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Both haemin and chlorophyll have the porphyrin ring, and their pigments are similar in chemical constitution.

Isolating the catalases in a crystalline form from an animal, a plant and a yeast, we demonstrated that they had haemin as their prosthetic group.

We minutely studied the prosperity and decline of plant catalase in the natural kingdom.

We proved that there was a very intimate relation between photosynthesis and catalase after we had repeatedly investigated the distribution of catalase in the body of the plant, prosperity and decline of plant catalase in a whole day, the relation between the catalase and the growth of vegetable, and the relation of the disappearance of chlorophyll and the catalase.

We found that there were great disparities in the optimum temperature between warm-blooded animals and cold-blooded ones, and between summer vegetables and winter ones. This was a very interesting finding of the biological adaptability to nature, and we demonstrated that the disparities between the optimum temperatures of the various catalases are due to the differences of the apo-enzymes, the protein parts of the enzyme molecules.

INTRODUCTION

Hydrogen peroxide, which is produced as an intermediate substance in the course of metabolism, has a very injurious effect on the cell-tissue.

It is a part assigned to the catalase to decompose hydrogen peroxide and prevent the physiological defects ; that is to say, the catalase is an enzyme which has the power to decompose hydrogen peroxide into water and oxygen, and to convert conc. monoethyl-hydroxide into acetaldehyde and alcohol, though in the latter case it acts only slightly, and every living tissue or organ has this catalase.

Many great results of the scientific research concerning animal catalase have been achieved by K. Zeile and H. Hellström[1], K.G. Stern[2], J.B. Sumner and A. L. Dounce[3,4] etc, and animal catalase has already been isolated as crystals, and its constitution has been cleared up by these scientists.

Though there are students of plant catalase such as M. Gračanin[5], H. v. Euler [6,7], A.C. Neish[8] and H.S. Olcott[9], the studies on plant catalase are in very poor way compared with studies on animal catalase.

Since 1939 we[10] have minutely studied the prosperity and decline of this enzyme in the vegetable kingdom, inquiring into the properties of enzyme, and have made a suc-

cess of isolating a highly purified catalase as crystals, and at the same time we added to our stock of knowledge the discovery about the mechanism of the photosynthesis.

METHODS

To determine catalase activities, one can adopt a method to measure the volume of oxygen which is generated under the chemical action of enzyme upon hydrogen peroxide, or a method to titrate hydrogen peroxide which remains undecomposed with potassium permanganate solution in presence of sulphuric acid[11] ($2KMnO_4+5H_2O_2+4\,H_2SO_4=2KHSO_4+2MnSO_4+8H_2O+5O_2$), or else by iodometry.

We adopted the iodometry, i.e., the method of titrating free iodine, generated under the action of potassium iodide upon hydrogen peroxide upon which catalase has not reacted, with sodiumthiosulfate[12,13].

To state in full, to 5 ml. of 0.02 N hydrogen peroxide solution was added 1 ml. of M/15 Sørensen's phosphate buffer (pH 6.8) and 1 ml. of catalase extract suitably diluted so that 10 to 20% of the peroxide was decomposed in two minutes at 25°C.

At the end of this period 10 ml. of 10% sulfuric acid was added.

The peroxide remaining was determined iodometrically ; 10 ml. of 1% potassium iodide and three drops of 1 N ammonium molybdate solution were added, and the liberated iodine was titrated 0.02 N sodium thiosulfate with starch as an indicator.

By measuring the amount of decomposed hydrogen peroxide (or rather, the amount of hydrogen peroxide which has not been decomposed), upon which 1 ml. of catalase solution extracted under definite conditions has acted, we can know the total activities of 1 ml. of various extractive matters.

As catalase acts monomolecularly in an early stage of action, we can compare the catalase activities of various samples by the following method to indicate the total activities of 1 ml. of various catalase extractive matters of every sample.

$$\text{Catalase activity } (f)=k\times 100$$

$$k=1/t\ \log\ a/(a-x)$$

k : velocity constant of the monomolecular reaction.

a : titrimetric value of 0.02 N $Na_2S_2O_3$ at zero minute of reaction.

$a-x$: titrimetric value of 0.02 N $Na_2S_2O_3$ after a fixed time of reaction.

t : time of reaction (0-2-4-6-8 minutes).

But we must in times compare the catalase activities by H. v. Euler and Josephson's method[14].

The purity of an enzyme preparation is expressed as

$$\text{Kat.-f.}=\frac{\text{velocity constant}}{\text{g. enzyme used in the test}}$$

To obtain the dry weight of the proteins of sample used, the sample was dia-

lyzed four times against 500 ml. of distilled water for 24 hours.

The sample was then dried up and made constant weight at 105°C and weighed in a semi-micro balance.

Optimum temperatures were measured by the method shown in the following.

To 5 beakers, each containing 5 ml. of 0.02 N hydrogen peroxide in 1/90 M phosphate buffer of pH 6.8 and kept at the temperature to be tested, was added 1 ml. of suitably diluted catalase solution.

After every 2, 4 and 6 minutes, stop the enzyme reaction with 10 ml. of 2 N H_2SO_4 and cool the beakers in an ice-water bath.

Make a blank test adding H_2SO_4 before addition of enzyme solution.

Then determine the remaining quantities of peroxide by iodometry and calculate the velocity constant.

Absorption spectra were measured by a Beckman spectrophotometer Model DU. using 1 cm Silica cell. For electrophoretic analysis, Tiselius apparatus was used.

Mobilities and apparent concentration ratios of each component were calculated at descending patterns according to Longsworth and Tiselius[15].

EXPERIMENTAL RESULTS

(A) Prosperity and Decline of Plant Catalase in the Natural Kingdom

(1) Experiments on the catalase activities in parts of vegetable

Examining the distribution of catalase in each part of vegetables, i.e., in the parts of rice-plant, barley, wheat, spinach, okra, sweet potato, amaranth, radish and Japanese cabbage, we found that catalase activities are strongest in the leaf part as

Table 1. Catalase activities corresponding to the parts of rice-plant, wheat and barley.

Parts	Sample, date of collection, lenght and the state of growth				
	Rice-plant (Asahi No. 1)			Barley (Shindo No. 3)	Wheat (Norin No. 21)
	July 20th 55 c.m. Tillering period	Sept. 14th 125 c.m. Earing period	Oct. 2nd 129 c.m. Ripening period	May 6th 111 c.m. Flowering period	May 14th 83 c.m. Fructifying period
Leaf (Point)		1467.38	912.16	832.50	1350.40
Leaf (Latter half)	810.04	1249.04	891.56	565.96	1222.00
Ear	—	123.34	56.91	102.52	357.13
Stem	40.76	17.44	6.91	30.35	43.28
Root	2.99	3.25	2.31	6.74	7.13

Table 2. Catalase activities corresponding to the parts of other various vegetables.

Parts	Sample, date of collection and length							
	Spinach	Okra	Sweet potato		Potato	Amaranth	Radish	Japanese cabbage
	Jan. 30th	July 14th	Okinawa No. 100 Oct. 20th	Beniaka Oct. 22nd	June 30th	Oct. 19th 30th	Oct. 12th	Nov. 5th
	20 c.m.	150 c.m.			57 c.m.	154 c.m.	25 c.m.	20 c.m.
Leaf	163.38	102.98	78.15	93.36	77.43	154.24	151.90	53.81
Petiole	15.08					29.02	14.20	1.89
Stem (vine)		2.25	3.48	4.21	23.04	23.35		
Root Upper part	21.61	5.21				5.92	12.31	1.21
Root Lower part	17.27		11.00	12.98	1.49		4.37	

compared with the ear, the stem and the root-part. (Tables 1 and 2)

Every organ or tissue of vegetable body is indeed conducting its respective vital function systematically and harmoniously as a whole, and it is by no means functioning separately ; but it is very interesting that the catalase activities of vegetable body distinctly vary with its parts, and this can be said to be an evidence of the existence of the intimate relations between the chlorophyll and the catalase.

(2) Prosperity and decline of plant catalase in a whole day

Observing the activities of plant catalase in a whole day we found that they rapidly increased from sun-rise, and attained to their highest degree at noon, then gradually decreased, and at sun-set they suddenly decreased. (Fig. 1, Tables 3 and 4)

Table 3. Prosperity and decline of catalase of barley in a whole day (May 22nd 1942, at fructifying period).

Time to collect samples (o'clock)	Temperature °C	Catalase activity (f.)	Remarks
4	11°	602.04	Before daybreak
8	15°	977.40	
12	25°	1273.95	Midday
16	22°	1173.38	
20	12°	758.25	Sunset
24	10°	504.99	Midnight

Table 4. Prosperity and decline of catalase of wheat in a whole day. (May 21st 1942, at fructifying period).

Time to collect samples (o'clock)	Temperature °C	Catalase activity (f.)	Remarks
4	11°	1019.15	Before daybreak
8	14°	1226.38	
12	24°	1433.40	Midday
16	22°	1321.52	
20	13°	1176.94	Sunset
24	11°	1024.03	Midnight

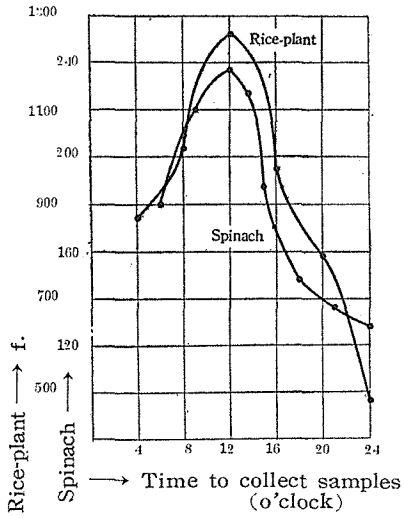


Fig. 1. Prosperity and decline of catalase activities of spinach and rice-plant in a whole day.

(3) Relation between the growth of vegetable and the catalase activities

The relation between the growth of plant and the catalase activities is also a very important question.

To solve this question we repeated many experiments from 1942 to 1944.

We gave the results of determination of rice-plant (Asahi No. 1), barley (Shindo No. 3) and wheat (Norin No. 21) at 6 periods, i.e., at tillering, pre-earing, earing, flowering, ripening and fructifying periods (see Figs. 2 and 3).

Generally surveying the results, we saw in all cases of rice-plant, barley and wheat, catalase activities of stem and root showed little difference among their growing periods, but in case of leaf part the catalase activities increased successively from tillering to pre-earing, and from pre-earing to earing period, and attained the

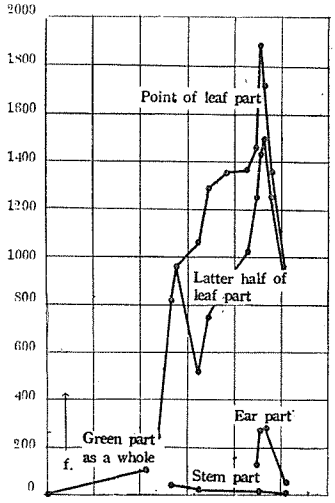


Fig. 2. Prosperity and decline of catalase activities of rice-plant in each growing period.

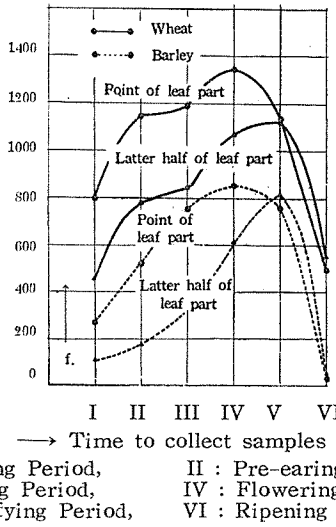


Fig. 3. Prosperity and decline of catalase activities of barley and wheat in each growing period.

highest peak at flowering period and then rapidly diminished.

(4) Pathological study upon the catalase activities of plant

Sometimes plant loses its health and has malfunction in its physiology from some exterior or interior causes and presents so-called symptoms.

As such a sick plant is not able to operate the normal photo-synthesis, there natu-

rally occurs differences in catalase activities between the sick plant and a normal one.

We show in Table 5, our experiments determined and compared the catalase activities of healthy leaf and a sick one using the leaves of Helminthosporium Disease of Rice-plant (Ophioblus Miyabeanus Ito et Kurib), of Potato leaf blight (Alternaria Solani (Ell. et Mart) Sor.), of French bean Anthracnose of scarlet runner (Glomerella Lindemuthianum (Sacc. et Magn.) Shear), and of Strawberry leaf Spot of leaf blight (Mycosphaerella Fragariae (Tul) Lindau).

From Table 5 we can clearly see that catalase activities conspicuously differ between spotted sick half and healthy one, though both are the leaves of plants grown under the same conditions.

The difference is of course influenced by the degrees of disease. We can even say that generally, before symptoms of disease come to appear, there was a change occurring in the body of plant, and the assimilating power gradually weakened, and the amount of chlorophyll produced could not attain the amount of it decomposed, and at last the leaf becomes yellow and show the symptoms of disease.

To make these processes clearer, we minutely experimented on a sick leaf afflicted with Helminthosporium disease of rice-plant.

Germinating rice-seeds in two groups of pots, and when the rice-plants became 30 cm. high, we inoculated them by spraying with purely cultured Ophiobolus Miyabeanus Ito et Kuribayashi ; and after they were infected with the disease, we determined catalase activities and chlorophyll, and compared them with the control.

The experimental results are shown in Table 6.

We believe that the biochemical study on the vegetable pathology is worth studying to solve various questions of physiological botany and that applying the results of the study, i.e., by means of determining catalase activities, we can examine the

Table 5. Influence of disease germs of plant on catalase activities.

	Rice-plant	Potato	French bean	Strawberry
	Helminthosporios	Leaf blight	Anthracnose of scarlet runner	Leaf spot of leaf blight
Healthy leaf	655.35	95.20	116.17	73.88
Sick leaf	423.21	32.05	92.39	44.05
Symptom of disease	Several more than ten grayish brown spots of 2mm. in diameter appeared and whole leaf became yellowish.	2 brown spots of 6mm. in diameter per one leaf appeared and surrounded by veins.	3 round light brown spots of 1mm. in diameter per one leaf appeared.	Several round spots of 3mm. in diameter appeared on the face of leaf.

Table 6. Influence of helminthosporium disease of rice-plant on catalase activities and chlorophyll.

Lapse of time (Day)	Sick leaf		Healthy leaf	
	Catalase activity (f.)	Chlorophyll (fresh matter) %	Catalase activity (f.)	Chlorophyll (fresh matter) %
0	308.14	0.185	308.14	0.185
2^{+}	272.11			
3	203.75	0.163	297.04	0.179
4	163.16			
5^{++}	144.04	0.151	298.60	0.181
6	120.53			
7^{+++}	100.89	0.115	295.08	0.180

$^{+}$: 30~40 brown spots (0.3mm in diameter) appeared on each leaf.
$^{++}$: Each spot grew elliptical (2mm X 0.5mm).
$^{+++}$: At last whole leaf became yellowish.

health of vegetables, and can take some measures to meet their diseases.

(5) Correlation between photo-synthesis and catalase

It can be easily deduced from the prosperity and decline of the plant catalase in a whole day, that the degree of the sunlight is closely connected with the catalase activities.

Collect several leaves of a size from a certain plant, and divide them into two groups, and let one group act the normal photo-synthesis and the other be wrapped in black paper and shut out the sunlight all day long, and then determine catalase activities of both groups. Or wrap the half part of leaf and shut out the sunlight, and then determine the catalase activities of each half of the leaf and compare them. We show the results of the above experiments in Table 7.

As can be easily found in Table 7, when the sunlight was shut out by the black paper, the catalase activities of the leaf conspicuously declined. In the same leaf, however, when we wrapped half of it lengthwise or breadthwise, the catalase activities of the part which was shut out from sunlight distinctly declined. In short, the photo-synthesis is closely connected with the catalase activities.

(6) Interrelation between vitamin C, catalase and chlorophyll

Formerly we reported the relation between the discolouration of amaranth and vitamin C content of reduced form and oxidised form in order to explain the role of vitamin C in vegetable body[16]. The amaranth is, it appears to us, a most adequate sample for the purpose of testing the relation between its chlorophyll and catalase activities.

It is a matter of course that, the discolouration of amaranth starts with the upper extremity and extends to the lower extremity, for example, a tricoloured amaranth, as it is growing, changes the colour of its upper green leaves first, and then its lower green leaves into yellowish red, and especially in case of a leaf, the discolouration extends from the base to the top of it. We collected tricoloured and purple red species of amaranth and examined catalase activities of leaves in almost equal size before and after discolouration.

The experimental results are shown in Table 8.

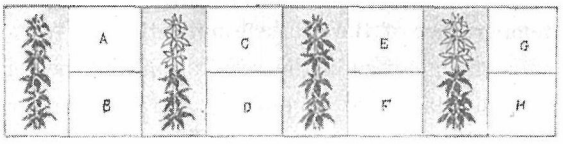
Table 7. Relation between the measure of sunlight and catalase activities.

	Potato			Pumpkin	
I		113.65	I		432.72
II		90.96	II		256.75
III		110.21	III		405.96
IV		96.78	IV		287.47

I : Control
II : Entirely wrapped
III, IV : Half wrapped

Table 8. Relation between discolouration of amaranth and catalase activities.

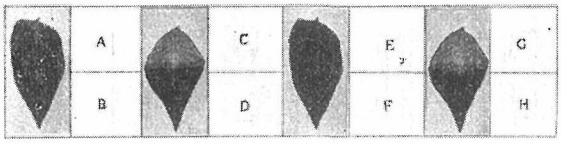
Tricoloured species				Purple red species			
Before discolouration		After discolouration		Before discolouration		After discolouration	
Green		Yellowish red		Dark purple		Red	
A	91.41	C	33.63	E	122.18	G	38.90
B	100.34	D	09.091	F	137.26	H	160.36
Green		Green		Dark purple		Dark purple	



As we can understand from Table 8, both the tricoloured and purple red species of amaranth had, before discolouration, almost the same catalase activities in the upper part and lower part of their leaves, but after discolouration began, and the colour of the upper part of leaves turned red or yellowish red, the discoloured part much differed from the non-discoloured part in catalase activities, i.e., catalase activities of the part which had lost its chlorophyll, diminished to 1/3~1/4 of those

Table 9. Relation between disappearance of chlorophyll and catalase activities.

Tricoloured species				Purple red species			
Before discolouration		Half discoloured		Before discolouration		Half discoloured	
Green		Yellowish red		Dark purple		Red	
A	127.39	C	37.68	E	103.73	G	30.57
B	134.42	D	160.41	F	104.11	H	170.73
Green		Green		Dark purple		Dark purple	



of the part which kept its chlorophyll.

These facts can also be observed in a leaf, and the results of the experiments on a leaf not discoloured yet, and on a half-discoloured one are given in Table 9.

It is clear from Table 9 that, before discolouration the catalase activities were almost equal in the upper part and in the lower part of both tricoloured and purple red species of amaranth, but after discolouration began, and when the amaranth lost a part of its chlorophyll and the colours of its leaves showed difference each other, catalase activities of the upper part (discoloured part) became 1/5～1/6 of those of the lower part (non-discoloured part). Very interesting it is that the parts of the same leaf so distinctly differ in catalase activities.

(B) Several Observations on the Properties of Catalase

(1) Optimum temperature of the catalase activities

The organic matter which is used in the respiration and the fermentation is originally produced by the assimilation of vegetables, and it stores great many energies.

In the respiration and the fermentation, these energies are made free and become the motive powers of all physiological actions and thus the anagenesis is in operation.

These free energies are not only spent as the kinetic energies, but also generate the heat and maintain the temperature of the warm-blooded animals.

The temperature of plants is almost equal to that of the environment, and naturally the reason is that the heat of plants is taken away from their wide sur-

face by the evaporation.

As the temperature of plants is absolutely controlled by the temperature around them, it is a matter of course that there is great difference between the temperature of winter plants and that of summer ones.

We undertook the following experiments expecting that we might find some difference in the optimum temperature of catalase activity between winter and summer.

We chose as examples of vegetables, spinach, Japanese cabbage, turnip and winter radish, and examined each optimum temperature of catalase activities of their green leaves.

We give the experimental results in Table 10.

Table 10. Experiments on the optimum temperature of the catalase activities.

Group		Sample	Optimum temperature (C)
A	Green leaf catalase	Spinach	about 15.0°
		Turnip	〃 20.0°
		Japanese cabbage	〃 17.5°
		Winter radish	〃 12.5°
B		Rice-plant	〃 35.0°~37.5°
		Wheat	〃 32.5°
		Summer radish	〃 35.0°
C	Liver catalase	Toad (as of October)	〃 12.5°~15.0°
		Tunny	〃 20.0°
		Oyster	〃 20.0°
		Snake	〃 25.0°
		(as of September) Eel	〃 30.0°
D		Cow	〃 40.0°
		Rabbit	〃 40.0°
		Snail	〃 40.0°
		Cat	〃 40.0°
		Cock	〃 50.0°

As we show in group A of Table 10, catalase of every sample acts most fittingly at the temperature lower than 20°C, i.e., catalase of spinach at 15.0°C, of Japanese

cabbage at 17.5°C, of turnip at 20.0°C and of winter radish at 12.5°C.

While, we measured and compared the optimum temperatures of catalase activities of each leaf part of the wheat, the summer radish, and the rice-plant; as we show the experimental results in group B of Table 10, we found that winter vegetables distinctly differed from summer vegetables in the optimum temperatures of their catalase activities.

Indeed it is natural that the optimum temperature of the plant catalase activity is not fixed at all and it is different according to the various species of plants, but in the same species, as the experimental results on radishes show clearly, the optimum temperature of the plant catalase activity conspicuously varies with the difference of the temperature of the seasons of cultivation such as winter and summer.

After the above experiments, we have tried to compare the optimum temperatures of the catalase activities of warm-blooded animals with those of cold-blooded animals[17].

The results of our study are such that the optimum temperatures of the catalase activities of cows, rabbits, snails and cats are around 40°C and those of cocks are such high temperature as 50°C, but on the contrary, those of toads are 12.5°C (as at October), those of snakes 25°C (as at September), those of tunnies and oysters 20°C, those of eels 30°C, and thus we have seen their optimum temperatures of the catalase activities are so remarkably different from each other (Table 10).

We adopted the following method to indicate the degree of destruction of catalase by heating according to the chemical kinetics.

$$H_T = 1 - \frac{K'}{K}$$

H_T : degree of destruction at each temperature.
K' : reaction velocity constant when they are heated.
K : reaction velocity constant when they are not heated.

Testing the stability of the plant catalase on heating, we found that the vegetables whose optimum temperatures are low, were much less stable on heating than those whose optimum temperatures are high ; that is to say, though the former lost its greater part of the plant catalase when heated to 55°C for only five minutes, the latter was reduced to the same extent when heated to the same degree for 25 minutes (Fig. 4).

This is the same with the animal catalase (Fig. 5).

It seems to us this is a very interesting discovery.

This discovery enables us to get a general idea of the mysterious phenomenon that the catalase adapts itself to the nature by changing its optimum temperature according to the temperature around the living things which controls the temperature of the plants and animals themselves.

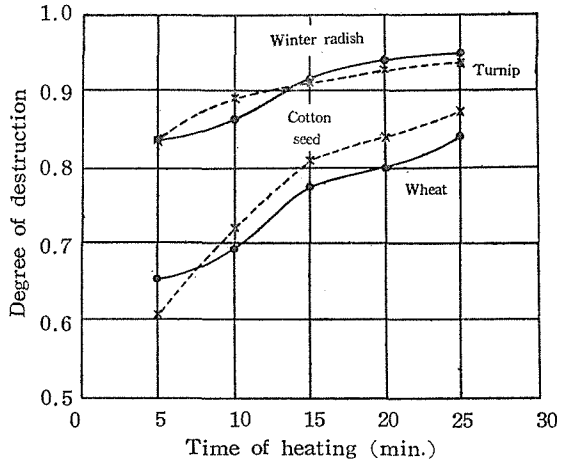


Fig. 4. Degree of destruction of plant catalase on heating (55°C).

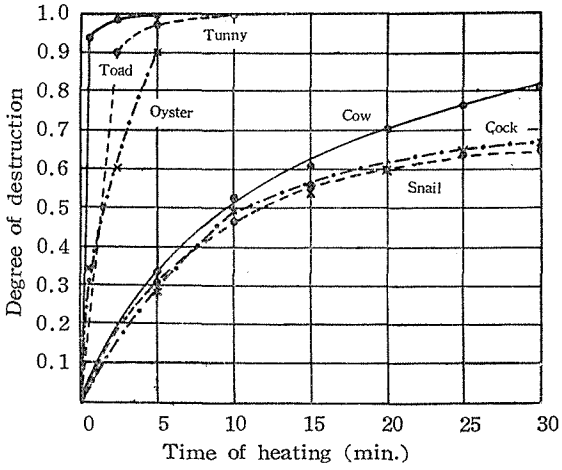


Fig. 5. Degree of destruction of animal catalase on heating (55°C).

But the enzyme solution used for this measurements was too crude to decide whether the difference was due to the protein part of enzyme itself or to ions or other proteins accompanied.

In order to investigate the problems above mentioned, we isolated crystalline catalases from both cow and toad livers.

Table 14 shows that these optimum temperatures of catalases are independent of enzyme purities. In other words, toad and cow liver catalases are different in their optimum temperature and these differences are same even if we measure such pure preparations as crystalline enzymes.

This is the same with the plant catalase, namely, the optimum temperature of the rice-plant leaf catalase (this plant grows in summer) was 35°C, but that of the spinach leaf catalase (this vegetable grows in winter) was 15°C, when we measured

their extracted solution. These optimum temperatures closely resemble the temperatures at which they grow.

Now, as we prepared the crystalline catalases from both spinach and rice-plant, we measured their optimum temperatures.

As we show in Table 14, the optimum temperature is independent of the enzyme purity.

These results indicate that the difference of the optimum temperature is attributed to that of each enzyme protein itself.

(2) The optimum hydrogen ion concentration

In order to make the optimum pH of the plant catalase clear, we experimented on the leaves of wheat, turnip, amaranth, Japanese cabbage, spinach and the cotton-seed.

We observed that the optimum hydrogen ion concentration of the plant catalase in every sample is near pH 7.0 (6.8~7.1).

This is the same with the animal catalase.

(3) Comparative researches in the stabilities of plant catalase and animal catalase

Formerly, Dr. K. Kondo, D. Yonezawa, H. Chiba and F. Kawai[18] succeeded in comprehending many important natures of the green leaf enzyme in general, after they had minutely and repeatedly studied on the plant carbonic anhydrase which had been regarded as one of the most unstable sorts of the green leaf enzyme, and accordingly as one of the most difficult sorts of enzyme to be isolated. Especially we should not forget that they found out the plant carbonic anhydrase was perfectly stable in 0.1 M NaCl solution though it was extremely unstable in water.

This finding must have greatly contributed to open the way for their success in isolating the plant carbonic anhydrase from a green leaf.

Though indeed the enzyme chemistry has made great progress, we have found that the researches in green leaf enzyme have been conducted very rarely, and notwithstanding that deep and thorough studies have been made on animal catalase, the researches in the plant catalase have made very slow progress.

We think this may be due to the fact that students have not often experimented on green leaves to study on the plant catalase, as the green leaf enzyme is very unstable in water and when the enzyme solution is diluted with water or dialysed against water, the enzyme activity declines conspicuously, and the green leaf tissues have never been regarded as a good sample for the studies on the enzyme.

In order to study the plant catalase, or above all in order to isolate it, we must first look for a good stabilizer.

We show in Fig. 6 for example, how the plant catalase is much more unstable than the animal catalase.

We have got the results shown in Fig. 6, comparing the prosperity and decline of activity of the animal catalase (the cow liver catalase) as time goes by, with

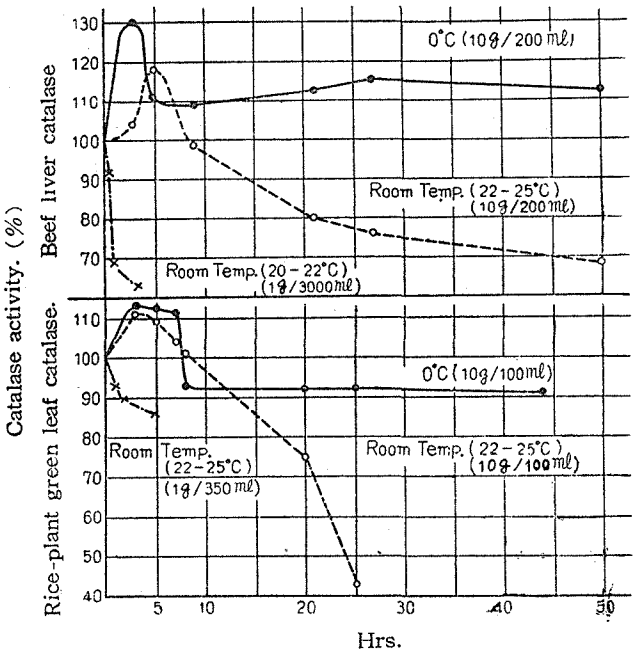


Fig. 6. Comparison of the stability of the solution of animal catalase with that of plant catalase.

Table 11. Experiments on the stabilizers of the green leaf catalase of the rice-plant.

Densities of salts	Catalase activity		
	Initial k	After 6 hrs. k	After 24 hrs. k
0.5 M Phosphate buffer (pH=7)	0.0425 (100%)	0.0393 (92.5%)	0.0377 (88.7%)
0.5 M NaCl	〃	0.0098 (23.0%)	0.0064 (15.2%)
0.5 M KCl	〃	0.0073 (17.2%)	0.00509 (12.0%)
0.5 M $MgSO_4$	〃	0.0334 (78.8%)	0.0094 (22.1%)
0.5 M $NaHCO_3$	〃	0.0423 (99.6%)	0.0377 (88.7%)
H_2O	〃	0.0316 (74.4%)	0.00857 (20.1%)

that of the plant catalase (the rice-plant green leaf catalase).

The stability naturally differs according to the dilution.

Our dilution of the solution of the cow liver was 10 g/200 ml., and it was half as dense as that of the rice-plant green leaf (10 g/100 ml); however, as indicated by the two curves showing the stabllities of their catalase activities at the room temperature in Fig. 6, plant catalase is obviously more unstable.

When we make the dilutions to such a degree as 1 g/3000 ml., the catalase is inactivated in a few hours.

As we show in Table 11, we compared the stability of the green leaf catalase of the rice-plant in the solution of 0.5 M phosphate buffer with each of its stabilities in the solution of 0.5 M NaCl, 0.5 M KCl, 0.5 M $MgSO_4$ and 0.5 M $NaHCO_3$, and found that the catalase was much more stable in the solution of 0.5 M phosphate buffer and 0.5 M $NaHCO_3$ than it was in that of 0.5 M NaCl, 0.5 M KCl and 0.5 M $MgSO_4$.

Then we extracted catalase from the tissues of green leaves with the solution of the above mentioned salts at such concentrations as 0.01, 0.05, 0.1 and 0.5 M, and compared the stabilities of the catalase in each of the cases.

As in Table 12 and in Fig. 7, we testified 0.01 M phosphate buffer was a very suitable stabilizer.

Then we examined the effect of the dialysis on the animal and plant catalase activities, and as we show in Table 13, when we dialysed the catalase against water for 48 hours, only 44.5% of the activities survived.

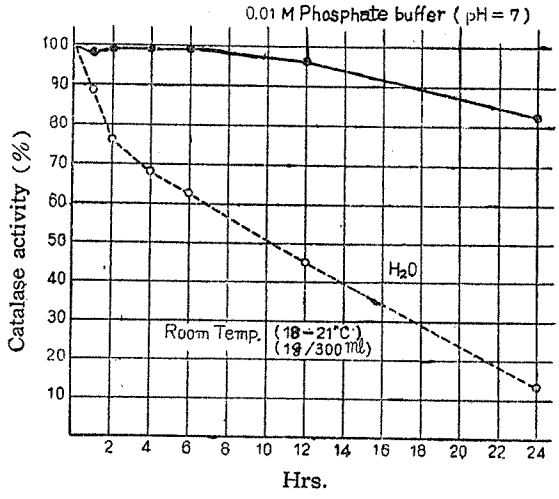


Fig. 7. Experiments on the stabilities of the rice-plant catalase.

But we found that when we dialysed the catalase against 0.01 M Na_2HPO_4 for 48 hrs., as much as 93.2% of the activities survived.

In case of the dialysis, NaCl cannot be used as a stabilizer on the catalase[26].

(4) Experiments on the influences of organic solvents upon catalase.

Table 12. Experiments on the stabilizers of the green leaf catalase of the rice-plant.

Densities of salts	Catalase activity		
	Initial k	After 6 hrs. k	After 24 hrs. k
0.01 M Phosphate buffer (pH=7)	0.0505 (100%)	0.0604 (119.6%)	0.0514 (101.8%)
0.05 M 〃	〃	0.0548 (108.5%)	0.0457 (90.5%)
0.1 M 〃	〃	0.0541 (107.1%)	0.0447 (88.5%)
0.5 M 〃	〃	0.0469 (92.9%)	0.0402 (79.6%)
0.01 M NaCl	0.0464 (100%)	0.0314 (67.7%)	0.0118 (25.4%)
0.05 M 〃	〃	0.0236 (50.8%)	0.0131 (28.2%)
0.1 M 〃	〃	0.0216 (46.6%)	0.0111 (23.9%)
0.5 M 〃	〃	0.0105 (22.6%)	0.0065 (14.0%)
0.01 M $NaHCO_3$	〃	0.0488 (105.2%)	0.0386 (83.2%)
0.05 M 〃	〃	0.0496 (106.9%)	0.0368 (79.3%)
0.1 M 〃	〃	0.0455 (98.1%)	0.0390 (84.1%)
0.5 M 〃	〃	0.0408 (88.0%)	0.0302 (65.1%)
H_2O	0.0505	0.0305 (65.7%)	0.0043 (9.2%)

Set at room temperature (18°~20°C). Dilution : 1 g/300 ml.

Table 13. Effect of dialysis.

	Initial activity k	After 48 hrs. k	Survived %
0.01 M Na_2HPO_4	0.0382	0.0356	93.2
0.05 M Na_2HPO_4	0.0382	0.0349	91.3
0.01 M $NaHCO_3$	0.0382	0.0346	90.6
0.05 M $NaHCO_3$	0.0382	0.0337	88.2
0.01 M NaCl	0.0382	0.0223	58.4
0.05 M NaCl	0.0382	0.0203	53.1
H_2O	0.0382	0.0170	44.5

Sample : green leaves of the rice-plant ; Dilution : 10 g/100 ml ; Temperature : 0~2°C.

Table 14. Experiments on the optimum temperatures of catalases in various stages of purification.

Spinach leaf catalase			Rice-plant leaf catalase		
Soln. Name	Kat.-f.	Opt. Temp.	Soln. Name	Kat.-f.	Opt. Temp.
A	14	15°C	A_1	28	35°C
B	70	15°C	B_1	40	35°C
C	180	15°C	C_1	430	35°C
D	2200	15°C	D_1	2260	35°C
E	3300	15°C	E_1	7120	35°C
F	15300	15°C			

Toad liver catalase			Cow liver catalase		
Soln. Name	Kat.-f.	Opt. Temp.	Soln. Name	Kat.-f.	Opt. Temp.
A_2	350	15°C	A_3	530	40°C
B_2	1100	15°C	B_3	2300	40°C
D_2	8600	15°C	D_3		40°C
E_2	21600	15°C	F_3	39500	40°C

It is said that many enzymes associate with cell components.

In green leaf, catalase also, exists in chloroplasts and it is not extracted by distilled water or buffer from them.

As association with particulate material is an unmitigated nuisance for isolation of highly purified enzymes, we tested various methods to make catalase into solution, for example, dioxane, picoline, ethanol, *n*-butanol and dry ice treatments.

We tested them on the effects upon the catalase activity and extractabilities, and found that *n*-butanol treatment was ideally suited to extract catalase from chloroplasts on the following three points.

Firstly, the catalase activity is not inactivated.

Secondly, the method has an excellent extractability.

Thirdly, the method is specific[27].

(C) Crystallization of Animal, Plant and Yeast Catalase

Since Sumner and Dounce[19] succeeded in crystallizing cow liver catalase in

1937, animal catalases have been isolated from several different sources in crystalline forms.

For example, this enzyme was crystallized from horse liver[20], sheep liver[21], rat liver[22] and beef erythrocytes[23].

Moreover, from a bacterium it was also purified[24].

Nowadays, it is believed that the animal catalase is most easily crystallized.

Then, it has become very interesting to investigate whether each crystalline catalase has the identical property or not, as we could prepare highly purified enzymes from various sources.

From this view-points, Herbert and Pinsent[24] isolated bacterial catalase in crystalline form.

Catalase activity is found in almost all kinds of plant materials so far as it has been investigated.

The amounts of catalase, however, are very low and the extracted enzyme solution is very unstable, compared with those of animals.

From these reasons, the plant catalase could not be crystallized yet by other students.

In 1942, the author[25] succeeded in crystallizing the enzyme from rice-plant leaves, using the dioxane method with which Sumner and Dounce[19] had crystallized the animal catalase for the first time in the world.

But the dioxane is too dear a solvent to be used for an enzyme purification in Japan, so that we have been trying to crystallize the plant catalase using no dioxane.

We[26,27] reported that the phosphate buffer was the best stabilizer of catalase, and that the butanol treatment adopted by Morton[28] to separate enzymes associated with insoluble particles was very suitable to extract catalase from chloroplasts.

Then, we have succeeded again in crystallizing the plant catalase from both spinach and rice-plant leaves, using this improved method[29].

In Fig. 8, the method used for the purification of the spinach leaf catalase is shown.

The total volume of the extracted solution was approximately 15.5 l. which had a Kat.-f. of about 14.

This Kat.-f. is very small compared with that of animal catalase shown in Figs. 10 and 11, i.e., the Kat.-f. of toad liver is 350 and that of cow liver 530 [30].

To this extracted solution, *n*-butanol was added to extract catalase from chloroplasts and to make a true solution. The yield of this treatment was 78%.

It is clear that the association of an enzyme with a certain particle is an unmitigated nuisance to isolate it in highly purified state.

To obtain readily the solution of catalase from chloroplasts in good yield is, then, a very important but difficult object.

From this viewpoint, the butanol treatment, we think, is a very suitable

Solution Name

Leaf-blade of Spinach (13 Kg)

wash, cut,
homogenize with 10 l. phosphate buffer (1/60M, pH 7.0)
press with cotton clothes

A — Green Suspension 15.5 l.

cool to 4°C
6.2 l. cold *n*-butanol (0.4 volume)
stir, 1 hour at 4°C
stand in an ice-box for 1 night
separate clear aqueous layer by siphon

B — Yellowish Clear Solution 13.5 l.

cool to 3°C
10.8 l. cold acetone (0.8 volume)
stir, 20 min., stand in an ice-box for 1.5 hrs.
centrifuge 3000 r.p.m. 5 min.

Precipitate

phosphate buffer (1/100M, pH 7.0) 1 l.
stir, 30 min.
stand in an ice-box for 1 night
centrifuge 3000 r.p.m. 1 hr.

C — Supernatant 1000 ml.

cool to 4°C
cryst. $(NH_4)_2SO_4$ 352 g (to 50% sat.)
centrifuge 3000 r.p.m. 30 min.

Precipitate

260 ml. distd. water
centrifuge 3500 r.p.m. 1 hr.

D — Supernatant 300 ml.

sat. $(NH_4)_2SO_4$ soln. 40 ml. (to 11.8% sat)
centrifuge

Supernatant

sat. $(NH_4)_2SO_4$ soln. 160 ml. (to 40% sat.)
centrifuge 4000 r.p.m. 20 min.

Precipitate

50 ml. distd. water
centrifuge 3500 r.p.m. 30 min.

E — Supernatant 50 ml.

sat. $(NH_4)_2SO_4$ soln. 10 ml. (to 16.7% sat.)
centrifuge 3500 r.p.m. 1 hr.

Supernatant

sat. $(NH_4)_2SO_4$ soln. 8 ml. (to 27% sat.)
centrifuge 3500 r.p.m. 30 min.

Precipitate

6 ml. distd. water
centrifuge 3500 r.p.m. 30 min.

F — Supernatant

stand in an ice-box for 2 days

Silkiness with stirring

Needle Crystals

Fig. 8. The method to prepare the crystalline spinach leaf catalase.

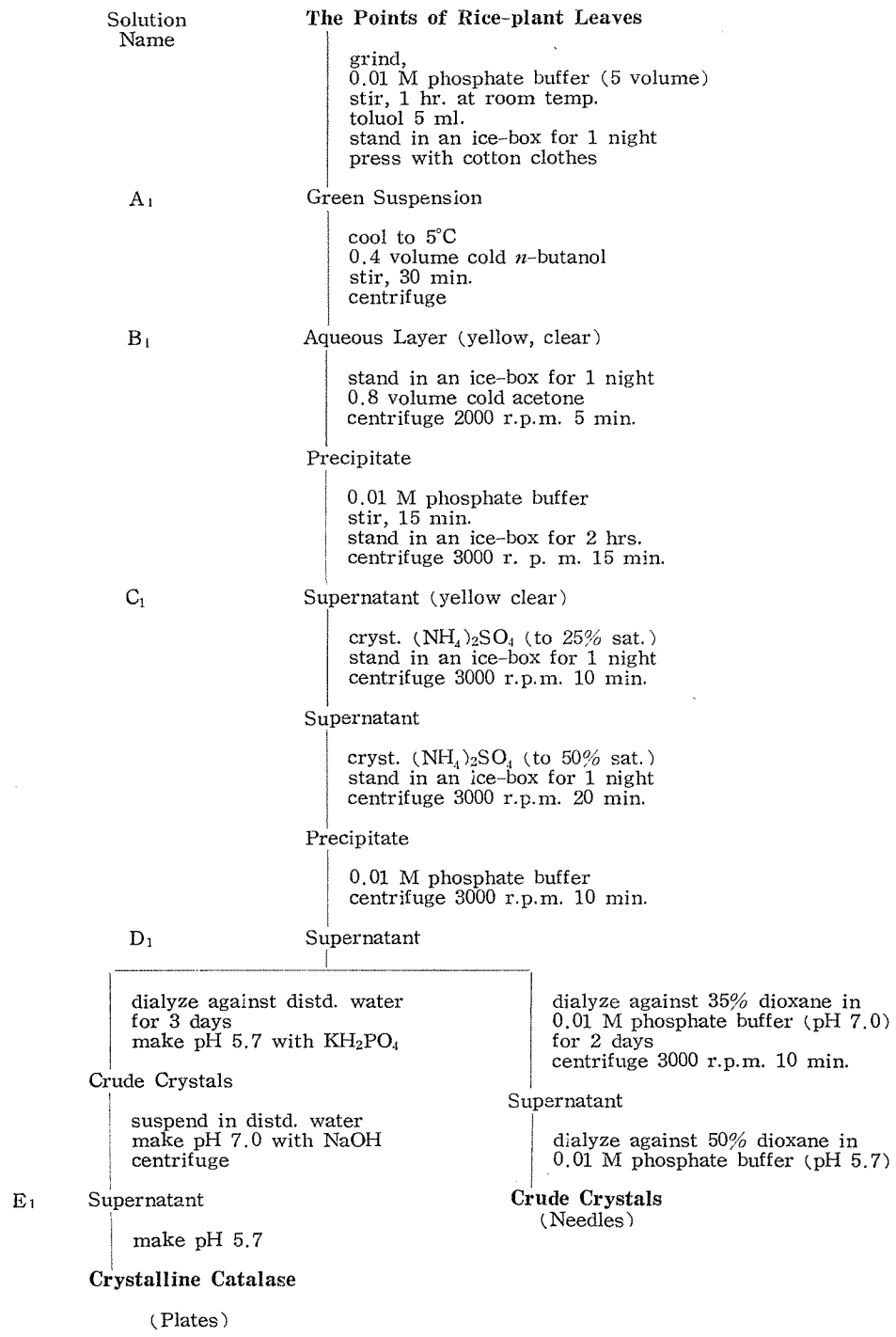


Fig. 9. The method to prepare the crystalline rice-plant leaf catalase.

Solution Name	
	Toad Liver
	wash, homogenize
	phosphate buffer (0.01 M pH 7.0)
	extract
	centrifuge
A_2	Supernatant Kat.-f. **350**
	0.8 volume cold acetone
	centrifuge
	Precipitate
	distd. water
	centrifuge
B_2	Supernatant Kat.-f. **1100**
	cryst. $(NH_4)_2SO_4$ (to 0.25 sat.)
	centrifuge
C_2	Supernatant
	cryst. $(NH_4)_2SO_4$ (to 0.5 sat.)
	centrifuge
	Precipitate
	distd. water
	centrifuge
D_2	Supernatant Kat.-f. **8600**
	stand in an ice-box
	Crystalline Catalase
	centrifuge
	Precipitate
	distd. water
	centrifuge
	Supernatant
	sat. $(NH_4)_2SO_4$ solution (to 0.5 sat.)
	centrifuge
	Precipitate
	distd. water
	centrifuge
	Pure Catalase Solution
E_2	Kat.-f. **21600**

Fig. 10. Scheme for preparing crystalline toad liver catalase.

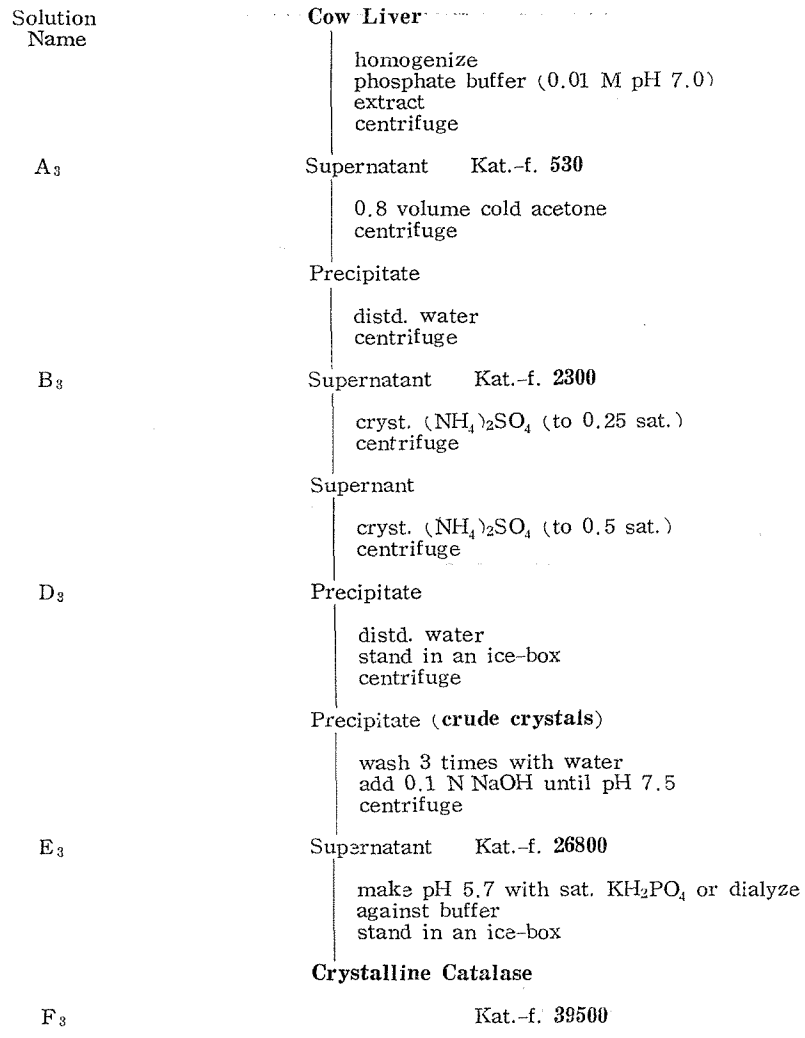


Fig. 11. Scheme for preparing crystalline cow liver catalase.

method.

After this treatment the method is the same as that of the animal catalase in Figs. 10 and 11.

The method used for purification of the leaf catalase of the rice-plant in shown in Fig. 9.

Only the points of rice-plant leaves at flowering period, were used for this purpose, as catalase activity was highest in this part and period.

The amounts of the rice-plant leaf catalase are greater than those of spinach catalase, so that the Kat.-f. of the extracted solution of the rice-plant is about two times as great as that of spinach.

The Kat.-f. 28 is, however, smaller than that of animals.

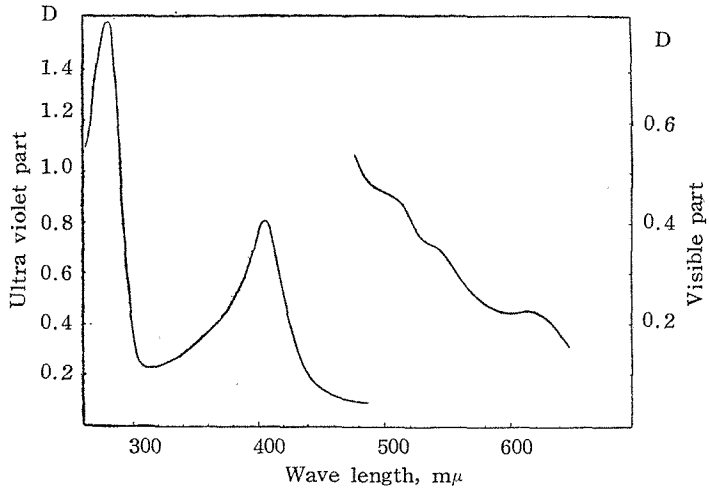


Fig. 12. Absorption spectrum of spinach leaf catalase (Kat.-f. 15300).

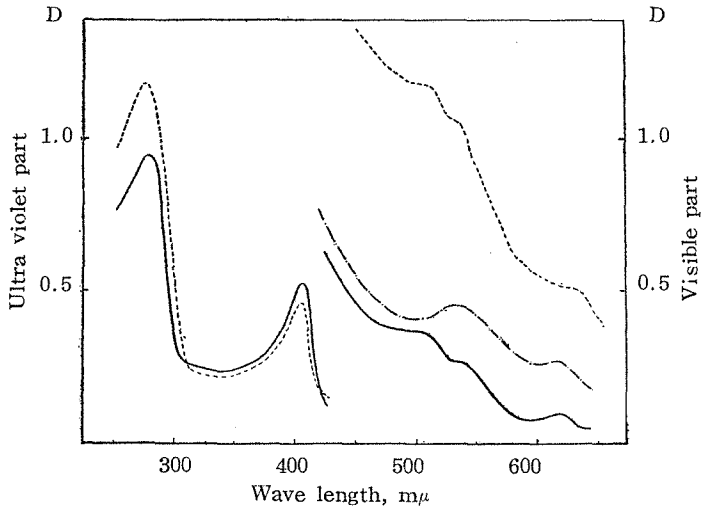


Fig. 13. Absorption spectra of various fractions of toad liver catalases in phosphate buffer (pH 7.0).
—·— Soln. B_2, - - - - - Soln. D_2, ——Soln. E_2.

The same method as the spinach purification was used.

In Fig. 12, the absorption spectrum of spinach leaf catalase (Kat.-f. 15300) is shown. It shows peaks at 278 and 405 mμ and shoulders at 505, 540 and 620 mμ, and it resembles that of animal catalases (Figs. 13 and 14), so that plant catalase also seems to have haemin as its prosthetic group.

Then, we succeeded in isolating catalase from baker's yeast into a crystalline form. We adopted the same method by which we had purified the plant catalase, i.e., the butanol treatment and the acetone and ammonium sulfate fractionations.

It was found that the crystalline enzyme thus obtained had the same prosthetic

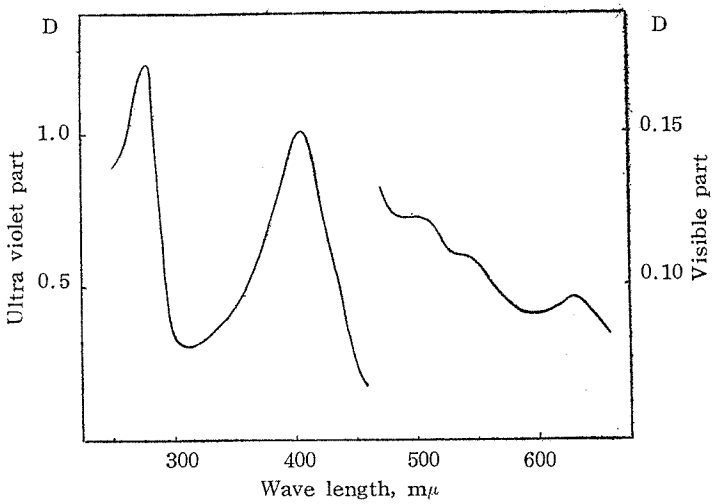


Fig. 14. Absorption spectrum of cow liver catalase. (Recrystallized crystal was disolved in distilled water containing a little amount of NaOH (pH 7.5)).

group as that of the animal and plant catalase.

SUMMARY

(1) Examining the distribution of catalase in various parts of vegetables, i. e., in the parts of rice-plant, barley, wheat, spinach, okra, sweet potato, potato, amaranth, radish and Japanese cabbage, we found that catalase activities are strongest in the leaf when compared with the ear, the stem and the root.

(2) Observing the activities of plant catalase in a whole day we found that they rapidly increased from sunrise, and attained their highest degree at noon; then they gradually decreased and at sunset they sharply decreased.

(3) In proportion to the growth of vegetable, its catalase activities increase, and attain their highest degree at the times of florescence and fructification.

(4) We found very interesting relations between the catalase and the discoloration caused by the disappearance of chlorophyll, and between the catalase activities and the etiolement.

(5) We minutely studied the interrelation among vitamin C, catalase and chlorophyll.

We pointed out that there was a close relation in quantity between plant catalase and chlorophyll, and that vitamin C was especially in close relation to the chlorophyll which had the central rôle in the mechanism of photosynthesis in chloroplast.

From the facts mentioned above, we concluded that plant catalase, chlorophyll and vitamin C play important rôles in photosynthesis.

(6) We observed that the optimum temperature of catalase of the winter vegetable is 12.5~20.0°C and that of summer one is 25.0~35.0°C, and it was our very interesting discovery that in vegetables, differing from the case of animals, their bodily temperature is much influenced by the atmospheric temperature, and the optimum temperature of plant catalase varies with the fluctuation of the bodily temperature.

(7) We purified the catalases of both spinach leaves and rice-plant leaves by the *n*-butanol treatment and isolated them into the crystalline forms.

The activity of the spinach leaf catalase has its optimum at 15°C, but that of the rice-plant leaf catalase at 35°C.

These optimum temperatures adapt to the circumstances where the plants grow, and they are independent of the enzyme purity.

(8) We have tried to compare the optimum temperatures of the catalase activities of warm-blooded animals with those of cold-blooded animals, and have seen that their optimum temperatures of the catalase activities are so remarkably different from each other.

(9) We have isolated catalase from the toad liver in a crystalline form.

Kat.-f. of the crystal is 21600 and its crystalline form is a square thin plate.

The activity of crystalline toad liver catalase has its optimum at 15°C and this temperature is the same as the optimum one of the extracted solution, namely, the optimum temperature is constant even if the enzymes are purified to take crystalline forms.

(10) The same experiments were made on the cow liver catalase and we found that the optimum temperatures of the cow liver catalase were 40°C, measuring both extracted solutions and crystalline enzymes.

(11) The absorption spectrum of the spinach leaf catalase is identical with that of the animal catalase.

We think that the plant catalase has haemin as its prosthetic group.

(12) Both haemin and chlorophyll have the porphyrin ring, and these pigments are similar in chemical constitutions.

It is very significant that both of them perform important physiological actions in animal and vegetable bodies and it can be said that biochemical researches in chlorophyll are very important subjects to be studied.

In conclusion the writer must express his sincere thanks to his respectable teacher Dr. K. Kondo for his sympathetic guidance and encouragement throughout the whole of the writer's studies.

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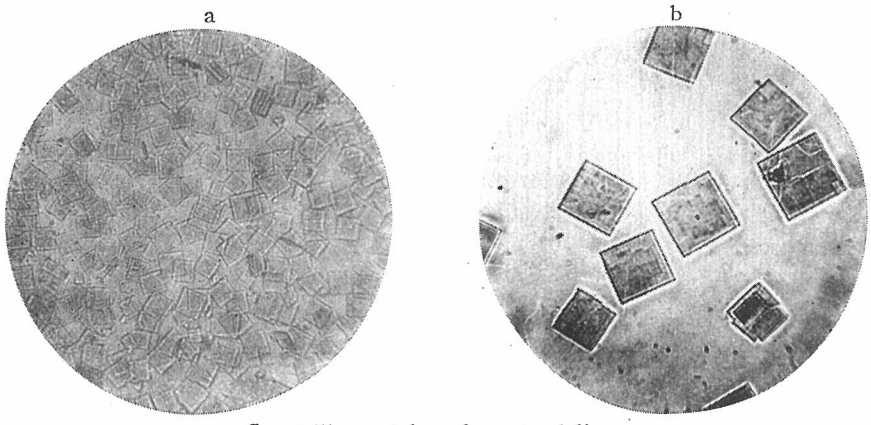
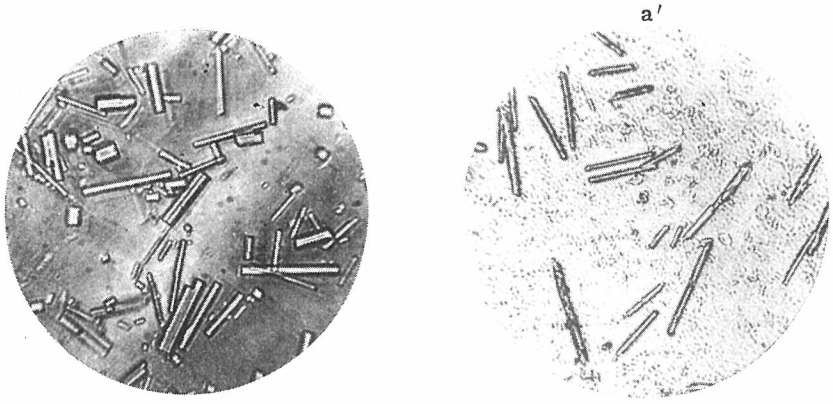
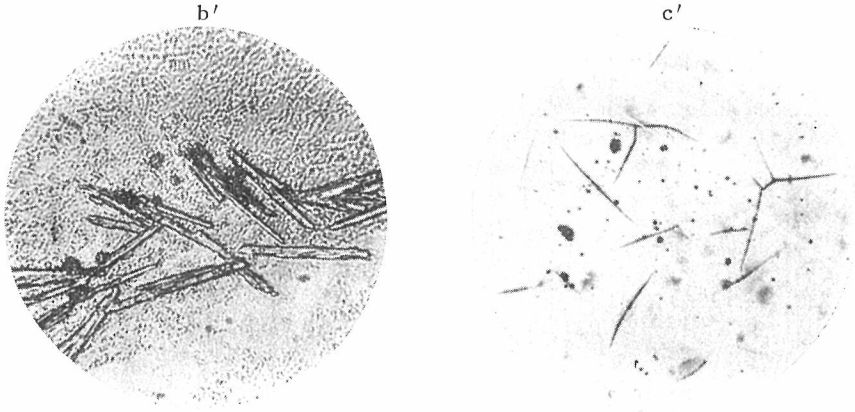


Crystalline catalase from toad liver.
(a…… × 300, b…… × 600)



Crystalline catalase from cow liver.
(× 400)



Crystalline catalase from rice-plant leaves.
a′, b′…… Crystallized from soln. E_3 (× 1000)
c′…… Crystallized by dialysis (× 200)