration was 2.2 x 10^{-2} M/lit. but Cholase was diluted 1:8000. Readings were taken at 5 minute intervals for 35 minutes. Michaelis constants (K_m) were determined according to Lineweaver and Burk (8) by plotting S/V against S. The K_m of Mur was determined in the spectrophotometer and that of DhMur in the manometric apparatus. The anticholinesterase effect of compounds was studied with the manometric technic. Cholase or red cell cholinesterase was the source of enzyme; ACh, benzoylcholine (BeCh) and butyrylcholine (BuCh) were used as substrates. The I_{50} values were

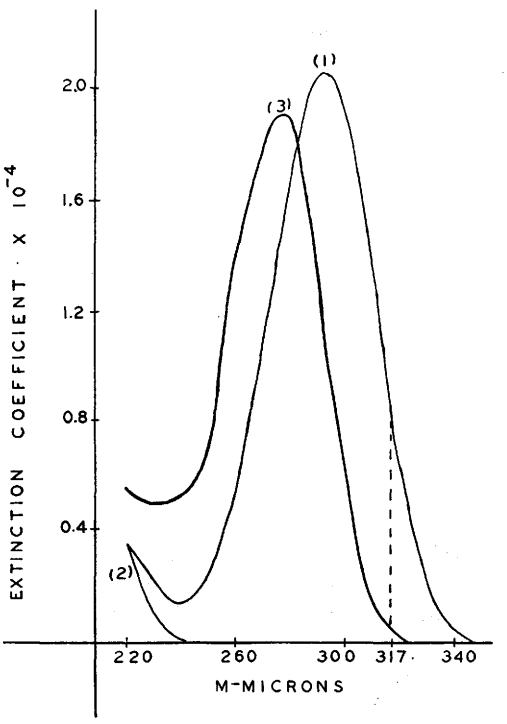

FIG. 2. U.V. absorption spectra of murexine (1), dihydromurexine (2) and urocanic acid (3) at pH 7.4.

‡ Cholase used in this study was kindly supplied by Dr. Edwin B. McLean of Cutter Laboratories, Berkeley, Calif.

TABLE I. Hydrolysis of Murexine and Dihydromurexine by Concentrated Human Plasma Cholinesterase (Cholase).

Substrate	Hydrolysis rate in Cholase (mol./ml Cholase/30 min.) Manometric method	Spectrophotometric method	K_m (M)/l
Murexine	1.3×10^{-3}	1.3×10^{-3}	1.1×10^{-5}
Dihydromurexine	2.3×10^{-2}		1.3×10^{-4}

calculated according to Wilson's(9) formula.

$$V/V_i = 1 + \frac{K_m}{K_i}\left[\frac{I}{K_m + S}\right].$$

Results. Red cell cholinesterase ("true" cholinesterase) did not hydrolyze either Mur or DhMur. The results obtained with hydrolysis of Mur and DhMur by Cholase are presented in Table I. This table shows that the hydrolysis rates of Mur determined with spectrophotometric and manometric methods were identical. DhMur was hydrolyzed as fast as ACh and 18 times faster than Mur. The spontaneous hydrolysis of Mur and DhMur was very slow under our experimental conditions. In calculating enzymatic hydrolysis rates, corrections were made for alkaline hydrolysis. The K_m of Mur was found to be an order lower than that of DhMur, indicating the higher affinity of Mur to this enzyme. The K_m of ACh (1.9×10^{-3} M/lit) indicates that its affinity to plasma cholinesterase is lower than those of Mur and DhMur. Prostigmine inhibited hydrolysis of Mur and DhMur by Cholase.

Table II demonstrates the inhibitory effect of Mur, DhMur and urocanic acid on human cholinesterases.

Mur inhibited the hydrolysis of ACh and BuCh by Cholase and that of ACh by red cell cholinesterase. Mur had almost no effect on hydrolysis of BeCh. DhMur did not inhibit Cholase in the Warburg experiments. Both Mur and DhMur are rather poor inhibitors of cholinesterases. For comparison the I_{50} of prostigmine, with ACh as substrate, is in the order of 10^{-8} M in Cholase and 10^{-7} M in red cell cholinesterase.

Discussion. Our results indicate that both Mur and DhMur are hydrolyzed by human plasma cholinesterase, but they are unaffected by "true" human red cell cholinesterase. Earlier, Erspamer(2,10) stated that Mur is not broken down by cholinesterases. This discrepancy between the findings of Erspamer and ours is probably due to the fact that the enzyme preparations (calf and rabbit blood and various tissue extracts) used by him contained mostly "true" cholinesterase.

It is of interest that there was an 18 fold difference between hydrolysis rates of Mur and DhMur. The faster hydrolysis rate of DhMur would explain its shorter duration of action and its lower toxicity in mice. The reason for the faster hydrolysis rate of DhMur cannot be explained as yet. It is possible that it is caused by a greater flexibility of the saturated side chain.

The failure of Mur to inhibit hydrolysis of BeCh by Cholase, as contrasted to other substrates, may be due to the low K_m (high affinity) of this substrate to human plasma cholinesterase.

The lack of inhibitory effect of DhMur against Cholase is probably due to its own rapid hydrolysis by this enzyme which causes its fast disappearance from the system.

Urocanic acid, the hydrolysis product of Mur, is also an intermediary breakdown product of histidine metabolism(11). It is, therefore, conceivable that Mur, the choline ester of urocanic ester, might normally occur

TABLE II. Inhibitory Effect of Murexine, Dihydromurexine and Urocanic Acid.

Substrate	Enzyme	I_{50} (M)/liter Murexine	Dihydromurexine	Urocanic acid
Acetylcholine	Cholase	8.2×10^{-4}	No inhibition	Negligible
	RChE*	3.6×10^{-3}	2.4×10^{-3}	No inhibition
Benzoylcholine	Cholase	Negligible	No inhibition	Negligible
Butyrylcholine	"	4.4×10^{-3}	No inhibition	No inhibition

* Red cell cholinesterase.

in mammals. Indeed, Kewitz(12) found traces of a choline ester of urocanic acid, probably Mur, in extracts of cervical ganglia of cattle and horse.

Summary. 1. Murexine (Mur) and dihydromurexine (DhMur) are both hydrolyzed by human plasma cholinesterase. The hydrolysis rate of DhMur is as fast as that of acetylcholine (ACh) and is 18 times faster than that of Mur. 2. Neither Mur nor DhMur are hydrolyzed by human red cell cholinesterase. 3. Both Mur and DhMur inhibit the hydrolysis of ACh by red cell cholinesterase. Mur also inhibits plasma cholinesterase.

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Determination of Serum and Tissue Cholesterol. (22992)

ROY G. HERRMANN. (Introduced by K. K. Chen)
The Lilly Research Laboratories, Indianapolis, Ind.

It was found by Swinnen(1) and by us that the direct ferric chloride method of MacIntyre(2) yielded high values for total cholesterol of the serum. Furthermore, this procedure was obviously not directly applicable to tissues. By combining this method with the extraction procedure of Abell(3), with minor modifications, a satisfactory method was obtained that eliminated the false high values but retained the desirable color stability and sensitivity.

Methods. 1. Alcoholic KOH; 6 ml of a 30% w/w KOH solution are added to 94 ml of redistilled absolute ethyl alcohol and well mixed. A white precipitate may form. This reagent should be prepared just before use. 2. Petroleum ether; 60-70°C boiling range and redistilled. 3. Glacial acetic acid; reagent grade glacial acetic acid is refluxed (2) over chromium trioxide for 3 to 4 hours and then redistilled. Start application of heat cautiously and with constant stirring until mixture is close to boiling point, then remove stirrer and install the reflux condensor. This precaution should be followed to prevent a possible violent exothermic reaction. 4. Color reagent; to about 150 ml of reagent grade sulfuric acid add 10 ml of 10% $FeCl_3 \cdot 6H_2O$ in redistilled glacial acetic acid and mix well, then dilute to 1 liter with concentrated sulfuric acid. 5. Standard cholesterol solution; 20 mg of cholesterol/100 ml of glacial acetic acid. *Tissue preparation*: To known weight of tissue, 100 to 200 mg, add 0.5 ml of 10% tetraethylammonium hydroxide and heat in boiling water bath, with occasional mixing, until solution is attained (about 45 min). Allow solution to cool to room temperature, then dilute to exactly 1 ml with distilled water. The use of tetraethylammonium hydroxide makes possible a further analysis of the sample for alkali earth metals. An aliquot of this is used for determination of cholesterol.

Procedure. Place 0.1 ml of serum (0.2 ml in the case of tissue) or less, as required by