PHOTOSYNTHESIS OF BARLEY LEAVES INFECTED WITH BARLEY STRIPE MOSAIC VIRUS

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ABSTRACT

The primary leaves of ten day old Hordeum vulgaris L. var. Blackhulless were inoculated with barley stripe mosaic virus. Approximately ten days later, third leaves of healthy and diseased plants were used for photosynthetic studies. The latter were based on 0_2 evolution or $C^{14}0_2$ uptake and indicated that such factors as light intensity, temperature, $C0_2$ partial pressure, and the units chosen for comparison of photosynthesis affected the rates by healthy and infected third leaves. Expression of 0_2 evolution on either an area or a fresh weight basis indicated that no significant differences existed between healthy and infected leaves. However on a chlorophyll basis, measurement of 0_2 evolution showed that infected leaves photosynthesized at a greater rate than healthy leaves. In contrast, expression of $C^{14}0_2$ uptake on a fresh weight basis revealed that healthy leaves incorporated more radioactivity than infected leaves. A study of three photochemical activities by chloroplasts from infected leaves showed that they reacted differently to the virus. Both the Hill reaction and $C^{14}0_2$ fixation rates by infected chloroplasts were lower than those of healthy chloroplasts. In contrast, photophosphorylation was greater in infected than healthy chloroplasts. The possibility is considered that virus-induced photosynthetic alterations could account for the reduced growth and vigor of BSMV-diseased plants.

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSMV	barley stripe mosaic virus
EDTA	ethylenediaminetetraacetic acid
glucose-6-P	glucose-6-phosphate
H	healthy
Hepes	N-2-hydroxyethylpeperazine-N'-ethane-sulfonic acid
I	infected
MES	2-(N-morpholine)ethanesulfonic acid
NADP	oxidized nicotinamide adenine dinucleotide phosphate
$NADPH_2$	reduced NADP
P_i	inorganic phosphate
6-PGA	6-phosphogluconic acid
PMS	phenazine methosulfate
PPO	2,5-diphenyloxazole
POPOP	1,4-bis-2-(5-phenyloxazolyl)benzene
RNA	ribonucleic acid
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane

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INTRODUCTION

The green plant is capable of photosynthetically assimilating inorganic minerals, carbon dioxide, and water into substances which are required for its growth and development. Consumption of these assimilates by the respiratory process provides the necessary energy to carry out such metabolic activities as the building up of pectic and cellulosic substances for cell walls, fat and starch reserves, and proteins and nucleoproteins for the protoplasm and the nucleus. Under normal conditions, numerous genetically programmed enzyme systems rigidly control these activities. This results in the maintenance of a well-balanced rhythm of growth, multiplication, and reproduction in the plants. However, many agents are present in the biosphere which are potentially capable of disrupting these regulatory systems (82, 89). In each case the end result is similar, namely the undue expenditure of host reserves with the concomitant expression of physiological disorders and/or structural abnormalities. With inanimate agents such as soil and meteorological conditions, the disruption of host mechanisms is generally temporary, and these readily revert to normal with the onset of conditions more favorable for growth. On the other hand, certain animate and viral agents, normally regarded as obligate parasites, tend to induce marked changes in the metabolic activities of the host. Striking changes in the respiratory

metabolism and, to a lesser degree, photosynthesis have been reported. Whereas the respiration of diseased plants has been studied extensively (21,23,39,43), the interactions of pathogen development and photosynthesis has received little attention (15,23,39,85).

The peculiar nature of virus-host interactions offer distinct advantages over such studies in bacterial or fungal diseases. In direct contrast to the latter, viral pathogens lack an independent metabolic activity. Despite this deficiency, infecting virus particles are still capable of disrupting host metabolic activities (15,23,39,85).

A literature survey revealed very few metabolic studies on the BSMV-barley host combination (39,68). Preiss (68) showed that maximum differences in respiration between infected and healthy plants occurred approximately 9 to 12 days after inoculation. At this time the third leaves of infected plants showed extensive chlorotic mottling as well as the appearance of a characteristic necrogenous 'V' shaped mark. These foliar symptoms suggested that the photosynthetic activities of infected plants must undoubtedly be altered and this investigation was undertaken to reveal the extent to which BSMV had affected the photosynthetic activities of susceptible barley plants.

LITERATURE REVIEW

Photosynthetic studies have generally stressed the effect of the pathogen on the chlorophyll content and on the overall rate of photosynthesis by the plant. However, biochemical studies of the photosynthetic apparatus in the chloroplast have received less attention.

The color changes typical of most diseases indicate either that chlorophyll is not synthesized at the same rate as in healthy plants or that some chlorophyll is destroyed as a consequence of infection. Peterson and McKinney (67) reported changes in the chlorophyll content and chlorophyllase activity in leaves infected with four viruses which produced mosaic symptoms of varying severity in tobacco. In uninfected leaves, chlorophyllase activity and chlorophyll content were directly proportional, whereas in infected leaves chlorophyllase was greatest in the leaves that contained the least chlorophyll. As all the pigments were not decreased by the same proportion, these authors suggested that chlorosis was the result of chlorophyll failing to mask the yellow pigments. Akai and Fukutomi (1,2) also reported differences between the protochlorophyll, chlorophyll a and b, carotene and xanthophyll contents of healthy and downy mildewed leaves of rice plants. In the healthy leaves, the pigments decreased with the descending order of the leaf arrangement, whereas in diseased plants they increased. The

difference in chlorophyll content between the healthy and diseased leaves was most marked in the newly developed leaves - the latter showing a yellowish tint. They concluded that the yellowish tint of diseased upper leaves was probably due to a decreased chlorophyll content and not to an increased carotenoid content. However, in 1925 Elmer, cited in Bawden's text Plant Viruses and Virus Diseases (12), reported that infection with tobacco mosaic virus doubled the concentration of carotene while decreasing the amounts of both chlorophyll and xanthophyll.

The chlorotic symptoms characteristic of many disease syndromes may actually result from an inhibition of chlorophyll production due to the secretion of metabolites by the invading pathogen. Ryan et al (73) found that culture extracts from certain clones of Alternaria tenuis Auct. induced albinism in citrus seedlings when the seeds were germinated in the extracts. Fuller et al (31) demonstrated that a metabolite secreted by this fungus only induced chlorosis in cotton seedlings when it was placed in contact with germinating cottonseed before the tissue was exposed to light. It was suggested that the inhibitor blocked biosynthesis of chlorophyll and did not actually destroy the pigment. Studies by Braun (17) on the action of the wildfire toxin of Psuedomonas tabaci (Wolf and Foster) F.L. Stevens

suggested that this toxin either destroyed chlorophyll or inhibited its synthesis. Spectroscopic and chemical analyses of the chlorophyll of toxin treated and untreated leaves, revealed that chlorophyll concentrations in treated tissues were much lower than in comparable untreated tissue. As the chlorophyll from both leaf types were spectroscopically similar, he concluded that the biological effect exerted by the toxin was not upon the chlorophyll molecule per se.

While invasion by a pathogen generally results in chlorosis and in a reduction in chlorophyll in restricted areas of the plant, infection may result in enhanced chlorophyll contents of infected areas or of whole leaves. Holmes (45) cited several examples of over-all color abnormalities in leaves affected by viral diseases. Wang (90) showed that "green islands" surrounding rust pustules on leaves of Pinto bean were the result of pigment retention in the host tissue within the area of influence of the parasite. Similarly, Fucikovsky (30) reported that the green rings surrounding lesions on soybean leaves caused by Cercospora sojina Hara were due to chlorophyll retention.

Generally, these changes in chloroplast pigments are associated with an alteration in the morphology of the chloroplast. Cytological studies of wheat leaves by Allen (5) showed that chloroplasts were much smaller in rust-affected

than in healthy leaves. However, electron microscopic studies of chloroplast alterations of this type have generally been restricted to virus infected plants.

Misawa and Ehara (60) reported that with the lapse of time after infection, chloroplasts from cucumber mosaic virus infected cucumber cells showed a marked distortion and degeneration. Six to eight days after inoculation, osmiophilic bodies in the chloroplast expanded and twelve days after inoculation, the chloroplasts appeared highly disorganized with the stromal material breaking down and numerous vacuolated areas forming. By this time the expanded osmiophilic bodies had faded away. Eighteen days after inoculation, the chloroplast membrane and stroma lamellae had collapsed; in addition the grana lamellae was greatly reduced. However, while this pattern of disintegrations was occurring, apparently normal chloroplasts could still be observed within the same cells.

Gerola et al (34,35,36) carried out a series of electron microscopic observations into ultrastructural alterations induced in chloroplasts of virus infected plants. They found that the lamellar system of parenchyma cell chloroplasts of wheat plants infected with maize rough dwarf virus were disarranged. The grana and intergrana lamellae were randomly oriented rather than regularly aligned

as in normal chloroplasts. They also found chloroplasts from leaves of Petunia hybrida Hort. infected with arabis mosaic virus were similarly disorganized and that chloroplasts isolated from Chinese cabbage leaves infected with turnip yellow mosaic virus were more deeply lobed, shorter and thicker than normal. These lobed portions often detached themselves from the chloroplasts to lie free in the cytoplasm. Similar degenerative changes in chloroplasts have been reported for other virus-host combinations such as barley stripe mosaic virus-barley (32), wheat striate mosaic virus-wheat (53), tobacco mosaic virus-tomato (79), turnip yellow mosaic virus-Chinese cabbage (19), sunflower mosaic virus-sunflower (9), California tobacco rattle virus-tobacco (22), and tobacco mosaic virus-Datura stramonium L. (18).

It is not surprising that plants with this loss of chlorophyll and/or breakdown of the chloroplast should suffer impaired photosynthesis. However, few measurements have been made on photosynthesis by leaves infected with obligate parasites (15,23,39). Sempio (78) remarked that as early as 1904 Montemartini had discovered a gradual attenuation of photosynthesis in leaves infected with certain groups of the Uredinales. Parris (66) compared the rates of apparent photosynthesis by diseased and healthy bean leaflets and found

that bean leaflets infected with Colletotrichium lindemuthianium (Sacc. and Magn.) Bri. and Cav. assimilated 24% less carbon dioxide than did the companion healthy leaflets on the same plant. However, bean leaflets infected with Erysiphe polygoni D.C. did not reduce normal assimilation until yellowing was present. Excessive yellowing was then accompanied by a pronounced reduction in assimilation as compared with the assimilation of healthy leaflets. In mildewed wheat leaves, Allen (4) observed an initial high rate of photosynthesis followed by a drop in both chlorophyll content and photosynthesis and, by the ninth or tenth day, photosynthesis reached a very low level. Similar trends of a stimulated photosynthesis followed by a subsequent decline were reported for the same host-pathogen combination by Sempio (77) and Scott and Smillie (76), and for the Helminthosporium blight of rice plants by Akai and Tanaka (3).

Edwards and Allen (25) have refined the earlier techniques (4) by photosynthetically feeding $C^{14}O_2$ to the powdery mildew-barley complex. Photosynthesis by the complex was then found to decrease after inoculation as compared with healthy leaves. In addition they found that the ethanol soluble metabolites of the infected host tissues differed only slightly from those of the healthy, the major differences being a decreased amount of sucrose and an increased amount

of malic acid and serine. Similar decrease in photosynthetic assimilation of carbon dioxide have been reported for Yellow Rust infection of wheat (24) and for potato plants infected by Phytophthora infestans (Mont.) d By. (33).

In contrast to this general finding of a decreased photosynthesis, several workers have presented evidence of a stimulated photosynthesis by host plants following pathogenic attack. Wang (90) showed that, in rusted wheat, fixation was depressed at sporulation sites, but was stimulated in the surrounding zone. Likewise, Livne (54) observed a marked stimulation in photosynthesis of young bean trifoliate leaves during periods when rust-infected unifoliate leaves were inhibited. A similar effect was also found in diseased wheat and safflower. Livne suggested that inhibition of photosynthesis in infected tissue could be compensated in part by stimulation of tissues at a distance from the infected leaf.

Experimental evidence of an altered photosynthetic pattern in plants invaded by phytobacterial pathogens is lacking, except for the work of Beckman et al (13). They found that the reduced photosynthesis in leaves of banana plants attacked by Pseudomonas solanacearum E.F. Sm. was attributed to water stress, and was readily overcome when this stress was eliminated.

Effects on photosynthesis have been studied with only

a few viruses. Owen (63,65) measured apparent photosynthesis of virus-infected plants and found in most instances that there was no effect until symptoms appeared. However, when he inoculated tobacco leaves with tobacco mosaic virus a 10-15% reduction in photosynthetic activity occurred within one-half hour after inoculation (64). Why this immediate effect on photosynthesis should occur has not yet been resolved particularly in view of the fact the activities of chloroplasts isolated from such plants failed to show effects until seven days after inoculation (96). A subsequent attempt to verify Owen's work failed (95). Roberts and Corbett (72) studied photosynthesis of tobacco plants infected with tobacco ringspot virus and when based on leaf area, photosynthesis was significantly reduced in necrotic tissue but not in recovered tissue. Photosynthesis was, however, significantly reduced in recovered leaves when the results were expressed on a chlorophyll basis. These authors therefore concluded that decreased photosynthesis in virus-infected plants was due to early changes in the enzymatic components of the chloroplast.

The effects of plant pathogens on enzyme controlled reactions of photosynthesis such as the Hill reaction, photophosphorylation and $C0_2$ fixation, have received very little attention. Spikes and Stout (81) measured the Hill reaction of sugar beet chloroplasts by a potentiometric technique as

a function of light intensity in order to determine whether the beet yellows virus affected the rate-limiting photochemical reaction, the rate-limiting dark reaction, or both. They found the rates of both processes were decreased by approximately 50%. Zaitlin and Jagendorf (96) reported a decrease in the Hill reaction and in photophosphorylation by chloroplasts isolated from tobacco leaves inoculated with tobacco mosaic virus. However, it was demonstrated that this loss of enzymatic activity was due to a secondary effect of virus infection. Diseased plants supplemented with nitrogen fertilizer failed to show a decreased Hill reaction despite a significant increase in virus multiplication. The authors suggested that virus multiplication had affected chloroplast activities by inducing a state of nitrogen stress. Goffeau and Bové (37) demonstrated alterations in the Hill reaction and in cyclic and noncyclic photophosphorylation in chloroplasts isolated from turnip yellow mosaic virus-infected leaves. Only with chloroplasts from local lesion leaves were these workers able to observe an increase in photosynthetic activity in comparison with chloroplasts from control plants and this increased activity occurred at a time when the virus was in an active state of multiplication in these leaves. In contrast, decreased activity was recorded for chloroplasts from systemically infected leaves of the same

plants. This was in agreement with the results of Spikes and Stout (81) and Zaitlin and Jagendorf (96). These activities have also been partially characterized in rusted and mildewed host plants.

Wynn (94) studied the influence of infection by Puccinia coronata Cda. f. sp. avenae on photosynthetic incorporation of P_i into ATP. Chloroplasts isolated from infected oat seedlings six days after inoculation were found to photophosphorylate ADP at essentially the same rate as chloroplasts from comparable healthy leaves. This was observed regardless of whether the chloroplasts had been obtained from a susceptible or a resistant variety. Wynn suggested, therefore, that the primary or "light" reactions of photosynthesis which are centered around this process were not altered by rust infection. Scott and Smillie (76) found that the capacity of leaves infected with powdery mildew for partial reactions of photosynthesis, such as the Hill reaction and the photoreduction of NADP, decreased during the later stages of infection. Associated with these decreased activities was a decrease in the activities of other chloroplast enzymes such as aldolase and NADPH-diaphorase.

Varied results have been reported for the fixation of CO_2 by chloroplasts and/or the carboxylative enzymes isolated from infected leaves. Farkas et al (28), working with

turnip yellow mosaic virus, found no effect on the rate of fixation, even when there was a breakdown of chlorophyll and chloroplast degeneration. Malca et al (57) attempted to correlate the reported decrease in photosynthesis by mildewed barley (75) with decreased activities of the photosynthetic carboxylative enzymes. The authors compared the activities of phosphoriboisomerase, phosphoribulokinase and ribulose-1, 5-diphosphate carboxylase in cell-free extracts of healthy and infected barley leaves. The activity of these enzymes decreased considerably in infected leaves soon after the appearance of symptoms. Similar changes to these have also been reported for susceptible leaves of maize infected with Helminthosporium carbonum Ullstrup (56).

MATERIALS AND METHODS

A. GROWTH AND INOCULATION OF PLANT MATERIAL

The barley variety H. vulgaris var. Blackhulless was used exclusively as the host material. Seed of this variety was kindly provided by Dr. R. G. Timian, United States Department of Agriculture, Fargo, North Dakota and the supply was increased by planting some of the original seed in field plots.

All laboratory experiments employed plants grown from seeds which had been planted in a loam-sand mixture in six-inch plastic pots. The pots were placed in an environmental control room (Coldstream Growth Chamber) where the seeds germinated and the resulting plants allowed to grow for ten days at which time they were inoculated. In this controlled environment the temperature was held at 20±1°C and the light intensity, at the level of the seedlings, was 1200 to 1500 ft-c from combined fluorescent and incandescant bulbs. A 16-hour photoperiod was used, followed by an 8-hour dark period.

An unknown strain of BSMV was isolated from plants grown from infected barley seed (H. vulgaris var. Plush) kindly provided by Dr. W.A.F. Hagborg, Canada Department of Agriculture, Winnipeg, Manitoba. The virus was maintained in Blackhulless barley seedlings.

The inoculation procedure employed for this study was

similar to the one followed by Preiss (68). Leaves showing pronounced symptoms were harvested from the infected stock plants and pulped in a mortar with buffer, in the ratio of 1 g leaf tissue to 1.5 ml of phosphate buffer, pH 7.2. The liquid, from which most of the tissue was removed by passage through a double layer of cheesecloth, served as the virus suspension. To ensure a pronounced symptom appearance, 600-mesh Carborundum powder was dusted lightly over the surface of the primary leaves and the virus suspension was then rubbed over the entire surface of the primary leaf with a pair of tongs fitted with sponge-like plugs. Control plants were rubbed in a similar way with an extract from healthy plants.

B. STUDIES OF APPARENT PHOTOSYNTHESIS

(a) Photosynthesis by Leaf Discs as Measured by Oxygen Evolution

(1) Plant material

Leaf discs were cut, from the third leaves of healthy and infected plants, with a number three cork borer from three locations on each leaf, namely the base, the middle, and the tip.

(2) Measurement of apparent photosynthesis

The methods employed for this study were essentially those used by Forsyth and Hall (29). Standard

Warburg manometers and flasks were used in a conventional Warburg apparatus. Ten leaf discs were weighed and placed in the main compartment of the flask. To retard water loss from the discs 0.5 ml of glass distilled water was placed in the sidearm. Pardee's CO_2 buffers (85) were placed in the centre well. A folded filter paper was placed in each centre well to increase the buffer surface exposed to the flask atmosphere. After a ten minute equilibration period, the lights were switched on, giving approximately 500 ft-c or 3000 ft-c of white light at flask level. Light intensities were measured with a Weston light meter.

Gas output measurements were made at ten minute intervals. Data obtained was calculated as the apparent photosynthesis on an area, a fresh weight, and a chlorophyll basis.

(b) Photosynthesis by Whole Leaves as Measured by $C^{14}O_2$ Fixation

(1) Apparatus

The apparatus for the fixation of $C^{14}O_2$ by whole leaves consisted of a system for the generation of labeled CO_2, an incubation chamber, and an outlet system for the absorption of unfixed $C^{14}O_2$ (91). The incubation chamber was partially immersed in water contained in a large beaker, the whole apparatus being placed on a magnetic stirrer. A coil of copper tubing, inserted into the beaker and attached to a cold water faucet, cooled the system.

(2) Procedure

Five gram lots of third leaves from healthy and infected plants were placed with their cut ends in separate vials containing water. After placing the vials in the incubation chamber, the lid was closed and the chamber was evacuated after all stopcocks had been closed. At zero time a few drops of concentrated sulfuric acid were introduced into 0.5 ml of $NaHC^{14}O_3$ containing 500 μC C^{14} to generate the $C^{14}O_2$. Approximately 1500 ft-c of white light was supplied to the leaves to promote photosynthetic CO_2 fixation. At the end of 20 minutes, the remaining $C^{14}O_2$ was flushed out and trapped in NaOH.

(3) Isolation of the radioactive fractions

The extraction procedure was essentially that employed by Edwards and Allen (25). The leaves were immediately removed from the incubation chamber, weighed, cut into small pieces, then placed in boiling 80% ethanol (1 g - 15 ml) and refluxed for 30 minutes. The supernatant liquid was decanted and the residues were ground in 80% ethanol in a mortar, then centrifuged at 7200 x g for 10 minutes. The supernatant liquid was decanted, the residue was ground and centrifuged a second time. The supernatants from each centrifugation were combined and made to 100 ml total volume. The residue from the last centrifugation was resuspended in

alcohol and then made to 100 ml total volume. The samples were designated as the alcohol soluble and insoluble fractions respectively.

(4) Assay of $C^{14}O_2$ incorporation

One-ml aliquots were removed from each of the fractions and pipetted into separate liquid scintillation vials along with 10 ml of scintillation solvent. The solvent system consisted of dioxane (833 ml), 2-ethoxyethanol (167 ml), naphthalene (50 g), PPO (4 g), and POPOP (0.1 g). The vial contents were counted in a Packard Co. Tri-Carb 3000 series, liquid scintillation counter over a background of 13-18 cpm.

C. PHOTOCHEMICAL ACTIVITIES OF ISOLATED CHLOROPLASTS

(a) Hill Reaction

(1) Preparation of the chloroplasts

Chloroplasts were isolated according to the method of Jagendorf and Wildman (49). Five gram samples of leaves from healthy and diseased plants were harvested, washed with distilled water, then blotted dry with absorbent tissue, and ground immediately in a chilled mortar with 15 ml of chilled 0.02 M phosphate buffer, pH 6.8, containing 0.05% KCl (86). The resulting brei was filtered through four layers of cheesecloth into prechilled centrifuge tubes

and centrifuged immediately at 1000 x g for 10 minutes. The centrifuged pellets were suspended in 5 ml of the same buffer, using a homogenizer operated at a very low speed. Centrifugation and resuspension were repeated a second time, except that the chloroplasts were resuspended in 2 ml of the buffer. The chloroplast suspensions were kept on crushed ice until needed. All operations were carried out in a cold room maintained at approximately $4^{o}C$.

(2) Assay system

The Hill reaction was measured manometrically as oxygen evolution in a Warburg apparatus operated at $15^{o}C$. Recrystallized p-benzoquinone was used as the hydrogen acceptor (8,86). The main compartment of the vessels contained 500 µg of chlorophyll in 2.8 ml of phosphate buffer, pH 6.8. Into the darkened sidearm was pipetted 0.2 ml of the quinone solution consisting of 40 mg of quinone in 10 ml of 0.01 N H_2SO_4 (8). After equilibration and tipping, the vessels were illuminated with approximately 3000 ft-c of light from a bank of four 300 Watt photoflood lamps. In experiments in which Hill reaction rates were studied as a function of light intensity, the light intensity was varied by altering the voltage to the light source with a Variac (48). The footcandles at each voltage were recorded with a Weston light meter. Oxygen evolution was measured for a 20 minute period (20,86).

(b) Photophosphorylation

The methods employed for the study of this photochemical activity were essentially those described by Avron (10).

(1) Preparation of the chloroplasts

Third seedling leaves were collected in 5 gram lots, cut into small pieces and placed in 20 ml of a chilled buffer containing 0.4 M sucrose, 0.05 M Tris, 0.01 M NaCl and 0.02 M sodium ascorbate, pH 7.8. The extraction process was carried out in an Omnimixer homogenizer operated at full speed for 5 seconds. The brei was pressed through four layers of cheesecloth into chilled centrifuge tubes and centrifuged at 200 x g for 1 minute. The supernatant liquid was decanted and recentrifuged at 2000 x g for 10 minutes. The pellet from this centrifugation was suspended in 2 ml of the grinding buffer and kept on crushed ice until needed. All manipulations were carried out at approximately $4^{o}C$.

(2) Purification of the P^{32} solution

The inorganic-P^{32} solution was refluxed for one hour at $100^{o}C$, then mixed with acid washed charcoal and filtered. After neutralization with 1 N NaOH, the filtrate was diluted with potassium phosphate buffer, pH 7.6, to give a solution of 2 μmoles of orthophosphate containing 1 μC P^{32} per 0.1 ml.

(3) Reaction system and procedure

The reaction was carried out in chilled, thick walled pyrex test tubes. Each tube contained in μmoles: Tris buffer (pH 7.8), 48; $MgCl_2$, 2; K_2HPO_4-KH_2PO_4, 2, containing 1 μC P^{32}; glucose, 30; and PMS, 0.05. In addition each tube contained 7 mμmoles of dicumarol, 200 μg (13 units) of commercial yeast hexokinase, and an aliquot of chloroplasts containing 500 μg of chlorophyll. The final volume was made to 3 ml with distilled water. Dark controls were carried out in tubes wrapped in aluminum foil.

The tubes were attached to a rack (44) which was fastened to a Warburg manometer stand. The latter was inserted in a refrigerated Warburg apparatus operated at 15°C. The reaction was started by adding the p^{32} and turning on the lights. The light source was provided by a bank of four 300 Watt photoflood lamps and was adjusted to provide 3000 ft-c of white light. After 1 minute the lights were turned off and the reactions were stopped by the addition of 0.3 ml of 20% TCA. The acidified reaction mixture was centrifuged and the supernatant liquid was saved for analysis of "organic phosphate".

(4) Assay of ATP^{32} formation

One-ml aliquots from the supernatant liquids were placed in pyrex glass tubes, 1.2 ml of acetone was added, and

the contents allowed to stand for 10 minutes. The total volume of the tube was made to 2.5 ml with water saturated with isobenzene-butanol (1:1 v/v). After phase separation, 0.8 ml of a molybdate reagent containing 5 g of ammonium molybdate and 40 ml of 10 N H_2SO_4 in 100 ml of solution was added. Following decantation of the upper layer, 0.02 ml of 0.02 M KH_2PO_4 and 7 ml of the isobutanol-benzene mixture were added to the lower layer. After phase separation the upper layer was again decanted and discarded. The aqueous layer was then used for radioactivity measurements.

Aliquots of 50 µl were removed from this aqueous layer, pipetted into aluminum planchets and dried down under infrared light. The planchets were counted directly with a Philips Scaler (Model PW 4035). Background and total counts were taken for every experiment.

The µmoles of ATP formed were calculated from the following equation:

$$\mu M\ ATP^{32} = \text{corrected cpm in planchet} \times 3.3 \times \frac{(\text{volume in flask after TCA addition})}{\text{volume used for assay}} \times \frac{(\mu M\ P_i \text{ in flask})}{\text{total cpm added}}$$

(c) CO_2 Fixation

(1) Preparation of the chloroplasts

The chloroplasts were isolated according to

the method described by Jensen and Bassham (50). Three solutions, designated A, B, and C, were used for the isolation and subsequent assay of the chloroplasts. Each solution contained the following: 0.33 M sorbitol; 0.002 M $NaNO_3$; 0.002 M $MnCl_2$; 0.002 M EDTA (dipotassium salt); 0.002 M sodium isoascorbate; and 0.0005 M K_2HPO_4. In addition each solution contained various concentrations of the Zwitterionic buffers recommended by Good et al (38). Solution A contained 0.05 M MES, pH 6.1 and 0.02 M NaCl; solution B contained 0.05 M Hepes, pH 6.7 and 0.02 M NaCl; and solution C contained 0.05 M Hepes, pH 7.6 and 0.005 M $Na_4P_2O_7$.

Five gram lots of healthy and infected third leaves were buffered with 30 ml of solution A and blended for 5 sec at high speed in a semimicro Monel homogenizing vessel on a Waring Blendor. The brei was pressed through four layers of cheesecloth into chilled centrifuge tubes and centrifuged for 50 sec at 2000 x g. The resulting pellet was suspended in solution B and kept on crushed ice until needed. All manipulations were carried out at approximately $4^{o}C$.

(2) Reaction system

CO_2 fixation was carried out in round bottomed pyrex tubes. To each tube was added an aliquot of chloroplasts containing 500 µg of chlorophyll and solution C to a volume of 0.98 ml. The reaction tubes were mounted on a

rack attached to a Warburg manometer stand and inserted in a refrigerated Warburg apparatus operated at 20°C. The tubes were preilluminated for three minutes. Illumination from a bank of four 300 Watt photoflood lamps provided 3000 ft-c of white light at tube level. At the end of the preillumination period, 0.02 ml of a 0.006 M sodium bicarbonate solution containing 24 μC $HC^{14}O_3$ was added to each tube to give a final volume of 1 ml. After 6 minutes of photosynthesis, the chloroplast reactions were stopped by the addition of 2 ml of methanol. A stream of air was then bubbled through each reaction system to remove the unfixed $C^{14}O_2$.

(3) Assay of $C^{14}O_2$ incorporation

Aliquots of 50 μl were removed from each tube and pipetted into separate liquid scintillation vials along with 10 ml of scintillation solvent. The vial contents were counted in a Packard Co. Tri-Carb 3000 series, liquid scintillation counter. The counts per minute were corrected for background and dark controls.

D. CHLOROPHYLL DETERMINATION

The chlorophyll contents of leaf discs were assayed according to the method of Von Abrams and Pratt (87). The discs were extracted in 80% acetone and the extract was made to a final volume of 10 ml. Optical density was read on a

Bausch and Lomb Spectronic 20 colorimeter at 665 mμ. Chlorophyll was then expressed in arbitrary units ($\mu g/cm^2$) as the product of optical density and the final volume (in ml) of the extract, x 10^3, divided by the initial weight (in mg) of the excised discs.

The chlorophyll content of chloroplast suspensions was determined by diluting 0.1 ml of the suspension to 20 ml with 80% acetone. The optical density was read at 652 mμ on a Bausch and Lomb Spectronic 20 colorimeter. The mg of chlorophyll per ml of original suspension was calculated from the equation given by Whatley and Arnon (93):

$$(\text{chlorophyll})_{mg/ml} = \text{O.D.} \times 5.8$$

When the concentrations of chlorophylls a and b were to be determined, the optical density was read at 645 mμ and 663 mμ on a Zeiss spectrophotometer (Model PM QII). The concentrations of chlorophyll a (c_a) and chlorophyll b (c_b) were calculated using the absorbancy index values of Arnon (6):

$$c_a = 12.717\ \text{O.D.}_{663} - 2.584\ \text{O.D.}_{645} = mg/L$$

$$c_b = 22.869\ \text{O.D.}_{645} - 4.670\ \text{O.D.}_{663} = mg/L$$

Absorption spectra of chloroplast suspensions were carried out in a Unicam recording spectrophotometer (Model SP 800).

EXPERIMENTAL RESULTS

A. SYMPTOMS OF THE BSMV DISEASE ON BLACKHULLESS BARLEY LEAVES

When the primary leaves of ten day old seedlings were inoculated, the first visible systemic symptoms usually appeared on the third leaf, after six days. These symptoms appeared initially as fine chlorotic lines along each side of the leaf-blade margin. As the disease progressed, the symptoms on the third leaf changed to a chlorotic mottling, with spotting and/or streaking. Occasionally, however, a pronounced bleaching occurred. Associated with these symptoms was a necrotic browning which occurred in stripes or broken stripes, with or without aggregation into a necrogenous 'V' shaped mark. The loss in chlorophyll occurred primarily in the area between the base of the leaf and the necrogenous 'V'.

Similar symptoms, except for the necrotic browning, were apparent on other leaves as they emerged. However, as the plants approached the heading stage, the newly emerged leaves showed a "recovery" from the disease in that the symptoms were limited to faint chlorotic lines.

At heading mature, virus-infected plants generally displayed four characteristics. These were stunting, a reduction of grain yield as indicated by the incompletely filled, smaller sized heads, a retardation, or delayed onset

of heading, and an incomplete emergence of the heads from the sheaths. Often the sheaths enclosing the heads of the diseased plants showed the spotting-type of symptom observed on the leaves. These symptoms agreed with those reported in the literature by other workers (41,47,58,59,61).

B. APPARENT PHOTOSYNTHESIS BY HEALTHY AND BSMV-INFECTED BLACKHULLESS BARLEY LEAVES

(a) Photosynthesis by Leaf Discs

(1) Rates of oxygen evolution during the first hour of measurements

The symptoms obtained on inoculation of the barley plants by BSMV, and recorded in the section on symptomalotogy, provided evidence that the disease induced considerable bleaching and necrosis in leaves of susceptible test plants. This suggested a destruction of chlorophyll and therefore a decreased photosynthetic activity in diseased plants.

Preiss (68) has shown that maximum differences in respiration between diseased and healthy plants, grown under controlled environmental conditions, occurred approximately ten days after inoculation. Also, by this time the third leaves of diseased plants showed extensive chlorotic mottling, as well as the characteristic necrogenous 'V' shaped mark. Consequently this leaf was chosen for comparative studies of photosynthesis between healthy and diseased

plants. However, since host-parasite studies are comparative in nature, interpretation of the experimental findings is dependent upon a consideration of such factors as the time after inoculation when the measurements were made, the physiological state of the plant, the leaves chosen for the study, and the mode of expression of the results. While these factors have been proposed for respiratory studies (62), they are considered to be of equal importance for photosynthetic studies. Therefore, they were considered in the design of the experiments.

To avoid the implications caused by light respiration (28,83,98), "apparent" photosynthesis was used to measure changes in the rates of photosynthesis induced by the virus. The rates were determined by measuring the oxygen evolved by ten disc samples taken from healthy and infected leaves respectively. The results were expressed on an area, fresh weight, and chlorophyll basis.

Table I shows the photosynthetic rates obtained from experiments carried out under saturating conditions of temperature (25^{o}C), light intensity (3000 ft-c), and CO_2 partial pressure (1%). In columns 2 and 3 numbered from the left, expression of the activities on an area basis indicated that the apparent photosynthesis was slightly less in infected than healthy tissues. However, this difference was not considered to be significant. For example, the mean activities

TABLE I. Apparent photosynthesis by healthy and BSMV-infected barley leaf discs expressed per units of area, fresh weight or chlorophyll.

Experiment	Oxygen evolved in 1 hr.						chlorophyll[a] content	
	µl/cm²		µl/100 mg		µl/µg chlorophyll		µg/cm²	
	H	I	H	I	H	I	H	I
1	40.2	35.4	37.9	32.6	2.31	3.19	17.4	11.1
	41.9	39.3	39.2	37.2	2.30	3.14	18.4	12.5
2	43.4	45.4	43.9	44.4	2.45	2.99	17.7	15.1
	47.4	38.2	42.2	37.2	2.71	2.97	17.5	12.8
3	38.7	38.2	37.8	33.3	2.22	3.89	17.4	9.8
	40.6	39.8	36.4	37.7	2.16	2.82	18.8	14.1
4	43.0	40.2	38.2	39.2	2.44	3.12	17.7	12.9
	44.6	38.4	40.4	38.3	2.56	3.06	17.4	12.5
5	42.5	40.2	38.3	35.4	2.61	3.00	16.3	13.4
	46.6	45.8	43.8	42.4	2.62	3.98	17.8	11.5
6	40.5	39.8	36.2	37.4	2.13	2.87	19.1	13.8
	45.0	43.2	40.8	39.4	2.37	3.27	19.0	13.3
7	40.2	39.5	35.4	39.8	2.31	3.18	17.8	14.8
	45.8	43.4	43.9	40.4	2.70	3.64	17.1	11.9
Mean	43.2	40.5	39.6	38.1	2.42*	3.16	17.8	12.8

[a] chlorophyll expressed arbitrarily (µg/cm²) as the product of optical density read at 665 mµ and the final volume (in ml) of the extract, X 10^3, divided by the initial weight (in mg) of the tissue. No correction was made for specific absorbance.

* differences significant at the 0.01 level by the t-test.

from seven experiments for healthy and infected discs were 43.2 µl and 40.5 µl respectively. On this basis the oxygen evolved by infected discs was approximately 94% of that from healthy discs when the oxygen evolution from healthy discs was made equal to 100%. When the apparent photosynthesis was expressed on a fresh weight basis, similar results were obtained as seen by a comparison of columns 4 and 5. However, a comparison made on a per unit chlorophyll basis indicated that infected tissues photosynthesized at a faster rate than healthy tissues. Columns 6 and 7 show that the mean activities of infected and healthy tissues were 3.16 µl and 2.42 µl per µg of chlorophyll respectively, giving a ratio of infected to healthy activities of 1.306. This was observed because infected discs contained less chlorophyll than the healthy discs as seen in columns 8 and 9 of Table I.

The data presented in Table I represented total oxygen evolution recorded for one hour. However, these totals did not take into account fluctuations in oxygen evolution by the tissues during this one hour period. Possibly a different interpretation could be made had another time period been chosen for the expression of the results. Since these totals represented six readings taken every ten minutes, oxygen evolution for similar readings from each of the seven experiments were averaged and reported in the form of a progress

curve. Figures 1A and 1B show that over a one hour period there were no significant differences ($p > 0.05$) between photosynthesis by healthy and infected tissues when the results were expressed per unit of area or per unit fresh weight. Figure 1C shows that on a chlorophyll basis the infected discs consistently evolved more oxygen than the healthy discs. The differences were found to be significant ($p < 0.01$).

The progress curves showed that a single measurement of the total oxygen evolved after one hour was a measure of the effect the virus had on the apparent photosynthesis by infected leaves. Therefore, in subsequent experiments oxygen evolution was determined at the end of a one hour period and the results were expressed on an area, fresh weight, or chlorophyll basis.

(2) Effect of environmental factors on the apparent photosynthesis

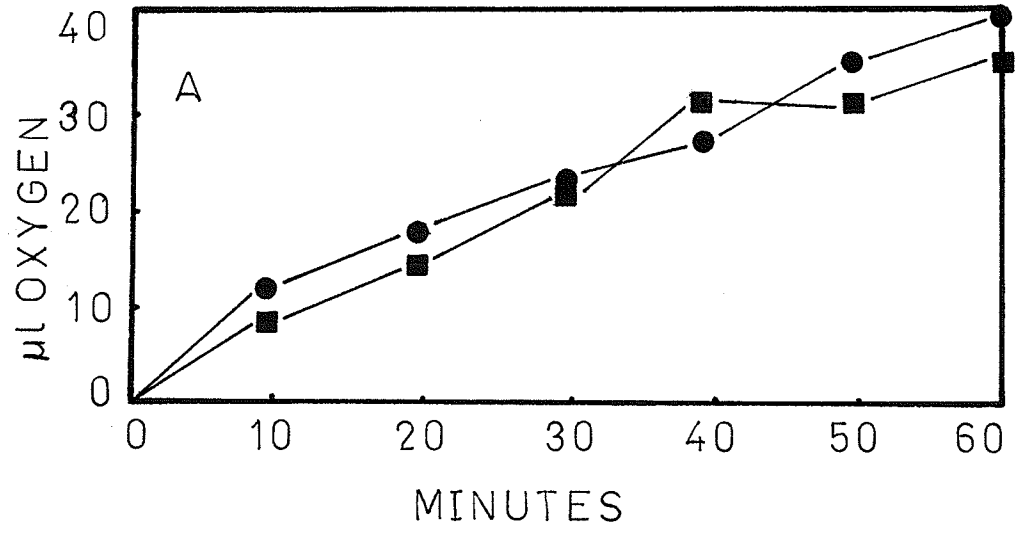
Photosynthesis consists of at least two different phases, a photochemical reaction requiring light and a biochemical reaction requiring carbon dioxide (14). Each reaction is affected by environmental factors such as light intensity, temperature, and carbon dioxide pressure. Studies were carried out to measure the influence these factors had on the rates of apparent photosynthesis in the third leaves of diseased plants.

Figure 1. Effect of virus infection on the apparent photosynthesis by healthy and BSMV-infected barley leaves.

A. area basis $\mu l/cm^2$
B. fresh weight basis $\mu l/100$ mg
C. chlorophyll basis $\mu l/ug$

● healthy

■ infected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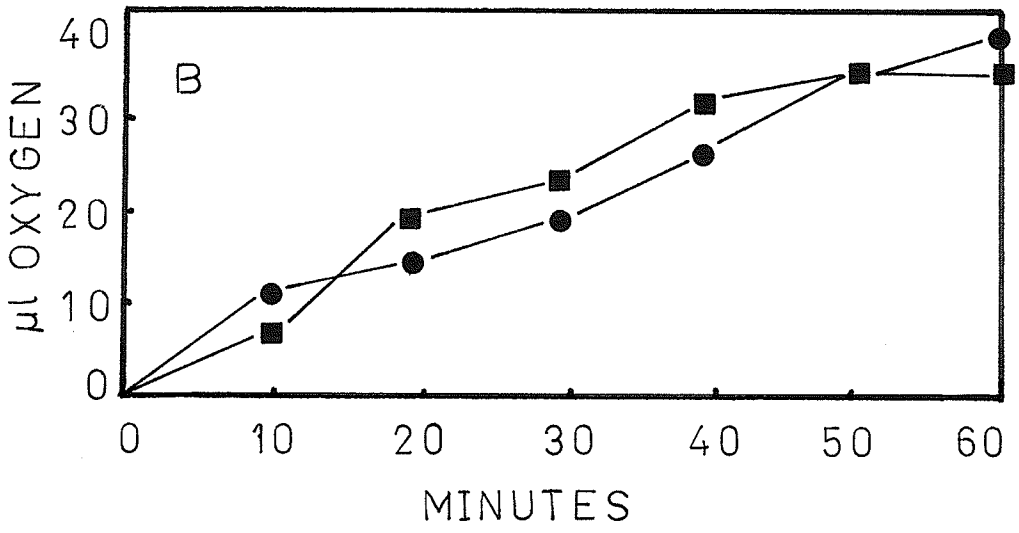
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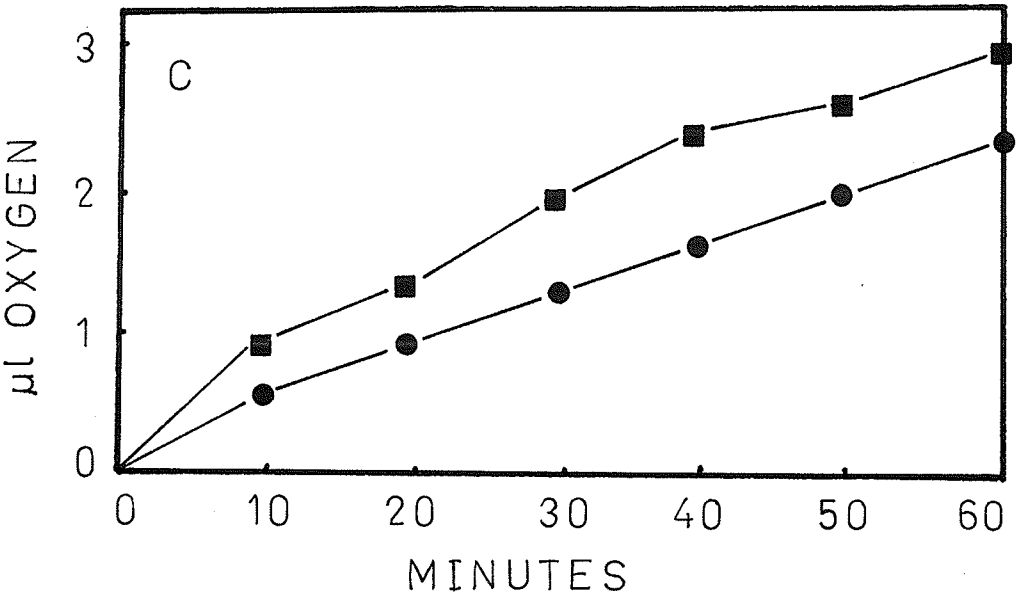
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(i) Effect of light intensity

Blackman (14) showed that the effect of light intensity on the photochemical reaction could be studied by maintaining constant those factors which affected the biochemical reactions. In the study reported here the temperature and carbon dioxide pressure were held constant while the effect of two different light intensities was studied. The apparent photosynthesis was expressed as µl of oxygen evolved in one hour on an area, fresh weight, and chlorophyll basis and is presented in Table II.

It may be seen that there was a significant effect of light intensity on the rates of apparent photosynthesis by healthy and infected leaf discs. However, at neither light intensity was there a significant difference ($p > 0.05$) in the rates between healthy and infected discs when the results were expressed as units of area or fresh weight. For example, at 500 ft-c the oxygen evolved by infected tissues expressed on an area and fresh weight basis was 91% and 96% respectively of that for healthy tissues. However, a comparison of these activities on a per unit chlorophyll basis indicated that infected tissues photosynthesized at a faster rate than healthy tissues. At 500 ft-c, the oxygen evolved by infected discs was found to be approximately 148% that evolved from healthy discs and at 3000 ft-c it was approximately 141%.

TABLE II. Effect of light intensity on apparent photosynthesis by healthy and BSMV-infected barley leaves[a].

Light intensity	Oxygen evolved in 1 hr.										
	µl/cm²			µl/100 mg			µl/µg chlorophyll			Chlorophyll[c] content µg/cm²	
ft-c	H	I	%[b]	H	I	%[b]	H	I	%[b]	H	I
500	13.1	12.3	91.3	13.1	12.6	96.2	0.76*	1.07	148.4	18.3	11.7
3000	43.2	40.5	93.7	39.6	38.1	96.2	2.42*	3.16	140.9	17.8	12.8

a figures in the table represent the means of seven to ten experiments.

b activities of infected leaf discs expressed as a percentage of the healthy controls.

c chlorophyll expressed arbitrarily (µg/cm²) as the product of optical density read at 665 mµ and the final volume (in ml) of the extract, X 10^3, divided by the initial weight (in mg) of the tissue.

* differences significant at the 0.01 level by the t-test.

This was observed because, as shown by the last two columns of Table II, healthy discs contained more chlorophyll than infected discs.

(ii) Effect of temperature

Studies were carried out to see how the biochemical reactions of photosynthesis were affected by temperature in diseased plants. The two phases of photosynthesis differ in their response to temperature. The photochemical phase of photosynthesis is known to be relatively insensitive to temperature changes, whereas the biochemical phase is greatly influenced by temperature. To test for the influence of temperature on the rates of apparent photosynthesis in healthy and infected leaf discs, experiments were designed in which the temperature was varied while the light intensity and carbon dioxide partial pressure were held constant. Oxygen evolution was determined from samples of ten discs and the results were expressed on an area, fresh weight, and chlorophyll basis. Table III shows the effect of four temperatures on apparent photosynthesis by healthy and infected leaf discs.

The study showed that as the temperature increased from 15°C to 30°C there was a marked increase in the amount of oxygen evolved. However, a comparison of the results on an area or a fresh weight basis revealed that no major differences existed between the rates for healthy and infected

TABLE III. Effect of temperature on apparent photosynthesis by healthy and BSMV-infected barley leaves[a].

Temperature	Oxygen evolved in 1 hr. µl/cm²			µl/100 mg			µl/µg Chlorophyll			Chlorophyll[c] content µg/cm²	
°C	H	I	%[b]	H	I	%[b]	H	I	%[b]	H	I
15	12.1	10.4	85.1	12.1	11.7	96.8	0.65*	0.94	144.4	19.2	12.8
20	21.7	22.6	104.3	22.2	22.3	100.0	1.33*	1.88	141.5	18.2	12.3
25	43.2	40.5	93.7	43.9	40.4	96.2	2.42*	3.16	130.6	17.8	12.8
30	50.8	49.8	98.0	50.9	50.9	100.0	2.76*	3.98	140.9	18.7	13.1

a figures in the table represent the means of seven or eight experiments.

b activities of infected leaf discs expressed as a percentage of the healthy controls.

c chlorophyll expressed arbitrarily (µg/cm²) as the product of optical density read at 665 mµ and final volume (in ml) of the extract, X 10^3, divided by the initial weight (in mg) of the tissue.

* differences significant at the 0.01 level by the t-test.

plants. For example, the values at 30°C showed that on an area basis 50.8 µl of oxygen were evolved by healthy discs as compared to 49.8 µl of oxygen by infected discs. The oxygen evolved by infected discs was approximately 98% of that by healthy discs when the latter was taken as 100%. Similar results were obtained when the values were expressed per unit of fresh weight. However, diseased plants compared to healthy plants showed a marked increase in their rates of apparent photosynthesis when expressed on a per unit of chlorophyll basis. This increased activity was of approximately the same magnitude at each temperature. For example, the oxygen evolved by infected tissues was 144%, 142%, and 141% of that by healthy tissues at 15°C, 20°C, and 30°C respectively. The value for 25°C was slightly lower than those obtained at the other three temperatures and this was attributed to a lower chlorophyll content in the healthy discs. Since the latter was used to calculate the rates on a per unit chlorophyll basis, expression of the infected tissue activity as a percentage of the healthy controls could explain the lower value.

(iii) Effect of carbon dioxide partial pressure

The biochemical reactions of photosynthesis are also affected by the carbon dioxide partial pressure. In view of the terminal oxidant function of carbon dioxide, a study

was carried out to test for its influence on the apparent photosynthesis by discs from leaves of diseased plants. The experiments were carried out under conditions of constant temperature and light intensity, with only the carbon dioxide partial pressure being varied. Oxygen evolution was recorded for samples of ten discs and was expressed on an area, fresh weight, and chlorophyll basis.

Table IV shows that the oxygen evolved increased with increased CO_2 concentrations regardless of the units chosen for expression of the results. On an area or fresh weight basis, the infected tissues evolved less oxygen than the healthy control for each CO_2 concentration. However, the differences were not considered to be significant. On a chlorophyll basis the infected tissue evolved more oxygen than the corresponding control for each CO_2 concentration tested. When the oxygen evolved by infected tissues is expressed as a percentage of the healthy controls the values obtained are those shown in columns 4, 7, and 10 numbered from the left-hand side of Table IV. On an area and a fresh weight basis a greater percentage of oxygen was evolved in healthy than in infected tissue, whereas the reverse was true for results expressed on a chlorophyll basis. Furthermore, the photosynthesis of infected tissue expressed as a percentage of healthy tissue decreased as the CO_2 partial pressure in-

TABLE IV. Effect of CO_2 partial pressure on the apparent photosynthesis by healthy and BSMV-infected barley leaves[a].

CO_2 pressure	Oxygen evolved in 1 hr. µl/cm^2			µl/100 mg			µl/µg chlorophyll			Chlorophyll[c] content µg/cm^2	
%	H	I	%[b]	H	I	%[b]	H	I	%[b]	H	I
0.2	9.1	8.2	90.1	9.2	8.2	90.0	0.48*	0.71	148.0	19.2	12.1
0.4	14.9	12.2	81.8	14.8	12.2	81.8	0.79*	1.08	136.7	18.9	11.3
0.6	17.8	14.1	79.2	16.2	13.4	82.7	0.96*	1.15	120.0	18.1	12.3
0.8	25.4	25.3	100.0	26.9	26.2	100.0	1.37*	2.22	162.0	18.2	11.4
1.0	43.2	40.5	93.7	39.6	38.1	96.2	2.42*	3.16	130.6	17.8	12.8

a figures in the table represent the means of three to seven experiments.

b activities of infected leaf discs expressed as a percentage of the healthy controls.

c chlorophyll expressed arbitrarily (µg/cm^2) as the product of optical density read at 665 mµ and the final volume (in ml) of the extract, X 10^3, divided by the initial weight (in mg) of the tissue.

* differences significant at the 0.01 level by the t-test.

creased to 0.6%, then it started to rise with further increases in the CO_2 concentration. The reason for the apparent rise in the rate of photosynthesis at the highest CO_2 concentrations cannot be explained.

The results show that environmental factors such as light intensity, temperature, CO_2 partial pressure, and the units chosen for photosynthesis have a pronounced effect on the rates for healthy and diseased plants. Expression of the oxygen evolved on an area or a fresh weight basis indicated that little or no differences existed between healthy and infected leaf discs. However, when the oxygen evolved was expressed on a unit chlorophyll basis, infected leaves were consistently more active than the healthy controls.

(b) Photosynthesis by Whole Leaves

The results from the previous apparent photosynthetic studies were based on the rates of oxygen evolution by healthy and infected leaf discs. However, the possibility of biased results arising from the selection of leaf positions for the removal of the discs as well as from the shock induced in the small discs by the cutting action of the cork borer could mean that leaf discs do not provide a true indication of the activities in whole leaves. Consequently, an experiment was designed to measure the relative rates of CO_2 fixa-

tion by replicate samples of detached third leaves from healthy and diseased plants. The leaves were exposed to an atmosphere containing $C^{14}O_2$ and were allowed to photosynthesize for 20 minutes. The results were expressed as counts per minute per gram fresh weight incorporated into the ethanol soluble and insoluble fractions and are given in Table V.

TABLE V. Distribution of activity (cpm) in ethanol soluble and insoluble fractions of healthy and BSMV-infected barley leaves.

Sample	Total counts			Activity[a] (cpm/g fresh wt) Soluble			Insoluble		
	H	I	%[b]	H	I	%[b]	H	I	%[b]
1	10,772	7,181	66.7	9,786	6,563	67.1	986	618	62.7
2	9,468	6,503	68.7	8,480	5,919	69.8	988	550	55.7
mean	10,120*	6,824	67.7	9,133*	6,241	68.5	987*	584	59.2

a corrected for background.

b activities of infected leaves expressed as a percentage of the healthy controls.

* differences significant at the 0.01 level by the t-test.

Table V shows that healthy leaves fixed more $C^{14}O_2$ than infected leaves, and that most of the activity was found in the alcohol soluble fraction. Thus, on a fresh weight

basis infected leaves were less efficient than healthy leaves in the fixation of CO_2. This decreased efficiency is probably associated with a reduced enzyme activity for CO_2 fixation in the infected leaves.

C. PHOTOCHEMICAL ACTIVITIES OF CHLOROPLASTS ISOLATED FROM LEAVES OF HEALTHY AND BSMV-INFECTED BLACKHULLESS BARLEY LEAVES

The photosynthetic apparatus has been shown to reside in the chloroplasts (7). By isolating chloroplasts and studying their photochemical activities, it was felt that the effect of virus infection on the photosynthetic capacities of susceptible plants might be better understood. This was done by measuring the intensity of the Hill reaction, photophosphorylation, and CO_2 fixation in chloroplasts isolated from healthy and infected leaves.

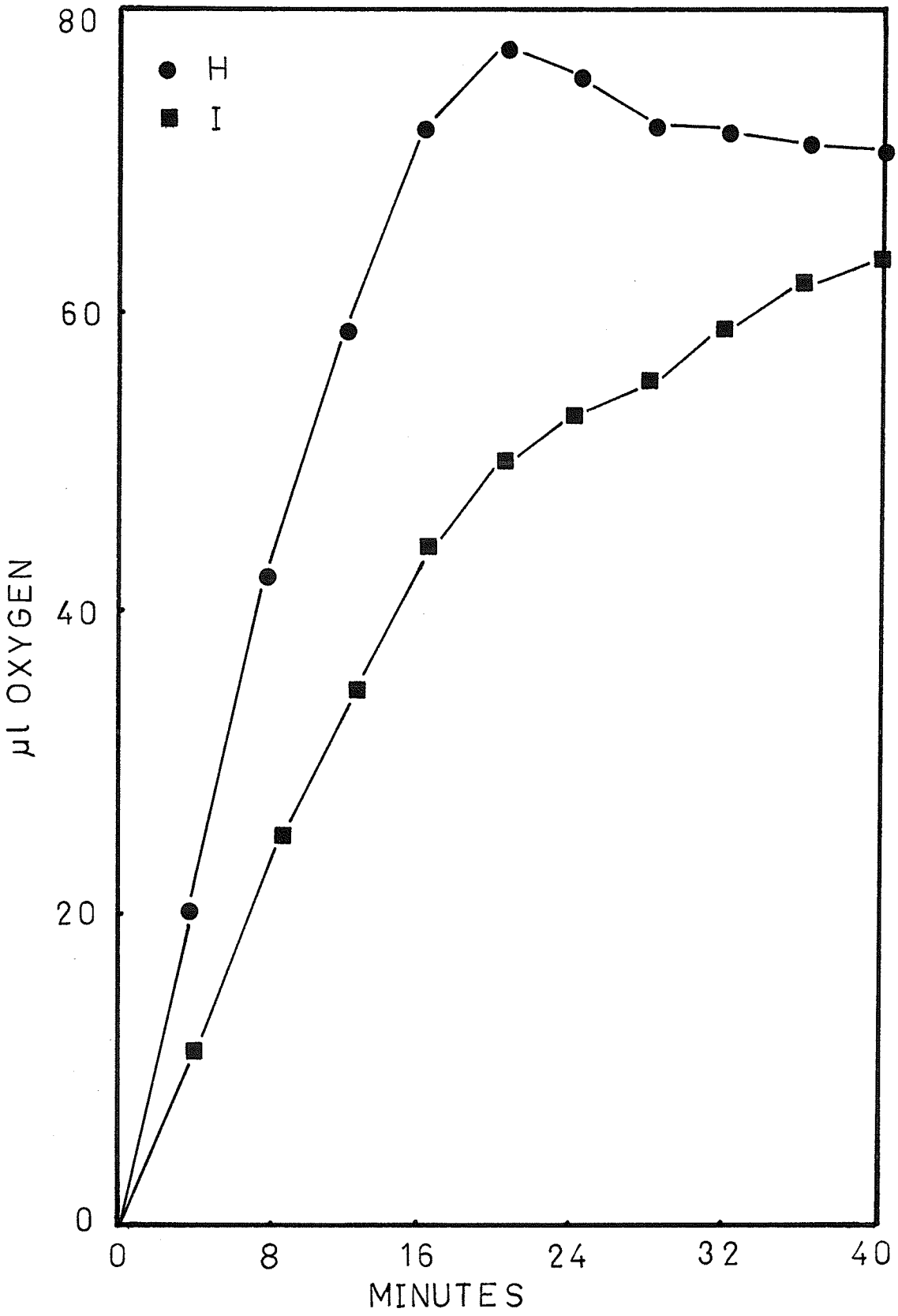
(a) Hill Reaction

A series of experiments were carried out to compare the Hill reaction activity of chloroplasts isolated from healthy and infected leaves under such conditions as time, pH, chlorophyll concentration, temperature, and light intensity.

(1) Time course of the reaction

Figure 2 shows a progress curve for the Hill reaction by healthy and infected chloroplasts. The reaction

Figure 2. Time course study on the Hill reaction by chloroplasts isolated from healthy and BSMV-infected barley leaves.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rate for the healthy chloroplasts was essentially linear for the first 20 minutes, then it decreased quickly to a more or less constant value. The infected chloroplasts, however, showed a continual increase in oxygen evolution, approaching the level of total oxygen evolution by the healthy chloroplasts after approximately 40 minutes.

(2) Effect of pH

The pH optimum of the healthy chloroplast reaction system shown in Figure 3 agrees with those reported in the literature (69). The curves for both healthy and infected systems peaked at approximately 6.8 to 7.0 pH units. However, the optimum appeared to be more sharply defined for the healthy than for the infected system.

(3) Effect of chlorophyll concentration

A preliminary analysis of the chloroplasts isolated for Hill reaction studies indicated that the infected leaves contained less chlorophyll than the healthy leaves. The average total chlorophyll content for the healthy leaves was 10.631 mg per 5 g fresh weight, consisting of 5.695 mg of chlorophyll a and 4.936 mg of chlorophyll b. This compared to an average total chlorophyll content in the infected leaves of 5.679 mg per 5 g fresh weight, consisting of 3.140 mg of chlorophyll a and 2.539 mg of chlorophyll b. The

absorption spectra of chloroplast suspensions used in a typical experiment are shown in Figure 4. The curves for the chlorophylls extracted from the two suspensions were spectroscopically similar, suggesting that the virus effect was not upon the chlorophyll molecule per se.

Figure 5 shows the relationship between chlorophyll concentration and Hill reaction activity. The reaction rates of both healthy and infected systems were essentially linear over the range of chlorophyll concentration tested.

(4) Effect of temperature

Table VI shows the relative rates of oxygen evolution as a function of four temperatures. The optimum temperature was 15°C and this agreed with the reported optimum for oxygen evolved by spinach chloroplasts (8).

TABLE VI. Effect of temperature on Hill reaction by chloroplasts isolated from healthy and BSMV-infected barley leaves.

Preparation	$\mu l\ O_2$ evolved in 4 minutes[a]			
	10°C	15°C	20°C	25°C
Healthy	7.4*	15.8*	8.4*	4.9*
Infected	4.9	11.6	6.1	3.0

a average values of two experiments.

* differences significant at the 0.01 level by the t-test.

Figure 3. Effect of pH on the Hill reaction by chloroplasts isolated from healthy and BSMV-infected barley leaves.

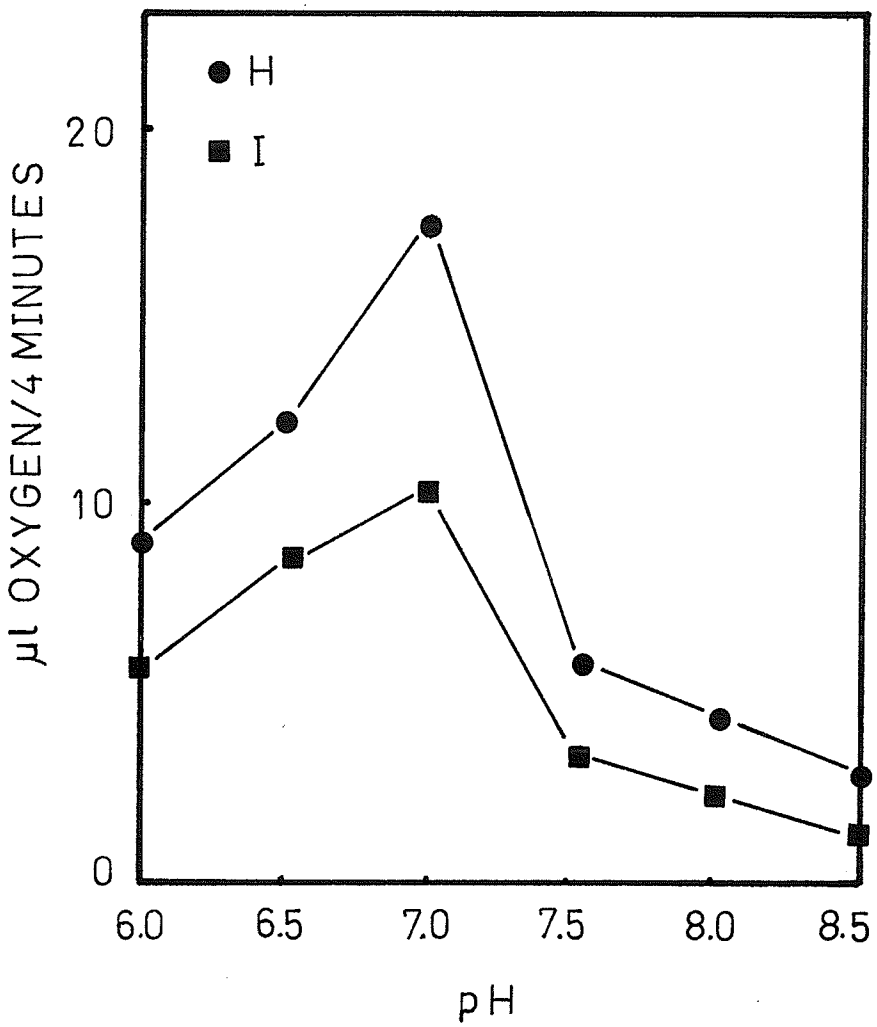
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Figure 4. Absorption spectra of chloroplast suspensions from healthy and BSMV-infected barley leaves.

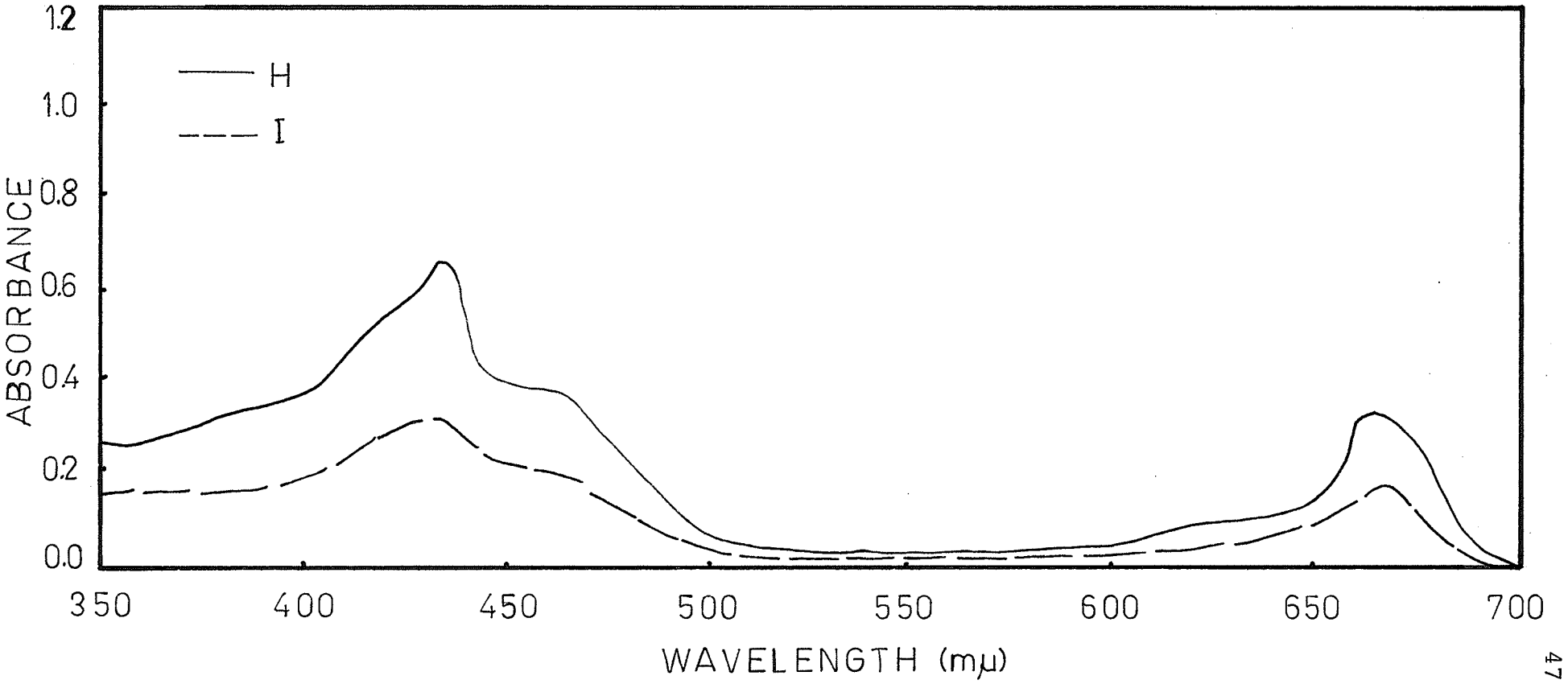
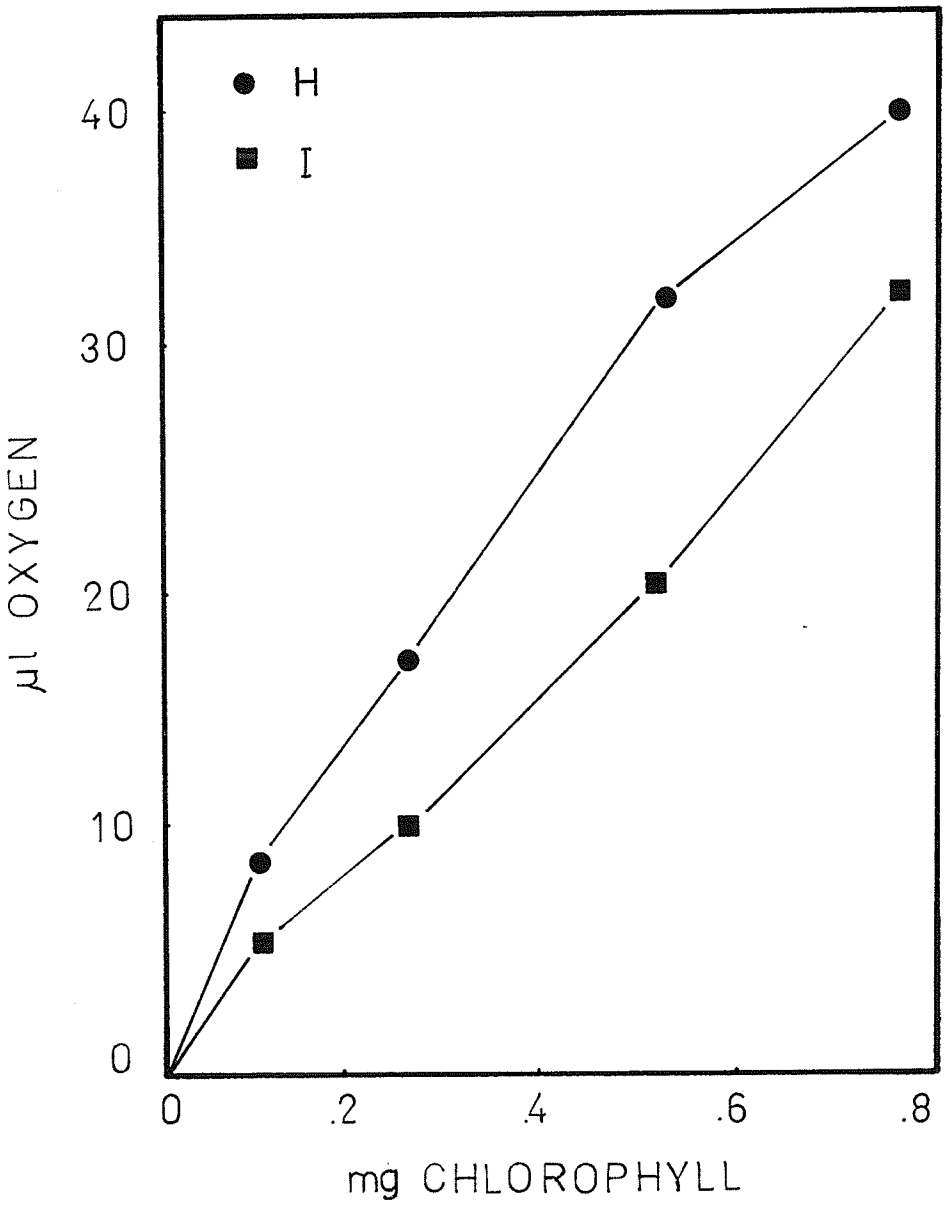


Figure 5. Effect of chlorophyll concentration on the Hill reaction by chloroplasts isolated from healthy and BSMV-infected barley leaves.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(5) Effect of light intensity

Lumry, Spikes and Eyring (56) have shown that by investigating the relationship between light intensities and Hill reaction rates, one could differentiate between effects related to the "light reactions" and those related to the "dark reactions". This relationship was described by the equation:

$$V = \frac{K_D \times I}{I + K_D/K_L}$$

where V = rate of reaction
I = light intensity
K_D = constant related to dark reaction
K_L = constant related to light reaction

By a rearrangement of terms the equation can be written as:

$$\frac{1}{V} = \frac{1}{K_L} \times \frac{1}{I} + \frac{1}{K_D}$$

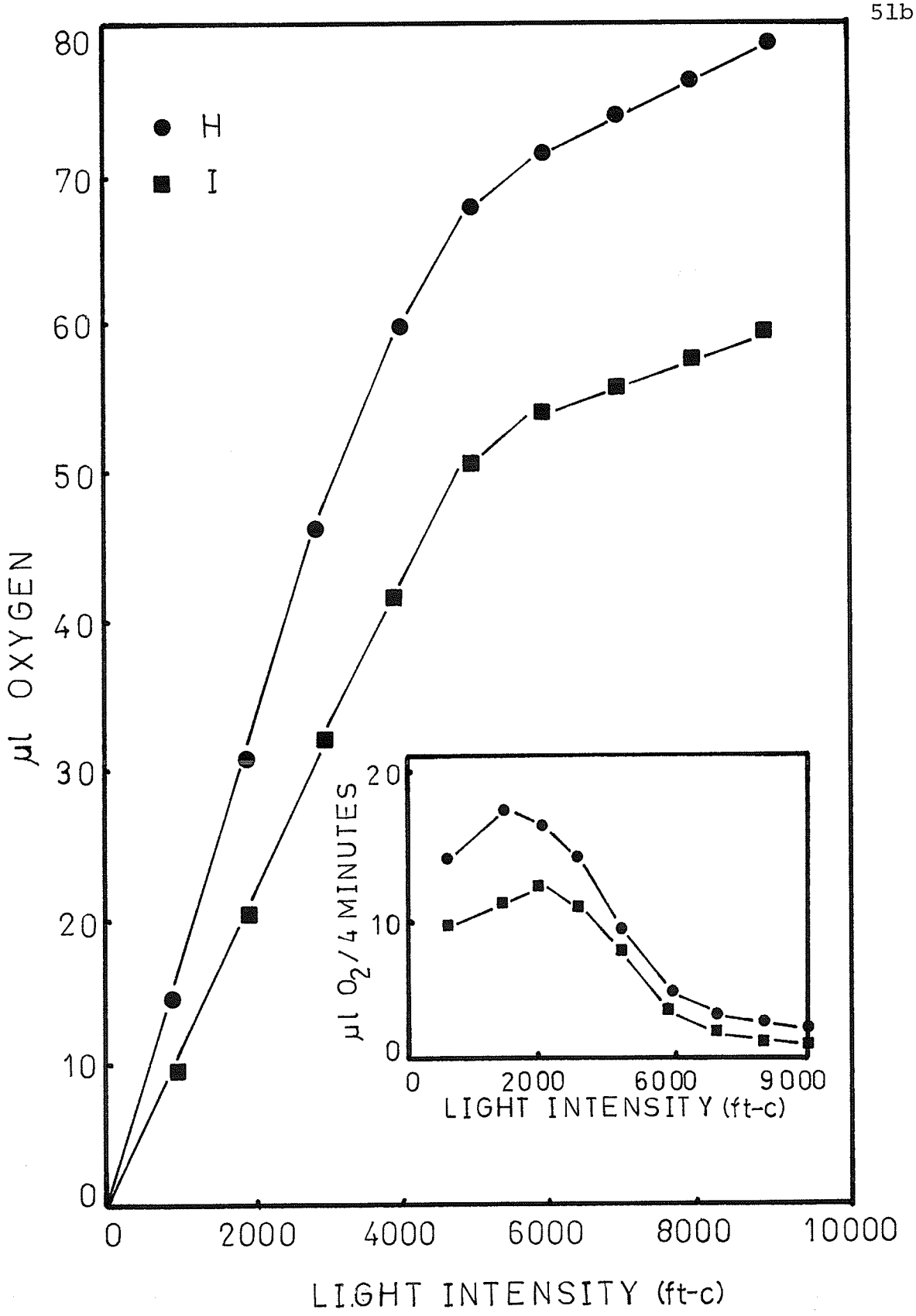
where $1/K_L$ = slope of the light reaction
$1/K_D$ = intercept of the dark reaction

Thus if one plots 1/V against 1/I, a straight line should be obtained. Conditions affecting the "dark reactions" will be reflected in the change in the intercept of the line, while conditions affecting the "light reactions" will be reflected in changes in the slope of the line (11). With these principles in mind, experiments were carried out with chloroplast fragments from leaves of healthy and dis-

eased plants in order to determine whether the virus affected the rate-limiting light reactions and/or the rate-limiting dark reactions.

Preliminary experiments were carried out to test the effect of light intensity on the Hill reaction. In these experiments the chloroplasts were subjected to a gradual increase in light intensity after every measurement of oxygen evolution. In Figure 6 oxygen evolution increased linearily with light intensities up to approximately 4000 ft-c, then it decreased at the higher light intensities. The decrease in activity was probably due to a combined effect of end-product inhibition by the quinone substrate coupled to a saturation of the rate-limiting "dark reactions" at the high light intensities. Rates of oxygen evolution per unit time were obtained from these curves and are shown in the insert of Figure 6. The rates indicate that light saturation was reached in the range of 2000 ft-c to 4000 ft-c. Therefore, subsequent experiments were carried out with light intensities ranging from 0 ft-c to 3000 ft-c. Four different light intensities were used to cover this range, the light intensity being held constant for each experiment. The results were reported as rates versus light intensities and are shown in Figure 7. The rates for both healthy and infected chloroplast systems increased with

Figure 6. Time course study showing the effect of increasing light intensity on the Hill reaction by chloroplasts isolated from healthy and BSMV-infected barley leaves. Light intensity was increased at four minute intervals. The insert shows the rates of oxygen evolution for each light intensity for each four minute reading period.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LIGHT INTENSITY (ft-c)
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µl O2/4 MINUTES
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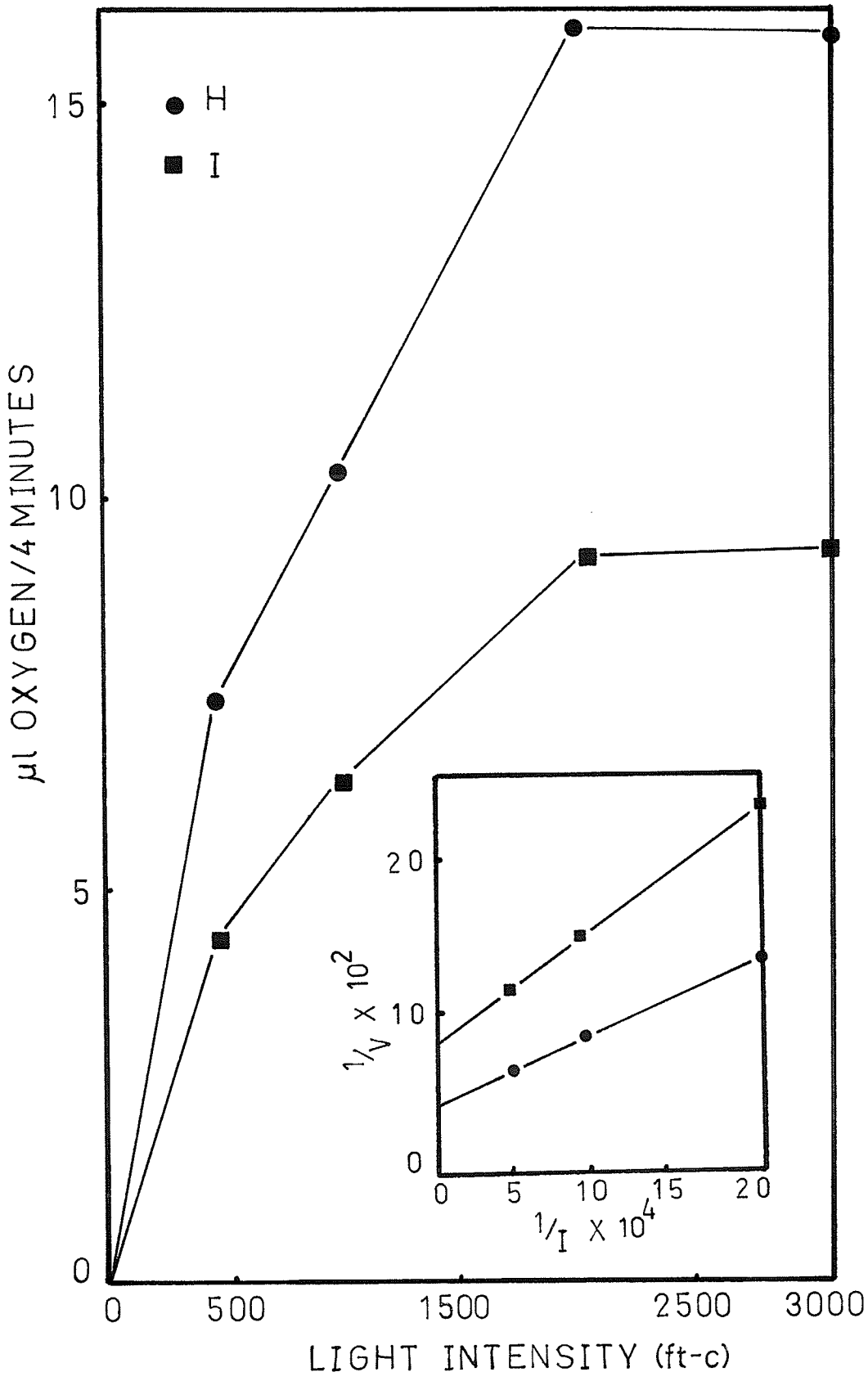
2000
6000
9000
LIGHT INTENSITY (ft-c)

Figure 7. Effect of light intensity on the Hill reaction by chloroplasts isolated from healthy and BSMV-infected barley leaves. The insert shows a reciprocal plot of rates vs light intensities. Each point represents the average values from three to five experiments.


H
I
15
10
5
0
μl OXYGEN/4 MINUTES
0
500
1500
2500
3000
LIGHT INTENSITY (ft-c)
20
10
0
1/V X 10^2
0
5
10
15
20
1/I X 10^4

increasing light intensity up to 2000 ft-c, then they became independent of light intensity. Reciprocal plots of rates vs light intensities are shown in the insert of Figure 7. With this treatment of the data it is evident that virus infection caused a change in both the "light and dark" reactions. The values for the rate parameters were calculated from the line of best fit determinants by the method of least squares (27) and are as follows:

healthy plants	K_L	1.96
	K_D	0.24
infected plants	K_L	1.25
	K_D	0.14

By expressing the infected K_L and K_D values as a percentage of the corresponding values from healthy plants, it can be shown that the "light and dark" reactions of diseased plants were decreased by approximately 36% and 42% respectively.

Since the three photochemical activities employed in this study to characterize photosynthesis are intimately associated, the decreased Hill reaction activity should be indicative of the expected response for the remaining photochemical activities.

(b) Photophosphorylation

Three methods were tried to measure this photo-

chemical reaction. The first method involved the measurement of the disappearance of inorganic phosphate from the reaction mixture. The second method measured the change in optical density at 340 mμ caused by the formation of $NADPH_2$ as a consequence of the following reaction sequence:

$$ADP + P_i \xrightarrow{1} ATP$$

$$ATP + glucose \xrightarrow{2} glucose\text{-}6\text{-}P + ADP$$

$$glucose\text{-}6\text{-}P + NADP \xrightarrow{3} 6\text{-}PGA + NADPH_2$$

where 1 = photophosphorylative enzymes
2 = hexokinase
3 = glucose-6-P dehydrogenase

The third method measured the incorporation of P^{32} into the ATP formed in photosynthesis. In direct contrast to the reports of Wynn (94), Hill and Walker (44), Kleese (51), Wessels (92), and Horecker and Wood (46), little or no success was achieved in this study after repeated applications of either of the first two methods of assay. Since the third method was the most sensitive chemically, it was anticipated that if any differences in photophosphorylation did exist between healthy and infected chloroplasts they would be revealed by P^{32} incorporation. The chloroplasts were supplemented with the necessary cofactors and allowed to incorporate inorganic P^{32}. The results were expressed as μmoles ATP formed per mg chlorophyll per hour and are shown in Table VII.

TABLE VII. Cyclic photophosphorylation by chloroplasts isolated from healthy and BSMV-infected barley leaves

Experiment	μM ATP/mg chl/hr[a]			
	healthy		infected	
	light	dark	light	dark
1	88.8[b]	45.6	135.6	58.8
2	374.4	172.8	676.8	307.2
3	302.4	64.8	585.6	266.4
4	322.4	150.0	594.0	195.6
5	246.0	55.2	390.0	100.8
6	312.0	98.4	452.4	55.2
7	51.6	271.2	603.6	1664.4
8	252.0	116.4	548.4	1633.2
9	98.4	74.4	67.2	168.8
10	117.6	229.2	404.4	144.0
11	73.2	91.2	468.0	1416.0
12	444.0	546.0	385.2	513.6
13	55.2	40.8	582.0	1843.2
14	183.6	342.0	133.2	367.2

a corrected for background

b values represent the averages of duplicate samples

A considerable variation was observed in the number of μmoles of ATP^{32} that were formed. For example, the number of μmoles of ATP^{32} formed in the light ranged from 52 to 444 for healthy and from 67 to 677 for infected chloroplasts. To this variation was added the additional problem of an excessive ATP formation in some of the dark controls, the values ranging from 41 to 546 μmoles for the healthy chloroplasts and from 55 to 1664 μmoles for the infected chloroplasts. Despite these difficulties chloroplasts from infected leaves showed a greater capacity to incorporate inorganic P^{32} into ATP than did the chloroplasts from healthy leaves. For example, in experiments 2 to 6 inclusive after correction for the dark controls, the means for the healthy and infected chloroplasts were 203.7 and 354.7 μmoles respectively. In these selected experiments, the BSMV-infected chloroplasts photophosphorylated ADP at approximately 178% of the rate of healthy chloroplasts when the latter was considered to present 100%.

(c) $\underline{C^{14}O_2}$ Fixation

The results obtained from the previous photochemical study were in direct contrast to the findings obtained from the Hill reaction study. Consequently, a series of experiments were conducted to determine the CO_2 fixation capacities of chloroplasts isolated from leaves of diseased plants.

The results were expressed as counts per minute per 0.5 mg chlorophyll and are reported in Table VIII. The table shows that the chloroplasts isolated for these experiments incorporated $C^{14}O_2$ but there was a wide range in, and a considerable over-lapping of, the data.

TABLE VIII. Fixation of $C^{14}O_2$ by chloroplasts isolated from healthy and BSMV-infected barley leaves

Experiment	Activity[a] (cpm/0.5 mg chlorophyll) healthy	infected	%[b]
1	4063[c]	2488	61.6
2	8602	5175	60.2
3	3753	2826	74.9
4	5265	3965	75.3
5	5062	3454	68.2
6	6093	4075	66.9
mean	5473*	3664	66.8

a corrected for background.

b activities of infected systems expressed as a percentage of the healthy controls.

c values represent the averages of duplicate samples.

* differences significant at the 0.01 level by the t-test.

For example, the counts ranged from approximately 3700 to 8600 cpm in healthy chloroplasts and from 2400 to 5100 cpm

in infected chloroplasts. Nevertheless, in each experiment the healthy chloroplasts were always more active than the infected chloroplasts. The difference in counts ranged from approximately 1000 cpm in experiments showing the least activity to about 3400 cpm in those experiments which showed the highest activity. When the averages for replications of each experiment were expressed as a percentage of the control, the infected chloroplasts photosynthesized at approximately 67% of the rate shown by the healthy chloroplasts. A reduction in the quantity or quality of the enzymes associated with either the event leading to the formation of the CO_2 acceptor compound or the actual CO_2 fixation process could account for these results.

DISCUSSION

Inoculation of the primary leaves of ten day old Blackhulless barley seedlings with a suspension of BSMV presumably created infection sites in the leaves (80). Upon interaction with the virus particles these sites were then converted into infection centres. In the latter, virus particles must have been synthesized until an infectious concentration was reached, at which time particles were translocated (68,79) to the newly emerging third leaf. The invasion of this leaf by virus particles was correlated with the appearance of chlorotic markings as early as six days after inoculation of the primary leaf and by the tenth day the third leaf showed extensive chlorosis and necrosis. These observations prompted photosynthetic studies since the loss of chlorophyll in these leaves suggested a decreased photosynthesis.

Apparent photosynthesis was measured by oxygen evolution from leaf discs or by $C^{14}O_2$ uptake by whole leaves, and the results were expressed on a per unit of area, fresh weight or chlorophyll basis. The apparent photosynthetic rates based on oxygen evolved by discs from the third leaves of healthy and infected plants were similar when expressed on an area or fresh weight basis. This was in direct contrast to the results of Owen (64) and of Roberts and Corbett

(72). Owen observed a 10-15% reduction in apparent photosynthesis expressed on a fresh weight basis within one-half hour after inoculation of tobacco leaves with tobacco mosaic virus. With other host-virus combinations, however, this reduction was not observed until after the symptoms had appeared (63,65). Roberts and Corbett found that on an area basis, photosynthesis by tobacco leaves infected with tobacco ringspot virus was significantly reduced in necrotic but not in symptom free tissue. However, photosynthesis was significantly reduced in symptom free tissue when it was expressed on a chlorophyll basis. In the present study, when photosynthesis was expressed on a chlorophyll basis, it was significantly increased in chlorotic BSMV-infected tissues as compared to green healthy tissues.

The possibility was considered, therefore, that the external factors under these experimental conditions were so far above saturation that they were inhibiting the photosynthetic activities of the healthy tissues or stimulating photosynthesis in the infected itssues. As a result the infected tissues may have approached the activities of the healthy tissues in terms of overall photosynthetic rate. Some support for this suggestion can be found in a paper by Schmid and Gaffron (74) whereby a chlorophyll deficiency in a mutant tobacco plant was compensated for by increased light

intensities. Since leaves of diseased plants usually have a reduced chlorophyll content, it was felt that perhaps a similar phenomenon had been observed in the present study. However, a subsequent study in which the experimental conditions were below saturation, still did not reveal a difference in photosynthesis between healthy and infected third leaves. The experimental results cannot be explained at present since it is difficult to understand how the chlorotic infected tissues could evolve as much oxygen as the green healthy tissues.

Photosynthetic activities of healthy and infected third leaves, measured by $C^{14}O_2$ uptake and expressed on a fresh weight basis, showed that infected leaves accumulated less radioactivity than healthy leaves. Similar results have been reported for other host-pathogen combinations (24, 25,33). The decreased photosynthetic assimilation was accounted for by a reduced enzyme activity associated either with the events leading to the formation of the CO_2 acceptor compound or with the actual CO_2 fixation process. However, in the absence of direct experimental evidence, two additional possibilities could be advanced to explain the experimental results. One possibility is that a decreased number of functional stomates in infected leaves resulted in a reduced uptake of CO_2. However, the reported increase

in oxygen uptake, as evidenced by the greater respiratory rate in BSMV-infected plants compared to healthy plants (39,68), argues against a decrease in the number of functional stomates in infected leaves. A more probable explanation centres about the photorespiratory activities of leaf tissue (26,83,98). A stimulated photorespiration in diseased plants could increase the internal CO_2 concentration above that in the atmosphere. This reversed CO_2 concentration gradient would certainly have a limiting effect on photosynthesis if the rates were based on the incorporation of atmospheric $C^{14}O_2$.

In the preceeding discussion it was stated that photosynthetic rates of healthy and diseased plants depended upon the methods used to measure photosynthesis and the units chosen to express photosynthetic activity. It is difficult to evaluate and compare reports of photosynthetic activity in diseased tissue as reported in the literature because the data obtained from such studies was usually expressed only on a fresh weight or an area basis (4,42,71,75). For example, Scott and Smillie (75) expressed their results on a fresh weight basis and found that photosynthesis by mildewed barley was greatly depressed in comparison to healthy barley. However, while they demonstrated that there was also a drastic decrease in chlorophyll content of the infected barley,

they did not express the photosynthetic rates of such plants on a per unit chlorophyll basis. Use of their data to calculate photosynthetic rates on a chlorophyll basis reveals that diseased plants were slightly more photosynthetically active than healthy plants. Despite these inconsistencies it is generally agreed that following pathogenic attack there is an early decline in the photosynthetic activities of the invaded host.

The localization of the photosynthetic apparatus in the chloroplast has facilitated studies of the effects of pathogens on the photosynthetic activities of diseased plants. However, many problems have been encountered in carrying out such studies. For example, it is widely recognized that the photosynthetic activity of isolated chloroplasts may be modified by changes in structure during extraction (53,54,69,88). Since these problems have been reported only for healthy plant chloroplasts, it is very likely that in the present study they were multiplied many fold during the isolation of diseased plant chloroplasts. Thus, some of the difficulties which were encountered during the studies of chloroplast activities from healthy and infected leaves may actually have been manifestations of these problems.

The study of the Hill reaction indicated a decreased

activity by diseased plants as compared to healthy plants. Since the activity of this reaction was determined on a chlorophyll basis, it was assumed that the enzymes associated with this process had declined more rapidly than the chlorophyll content. Similar conclusions have been reported by Roberts and Corbett (72) and by Zaitlin and Jagendorf.(96) In this respect future studies of chloroplast enzymes such as photosynthetic pyridine nucleotide reductase, aldolase and NADPH-diaphorase might help to substantiate this assumption.

The decreased rates in the "light and dark" reactions of the Hill reaction suggested that BSMV-infection exerted a direct effect on chloroplast activities. A similar observation was reported by Spikes and Stout (81) for beet-yellows virus infected sugar beet plants. Reports of a decreased Hill reaction were also reported by Scott and Smillie (76) for mildewed barley and by Zaitlin and Jagendorf (96) for tobacco mosaic virus infected tobacco plants. However, the latter workers found that under certain conditions the addition of supplemental nitrogen to virus infected tobacco plants overcame the previously observed photosynthetic alterations. They suggested that virus multiplication could affect the photosynthetic process indirectly by inducing a state of nitrogen stress. It would be interesting to see

what effects BSMV-infection would have on barley plants treated with supplemental nitrogen fertilizer.

The many difficulties which were reported in the study on photophosphorylation by isolated barley chloroplasts were not encountered (unreported observations) when isolated spinach chloroplasts were tested. In fact, when P^{32} incorporation into ATP was followed with spinach chloroplasts, the results obtained were similar to those reported by Avron (10). It was apparent that chloroplasts capable of carrying out an active photophosphorylation could not be obtained from barley leaves using a procedure developed for spinach. In the present study therefore, it was concluded that sub-optimal conditions were employed for the isolation and subsequent testing of barley chloroplasts.

Despite these difficulties the liberal interpretation was made that infected chloroplasts showed a stimulated capacity to photophosphorylate ADP. This was in direct contrast to the findings of Wynn (94), of Zaitlin and Jagendorf (96) and of Goffeau and Bové (37). Whereas Wynn observed no change in photophosphorylation by rust-infected oat chloroplasts, both Zaitlin and Jagendorf and Goffeau and Bové reported decreases in ATP formation in virus-infected chloroplasts. However, while the latter workers observed a decreased photophosphorylation in systemically infected Chinese

cabbage leaves, local lesion leaves of the same plants actually showed a stimulated ability to incorporate P^{32} into ATP. They postulated that the virus had induced the destruction of some hypothetical regulatory mechanism which had previously prevented the chloroplasts from functioning at full rate. Since the increased ATP synthesis occurred at the time of active virus multiplication in these leaves, it was further suggested that the increased supply to ATP could be used for virus-RNA synthesis within the chloroplasts. This hypothesis has in fact been recently strengthened by the experiments of Bové et al (16).

In the present study it is difficult to accept that BSMV infection induced a stimulated cyclic photophosphorylation when it had already induced a decreased Hill activity. The localization of the photophosphorylative process between the oxygen evolving PHOTOSYSTEM II and CO_2 reducing PHOTOSYSTEM I (70) demands that any reduction in electron generation by the Hill reaction results in decreased activities of the two remaining reactions. Studies with a specific cyclic photophosphorylative inhibitor such as desaspidin could help to clarify the effect of BSMV infection on the photophosphorylative capacity of barley chloroplasts.

Isolated "whole" chloroplasts from healthy and infected leaves were shown to be capable of incorporating $C^{14}O_2$.

However, in view of the low incorporation it seems apparent that the chloroplasts were damaged and therefore possessed a limited capacity to incorporate CO_2. Nevertheless, the consistently lower rate of incorporation by infected chloroplasts in comparison to healthy chloroplasts tends to substantiate the previously observed reduced photoassimilation of $C^{14}O_2$ by whole leaves. That these results could be accounted for by a decreased carboxylative enzyme activity in infected plants appears to be supported indirectly by the studies of Malca et al (56,57). They reported a decreased rate of $C^{14}O_2$ incorporation by the ribulose-1,5-diphosphate carboxylase enzyme isolated from infected plants. However, the results reported in the present study and those of Malca et al both appear to be in direct contrast to those of Farkas et al (28). These workers reported that the enzymes for CO_2 fixation in Chinese cabbage leaves were unaffected by turnip yellow mosaic virus infection. It would be interesting to see if the activity of the ribulose-1,5-diphosphate carboxylative enzyme was altered upon BSMV infection of susceptible barley plants.

The purpose of this investigation has been to characterise the photosynthetic capacities of the third leaves from BSMV-infected plants. In this respect, it is suggested

that the decreased photosynthetic assimilation of $C^{14}O_2$ by whole infected third leaves and the associated decrease in activity of such chloroplast reactions as the Hill reaction and $C^{14}O_2$ fixation could result in a reduced supply of photosynthate for the plant, thereby affecting the growth and vigor of such plants.

SUMMARY

1. The primary leaves of ten day old Blackhulless barley plants were inoculated with a suspension of BSMV.

2. Pronounced visual symptoms indicative of a systemic virus invasion of the third leaves of such plants were observed ten days later.

3. Apparent photosynthesis by leaf discs obtained from the third leaves of healthy and infected plants was measured by oxygen evolution and the results were expressed on an area, fresh weight, and chlorophyll basis. On either an area or a fresh weight basis the results indicated no significant differences between healthy and infected tissues. However, on a chlorophyll basis, infected tissues photosynthesized at a greater rate than healthy tissues. This was observed despite the fact that healthy tissues possessed more chlorophyll than infected tissues.

4. A similar trend was observed when photosynthesis by healthy and infected leaf discs was compared under different conditions of light intensity, temperature, and CO_2 partial pressure.

5. The photosynthetic assimilation of $C^{14}O_2$ by whole leaves from healthy and infected plants indicated that, on a fresh

weight basis, healthy leaves fixed more $C^{14}O_2$ than infected leaves.

6. A kinetic study of the Hill reaction indicated that chloroplasts isolated from infected leaves possessed a decreased capacity to evolve oxygen when compared with chloroplasts isolated from healthy leaves. This was most evident from a study on the effect of light intensity on Hill reaction activity which indicated that the rate-limiting photochemical reactions and the rate-limiting dark reactions were decreased by approximately 36% and 42% respectively in infected chloroplasts.

7. The photophosphorylative capacity of infected chloroplasts was found to be approximately 178% of that exhibited by healthy chloroplasts.

8. Chloroplasts isolated from infected leaves were found to incorporate $C^{14}O_2$ at approximately 67% of the rate shown by the chloroplasts isolated from healthy leaves.

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