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SYNTHESIS OF TRITIUM-LABELED PHYTOHORMONES

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A method has been developed for obtaining tritium-labeled analogues of β-indolylacetic acid and of 6-benzylaminopurine by the method of isotopic catalytic exchange. The localization of cytokinin- and auxin-binding proteins in cotton seedlings has been investigated.

One of the main methods of identifying receptor proteins in plant and animal tissues is that of isotopic indicators using labeled analogues of corresponding hormones possessing a high molar activity – not less than 10 Ci/mole [1]. In the present paper we describe the introduction of a tritium label into β-indolylacetic acid (IAA) and 6-benzylaminopurine (BAP) and the localization of the corresponding hormone-binding proteins in cotton seedlings.

The generally adopted method of introducing a tritium label into complex organic compounds is the method of catalytic $^1H \rightarrow {}^3H$ exchange. The intensity of the exchange reaction, the degree of labeling with tritium, and the final specific activity are determined by a number of factors, among which the most important is the efficiency of the catalyst [2].

Table 1 gives the dependence of the efficiency of isotopic exchange on the nature of the catalyst. It can be seen from Table 1 that the highest value of the molar ratio activity (A_{mol}) was achieved in the catalyst PdO/Al_2O_3. Isotopic exchange also took place in the presence of organic compounds, pyridine being more effective than triethylamine. The addition of triethylamine to the solid-phase catalyst PdO scarcely changed the value of A_{mol} for IAA and BAP. A subsequent investigation of the dependence of isotopic exchange on the amount of catalyst showed that an increase in the ratio of catalyst to compound raised the molar activity of IAA and, to a smaller degree, that of BAP (Table 2).

It must be mentioned that with an increase in the amount of catalyst the nonspecific chemosorption of BAP and IAA on its surface rose. This phenomenon, together with radiolysis, lowered the yield of the desired product, thereby limiting the use of large amounts of catalyst.

The nature of the solvent also had a definite influence on the resulting activity (Table 3).

As can be seen from Table 3, the molar activities of ^3H-BAP and ^3H-IAA increased substantially in dioxane, particularly when triethylamine was added to the reaction mixture. The high rate of exchange in an alkaline medium can be explained by the assumption that, together with protonated forms, nonprotonated forms of benzopyrrole and of benzylaminopurine

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TABLE 1. Influence of Various Catalysts on Isotopic Exchange in BAP and IAA in Dimethylformamide

Catalyst	Characteristics of the purification					
	yield, %		μCi		A_{mol}, Ci/mole	
	BAP	IAA	BAP	IAA	BAP	IAA
5% Pd/Al_2O_3	24,0	21,1	1,56	1,22	1,30	1,16
PtO_2	26,4	27,8	0,11	0,73	0,10	0,53
5% $Pd/BaSO_4$	80,0	65,4	3,0	2,31	0,34	1,04
PdO	66,0	41,1	1,60	1,15	0,42	0,20
$C_{36}H_{30}Cl_2P_2Pd$	51,0	28,2	0,78	0,72	0,31	0,22
$(PhP)_3PdCl$	23,1	26,0	0,42	0,57	0,36	0,28
C_2H_5N(10	98,3	90,0	1,25	1,22	0,25	0,05
$(C_2H_5)_3N$(10	98,1	93,4	0,44	0,38	0,09	0,02
$PdO+(C_2H_5)_3N$	52,2	37,8	1,05	1,02	0,4	0,21

TABLE 2. Dependence of the Isotopic Exchange of BAP and IAA in Ethanol on the Amount of Catalyst

Compound	Catalyst PdO/Al_2O_3, mg	Af. purification		
		yield, %	μCi	A_{mol}, Ci/mole
IAA	10	11,2	0,04	0,14
BAP	10	14,8	0,34	0,92
IAA	20	14,0	0,07	0,19
BAP	20	16,8	0,45	1,10
IAA	30	15,1	0,15	0,40
BAP	30	16,2	0,53	1,43
IAA	40	16,8	0,25	0,60
BAP	40	7,0	0,10	0,59

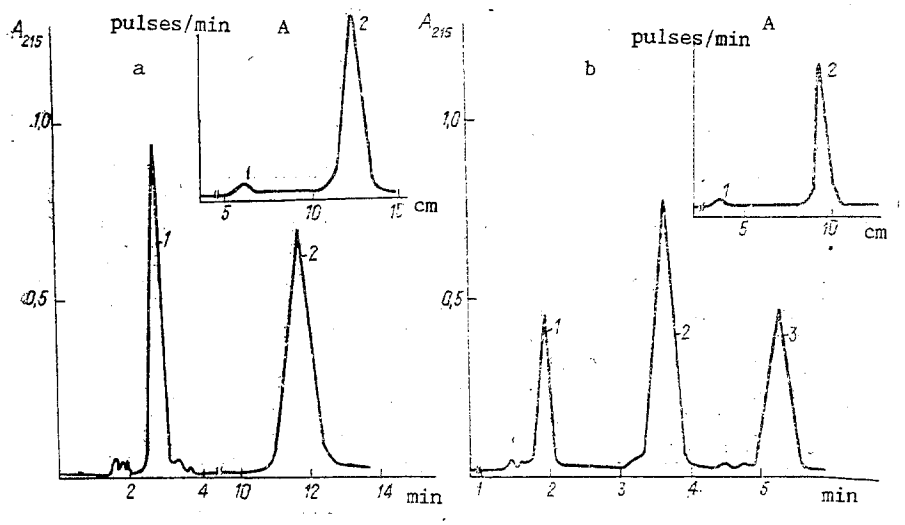

Fig. 1. Preparative isolation of ^{3}H-BAP (a) (peak 2) and of ^{3}H-IAA (b) (peak 2) by the HPLC method: A) radiochromatogram of ^{3}H-BAP and of ^{3}H-IAA after purification.

participate in the exchange reaction. The higher rate of inclusion of tritium in BAP is apparently connected with the fact that in strongly alkaline solutions the direct nucleophilic attack of the C_8 group by $-O^3H$ ions accompanied by isotopic exchange becomes effective [3].

It may be concluded from what has been said above that the isotopic exchange reaction takes place most effectively in dioxane in the presence of the heterogeneous catalyst PdO/Al_2O_3 and a strong organic base.

The preparative production of ^{3}H-BAP and ^{3}H-IAA was carried out under these conditions using tritium water with a high molar activity. To free the labeled phytohormones from radiolysis products we used the HPLC method (Fig. 1). The molar activity after the purification of ^{3}H-BAP was 24.0 Ci/mmole, and that of ^{3}H-IAA was 9.7 Ci/mmole.

The results of radiochromatographic analysis (Fig. 1, A) showed that the use of this method permits isotopically labeled products with a radiochemical purity of not less than 97% to be obtained.

The ^{3}H-IAA and ^{3}H-BAP obtained were used in experiments on the determination of the localization of the corresponding hormone-binding proteins in three-day cotton seedlings (Table 4).

It was found that the largest amount of cytokinin-binding proteins (CBPs) was localized in the nuclear fraction of the radicles and in the supernatant after 18,000 g, containing the membranes of the endoplasmic reticulum. Auxin-binding proteins (ABPs) were localized

TABLE 3. Catalytic Isotopic Exchange of BAP and IAA in Dioxane

Catalyst	Characteristics after purification: yield, %		μCi		Amol, Ci/mole	
			BAP	IAA	BAP	IAA
5% PdO/Al_2O_3	64,4	34,7	7,5	1,6	2,3	0,92
5% PdO/Al_2O_3 + 1% $(C_2H_5)_3N$	48,6	41,1	3,9	1,4	2,7	0,68
5% PdO/Al_2O_3 + 5% $(C_2H_5)_3N$	53,4	45,5	4,9	3,2	3,1	1,41
5% PdO/Al_2O_3 + 10% $(C_2H_5)_3N$	61,0	54,3	3,1	1,8	1,7	0,66

TABLE 4. Specific Binding of Labeled Phytohormones (pmole/mg of protein) with Fractions Obtained as the Result of the Successive Centrifugation of Homogenates from Leaves (A) and Radicles (B) of Three-day Cotton-plant Seedlings

Fraction	BAP		IAA	
	A	B	A	B
1000 × g precip.	8,6	28,2	2,3	1,4
18,000 × g precip.	5,1	18,9	3,4	2,1
Supernatant after 18,000 × g	23,2	0,05	1,8	0,5

predominately in the leaves, the largest amount being in the fractions of plasmatic membranes, which agrees with the results for the ABPs from maize coleoptiles [4, 5]. The fractions revealed can be used for the preparative isolation of the hormone-binding proteins. Thus, the method of introducing a tritium label into BAP and IAA that has been developed enables their radioactive analogues to be obtained with a high molar activity that is sufficient for the identification of the corresponding hormone-binding proteins of the cotton plant.

EXPERIMENTAL

Model Experiments to Determine the Optimum Conditions for Introducing a Tritium Label. A glass reaction ampul was charged with a solution of 2-5 μmole of the compound under investigation in 100 ml of an organic solvent, 30 mg of catalyst, and 15 μl of tritium water with a molar activity of 40 Ci/mmole. In the case of oxide catalysts, they were first reduced with a mixture of tritium and hydrogen (1:1000) in the same organic solvent and then the compound under investigation and the necessary amount of tritium water were added. The reaction mixture was stirred with a magnetic stirrer for 3 h. All the initial compounds and catalysts had previously been dried in a vacuum desiccator over phosphorus pentoxide.

The following catalysts were used in the experiments: 5% PdO/Al_2O_3, 5% $Pd/BaSO_4$, PdO, $C_{36}H_{30}Cl_2Pd$, and $(PhP)_3PdCl$. After the end of the reaction, the catalyst was separated off by centrifugation. Labile tritium was eliminated by distillation with ethanol in vacuum.

Preparative Synthesis. IAA or BAP (5 μmole) was dissolved in 100 μl of anhydrous dioxane with the addition of 5-6% of tritium water with a high molar activity. The 5% tritium water was obtained by the oxidation of gaseous tritium with palladium oxide in an organic solvent. The latter was then lyophilically refrozen into the reaction ampul containing the compound in which the tritium label was to be introduced. After the end of the reaction the solvent containing the tritium water was lyophilically refrozen into another ampul. Then the labeled tritium was eliminated as described above. The products of the exchange reaction were analyzed by thin-layer chromatography on Silufol in the chloroform–methanol (18:2) system, and on cellulose (Merck) in the tert-butanol–methyl ethyl ketone–water–ammonia (40:30:20:10) system. The chromatograms obtained were scanned on a NZQ-901 radiochromatograph (Czechoslovakia).

The desired compounds were isolated from the reaction mixture by reversed-phase chromatography on a Separon S-6XC column (Czechoslovakia), 7 μm. The dimensions of the column were 3.3 × 150 mm. The rate of elution was 5 ml/min and the pressure 8 MPa. To isolate the ^{3}IAA we used a 20 mM solution of ammoniumacetate in 15% methanol, and for the isolation of the ^{3}H-BAP a 20 mM solution of ammonium acetate in 40% methanol. The retention times of standards were 3.8 and 11.4 min, respectively. Detection was carried out at wavelength of 254 nm.

Determination of the Localization of Hormone-Binding Proteins. Three-day seedlings of a cotton plant of variety 108-F were homogenized in buffer (50 mM Tris-HCl, 50 mM $MgCl_2$, 5 mM EDTA, 250 mM sucrose, pH 7.8). The homogenate was filtered through two layers of nylon tissue and was then centrifuged.

The experiments on binding were carried out by the method of filtration on Synpor nitrocellulose filters (Czechoslovakia). Microtest-tubes with a volume of 200 ml were each charged with 10 µl of ^{3}H-IAA or ^{3}H-BAP in a concentration of 10^{-6} N, 10 µl of water (in the control, 10 µmole of unlabeled phytohormone in a concentration of 10^{-3} M), and 80 µl of the solution of the protein under investigation. The mixture was incubated at 20°C for 30 min and was filtered under vacuum. The filters were washed with water and dried, and their radioactivities were determined in a ZhS-107 toluene scintillator on a Beta-1 counter.

Protein concentrations were determined by Bradford's method [6].

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ISOLATION AND CHARACTERIZATION OF PHYTOKININ-BINDING PROTEINS FROM THE COTTON PLANT

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A highly purified site of a cytokinin-binding protein with a K_D value for ^{3}H-BAP of 4.3 nM has been isolated from cotton-plant seedlings by the methods of biospecific and hydrophobic chromatography. According to the results of electrophoretic analysis under denaturing conditions its molecular mass is 43 kDa.

Cytokinin-binding proteins (CBPs) of plants play an important role in the molecular mechanism of the reception and transmission of a hormonal signal. It is assumed that they form with cytokinin a hormone-receptor complex which initiates a cascade of biochemical reactions determining the physiological response of the plant organism to the action of the given hormone.

The first communication on the isolation of proteins reversibly binding with kinetin and with benzylaminopurine (BAP) appeared in 1970 [1]. At the present time, voluminous experimental material has accumulated on the isolation and characterization of CBPs from various plants — wheat, barley, maize, mungo beans, squash, tobacco. It has been shown that it is mainly soluble CBPs with molecular masses of 40-45 kDa and binding constants with zeatin, K_D, of 10^{-7}-10^{-8} M, that are localized in the vegetative organs of cereals [2, 3]. In addition to soluble proteins, CBP sites have been detected in fractions of the endoplasmic reticulum [4].

The present work was devoted to the isolation, chromatographic purification, and characterization of the CBPs of the water-soluble fraction of proteins from cotton seedlings.

One the problems arising in the isolation of proteins from the cotton plant is the presence in a plant tissue homogenate of a large amount of various low-molecular-mass compounds, some of which — tannins, phenols, phytoalexins — may interact with proteins and inactivate them. Furthermore, the extract also contains endogenous cytokinins saturating the active sections of the receptor proteins and preventing their binding with the ligands of biospeci-

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