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DRUG INDUCED CHANGES IN FREE, LABILE AND STABLE ACETYLCHOLINE OF GUINEA-PIG BRAIN*

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Abstract—A simplified procedure to separate free, labile and stable acetylcholine (ACh) fractions from guinea-pig cerebral cortex is described. The time course of the effect exerted by some drugs on the three fractions is investigated. ACh-depleting agents, as pentylenetetrazol and scopolamine, mainly decrease both "bound" (labile and stable) fractions. Both 3 hr after a single dose and following sub-acute treatment with scopolamine, choline acetyltransferase activity increases: at this time the depleting effect of the drug on the labile fraction is less evident. These findings suggest that stable ACh represents the pool actually available for release, whilst labile ACh is the newly synthetized transmitter, designed to refill the vesicular stores. Anaesthetic agents, as thiopental and γ-hydroxy-butyric acid, increase only the free and labile fractions.

(i) the stable pool seems to be saturated even in the waking animal, and (ii) although the transmitter release is reduced by anaesthesia, the synthesis continues, so that the free and labile fractions increase. Eserine mainly affects "free" ACh and increases both bound fractions in a parallel fashion; the drug does not change, however, the net depleting effect of pentylenetetrazol. These findings, discussed on the basis of Whittaker's scheme of intraneuronal transmitter compartmentation, agree with the view that the three subcellular ACh fractions have different physiological meanings.

INTRODUCTION

THE DEVELOPMENT of subcellular fractionation techniques combined with electrophysiological and electronmicroscopic investigations[1–6] gave rise to the hypothesis[7] that brain acetylcholine (ACh) is stored in three neuronal compartments: (i) the somato-axonic structures; (ii) the synaptosomial cytoplasm; (iii) the vesicles. These could correspond to, respectively, the so-called free, labile and stable ACh fractions, separated by means of suitable fractionation procedures. Some doubts, however, exist on the significance of free ACh: it could either originate from somato-axonic structures and ruptured synaptosomes, or it could, at least in part, represent a leakage of neurotransmitter from the particulate fractions. The investigations carried out on the intracellular distribution of choline acetyltransferase (EC 2.3.1.6, ChAc)[8,9] strengthen the idea that ACh synthesis occurs in the cytoplasm; the neurotransmitter is subsequently stored in the vesicles, at such a high concentration[10] that any further synthesis is inhibited,[11] even if part of the enzyme would be present in the vesicles or adsorbed to them.[12,13]

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Recent experiments show that vesicular ACh does not easily exchange with the cytoplasmic synaptosomial ACh pool;[14] this second compartment can slowly exchange only with extraneuronal ACh, present at high concentrations in the medium. Taking into account that in normal conditions the cholinesterases present in the axonal membrane make the reuptake of intact ACh unlikely, the following temptative[9] picture may be suggested:[15,16] the ACh synthetized in the cytoplasm is designed to reintegrate vesicular stores, i.e. the pool actually available for release. After the release, ACh acts on the membrane receptors and undergoes hydrolysis: choline reuptake, in turn, ensures part of the substrate required for the resynthesis. The amounts of somato-axonic, synaptosomial and vesicular ACh (and the ratios between them) may therefore depend both on the firing rate of cholinergic neurones and on the efficiency of ACh synthesis, storage and release.

Up to today only a few investigations were carried out with the aim of assessing whether pathological conditions or drug treatment could modify the pattern of ACh subcellular distribution.[17,18]

In this report we are attempting to check the hypothesis advanced by Whittaker[7,16] by analysing the time course of the changes in free, labile and stable ACh brought about by drugs which either exert an overall change in the CNS functional level or act on some steps of ACh action and metabolism. A preliminary account of this investigation was presented at the Italian Pharmacological Congress, Palermo 1965.[19]

EXPERIMENTAL

Guinea-pigs of both sexes, weighing 300–400 g, were used. Beheading of animals, removal of brain and preparation of samples were carried out in a refrigerated room at 0–2°. The procedure was as follows:

the right and left sides of the cerebral cortex were removed as quickly as possible, weighed and plunged into an ice-cold solution of sucrose 0·32 M plus eserine 1×10^{-5} g/ml to obtain a 10 per cent final concentration of tissue. The tissue was homogenized in a glass-teflon homogenizer according to Aldridge,[20] clearance 0·25 mm, at 840 revs/min for 30 sec.

Microscope observation showed almost complete rupture of the cells. Part of the homogenate was used to extract total ACh (S_t, see below), the remainder was centrifuged at 100·000 *g* for 1 hr in a refrigerated centrifuge model MSE super speed 50. The supernatant (S_1) was collected and stored at −20° until the bioassay of "free" ACh was performed. The precipitate was resuspended in ice-cold solution of sucrose 0·032 M plus eserine 1×10^{-5} g/ml, turning the pestle of the homogenizer two or three times up and down, by hand. After 20 min, the suspension was centrifuged at 100·000 *g* for 1 hr. The supernatant (S_2) was collected, brought to 0·32 M by adding concentrated sucrose solution and stored at −20° until the bioassay of the "labile" ACh was performed. Then the second precipitate was resuspended in 0·32 M sucrose solution, brought to pH4 with McIlvain buffer[21] and boiled for 10 min. After centrifuging at 5000 revs/min for 20 min, the supernatant (S_3) was collected, buffered at pH7 and employed for the "stable" ACh bioassay. A similar procedure was followed for extracting total ACh from part of the first homogenate of the whole tissue (S_t).

Standards were prepared by adding known AChCl amounts plus eserine 1×10^{-5} g/ml to part of S_1, S_2, S_3 and S_t supernatants previously kept at 37° for 30 min at pH 10.

The bioassay was carried out on eserinized frog rectus abdominis muscle. The values of free, labile and stable ACh were used to calculate, in each experiment, the particulate (labile + stable)/free ACh ratio (P/S), and labile/stable ACh ratio (L/ST). The percent recovery of ACh at the end of fractionation procedure was determined by the ratio $(S_1 + S_2 + S_3) \times 100/S_t$.

In some experiments the ChAc activity of the cerebral cortex was determined according to Bull *et al.*;[22] the acetone powder samples were prepared according to Bianchi *et al.*[23] Boeringher reagents and substrates were used; the bioassay was performed on the frog rectus abdominis muscle and the activity expressed as μg of AChCl formed/hr/g of fresh tissue. Freshly prepared solutions of scopolamine bromide, eserine sulphate, thiopental sodium, gamma-hydroxybutyric acid (sodium salt) (γ-OH), pentylenetetrazol (PTZ) were injected i.p. In the text the dose and concentrations are given as salts.

RESULTS

(A) *Control of the method and acetylcholine distribution in the cerebral cortex of normal guinea-pigs*

The estimation of total ACh was made so as to control whether hydrolysis or synthesis occurred during the experimental procedure. In most of the experiments the percentage recovery of ACh ranged between 105 and 95 per cent.

A few experiments, showing a recovery below 80 per cent or above 120 per cent, were discarded. Our findings on ACh distribution are well in agreement with the figures reported by Whittaker;[2] free ACh was 18 per cent and both labile and stable fractions were 41 per cent of the total amount (Tables 1 and 2, controls). The normal particulate:free (P:S) and labile:stable (L:ST) ratios were 4·66 and 1·09 respectively.

(B) *Effect of drugs which lower total brain acetylcholine*

PTZ was injected i.p. at 80 mg/kg as 5% solution in H_2O: propyleneglycol 1:1; convulsive seizures began about 2 min after injection and occurred repeatedly for some minutes. Some of the animals were killed 30 sec, some 3 and others 6 min after the first convulsion. In this last group some guinea-pigs were recovering, others were almost dead, with clear signs of deep post-seizure neurodepression. The brains of dying animals, examined separately, showed nearly normal ACh levels; in the other groups the amount of total ACh was significantly below the control values (Table 1). The reduction only concerned bound ACh (labile and stable), so that the P:S ratio decreased. The L:ST ratio decreased 3 mins after the onset of seizures. This is in agreement with the observations of Kurokava *et al.*[17] who found that, on mice having spontaneous convulsions, the decrease in the labile ACh fraction prevailed on the others. Scopolamine 5 mg/kg i.p. did not cause any evident behavioural change in the animals at any time, but as early as 2 min after injection the ACh content fell to very low levels, because of the strong reduction in both the bound fractions. Free ACh was also involved at 5, 20, 60 min after injection. Its change, however, was not so evident as that shown by both the bound fractions; the P:S ratio was therefore significantly decreased (Table 1).

Three hr after scopolamine, when the depleting effect of the drug was vanishing, only bound ACh remained below the control levels; at this time, as occurred 1 hr

TABLE 1. TIME COURSE OF THE EFFECT EXERTED BY SCOPOLAMINE (5 mg/kg, i.p.) AND PENTYLENETETRAZOL (80 mg/kg, i.p.) ON TOTAL AND FRACTIONAL ACETYLCHOLINE (μg/g $\pm$ S.D.) OF GUINEA-PIG CEREBRAL CORTEX

Treatment	Time of killing after injection (no. of animals)	Acetylcholine μg/g $\pm$ S.D.					Ratio $\pm$ S.D.	
		S_1 (free)	S_2 (labile)	S_3 (stable)	S_t (total)	% recovery $\pm$S.D.	P/S	L/ST
Controls	(23)	0·567±0·124	1·319±0·188	1·219±0·205	3·028±0·392	102·8±10·6	4·66±1·04	1·09±0·17
Scopolamine 5 mg/kg	2 min (8)	0·561±0·147	0·883±0·129‡	0·878±0·141‡	2·312±0·229‡	100·7±9·9	3·31±1·01†	1·01±0·16
	5 min (10)	0·430±0·106†	0·780±0·178‡	0·720±0·165‡	1·853±0·358‡	104·5±6·4	3·59±0·83†	1·09±0·18
	20 min (10)	0·430±0·094†	0·712±0·165‡	0·646±0·144‡	1·769±0·337‡	101·1±13·7	3·23±0·81‡	1·11±0·22
	60 min (8)	0·355±0·066‡	0·757±0·276‡	0·568±0·170‡	1·643±0·477‡	102·0±7·1	3·82±1·34	1·32±0·26†
	180 min (9)	0·502±0·086	1·051±0·231†	0·834±0·158‡	2·427±0·471†	98·8±8·4	3·79±0·63*	1·27±0·24*
	24 hr (7th daily dose) (7)	0·585±0·130	1·212±0·246	1·222±0·354	2·871±0·610	102·3±8·5	4·08±0·96	1·15±0·35
	1 hr (8th daily dose) (8)	0·555±0·132	1·056±0·115†	0·748±0·137‡	2·407±0·276†	98·1±5·0	3·39±0·67†	1·46±0·40‡
Pentylenetetrazol 80 mg/kg	30 sec (12)	0·513±0·107	0·854±0·090‡	0·750±0·131‡	2·144±0·277‡	98·8±6·3	3·19±0·46‡	1·16±0·20
	3 min (8)	0·608±0·102	0·860±0·116‡	0·931±0·110‡	2·357±0·148‡	102·3±7·5	3·03±0·73†	0·92±0·13*
	6 min (10)	0·516±0·087	0·752±0·159‡	0·756±0·132‡	1·996±0·260‡	101·3±2·3	2·98±0·68‡	1·00±0·21
	6 min (dying) (9)	0·653±0·098	1·290±0·160	1·122±0·164	3·020±0·304	101·5±1·4	3·77±0·77*	1·16±0·20

The % recovery and the ratios particulate/free ACh (P/S) and labile/stable ACh (L/ST) were calculated in each experiment. In brackets the number of the animals.

* = Significantly different from the controls value, $P < 0·05$.

† = $P < 0·01$.

‡ = $P < 0·001$.

TABLE 2. TIME COURSE OF THE EFFECT EXERTED BY THIOPENTAL (20 mg/kg, i.p.), γ-HYDROXY-BUTYRIC ACID (1 g/kg, i.p.) AND ESERINE (1 mg/kg, i.p.) ON TOTAL AND FRACTIONAL ACETYLCHOLINE (μg/g ± S.D.) OF GUINEA-PIG CEREBRAL CORTEX. THE EFFECTS OF PENTYLENETETRAZOL IN THIOPENTAL AND ESERINE-PRETREATED ANIMALS ARE ALSO SHOWN

Treatment	Time of killing after injection (no. of animals)	Acetylcholine μg/g ± S.D. S_1 (free)	S_2 (labile)	S_3 (stable)	S_t (total)	% recovery ± S.D.	Ratio ± S.D. P/S	L/ST
Controls	—— (23)	0·567±0·124	1·319±0·188	1·219±0·205	3·028±0·392	102·8±10·6	4·66±1·04	1·09±0·17
Thiopental 20 mg/kg	5 min (8)	0·543±0·069	1·215±0·145	1·095±0·128	2·872±0·401	99·8±6·7	4·27±0·49	1·11±0·10
	10 min (10)	0·747±0·105†	1·406±0·204	1·314±0·163	3·400±0·449*	102·1±5·0	3·79±0·82*	1·08±0·24
	15 min (8)	0·898±0·189‡	1·750±0·250‡	1·301±0·240	3·718±0·617‡	106·7±6·2	3·49±0·77†	1·37±0·23‡
	30 min (10)	0·724±0·075†	1·149±0·147*	1·335±0·134	3·160±0·201	101·6±4·1	3·48±0·41†	0·86±0·15†
	60 min (12)	0·722±0·107†	1·248±0·151	1·146±0.100	3·052±0·100	102·3±3·4	3·41±0·63‡	1·08±0·17
Thiopental 20 mg/kg + Pentylenetetrazol 80 mg/kg 12 min later	15 min (12)	0·492±0·134	0·859±0·224‡	0·765±0·175‡	2·050±0·500‡	103·7±7·3	3·46±0·91†	1·11±0·09
Gamma-hydroxy-butyric acid 1 g/kg	60 min (8)	0·877±0·145‡	1·968±0·390‡	1·331±0·246	4·368±0·684‡	95·7±7·7	3·76±0·35*	1·48±0·12‡
	1 hr (5th daily dose) (11)	1·053±0·156‡	2·104±0·238‡	1·559±0·175‡	4·970±0·499‡	96·1±7·0	3·52±0·51†	1·35±0·19‡
	12 hr (5th daily dose) (11)	0·582±0·186	1·359±0·218	1·028±0·169†	2·912±0·532	102·3±4·8	4·38±1·23	1·32±0·12‡
Eserine 1 mg/kg	20 min (8)	1·040±0·262‡	1·662±0·261‡	1·493±0·132†	4·262±0·592‡	98·9±6·3	3·17±0·76‡	1·10±0·15
	180 min (8)	0·570±0·114	1·427±0·263	1·211±0·145	3·350±0·400	95·9±9·6	4·74±0·95	1·16±0·15
Eserine 1 mg/kg Pentylenetetrazol 80 mg/kg 17 min later	20 min (12)	1·105±0·243‡	1·462±0·258	1·180±0·171	3·479±0·447*	107·6±8·6	2·50±0·59‡	1·24±0·18*

The % recovery and the ratios particulate:free ACh (P:S) and labile:stable (L:ST) were calculated in each experiment. In brackets the number of the animals.
* = Significantly different from the controls value, $P < 0·05$.
† = $P < 0·01$.
‡ = $P < 0·001$.

after, the L:ST ratio was significantly higher than that in the controls, following the persistent reduction in the stable fraction.

In order to ascertain whether repeated drug injections were associated with changes in the pattern of the depleting effect, some guinea-pigs received scopolamine 5 mg/kg, i.p. every day for 8 days. Some of the animals were killed immediately before the eighth injection, the others 1 hr after. No evident change in total and fractional ACh was found 24 hr after the seventh injection; the depleting effect of the drug was still significant 1 hr after the eighth dose, but it was smaller than that detected after the single injection (Table 1). It is interesting to point out that ChAc activity in the cerebral cortex increased both 3 hr after the single injection and 24 hr after 7th dose: the enzymatic activity in sixteen controls was 1010 ± 170 (S.D.) μg of ACh formed/g of fresh tissue/hr; in ten animals, 3 hr after injecting the drug, it was 1383 ± 336 ($P < 0{\cdot}01$) and in 7-day-treated guinea-pigs (seven animals) it was 1211 ± 163 ($P < 0{\cdot}01$). These results confirm our previous findings[24] and explain why the depleting effect of the eighth injection was reduced and why the L:ST ratio was increased 3 hr after the single dose.

(C) *Effect of drugs which increase total brain acetylcholine*

Following the anaesthetic dose of thiopental (20 mg/kg, i.p.), the changes in the ACh fractions were very complex. Free ACh increased more or less proportionally to total ACh up to its maximum at the 15th minute when the neurodepression was at the peak; moreover, it was still significantly higher 1 hr after the drug, when the animals recovered and total ACh levels approached normal values. Bound ACh did not change appreciably till 10 min after injection; at the 15th minute it increased to over 20 per cent, only the labile fraction being affected; the stable fraction remained just a little above the control level (Table 2).

In waking animals (30 min after thiopental) the amount of stable fraction was still normal, but the labile fraction fell below. Consequently the L:ST ratio increased at the 15th minute and decreased at the 30th minute. The P:S ratio was reduced at the various times as a consequence of the considerable change occurring in the free fraction. A similar pattern of distribution changes, i.e. increase exclusively of free and labile ACh, was given by γ-OH acid anaesthesia (1 g/kg i.p.). In this case the neurodepression peak was reached after 60 min and lasted for several hours; the increase in total ACh and in free and labile fractions was more evident than after thiopental (Table 2). The recovery from this type of anaesthesia was not investigated because the waking state was reached slowly, as seen by the recovery of the righting reflex and of the normal behaviour.

The fact that a single injection of an anaesthetic drug did not modify the stable ACh pool was in agreement with previous findings reported by Kurokava *et al.*[17]

It was considered of some interest to test whether prolonged neurodepression would or would not be able to modify the stable fraction. With this aim, twenty-two guinea-pigs were injected with γ-OH 1 g/kg every morning for 5 days. Some of the guinea-pigs were sacrificed 1 hr after the 5th injection, some 12 hr later. The effect of repeated anaesthetic drug administration on total ACh was greater than that of one single injection, but the changes in the P:S and L:ST ratios were similar. At variance with the acute treatment, the stable fraction increased 1 hr after the 5th dose and fell below the normal values after 12 hr. This finding suggests that during prolonged

repeated neurodepression the cholinergic neurones temporarily acquire the ability of increasing the compartment of stable ACh and that in the waking state this ability is soon lost. In this condition there is no concomitant change in the ACh synthesizing activity: ChAc in the cerebral cortex, determined 12 hr after the 5th dose, was in fact 1078 ± 71 (five animals) against 1010 ± 70 in sixteen guinea-pig controls. The pattern of the changes in ACh subcellular distribution induced by eserine was substantially different from that brought about by the anaesthetic drugs. eserine 1 mg/kg i.p. gave rise to only mild signs of muscarinic excitation, as increased salivation, without any apparent behavioural effect. Concomitantly, the increase in total brain ACh was associated to the increase in all the three ACh fractions (Table 2). The drug affected more the free than the bound ACh so that the P:S ratio decreased; unlike the anaesthetic drugs, eserine did not modify the L:ST ratio, both the bound pools increasing in a parallel fashion. The recovery to normal values was reached 3 hr after injection. These findings seem to show that the increase in the stable compartment may be obtained more easily with the inhibition of esterases than with the depression of neuronal activity.

(D) *The effect of pentylenetetrazol in eserine- and thiopental-pretreated animals*

These experiments were performed in order to obtain further information on the significance of drug induced changes in ACh subcellular distribution. PTZ was chosen so as to increase ACh release by stimulating the brain stem corticipetal structures[25] without directly affecting the ACh metabolism or its physiological actions. One group of guinea-pigs received eserine 1 mg/kg and, 17 min later, PTZ 80 mg/kg. The animals were killed at 20th minute, i.e. 1 min after beginning of convulsions. The total ACh levels were significantly higher than in the controls, but lower than in animals receiving only eserine. The labile and stable pools showed changes in the opposite sense, so that the L:ST ratio increased. The most prominent feature was the extremely high value of free ACh: P:S ratio was halved with respect to the normal value (Table 2).

Other guinea-pigs received thiopental 20 mg/kg and, 12 min later, PTZ 80 mg/kg. Within 3 min the animals woke up, recovered their righting reflex but had no convulsions. At this moment they were killed: as shown in Table 2, total and fractional ACh values were about the same as in the animals killed 3–6 min after receiving PTZ alone. The drug was therefore able to fully overcome both the neurodepression and the biochemical changes brought about by Thiopental in the ACh stores.

DISCUSSION

In the simplified procedure we employed to separate brain ACh into three subcellular fractions, the amount of ACh present in the coarse precipitate contributes to the values of labile and stable pools found by us. In this coarse precipitate, however, ACh is only 12 per cent of the total; therefore this small amount cannot appreciably influence the detection of the actual changes occurring in the labile and stable pools. The use of two high-speed centrifugations simplifies and shortens the experimental procedure, giving fractional values of free, labile and stable ACh which are in agreement with those determined by Whittaker[2] in guinea-pig brain. Both scopolamine and PTZ are known to increase ACh release from the exposed cerebral cortex, thus lowering its tissue levels.[26–31]

These drugs do not seem to directly affect neurotransmitter synthesis and hydrolysis;[28] the effect of PTZ may be referred to the stimulation of the brain stem and cerebral structures[25] where scopolamine seems to act by interrupting a negative feedback mechanism on the cortical cholinergic nerve-endings, a mechanism which is linked to or promoted by nerve-conducted activity.[32,33]

From our findings, bound ACh appears to be more affected than free ACh, in agreement with the recent data reported by Crossland and Slater.[18]

If the stable fraction represents the immediately available store for release,[16] a high equilibrium rate must exist between the labile and the stable pools, to compensate for the increased neurosecretion: in fact the changes in the L:ST ratio are not very impressive. It is interesting to note that the effect in the labile ACh is less evident 1–3 hr after scopolamine and following subacute treatment. In these cases ChAc activity increases: this observation gives indirect support to the view that ACh synthesis mostly occurs outside the vesicles, at least in the guinea-pig brain.

The pattern of the free ACh changes occurring after scopolamine and PTZ is in agreement with Whittaker's suggestion that this fraction is not a simple artifact.

Free ACh cannot in fact: (i) be referred to the neurotransmitter released in the synaptic clefts because, otherwise, it should have increased both after scopolamine and after PTZ, (ii) originate from the mechanical disruption of some synaptosomes, because its levels should always have been proportional to those of total and bound ACh. As a matter of fact, a certain reduction occurs after scopolamine, when a strong depletion of both the bound fractions takes place, but this result may be better explained by the attractive hypothesis that some kind of equilibrium exists between the intraneuronal free and labile pool.

The recent evidence[34] that free ACh is particularly sensitive to some choline antagonists further supports the view that it originates from somato-axonic structures.

Other arguments in favour of the functional significance of ACh subcellular fractions are given by the findings obtained with anaesthetic drugs and with eserine. All these drugs increase brain ACh levels,[35–37] the former by reducing its release, the latter by inhibiting its hydrolysis. Both thiopental and γ-OH increase exclusively the free and labile fractions: it seems therefore that: (i) in the normal waking guinea-pigs the stable pool is saturated so that, when the transmitter release is reduced by drugs, it cannot increase further, (ii) during anaesthesia, ACh synthesis continues at a higher rate than that of the storage and release processes thus increasing the labile and free ACh pools, clearly not saturated in the waking animals.

Another point of interest is afforded by the absence of any change in ChAc activity after the repeated, long-lasting neurodepression produced by the 5-day treatment with γ-OH: probably some changes (inductive?) easily occur only when the ACh release increases, i.e. after scopolamine, but not when the release decreases.

The essential role played by esterases in ACh subcellular distribution is pointed out by the results obtained in eserine-treated animals.

Twenty min after injection, the ACh content of the cerebral cortex had a 40 per cent increase. Bearing in mind the relationship between cholinesterase inhibition and increase in brain ACh,[38] the degree of inhibition reached in our experiments was most likely not above 30–40 per cent.

In these conditions, the extremely high values of free ACh may mainly be referred

to the reduced hydrolysis of the neurotransmitter present on the synaptic clefts. Accordingly, these values are still higher in animals treated with Eserine plus PTZ. On the other hand, cholinesterases seem to control the size of both the bound pools, as judged by the consistent increase in the labile and stable fractions. Taking into account that eserine, according to Bertels-Meenws *et al.*,[32] seems to deplete part of the neurotransmitter stores, the observed changes would have been greater with an irreversible esterase inhibitor. In any case, the esterase inhibition determines an intricate condition because it may enable a back-diffusion of outer ACh into the neuronal structures[39] and certainly modifies the significance of free fraction. Nevertheless, the drug does not counteract the depleting effect of PTZ: the net reduction in the total and stable ACh detected 3 min after this drug, both in normal and in eserine-pretreated guinea-pigs, was about 700–800 ng/g and 300 ng/g respectively, in comparison with the control values (normal and eserine-treated animals, see Tables 1 and 2). Obviously, the effect of PTZ was more dramatic when the drug cancelled the Thiopental neurodepression.

In conclusion, our results fit well into and can be explained by Whittaker's scheme of ACh intracellular compartments: the free, labile and stable pools appear to have different functional meanings. Free ACh, although it is the less characterized fraction, probably originates from somato-axonic structures, unless the animals receive esterase inhibitors. An equilibrium seems to exist between free and labile pools, but in some circumstances, as in the first stages of thiopental anaesthesia and in the brief period of PTZ convulsions, only one of the pools is affected.

The P:S ratio always decreases, either owing to the deeper depletion of the bound pools or by the greater increase of free ACh. Labile ACh seems to represent the newly synthetized neurotransmitter in the synaptosomial cytoplasm. A high equilibrium rate ensures the refillment of the vesicular pool. Moreover, in the course of a sustained increase of release, as after scopolamine, subsidiary enzymatic mechanisms compensate for the changes in the synthesis rate. Consequently, the L:ST ratio may vary in opposite directions during drug effect.

The stable pool is saturated in the normal, waking animals, so that it does not increase during anaesthesia. Its prevailing depletion after PTZ and scopolamine confirms that it represents the pool immediately available for release. No doubt definite conclusions concerning the links between the three compartments and the effect of drugs on them, may be reached only by labelling the neurotransmitter present in the compartments themselves, in different degrees.

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