Choline Acetyltransferase in the Nettle *Urtica dioica* L.

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Extracts of acetone-dried powders prepared from nettle leaves were shown to catalyse the synthesis of acetylcholine. The specific activity of the enzyme in these extracts is of the same order as that of extracts from mammalian sources, such as ox brain, and the effects of temperature and pH are similar to those reported for mammalian choline acetyltransferase. Synthesis is not restricted to the younger leaves but appears to be continuous up to senescence.

Acetylcholine has been found in a number of plant species (see Jaffe, 1970, for a summary), but is particularly plentiful in the nettle, where the concentration in the stinging hairs has been estimated to be greater than 50 mM (10 mg/ml) by Emmelin & Feldberg (1947). This is interesting because acetylcholine is one of the components of the hair which produces the stinging reaction. The other component is histamine, which is also present in the hair fluid but at less than one-tenth of this concentration. The sting produced by the hairs can be imitated by the intradermal injection in man of a mixture of acetylcholine and histamine, though not by either given separately. It is also interesting because the high concentrations of acetylcholine in nettle hairs may be comparable with the concentrations in the stores in cholinergic nerve endings in animals. The processes for making and storing acetylcholine in the nettle are therefore worth comparing with those of animals, but nettles do not appear to contain cholinesterases, which makes the bioassay of acetylcholine formed in nettle extracts much easier than in extracts from animal tissues. Further interest in the synthesis of acetylcholine in plants arises from the suggestions which have been made that it may be concerned with phytochrome-mediated processes (Jaffe, 1970).

In the present paper we describe a preliminary study of the synthesis of acetylcholine in plants, by using acetone-dried powders prepared from nettles, and a comparison of the results with those of purified choline acetyltransferase prepared from rat brain (Glover & Potter, 1971) and rabbit brain (Bull & Oderfeld-Nowak, 1971).

Materials and Methods

Nettle extracts

Local nettle rhizomes were obtained and cultivated in the greenhouse.

Leaf homogenates and acetone-dried powders were normally prepared from the top of the nettle stem

bearing leaves 3 cm or less in length including the terminal bud. Other extracts are described in the text.

Homogenates obtained by grinding nettles in buffer were unsatisfactory for two reasons. First, they contained large amounts of acetylcholine and histamine already synthesized, and, secondly, they contained large quantities of polyphenol oxidase activity, which could not be completely inactivated by ascorbic acid and dithiothreitol or polyvinylpyrrolidone. We have therefore prepared acetone-dried powders of nettle leaves by the usual method (Green *et al.*, 1937). Even these contained some polyphenol oxidase activity and substrate, but this could be suppressed by adding ascorbic acid and dithiothreitol in the concentrations given below. The complete extraction medium for the acetone powders had the following composition: 0.05 M-potassium phosphate buffer, 0.1 M-KCl, 2.5 mM-MgCl₂, 2.5 mM-EDTA, 0.1 M-ascorbic acid and 0.65 mM-(0.1 mg/ml) dithiothreitol; the pH was 7.6. The acetone-dried powder was ground with the extraction medium (50 mg/ml) in a pestle and mortar, squeezed through cheese-cloth, centrifuged at 1000g for 20 min and the supernatant was passed down a column of polyacrylamide gel (Bio-Gel P-4; exclusion limit mol.wt. 3600) to remove final traces of acetylcholine and histamine. This gel had previously been soaked for 24 h in a solution (subsequently referred to as solution A) consisting of 0.05 M-potassium phosphate buffer containing 0.1 M-KCl, 2.5 mM-MgCl₂ and 2.5 mM-EDTA; the pH was 7.6. The eluate was placed in a dialysis bag in contact with moist polyethylene glycol 4000 and left to concentrate approximately threefold. All of these preparations were performed in a cold room (4°C). If necessary the final solution was stored at -20°C.

Assay of choline acetyltransferase (EC 2.3.1.6) activity

The usual assay mixture consisted of 0.1 ml of choline chloride (0.03 M prepared in solution A described above), 0.1 ml of 5 mM-acetyl-CoA and 0.3 ml

of the eluate containing the choline acetyltransferase from the nettle, making a total volume of 0.5 ml. The reaction was normally started by the addition of the acetyl-CoA and was stopped after incubation for a suitable time at a suitable temperature (e.g. 10 min at 30°C) by the addition of 0.1 ml of 3 M-HCl followed after 5 min by 0.1 ml of 3 M-KOH to restore the pH to neutrality. Water (0.3 ml) was then added to bring the total volume to 1.0 ml and the solutions were kept in ice until assayed.

The acetylcholine formed was assayed on the isolated guinea-pig ileum in Tyrode's solution containing mepyramine (5 μ M). Full details of the composition of Tyrode's solution and the procedure for conducting the assay and for calculating the results are given by Edinburgh Staff (1970). Because Emmelin & Feldberg (1947) reported that the nettle does not appear to contain any cholinesterase activity we did not consider it necessary to conduct the assay in the presence of any cholinesterase inhibitor. In confirmation of their observations we could not detect any significant loss in activity of the solutions during the time required for the bioassays (up to 3 h). This greatly simplified and shortened the assay, making it possible to work with the guinea-pig ileum, with doses applied once every 2 min, rather than with a preparation such as eserinated leech muscle and doses given only once every 20 min.

Each sample was tested, diluted if necessary, in a four-point assay of latin-square design and the concentration in the reaction mixture was calculated, together with the fiducial limits ($P = 0.05$) for the estimate. These limits were usually $\pm 10\%$ but varied slightly depending on the sensitivity of the particular piece of ileum used in the assay. Full details are given by Edinburgh Staff (1970).

Protein was determined by the method of Lowry *et al.* (1951), with dry bovine serum albumin powder (Calbiochem, Los Angeles, Calif., U.S.A.) as a standard.

Results

Controls

Enzyme assay reaction mixtures from which the acetyl-CoA was omitted, or where it was added but the nettle extract had been boiled, did not produce any detectable effects on the guinea-pig ileum. Mixtures from which the choline was omitted were also inactive.

At pH 7.6 and at temperatures of up to 35°C the reaction rate was constant for at least 20 min (Table 1), although the reaction rate decreased thereafter. At 30°C there was no significant difference in the amount of acetylcholine formed whether acetyl-CoA was added initially and incubated for 10 min, or

whether the reaction mixture minus acetyl-CoA was incubated for 40 min before adding the acetyl-CoA and then incubated for 10 min. This suggests that there was no detectable inactivation of the enzyme during the 40 min period. In a similar experiment in which all the constituents of the reaction mixture except choline were added and choline was used to start the reaction either initially or after 40 min pre-incubation, there was less activity after the 40 min pre-incubation, suggesting that in the experiments lasting longer than 20 min the non-linearity was due to the hydrolysis of the acetyl-CoA. In experiments lasting up to 20 min, doubling the concentration of acetyl-CoA had little effect (Table 1).

Specific activity

During the preparation of the nettle extract and its storage, variable amounts of protein became precipitated. The most extensive precipitation occurred when the preparation was frozen and thawed, although this was accompanied by no appreciable loss of activity. This behaviour, however, means that when activities are expressed on a protein basis they are rather variable and range between 0.76–1.12 nmol/min per mg of protein. When expressed in terms of the weight of fresh material the enzyme activity varied according to the source of the nettles used and ranged from 5.8–10.2 nmol/min per g fresh wt. The specific activity of the acetone-dried powder extract, in terms of weight of protein is of the same order as that of homogenates from ox brain striate nuclei, 0.28 nmol/min per mg of protein (Glover & Potter, 1971).

Effect of pH and temperature

The optimum temperature for a 10 min incubation period was about 40°C (Table 1). Above this temperature the rate of acetylcholine synthesis declined sharply. Reaction mixtures incubated above 45°C became turbid.

Comparison of the synthesis of acetylcholine at different pH values indicated that it was greater in more alkaline conditions. Instead of a sharp pH optimum the rate increased to a plateau above pH 7.5 (Table 1); synthesis at pH values greater than 9.0 was not tested. In these conditions both acetyl-CoA and acetylcholine may be appreciably hydrolysed and the enzyme denatured so that the cause of any loss of activity could not be determined without considerable further investigation. The synthesis was apparently lower in 0.1 M-potassium phosphate buffer than in 0.1 M-glycylglycine buffer, an effect which was also observed with phosphate and Tris buffers with the mammalian enzyme (Glover & Potter, 1971).

Table 1. *Acetylcholine synthesis by extract of acetone-dried powders obtained from nettles*

Estimates of the acetylcholine concentrations (μM) in the reaction mixture at the end of the experiment are given. The fiducial limits ($P = 0.05$) were approximately $\pm 10\%$ but varied slightly depending on the sensitivity of the piece of ileum used in the assay. The amount of acetylcholine synthesized (nmol) in the assay in the time stated can be calculated by dividing the numbers in the Table by 2. The results can only be compared within each experiment. Unless otherwise stated, the conditions of the experiments are as described in the text.

Effect of acetyl-CoA concentration		Incubation time ...	Concn. of acetylcholine (μM)	
Experiment 1	Temperature ($^{\circ}\text{C}$)		10min	20min
Concn. of acetyl-CoA (mM)				
1	30		34.3	73.7
2	30		49.0	73.9
Effect of temperature				
Temperature ($^{\circ}\text{C}$)			Experiment 1	Experiment 2
0			2.0	
15			5.6	
25			10.7	16.2
30				23.3
35			22.9	34.8
40				38.2
45			16.8	19.7
50				5.8
55			1.0	
Effect of pH				
Buffer	pH		Experiment 1	Experiment 2
Potassium phosphate	6.5		27.0	37.5
	7.0		33.1	43.1
	7.5		33.6	41.0
	8.0		33.8	44.5
Glycylglycine	8.0		41.2	52.9
	9.0		45.4	61.7

Table 2. *Production of acetylcholine in relation to the age of leaf*

Acetone-dried powders were made from: 1, the tip of stem bearing leaves less than 3cm; 2, the first pair of fully expanded leaves; and 3, the last pair of leaves.

Leaf sample	Concn. of acetylcholine (μM) after 10min	Amount of acetylcholine synthesized (nmol/min per g fresh wt.)
1	36.6	6.7
2	13.0	1.7
3	10.7	1.7

Enzyme distribution in the plant

As most, if not all, of the acetylcholine in the nettle leaf is in the stinging hairs it is reasonable to suppose that the enzyme synthesizing it resides there also. It is not, at the moment, practicable to test this because of the difficulties of obtaining leaves free from stinging hairs and because there is no method

sensitive enough to detect the enzyme in the hairs. A comparison was, however, made of acetone powders prepared from leaves of three different ages: 1, the top of the nettle stem bearing leaves shorter than 3cm including the terminal bud; 2, the first pair of fully expanded leaves; and 3, the last pair of fully expanded leaves. The results are given in Table 2.

Spinach extracts

Among plants other than nettles that contain acetylcholine, spinach is a good source (Appel & Werle, 1959). However, we were unable to detect any synthesis of acetylcholine with acetone-dried powders made from the leaves of mature plants, although on other occasions we detected acetylcholine (along with histamine) in extracts of spinach leaves.

Discussion

We are not aware of any previous reports of studies of choline acetyltransferase activity in plants. The preliminary results reported here suggest that the enzyme in plants is very similar to the mammalian enzyme described by Glover & Potter (1971). There appear to be slight differences, however, particularly in the effects of temperature. The mammalian enzyme had an optimum activity at about 45°C compared with 40°C for the nettle extracts and was inactive at 0°C whereas nettle extracts appear to have some activity at this temperature. The effect of pH is strikingly similar however. The enzyme activity is maintained in the nettle leaves at all ages up to the oldest obtainable, namely the oldest leaves just before flowering. Although the results show that there is a considerable decrease in activity in terms of weight of fresh material, this is more apparent than real,

because the leaves expand more than fourfold from the largest leaves of the youngest sample to the fully expanded state, so that the stinging hairs per area of leaf, and so per g fresh weight, will have decreased. The agreement of the results with the two ages of fully expanded leaves is very close. It seems, then, that the synthesis of acetylcholine continues throughout the life of the leaf. The fact that the enzyme was not detectable in the older leaves of spinach suggests that synthesis in this plant is not continued.

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