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Relationship Between Biological Activity and Structure of Alantolactone

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Alantolactone의 構造와 生物學的 活性

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ABSTRACT

To elucidate the relationship between chemical structure and biological activity of alantolactone, and also to investigate the relationship between the growth of cells and the respiration of *Chlorella pyrenoidosa* affected by alantolactone, alantolactone and isoalantolactone were isolated from *Inula helenium* L., and di-, and tetrahydroalantolactones were prepared by the hydrogenation.

At a concentration of 5×10^{-5}M alantolactone, the growth rate of *Chlorella* was greately reduced. The viability of cells was also reduced over 50% within 2 hr at a concentration of 2.5×10^{-4}M alantolactone. However, oxygen uptake was increased by 20% over 3 hr. And $^{14}CO_2$ production from glucose-1-^{14}C, glucose-6-^{14}C and ^{14}C-acetate-U.L. was also increased by alantolactone. Biological activity of alantolactone was significantly reduced by cysteine, reduced glutathione or cystine but not by tryptophan or histidine.

It was detected by spectrophotometrically and by TLC that alantolactone was also reacted with thiols except cystine. The solution of alantolactone reacted with thiol gave the UV absorption spectrum of α-saturated γ-lactone, and most of SH groups were disappeared by the addition reaction. From the reaction mixture of alantolactone and cysteine, a lactone adduct was isolated and purified.

Isoalantolactone had shown similar activity as alantolactone, however, it was appeared that di-, and tetrahydroalantolactones were not only inactive biologically but also *in vitro*.

It was concluded that there was no correlationship between increased respiration rate and mortality of *Chlorella*. During the respiration TCA cycle was activated, however it was uncertain that the activation of EMP or HMP was also appeared. Alantolactone and isoalantolactone were biologically active compounds but others were inactive. The reactivity of α-methylene γ-lactone moiety toward SH group was principally responsible for its biological activity in sesquiterpene lactones.

INTRODUCTION

It was believed for a long time that a certain chemical compound, which possesses a specific biological activity has its specific molecular structure and that others with similar structures will also have similar biological activities (Takagi and Osawa, 1963). Such an idea has not changed till now, though more supporting evidences are being cited. Today, with the rapid progress of biological sciences, especially molecular biology, and with more concentrated interests in the structure-biological activity relationship, more systematic studies are being carried out in many fields including biochemistry, physiology, chemistry and pharmaceutical sciences. As a result, our traditional and superficial concepts on the problem of the structure-activity relationship have been changed.

One of the most representative examples is the relationship between the activity of auxin and the molecular structure of auxins. It was beyond controversy that indole acetic acid and its derivatives had the activity of auxin (Thimann, 1958). Later many compounds such as phenoxy acids (Zenk, 1962), benzoic acids (Keitt, Jr. and Baker, 1966), naphthalene acids (Porter and Thimann, 1965), and other thiocarbamate compounds (Velstra, 1944) whose chemical structures are quite different from that of IAA were proved to have the activity of plant growth regulator. This presented the necessity to reinvestigate the relationship between the structure and activity of auxins. Thus it had become inevitable to examine this problem in the viewpoint of some peculiar structural properties rather than that of the resemblance in chemical structure (Price, 1970; Wain, 1953).

Encouragingly, solutions of this problem are continuously provided in case of auxin (Thimann, 1969). But the interrelationship between the structure and biological activity of many useful compounds such as gibberellin (Lang, 1970; Yomo, 1971), and anticancer substances (Goodman and Gilman, 1968), is not yet fully explained.

More than a hundred of sesquiterpene lactones are found today (Asplund and McKee, 1972; Inayama et al., 1973; Jeremic et al., 1973; Kupchan et al., 1971; Shibaoka et al., 1967b; Yoshioka et al., 1970). Among them, many are known to have antineoplastic activities (Bialecki et al., 1973; Kupchan et al., 1966; Kupchan et al., 1971; Lee et al., 1971; Lee et al., 1972; Lee et al., 1973; Petitt and Cragg, 1973), phytotoxicities to inhibit germination and growth, or activity to promote the differentiation of root formation in plants (Kwon, 1973; Shibaoka et al., 1967a; Yamaki et al., 1966). It is also known that most of such active compounds are α-unsaturated γ-lactones (Cavallito and Haskell, 1945; Kupchan et al., 1971; Lee et al., 1971). At the same time, biologically inactive unsaturated lactones and active saturated lactones are also reported from time to time (Mitchell et al., 1970; Lee et al. 1973). This is the reason why it seems to be unreasonable to say that the biological acitvity of sesquiterpene lactones is attributable to α-unsaturated γ-lactone.

It is well known that the activity of lactones is inhibited by nucleophiles such as thiol group (Black, 1966; Jones and Young, 1968; Kupchan et al., 1970a; Kupchan et al., 1970b). But it is uncertain whether the reactivity of lactone with thiol is the actual cause of its biological effect or not regarding to the fact that α-saturated lactones can possess biological activities too.

As can be seen above, the structure-activity relationship in sesquiterpene lactones still remains to be understood. Besides, most of the α-unsaturated lactones are known to be cytotoxic. Thus it was believed to be meaningful enough to elucidate this problem, and an attempt to solve this problem was made with alantolactone, the most simple sesquiterpene lactone in structure (Marshall and Cohen, 1964). Alantolactone is one of the earliest known sesquiterpene lactone widely distributed in plant kingdom, Compositae, exist[illegible] together with isoalantolactone and dihydro[illegible]

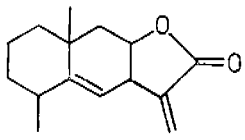
lactone (Colline-Asselineau and Bory, 1958). It has also high cytotoxicity inhibiting the growth of bacteria, yeast, fungi, helminth and plants (Dalvi et al., 1971; Kim et al., 1961; Kwon et al., 1973; Kwon, 1973; Kwon, 1974a; Olechnowicz-Stepien and Stepien, 1963; Yudovich, 1962). Its ability to cause allergy in human body was reported recently (Mitchell et al., 1970). But isoalantolactone has been known to be inactive in causing allergy, though it could inhibit the growth of plants (Kwon, 1974b).

In this experiment, effect of alantolactone and its derivatives on the growth and the respiration of *Chlorella* was examined together with the investigation of the relationship between the respiration and cell multiplicity affected by alantolactone. For this purpose, alantolactone and isoalantolactone were isolated from plant root. Then di-, and tetrahydroalantolactones were prepared by hydrogenation of lactones.

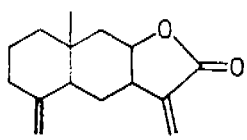
MATERIALS AND METHODS

Analysis

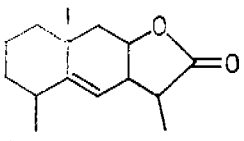
Melting points of alantolactone and its derivatives were determined using Mitamura Ricken MRK. Elementary analysis was carried out using titriplex or glucose as a standard. UV-absorption curves were obtained using Shimazu MPS-50L. IR spectra were determined by Japan Spectroscopic IR-S. Gas liquid chromatography was carried out using Yanagimoto GCG-5DH. NMR spectrum of alantolactone was detected using Varian HA100 with $CDCl_3$ as a solvent.



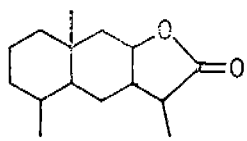
alantolactone



isoalantolactone



dihydroalantolactone



tetrahydroalantolactone

Isolations of alantolactone and isoalantolactone

Alantolactone and isoalantolactone used in this experiment were isolated from Inula Radix(*Inula helenium* L.). A component obtained by steam-distillation was column chromatographed to fractionate alantolactone and isoalantolactone. A column(4×150cm) was packed with 12.5% $AgNO_3$ impregnated silica gel(Dalvi et al., 1971; Terauchi et al., 1970). About 1g of the sample was applied in the column and was eluted with benzene. Each of 30m*l* fractions was thin layer chromatographed (Woo, 1972), and the fractions containing alantolactone and isoalantolactone were combined, respectively. After removal of eluent, recrystallization from ethanol gave pure compounds, as needles.

alantolactone; $C_{15}H_{20}O_2$, m.p. 79—80°C(78.5—80° C, Marshall and Cohen, 1964),
calculated ; C, 77.55; H, 8.68,
found ; C, 77.81; H, 8.23,
NMR spectra; $\delta^{CDCl_3}_{TMS}$=6.20(H-13 doublet), 5.61(H-13 doublet), 5.16(H-6 doublet), 4.83(H-8 quintet), 3.57(H-7 multiplet), 1.25(C-10 CH_3) and 1.11ppm(C-4 CH_3 doublet).

isoalatolactone; $C_{15}H_{20}O_2$, m.p. 111—113°C. (112-113°C, Marshall and Cohen, 1964; 111—113°C, Colline-Asselineau and Bory, 1958).
calculated ; C, 77.55; H, 8.68,
found ; C, 77.78; H, 8.36.

Preparations of di-, and tetrahydroalantolactones

Dihydroalantolactone(250mg) was prepared by hydrogenation of alantolactone(320mg) over paladium adsorbed to active carbon as a catalyst in ethanol. The reaction was continued at room temperature until 1 mole equivalent of hydrogen was taken up(Barton and De Mayo, 1957). After the catalyst was removed, recrystallization from

methanol gave pure dihydroalantolactone, as a short needle.

dihydroalantolctaone; $C_{15}H_{22}O_2$, m.p. 132—134°C, (132—132.5°C, Marshall and Cohen, 1964; 133.5—134°C, Ukida and Nakazawa, 1960),
calculated ; C, 76.88; H, 9.46,
found ; C, 77.16; H, 9.13.

Likewise, 200mg of tetrahydroalantolactone was obtained by hydrogenation of isoalantolactone(290 mg) until 2 moles equivalent of hydrogen was consumed.

tetrahydroalantolactone; $C_{15}H_{24}O_2$, m.p. 144—145°C(141—143°C, Ukida and Nakazawa, 1960),
calculated ; C, 76.23; H, 10.24,
found ; C, 76.54; H, 9.88.

Each of the lactones showed different single peak on GLC, respectively.

Growth experiments

The high temperature strain of *Chlorella pyrenoidosa* Chick was maintained on the proteose agar receiving light of 50—100 ft-C intensity at room temperature(Starr, 1964). For the growth experiment, inorganic salt medium(Devlin and Galloway, 1968) was used and illumination of the cells was continuously provided at 25°C by two banks of fluorescent lamps giving light intensity of 500 ft-C at the surface of the cultures. The cultures were frequently shaken with hand. Under this condition, stationary phase of cultures was reached within 60—72hr.

The addition of alantolactone to the culture flask was carried out as an alcoholic solution. The concentration of alcohol was not exceeded 0.75% in the medium, and the same quantity of ethanol was added into the control so that no effect of alcohol could interfere with the results of the experiment.

The effect of alantolactone on the growth of *Chlorella* was expressed as the change of cell numbers compared to the control. Cell number was counted with hemacytometer.

Respiration measurements

Consumption of oxygen was measured by the conventional Warburg technique, at 25°C, with cells of *Chlorella* in 0.067M phosphate buffer *p*H 7.2, and 20% KOH in the center well. Before the measurement of oxygen uptake, cells of *Chlorella* were suspended in 0.067M phosphate buffer *p*H 7.2, and aerated for 6 hr at 25°C under the dark condition. At the end of aeration, cells were harvested again by centrifugation and resuspended in the buffer for respiration measurement. Lactones were suspended in 0.5% CMC solution which was proved to have no effect on the respiration of cells. Respiration rate was expressed per unit mg of cell protein. After chlorophyll was disintergrated by adding KOH to the cell suspension and placing it under the strong light for 2—3 hr, protein content was determined by Lowry procedure using bovine serume albumine as a standard (Lowry et al., 1951).

Incorporation of radioactivity into CO_2

The cell respiration was increased in the presence of alantolactone or isoalantolactone. In order to trace the changes in the respiratory system of *Chlorella*, cells were incubated in Warburg flasks as above, and supplied with labelled substrates, at 10mM. Carbon dioxide was trapped in KOH and ^{14}C content was determined as a $Ba^{14}CO_3$ by gas flow Geiger tube(Kwon et al., 1973). The effect of self absorption was not corrected. Following the extraction of cells with hot ethanol, the aliquot and insoluble residues were counted to determine radioactivity present.

RESULTS AND DISCUSSION

To elucidate the relationship between activity and structure, the effects of alantolactone and its derivatives on the growth and the respiration

CMC, carboxymethylcellulose; EMP, Embden-Meyerhof-Pathway; GLC, gas liquid chromatography; HMP, hexose monophosphate pathway; IR, infrared; NMR, nuclear magnetic resonance; PDS, 2, 2′-dithiodipyridine; TCA, tricarboxylic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; 2-TP, 2-thiopyridone.

of *Chlorella* were examined.

Alantolactone(5×10^{-6}M) reduced the growth of the cells by about 50%, and at the higher concentration, 5×10^{-5}M, the growth of cells was completely retarded, while di-, and tetrahydroalantolactones showed no effects on the growth. However, isoalantolactone was revealed having the same activity as alantolactone(Fig. 1; Table 1). And it is also shown that alantolactone has a strong inhibitory action on the viability of *Chlorella*(Fig.2). The viability of cells was reduced by more than 90% in the treatment of alantolactone (2.5×10^{-4}M) for only 30 minutes, and the treatment of the lactone for more than 2 hr was proved to have no excessive effect.

Such an inhibitory effect of alantolactone on the growth of cells could also be observed in yeast, HeLa, and seedlings of *Phaseolus*(Dalvi et al., 1971; Kwon, 1973; Woo, 1972). And it could be considered that cytotoxicity of alantolactone was one of the common characteristics of biologically active sesquiterpene lactones.

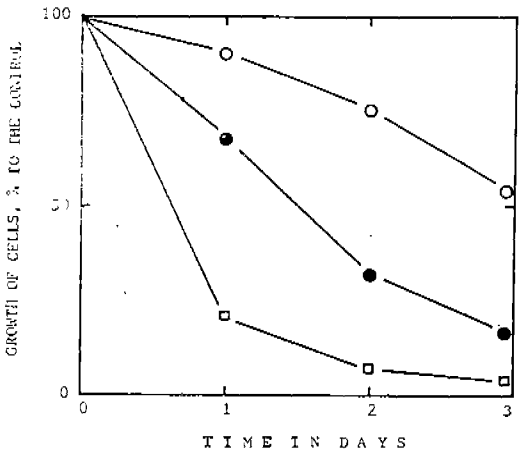


Fig 1. Growth of Chlorella at the different concentrations of alantolactone.

Cells were cultured in 100m*l* flask containing 30m*l* inorganic medium under the continuous illumination at 25°C. The growth rate of the cells was calculated by the cell count using hemacytometer.

Concentrations of alantolactone;

○···○, 5×10^{-6}M, ●···●, 2.5×10^{-5}M, □···□, 5×10^{-5}M.

Table 1. Effect of alantolactone on the growth of Chlorella

	Cell numbers, $\times10^7$ cells/m*l*				
	Conc $\times5\cdot10^{-6}$M				
Additions	none	0.1	1.0	5.0	10.0
alantolactone	73.42	74.02	35.07	13.11	0.96
isoalantolactone	73.42	70.54	33.25	12.42	1.43
dihydroalantolactone	73.42	—	72.16	—	73.53
tetrahydroalantolactone	73.42	—	74.06	—	72.86
alantolactone and cysteine	73.42	72.51	49.87	38.36	9.44
isoalantolactone and cysteine	73.42	71.78	51.26	36.23	10.47
alantolactone and tryptophan	73.42	—	30.97	—	0.85
alantolactone and histidine	73.42	—	37.45	—	1.08
cysteine	73.42	74.28	71.88	75.22	75.80
tryptophan	73.42	—	75.40	—	74.28

The growth condition was the same as that shown in Fig. 1. Initial number of cells in the cultures was 2.46×10^6 cells/m*l*. The concentration of amino acids was maintained 3 times higher than that of alantolactone.

It was reported that the biological activity of α-unsaturated γ-lactones could be reduced by the presences of nucleophiles (Black, 1966; Cavallito et al., 1945; Dickens and Cooke, 1965; Kupchan et al., 1970a; Kupchan et al., 1970b). Cysteine, histidine and tryptophan were used as nucleophiles with alantolactone in this experiment. An antagonistic relation between alantolactone and cysteine for the growth of *Chlorella* was obviously observed by simultaneously supplied cysteine (Table 1). Amino acids having no SH group did not show any effect, while cystine reduced the effect of alantolactone at a half concentration of the case of cysteine.

When the cells were treated with cysteine before or after the treatment of alantolactone, the inhibition by 10hr treatment of alantolactone (25×10^{-5}M) was not reduced by successive addition of cysteine (7.5×10^{-5}M), but 10hr pretreatment of cysteine reduced effect of post addition of alantolactone(Table 2 and 3). It is well known that α-

unsaturated γ-lactone can react with nucleophiles (Black, 1966; Kupchan et al., 1970a). It seems that alantolactone has a high reactivity towards SH group but not animo group. And it may be true that alantolactone combined with cysteine loses its biological acitvity, thus alantolactone taken up by cells reacts with SH groups of the cell components resulting in the inactivation of cell components and inhibition of cell growth.

Cells of *Chlorella* showed increase in respiration by over 20% when supplied with alantolactone(5×10^{-5}M), while di-, and tetrahydroalantolactones showed no effect at all (Fig.2; Table 4). Such increased phase of respiration continued for over 3 hr. It is evident that oxygen consumtion of cells is not only increased by alantolactone, but the production of carbon dioxide is also promoted by it. When the cells were incubated with ^{14}C-glucose-U. L. or ^{14}C-acetate-U.L. which were used as substrate, the labelled carbon dioxide production was increased by 36% at 5×10^{-5}M of alantolactone(Table 5 and 6). The rate of $^{14}CO_2$ production exceeded the consumption of oxygen by alantolactone. However, the respiratory quotient was constant in the presence of alantolactone(Kwon et al., 1973).

Cysteine acted as an antagonist on the respiratory stimulation of alantolactone in some extent as in the growth of cells. Besides the respiration of aged-slices of potato was activated(Kwon et al., 1973), it was also noted that alantolactone promoted the respiration of *Chlorella* too, when glucose or acetate was used as a substrate(Fig. 3). However,

Table 2. Interactions between alantolactone and cysteine in the growth of Chlorella

Additions		Cell numbers,
First	Second	$\times10^7$ cells/m*l*
none	none	29.23
none	alantolactone	9.16
cysteine	none	28.37
cysteine	alantolactone	20.51

The growth condition was the same as that shown in Fig. 1. Ten hours later, after the first addition of cysteine, alantolactone was added into the cultures. The concentration of alantolactone and cysteine were 2.5×10^{-5}M and 7.5×10^{-5}M, respectively. Initial number of cells was 1.87×10^6 cells/m*l*. The period of incubation was 64hr.

Table 3. Interactions between alantolactone and cysteine in the growth of Chlorella

Additions		Cell numbers,
First	Second	$\times10^7$ cells/m*l*
none	none	28.44
alantolactone	none	3.27
none	cysteine	29.54
alantolactone	cysteine	3.88

The growth condition was the same as that shown in Fig. 1. Ten hours after the first addition of alantolactone, cysteine was added into the cultures. Initial number of cells was 2.12×10^6 cells/m*l*. Incubation time was 64 hr. The concentrations of the chemicals were the same as those in Table 2.

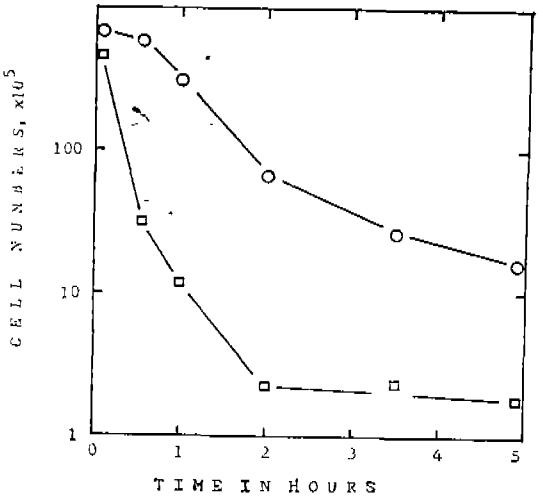


Fig. 2. Effect of alantolactone on the viability of Chlorella.

The growth condition was the same as that shown in Figure 1. Cell suspension (1.4×10^8 cells/m*l*) in phosphate buffer (0.067M, *p*H 7.2 containing alantolactone, 2.5×10^{-4}M) was incubated at 25°C. At each different time, 0.5m*l* of the cell susp. was removed and transferred to the fresh medium, and cultured for days. In the case of cysteine pretreatment, cell susp. containing cysteine(7.5×10^{-4}M), was incu bated for 1hr, then cells were removed by centrifugation, resuspended into the fresh buffer solution.
○···○, cysteine pretreated,
□···□, no pretreatment.

Table 4. The respirations of Chlorella in the presence of lactones

Additions	Oxygen consumption, $O_2\mu l$/mg protein 1	2	3hr
none	15.3	38.7	60.2
alantolactone	16.0	41.5	73.4
isoalantolactone	15.2	43.6	71.3
dihydroalantolactone	15.8	37.2	58.3
tetrahydroalantolactone	15.0	38.1	61.6
alantolactone and cysteine	15.2	40.3	64.6
isoalantolactone and cysteine	14.5	38.2	63.4
cysteine	16.0	37.4	58.9

The experimental method was same as that in Fig. 3. Glucose was added as a substrate into the reaction vessel. The concentrations of alantolactone and amino acids were 5.0×10^{-5}M and 1.5×10^{-4}M, respectively. Cell protein in the vessel was 6~9mg.

Table 5. Effect of cysteine on the stimulatory action of alantolactone in the respiration of Chlorella

Treatment	Addition	^{14}C-glucose-U.L. $O_2\mu l$/mg protein	cpm in CO_2	^{14}C-acetate-U.L. $O_2\mu l$/mg protein	cpm inCO_2
none	none	34.5	3127	41.6	9428
none	alantolactone	39.2	4263	48.6	12665
cysteine	none	33.6	3008	42.0	9807
cysteine	alantolactone	36.3	3567	44.1	11078

Incubation conditions were same as those in Fig.3 with specific activity of glucose(0.5μ Ci/20μmoles) and acetate(0.3μCi/50μmoles). Four hours after the starvation, cysteine (1.5×10^{-4}M) was added into the cell suspension and the starvation was continued for 1hr. Concentration of alantolactone was 5.0×10^{-5}M. Cell protein was 5~7mg and incubation time, 2hr. Labelled CO_2 retained in KOH was transferred into the $Ba(OH)_2$ solution and $BaCO_3$ was counted to determine radioactivity presence.

the respiration of fresh slices of potato or yeast was not affected by alantolactone. Moreover it suppressed the changes of respiratory systems in the slices during the aging processes in the presence of alantolactone(Kwon et al., 1973).

Thus it was postulated that the stimulation of respiration described above resulted from the special characters of the respiratory system of *Chlorella* and aged-slice of potato rather than from

Table 6. Utilization of labelled glucose in the respiration of Chlorella in the presence of alantolactone

	Alantolactone $\times5\cdot10^{-6}$M	Oxygen consumption $O_2\mu l$/mg protein	Radioactivity in CO_2 cpm/mg protein
glucose-1-^{14}C	none	14.2	3484
	0.1	14.1	3628
	1.0	16.4	3760
	10.0	17.6	4682
glucose-6-^{14}C	none		3172
	0.1		3486
	1.0		4214
	10.0		4336

Incubation conditions were same as those in Table 3 with specific activity of glucose, 0.5μCi/20μmoles. In duplicate, the amount of cell material varied to 5~8mg protein. Incubation time was 60min.

the properties of lactones. Such an idea is based on the facts that the cytotoxicity of sesquiterpene lactones varies depending upon the test materials and that a few of lactones can inhibit the growth of cells while it promotes the differentiation and cell divisions in other cases(Bialecki et al., 1973; Dalvi et al., 1971; Kupchan et al., 1971; Pettit and Cragg, 1973; Shibaoka et al., 1967b). However, there is a report that the sesquiterpene lactones from sagebrush can stimulate the respiration of *Cucumis sativa*(McCahon et al., 1974).

Comparing to the control, the radioactivity in CO_2 and oxygen consumption were increased by alantolactone in the respiration of *Chlorella* when acetate was added as a substrate(Table 5). It is postulated that TCA cycle in *Chlorella* is activated by alantolactone.

The generation of $^{14}CO_2$ was promoted to the same extent by alantolactone when either glucose-1-^{14}C or glucose-6-^{14}C was supplied as substrate (Table 6). It was meant by facts that both EMP and HMP were present in the cells of *Chlorella* (Devlin nad Galloway, 1968), and that the activities of both pathways were increased by alantolactone. However, it was not evaluated whether EMP and HMP were really activated or only TCA

cycle was activated resulting in the superficial appearance of the activation of EMP and HMP. The only thing which can be said clearly is that either EMP or HMP was not inhibited by alantolactone.

The ability of *Chlorella* to multiply was inhibited permanently in short period after the addition of alantolactone, while the increased state of respiration of cells continued much longer after the multiplicity of cells was completely inhibited (Fig. 2; Table 4). This indicates that the increased respiration is not the reason in *Chlorella* to lose its ability to multiply.

Alantolactone promoted the incorporations of ^{14}C from exogeneous ^{14}C-glucose-U.L. into CO_2 or alcohol soluble fractions. But the radioactivity in alcohol insoluble fractions was reduced by it(Table 7). With regard to this fact and other evidences that alantolactone inhibits the biosynthesis of hydrolylases in *Phaseolus*(Dalvi et al., 1971) and the formation of new respiratory systems in potato slices(Kwon et al., 1973; Nakano and Tadashi, 1970; Romberger and Norton, 1961), it is believed that alantolactone can inhibit some biosynthetic processes in *Chlorella*.

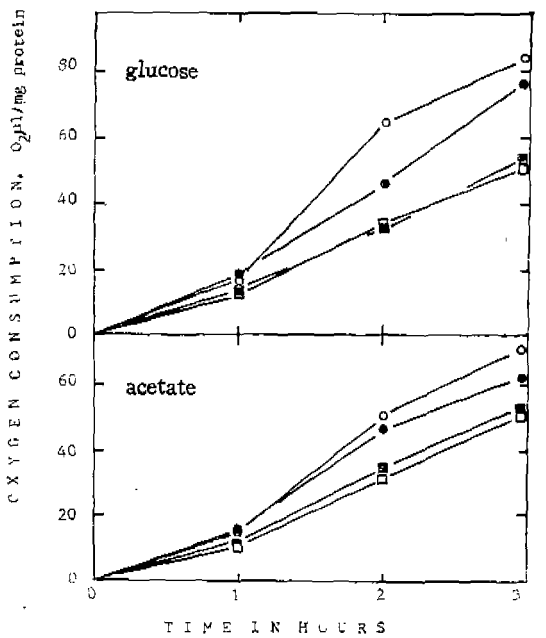


Fig. 3. Effect of alantolactone on the respiration of Chlorella.

Each vessel contained cell suspension(4—8mg as protein) in 2.2m*l* of phosphate buffer(120μmoles) *p*H7.2. Glucose was added at a conc. of 10μmoles/m*l* and acetate, 20μmoles/m*l*. Alantolactone in 0.5% CMC put into the side arm. The center well contained 0.2m*l* of 20%KOH, and the temp. was 25°C. Conc. of alantolactone: ○···○, 5×10^{-4}M; ●···●, 5×10^{-5}M; ■···■, 5×10^{-6}M; □···□, control.

Table 7. Effect of alantolactone on the incorporation of ^{14}C from labelled glucose into CO_2 and cell materials in Chlorella

Alantolactone $\times5\cdot10^{-5}$M	cpm			Oxygen consumption $O_2\mu l$/mg protein
	CO_2	EtOH sol.	EtOH insol.	
none	10344	517	12596	40.6
5.0	13966	529	10316	42.2
10.0	15503	537	10356	46.8

Experimental condition was same as that in Table 3 with specific activity of glucose-^{14}C, 4μCi/20μmoles. Two hours after incubation, an aliquot of the reaction mixture was removed, centrifuged briefly at 1000×g and washed twice in the medium used for incubation with 100mM nonlabelled substrate. Cells were then extracted twice with hot ethanol, the extracts combined. The aliquot and the ethanol insoluble residue were counted for their radioactivity. The cell material was 12.3mg protein.

For the investigation of reactivity of alantolactone with cysteine, the interactions between lactones and cysteine were determined spectrophotometrically(Kupchan et al., 1970a). A 10^{-2}M of lactones in THF was respectively added to 10^{-4}M solution of L-cysteine in 0.067M phosphate buffer, *p*H 7.2, prepared in a 1cm quartz cell, and the resultant solution was mixed rapidly. After an appropriate reaction time in the constant temperature, 30°C, the SH content was measured by quenching the reaction with an excess of a THF solution of PDS, which reacts with cysteine to give 2-TP. The amount of 2-TP(molar extinction, 7.06×10^3 at *p*H7.2) produced was measured at 343nm and from the result the quantity of cysteine-addition product of lactones was calculated.

The reactivity of alantolactone and isoalantolactone with thiols in cell free homogenates was

also examined(Grassetti and Murray, Jr., 1967). The homogenates of *Chlorella* and rat liver were respectively mixed with alantolactone and incubated at 40°C for a certain reaction time. A solution of PDS was added to the mixture followed by centrifugation and the SH content in the supernatant was measured spectrophotometrically.

Alantolactone and isoalantolactone reacted with thiols (Fig. 4), while di-, and tetrahydroalantolactones were proved to have no reactivity. Reduced glutathione also reacted with alantolactone, as cysteine did. The hydrogen ion concentration of the solution was very important factor at the reaction. The reaction rate was most rapid in physiological *p*H(Friedman et al., 1965). At this condition, about 2.3 mμmoles of cysteine was reacted with alantolactone in a minute. However, alantolactone was believed to have no large affinity to SH groups in cell free homogenates. Only 0.85% of thiols in *Chlorella* homogenate reacted with alantolactone

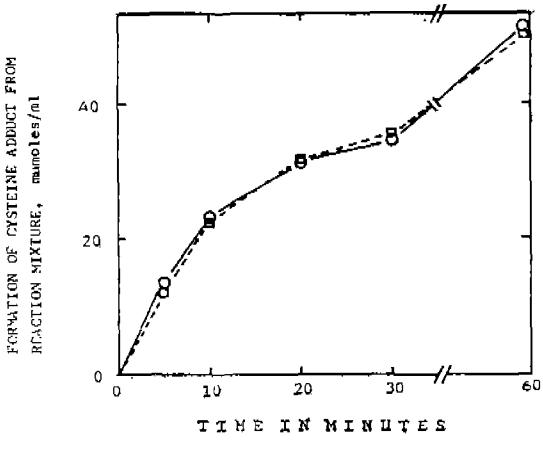


Fig. 4. Formation of cysteine adduct in the mixture of alantolactone and cysteine.

One tenth m*l* of 10^{-2}M alantolactone in THF was mixed with 4.9m*l* of 10^{-4}M cysteine in phosphate buffer, 0.067M, *p*H 7.2, and incubated for a certain period. Then 5m*l* of 10^{-3}M PDS in THF was added into the mixture. After 5 min incubation absorbance of the mixture was read at 343nm against a blank containing no PDS. Formation of the adduct corresponded to the formation of 2-thiopyridone(2-TP).
○···○, alantolactone, □···□, isoalantolactone.

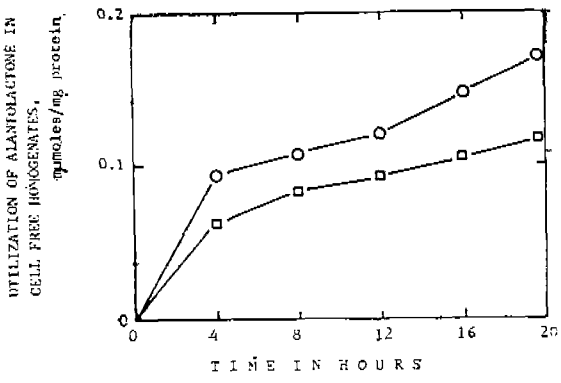


Fig. 5. Reactivity of alantolactone with thiol in the cell free homogenates.

Cells harvested from the culture and tissue from rat liver were homogenized in cold phosphate buffer, 0.067M, *p*H 7.2, and heated in water bath at 90°C for 5 min, then cooled rapidly. Homogenates containing alantolactone(2.5×10^{-4}M) were incubated and PDS was added into the homogenates, then absorbance at 343nm was read. ○···○, Chlorella, □···□, rat liver.

(Fig. 5), which was much lower value compared to the one by free cysteine.

And another attempts for the elucidation of the interactions between lactones and nucleophiles were examined. The reaction of lactones with thiols in alcohol could also be detected qualitatively by measuring UV spectra. On the basis of this result, the reactions of alantolactone or isoalantolactone with other nucleophiles such as cystine, reduced glutathione, histidine and glycine were examined. Alantolactone and isoalantolactone showed in the UV spectra strong absorption in the range of 210—220nm due to the α-unsaturated γ-lactone, as shown in Fig. 7. No peaks in this region of UV spectrum of dihydroalantolactone showes that α-methylene γ-lactone moiety was saturated. When lactones were reacted with cysteine, this absorption also disappeared (Barton et al., 1950; Steele et al., 1959). The mixture of alantolactone and other amino acids, cystine, histidine, tryptophan, or glycine, gave no changes in UV absorption pattern, although the mixture of reduced glutathione and alantolactone gave same

changes as well as cysteine. It is postulated that α-unsaturated γ-lactone moiety in alantolactone or isoalantolactone was easily reacted with thiol groups.

It was confirmed by following experiment. The 50% alcoholic solution of alantolactone-nucleophile mixture (10^{-3}M) was incubated for 24 hr at room temperature, concentrated under the reduced pressure at 40°C, and then was thin layer chromatographed. The formation of a new spot on the TLC was detceted by H_2SO_4, ninhydrin, and UV light(Woo, 1972). Among all the amino acids and guanosine tested only cysteine and glutathione could react with alantolactone (Fig. 6). Cystine, tryptophan, histidine, and guanosine appeared to be completely inactive. In order to examine the chemical and biological properties of the new products, following preparations were made; Each of the lactones was dissolved in ethanol at 30°C. Then small amount of cysteine was slowly

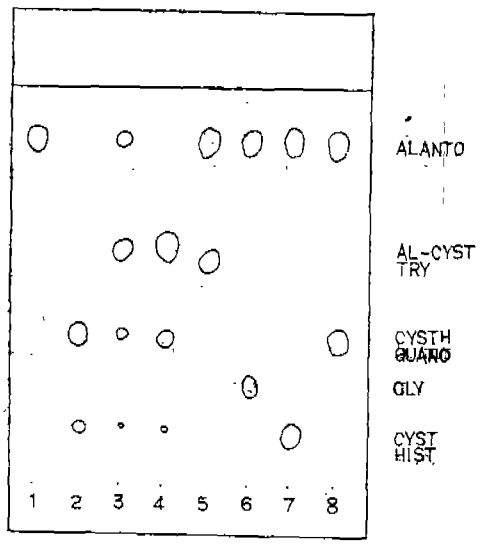


Fig. 6. Thin-layer chromatogram of cysteine adduct of alantolactone.

1; alantolactone, 2; cysteine, 3; alantolactone and cysteine (1 : 1, mole/mole), 4; alantolactone cysteine (1 : 3), 5; alantolactone and tryptophane, 6; alantolactone and glycine, 7; alantolactone and histidine, 8; alantolactone and guanosine. (Reproduced from ref. Kwon, 1974a)

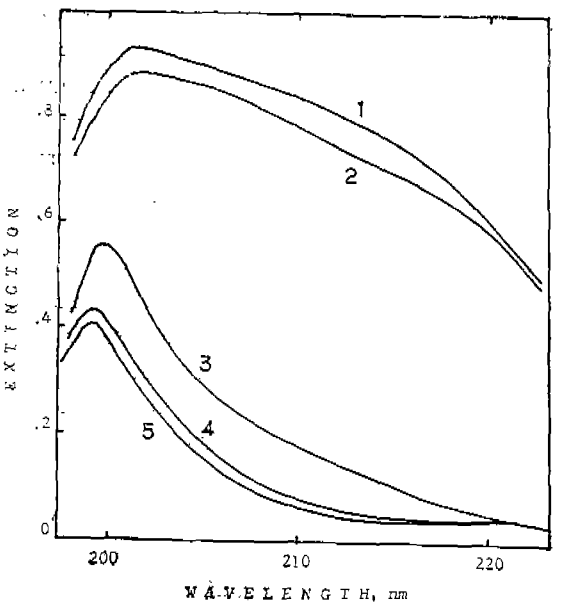


Fig. 7. Ultraviolet absorption spectra of alantolactone and its derivatives.

The concentration of the lactones in EtOH varied, $6.72\times10^{-5}\sim1.16\times10^{-4}$M. 1, alantolactone; 2, isoalantolactone; 3, alantolactone cysteine adduct; 4, dihydroalantolactone; 5, isoalantolactone cysteine adduct.

added to the solution until the quantity of cysteine reached up to 0.7 mole equivalent of lactone. Such process reduced most effectively the formation of cystine. After 2hr, about 5 times as much of cold distilled water was added into the reaction mixture. The precipitates were recrystallized from methanol to give pure crystals of cysteine adducts of alantolactone and isoalantolactone, respectively.

alantolactone cysteine adduct: $C_{18}H_{27}O_4NS$, m.p. 208—209°C,

calculated; C, 61.16; H, 7.70; N, 3.96,

found ; C, 59.72; H, 8.04; N, 4.12,

isoalantolactone cysteine adduct; $C_{18}H_{27}O_4NS$, m.p. 200—202°C,

calculated; C, 61.16; H, 7.70; N, 3.96,

found ; C, 60.23; H, 7.36; N, 4.02.

By the elementary analysis it was revealed that each one mole of lactones reacted with one mole of cysteine, and these compounds gave UV spectra

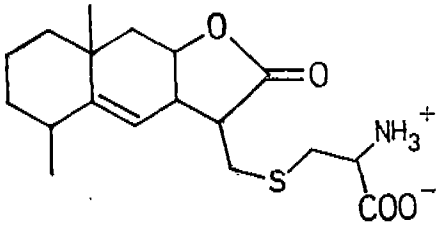
as same as the one from the mixture of alantolactone and cysteine or isoalantolactone and cysteine, and also same as dihydroalantolactone (Fig. 7).

Alantolactone showes absorption bands in IR spectrum(in KBr) at 1740(γ-lactone) and 1653cm^{-1} (α-methylene); isoalantolactone, at 1750(γ-lactone), 1665(isolated $=CH_2$), and 1635(α-methylene) and a strong band at 886cm^{-1}(isolated $=CH_2$). No absorption band at 1600—1700cm^{-1} could be read on the IR spectra of dihydroalantolactone and tetrahydroalantolactone. Cysteine adduct of alantolactone showes strong bands at 1765(γ-lactone), 1630(NH_3^+) and 1601cm^{-1}(COO^-). And the adduct of isoalantolactone showes at 1750 (γ-lactone), 1640(NH_3^+), 1595(COO^-) and 858cm^{-1}(isolated $=CH_2$). A strong band at 1255—1265cm^{-1} could be read only at the spetra of alantolactone and isoalantolactone(Fig. 8). On the other hand, no absorbance at 1653—1653, 1255, and 885—886 cm^{-1} could be read on the IR spectra of the derivatives, which were derived from α-methylene or conjugated lactone moiety of the lactones(Kanazawa et al., 1958). In the case of isoalantolactone cysteine adduct, there are an evidence that C—4 methylene do not react with cysteine because it still showes band at 886cm^{-1}(isolated $=CH_2$). It is another evidence that not C—4 methylene but α-methylene moiety of isoalantolactone reacts with hydrogen or cysteine at the addition processes (Barton et al., 1960).

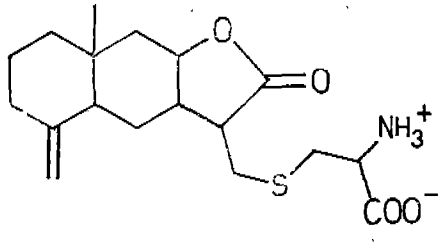
Unlike dihydro-, or tetrahydroalantolactone, alantolactone can be combined with cysteine *in vitro* (Fig. 4 and 6) and reacted with thiols in cell homogenates (Fig. 5), while the activity of alantolactone is effectively reduced by cysteine in the cells(Fig. 2; Table 2 and 4). This was thought to indicate the ability of alantolactone to react with thiols in the cells. The reason why cystine affected the biological action of alantolactone to the same extent as cysteine (molar equivalent) in the cells, but did not react with it at all *in vitro*, is presumed to be such that alantolactone combines only with the thiol group of cysteine. In the cells, however, cystine will be converted to cysteine by $NADH_2$ L-cysteine oxidoreductase, acquiring the ability to react with alantolactone(Black, 1966; Shibaoka et al., 1967a; Romano and Nickerson, 1954).

Regarding to the reaction of alantolactone and SH group, free cysteine or reduced glutathione reacts easily with the lactone, while only a small fraction of thiol groups in cell homogenates reacts with it(Fig. 4 and 5). Such a difference in the reactivities might be originated from the fact that the reactivity of SH group in free cysteine solution and in a protein solutions(in protoplasm) can be different. It is because the reactivity of SH group in free cysteine is altered by the changes of *p*H, and some of thiols in protein can be protected by certain molecules or ions, or tertiary structure of the protein. However, if one mole of thiol in protein react with a lactone, it may profoundly alter the activity of the protein(Friedman et al., 1965; Hanson et al., 1970; Kemp and Forest, 1968).

The reason why alantolactone reacts actively with only SH group among the nucleophiles can be explained as that only SH group has the reactivity



alantolactone
cysteine adduct



isoalantolactone
cysteine adduct

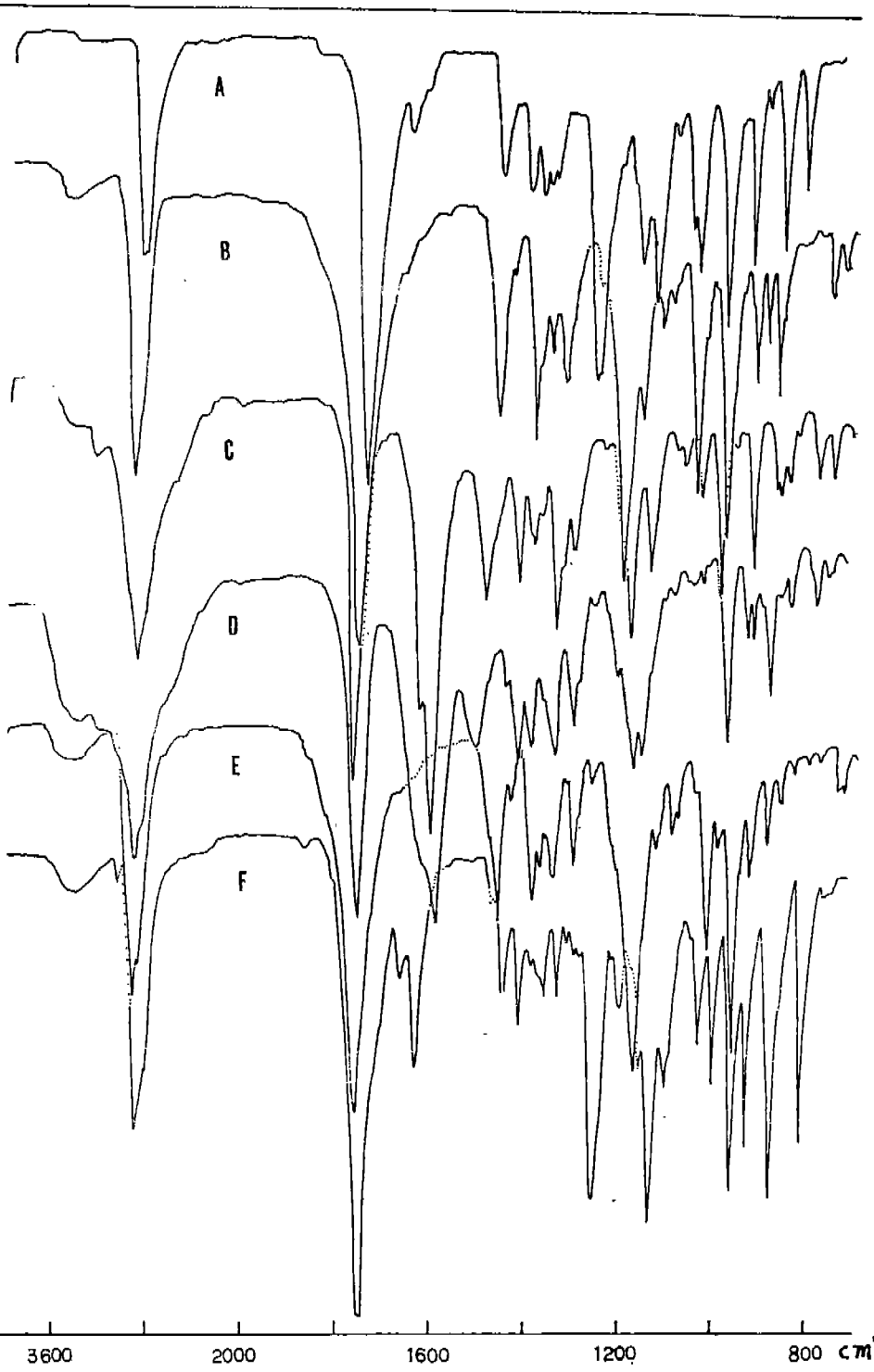


Fig. 8. Infrared absorption spectra of alantolactone and its derivatives in KBr.

A; alantolactone, B; dihydroalantolactone, C; alantolactone cysteine adduct, D; isoalantolactone cysteine adduct, E; tetrahydroalantolactone, F; isoalantolactone.

with alantolactone in the physiological *p*H range (Fig. 6). Moreover, it would be very difficult problem to say that biological activity of alantolactone in the cells is manifested in the way to react with only thiol groups of protein.

In this experiments, the activities of alantolactone and isoalantolactone were proved to be (nearly) identical, which was thought to be very natural regarding to the structures and properties of both compounds(Table 1 and 4; Fig. 4, 6, 7, and 8) even though some worker proposed that isoalantolactone was inactive. It seems that their results are false negative because the most of sesquiterpene lactones which have the antineoplastic actions *in vitro* also have been revealed to be inactive *in vivo*. And some biological effects of a certain compound can be observed depending upon the selections of experimental methods or materials. The possibility that lactones do not react with nucleophiles in mammals is not completely excluded(Mitchell et al., 1970; Stier, 1973).

Dihydro-, and tetrahydroalantolactones were proved to be quite inactive in this experiments. But there are reports that in other sesquiterpene lactones, even saturated lactones can manifest considerable activities. Such a result is supposed to be quite reasonable because it is possible the activities of sesquiterpene lactones are not always localized on lyon the α-unsaturated lactone moiety, but also on other groups in the molecule (Lee et al., 1972; Lee et al., 1973).

CONCLUSION

From the results of this experiments, it can be concluded as follows:

It is certain that alantolactone inhibits the growth but promotes the respiration of *Chlorella pyrenoidosa*. Alantolactone promotes TCA cycle in the cells inevitably. And it is certain that either EMP or HMP is not inhibited by it.

The active moiety of alantolactone is assured to be only the unsaturated lactone moiety considering no biological effects of di-, and tetrahydroalantolactones was observed. Both alantolactone and isoalantolactone were proved to be similarly active. Therefore it is proposed that isoalantolactone can be in the cate gory of inactive sesquiterpene lactone. Though alantolactone reacts with thiol group in the absence of enzyme, it is not certain whether the biologicl aactivity of the lactone is only derived from such a reactiviy or not.

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