SECTION A, BIOLOGICAL SCIENCES

Subsection Botany

Effects of Light and 2,4-Dichlorophenoxyacetic Acid on the Glutathione Reductase and other NADP-Utilizing Enzymes of Excised Cotton Cotyledons[1]

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Auxins have been reported to have effects on the oxidation-reduction state of ascorbic acid and sulfhydryls in plant tissue (Marré and Arrigoni, 1957; Key and Wold, 1961; Key, 1962). The suggestion was made that low auxin concentrations may produce reduced conditions correlated with rapid growth and high auxin concentrations may produce an oxidized state correlated with a low growth rate (Marré and Arrigoni, 1957). More extensive research (Lin and Key, 1964) indicated that for a number of plant tissues there were no significant changes in ascorbic acid and dehydroascorbic acid which could be correlated with growth after 2,4-dichlorophenoxyacetic acid (2,4-D) treatment. However, there was a tendency for the ratio of ascorbic acid to dehydroascorbic acid to shift toward the oxidized state when plant tissue was treated with high levels of 2,4-D. Other research (Key, 1962) showed that the green leaf tissue of cucumber seedlings reflected an oxidized condition with less ascorbic acid and more dehydroascorbic acid occurring in the leaf after several hours treatment with herbicidal levels of 2,4,5-trichlorophenoxyacetic acid and 2,4-D.

We have been interested in the effects of herbicidal concentrations of 2,4-D on excised cotyledons of green cotton seedlings. The research presented in this paper shows that herbicidal concentrations of 2,4-D produces an increased level of oxidized glutathione (GSSG) and free cystine and a decreased level of reduced glutathione (GSH) and cysteine in excised cotton cotyledon tissue. Some additional experiments relative to some pertinent enzyme systems which may be involved in the oxidation-reduction changes are also presented.

Materials and Methods

Cotton ('Acala 44') was germinated and grown and excised cotyledons were cultured as previously described (Basler, 1963).

For studies involving determinations of the effects of 2,4-D on the GSH and GSSG content of tissue, six cotyledons per sample were cultured for 48 hr in 20 ml of a minus-sulfur nutrient solution containing 33μc of carrier-free ^{35}S as H_2SO_4 and 0 or 10^{-4} M 2,4-D adjusted to pH 5.8 with $KHCO_3$. The samples were cultured in the dark or in 550 ft-c of fluorescent light. At the end of the culture the samples were washed six times and stored at —15 C.

The sulfhydryl and disulfide components of the tissue were determined by reaction of the sulfhydryl components with N-ethylmaleimide and subsequent chromatography using a method similar to that previously

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described (Lee and Samuels, 1962). The frozen samples were ground in 10 ml of pH 5.8, 0.5 M phosphate buffer containing 5 moles of EDTA and 20 m moles of N-ethylmaleimide. Each sample was allowed to stand at room temperature for one hour to promote reaction of the sulfhydryls with N-ethylmaleimide, and then brought to 2 C and made to 5% trichloroacetic acid to precipitate the protein fraction which was removed by centrifugation. The supernatant fraction containing the free disulfides and sulfhydryl-N-ethylmaleimide adducts was washed 2 times with equal volumes of diethyl ether and made to 15 ml total volume. The supernatant fraction (0.01 ml portions) was chromatographed on thin-layer chromatograms of silica gel using phenol-water (86:15 v/v) as a solvent. Known amounts of unlabeled methionine, cystine, and cysteine-N-ethylmaleimide and GSH-N-ethylmaleimide adducts were added as carriers to each fraction before chromatography. These spots were then located after chromatography by spraying the chromatogram with ninhydrin and by autoradiography. The spots were then removed from the chromatogram and counted by liquid scintillation counting. Due to inadequate separation on the chromatogram the spots of GSSG and cystine and the spots for GSH-N-ethylmaleimide and cysteine-N-ethylmaleimide adducts were combined and counted together.

The effects of 2,4-D on the level of glutathione reductase in cultured tissue was determined. Samples consisting of six cotyledons were cultured in the light and dark in 0 and 10^{-3} M 2,4-D. The samples were ground in a glass homogenizer and made to 10 ml with pH 7.5, 0.1 M tris containing 5 mM $MgCl_2$. The reaction mixture contained 0.5 ml of the homogenate as enzyme, 10^{-3} M GSSG and 10^{-3} M NADPH in a total volume of 1.5 ml. The enzyme reactions were started immediately after grinding the sample. The reactions were stopped by boiling for two minutes. The denatured protein was centrifuged and the glutathione reductase activity was determined by titrating the GSH in the supernatant with salyrganic acid as previously described (Klotz and Carver, 1961).

The levels of the enzymes glucose-6-phosphate dehydrogenase (G-6-PD), NAPD-malic dehydrogenase and isocitrate dehydrogenase were determined in 2,4-D-treated tissue. Six cotyledons were cultured in 0 or 10^{-3} M 2,4-D for 48 hr in the light and then ground in pH 7.5, 0.1 M tris containing 5 mM $MgCl_2$ and made to 10 ml. The NADPH produced by the above enzymes was measured by a coupled enzyme reaction in which the NADPH was utilized by glutathione reductase to reduce GSSG in the reaction mixture. The GSH produced was then titrated with salyrganic acid. The reaction mixtures contained 10^{-3} M NADP, 10^{-3} M GSSG, 0.03 mg glutathione reductase (6 enzyme units) and 3.3×10^{-3} M of either glucose —6-phosphate, malic acid, or isocitric acid as substrate in pH 7.5, 0.1 M tris and 0.5 ml of the homogenate as the enzyme source. The total volume of the reaction mixtures was 2 ml. In addition, the reaction mixture for isocitrate dehydrogenase contained 4.4×10^{-4} M $MnCl_2$. The enzyme reactions were started immediately after grinding and were stopped by boiling for 2 minutes. The denatured protein was centrifuged and the GSH in the supernatant was titrated with salyrganic acid. The enzyme reaction rates were determined from rates established during the initial 15 minutes of reaction.

Results and Discussion

The effects of 10^{-3} M 2,4-D on the oxidation-reduction state of some components of excised cotyledon tissue are shown in Table 1. In these experiments 2,4-D produced a more oxidized condition so that the newly synthesized GSSG and cystine occurred in higher concentrations and the GSH and cysteine occurred in lower concentrations in treated tissue after 48 hr of culture. These changes were more extensive in samples cultured in the light than those cultured in the dark.

TABLE I. EFFECTS OF 2,4-D ON THE DISTRIBUTION OF ^{35}S IN THE FREE CYSTINE PLUS GSSG AND FREE CYSTEINE PLUS GSH FRACTIONS OF EXCISED COTYLEDONS AFTER 48 HR CULTURE IN DARK AND LIGHT OF 550 FT-C.

Culture	Counts per minute		
	cystine + GSSG	cysteine + GSH	Ratio oxidized/reduced
Dark, O M 2,4-D	182	156	1.17
Light, O M 2,4-D	280	178	1.57
Dark, 10^{-3} M 2,4-D	316	139	2.29
Light, 10^{-3} M 2,4-D	432	95	4.62

The data, which showed 2,4-D to be effective in promoting increases in GSSG and cystine, appear to be in accord with other data (Key, 1962) showing that 2-4-D promoted an oxidized condition exemplified by low levels of ascorbic acid in cucumber leaves. This effect may be a general one on green leaf tissue and as such might be of importance in the interpretation of the toxic mechanism of the auxin-like herbicides. Therefore, some experiments were designed which might reveal some of the mechanisms involved in the action of 2,4-D in producing the oxidized state in the glutathione components of plant tissue. The central enzymes utilized in plants to regulate the oxidation-reduction level of glutathione appear to be glutathione reductase, which utilizes NADPH to reduce GSSG, and dehydroascorbic acid reductase, which utilizes dehydroascorbic acid to oxidize GSH. The possibility exists that NADPH could be a limiting factor in the reduction of GSSG if 2,4-D were to have adverse effect on the dehydrogenase enzymes utilized in the reduction of NADP. Therefore, the effects of 2,4-D and light on the glutathione reductase and G-6-PD enzymes have been studied in some detail.

Preliminary experiments showed that 2,4-D at concentrations as high as 3.3×10^{-3} M did not inhibit the action of a partially purified glutathione reductase enzyme in *in vitro* experiments. Other experiments were designed to test the possibility that 2,4-D-treated cotyledons may produce inhibitors of the action of gluthathione reductase. These experiments showed that boiled extracts of cultured cotyledons have inhibitors of this reaction but this inhibition was no greater in 2,4-D-treated tissue than untreated tissue. Therefore, other experiments were conducted to determine the effects of high concentrations of 2,4-D on the level of glutathione reductase in cultured cotyledons. These data are shown in Figure 1 where it is shown that 2,4-D does affect the level of glutathione reductase but not in a direction which would explain the accumulation of GSSG in treated tissue. Rather than decreasing the level of glutathione in treated tissue, 2,4-D greatly enhanced the level of this enzyme. Light also appears to affect the level of glutathione reductase during culture. In the absence of 2,4-D, light inhibited the accumulation of glutathione reductase. In the presence of 2,4-D, samples cultured in the light invariably had more glutathione reductase at the end of 48-hr culture than those cultured in the dark. However, the initial rate of accumulation was as great in samples cultured in the dark.

The data showed that 2,4-D treatment increased the level of glutathione reductase in cultured tissue. A lack of glutathione reductase does not appear to be the cause of the accumulation of GSSG in 2,4-D-treated tissue. Since NADPH possibly could be the limiting factor in the reduction of GSSG, some experiments were conducted to determine the influence of 2,4-D on some of the dehydrogenase enzymes involved in the

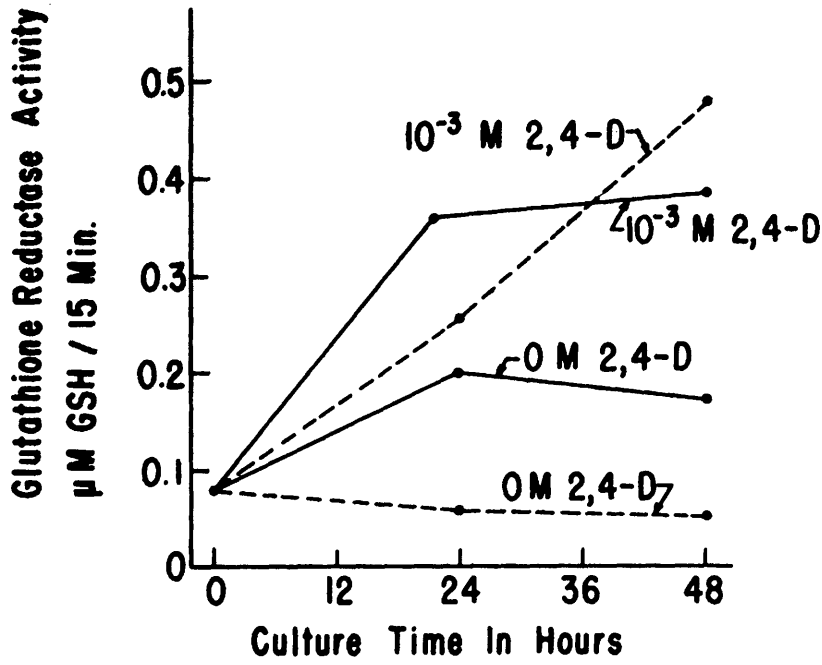

Figure 1. The activity of glutathione reductase in cell-free homogenates of cotyledons after treatment of cotyledons with light and 2,4-D during culture. Solid lines represent samples cultured in the dark and dashed lines represent samples cultured in fluorescent light of 550 ft-c. The curves represent the averages of three determinations.

reduction of NADP in cotyledons. During culture in the light, 2.4-D treatment greatly increased the concentration of G-6-PD, NADP-malic dehydrogenase and isocitrate dehydrogenase. The detailed data for G-6-PD is shown in Figure 2. G-6-PD was greatly increased in the light- and dark-cultured samples by 2,4-D treatment during the first 24 hr of culture. Others have shown an increase in this enzyme in corn root tissue (Black and Humphreys, 1962) and cucumber stem tissue (Key, 1962) after 2,4-D treatments. This increase in G-6-PD levels does not appear to be a direct cause of the accumulation of GSSG in treated tissue since high concentrations of this enzyme should lead to a higher rate of reduction of NADP and thus a higher rate of reduction of GSSG. One possibility is that NADPH is a limiting factor in the reduction of GSSG by virtue of being used preferentially in other systems. A large increase in respiration of cotton cotyledons effected by high concentrations of 2,4-D has been shown (Basler and Nakazawa, 1961). The available NADPH could be used as an electron donor for this increased process of respiration. Recent studies have shown that NADPH may be used as readily as NADH in respiration of plant tissue (Ragland and Hackett, 1965).

Light also affected the concentration of G-6-PD in cultured tissue. In the absence of 2,4-D, light inhibited the accumulation of this enzyme (Figure 2). In the presence of 2,4-D, G-6-PD accumulated rapidly and with equal facility in samples cultured in light or dark conditions during

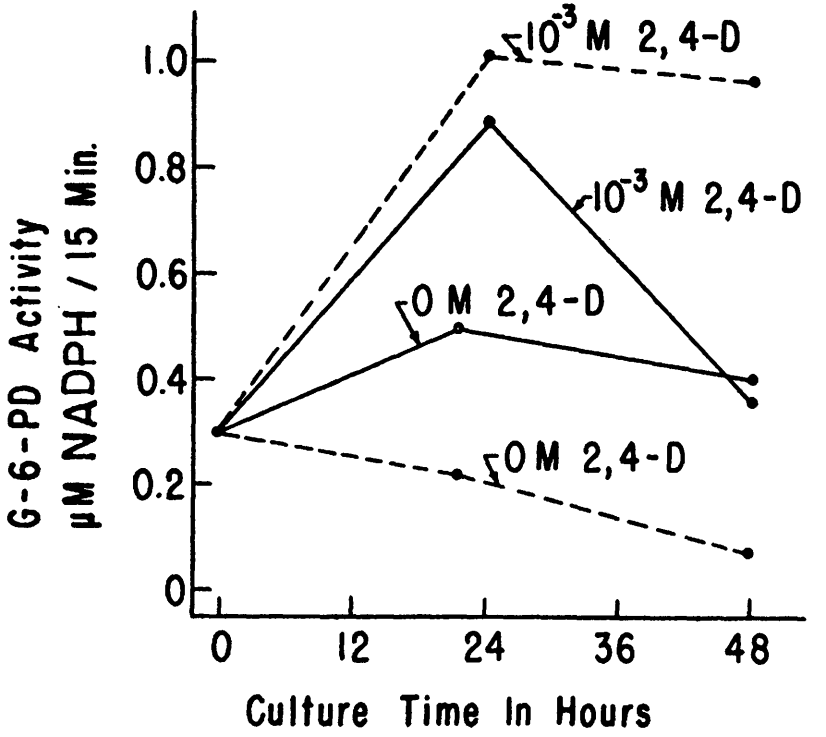

Figure 2. The activity of glucose-6-phosphate dehydrogenase in cell-free homogenates of cotton cotyledons after treatment of cotyledons with light and 2,4-D during culture. Solid lines represent samples cultured in the dark and dashed lines represent samples cultured in fluorescent light of 550 ft-c intensity. The curves represent averages of three determinations.

the first 24 hr. During the second 24 hr of culture in 2,4-D, those cotyledons maintained in the dark rapidly lost G-6-PD. The reason for this loss is not readily apparent. One possibility is that some factor which is produced in the light and allows 2,4-D to enhance synthesis becomes limiting after a number of hours in the dark so that the normal degradation rate of G-6-PD, in the absence of synthesis, caused the concentration of the enzyme to decrease. Light has been shown to be necessary for the synthesis of NADP-linked glyceraldehyde-3-phosphate dehydrogenase (Margulies, 1965). The process was mediated by the phytochrome system and was independent of photosynthetic ability in bean leaf tissue. The possibility that the active form of phytochrome may be necessary for the synthesis of G-6-PD in cotton cotyledons was studied and the results are shown in Figure 3. Samples of cotyledons were cultured for 24 hr in the light and dark before 2,4-D was added to the culture medium. When 2,4-D was added 24 hr after the beginning of culture there was very little enhancement of accumulation of G-6-PD in the dark-cultured samples. However, there was even less response to 2,4-D in those samples cultured in the light. This indicates that activated phytochrome is not the limiting factor for synthesis of G-6-PD during the second 24 hr of culture. Rather, there appears to be some factor which is supplied

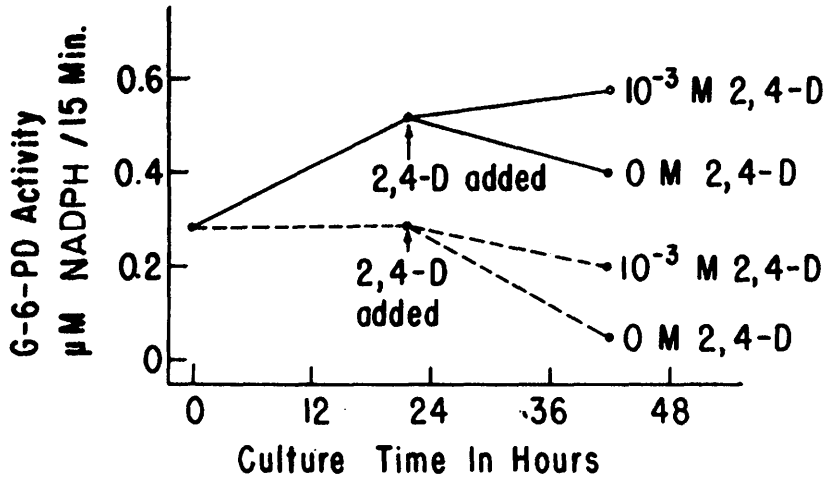

Figure 3. The activity of glucose-6-phosphate dehydrogenase in cell-free homogenates of cotton cotyledons after treatment of cotyledons with light and 2,4-D during culture. 2,4-D was added to some samples 24 hr after the beginning of culture. Other samples were cultured for 42 hr in the absence of 2,4-D. Solid lines represent samples cultured in the dark and dashed lines represent samples cultured in fluorescent light of 550 ft-c intensity. The curves represent averages of three determinations.

to the cotyledons from the roots or stems of the intact seedlings which either is necessary for the synthesis of G-6-PD or is inhibitory to the breakdown or inactivation of the enzyme. This factor appears to be less stable in the light than the dark in excised cotyledons cultured in the absence of 2,4-D, but is more stable in excised cotyledons in the light when 2,4-D is present.

SUMMARY

The treatment of excised cotton cotyledons with 10^{-3} M 2,4-D caused a more oxidized condition in the glutathione components of the tissue. The accumulation of oxidized glutathione did not appear to be due to the effects of 2,4-D on the activity or concentration of glutathione reductase or the glucose-6-phosphate dehydrogenase concentration in the tissue. The concentration of both of these enzymes was greatly increased by treatment of the cotyledons with 2,4-D. Light was inhibitory to the accumulation of these enzymes in cultured cotyledons. The 2,4-D-enhanced accumulation of these enzymes was not inhibited by light.

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