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Effects of Some Heavy Metals(Al, Cd, Hg, and Pb) on ATP Content in Plant Leaves*

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植物葉의 ATP含量에 미치는 重金屬(Al, Cd, Hg 및 Pb)의 影響

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ABSTRACT

The present study was carried out to estimate the comparative effects of hydroponic heavy metals (Al, Cd, Hg, and Pb) on ATP content in plant leaves grown with Hoagland solution under green house condition. The two plants, kidneybean (*Phaseolus vulgaris* L.) and buckwheat (*Fagopyrum esculentum* Mönch), showed similar inhibitory effect of heavy metals on ATP content in order of Hg, Cd, Pb, and Al. But the overall inhibitory effect was greater in kidneybean than in buckwheat.

The affinity of heavy metals, *in vitro*, toward the enzyme (luciferin-luciferase) is in order of Hg, Al, Cd, and Pb, similar to that toward ATP. The results showed that the inhibitory effect of heavy metals on ATP hydrolysis is mainly due to the coordination of heavy metals with enzyme than ATP.

INTRODUCTION

Several reports have been published on the study of toxicity of heavy metals to selected plant species in respect of growth inhibition, heavy metal content, and induced symptoms. Recently some studies were undertaken to characterize the correlation of aluminum content with ATP content or ATPase activity (Clarkson, 1965, 1966b; Wright, 1953). It has been generally accepted that some metals (*e.g.*, Ag, Cd, Cu, Hg, Li, Pb, Zn, etc.) inactivate certain essential functional groups, especially thiol group of enzymes (Frobisher, 1968), and coordinate with the ATP, because of the high affinity of the phosphate groups for binding divalent cations (Lehninger, 1970).

The purpose of present study was mainly to estimate the comparative effects of hydroponic heavy metals on ATP content in plant leaves. We also carried out other experiments to determine the inactivating effect of heavy metals on chemical ATP, leaf extracted ATP and luciferase, an ATP hydrolysis enzyme.

MATERIALS AND METHODS

Plant Materials The kidneybean(*Phaseolus vulgaris* L.) and buckwheat (*Fagopyrum esculentum* Mönch) seeds were germinated and the seedlings were grown in sand for 15 days by irrigating with Hoagland solution daily. The seedling roots were

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washed with distilled water and 4 to 5 seedlings were subsequently transplanted to each of 500ml flasks containing 450ml Hoagland solution of various heavy metal concentrations. The hydroponic heavy metal concentrations of 0, 0.1, 1, 10, 100 and 1000 ppm for each of Al, Pb, Hg and Cd treatments were prepared from $AlCl_3$, $PbNO_3$, $HgCl_2$ and $CdCl_2$, in Hoagland solution, respectively. The flasks were wrapped around with aluminum foil and immersed in water-bath filled with overflowing tap water. During the hydroponic culture, vigorously aerated hydroponic solutions were changed every five days. The leaves of seedlings were harvested after they had grown 15 days in each heavy metal solution. The harvested leaves were stored immediatly in refregerator and usually used within two days of excision. The experimental plants were grown during June and July normally in green house condition.

ATP Content Measurement Fresh leaves (3g) were boiled with 30ml tris buffer of pH 7.4 for three minutes to kill the cells rapidly and inactivate the ATPase contained in the leaf cells themselves. The boiled leaf solution was replenished for evaporated moiety to 30ml by adding some precooled tris buffer solution and thereafter transferred to ice-jacketed homogenizer vessel. After homogenizing at 4500rpm, at 0°C, for 10 minutes, the homogenate was filtered through two layers of gauge. The ATP content of filtrate was determined by using a SAI Technology Co. 2000 ATP-Photometer which measures the fluorescence of 560–580mμ wavelength emitted from the reaction cuvette by hydrolysis of ATP contained in reaction mixture. The reaction mixture was prepared by adding 0.5ml enzyme solution to the reaction cuvette containing 0.5ml of sample filtrate. After mixing the components vigorously for three seconds, the reaction cuvette was immediately settled in the chamber of ATP-Photometer to measure the fluorescence. The counting was started within 10 seconds after the enzyme was added to the reaction cuvette. The ATP content of filtrate was computed from the peak reading of ATP-Photometer by referring it to the standard curve graphed with chemical ATP. All the manipulations after boiling were conducted at 0°C.

Enzyme Preparation To determine the inhibitory effects of heavy metals on ATP hydrolysis, the luciferin-luciferase, a kind of ATPase, was used. The luciferin-luciferase was from a crude extract of 50mg of firefly lanterns premixed with buffer and desiccated. The enzyme solution was prepared for use by adding 15ml tris buffer solution of pH 7.4 to the firefly extract, storing overnight to reduce the background, filtering prior to use. All enzymes and chemicals used were products of Sigma Chemical Co.

Interactions of Heavy Metals with Extracted ATP or Chemical ATP The mixture for assay was prepared by adding to heavy metal solutions of various concentrations in photometer cuvette, either 0.5ml of leaf extracted ATP or 0.5ml of 10^{-4}ppm chemical ATP solution. After shaking vigorously the cuvette for one minute, 0.5ml enzyme solution was added to it. The one minute delay was allowed for interactions of heavy metal with ATP to inhibit ATP hydrolysis if it could be happened. The reaction mixture with a final volume of 1.5ml was used to determine the ATP hydrolysis. The counting was started within 10 seconds after the enzyme was added.

Interactions of Heavy Metals with Luciferase Enzyme solution (0.5ml) was mixed with 0.5ml heavy metal solution of various concentrations. After shaking the mixture for one minute, 0.5ml of 10^{-4}ppm ATP solution was added to it. Measuring of fluorescence emitted by ATP hydrolysis was as mentioned above.

RESULTS AND DISCUSSION

Inhibitory Effect of Heavy Metals on ATP Content The ATP content of kidneybean and buckwheat leaves pretreated with various hydroponic heavy metal concentrations for 15 days were graphed (Fig. 1). The ATP concentration in kidneybean and buckwheat leaves, treated with 100 ppm Al, were increased by 72 and 6%, respectively, over the controls. These results corresponded to the

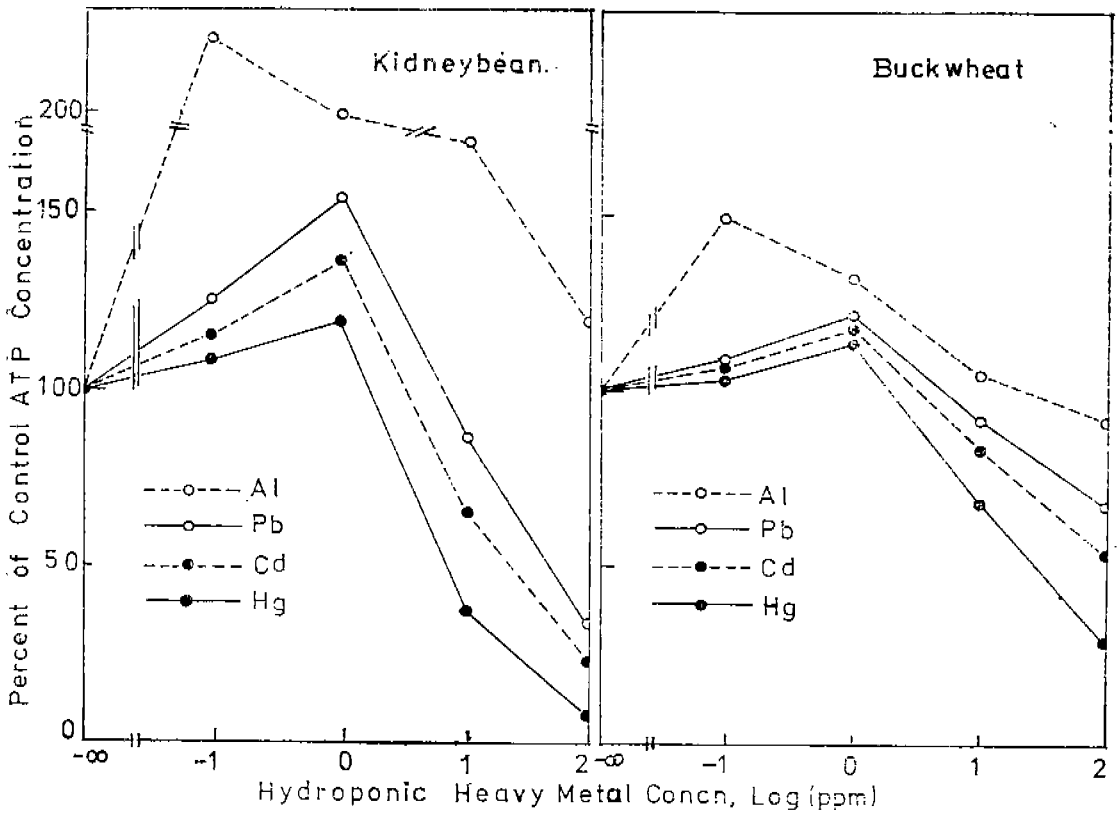

Fig. 1. Effect of the heavy metals on ATP content in the leaves of kidneybean and buckwheat.

previous reports (Clarkson, 1965, 1966b; Wright, 1943). They suggested that the effect of Al on phosphate metabolism was, at least in the first stage, the inhibition of usage of ATP in hexose-phosphorylation.

There are a number of reports on Al tolerance and Al toxicity related to Ca or P-deficiency. Long *et al.* (1973) showed large difference between genotypes in their tolerance to high levels of Al in solution. A pearl millet cultivar was not affected by Al levels up to 16 ppm, while an oat cultivar was considerably affected by only 1 ppm Al in the nutrient solution. But in general it appeared that low levels of 3 to 10 ppm Al in solution stimulated rice root growth, whereas at higher levels of Al root growth was markedly reduced in all cultivars (Howlder and Cadavid, 1976). Edwards and Horton (1977) reported that, as Al concentrations increased, the uptake rates for Ca and P decreased but that for K increased, and Al toxicity in peaches may be related to a reduction of Ca uptake. Other reports also suggested that the Al toxicity was the same as P-deficiency (Clarkson, 1967; Foy and Brown, 1963) and Ca-deficiency (Foy *et al.*, 1969). Evidently, Al binds P on the surface of plant roots (Clarkson, 1965, 1966a, 1966b). These results indicate that less P is available for metabolic activities within the cells. However Al tolerant cultivars had higher levels of P and Ca and lower levels of Al in the shoots than the susceptible ones(Howlder and Cadavid, 1976).

From the results of present experiments, it could be concluded that the increased ATP content by Al treatment even up to 100 ppm level might be associated with an inhibitory effect of Al on acid phosphatase and ATPase, resulting inhibition of usage of ATP in sugar phosphorylation. Although the Ca and P-deficiency induced by Al treatment could not be left out of consideration, in view of the fact that Ca is a cofactor of some enzyme in-

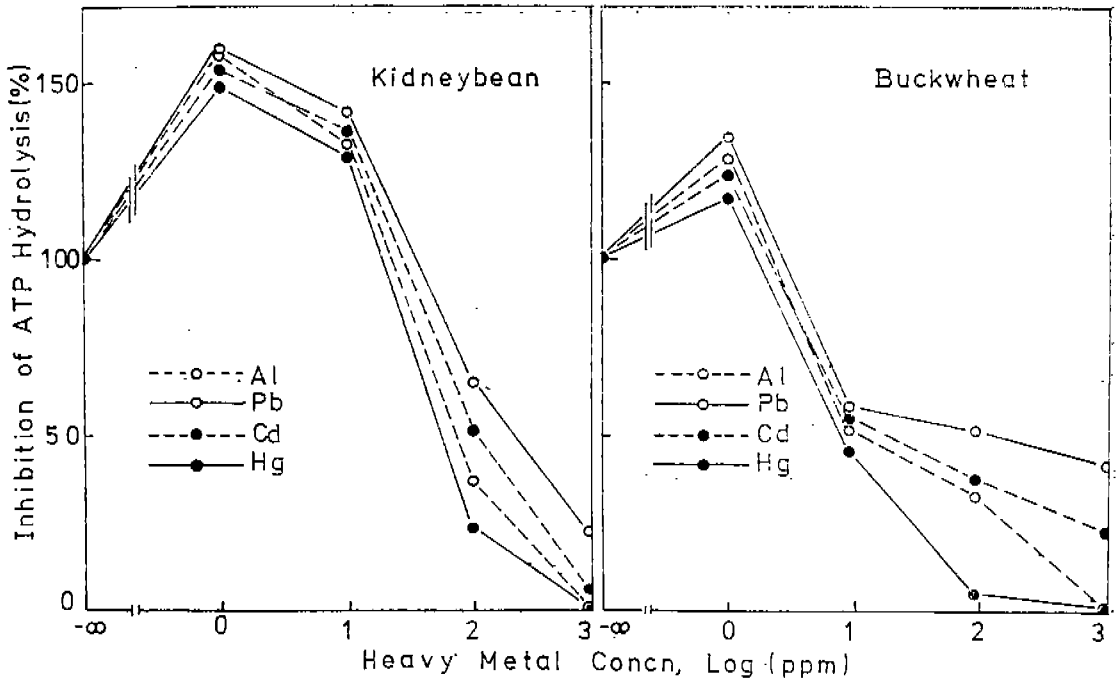

Fig. 2. The inacitvation of extracted-ATP by the heavy metals. The extracted-ATP was prepared from leaf extracts of untreated control plants and mixed with each heavy metal of various concentrations. One minute later, luciferase was added to determine the ATP hydrolysis level of the final mixture as described in the "Methods."

volved in ATP hyrolysis (Noggle and Fritz, 1976), the Ca-deficiency could be, at least partly, consistent with the increase of ATP content in leaves.

Pb, Cd and Hg treatment with each 100 ppm reduced the ATP content in kidneybean leaves to 87, 65 and 34% and in buckwheat leaves to 93, 84 and 68% of control, respectively. The inhibition effect of each heavy metal on ATP content was similar to the tendency of that on respiration, reported in the previous study (Sung, 1979). Relatively, few reports characterized the effect of hydroponic heavy metal concentrations on ATP content of the plant leaves. Keck(1978) reported that the ATP levels in oat root segments, treated with 1 mM $CdSO_4$ in aerated Hoagland solution, declined to 75 % of control in 2hr and to 20% of control levels in 24hr. These decrease in ATP levels in 2hr is similar to the decrease in respiration as measured by CO_2 evolution. The published reports (Jacobs *et al.*, 1959; Mustafa and Cross, 1971) suggested that Cd^{2+} uncouples oxidative phosphorylation and blocks electron transports which are assumed to be closely related with ATP production. The previous work with *Chlamydomonas* suggested that it might be a possible factor to reduce the ATP content by lead that the lead salts added to the culture media cause precipitaiton of $Pb_3(PO_4)_2$, effectively removing phosphate from solution (Schulze and Brand, 1978). They demonstrated that cells could not survive when the amount of Pb^{2+} in the culture solution exceeded the equivalence of phosphate.

From the results of present experiments and the previous reports, it may be concluded that the effect of heavy metals on ATP of the leaves are, at least partly, due to the inactivation of enzyme essential for ATP production by heavy metals. In the comparison, the two plants were similar to each other in respect of inhibitory effects of heavy metals on ATP content in order of Hg, Cd, Pb and Al. But the overall inhibitory effect was greater in kidneybean than in buckwheat. It suggests that

heavy metal tolerance is greater in buckwheat than in kidneybean.

Interaction of Heavy Metals with Chemical ATP or Leaf Extracted ATP From the results of measuring the extent of ATP inactivation by ATP-heavy metal complex formation, the interaction of each heavy metal with leaf extracted ATP (Fig. 2) and it with chemical ATP (Fig. 3) could be compared.

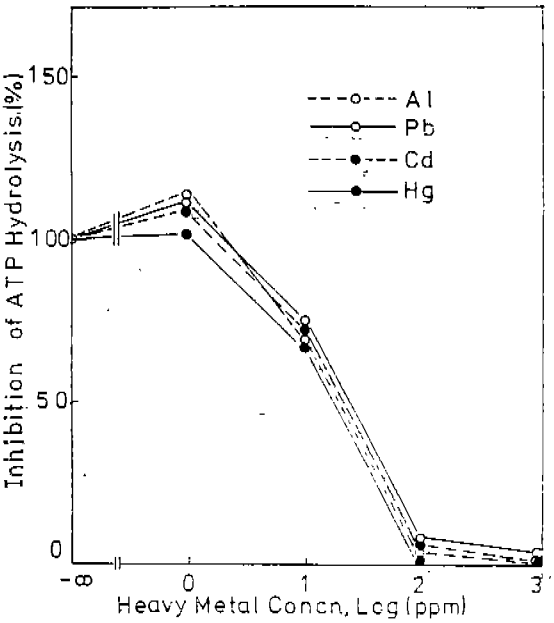

Fig.3. ATP inactivation by the heavy metals. Chemical-ATP was mixed with each heavy metal of various concentrations. The next procedure was the same as described in the ligend for Fig. 2.

Leaf extracted ATP treated with 100 ppm Pb, Cd, Al and Hg was inactivated in buckwheat to 52, 37, 32 and 5%, and in kidneybean to 62, 53, 38 and 22% levels of control values, respectively. In either case, the inactivating effect of each heavy metal on extracted ATP was shown in order of Hg, Al, Cd and Pb. In the case of interactions between each heavy metal and chemical ATP (Fig. 3), the inactivating effects of each heavy metal on ATP level were in order of Hg, Al, Cd and Pb, *i.e.*, 97.6, 97.2, 95 and 91.5%, a tendency similar to that on extracted ATP. In any case, the ATP inactivating effects of the heavy metals showed the same tendency with each other, although the overall inactivating effect was higher on the chemical ATP than on extracted ATP. It may be inferable from the results that there are some quantity of ligands besides ATP in the leaf extracts which reduce the free heavy metal concentrations in the reaction mixture, and therefore mitigate the ATP inactivation.

In this experiment, it seemed that the inhibition of ATP hydrolysis might be due to not only ATP-heavy metal complex but also enzyme-heavy metal complex. Especially, if the amount of heavy metal added to ATP solution exceeds the molecular equivalancy of ATP as the condition of present experiment with over 100 ppm heavy metal, it was assumed that there is good degree of possibility in interaction of heavy metal with enzyme, although we could not measure it quantitatively in this experiment. It is also noticeable that Al exhibited higher inactivating level, although in the case of hydroponic treatment it increased the ATP content in plant leaves. As the conclusion, the order of each heavy metal effect on ATP inactivation *in vitro* condition is the same as that in the hydroponic condition as Hg, Cd and Pb, except Al which is the only trivalent cation used in present experiment.

Interaction of Heavy Metals with Enzyme The inhibition of enzymatic activities by the heavy metals were determined (Fig. 4).

Metals, particulary Hg, Cu and Ag are general enzyme inhibitors. Spalding(1979) reported that concentrations of Hg or Cu of 25 ppm, added to the litter extracts, were adequate to inhibit most enzymatic activities. Venugopal and Luckey(1978) demonstrated that Pb can form the stable complex with free thio-, carboxylate- and phosphate- carrying ligands of biopolymers although the existence of lead chelates in biological fluid is not confirmed. Mustafa and Cross(1971) demonstrated that Cd inhibits plasma membrane and mitochondrial ATPases isolated from animal cells. Keck(1978) showed that the segments of oat roots which had been exposed to 1 mM $CdSO_4$ solution exhibited decreased respiratory rates, ATP levels, membrane-bound ATPase activity, and reduced K^+ fluxes.

From those results, he suggested that the primary site of Cd^{2+}-root interaction is the K^+ carrier(ATPase), bound to the membrane and uses ATP as an energy source. Cd was also known to be either inhibitor or activator of enzyme such as nitrate reductase or the ion stimulated ATPase (Lee *et al.*, 1976; Maths, 1975; Valles and Ulmer, 1972), both of which are believed to be closely associated with ion uptake (Hodges, 1973; Neyra and Hageman, 1975). According to the present experiment, the affinity of heavy metals toward the enzyme is in

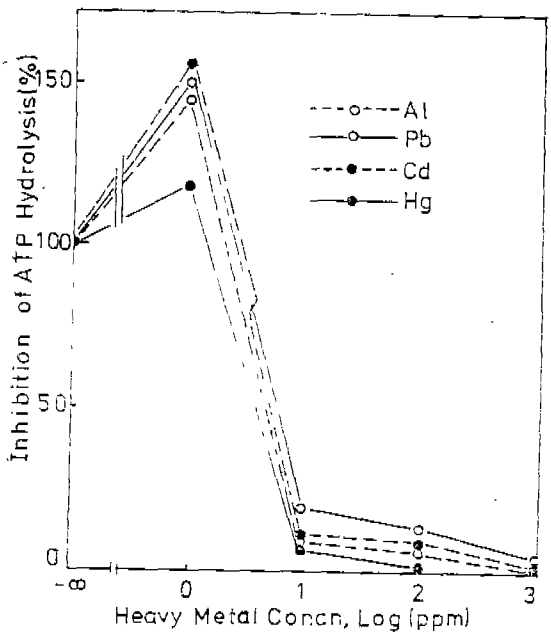

Fig. 4. Enzyme inactivation by the heavy metals. Luciferase was mixed with each heavy metal of various concentrations. One minute later, chemical-ATP was added to determine the ATP hydrolysis level as described in the "Methods."

order of Hg, Al, Cd and Pb, similar to the affinity order of heavy metals toward ATP. However the heavy metals inactivated more markedly the enzyme than ATP as shown at 10 ppm of each metal concentrations.

In the case of the interactions of heavy metals with ATP or enzyme, the heavy metal concentrations of lower than 1 ppm exhibited enhancing effects and Hg always exhibited the greatest affinity to ATP and enzyme. Venugopal and Luckey(1978) also reported that among the metals, Hg has the greatest affinity toward thio groups and the affinity of Hg toward reactive group is in order of SH, $CONH_2$, NH_2, COOH and PO_4. In general, it may be concluded that the inhibitory effect of heavy metals on ATP hydrolysis is mainly due to the inactivation of heavy metals with enzyme than ATP.

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摘 要

강낭콩(*Phaseolus vulgaris* L.) 및 메밀(*Fagopyrum esculentum* Mönch)의 種子를 15日間 Hoagland solution으로 砂耕栽培하였다. 各 重金屬(Al, Cd, Hg 및 Pb)의 濃度를 Hoagland solution으로 0~1000 ppm 범위에서 6個 濃度區로 만들고 이것을 500 m*l* 삼각 후라스크에 넣어, 砂耕栽培한 植物을 여기에 移植하여 15日間 水耕栽培하였다. 植物葉의 ATP含量에 미치는 各 重金屬의 影響은 Hg>Cd>Pb>Al의 順으로, Hg의 影響이 가장 크고 Al의 影響이 가장 작았다. *In vitro* 條件에서 ATP 및 酵素의 inactivation에 미치는 各 重金屬의 影響은 Hg>Al>Cd>Pb의 順으로 Hg의 影響이 가장 크고 Pb의 影響이 가장 작았다. ATP의 加水分解에 미치는 重金屬의 抑制的 影響은 重金屬에 依한 ATP의 不活性化 보다도 ATPase(luciferase)의 不活性化에 起因하는 것으로 나타났다. 그러나 重金屬의 濃度가 낮은 경우는 ATP의 加水分解가 促進되었다.

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