Plant Physiol. (1987) 85, 570–574
0032-0889/87/85/0570/05/$01.00/0

Increased Levels of Peroxisomal Active Oxygen-Related Enzymes in Copper-Tolerant Pea Plants[1]

Received for publication February 10, 1987 and in revised form July 2, 1987

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ABSTRACT

The effect *in vivo* of high nutrient levels of copper (240 micromolar) on the activity of different metalloenzymes containing Cu, Mn, Fe, and Zn, distributed in chloroplasts, peroxisomes, and mitochondria, was studied in leaves of two varieties of *Pisum sativum* L. plants with different sensitivity to copper. The metalloenzymes studied were: cytochrome *c* oxidase, Mn-superoxide dismutase (Mn-SOD) and Cu,Zn-superoxide dismutase I (Cu,Zn-SOD I), for mitochondria; catalase and Mn-SOD, for peroxisomes; and isozyme Cu,Zn-SOD II for chloroplasts. The activity of mitochondrial SOD isozymes (Mn-SOD and Cu,Zn-SOD I) was very similar in Cu-tolerant and Cu-sensitive plants, whereas cytochrome *c* oxidase was lower in Cu-sensitive plants. Chloroplastid Cu,Zn-SOD activity was the same in the two plant varieties. In contrast, the peroxisomal Mn-SOD activity was considerably higher in Cu-tolerant than in Cu-sensitive plants, and the activity of catalase was also increased in peroxisomes of Cu-tolerant plants. The higher activities of these peroxisomal active oxygen-related enzymes in Cu-tolerant plants suggest the involvement of reactive oxygen intermediates (O_2^-, $OH^\cdot$) in the mechanism of Cu lethality, and also imply a function for peroxisomal Mn-SOD in the molecular mechanisms of plant tolerance to Cu in *Pisum sativum* L.

Different molecular mechanisms have been proposed to explain the tolerance of certain higher-plant species to high concentrations of heavy metals which are toxic for most plants. These mechanisms mainly imply modifications of membrane and cell wall properties, compartmentation of metals in vacuoles, immobilization of metals in cell walls, and adaptive mechanisms of metabolic and enzymic nature (12, 30). In addition, inducible metallothionein or metallothionein-like complexes have also been postulated to be involved in the mechanism of tolerance of certain plants to copper (12, 18) and cadmium (28).

Although the primary toxic action of copper ions on plant growth apparently takes place in roots, by means of alterations in cell membrane properties (30), high levels of this metal can also affect the metabolism of plant leaves. The effect of excess Cu on the photosynthetic apparatus of spinach plants was studied by Baszyński *et al.* (3), and Lolkema and Vooijs (13) have investigated the effect of high copper concentrations on plastocyanin synthesis in *Silene cucubalus* leaves. Moreover, *in vitro* experiments on the phytotoxic action of copper on the photosynthetic electron transport system of isolated chloroplasts have also been conducted (20, 26).

However, there is a paucity of information on the activity response of metalloenzymes present in different cell compartments of plant leaves to an enhanced pool of nutrient copper. Comparative studies of the activity of metalloenzymes in different cell organelles of copper tolerant and nontolerant plants grown in high Cu nutrient levels would allow deeper insights into the molecular mechanisms of intracellular responses to plant toxicity and metal tolerance. These types of studies may also give information on possible alterations of metalloenzyme characteristics in metal-tolerant plants and on the adaptive nature of enzymes to metal stress in certain plants.

The induction of a Mn-SOD[3] in leaves of pea plants by high nutrient levels of zinc and manganese has been recently demonstrated (7), as well as the induction of Fe-superoxide dismutases in lemon leaves by iron (25). This suggested a possible involvement of the metalloenzyme family of SODs in the mechanism of plant tolerance to metal toxicity. SODs (EC 1.15.1.1) are a group of metalloenzymes that catalyze the disproportionation of superoxide free radicals (O_2^-), produced in certain cellular loci, and appear to play an important role in protecting cells against the indirect lethal effect of O_2^- radicals (17). There are essentially three types of SODs containing either Mn, Fe, or Cu plus Zn as metal prosthetic groups (17).

Leaves of pea plants contain three electrophoretically distinct SODs, a Mn-containing and two Cu,Zn-containing isozymes, named I and II in order of increasing mobility (7). The isozyme Mn-SOD has been fully characterized (24), as well as the two pea leaf Cu,Zn-SODs (9). By studies of subcellular distribution of SODs in pea leaves the presence of Mn-SOD has been demonstrated both in mitochondria and peroxisomes (6, 19) whereas isozyme Cu,Zn-SOD II was located in chloroplasts (15), and Cu,Zn-SOD I appeared to be distributed between mitochondria and the cytosol (19). This location of SOD isozymes in different cell compartments makes this metalloenzyme system a valuable tool for probing plant metal tolerance at the subcellular level, and this is particularly interesting in the case of copper considering that high concentrations of this metal have been reported to induce the generation of O_2^- radicals, the substrate of SODs, in isolated chloroplasts (20).

In this work, using two varieties of pea plants with different sensitivity to copper, the effect *in vivo* of high nutrient levels of copper on the activity of metalloenzymes from chloroplasts (Cu,Zn-SOD II), mitochondria (Mn-SOD, Cu,Zn-SOD I, and Cyt *c* oxidase) and peroxisomes (catalase and Mn-SOD) is studied.

[1] Supported by grant 603/275 from the CAICYT-CSIC (Spain).

[2] Recipient of a research fellowship from the Caja General de Ahorros y Monte de Piedad de Granada and CSIC.

[3] Abbreviations: Mn-SOD, manganese-containing superoxide dismutase; NBT, nitroblue tetrazolium; SOD, superoxide dismutase; Cu,Zn-SOD, copper-zinc-containing superoxide dismutase.

MATERIALS AND METHODS

Plant Material. Different commercial varieties of peas (*Pisum sativum* L.), obtained from Royal Sluis (Enkhuizen, Holland), were grown in optimum nutrient solutions (7) containing different concentrations of $CuSO_4$ in order to study their susceptibility to this metal. Plants were first grown under aeration in a growth chamber, Conviron PGW-36, under optimum conditions (7) for 7 d, and then they were transplanted to similar media supplemented with different Cu(II) concentrations (0.8–320 μM) and grown for 14 d. The content of micronutrients in nutrient media was determined by atomic absorption spectrometry. Plants were harvested and plant growth was determined by measuring fresh weight of leaves, fresh weight of shoots, foliar area (in cm^2), dry weight, and fresh weight of roots.

Purification of Cell Organelles. In pea leaves of the selected varieties, chloroplasts, peroxisomes, and mitochondria were isolated essentially by the method described by Schwitzguébel and Siegenthaler (23). All operations were performed at 0 to 4°C. In leaves of each plant variety, two independent procedures were followed for the purification of chloroplasts, and mitochondria plus peroxisomes, respectively. Highly purified intact chloroplasts were obtained by differential and Percoll density-gradient centrifugation (21–60%; v/v) as described earlier (15). Percoll was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Intact peroxisomes and mitochondria were isolated by differential centrifugation followed by centrifugation in discontinuous density-gradients of Percoll (15–53%; v/v) (19). After centrifugation, the two separate gradients (one for chloroplasts, and another for peroxisomes plus mitochondria) were fractionated by upward displacement with 45% sucrose (w/w) using an Isco fractionator model 185 equipped with an optical unit and an absorbance detector. The transmittance of eluted samples was monitored at 280 nm and enzyme activities were measured in the different fractions collected (1.5 ml).

Assays. Catalase (EC 1.11.1.6), Cyt *c* oxidase (EC 1.9.3.1), and Chl were used as markers for peroxisomes, mitochondria, and chloroplasts, respectively. Catalase was determined according to Aebi (1), and Cyt *c* oxidase was assayed as described by Schnarrenberger *et al.* (22). Chl and proteins were determined according to Arnon (2) and Bradford (4), respectively. Measurements of the density of the Percoll solutions were carried out at room temperature using an Atago refractometer.

In fractions from Percoll density-gradient centrifugation, the SOD activity determinations were carried out quickly and samples were appropriately diluted in order to reverse the Percoll inhibitory action on SODs (8). Total SOD activity was determined by the ferricytochrome *c* method using xanthine/xanthine oxidase as the source of superoxide radicals (14). SOD isozymes were separated by polyacrylamide disc gel electrophoresis on 10% gels at pH 8.9 according to Davis (5). Prior to electrophoresis, organelle samples were about 2- to 5-fold diluted with 50 mM phosphate buffer (pH 7.8) containing Triton X-100 (0.1% final concentration) to solubilize bound superoxide dismutases. SODs were localized on the gels by the method of the NBT reduction by O_2^- radicals generated photochemically (29). The isozyme activity was quantitated by recording the transmittance of the gels in a Vernon PHI-6 densitometer and calculating the areas under the transmittance peaks. Metal concentrations of intact leaves were estimated by atomic absorption spectrometry with a Perkin-Elmer model 503.

RESULTS

The range of copper nutrient concentrations used in preliminary experiments (0.8–320 μM Cu^{2+}) allowed discrimination between a *Pisum sativum* L. variety tolerant to high metal concentrations (var Lincoln), and another variety relatively sensitive to copper (var Granada). The effect of different Cu nutrient concentrations on the growth of the two *Pisum sativum* varieties, expressed as fresh weight of leaves, is shown in Figure 1, and similar results were obtained when plant growth was expressed either as dry weight, foliar area, fresh weight of shoots, or fresh weight of roots. On the basis of these results, we decided to investigate the effect of 240 μM Cu(II) nutrient concentrations, which strongly inhibited plant growth in Cu-sensitive plants, on the activity of different metalloenzymes containing Cu, Mn, Fe, and Zn, distributed in chloroplasts, peroxisomes, and mitochondria of both *Pisum sativum* varieties. In preliminary experiments, Cu-tolerant and Cu-sensitive pea plants were grown under normal copper nutrition (1 μM Cu^{2+}) (7), and it was found that the activity of metalloenzymes from chloroplasts (Cu,Zn-SOD II), mitochondria (Mn-SOD, Cu,Zn-SOD I, and cytochrome *c* oxidase) and peroxisomes (catalase and Mn-SOD) were very similar in both plant varieties (results not shown).

A purification procedure of peroxisomes and mitochondria from Cu-tolerant and Cu-sensitive plant leaves, representative of different independent experiments carried out, is shown in Figure 2. Peroxisomes were located in the Percoll density-gradients in fractions 16 to 20, at an average equilibrium density of about 1.092 g/cm^3, characteristic of these intact organelles in Percoll solutions (19, 23). Purified peroxisomes were well separated from mitochondria which, as shown by the Cyt *c* oxidase activity, banded in fractions 8 to 12 at an average density of about 1.069 g/cm^3, nearly identical to that density reported for mitochondria isolated from spinach and pea leaves using the same method (19, 23). Mitochondria were sometimes contaminated with peroxisomal activity probably derived from partially broken peroxisomes co-eluting with the mitochondrial fraction (21). The major part of chloroplasts was removed in the first differential centrifugation step of the method used (19) and the remaining broken chloroplasts banded on top of the gradient.

In general, in the Cu-sensitive variety the intracellular metalloenzymic activities were lower than in Cu-tolerant plants. The total SOD activity in peroxisomes (Mn-SOD) was considerably higher in Cu-tolerant than in Cu-sensitive plants, and the activity of catalase was also increased in peroxisomes of Cu-tolerant plants, though not as dramatically as in the case of Mn-SOD. In mitochondria, the activity of Cyt *c* oxidase was also lower in Cu-sensitive plants compared to Cu-tolerant plants, whereas the total SOD activity of those organelles was of the same order in both plant varieties.

The purification of chloroplasts from Cu-tolerant and Cu-sensitive plant leaves is shown in Figure 3 and is representative

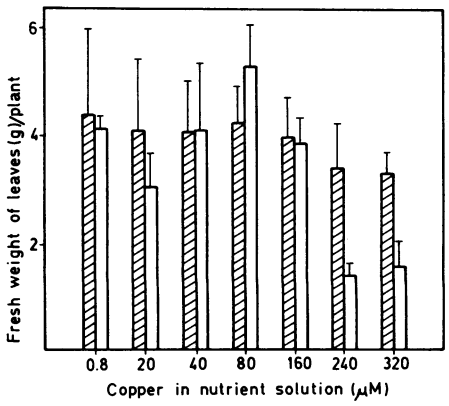

FIG. 1. Plant growth of a sensitive (open columns) and a copper-tolerant (hatched columns) variety of *P. sativum* L. expressed as fresh weight of leaves after a growth period of 14 d on a range of Cu-concentrations in the nutrient solution. Columns represent the average of five plants ± SE.

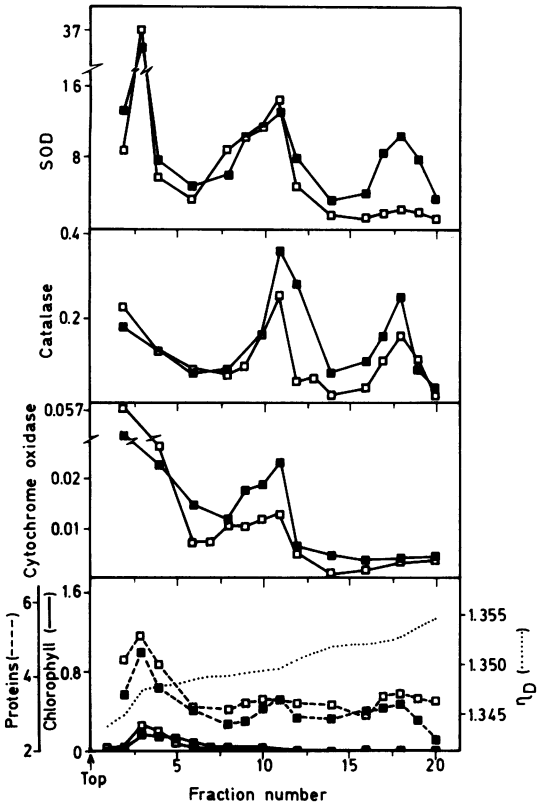

FIG. 2. Purification of peroxisomes and mitochondria from leaves of Cu-tolerant and Cu-sensitive *P. sativum* L. plants. Plants were grown in nutrient solutions containing 240 μM Cu^{2+} and leaf organelles were isolated by differential and Percoll density-gradient centrifugation (19). SOD activity is expressed as unit/ml (14) and the activity of catalase and Cyt *c* oxidase is given as μmol × min^{-1} × ml^{-1}. Proteins and Chl are expressed as mg × ml^{-1}. (■), Cu-tolerant plants; (□), Cu-sensitive plants.

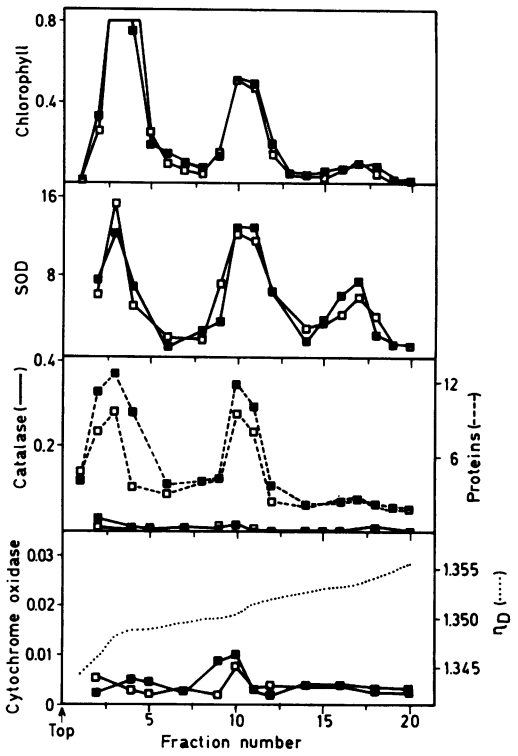

FIG. 3. Purification of chloroplasts from leaves of Cu-tolerant and Cu-sensitive *P. sativum* L. plants. Leaves from the same plants described in Figure 2 for the isolation of peroxisomes and mitochondria were used. Chloroplasts were isolated by differential and Percoll density-gradient centrifugation (15). Enzymic activities, and protein and Chl contents were expressed as described in Figure 2. (■), Cu-tolerant plants; (□), Cu-sensitive plants.

of different independent isolation experiments carried out. By the method used, two highly intact chloroplast bands were obtained (fractions 9–12 and 15–18) with percentages of intactness in the range 78 to 85%, the higher-density chloroplast band (1.094 g/cm^3) being rather pure whereas the other band was slightly cross-contaminated with mitochondria (15). In *Pisum sativum* plants tolerant and sensitive to copper, the Chl contents did not show variations, and these were only very slight in the case of the SOD activity of chloroplasts (isozyme Cu,Zn-SOD II). The SODs present in mitochondria were separated by polyacrylamide gel electrophoresis and quantitated by densitometry, all of the fractions defining the mitochondrial peak being analyzed. The isozyme activities of Mn-SOD and Cu,Zn-SOD I in mitochondria, and of Mn-SOD in peroxisomes are shown in Figure 4. The activity of mitochondrial SODs, in contrast to peroxisomal Mn-SOD, were very similar in Cu-tolerant and Cu-sensitive plants. The concentration of metals in leaves of pea plants sensitive and tolerant to copper is represented in Table I. When plants were grown under normal copper nutrition, the metal concentrations in leaves from Cu-tolerant and Cu-sensitive plants were very similar except for iron (difference significant at $P < 0.01$). However, the growing in nutrient solutions containing high copper levels showed that metal concentrations were bigger in Cu-tolerant plants than in Cu-sensitive ones, and the differences found were statistically significant for Cu ($P < 0.001$), Mn ($P < 0.001$), Fe ($P < 0.01$), and Zn ($P < 0.05$). Attempts were made to determine the compartmentation of metals in cell organelles from pea plants sensitive and tolerant to Cu, by measuring the metal concentrations in the Percoll gradient frac-

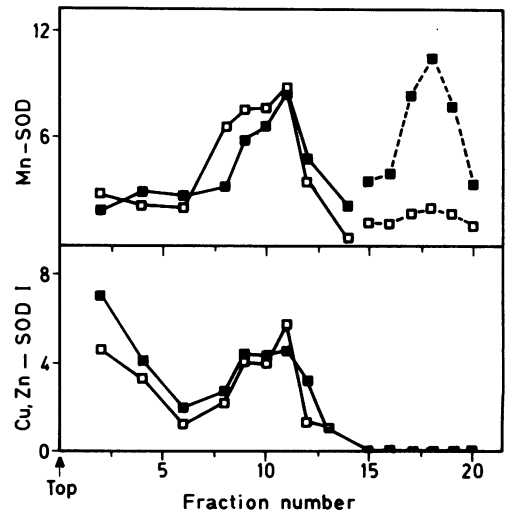

FIG. 4. Activity of individual superoxide dismutase isoenzymes in mitochondria and peroxisomes from leaves of Cu-tolerant and Cu-sensitive pea plants. Organelles were purified as described in Figure 2 and SODs were separated by polyacrylamide disc gel electrophoresis followed by activity staining. Gels were scanned with a densitometer and the percentage of each isozyme was calculated by integration of the SOD activity areas under the transmittance peaks. Isozyme activities were obtained by multiplying each percentage by the total SOD activity of fractions. (■), Cu-tolerant plants; (□), Cu-sensitive plants. Peroxisomal Mn-SOD is represented by the discontinuous line.

Table I. *Metal Concentration in Leaves from Cu-Tolerant (cv Lincoln) and Cu-Sensitive (cv Granada) Pea Plants grown in Nutrient Solutions Containing Normal (about 1 μM) and High (240 μM) Copper Concentrations*

Metal concentrations were estimated by atomic absorption spectrometry. Each value represents mean ± SE of three samples.

Plant	Cu supply	Fe	Cu	Mn	Zn
	μM		μg/g dry matter		
Tolerant	1	134 ± 8.8	12.2 ± 1.3	72.4 ± 6.9	40.7 ± 4.7
	240	80.5 ± 4.2	72.8 ± 2.3	122 ± 11.9	22.4 ± 2.2
Sensitive	1	115 ± 7.7	10.3 ± 0.8	71.3 ± 3.3	36.4 ± 4.3
	240	61.4 ± 6.2	47.1 ± 5.3	62.9 ± 6.1	17.2 ± 0.9

tions. However, this approach had to be abandoned due to the high concentration of metals that were detected in the commercial Percoll reagent ordinarily used for the preparation of density-gradients. These metal contaminants, mainly Cu, Fe, Mn, and Zn, could not be removed by treatment of Percoll with Chelex 100 and gave very high backgrounds, precluding the determination of the low metal concentrations commonly present in cell organelles.

DISCUSSION

The results reported here show that in leaves of *Pisum sativum* L. plants sensitive to copper, several intracellular metalloenzymic activities studied are, in general, lower than those present in Cu-tolerant plants. The only metalloenzymes that did not show significant variations in both plant populations were isozymes Cu,Zn-SOD I and Cu,Zn-SOD II which are located in mitochondria and chloroplasts, respectively. In these two cellular organelles the production of superoxide free radicals has been clearly demonstrated (11, 17), and the induction of SOD isozymes in response to enhanced rates of intracellular O_2^- production has been described in different organisms (11, 17).

The fact that the activity levels of mitochondrial and chloroplastid Cu,Zn-SODs reported here are very similar to those of pea plants grown under optimum copper nutrition suggests that in the two varieties studied Cu toxic levels apparently do not bring about O_2^--derived induction of SOD isozymes. This assumption would agree with other *in vivo* experiments with spinach plants that showed a lack of copper toxicity on the photosynthetic activity of chloroplasts of Cu-treated plants (3), in contrast to isolated spinach chloroplasts where Cu-mediated lipid peroxidation of photosynthetic membranes by O_2^--derived $OH^\cdot$ free radicals has been demonstrated (20). On the other hand, Lolkema and Vooijs (13) have shown that copper does not accumulate *in vivo* in the chloroplasts of either sensitive or tolerant plants of *Silene cucubalus*, and no difference in plastocyanin content was detected between tolerant and sensitive plants as a result of high copper concentrations.

The higher activities of the peroxisomal enzymes (Mn-SOD and catalase) found in Cu-tolerant plants, suggest that peroxisomes, and active O_2-related enzymes in particular, might have a certain role in the molecular mechanisms responsible for the plant tolerance to Cu toxicity. In recent experiments conducted in our laboratory, we have demonstrated the production of superoxide free radicals (O_2^-) in isolated peroxisomes by certain endogenous metabolites of peroxisomes (LM Sandalio, unpublished results). On the other hand, an increase in the peroxisomal concentration of copper, could under appropriate conditions, originate the generation of the vastly reactive OH radicals by reaction of O_2^- with H_2O_2 through a metal-catalyzed Haber-Weiss reaction (11). Therefore, Cu-tolerant plants could have evolved a protection mechanism against the production in peroxisomes of O_2^--dependent toxic species by high levels of copper by inducing the peroxisomal Mn-SOD and catalase activities. In this way, O_2^- radicals and H_2O_2 could be effectively removed, and the eventual formation of $OH^\cdot$ radicals, highly toxic for biological membranes, avoided.

Inducible metallothioneins or metallothionein-like complexes have been proposed to play a role in detoxification of copper in different Cu-tolerant plants (12, 18). In fact, these low mol wt metalloproteins have important features in common with the metalloenzyme family of superoxide dismutases. Metallothioneins are able to scavenge free hydroxyl ($OH^\cdot$) and superoxide (O_2^-) radicals thus showing mimic SOD activity (27), and this property suggests that metallothioneins could have a certain role in cellular metabolism as secondary protective agents against the eventual Cu-derived production of O_2 free radicals. On the other hand, superoxide dismutases have been demonstrated in recent years to be metal-inducible, similar to animal and plant metallothioneins. In bacteria, the induction of SOD isozymes by Fe and Mn salts has been described (16), and Cu also induced a Cu,Zn-SOD isozyme in yeast (10). In higher plants, the induction of different SOD isozymes by Fe(II) has been reported in *Citrus limonum* R. (25) and a Mn-containing SOD was found to be induced in *Pisum sativum* L. by high nutrient levels of Zn and Mn (7).

The results described in this work indicate a role for peroxisomes in plant cellular metabolism related to Cu toxicity and imply the involvement of active O_2 species, possibly generated in these oxidative organelles, in the mechanism of Cu lethality. Though further experiments are still necessary, superoxide dismutases appear to have a certain role in the molecular mechanism of plant tolerance to Cu in *Pisum sativum*, possibly participating in conjunction with metallothioneins, another family of metalloproteins with whom they share important functional properties.

Acknowledgments—The authors wish to thank Miss M. D. Mirasol for her technical assistance, and Mr. M. Martínez for drawing the figures.

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