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Immunochemical Approach to the Problem of Differential Determination of Natural Forms of Abscisic Acid

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Abstract—An original modification of the standard ELISA procedure for differential determination of different forms of abscisic acid (ABA) is proposed. It is shown that endogenous forms of ABA may be quantitatively determined in plant tissues subjected to minimal treatment, without purification of the hormones and their chemical modification. The modification has been approved when analyzing changes in the content of different ABA forms in plant tissues differing in physiological activity. Quantitative differential determination of changes in the content of different ABA forms has been performed in ovaries of *Triticum aestivum* L. and *Taraxacum officinale* Web. in the period of activity of the ovule (from the moment of its activation to the beginning of division). It is shown that, despite the different types of reproduction in the species studied (amphimixis and apomixis), the time course of changes in the content of different forms of ABA in ovaries is similar, which is suggestive of a correlation between the activity of endogenous hormonal system and chronology of main events (e.g., the beginning of endospermogenesis) of the reproductive cycle.

Key words: ELISA, immunochemical method, endogenous hormones, (+)-2-*cis*,4-*trans*-abscisic acid, (±)-2-*cis*,4-*trans*-abscisic acid

The hormonal system is one of the main systems that ensure integrity of the plant throughout its life, during both regular (growth and reproduction) and irregular phases (temperature, water, salt, transpiration, photoperiodic, and other types of stress) [1-3]. Among the five major groups of phytohormones (abscisins, auxins, gibberellins, cytokinins, and ethylene), abscisic acid (ABA) takes a special place, because this is the only hormone that can serve as agonist with respect to all the other hormones. This fact accounts for a special role of ABA, its presence in all tissues and organs in all phases of the life cycle of plants, a complicated mode of interaction with other hormones, and a broad range of morphophysiological expression. The protective and adaptive functions of ABA in stress and under changing ecological conditions are of particular importance [4].

Currently, the most promising method for study of phytohormone dynamics is enzyme-linked immunosorbent assay (ELISA); it allows quantitative and highly sensitive determination of the content of hormones in any plant tissues and organs in the course of their growth and development [5-7]. However, the published immunochemical methods of assay of ABA do not allow different endogenous forms of this hormone—free (active) and bound (reserve, inactive)—to be determined differentially, because the antibodies used for this purpose equally interact with either form of ABA [8]. It should be noted that differential determination of free ABA is possible only in the case of fractionation of samples [8-10], which may cause significant biases in the results of analysis. In view of this, it requires conducting parallel measurements of a radioactively labeled control ABA sample. In addition, an ample amount of biological material should be accumulated in view of a low level of this phytohormone in plants and small sizes of studied tissues and organs (e.g., when studying ovaries, seed-buds, and embryo). Earlier, we described a new immunochemical approach for quantitative differential determination of zeatin and zeatin riboside [11, 12], which is based on the

Abbreviations: ELISA) enzyme-linked immunosorbent assay; ABA) abscisic acid; ABH-ABA) 4′-*p*-aminobenzoylhydrazone (±)-2-*cis*,4-*trans*-ABA; BSA) bovine serum albumin; OV) ovalbumin; TPB) 0.01 M potassium-phosphate buffer, pH 7.4, containing 0.1 M NaCl and 0.1% Triton X-100.

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use of two immune-enzyme systems differing in the specificity for the assayed zeatin forms. The proposed method has been successfully used for studying the dynamics of zeatin and zeatin riboside in the reproductive organs and tissues of *Triticum aestivum* L. and *Taraxacum officinale* Web. in the period of egg activity [13, 14]. The main advantage of the proposed approach is the possibility to use extremely small quantities of biological material when the purification and separation of a sample to the fractions of zeatin and zeatin riboside is hampered due to difficulties in accumulation of biological material. Similar problems are encountered in assaying other phytohormones as well.

In this work, we demonstrated the possibility of using the immunochemical approach, proposed by us earlier, for differential quantitative determination of different natural forms of ABA, which allows selective, quantitative, and highly sensitive determinations of the hormone to be performed, eliminating the stage of preliminary fractionation of the study sample with subsequent determination of the free and bound forms of ABA in different extracts of reproductive organs and tissues.

MATERIALS AND METHODS

The natural and synthetic isomers of 5-(1-hydroxy-2,6,6-trimethyl-4-oxocyclohexen-2-yl-1)-3-methylpentadien-2,4-ic acid (ABA), used in this study, were as follows: (+)-2-*cis*,4-*trans*-ABA, an endogenous free form of ABA (Aldrich, USA); (±)-2-*cis*,4-*trans*-ABA, a synthetic form of the hormone (Aldrich); and the methyl ester of (±)-2-*cis*,4-*trans*-ABA, a synthetic analog of the reserve form of the hormone (Sigma, USA). In addition, we synthesized 4′-*p*-aminobenzoylhydrazone of (±)-2-*cis*,4-*trans*-ABA (ABH-ABA), a chemical derivative of the synthetic form of the hormone, for obtaining ABA conjugated with protein carriers.

ABH-ABA was obtained as described in [15]. Briefly, *p*-aminobenzoylhydrazine (Aldrich) was recrystallized in boiling ethanol and dried under vacuum at room temperature. Then, 30 mg of *p*-aminobenzoylhydrazine was dissolved in 6 ml of methanol containing 1 M acetic acid. To the thus obtained solution was added 30 mg of (±)-2-*cis*,4-*trans*-ABA dissolved in 4 ml of the same solution. The mixture was incubated for three days in the dark under a nitrogen atmosphere with stirring. Then, the solvent was removed by evaporation under vacuum at room temperature. After dissolving the dry powder in 10 ml of 0.1 M sodium-borate buffer (pH 9.0), an equal volume of ethyl acetate was added. The extraction of ABH-ABA with an equal volume of ethyl acetate was performed twice. Pooled fractions of the organic extract were concentrated and purified by thin-layer chromatography on Silica gel plates in the system toluene–ethyl acetate–methanol (2.5 : 7.5 : 1.5). The adsorption zone with the R_f 0.40 (visualized using a UV lamp) was collected, and ABH-ABA was eluted with the methanol–ethyl acetate (4 : 6) mixture. Solvent was removed by evaporation under vacuum at room temperature. The yield of ABH-ABA was 78%.

(±)-2-*cis*,4-*trans*-ABA and ABH-ABA were used for obtaining immunogens and conjugated with different protein carriers, such as bovine serum albumin (BSA) and ovalbumin (OV).

To obtain the conjugate BSA–ABA, 100 mg of (±)-2-*cis*,4-*trans*-ABA was dissolved in 2 ml of freshly distilled absolute dimethylformamide. The solution was mixed with 2 ml of a mixture of 100 mg of N,N′-cyclohexylcarbodiimide and 50 mg of N-hydroxysuccinimide in dimethylformamide. The mixture was stirred at room temperature for 90 min. The precipitate was separated by centrifugation at 8000 rpm for 10 min. Supernatant, containing ABA with an activated carboxyl (C1) group, was mixed with 10 ml of 0.2 M potassium phosphate buffer supplemented with 100 mg of BSA. The mixture was incubated under stirring at 20°C for 1 h and at 4°C overnight. The precipitate was separated by centrifugation at 8000 rpm for 10 min. The supernatant was dialyzed against distilled water at 4°C for 3 days. Protein concentration in the synthesized immunogen was determined by the Lowry method. The degree of modification of the amino groups of the protein was determined by titration with trinitrobenzene sulfacid [16].

The conjugate BSA–ABH-ABA was obtained as described in [15]. Briefly, 20 mg of ABH-ABA was suspended in 0.5 ml of methanol, and the suspension was mixed first with 1 ml of 0.1 M HCl. Then, 150 μl of aqueous solution of sodium nitrate (25 mg/ml) was slowly added with stirring. Diazotization of ABH-ABA was conducted for 20 min. Thereafter, the bright yellow solution was added drop by drop to cooled BSA solution (60 mg of BSA dissolved in 3 ml of 0.25 M sodium borate buffer, pH 9.2). The mixture was incubated at 4°C for 16 h. The resulting orange solution was dialyzed against 0.25 M sodium borate buffer (pH 9.2) for 24 h and against water for 48 h. Protein concentration in the conjugate was determined by the Lowry method. The protein/hormone ratio in the synthesized conjugate was determined spectrophotometrically at 275 nm.

Rabbits were immunized for two to three months by injecting 1 mg of the conjugates BSA–ABA or BSA–ABH-ABA with a two-week interval. The first immunization was performed by injecting the conjugates mixed with complete Freund's adjuvant (1 : 1) subcutaneously into the withers. For the second immunization, incomplete Freund's adjuvant was used; injections were made subcutaneously into several points on the belly. Subsequent immunizations were performed by injecting pure conjugate into the hind leg muscle. Blood samples were taken from the auricular vein 8-10 days after the seventh immunization [12].

The antisera obtained were characterized using the conjugates of OV with (±)-2-*cis*,4-*trans*-ABA and ABH-ABA. The antigens were conjugated with OV according by the procedures that were similar to the methods used to obtain the immunogens. Molar ratio OV/ABA in the reaction mixture was varied so as to obtain conjugates of various composition.

Antisera were titrated using successive double dilutions on polyester plates with high absorption capacity (Nunc Maxi Sorb, Denmark) with a preliminarily adsorbed antigen OV–ABA or OV–ABH-ABA (0.2 ml, 0.5 μg/ml in 0.02 M sodium-carbonate buffer, pH 9.5, 4°C, 12 h). After incubation of antisera with titrated solution at 37°C for 90 min, plates were washed four times with 0.01 M potassium phosphate buffer (pH 7.4) containing 0.1 M NaCl and 0.1% Triton X-100 (TPB; 0.2 ml per well). Then, 0.2 ml of horseradish peroxidase-labeled anti-species antibodies (Sigma) in TPB (pH 7.4) was added to each well (1 nM, as determined by the marker enzyme). After incubating plates at 37°C for 60 min and subsequent washing unbound reagents four times with 0.2 ml of TPB (pH 7.4), 0.15 ml of freshly prepared substrate solution (4 mg of *o*-phenylenediamine and 4 μl of 30% hydrogen peroxide in 10 ml of 0.1 M sodium citrate buffer, pH 5.0) was added to each well. After 60 min of incubation, the reaction was stopped by addition of 50 μl of 4 M sulfuric acid to each well, and the optical density was measured at 490 nm on a Uniplan AIFR-01 vertical spectrophotometer for 96-well plates (Russia) [17].

The study was performed with the amphimictic species *Triticum aestivum* L. and apomictic species *Taraxacum officinale* Web., in which the egg enters division either after fertilization (*T. aestivum*) or in the absence of fertilization (*T. officinale*). In the study we used: a) wheat ovaries with eight-nucleus mature embryo sac before fertilization (stage 1), as well as ovaries 12 and 24 h after fertilization, which corresponded to the stages of interphase (stage 2) and beginning of division (stage 3) of the zygote, respectively; and b) dandelion ovaries at the stage of mature embryo sac (stage 1), as well as ovaries at the stages of interphase of a parthenogenetically developing egg (stage 2) and its first division (stage 3). The ovaries isolated from flowers (50 mg) were homogenized in 0.5 ml of 0.01 M potassium phosphate buffer (pH 7.4) containing 0.1 M NaCl, 0.01 M sucrose, and 0.1% Triton X-100. The homogenate was centrifuged at 10,000*g* for 10 min. The supernatant was diluted with TPB and analyzed using two competitive ELISA systems. When determining concentrations of different ABA forms in ovary extracts, we used a narrow range of the calibration curves (at most $3 \cdot 10^{-9}$ M ABA), which was close to linear (Figs. 1 and 2). Fluctuations of the optical density values from the maximum value were approximately 40%, which allowed ABA concentration to be reliably determined within the narrow range specified. We analyzed plant extracts diluted with TPB by 100 and more times. When assessing the results, we took into consideration only those points that fell on the linear part of the calibration curves.

The total and effective concentrations of endogenous forms of ABA in aqueous plant extracts were determined by competitive ELISA using labeled anti-species antibodies. The conjugates of OV with (±)-2-*cis*,4-*trans*-ABA and ABH-ABA, containing three or four molecules of the hormone per protein molecule, were used as solid-phase-immobilized antigens. Antigens were adsorbed on polystyrene plates with high adsorption capacity (Nunc Maxi Sorb) in a volume of 0.2 ml at a protein concentration of 0.5 μg/ml (0.02 M sodium carbonate buffer, pH 9.5, 4°C, 12 h). After incubation, wells were washed three times with TPB (0.3 ml), and 0.1 ml of solution of the sample analyzed (juice at a dilution of 1 to 1000), or 0.1 ml of standard sample of (+)-2-*cis*,4-*trans*-ABA within a concentration range to $1 \cdot 10^{-7}$ M was added to each well. Then, 0.1 ml of solution of a homologous antiserum at a dilution of 1 : 40,000 in TPB was added. To evaluate the competition between the determined and immobilized antigens for antibody binding sites, plates were incubated at 4°C overnight, the unbound components of the analytical system were removed, and wells were washed three times with 0.3 ml of TPB. Then, 0.15 ml of AB_{-2}–HRP (Sigma) in TPB (pH 7.4) (1 nM, as determined by the marker enzyme) was added. After incubation (37°C, 60 min), unbound reagents were removed by washing four times with 0.3 ml of TPB (pH 7.4), and 0.15 ml of freshly prepared substrate solution was added.

RESULTS AND DISCUSSION

By titrating antisera on various supports (OV, OV–ABA, and OV–ABH-ABA), we demonstrated high specificity of interaction of all antisera obtained in different immunization cycles with all homologous supports synthesized. When adsorbed OV was used as a control, the level of the baseline signal did not exceed 2-5% from the signal recorded on a homologous support OV–hormone. No significant differences between homologous supports with different OV/ABA molar ratio (in experiments on antiserum titration, 3-10) were revealed. In the course of immunization, antiserum titers successively increased from 1 : 500 to 1 : 80,000 (by titer of an antiserum is meant its dilution at which the value of optical density recorded at 490 nm is 1.0). In some experiments, it was found that the binding of antibodies with homologous immobilized conjugates OV–ABA and OV–ABH-ABA was inhibited by addition of an analogous conjugate OV–hormone to solution, which is indicative of a reversible mode of immunochemical interaction in the analytic systems studied. Addition to the system of a heterologous conjugate affected the interaction between antibodies and immobilized homologous conjugate much less significantly, which corroborates the differences in

the specificity of the obtained antisera with respect to the conjugated supports used in the study. In addition, when antisera against ABA were titrated on heterologous supports, their titer markedly (more than 100-fold) decreased. This finding suggests that spatial orientation of a covalently immobilized hormone molecule relative to the protein globule of the support is important for the interaction with anti-ABA antibodies of different specificity. In view of this, in further experiments we considered only two homologous analytic systems of competitive ELISA for ABA determination. System 1 consisted of the antiserum against BSA–ABA and the support OV–ABA (Fig. 1); system 2, the antiserum against BSA–ABH-ABA and the support OV–ABH-ABA (Fig. 2). The first system is based on the immune reagents obtained by covalent immobilization of (±)-2-*cis*,4-*trans*-ABA through the carboxy group (C1) in the hormone molecule. In the second system, carbonyl (C4′) derivatives of ABA were used. This caused significant changes in the spatial location of functional groups of ABA, which had polar orientation relative to the protein globule of the support and were accessible for the interaction with the active sites of antibodies. Natural inactive bound form of ABA is a carboxyl (C1) derivative of the hormone (glycosides), i.e., esterification of the -C_1OOH group of the phytohormone molecule leads to the loss of hormonal activity of ABA. It is known that the nature of response of a plant to ABA depends on the concentration of the latter [4]. The ratio between the active form of the hormone (free ABA) and the reserve form (esterified ABA) is also important [2]. Heterogeneity of endogenous forms of ABA with different physiological activity determines the importance of solving the problem of differential quantitative determination of different natural forms of the hormone in the course of plant ontogeny. This problem may be solved on the basis of immunochemical studies of different analytical systems of ELISA, with the purpose of designing new, more advanced techniques of quantitative determination of different natural forms of ABA. Earlier, a similar immunochemical approach was proposed and successively used for differential quantitative determination of zeatin and zeatin riboside [11-14]. In this study, we sought to solve the problem of differential quantitative determination of different endogenous forms of ABA on the basis of immunochemical study of two competitive ELISA systems differing in specificity for free and bound forms of ABA. Note that the analytical systems chosen included only homologous conjugate–antiserum pairs, which was determined by a much greater specificity of immunochemical interaction in homologous ELISA systems, by contrast to alternative heterologous systems antiserum against BSA–ABA/support OV–ABH-ABA and antiserum against BSA–ABH-ABA/support OV–ABA. The above-mentioned heterologous analytical ELISA systems were not modeled in this study because of a low titer of the antibodies used for heterologous supports. The principle of competitive ELISA is the same for the first and second analytical systems; it consists in competition of the determined and immobilized antigens for the antibody binding sites. After the onset of equilibrium in the system and separation of the components of immunochemical reaction, the complex antigen–antibody formed on polystyrene surface is detected using enzyme-labeled anti-species antibodies. The use of a specific substrate system allows the concentration of marker enzyme to be determined [17]. Enzymatic activity, measured on the surface of polystyrene support, is proportional to the concentration of the complex (immobilized antigen–antibody–anti-species antibodies–enzyme); therefore, it may serve as a characteristic of ABA content in the solution studied. The first analytical homologous system of competitive ELISA was characterized by a high specificity both for the original (±)-2-*cis*,4-*trans*-ABA and its methylated form (methyl ester of (±)-2-*cis*,4-*trans*-ABA). The behavior of both forms of ABA in this analytical system was identical within the studied range of hormone concentrations (up to $1 \cdot 10^{-8}$ M) (Fig. 1, curves *1* and *2*), which is related to the closeness of chemical structures of the immunogen and antigens. The analytical system based on OV–ABA and antiserum against BSA–ABA has been studied sufficiently well and described in the literature [8, 10]. Our results are in good agreement with the published data [8]. In this case, esterification of the carboxy group (C1) of ABA molecule was not revealed with the antibodies specific for functional groups of ABA that had a polar orientation relative to the carboxy (C1) group of the hormone molecule. The degree of cross-reactivity for the methyl ester of (±)-2-*cis*,4-*trans*-ABA, calculated in this case for (±)-2-*cis*,4-*trans*-ABA, was 100%. Thus, a key characteristic of the first analytical system is the identity of behavior of free and bound forms of ABA during inhibition of formation of the complex between antibodies and immobilized antigen. Therefore, the effective concentration of ABA, determined using the first test system, almost equally reflects the content of both free and reserve forms of ABA, i.e., in fact is the total concentration of the phytohormone. However, this analytical system does not allow the free and bound forms of ABA to be determined differentially without preliminary fractionation of sample analyzed, because the antibodies used in this system almost equally interact with either form of the hormone, as seen from the comparison of curves *1* and *2* in Fig. 1. Differential determination of different forms of ABA is possible only in the case of fractionation of study sample, which may bias the results of analysis due to a low content of the hormone in plant tissues. At present, this approach is used in research practice [9, 10] because of the absence of alternative methods.

Formation of the complex between OV–ABH-ABA and antibodies against BSA–ABH-ABA in the second analytical system is inhibited by free and reserve forms of ABA to different extent (Fig. 2, curves *1* and *3*). Maximal

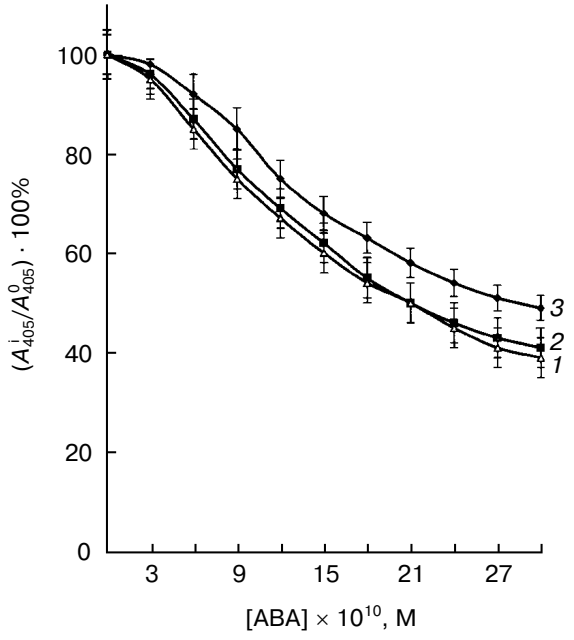

Fig. 1. Dependence of optical density of the product of enzymatic oxidation of *o*-phenylenediamine with oxygen peroxide on antigen concentration in solution of competitive solid-phase ELISA: (±)-2-*cis*,4-*trans*-ABA (*1*); methyl ester of (±)-2-*cis*,4-*trans*-ABA (*2*); (+)-2-*cis*,4-*trans*-ABA (*3*). The first system of ELISA based on BSA–ABA-specific antibodies. The OV–ABA conjugate was used as an antigen immobilized on polystyrene (Nunc Maxi Sorb). The abscissa shows the concentration of phytohormones in solution. Ordinate shows A^i_{490}/A^0_{490} (%), where A^i_{490} is optical density of the product of enzymatic reaction, with the phytohormone being used at a standard concentration, and A^0_{490} is optical density of the product of enzymatic reaction in the absence of the phytohormone in the immunochemical system.

inhibitory effect on the binding of antibodies with the immobilized antigen was observed in the case of free form of (±)-2-*cis*,4-*trans*-ABA (Fig. 2, curve *3*), i.e., in the case of the antigen that is most close in chemical structure to the immunogen BSA–ABH-ABA used in this system (that is, when the carboxy (C1) group in the hormone molecule is not modified). The degree of cross-reactivity of the esterified form of ABA (methyl ester of (±)-2-*cis*,4-*trans*-ABA), calculated with respect to the control antigen (±)-2-*cis*,4-*trans*-ABA, was only 9%. It is very important that the second analytical system is characterized by significant difference in the specificity for the free and esterified forms of ABA. In this case, antibodies were specific for the antigens whose surface had a polar orientation relative to the protein support, including the carboxyl (C1) group of the ABA molecule. Obviously, chemical modification of the carboxyl (C1) group of ABA molecule should have a strong effect on the interaction of the hormone with such antibodies. This effect may result from the steric inconsistence between the antigenic epitope and the active site of antibodies, as well as from the influence of quenching the charge of the carboxyl (C1) group resulting from its esterification on the structure of resonance electron density in the ABA molecule. The second test system, characterized by a much greater specificity towards free ABA compared to its esterified form, allows the determination of the effective concentration of endogenous ABA, which reflects primarily the content of free form of the hormone with an unmodified carboxy group. However, ABA concentration determined by this method is not precise because it includes a certain contribution of the reserve form of ABA, which should be also taken into account when performing measurements. The sensitivity of determination of (±)-2-*cis*,4-*trans*-ABA in buffer solution was $1.0 \cdot 10^{-9}$ M, with relative error being no more than 13% within the whole range of concentrations of (±)-2-*cis*,4-*trans*-ABA on the calibration curve.

Thus, the use of the second analytical system allows determining the effective ABA concentration, $(ABA)^2_{ef}$, which primarily reflects the content of the free form of the hormone, $(ABA)_f$. However, the use of the first analytical system makes it possible to determine the total concentration of the main natural forms of ABA, $(ABA)^1$,

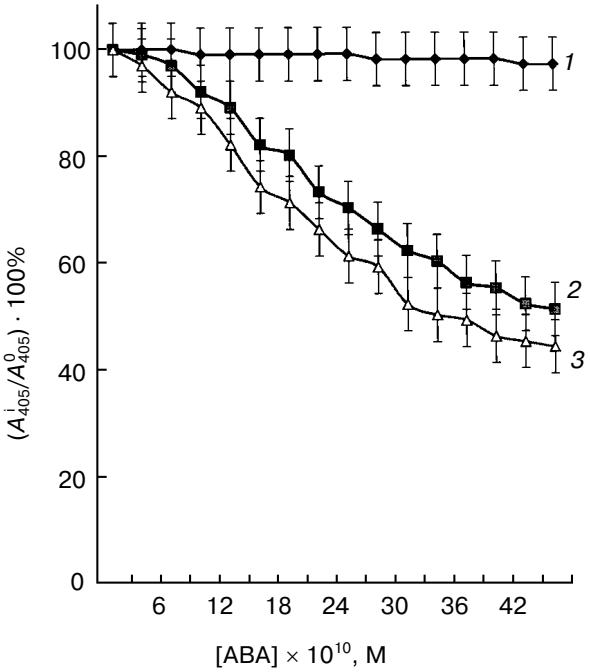

Fig. 2. Dependence of optical density of the product of enzymatic oxidation of *o*-phenylenediamine with hydrogen peroxide on antigen concentration in solution of competitive ELISA system: methyl ester of (±)-2-*cis*,4-*trans*-ABA (*1*); (+)-2-*cis*,4-*trans*-ABA (*2*); (±)-2-*cis*,4-*trans*-ABA (*3*). The second ELISA system was based on BSA–ABH-ABA-specific antibodies. The conjugate OV–ABH-ABA was used as an antigen immobilized on polystyrene (Nunc Maxi Sorb). The abscissa shows concentration of phytohormones in solution, and the ordinate shows A^i_{490}/A^0_{490} (%), where A^i_{490} is optical density of the product of enzymatic reaction when the phytohormone was used in standard concentration.

in the specimen analyzed. Taken together, the two analytical systems allow the sought concentrations of free and bound forms of ABA to be determined based on the measured total and effective concentrations of endogenous forms of ABA:

$$(ABA)^1 = (ABA)_f + (ABA)_b, \quad (1)$$

$$(ABA)^2_{ef} = n\cdot(ABA)_f + m\cdot(ABA)_b, \quad (2)$$

where n and m are the coefficients characterizing the contribution of different endogenous forms of ABA to the measured values of effective total concentration of ABA; $(ABA)_f$ is the molar concentration of free ABA; and $(ABA)_b$ is the molar concentration of the reserve (bound) form of ABA. Apparently, when free ABA is used as a standard for plotting the calibration curve in the second analytical system, the coefficient n in Eq. (2) is 1, i.e., at extreme ratio $(ABA)_b/(ABA)_f = 0$. Therefore, in the absence of the bound form of ABA in the standard, the free form of ABA is measured directly. In this case, the effective concentration $(ABA)^2_{ef}$ reflects the actual concentration of the free form of ABA. Thus, when using the free form of ABA as a calibration standard, Eq. (2) may be written in the following form:

$$(ABA)^2_{ef} = (ABA)_f + m\cdot(ABA)_b. \quad (3)$$

Tests for opening (Table 1) for the second analytical test system using standard samples of free and ethylated forms of ABA at concentrations less than 3.0 nM, which differed in the proportion of free and bound forms of ABA, allowed the contribution of the bound form of ABA to the effective total concentration of endogenous ABA, $(ABA)^2_{ef}$, i.e., the coefficient m, to be determined. Under boundary conditions, when the free form of ABA is absent in the

Table 1. Dependence of the coefficient m on the concentration and ratio between (±)-2-*cis*,4-*trans*-ABA and methyl ester of (±)-2-*cis*,4-*trans*-ABA in the standard ($N = 10$, the number of analytical replicates)

Initial concentration of (±)-2-*cis*,4-*trans*-ABA, M $(ABA)_f$	Initial concentration of methyl ester of (±)-2-*cis*,4-*trans*-ABA, M $(ABA)_b$	Determined total concentration of ABA, $(ABA)^1$, M (system 1)	Determined effective total concentration of ABA, $(ABA)^2_{ef}$, M (system 2)	m_{mean}
$1.0 \cdot 10^{-9}$	0	$0.9 \cdot 10^{-9}$	$0.9 \cdot 10^{-9}$	
$2.0 \cdot 10^{-9}$	0	$1.9 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	
$3.0 \cdot 10^{-9}$	0	$3.1 \cdot 10^{-9}$	$3.0 \cdot 10^{-9}$	
$1.0 \cdot 10^{-9}$	$1.0 \cdot 10^{-9}$	$2.1 \cdot 10^{-9}$	$1.1 \cdot 10^{-9}$	0.10
$2.0 \cdot 10^{-9}$	$1.0 \cdot 10^{-9}$	$3.2 \cdot 10^{-9}$	$2.2 \cdot 10^{-9}$	0.12
$3.0 \cdot 10^{-9}$	$1.0 \cdot 10^{-9}$	$4.2 \cdot 10^{-9}$	$3.2 \cdot 10^{-9}$	0.09
$1.0 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	$3.1 \cdot 10^{-9}$	$1.3 \cdot 10^{-9}$	0.11
$2.0 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	$4.2 \cdot 10^{-9}$	$2.2 \cdot 10^{-9}$	0.09
$3.0 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	$5.3 \cdot 10^{-9}$	$3.3 \cdot 10^{-9}$	0.10
$1.0 \cdot 10^{-9}$	$3.0 \cdot 10^{-9}$	$4.2 \cdot 10^{-9}$	$1.4 \cdot 10^{-9}$	0.10
$2.0 \cdot 10^{-9}$	$3.0 \cdot 10^{-9}$	$5.2 \cdot 10^{-9}$	$2.4 \cdot 10^{-9}$	0.11
$3.0 \cdot 10^{-9}$	$3.0 \cdot 10^{-9}$	$6.4 \cdot 10^{-9}$	$3.4 \cdot 10^{-9}$	0.11
0	$1.0 \cdot 10^{-9}$	$1.0 \cdot 10^{-9}$	$0.1 \cdot 10^{-9}$	0.10
0	$2.0 \cdot 10^{-9}$	$2.1 \cdot 10^{-9}$	$0.2 \cdot 10^{-9}$	0.10
0	$3.0 \cdot 10^{-9}$	$2.9 \cdot 10^{-9}$	$0.3 \cdot 10^{-9}$	0.10

Table 2. Test for opening ($N = 10$, the number of analytical replicates) for determination of (±)-2-*cis*,4-*trans*-ABA and methyl ester of (±)-2-*cis*,4-*trans*-ABA at different ratio of hormones in the standard

Initial concentration of (±)-2-*cis*,4-*trans*-ABA, M $(ABA)_f$	Initial concentration of methyl ester of (±)-2-*cis*,4-*trans*-ABA, M $(ABA)_b$	Determined concentration of (±)-2-*cis*,4-*trans*-ABA, M $(ABA)_f$	Determined concentration of methyl ester of (±)-2-*cis*,4-*trans*-ABA, M $(ABA)_b$	Opening of (±)-2-*cis*,4-*trans*-ABA, % $(ABA)_f$	Opening of methyl ester of (±)-2-*cis*,4-*trans*-ABA, % $(ABA)_b$
$1.0 \cdot 10^{-9}$	0	$0.9 \cdot 10^{-9}$	0	90	
$2.0 \cdot 10^{-9}$	0	$1.9 \cdot 10^{-9}$	0	95	
$3.0 \cdot 10^{-9}$	0	$3.1 \cdot 10^{-9}$	0	103	
$1.0 \cdot 10^{-9}$	$1.0 \cdot 10^{-9}$	$1.0 \cdot 10^{-9}$	$1.1 \cdot 10^{-9}$	100	110
$2.0 \cdot 10^{-9}$	$1.0 \cdot 10^{-9}$	$2.1 \cdot 10^{-9}$	$1.1 \cdot 10^{-9}$	105	110
$3.0 \cdot 10^{-9}$	$1.0 \cdot 10^{-9}$	$3.1 \cdot 10^{-9}$	$1.1 \cdot 10^{-9}$	103	110
$1.0 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	$1.1 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	110	100
$2.0 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	$2.2 \cdot 10^{-9}$	100	100
$3.0 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	$3.1 \cdot 10^{-9}$	$2.2 \cdot 10^{-9}$	103	110
$1.0 \cdot 10^{-9}$	$3.0 \cdot 10^{-9}$	$1.1 \cdot 10^{-9}$	$3.1 \cdot 10^{-9}$	110	103
$2.0 \cdot 10^{-9}$	$3.0 \cdot 10^{-9}$	$2.0 \cdot 10^{-9}$	$3.2 \cdot 10^{-9}$	100	106
$3.0 \cdot 10^{-9}$	$3.0 \cdot 10^{-9}$	$3.0 \cdot 10^{-9}$	$3.4 \cdot 10^{-9}$	100	113
0	$1.0 \cdot 10^{-9}$	0	$1.0 \cdot 10^{-9}$		100
0	$2.0 \cdot 10^{-9}$	0	$2.1 \cdot 10^{-9}$		105
0	$3.0 \cdot 10^{-9}$	0	$2.9 \cdot 10^{-9}$		97

Note: Antigen opening (%) = (determined ABA concentration/actual ABA concentration) × 100%.

sample (i.e., when $(ABA)_f = 0$) and the free form of (±)-2-*cis*,4-*trans*-ABA is used as a calibration standard, the determined effective concentrations in samples with the use of standard calibration curves plotted using free ABA in the second analytical system are significantly underestimated (Table 1). This is due to a lower specificity of the second analytical system for the reserve form of ABA. In this case, the coefficient m, which reflects the contribution of the reserve form of ABA to the effective total concentration of the phytohormone, may be determined

$$m = (ABA)^2_{ef}/(ABA)_b \quad (4)$$

at $(ABA)_f = 0$ in the tested sample and with the use of free ABA as a calibration standard (Table 1). When the tested sample contained a mixture of free and bound forms of ABA, the coefficient m was calculated according to Eq. (5):

$$m = 1 - [(ABA)^1 - (ABA)^2_{ef}]/(ABA)_b. \quad (5)$$

Thus, when the value of the coefficient m is known (0.1 ± 0.02), the original system of Eqs. (1) and (2) will take the final form:

$$(ABA)^1 = (ABA)_f + (ABA)_b, \quad (6)$$

$$(ABA)^2_{ef} = (ABA)_f + (0.1 \pm 0.02)\cdot(ABA)_b. \quad (7)$$

The obtained system of Eqs. (6) and (7) allows calculating the actual concentrations of the free and bound forms of ABA based on the experimentally determined parameters—total concentration $(ABA)^1$ and effective concentration $(ABA)^2_{ef}$, using in the two ELISA systems free form of ABA as a calibration standard (Table 2):

Table 3. Test for opening of different standards of ABA using calibration curves plotted using different forms of the hormone for the system BSA–ABA/OV–ABA (system 1) ($N = 25$, the number of analytical replicates)

Hormone used for plotting calibration curve	Composition of standard phytohormone, M	Measured hormone concentration, M (mean value)	ABA opening, %
(+)-2-*cis*,4-*trans*-ABA	$1.0 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$1.1 \cdot 10^{-9}$	110
	$1.5 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$1.5 \cdot 10^{-9}$	100
	$2.0 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$1.9 \cdot 10^{-9}$	95
	$2.5 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$2.3 \cdot 10^{-9}$	92
(+)-2-*cis*,4-*trans*-ABA	$1.0 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$1.7 \cdot 10^{-9}$	170
	$1.5 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$2.5 \cdot 10^{-9}$	166
	$2.0 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$3.2 \cdot 10^{-9}$	160
(±)-2-*cis*,4-*trans*-ABA	$1.0 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$1.0 \cdot 10^{-9}$	100
	$1.5 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$1.4 \cdot 10^{-9}$	93
	$2.0 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$2.1 \cdot 10^{-9}$	105
	$2.5 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$2.6 \cdot 10^{-9}$	104
(±)-2-*cis*,4-*trans*-ABA	$1.0 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$0.6 \cdot 10^{-9}$	60
	$1.5 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$0.9 \cdot 10^{-9}$	60
	$2.0 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$1.1 \cdot 10^{-9}$	55
	$2.5 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$1.4 \cdot 10^{-9}$	56

Table 4. Test for opening different ABA standards using calibration curves plotted using different forms of the hormone for the system BSA–ABH-ABA/OV–ABH-ABA (system 2) ($N = 25$, the number of analytical replicates)

Hormone used for plotting calibration curve	Composition of standard phytohormone, M	Measured hormone concentration, M (mean value)	ABA opening, %
(+)-2-*cis*,4-*trans*-ABA	$0.6 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$0.7 \cdot 10^{-9}$	117
	$1.2 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$1.1 \cdot 10^{-9}$	92
	$1.8 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$1.7 \cdot 10^{-9}$	94
	$2.4 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$2.5 \cdot 10^{-9}$	104
(+)-2-*cis*,4-*trans*-ABA	$0.6 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$0.9 \cdot 10^{-9}$	150
	$1.2 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$1.7 \cdot 10^{-9}$	142
	$1.8 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$2.6 \cdot 10^{-9}$	144
	$2.4 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$3.4 \cdot 10^{-9}$	142
(±)-2-*cis*,4-*trans*-ABA	$0.6 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$0.7 \cdot 10^{-9}$	117
	$1.2 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$1.3 \cdot 10^{-9}$	108
	$1.8 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$1.8 \cdot 10^{-9}$	100
	$2.4 \cdot 10^{-9}$ (±)-2-*cis*,4-*trans*-ABA	$2.3 \cdot 10^{-9}$	96
(±)-2-*cis*,4-*trans*-ABA	$0.6 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$0.4 \cdot 10^{-9}$	67
	$1.2 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$0.8 \cdot 10^{-9}$	67
	$1.8 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$1.3 \cdot 10^{-9}$	72
	$2.4 \cdot 10^{-9}$ (+)-2-*cis*,4-*trans*-ABA	$1.7 \cdot 10^{-9}$	71

$$(ABA)_b = [(ABA)^1 - (ABA)^2_{ef}]/(1 - m), \quad (8)$$

$$(ABA)_f = [(ABA)^2_f - m \cdot (ABA)^1]/(1 - m). \quad (9)$$

The determination of the concentrations of free and bound forms of ABA in buffer at various proportions of the hormones in the standard allowed us to calculate the reliability of determination of the different forms of the hormone using this method, because the found concentrations of different forms of ABA were always compared to their initial (actual) concentrations. Thus, tests for opening (Table 2) showed that the opening of the free form of (±)-2-*cis*,4-*trans*-ABA varied within the range of 90-110%. The opening of the bound form of (±)-2-*cis*,4-

trans-ABA varied within the range of 97-113%. By antigen opening is meant the ratio between the found antigen concentration and its actual concentration, expressed in percent. Importantly, with regard for the fact that the natural isomer (+)-2-*cis*,4-*trans*-ABA is very expensive, in this work we performed immunization using only the synthetic racemate (±)-2-*cis*,4-*trans*-ABA. In this case, immunization of animals with the immunogens BSA–(±)-2-*cis*,4-*trans*-ABA and BSA–ABH-(±)-2-*cis*,4-*trans*-ABA yields antibodies both against (+)-2-*cis*,4-*trans*-ABA and (–)-2-*cis*,4-*trans*-ABA forms. Note that the ratio between the concentrations and the affinity of the generated antibodies to each optical isomer of 2-*cis*,4-*trans*-ABA remained unknown. In addition, the specificity of the antibodies against both isomers of 2-*cis*,4-*trans*-ABA with respect to the alternative isomer was also unknown. We showed that both analytical systems, obtained using the reagents of the basis of ABA racemate, exhibited a lower specificity to the optically active natural form (+)-2-*cis*,4-*trans*-ABA (60 and 68% in the case of the first and second system, respectively). Thus, using (±)-2-*cis*,4-*trans*-ABA as a calibration standard, it is impossible to determine the actual content of natural isomer, (+)-2-*cis*,4-*trans*-ABA, in samples analyzed, which is clearly demonstrated by the data summarized in Tables 3 and 4. In this case, the values of the hormone concentration calculated using the calibration curve plotted using (±)-2-*cis*,4-*trans*-ABA will de underestimated. As seen from the data summarized in Tables 3 and 4, to determine the natural form of the hormone, measurements should be performed using the natural isomer (+)-2-*cis*,4-*trans*-ABA, which will interact both with the specific antibodies (steady-state binding constant, K_1 (M^{-1})) and with the antibodies against the alternative optical form (–)-2-*cis*,4-*trans*-ABA (steady-state binding constant, K_2 (M^{-1})). Apparently, K_2 will not be greater that K_1. Therefore, the population of antibodies against (–)-2-*cis*,4-*trans*-ABA may be regarded as a low-affinity faction of antibodies against the natural form (+)-2-*cis*,4-*trans*-ABA. In this case, the calibration curve obtained using (+)-2-*cis*,4-*trans*-ABA as a standard will have a broader range of concentrations of the calibration standard than the calibration curve plotted using the racemate (±)-2-*cis*,4-*trans*-ABA. This limitation may be eliminated by using (+)-2-*cis*,4-*trans*-ABA for obtaining immune reagents. Due to the high content of (+)-2-*cis*,4-*trans*-ABA (10^{-6}-10^{-8} M) in different organs and tissues of plants, the immune reagents obtained may be effectively used in many biochemical studies. In our case, the sensitivity of the calibration curves for (+)-2-*cis*,4-*trans*-ABA did not exceed 1 nM, which completely encompassed the physiologically active range of concentrations of this hormone. When extrapolating the results for the ethyl ester of the racemate (±)-2-*cis*,4-*trans*-ABA, obtained in this study, on the natural reserve form (+)-2-*cis*,4-*trans*-ABA, a similar behavioral pattern of it may be assumed, as in the case of free form of natural isomer (+)-2-*cis*,4-*trans*-ABA, compared to the esterified racemate (±)-2-*cis*,4-*trans*-ABA. Therefore, the specificity of the systems in the case of the natural bound form of (+)-2-*cis*,4-*trans*-ABA should decrease proportionally relative to the esterified racemate (±)-2-*cis*,4-*trans*-ABA. Unfortunately, these assumptions, which seem most probable to us, have not yet been tested experimentally in this work due to financial limitations and a high price of different natural isomers of ABA. Because of the absence of the esterified form of (+)-2-*cis*,4-*trans*-ABA, we performed the tests for opening only for the specimens containing solely the free form of (+)-2-*cis*,4-*trans*-ABA (Tables 3 and 4). Our results confirmed the high percentage of opening (92-117%) of the natural form (+)-2-*cis*,4-*trans*-ABA in samples at different phytohormone concentrations in solution.

The possibility to use in practice the developed method of differential quantitative determination of the

Table 5. Content of free and bound forms of ABA (M) in ovary extracts (1 : 10) of *Triticum aestivum* L. and *Taraxacum officinale* Web. at three early stages of embryogenesis (N = 20, the number of analytical replicates; n = 100, the number of biological replicates, p = 0.998)

Triticum aestivum

Embryogenesis stage	Concentration of free form of ABA × 10^8, M	Concentration of bound form of ABA × 10^8, M
1st (mature embryo sac before fertilization)	10.4 ± 1.8	2.6 ± 0.5
2nd (interphase of zygote)	9.2 ± 1.7	2.4 ± 0.4
3rd (division of egg)	4.5 ± 0.8	2.7 ± 0.6

Taraxacum officinale

Embryogenesis stage	Concentration of free form of ABA × 10^8, M	Concentration of bound form of ABA × 10^8, M
1st (mature embryo sac)	8.4 ± 1.7	1.4 ± 0.4
2nd (interphase of parthenogenetic egg)	9.6 ± 2.0	1.6 ± 0.5
3rd (division of parthenogenetic egg)	6.3 ± 1.1	1.8 ± 0.6

main endogenous forms of ABA was demonstrated using the amphimictic species *Triticum aestivum* L. and apomictic species *Taraxacum officinale* Web. as examples. This was the first qualitative differential study of changes in the relative content of free and bound forms of ABA *in vivo* in wheat and dandelion ovaries in the period of egg activity, intended to reveal a correlation between the activity of the hormonal system and the main events of this stage of reproduction, as well as to determine the possibility of hormonal induction of these events (Table 5). Our results indicate that, in the period of egg activity, wheat ovaries contained predominantly free ABA, the content of which was approximately threefold greater than that of the bound form of ABA. By the end of this period, the prevalence of free over bound form was retained, decreasing to 1.5 as the total ABA content decreased by a factor of 2. In the dandelion, the content of free ABA at the beginning of the period of egg activity was fourfold greater than the content of the bound form. By the end of this period, the prevalence was retained but decreased threefold, with the total content of ABA decreasing only slightly.

Based on the results obtained in this study, we performed a comparative study of the dynamics of the proportion of different natural hormonal forms within one class, which differ in their physiological activity. The data on changes in the content of free and bound forms of ABA in ovaries of *Triticum aestivum* and *Taraxacum officinale*, obtained using the developed procedure of ELISA (Table 5), allowed us to postulate that change in the hormonal state of the ovary out of the period of egg activity also takes place within this period, irrespective of reproduction type (amphimixis in *Triticum aestivum* and apomixis in *Taraxacum officinale*). The data also suggest that the quantitative pattern of this change does not depend on reproduction type as well. However, in qualitative respect, the observed tendency is expressed differentially, which is possibly indicative of different quantitative pattern of regulation of the main events of the reproductive cycle, determined by the presence and absence of egg fertilization. Irrespective of either of them, endospermogenesis takes place in ovaries of both species. In our opinion, this factor is manifested in the general mode of qualitative changes in the hormonal state with respect to relative content of free and bound forms of ABA.

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