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THE EFFECT OF SOME DRUGS ON THE "FREE" AND "BOUND" ACETYLCHOLINE CONTENT OF RAT BRAIN

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A number of drugs alter the acetylcholine content of the brain. In some instances the origin of this effect is clear. Thus the increase in the brain acetylcholine content which occurs during anaesthesia is probably caused by a decreased liberation of the transmitter and the similar change which follows administration of eserine is caused by the preservation of acetylcholine released in the course of normal neuronal activity. In other instances, however, it is less easy to see why the acetylcholine content has altered and it is correspondingly more difficult to determine whether the observed effect is the cause or the result of the drug's action. A study of the effects of the drug on the cholinesterase and choline acetyltransferase systems *in vitro* may provide additional clues but these are often insufficient to provide an answer to the question whether the action of the drug can be attributed to its intervention in the processes controlling the production, release or inactivation of acetylcholine. With these considerations in mind, an attempt has been made to obtain more information by partitioning the acetylcholine of the brain into "free" and "bound" components and determining the effects of some drugs on these acetylcholine fractions. The results of this study are presented here.

No assertion is made concerning the identity of the "free" and "bound" acetylcholine fractions but the results do reveal a regularity in the response of the fractions to different types of drugs and suggest that simple fractionation of the brain acetylcholine in this way may lead to a substantial increase in our knowledge of the mode of action of centrally acting drugs.

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METHODS

Female Wistar rats weighing from 80 to 100 g were killed by decapitation and the excised brains were gently homogenized in ice cold normal saline solution containing eserine sulphate (15 μg/ml.) and cupric chloride (17 μg/ml.). The volume of medium used was 5 ml./g wet weight of brain. The homogenizer used in these experiments had a stainless steel blade rotating at a variable speed in a 15 ml. vortex flask. The speed controller on the machine was replaced by a calibrated Variac rheostat which permitted a more precise regulation of speed. Preliminary experiments had shown that with the homogenizer used a speed of 1,200 rev/min was the minimum required to obtain a thorough disintegration of brain tissue. The amount of acetylcholine extracted from the brain in these conditions rose rapidly during the first 2.5–3.0 min of homogenization and thereafter rose very slowly.

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Throughout this paper "free" acetylcholine, therefore, refers to that portion of the brain's total acetylcholine which is extracted by homogenization at 1,200 rev/min for 3 min and at 0° C in the medium described. The purpose of the cupric chloride is to prevent synthesis of acetylcholine which may otherwise occur during extraction in saline media. After extraction of the "free" acetylcholine in this way, the homogenate was centrifuged and the supernatant solution was adjusted to pH 4 with 0.5N hydrochloric acid.

Extraction of "bound" acetylcholine

The residue left after extraction of "free" acetylcholine was ground for 5–10 min with an acid-ethanol solution at 0° C. The solution was made by adding 2 ml. of glacial acetic acid to 1 l. of ethanol; it was added to the brain tissue in the ratio of 5 ml. of solution to every g of original brain tissue. The mixture was allowed to stand for 20 min and was then centrifuged. The supernatant was decanted and the residue washed twice with 75% ethanol containing glacial acetic acid 15 ml./l. (2.5 ml./g of brain). The supernatants were combined, diluted with 5 ml. of water and the volume reduced to 1–2 ml. in a stream of air at 35°–45° C. The pH of the solution was adjusted to 4 with 0.5N sodium hydroxide and the extracts were stored at 4° C until they were assayed.

Assays were performed using the frog rectus abdominis muscle sensitized with neostigmine bromide (10^{-6}). The method originally due to Feldberg (1945) was used to compensate for the presence in the extracts of material which might otherwise increase the response of the frog rectus to acetylcholine.

Drugs used were pentobarbitone sodium (Nembutal), urethane, chloral hydrate, eserine sulphate, neostigmine methylsulphate, atropine sulphate, morphine sulphate and nalorphine hydrobromide, all administered by intraperitoneal injection. Tremorine dihydrochloride was given subcutaneously and ether was administered from a pad of cotton wool. Doses of drugs are expressed in terms of their salts and are given in the appropriate tables.

RESULTS

The effects of anaesthetic drugs on the brain content of "free" and "bound" acetylcholine are shown in Table 1. It can be seen that each drug produced increases in the amounts of both "free" and "bound" acetylcholine but these changes were of equal proportionate magnitude. Consequently the ratio of free to total acetylcholine was not altered.

TABLE 1

EFFECT OF ANAESTHESIA ON THE "FREE" AND "BOUND" ACETYLCHOLINE CONTENT OF RAT BRAIN

Results (means and standard errors) are expressed as μg/g fresh tissue. Each drug was administered by intraperitoneal injection and the animals were killed after 30 min of anaesthesia. N.S., not significant.

Drug	Dose (mg/kg)	No. of rats		Free	Bound	Total	Free/ Total
None	—	18		0·40±0·03	2·82±0·14	3·22±0·13	12·8±1·1
Pentobarbitone	50	11		0·68±0·08	4·56±0·35	5·24±0·32	14·6±1·8
			Percentage change	+70	+62	+63	+14
			Significance (*P*)	<0·001	<0·001	<0·001	N.S.
None	—	6		0·58±0·09	3·24±0·12	3·82±0·13	15·0±2·2
Chloral hydrate	350	6		0·83±0·13	5·07±0·31	5·90±0·35	14·0±1·8
			Percentage change	+43	+56	+54	−7
			Significance (*P*)	N.S.	<0·001	<0·001	N.S.
None	—	4		0·31±0·03	3·16±0·05	3·46±0·05	8·8±0·6
Urethane	1,000	4		0·47±0·08	5·16±0·34	5·63±0·31	8·5±1·6
			Percentage change	+52	+63	+63	−3
			Significance (*P*)	N.S.	<0·001	<0·01	N.S.
Ether	—	4		0·43±0·07	4·08±0·14	4·51±0·17	9·5±1·
			Percentage change	+39	+29	+30	+7·5
			Significance (*P*)	N.S.	<0·001	<0·001	N.S.

TABLE 2

EFFECT OF MORPHINE AND NALORPHINE ON THE "FREE" AND "BOUND" ACETYL CHOLINE CONTENT OF RAT BRAIN

Results (means and standard errors) are expressed as μg/g fresh tissue. Each drug was administered by intraperitoneal injection. N.S., not significant.

Drug	Dose (mg/kg)	No. of rats		Free	Bound	Total	Free/ Total
None	—	7		0·36±0·03	2·50±0·11	2·86±0·12	12·7±0·7
Morphine sulphate	100	8		0·29±0·02	3·31±0·15	3·59±0·16	7·7±0·5
			Percentage change	−22	+32	+26	−39
			Significance (*P*)	<0·05	0·001	<0·01	<0·001
Morphine + nalorphine	100 20	6		0·32±0·03	2·34±0·06	2·66±0·13	12·0±1·1
			Percentage change	−10	−6	−6	−6
			Significance (*P*)	N.S.	N.S.	N.S.	N.S.

The administration of morphine (Table 2) produced similar effects to the anaesthetics inasmuch as the amounts of "bound" and total acetylcholine were increased. The amount of "free" acetylcholine was reduced, however, and as a consequence of these changes the ratio of "free" to total acetylcholine was also reduced. When morphine was given in combination with nalorphine the changes in "free" and "bound" acetylcholine did not occur.

The changes which occurred in the "free" and "bound" acetylcholine content of brain following the administration of tremorine, eserine, neostigmine and atropine are

TABLE 3

EFFECT OF TREMORINE, ESERINE, NEOSTIGMINE AND ATROPINE ON THE "FREE" AND "BOUND" ACETYLCHOLINE CONTENT OF RAT BRAIN

Results (means and standard errors) are expressed as μg/g fresh tissue. N.S. not significant.

Drug	Dose (mg/kg)	No. of rats		Free	Bound	Total	Free/ Total
None	—	6		0·43±0·04	2·88±0·13	3·31±0·12	13·1±1·2
Tremorine	75	6		0·78±0·09	3·75±0·2	4·53±0·33	17·5±2·2
			Percentage change	+81	+30	+37	+34
			Significance (*P*)	0·006	0·038	0·001	0·01
None	—	18		0·40±0·03	2·82±0·14	3·22±0·13	12·8±1·1
Eserine	0·75	10		0·77±0·06	3·60±0·22	4·37±0·21	18·0±1·8
			Percentage change	+93	+28	+36	+41
			Significance (*P*)	<0·001	0·005	<0·001	0·01
None	—	8		0·33±0·02	3·21±0·08	3·53±0·08	9·2±0·6
Neostigmine	0·5	8		0·49±0·04	4·18±0·32	4·67±0·33	10·7±1·0
			Percentage change	+49	+30	+32	+16
			Significance (*P*)	<0·01	<0·05	<0·01	N.S.
None	—	4		0·38±0·04	3·22±0·33	3·60±0·28	10·9±1·5
Atropine	25	4		0·40±0·05	2·04±0·15	2·44±0·18	16·3±1·6
			Percentage change	+5	−36	−32	+50
			Significance (*P*)	N.S.	<0·02	<0·02	<0·05
None	—	4		0·37±0·03	3·33±0·36	3·70±0·37	10·2±1·1
Tremorine + atropine	75 5	5		0·53±0·04	2·74±0·20	3·26±0·22	16·3±1·2
			Percentage change	+43	−18	−12	+60
			Significance (*P*)	0·01	N.S.	N.S.	0·02
Eserine + atropine	0·75 20	5		1·00±0·05	3·49±0·58	4·50±0·62	23·9±3·1
			Percentage change	+170	+5	+21	+134
			Significance (*P*)	0·001	N.S.	N.S.	0·01

shown in Table 3. Both tremorine and eserine produced increases in the amounts of "free," "bound" and total acetylcholine but the "free" fraction was increased to a greater extent than the bound acetylcholine. As a result the ratio of "free" to total acetylcholine was increased. The changes produced by neostigmine were very similar to those produced by eserine but were not of the same magnitude and consequently the increase in the ratio of "free" to total acetylcholine did not reach statistical significance. The effect of atropine was to decrease the "bound" acetylcholine fraction without producing any change in the amount of "free" acetylcholine.

When tremorine and eserine were given in combination with atropine, the "free" acetylcholine increased while the "bound" acetylcholine did not change. The ratio of "free" to total acetylcholine was significantly increased.

DISCUSSION

The results presented in this paper make it clear that different groups of drugs may differ in the extent to which they affect the "free" and "bound" fractions of brain acetylcholine. These differences (which are summarized in Table 4) would not have been evident if only the total acetylcholine content of the brain extracts had been assayed and they could not, in general, have been predicted from a knowledge of the action of the drugs on the choline acetyltransferase and cholinesterase systems of the brain.

TABLE 4

SUMMARY OF THE EFFECTS OF DRUGS ON THE "FREE" AND "BOUND" ACETYLCHOLINE CONTENT OF RAT BRAIN

↑ = increase; ↓ = decrease; → = no change.

	Free	Bound	Total	Free/Total
Anaesthetics	↑	↑	↑	→
Morphine	↓	↑	↑	↓
Eserine	↑	↑	↑	↑
Tremorine	↑	↑	↑	↑
Atropine	→	↓	↓	↑

All the anaesthetics tested caused an increase in the acetylcholine content of the brain but because the "free" and the "bound" fractions were affected to the same relative extent no change in the "free": total ratio occurred. It is a well-established fact that anaesthesia is associated with an increase in the acetylcholine content of brain (Tobias, Lipton & Lepinat, 1946; Richter & Crossland, 1949; Crossland & Merrick, 1954) and it is generally accepted that this increase is the result of a reduced release of acetylcholine consequent on the decreased neuronal activity. Direct evidence for a reduction in acetylcholine release comes from the work of MacIntosh & Oborin (1953), Mitchell (1963) and Beleslin, Carmichael & Feldberg (1964). It is not immediately obvious why anaesthesia should affect the "free" and "bound" acetylcholine fractions to the same extent and no useful purpose would be served by discussing the possibilties in detail because the physiological significance of the "free" and "bound" fractions is not yet clear. We tentatively suggest, however, that this pattern of change in the two fractions is characteristic of conditions in which central nervous activity is reduced by drugs which do not have any direct effect on the systems responsible for the synthesis, release or destruction of acetylcholine. If this is a valid generalization it would suggest that

morphine—a central depressant which increases the total acetylcholine content of brain but reduces the content of " free " ester—does interfere with these processes. There is a considerable amount of evidence that this is so. Morphine reduces acetylcholine release in the isolated guinea-pig ileum (Paton, 1957; Schaumann, 1957) and in the brain (Beleslin & Polak, 1965; Beleslin, Polak & Sproull, 1965) and the results presented in this paper are consonant with the view that, after morphine administration, acetylcholine release is reduced by a direct action of the drug rather than as a simple consequence of reduced neuronal activity. The effects of nalorphine are particularly interesting in this connection. Nalorphine, as is well known, reverses the actions of morphine. It also abolishes the effects of morphine on the " free " and "bound " acetylcholine fractions of brain (Table 2). Moreover Shaw & Bentley (1952) observed that the depressant effects of morphine were antagonized by eserine, which increases the amount of " free " acetylcholine in the brain. These facts, taken together, suggest that the depressant effects of large doses of morphine may be the result of a reduction in the rate of release of acetylcholine.

The present results provide additional information concerning the possible mode of action of tremorine. It was already known that the onset of tremors induced by tremorine coincided with an increase in the acetylcholine content of brain (Pepeu, 1963; Holmstedt, Lundgren & Sundwall, 1963). It had also been reported that doses of atropine which prevented the actions of tremorine also prevented the increase in the brain acetylcholine content (Holmstedt, Lundgren & Sundwall, 1963). It is clear from the figures presented in Table 3, however, that atropine only causes a diminution in the " bound " acetylcholine fraction. Tremorine increases the content of both " free " and " bound " acetylcholine in the brain. When the two drugs are given together the " bound " acetylcholine content falls, presumably because the depressant effect of atropine on this fraction more than compensates for the effect of tremorine but the amount of free acetylcholine increases to the same extent as when tremorine is given alone. Nevertheless, because the " free " fraction forms but a small proportion of the total brain acetylcholine content, the latter does not increase. It seems reasonable to conclude that the action of atropine in preventing the effects of tremorine does not lie entirely in its ability to prevent an increase in the total acetylcholine content of brain. At least part of its action seems to reside simply in its ability to block the muscarinic actions of the increased amounts of available acetylcholine which are reflected in the elevated content of " free " acetylcholine.

Although tremorine does not inhibit cholinestrase it produces effects (salivation and tremors) which are similar to those seen after administration of an anticholinesterase. A similarity between the electroencephalographic effects of tremorine and eserine has also been reported (Everett, 1964). It is therefore interesting to note (Table 3) that eserine and tremorine have strikingly similar effects on the " free " and " bound " acetylcholine of the brain. The pharmacological actions and the acetylcholine changes produced by tremorine and eserine are similarly affected by atropine.

Neostigmine had effects on the " free " and " bound " acetylcholine which though qualitatively similar, were quantitatively less marked than those produced by eserine. The behavioural changes were also less intense. The less obvious effects of neostigmine in comparison with those of eserine are presumably due to the less effective penetration of the drugs into the brain tissue.

It is suggested that the fractionation of brain acetylcholine into its "free" and "bound" components may provide a useful additional means of elucidating the modes of action of drugs which interact with cholinergic mechanisms in the brain.

SUMMARY

1. Changes in the "free" and "bound" acetylcholine content of brain following the administration to rats of drugs which act on the central nervous system are described.

2. Anaesthetics, morphine and anticholinesterase compounds increase the total amount of acetylcholine in brain but have different effects on the ratio of "free" to total acetylcholine.

3. The results reveal a close similarity between the effects of eserine and tremorine on the "free" and "bound" acetylcholine content of brain.

4. An excess of "free" acetylcholine seems to be implicated in the production of tremor by tremorine.

5. The effects of atropine and morphine on "free" and "bound" acetylcholine may provide further information regarding some of the actions of these drugs on the central nervous system.

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