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EFFECT OF TWO HEMICHOLINIUMS ON THE DISPOSITION AND DISTRIBUTION OF ENDOGENOUS FREE CHOLINE IN ANAESTHETIZED RABBITS

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1 The effects of hemicholinium no. 3 (HC-3) and its *p*-terphenyl analogue (TPHC-3) on the disposition and distribution of free choline were studied in rabbits under pentobarbitone anaesthesia. Free choline was determined by bioassay.

2 The respiration of animals given 0.35 μmol/kg of HC-3 was hardly affected; however, 4 out of 6 rabbits given the same dose of TPHC-3 exhibited varying degrees of respiratory impairment. All animals that received 2 injections (1 h apart) of either HC-3 (1.4 μmol/kg) or TPHC-3 (0.7 μmol/kg) developed respiratory difficulty about 1 h after the second injection.

3 The respiratory distress was accompanied by a 2 to 15-fold rise in plasma choline concentration. This rise has been attributed to hypoxia.

4 In experiments in which choline has been infused it was observed that HC-3 could impair the animal's ability to dispose of exogenous choline. In control rabbits neither HC-3 nor TPHC-3 produced changes in plasma choline concentrations unless the respiration was depressed.

5 Either HC-3 or TPHC-3 (both at 0.35 μmol/kg) significantly ($P < 0.05$) reduced the kidney choline concentration by 40% and 30% respectively; both hemicholiniums raised the lung choline concentration by about 35%. Only TPHC-3 caused a significant rise (40%) in liver choline. The choline concentrations in other tissues were unaffected by the hemicholiniums.

Introduction

The hemicholiniums, especially hemicholinium-3 (HC-3) (Schueler, 1955) have been much used for the detection and study of cholinergic nerves. The basis for their pharmacological actions is generally believed to be an interference with the access of choline through cellular barriers to the sites where it is acetylated. They thus interrupt synaptic transmission when the stores of the transmitter acetylcholine are depleted (see Bowman & Marshall, 1972). The action of HC-3 on the transport of choline is most clearly seen on tissue preparations containing intact cells where there are more restrictive barriers to the free movement of choline (Gardiner, 1961).

In the intact animal changes are therefore to be anticipated in the distribution and disposition of free choline during hemicholinium intoxication. Sites or tissues that normally obtain their choline requirements as free choline would be expected to become depleted; those other organs or tissues supplying the base might conversely be expected to show raised levels. Knowledge of the changes should be of assistance in the study of the normal metabolism and function of choline as well as of the details of hemicholinium lethality.

The experiments described here were conducted in rabbits; changes in the plasma and tissue concentrations of endogenous free choline were followed during the development of intoxication with HC-3 or its *p*-terphenyl analogue (TPHC-3) and measurements were made of the disposition of exogenous choline in the presence of HC-3. The tissues studied were plasma and erythrocytes of arterial blood, skeletal muscle, heart, brain, lung, liver, kidney and small intestine. The results of parallel experiments conducted over the same period but without the use of the hemicholinium have been published previously (Gardiner & Gwee, 1974). Preliminary results of this study were presented at the 5th International Congress of Pharmacology (Gardiner & Gwee, 1972).

Methods

Adult rabbits of either sex weighing 1.3–1.6 kg were used. The animals were anaesthetized with sodium pentobarbitone (50 mg/kg i.v.). The preparation of the

animals, general experimental and analytical procedures, and choline bioassay have been described previously (Gardiner & Gwee, 1974).

The hemicholinium HC-3 was prepared as described by Long & Schueler (1954) with modifications introduced by V.B. Haarstadt & F.W. Schueler (personal communication). The *p*-terphenyl analogue of HC-3 (TPHC-3) was prepared similarly (Lee, 1969) and it was used in the form of the monohydrate, recrystallized from ethanol, $C_{30}H_{38}O_4N_2Br_2.H_2O$, molecular weight 668.46.

Results

Plasma choline concentration after hemicholinium treatment

All six animals given HC-3 0.35 μmol/kg and two out of six given the same dose of TPHC-3 exhibited no signs of intoxication over a 3 h period. The other four rabbits receiving TPHC-3 showed respiratory impairment approximately 60 min after the drug was injected: periods of slow decline of the respiratory amplitude were terminated by a large gasp and followed by a temporary return to normal amplitude. As the intoxication proceeded the gasps became more frequent and the fall in amplitude became quicker; the rate of respiration also decreased. The effect was maximal at 1.5 to 2 h, after which the respiration recovered. The animals were breathing normally at the end of the experiment.

The plasma choline concentration remained steady in the control animals and in those given a hemicholinium whose respiration was unaffected. In the 4 animals which showed respiratory impairment after 0.35 μmol/kg of TPHC-3 there were 2 to 4-fold increases in the plasma choline concentrations during the periods when the respiratory difficulties were most severe. The choline concentrations decreased again as respiration improved.

Table 1 and Figure 1 show the changes in plasma choline concentrations which followed the injection of higher doses of the hemicholiniums sufficient to produce complete or almost complete respiratory failure. In these experiments two intravenous injections of either HC-3 (1.4 μmol/kg) or TPHC-3 (0.7 μmol/kg) were given 1 h apart. In two rabbits, after the first dose, a mild stimulation of the respiration was seen. One hour after the second injection the progressive respiratory distress characteristic of hemicholinium intoxication developed. The respiratory disturbances were followed by a rise in the plasma choline concentration, the terminal values being 8 to 15-fold greater than the basal values. The increase in choline concentration was greatest in those animals whose respiration was most severely affected.

Effect of hemicholinium-3 on continuous infusions of choline

In the experiments described above it appeared that increases in plasma choline concentration which occurred after the administration of a hemicholinium may have been the consequence of the respiratory impairment rather than a direct action of the drug. To investigate whether, in the absence of respiratory distress, HC-3 could cause a rise in plasma choline concentration, choline was infused intravenously in the absence and presence of HC-3. The plasma choline concentrations were measured every 5 minutes. In other experiments, in the absence of a hemicholinium compound, it was observed that the rate of the choline infusion had to be larger than 400 nmol kg^{-1} min^{-1} to cause a significant rise in the plasma choline con-

Table 1 Plasma choline concentration following intravenous injection of hemicholinium no. 3 (HC-3, 2 × 1.4 μmol/kg) or its *p*-terphenyl analogue (TPHC-3, 2 × 0.7 μmol/kg)

	Plasma choline concentration (nmol/ml)			
	Before first injection	*After hemicholinium*		
		1st h	*2nd h*	*3rd h*
			HC-3 (2 × 1.4 μmol/kg)	
1	5.2 ± 0.2	6.5 ± 1.1	13 ± 5.2	40 ± 1.9**
2	7.6 ± 1.1	10.0 ± 1.0	17 ± 1.2**	49 ± 13**
3	8.4 ± 0.9	9.5 ± 0.4	14 ± 3.1	47 ± 5.1**
			TPHC-3 (2 × 0.7 μmol/kg)	
1	6.4 ± 0.6	8.3 ± 0.3*	20 ± 2.4**	63 ± 1.2**
2	9.8 ± 0.8	13.0 ± 1.6	25 ± 6.7	69 ± 5.2**
3	5.5 ± 0.3	6.9 ± 0.5	20 ± 6.0	77 ± 11**

After collection of control samples, the first injection of HC-3 or TPHC-3 was given followed by the second injection 1 h later. Each value is the mean ± s.e. mean of four samples taken at 15 min intervals. Significance of difference from pre-injection value: *$P < 0.05$; **$P < 0.001$.

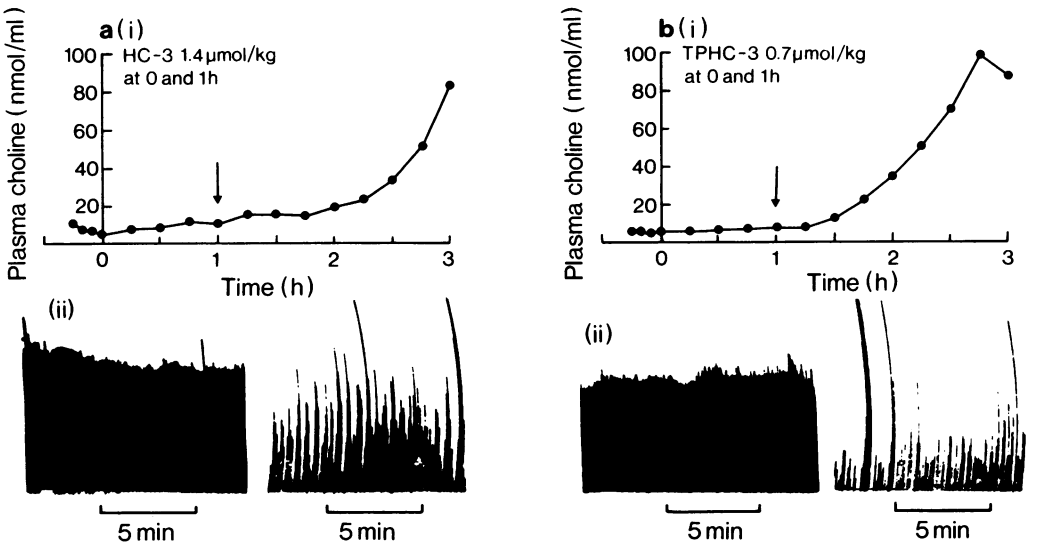

Figure 1 Plasma choline concentration and respiratory activity of (a) rabbit given hemicholinium no. 3 (HC-3) and (b) another rabbit given its *p*-terphenyl analogue (TPHC-3) (ai) HC-3 (1.4 μmol/kg) injected at zero time and 1 h later at arrow. (bi) TPHC-3 (0.7 μmol/kg) injected at zero time and 1 h later. (ai) and (bi) show the plasma choline concentrations. (aii) and (bii) are examples of the kymograph tracing produced by a piston recorder connected to the side arm of a tracheal cannula. First tracing: respiratory movements during the pre-injection period; second tracing: after the injection when the respiration was most severely affected.

centration (Gardiner & Gwee, 1974). In the present experiments therefore choline was infused at rates of 200, 400, 800 and 1200 nmol kg^{-1} min^{-1} for 15 min each. HC-3 (1.4 μmol/kg) was injected 30 min before the start of the first choline infusion period. The infusions were completed before any depressant effect on the respiration was detectable. After HC-3 (1.4 μmol/kg) had been given, the plasma choline concentration was clearly raised in three rabbits out of four when choline was infused at 200 nmol kg^{-1} min^{-1} and in all four when the rate was 400 nmol kg^{-1} min^{-1} (see Table 2).

Table 2 Effect of hemicholinium no. 3 (HC-3) on the plasma choline concentration during the intravenous infusion of choline

	Plasma choline concentration (nmol/ml)				
	Control	R_1	R_2	R_3	R_4
			Without HC-3		
1	21.3 ± 0.9	26.0 ± 2.3	23.0 ± 1.0	35.6 ± 1.0**	45.2 ± 1.0**
2	10.8 ± 1.0	11.0 ± 0	15.0 ± 0.4**	23.9 ± 1.7**	36.5 ± 0.3**
3	12.4 ± 0.9	14.5 ± 1.7	13.9 ± 1.3	22.5 ± 0**	40.0 ± 5.0**
4	10.1 ± 0.7	11.3 ± 0.3	12.7 ± 0.7*	17.4 ± 0.8**	31.0 ± 1.0**
			With HC-3		
5	9.4 ± 1.4	9.7 ± 0.3	23.3 ± 0.3**	32.9 ± 1.0**	43.0 ± 1.7**
6	6.3 ± 0.2	9.2 ± 0.6	15.5 ± 1.3**	21.6 ± 1.3**	43.7 ± 0.8**
7	7.3 ± 0.3	8.7 ± 0.3*	13.5 ± 2.3*	20.5 ± 0.5**	34.7 ± 0.3**
8	11.9 ± 0.8	16.6 ± 1.0*	26.5 ± 2.2**	32.2 ± 1.2**	61.7 ± 1.7**

Choline was infused for 15 min at each of four rates (R_1) 0.2, (R_2) 0.4, (R_3) 0.8 and (R_4) 1.2 μmol kg^{-1} min^{-1}. Arterial blood samples were taken at 5 min intervals, four during the control period and three during each of the choline infusions. The values are means ± s.e. mean of all samples taken during each period. In experiments 5 to 8 HC-3 (1.4 μmol/kg) was injected intravenously immediately after the last control sample was taken; the infusion was started 30 min later.

Significance of difference from control values: *$P < 0.05$; **$P < 0.001$.

Choline in the erythrocytes

The concentration of choline in the erythrocytes of arterial blood did not change throughout the experiments in either the control animals or any of those given a hemicholinium.

Tissue choline concentrations after hemicholinium treatment

The concentrations of choline in some tissues of rabbits which were given HC-3 or TPHC-3 (0.35 μmol/kg) and were killed 3 h later are shown in Table 3. Lung choline was raised by about 35% after either HC-3 or TPHC-3 and choline in the kidney was reduced by 30% (TPHC-3) or 40% (HC-3). In the liver only TPHC-3 caused a rise (40%) in the choline concentration. The concentrations of choline in muscle, heart, brain and the terminal portion of the small intestine were unaffected by the administration of either hemicholinium. The choline concentrations in the control rabbits were reported previously (Gardiner & Gwee, 1974). In those experiments it was shown that the delay between the death of the animal and the extraction of tissue samples for choline did not produce any appreciable change in the choline content of the liver, lung and kidney. The results obtained here cannot therefore be accounted for by *post mortem* changes.

Discussion

In the animals whose respiration was appreciably depressed by the hemicholinium, the 2 to 15-fold rise in plasma choline concentration was unlikely to have been due to gradual accumulation of choline consequent upon a direct inhibitory action of hemicholinium on choline transport mechanisms, as the rise in plasma choline did not precede the development of respiratory difficulty. We consider it likely that hypoxia caused the observed rise in plasma choline concentration. This idea is supported by the experiments of Chan (1971) and Gardiner (1975) in rabbits. They showed that the development of tissue hypoxia produced by the administration of cyanide or of gas mixtures deficient in oxygen, massive haemorrhage, or following respiratory arrest induced by (+)-tubocurarine, was accompanied by rises in the concentration of choline. Gardiner (1975) further showed that the rises following (+)-tubocurarine of TPHC-3 were prevented if the animals were artificially resuscitated.

Our results do not negate the idea that hemicholinium toxicity is primarily due to impaired acetylcholine synthesis at cholinergic junctions. This effect occurring at the neuromuscular junctions of respiratory muscles may well be responsible for, or at least contribute to, the impairment of breathing. However, inhibition of choline transport at such junctions is clearly insufficient to account for the observed rise in plasma choline concentration which, as our experiments show, is secondary to the hypoxia produced.

HC-3 in a dose (1.4 μmol/kg) that did not produce respiratory depression, did not impair the ability of animals to dispose of infused exogenous choline. In HC-3-treated animals the plasma choline concentration started to rise during the infusion of choline 200 nmol kg^{-1} min^{-1} whereas infusion rates of greater than 400 nmol kg^{-1} min^{-1} were required in untreated animals (Gardiner & Gwee, 1974). It is probable therefore that the entry of free choline, from the diet and from sites of synthesis, into the circulation is normally well below a rate of 200 nmol kg^{-1} min^{-1} but that during hemicholinium-induced respiratory distress, the rate of entry of choline from tissues into the circulation exceeds 200 nmol kg^{-1} min^{-1}.

The hemicholiniums, at a dose of 0.35 μmol/kg produced variable effects on the tissue disposition of choline. The choline concentration of the brain, intestine, skeletal muscle or heart was not appreciably

Table 3 Effect of hemicholinium no. 3 (HC-3) or its *p*-terphenyl analogue (TPHC-3) on the concentration of choline in rabbit tissues

	Choline concentration (nmol/g tissue)						
Treatment	*Muscle*	*Heart*	*Brain*	*Lung*	*Liver*	*Ileum*†	*Kidney*
Control	19 ± 2.2	84 ± 8	200 ± 14	320 ± 14	390 ± 30	430 ± 40	500 ± 25
HC-3 0.35 μmol/kg	12 ± 1.6	68 ± 10	150 ± 17	420 ± 62*	320 ± 29	510 ± 57	300 ± 21**
TPHC-3 0.35 μmol/kg	19 ± 2.9	100 ± 7	210 ± 19	410 ± 47*	560 ± 44**	520 ± 57	360 ± 29**

In the animals given HC-3 or TPHC-3 (0.35 μmol/kg i.v.) each value is the mean ± s.e. mean from six animals. The values for normal animals have been reported previously (Gardiner & Gwee, 1974) and were obtained from analyses on 20 or 26 animals. The animals were killed 3 h after the drug injection. †: terminal small intestine.

Significance of differences from control values: *$P < 0.05$; **$P < 0.001$.

affected. This may reflect a lack of susceptibility of the choline uptake mechanisms in these tissues to the action of the hemicholiniums. Alternatively, these tissues may receive their supply of choline not in the free form but, rather, as a derivative whose transport is unaffected by the hemicholinium. There is evidence, for example, that the brain receives choline in a phospholipid form (Björnstad & Bremer, 1966; Ansell & Spanner, 1971). The rise in the choline concentration of the liver caused by TPHC-3 is puzzling.

The kidney choline concentration was significantly decreased by HC-3 (40%) and TPHC-3 (30%). This might be due to the hemicholinium inhibiting the tubular reabsorption of choline in the glomerular filtrate as shown by Vander (1962) and Acara & Rennick (1973). The intracellular choline concentration can then be expected to fall, since the choline will continue to undergo metabolism without being replaced.

Hemicholinium treatment significantly increased the lung choline concentration by about 35%. The lungs, unlike most other tissues, require preformed choline for the synthesis of lecithin (Spitzer, Norman & Morrison, 1969; Gluck, Kulovich, Eidelman, Cordeiro & Khazin, 1972) which forms the major phospholipid component in the lung surfactant system (Klaus, Clements & Havel, 1961; Gluck, Motoyama, Smits & Kulovich, 1967). It is possible that the hemicholinium inhibited the incorporation of choline into lecithin with resultant accumulation of choline in the tissues. An alternative explanation is that the lung normally releases choline into the circulation and that this release process is probably impaired by the hemicholinium, again resulting in accumulation of choline in the tissues. The situation is analogous to the reduced loss of choline from the cerebrospinal fluid in HC-3-treated cats (Gardiner & Domer, 1969). It is not yet possible to distinguish between the two alternatives; although it is known that the lungs readily take up choline from the circulation (Gardiner & Paton, 1972; Gardiner & Gwee, 1974), it is not known how or in what form it is subsequently lost from them.

The effect of the hemicholinium on the disposition of choline in the lung has important implications which might help to explain some of the unusual features seen in hemicholinium intoxication. If the increase in lung choline concentration can be shown to result from inhibition of lecithin synthesis by the hemicholinium, then changes in the surfactant properties of the lung can be expected and this could yet be an additional mechanism contributing to the complex action of the hemicholiniums on the respiration.

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