The synthesis of acetylcholine by plants

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(Received 19 September 1980/Accepted 26 September 1980)

Choline acetyltransferase was demonstrated in nettles (*Urtica dioica*), peas (*Pisum sativum*), spinach (*Spinacia oleracea*), sunflower (*Helianthus annuus*) and blue-green algae by using a Sepharose–CoASH affinity column. The column effected a 1500-fold purification of the enzyme from nettle homogenates and was required for demonstrating activity in the other higher plants. Demonstration of the enzyme in blue-green algae suggests that acetylcholine was a biochemical necessity in the earliest photosynthetic organisms.

Evidence that the concentration of acetylcholine in plants is light-regulated has focused interest on the synthesis of acetylcholine by plants (Jaffe, 1970; Hartmann & Kilbinger, 1974). However, the acetylcholine-synthesizing enzyme choline acetyltransferase (EC 2.3.1.6) has been demonstrated only in one plant, the common nettle (*Urtica dioica*), which is known to have a high content of acetylcholine and was used by Barlow & Dixon (1973) to demonstrate and define some characteristics of the enzyme. Our experiments show that choline acetyltransferase is difficult to detect in plant tissues; an affinity-chromatographic purification was necessary to detect and measure its activity in certain plants. Our methods and the results obtained with several plant species should now facilitate a new approach to the photoregulation and function of acetylcholine in plants.

Experimental

Materials

Higher plants used were: the common nettle (*Urtica dioica*); the garden pea (*Pisum sativum*); spinach (*Spinacia oleracea*); sunflower (*Helianthus annuus*). All were grown in the greenhouse under natural light supplemented by fluorescent lamps for 16 h/day. Blue-green algae of the species *Oscillatoria agardhii* were grown by the method of Daley & Brown (1973) and harvested in the exponential phase of growth.

Sephadex G-10, DEAE-Sephadex, Sephacryl S-200 and activated CH-Sepharose 4B were purchased from Pharmacia (Canada) Ltd., Dorval, Quebec, Canada. CoA (85–90% pure), 5,5'-dithio-bis-(2-nitrobenzoic acid), polyvinylpyrrolidone, citrate synthase (EC 4.1.3.7), and eel acetylcholinesterase (EC 3.1.1.7) were purchased from Sigma

Chemical Company, St. Louis, MO, U.S.A. Econo-fluor scintillation 'cocktail', and [^3H]acetic anhydride (sp. radioactivity 50 mCi/mmol) were purchased from New England Nuclear Corp., Lachine, Quebec, Canada. Of the choline acetyltransferase inhibitors used, bromoacetylcholine perchlorate was synthesized by the procedure of Chiou & Sastry (1968), and the 4-styrylpyrimidine derivatives were supplied by Calbiochem–Behring Corp., La Jolla, CA, U.S.A. Proteins were measured with the assay kit supplied by Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada.

All column and concentration steps were done in a Coldstream refrigerated cabinet at 4°C. For concentration steps, an Amicon stirred cell, model 52, with Diaflow PM 30 ultrafiltration membrane was used. Radioactivity was measured with a Beckman LS-9000 liquid-scintillation counter programmed to yield d.p.m. directly.

Extraction and purification procedures

With higher plants, homogenates were prepared by using growing tips with only partly expanded leaves. Usually, 5–10 g of growing tips were homogenized in 100 ml of buffer; with peas, up to 15 g of tissue was used. The buffer (buffer A) contained 25 mM-sodium phosphate, 25 mM-EDTA, 100 mM-ascorbic acid, 1% (w/v) polyvinylpyrrolidone, final pH 7.6. The tissues were first chopped in a Waring blender, then homogenized in a Polytron homogenizer, and centrifuged at 40 000 g for 30 min. The supernatant (50 ml) was concentrated to half the original volume with the Amicon stirred cell under N_2 at 31.7 kg (70 lb/in 2 ; 4.83 kPa) pressure. The concentrate (20–25 ml) was applied to a column (56 cm $\times$ 2.5 cm) of Sephadex G-10 equilibrated and eluted with buffer (buffer B) containing

25 mM-sodium phosphate, 25 mM-EDTA, pH 7.6. Eluted fractions that contained protein (detected by u.v. absorbance) were combined and concentrated up to 5-fold. A 5 ml portion of this concentrate was applied to a CH-Sepharose 4B-CoASH affinity column (15 cm $\times$ 2.5 cm), followed by buffer B until all unbound protein was eluted. Finally, the bound protein was eluted from the column with a linear 0–1.0 M-NaCl gradient in the same buffer. Individual 3.6 ml fractions collected from the column were assayed for choline acetyltransferase activity; the active fractions were pooled, concentrated and used to determine the specific activity of the enzyme.

With blue-green algae, a homogenate of about 10% (wet w/v) was made up in buffer A (a mild sonication being used to break up the filaments), followed by passage through a French press at 862.5 kPa (12 500 lbf/in²). This homogenate was then centrifuged and processed through the two columns in the same way as the higher-plant homogenates.

Housefly (*Musca domestica*) heads were collected from 3–4-day-old flies, homogenized in buffer (100 mM-sodium phosphate/100 mM-NaCl, pH 7.8), centrifuged at 100 000 *g* for 30 min, and the supernatant processed on the two columns, as described above.

Preparation of the affinity resin

The affinity resin was prepared by the general method of Pharmacia Fine Chemicals (1979) for ligands immobilized on Sepharose 4B. For our purpose, we allowed 750 mg of CoA to react with 15 g of activated CH-Sepharose 4B. The affinity column was reduced with 1 mg of DL-dithiothreitol/ml in buffer B to ensure that CoA was restored to the free-thiol form before each run; the column was then washed with buffer alone to remove excess dithiothreitol.

Preparation of [³H]acetyl-CoA

Acetyl-CoA was prepared by the method of Simon & Shemin (1953), and the product was purified further and assayed essentially by the method of Hebb *et al.* (1975). The specific radioactivity of the purified [³H]acetyl-CoA varied from 12 mCi/mmol to 24 mCi/mmol, depending on the initial specific radioactivity of the [³H]acetic anhydride purchased.

Choline acetyltransferase assay

The assay system was essentially that of Fonnum (1975), but the volume and concentration of the incubation components were as described by Morris *et al.* (1971). The incubation mixture contained sodium phosphate buffer, pH 7.5 (33 mM), NaCl (500 mM), eserine sulphate (0.13 mM), choline chloride (10 mM), [³H]acetyl-CoA (0.5 mM) and enzyme solution (50 μ l); the final volume was

125 μ l. The reported activities for choline acetyltransferase are means of duplicate determinations after correction for control incubations without choline. Other controls used periodically were inclusion of acetylcholinesterase and of choline acetyltransferase inhibitors in the incubation mixture to determine whether the labelled product was indeed acetylcholine and whether the synthesis of labelled product was indeed catalysed by choline acetyltransferase.

Results and discussion

With nettles, and by using the 40 000 *g* supernatant or the Sephadex G-10 concentrate, we confirmed the presence of choline acetyltransferase activity as reported by Barlow & Dixon (1973). However, the same methods failed to reveal activity in peas, sunflower and pumpkin (*Cucurbita pepo*), all of which have been shown to contain acetylcholine (Hartmann & Kilbinger, 1974). With the latter plants we regularly obtained the same or higher radioactivities in our control incubation mixtures without choline as we did in our incubation mixtures with choline. With peas, for example, when acetylcholinesterase or choline acetyltransferase inhibitors were included in incubation mixtures without choline, the radioactivity remained the same, indicating that the labelled product was not acetylcholine and that its production was not catalysed by choline acetyltransferase. We concluded that pea extracts contained an enzyme capable of splitting off a portion of [³H]acetyl-CoA to form a molecule carrying the label and with the positive charge required for the cationic separation of acetylcholine by Fonnum's (1975) assay. Attempts to separate this putative enzyme from choline acetyltransferase by conventional methods failed. Finally, a CH-Sepharose 4B-CoASH affinity column was prepared, tested with a known source of choline acetyltransferase (housefly heads), and used successfully to isolate choline acetyltransferase and measure its activity in plants.

Nettles

Fig. 1 shows the elution profile for choline acetyltransferase from the affinity column. The elution of bound choline acetyltransferase was initiated at 0.2 M-NaCl and showed a peak at about 0.4 M; choline acetyltransferase from the other higher plants tested also eluted at these values. With nettles, the affinity column effected a 1500-fold increase in the specific activity over that of the Sephadex G-10 concentrate. The rates of acetylcholine synthesis at various temperatures were measured by using the pooled and concentrated active fractions from the affinity column. Rates of acetylcholine synthesis were linear for at least

30 min up to 40°C; the temperature optimum was sharply delineated at 40°C.

Peas

Choline acetyltransferase activity in extracts of peas was demonstrated only in effluents from the affinity column. The elution profile from the affinity column was almost identical with that for nettles, with peak activity at 0.35 M-NaCl. However, at the peak activity, the rate of acetylcholine synthesis was one-tenth that for nettles. The pooled and concentrated active fractions from the affinity column gave linear rates of acetylcholine synthesis

for at least 40 min up to 40°C; the maximum activity occurred at 40°C.

Other plants and neural tissue

Table 1 compares the choline acetyltransferase activity of nettles and peas with that of other selected plants and neural tissue. With nettles the activity was strikingly higher than with the other plants, and was equal to that in housefly heads, which are known to be a rich source of the enzyme (Smallman, 1956). Extracts of blue-green algae were strongly pigmented and these pigments were retained by the affinity column, which failed to yield any obvious peak of choline acetyltransferase activity. However, the Sephadex G-10 concentrate yielded a value for choline acetyltransferase activity that was the same as that obtained with spinach after the affinity column, suggesting that the comparable value for blue-green algae is considerably higher than the reported value.

Barlow & Dixon (1973) reported the specific activity of choline acetyltransferase in extracts of acetone-dried powders of nettles as $0.76\text{--}1.12\ \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}$ of protein⁻¹; our comparable preparation was the supernatant of nettle homogenate, which yielded a specific activity of $0.85\ \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. We confirm also their findings that the optimum temperature is 40°C and that the choline acetyltransferase activity of nettles is similar to that of nervous tissue. However, our introduction of the affinity column yielded specific activities at least 100-fold greater than they obtained.

The only previous reference to the use of a CoASH-Sepharose affinity column is a preliminary communication by Ryan & McClure (1976), who reported a 6500-fold purification of choline acetyltransferase from bovine caudate nucleus. Rossier (1977) has emphasized the importance of affinity chromatography for the purification of choline acetyltransferase; the column described and used by

Fig. 1. Elution profile for choline acetyltransferase activity of nettles from the CoASH-Sepharose affinity column

The procedure was that detailed in the Experimental section. The arrow indicates the initiation of the linear NaCl gradient for the elution of bound protein. The choline acetyltransferase activities of the fractions were determined by Fonnum's (1975) assay at 40°C.

Table 1. Choline acetyltransferase activities in selected plants and neural tissue

For blue-green algae, values were obtained with a concentrate of protein fractions from the Sephadex G-10 column. All other values were obtained with concentrates of pooled active fractions from the affinity column. Details of preparation of homogenates and columns, and of the choline acetyltransferase assay are given in the Experimental section.

Source	Choline acetyltransferase activity (nmol of acetylcholine synthesized · $\text{min}^{-1}\cdot\text{ml}^{-1}$)	Protein ($\mu\text{g}/\text{ml}$)	Specific activity (nmol of acetylcholine synthesized · $\text{min}^{-1}\cdot\text{mg}$ of protein ⁻¹)
Nettle	1.067	1.0	1067.0
Pea	0.108	5.7	18.94
Spinach	0.016	3.5	4.53
Sunflower	0.026	4.1	6.26
Blue-green algae	0.016	101.0	0.16
Housefly head	1.21	1.2	1008.0

us for the purification of choline acetyltransferase from plants may prove to have wider applications.

The choline acetyltransferase activity in nettles is approximately two orders of magnitude greater than in the other higher plants tested. This difference is paralleled by the acetylcholine content: the stinging hairs and leaf tissue of nettles contain 15 μmol and 88 μmol of acetylcholine/g fresh weight respectively (Emmelin & Feldberg, 1947); the corresponding values for pea, spinach, and sunflower are in the range 7–8 μmol (Hartmann & Kilbinger, 1974). Since acetylcholine with histamine causes the urticaria of nettle sting (Emmelin & Feldberg, 1947), the high acetylcholine content of nettles may provide protection against herbivores, thus conferring a selective mechanism for this characteristic. Finally, our demonstration of choline acetyltransferase in blue-green algae suggests that acetylcholine was a biochemical necessity in the earliest photosynthetic organisms.

We thank Ms. Patricia Garland for technical assistance, and the National Research Council of Canada for support under grant no. A2394 to B. N. S.

References

- Barlow, R. B. & Dixon, R. O. (1973) *Biochem. J.* **132**, 15–18
- Chiou, C. Y. & Sastry, V. B. (1968) *Biochem. Pharmacol.* **17**, 805–815
- Daley, R. J. & Brown, S. R. (1973) *J. Phycol.* **9**, 395–401
- Emmelin, N. & Feldberg, W. (1947) *J. Physiol. (London)* **106**, 440–455
- Fonnum, F. (1975) *J. Neurochem.* **24**, 407–409
- Hartmann, E. & Kilbinger, H. (1974) *Experientia* **30**, 1387–1388
- Hebb, C., Mann, S. P. & Mead, J. (1975) *Biochem. Pharmacol.* **24**, 1007–1011
- Jaffe, M. J. (1970) *Plant Physiol.* **46**, 768–777
- Morris, D., Maneckjee, A. & Hebb, C. (1971) *Biochem. J.* **125**, 857–863
- Pharmacia Fine Chemicals (1979) *Affinity Chromatography: Principles and Methods*, pp. 19–22, Pharmacia, Uppsala
- Rossier, J. (1977) *Int. Rev. Neurobiol.* **20**, 284–331
- Ryan, R. L. & McClure, W. O. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35**, 1647
- Simon, E. J. & Shemin, D. (1953) *J. Am. Chem. Soc.* **75**, 2520
- Smallman, B. N. (1956) *J. Physiol. (London)* **132**, 343–357