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The identification of acetylcholine and choline in oat seedlings by gas chromatography and nuclear magnetic resonance (NMR)

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Abstract

Four methods of isolation and purification of choline esters from green 7-day-old oat (*Avena sativa* L. cv. Diadem) seedlings were tested. The results showed that the best recovery of acetylcholine and choline from plant tissues was obtained using an extraction solution composed of 15% 1N formic acid and 85% acetone followed by precipitation of both these substances with ammonium reineckate. The presence of acetylcholine and choline in the plant extracts was confirmed by nuclear magnetic resonance (NMR) and gas chromatography. In the case of gas chromatography, after isolation and purification of the studied compounds from the plant material, estrification of choline followed by N-demethylation of acetylcholine and estrified choline were performed. The demethylation reaction was conducted in a reaction mixture of 50 mM sodium thiophenolate and 25 mM thiophenol in anhydrous acetone. After its completion, the mixture was removed with pentanone and the demethylated esters were extracted into chloroform.

Key words: *acetylcholine, choline, gas chromatography, ^{1}H-NMR, Avena sativa*

INTRODUCTION

Acetylcholine, which is present in some animal cells, has also been found in the tissues of many plants (Miura and Shih 1984). Initially, the occurrence of this animal neurotransmitter in plants was studied using various biotests (Jaffe 1979, Hartmann 1971, Satter et al. 1972, Kopce-

wicz et al. 1977) widely used in animal physiology. These biotests are characterized by high sensitivity, however, their reliability in testing plant extracts has been questioned (Hartmann and Kilbinger 1974a). It was found that the isolated animal organs used in these tests are not sensitive only to acetylcholine, and that other pharmacologically active compounds present in the plant extracts can reduce or increase their sensitivity (Hoshito and Oota 1978). The method of chromatographic determination of choline esters from rat brain extracts developed by Jenden et al. (1968) is characterized by a similar sensitivity as biotests. Simultaneous testing of acetylcholine by a biotest and the chromatographic method in the same extract showed that the pharmacologically active substance in the biotest can be quantitatively determined in a gas chromatograph (Hanin and Jenden 1969). In addition it was found that by using gas chromatography, several choline esters can be determined at the same time and that the sensitivity of this method, in contrast with biotests, does not depend on the presence of other pharmacologically active compounds in the extract.

So far, the chromatographic method of determining acetylcholine has only sporadically been used in studies on plants. It was employed for the first time in such studies by Hartmann and Kilbinger (1974a, b), and 10 years later, Miura and Shih (1984) used it to demonstrate the universal occurrence of acetylcholine in the plant kingdom.

The mentioned authors paid little attention to the method of isolation and purification of acetylcholine and choline from plant tissues. For this reason it seemed purposeful to test several selected methods of extraction and purification of choline esters, used in studies on animal tissues, to see which of them gives the best results on plant material. The presence of choline esters in plant extracts was studied using a modified gas chromatography method, and for the first time, nuclear magnetic resonance was used for this purpose.

MATERIAL AND METHODS

PLANT MATERIAL

Oat seedlings (*Avena sativa* L. cv. Diadem) were used in these experiments. Oat grains were soaked for 1 hr in distilled water and then sowed in plastic trays containing moist, sterilized sawdust. The trays were placed for 4 days in a dark thermostat (at 25°C). The seedlings were than illuminated for the following three days with white light. The seedling tips were used in the study.

ISOLATION OF ACETYLCHOLINE AND CHOLINE

Isolation of acetylcholine and choline was performed using four different methods. The first two were used in determination of choline esters by pyrolysis, the other two in N-demethylation of these compounds. In addition, they differ from each other in the manner of extraction and precipitation of the choline esters from the homogenates.

Procedure one (according to Muruyama et al. 1979, modified)

From 1 to 2 g of tissue were placed in a mortar, liquid nitrogen was immediately poured on and the material ground to a powder. The triturated tissue was transferred to a homogenizing tube and homogenized for 20 s in 4 cm^3 of a mixture containing 15% 1N formic acid and 85% acetone. After cooling for 30 min in an ice bath, the homogenate was centrifuged for 20 min at 0°C at 15000 × g. The supernatant was washed twice with diethyl ether, and after discarding the upper ether layer, the lower aqueous layer was dried in a vacuum. The sediment was dissolved in 0.1 cm^3 of acetonitrile and 0.3 cm^3 propionyl chloride was added. The tubes and their contents were placed for 40 min in a water bath at 60°C. After completion of estrification, acetonitrile and propionyl chloride were removed with a stream of nitrogen and the precipitate was dissolved in 0.2 cm^3 of distilled water. Next, 0.02 cm^3 of a solution of I_2+KI were added and mixed. After brief centrifugation, the precipitate was dissolved in 0.05 cm^3 of acetonitrile. Five mg of the ion-exchange resin, AG 50W-× 8 (100–200 mesh) were added to the solution and after 1 min of agitation followed by centrifugation, the supernatant was transferred to Eppendorfer tubes, in which the N-demethylation reaction was conducted after evaporation of the acetonitrile.

Procedure two (according to Green and Szilagyi 1974, modified)

From 1 to 2 g of oat seedlings, after previous pulverization in liquid nitrogen, were placed in a homogenizing tube with 4 cm^3 acetonitrile and homogenized for 20 s. The suspension was placed for 30 min in an ice bath, then centrifuged for 20 min at 0°C and 15 000 × g. The supernatant was transferred to conical centrifuge tubes to which 0.5 cm^3 of HCl, pH 4–5 containing 5% Na_2SO_4 and 11% $CaCl_2$ was added. An equal volume of a mixture of diethyl ether and toluene (2:1 v/v) was added to the tubes, their contents shaken, centrifuged for 3 min at 1000 × g and the upper (organic) layer of the supernatant discarded. Hexane (twice the volume of the lower layer) was added to it,

and after shaking and centrifugation, the upper layer was again discarded and the remaining hexane removed with a stream of nitrogen. The lower layer was transferred to Eppendorfer tubes and 0.2 cm^3 of I_2+KI per gram of homogenized tissue added. The mixture was kept for 20 min in an ice bath, then centrifuged and the upper, soluble phase was discarded. The precipitate was dissolved in 1 cm^3 of acetonitrile and 20 mg of the ion-exchange resin, AG 50W-$\times$8 (100–200 mesh) were added. The contents of the tube were agitated for several minutes, centrifuged and the supernatant, after being transferred to new tubes, evaporated with a stream of nitrogen.

Procedure three (according to Jenden and Hanin 1974)

After collection and weighting of 1–2 g of oat seedling tissues, they were immediately submerged in liquid nitrogen. The tissue, after pulverization in a mortar, was homogenized for 20 s in 5 cm^3 cold 0.4 N perchloric acid and the homogenate was cooled for 30 min in an ice bath. The homogenate was then centrifuged for 30 min at 0°C and 15 000 $\times$ g. The supernatant was transferred to centrifugation tubes and centrifuged after the pH had been adjusted to 4.2–4.4 with 7.5 M potassium acetate. The supernatant was transferred to new tubes and to each 1 cm^3 of its volume, 0.1 cm^3 of a 1 mM solution of tetraethylammonium chloride and 1 cm^3 2% ammonium reineckate were added, mixing after the addition of each of these solutions. The mixture was chilled for 40 min in an ice bath. After centrifugation, the supernatant was discarded and the precipitate dried in a vacuum. To the dry precipitate, 1 cm^3 of 5 mM silver p-toluenesulfonate in acetonitrile was added, the contents, after being broken up, were thoroughly mixed and centrifuged. The supernatant was transferred to Eppendorfer tubes and completely dried in a stream of nitrogen.

Procedure four (according to Hanin and Jenden 1969)

The collected material (1–2 g) was immediately immersed in liquid nitrogen, pulverized in a mortar and then transferred to homogenation tubes. A cold extraction mixture containing 15% 1 N formic acid and 85% acetone was added in the proportion of 4 cm^3 per gram of tissue to the tubes and homogenation was conducted for 20 s. The homogenate was chilled for 30 min in an ice bath, after which it was centrifuged for 30 min at 0°C at 15 000 $\times$ g. The supernatant was transferred to new tubes and washed with an equal volume of diethyl ether. After brief

centrifugation, the ether phase was discarded. The washing was repeated twice, and the ether remaining after centrifugation was removed with a stream of nitrogen. To each cm^3 of the aqueous phase of the homogenate, 0.1 cm^3 of 1 mM tetraethylammonium chloride and 1 cm^3 2% ammonium reineckate were added. The contents of the tubes were agitated and then chilled for 40 min in an ice bath and centrifuged for 20 min at 0°C. The precipitate was dried in a vacuum after which it was dissolved in 1 cm^3 5 mM silver p-toluenesulfonate in acetonitrile and after agitation for 1 min., centrifuged. The supernatant was transferred to Eppendorfer tubes and after being dried with nitrogen, subjected to estrification and N-demethylation.

ESTRIFICATION OF CHOLINE

Estrification of choline was conducted according to the methods described by Muruyama et al. (1979) and Jenden and Hanin (1974). In the case of the first method, 0.1 cm^3 acetonitrile and 0.3 cm^3 propionyl chloride were added to the tubes containing the purified plant extract. After mixing, the tubes and their contents were placed for 40 min in a water bath at 60°C. After completion of estrification, acetonitrile and propionyl chloride were removed with a stream of nitrogen, and the precipitate dried in vacuum. This method was used for estrification of choline obtained from oat tissues by methods 1 and 2, whereas for that obtained by procedures 3 and 4, the method described by Jenden and Hanin (1974) was used. After precipitation of the reineckate ions with silver p-toluenesulfonate and centrifuging off the precipitate, the supernatant was transferred to new tubes to which 50 mm^3 of propionyl chloride were added. The tubes were tightly closed and left for 5 min at room temperature. The contents of the tube were evaporated with a stream of nitrogen and the precipitate dried in a dessicator.

N-DEMETHYLATION OF ACETYLCHOLINE AND CHOLINE

After completing estrification of choline, 0.5 cm^3 50 mM sodium thiophenolate (synthesized according to the method given by Jenden and Hanin (1974)) and 25 mM thiophenol in anhydrous butanone were added to the dry precipitate, and the air immediately removed from the tubes with a stream of nitrogen. The tubes were closed tightly and their contents mixed thoroughly, after which they were placed for 45 min in a water bath at 80°C. After cooling, 0.1 cm^3 of 0.5 M citric acid added to the tubes, mixed briefly and 2 cm^3 pentane added, next the entire mixture was shaken for 1 min. After centrifugation, the upper,

organic phase was discarded and washing with pentane was repeated twice more. Traces of pentane were removed with a stream of nitrogen, and the tubes with the remaining aqueous phase were placed in an ice bath after which 50 mm^3 chloroform and 100 mm^3 of a mixture of 2 M ammonium citrate and 7.5 M ammonium hydroxide were added. The contents of the tube were vigorously shaken for 2 min. After centrifugation, 1 mm^3 of the lower, chloroform, phase was taken for gas chromatography analysis.

CHROMATOGRAPHIC ANALYSIS

The chromatographic determination of choline and acetylcholine was carried out on a 1 meter glass silanized column filled with 10% OV-17 and 10% Triton X-100 on Gas Chrom Q 100/200 mesh (Kosh et al. 1979). A Chromotron GCHF 18.3 chromatograph equipped with a flame-ionization detector (FID) was used. The following gas flows were used: N_2 — 40 $cm^3 \times min^{-1}$, H_2 — 40 $cm^3 \times min^{-1}$, air 400 $cm^3 \times min^{-1}$, the doser temperature was 250°C, detector temperature 250°C, and column temperature 90°C. The retention time and peak areas were recorded automatically using an Ursalyt integrator (Chromatron) coupled with the chromatograph.

NUCLEAR MAGNETIC RESONANCE ANALYSIS OF PLANT EXTRACTS

The ^{1}H-NMR spectra of acetylcholine and choline as well as plant extracts were obtained on a Tesla BS 567A impulse spectrometer (100 MHz). A mixture of deuterated chloroform ($CDCl_2$) and dimethylsulfoxide (DMSO-D_6) at a 5:1 v/v ratio was used as the solvent. Tetramethyl silane (TMS) was the standard. The plant extracts were obtained from 7-day-old, green oat seedlings. Acetylcholine and choline were isolated from 100 g of these seedlings by the methods described above.

RESULTS

Initially, the chromatographic determination of choline and acetylcholine was done on standards. The N-demethylation of these compounds was carried out according to the procedure of Jenden and Hanin (1974). It was already found in the initial experiments that the synthesized sodium thiophenolate had a poor solubility in anhydrous butanone, In order to improve its solubility it was necessary to give greater amounts of acetic acid than the N-demethylating agent should contain. This caused

a fall in the efficiency of the demethylation reaction and poor reproducibility of the results.

Only after all of the reagents used in the demethylating agent had been highly purified was it possible to obtain sodium thiophenolate which was well soluble in butanone. In addition it was found that sodium thiophenolate is much more soluble in acetone than butanone. The demethylation reaction proceded much more efficiently in an acetone solution and was more reproducible. The efficiency of this reaction at 50°C increased in time and attained its maximum after 100 min of incubation of acetylcholine with the demethylating solution (sodium thiophenolate + thiophenol) (Fig. 1). After substituting acetone for butanone, the problem

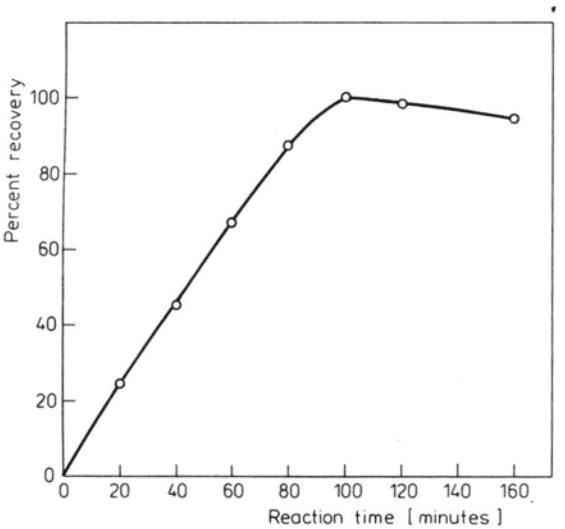

Fig. 1. N-demethylation of acetylcholine chloride (50 nM) by sodium thiophenolate (50 mM) in acetone at 50°C

arose of removing the demethylating mixture after completion of the reaction. It turned out that pentane, which in the original method (Jenden and Hanin 1974) is used to remove this mixture, was ineffective when acetone was used. A part of the demethylating mixture remained in the tube and made chromatographic analysis impossible. Due to this, it was attempted to replace pentane with other solvents. Butanone, hexane and hexanone, similarly as pentane, did not remove the demethylating mixture completely. Only when pentanone, regardless of the isomer, was used were good results obtained. The remaining steps of the procedure as described by Jenden and Hanin (1974) were not changed.

The modified method of N-demethylation was characterized by high sensitivity (below 0.1 nM) and reproducibility of results. Regardless of the temperature of the column, the acetylcholine standard curve was characterized

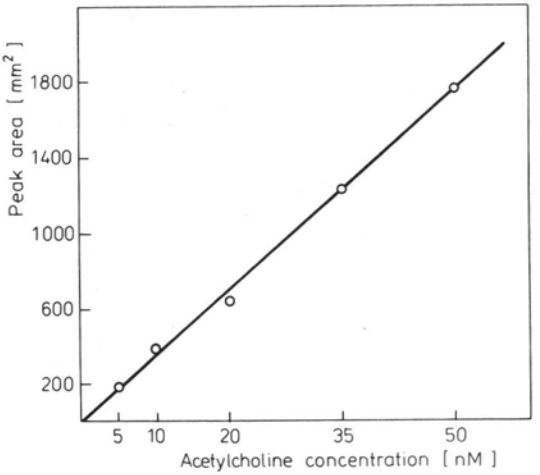

Fig. 2. Acetylcholine standard curve. The column was filled with 10% OV-17 and 10% Triton X-100 on Gas Chrom Q 100/200 mesh. Column temperature 90°C

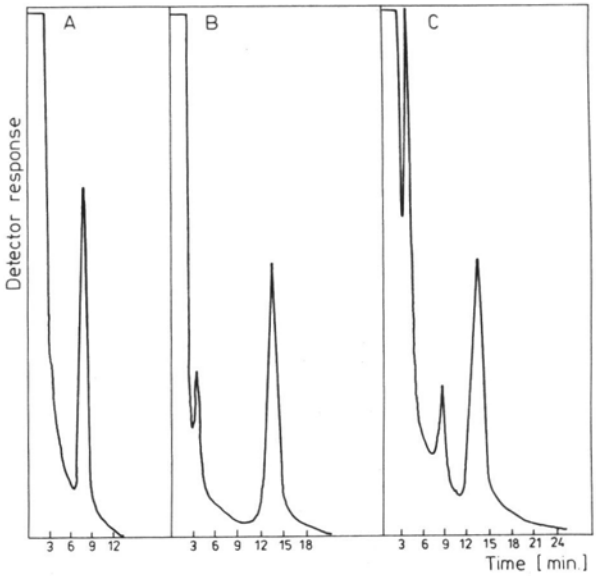

Fig. 3. Chromatograms of acetylcholine (25 nM) — A, choline (25 nM) — B and a chromatogram of a plant extract

by a linear course of the N-demethylation reaction in the 0 to 50 nM range of this compounds (Fig. 2). Reduction of the column temperature to 90°C allowed the volume of the sample to be reduced to 1 mm^3.

Both methods of estrification of choline produced peaks on the chromatogram which corresponded to demethylated propionylcholine. They were characterized by a similar retention time (Rt 12.56). This time was decidedly different from that for retention of peaks corresponding to

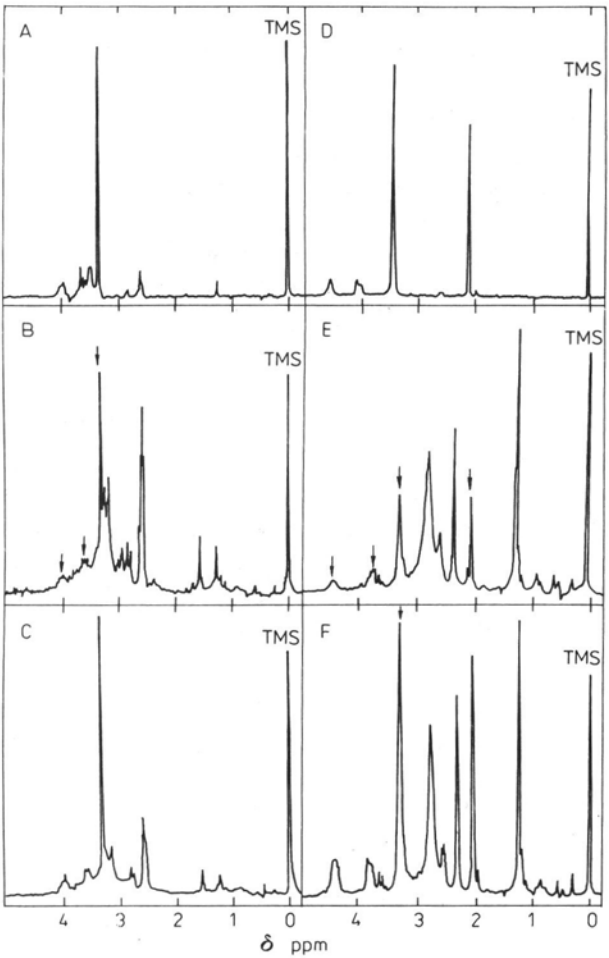

Fig. 4. ^{1}H-NMR spectra of acetylcholine and choline (D and A, respectively), plant extracts (B and E) and plant extracts to which choline (C) and acetylcholine (F) had been added. Detailed description in the text

demethylated acetylcholine (Rt 7.49), which allowed both of these compounds to be assayed simultaneously in the plant extracts (Fig. 3A-C).

After establishing the conditions under which the propionylation and demethylation of standard choline and acetylcholine should be carried out, this method was applied to both of these compounds in plant material. Four methods of isolation and purification of choline and acetylcholine were tested. Not all of them turned out to be equally suitable for isolation and purification of choline and its esters from oat seedling tissues. The first tested procedure was impractical due to its long duration. The remaining three tested methods required about the same length of time to be carried out, however they differed in the recovery of acetyl-

choline and choline from the tissues. Simultaneous studies done on the same plant material showed that the most acetylcholine was recovered from oat seedling tissues using the fourth tested procedure. The second tested method gave somewhat better results than the third. The use of the fourth procedure for the isolation and purification of choline esters from plant extracts and their subsequent propionylation and demethylation gave two peaks in gas chromatography. The first of them (Rt 7.49) corresponded to demethylated acetylcholine, whereas the second (Rt 12.56) demethylated, previously propionylated choline (Fig. 3C). This was found by comparison of the retention times of these peaks with the appropriate standards (Fig. 3A–B).

The purity of the plant extracts obtained using the tested methods of isolation and purification of choline esters was determined by nuclear magnetic resonance.

Initially, the spectra of standard choline and acetylcholine were analyzed. The ^{1}H-NMR spectra of both of these substances were in agreement with their catalogue spectra (Aldrich Library of NMR spectra; 1, 347C; 1, 571A). In the case of choline, three peaks were obtained with chemical shifts of 3.32, 3.60, 4.00 ppm, corresponding to proton signals from the following groups, respectively: $(CH_3)_3N^+$-; -CH_2OH and -CH_2-. In the case of acetylcholine, in addition to the analytical band (3.32 ppm), characteristic also for choline, the presence of 3 additional peaks with chemical shifts of 2.10, 4.00 and 4.50 ppm were also found. These peaks corresponded to signals from protons in the following groups: -OOC-CH_3; -CH_2- and -CH_2- (present in the acetate group). The signal making it possible to distinguish between acetylcholine and choline was the signal from the proton present in the acetate group of acetylcholine (Fig. 4A and 4D).

In the extracts isolated by the second procedure, the presence of the analytical band was found, however the signal from the acetate group was not observed (Fig. 4B). The addition of standard choline to the studied extracts caused an increase in the intensity of the proton signals characteristic for choline (Fig. 4A-C). In these extracts, the presence of acetylcholine was not found by gas chromatography, whereas the presence of choline was. These studies were conducted 24 hours after completion of isolation and purification of choline esters from oat seedlings.

The ^{1}H-NMR spectra of plant extracts isolated according to procedure four contained many more peaks in comparison with the extracts purified according to procedure two. Among the observed peaks, the presence of the analytical band and a signal from a chemical shift similar to that of an acetate group were found (Fig. 4E — arrows). A signal from a chemical shift similar to the proton signal from the choline -CH_2-OH was also seen. The addition of an internal standard (acetylcholine) to the analyzed

sample caused an increase in the intensity of the signals characteristic for acetylcholine (Fig. 4D and 4F).

DISCUSSION

Gas chromatography is presently commonly used in the analysis of choline esters in animal material (Hasegawa et al. 1982, Singh and Drewes 1985). In the case of plant material, this method has been employed several times (Hartmann and Kibinger 1974a, b, Miura and Shin 1984). In the studies conducted in the present paper, it has been found that the methods of isolation, purification and determination of choline and acetylcholine applied to animal tissues can be succesfully applied to plant tissues as well. In the case of all four tested methods, the presence of choline and acetylcholine was found in the plant extracts. Some of these methods had already been applied to plant material (Hartmann and Kilbinger 1974a, b, Miura and Shih 1984). However, no experiments had been conducted to determine which of the methods developed for animal tissues is the most appropriate for plant material. Of the 4 selected methods of isolation and purification of choline esters, only one (due to its tediousness) was shown to be unsatisfactory.

The presence of choline and acetylcholine in plant extracts was determined by nuclear magnetic resonance (^{1}H-NMR) and gas chromatography. The former of these methods, never before used for this purpose, was shown to be very good in the evaluation of the purity of the extracts and in the identification of choline and acetylcholine in them. The results of this study indicate that the purest extracts were obtained using the method of isolation of the studied compounds based on acetonitrile and precipitation with I_2+KI (procedure two). This method, used by Green and Szilagyi (1974) for the assay of choline derivatives in animal tissues by pyrolysis, has several drawbacks. First of all, acetonitrile, used to extract the studied compounds, is toxic. Secondly, as shown by both methods used to identify choline esters, when the procedure of purification of acetylcholine was prolonged for any reason, its decomposition occurred. It is thought that the iodine used to precipitate the studied compounds caused the decomposition of acetylcholine to choline.

The method of isolation of acetylcholine and choline from oat seedling tissues using a mixture of formic acid and acetone, although not providing such pure extacts as in the case of the acetonitrile method, was more practical. During the removal of formic acid and acetone from the homogenate with diethyl ether, other organic substances, including chlorophyll, were removed as well. In addition, this method provided the highest

concentration of choline esters in the aqueous phase of the homogenate, which increased the effectivity of their precipitation with ammonium reineckate. The presence of choline and acetylcholine in the extracts obtained by this method was confirmed by both gas chromatography and NMR.

Certain difficulties arose in the determination of acetylcholine and choline in oat tissues by gas chromatography while preparing the demethylating mixture. They were overcome by the very exact purification of all of the chemicals used in it. Also, the use of the more polar acetone in the place of butanone increased the solubility of sodium thiophenolate — a component of the demethylating mixture — in this ketone. As had been found beforc (Jenden et al. 1968), dimethylformamide can also be used for this purpose. Due to the substitution of the more polar acetone for butanone, pentane used to remove the demethylating mixture became little effective. Among the tested solvents, pentanone was shown to be the best. The polarity of this solvent could enhance the removal of both acetone and thiophenolate from the reaction medium.

The use of a modified method of assaying acetylcholine and choline in oat tissues gave a sensitivity comparable to that of biotests (Jaffe 1979, Hartmann 1971, Satter et al. 1972, Kopcewicz et al. 1977) and of gas chromatography used by other authors engaged in the determination of these substances in plant and animal material (Jenden and Hanin 1974, Miura and Shih 1984). The wider use of gas chromatography in studies conducted on plants will allow the better understanding of the function of acetylcholine in these organisms.

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Identyfikacja acetylocholiny i choliny w siewkach owsa metodą chromatografii gazowej i magnetycznego rezonansu jądrowego

Streszczenie

Przetestowano cztery metody izolacji i oczyszczania estrów cholinowych z tkanek 7-dniowych, zielonych siewek owsa (*Avena sativa* L. cv. Diadem). Stwierdzono, że największy odzysk actylocholiny i choliny z tkanek roślinnych uzyskiwano po zastosowaniu mieszaniny ekstrakcyjnej, składającej się w 15% z 1 kwasu mrówkowego i w 85% z acetonu, i po wytrąceniu obu tych substancji solą amonową Reinecka. Obecność acetylocholiny i choliny w uzyskanych ekstraktach roślinnych potwierdzono metodą magnetycznego rezonansu jądrowego oraz chromatografii gazowej. W przypadku stosowaniu chromatografii gazowej, po wyizolowaniu i oczyszczeniu badanych substacji z materiału roślinnego, przeprowadzono reakcję estryfikacji choliny a następnie N-demetalację acetylocholiny i zestryfikowanej choliny. Reakcję demetylacji przeprowadzono w mieszaninie 50 mM tiofenolanu sodu i 25 mM tiofenolu w bezwodnym acetonie. Następnie mieszaninę usuwano przy użyciu pentanonu a zdemetylowane estry cholinowe ekstrahowano do chloroformu.