INTERRELATIONSHIPS OF CATALASE, PEROXIDASE, HEMATIN, AND CHLOROPHYLL [1]

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The formation of chlorophyll in the plant has long been known to depend upon an adequate supply of iron and, although it contains magnesium in its porphyrin group, the striking similarity between the prosthetic groups of chlorophyll and hemoglobin allows similar biosynthetic pathways (14) to be postulated.

The iron-porphyrin enzymes, catalase and peroxidase, have attracted attention in this field. The dependence of catalase formation in the leaf on the iron supply to the plant has been repeatedly demonstrated (1, 5) and the catalase and peroxidase activities of soybean leaves have been shown to be depressed by heavy metal toxicity (30).

Over the past few decades interest has centered on the cytochromes as pathways of electron transfer. Hill and Scarisbrick (16) have devised a method for estimating the total hematin compounds in leaves; Sironval (27) has used this method to show an intimate relationship between these pigments and chlorophyll; other work, especially that concerned with genetical variegation (12, 24), has pointed to a relationship between catalase activity and chlorophyll formation.

Though classical views assign to catalase and peroxidase the function of protecting the organism from the harmful effects of hydrogen peroxide, recent work suggests that catalase and peroxidase play an active part in metabolism, especially so the finding that peroxidase will oxidize a wide variety of biologically occurring compounds in the presence of manganese (2, 13, 18, 19) and that catalase acts as an inhibitor of these reactions. Kamerbeek (17) has also shown that dwarf varieties of plants show much higher peroxidase activities than the corresponding tall varieties, an inverse relationship between growth rate and peroxidase activity being inferred.

Recent studies on the iron nutrition of plants have shown that there is an interdependence among the elements phosphorus, iron, potassium, and calcium (10). Such relationships are strikingly apparent in variegated plants (9) and also in plants suffering from heavy metal toxicity (8). Comparative studies of chlorophyll, catalase, peroxidase, and total heme pigments have accordingly been made in relation to the mineral nutrition of normal mustard plants and of various plants suffering from chlorosis caused by heavy metal toxicity, genetical, or other causes.

METHODS

As described in a previous communication (10), mustard plants (*Sinapis alba*) were grown in nutrient culture containing three levels of iron (Fe_1, Fe_2, Fe_3, resp., 0.1, 0.5, and 2.5 ppm Fe); four ratios of potassium to calcium (K_1, K_2, K_3, K_4, resp. 12/1, 10/3, 7/6, 3/10 meq./1); two ratios of phosphorus to nitrogen (P_1, P_2, resp., 1/11, 2/10 meq/1) such that the sums of the cations and the sums of the anions remained constant. Sodium, magnesium, sulphate, and chloride were supplied in constant amounts; iron was added as the ethylene diamine tetra-acetic acid (EDTA) chelate. These treatments were arranged in a $4 \times 3 \times 2$ factorial design and with two replicates, 48 determinations could be made. When the plants were 3 weeks old, fully expanded upper leaves were taken for determination of catalase and peroxidase activities, chlorophyll and hematin according to methods briefly outlined below. Growth and mineral composition of such plants is discussed elsewhere (10). Other plants were grown or obtained as indicated in the text.

Estimation of chlorophyll was performed according to Comar, Benne, and Buteyn (6). The residue (after extraction of the chlorophyll with acetone which will be termed "acetone residue") was used to determine total hematin contents of the samples. The method of estimating hematin differed from that of Hill and Scarisbrick (16), in that pyridine was added to give the strong absorption of the pyridine hemochrome at 556 mμ, and a photoelectric spectrophotometer (Hilger Uvispek, with glass prism) was used to measure the absorption. Acetone residue (0.1 g) from leaves stripped of midribs was thoroughly ground with 30 mg sodium hydrosulphite in a mortar. This was then mixed with 1.8 ml pyridine/water (2/1 v/v mixture) in the mortar, and the resulting slurry transferred to a 0.5 cm cell. This cell was placed close to the photocell in the spectrophotometer; absorbance readings (A_λ) were made at 570, 555, and 540 mμ. Filter paper was used in place of the reference cell to compensate for light scattering. Its position and thickness were adjusted to give a low absorbance reading at 570 mμ. A slit width of 0.3 mm (equivalent

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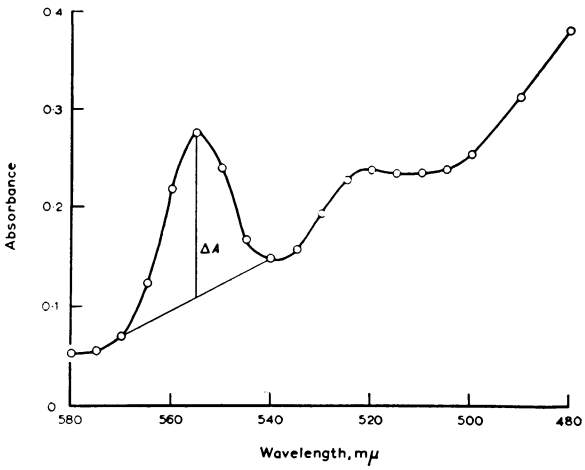

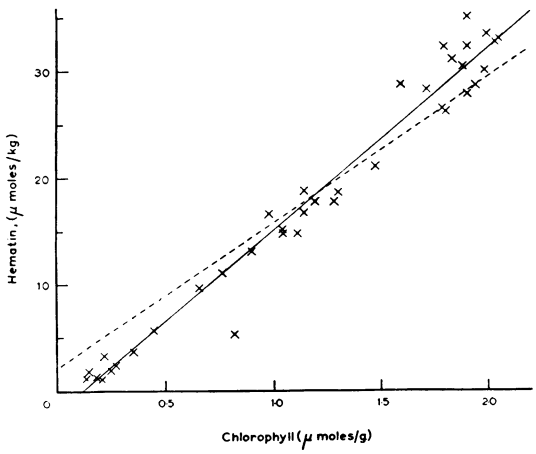

FIG. 1. Absorption spectrum of the pyridine hemochromes in a leaf acetone residue.

FIG. 2. Relationship between hematin and chlorophyll contents of fresh mustard leaves. The full line represents the total regression equation and the dashed line represents the residual regression equation after eliminating differences due to Fe.

to a bandwidth of 1.5 mμ) was used. Figure 1 shows the absorption spectrum of such a slurry, in which the α and β bands of pyridine hemochrome are clearly defined. The height of the α peak above the background, $\triangle A$, is given by $\triangle A = A_{555} - (A_{540} + A_{570})/2$; from this the hematin content was obtained by comparison with a standard. This standard was prepared by adding denatured hemoglobin (4), incorporated in the pyridine/water mixture, to an acetone residue low in hematin. The hemoglobin solution was prepared by lysis of blood cells, and was standardized spectrophotometrically as oxyhemoglobin, for which A^{576}_{mM} was taken as 15.8 (20). The addition of 0.047 μmoles denatured hemoglobin increased the $\triangle A$ (measured on the slurry) from 0.02 to 0.31. From this the concentration of hematin compounds in fresh leaf was calculated as $\triangle A \times 1.62 \times R \times 10^3$ μmoles/kg, where R is the yield of acetone residue from 1 g fresh leaf. Measured $\triangle A$ values ranged from 0.01 to 0.28. Replicate measurements on the same acetone residues indicated a 4.1 % coefficient of variation in $\triangle A$. The accuracy of the hematin estimation is uncertain, as the standard used is not strictly comparable to the samples analyzed. The pyridine hemochrome is uniformly dispersed in the standards, but may be present as insoluble aggregates in the usual acetone residue slurry; if so, our estimates are probably too low by a constant factor (21). Again figure 1 shows that the background scattering does not vary linearly with wave length, as assumed in the calculation: the $\triangle A$ values should probably be about 0.01 units higher than calculated, and the corresponding hematin contents in the fresh leaf should be higher by about two μmoles/kg. In spite of these uncertainties, the method allows a rapid routine comparison of hematin contents in leaves.

Measurement of catalase was made according to the perborate method of Feinstein (15). Leaf tissue (0.5 g) was crushed in a mortar with 5 ml of pH 6.8 phosphate buffer; the macerate then was squeezed through muslin. A series of flasks containing 5 ml of 1.5 % sodium perborate + 1.5 ml phosphate buffer (pH 6.8) was prepared. At zero time 1 ml of macerate was pipetted into each flask; the reaction was stopped in successive flasks after 1, 2, 3, 4, and 5 minutes by rapidly adding 10 ml 2 *N* sulfuric acid. For measurements at zero time the sulfuric acid was added first. The remaining perborate was then titrated with 0.05 *N* potassium permanganate to the first pink color which lasted for 30 seconds. The activity, A, was then calculated according to the formula of Patterson (25) using:

$$r = \frac{4y_4 + 3y_3 - y_2 - 6y_1}{4y_3 + 3y_2 - y_1 - 6y_0} \quad \text{for } t = 0, 1, 2, 3, 4$$

$$\text{or } r = \frac{4y_5 + 4y_4 + 2y_3 - 3y_2 - 7y_1}{4y_4 + 4y_3 + 2y_2 - 3y_1 - 7y_0} \quad \text{for } t = 0, 1, 2, 3, 4, 5$$

where y_0, y_1 . . . . . y_5 are readings at times t_0, t_1 . . . . . t_5 in minutes, $K = -\log_{10} r$ and $A = K/w$ where w is the fresh weight of tissue used, in grams.

Peroxidase activity of leaf tissue was estimated according to the method of Derx (11) as used by Kamerbeek (17), in which 0.5 g of fresh material is thoroughly ground in a porcelain mortar in 96 % alcohol with 0.1 g acid-washed sand and filtered on a 2 inch diameter Buchner funnel with suction using a fine filter paper (Whatman 531). The residue is mixed with 5 ml citrate buffer, left to stand for 1 hour, and filtered as before. Two flasks were then prepared: one contained 1 ml 0.1 *N* H_2O_2, 0.5 ml *o*-toluidine (0.05 %), and 22.5 ml citrate buffer; the other contained 0.5 ml 0.01 *N* ascorbic acid, 4.5 ml

TABLE I

PEROXIDASE VALUES (U.N.P. × 10^4) OF LEAVES OF MUSTARD PLANTS GROWN AT 3 LEVELS OF FE, 4 RATIOS OF K TO CA, AND 2 RATIOS OF P TO N

	Fe_1	Fe_2	Fe_3	P_1	P_2	MEAN
K_1	44	64	63	58	56	57
K_2	47	61	76	62	61	61
K_3	45	63	56	57	53	55
K_4	33	48	53	46	43	45
		(± 4.9)		(± 4.0)		(± 2.9)
P_1	44	60	62			56
P_2	40	58	62			53
		(± 3.5)				(± 2.0)
Mean	42	59	62			
		(± 2.5)				

Standard errors are given in brackets

citrate buffer and 1 ml of the enzyme solution. After equilibration in a water bath at 25° C, the contents of the two flasks were mixed and the time taken for the formation of the blue color noted. From the time taken, the activity (Unités normale de Peroxidase) is calculated according to the equation $\text{U.N.P.} = \frac{150}{a\,t}$ where a is the weight of material in milligrams and t is observed time in seconds.

RESULTS

The statistical treatment of the readings obtained for the peroxidase measurements on leaves of mustard plants grown in a 48 crock experiment are presented in table I. It is quite evident that the peroxidase activity of the mustard leaf is dependent upon the iron supply, since an increase in the iron content of the nutrient medium from 0.1 (Fe_1) to 0.5 ppm (Fe_2) causes a rise of 40 % in the peroxidase value. Further increase of iron to 2.5 ppm (Fe_3) causes only a small increase in the peroxidase value. More remarkable is the apparent relationship between peroxidase activity and the K/Ca ratio of the nutrient medium. The highest K/Ca ratio (K_4) is associated with lowest peroxidase activity.

Catalase activity was dependent only upon the iron level. There was no statistically significant effect of either K/Ca or P/N ratio (table II). But whereas peroxidase showed only a relatively small increase with increase in the iron level, catalase values showed a threefold increase on going from level 1 to 2 of iron, and a fivefold increase on going from level 1 to 3.

The statistical treatment of the chlorophyll and hematin measurements (made on another 48 crock experiment) are presented in tables III and IV. Chlorophyll content increases considerably as the level of iron is raised; fourfold increases were recorded from level 1 to level 2, and eightfold increases from level 1 to level 3 of iron. Apart from a slight depression of chlorophyll content at the highest potassium-calcium ratio (K_4) there was little effect shown by other variants.

Since few measurements of hematins were obtained for the lowest iron level, results of only the two highest iron levels could be considered statistically; these (table IV) showed a closely similar pattern to chlorophyll. If the actual measurements of chlorophyll and hematin are compared by the method of least squares, a rectilinear relationship is obtained (fig 2).

Measurements of chlorophyll and hematin were also made on leaves of mustard plants grown in nutrient culture as well as on leaves of sugar beet plants grown in sand culture. They were given nickel (2

TABLE II

CATALASE ACTIVITY (A) OF LEAVES OF MUSTARD PLANTS GROWN AT 3 LEVELS OF FE, 4 RATIOS OF K TO CA, AND 2 RATIOS OF P TO N

	Fe_1	Fe_2	Fe_3	P_1	P_2	MEAN
K_1	0.43	1.50	1.96	1.40	1.20	1.30
K_2	0.44	1.72	2.11	1.21	1.63	1.42
K_3	0.37	1.31	2.36	1.33	1.36	1.35
K_4	0.52	1.21	2.19	1.23	1.38	1.31
		(± 0.254)		(± 0.207)		(± 0.146)
P_1	0.44	1.51	1.93			1.29
P_2	0.44	1.36	2.38			1.39
		(± 0.179)				(± 0.104)
Mean	0.44	1.44	2.16			
		(± 0.127)				

Standard errors are given in brackets

TABLE III

CHLOROPHYLL (μMOLES/G) CONTENT OF LEAVES OF MUSTARD PLANTS GROWN AT 3 LEVELS OF FE, 4 RATIOS OF K TO CA, AND 2 RATIOS OF P TO N

	Fe_1	Fe_2	Fe_3	P_1	P_2	MEAN
K_1	0.30	0.93	1.88	1.11	0.97	1.04
K_2	0.22	1.19	1.90	1.10	1.12	1.11
K_3	0.28	1.16	1.88	1.21	2.13	1.11
K_4	0.17	0.92	1.89	0.91	1.07	0.98
		(± 0.055)		(± 0.045)		(± 0.031)
P_1	0.28	1.03	1.92			1.07
P_2	0.21	1.07	1.85			1.04
		(± 0.039)				(± 0.022)
Mean	0.25	1.05	1.89			
		(± 0.028)				

Standard errors are given in brackets

TABLE IV

HEMATIN CONTENT (μMOLES/G) OF LEAVES OF MUSTARD PLANTS GROWN AT 3 LEVELS OF Fe, 4 RATIOS OF K TO Ca, AND 2 RATIOS OF P TO N

	Fe_2	Fe_3	P_1	P_2	MEAN
K_1	11.5	31.6	23.5	19.7	21.5
K_2	18.0	30.5	24.1	24.5	24.3
K_3	17.1	31.2	25.5	22.8	24.1
K_4	12.7	29.5	18.9	23.3	21.1
	(± 1.49)		(± 1.49)		(± 1.05)
P_1	14.7	31.3			23.0
P_2	15.0	30.2			22.5
	(± 1.05)				(± 0.75)
Mean	14.8	30.7			
	(± 0.75)				

Standard errors are given in brackets

ppm) as the sulphate or EDTA chelate with iron supplied as the chloride or EDTA chelate (8). The results are given in tables V and VI. A similar rectilinear relationship between these two components can be shown for these results, though some aberration occurs at lower values.

Catalase and peroxidase activities of leaves of a similar set of mustard plants grown in the presence of nickel are shown in table V. Since the colorless areas of variegated plants have similar P/Fe and K/Ca ratios to iron deficient plants (9) measurements of total hematin, chlorophyll, catalase, and peroxidase were made on such leaves (table VII).

DISCUSSION

The levels of all four of the cellular components studied showed a clear relationship to the amount of iron supplied to the mustard plants. It is evident, however, that whereas catalase and chlorophyll were practically absent when the iron supply was low, and increased several fold when the iron supply was increased, peroxidase was present in considerable amounts even at the lowest iron level. Peroxidase therefore appears to be an essential component of living matter and is synthesized preferentially when iron supply is inadequate. Peroxidase content also shows an inverse relationship to the potassium-calcium ratio in the nutrient solution.

The similar behavior of catalase and chlorophyll, noted here, has often been observed. This probably implies closely associated pathways of biosynthesis, as is clearly shown in the results of Mikhlin and Mutuskin (23). They find an inverse relationship between peroxidase on the one hand and catalase and chlorophyll on the other. Schwarze (26) has argued against an inverse relationship between catalase and peroxidase in plant tissue. Inspection of his results shows, however, that though there may be little dif-

TABLE V

CHLOROPHYLL AND HEMATIN, CATALASE (A) AND PEROXIDASE (U.N.P. × 10^4) IN MUSTARD LEAVES GROWN IN PRESENCE OF NICKEL (2 PPM) AS SULPHATE OR EDTA CHELATE (NiV) WITH IRON (2 PPM) ADDED AS CHLORIDE OR EDTA CHELATE (FeV)

TREATMENT	% ACETONE RESIDUE	HEMATIN (μMOLE/KG) FRESH	CHLOROPHYLL (μMOLE/G) FRESH	CATALASE (A)	PEROXIDASE (U.N.P. × 10^4)
1. $NiSO_4$ + $FeCl_3$	12.4	11.4	0.38	0.755	209
2. $NiSO_4$ + FeV	13.1	20.9	0.79	1.945	399
3. NiV + $FeCl_3$	10.7	36.8	1.81	0.525	72
4. NiV + FeV	10.2	36.8	1.88	1.975	102
5. $FeCl_3$ Control	7.0	12.3	0.66	0.655	143
6. FeV Control	9.3	36.8	2.21	2.500	73
Standard error	...	...	...	± 0.089	± 22

TABLE VI

CHLOROPHYLL AND HEMATIN IN FRESH LEAVES OF NORMAL (A) AND NICKEL-TOXIC (B) SUGAR BEET LEAVES

	TREATMENT	% ACETONE RESIDUE	HEMATIN (μMOLE/KG) FRESH	CHLOROPHYLL (μMOLE/G) FRESH
A	Healthy green	8.4	20.5	1.49
A	" "	10.5	24.1	1.47
B	Slight chlorosis	10.7	15.9	0.79
B	Intermediate chlorosis	8.3	12.3	0.63
B	Severe chlorosis	9.7	6.8	0.00

TABLE VII

CHLOROPHYLL, HEMATIN, CATALASE (A) AND PEROXIDASE (U.N.P. × 10^4) OF GREEN (G) AND ALBINO (W) PORTIONS OF VARIEGATED LEAVES OF VARIOUS PLANTS

LEAF		% ACETONE RESIDUE	HEMATIN (μMOLE/KG) FRESH	CHLOROPHYLL (μMOLE/G) FRESH	CATALASE	PEROXIDASE
Bougainvillea glabra	G	17.4	36.4	1.88	2.28	61.2
" "	W	10.0	10.0	0.08	0.22	75.0
Sambucus nigra	G	13.7	24.1	1.90	2.90	42.9
" "	W	9.5	11.4	0.03	0.69	52.1
Hosta fortunei	G	...	...	...	4.24	50.0
" "	W	...	...	...	0.32	64.0
Chlorophytum comosum	G	9.8	16.9	1.12	1.60	25.0
" "	W	6.1	3.2	0.03	0.58	73.0
Spiraea japonica	G	20.2	23.8	1.17	1.10	27.8
" "	W	14.8	8.8	0.01	0.55	104.0

ference between the peroxidase activity of green and chlorotic tissue, catalase is always considerably lower in chlorotic tissue, whether chlorosis is due to nutrient deficiency, genetical causes, or even darkness. Our results show that irrespective of the cause of chlorosis, the peroxidase-catalase ratio is always higher in plants showing an iron-deficiency type of chlorosis. Such a relative relationship could also explain apparent anomalies of catalase and peroxidase activity reported by Appleman (3) and Woods and Dubuy (31).

Sironval (28) has found no simple relationship between peroxidase, catalase, and chlorophyll. He noticed, however, that the activities of catalase and peroxidase at different stages in development of the leaf "thus vary in an inverse manner, as if they rearrange themselves during the development of the leaf", conclusions conforming with those found here.

Lundegardh (22) has discussed the possibility of peroxidase playing a role in terminal oxidation of the cell. He mentioned its insensitivity to inhibitors such as azide and carbon monoxide. His calculations suggest that respiration due to peroxidase proceeds at so low an intensity as to make little difference to the cyanide-sensitive respiration. He has noted, however, that high concentrations of oxygen reduce the inhibiting effect of cyanide on respiration; it may well be that the in vivo hydrogen peroxide concentration is so low that peroxidase behaves differently from in vitro experiments where the hydrogen peroxide concentration is high. The presence of catalase in the living cell ensures a low peroxide concentration; Kenten and Mann (19) have found catalase to be inhibitory to the peroxidase-manganese oxidizing system. Some participation of peroxidase in terminal oxidation cannot, therefore, be excluded.

Manganese is connected intimately with iron metabolism; in the plant their relative amounts are of importance (29). Excess of manganese causes an iron-deficiency chlorosis while a deficiency of manganese causes an iron toxicity chlorosis (8). It would appear then that manganese plays an important part in oxidation. Apart from the studies of Kenten and Mann (19), however, no mechanism of oxidative metabolism involving manganese has been suggested.

The rectilinear relationship between chlorophyll and total hematin (full line in fig 2) has been reported by Sironval (27) and by Davenport (7). In agreement with these workers, we find the ratio of chlorophyll to hematin molecules to be about sixty to one. The relationship can be represented by the total regression equation:

$$y = 17.05x - 1.84.$$

where y represents the hematin and x the chlorophyll values. This appears to show that hematin declines to zero before chlorophyll disappears entirely. This may be due to the approximation used in correcting for background scattering as indicated under methods. An analysis of covariance was carried out in which Fe was the only factor of classification considered. This showed that differences in hematin due to the level of iron are greater than can be accounted for by the differences in chlorophyll. The regression for the relationship within the iron levels is less steep and given by $y = 13.78x + 2.16$ (shown by a dashed line in fig 2). Thus a positive hematin value is indicated when chlorophyll is zero.

In any case it is clear that little hematin remains at low chlorophyll levels when chlorosis is due to iron deficiency. This is in contrast to the results obtained with chlorotic tissue from variegated leaves (table VII), and with leaves of mustard (table V) and sugar beet (table VI) in which chlorosis is due to nickel toxicity. Here significant amounts of hematin compounds remain even when chlorophyll is almost absent, although the hematin levels are always much lower in chlorotic than in corresponding green and healthy tissue, in accordance with earlier findings (9).

Summary

A study was made of the catalase and peroxidase activities as well as of chlorophyll and total hematin contents of mustard plants. The plants were grown in nutrient culture with different iron, potassium, calcium, phosphorus, and nitrogen levels. Leaves of nickel-toxic and variegated plants were also studied. Peroxidase activity showed only small variations between chlorotic and green leaves, whereas catalase activity was several fold greater in green than in chlorotic leaves. A linear relationship of 60:1 between chlorophyll and hematin was found.

A spectrophotometric method is reported for determining total hematin compounds as pyridine hemochrome in acetone residues derived from leaves.

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