STUDIES ON CHOLINE ACETYLASE (I)

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The discovery of choline acetylase (1) has revealed the position of acetylcholine (ACh) in general metabolism. In view of the fact that certain drugs including narcotics and convulsants have some effects on ACh metabolism of brain, it is reasonable to suppose that some of these drugs might associate with the activity of choline acetylase.

In this paper, we have studied several problems regarding choline acetylase, especially the effects of certain drugs on this enzyme.

METHOD

The method was essentially the same as that of Nachmansohn (2), but in some respects we adopted new procedures, especially in the preparation of enzyme material.

(1) *Enzyme source.* Almost all the experiments were conducted by means of freeze-dried powder of rat or guinea pig brain. The freeze-dried powder weighed 21-22 % of the original fresh tissues, while the acetone-dried powder 19-20 % thereof.

(2) *Extraction and incubation.* One hundred mg. of dried tissue was ground to fine powder, to which 5 cc. of ice-cold solution A (*see below*) was added, mixed up and, after being left as it is for about 15 minutes at 5°C, centrifuged at 1500 × gravity for 15 minutes. To its separated supernatant solution B (*see below*) of equal volume was added, just before the incubation was carried out aerobically at 37°C for 20-150 minutes.

Solution A		Solution B	
KCl	0.15M	ATP	0.003-0.006 M
NaCl	0.04	Choline	0.01
MgCl ₂	0.004	Citrate	0.02
NaF	0.01	Drugs	Twice that of the required concentration
Na ₂ HPO ₄	0.032		
KH ₂ PO ₄	0.008		
Eserine	0.003		
Cysteine	0.006		

pH was adjusted at 6.8

RESULTS

I. Preparation of Enzyme

1) *Comparative examination on various preparations of enzyme.* In order to know the availability of the freeze-dried powder as an enzyme source of choline acetylase, it was compared with acetone dried powder and fresh tissue.

The result, as summarized in Table 1, showed that amount of ACh synthesized during the first 20 minutes' incubation with freeze-dried powder of rat brain as well as guinea pig brain surpassed at the significant level of 0.05 that with acetone dried powder.

TABLE 1. ACh synthesis by various enzyme materials

Enzyme sources		Acetone dried powder			Freeze-dried powder			Fresh tissues			
Time		20 ^m	1 ^h	2.5 ^h	20 ^m	1 ^h	2.5 ^h		20 ^m	1 ^h	2.5 ^h
Rat brain	I	72(360)	250(1250)	500(2500)	98(480)	283(1330)	390(1840)	a	—	46	48
	II	86(430)	178(890)	420(2100)	95(445)	168(790)	358(1680)	b	28	30	30
								c	47	81	93
Guinea pig brain	I	63(315)	118(590)	210(1050)	78(365)	184(875)	285(1350)				
	II	68(340)	120(600)	272(1360)	87(405)	141(665)	298(1415)				

Figures refer to amount of ACh in $\mu\text{g./g.}$ fresh tissue ($\mu\text{g./g.}$ powder shown in parenthesis). Acetone dried powder and freeze-dried one were prepared from the same animal.

(2) *Effect of pH on ACh synthesis.* As shown in Fig. 1, optimum pH for ACh synthesis with freeze-dried powder extracts of rat brain was about 7.4 in the earlier stage of incubation but in the later stage it shifted to acid side.

FIG. 1. Influence of pH on ACh synthesis.
Ordinate: Amount of ACh. Abscissa: pH.

(3) *Determination of ACh.* The amount of synthesized ACh was determined biologically by eserinizated *M. rectus abdominis* of *Rana nigromaculate*. After 20, 60 and 150 minutes' incubation, a small part of incubated mixture was diluted at first.

with 30 volumes of K-free frog-Ringer solution and then with normal frog-Ringer solution until the suitable concentration of ACh for testing muscle, viz. 0.025–0.1 $\mu\text{g. cc.}$, was obtained. Then the response of muscle was observed by the use of this diluted solution. ATP, contained in the mixture, has a sensitizing effect on the response of muscle to ACh, so that the values calculated from the response of muscle are always a little larger than the genuine ones. A correction therefor was made: the calculated amount of ACh in the mixture per cc. was decreased by 0.1 $\mu\text{g.}$ or 0.05 $\mu\text{g.}$ when the test was performed by 50 or 100 times the diluted solution. In case where more than 100 times diluted solution was used, correction was omitted.

The amount of ACh was expressed in terms of ACh-chloride equivalent.

II. Distribution of Choline Acetylase in Central Nervous System

Rat's or guinea pig's central nervous system was dissected into the following five parts.

- A. Almost all parts of cerebral cortex, including the greater part of cerebral medulla and a small part of basal ganglia (the greater part of Ncl. amygdalae and a small caudal part of Claustrum, Putamen and Ncl. caudatus. The total amount of these parts corresponds to only one-tenth of the total basal ganglia).
- B. Diencephalon, ammonhorn, the greater part of basal ganglia and a small part of cerebral cortex.

TABLE 2. ACh synthesis in central nervous system ($\mu\text{g./g. powder. hr.}$)

Region \ Species	Rat		Dog	Guinea pig
A	255	215	260	275
B	545	670	610	680
C	785		560	440
D	50	65	45	115
E	530	500	370	405

As for A, B, C, D and E, see the text.

C. Mesencephalon, medulla and pons.

D. Cerebellum.

E. Spinal cord.

B was separated from A as indicated in Fig. 2, in order to leave basal ganglia in B. Fila olfactoria was omitted.

As for dog, A represents almost pure cortex.

As shown in Table 2, the amount of synthesized ACh was the largest

in brain stem, less in spinal cord and cerebrum, and least in cerebellum.

III. Effect of Drugs on Choline Acetylase

A number of drugs, including convulsants and narcotics were studied with respect to their effect on ACh synthesis by choline acetylase obtained from brain freeze-dried powder (Table 3). ACh determination by M. rectus abdominis of frog was so accurate as to detect even the difference of 3% between two samples. For each drug two controls were tested and when the standard deviation between the two controls was larger than 2%, this experiment was given up. While in case of deviation less than 2%, the mean value of the two was adopted as control. When the amount of synthesized ACh of sample with test drug was larger or smaller than the control by 5% more, the drug was regarded as a significant one.

TABLE 3. Influence of drugs on ACh synthesis

A) Narcotics

Drugs \ Concentration	1 mM	0.1 mM	0.01 mM	Controls
Chloroform	286(100)	286(100)	280(97)	286△
Urethane	520(98)	520(98)	520(100)	520
Barbital-Na	550(90)	610(100)	590(97)	610
Pentobarbital	621(102)	618(101)	602(99)	608
Evipan-Na	-	342(101)	346(102)	240△
Thiopentothal	248(96)	256(98)	262(101)	260△
Eunarcon*	256(102)	260(103)	252(100)	252△
Phenobarbital	258(100)	264(102)	254(98)	258△
Morphine	302(97)	314(101)	314(101)	310
KBr	495(98)	520(104)	-	504

* Sodium of Isopropyl-β-bromallyl-N-methyl-malonylurea

B) Convulsants

Drugs \ Concentration	1 mM	0.1 mM	0.01 mM	Controls
Picrotoxin	normal mixture	-	200(98)*	205△
	NaCl 0.12M, KCl 0.02M	-	205(104)*	195△
Strychnine	695(100)*	725(104)**	705(101)***	695
Cardiazol	572(101)	570(101)	565(100)	565
Aminocordine (Coramine)	570(101)	575(102)	565(100)	565
Trans-π-Oxocampher (Vitacampher)	438(91)	480(99)	482(103)	483
Caffeine	474(91)	518(99)	538(103)	522
Phenol	306(99)	310(100)	314(101)	310△
Benzedrine	525(93)	580(103)	560(99)	565

* 10 μg/ml, ** 1 μg/ml, *** 0.1 μg/ml.

C) Autonomic agents

Drugs	Concentration	1 mM	0.1 mM	0.01 mM	Controls
Adrenaline		540(91)	556(93)	585(98)	595
Nor-Adrenaline		513(90)	570(99)	570(99)	573
Dibenamine		105(60)	174(98)	180(102)	177△
Benzylimidazoline		495(101)	482(98)	470(96)	490
Ergotamine		250(103)*	240(99)**	250(103)***	243△
Atropine		-	250(102)	240(98)	245△
C ₆		540(100)	540(100)	550(102)	540
TEAB		505(103)	485(99)	482(98)	490

* 50 µg/ml, ** 5 µg/ml, *** 0.5 µg/ml.

D) Other drugs

Drugs	Concentration	Inhibition	Drugs	Concentration	Inhibition
Histamine		-	Vitamin B ₁	{1 mM 0.1 0.01 }	+
Benadryl		-	Vitamin B ₂		-
Veratrin	{100 µg 10 1 }	-	Vitamin B ₆		-
Guanidine		-	Vitamin C	{1 mM 0.1 0.01 }	+
d-Tubocurarine	{0.01 mM 0.001 }	-	Vitamin P		-
C ₁₀	{0.1 mM 0.01 0.001 }	-	Vitamin K ₂	{1 mM 0.1 0.01 }	++
Cocaine		-	Estrone (Estradiol Estrotriol)		+
Procaine		-	Progesterone		-
Digitoxin		-	Thyroxin	{0.1 mM 0.01 0.001 }	-
Digoxin		-	Insulin	{4 unit 0.4 0.04 }	-
g-Strophanthin		-	Pituitrin	{0.1 unit 0.01 }	-
Quinine	{1 mM 0.1 mM 0.01 }	+			
Quinidine		-			
Aconitin		-			
Procaine-amide		-			
Papaverine		-			

Figures indicate the amount of ACh (µg./g. powder. hr.); those in parentheses percentage of controls.

Ratio of inhibition: + 5--10%, ++ 10--20%, +++ 20--30%. Substrate (citrate): 10mM (△ 2.5mM).

IV. Effect of Metabolites

Effects of some metabolites, especially those belonging to Krebs' cycle, on ACh synthesis by choline acetylase were observed. In this case we must consider some possibility that these metabolites themselves are also acetyl donors. We, therefore, observed the effects of these metabolites on the enzyme system without citrate,

Result, as shown in Table 4, showed that ACh synthesis without citrate was accelerated moderately by acetate and slightly by glutamate.

TABLE 4. Influence of metabolites on ACh synthesis by choline acetylase

	Citrate as substrate	Without substrate
Control	100	100
Citrate	—	375
Acetate	101	185
Lactate	90	88
Malate	89	90
Fumarate	86	90
Succinate	86	79
α -Ketoglutarate	13	24
Pyruvate	16	25
Glutamate	129	103(20 m : 113)
Monofluoroacetate	94	95
Malonate	102	—

Figures are shown in percentage of control. Incubated for 60 min..

DISCUSSION

1) Preparation of enzyme material

Indeed, acetone dried powder is a convenient source for choline acetylase. It is ready prepared and preserved; besides its cholinesterase content is very low (3). There is, however, no assurance that even a small part of ACh synthesizing system is not inactivated by acetone treatment. Accordingly we studied the relative yields of ACh by three enzyme sources; freeze-dried powder, fresh tissue and acetone dried powder. It was shown that the amount of synthesized ACh with acetone dried powder was less than that with freeze-dried powder, specifically in the earlier stage of incubation. In the later stage of incubation lasting for more than 60 minutes, acetone dried powder exceeded freeze-dried one in yielding ACh. This tendency is not so remarkable in guinea pig brain. The reason for this superiority of acetone dried powder in longer incubation has not been yet adequately explained, but the inactivation of ATP-ase by acetone may account for it to a certain degree.

The amount of synthesized ACh with fresh tissue extracts is far less than the other two. Though the reason is not yet clarified, it may be partly due to adherence of the enzyme to cell structure.

2) pH

In longer period of incubation in weak alkaline solution, the rate of ACh synthesis was shown to go on decreasing; this may be due to the inactivation of the enzyme system or/and hydrolysis of ACh in alkaline medium. In experiments on choline acetylase, we should be careful of pH, particularly when drugs to be tested are added.

3) *Distribution of choline acetylase in central nervous system*

There is a considerable difference in the amount of synthesized ACh according to the anatomical regions in the central nervous system. Table 5 shows the relative distribution of ACh, cholinesterase and choline acetylase in the central nervous system. The general trend of quantitative distribution of ACh seems to be in close parallel with that of choline acetylase, and is not so with that of cholinesterase. This suggests that the amount of ACh in brain may depend upon amount or activity of choline acetylase.

TABLE 5. Distribution of ACh, cholinesterase and choline acetylase

Resion	ACh (Rat) ⁽⁴⁾	Region	Cholinesterase (Dog) ⁽⁵⁾	Region	Choline acetylase (Rat, Dog, Guinea pig)
Pallium	100	Cortex	100	A	100
Brain stem	150	Diencephalon	550	B	200-250
				C	200-250
Cerebellum	30	Cerebellum	1000	D	20- 40
		Spinal cord	150	E	150-200

Figures are shown in percentage of cortex or pallium.

4) *Effects of drugs on choline acetylase*

There are now good evidences that narcotics increase ACh content in brain, although the effect of convulsants is a complicated matter (6-8). As regards the effect of these drugs on enzymatic ACh synthesis, we find but a few papers. Recently McLennan and Elliot (9) published their studies on this problem by using brain slices. They revealed that both narcotics and convulsants presented an accelerating effect on ACh formation at low concentration and an inhibiting effect at high concentration.

In contrast with the above results, we demonstrated that both narcotics and convulsants have no effect on the ACh synthesis by choline acetylase. Thus, the enzymatic process differs from the metabolism of living tissue. Evidence already obtained indicates that narcotics have an inhibitory influence on respiration and ACh formation depends partially upon the latter. Therefore the effect of drugs on ACh formation in slices may be due to their effect on respiration, though the detailed mechanism is not yet made clear.

We can not agree with the preliminary report of McLennan and Elliot (9), announcing that narcotics and convulsants exerted an effect on enzymatic synthesis similar to that observed in slices.

We have found only a few of drugs which gave a remarkable effect on choline acetylase. The inhibiting action of dibenamine on choline acetylase may not relate with its pharmacological action. The inhibiting action of vitamin K has been reported by Nachmansohn (10).

5) *Acetyl donor*

There is no agreement whether the acetyl donor is acetate or citrate. While

Nachmasohn (10) assumed only acetate to be a unique acetyl donor, other workers (11, 12) have been apt to admit citrate as a chief one. The present results showed that citrate was an excellent acetyl donor, while acetate was also utilized by guinea pig brain.

Among the usual metabolites, especially some members of Krebs' cycle, we could not find so remarkable accelerator as citrate and acetate. The inhibition caused by succinate, fumarate, lactate and malate was found to be so slight that it seems to have no physiological meaning.

The strongly inhibiting action of pyruvate and α -ketoglutarate on ACh synthesis was already reported by Nachmansohn (3).

Both malonate and monofluoroacetate have no effect on ACh synthesis with citrate. This means that ACh synthesis from citrate does not pass through Krebs' cycle.

Glutamate, noticed by Nachmansohn (1) already at the time of discovery of this enzyme as an accelerator on ACh synthesis, is not yet clarified as to its role in ACh synthesis. According to this experiment, the matter remains to be studied.

SUMMARY

1. Narcotics and convulsants have no effect on acetylcholine synthesis by choline acetylase. Among the drugs tested hereby, dibenamine and vitamin K were proved to have some inhibiting effect on acetylcholine synthesis.

2. Acetylcholine synthesis by choline acetylase obtained from freeze-dried powder of guinea pig brain was remarkably accelerated by citrate, moderately by acetate and slightly by glutamate.

3. Distribution of choline acetylase in central nervous system of guinea pig, rat and dog, estimated from the amount of synthesized acetylcholine, was given in order of amount: brain stem > spinal cord > cerebrum >> cerebellum.

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