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ATTEMPTS TO DEMONSTRATE INCORPORATION OF LABELLED PRECURSORS INTO ACETYLCHOLINE BY *PHASEOLUS VULGARIS* SEEDLINGS

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; acetylcholine biosynthesis; acetate; choline; hypocotyl hook; red light.

Abstract—The labelling of acetylcholine was investigated in bean seedlings (*Phaseolus vulgaris*) and hypocotyl hooks. Labelling could only be found after red light irradiation but not in the dark. Acetate was a much better precursor than choline. Glucose gave no detectable amounts of acetylcholine labelling during 24 hr of incubation. The rate of acetylcholine labelling was higher in whole seedlings than in hypocotyl hook preparations.

INTRODUCTION

Acetylcholine (ACh) is widely distributed in nature. Although its only well known function is that of neurotransmitter, it is found in many plants [1, 2], in organisms without a nervous system like Protozoa and in some non-nervous tissues of animals, e.g. placenta. The ACh levels of plant tissues are regulated by light. The effect of light is mediated by the phytochrome system [1, 3]. In spite of great efforts to elucidate the physiological functions of ACh in these situations, they remain unclear and controversial. In the absence of information about the synthesis, storage and degradation of ACh under different developmental conditions, it is very difficult and speculative to discuss its function. Therefore the answer to the question of how ACh acts hinges to some extent on a better understanding of how biosynthesis of ACh occurs.

Where the properties of ACh hydrolysis are rather well understood [1, 4], there is much less information about the first steps of biosynthesis and the properties and intracellular location of the enzymes involved. It is known that cholineacetyltransferase from animal tissues catalyses the formation of ACh from two substances, choline and acetyl-Co-A [5]. Cholineacetyltransferase activity could also be demonstrated in plants, among others stinging nettle [6], pea bud extracts, cauliflower, and bean hypocotyl hooks [7; Jaffe, personal communication]. Nothing is known about the pathway of ACh-biosynthesis of plant tissues *in vivo*. The main problem is the quantitative determination of ACh levels. A sensitive and specific method for the ACh analysis is gas chromatography [8], but this method is not suitable for routine plant ACh determinations as so much plant material and so many separation steps are necessary. We have tested the incorporation of some radioactive tracers into ACh using etiolated seedlings and hypocotyl hooks of beans to get some preliminary information about the possibility of using radiotracers for ACh determination.

Table 1. Recovery of acetylcholine-[1-^{14}C] and choline-[methyl-^{14}C] added to deep frozen hypocotyl hooks and prepared as described in Experimental

	Acetylcholine-[1-^{14}C] % recovery	Choline-[methyl-^{14}C]
Homogenate	96.9 ± 63	96.7 ± 5.7
after evaporation	69.0 ± 6.5	73.6 ± 6.5
Electrophoresis		
Origin	17.1 ± 2.3	14.7 ± 1.7
ACh band	74.3 ± 6.1	0.9 (1.1%) ± 0.03
Choline band	0.1 ± 0.01	78.7 ± 5.8
Fonnum extract		
Organic phase	96.6 ± 6.5	87.3 ± 5.8
Water phase	3.4 ± 0.4	12.7 ± 0.8
Electrophoresis of organic phase		
Origin	28.1 ± 6.3	14.2 ± 2.1
ACh band	41.1 ± 2.8	15.8 (23.1%) ± 0.9
Choline band	0	68.4 ± 5.3

The recovery of the homogenate and the residue was calculated from the total radioactivity of the radiolabelled ACh. The recovery after Fonnum extraction was calculated from the radioactivity of the cleared extract. The overlapping of choline in the acetylcholine-band is shown in parentheses. The numbers present the mean values of 4 experiments.

RESULTS

Recovery of acetylcholine

Owing to the small amounts of ACh present in biological tissues the quantitative isolation of unhydrolysed ACh is very difficult. The problem of the isolation of ACh has become increasingly important with the introduction of radiolabelled acetylcholine. Table 1 shows the recovery of ACh-[1-^{14}C] during the standard

extraction procedure. We found in the cleared homogenate before evaporation an overall recovery of about 97% and a recovery of about 69% after evaporation. It is difficult to redissolve the residue after concentration and to avoid degradation of ACh even under nitrogen. This loss was about 25%. The extraction of the homogenate by the method of Fonnum [9] gave in the organic layer 96.6% and in the water phase 3.4% recovery of the radiolabelled ACh. Using the Fonnum organic phase for electrophoresis we recovered 41% in the ACh-band and 28% at the origin. The remaining radioactivity could not be located. There was no overlapping with the choline band nor detectable radioactivity in other bands of the pherogram. After separating the concentrated homogenate by electrophoresis, we found 17% of the total amount at the origin and 74% in the ACh band. Therefore we chose this method for analysing our samples. The problems of this procedure are emphasized by the results which we found with choline chloride-[methyl-^{14}C]. We determined a choline overlap of 23% using the organic phase of Fonnum extract but with the other method of sample preparation only an overlap of 1%. These values remained constant depending on different concentrations. The data were calculated on this basis.

Test of different precursors

The incorporation of radiolabelled acetate and choline into ACh can be seen from Fig. 1. With acetate as the precursor, we observed a measurable amount of radiolabelled ACh only with red light irradiation. ACh could first be detected after 4 hr incubation. In the dark there were only traces of radiolabelled ACh detectable even after 36 hr incubation. In contrast to ACh, choline was quickly radiolabelled from acetate in the dark. The amount of radiolabelled choline in the dark remained higher during the whole incubation time than in red light. Under the red light condition a small peak appeared after 6 hr and thereafter remained at a constant level. Using choline as precursor we found the same light mediated ACh-radiolabelling, but with the remarkable difference that radiolabelled ACh could be detected first only after 36 hr, more than 30 hr later than with acetate.

The same precursors were tested with hook preparations for 24 hr (Table 2). We found radiolabelling of ACh only with acetate as a precursor. With glucose and choline no radiolabelled ACh was detected after 24 hr incubation. There was radiolabelling of Fonnum-extractable substances after incubation with glucose-[U-^{14}C] (Table 2) but the electrophoresis of these samples gave no measurable amounts of choline or acetylcholine. There was some radioactivity at the origin and in other parts of the pherogram, but also a high loss of radioactivity.

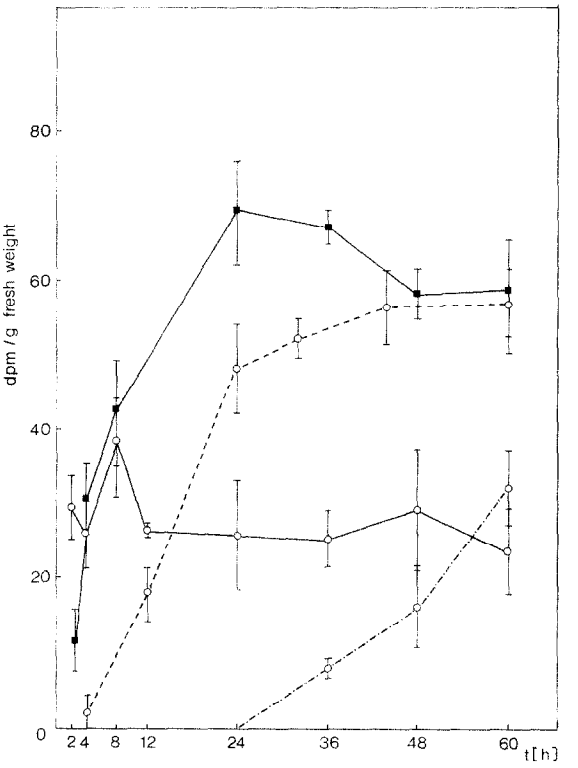

Fig. 1. Labelling of acetylcholine and choline using acetate-[1-^{14}C] and choline-[methyl-^{14}C] as precursors. Precursors were applied with an activity of 25 μCi/50 ml to a 6-day-old culture of etiolated bean seedlings. The seedlings either remained in the dark or they were irradiated with continuous red light. The graph shows the mean values and the standard deviations of 4 experiments. ■——■, Precursor acetate-[1-^{14}C], choline labelling in the dark; O——O, precursor acetate-[1-^{14}C], choline labelling in continuous red light (λ 660 nm); O- - - -O, precursor acetate-[1-^{14}C], acetylcholine labelling in continuous red light (λ 660 nm); O-·-·-O, precursor choline-[methyl-^{14}C], acetylcholine labelling in continuous red light (λ 660 nm).

Table 2. Efficiency of different precursors for acetylcholine labelling in etiolated hypocotyl hooks

Hypocotyl hooks incubation in red light (hr)	Acetate-[1-^{14}C] Choline %	ACh %	Choline-[methyl-^{14}C] Choline %	ACh %	Glucose-[U-^{14}C] Fonnum extract Organic %	Water %
2	0.04	—	72.8	—	0.8	87.0
4	0.24	—	66.5	—	0.6	93.6
8	0.36	0.21	80.2	—	1.1	93.8
12	0.39	0.41	76.7	—	0.6	86.6
24	0.41	0.46	77.3	—	0.9	91.2

The hypocotyl hooks were prepared from 8-day-old seedlings. The precursors were applied to the medium with the following activities: acetate-[1-^{14}C] (10 μCi/25 ml), choline-[methyl-^{14}C] (10 μCi/25 ml), glucose-[U-^{14}C] (10 μCi/25 ml). The overlap in the ACh band by choline label was subtracted. The experiment was repeated four times with the same results. The results are expressed as a % of the radiolabelling of the extract used for Fonnum separation. The electrophoresis of the organic Fonnum extracts from plants fed with glucose-[U-^{14}C] showed no radiolabelling in the choline and ACh band.

DISCUSSION

Our experiments show that it is possible to radiolabel ACh. The best method for the determination of ACh at present is by acid extraction and electrophoresis of the concentrated extracts. There are problems with the electrophoresis of the organic Fonnum extract. We found a high amount of non-separated substances at the origin and some overlap of choline in the ACh band. Therefore it was not possible to use the organic phase from the Fonnum extraction directly for high voltage electrophoresis. This may depend on the acetylcholine-tetraphenylboron complex which is insoluble in aqueous solutions but soluble in some organic solvents such as nitriles and ketones. We do not know what happens after drying the organic acetylcholine tetraphenylboron complex on the electrophoresis paper. It seems to be necessary to isolate acetylcholine from the organic phases and to separate it in aqueous solutions.

In order to get information about the biosynthesis of ACh or the ACh level in plant tissues, it is necessary to determine the specific activity of ACh. This should be possible by radio-gas chromatography. At present, therefore, our data allow us to say something about the precursors which are well or poorly used for radiolabelling of ACh but nothing about the biosynthesis of ACh.

We could detect radiolabelled ACh only when the seedlings or hypocotyl preparations were illuminated with light. The results were consistent with the gas chromatographic determinations. We were unable to find ACh in etiolated seedlings of different plants [2] but we found a strong dependence of ACh levels on light. The light effect was transmitted via phytochrome [3, 10]. Biro [7] was further able to show phytochrome dependence of ACh synthesis with different cell fractions *in vitro*. In our *in vivo* experiments with different precursors, acetate gave the highest amount of radiolabelled ACh. It therefore appears to be used for acetylcholine synthesis better than choline. The delay in radiolabelling of ACh by radiolabelled choline indicates that choline may be primarily needed by the seedlings for metabolic pathways other than the acetylation reaction to acetylcholine. One can detect high radiolabelling of organelles and membrane fractions after choline incubation (Hartmann, unpublished). There are many papers which deal with choline as a precursor [11, 12] but all describe the use of choline as a precursor for lipids. There is little information about choline turn-over and the relation between choline biochemistry and cell physiology in plants. Our data show a remarkable difference in choline radiolabelling depending on light and dark conditions.

Some results from animal tissues show that both acetate and choline are bad precursors for acetylcholine compared to glucose and pyruvate [5]. However, we could not detect radiolabelled ACh after using glucose as precursor even after 24 hr incubation. There were Fonnum-extractable radiolabelled compounds but these unidentified substances were not choline or acetylcholine. The results presented also show that the application of precursors to the intact seedlings gives a higher rate of labelled ACh than when hook preparations are used. The reason for this difference is not as present explicable, but it suggests that the capacity of different plant organs and tissues to synthesize ACh should be tested.

EXPERIMENTAL

Bean seedlings (*Phaseolus vulgaris* L. cv St. Andreas Erste Ernte) were grown in hydroponic culture in total darkness with a constant room temp. of 20°. The medium used was KCl (10.0 mM); $CaCl_2 \times 2\ H_2O$ (0.01 mM); $MgSO_4 \times 7\ H_2O$ (0.05 mM); KH_2PO_4 (1.0 mM); Fe(III)-citrate (0.008 mM), pH 5.2. In order to reduce contamination, the medium was autoclaved and the bean seeds sterilized by 70% EtOH and the vessels by UV irradiation. The tracers were applied after 6 days with a syringe into fresh medium. Half of the seedlings were left in the dark, while the others were irradiated with red light 660 nm (Philips fluorescent tubes TL 40/15 with red Plexiglas Nr. 501; Röhm and Haas, Darmstadt, with an irradiance of 4.8 W/m^2). We irradiated the seedlings continuously during the incubation. After incubation with the tracer, the bean seedlings were collected and the hypocotyl hooks decapitated, cut into small pieces and deep frozen in liquid N_2. All manipulations were carried out under dim green light (Philips TL 20 W/17, fluorescent tube with two Plexiglass filters one Nr. 602 and one Nr. 700, Röhm and Haas, Darmstadt, 0.2 W/m^2). The deep frozen hook pieces were homogenized with a pestle and mortar under liquid N_2 and then extracted with buffer (1:4) at 0–4°. The extraction buffer was 0.2 M NaOAc–HOAc (pH 3.8). At this pH the autohydrolysis of ACh is reduced and cholinesterases are inhibited. The addition of eserine, a potent inhibitor of animal and plant cholinesterase, did not increase the amount of extractable ACh. The homogenate was filtered through 2 layers of a nylon net (pore size 55 μm), the filtrate was then cleared by centrifugation (15 min, 20000 *g*) and the supernatant used for determinations. The analysis of the extracted samples was made by the following methods:

(1) The extract from *ca* 25 hook prepns was concd *in vacuo* to dryness at 35° and then dissolved in 1 ml NaOAc–HOAc buffer (pH 3.8). This soln was separated by high voltage electrophoresis (buffer: 0.3 M Py–0.66 M HCOOH, pH 4.6, 1500 V, 1.5 hr). If the labelling was high enough the pherograms were analysed with a chromatogram scanner for radioactivity, otherwise the acetylcholine bands were cut out. The positions of these substances were determined by chromatographing pure ACh and choline, and by using Dragendorff reagent for detection. The paper strips were analysed by the method of ref. [13], rolled up into a cylinder, put into specially prepared Eppendorf reaction tubes, soaked with 250 μl acetate buffer, pH 3.8 and centrifuged at 27000 *g* for 2 min. The extraction of radioactive compounds by this method was more than 98% and it was not necessary to repeat the extraction procedure. The solns extracted from the pherogram paper cylinders were measured by scintillation counting. We used Unisolve I (Zinsser, Frankfurt) as scintillation cocktail. The dpm values were calculated with the ESR method. The cleared acid extracts were further extracted by a modified Fonnum method [9].

(2) 200–500 μl of the extracts were diluted to 2.0 ml. The soln was shaken for 1 min with 3 ml Na-tetraphenylboron (Kalignost) (15 mg/ml) which was dissolved in a mixture of 3-heptanone and acetonitrile (5:1). The soln was centrifuged for 10 min (2000 *g*) to separate the phases. The organic phase contained up to 97% of all ACh and 87% of all choline. These extraction values were repeatable (Table 1). The organic layer was evapd, redissolved in acetonitrile and used for electrophoresis and radioassay under the same conditions as described for method 1. Short incubation experiments of 24 hr were made with cut off hypocotyl hooks using an aerated incubation medium with the same salts as the growing medium.

Radiochemicals were obtained from Amersham Buchler (Braunschweig) (acetylcholine-chloride-[1-^{14}C] sp. act. 13.4

mCi/mmol, choline chloride-[methyl-^{14}C] sp. act. 54.4 mCi/mmol, glucose-[U-^{14}C] sp. act. 274 mCi/mmol) and from Höchst (Frankfurt) (acetic acid-[1-^{14}C] sodium salt sp. act. 50.7 mCi/mmol).

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