Gas-Liquid-Chromatographic Determination of Light-Dependent Acetylcholine Concentrations in Moss Callus

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Acetylcholine was identified and quantified by g.l.c. in moss callus. The acetylcholine concentrations in the moss callus were found to be regulated by phytochrome. The highest concentrations of acetylcholine occurred after irradiation with red light. Moss callus grown in the dark contained no acetylcholine.

Acetylcholine has been detected in a number of higher and lower plants (Whittaker, 1963; Jaffe, 1970). The physiological significance of acetylcholine in plants is not yet clear. It has been suggested that acetylcholine concentrations in mung bean (*Phaseolus aureus* L.) (Jaffe, 1970) and moss callus (Hartmann, 1971) are related to phytochrome-mediated processes. Hitherto, acetylcholine in plants has always been determined by bioassay. Bioassay procedures are very sensitive but lack specificity and are susceptible to artifacts.

In the present study we have measured the acetylcholine concentration in moss callus by g.l.c. (Hanin & Jenden, 1969). This method, as modified by Kilbinger (1973), allows a specific and sensitive determination of small amounts of acetylcholine in tissue extracts. We have also determined the changes in acetylcholine concentrations in moss callus under different irradiation conditions.

Materials and Methods

Plant material

Moss callus is a regeneration form from the seta of the sporophyte of the hybrid *Funaria hygrometrica* × *Physcomitrium piriforme* (Bauer, 1963).

Moss callus was grown on a sterile medium in a climatic chamber (23°C). The culture medium and the irradiation apparatus are described by Hartmann (1973). The moss callus was irradiated for 10 days by continuous repetition of either 5 min red light–30 min dark, or 5 min red light–5 min far-red light–25 min dark, or 16 h white light–8 h dark. After the last irradiation the callus was kept in the dark for 15 h, then collected and deep-frozen. In one type of experiment the moss callus was irradiated for 3 days by continuous repetition of 16 h red light–8 h dark. In these experiments the moss callus was kept in the dark for 6 days between the end of the red-

light treatment and the collection of the callus.

Red light of $\lambda_{\max}$ 660 nm was supplied by a combination of Philips TL 40W/15 fluorescent tubes (Deutsche Philips G.m.b.H., Hamburg, Germany) and a Plexiglas filter no. 501 (Röhm, Darmstadt, Germany). The irradiance of red light was $0.46 \text{ mJ} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$. Far-red light of $\lambda_{\max}$ 730 nm was supplied by a combination of reflecting tubular lamps (Radium 60W; Radium, Wipperfurth, Germany) and two blue Plexiglas filters (no. 627) and a red one (no. 501) (Röhm). The irradiance of far-red light was $0.42 \text{ mJ} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$. White light was supplied by Philips TL 40W/34 fluorescent tubes. The irradiance of the white light was $0.17 \text{ mJ} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$.

Determination of acetylcholine

Each acetylcholine determination was done with about ten callus samples. Ten culture tubes contained about 5 g fresh wt. of moss callus. The deep-frozen moss callus was homogenized with an Ultra-Turrax apparatus (Janke & Kunkel K.G., Staufen i.Br., Germany) in 10 ml of ice-cold 0.4 M-HClO₄. The homogenate was kept in ice for 60 min and then centrifuged (3500g) for 15 min at 0°C. The supernatant was kept and the pellet resuspended for 15 min in 5 ml of 0.4 M-HClO₄. After re-centrifuging, both supernatants were combined. To 5 ml of the combined supernatants propionylcholine was added as internal standard in a concentration similar to the expected concentration of acetylcholine in the sample (1–300 nmol of propionylcholine). The pH of the solution was then adjusted to 4.5 with 5 M-K₂CO₃. After centrifuging (3500g) for 15 min at 0°C the supernatant was transferred to a chilled centrifuge tube containing 0.2 ml of tetraethylammonium chloride (1 mM). The choline esters were precipitated as reineckates, treated with the ion-exchange resin Biorex 9 (Bio-Rad Laboratories, München, Ger-

many), demethylated and analysed by g.l.c. as described previously (Hanin & Jenden, 1969; Kilbinger, 1973). Peaks were electronically integrated

with a Hewlett-Packard 3370b integrator. The quantity of acetylcholine in the sample was evaluated from the ratio of the areas of the peaks corresponding to acetylcholine and propionylcholine. Calculations were based on duplicate gas-chromatographic determinations from each sample.

Results

Identification of acetylcholine

A typical chromatogram obtained from a moss callus extract to which propionylcholine had been added as internal standard is shown in Fig. 1. Endogenous propionylcholine was not detected in the moss callus. The authenticity of the compound in the moss callus as acetylcholine was shown by several types of experiments.

(1) The retention time relative to propionylcholine was identical for acetylcholine from callus extract and authentic acetylcholine, and the addition of known amounts of acetylcholine to the extract resulted in a nearly quantitative recovery ($88 \pm 7\%$; $n = 5$) of the added acetylcholine.

(2) In two experiments the moss callus was extracted with 0.1M-KH₂PO₄ adjusted to pH 7.2 with 2M-NaOH. A sample (4ml) of the extract was incubated for 30min at 30°C with 10units of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7; Serva, Heidelberg, Germany) and subsequently analysed as described in the Materials and Methods section. In these samples no acetylcholine was detected.

(3) In two other experiments the neutralized HClO₄ extract was submitted to paper chromatography (Schleicher and Schüll 2043b; ascending system; butan-1-ol-ethanol-acetic acid-water, 8:2:1:3, by vol.). Choline and acetylcholine were applied as reference compounds and stained with Dragendorff's reagent (Gasparic, 1958). The zone on the chromatogram with the same R_F value as authentic acetylcholine was eluted with 2ml of 0.1M-NaH₂PO₄ buffer (pH 4.4). The g.l.c. of the eluate yielded a peak whose relative retention time was identical with that of authentic acetylcholine.

Fig. 1. Gas chromatogram of moss callus extract

A, acetylcholine; P, propionylcholine (50nmol) added as internal standard. The electrometer setting was 10nA. Chromatographic conditions: silanized glass column (1.80m $\times$ 2mm) packed with 7% Carbowax 20M plus 5% KOH on Gas-Chrom Q (100-120 mesh). The temperature of the column was 110°C, of the injection port 200°C, and of the alkali flame detector 400°C. The flow rates were: helium 65ml/min, air 170ml/min and hydrogen 27ml/min.

Table 1. *Acetylcholine content of moss callus*

(a) Acetylcholine content of moss callus in relation to prolonged irradiation. Moss callus was irradiated as described in the Materials and Methods section. (b) Acetylcholine concentration after short-time irradiation with red or red-far-red light. Results are means $\pm$ S.E.M. of the numbers of acetylcholine determinations in parentheses. The lower limit of sensitivity of this method is 50 pmol of acetylcholine, which is equivalent to 0.03 nmol/g fresh wt. of moss callus.

Treatment	Acetylcholine (nmol/g fresh wt.)
(a) 10 days repeated cycles of 5 min red–30 min dark	124.12 $\pm$ 23.75 (4)
10 days repeated cycles of 5 min red–5 min far-red–25 min dark	2.23 $\pm$ 0.85 (6)
10 days repeated cycles of 16 h white light–8 h dark	34.35 $\pm$ 4.41 (5)
10 days dark	Nil (<0.03) (3)
3 days repeated cycles of 16 h red light–8 h dark, followed by 6 days dark	0.33 $\pm$ 0.14 (3)
(b) 6 days dark–30 min red light	6.26 $\pm$ 1.60 (3)
6 days dark–30 min red light–10 min far-red light	0.11 $\pm$ 0.03 (4)

Influence of irradiation on the acetylcholine content of moss callus

In a first series of experiments the acetylcholine content was determined in moss callus which was irradiated for 10 days with different irradiation programmes. Moss callus irradiated with red light contained 56 times as much acetylcholine per g fresh wt. as did the callus irradiated with red-far-red light (Table 1). The acetylcholine content of the callus grown under standard conditions in white light was only 27% of the amount found after red-light irradiation. In moss callus grown in darkness no acetylcholine was detected. If the dark period was preceded by 3 days of red-light irradiation the moss callus contained small amounts of acetylcholine.

In another series of experiments the effect of short-time irradiations with red and red-far-red light on the acetylcholine concentration of moss callus grown in the dark was investigated. After 6 days of darkness the callus was irradiated for either 30 min with red light or for 30 min with red followed by 10 min of far-red light. After these short-time irradiations the acetylcholine content in moss callus was only 5% of that after the respective prolonged irradiation in the experiments with red and red-far-red light.

Discussion

The investigation verified that the pharmacologically active substance in moss callus (Hartmann, 1971) is identical with acetylcholine. We further showed that the acetylcholine concentration is regulated by phytochrome. The effect of red light on the acetylcholine concentration in the moss callus could be reversed by exposure to far-red light in both short and long-term irradiations (Table 1).

Two possible models exist for the involvement of

acetylcholine in the action of phytochrome. Jaffe (1970) and Yunghans & Jaffe (1972) suggested that acetylcholine is a highly specific hormone linked to the primary action of the far-red-absorbing form of phytochrome holochrome (P_{fr}), whereas Kasemir & Mohr (1972), Satter *et al.* (1972) and Tanada (1972) believe that acetylcholine unspecifically interferes with a phytochrome-regulated process. The main question is whether acetylcholine is involved in the short-term effects of phytochrome. Our experiments show that, with short-time irradiations, little acetylcholine is formed. If the synthesis of acetylcholine is a long-term effect and depends on the duration of irradiation, it is difficult to explain the phytochrome-mediated fast responses (Haupt, 1972a,b). An alternative explanation for our results is that acetylcholine is an intermediate in the biosynthesis of acetyl-CoA. This pathway is regulated by light (Hendricks, 1963). More experimental information is needed to confirm this latter possibility, and the gas-chromatographic determination method for acetylcholine appears to be a valuable tool in the elucidation of this mechanism.

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