the amounts of cosynthetase in hemolyzates from four mature porphyric cattle have been found to be much lower than the amounts in hemolyzates from four mature normal animals.

The assay for uroporphyrinogen III cosynthetase in crude tissue extracts depends on proportionality between the percentage of isomer III in the reaction product and the amount of the cosynthetase preparation added, under conditions where the total amount of uroporphyrinogen (I + III) is kept constant (*2*). Heparinized blood, drawn at the same time from a porphyric animal and a normal control, was the source of the cosynthetase used for these experiments. The specimens were frozen in solid CO_2 and maintained at −20°C until assayed. The paired porphyric and normal samples were assayed simultaneously, 2 to 5 days after the blood was drawn. Thawed hemolyzates were diluted with three volumes of 0.05*M* potassium phosphate buffer, *p*H 7.65. Portions of this dilution were incubated for 30 minutes at 31°C with 50 μmole of potassium phosphate buffer, *p*H 7.65, 0.12 μmole of tritium-labeled porphobilinogen, and enough uroporphyrinogen I synthetase, prepared from mouse spleen (*2*), to catalyze the formation of 5 mμmole of uroporphyrinogen (I + III), in a volume of 0.5 ml. The reaction was stopped with 0.035 ml of acetic acid. The uroporphyrinogen formed was oxidized to the porphyrin with 0.01 ml of 0.2*M* iodine in 0.3*M* KI, isolated on talc, esterified with methanolic H_2SO_4, and extracted into chloroform (*1*). The methyl esters were separated chromatographically (*6*), eluted from the paper with toluene, and counted in a liquid-scintillation system. Cosynthetase activity is expressed as the percentage of isomer III in the total ester recovered.

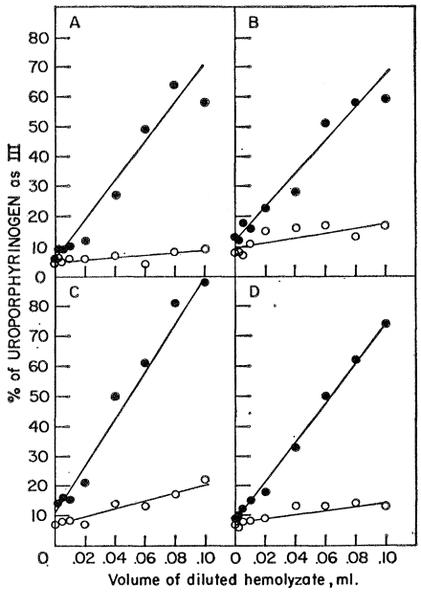

Fig. 1. Measurement of uroporphyrinogen III cosynthetase activity in diluted hemolyzates from normal and porphyric cattle. Closed circles, normal animals; open circles, porphyric animals. Hemoglobin determinations on the porphyric animals gave: 7.8, 11.9, 12.9, and 13.0 g per 100 ml of blood for porphyric animals A, B, C, and D, respectively; and 9.9, 9.1, 16.0, and 10.8 g per 100 ml of blood for the corresponding controls. The porphyric animals of pairs B and D and the normal animal of pair C were mature bulls; the others were mature cows.

The results obtained in the four paired assays are shown in Fig. 1. The assays on all the normal animals gave a linear relationship, rising as high as 89 percent of the product as isomer III with 0.1 ml of diluted hemolyzate (control animal C). In each case, the hemolyzate from the porphyric animal had much less effect than did the hemolyzate from the normal animal on the isomer composition of the uroporphyrinogen formed by synthetase from mouse spleen. In porphyric animals A and D, the cosynthetase activities of the amounts of hemolyzate tested were not significantly greater than the blank without added hemolyzate. The increment in percentage of III produced by 0.1 ml of diluted hemolyzate was from four to ten times greater in the normal than in the porphyric animals.

The hemolyzates from the porphyric animals did not interfere with the cosynthetase activity of hemolyzates from the normal animals. For example, in one experiment with 0.04 ml of the diluted hemolyzate from the normal animal D, the product contained 36 percent of isomer III; with 0.06 ml from the porphyric animal D, it contained 12 percent of III; and with the two combined, 39 percent of III. The product in the blank without hemolyzate contained 8 percent of III. Also, the hemolyzate from this porphyric animal did not inhibit the activity of cosynthetase prepared from mouse spleen (*2*).

Control experiments demonstrated that the amounts of hemolyzate assayed for cosynthetase did not have detectable synthetase activity under the reaction conditions, and did not stimulate or inhibit the mouse spleen synthetase. The amount of endogenous uroporphyrinogen which could be recovered as the methyl ester from the volumes of hemolyzate used for assay were less than 2 percent of the amounts formed in the reaction mixture.

The low concentrations of measurable cosynthetase in the hemolyzates of porphyric animals are thus probably due to a true lack of the cosynthetase activity, rather than to the presence of an inhibitor.

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2 May 1968; revised 26 June 1968

Choline Acetyltransferase: Regional Distribution in the Abdominal Ganglion of Aplysia

Abstract. *Using a new microassay, we have determined the properties and the regional distribution of choline acetyltransferase in the abdominal ganglion of* Aplysia. *Enzyme concentrations in homogenates of groups of cells and in single identified cells indicate that neurons which function as neurosecretory cells, and which do not form chemical synapses with other cells or with peripheral structures, have little or no ability to synthesize acetylcholine; neurons which are involved in visceromotor integrations, and which connect with each other or with the periphery, have a substantial concentration of the enzyme.*

The abdominal ganglion of the marine mollusk *Aplysia californica* is particularly useful for the biochemical analysis of individual cells. Most of the neurons in this ganglion are large, some reaching 1 mm in diameter, and many are located near the ganglion's surface. The cells are readily seen under the dissecting microscope, and intact cell bodies without glial contamination can be isolated by manual dissection. Moreover, 30 of the 1000 to 2000 neurons in the ganglion can be iden-

tified reproducibly by their characteristic morphological and electrophysiological properties (*1*, *2*). Although there are abundant physiological and pharmacological data on this ganglion, no systematic, biochemical studies have been made. For example, there is considerable pharmacological evidence to indicate that in this ganglion acetylcholine is a chemical transmitter for both excitation and inhibition (*2*, *3*). Although Bacq (*4*) noted the presence of acetylcholine in *Aplysia* in 1935, there has been no direct analysis of its synthesis or distribution in the ganglion. Using a new microassay for choline acetyltransferase (E.C. 2.3.1.6), we have demonstrated the last step in the synthesis of acetylcholine, studied some of the properties of the transferase, and examined its regional distribution in the ganglion.

A cut was made in the connective tissue sheath through which clumps of neurons were collected with a micropipette; these were then transferred to a small, ground-glass tissue grinder (Micro-metric Instrument Co., Cleveland, Ohio) and homogenized at *p*H 7.4 in the cold in about 0.5 ml of 0.02*M* sodium phosphate buffer. Following earlier methods for assaying choline acetyltransferase (*5*), we have used ^{14}C-acetyl coenzyme A to label the acetylcholine formed (*6*). Radioactivity in acetylcholine was separated from labeled acetyl coenzyme A by high-voltage electrophoresis (*7*). Homogenates were kept on ice. Assays were made within an hour of the dissection.

With this assay we could readily detect the formation of ^{14}C-acetylcholine in the ganglion. For 30 minutes, acetylcholine was produced at a linear rate; by 1 hour, the rate of synthesis had diminished (Fig. 1). The initial rate at which the product appeared was proportional to the concentration of the enzyme.

Some of the properties of the *Aplysia* choline acetyltransferase were studied with homogenates and acetone powders (*8*) of this ganglion. We could find no indication in our preparation that the enzyme was bound to a particulate or membrane fraction, an association observed in the nerve endings of some other species (*9*). All of the enzyme in the homogenate was recovered in the supernatant after centrifugation for 30 minutes at 111,000*g*. The inclusion of ethylenediaminetetraacetate in the assay mixture enhanced enzyme activity. This compound also appeared to stabilize the enzyme, for activity was rapidly lost during incubations in the absence of substrate. Neutral detergents (0.5 percent Triton X-100 or Nonidet) were without effect. As shown previously for enzyme from other sources (*10*), activity was lost after exposure to low concentrations of alkylating agents (iodoacetate and *N*-ethylmaleimide) and *p*-hydroxymercuribenzoate. Nonetheless, the addition of sulfhydryl-protecting reagents (cysteine, 2-mercaptoethanol, or dithioerythritol) did not result in greater activity.

Table 1. Regional distribution of choline acetyltransferase in the abdominal ganglion of *Aplysia*. Clumps of the different cell types were dissected from the ganglion of 200- to 400-gram *Aplysia*, leaving a residual core, the neuropil, consisting of axons and nerve endings. The various cell fractions and the neuropil were homogenized and assayed as described in the legend to Fig. 1. Protein was always greater than 50 μg per milliliter of homogenate. The protein concentration of coelomic fluid was 0.5 mg/ml. The values for the specific activity of acetylcholine synthesis were calculated from 10-μl samples removed after 30 minutes of incubation; they are listed with their standard errors. In parentheses are the numbers of independent determinations from different ganglia.

Regions	Acetylcholine synthesis (nmole/hour per mg protein)
Bag cells	45 ± 16 (11)
White cells	0 (8)
Pigmented cells	248 ± 16 (11)
Neuropil	344 ± 47 (3)
Coelomic fluid	0 (1)

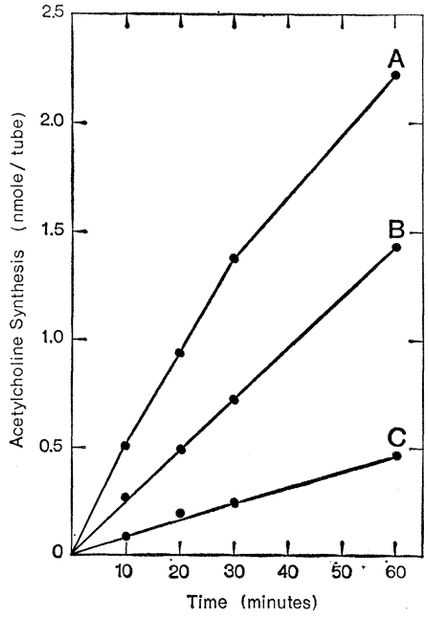

Fig. 1. Time course of acetylcholine synthesis. After dilution with 0.02*M* sodium phosphate buffer, *p*H 7.4, homogenates of the pigmented cell fraction of an abdominal ganglion from *Aplysia* were incubated at 35°C with ^{14}C-acetyl coenzyme A, 20 m*M* choline, 1 m*M* EDTA, 0.2*M* NaCl, and 0.02*M* phosphate buffer, *p*H 7.4, in a final volume of 50 μl in the bottom of a conical glass tube. Samples of 10 μl were pipetted onto paper, air-dried, and subjected to high-voltage electrophoresis at *p*H 4.7 (*7*). The areas (2 by 2 cm) of the electropherogram containing the radioactive product were cut out and counted directly by liquid scintillation. Using egg-white lysozyme as a standard, we measured protein by the Lowry method for small amounts of protein (*16*). The amounts of protein per tube were: *A*, 12.5 μg; *B*, 6.2 μg; and *C*, 2.1 μg.

The product formed was identified as acetylcholine. Its synthesis was dependent upon the addition of choline. Inclusion of 0.1 m*M* eserine and 0.5 m*M* diisopropylfluorophosphate, inhibitors of acetylcholinesterase, resulted in a slight increase in the amount of product formed after a 30-minute incubation, and a greater increase (about 10 to 20 percent) after 1 hour. The radioactive material formed during the incubation was located on electropherograms by radioautography with x-ray film (*7*); it corresponded in position to acetylcholine, which was identified after the electropherogram was sprayed with Dragendorf's reagent (*12*). During electrophoresis the product was separated from acetyl coenzyme A by about 10 cm. No other radioactive material that migrated toward the cathode was detected by radioautography. The radioactive product was characterized further after elution from electropherograms. It migrated with acetylcholine during thin-layer chromatography on cellulose (*13*). As expected of acetylcholine, the product was quantitatively precipitated as the reineckate and was completely hydrolyzed at 70°C in 15 minutes in 0.5*M* NaOH. Although the enzyme is not completely specific for choline, it catalyzed the acetylation of related

amines at considerably lower rates. Similar results for the enzyme from other sources have been reported (*10*).

The neurons of the abdominal ganglion have been grouped into three cell types: white cells, bag cells, and pigmented cells (Fig. 2) (*1*). The white cells and the bag cells have the morphological characteristics of neurosecretory neurons. These cells produce characteristic granules and send their processes into the connective tissue sheath which surrounds the ganglion and which functions as a neurohaemal organ (*1, 14*). Furthermore, these neurons do not seem to form chemical synapses with other cells or with peripheral organs (*1*), and we would not expect them to synthesize acetylcholine. By contrast, the pigmented cells, which are involved in visceromotor integration, have direct connections with each other and also innervate the gills, siphon, heart, and sexual organs (*2, 15*). It is among these neurons that we would expect to find cells able to synthesize the transmitter.

Choline acetyltransferase was detected in both the pigmented-cell region and in the neuropil (Table 1). No significant activity was found in the white cells. Bag cells had a low and variable concentration of the enzyme. The variation was possibly due to contamination of this fraction with nerve endings and fibers which course through the bag-cell region (Fig. 2). The low activity in white cells and bag cells was not due to high concentrations of acetylcholinesterase or any inhibitors of synthesis. When mixed with homogenates of pigmented cells, extracts of the white cells or of the bag cells did not interfere with the detection of acetylcholine synthesis. Coelomic fluid, which bathes the ganglion, had no enzymatic activity.

Table 2. Acetylcholine synthesis in single identified cells of the abdominal ganglion of *Aplysia*. The pigmented cell, R2, and the white cell, R14, were identified by eye in ganglia from 200- to 400-gram *Aplysia*. The pigmented cell L10 was identified electrophysiologically by its connection to L3 (*1*). The contents of the cells were assayed for choline acetyltransferase as described in the legend to Fig. 1. Using the formula for the volume of an ellipsoid, we calculated cell volumes from estimates of the cell axes aided by a hemocytometer and a micrometer scale fitted to the ocular of the microscope. Measurements are presented with their standard errors. In parentheses are the numbers of independent determinations.

Cells	Volume (nl)	Acetylcholine synthesis	
		Nmole/hour per cell	Nmole/hour per nl cell volume
		White	
R14	41 ± 9 (7)	< 0.04(8)	0
		Pigmented	
L10	32 ± 5 (10)	0.68 ± 0.16(9)	0.021
R2	165 ± 10(9)	2.26 ± .18(16)	.014

We have also detected the synthesis of acetylcholine in single cells (Table 2). When the connective tissue sheath is cut, cell bodies tend to protrude. By gentle manipulation, the cell may be freed from the rest of the ganglion by severing the axon. The cell body is then promptly sucked up in buffer into a micropipette and transferred either to a small tissue grinder for homogenization or directly to a conical glass tube, where the cell is ruptured by freezing and thawing five times. We obtained similar results using both methods of disrupting the cells.

As with clumps of white cells, no synthesis was obtained with the large single white cell, R14. Two individual pigmented cells were examined, and both synthesized acetylcholine. The cell body of the giant cell, R2, had about the same concentration of the enzyme as the pigmented-cell region collectively; R2 contained 7.4 ± 0.6 μg of protein, which is enough to be measured by the Lowry method (*16*). Synthesis of acetylcholine was also seen with L10 (*17*), a pigmented cell that has about one-fifth the volume of the giant cell R2, as judged by microscopic estimation. If our approximations of the cell volumes are correct, and if both cell bodies contain about the same concentration of protein, then R2 and L10 have similar concentrations of choline acetyltransferase.

As far as the groups of neurons are concerned, we have found good correlation between the functional architecture of the ganglion and the distribution of choline acetyltransferase in the bag-cell, white-cell, and pigmented-cell regions. Acetylcholine synthesis appeared, however, to be more extensive among the individual pigmented cells than anticipated. While it is axiomatic that a cholinergic neuron synthesize acetylcholine, it is not at all certain that the ability to synthesize acetylcholine should be taken as proof that it is used as a transmitter. Neurophysiological and pharmacological data indicate that L10 is cholinergic (*2*). Our results show

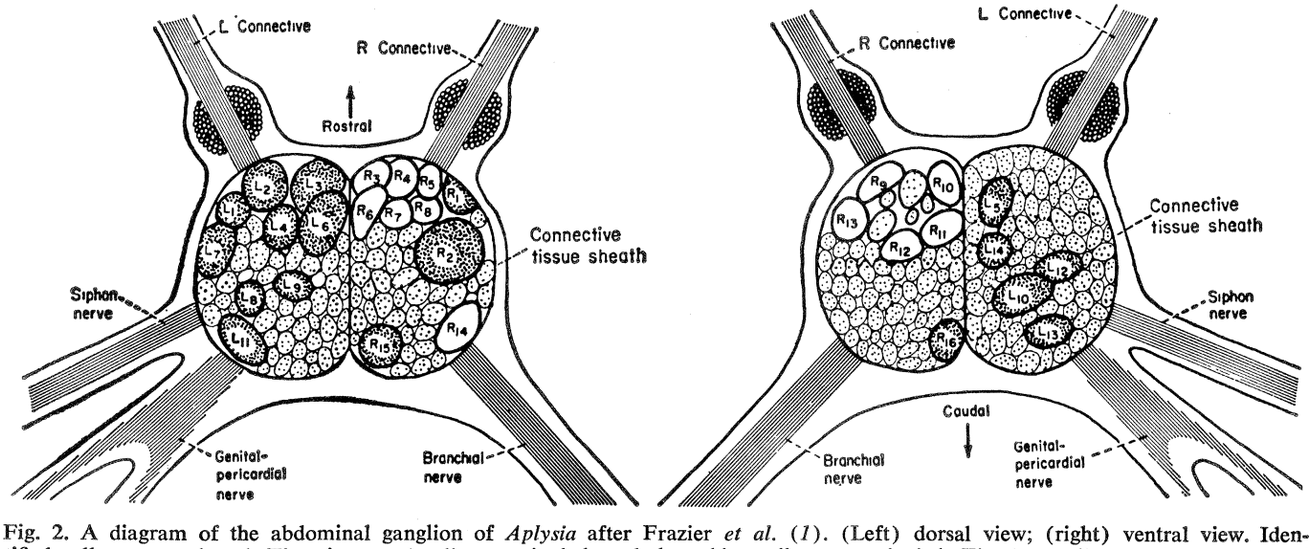

Fig. 2. A diagram of the abdominal ganglion of *Aplysia* after Frazier *et al.* (*1*). (Left) dorsal view; (right) ventral view. Identified cells are numbered. The pigmented cells are stippled, and the white cells are unshaded. The bag cells are shown clustered around the right and left connectives.

that this cell contains choline acetyltransferase at a concentration slightly greater than the highest activity reported for cholinergic mammalian axons (for example, ventral spinal roots) (*18*). However, there is evidence in one other species of *Aplysia* that the pigmented cell R2, which has an enzyme concentration comparable to that of L10, mediates an action which is not cholinergic. A branch from R2 to the giant cell in the left pleural ganglion produces a postsynaptic potential which was not blocked by curare and was not simulated by acetylcholine applied iontophoretically (*19*). Although these pharmacological data are not conclusive, they suggest that R2 may be a neuron which is not cholinergic, but which nonetheless has the capacity to synthesize acetylcholine. Alternatively, it is possible that R2 releases acetylcholine in addition to a noncholinergic transmitter.

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17 April 1968

Centrifugation of Mammalian Cells on Gradients: A New Rotor

Abstract. *A short-arm rotor increases separation of viable mammalian cells, from mixtures, by low-speed centrifugation; continuous Ficoll density gradients in tissue-culture media are used. We describe the theory and experimental demonstration of the superior separation achieved with this new rotor.*

Ability to isolate single species of cells from mammalian tissues would be widely useful because experimentation on specific cell types within whole tissues is complicated by the cells' intimate association with other cells differing markedly in structure and function. We are currently developing methods for resolution of disaggregated solid tumors into fractions containing purified suspensions of such things as viable malignant cells, fibroblasts, reticuloendothelial cells, and inflammatory cells.

Viable mammalian cells of predetermined diameters and densities may be sedimented at predictable rates (*1*) on density gradients of Ficoll (*2*) in tissue-culture medium; the computer-integrated differential sedimentation equation is used for determination of the optimal speed and duration of centrifugation. Anderson's low-speed A-XII zonal rotor is used.

We have now devised a similar system for the predictable separation of cells by gradient centrifugation; ordinary, parallel-walled polycarbonate centrifuge tubes of 100-ml capacity are used in the International Equipment Company centrifuge, model PR-2. This system has the advantage of easily maintained sterility, and permits multiple experiments in less time. Ehrlich ascites tumor cells and HeLa cells, separated from mixtures by this procedure, have been subsequently regrown in mice and in tissue culture, respectively. Rabbit thymocytes and Ehrlich ascites tumor cells also have been separated from mixtures in 98- to 100-percent purity.

We now report theoretical and experimental verification of the fact that greater separation of particles, sedimenting in solution in a centrifuge tube, can be achieved by movement of the centrifuge tube closer to the axis of revolution.

Consider a system consisting of two spherical particles, 1 and 2, sedimenting in a homogeneous solution of given density ρ_S and viscosity η, in which particle 2 sediments faster by virtue of its larger diameter a or greater density, or of both (*3*, *4*). After setting of the angular velocity during centrifugation (ω) constant, let both particles start sedimenting at a specific distance r_0 from the axis of revolution. Proceed for the time (t) that it takes particle 2 to travel distance l from r_0. During this time, particle 1 sediments to a position r_1 intermediate between r_0 and (r_0+l). The differential sedimentation equation states that the velocity of either particle is

$$\left(\frac{dr}{dt}\right)_i = C_i r$$

where

$$C_i = \frac{a^2_i(\rho_i - \rho_S)\,\omega^2}{18\eta}$$

Therefore

$$t = \frac{1}{C_2} \ln \frac{r_0 + l}{r_0} = \frac{1}{C_1} \ln \frac{r_1}{r_0}$$

and

$$r_1 = r_0 \left(1 + \frac{l}{r_0}\right)^{\alpha}$$

where

$$\alpha = C_1 / C_2$$

Distance S between the two particles after time t is therefore

$$S = r_0 + l - r_0 \left(1 + \frac{l}{r_0}\right)^{\alpha}$$