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THE EFFECT OF TIBA AND 2,4-D ON GROWTH AND METABOLIC PROCESSES OF SOYBEAN HYPOCOTYL SECTIONS

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Abstract—2,3,5-Triiodobenzoic acid (TIBA) at concentrations of 50 and 100 ppm inhibits the 2,4-dichlorophenoxyacetic acid (2,4-D) induced growth of soybean hypocotyl sections by 30–60 per cent. These TIBA levels also decrease 2,4-D uptake from the incubation medium by less than 25 per cent which should not be sufficient to cause the observed growth retardation. TIBA causes a decrease in tissue dry wt. which cannot fully be accounted for by inhibition of sucrose uptake. TIBA accumulates in the tissue in proportion to the external concentration and is metabolized to a slight extent when 2,4-D is present. DNA, RNA, and protein synthesis rates were decreased by TIBA while only the tissue RNA level declines. The rates of synthesis of transfer, ribosomal, and messenger RNAs are apparently inhibited to the same degree while respiration is not affected appreciably Microbial contamination appears to be insignificant in the system utilized

INTRODUCTION

A SERIOUS effort is being made to determine the value of the use of TIBA in crop and fruit production.[1,2] Even though this plant growth regulator has been studied for some time, little is known concerning its biochemical mode of action. TIBA has been shown to affect plant growth and morphology markedly.[3–5] Kuse[6] concluded that auxin transport was inhibited by TIBA since treated plants became stunted and lost apical dominance. Later it was shown directly that TIBA does interfere with polar auxin transport.[7,8]

Respiration of tomato stem slices was depressed to 11 per cent of the control level when incubated in a 1000 ppm solution of TIBA.[9] TIBA at a low level (0·01 ppm) increased respiration of cultured virus tumor tissue from *Rumex acetosa* by about 30 per cent while a higher TIBA level (100 ppm) inhibited respiration by about 20 per cent.[10]

The studies reported here were initiated to determine which processes are affected in soybean tissue when TIBA is used to inhibit auxin (2,4-D) induced growth. An etiolated soybean stem section growth system[11] was employed and the processes examined were growth, respiration, 2,4-D uptake and release, TIBA and sucrose uptake, and DNA, RNA, and protein synthesis. In most experiments these processes were studied using 2,4-D at 5 ppm and TIBA at 50 or 100 ppm.

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RESULTS

Growth. The rates of fresh wt. increase of the hypocotyl sections were nearly constant for 8 hr and then declined steadily until very little increase could be noted after 24 hr with all treatments. Usually, the maximal 2,4-D induced growth (induced by 5–20 ppm 2,4-D) was about 56 per cent in 10 hr and 85 per cent in 24 hr. The controls (no 2,4-D added) grew about 19 and 28 per cent during the same respective time periods.

TIBA, at concentrations lower than 10 ppm did not inhibit the 2,4-D stimulated growth of hypocotyl sections (Fig. 1). However, at 50 and 100 ppm TIBA inhibited 2,4-D stimulated growth when 2,4-D was present at either 5 ppm (Fig. 1) or at 1 and 20 ppm.

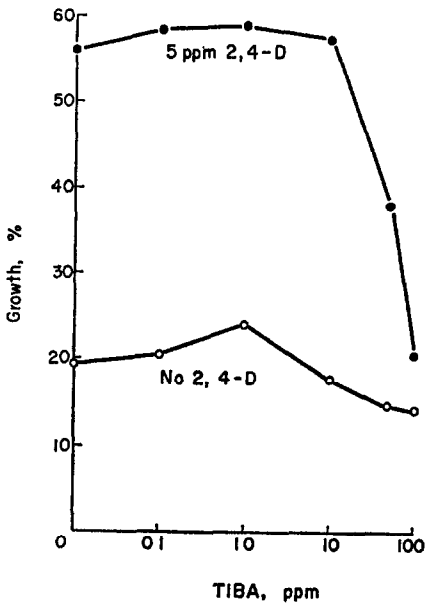

FIG 1. EFFECT OF TIBA AND 2,4-D ON THE GROWTH (% INCREASE IN FRESH WT) OF SOYBEAN HYPOCOTYL SECTIONS DURING A 10 hr INCUBATION PERIOD.

Tissue dry wt. was measured after an incubation period of 8 hr under standard conditions The dry wt. of the control (no 2,4-D present) sections increased by 4·5 per cent during this period while the fresh wt. increased by 15 per cent. The 2,4-D concentrations of 1, 5 and 20 ppm stimulated the dry wt. increase up to 8 per cent while the fresh wt. increase was stimulated to 40–60 per cent. One and five ppm TIBA had no effect on the tissue dry wt. At 50 and 100 ppm, however, it caused the dry wt. to decline by 5 per cent during the 8 hr incubation period with 2,4-D absent or present at 5 ppm.

Sucrose accumulation. Any tissue dry wt. increase must be due to sucrose or potassium phosphate uptake since these substances are the only ones available in the medium. There was 50 mg of sucrose and 3·4 mg of potassium phosphate present in the 5 ml of incubation solution used. Analysis of the medium shows that no phosphate is taken up, but experiments with ^{14}C-sucrose show that it is taken up rapidly (Table 1). In the presence of 5 ppm 2,4-D, sucrose is taken up in a rapid and linear fashion for the entire incubation (22 hr). If 2,4-D is absent, however, the uptake decreased after 4 hr while TIBA at 100 ppm decreased sucrose accumulation very markedly.

Tissue dry wt. changes and sucrose accumulation measured after an 8 hr incubation are given in Table 1. When 2,4-D was present alone, the dry weight increase of 6 7 mg could be accounted for in slight excess by the uptake of 7·3 mg of sucrose. The TIBA treated tissues, however, took up from 2·6–3·0 mg of sucrose, but lost from 2·1–2·8 mg of dry wt. This leaves an unaccounted for loss by the tissue of 4·7–5·8 mg with TIBA present.

2,4-D *and* TIBA *uptake.* Experiments were performed to determine the effect of TIBA on 2,4-D uptake since the growth inhibiting effect of TIBA might be due to a reduced level of 2,4-D in the hypocotyls.

The levels of 2,4-D accumulated by soybean hypocotyls when incubated with ^{14}C-2,4-D increased during the first 4 hr and then decreased sharply (Fig. 2). The rate of 2,4-D uptake

TABLE 1. THE EFFECT OF TIBA AND 2,4-D ON TISSUE DRY WT AND SUCROSE UPTAKE*

Treatment 2,4-D (5 ppm)	Treatment TIBA (100 ppm)	Fresh wt increase (%)	Dry wt. change (%)	Dry wt. change (mg)	Sucrose uptake (mg)	Tissue loss† (mg)
		20	+ 4 8	+2 8	4 9	2 1
	+	17	− 3 4	−2 1	2·6	4 7
+		58	+11 0	+6 7	7·3	0 6
+	+	35	− 4 8	−2 8	3 0	5 8

* Measured in duplicate after incubation for 8 hr with 2 μc ^{14}C-μl-sucrose.
† Dry wt. loss not accounted for by the sucrose accumulated.

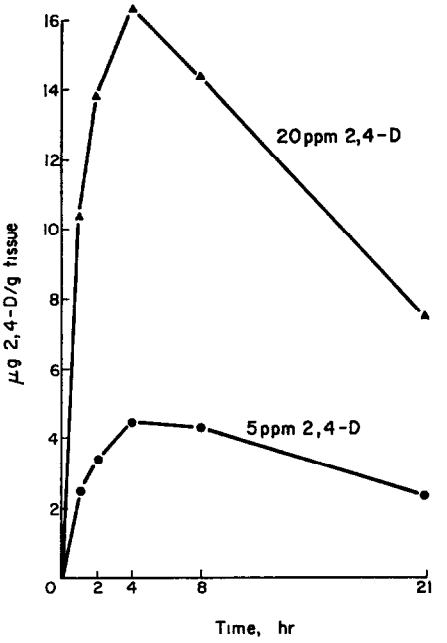

FIG. 2. ACCUMULATION OF 2,4-D IN SOYBEAN HYPOCOTYL SECTIONS WITH 2,4-D PRESENT AT 5 AND 20 ppm AFTER INCUBATION WITH ^{14}C-2,4-D THE SECTIONS WERE REMOVED FROM THE MEDIUM, RINSED AND HOMOGENIZED IN WATER AND AN ALIQUOT WAS COUNTED.

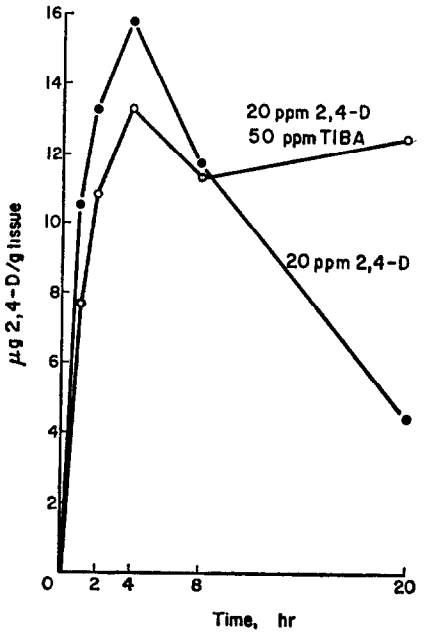

FIG. 3. THE EFFECT OF TIBA ON THE ACCUMULATION OF 2,4-D IN SOYBEAN HYPOCOTYL SECTIONS MEASURED BY INCUBATING IN THE PRESENCE OF ^{14}C-2,4-D. AT THE TIMES SHOWN THE SECTIONS WERE REMOVED AND THE RADIOACTIVITY PRESENT MEASURED AS IN FIG. 2.

as measured for 1 hr periods, is high for the first 3 hr and then decreases markedly. This uptake rate data is what would be expected from the accumulation levels shown in Fig. 2.

TIBA (50 ppm) somewhat inhibited 2,4-D accumulation by soybean stems during the first 4 hr of incubation (Fig. 3). After 8 hr, however, the TIBA treated stems contain more 2,4-D than the untreated ones. TIBA treatment likewise inhibits the rate of uptake of 2,4-D by about 20 per cent during the first 3 hr; thereafter, the TIBA treated sections take up the labeled 2,4-D more rapidly than those treated with 2,4-D alone.

When soybean sections which had previously been incubated for 1 hr with ^{14}C-2,4-D are placed in medium without ^{14}C-2,4-D the radioactivity is released into the external medium rapidly for the first 30 min. Thereafter the ^{14}C appeared in the medium at a linear rate for the next 4 to 5 hr at which time the tissue ^{14}C concentration about equaled that of the external medium. The higher the original internal ^{14}C level, the higher the release rate. The presence of 2,4-D or TIBA in the medium at the usual levels did not effect the release rate appreciably.

The uptake of TIBA from the medium was measured using ^{14}C-TIBA and the results are shown in Fig. 4. The TIBA is taken up rapidly and reaches the peak levels between 4 and 8 hr, then the tissue levels decline somewhat. The peak levels attained in the tissue are proportional to the TIBA concentrations present in the medium at least for those concentrations shown in Fig. 4 (20, 50 and 100 ppm). It is also evident that the presence of 5 ppm 2,4-D in the medium increases TIBA accumulation somewhat.

All the radioactivity found in the TIBA treated tissues chromatographed identically to the ^{14}C-TIBA standard except in treatments where 2,4-D was present. Then 3–24 per cent of the radioactivity remained near the origin after chromatography while the remainder

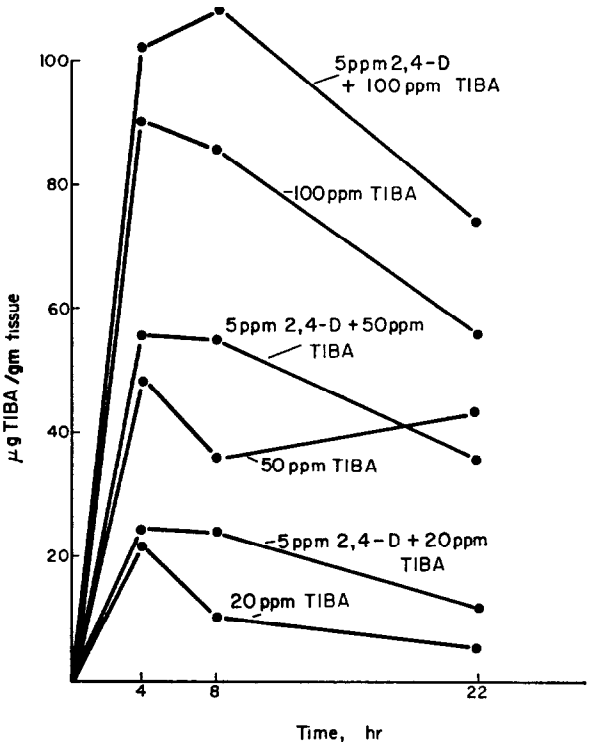

FIG. 4. ACCUMULATION OF TIBA IN SOYBEAN HYPOCOTYL SECTIONS IN THE PRESENCE OR ABSENCE OF 2,4-D. THE UPTAKE WAS MEASURED BY INCUBATING WITH ^{14}C-TIBA FOR THE PERIODS SHOWN AND THE RADIOACTIVITY WITHIN THE TISSUE WAS MEASURED AS IN FIG. 2.

chromatographed like TIBA. The material near the origin appears similar to an unidentified metabolite noted in animals.[12]

Respiration. Since the mode of action of TIBA might be concerned with respiration, the effect of TIBA on the rates of respiration were measured. Results of a typical experiment are given in Fig. 5.

When compared with untreated tissues, those treated with 50 ppm TIBA showed slightly depressed respiration for the first few hours (Fig. 5). After 22 hr, however, the TIBA treated sections respired more than untreated sections. The presence of 2,4-D was necessary for sustained oxygen uptake. TIBA had little effect on oxygen uptake in the presence of 2,4-D.

Nucleic acid and protein synthesis. The effect of TIBA on nucleic acid and protein synthesis was investigated by measuring incorporation of ^{14}C-labeled precursors into the appropriate macromolecules (^{14}C-thymidine, ^{14}C-uridine, and ^{14}C-L-leucine into DNA, RNA and protein, respectively).

The first effect of TIBA (100 ppm) was found to be an inhibition of the uptake of the ^{14}C-labeled compounds (Table 2). This inhibition of uptake resulted in a lower ^{14}C-precursor concentration within the tissue so that the incorporation values alone would appear to be invalid comparisons of the synthesis rates. Percent incorporation values should allow a better comparison of the synthesis rates under these conditions. These values

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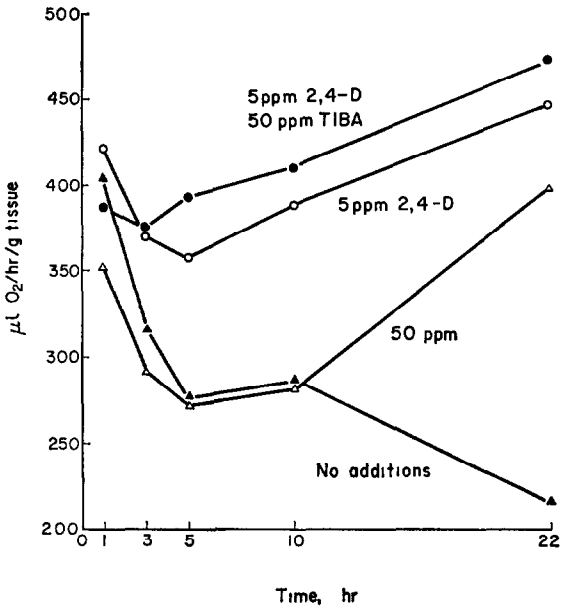

FIG. 5. RESPIRATION OF SOYBEAN HYPOCOTYL SECTIONS FIVE OR TEN HYPOCOTYL SECTIONS WERE INCUBATED IN STERILE VESSELS WITH KOH IN THE CENTERWELL IN A GILSON DIFFERENTIAL RESPIROMETER READINGS WERE MADE AT 5 min INTERVALS FOR A PERIOD OF 1 hr TO DETERMINE EACH POINT SHOWN THESE CONDITIONS ALLOWED A NORMAL GROWTH RATE

represent the per cent of ^{14}C-labeled compound in the tissue which was incorporated into acid insoluble material.

When the hypocotyl sections were incubated for 6 hr with the ^{14}C-labeled precursors, TIBA (100 ppm) inhibited uptake and incorporation of all three ^{14}C-labeled compounds (Table 2). The tissue growing in the presence of 2,4-D alone synthesized the 3 macromolecules at a greater rate than if 2,4-D is absent (percent incorporation is assumed to be a measure of synthesis rate). Longer incubation periods allow this synthesis enhancement caused by 2,4-D alone to be even greater.

If the 6 hr incorporation periods are started by the addition of the ^{14}C-precursors to the medium 6 or 21 hr after the incubation of the sections has begun we find incorporation results similar to those obtained when the ^{14}C-precursors were present from time zero (Fig 6). Figure 6 displays the present incorporation values obtained during the three incorporation time periods with 2,4-D present alone or with 2,4-D and TIBA present together. Clearly TIBA inhibits the synthesis of DNA and RNA at all times. Protein synthesis is likewise inhibited, but to a lesser extent.

Figure 7 shows the quantities of nucleic acids and protein present in the hypocotyl sections as measured by chemical means during the incubation period. The RNA level was depressed markedly by TIBA, but increased by 2,4-D alone. The presence of 2,4-D alone also increases the DNA level and may increase the protein level by 24 hr. The DNA and protein levels change little when TIBA is present.

When ^{14}C-uridine labeled RNA isolated from TIBA treated and untreated tissues was analyzed by sucrose density gradient sedimentation, the ^{14}C and absorbancy tracings coincide quite closely. The specific activity of the RNA isolated from the TIBA treated tissue is lower, however, as measured by the ratio of cpm to the absorbancy at 260 nm.

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TABLE 2. THE EFFECT OF TIBA AND 2,4-D ON INCORPORATION OF ^{14}C-LABELED PRECURSORS INTO DNA, RNA AND PROTEIN

Precursor	Treatment 2,4-D (5 ppm)	Treatment TIBA (100 ppm)	Growth (% increase in fresh wt.)	Precursor uptake (mμmoles/gm)	Precursor incorporation (mμmoles/gm)	% Incorporation (% of uptake which was incorporated)
			DNA			
^{14}C-Thymidine			14	3·66	0·187	5·1
		+	14	2·57	0·095	3·7
	+		38	4·46	0·241	5·4
	+	+	22	2·82	0·079	2·8
			RNA			
^{14}C-Uridine			13	6·51	0·371	5·7
		+	12	4·18	0 100	2·4
	+		37	9·09	0·682	7·5
	+	+	23	4·37	0·114	2 6
			Protein			
^{14}C-L-Leucine			12	3·10	2·70	87
		+	7	1·47	0·81	55
	+		42	5·13	4·62	90
	+	+	22	1·95	1 01	52

The hypocotyl sections were incubated for 6 hr with ^{14}C-thymidine (1 6 μc, 0 03 μmole), ^{14}C-uridine (1 0 μc, 0 02 μmole), and ^{14}C-L-leucine (0 1 μc, 0·012 μmole) for incorporation into DNA, RNA, and protein respectively. After incubation the sections were rinsed, blotted, weighed, and then homogenized in 0·15 M NaCl, 0 015 M Na citrate pH 7·5 and filtered through miracloth. An aliquot was counted and two were mixed with cold 5% trichloracetic acid. After 30 min on ice the samples were filtered through Millipore HA filters (24 mm dia., 0·45 μ pore size) and were washed twice with cold trichloroacetic acid. The filters were dried and counted. The % incorporation valued shown represent the % of the radioactivity present in the tissue which was incorporated into acid-insoluble material.

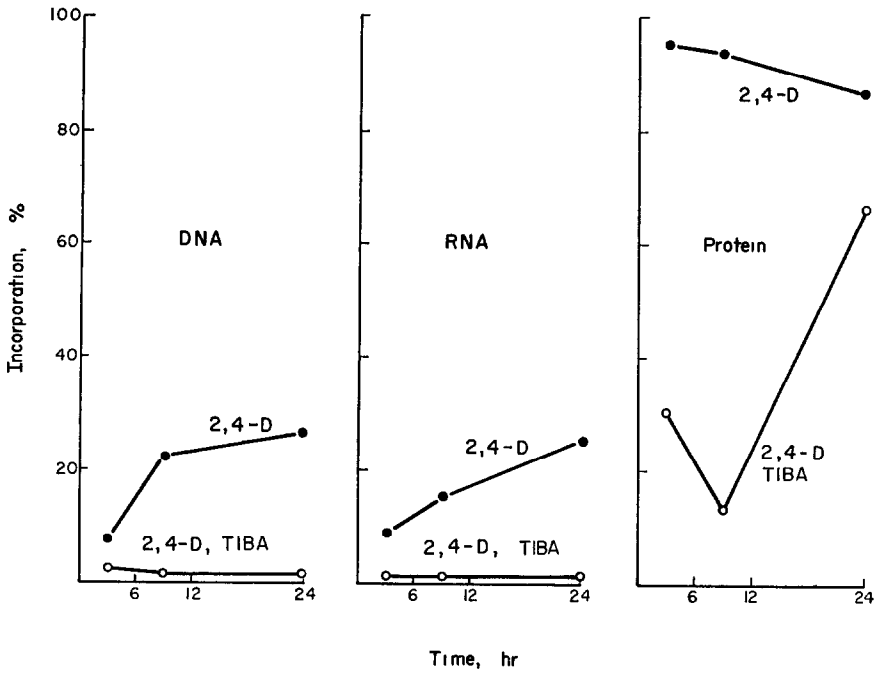

FIG 6. INCORPORATION OF ^{14}C-PRECURSORS INTO DNA, RNA, AND PROTEIN BY SOYBEAN HYPOCOTYL SECTIONS IN THE PRESENCE OF 5 ppm 2,4-D ALONE (●) OR 5 ppm 2,4-D PLUS 100 ppm TIBA (○). THE SECTIONS WERE INCUBATED FOR 6 hr PERIODS WITH THE ^{14}C-PRECURSORS AND THE INCORPORATION DETERMINED AS IN TABLE 2.

RNA, labeled with ^{14}C-uridine, was analyzed also by chromatography on MAK columns. Here again ^{14}C and absorbancy coincided well. The only difference noted in the ^{14}C and absorbancy profiles for the RNA samples in this case, as with the sedimentation experiments, is that the TIBA treated RNA has a lower specific activity for all fractions.

DISCUSSION

Since microbial contamination can invalidate incorporation experiments such as these[13] we present evidence which indicates that these experiments were not significantly contaminated. Since microorganisms usually respire rapidly, we measured the oxygen uptake of incubation solutions which had been previously incubated for 22 hr with stem sections. The greatest uptake measured was 2 μl/hr or 0 6 per cent that measured for the soybean tissue.

Secondly, contaminating microorganisms if present in significant quantities, would very likely incorporate a high percentage of the added ^{14}C-labeled compounds into their cellular materials.[13,14] Thus, bacterial RNA would be expected to be highly labeled when ^{14}C-uridine was present during the incubation. Sucrose density gradient sedimentation and MAK chromatography can allow one to distinguish higher plant ribosomal (pea and potato) from bacterial (*Escherichia coli* and *Bacillus subtilis*) ribosomal RNA since their

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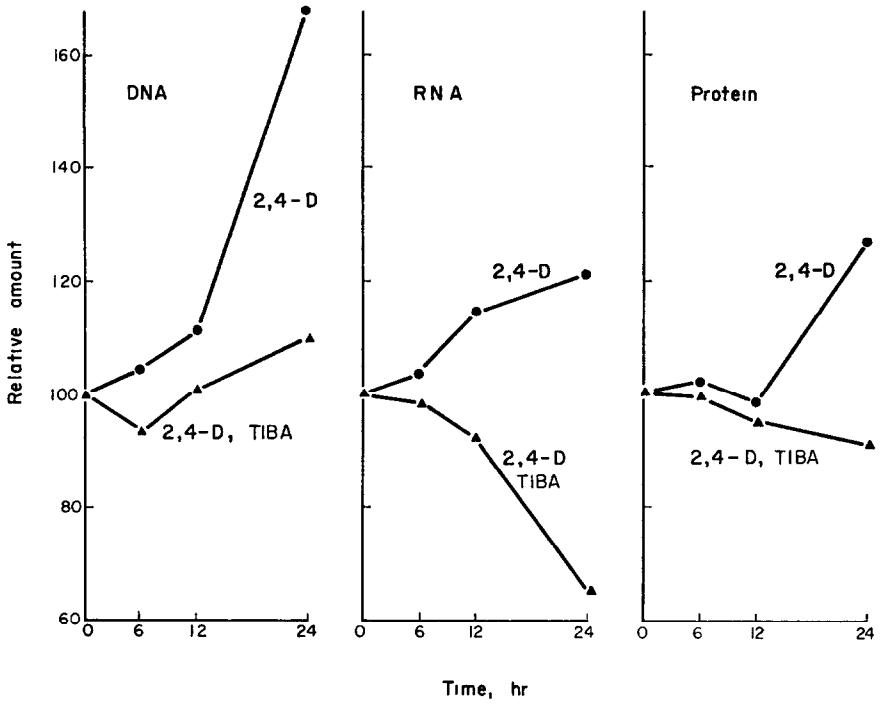

FIG 7 RELATIVE AMOUNTS OF DNA, RNA, AND PROTEIN PRESENT IN SOYBEAN HYPOCOTYL TISSUE AFTER INCUBATION WITH 5 ppm 2,4-D (●) OR 5 ppm, 2,4-D PLUS 100 ppm TIBA (▲). IN EACH CASE THE ORIGINAL TISSUE LEVEL IS SET EQUAL TO 100

profiles differ.[15,16] Since soybean and pea ribosomal RNA possess almost identical sedimentation properties (unpublished), sedimentation and MAK chromatography should allow the determination of contamination in these experiments also. When ^{14}C-uridine labeled soybean RNA was analyzed by sucrose density gradient sedimentation and by MAK chromatography no deviation between radioactivity and absorbancy was ever found. This indicates little or no contamination.

We have shown that TIBA at growth-inhibiting concentrations does inhibit 2,4-D uptake. However, as shown in Fig. 3, the 2,4-D level is not depressed by more than 25 per cent at any time by TIBA. Since 5 and 20 ppm, 2,4-D illicit the same growth response, yet the tissue 2,4-D concentration for 5 ppm 2,4-D is less than half that for the higher level (Fig. 2), we conclude that the slight lowering of the tissue 2,4-D level by TIBA is insufficient to cause the growth inhibition.

It is evident that since the tissue fresh wt. increases proportionately more than the dry wt. during incubation (Table 1) that the enlargement measured is largely due to water uptake.

The reason for the decrease in dry wt. of the TIBA treated tissue is not clear. TIBA does decrease the uptake of sucrose (Table 1), but apparently not sufficiently to cause the observed weight loss. This loss in weight might be explained by a TIBA induced increase in energy usage, but data presented here shows that TIBA does not increase respiration significantly during an 8 hr incubation period. Since sucrose uptake was inhibited markedly by

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[16] A E SOBOTA, C. J. LEAVER and J. L. KEY, *Plant Physiol.* **43**, 907 (1968).

TIBA, perhaps less of the exogenous sucrose was used as energy by the TIBA treated tissues resulting in more of the tissue dry weight being used for this purpose. Another possibility is that the TIBA treated tissue might have lost some cellular material into the medium. This possibility was not explored, however.

Since TIBA does not affect soybean tissue respiration rates appreciably, it would appear that this compound does not manifest its growth retarding effect by altering respiration. In addition to the data reported here, Malhotra and Hanson[17] found that TIBA did not uncouple oxidative phosphorylation in isolated corn mitochondria even when present at the very high level of 10^{-3} molar, but did reduce both oxygen and phosphate assimilation markedly.

In our experiments oxygen consumption did not parallel the growth rate. The data in Fig. 5 shows that the oxygen consumption with 2,4-D present is greatest after 22 hr of incubation at which time growth had almost ceased.

The synthesis rates of DNA, RNA and protein are depressed by growth inhibiting TIBA concentrations with protein being least affected (Fig. 6). This evidence suggests that nucleic acid synthesis is affected more directly by TIBA than is that of protein, and that inhibition of RNA synthesis results in less growth.

When ^{14}C-uridine-labeled RNA was fractionated by sucrose density gradient sedimentation and MAK column chromatography, no apparent alteration in labeling pattern was observed due to TIBA treatment of the donor plant tissue other than a uniformly lower specific activity. It appears then that TIBA depresses the synthesis of transfer, ribosomal, and messenger RNA to approximately the same extent.

EXPERIMENTAL

Soybean hypocotyls (*Glycine max*, var. Harosoy 63) were prepared and about 1 gm were incubated in sterile media in a similar fashion to that described by Key and Shannon [11]

The dimethylamine salt of TIBA and the 2,4-D acid were used. Ring labeled ^{14}C-2,4-D (Calbiochem, 0·2 μc/μmole), carboxyl labeled ^{14}C-TIBA- (gift of L. Spitznagle, Purdue University, 0 2 μc/μmole) and ^{14}C-thymidine, ^{14}C-uridine, ^{14}C-L-leucine, and uniformly labeled ^{14}C-sucrose (ICN Corp) were the radioactive compounds employed.

Dry wt. were determined from sections which had been dried for 48 hr at 60°. MAK column chromatography was carried out as described by Mandell and Hershey [18] Radioactivity measurements were made in Bray's[19] solution by liquid scintillation spectrometry.

To determine if TIBA was altered after entering the tissue, homogenates of tissues which had been incubated with ^{14}C-TIBA were acidified to pH 1 0 with HCl and extracted 4 × 50 ml of Et_2O. The Et_2O extract was evaporated to dryness and the residue dissolved in a small amount of acetone for application to silica gel G TLC plates for analysis. The plates were developed with petroleum ether–propionic acid (10·1, v/v).

DNA, RNA and protein were measured chemically after fractionation of the final precipitate obtained by the Key and Shannon procedure.[11] This precipitate was incubated overnight at 37° with 1 ml 0·3 N KOH. An equal vol of cold 0 8 M $HClO_4$ was added and the tubes were placed on ice for 1 hr. After centrifugation, the supernatant was decanted and saved for the chemical determination of RNA.[20] The precipitate was washed with 1 ml of cold 0 5 M $HClO_4$ and the supernatant was added to the RNA solution above. After the addition of 1 ml of 0·5 M $HClO_4$ to the precipitate, the tube was heated to 95° for 10 min and then cooled on ice for 1 hr The resulting precipitate was collected by centrifugation and the supernatant decanted and saved for the chemical determination of DNA.[21] The precipitate was then suspended in 2 ml of 0 3 N KOH and the amount of protein present determined chemically.[22]

Acknowledgement—We are indebted to Philip Moy for performing the TLC analysis

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