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Biosynthesis and secretion of catalytically active acetylcholinesterase in *Xenopus* oocytes microinjected with mRNA from rat brain and from *Torpedo* electric organ

(bioassay/half-life/scarce-enzyme expression)

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ABSTRACT **A novel technique was developed for monitoring the level of the mRNA species that direct the synthesis of acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.7), using microinjected *Xenopus* oocytes as a translation system. When injected with poly(A)-containing RNA from whole rat brain or rat cerebellum and from electric organ of *Torpedo ocellata*, *Xenopus* oocytes synthesize and secrete catalytically active cholinesterase. The newly synthesized enzyme, which is mostly secreted into the oocytes incubation medium, appears to be primarily AcChoEase because it is inhibited by the specific inhibitor BW 284C51. The new enzymatic activity can be detected after injection of as little as 12.5 ng of poly(A)-containing RNA per oocyte, and there is a linear dependence of the oocytes' ability to form AcChoEase on the amount of injected RNA. The AcChoEase mRNA displays a $\tau_{1/2}$ of about 10 ± 3 hr in injected oocytes. The abundance of AcChoEase mRNA in the total nonfractionated mRNA injected was calculated to be *ca.* 1×10^{-5}, a value similar to the level of AcChoEase protein determined in rat brain. The combination of the high turnover number of AcChoEase, the efficiency of the oocyte system, and the sensitivity of the assay used thus permit the accurate monitoring of the scarce mRNA species that direct the synthesis of this enzyme.**

The principal role of acetylcholinesterase (AcChoEase; acetylcholine acetylhydrolase, EC 3.1.1.7) is believed to be the termination of impulse transmission by hydrolysis of the neurotransmitter acetylcholine (AcCho), and development of cholinergic synapses is indeed accompanied by accumulation of this enzyme. The biosynthesis of AcChoEase is of interest for a number of reasons. It is a synaptic enzyme whose regulation and assembly may involve both presynaptic and postsynaptic control (1–8). It exists in multiple molecular forms, differing in their localization, mode of association with the surface membrane, regulation, and presumably physiological function (1, 5, 9–11). Moreover, it may be expected that certain of these molecular forms will undergo novel modes of posttranslational processing and modification in the course of their integration into the functional synapse.

A number of groups have studied the appearance and regulation of AcChoEase in cultures of nerve and muscle. Thus, it has been shown that after irreversible inhibition of existing enzyme with organophosphate anticholinesterases, protein synthesis inhibitors arrest reappearance of activity, providing proof that *de novo* synthesis is involved (12–15). Reappearance of low molecular weight forms of the enzyme (monomer and dimer) precedes that of more complex forms (3, 12, 15), suggesting that the former serve as precursors of the latter. Rotundo and Fambrough (16) have recently, using this approach, analyzed in detail the kinetics of appearance, insertion into the plasma membrane, and secretion of AcChoEase in muscle cultures.

Meedel and Whittaker (17) have shown that increases in AcChoEase activity during the early stages of embryogenesis of the ascidian, *Ciona intestinalis*, are inhibited by actinomycin D and have suggested a possible role for mRNA production in the ontogeny of AcChoEase.

In vitro studies of AcChoEase biosynthesis as directed by mRNA are difficult to carry out because the enzyme comprises only a minor fraction of the total tissue protein. However, the high turnover number of the active enzyme (18) permits the detection of minute amounts of active AcChoEase (19). This raised the possibility that a bioassay could be developed for the production of active AcChoEase as directed by mRNA. Because *in vitro* systems like reticulocyte lysate or wheat germ extract are of limited duration and deficient in their ability to perform posttranslational processing and to produce biologically active proteins (20), we chose to develop such a bioassay in microinjected *Xenopus* oocytes.

The *Xenopus* oocyte system (21) offers an attractive experimental approach to this issue for a number of reasons. It has been extensively used as an efficient translation system for a variety of microinjected mRNAs (22), correctly performing translation (21), processing (23), and various posttranslational modifications (24); in the case of mRNAs directing synthesis of secretory proteins, *Xenopus* oocytes secrete the correct translational products (25–28) and, finally, in a number of cases, the injected mRNAs have been translated into biologically active products (26, 29–31).

We demonstrate in this report that *Xenopus* oocytes synthesize and secrete catalytically active AcChoEase upon microinjection with poly(A)-containing RNA prepared from whole rat brain, from dissected rat cerebellum, and from *Torpedo* electric organ. We further define the stability and efficacy of the AcChoEase mRNA from rat brain in the microinjected oocytes and, on the basis of the amounts of mRNA injected and AcChoEase secreted, present calculations demonstrating that AcChoEase mRNA is a scarce mRNA species.

MATERIALS AND METHODS

Sprague–Dawley rats (10 days old) were obtained from the Weizmann Institute animal facilities, and embryos of *Torpedo ocellata* were provided by D. Michaelson of Tel Aviv Univer-

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Abbreviations: AcCho, acetylcholine; AcChoEase, acetylcholinesterase; ChoEase, cholinesterase.

sity. Tissues (whole brain, cerebellum, or electric organ) were dissected on ice and stored at −70°C until use. RNA was extracted with phenol (32), and DNA and tRNA were removed by sodium acetate wash (33). Poly(A)-containing RNA was prepared by oligo(dT)-cellulose chromatography at 4°C (34).

Adult *Xenopus laevis* females were obtained from the South African Snake Farm (Fish Hoek, South Africa). The toads were anaesthesized by cooling in ice, ovarian lobes were removed, and individual oocytes were dissected and staged (35). Stage 6 oocytes were microinjected with the indicated amounts of RNA in 50 nl of H_2O and incubated in groups of 10 single oocytes in 100 μl of Barth medium (21). Control oocytes were injected with equal volumes of Barth medium. Incubation was at 21°C in a humidified atmosphere. After the appropriate incubation period, the oocyte medium was removed and either analyzed immediately or stored at −20°C, at which temperature AcChoEase levels remained unchanged for at least 20 days. Intracellular cholinesterase activity, which can include not only AcChoEase but also other cholinesterases, was determined in oocyte homogenates prepared in 10 vol of 0.01 M Tris·HCl (pH 7.5) containing 1 M NaCl and 1% Triton X-100.

Cholinesterase activity was determined in 30-μl aliquots of oocyte extract or incubation medium according to Johnson and Russell (19). Briefly, samples were incubated in 5-ml scintillation vials in 0.05 M Tris·HCl (pH 7.5) containing 0.12 M NaCl and 300 nmol of [^{3}H]acetylcholine (AcCho; Radiochemical Centre, Amersham) containing about 60,000 cpm. The final reaction volume was 100 μl. Incubation was at 21°C for the indicated times. In the course of the prolonged incubations needed to assay newly synthesized AcChoEase in the oocyte incubation medium (up to 48 hr), both acetic acid and AcCho appear to decrease in amount because of metabolic processes that we have not characterized, both in control and in RNA-injected oocytes. However, these processes are completely arrested by inclusion of *p*-chloromercuriphenylsulfonic acid (1 mM; Sigma) in the assay medium, which, as expected (36), does not affect AcChoEase activity. The reaction was terminated by addition of 100 μl of 1 M monochloroacetic acid containing 2 M NaCl and 0.5 M NaOH. Protonated acetic acid was extracted into 4 ml of scintillation cocktail consisting of isoamyl alcohol and toluene (1:10) with 4.0 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl Popop) per liter of cocktail. Extracted [^{3}H]acetate was measured in a Packard β-counter. Background samples, with equal volumes of reaction cocktail, contained 30 μl of H_2O and were similarly incubated.

RESULTS

When injected with poly(A)-containing mRNA from rat cerebellum (37), *Xenopus* oocytes secrete cholinesterase activity into their incubation medium. Up to 40 nmol of AcCho per oocyte was degraded during a 50-hr incubation of RNA-injected oocyte medium. In the medium of control-injected oocytes, into which the secretion of cholinesterase activity is somewhat enhanced as compared with noninjected oocytes, about 15 nmol of AcCho were degraded per oocyte during this period. Spontaneous AcCho degradation occurring under the reaction conditions used amounts to about 10 nmol for an equal volume of Barth medium. Thus, it appears that microinjection of oocytes with cerebellar mRNA enhances the cholinesterase activity secreted by a factor of 6 (Fig. 1).

The presence of endogenous cholinesterase activity in noninjected *Xenopus* oocytes is compatible with the earlier report of AcCho receptors in the membrane of *Xenopus* oocytes (38). The endogenous oocyte enzyme may consist of two components. It is completely inhibited by BW 284C51, a specific inhibitor of AcChoEase (39) and it is *ca.* 50% inhibited by tetraisopropylpyrophosphoramide, a specific inhibitor of pseudocholinesterase (acylcholine acylhydrolase, EC 3.1.1.8; ref. 39).

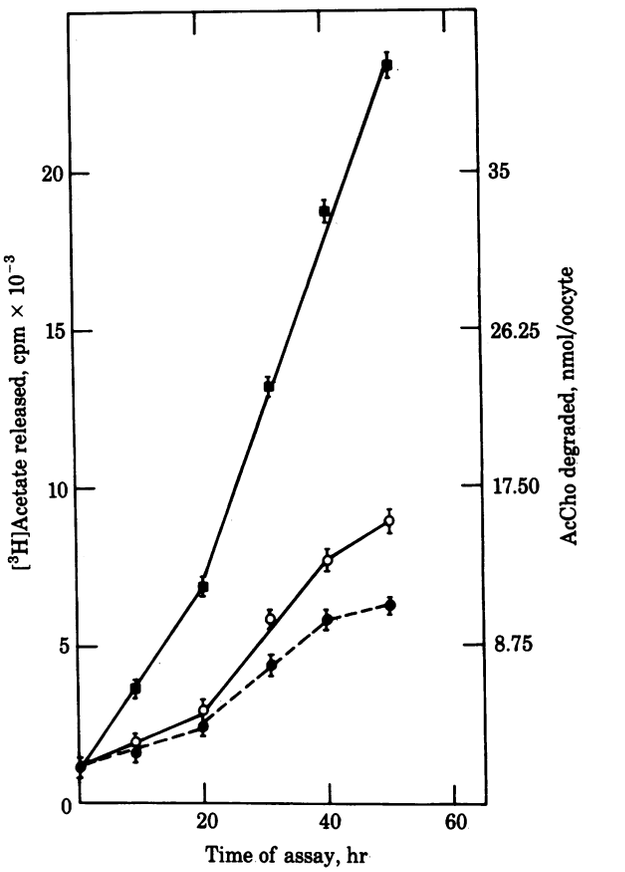

FIG. 1. Kinetics of AcCho degradation by the incubation medium of *Xenopus* oocytes injected with rat cerebellum mRNA. Oocytes were microinjected with 200 ng of mRNA each and incubated for 20 hr. Cholinesterase activity was determined in duplicate samples of medium of mRNA-injected oocytes (■), control oocytes (○) and H_2O (●). Samples were incubated with [^{3}H]AcCho for the times indicated.

The newly synthesized cholinesterase activity secreted by the oocytes appears, in contrast to the endogenous oocyte enzyme, to display properties of AcChoEase because it is completely inhibited by 0.1 mM BW 284C51 and by eserine, a general cholinesterase inhibitor (40), but not by tetraisopropylpyrophosphoramide (Table 1). The observation that the newly synthesized enzyme is wholly BW 284C51-sensitive and

Table 1. Inhibition by anticholinesterases of cholinesterase activity excreted from injected oocytes

Inhibitor	AcCho degraded, pmol/min per oocyte	% of initial activity
None	9.7 ± 2.7	100
BW 284C51	0.2 ± 0.3	2 ± 3
Tetraisopropyl-pyrophosphoramide	10.3 ± 0.7	106 ± 7
Eserine	1.0 ± 0.2	10 ± 2

Microinjection was performed with total rat brain RNA, and cholinesterase activity in the medium was determined. Reaction mixtures contained the appropriate inhibitor at 0.1 mM final concentration and were incubated for 24 hr. Tetraisopropylpyrophosphoramide was preincubated with the samples for 30 min before addition of the [^{3}H]AcCho. Activity in the medium of control oocytes was subtracted. Data represent the average (mean ± SD) of three experiments, in each of which duplicate assays were performed.

is insensitive to tetraisopropylpyrophosphoramide is compatible with the fact that AcChoEase seems to be the predominant AcCho-hydrolyzing enzyme in rat brain (41). Thus, under our assay conditions, hydrolysis of AcCho by extracts of whole rat brain is inhibited more than 90% by 0.1 mM BW 284C51 and less than 10% by 0.1 mM tetraisopropylpyrophosphoramide. Moreover, we find that these extracts hydrolyze butyrylthiocholine at <10% of the rate at which they hydrolyze acetylthiocholine.

The mRNA-induced AcChoEase activity could clearly be detected as early as 5 hr after injection, resembling the early appearance of globin (21), interferon (26), and plasminogen activator (31) in microinjected oocytes. AcChoEase accumulates in the incubation medium of microinjected oocytes for over 20 hr. After this period, the level of the enzyme in the medium of microinjected oocytes either reaches a plateau (Fig. 2 *Left*) or slightly decreases (Fig. 2 *Right*), perhaps as a result of enhanced secretion of endogenous oocyte proteases, whose level differs among various frogs (42). Indeed, AcChoEase activity decays at a variable slow rate (at about 3% per hr) also in samples of medium that were removed and further incubated. From these experiments, together with two others (not shown), an average half-life ($\tau_{1/2}$) of 10 ± 3 hr (mean ± SD) could be calculated for the AcChoEase mRNA, which appears to be similar to the average *in vivo* half-life of polyadenylated mRNAs of mammalian origin (43).

The newly synthesized cholinesterase, as well as the endogenous oocyte activity, is mostly secreted into the incubation medium (Table 2). The ratio of secreted to intracellular cholinesterase was similar in control and injected oocytes.

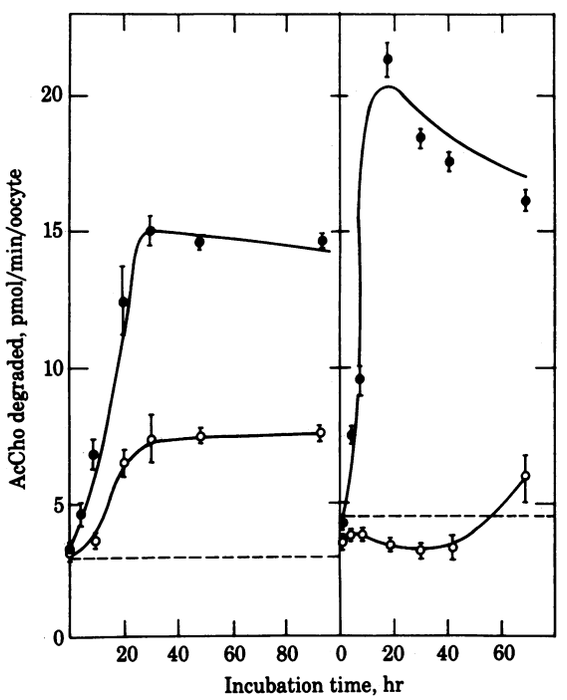

FIG. 2. Kinetics of excretion of AcChoEase activity from oocytes microinjected with rat brain mRNA. Oocytes were microinjected as described in the legend to Fig. 1. At the times indicated on the abscissa, incubation medium was removed from the microinjected oocytes and assayed (24 hr) for AcChoEase activity. Medium was changed every 20 hr, and cholinesterase activity for longer periods is the cumulative sum of added activities. Assays were carried out in triplicate. Two experiments, performed with oocytes from different frogs, are shown (*Left* and *Right*). ---, Background degradation of AcCho; ○, AcChoEase activity in medium of control oocytes; ●, activity in medium of mRNA-injected oocytes.

Table 2. Excretion of cholinesterase activity from microinjected oocytes

Activity	Intracellular cpm/sample	Intracellular pmol/min/oocyte	Medium cpm/sample	Medium pmol/min/oocyte	% Excreted
In RNA-injected oocytes	1544	0.80	9879	5.14	86
In control oocytes	472	0.24	2770	1.44	85
Newly synthesized	1072	0.56	7109	3.70	87

Oocytes were injected with 50 ng of rat cerebellum mRNA. Control oocytes were injected with Barth medium. Incubation was for 20 hr. Cholinesterase activity was determined in triplicate in samples of oocyte homogenate or incubation medium of the microinjected oocytes. Mean values are shown. Newly synthesized cholinesterase is the difference between that in the RNA-injected oocytes and controls.

Based on the observation that maximal levels of cholinesterase are detected in the oocyte incubation medium 20 hr after injection, increasing amounts of cerebellar mRNA were injected into oocytes, and the amount of cholinesterase activity accumulating in the medium during this period was measured to determine the range in which the level of newly synthesized cholinesterase corresponds linearly to the amount of microinjected mRNA. Considerable amounts of mRNA-directed cholinesterase activity are observed in the medium of oocytes microinjected with as little as 12.5 ng of nonfractionated poly(A)-containing mRNA per oocyte (Fig. 3). The level of induced enzyme increases with increasing amounts of poly(A)-containing mRNA, and 50 ng of mRNA appeared to saturate the capacity of the oocyte to synthesize AcChoEase. The limited capacity of the oocytes to express microinjected mRNA is in agreement with previous reports (44), and the decrease observed in the level of newly synthesized AcChoEase in oocytes injected with

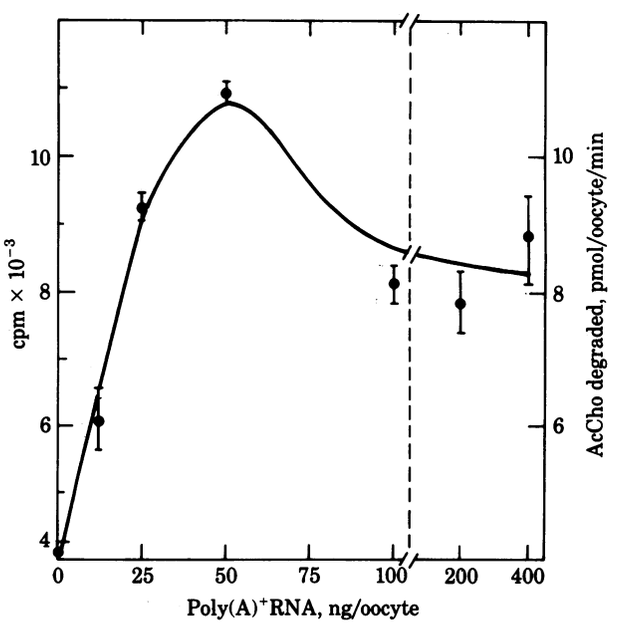

FIG. 3. Dose response of cholinesterase production in oocytes microinjected with different amounts of rat cerebellum mRNA. Oocytes were microinjected with the indicated amounts of rat cerebellum mRNA in 50 nl of H_2O. Incubation was for 20 hr. Cholinesterase activity was assayed (24 hr) in triplicate samples. Deviation from the average is indicated by bars.

Table 3. Excretion of cholinesterase activity from oocytes injected with mRNA from *Torpedo* electric organ and rat cerebellum

Source of mRNA	[^{3}H]AcCho degraded		Cumulative newly synthesized AcChoEase	
	cpm/sample	pmol/min/oocyte	cpm/sample	nmol/oocyte
Rat cerebellum	3816 ± 1629	2.2 ± 0.9	2671	.4.26
Torpedo electric organ	7039 ± 1372	4.1 ± 0.8	5894	9.56
Barth medium	1145 ± 1036	0.7 ± 0.6	—	—

Oocytes from a single frog were microinjected with 50 ng each of poly(A)-containing RNA from rat cerebellum or from electric organ dissected from 6-cm-long embryos of *Torpedo ocellata*. Incubation was for 45 hr. Degradation of [^{3}H]AcCho was determined in triplicate. Mean values ± SD are shown.

more than 50 ng of mRNA may indicate competitive inhibition exerted by more abundant mRNA species. The level of excreted activity in the medium of oocytes injected with up to 50 ng of nonfractionated mRNA reflects, therefore, the abundance of AcChoEase mRNA in the injected mRNA population.

The production and excretion of AcChoEase in oocytes also has been examined with mRNA prepared from the AcChoEase-enriched electric organ of *Torpedo ocellata*. When injected into the oocytes, electric organ mRNA directed the secretion of cholinesterase capable of degrading 5.0 pmol of AcCho per ng of injected mRNA per hr, whereas one ng of mRNA from rat cerebellum induced degradation of 2.6 pmol of AcCho per hr (Table 3).

DISCUSSION

On the basis of the levels of AcChoEase activity detected in whole rat brain and various dissected regions (45, 46) and on the basis of the turnover number of the rat enzyme (18), it can be calculated that AcChoEase accounts for as little as 1×10^{-5} of total brain protein. The scarcity of AcChoEase and, hence, of the mRNA species that direct(s) its synthesis have prevented, so far, studies of AcChoEase biosynthesis at the translational level. To overcome these difficulties, we combined the use of *Xenopus* oocytes as a translation system with a sensitive radioassay for the detection of AcChoEase activity. Each mRNA molecule undergoes hundreds of translation cycles in the injected oocyte (21, 47). The newly synthesized AcChoEase is then incubated for many hours in the presence of labeled AcCho. The amplification of activity thus provided permits the accurate detection and quantification of minute amounts of AcChoEase mRNA prepared from various biological sources, such as the electric organ of *Torpedo* or dissected regions of rat brain.

In the linear range of dose response, one may assume that the injected mRNA is being translated fully, at a rate which, for globin (21) and for ovalbumin mRNA (47), has been found to be similar to the *in vivo* rates of translation of these and many other mRNA species. Because the turnover number of AcChoEase has been measured (18), one may calculate the fraction that this particular mRNA species represents out of the total injected mRNA by measuring the amount of its specifically directed and catalytically active protein product.

When *in vivo* labeled mengovirus RNA was injected into *Xenopus* oocytes, ≈10–20% remained trichloroacetic acid-insoluble 10 hr after injection (43, 48), in agreement with earlier findings of others (49). When iodinated mRNA from rat cerebellum was injected into the oocytes, about 15% of the injected radioactivity remained trichloroacetic acid-insoluble 20 hr after injection (unpublished observations). Therefore, it would be reasonable to assume that the oocytes still include about 10% of the injected RNA at this time in a functional state, which amounts to 20 ng per oocyte for the experiments presented in Fig. 2. Assuming a turnover time of about 270 μsec (18), a single catalytic site of AcChoEase degrades 6×10^{-12} nmol of AcCho per sec. During the 10-hr period of linear increase in accumulated cholinesterase activity (from 5 to 15 hr after injection) it can be calculated that a single oocyte synthesizes an amount of enzyme sufficient to degrade about 4×10^{-3} nmol of AcCho per min (see Fig. 2). The number of catalytic sites that have been produced is therefore: $4 \times 10^{-3} \div (60 \times 6 \times 10^{-12}) = 1.1 \times 10^7$ sites per oocyte. This number of sites could have been translated during this 10-hr period by 1.1×10^5 molecules of AcChoEase mRNA, assuming a 6-min duration for the elongation process required to synthesize a full-length polypeptide chain of the enzyme (47) and disregarding the minor decay of newly synthesized enzyme. AcChoEase contains about 800 amino acid residues in its catalytic polypeptide chain (50); therefore, its mRNA should be of at least M_r 80,000. Thus, the calculated number of 1.1×10^5 molecules of AcChoEase mRNA represents about 0.2 pg. The fraction of mRNA that directs the biosynthesis of catalytically active AcChoEase is, hence, of the order of about 1×10^{-5} of the total mRNA injected. A large fraction of rat brain mRNA complexity has been reported to represent scarce mRNA species (51), and AcChoEase mRNA belongs, therefore, to this class. Implicit in these calculations are the assumptions that elongation rates of nascent polypeptide chains of different proteins are approximately similar and that the limiting factor in translation is the amount of mRNA.

The calculated fraction of AcChoEase mRNA is similar to the fraction that AcChoEase protein represents out of total brain protein as estimated above, and the half-life of AcChoEase mRNA is similar to the *in vivo* turnover rate of other mammalian mRNAs (43). Therefore, the heterologous oocyte system appears to carry out efficiently both the translational and the post-translational processes required to obtain this scarce enzyme in its catalytically active form. However, it should be emphasized that different patterns were observed for the accumulation of AcChoEase in the incubation medium of oocytes from different frogs. This variability between experiments might partially be explained by the heterogeneity in the proteolytic activities secreted by oocytes from different frogs (42). Proteolytic degradation also might explain the different levels of newly synthesized and of endogenous AcChoEase that have been observed to be secreted by injected and control oocytes in various experiments.

Most of the newly synthesized AcChoEase is secreted from the injected oocytes into their incubation medium. Various studies on ganglia (52) and on neuronal cells in culture (53) have shown that much of the AcChoEase produced is secreted into the external medium. Therefore, our findings are in line with earlier observations, showing that *Xenopus* oocytes accumulate in vesicles (27) and secrete into the medium exclusively secretory products like interferon (26), milk proteins (25) and plasminogen activator (31) but not nonsecretory proteins such as histones or globin (25). In the systems mentioned above, much of the AcChoEase secreted is detected on sucrose gradients as oligomeric species (52, 53). Preliminary experiments suggest that the AcChoEase secreted by oocytes microinjected with rat brain mRNA is also primarily in an oligomeric form (unpublished observations).

The bioassay described herein can be used to monitor the level of AcChoEase biosynthesis in various tissues, in which the

appearance of this scarce protein is correlated with physiological function. Furthermore, it can be exploited to investigate the posttranslational processes involved in the formation of the various forms of this enzyme. Finally, a quantitative bioassay for AcChoEase mRNA is a necessary requirement for the preparation and amplification of a cDNA probe and the analysis of the genetic elements involved in the expression of this enzyme.

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