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Studies on Cholinesterase

6. THE SELECTIVE INHIBITION OF TRUE CHOLINESTERASE *IN VIVO*

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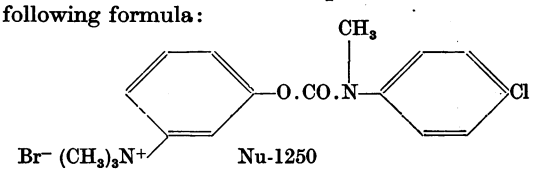
(*Received* 27 *September* 1948)

When the distinction was made between two types of cholinesterase (ChE) in the animal body, these enzymes were called true cholinesterase and pseudo-cholinesterase (Mendel & Rudney, 1943*a*). The name pseudo-cholinesterase was chosen because it was deemed unlikely that this enzyme could deal effectively with acetylcholine *in vivo* in view of its relative inactivity towards low concentrations of that substrate. To determine the validity of this assumption Hawkins & Gunter (1946) employed a selective inhibitor of pseudo-cholinesterase, the prostigmine analogue Nu-683. *In vivo* experiments with this compound showed that a prolonged and almost complete inhibition of pseudo-cholinesterase in plasma and tissues does not evoke any reaction indicative of the accumulation of acetylcholine. Thus it appeared that in conditions in which the activity of true cholinesterase is relatively unimpaired pseudo-cholinesterase plays no essential role in the removal of acetylcholine *in vivo*.

The possibility remained, however, as these workers pointed out, that an auxiliary role might be assumed by pseudo-cholinesterase, namely, that of serving as a substitute for true cholinesterase whenever the activity of the latter enzyme is impaired. Proof of this hypothesis has hitherto not been put forward. It has awaited the finding of a compound which not only possesses the property of acting as a selective inhibitor of true cholinesterase, but which can also be administered *in vivo* without eliciting any effects other than those attributable to its cholinesterase-inhibiting action.

Selective inhibitors of true cholinesterase have been described previously. Zeller & Bissegger (1943) have shown that high concentrations of caffeine inhibit the cholinesterases of erythrocytes and brain, two examples of true cholinesterase (Mendel & Rudney, 1943*a*, *b*), without significantly affecting the cholinesterase activity of human plasma which contains mainly pseudo-cholinesterase (Mendel, Mundell & Rudney, 1943). Recently, Adams & Thompson (1948) have demonstrated that certain 'nitrogen mustards', particularly di-(2-chloroethyl) methylamine (DDM), act as selective inhibitors of true cholinesterase.

The present communication deals with a cholinesterase inhibitor which, apart from possessing a selective inhibitory action on true cholinesterase, produces no effects *in vivo* that could not be attributed to the accumulation of acetylcholine. This compound, a prostigmine analogue, the *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Nu-1250; Aeschlimann & Stempel, 1946), has the following formula:



Nu-1250

The aforementioned properties of Nu-1250 suggested its use as a tool in determining whether pseudo-cholinesterase can assume an auxiliary role in the hydrolysis of acetylcholine when the activity of true cholinesterase is impaired. The results of experiments dealing with this question are reported here.

METHODS

Substrates

Acetylcholine chloride (Ach), Hoffmann-La Roche, Basel, Switzerland. Benzoylcholine chloride (Bch), kindly supplied by Hoffmann-La Roche, Inc., Nutley, N.J. Acetyl-β-methylcholine chloride (Mch), Merck's Mecholyl.

In all cases, the final concentration of substrate in the reaction mixture was sufficient to afford complete saturation of the enzyme, viz. 0·0012 M-Ach for true cholinesterase, 0·06 M-Ach for pseudo-cholinesterase, 0·03 M-Mch and 0·006 M-Bch for the same two enzymes respectively (Mendel *et al.* 1943).

Estimation of enzyme activity

The activity of the cholinesterases was measured manometrically by Warburg's method at 37·5° in a medium containing 0·025 M-$NaHCO_3$ saturated with 5% CO_2 in N_2 (pH 7·4). Blank determinations were run for each substrate to estimate the extent of non-enzymic hydrolysis.

The enzyme preparation was placed in the main compartment of the Warburg vessel, the substrate in the side arm, and the inhibitor (0·1 ml.) was added to the enzyme-bicarbonate mixture in the main compartment. The total fluid volume was 5 ml. A 15 min. period was allowed for the attainment of thermal equilibrium, following which the substrate was tipped in from the side arm. Readings were started 5 min. later, and were repeated at appropriate intervals for the next 20 min., or, in cases where low substrate concentrations were used, until the rate of the reaction began to fall.

Source of enzyme

Plasma. Freshly drawn, oxalated blood was used. The cells were separated from the plasma by centrifugation. The volume of plasma used (0·1–1·0 ml.) depended on the activity, which varied from one species to another. Following the injection of the inhibitor into experimental animals, the activities of the cholinesterases were measured in undiluted plasma to prevent the inhibitor-enzyme complex from dissociating upon dilution. This procedure necessitated correction for the retention of CO_2 (Warburg, 1925).

Erythrocytes. After removal of the plasma, the cells were washed three times with 0·9% NaCl, and haemolysis was then brought about by the addition of three times their volume of distilled water. 0·2 ml. of the haemolysate was used in the estimation of enzyme activity.

Fractionated rat plasma. The plasma of six male rats was brought to 0·5 saturation with $(NH_4)_2SO_4$. After 30 min. the mixture was centrifuged and the supernatant fluid (*S*) decanted. The precipitate was then washed with 0·4 saturated $(NH_4)_2SO_4$ for 20 min., centrifuged, and the remaining precipitate (*P*) suspended in a small volume of water. Both *S* and *P* were dialyzed against tap water for 18 hr. Using Mch and Bch as substrates, it was found that fraction *S* contained pseudo-cholinesterase but no true cholinesterase, whilst fraction *P* contained the true cholinesterase, but only a trace of pseudo-cholinesterase.

Dog brain. The brain was removed as soon as possible after death, and a suspension of the nucleus caudatus was prepared by grinding the tissue in three times its weight of distilled water. The suspension (0·1 ml.) was used in the determination of enzymic activity.

Dog pancreas. The gland was obtained from the same animal from which the brain had been taken. The larger blood vessels and fibrous tissue were removed, and a suspension was prepared by grinding the pancreas with nine times its weight of distilled water in a Waring Blendor and subsequently straining it through a fine sieve. The suspension (0·1 ml.) was used in the experiments reported.

Inhibitor

A stock solution of Nu-1250 (10^{-3} M) in distilled water was prepared every second day. Subsequent dilutions were made from this solution as required. In contrast to the experiences with di*iso*propyl fluorophosphonate (DFP) and DDM, no significant change in potency was evident within a 36 hr. period. (Mol.wt. of Nu-1250 = 3994.)

RESULTS

(A) In vitro *experiments*

The following preliminary experiments were performed to assess the suitability of Nu-1250 as a tool for testing whether pseudo-cholinesterase can play an auxiliary role in the hydrolysis of acetylcholine *in vivo*:

(1) In order to find a suitable animal for the proposed *in vivo* experiments, the margin between the sensitivities of true cholinesterase and pseudo-cholinesterase in various species towards Nu-1250 was determined.

(2) Since acetyl-β-methylcholine and benzoylcholine were to be used as substitutes for acetylcholine in estimating the activities of the two cholinesterases separately (Mendel *et al.* 1943), it was necessary to ascertain whether the inhibitions by Nu-1250 of the hydrolysis of these substrates and of acetylcholine are identical.

The results of these investigations appear below.

(1) The inhibition by Nu-1250 of the cholinesterases in the plasma and tissues of various species is summarized in Table 1, the values obtained with each concentration of the inhibitor representing the average of several determinations. It can be seen that Nu-1250 acts as a selective inhibitor of true cholinesterase. However, the margin between the sensitivities of the true cholinesterase and pseudo-cholinesterase in the plasma and tissues varies from species to species. In the case of human subjects (section A) the activity of true cholinesterase (erythrocytes) is at least a thousand times more sensitive to the inhibitory action of Nu-1250 than is the pseudo-cholinesterase (plasma); with the tissues of the dog this margin drops to about twenty times, whilst in the case of the true cholinesterase and pseudo-cholinesterase of horse blood, a margin of approximately five times is obtained. The rat seemed ideal for *in vivo* experiments because the true cholinesterase in the plasma of this animal is at least a thousand times more sensitive to Nu-1250 than is the pseudo-cholinesterase.

Table 1. *Percentage inhibition by Nu-1250 of the cholinesterases in the plasma and tissues of various species**

Section	Enzyme source	Substrate: Nature	Substrate: Concentration (M)	Concentration of Nu-1250 (M): 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	5×10^{-8}	10^{-8}	5×10^{-9}	10^{-9}
A	Human erythrocytes	Ach	0·0012	—	—	100	96	84	—	66	60	34
		Mch	0·03	—	—	—	—	86	—	—	56	—
	Human plasma	Ach	0·06	97	58	29	16	—	—	0	—	—
B	Horse erythrocytes	Ach	0·0012	—	—	—	100	97	93	58	—	—
	Horse plasma	Ach	0·06	—	—	100	97	70	66	—	—	—
C	Dog brain (nucleus caudatus)	Ach	0·0012	—	—	100	95	92	86	—	—	—
	Dog pancreas	Bch	0·006	—	—	88	83	53	14	—	—	—
	Dog plasma	Mch	0·03	—	—	—	—	93	87	—	—	—
		Bch	0·006	—	—	—	—	54	32	—	—	—
D	Fractionated rat plasma*											
	Precipitate (*P*)	Mch	0·03	—	—	100	100	100	91	82	69	13
		Ach	0·0012	—	—	—	—	—	—	—	72	—
	Supernatant fluid (*S*)	Bch	0·006	—	81†	65	45	31	24	6	—	—
		Ach	0·06	—	76†	—	43	—	—	—	—	—

* See Methods section for details of procedure and explanation of abbreviations used.
† Nu-1250 concentration used here was 3×10^{-5} M.

(2) Experiments with human erythrocytes, horse erythrocytes and the precipitate (*P*) from fractionated rat plasma (sections A, B and D of Table 1) showed that when acetyl-β-methylcholine was employed as substrate the inhibition of the activity towards that compound effected by Nu-1250 mirrored the inhibition displayed when the natural substrate acetylcholine was used. Hence, it was deemed valid to consider the inhibition of the activity towards acetyl-β-methylcholine as a reflexion of the inhibition of the acetylcholine hydrolysis brought about by true cholinesterase. Similar results with the supernatant fluid (*S*) from fractionated rat plasma (section D, Table 1), which had been shown to contain pseudo-cholinesterase only, justified substituting the inhibition of the activity towards benzoylcholine for the inhibition of the acetylcholine hydrolysis brought about by pseudo-cholinesterase.

(B) In vivo *experiments*

Since the true cholinesterase in the plasma of rats was found to be about a thousand times more sensitive to the inhibitory action of Nu-1250 than the pseudo-cholinesterase (see Table 1, section D), the rat seemed most suitable for the *in vivo* experiments. The only difficulty appeared to be that a comparatively large volume of blood (2·5 ml.) had to be obtained before the injection of the inhibitor in order to determine the normal levels of the cholinesterases in the plasma. However, Sawyer & Everett (1946) have shown that in adult male rats both cholinesterases remain fairly constant over long periods of time, and preliminary experiments in the present series revealed that after the removal of 2·5 ml. of blood from rats weighing about 300 g. an interval of 2 weeks sufficed for the regeneration of both enzymes to previous levels. Therefore, adult male rats, weighing at least 300 g. were used in the following experiments:

Blood (2·5 ml.) was removed by heart puncture under light ether anaesthesia. The activities of the cholinesterases in the plasma obtained from this sample served as controls against which the magnitude of the inhibition of these enzymes was gauged, when an effective dose of Nu-1250 was injected 2 weeks later. The effectiveness of the dose was determined by the appearance of well defined symptoms indicative of acetylcholine accumulation, viz. chewing movements, yawning, and extensive and violent fibrillary twitching of the voluntary muscles. Upon their appearance the rats were killed by exsanguination through the carotid artery, and the blood was collected in a beaker containing sodium oxalate (*c.* 20 mg.). The susceptibility of the complex between the cholinesterases and Nu-1250 to dissociation upon dilution necessitated the measurement of enzymic activity in the undiluted plasma of these animals.

Table 2 gives the cholinesterase levels in the plasma of six rats which displayed well defined symptoms of acetylcholine accumulation following the intraperitoneal injection of Nu-1250. It will be seen that the true cholinesterase is inhibited 75–89 %, whereas the activity of the pseudo-cholinesterase is but insignificantly affected. Thus,

Table 2. *Inhibition of the cholinesterases in the plasma of rats injected with Nu-1250*

(Activities are expressed as μl. CO_2 evolved in 20 min. by 1 ml. plasma with Mch and Bch as substrates for true ChE and pseudo-ChE, respectively.)

			True cholinesterase			Pseudo-cholinesterase		
Rat no.	Wt. (g.)	Wt. Nu-1250 injected (mg.)	Activity before injection (μl. CO_2)	Activity after injection (μl. CO_2)	Inhibition (%)	Activity before injection (μl. CO_2)	Activity after injection* (μl. CO_2)	Inhibition (%)
1	330	0·066	52·5	13·2	75·0	49·2	48·0	2·5
2	335	0·066	44·0	6·0	86·0	36·0	32·0	11·0
3	350	0·070	45·0	7·2	84·0	29·0	27·3	6·0
4	300	0·080	44·3	4·8	89·0	34·6	30·4	12·0
5	360	0·158	43·0	5·2	87·5	35·0	31·2	10·0
6	395	0·170	44·5	6·0	86·5	44·3	37·3	15·0

* Corrected for CO_2 retention.

in spite of its virtually undiminished activity, pseudo-cholinesterase is unable to prevent the effects of acetylcholine accumulation when the true cholinesterase is inhibited.

To exclude the possibility that the reaction of the experimental animals was due to an effect of Nu-1250 not related to acetylcholine accumulation, (+)-tubocurarine, which blocks the response of skeletal muscles to acetylcholine, was administered to a series of rats when the fibrillary twitching was at its height. The intraperitoneal injection of (+)-tubocurarine (1·25 mg./kg.) arrested the fibrillation almost immediately, thus proving that the symptoms elicited by Nu-1250 were due to the accumulation of acetylcholine.

DISCUSSION

As Adams & Thompson (1948) have pointed out, cholinesterase inhibitors may be divided into three main groups: (1) compounds which inhibit pseudo- and true cholinesterase to the same extent; (2) compounds which act selectively on pseudo-cholinesterase; and (3) selective inhibitors of true cholinesterase. Group (1) includes eserine and prostigmine (Hawkins & Mendel, 1946); group (2) includes pyrazolone derivatives (Zeller, 1942), percaine (Zeller & Bissegger, 1943), tri-*o*-cresylphosphate (Mendel & Rudney, 1944), certain unauthenticated curare preparations (Harris & Harris, 1944), the prostigmine analogue Nu-683 (Hawkins & Gunter, 1946), di*iso*propyl fluorophosphonate (Hawkins & Mendel, 1947), and *N*-diethylaminoethylphenothiazine (2987 R.P.) (Gordon, 1948); group (3) includes caffeine (Zeller & Bissegger, 1943), certain 'nitrogen mustards', especially di-(2-chloroethyl)-methylamine (DDM) (Adams & Thompson, 1948) and the prostigmine analogue Nu-1250.

By selectively inhibiting pseudo-cholinesterase *in vivo* Hawkins & Gunter (1946) demonstrated that this enzyme plays no essential role in the hydrolysis of acetylcholine in the animal body. Whether an auxiliary role might be assumed by pseudo-cholinesterase in cases where the activity of true cholinesterase is impaired could not be determined by these authors, because no suitable selective inhibitor of true cholinesterase was available at that time. The results outlined in the present paper, however, permit the conclusion that pseudo-cholinesterase is incapable of playing even an auxiliary role in preventing the accumulation of acetylcholine *in vivo*. Consequently, the appearance of symptoms indicative of acetylcholine poisoning following the administration of a cholinesterase inhibitor from any one of the aforementioned groups must be directly related to the action of the inhibitor on true cholinesterase. Moreover, an inhibition of approximately 75% of the activity of true cholinesterase must be exceeded before the onset of symptoms of acetylcholine accumulation can be expected, since the experiments of Gunter & Mendel (1945), Hawkins & Gunter (1946) and Hawkins & Mendel (1947) have shown that a surplus of true cholinesterase exists in the animal body. It would seem therefore that if attempts are made to correlate the pharmacological effects of a compound with its ability to act as a cholinesterase inhibitor, they will be of no avail, unless the cholinesterase inhibitory potency expresses the degree of inhibition of the true cholinesterase.

SUMMARY

1. The prostigmine analogue, *N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide (Hoffmann-La Roche Nu-1250), was found to be a selective inhibitor of true cholinesterase.

2. Inhibition of true cholinesterase in rats brought about by the injection of Nu-1250 elicits symptoms indicative of acetylcholine accumulation, in spite of the undiminished activity of pseudo-cholinesterase. Thus, pseudo-cholinesterase is not essential to the hydrolysis of acetylcholine *in vivo*, as previous experiments have shown, nor is it capable of assuming even an auxiliary role in this process when the activity of the true cholinesterase is impaired.

We are indebted to Dr J. A. Aeschlimann and Hoffmann-La Roche Inc., Nutley, N.J. for making available to us the Nu-1250 used in these experiments.

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The Absorption of Vitamin A in Ruminants and Rats

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(*Received* 20 *August* 1948)

Drummond, Bell & Palmer (1935) and McCoord, Breese & Baum (1943) demonstrated an increased concentration of vitamin A in the lymph collected from the thoracic duct, after oral administration of the vitamin. Popper & Volk (1944) observed a fluorescence typical of vitamin A in the lacteals of the rat following dosage. Radice & Herraiz (1947) confirmed the results of Popper and claimed that they had observed a similar fluorescence in portal blood. These findings suggested that vitamin A may be absorbed by two different routes, as has been described for fats by Frazer (1946).

In the present study, both the portal blood and the lymph were examined as possible pathways of absorption, in order to ascertain the relative importance of the two routes. The experiments were performed on three species, oxen, sheep and rats, by dosing them with vitamin A and estimating the vitamin in systemic and portal blood, and in lymph glands from various regions of the body. The samples were collected as soon as possible after slaughter of the animals.

EXPERIMENTAL

Treatment of animals

The animals were given vitamin A (5000 i.u./kg. body wt.) in the form of halibut liver oil, by mouth. Doses were prepared for bullocks and sheep by emulsifying the halibut liver oil with reconstituted separated milk by means of a Waring Blendor. Rats, fasted for 12 hr., were dosed from a precision pipettè with undiluted oil.

Bullocks. Fourteen Ayrshire and two Friesian bullocks weighing 150–250 kg. each were dosed at different times, ranging from 2 to 24 hr., before slaughter. The total bulk of the dose was 750 ml. Before dosing, samples of jugular blood were taken into oxalate, and after dosing, samples of jugular and portal blood were collected, usually within 2 and not exceeding 5 min. after slaughter. All blood