Relation between Occurrence of Choline Acetylase and
Appearance of Synthetic Activity of Acetylcholine in Eggs of
Rice Stem Borer, *Chilo suppressalis*, and Cabbage Armyworm,
Barathra brassicae, during Embryonic Development¹

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INTRODUCTION

It has been reported in a previous paper that the content of the acetylcholine (ACh)-like substance found in the eggs of the rice stem borer, *Chilo suppressalis*, and the cabbage armyworm, *Barathra brassicae*², varied throughout the embryonic development of the eggs. The ACh-like substance was detected first 24 hours after oviposition. The content of the substance increased rapidly in the succeeding stages, reaching a maximum value about 60 hours after oviposition, and began to decrease about 30 hours before hatching. Finally the substance could not be detected at all at about several hours prior to hatching (CHINO & YUSHIMA, 1953). It has been also reported previously that the ACh synthetic activity of the embryo was not detected in cell-free extracts of these eggs during the first 20 hours after oviposition, but was found at 24 hours after oviposition, and increased with the succeeding of embryonic development (YUSHIMA, 1957). The experiments to be reported in this paper show clearly the reason why the older stages of these eggs are able to synthesize ACh but early stage eggs are unable to do.

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MATERIALS AND METHODS

Preparation of acetone powder of insect eggs. The eggs at several different stages of embryonic development were homogenized with 50 holds of cold acetone (-10°C) in a glass homogenizer. The homogenate was separated by centrifugation at 3,000 r.p.m. for 5 minutes. The precipitate was left at room temperature until the acetone had evaporated.

Preparation of rabbit brain acetone powder. After decapitation, the rabbit brain was weighed as soon as possible, and homogenized with 20 holds of cold acetone in a Waring-Blender. After the homogenate was separated by filtration with a Buchner's funnel, the resulting precipitate was washed twice with cold acetone, and the acetone was evaporated at room temperature.

Preparation of crude coenzyme A. Crude coenzyme A (CoA) was prepared from rabbit liver according to the method described by Lipmann and Kaplan (1948). The liver was weighed as soon as possible after decapitation and minced with 2 holds of distilled water in a Waring-Blender. The minced liver was boiled for 5 minutes, chilled with ice, and then filtered by Buchner's funnel. The filtrate was adjusted to pH 2.0 with 3 N

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² *Mamestra* is used in U.S.A. as the generic name of this species.

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HCl, 20 holds of cold acetone were added, and it was kept in a chill box for 15 minutes. After centrifugation for 10 minutes at 3,000 r.p.m., the resulting precipitate was washed twice with cold acetone. Then acetone was evaporated under vacuum desiccation. The Lipmann's unit was not determined in the present experiments, but it is expected from the other series of experiment that the number of unit is 0.3 to 0.5 per mg of dried precipitate.

Dialysis. Dialysis was carried out at 2°C with 0.08 M KCl from a cellophane membrane for 8 hours, then large excess of cold acetone was added to the residue. The yielding precipitate was washed twice with cold acetone. Then, acetone was evaporated under vacuum desiccation.

Reaction mixture. The proportion was essentially the same as that shown in a previous paper (YUSHIMA, 1957) except that the concentration of ATP. Reagent A contained 0.08 M KCl, 0.04 M NaCl, 0.004 M $MgCl_2$, 0.032 M Na_2HPO_4 , 0.008 M KH_2PO_4 , 0.003 M eserine-S and 0.06 M cysteine-Cl. Reagent B contained 0.01 M choline-Cl, 0.02 M Na-acetate and less than 0.04 M ATP-Na. (ATP-Ca was prepared from rabbit muscle using the method of Szent-Györgyi (1948), and transformed to ATP-Na using an ion exchange resin. The original concentration of ATP-Ca was about 0.04 M, however, the concentration of yielding ATP-Na was not determined in this experiment.)

Ten mg of acetone powder was extracted with 5 ml of reagent A, and centrifuged for 5 minutes at 3,000 r.p.m. to remove precipitate. The an equivalent volume of reagent B was added to the supernatant liquid. The reaction was carried out at 37.5°C and at pH 6.8 for 50 minutes.

Detection of ACh. The biological method using an eserinated frog rectus was employed for the detection of ACh (FUJII & CHINO, 1955). The method has also been described in a detail in previous paper (YUSHIMA, 1957).

RESULTS

The determination of ACh produced by the reaction was carried out at each 10

minutes from 10 to 50 minutes after incubation. In Tables listed in this paper the abbreviation (+) indicates that the increase of ACh content was observed in reaction mixture during the incubation, and (−) indicates that no change occurred in ACh value during the course of incubation.

The results of quantitative experiments will be reported in future papers.

Preliminary experiment using rabbit preparation. The results are summarized in Table 1.

Table 1. Preliminary experiment on acetylcholine synthesis by acetone powder prepared from rabbit tissues.

Enzyme preparation	Detection of ACh
Acetone powder of rabbit brain	+ *
Crude CoA †	− *
Dialyzed acetone powder of rabbit brain	−
Dialyzed acetone powder of rabbit brain plus crude CoA	+

* Abbreviations defined in the text.

† Ten mg of crude coenzyme A was used.

The results showed that coenzyme A (CoA) contained in the acetone powder of rabbit brain was completely removed by dialysis for 8 hours, and that the crude CoA prepared from rabbit liver did not contain any traces of choline acetylase (ChAase). Because the ACh synthetic activity of the dialyzed acetone powder of the rabbit brain was reactivated by addition of crude CoA, it is apparent that ChAase remained in the dialyzed acetone powder of the rabbit brain. In the following section, the term "CoA" means a crude coenzyme A prepared from rabbit liver and "ChAase" indicates the dialyzed acetone powder of the rabbit brain.

Synthesis of ACh by acetone powder of insect eggs. The experiments were performed under design indicated in Table 2, where the results were also summarized.

As has been reported previously from studies on ACh synthesis by extracts of the fresh eggs (YUSHIMA, 1957), the acetone powder prepared from the eggs within 20 hours after oviposition could not synthesize ACh at all. The activity of ACh synthesis in the acetone powder was effected by adding ChAase, but the addition of CoA had no

effect. These results lead to the following conclusion that CoA is present but ChAase is lacking in the eggs for the first 24 hours after oviposition.

Table 2. Synthesis of acetylcholine by acetone powder prepared from eggs of the rice stem borer and the cabbage armyworm (I).

Enzyme preparation		Adding enzyme	Age of egg (after oviposition)	
			15~20 h	60~80 h
Acetone powder of	{ rice stem borer egg	{ none	—	+
		{ CoA *	—	+
		{ ChAase*	+	+
	{ cabbage armyworm egg	{ none	—	+
		{ CoA	—	+
		{ ChAase	+	+
Dialyzed acetone powder of	{ rice stem borer egg	{ none	—	—
		{ CoA	—	+
		{ ChAase	—	—
	{ cabbage armyworm egg	{ none	—	—
		{ CoA	—	+
		{ ChAase	—	—

*Terms defined in the text, and abbreviations (+) and (—) are detailed in the text.

The acetone powder prepared from later stages of eggs, 60~80 hours after oviposition, had a synthetic activity of ACh. The activity was lost by dialysis for 8 hours, and the lost activity was reactivated by adding of CoA. Accordingly, the dialyzed factor might be CoA itself.

These results were also supported by the experiments showing in Table 3. The dialyzed acetone powder prepared from the eggs of early stages did not show the synthetic activity even the dialyzed acetone powder of the later stage eggs was added.

Table 3. Synthesis of acetylcholine by acetone powder prepared from eggs of the rice stem borer and the cabbage armyworm (II).

	Dialyzed acetone powder of	Acetone powder of	Det. of ACh
Rice stem borer	{ 15~20h plus 60~80h	{ 60~80h plus 15~20h	—
	{ 60~80h plus 15~20h		+
Cabbage armyworm	{ 15~20h plus 60~80h	{ 60~80h plus 15~20h	—
	{ 60~80h plus 15~20h		+

The abbreviation (h) indicates number of hours after oviposition, and (—), (+) in Det. of ACh are defined in text.

On the other hand, the activity of the dialyzed acetone powder of the later stage egg was recovered completely, when the acetone powder of early stage undialyzed was added.

DISCUSSION

Smallman(1956)reported that the synthesis of ACh in the extracts of acetone powder of blowfly head, *Lucilia sericata*, was shown to proceed by a two-step process; in the first step, an intermediate conforming with properties of acetyl CoA is formed under the presence of CoA, ATP, and acetyl-groups. The second step, involves the acetylation of choline, catalyzed by the terminal enzyme, ChAase. In this experiment, the eggs of the rice stem borer and the cabbage armyworm also required CoA and ChAase for synthesis, and formed ACh under the presence of choline, ATP and acetate.

Augustinsson and Grahn (1954) have presented evidence for two unidentified choline molecules beside ACh in the head of honey bee. In contrast to their result, Chefurka and Smallman (1955; 1956) determined that the extracts from the house fly, *Musca domestica*, contained only one ester of choline. This choline ester shown to be identical with ACh by many methods including infrared spectrophotometry. In previous papers, it has been suggested that the high amount of ACh-like substance contained in the eggs of the rice stem borer and the cabbage armyworm might be ACh itself, a conclusion based on the chemical or pharmacological action, and on the knowledge of the relationships between quantitative fluctuation of the ACh-like substance and the changes of the cholinesterase (ChEase) activity during the embryonic development (YUSHIMA & CHINO, 1953; CHINO & YUSHIMA, 1953, 1954). ACh in these eggs is not found in the first 20 hours after oviposition, a fact in good agreement with the changes of rate of ACh synthesis *in vitro* during the embryonic development (YUSHIMA, 1957). In the present paper, the reason why the early stage eggs had no the synthetic activity is suggested. That is, though the early stage eggs had CoA even at the time of oviposition, there

is no ChAase within the first 20 hours after oviposition. In conclusion, there is no doubt that the insect including the egg contains ACh as a choline ester.

The type of fluctuation of ACh in the eggs of the insects can be divided into three types. The first type is characterized by high amounts of ACh in some embryonic stages, for example, the eggs of the rice stem borer and of the cabbage armyworm, etc. (YUSHIMA, 1956). The second type was found recently by Chino (1957), in the egg of the silkworm, *Bombyx mori*. He found that the early stages of the egg contained no ACh, but with the differentiation of the nervous system he found ACh in concentrations of only few gammas per gram of fresh material even at the maximal level. The third type was observed in the egg of the paddy borer, *Schoenobius incertulas*, where the combined form of the first type and the second type was observed clearly (YUSHIMA, 1956a). In the first types, it is possible that additional ACh resulting from the differentiation of the nervous system may also appear, but this is too difficult to distinguish experimentally. The fluctuations of ACh and the related factors were presented in diagrammatically in Figs. 1~3.

In the first type, ChAase was found in earlier egg stages than ChEase. On the contrary, the appearance of ChAase in eggs belonging to the second type was roughly same that of ChEase (CHINO, 1957). In a previous paper it has been shown that the first appearance of the ChEase in the eggs of the Lepidopterous insects had no relation to the differentiation of the neuroblast (CHINO & YUSHIMA, 1954). Recently, these results were supported by the results obtained from the peach tree borer, *Sannionidza exitiosa*, and the large milkweed bug, *Oncopeltus fasciatus* (SMITH, 1956), where the first appearance of ChEase was detected at two and one days prior to hatching, respectively. Consequently, the difference in timing of the first appearance of the ChAase and ChEase caused those different forms in the fluctuations of ACh content already mentioned above. (The diagram for the third

Fig. 1. The first type fluctuation of acetylcholine in insect eggs. (Dotted line is speculation.)

Fig. 2. The second type fluctuation of acetylcholine in insect eggs.

Fig. 3. The third type fluctuation of acetylcholine in insect eggs. (* speculation)

type presented in Fig. 3 contains the speculations because the experiments are incomplete.)

In a previous paper (YUSHIMA, 1956), the author suggested that the fluctuation found in the first type does not bear any relationship to the nervous system but that in the second type is closely related to neural development. It seems to be that the fluctuation found in the second type has no connection with the rate of activity of ACh synthesis or the activity of ChEase.

SUMMARY

1. Coenzyme A and choline acteylase are essential for the synthesis of acetylcholine in the eggs of the rice stem borer, *Chilo suppressalis*, and of the cabbage armyworm, *Barathra brassicae*.

2. The absence of synthetic activity of acetylcholine in these eggs of early stage is due to the lack of the terminal enzyme, choline acetylase, nevertheless coenzyme A is present during the course of their embryonic development.

3. The fluctuations of acetylcholine and the related factors in the eggs of insects are shown in diagrammatically.

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摘 要

ニカメイチュウおよびヨトウムシの卵における胚子発育に伴う
コリンアセチラーゼの出現とアセチルコリン合成能の発現との関係

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ニカメイチュウおよびヨトウムシの産卵後時間以内の卵では、アセチルコリンが発見できず(CHINO & YUSHIMA, 1953), またアセチルコリン合成能もない(YUSHIMA, 1957)。本報告ではこの理由について究明した。

すなわち、昆虫におけるアセチルコリンの合成には他の動物ですでに知られているように、coenzyme A と、反応を完結させる Choline acetylase の二つの酵素が絶対に必要である。ところが、産卵後 24 時間以内の卵

には coenzyme A は存在するのに choline acetylase はこの時期以後になって出現し、これに伴って同時にアセチルコリン合成能も生じてくることを確かめた。

上記の事がらを根拠として、今までに見出されている昆虫卵の胚子発育に伴うアセチルコリンの消長(CHINO & YUSHIMA, 1953; CHINO, 1957; YUSHIMA, 1956, 1956 a) を三つのタイプにわけ、それらの三つのタイプの現われる理由を図式的に示した。