SECTION A, BIOLOGICAL SCIENCES

Subsection Botany

Interaction of 2,4-Dichlorophenoxyacetic Acid, Ethylene Chlorohydrin and Glutathione in the Stability of the Microsomal Ribonucleic Acid of Excised Cotton Cotyledons[1]

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The nucleic acids of isolated plant tissues were first shown to be affected by plant growth regulators by Silberger and Skoog (1953). They showed that indoleacetic acid treatment would increase the level of ribonucleic acid (RNA) and deoxyribonucleic acid in tobacco pith tissue. Recently, experiments with excised stems of cucumber seedlings (West, Hanson and Key, 1960), and excised cotton cotyleions (Basler and Nakazawa, 1961) showed that the RNA of the soluble protein and microsome fractions disappeared during culture and that 2,4-dichlorophenoxyacetic acid would prevent this loss of RNA. Other experiments (Chrispeels and Hanson, 1962) showed that the RNA of soybean hypocotyls was increased 175 percent over the control in the microsome fraction at 48 hours after spraying with 2,4-D. It was hypothesized that the herbicide renews nuclear activity in the tissue leading to a synthesis of RNA and protein and that the cytochemical basis of auxin-herbicide action lies in a reversion to a meristematic metabolism.

These experiments have revealed those sites within the protoplasm of the cell which have net changes in RNA level with 2,4-D treatment. However, the experimental data do not show that this response is unique to 2,4-D or other auxin treatments and these data will be necessary before any phase of auxin action can be ascribed to effects on nucleic acid metabolism of any particular site in the cell. The present paper presents data on the response of cotton cotyledon RNA to a number of growth regulators. The effects of ethylene chlorohydrin (ECH), reduced glutathione (GSH) and oxidized glutathione (GSSG) were examined in detail in these studies.

Materials and Methods

Cotton ('Acala 44') was germinated and grown in vermiculite wet with Shive's R5S2 nutrient solution which had been adjusted to pH 5.5 with NaOH. These plants were grown at 28C under continuous fluorescent light of about 700 ft-c. When the plants were 9 days old, cotyledons were removed for treatment. A treatment sample consisted of six cotyledons (about 2 gm fresh weight) floated in 10-cm Petri dishes containing 20 ml of Shive's nutrient solution at pH 5.5 or Shive's nutrient solution containing various concentrations of the growth regulator being studied. Potassium bicarbonate was added to adjust the pH of the acidic growth regulators.

The culture period lasted for 48 hours at 28C under continuous fluorescent light of about 700 ft-c. Samples were analyzed before, as well as at the end of, the culture period.

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At the time of analysis the samples were washed with distilled water and ground with a mortar and pestle in 0.05 M, pH 7.0 phosphate buffer containing 0.25 M sucrose. The total volume of the homogenate was made to 10 ml. The homogenate was centrifuged for 40 minutes at 27,000 x g and the precipitate was discarded. The supernatant, containing the soluble protein and the microsome fractions, was used for RNA analysis.

The RNA was determined by the methods of Ogur and Rosen (1952). Three ml of the soluble protein and microsome fraction were precipitated with cold 70% ethyl alcohol containing 0.1 N $HC10_4$; these precipitates were then washed with a 3:1 mixture of ethonol-ether and then extracted with 0.5 N $HC10_4$ for 30 minutes at 80C. Their extinction coefficients (Ogur and Rosen, 1952) were used for estimating the RNA of the hot acid extract on a Backman DU spectrophotometer.

Microsomes, which were isolated for the ribonuclease reaction studies, were prepared from 70 gm of 9-day-old cotton cotyledons ground in 100 ml of 0.05 M, pH 7, phosphate buffer containing 0.25 M sucrose. The homogenate was centrifuged at 27,000 x g for 40 minutes and the precipitate was discarded. The supernatant was subjected to centrifugation for 1 hour at 100,000 x g. The resulting precipitate containing the microsomes was washed once with the buffer solution and then made to 25 ml. This preparation, containing 160 μg of RNA per gram of original fresh weight, was used as the enzyme to test the effects of ethylene chlorohydrin on the endogenous ribonuclease activity or breakdown of the microsomal RNA. A reaction mixture contained 1 ml of enzyme, 1 ml of various concentrations of ethylene chlorohydrin, 1 ml of 0.3 m KCL or water to make a total of 6 ml. The reaction mixture was maintained at 26C. One ml samples were removed at 30-minute intervals and assayed for RNA content as described above. An enzyme prepared from 86 gm of cotton cotyledons and containing 294 μg of RNA per gm of original fresh weight was made to 25 ml and used in similar reaction mixtures to test the effects of GSH and GSSG on the breakdown of RNA in isolated microsomes.

Results and Discussion

The effects of a number of growth regulators on the RNA level of the soluble protein and microsome fraction during a 48-hour culture period are shown in Table I. Indoleacetic acid, indolebutyric acid and coumarin were active in preventing the breakdown of RNA in this fraction. However, they were not as effective as 2,4-D. Gibberellic acid had no observable effects.

TABLE I. EFFECTS OF VARIOUS CHEMICALS IN THE NUTRIENT MEDIA OF EXCISED COTTON COTYLEDONS ON THE RNA OF THE SOLUBLE PROTEIN FRACTION DURING A 48-HOUR CULTURE PERIOD.

Chemical (10^{-3} M)	RNA as % of the 48-hour control*
Control	100
2, 4-D	214
Ethylene chlorohydrin	325
Indole-3-acetic acid	144
Indolebutyric acid	165
Coumarin	135
Maleic hydraxide	119
Gibberellic acid	101

*The level of RNA in the control at the beginning and at the end of culture was 223 and 115 μg of RNA per gram of fresh weight tissue.

Ethylene chlorohydrin treatment had a marked influence on the level of RNA resulting in the accumulation of RNA above the initial level for this tissue (Table I). The effects of ECH and the interactions with 2,4-D were studied in more detail and the data are shown in Table II. These data show that ECH will augment 2,4-D in its effect and that ECH, like 2,4-D, is effective only at fairly high concentrations.

TABLE II. EFFECTS OF VARIOUS CONCENTRATIONS OF 2,4-D AND ETHYLENE CHLOROHYDRIN ON THE RNA OF THE SOLUBLE PROTEIN OF EXCISED COTTON COTYLEDONS AFTER 48-HOURS OF CULTURE.

Molar Concentration	μg RNA*/ gm Fresh Weight			
ethylene	Molar 2,4-D			
chlorohydrin	0	10^{-5}	10^{-4}	10^{-3}
0	70	110	140	290
10^{-5}	60	70	180	300
10^{-4}	70	100	150	300
10^{-3}	210	390	420	460

*The level of RNA in the soluble protein of this tissue at the beginning of culture was 360 μg RNA per gram of fresh weight tissue.

There are a number of physiological responses which occur in plant tissue upon treatment with ECH which resemble the responses of plant tissue to treatment with 2,4-D. It was shown that ECH would decrease protoplasmic viscosity of *Elodea* at low concentrations (0.5-2%) while higher concentrations or longer exposure periods would increase viscosity (Northen, 1946), and that 2,4-D at low concentrations also decreased the viscosity of *Spirogyra* while higher concentrations or longer exposure times increased viscosity (Carroll, 1949). A postulate (Northen, 1942) was made that auxins and other protein dissociating agents elicit parallel responses in respiration and other enzyme activities because of the activation of enzymes on dissociation of the protein units possibly by the production and exposure of sulfhydryl groups which may act as activators of some of the respiratory enzymes. It was shown that ascorbic acid and sulfhydryl in GSH and protein were increased on 2,4-D treatment (Key and Wold, 1961; Marre and Arrigoni, 1957), and that both ascorbic acid and GSH increased in potato tubers upon treatment with ECH (Guthrie, 1937).

There are also a number of plant responses in which 2,4-D and other auxins and ECH may show antagonistic effects or affect a physiological response in opposite directions. Examples may include effects in the abscission of *Coleus* (Hall, 1952), sprouting of potatoes (Crocker, 1948) and sweetpotatoes (Hall and Greig, 1956) and flowering in cocklebur (Hamner and Bonner, 1938; Kudairi and Hamner, 1954).

Recent work (Morgan and Hall, 1962) showed that 2,4-D will stimulate the production of ethylene in cotton plants as much as 26-fold and it was suggested that this interaction may represent a parallel mode of action of the two regulators which results in both producing many similar symptoms in susceptible plants. It is possible that 2,4-D may elicit the production of some substance capable of acting in a manner similar to that of ECH to prevent the loss of RNA in the microsome fraction of cotton cotyledon tissue. Isolated microsomes were shown to break down rapidly in the presence of potassium ions, possibly due to the activity of endogenous ribonuclease, and 2,4-D would not prevent this breakdown of isolated microsomes (West and Hansen, 1960). The effects of ECH on the breakdown of isolated cotton cotyledon microsomes was tested in the

present work and the results are shown in Table 3. ECH had no observable effect on the rate of breakdown of the RNA of these particles either in the absence or presence of K ions. Thus, ECH apparently does not have a direct blocking effect on ribonuclease in preventing the loss of RNA in the microsome fraction under tissue culture conditions.

TABLE III. EFFECTS OF ETHYLENE CHLOROHYDRIN AND POTASSIUM CHLORIDE ON THE *in vitro* BREAKDOWN OF THE RNA IN ISOLATED MICROSOMES OF COTTON COTYLEDON TISSUE.

Molar KCl	Loss of acid precipitable RNA µg / 60 minutes reaction time				
	Molar ethylene chlorohydrin				
	0	10^{-5}	10^{-4}	10^{-3}	10^{-2}
0	11.0	11.2	10.4	10.8	11.0
0.05	39.0	38.0	38.0	36.0	40.0

Glutathione was shown to be effective in forcing dormant buds of potatoes and the suggestion was made that it may act as one of the effective intermediate chemicals in the forcing action of ECH (Guthrie, 1940). Reduced GSH has also been shown to augment the growth of plant tissues (Marre and Arrigoni, 1957) and was shown to be increased in plant tissues by 2,4-D treatment (Marre and Arrigoni, 1957; Key and Wold, 1961). Some determinations of the interactions of 2,4-D and GSH as they affect the RNA level of the soluble protein and microsome fraction were made in the present study (Table IV). GSH in the presence of 2,4-D was found to be one of the most effective methods of preventing a loss of RNA. This treatment actually resulted in a net accumulation of RNA above the initial level in the soluble protein and microsome fractions. However, GSH had no observable effect in the absence of 2,4-D and treatment with the sulfhydryl poison N-ethylmaleimide at 10^{-4} M, both in the presence and absence of 2,4-D, had no effect on the RNA level. These results indicate that GSH, which may be produced in the tissue by 2,4-D treatment, is not singly involved in the prevention of loss of RNA. The suggestion was made (Marre and Arrigoni, 1957) that changes in the ratio of reduced GSH to oxidized glutathione were in themselves capable of influencing growth. However, they pointed out that reduced GSH when added alone produced only slight increases in growth.

TABLE IV. EFFECTS OF VARIOUS CHEMICALS IN THE NUTRIENT MEDIA OF EXCISED COTTON COTYLEDONS ON THE RNA OF THE SOLUBLE PROTEIN FRACTION DURING A 48-HOUR CULTURE PERIOD.

Chemical	RNA as % of the 48-hour control*
Control	100
2,4-D (5×10^{-6}M)	177
2,4-D (5×10^{-6}M) + Glutathione (10^{-3}M)	328
Glutathione (10^{-3}M)	102
N-Ethylmaleimide (10^{-4}M)	107
2,4-D (5×10^{-6}M) + N-Ethylmaleimide (10^{-4}M)	189

*The level of RNA in the control at the beginning and at the end of culture was 284 and 102 µg RNA per gram of fresh weight tissue.

GSH, which is produced in the cell upon 2,4-D treatment, may also affect the RNA level in the microsome fraction. It has been shown that the activity of certain ribonucleases is increased by disulfide bonding in the enzyme protein chain (White, 1960). An excess of GSH could interact and disrupt disulfide bonding in protein and thus inhibit ribonuclease activity. In the present studies the effects of GSH and GSSG on the breakdown of the ribonucleic acid of isolated microsomes was determined and the results are shown in Table V. GSH inhibited the K ion-induced breakdown of RNA while GSSG was without effect or slightly stimulatory in those samples in which K ion was not added. These data suggest that 2,4-D prevents RNA breakdown in intact tissue by effecting a reduced state in the sulfhydryl components of the tissue.

TABLE V. EFFECTS OF REDUCED AND OXIDIZED GLUTATHIONE, 2,4-D AND KCl ON THE *in vitro* BREAKDOWN OF THE RNA IN ISOLATED MICROSOMES OF COTTON COTYLEDONS.

Chemical Treatment*	Loss of acid precipitable RNA µg / 60 minutes reaction time
None	11
KCl	79
KCl + 2,4-D	85
KCl + GSH	28
KCl + GSH + 2,4-D	28
KCl + GSSG	57
KCl + GSSG + 2,4-D	62
GSSG	31

*The chemicals were added to the reaction mixture in the following molar concentrations: KCl, 0.1 M; GSH, 10^{-2}M; GSSG, 5 x 10^{-4}M; 2,4-D, 10^{-5}M. The pH of all additives were adjust to 7 by adding appropriate amounts of Tris buffer. The total reaction mixture was 6 ml.

SUMMARY

The effects of a number of chemicals on the RNA of the soluble proteins and microsomes fractions of cotton cotyledon tissue during a 48-hour culture period were determined. Previous work had shown that 2,4-D would prevent the loss of RNA in the microsome and soluble protein fraction during culture. The present work showed that other growth regulators including indoleacetic acid, indolebutyric acid and coumarin, were effective in stabilizing the RNA level of these fractions, although not as effective as 2,4-D. Ethylene chlorohydrin was just as effective as 2,4-D in preventing the loss of RNA during culture, but like 2,4-D ethylene chlorohydrin was not effective in blocking the ribonclease activity of isolated microsomes. Glutathione added to the culture medium in the presence of 2,4-D was very effective in preventing a loss of RNA in the soluble protein and microsome fractions but was without effect when added alone. N-Ethylmaleimide, a sulfhydryl poison, was not effective in altering the RNA level when added either in the absence or presence of 2,4-D to intact tissue. However, GSH was effective in inhibiting the breakdown of RNA in isolated microsomes and GSSG was either without effect or slightly stimulatory in some cases.

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