THE EFFECT OF NUTRIENT DEFICIENCIES ON THE HILL REACTION OF ISOLATED CHLOROPLASTS FROM TOMATO

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Summary

Tomato plants were grown deficient in each known essential macro- and micronutrient (except chlorine) and the effect of each deficiency on the Hill reaction activity of chloroplasts isolated from these plants was studied.

All deficiencies, except that of iron, resulted in chloroplasts with reduced Hill reaction activity per unit chlorophyll. Among the micronutrients, deficiencies of manganese, copper, zinc, boron, or molybdenum caused impairment of activity in that decreasing order of severity.

In no case was the activity of chloroplasts from a deficient plant enhanced by the addition of the missing nutrient to the chloroplast suspension.

A comparison of the absorption spectra of suspensions of chloroplasts from healthy and deficient plants, and of 80 per cent. acetone extracts of these suspensions, revealed no shifts in the absorption peaks, and no gross change in the relative proportions of the major components. In some deficiencies a small but significant decrease was observed in the ratio of chlorophyll *a* to chlorophyll *b*.

Chloroplasts from healthy and deficient plants were assayed over a range of limiting light intensities up to saturation, and the effect of light intensity on the degree of impairment was recorded. In some cases, the degree of impairment due to deficiency was measured at 30 and 10°C with saturating light intensity.

Some tentative conclusions are drawn concerning the site of the impaired component of the Hill reaction associated with a number of the deficiencies.

I. INTRODUCTION

Manganese deficiency causes a reduction in the rate of photosynthesis per unit amount of chlorophyll in a number of algae. This is true whether photosynthesis is measured as CO_2 fixed, O_2 evolved in presence of CO_2, or O_2 evolved in the presence of an artificial oxidant such as a quinone (Pirson 1937; Pirson, Tichy, and Wilhelmi 1952; Arnon 1954; Eyster, Brown, and Tanner 1956). This evidence indicates that manganese is essential, either directly or indirectly, for the Hill reaction (the photolysis of water, and the evolution of oxygen accompanied by the reduction of an artificial oxidant). Kessler (1955) demonstrated that in the green alga *Ankistrodesmus* manganese deficiency causes no reduction in the rate of the initial photolytic reaction, but is involved rather in the evolution of O_2 from the products of photolysis.

Evidence concerning the role of manganese, and other nutrients, in the Hill reaction of higher plants is meagre. Gerretsen (1949) has shown that CO_2 assimilation per unit leaf area of oat leaves was reduced in manganese deficiency. He also found that breis of manganese-deficient leaves without any added oxidant formed less peroxides upon illumination that those of normal leaves (Gerretsen 1950). Mehler

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(1951) found that manganous ions stimulated oxygen uptake by isolated chloroplasts in the presence of catalase and ethanol. Manganous ions have been shown to stimulate CO_2 fixation, but not the Hill reaction, of isolated chloroplasts from spinach (Allen *et al.* 1955).

Little is known about the effects of other nutrient deficiencies on the Hill reaction, especially in higher plants. Kessler (1955, 1957) has shown that phosphate deficiency results in lowered photosynthesis and photoreduction in intact *Ankistrodesmus*, whilst iron deficiency lowers the rate of photoreduction much more than photosynthesis.

The aim of the present work was to test whether manganese deficiency in a higher plant results in a reduction in the Hill reaction activity as measured in isolated chloroplasts. Having determined that this was the case, the effect of other nutrient deficiencies on the Hill reaction was also studied in order to test whether the effect of manganese deficiency is specific.

Since most of the work in this field to date has been carried out with intact algae, it has not been possible to decide whether the effects observed were due to direct or indirect participation in the Hill reaction of the nutrients concerned. By working with chloroplasts isolated from deficient tissues, rather than with intact cells, it was possible to attempt direct restoration of the Hill reaction *in vitro*.

II. Methods

(a) *Water Culture Methods*

Tomato plants (*Lycopersicon esculentum* Mill., cv. Bonny Best) were grown in nutrient solutions in a glass-house in which the air temperature was controlled to approximately 25°C by day and to 20°C by night. For studies of micronutrient deficiencies, seedlings were germinated over distilled water and then transferred to nutrient solution of the composition described by Tsui (1948). For macronutrient deficiency studies seeds were germinated in vermiculite and transferred to nutrient solution of the composition described by Hoagland and Arnon (1950). Each water-culture vessel (3-l. "Pyrex" beakers) held 12 plants, and the nutrient solutions, which were aerated continuously, were not changed during the 2–3 weeks growing period.

Nutrient solutions deficient in molybdenum were prepared by the method of Gentry and Sherrington (1950). Solutions deficient in copper, zinc, and manganese were prepared by the method of Stout and Arnon (1939). Solutions deficient in boron were prepared by dissolving A.R. grade salts in water from a stainless steel still. Boron contamination was further reduced by lining the beakers with thin polythene bags. Iron-deficient solutions were prepared from A.R. grade salts dissolved in distilled water.

In order to obtain plants of a convenient size it was necessary to add a supplement of the missing nutrient routinely to certain deficient cultures. The iron-deficient cultures were supplemented with a final concentration of 0·143 μM Fe. Similarly, 0·182 μM B, 0·035 mM K, 0·01 mM Ca, 0·056 mM N, 0·0133 mM Mg, and 0·0133 mM P was added to the corresponding deficient cultures.

(*b*) *Preparation of Chloroplasts*

For each chloroplast preparation sufficient plants were harvested to yield 2–5 g fresh weight of leaves. All the leaves of each plant (excluding cotyledons) were used. In order to minimize day-to-day variation due to diurnal fluctuations in the Hill reaction activity (Hill and Scarisbrick 1939; A. W. Galston and J. Miller, personal communication) plants were harvested at approximately the same hour (9.00 a.m.) each day.

The procedure adopted for the preparation of chloroplasts followed closely that of Jagendorf and Evans (1957). The major departure from their method was the omission of polyvinylpyrrolidone from the initial grinding solution. Leaves were macerated in 60 ml of solution containing sucrose (0·30M), KCl (0·01M), potassium phosphate (0·05M, pH 8·5), and sodium ethylenediamintetra-acetate (0·01M, pH 8·5). After removal of the cell debris by filtration through four layers of fine cloth, chloroplasts were sedimented by centrifugation at 1000 *g* for 10 min. The chloroplast pellet was suspended in 40 ml of a solution containing sucrose (0·30M), KCl (0·01M), and potassium phosphate (0·05M, pH 7·3) and again sedimented at 1000 *g* for 10 min. The pellet was resuspended in the latter solution. This chloroplast suspension was then used for the determination of Hill reaction rates and chlorophyll content.

Chloroplasts prepared in this way were examined under phase-contrast at ×1600 magnification. The chloroplasts appeared predominantly intact and undamaged, and were accompanied by insignificant amounts of other cell constituents excepting starch. The amount of starch in each preparation varied with the starch content of the leaf samples. Chlorophyll assays carried out on chloroplast preparations and on whole plants indicated that more than one-third of the total plant chlorophyll was contained in the chloroplast preparation.

Our aim in these experiments was to compare the activity of chloroplasts from deficient plants at an early stage of deficiency, with those from the appropriate healthy control plants. Comparisons between grossly deficient and healthy plants were avoided because of the extreme difference in size and morphology. Plants were harvested as soon as each deficiency was evidenced either by reduced fresh weight of whole tops, or by the commencement of characteristic deficiency symptoms. In most cases subsequent harvests were made within a period of 7 days.

(*c*) *Assay of Hill Reaction Rates*

These assays were carried out according to the procedure of Jagendorf and Evans (1957). This involves measuring the change in optical density (O.D.) at 620 mμ upon illumination for 45 sec of a reaction mixture containing chloroplasts, approximately 0·1M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7·3, and 0·07 μmoles of *o*-chlorophenol-2,6-dichloroindophenol (B.D.H.) in a total volume of 3·0 ml. Illumination at more than saturating intensity was provided by a 250-W "Photoflood" lamp. The amount of chloroplasts (≡2–10 μg chlorophyll) in the reaction mixture was varied so as to give an O.D. change of 0·09–0·120 upon illumination for 45 sec. O.D. measurements were made in a Unicam SP 600 spectrophotometer and, unless otherwise noted, assays were carried out at 30°C.

Where a range of light intensities was required, the distance between the reaction vessel and the light source was varied. Light intensities were determined with an E.E.L. photoelectric illuminance meter.

Phosphate buffer used in the preparation of the chloroplasts and Tris buffer used in the Hill reaction assay were extracted with 8-hydroxyquinoline in chloroform in order to remove heavy metal contaminants.

Total chlorophyll concentration, and the relative proportions of chlorophyll *a* and *b* were calculated from the O.D. at 663 mμ and 645 mμ of an 80 per cent. acetone extract of the chloroplast suspension, as described by Arnon (1949). Hill reaction rates are expressed as the change in optical density at 620 mμ per 45 sec per

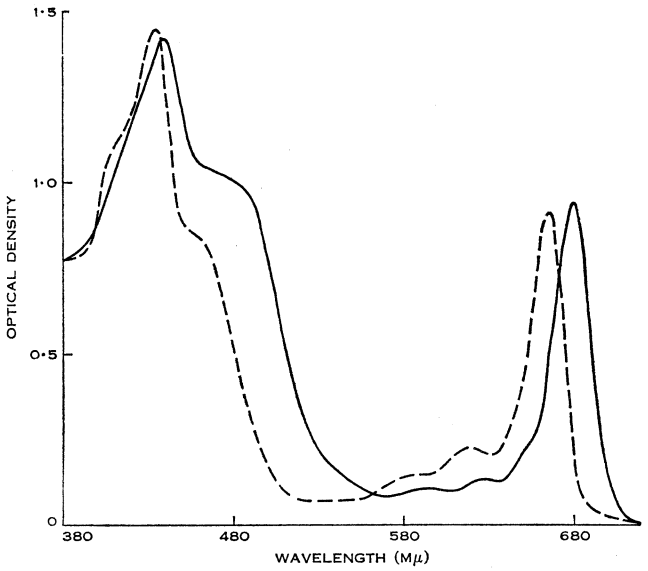


Fig. 1.—Absorption spectrum of isolated chloroplasts in sucrose–KCl–phosphate solution (———), and of an 80 per cent. acetone extract of isolated chloroplasts (– – – –).

mg chlorophyll. The expression of rates on the basis of chlorophyll present in the chloroplast preparation serves to eliminate the varying effects of the deficiencies on the chlorophyll status of the plants.

(*d*) *Spectral Examination of Chloroplast Pigments*

The absorption spectrum of normal and deficient chloroplasts was compared in two ways (1) by direct measurement on the chloroplast suspensions, and (2) by an examination of an 80 per cent. acetone extract of the chloroplast suspensions. In both cases measurements were made in an Optica CF4 spectrophotometer.

For the first method a strip of matt plastic tracing paper ("Astrafoil", 0·01 in. in thickness) served as the standard against which the optical density of the chloroplast suspension was measured. This tracing paper had a constant optical density, relative to air, over the range from 380 to 710 mμ, and served as a reproducible

standard. The chloroplast suspensions prepared as described above were diluted with additional sucrose–phosphate–KCl solution until at 680 mμ the suspensions from both healthy and deficient plants had optical densities of approximately 1, relative to the "Astrafoil" standard. The cuvettes containing the chloroplast suspensions were routinely placed at the back of the carriage-holder where they were as close as possible to the photocell.

Measurements of optical density were then made at 5-mμ intervals over the range from 380 to 710 mμ. A comparison with published spectra (Latimer 1959) and with the spectrum from the acetone extract of the chloroplast suspensions (Fig. 1) indicates that under these conditions in this instrument a representative portion of the scattered light is measured.

For the second method of comparison an 80 per cent. acetone extract of the chloroplast suspensions was prepared as described by Arnon (1949). Measurements were again made at 5-mμ intervals over the range from 380 to 710 mμ, using 80 per cent. acetone as the reference.

III. Results

(*a*) *Effect of Micronutrient Deficiencies*

The Hill reaction activity of chloroplasts from leaves of tomatoes deficient separately in each micronutrient was compared with that of chloroplasts from corresponding healthy control plants (Table 1). Deficiencies of manganese, copper, zinc, boron, or molybdenum resulted in impaired chloroplast activity per unit amount of chlorophyll in that decreasing order of severity. Iron deficiency was an exception since it consistently yielded chloroplasts which had slightly higher Hill reaction activity than chloroplasts from healthy plants when assayed at saturating light intensity. In one experiment boron deficiency did not cause marked reduction in activity at incipient deficiency.

The results in Table 1 were obtained from a number of experiments carried out over the course of 6 months (April to September, 1959). In some experiments successive harvests were made at 2- or 3-day intervals following the first detectable signs of deficiency. A considerable day-to-day variation was noted in the absolute Hill reaction rates of chloroplasts isolated from both healthy and deficient plants. Preparative and assay procedures contributed a small fraction of this variation. For example, four replicate preparations from uniform healthy plants harvested at the one time gave activities of 25·1, 22·3, 22·1, and 24·8 units. The major variation in rates appeared to arise from at least three sources: (1) a seasonal variation—in midwinter, especially if natural light was relatively poor, low activities were obtained; (2) a maturation effect—the activity of chloroplasts appeared to increase with increasing age of the plant up to about 3 weeks; (3) a diurnal fluctuation—this was brought to our attention by A. W. Galston and J. Miller (personal communication) and confirmed by us. Within the first few hours of daylight there is a rapid, almost twofold, rise in activity, followed by a decline throughout the remainder of the day. The extent and time scale of this diurnal fluctuation appears to be dependent on prevailing lighting conditions. This diurnal fluctuation probably accounts for most of the variation between harvests found in our experiments.

TABLE 1

EFFECT OF MICRONUTRIENT DEFICIENCIES ON THE HILL REACTION ACTIVITY OF ISOLATED CHLOROPLASTS

Light intensity = 2420 f.c. Hill reaction activity expressed as ΔO.D. at 620 mμ/mg chlorophyll/45 sec

Plant Material from which Chloroplasts were Isolated	Hill Reaction Activity									$\frac{\text{Activity of "Deficient" Chloroplasts}}{\text{Activity of "Control" Chloroplasts}} \times 100$								
	Expt. I			Expt. II			Expt. III		Expt. IV	Expt. I			Expt. II			Expt. III		Expt. IV
	Harvest No.			Harvest No.			Harvest No.		Harvest No.	Harvest No.			Harvest No.			Harvest No.		Harvest No.
	1	2	3	1	2	3	1	2	1	1	2	3	1	2	3	1	2	1
Manganese-deficient	5·5	7·9	10·1	8·1	11·9	14·3	9·7	10·3	11·0	39	43	35	41	38	46	42	55	32
Control	14·3	18·4	28·6	19·7	31·6	31·1	23·4	18·7	34·2									
Copper-deficient	10·0	8·3	10·4	21·1	14·9					60	48	60	60	60				
Control	16·8	17·3	17·5	35·1	24·7													
Zinc-deficient	26·2	20·6		16·6			14·3			65	66		60			72		
Control	40·6	31·1		27·7			19·8											
Boron-deficient	24·7	23·0		21·8	18·6		15·8		22·9	73	74		95	74		64		77
Control	34·1	31·9		23·0	24·5		24·7		29·7									
Molybdenum-deficient	17·3	31·1	25·7	20·9			24·7		17·7	73	77	83	92			80		73
Control	23·7	40·6	31·1	22·7			31·9		24·1									
Iron-deficient	33·2	27·5	25·3	35·5			30·1			102	111	111	112			115		
Control	32·4	24·8	22·8	31·7			26·2											

Because of this variation in absolute rates, the activity of chloroplasts from deficient plants is also expressed as a percentage of the activity of chloroplasts from the appropriate control plants (Table 1). It can be seen that on a percentage basis the effect of a deficiency on chloroplast activity is fairly constant under our experimental conditions. This suggests that the degree of fluctuation of Hill reaction activity is of the same order in chloroplasts from both deficient and healthy plants.

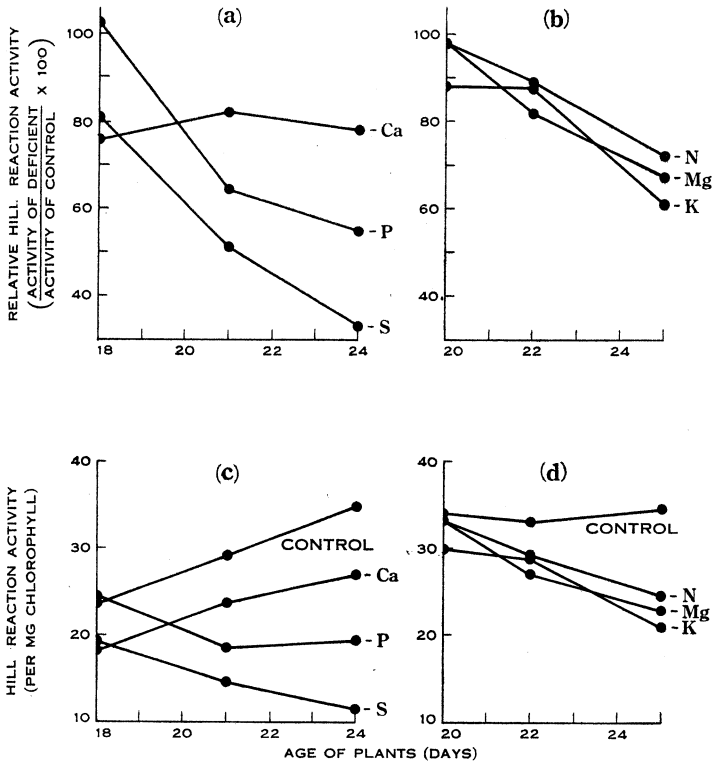


Fig. 2.—Effect of macronutrient deficiencies on Hill reaction activity of chloroplasts isolated from plants at intervals following the onset of deficiency: (*c*), (*a*), absolute activities (per mg chlorophyll) and relative activities (percentage of control value) respectively of chloroplasts isolated from plants deficient in calcium, phosphorus, or sulphur; (*d*), (*b*), absolute and relative activities respectively of chloroplasts isolated from plants deficient in nitrogen, magnesium, or potassium.

(*b*) *Effect of Macronutrient Deficiencies*

Figure 2 shows the results of two experiments in the first of which chloroplasts were prepared from plants deficient in either calcium, phosphorus, or sulphur (Figs. 2(*a*) and 2(*c*)). Three harvests were made in the 6-day period following the first detectable sign of deficiency. The results of a similar experiment in which three harvests were made of plants deficient in either nitrogen, magnesium, or potassium during the 5-day period following the first sign of deficiency are also shown in Figures 2(*b*) and 2(*d*).

A deficiency of any macronutrient resulted in a reduction, in some degree, of the Hill reaction activity per mg chlorophyll. It is not possible to compare the relative inhibitory effect of each deficiency in lowering the Hill reaction activity, because the plants are not necessarily under the same degree of nutritional stress. However, it is apparent from the trends in these two experiments that chloroplast activity, relative to that of control chloroplasts, declines rapidly at the onset of a deficiency of any one macronutrient (Figs. 2(*a*) and 2(*b*)). These trends were confirmed in other similar experiments. Deficiencies of sulphur and phosphorus led to the most rapid deterioration of activity under our experimental conditions.

TABLE 2

EFFECT OF LIGHT INTENSITY OF THE ASSAY ON THE DEGREE OF IMPAIRMENT OF THE HILL REACTION DUE TO MICRONUTRIENT DEFICIENCIES

Temperature = 20°C. Hill reaction activity expressed as ΔO.D. at 620 mμ/mg chlorophyll/45 sec

Light Intensity (f.c.)	$\frac{\text{Activity of "Deficient" Chloroplasts}}{\text{Activity of "Control" Chloroplasts}} \times 100$					
	−Mn	−Mo	−Fe	−B	−Zn	−Cu
2420	54	73	100	74	65	59
600	35	71	78	88	68	65
400	33	76	78	90	74	69
200	35	81	84	103	77	72
120	38	76	77	100	80	73

It is of interest that the effects of macronutrient deficiencies is accentuated markedly during the 5–6-day period covered by the harvests, whereas over this period the relative inhibition due to micronutrient deficiencies is fairly constant except in one case of boron deficiency, This suggests that from the visible onset of a deficiency the stress imposed by a deficiency of a macronutrient causes more rapid changes in metabolism than does a micronutrient deficiency.

(*c*) *Effect of Light Intensity of the Assay on the Degree of Impairment*

In order to determine whether the impairment caused by each deficiency was associated with a "light" or a "dark" step, chloroplasts from deficient and healthy plants were assayed for Hill reaction activity at a range of light intensities between 120 and 2420 f.c. At the lowest light intensity (120 f.c.) the activity of normal chloroplasts was reduced to about one-seventh of the saturation value. The activity of chloroplasts from deficient plants, relative to that of the appropriate control plants, is shown in Tables 2 and 3 for micronutrient and macronutrient

deficiencies respectively. It was found that chloroplasts from deficient plants fall into two categories, viz. (1) those in which the activity is impaired to the same, or even to a greater, extent at low light as at saturating intensities, and (2) those in which the degree of impairment is reduced at lower light intensities.

Among the micronutrients, manganese and molybdenum deficiencies resulted in chloroplasts in the first category whilst boron, zinc, or copper deficiency yielded chloroplasts which are in the second category (Table 2). Iron deficiency yielded chloroplasts which, although unimpaired at saturating intensities, consistently

TABLE 3

EFFECT OF LIGHT INTENSITY OF THE ASSAY ON THE DEGREE OF IMPAIRMENT OF THE HILL REACTION DUE TO MACRONUTRIENT DEFICIENCIES

Temperature = 20°C. Hill reaction activity expressed as ΔO.D. at 620 mμ/mg chlorophyll/45 sec

Light Intensity (f.c.)	$\frac{\text{Activity of "Deficient" Chloroplasts}}{\text{Activity of "Control" Chloroplasts}} \times 100$					
	−S	−Mg	−N	−Ca	−K	−P
2420	54	60	79	73	73	58
600	45	62	71	74	79	60
400	42	63	67	75	86	64
200	45	67	67	72	94	75
120	—	67	66	74	102	81

showed inhibition at all limiting intensities. This set of experiments with micronutrient deficiencies was repeated at least four times for each deficiency, and, although the magnitude of the effects varied, the nature of the effect was consistent in each case.

Chloroplasts from plants deficient in sulphur, magnesium, nitrogen, or calcium showed approximately the same or increased impairment at low as at high light intensities, while with chloroplasts from potassium- or phosphorus-deficient plants the degree of impairment was reduced at low light (Table 3). However, in the case of chloroplasts from phosphorus-deficient and potassium-deficient plants the effect of light intensity was not consistent. In each case one out of four replicate experiments yielded chloroplasts whose behaviour with varying light intensity was the opposite of that shown in Table 3. It is possible that in these cases the state of the chloroplast alters with the stage of the deficiency.

The implications of these light-intensity experiments as to the impaired component of the Hill reaction associated with each deficiency will be considered in Section IV.

(*d*) *Effect of Temperature of the Assay on the Degree of Impairment*

The Hill reaction activity of chloroplasts from plants deficient in manganese, molybdenum, sulphur, calcium, magnesium, or nitrogen was tested at both 30 and 10°C with saturating light intensity. In all cases the percentage activity relative to that of control chloroplasts, was constant at both temperatures. Lowering the temperature from 30 to 10°C reduced the absolute activity of both control and deficient chloroplasts by approximately one-half.

TABLE 4

MEAN VALUES OF THE RATIO OF CHLOROPHYLL *a* TO CHLOROPHYLL *b* IN ISOLATED CHLOROPLASTS FROM HEALTHY AND DEFICIENT TOMATO PLANTS

Significant differences between control and deficient values indicated by asterisks

Deficiency	$\frac{\text{Chlorophyll } a}{\text{Chlorophyll } b}$		$\frac{\text{Chlorophyll } a}{\text{Chlorophylls } (a+b)} \times 100$	
	Control	Deficient	Control	Deficient
Iron	3·078	3·116	75·39	75·70
Copper	3·516	3·305*	77·86	76·77
Molybdenum	3·128	2·985**	75·78	74·91
Boron	3·064	2·914**	75·39	74·45
Manganese	3·192	2·823***	76·15	73·84
Zinc	3·087	2·914***	75·53	74·25
Magnesium	3·160	3·201	75·96	76·20
Calcium	3·149	3·225	75·90	76·33
Nitrogen	3·092	2·831**	75·56	73·90
Sulphur	3·222	2·901**	76·32	74·37
Phosphorus	3·240	2·636***	76·42	72·50
Potassium	3·189	2·722***	76·13	73·13

* Difference significant at $P<0·05$.
** Difference significant at $P<0·01$.
*** Difference significant at $P<0·001$.

(*e*) *Attempted Restoration of Hill Reaction Activity* in vitro

For each deficiency which resulted in chloroplasts with reduced Hill reaction activity an attempt was made to restore the activity by the addition of the missing nutrient to the isolated chloroplast preparation. In no case was activity enhanced by this treatment. Macronutrients were added to the assay mixture at 10^{-2}M final concentration, and micronutrients were tested from 3×10^{-6} to 3×10^{-4}M final concentration.

(*f*) *Effect of Nutrient Deficiencies on the Pigment Composition of Chloroplasts*

The results of a large number of assays of chloroplast suspensions showed that all deficiencies except iron, magnesium, and calcium caused a small but significant reduction in the ratio of chlorophyll *a* to chlorophyll *b* (Table 4). However, these

changes were small compared with the reduction in Hill reaction activity associated with the various deficiencies. Expression of activities on the basis of chlorophyll *a* alone did not substantially alter the percentage reduction in activity due to deficiency.

Direct spectrophotometric examination of intact chloroplasts isolated from healthy plants gave an absorption spectrum essentially similar to that described by Latimer (1959) for *Chlorella* (Fig. 1). Comparison of healthy and deficient chloroplasts gave no evidence of any shifts in the absorption maximum of chlorophyll *a*. Furthermore, no pronounced changes were found in the shorter wavelengths where at least part of the absorption is due to carotenes. An examination of the spectra of the 80 per cent. acetone extracts of chloroplasts from healthy and deficient plants also failed to reveal any gross changes (other than the chlorophyll *a*: chlorophyll *b* ratio) in the relative heights of the major absorption peaks.

IV. Discussion

These results demonstrate that manganese deficiency in a higher plant, as in the case of algae (Eyster, Brown, and Tanner 1956), leads to a lowered Hill reaction rate per unit chlorophyll. On extending this investigation to other nutrient deficiencies it was found that this effect is by no means specific. A deficiency of any single macronutrient, or of any micronutrient other than iron, in some way induces changes which result in chloroplasts with reduced Hill reaction activity per unit of chlorophyll (Table 1; Fig. 2).

A reduction of Hill reaction rates as a result of a nutrient deficiency could be brought about in one or more of three ways. The deficient nutrient could be required as such directly in the Hill reaction, it could be a part of a more complex component of this reaction, or it could be essential in some reaction which in turn affects the formation of a Hill reaction component. In the last instance, the site of action of the nutrient could be outside the chloroplast. Since in no instance were impaired chloroplasts reactivated by the addition of the deficient nutrient *in vitro*, it can be concluded that the lowered activities observed were not due to lack of the deficient nutrient ion as such in the Hill reaction. If these are involved in this reaction they are present in adequate amounts even in washed chloroplasts from deficient plants. Photochemical reactions which respond to added manganous ions, such as those described by Gerretsen (1950) and by Mehler (1951), do not appear to be involved in the Hill reaction as measured by dye reduction.

Some of the effects which we have observed could be due to the association of natural inhibitors of the Hill reaction with certain deficiencies. Clendenning (1957) has reviewed evidence that naturally occurring compounds such as tannins can irreversibly inactivate chloroplasts during isolation. Cell sap of low pH is another potential source of inactivation. It is possible that both these factors could be affected by a nutrient deficiency and be responsible for lowered activities in isolated chloroplasts. Since the chloroplasts underwent a high dilution during isolation it is unlikely that any of the observed effects of deficiencies are due to reversible natural inhibitors. Consistent with this, when chloroplasts from plants deficient in any of the micronutrients were combined with normal chloroplasts their activities were additive.

By expressing Hill reaction activities on the basis of chlorophyll present in the chloroplast preparations we have sought to eliminate the effects of the various deficiencies on the formation of chlorophyll itself. Thus sulphur and iron deficiencies, although both characteristically resulting in reduced chlorophyll status, have markedly different effects on the Hill reaction rate per unit of chlorophyll. Sulphur deficiency apparently causes more drastic reduction in some other component of the Hill reaction than in chlorophyll, whilst iron deficiency does not (Fig. 2; Table 1). Comparison of activities on this basis implies that such chlorophyll as is formed in deficient plants is strictly comparable with that in healthy plants. The presence of abnormal, photochemically less active chlorophyll in deficient plants could result in an apparent reduction in other components of the Hill reaction. Any abnormality of the chlorophyll or its associated protein might be expected to result in a change in the characteristic absorption spectrum. However, examination of both the chloroplast suspensions and the 80 per cent. acetone extracts of these suspensions revealed no detectable qualitative difference between chloroplasts from healthy plants and from plants deficient in any nutrient. Although a small quantitative reduction was found in the proportion of chlorophyll *a* in the total chlorophyll under a number of deficiencies (Table 4), this was too slight to account for the reduced Hill reaction activities in terms of chlorophyll *a* as the only functional chlorophyll fraction. The differences in Hill reaction activity found in these experiments therefore do not appear to be explicable in terms of a grossly abnormal pigment complement resulting from nutrient deficiency. It is possible, however, that changes more subtle than those we have measured occur in both chloroplast composition and structure in deficient plants. Such changes could lead to reduced photochemical activity.

Bogorad *et al.* (1959) have reported that iron-deficient *Xanthium* leaves contain chloroplasts which have a granular disorganized appearance instead of the normal, highly organized lamellar structure of chloroplasts from healthy plants. However, the present results have shown that the chloroplast fraction of iron-deficient plants has a Hill reaction activity per unit of chlorophyll, at saturating light intensity, comparable with that of normal plants. It appears that highly organized chloroplast structure is not a prerequisite for Hill reaction activity, since small fragments of chloroplasts and even digitonin extracts of chloroplasts are known to retain this activity (Eversole and Wolken 1958).

The detailed mechanism of the steps which make up the Hill reaction is quite unknown. However, the overall reaction is known to consist of three components, viz. (1) the "light" step involving photochemical reactions through which water is split and an oxidized and reduced product are formed; (2) the "dark", enzymatic steps of the hydroxyl sequence which terminates in the liberation of molecular oxygen; and (3) the "dark", enzymatic steps in the reducing sequence whereby the reducing power is transferred to the added artificial oxidant—indophenol dye, (see diagram, p. 454). A decrease in the rate of any one of these reactions could result in a decreased overall Hill reaction rate (Brugger and Franck 1958). In order to obtain evidence as to which of these components is impaired by each deficiency, the effect of light intensity on the degree of impairment was studied. As a generalization, inhibitors of photosynthesis whose effects are lessened at low light intensities are

said to affect dark reactions, while those which are equally inhibitory at both high and low light intensities affect photochemical reactions (Rabinowitch 1945). However, the work of Gaffron and others on the effect of inhibitors on photoreducing algae demonstrates that this concept must be modified. Four inhibitors of photosynthesis, hydroxylamine (Weller and Franck 1941; Gaffron 1942), *o*-phenanthroline, phthiocol (Gaffron 1945*a*, 1945*b*), and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Bishop 1958) have been shown to act on the dark reactions of the oxygen evolution sequence. This is indicated by the fact that, at certain concentrations of these compounds, photosynthesis is inhibited while photoreduction is unaffected. In all cases photosynthesis is inhibited at both high and low light intensity. These examples prompt the generalization (Brugger and Franck 1958) that any factor which impairs the oxygen evolution sequence will, like an inhibitor of the photochemical step, result in inhibition of photosynthesis at all light intensities. It follows that, in the Hill reaction, inhibition which is dependent on light intensity will occur only when a dark reaction in the reductive sequence to indophenol dye is affected.

On this basis the present results (Tables 2 and 3) suggest that deficiencies of zinc, copper, or boron and probably postassium and phosphorus caused the impairment of some dark reaction in the reducing sequence to the indophenol dye, since in these cases the degree of impairment was reduced at low light intensities. On the other hand, with chloroplasts from plants deficient in either manganese, molybdenum, nitrogen, sulphur, calcium, or magnesium the impairment was as great, or greater, at low light intensities. This suggests that in these deficiencies either a light step, or a dark step involved in oxygen evolution has been affected. Chloroplasts from iron-deficient plants should probably be included in the latter category since, in this case, a reduction in Hill reaction activity was apparent only at limiting light intensities. This is consistent with the suggestion (Hill and Davenport 1952; Kamen 1956) that cytochrome *f* functions as an intermediate in the oxygen evolution sequence.

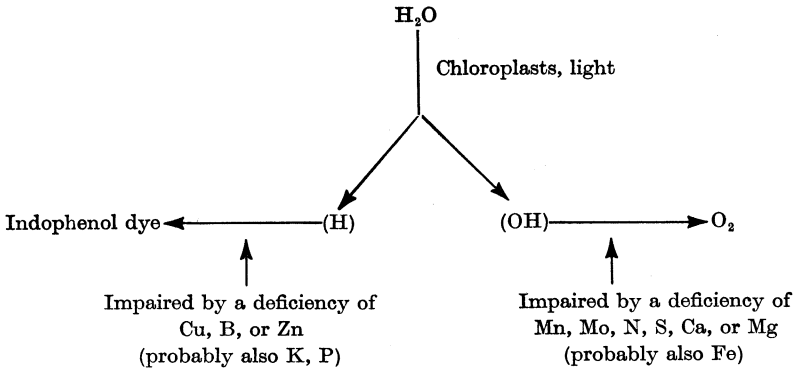
It should be possible to discriminate between impaired photochemical and impaired enzymatic reactions by virtue of their widely different temperature coefficients. Thus the Q_{10} of an enzymatic reaction is generally between 1 and 2, whereas the rate of a photochemical reaction is unaffected by temperature.

Under saturating light intensity, a decrease in temperature would result in a decrease in the Hill activity of the control chloroplasts. If an impaired enzymatic reaction is responsible for the reduced overall activity, a decrease in temperature would result in a decreased Hill activity. Since the rates of the control and enzymatically impaired chloroplasts are both reduced, no significant change in the relative rates would be observed by increasing the temperature. If a light step were impaired, on the other hand, a decrease in temperature would have no effect on the rate of the impaired reaction. Therefore a reduction in the relative degree of impairment should be observed by lowering the temperature.

The activity of those chloroplasts which showed undiminished degree of impairment at low light intensities was compared with that of normal chloroplasts at 30 and 10°C at saturating light intensity. (In the control chloroplasts the rate of the Hill reaction at 10°C was approximately one-half the rate at 30°C.) In no case was the degree of impairment affected by the change in temperature. This infers

that these deficiencies (manganese, molybdenum, nitrogen, sulphur, calcium, or magnesium) caused a reduction in the rate of some enzymatic reaction in the oxygen evolution sequence.

These conclusions concerning the site within the Hill reaction of the impairment caused by each deficiency can only be tentative. They are based on the assumption that each nutrient deficiency affects a single part of the Hill reaction. This is unlikely to be the case, especially for the macronutrients whose functions are manifold. Where a deficiency affects more than one site in the Hill reaction, only that site which is most impaired will be detected. With some nutrients the effect may vary with the degree of deficiency. However, the conclusions serve as a useful starting point for more detailed work on the effect of these nutrient deficiencies on the Hill reaction. They may be summarized as follows:



With respect to manganese deficiency these results are in accord with those found in studies on algae. Pirson, Tichy, and Wilhelmi (1952) and Arnon (1954) showed that the inhibition of photosynthesis caused by manganese deficiency is independent of light intensity. Working with cultures of the photoreducing algae *Ankistrodesmus*, Kessler (1955, 1957) demonstrated that manganese deficiency reduced photosynthesis but not photoreduction, and concluded that manganese was important in a dark reaction early in the hydroxyl sequence. Our results to date do not permit any conclusions as to the relative position in the hydroxyl sequence of the impairment caused by manganese deficiency in higher plants, but it is hoped to elucidate this in experiments now in progress.

It should be emphasized that the mere demonstration of a reduced metabolic activity associated with a nutrient deficiency in no way implies a direct function of this particular nutrient in that activity (cf. Pirson 1958). The effects observed could arise as an indirect consequence of the nutrient's direct action. It is felt that, to date, there is no unequivocal evidence of *direct* participation of any nutrient in the Hill reaction, although such a function has been claimed for manganese (Kessler 1957). With intact cells, as used by Kessler, the possibility of indirect action cannot be excluded, and in the present experiments no reactivation was found upon addition of manganous salts to isolated chloroplasts from manganese-deficient plants. The possibility remains, of course, that manganese participates in the Hill reaction in a complex molecule, such as a mangano-protein, which cannot be formed in isolated

chloroplasts. Indeed, the rapid restoration of photosynthesis upon addition of manganous salts to a manganese-deficient culture of *Scenedesmus* (Arnon 1954), and the increased manganese requirement of *Chlorella* under autotrophic as compared with heterotrophic conditions (Pirson and Bergmann 1955; Eyster *et al.* 1958) both suggest that manganese does participate directly in some photosynthetic reactions.

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