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Formation of a Complex Between a Derivative of the Plant Hormone Indoleacetic Acid and Ribonucleic Acid from Pea Seedlings

N. P. KEFFORD, RAVINDAR KAUR-SAWHNEY
and A. W. GALSTON

Department of Biology, Josiah Willard Gibbs Research Laboratories, Yale University, New Haven, Connecticut, USA

Incubation of the plant growth hormone indole-3-acetic acid (IAA) with peroxidase yields a substance of unknown structure (IAA′) which forms a complex with purified RNA from pea shoots, at pH values of 4.8 or below. Once formed at low pH values, the complex is stable at higher pH values. The complex is not formed if IAA itself is added to RNA, or if the RNA is first treated with ribonuclease.

The plant growth hormone, auxin, has a basic role in the regulation of many processes in plant growth and development. Indole-3-acetic acid (IAA), which is probably the major native auxin, is effective at the relatively low concentrations of 10^{-8} to 10^{-5} M. Thus, for some time, some basis for amplification has been sought as part of the mechanism of action of this auxin. An effect of IAA upon or through a catalytic macromolecule would provide one obvious means of amplification. Siegel and Galston[1] found evidence for the attachment of IAA to a macromolecule in pea tissues. This phenomenon was later resolved by Meudt and Galston[2] into two component reactions. These were (a) the conversion of IAA to a derivative of unknown structure (to be referred to as IAA′) by a protein fraction of pea homogenate and (b) the attachment of IAA′ to another macromolecular fraction which resembled ribonucleic acid (RNA). These findings suggested that in plant tissues, IAA may be activated, then attached to RNA. This could be part of the system of amplification possibly required for the growth regulating functions of IAA. In this paper the nature of this interaction between RNA and IAA′ is studied.

METHODS

Plant material. Pea (*Pisum sativum* cv. Alaska) seedlings grown in darkness at 26°C for seven days were used.

Isolation of RNA. A modification of the method of Gierer and Schramm[3] was used. Acetone powder of 50 g of frozen pea shoots was stirred with 500 ml of 0.01 M pH 8 Tris buffer at

4°C for 30 min and the mixture emulsified with 500 ml of phenol. The emulsion was stirred at 20°C for one hour and was then broken by centrifugation in the cold. The supernatant fraction was removed and emulsified with a further 50 ml of phenol. After centrifugation the supernatant fraction (now 600 ml) was removed. To this fraction was added 60 ml of 20 % potassium acetate solution (pH 5), then 1800 ml of cold ethanol. The precipitate, collected by centrifugation, was freed of phenol by two further precipitations with ethanol after redissolving in 200 ml of Tris buffer. For use, the precipitate of RNA was dissolved in 0.1 M phosphate buffer pH 7. The concentration of RNA is given as the equivalent number of grams of pea acetone powder per 1 ml of reaction mixture. In one experiment the RNA was pretreated with ribonuclease (Sigma Chemical Co., bovine pancreas, 5 × crystallized) at pH 7 for 30 min at 25°C.

Formation of IAA′ and its reaction with RNA. Meudt and Galston[2] found that IAA′ could be formed from the following defined mixture: 10^{-6} M crystalline horseradish peroxidase (Worthington Biochemical Corp.), 10^{-4} M 2,4-dichlorophenol, 10^{-4} M manganous chloride and 10^{-4} M or 2×10^{-4} M IAA in 0.01 M phosphate buffer pH 7. The mixture was incubated at 25°C for 20 min. For convenience in the text, the concentration of IAA′ is given as the concentration of the IAA used in its preparation. During the last 10 min of the above incubation, RNA was added, then the mixture was cooled to 4°C.

Precipitation of RNA and detection of indolic compounds. Aliquots of 2 ml of reaction mixtures were made 0.2 M with perchloric acid, yielding a precipitate. The precipitate was centrifuged, washed twice with 0.2 M perchloric acid, dissolved in 2 ml of 0.1 M phosphate buffer pH 7 and treated with 2 ml of Salkowski reagent (0.01 M ferric chloride in 14 N sulphuric acid). After 70 min the optical density of the pink color produced by IAA or IAA′ was measured in a Klett colorimeter using a green filter (Corning 54).

Dialysis of RNA-IAA′ mixtures. Dialysis was used to detect the formation of macromolecular complexes of IAA′. RNA-IAA′ mixture was added to an equal volume of cold 0.1 M phosphate-citrate buffer of known pH value. Aliquots of 4 ml were then dialyzed in cellophane dialysis tubing at 4°C for 24 h against 4 × 50 ml of 0.01 M buffer of the same or different pH. After dialysis, 2 ml of the bag contents were made 0.2 M with perchloric acid. The precipitated material was centrifuged, washed twice with 2 ml of 0.2 M perchloric acid, dissolved in 2 ml of 0.1 M phosphate buffer and treated with Salkowski reagent.

Gel filtration of RNA-IAA′ mixture or a pea acetone powder extract-IAA′ mixture. Twin columns, 2 × 30 cm, of the dextran gel Sephadex G-50, medium mesh (Pharmacia, Sweden),

Table 1. Interaction of IAA′ and RNA and of IAA and RNA shown by precipitation of RNA with perchloric acid.

Reaction mixture			
Ribonucleic acid g equiv./ml	IAA M × 10^4	IAA′ M × 10^4	Salkowski reaction Klett scale divisions
–	–	–	0.0
0.1	–	–	2.0
–	1.0	–	1.0
–	2.0	–	1.0
–	–	1.0	1.5
–	–	2.0	13.0
0.1	1.0	–	2.0
0.1	2.0	–	4.0
0.1	–	1.0	24.0
0.1	–	2.0	47.0

Table 2. Effect of preincubation with ribonuclease of the interaction of RNA and IAA′.

Reaction mixture			Salkowski reaction Klett scale divisions
Ribonucleic acid g equiv./ml	IAA′ $M \times 10^4$	Ribonuclease μg/ml	
–	–	–	0.0
0.1	–	–	0.4
–	1.0	–	4.0
0.1	1.0	–	33.0
–	–	0.5	0.1
0.1	–	0.5	4.0
–	1.0	0.5	5.5
0.1	1.0	0.5	11.5

were prepared in phosphate buffer pH 7. To one was added 18 ml of a mixture of 0.1 g/ml RNA and 10^{-4} M IAA′, to the other was added 18 ml of 10^{-4} M IAA′. The columns were developed with 0.01 M phosphate buffer at 4°C. Approximately 5 ml fractions were collected and their ultraviolet absorption spectra and Salkowski reactions, without prior perchloric acid treatment, were recorded. In another experiment the same columns were used. To one column, 5 ml of a mixture of phosphate buffer extract of 3.0 g of pea acetone powder and 10^{-4} M IAA′ was added and to the other 5 ml 10^{-4} M IAA′. The columns were developed and the fractions assayed as above.

RESULTS

When RNA from peas was incubated with IAA′, then precipitated with perchloric acid (Table 1) the precipitated material gave a positive reaction with the Salkowski reagent. Thus, an indolic compound was precipitated together with the RNA. In the same experiment, no Salkowski color was obtained if RNA was incubated with unchanged IAA, then precipitated with perchloric acid.

Table 3. Salkowski reaction of RNA precipitated by perchloric acid or alcohol following reaction with IAA′.

Reaction mixture		Salkowski reaction Klett colorimeter units	
RNA g equiv./ml	IAA′ $M \times 10^4$	Perchloric acid precipitation	Alcohol precipitation
–	–	0.0	0.0
0.1	–	1.5	2.5
–	1.0	2.0	4.5
–	2.0	5.5	1.5
0.1	1.0	19.5	4.5
0.1	2.0	36.5	4.5

Table 2 shows that the incubation of RNA with ribonuclease reduced the Salkowski color to a figure expected from the controls. Ribonuclease treatment of the RNA also reduced the amount of material precipitated by perchloric acid and increased the absorption at 260 mμ of the supernatant fraction.

If, after incubation with IAA′, RNA was precipitated with 70 % ethanol containing 0.05 M potassium acetate, no Salkowski reaction was obtained from the precipitated material (Table 3). The contrasting effects of perchloric acid and alcohol as precipitants suggested that the attachment of IAA′ to RNA might be effected by the hydrogen ion concentration. A study of the effect of pH upon the binding of IAA′ to RNA was made by incubating these substances together or separately at pH 7, adding 2 ml aliquots to 2 ml of phosphate-citrate buffers of pH range 2.2 to 6.8, and then dialyzing these mixtures against 0.01 M buffer of the same pH as that which had been added. After dialysis, the Salkowski reactions of the material in the dialysis bags precipitated by perchloric acid were recorded. The data in Fig. 1 show the binding of IAA′ to RNA, as indicated by the Salkowski positive reaction of the non-dialysable material, occurred only at pH values below 4.8.

The effect of pH upon RNA-IAA′ complex, already formed at pH 2.2, has also been studied. RNA and IAA′ were incubated together at pH 7 and added to an equal volume of 0.1 M phosphate-citrate buffer, pH 2.2. Aliquots of 4 ml were then dialyzed against 0.1 M buffers over the pH range 2.2 to 6.8. The amount of nondialyzable, indolic material, as indicated by the Salkowski reaction, was unchanged by dialysis against any of the buffers up to at least pH 6.8.

Gel filtration has been used, in addition to dialysis, for the separation of large and small molecules. The behavior of a mixture of RNA and IAA′ and IAA′ alone on Sephadex columns, developed with phosphate buffer at pH 7, is shown in Fig. 2, A and B. The RNA, as indicated by absorption at 260 mμ, passed

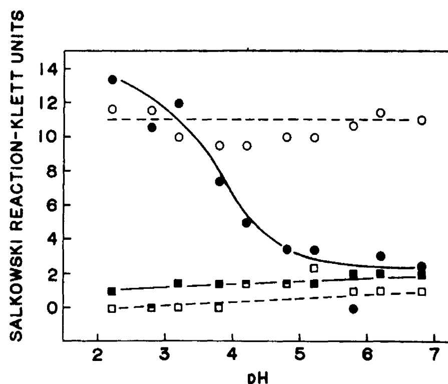

Fig. 1. Effect of pH upon the formation (full lines and symbols) and the stability (broken lines and open symbols) of the RNA-IAA′ complex. The optical density of the Salkowski reaction upon RNA plus IAA′ (circles) or IAA′ alone (squares) after dialysis against buffers of various pH values. In treatments represented by open symbols and broken lines, the RNA plus IAA′ or IAA′ alone were treated with buffer pH 2.2 prior to dialysis.

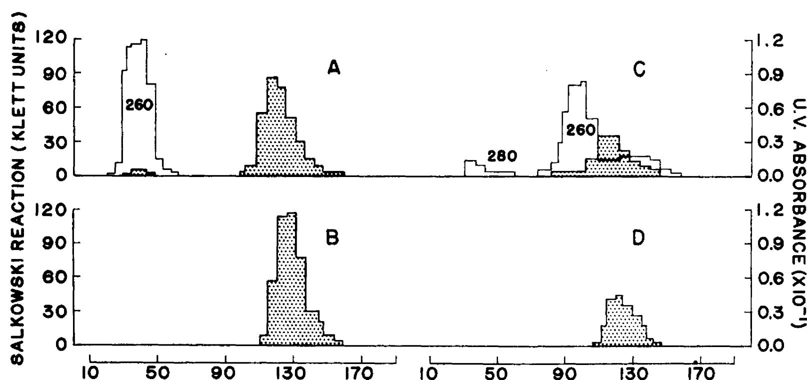

Fig. 2. The absorption at 260 or 280 mμ and the Salkowski reaction (stippled) of fractions from gel filtrations using buffer pH 7 as eluant. A and B represent twin columns to which were applied RNA plus IAA′ (A) or IAA′ alone (B), C and D represent subsequent runs on the same columns, this time a buffer extract of pea shoot acetone powder plus IAA′ (C) or IAA′ alone (D) were applied.

through the column near the solvent front and was clearly separated from the bulk of the Salkowski reacting material. The latter was eluted from the column at the same rate in the absence and presence of RNA except for a small, possibly insignificant quantity which travelled with the RNA peak.

The behavior of a buffer extract of pea shoot acetone powder incubated with IAA′, and of IAA′ alone, on the columns used in the previous experiment, is shown in Fig. 2, C and D. In this case, the first material eluted from the column holding extract of pea acetone powder had maximum absorption at 280 mμ and was presumed to be protein. This was followed by RNA-like material with a maximum absorption at 260 mμ. The Salkowski reacting material overlapped with the 260 mμ absorbing material and was eluted by the same volume in the absence and presence of acetone powder extract. Thus, these gel filtration experiments provide no evidence for the formation of the RNA-IAA′ complex at the higher pH values.

DISCUSSION

Purified RNA from peas has been shown to complex with a derivative of IAA, but not with IAA itself. The ability of the RNA preparation to complex with IAA′ was destroyed by treatment with ribonuclease, confirming that the complexing macromolecule is RNA. Both dialysis and gel filtration show that the complex between RNA and IAA′ is not formed at pH 7. It is, however, formed at pH values less than 4.8 and is subsequently stable to higher pH values. The formation of the complex only at low pH values makes its role in physiological processes questionable.

These results bear on earlier investigations of the interactions between IAA′ and macromolecules. The association between RNA-like material and IAA′, observed by Meudt and Galston[2] during the gel filtration of mixtures of IAA′ and

extract of pea acetone powder, were probably due to a coincidence of elution volumes. The difference found in the present experiments between the elution volume for purified RNA and the 260 mμ absorbing material from incubated acetone powder extract suggests some differences between the purified RNA and the RNA-like material of acetone powder extract.

The failure of Andreae and van Ysselstein[4] to detect a Salkowski positive macromolecular fraction in IAA treated pea tissue, as had been done by Siegel and Galston[1], may be explained by the respective use of alcohol and trichloroacetic acid as precipitant for the macromolecules.

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REFERENCES

1. Siegel, S. M. and Galston, A. W. *Proc. Natl. Acad. Sci. U. S.* **39** (1953) 1111.
2. Meudt, W. J. and Galston, A. W. *Plant Physiol. Suppl.* **37** (1962) xiv. (Full paper submitted for publication, March, 1963).
3. Gierer, A. and Schramm, G. *Z. Naturforsch.* **11B** (1956) 138.
4. Andreae, W. A. and van Ysselstein, M. W. H. *Plant Physiol.* **35** (1960) 225.

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