BIOCHEMICAL AND NUTRITIONAL STUDIES ON POTASSIUM V. OXYGEN UPTAKE OF THE CELL PARTICULATES IN THE HIGHER PLANT

BIOCHEMICAL AND NUTRITIONAL STUDIES ON POTASSIUM V. OXYGEN UPTAKE OF THE CELL PARTICULATES IN THE HIGHER PLANT

By

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I. Introduction

We have studied on the potassium problem from the stand point of the biochemical properties and the nutritional effects in the higher plants, using the whole organs such as leaves or roots, thus investigations were performed to elucidate the growth, the potassium content, the carbohydrate metabolism and the oxygen uptake of the intact plants under the cultural conditions of various potassium levels (27, 28, 29).

For more detailed research to clear the biochemical properties of potassium, it is necessary to investigate the potassium participation through the mechanism of the cell or particulates. In this report, the investigations were advanced to the separation and the isolation of cell particulates, chloroplast or mitochondria, and to the biochemical properties of particulates, mainly oxygen uptake relating to the role or the distribution of potassium.

Generally speaking, the cell particulates in the plant consist of nuclei, plastids, mitochondria and other microparticulates. Above all, the chloroplast is the place of the photosynthesis, and is considered to be the most important larger particulate in the plant leaves. Many excellent researches such as Hill reaction, CO_2 fixation, photosynthetic phosphorylation (24) and carbon path way in the synthesis of organic compounds (25) have been investigated in this field. In the field of zoology on the other hand, there was an early recognized conception that mitochondria contain the major enzymes of the respiration chain and

work as the center of metabolic reaction (26). But the true character of plant respiration in connection with photosynthesis is not fully solved, and the most accepted consideration is that mitochondria in the plant particulates have close relation with the respiration reaction.

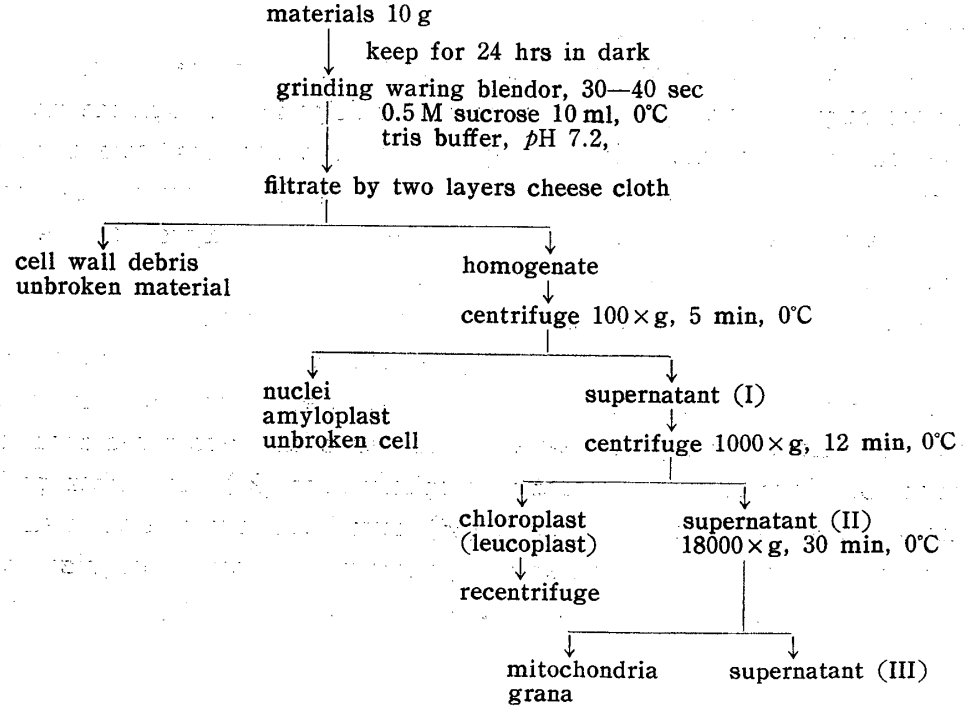
Thus we tried to find the mechanism of plant respiration through controling the nutritional status of potassium, as potassium deficiency brings in the severe incrcease of respiration of the higher plants.

II. Experiments and Results

(1) *Separation Method of Cell Particulates*

As materials, barley seedings cultured usually in sand, and other plants collected in the field were used. At first adopting the differential centrifugation, the separation of chloroplast and mitochondria was performed with the various conbinations of the centrifugal power and time, so as to decrease the contamination of mitochondoria and amyloplast in the chloroplast fraction and chloroplast fragment in the mitochondria fraction. By staining mitochondria with Janus green B, and nuclei with methyl blue, on each separated fraction, the material was observed under the microscope to show the contamination of the other particulates. The white granules of amyloplast were cosedimented with the chloroplast, so to remove them from the intact leaves, the plant materials were left in the dark, for 24 hours, as the break down of chlorophyll was observed over longer dark treatment. First and low centrifugation was done at 100—150×g

Table 1. Isolation method of cell particles



for 5 min. to remove the amyloplast and the unbroken debris. The most difficult problem was in the separation of mitochondria from the green plant material to eliminate the contamination of chloroplast fragments derived for the grinding procedure. This will be mentioned afterwards.

Separation method by means of differential centrifugation was done, as shown in Table 1 and then estimated were the mineral contents of the particulates, and activity of oxygen uptake in each fraction.

(2) *Mineral Content and Oxygen Uptake of the Chloroplast isolated by Different Grinding Methods.*

It is possible to assume that the biochemical properties of the isolated particulates differed by grinding methods or medium. It seems to be an important problem to keep the activity as similar as in intact leaves for the separation of cell particulates.

As the grinding methods, the mortar or Waring blendor available in our laboratory was used, and 10 per cent neutral lead acetate, 0.25 M sucrose solution so called isotonic solution of the plant cell and distilled water was adopted as the grinding medium. After the chloroplast isolated within the range of the centrifugal power and time shown in Table 2. the mineral content was determined by the method of flame photometer for potassium, sodium, calcium, by Allen's method for phosphorus and titan yellow method for magnesium. Oxygen uptake was measured by Warburg's manometry at 20°C, in the dark. By hand mortar it was difficult to get uniform grinding and the yield of the isolated chloroplast was decreased from the breakdown of them. By the short grinding with the Waring blendor, the chloroplast could be prepared at a high level of 60 mg dry weight from 7 g intact leaves.

Potassium, sodium, phosphorus, magnesium content in the isolated chloroplast was altered by the method or medium on the grinding, and the use of neutral lead acetate as the protein precipitator resulted in abnormally high magnesium content in the isolated chloroplast, probably due to the precipitate of a part of the sap protein conjugated with magnesium. On the other hand, by the distilled water, considerable amounts of potassium, phosphorus and magnesium were

Table 2. Mineral contents of chloroplast isolated by some grinding methods and mediums.

Grinding methods	Grinding mediums	K	Na	P	Mg	Ca
Mortar	Lead acetate	0.12	0.06		0.17	0.08
	Isotonic sucrose solution	0.16	0.04	0.29	0.12	0.08
	Distilled water	0.14	0.06	0.24	0.08	0.08
Waring blendor	Lead acetate	0.30	0.07		0.35	0.11
	Isotonic sucrose solution	0.32	0.07	0.43	0.21	0.11
	Distilled water	0.24	0.06	0.34	0.11	0.11

released. The use of 0.25 M isotonic sucrose solution gave a good result as compared with the above mediums from the point of mineral content. (Table 2)

Oxygen uptake of the homogenate ground at a low temperature of 5℃ by means of the chilled Waring blendor or mortar is shown in Fig 1. A high and suitable value was obtained by the Waring blendor. This may have resulted from the difference of the time required and the degree of grinding. (Fig. I)

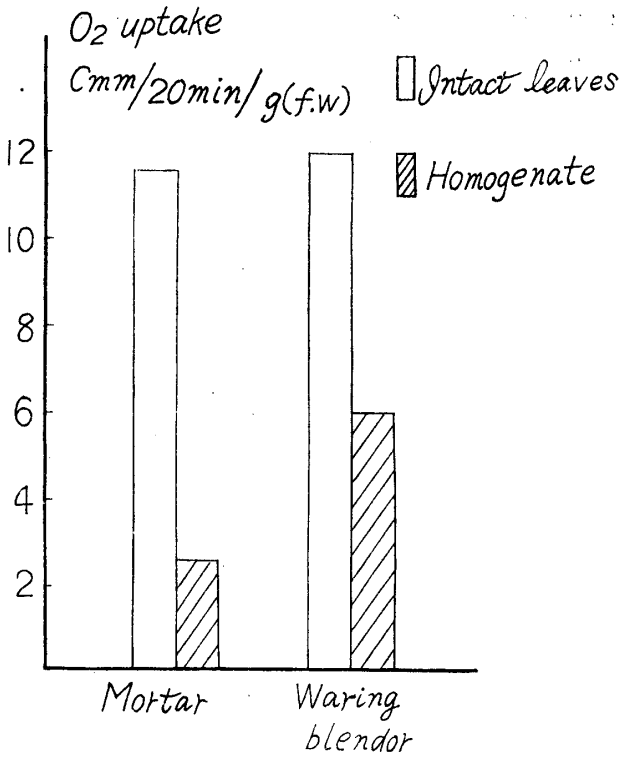


Fig. 1 Oxygen uptake of homogenates made by different grinding methods.

(3) *Mineral Content and Oxygen Uptake of the Chloroplast Isolated by Various Hydrogen Ion Concentrations.*

It was proved that the mineral content in the isolated chloroplast changed with the condition of osmotic pressure of the medium, and judged from the nature of cell membrane, it may also be suggested that the mineral content in

Table 3. Potassium and sodium content in the isolated chloroplast, influenced by the hydrogen ion concentration of grinding medium.

*p*H	K (mg)	Na (mg)
3.1	0.04	0.01
4.5	0.15	0.06
6.2	0.16	0.06
8.8	0.12	0.02
9.8	0.04	0.02

Isolated from 10 g intact leaves and *p*H controlled by dil. HCl, $Ca(OH)_2$

the isolated chloroplast is altered according to the hydrogen ion concentration of the medium. The results are shown in Table 3. Corresponding with the previous investigations, potassium was easily leaked out from the intact leaves, or roots, and from this experiment potassium or sodium moved freely from the chloroplast at high or low hydrogen ion concentration.

As to the physiological state of the chloroplast isolated by the some treatment, oxygen uptake was estimated as usual, and this influene of hydrogen ion concentration clearly appeared as shown in Fig. 2. The most suitable hydrogen ion concentration for the grinding medium was near to *p*H 6.8. (Fig. 2)

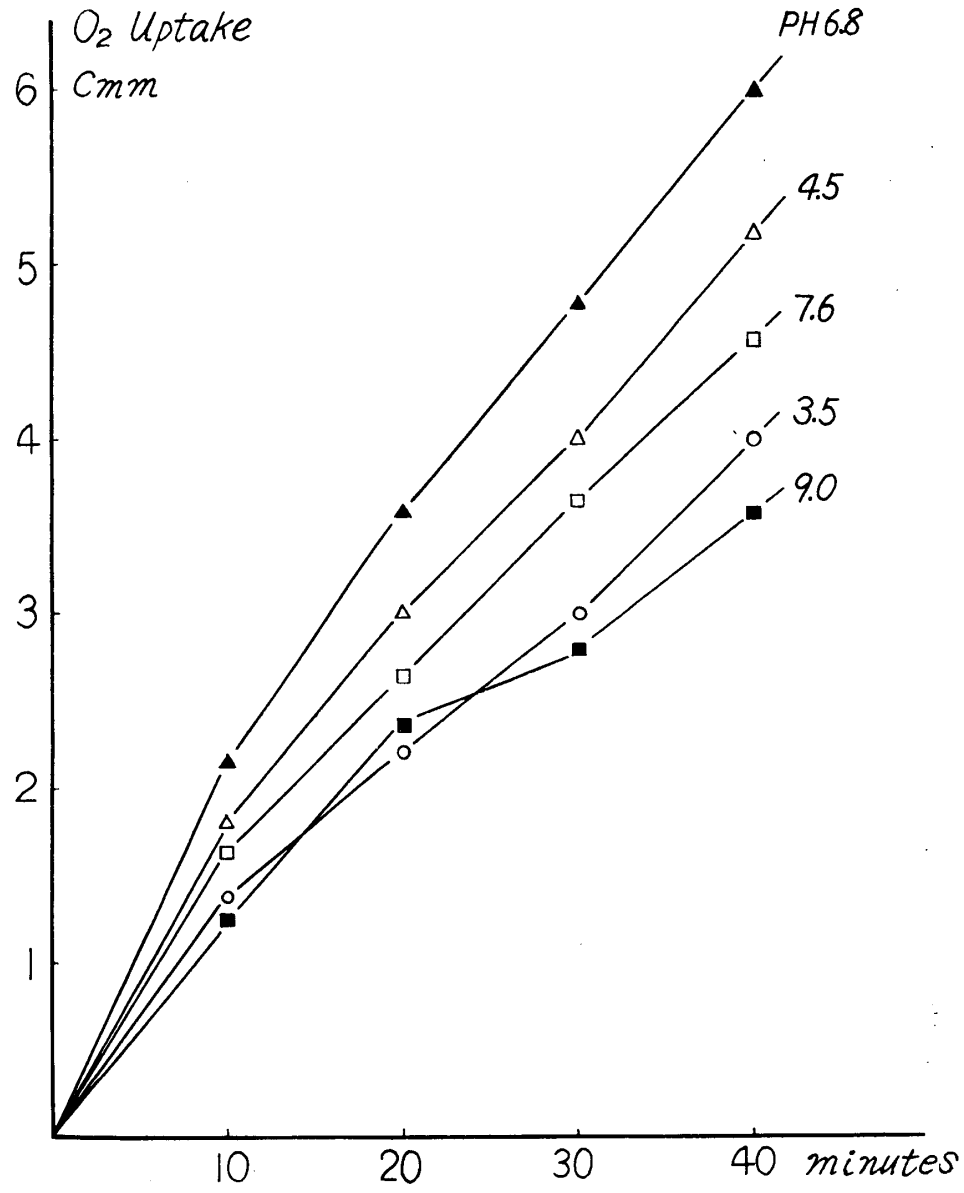


Fig. 2 Oxygen uptake of chloroplast isolated by various hydrogen ion concentration of medium from 7 g intact leaves.

(4) *Yield of the Chloroplast Fraction and Contamination of Chloroplast Fragment to the Mitochondria Fraction.*

After the treament of grinding and differential centrifugation, the distribution of chlorophyll in each separated fraction was determined to know the yield of the isolated chloroplast and the chloroplast fragment contaminated in the other fraction, adopting the Guthrie method for the analysis of chlorophyll (19). At first 19.5 mg of total chlorophyll contained in the intact leaves was withdrawn at the level of 65 per cent in the homogenate after grinding and filtrating with two layer cheese clothes. Next, by the treatment of low centrifugation, at $100 \times g$ for 5 min 0.7 mg of chloroplast precipitated, and in the chloroplast fraction, at 1000 g for 12 min, 9.4 mg of chlorophyll was obtained at 48 per cent level as compared with that of the intact leaves. Finally chlorophyll contamination in the mitochondrial fraction involved 3 mg of chlorophyll corresponding to 15 per cent of the intact leaves. Thus contamination is considerably high, so perhaps by this means, it may be impossible to separate the mitochondria in the pure state.

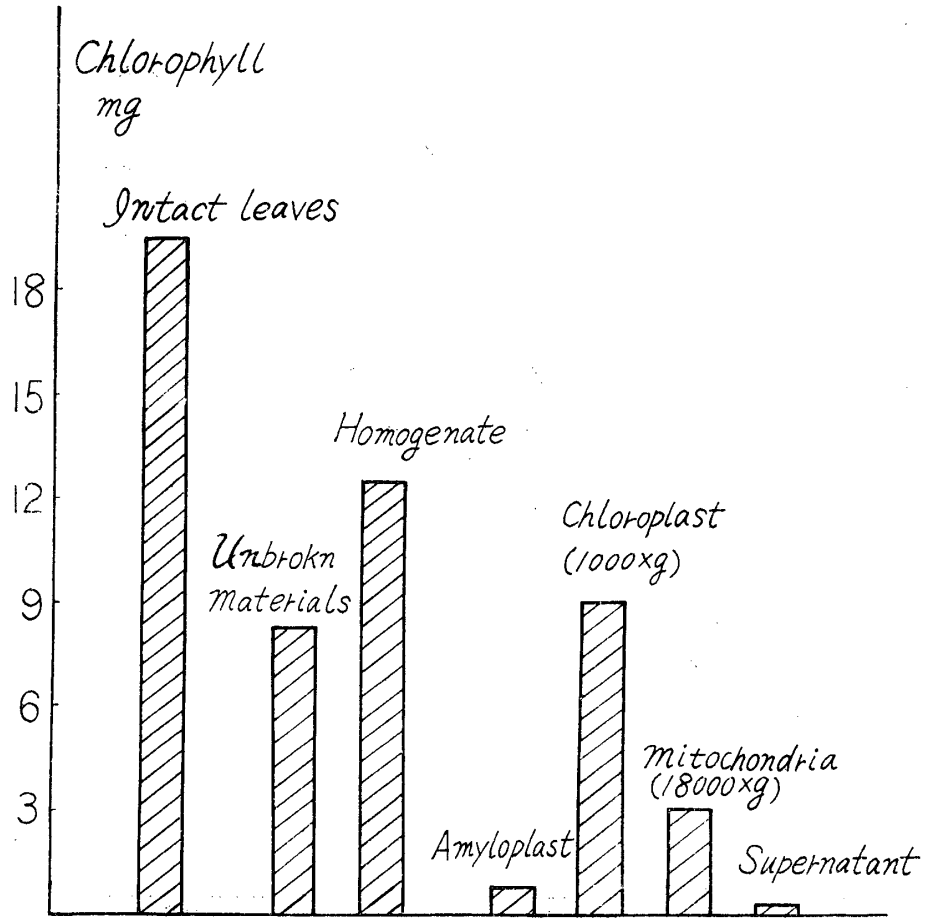


Fig. 3 Distribution of chlorophyll in each fraction separated from barley leaves (1 g). Yield of chloroplast and contamination into other fractions is determined.

(5) *The Yield of Chloroplast fraction and Mineral Content in the Chloroplast Isolated from the Leaves of Some Different species of Higher Plants.*

The yield of the chloroplast fraction was determined by the same procedure, using several plant species, and the results are shown in Table 4.

Taele 4. Yield of the chloroplast fraction isolated from the leaves of some different species of plant, measured from the chlorophyll content.

Species	Chlorophyll (mg) in intact leaves (1 g)	Chlorophyll (mg) in isolated chloroplast	Yield of (%) isolated chloroplast
Sweet potato	1.97	0.88	44.8
Kyona	1.66	0.81	48.6
Taisai	1.52	0.67	44.3
Radish	1.39	0.74	53.7
Tobacco	1.65	0.54	34.2
Potato	1.60	0.66	43.0
Wheat	1.85	0.85	45.7

Chloroplast fractions were isolated at the 34—53 per cent order of intact leaves and in this case a few amylopast were mixed in the chloroplast fraction in the species of potato or sweet potato.

The mineral contents in the isolated chloroplast were also examined as shown in Table 5 and the ratio of the mineral content contained in the isolated chloroplast compared with the intact leaves was calculated from the yield of chloroplast fraction and mineral content of the isolated chloroplast. (Table 6)

Table 5 Mineral content of chloroplast fraction isolated from some different species of plant leaves.

Species	P		K		Ca		Mg	
	Intact leaves	Isolated chloroplast	Intact leaves	Isolated Chloroplast	Intact leaves	Isolated Chloroplast	Intact leaves	Isolated Chloroplast
Kyona	3.75	0.38	24.01	0.09	3.40	0.66	3.60	0.44
Taisai	3.15	0.29	26.91	0.12	2.90	0.20	2.20	0.29
Tobacco	5.03	0.27	35.60	0.16	3.20	0.30	2.35	0.26
Potato	4.25	0.23	52.16	0.16	4.60	0.59	2.25	0.30

Mineral content 1/10 mg per 1 g fresh materials.

Table 6. Ratio of mineral contents in the whole chloroplast compared with the intact leaves, ratio as calculated from Tables 4, 5.

Species	P (%)	K (%)	Ca (%)	Mg (%)
Kyona	20.5	0.8	39.0	25.1
Taisai	20.8	0.9	14.7	29.6
Tobacco	15.8	1.3	28.4	56.0
Potato	12.4	1.7	30.0	26.5

The mineral content in the intact leaves differed widely according to the species of plant or the kind of elements. In the whole chloroplast the content

mainly varied by the kinds of elements, that is, magnesium was contained most abundantly in the chloroplast at the level of 25.1—56.0 per cent, then next came calcium at the level of 14.7—39 per cent phosphorus at the level of 12.4—20.8 per cent, and potassium content was the minimum at the level of 0.8—1.7 per cent. Magnesium and calcium was condensed in the chloroplast, similar to nitrogen but on the other hand the possium content was not so high in the chloroplast. Thus it is considered that most of potassium was contained in the cell sap and moved easily into or out of the chloroplast. In the next report the affect of the surrounding potassium concentration to the oxygen uptake of the isolated chloroplast will be mentioned (30).

(6) *The Oxygen Uptake in Each Separated Fraction.*

As mentioned above, the respiratory pathway and the place of respiratory reactions in connection with photosynthetic mechanism was not fully solved. In this respect, problem will be presented on the oxygen uptake in the particulates fractionated from the cell.

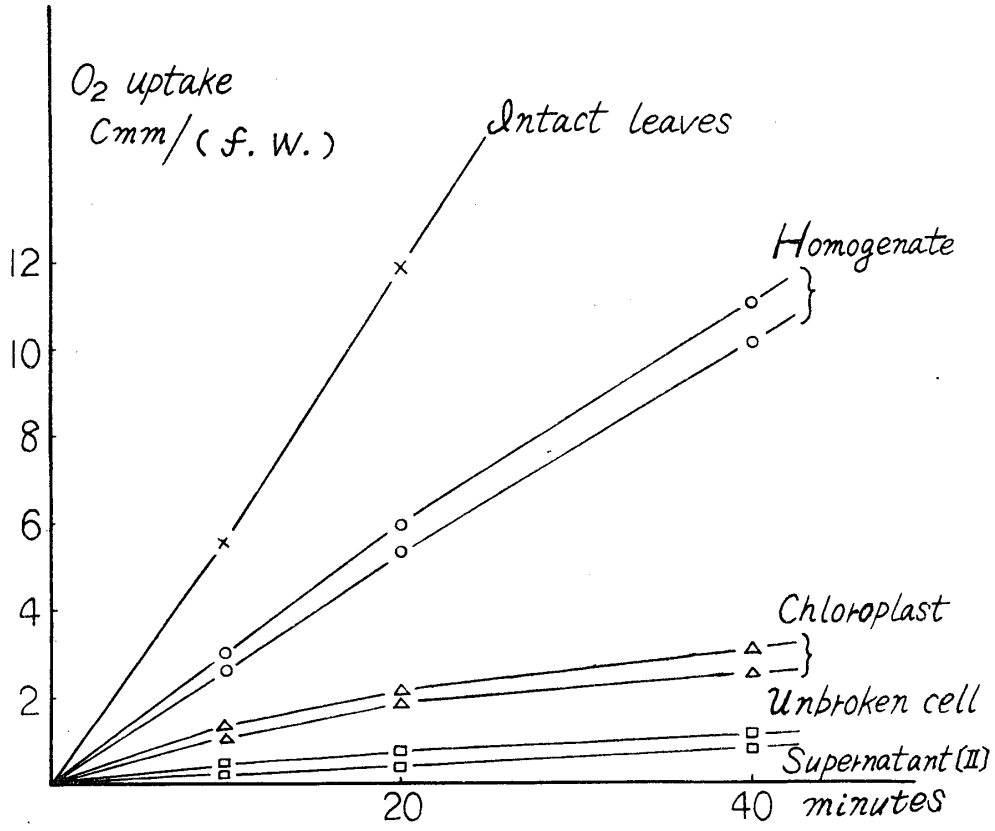


Fig. 4 Oxygen uptake of various factions isolated from barley seedling.

Fig. 4 shows the oxygen uptake of the fraction immediately after the separation. These experiments were performed under the same condition men-

tioned before and the addition of substrate or enzyme was avoided to know the autonomous oxygen uptake.

The highest autonomous oxygen uptake was observed in the chloroplast fraction among the particulates immediately after the separation, and the data are demonstrated in Fig. 5 and Fig. 6. In this case it would be supposed that the complexity in the level or ratio of activity, depends upon the imperfect system caused by the grind or differential centrifugation. But it may be emphasized that the autonomous oxygen uptake of chloroplast or leucoplast is higher than the mitochondria isolated from leaves or roots.

The fall of oxygen uptake in the process of measuring at 20°C in the dark is shown in Fig. 5; it comes down to less than at the level of 80 per cent, after ten minutes in the particulates isolated from the leaves. The same tendency was also observed in the root particulates though not so clear as compared with the cases of the leaves. This observation differed from the general conception. The experiments should be extended to pick up the several species to determine whether this phenomenon is only restricted to the barley seedling used in this experiment, or whether the oxygen uptake of the isolated chloroplast has a character similar with the respiration of the intact plant.

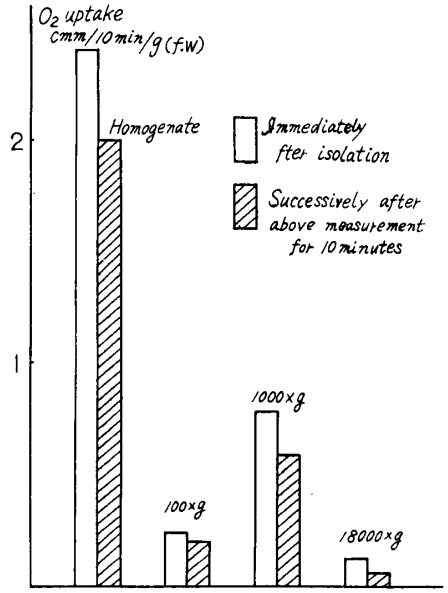


Fig. 5 Diminution of oxygen uptake in each fraction isolated from barley leaves, for the process of of 10 min. measurement.

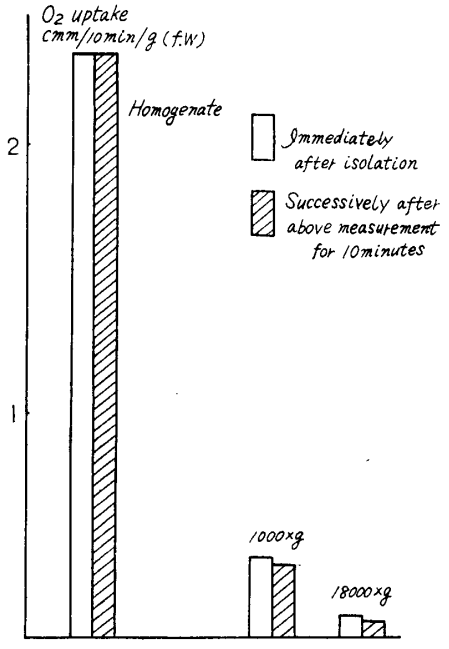


Fig. 6 Diminution of oxygen uptake in each fraction isolated from barley roots, for the process of 10 min. measurement.

(7) ***Oxygen Uptake of the Chloroplast Isolated from the Various Species.*** Potato, sweet potato, radish, tobacco, taisai, (*Brassica chinenis*), and kyona (*Brassica japonica*), cultivated usually in the field were used and autono-

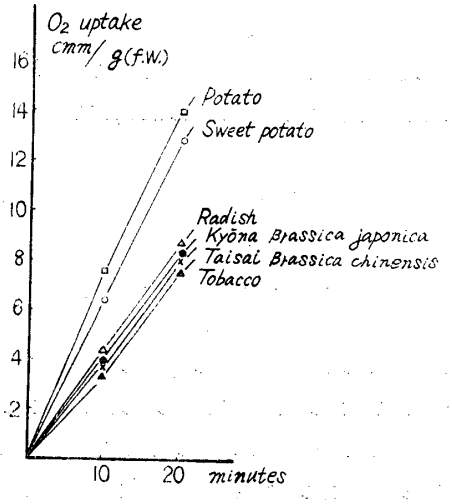


Fig. 7 Oxygen uptake of intact leaves in some species

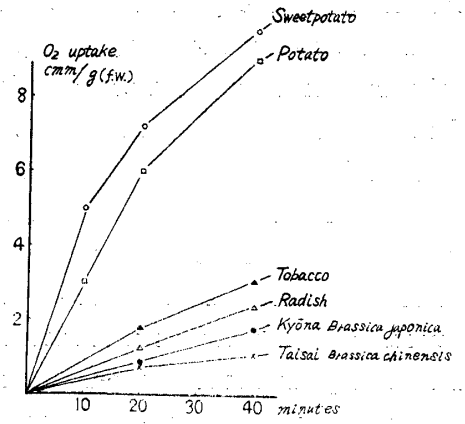


Fig. 8 Oxygen uptake of homogenate obtained from some species.

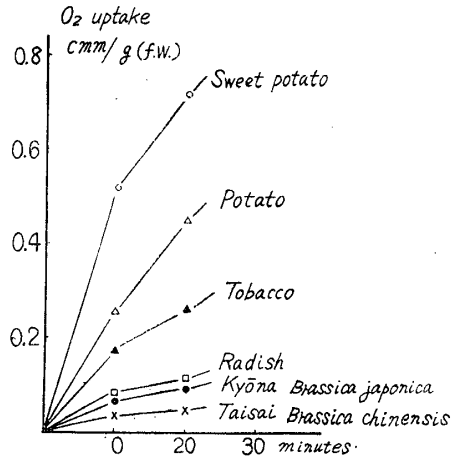


Fig. 9 Oxygen uptake of chloroplast isolated from some species.

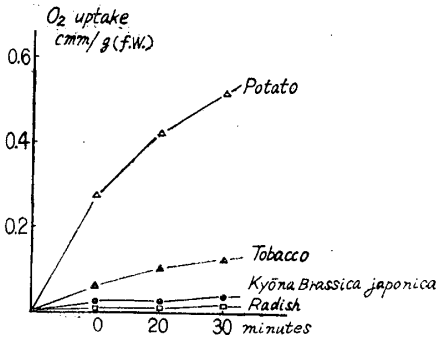


Fig. 10 Oxygen uptake of mitochondria isolated from some species.

mous oxygen uptake of the intact leaves, as the source of chloroplast homogenate, macerated leaves, isolated chloroplast and mitochondria were determined by the manometry method. Oxygen uptake of homogenate was decreased to less than half of that of the intact leaves by maceration and filtration treatment. Although considerably high oxygen uptake was still observed in the potato mitochondrial fraction, in general the oxygen uptake of the chloroplast fraction was more active than that of the mitochondrial fraction in the other species.

The data are shown in Figs. 7-10. From the results it may be concluded that autonomous oxygen uptake of the isolated chloroplast was kept in an active state in most plant species, as in that of the barley seedling.

III. Discussion

From the investigation of the biochemical behaviour of particulates by the centrifugation of cell component, several important principles should be considered. At first the identity of the fractions should be established from the cytological and morphological stand points, and a high degree of purity and high yield(23) of the isolated cell component by the separation procedure.

Secondly it is necessary to achieve the separation keeping the material in an active state during the isolation procedure. The cell particulates of the higher plant was isolated by some investigators as Menke(1), Neish(2), Granick(3), Chibnal(4). The isolation procedure involoved the mild grinding, the pressing out juice and the taking of the residue in 1/13 M primary calcium phosphate. The extract was separated by centrifugation, then the sediment was taken up in phosphate solution and the larger particles precipitated again by slow centrifugation at about $250 \times g$. Plastids were then removed from the supernatant solution by higher speed centrifugation at approximately $2220 \times g$. Granick adopted a slightly hypertonic sucrose solution as the grinding medium or suspending solution. Du Buy, Woods and Lackey(5) reported on the isolation of plastids and mitochondria from *Nicotiana* and *Louicea* leaves, comprising the mild disintegration of leaves in 0.15—0.5 M sucrose especially by a waring blendor and the filtration through glass wool followed by centrifugation at $120 \times g$, yielded almost entirely only the plastids. French and Milner(6) adopted a similar procedure of grinding and centrifuged the material 15 min at $12000 \times g$, treated with low temperature at 0°C, and obtained a heavy layer of starch and nuclei at the bottom of the tube with a layer of chloroplast material above. The green layer of chloroplast material was removed from the starch with the spatula, and the whole and broken chloroplast were then resuspended in the water or other medium.

From the stand point of biochemical research on the cell particulates it is a serious problem to separate the cell particulates in the active and pure state.

Recently the method of the separation has been improved and become suitable for each research purpose, and the morphological identification also has been performed. John, Madison(7) in the study of distribution of phosphorylase in tobacco leaves, made the preparation by grinding in a mortar with *p*H 6.2, 0.01 M malic buffer and fine sand, and the filtrated debris was centrifuged lightly at 250×g for 3 min and the residue, principally starch, was discarded. The supernatant was centrifuged supernatant was centrifuge for 20 minutes at 800×g. Miller and Evans(8) investigated on the influence of various concentrations and kinds of salts on the activity of cytochrome oxidase associated with particulate preparations to show the characteristics of mitochondria from the roots of certain higher plant species. In this experiment, as the medium 0.3 M sucrose and 0.05 M triamino methane buffer were used and the grinding was made with both the cold mortar and Ten Broeck homogenizer, after centrifuged 1000×g for 15 minutes. The supernatant was decanted and centrifuged at 20,000×g for 15 minutes, and the preparation was still desalted.

Arnon *et al*(9) investigated the photo-phosphorylation or CO_2 fixation on the isolated chloroplast and its fragment from the spinach leaves. They prepared the materials by grinding sliced leaf blades in a large ice cold mortar with ice cold 0.35 M NaCl and cold sand; centrifugation was made first at 200×g for 1 min, and next at 1000×g for 7 min to precipitate the whole chloroplast, and it was purified by recentrifugation. Finally the chloroplast fragment was obtained at 14000×g, for 10 min centrifugation, after the remaining chloroplast was left at 18000 g for 1 min. Each fraction separated was observed morphologically.

Ohmura(10) made a research similar to Arnon *et al*; remarking on the inactivation of oxygen consumption and uptake of inorganic phosphate in the green particulate fraction of spinach leaves. They used 0.45 M sucrose, 0.05 M sorbitol borate buffer *p*H 7.2, 0.03 M EDTA, 0.03 M potassium citrate mixture as a grinding solution and centrifuged the material at 500×g for 5 min, next at 12000×g for 20 min. differentially.

Chiba and Sugawara(11) confirmed the nucleic acid content in the chloroplasts isolated from spinach and tobacco leaves. To prevent the contamination of nuclei to chloroplast fragment, centrifugal precipitation was repeated several times with a procedure similar to that of Arnon *et al.*

For the isolation of mitochondria in the higher plant, roots and potato tubers, etiolated plants, germinating seedlings, fruits were commonly used as the materials. As the first step the same method as chloroplast isolation was adopted, and the final differential centrifugation differed according to the investigators or materials as shown in Table 7.

Briefly, caution on the practical isolation must be given for the following items, variety of cell particulates, grinding method and medium, range of centrifugal power, time of centrifuge, confirmation of contaminated particles and yields

Table 7. Isolation methods of mitochondria

Investigator	Materials	Grinding medium	Range of centrifugal power and time
Howard F.D. Yamaguchi M. (12)	Pepper fruit	0.1 M phosphate buffer *p*H 6.8 0.4 M sucrose, 0.01 M EDTA	500×g−16000×g 5min. 15min.
Stanley R.G. Conn E.E. (13)	Sugar pine germinating seedling	0.1 M phosphate buffer *p*H 7.0, 0.5 M sucrose (1MNaOH)	2000×g−20000×g 5 min. 15 min.
Freebairm H.T. Remmert L.F. (14)	Cabbage	0.1 M potassium phosphate buffer pH 7.2, 10 M sucrose, 0.01 MEDTA	3000×g−14000×g 10 min. 15 min.
Forti G. (15)	Etiolated pea internodes	0.45 M sucrose	1000×g−20000×g 10-20 min. 20 min.
Avron M. Biale J.B. (16)	Avocado fruit	0.05 M phosphate buffer 0.25 M sucrose	500×g−17000×g 5 min. 15 min.
Liberman M. Biale J.B. (17)	Sweet potato tubers	0.5 M sucrose	1000×g−14000×g 5 min. 15 min.
Webster G.C. (18)	54 species	0.1 M K_2HPO_4	1000×g−16000×g 15 min. 30 min.

respectively, the chilling or the addition of the stabilizer also should be considered for the prevention of inactivation or enzymatic denaturation.

The isolation of the chloroplast from the plant leaves is commonly made to study the photosynthetic reaction and related process and to investigate other process. There are some problems on the isolation and the distinguishment of the whole chloroplast from its fragment.

The contamination will take place, as the mitochondrial size is similar to that of the chloroplast fragment. In such isolation, the original phase of each particle could not be confirmed. Chloroplast isolation established in this experiment is similar to the method of Miller and Evans(8), Arnon *et al*,(9) Chiba and Sugawara(11), yielding a high rate of the whole chloroplast, but some amyloplast contamination conld not be avoided owing to the range of centrifugal power or other means. Relating to the mitochondrial isolation, as reported by Millerd(19), the pure mitochondrial separation from the green leaves was difficult because of the degradation of chloroplast taking place during the grinding process and its fragment contaminating is expected with the mitochondrial fraction by the process of centrifugation. Even the materials used in mitochondrial separation which do not contain the chloroplast but leucoplast or chromoplast, may be mixed with the mitochondrial fraction. In fact, from the result obtained in this experiment, about 15 percent of total chloroplast contaminated in the mitochondrial fraction. Already Menke(1) and Neish(2), had investigated the levels of

mineral content in chloroplast isolated from several species, the potassium content was found at first by Menke, but denied by Neish. From this result, a little potassium was found in the particulates varying with the levels by grinding means and mediums or hydrogen ion concentration of the grinding solution. This fact suggests that the potassium moves freely into and out of the particulates as the inner or outer conditions change. As to phosphorus, magnesium and sodium, a small part of the whole content was similarly lost during the isolation, and magnesiun and calcium were contained at a high level in the chloroplast. There were many remarkable researches on the biochemical reaction regarding the cell particulates but only a few are found concerning the autonomous oxygen uptake of isolated particulates.

Warburg, and Lüttgens(20) reported that the green extract showed the respiration which rapidly declined in oxygen uptake, and carbon monoxide reduced it by 50 per cent. From the observation that the oxygen consumption increased by the addition of polyphenol, pyrocatechol, etc it is considered that oxygen was transfered by a copper oxidase. Similarly Clendenning and Gorham(21) observed the oxygen absorption in crude leaf macerates of spruce and other gymnosperms, or in crude chloroplast suspension of the bean leaves.

Gerretsen(22), in the crude avena chloroplast observed the same phenomena. The results obtained from the barley seedling and other species were that the most autonomous oxygen uptake as found in the chloroplast or leucoplast can be compared with the mitochondrial fraction with the exception of the potato mitochondria. The decrease of oxygen uptake during a short experimental process was also reported in the Hill reaction of photosynthesis, and the cause was not proved. As a further problem, different from the case of the animal, mitochondria are not the major particulate in the higher plant cell, the mechanism of oxygen uptake in the chloroplast relating to potassium must be fully investigated.

IV. Summary

The isolation of intercelullar particulates was examined to clarify the biochemical properties of the particulate relating to the role of potassium or its distribution in the cell. As materials barley and several other plant species were used. The isolation test was initially performed and the influence of the centrifugal power and the duration time was examined, the identification of contamination, the effect of grinding methods and medium on the mineral content, and then the oxygen uptake of particulates was determined. The mineral contents of the isolated chloroplast, especially potassium, phosphorus, magnesium, and sodium were found to be easily lowered by the conditions of isolation. The activity of the isolated chloroplast expressed as the oxygen uptake was also changed widely by the method of isolation, that is, grinding means or medium, and hydrogen ion concentration. The yield of the chloroplast and the contami-

nation of the chloroplast fragments to the mitochondrial fractions were examined from the distribution of chlorophyll in each fraction by using the separation method, modified in this experiment. The chloroplast fraction was obtained at the good yield of 48 per cent in barley leaves and 34-53 per cent in other species. In some species a few amyloplast were mixed in the chloroplast fraction. On the other hand the mitochondrial fraction was obtained including the chloroplast fragment at the order of 15 per cent of total chloroplast, because by this grinding means the mitochondria fraction could not be separated successfully.

The mineral content was measured in the isolated chloroplast and in the intact leaves, to calculate the ratio of the content of the elements in and outside the particulate. Magnesium and calcium were found to be condensed in the chloroplast, on the other hand potassium content was found to be lower in it, so it may be considered that most of potassium was contained in the cell sap, and then moved easily into or out of the chloroplast. Autonomous oxygen uptake in each separated fraction was measured, and the most active oxygen uptake was found in the chloroplast fraction in barley seedling. The same tendency was also observed in the other species. This was contrary to the general conception that mitochondria is a place of respiratory reaction in the animal tissue.

As the isolation method of cell particulates had been performed by many investigators, their isolation methods were summarized, discussed and compared with our method. Further more, the investigation on the nature of oxygen uptake in the chloroplast fraction and role of potassium through the respiration of particulates is an important problem for the future.

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