Recovery of Labeled Ribonucleic Acid Following Administration of Labeled Auxin to Green Pea Stem Sections[1]

F. E. Bendaña,[2] A. W. Galston, R. Kaur-Sawhney, and P. J. Penny
Josiah Willard Gibbs Research Laboratories,
Department of Biology, Yale University, New Haven, Connecticut

Introduction

Recent experiments with vertebrates (14, 17, 21, 36), insects (6, 10) and plants (2, 18, 19, 26, 28, 34) have shown that RNA and protein synthesis are involved in the action of a wide variety of hormones. In particular, the blockage of hormone action by low concentrations of the RNA polymerase inhibitor, actinomycin D, indicates a connection between hormone action and the de novo synthesis of nucleic acids. It thus appears that the problem of explaining the molecular mechanisms involved in initial hormone action could be attacked by a study of the detailed mechanisms connecting hormones with nucleic acid metabolism.

Previous work from this laboratory (12, 16) has shown that a partial oxidation of the plant growth hormone indole-3-acetic acid (IAA) can complex in vitro with RNA extracted from growing pea stem segments. This finding led us to inquire whether similar complexes between auxin metabolites and nucleic acids could be formed in vivo.

Materials and Methods

Ten mm long subapical stem sections derived from 14 or 15-day-old light grown pea seedlings, var. Alaska (11) were incubated in petri dishes under fluorescent light (ca. 500 ft-c) in 10 ml of solution containing IAA, generally 10^{-4} M, 1 % sucrose and 0.01 M K phosphate buffer, pH 6.0. This high concentration of IAA, which would be supraoptimal and inhibitory if fed to the conventionally employed etiolated sections, is on the ascending limb of the dose-response curve for green sections (13). It is this fact, we believe, which renders our tracer experiments both technically feasible and physiologically meaningful. To work with stimulatory levels of IAA in etiolated tissues (i.e. ca. 10^{-6} M) would mean impossibly low levels of radioactivity in isolated fractions. To work with high levels (10^{-4} M) of IAA with etiolated tissue would mean injury and growth inhibition, rather than growth promotion.

After various time intervals ranging from 1 to 18 hours the sections were removed and washed, and their length and fresh weight measured. They were then stored in a deep freeze prior to extraction of RNA. To insure stability of RNA during the extraction procedure some investigators add ribonuclease inhibitors, such as bentonite (18, 19). We found no need to do so since exogenous RNA supplied to our homogenate fractions in the presence of phenol could be quantitatively recovered. A further check involved a direct determination of ribonuclease activity in the pea stem and in fractions thereof. Although we can readily detect ribonuclease activity in one 5-mm stem section, no activity could be detected in any of the fractions in the flow sheet described, in which one hundred 10-mm sections were homogenized in the presence of phenol (20). The frozen plant material (ca. 3 g) was homogenized to a still-frozen slurry with a prechilled mortar and pestle in 2 volumes of freshly redistilled phenol (90:10 with Tris, v/v) and an equal volume of 0.01 M Tris-HCl buffer, pH 8.0. The homogenate was permitted to stand at room temperature for 1 hour, centrifuged at 4 ± 1° for 20 minutes at 3500 × *g*, the phenol-water layer removed and extracted 4 × at room temperature for 1 hour with 0.01 M Tris-HCl buffer, pH 8.0. The RNA in the combined aqueous layers was precipitated by the addition of 2 % (final conc) potassium acetate and 2.5 to 3.0 volumes of cold ethanol (95 %). This mixture was allowed to stand at 4 ± 1° overnight, and the white flocculent precipitate harvested by centrifuging the mixture at 20,000 × *g* for 20 minutes in the cold. The precipitate was dissolved in 2 ml of Tris-HCl buffer, pH 8.0, and centrifuged at 30,000 × *g* for 20 minutes to remove any debris. The supernatant fraction was again treated with 2 % potassium acetate and 2.5 to 3.0 volumes of cold ethanol and allowed to stand 30 minutes in an ice bath, after which the precipitate was again harvested by centrifugation and redissolved in 1 ml of Tris. This procedure was repeated 3 more times to yield a purified RNA (Ppt IV), which had a constant spectrum and specific activity. Reextraction of this fraction with phenol changed neither its spectrum nor its radioactivity. All work reported here was performed on Ppt IV, whose isolation is summarized in figure 1.

Ribonucleic acid was hydrolyzed with 0.3 M KOH at 37° according to the technique of Davidson and

[1] Received January 11, 1965.
[2] United States Public Health Service Postdoctoral Fellow. Present address: Departamento de Biologia, Universidad Nacional de Nicaragua, Leon, Nicaragua, Central America.

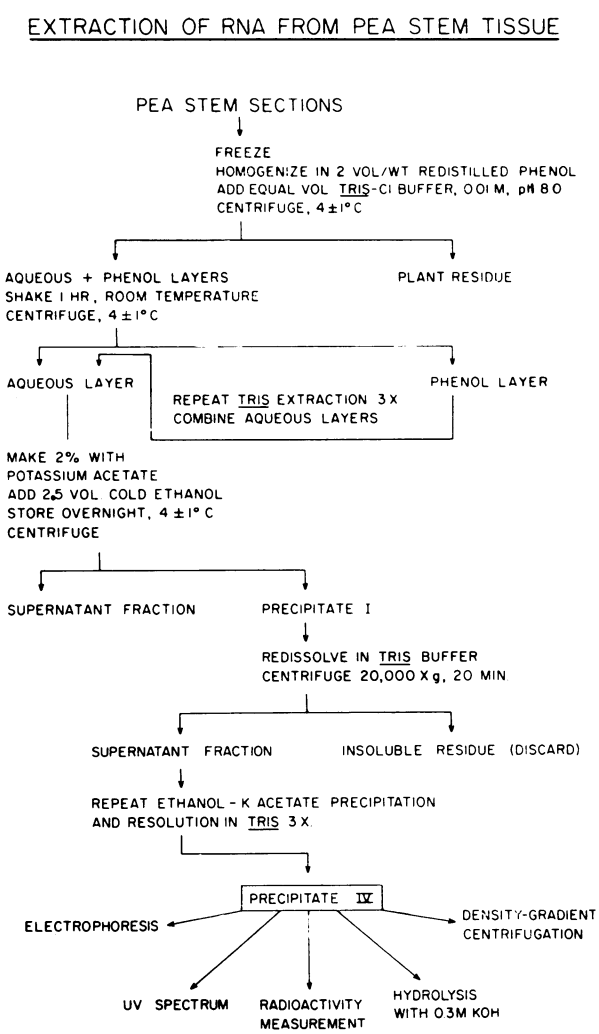

FIG. 1. Scheme for the isolation of pea RNA.

Smellie (7) and the monoribonucleotides separated electrophoretically by a slight modification of the Markham and Smith technique (23). The buffer used was 0.05 M ammonium formate-formic acid, pH 3.4, containing 5 % sucrose to decrease trailing of spots. The electrophoretic separation was performed with a Spinco Duostat and Durrum-type cell, with a voltage gradient of 9.8 v/cm, applied for 4 hours. The nucleotides were detected and marked under a UV lamp; the spots were then eluted with 0.01 N HCl, spectrally characterized and counted. Thin-layer chromatography was also used to separate the nucleotides. The adsorbent was MN cellulose powder (300 G/DEAE cellulose) sold by Brinkmann, Inc. The developer was .01 N HCl, as recommended by Randerath (27). The C^{14}-IAA used in most of the experiments was a carboxyl-labeled product (16.9 mc/mM) synthesized by our colleague B. B. Stowe (31). Both gas flow and scintillation counters were used for measurement of the radioactivity of the various fractions.

Results

Incorporation of Label into RNA. The fraction designated as precipitate IV was found to have the spectral characteristics of RNA (fig 2) and to be completely hydrolyzable by crystalline ribonuclease. When extracted from stem tissues incubated with C^{14}-IAA, precipitate IV was also radioactive. The specific activity of this fraction was found to be unaltered by dialysis in the presence of cold IAA and by extraction with such organic solvents as ethanol, ethyl ether, chloroform and to further phenol extractions. No DNA could be detected in precipitate IV by the *p*-nitrophenylhydrazine method of Webb and

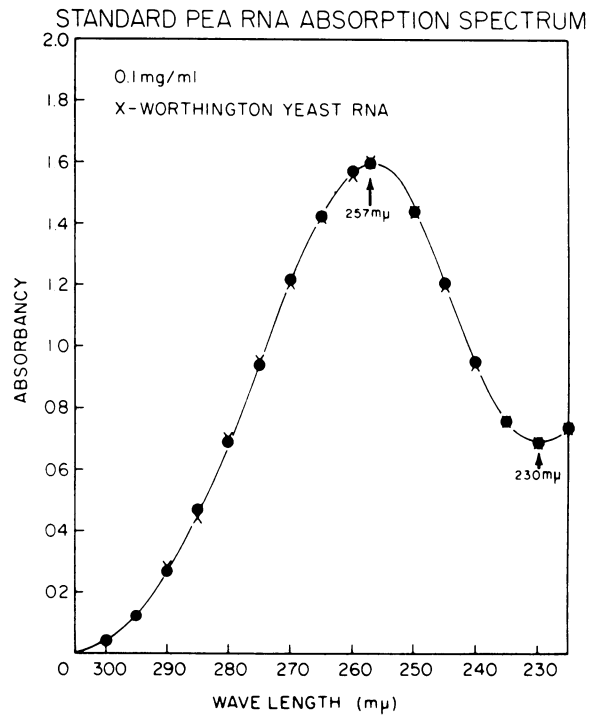

FIG. 2. Comparison of the spectral characteristics of pea and yeast RNA. RNA was dissolved in 0.01 M Tris-HCl buffer, pH 8.0, and characterized in a model 350 Perkin Elmer recording spectrophotometer.

Levy (35), no protein was detected by the Folin reagent (22) nor was any loss in material or specific activity found after treatment with 2-methoxyethanol, a material used by Kirby (20) to remove polysaccharides. It was thus concluded that the radioactivity found was an integral part of extracted RNA. The kinetics of incorporation of label from carboxyl C^{14}-IAA into precipitate IV are shown in figure 3.

The uptake of label from C^{14}-IAA into plant tissue rises as the concentration of IAA is increased

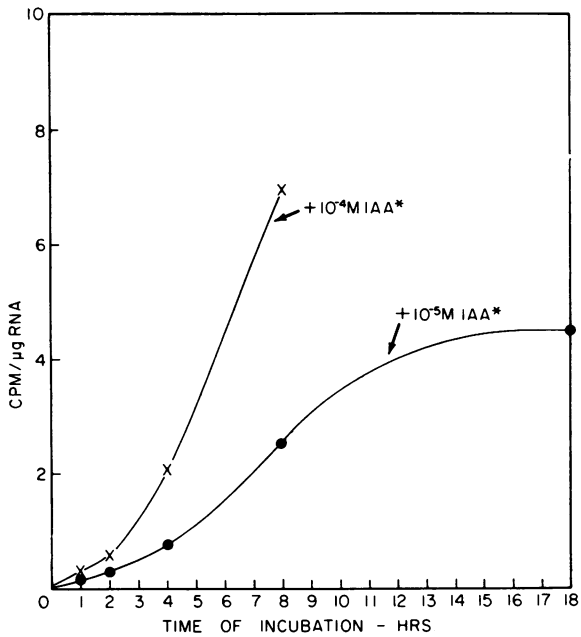

FIG. 3. Kinetics of incorporation of radioactivity from IAA into RNA as a function of IAA concentration.

(table I) and with increasing length of the incubation period (table II). The data of table II show also that under 5 % of the total counts supplied were absorbed into the tissue during a 4-hour period, and that, at any time chosen, under 1 % of the absorbed counts are found in RNA. The remainder of the counts must be located in other metabolites of IAA,

Table I. *Relation Between Concentration of Applied Carboxyl-Labeled IAA (13.5 mc/mM) and Labeling of Extracted RNA (Precipitate IV).*

Incubation time = 4 hours.

IAA (M)	% Increase in fr wt	Cpm per µg RNA
Control	15.4	0
10^{-6}	30.8	0.48
10^{-5}	46.2	1.19
10^{-4}	61.6	6.18
10^{-3}	73.0	27.6

not further investigated here. Such metabolites must include CO_2 or other volatile products which are not accounted for in sections or solution.

To investigate the specificity of IAA and of the carboxyl group as donors of label to RNA, methylene-labeled IAA and carboxyl- and methylene-labeled 2,4-dichlorophenoxyacetic acid (2,4-D), were fed to tissue and the resulting RNA isolated and counted. Table III shows that IAA is approximately 10 times more effective as a label donor than is 2,4-D, and that in each compound, carboxyl is 2 to 3 times more effective than methylene. This naturally raised the question of possible dismutation of the labeled compounds to smaller fragments, which could then be recycled, either photosynthetically or heterotrophically, to yield components of RNA. For comparison, therefore, labeled CO_2 was fed, in the presence of unlabeled 10^{-4} M IAA, to insure equality of the growth response. Like C^{14}-IAA, $C^{14}O_2$ is incorporated into RNA, but the pattern of labeling

Table II. *Kinetics of Uptake of Label from IAA into Sections and Incorporation into RNA*

Initial IAA was 6×10^{-5} M; specific activity 16.9 mc/mM. 100 sections per treatment.

	Incubation medium					
Time of treatment (hr)	Cpm per 4 µl	Total corrected cpm × 10^6	% of counts remaining	Cpm as IAA in 4 µl*	Total corrected cpm as IAA × 10^6	% of original IAA remaining
0	10,040	25.1	100	...	25.1	100
¼	8617	21.54	85.81	8880	22.20	88.44
½	8633	21.58	85.97	8544	21.36	85.09
1	8412	21.03	83.78	8202	20.50	81.67
2	8263	20.65	82.27	6873	17.17	68.40
4	7545	18.86	75.13	5115	12.78	50.91

	Sections			RNA (precipitate IV)		
Time of treatment (hr)	Cpm in 2 sections	Total cpm in all sections	% of counts absorbed	mg RNA	cpm/µg RNA	Cpm in RNA/cpm absorbed × 100
0	...	...	...	3.02	...	...
¼	1734.9	86,745	0.345	3.21	...	...
½	3526.3	176,315	0.702	3.74	0.3	0.636
1	9076.1	453,805	1.807	3.59	0.7	0.553
2	11,770	588,100	2.344	3.50	1.36	0.808
4	23,188.3	1,159,415	4.619	3.48	3.10	0.930

* 4 µl spotted on TLC and only cpm in R_F of IAA used.

Table III. *Comparative Effectiveness of Differentially Labeled Auxins as Donors of Label to RNA*

Sections were incubated for 18 hours before RNA was extracted. Growth, as measured by the increase in fresh weight, was approximately equal in all 4 groups. All auxins supplied at 10^{-4} M.

Source of label	Specific Activity μc/mg	Cpm added × 10^6	Total cpm in RNA fraction	Total RNA (mg)	cpm/μg RNA	Correction factor IAA-$1C^{14}$ = 1	Corrected cpm/μg RNA	Relative cpm/μg
IAA-$1C^{14}$	96.6	36.9	150,520	3.150	47.78	1	47.78	100.00
IAA-$2C^{14}$	16.2	6.2	10,640	2.900	3.66	5.95	21.82	45.66
2,4-D-$1C^{14}$	8.9	4.3	2,240	3.335	0.67	8.58	5.77	12.07
2,4-D-$2C^{14}$	19.2	9.4	640	2.875	0.22	3.29	0.73	1.52

from the 2 sources is quite different. RNA obtained from sections fed IAA show a great preponderance of label in the adenylate and cytidylate regions, while RNA from sections fed CO_2 show a much more symmetrical distribution of label in the 4 nucleotides. The incorporation of label from both IAA and CO_2 was markedly inhibited by 10 μg/ml actinomycin D (table IV), while the incorporation of label from $C^{14}O_2$ was unaffected by the presence of IAA and therefore of growth. It should be noted that auxin-induced growth is totally inhibited by the 10 μg/ml actinomycin D (AMD) treatment. That some incorporation of $C^{14}O_2$ into RNA should be observed even in the presence of AMD is not unusual, since it is well known (24, 32) that polyphosphorylase, the enzyme responsible for the addition of the terminal trinucleotide to s-RNA, is not inhibited by AMD. Although this reasoning may also be used to explain some of counts found in the C^{14}-IAA fed sections, approximately 18 cpm/μg RNA cannot be explained in this way (table IV). These data are interpreted as meaning that some of the counts going from fed IAA to extracted RNA arise from recycling of CO_2 or some other fragment of IAA, but

Table IV. *Effect of Actinomycin D (AMD) on Growth of Green Pea Stem Sections and Labeling of RNA.*

The sections were grown for 18 hours. IAA concentration 10^{-4}M; AMD concentration 10 μg/ml.*

Treatment			% Increase in fr wt	Cpm/μg RNA	Inhibition (cpm/μg RNA) due to AMD
IAA	CO_2	AMD			
...	...	−	35.4	...	
Labeled	Unlabeled	−	122.0	30.6	
Labeled	Unlabeled	+	38.4	24.6	6.00
...	Labeled	−	21.3	12.5	
...	Labeled	+	11.3	6.7	5.8
Unlabeled	Labeled	−	113.0	11.3	
Unlabeled	Labeled	+	33.6	5.5	5.8

* This concentration of AMD inhibits all auxin induced growth measured as percentage increase in fresh weight.

that the remainder of the incorporation occurs by way of a more direct pathway, such as direct coupling of IAA to preexisting RNA.

Isolation of IAA from the Labeled RNA. To investigate the possibility that IAA might also bind per se to RNA in a form recoverable from the complex, a sample of labeled Ppt IV was hydrolyzed with KOH, as described above, and also by the Na_2CO_3 method devised by Zamecnik et al. (37). After hydrolysis with KOH, the material was acidified to pH 2.8 to 3.0 with 0.01 N HCl and rapidly extracted 3 × with peroxide-free ethyl ether. While pure IAA partitioned 2.5 % into the aqueous layer and 97.5 % into the organic layer, the radioactivity of the complex partitioned 65 % into the aqueous layer and 35 % into the ethereal layer. This indicates that the label now exists in several different molecular species. The ethereal layer was taken down to dryness in a rotary flash evaporator at room temperature. The residue was taken up in 0.5 ml of absolute ethanol and separated by thin layer chromatography on silica gel, the solvent system being methyl acetate-isopropanol-25 % ammonium hydroxide (45:35:20, v/v/v). The gel from indicated areas of the plates was scraped off and subjected to liquid scintillation counting according to the technique of Snyder and Stephens (30). Under these conditions, where the R_F of IAA is 0.47 to 0.50, the major peak of radioactivity of the recovered silica gel occurred at the same position (fig 4). In another experiment, spraying the thin layer plate with the Prochazka reagent (27) produced a spot at the locus of IAA which was readily detected under an ultraviolet light and had an appearance identical with similarly-treated authentic IAA. A positive Prochazka reaction at the locus of IAA was also found in unextracted alkaline hydrolyzates of the RNA and in ether extracts thereof. Control RNA, or RNA from $C^{14}O_2$ fed sections yielded no such spot or counts in their hydrolyzates when chromatogrammed. These results indicate that IAA or some metabolite of IAA complexes with a RNA fraction during growth of pea stem tissue.

Physical Characterization of the Labeled RNA. Labeled RNA derived from sections fed carboxyl-labeled IAA was separated in a sucrose density gradient (0–25 %) according to the method of Brakke

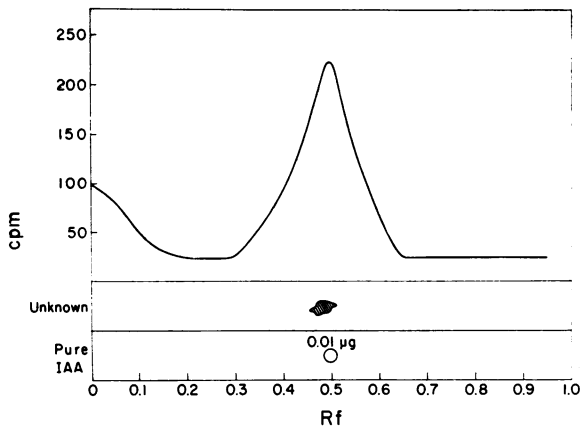

FIG. 4. Recovery of IAA from hydrolyzed RNA as shown by thin-layer chromotography on silica gel. Graphic representation of the average of 5 experiments. The pattern was obtained by removing the gel and determining the radioactivity at each R_F by liquid scintillation counting. IAA: specific activity 13.5 mc/mM; 170 cpm/mµg IAA; average cpm at R_F 0.5 was 250 cpm, or about 1.46 mµg IAA (8.3×10^{-10} moles). RNA content per spot applied = 30 µg (average M.W.4s RNA = 30,000, about 80 nucleotide residues) or about 10^{-9} M. RNA: IAA counts ratio = $(10^{-9})/(8.3 \times 10^{-10}) = 1.20$. Therefore we have found approximately 1.0 mole of IAA per mole 4s RNA or one IAA per 80 nucleotide residues.

(4), using a Spinco model L ultracentrifuge and an SW25.1 rotor, at 24,000 rpm for 8 hours, in 0.01 M Tris-HCl buffer, pH 8.0 and 0.001 M $MgCl_2$. From the very first detection of labeled RNA, the 4S fraction was most heavily labeled, and even after 18 hours of incubation in labeled IAA, the 16S and 28S ribosomal peaks were unlabeled (fig 5). There is

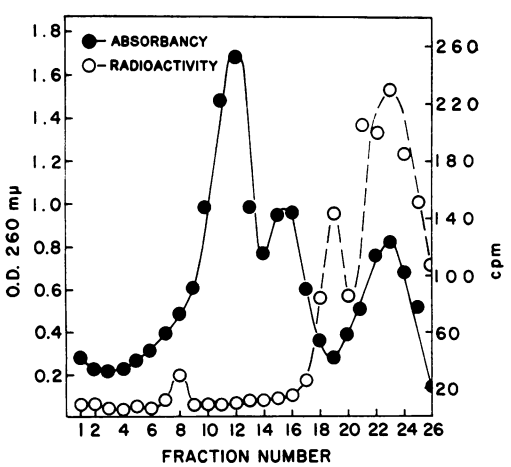

FIG. 5. Centrifugal profile of pea RNA in a 0 to 25 % sucrose density gradient. One ml fractions were collected and a complete absorption spectrum obtained for each. From right to left, the 3 absorbancy peaks represent 4S, 16S and 28S fractions. These figures were obtained by comparison with known rat liver RNA.

also a peak of labeling, but not of absorbancy, in the region between 4S and 16S, usually designated as messenger RNA. This peak would appear to have very high specific activity.

The question arises, is IAA initially incorporated into a 4S RNA fraction, or is it first incorporated into a heavier fraction, which is then broken down to 4S size? This was investigated by comparing the centrifugal distribution of radioactivity in labeled RNA produced from simultaneously applied C^{14}-IAA (13.5 mc/mM; 7.5 µc added) and H^3-uridine (4.6 c/mM; 100 µc added). RNA was obtained and separated centrifugally as previously described. A 2-channel scintillation spectrometer (ANS, Inc., Wallingford, Connecticut) permitted simultaneous determination of H^3 and C^{14} in each centrifugal fraction (fig 6). The counting efficiencies were as follows: in the H^3-channel, 15.7 % with a 2.64 % carryover from C^{14}; in the C^{14} channel 61.34 % with a carryover of less than 0.09 % from H^3. All plotted values were corrected for background, carryover and quenching.

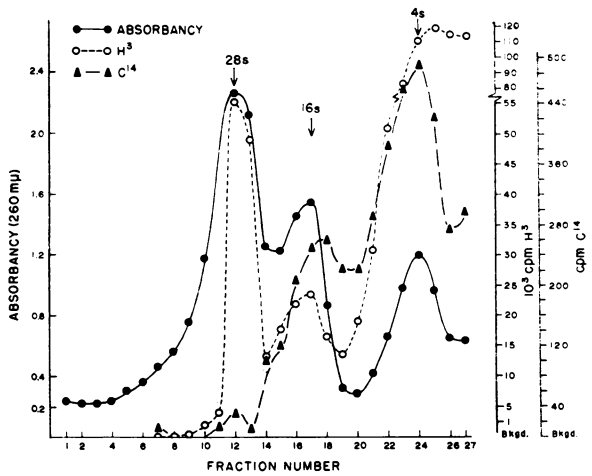

FIG. 6. Centrifugal profile and labeling pattern of pea stem RNA, obtained from sections simultaneously incubated for 6 hours with 100 µc H^3-uridine (specific activity 4.63 c/mM) and 1-C^{14}-IAA, (specific activity 13.5 mc/mM). Sucrose gradient 0 to 25 % in Tris-HCl, (0.01 M pH 8.0, 10^{-3} M $MgCl_2$. ●----●, absorbancy units; ○----○, corrected cpm of tritium; ▲----▲, corrected cpm of carbon-14. Uridine is incorporated into all types of RNA while C^{14} from IAA is associated mainly with the lighter fractions even after this long incubation period.

It is obvious that tritiated uridine finds its way into all RNA fractions after a 6-hour incubation period. However, C^{14} from IAA, as previously found, is associated mainly with the 4S peak, secondarily with a peak in the usual vicinity of messenger RNA, and practically not at all with the 28S ribosomal peak. In the 28S peak, the molar ratio of uridine to IAA incorporation was approximately

8.0. However, in the 4S and 16S regions the corresponding figure was less than 0.6. This substantiates the view that IAA is preferentially incorporated into light RNA fractions, and speaks against the large-scale unspecific incorporation of degradation products of IAA into general RNA synthesis.

Discussion

There are now several reports in the literature describing the binding of steroid hormones (25, 29, 33) and carcinogenic hydrocarbons (3, 8) to nucleic acids. In addition, there are reports that synthetic plant growth substances such as maleic hydrazide (5) and kinins (9) may be incorporated into nucleic acids, where they substitute respectively, for normal pyrimidines and purines. The present report of the binding of IAA with RNA, while it is the first indication of complex formation between a native plant growth hormone and nucleic acid, falls into an established framework. It is also instructive to note that mitomycin and porfiromycin are believed to bind to nucleic acid only after reduction to an aromatic indole (15).

It is clear that carboxyl-labeled IAA donates label to the isolated precipitate IV in 2 ways: A) direct binding of intact IAA with RNA, from which form it is recoverable as such; and B) dismutation, probably into CO_2, followed by reutilization of the split products in de novo purine and pyrimidine biosynthesis. Neither the nature of the linkage between RNA and IAA nor the reason for the relatively heavy labeling of cytidylate and adenylate regions are clear. In view of the known turnover of cytidylate and adenylate at the amino acid acceptor end of transfer RNA (1) it is tempting to postulate an attachment of IAA at this point. We hope to obtain further information relative to this possibility.

If a complex between IAA and RNA does occur in vivo, what may be its significance and role in growth promotion? There are 3 obvious possibilities: A) The hormone, through complex formation with RNA, could be involved in removing it from the surface of another molecule, such as template DNA or ribosomal RNA. We have as yet no evidence relevant to this possibility. B) The formation of the hormone-RNA complex could confer upon the RNA greater stability toward degradative agents, such as ribonuclease. In support of this possibility, we have in fact already observed progressively slower ribonuclease action on RNA isolated from stem sections incubated for increasing periods with 10^{-4} M IAA. These results will be reported later in detail. C) The possibility also exists that the IAA molecule, or portion thereof, may possess informational value when attached to a particular locus in an RNA chain. The general conformational similarity between the indole nucleus and the purine nucleus makes this conjecture at least a possibility worth entertaining.

Summary

C^{14} from carboxyl-labeled indole-3-acetic acid (IAA) fed to excised, growing green pea stem sections is progressively incorporated into RNA extractable by phenol. The kinetics of incorporation resemble the kinetics of growth induction by IAA. C^{14} from methylene-labeled IAA and from carboxyl and methylene-labeled 2,4-dichlorophenoxyacetic acid were also incorporated into RNA, but with lower efficiency than from carboxyl-labeled IAA. Both growth and C^{14} incorporation into RNA were inhibited by 10 μg/ml actinomycin D. The label was incorporated into a light (4S) RNA fraction as shown by sucrose density gradient centrifugation, while tritiated uridine simultaneously applied was incorporated into ribosomal peaks as well. Alkaline hydrolysis, followed by separation of the nucleotides by paper electrophoresis and thin layer chromatography showed that the bulk of the C^{14} label is associated with adenylate and cytidylate regions. Some activity from fed labeled IAA was also detected in the form of unchanged IAA recovered from the alkaline hydrolyzate. It is concluded that some IAA couples per se with soluble RNA while some of it is degraded, and the split products including CO_2 are used in resynthesis of RNA components.

Acknowledgments

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