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Chloroplast membrane alterations in triazine-resistant *Amaranthus retroflexus* biotypes

(triazine herbicides/diuron/photosystem II/pesticide resistance)

CHARLES J. ARNTZEN*, CATHY L. DITTO*, AND PHILIP E. BREWER†

*U.S Department of Agriculture/SEA, Department of Botany, University of Illinois, Urbana, Illinois 61801; and †Department of Agronomy, University of Illinois, Urbana, Illinois 61801

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ABSTRACT **The effectiveness of diuron, atrazine, procyazine, and cyanazine were compared in controlling growth of redroot pigweed (*Amaranthus retroflexus* L.) in hydroponic culture. A very marked differential inhibition response was observed for atrazine between resistant and susceptible biotypes. Procyazine and cyanazine exhibited less dramatic differential responses, whereas diuron was equally effective in controlling growth in both biotypes. Photosystem II activity of chloroplasts from both triazine-resistant and triazine-susceptible biotypes was inhibited by diuron but only the chloroplasts from triazine-susceptible biotypes were inhibited significantly by atrazine. The photochemical activity of chloroplasts from triazine-resistant biotypes was partially resistant to procyazine or cyanazine inhibition. The parallel lack of diuron differential effects, partial procyazine and cyanazine differential response, and very marked atrazine differential response in both whole plant and chloroplast assays indicates that the chloroplast is the site of selective herbicide tolerance in these triazine-resistant redroot pigweed biotypes.**

Photosystem II photochemical properties were characterized by analysis of chlorophyll fluorescence transients in the presence or absence of herbicides. Data with susceptible chloroplasts indicated that both diuron and atrazine inhibit electron flow very near the primary electron acceptor of photosystem II. Only diuron altered the fluorescence transient in resistant chloroplasts. In untreated preparations there were marked differences in the fast phases of the fluorescence increase in resistant vs. susceptible chloroplasts; these data are interpreted as showing that the resistant plastids have an alteration in the rate of reoxidation of the primary photosystem II electron acceptor. Electrophoretic analysis of chloroplast membrane proteins of the two biotypes showed small changes in the electrophoretic mobilities of two polypeptide species. The data provide evidence for the following herbicide resistance mechanism: genetically controlled modification of the herbicide target site.

The occurrence of biological resistance to pesticides has been frequently reported. Until recent years, however, there has been little indication of induced herbicide resistance in naturally occurring plant species subject to chemical weed control. An exception to this trend occurred when Ryan (1) reported failure of atrazine [2-chloro-4-ethylamino-6-(isopropylamine)-*s*-triazine] and simazine [2-chloro-4,6-bis(ethylamino)-*s*-triazine] in controlling a common groundsel (*Senecio vulgaris* L.) biotype in a nursery where simazine had been used once or twice annually over the previous 10 years. Radosevich and Appleby (2) subsequently demonstrated that the groundsel resistance extended to a wide range of different triazine herbicides. More recently there have been reports of the appearance of triazine-resistant redroot pigweed (*Amaranthus retroflexus* L.) and common lamb's-quarters (*Chenopodium album* L.) biotypes in areas that had been under triazine treatment for several years (3–5).

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The mechanism(s) that produces triazine resistance in the above-mentioned weed biotypes has been investigated. Differential uptake or translocation and differential rates of metabolism of the triazines by susceptible vs. resistant biotypes have been ruled out as the selection mechanisms for common groundsel (2), common lamb's-quarters (6, 7) and redroot pigweed (6). Because triazines are known to inhibit photosynthetic electron transport, it was of special interest that net photosynthesis as measured by CO_2 uptake was found to be inhibited by triazines in susceptible but not resistant biotypes of common groundsel (8) and redroot pigweed (4). Moreover, Radosevich found that light-induced dichloroindophenol (Cl_2indophenol) reduction by isolated chloroplasts from resistant common groundsel, common lamb's-quarters, or redroot pigweed biotypes was not inhibited by added atrazine, whereas the susceptible chloroplasts were inhibited (6, 8). It was suggested that the triazines could be weakly bonded to form an inactive conjugate by compounds in the resistant chloroplast preparation or, alternatively, that the resistant chloroplast envelope could exclude the herbicide (6, 8). We initiated the present study to examine in greater detail the mechanism(s) of chloroplast resistance to triazines in redroot pigweed and to determine whether fundamental differences in the susceptible vs. resistant chloroplasts could be detected aside from the differential activity of triazines.

MATERIALS AND METHODS

Seeds of triazine-resistant and triazine-susceptible redroot pigweed were provided by Homer LeBaron (CIBA–GEIGY, Greensboro, NC). The resistant seeds had been collected in two sites: Whatcon County, Washington, in 1973, and Baltimore County, Maryland, in 1976. Susceptible seed was provided from collections in Whatcon County, Washington, and Allegany County, Maryland; in addition, susceptible plant material collected locally in Illinois was used in some experiments.

Hydroponic Plant Culture. For analysis of herbicide selectivity, redroot pigweed seedlings were exposed to herbicides while being grown in aerated hydroponic culture. Seeds were germinated and grown in vermiculite moistened with 25% Hoagland's solution. After 11–12 days, seedlings in the third or fourth leaf stage were transferred to plastic cups containing 750 ml of 50% Hoagland's solution. Four plants per cup were supported over the solution via a perforated cup cover and sponge rubber plugs around each stem. The seedlings were maintained in nutrient solution for 3 days (until the sixth or seventh leaf stage) before herbicides at the appropriate concentrations were added to the cups. During seedling culture, the temperature regime was held at 25/20°C day/night during seedling establishment and 30/20°C during herbicide uptake. Light intensities at leaf level averaged 430 microeinsteins m^{-2}

Abbreviation: Cl_2indophenol, dichloroindophenol.

sec^{-1} (1 einstein = 1 mol of photons) with 16-hr light/8-hr dark cycles. The solutions for hydroponic culture were changed every second day; fresh herbicide solution was made up at original herbicide concentration levels. Treated seedlings were harvested after 6 days of herbicide treatment. Injury ratings were assigned as an average value for the four plants of each cup at the time of harvest.

Photochemical Assays with Isolated Chloroplasts. Chloroplasts were isolated from 4-week-old seedlings grown in soil in a greenhouse. Stroma-free thylakoids were prepared as described (9) and were suspended to 1 mg of chlorophyll per ml in 0.1 M sorbitol/10 mM NaCl/10 mM $MgCl_2$/10 mM Na *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) (pH 7.8) and held on ice until assay. The kinetics of light-dependent reduction of Cl_2indophenol and measurements of chlorophyll fluorescence were as described (9).

Chloroplast membrane samples used for electrophoretic analysis were washed twice with 0.75 mM EDTA to remove the extrinsic membrane proteins. Washed membranes were lipid-extracted and solubilized with sodium dodecyl sulfate according to the procedure of Hoober (10). Linear 10–20% gradient slab gels were prepared according to the procedure of Henriques and Park (11). Gels were run at 12 mA for 15 hr, using pyronin Y as a tracking dye. The gels were stained according to procedures of Fairbanks *et al.* (12) and dried as described by Maizel (13).

RESULTS

Differential Herbicide Effects in Whole Plant Studies. Atrazine-susceptible and atrazine-resistant seedlings derived from seed collected in the state of Washington were subjected to herbicides included in hydroponic culture media (Table 1). Visual observations and fresh or dry weight measurements demonstrated that both biotypes were equally susceptible to diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. The estimated I_{50} values (herbicide concentration required to elicit 50% of maximal effects under the defined experimental conditions) are primarily based on dry weight determinations because this growth variable has previously been found to have the greatest reproducibility (14). The two biotypes differed greatly with respect to triazine susceptibility. Whereas 0.4 μM atrazine in the nutrient solution gave 50% inhibition of growth of susceptible seedlings, more than a 100-fold increase in concentration was needed to give similar growth inhibition in the resistant biotype. Procyazine, 2-{[4-chloro-6-(cyclopropylamino)-1,3,5-triazine-2-yl]amino}-2-methylpropanenitrile, and cyanazine, 2-{[4-chloro-6-(ethylamino)-*s*-triazine-2-yl]amino}-2-methylpropionitrile, were also differentially inhibitory in the two biotypes. These Cl-substituted triazines, which differ from atrazine in alterations in side chain substitutions, exhibited I_{50} values that were only 10-fold greater in the resistant biotypes than in the susceptible biotypes.

Differential Herbicide Effects on Electron Transport in Isolated Chloroplasts. Photosystem II-mediated reduction of Cl_2indophenol was monitored at various herbicide concentrations in preparations of stroma-free thylakoid membranes isolated from Washington resistant or susceptible biotypes (Fig. 1). Very small differences were observed in the effectiveness of diuron in inhibiting electron transport in the susceptible vs. resistant biotypes (I_{50} = 0.04 μM vs. 0.08 μM, respectively). The I_{50} value for atrazine inhibition in susceptible chloroplasts was 0.3 μM. In resistant chloroplasts, 50% inhibition of electron transport could not be achieved within the solubility range of atrazine.

The activity of atrazine, procyazine, and cyanazine on electron transport in two resistant biotype chloroplasts was compared to susceptible biotype chloroplast samples. Both the Maryland and Washington resistant chloroplasts were strongly resistant to atrazine inhibition within the solubility range of the herbicide. I_{50} values for cyanazine and procyazine could be calculated, however. The I_{50} values for susceptible chloroplasts (regardless of the seed source) were near 0.70 μM for cyanazine and 0.68 μM for procyazine. In resistant samples (from seed collected in Maryland or Washington), the I_{50} values for these two herbicides averaged 36 μM and 18 μM, respectively.

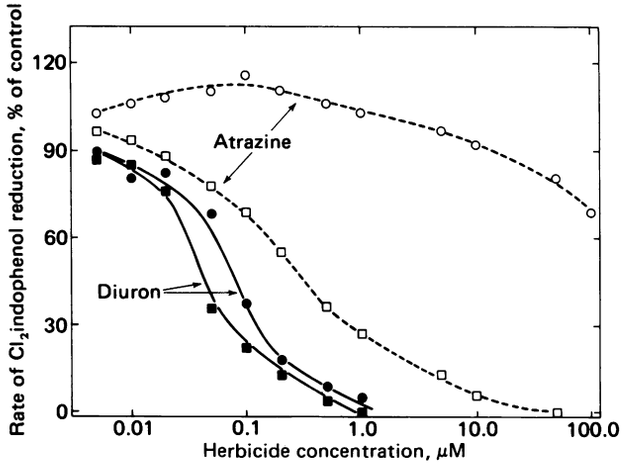

FIG. 1. Effect of diuron or atrazine on photoreduction of Cl_2indophenol by isolated chloroplasts from 4-week-old atrazine-susceptible (■, □) or atrazine-resistant (●, ○) biotypes of *A. retroflexus* L. collected in the state of Washington. Reaction mixtures contained 50 mM sodium phosphate (pH 6.8), 5 mM NH_4Cl, 10 mM $MgCl_2$, 30 μM Cl_2indophenol, and 10 μg of chlorophyll in a 2-ml volume, and either diuron or atrazine was added to give the final herbicide concentrations indicated. Control rates were 230 and 280 μmol of Cl_2indophenol reduced per mg of chlorophyll per hr for resistant and susceptible chloroplasts, respectively.

Analysis of Fluorescence Induction Transients. Analysis of chlorophyll fluorescence is now a widely used tool for monitoring pigment characteristics and photochemical properties of chloroplasts *in vitro* and *in vivo*. The rate of the time-dependent increase in intensity of fluorescence from dark-adapted chloroplasts is known to be stimulated by inhibitors of electron transport that act on the reducing side of photosystem II (15). This was verified for chloroplasts from an atrazine-susceptible biotype (Fig. 2 *left*); diuron and atrazine acted in a virtually identical fashion in increasing the rate of fluorescence rise. In contrast, diuron, but not atrazine, stimulated the rate of increase of the fluorescence rise in resistant-biotype chloroplasts (Fig. 2 *right*).

In the experiments utilizing fluorescence transient analysis, it was noted that the initial, very rapid changes in fluorescence (during the first 50 msec after onset of illumination) varied between susceptible and resistant biotypes. During illumination of dark-adapted chloroplasts, the fluorescence increase from the "original" level of fluorescence observed immediately after the onset of illumination (the F_O level) to the slightly higher "intermediate" value (F_I) was dramatically larger in the resistant chloroplasts (compare F_I of Fig. 2 *left* with that of Fig. 2 *right*). To determine whether the differences in fluorescence transients in isolated chloroplasts were due to an inherent characteristic of the membranes or due to some modification of the membranes suffered during isolation, we recorded *in vivo* fluorescence transients. The relative F_O and F_I values of the *in vivo* transients were reproducible within a population of seedlings of the same age grown under the same environmental conditions. When the fast (O → I) portion of the fluorescence transient was expressed as a proportion of the total variable

Table 1. The inhibitory effect of various concentrations of herbicides upon triazine-susceptible and -resistant *Amaranthus retroflexus* L. seedlings

Triazine-susceptible				Triazine-resistant			
Herbicide, μM	Visual injury rating	Dry weight (g/4 plants)	Estimated I_{50} value	Herbicide, μM	Visual injury rating	Dry weight (g/4 plants)	Estimated I_{50} value
Control	0	1.88 (100)	—	Control	0	1.86 (100)	—
Diuron				Diuron			
0.2	0	1.72 (91)		0.2	0	1.72 (93)	
0.3	0	1.72 (91)		0.3	0	1.40 (75)	
0.4	40	0.92 (49)	0.4 μM	0.4	15	1.00 (54)	0.4 μM
0.5	50	0.67 (36)		0.5	35	0.73 (39)	
0.6	88	0.44 (23)		0.6	75	0.41 (22)	
Atrazine				Atrazine			
0.2	0	2.05 (109)		0.5	0	2.12 (114)	
0.4	35	0.86 (46)		1.0	0	1.68 (90)	
0.6	50	0.56 (30)	0.4 μM	5.0	0	1.84 (99)	50.0 μM
0.8	65	0.41 (22)		10.0	0	1.54 (83)	
1.0	82	0.36 (19)		50.0	25	0.88 (44)	
Cyanazine				Cyanazine			
1.0	0	2.08 (111)		0.5	0	2.04 (110)	
1.5	0	1.84 (98)		1.0	0	1.46 (79)	
2.0	40	1.00 (53)	2.5 μM	5.0	0	1.48 (80)	30.0 μM
2.5	40	0.94 (50)		10.0	0	1.64 (88)	
3.0	70	0.52 (28)		50.0	45	0.77 (39)	
Procyazine				Procyazine			
1.0	0	1.80 (96)		0.5	0	1.84 (99)	
1.5	0	1.98 (105)		1.0	0	1.74 (94)	
2.0	0	1.33 (71)	2.5 μM	5.0	0	1.58 (85)	20.0 μM
2.5	45	0.86 (46)		10.0	0	1.25 (67)	
3.0	70	0.58 (31)		50.0	70	0.46 (25)	

Injury ratings (0–100 scale) were based upon the extent of chlorosis, leaf necrosis, or morphological alterations with 0 being no visual injury and 100 indicating seedling death. Values in parenthesis for dry weight are percentage of control. The estimated I_{50} value is based upon dry weight determinations because earlier studies have shown this to be the most reproducible analysis variable (14).

fluorescence, the atrazine-resistant seedlings were always found to have a higher value (i.e., a more rapidly rising transient to the F_I level).

Analysis of Chloroplast Membrane Polypeptides. Comparisons of membrane polypeptides of resistant and susceptible chloroplasts were conducted by using concentration-gradient polyacrylamide slab gel electrophoresis of sodium dodecyl sulfate solubilized proteins (Fig. 3). Almost all chloroplast

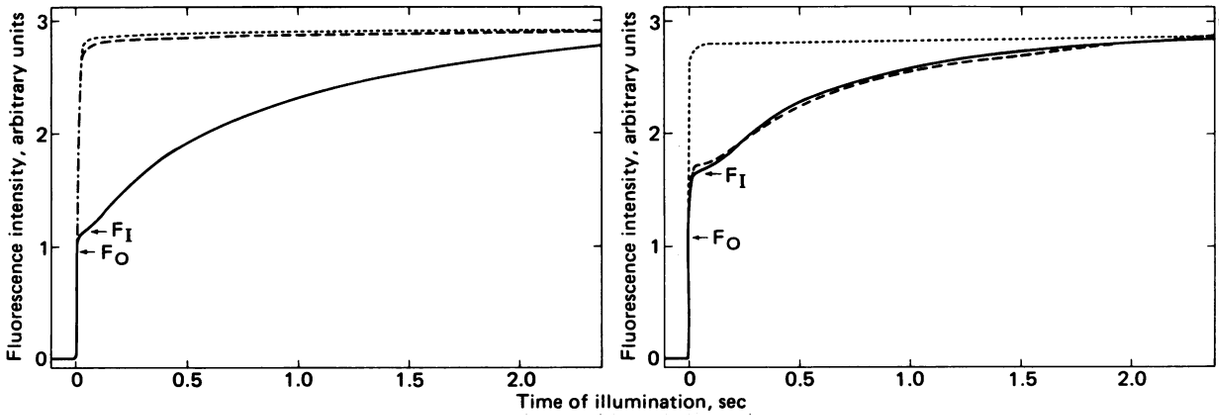

FIG. 2. Chlorophyll fluorescence transient changes observed upon illumination of dark-adapted chloroplasts isolated from atrazine-susceptible (*Left*) and -resistant (*Right*) *A. retroflexus* L. seedlings. Reactions contained 20 μg of chlorophyll, 0.1 M sorbitol, 10 mM NaCl, 10 mM $MgCl_2$, and 10 mM Na Tricine (pH 7.8) in 2-ml volumes. F_O levels of fluorescence were determined within 1 msec after full shutter opening. The F_I level of fluorescence in untreated samples was calculated from the fluorescence intensity after 50 msec of sample illumination. Diuron or atrazine was added to a final concentration of 10 μM to the isolated chloroplast suspensions, which were then incubated for 1 min in the dark at 4°C before fluorescence analysis. The transients presented are direct tracings of data obtained by x,y plotting of the rapid fluorescence changes recorded with a Nicolet digital analyzer. Chloroplasts used in these experiments demonstrated rates of Cl_2indophenol reduction of 250 and 300 μmol reduced per mg of chlorophyll per hr for resistant and susceptible chloroplasts, respectively. —, Control chloroplasts; - - -, 10 μM atrazine; ····, 10 μM diuron.

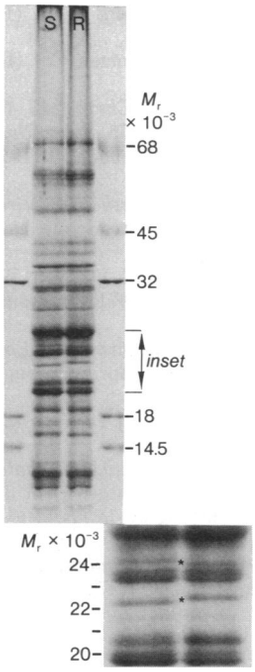

FIG. 3. Polypeptide profiles of intrinsic chloroplast membrane proteins from atrazine-susceptible (S) and atrazine-resistant (R) biotypes of *A. retroflexus* L. as revealed by slab gel electrophoresis. Parallel migration of all polypeptides in the two samples is evident except as indicated by ★ in *Inset*. Protein standards of known indicated molecular weight (M_r) were run on either side of the chloroplast samples.

membrane polypeptides of the two biotypes exhibited parallel migration. The only region of the gel that revealed any protein mobility differences is shown in the *inset* in Fig. 3. Polypeptides migrating with apparent molecular weights of 23,900 and 22,200 in the susceptible chloroplast sample can be compared to those of 24,100 and 22,000 in the resistant chloroplast sample. Chloroplast membrane proteins isolated from susceptible biotype seedlings from Maryland, Illinois, or Washington were identical in these experiments.

DISCUSSION

Relationship of Differential Triazine Effects upon Seedling Growth and Chloroplast Hill Reactions. Correlations between *in vivo* and *in vitro* effects of the four herbicides used in this study strongly suggest that differential herbicide activity on whole plant growth is determined by chloroplast membrane properties. Whereas diuron was equally effective in controlling plant growth in both susceptible and resistant plants, procyazine and cyanazine were 1/10th as active in the resistant plants and atrazine was 1/100th as active in the resistant plants. The same trend was seen in herbicide inhibition of electron transport activity of the isolated chloroplasts: diuron was quite similar in activity in both samples; procyazine and cyanazine were less active in the resistant chloroplasts; atrazine showed maximal differential effects. It should be emphasized that direct comparisons of absolute herbicide I_{50} values between *in vivo* and *in vitro* studies are not justified. The comparisons we note herein are those that relate *relative* resistance levels in the two systems of analysis. The data indicate that the degree of herbicide tolerance in whole plants is directly related to the effectiveness of the herbicide in limiting photosynthetic electron transport in the different biotypes.

The Mode of Action of Diuron and Atrazine on Photosynthetic Electron Transport. It is now generally accepted that diuron and atrazine block electron transfer between the primary electron acceptor of photosystem II and the secondary electron acceptor pool of the photosynthetic electron transport chain connecting photosystems II and I (15–17). It was suggested that the herbicides may interact with a target protein in the chloroplast membrane (18, 19); mild trypsin digestion of chloroplast membranes results in appearance of diuron-insensitive photosystem II activity (20). Low levels of diuron (≈1 diuron per 400 chlorophylls) have been found to block an acid-induced reversible decrease in midpoint potentials of cytochrome *b*-559 of chloroplast membranes. This may indicate that diuron binds directly to cytochrome 559, although binding of the herbicide to another protein in the photosystem II complex near the cytochrome cannot be ruled out (17). The use of radiolabeled herbicides in binding studies has led to the conclusion that the triazines and phenylureas interfere with the same electron carrier of the photosynthetic electron transport chain. The compounds bind to specific sites that exist at a concentration of approximately one site per photosynthetic electron transport chain (21).

Our data, which compare atrazine and diuron effects on fluorescence transients in susceptible chloroplasts, are in complete agreement with the above-mentioned theories of the identical mode of action of the two herbicides. Both herbicides were found to induce virtually identical rapid increases in the fluorescence rise, indicating an inhibition of electron flow at a point very near the photosystem II primary electron acceptor. In contrast, the data for the two herbicides in resistant plants were directly contradictory. Diuron continued to induce a rapid increase in fluorescence, indicating that the herbicide-binding site was present. Atrazine, however, had almost no effect on the fluorescence transient, indicating no effect of the herbicide on electron transport. The contradictory data can be resolved when it is assumed that the protein involved in herbicide binding is genetically altered in the resistant plants such that it has a lowered binding affinity for the triazines but a near normal binding affinity for diuron. Because the herbicide binding protein is thought to be a part of the photosystem II complex, such a change could alter the functional properties of this photosystem. Modified fluorescence increase characteristics were, in fact, observed in the resistant chloroplasts.

Alterations in Atrazine-Resistant Chloroplast Lamellae besides Herbicide Binding Properties. Comparisons of the rapid initial portion of the fluorescence increase in susceptible and resistant biotype chloroplasts showed a much higher "intermediate" (F_I) level in the resistant plastids. This implies a change in the rate of reoxidation of the primary photosystem II electron acceptor by the secondary electron pool. Because this is the electron transport step normally affected by the triazine or phenylurea herbicides (15–17), it seems possible that the alteration in electron transport processes could easily be related to a modification in a membrane component that normally binds the herbicide.

The data discussed above lead to the conclusion that a component (or components) of the photosystem II complex is altered in resistant chloroplasts. Polypeptide analysis of the chloroplast membranes did reveal that two proteins of molecular weights near 22,000 and 24,000 migrated slightly differently when the resistant and susceptible samples were compared by electrophoretic separation. We have observed that these proteins are absent from detergent-derived photosystem I submembrane fragments but are present in photosystem II-enriched preparations (data not shown). We emphasize, however, that the small change in apparent molecular weight of these polypeptides is only correlative with altered triazine herbicide activity; we need other research approaches to determine whether these proteins are actually involved in specific herbicide binding.

This study has presented evidence for a genetically controlled change in the target site for a herbicide. The fact that a change in photosystem II properties can be detected by nondestructive chlorophyll fluorescence analysis techniques opens new avenues for genetic or physiological study of this characteristic. Because specific herbicide resistance mechanisms are of great potential importance in crop production, further evaluation of this herbicide resistance mechanism is in order.

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