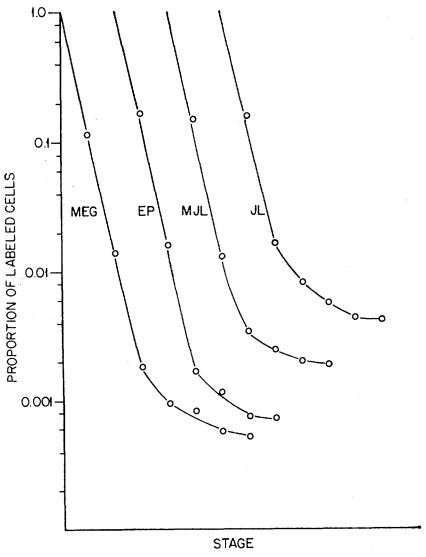

Fig. 2. Determination of the proportion of inagglutinable cells in four normal donors, with respect to lima bean anti-A lectin. The corrected proportion of labeled cells in the supernatant is plotted against the stage. M.E.G. belongs to group A_1B; the others to A_1. The phenotypes of the remaining labeled cells are accordingly B or O (*5*).

sion of free cells is in contrast to the entrapment seen when mixtures are centrifuged (*5*). Clearly, the dilution series is a likely source of error unless it is checked in reconstruction experiments.

To determine the proportion of naturally occurring inagglutinable cells with respect to a given agglutinin, 5 ml of washed cells were labeled and then washed six to eight times in 40-ml aliquots of saline to remove unbound chromate. Labeled cells release small amounts of Cr^{51}, partly by invisible hemolysis and partly by elution with a half-time of 75 days (*6*). Washing was considered sufficient when the supernatant activity was less than 10^{-4} that of an equal volume of cells. The initial reaction mixture (brought to a volume of about 40 ml) contained sufficient agglutinin to give massive agglutination within a few minutes. The components were mixed and sampled quickly before agglutination. The first sample usually required a dilution of 1:1000 for counting, a step facilitated by lysis in water. Inclusion of suspending fluid in the sample introduces no error early in the experiment since the activity in solution at this time is negligible compared with that in cells. Subsequent stages followed the protocol described for the reconstruction experiment (except, of course, that no further labeled cells were added). The total volume decreased 1 to 3 ml per stage but rarely became a limiting factor. Hemolysis during the experiment did not significantly decrease the number of labeled cells but often released enough activity during the first stage to exceed that of the few labeled cells remaining in the last stages. From the third stage on, therefore, the cells must be separated from suspending fluid before samples are counted. Chromium released from cells does not contaminate the carrier. Sedimentable activity other than that in erythrocytes was checked by lysis of pellets with 1 percent acetic acid. As in fresh cells, 80 percent or more of the label was released regardless of the stage tested, indicating that all the sedimentable label is equivalent to erythrocytes.

Progress of the experiment may be visualized in a semilogarithmic plot of stage versus ratio of the corrected activity to initial activity. Figure 2 shows experiments on three AB and one A blood with lima bean anti-A lectin. During the first two or three stages, the proportion of labeled cells removed per stage is remarkably constant; then it decreases as the inagglutinable fraction is approached. After the cells that react with a given antibody have been exhausted, the addition of an antibody of different specificity may result in a further removal of cells (*7*).

Activities of the initial reaction mixtures were in the neighborhood of 2×10^6 count/min ml. These were achieved with Abbot Rachromate containing about 0.03 mg of Cr per milliliter (specific activity about 30 mc/mg of Cr), mixed in 1:5 ratio to cell volume in concentrated saline suspensions and held at room temperature for about 30 minutes. Chromium uptake was less than one-tenth that necessary to increase the mechanical fragility of the erythrocytes (*6*). The inagglutinable cell levels indicated (0.0005 to 0.004) far exceed levels to which the method may be adapted with confidence. With maximum uptake, larger amounts of blood, higher specific activity of Cr, last-stage sampling of all cells, and counter operation at near-background levels, measurement of inagglutinable cells in the proportion of 10^{-6} should be entirely feasible.

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Carbon Monoxide in Green Plants

Abstract. Green plants grown in a closed, illuminated system liberate small quantities of carbon monoxide. Similarly, finely divided powder and chlorophyll extracts of green plants, when illuminated in an environment of oxygen and water, will yield small quantities of carbon monoxide as well as certain aldehydes. The component of the light spectrum which is absorbed in photosynthesis (480 to 680 mμ) was found to be responsible for the CO and aldehyde phenomena.

The literature contains numerous reports concerning the effects of carbon monoxide on the growth and development of plants subjected to varying concentrations of this gas. It is generally agreed that CO in very high concentrations inhibits growth and development, whereas with respect to low concentrations of this gas, the reports are somewhat controversial.

Concerning the natural occurrence of carbon monoxide in green plants, very little work has been reported. In a short report Langdon (*1*) observed CO in the floater of Pacific Coast kelp, *Heterocystis lulkeanea,* and considered it a by-product of respiration. He states that no gas was formed when the plant was killed, and that none was formed when the plant was macerated and allowed to undergo autolysis or decomposition. Rigg (*2*) repeated Langdon's observation on the bladder of sea kelp and also interpreted the CO as a by-product of respiration. Metz and Sjöstrand (*3*) and Sjöstrand (*4*) have reported the endogenous formation of CO in mammals and have attributed the presence of the gas to the oxidation of some methane group in the hemoglobin molecule by the addition of ascorbic acid, with formation of choleglobin and release of CO.

This report concerns the observation and measurement of carbon monoxide in a number of green plants and the possible significance of its presence. The investigation stemmed from observation of unusually high carbon monoxide content in a specimen of human muscle tissue into which a considerable quantity of green vegetation had been impacted during an aircraft accident.

A method for CO determination by means of carbon monoxide sensitive indicator tubes has been developed (*5, 6*) which can be used to determine CO concentrations as low as 2.5 ppm. This sensitivity was also obtained with the Liston-Becker model 15-A infrared CO analyzer. The method is based on the homogenization of a few grams of tissue in a gastight, nitrogen-filled homogenizer in the presence of 30 percent potassium ferricyanide $[K_3Fe(CN)_6]$, sodium acetate (*p*H 6), and caprylic alcohol. This is a standard procedure for releasing carbon monoxide from blood. Aliquots of the gas from the homogenizer were removed

and analyzed for CO with the Liston-Becker infrared CO analyzer and with CO sensitive indicator tubes (*6*) in accordance with methods now employed by the U.S. National Bureau of Standards. The CO is expressed as milliliters of CO per 100 grams of tissue at standard temperature and pressure, dry.

Various structural portions of fresh green vegetation — including shrubs, grasses, weeds, evergreen and deciduous trees, and algae—were used in this investigation. The samples included leaves, stems, fruits, and roots. The investigation was subsequently extended to a study of dried green leaves, extracts of dried green leaves, and green plants growing in a closed system. A series of color filters was used to determine the spectral components essential to the liberation of CO by the plant systems.

The results of the analyses of a number of fresh plant structures for carbon monoxide are presented in Table 1. These plant components, obtained between the hours of 10 A.M. and 2 P.M., reveal different CO levels—from a high of 2.1 ml/100 g in alfalfa to none in bleached celery leaves. There was no significant difference in the CO content of alfalfa leaves collected at various times during a 24-hour period. Further evidence of liberation of carbon monoxide from green plants was obtained by shaking 3 kg of chopped, fresh alfalfa leaves with the ferricyanide acetate buffer caprylic alcohol in a large glass jar filled with air for a period of 30 minutes. The gas was drawn off, and the vessel was refilled with air and shaken again. The process was repeated until 6 lit. of gas was obtained. This gas was subsequently scrubbed with bromine water, potassium hydroxide, and, finally, sulfuric acid, before analysis for CO. The percentages of CO concentration, as determined by the indicator tube method, the Liston-Becker infrared analyzer, and the phosphorus pentoxide method (*7*), were 0.344 ± 0.0030, 0.344 ± 0.0025, and 0.345 ± 0.0030, respectively.

Table 1. Carbon monoxide content of plant structures. Values are averages for ten samples obtained near midday.

Plant	CO (mg/100 g of tissue)
Leaves	
Alfalfa	2.100 ± 0.23
Cotton	0.248 ± 0.039
Sage plant	0.745 ± 0.063
Pigweed	0.212 ± 0.023
Morning-glory	0.176 ± 0.014
Cedar	0.368 ± 0.017
Ligustrum	0.487 ± 0.029
St. Augustine grass	0.048 ± 0.017
Combs grass	0.515 ± 0.093
Lettuce	0.001
Celery	No trace
Stems	
Pigweed	0.022 ± 0.002
Alfalfa	0.398 ± 0.05
Spirogyra	0.049 ± 0.008
Carrot	No trace
Pecan husk (green)	0.003

Three mice were exposed to this gas for 20 minutes. They showed a blood CO level of 71.0 ± 2.1 percent, whereas control mice in air showed blood CO levels of 0.20 ± 0.02 percent.

With the "flour" obtained from milling oven-dried (130°F) alfalfa leaves, the following observations were made. (i) Relatively low concentrations of CO were released when the dry flour was placed in an oxygen-filled flask and exposed to sunlight. (ii) Larger quantities of CO (1 ml/g) were released when the illuminated flask contained water in addition to the oxygen and plant flour. (iii) No CO was produced when oxygen was replaced by some inert gas such as helium or nitrogen. (iv) No CO was produced in flasks containing leaf flour, oxygen, and water if the flasks were kept in the dark. (v) Aldehydes were produced, along with CO, in the illuminated flasks containing leaf flour, oxygen, and water. (vi) The carbon monoxide phenomenon was not inhibited by a number of enzyme inhibitors, including cyanides, fluorides, acids, and alkalis. (vii) The effective spectral component for the CO and aldehyde formation lies primarily between 480 and 680 mμ.

Flasks containing hydrated leaf flour and rendered bacteriologically sterile by autoclaving gave results comparable to those obtained with nonsterile systems. This may be observed in Table 2. It is evident that the CO is liberated from those flasks which contain oxygen and are illuminated. Numerous experiments have shown that oxygen is consumed by the hydrated flour in darkness as well as in light but that the CO and aldehydes occur only when the system is illuminated.

Experiments with pigments extracted from leaf flour with various organic solvents gave results similar to those obtained with the leaf flour. The greater CO yield was obtained from the chlorophyll fractions. These pigments were separated from the organic solvents by vacuum distillation, placed in flasks containing oxygen and water, and subsequently illuminated by direct sunlight. The flour remaining after pigment extraction failed to yield CO when it was exposed to light.

R. D. Gafford (*8*), using a closed, illuminated system containing an algae, *Anacystis nidulans,* has shown that CO is evolved from the system during illumination and has obtained CO concentrations as high as 800 ppm during a period of a few days. The accumulated CO appears as a linear function of time, under constant illumination and in the presence of O_2. I made similar observations when large, thin polyethylene-lined cellophane bags were tied securely around branches of trees in such a manner as to make a transparent, gastight environment for the leaves. These bags were filled with various mixtures of O_2, N_2, and CO_2, and samples were removed for analysis under conditions of illumination and darkness. Analysis of the gas at timed intervals revealed accumulation of CO *only* in the illuminated bags containing oxygen. Over a 3- to 4-day period the CO concentration rose to a value of 800 to 900 ppm.

Table 2. Carbon monoxide, O_2, and CO_2 content of gas after 24 hours of illumination. Values are averages of five determinations.

Initial mixture	CO (ml/100 g)	CO (%)	CO_2 (%)	O_2 (%)
100% O_2	70.80	0.30	1.92	−6.80
81.64% O_2; N_2	69.90	0.29	1.60	−7.24
61.68% O_2; N_2	47.60	0.201	1.86	−6.63
39.07% O_2; N_2	46.60	0.195	1.60	−6.67
20.18% O_2; N_2	29.51	0.127	1.59	−4.78
10.35% O_2; N_2	17.92	0.075	1.09	−3.56
4.99% O_2; N_2	11.86	0.052	0.92	−3.43
3.4% O_2; N_2	7.270	0.034	0.45	−2.36
1% O_2; N_2	6.200	0.025	0.36	−0.85
100% N_2	0.247	0.001	0.08	0.12
100% O_2 (light-shielded flask)	2.470	0.010	0.00	−2.82
100% O_2 (chlorophyll extract)	14.180	0.130	1.00	−2.28
100% O_2 (dechlorophyllated)	14.17	0.135	1.76	−6.56

In a final series of experiments, seedlings of *Avena sativa* were dark-grown, and the leaves were harvested and dried in an electric oven. The flour from these plants did not yield CO or aldehydes when it was placed in flasks containing water and oxygen and illuminated in direct sunlight.

The fact that carbon monoxide is observed in injured plants does not necessarily mean that it is present in the normal plant. The experiments, however, seem to point to the natural occurrence of a CO "generator" or precursor substance in green plants, the generation and liberation being brought about through some photodegredative activity involving the chlorophyll system. It appears to occur both in vivo and in vitro and requires light and oxygen.

From the practical side, it would seem that there is a possibility of CO asphyxiation in the proper environment of green, damaged vegetation. In any proposed human-algae closed system, provisions may have to be made to eliminate the CO gas evolved from the plant systems. Even though I have made no measurements by way of verification,

it would seem reasonable to postulate the occurrence of higher blood CO levels in herbivorous animals consuming sizable quantities of green vegetation than in nonvegetarian animals.

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26 January 1959

Nemin: a Morphogenic Substance Causing Trap Formation by Predaceous Fungi

Abstract. Broths in which the nematode *Neoaplectana glaseri* had developed axenically caused the mycelium of the predaceous fungus *Arthrobotrys conoides* to differentiate into traps. The active principle was extracted from worm-free culture filtrates and named "nemin." The identity of nemin remains to be established.

Recent reviews by Duddington (*1*) summarized our present knowledge of predaceous fungi. These remarkable microorganisms can capture and kill nematodes by means of traps formed in response to the presence of their prey. The fact that they do not form traps when grown in pure culture, but do so in the presence of nematodes, suggests that some morphogenic substance produced by the worms is responsible for differentiation of the fungus mycelium into traps. Evidence substantiating this was obtained by Comandon and De Fonbrune (*2*) and by Lawton (*3*), who demonstrated that water in which nematodes had been suspended induced trap formation. The list of nematode-free preparations capable of causing predaceous fungi to form traps has been extended to include various animal sera and tissue extracts (*4*). The active principle in water in which nematodes had been suspended was destroyed by boiling (*2*), whereas that in guinea pig serum was thermostable and not affected by alcohol (*5*). The nature of the substance or substances causing trap formation has not been determined.

Table 1. The ability of culture filtrates of *N. glaseri* to cause trap formation by the predaceous fungus *A. conoides.*

Filtrate dilution	Trap formation	
	Uninoculated medium	Inoculated medium
0	–	++
1/5	–	++++
1/10	–	++
1/50	–	+
1/100	–	+
1/200	–	–
1/400	–	–

The nematode *Neoaplectana glaseri* and the predaceous fungus *Arthrobotrys conoides* (*6*) were employed in the present investigations. *Neoaplectana glaseri* was cultivated axenically in meat infusion broth supplemented with raw liver extract. The composition of the medium and the method of culturing the nematode were described by Stoll (*7*). Nematode populations were measured by direct microscopic counts of appropriate dilutions of the broths. Worm-free preparations were obtained by double filtration of broth samples through sterilized filter paper (H. Reeve Angel No. 802) under aseptic conditions. The activity of filtrates was determined by a simple dilution assay. Aliquots of the culture filtrates were diluted quantitatively in a series of sterilized water blanks, and 1 ml of each dilution was added to the surface of petri dishes containing 20 ml of maize-meal agar on which *Arthrobotrys conoides* had developed for 4 days at 28°C. The plates were returned to the incubator and examined microscopically (× 100) for trap formation 24 and 48 hours after treatment. The extent to which various dilutions of the culture filtrates caused trap formation by the fungus was recorded. Activity is reported as dilution units, the reciprocal of the highest dilution of a culture filtrate that caused the fungus to form traps.

When a washed suspension of living nematodes was added to the surface of petri dishes on which *A. conoides* had developed, the mycelium differentiated, producing networks of hyphal loops in which numerous worms were captured and destroyed. No traps were formed on plates which did not receive nematodes.

The activity of a broth freed of nematodes and assayed by the procedures described is shown in Table 1. The culture was 9 months old when it was tested. It had been inoculated with approximately 100 worms and supported a population of 110×10^3 nematodes after 6 weeks' incubation. The culture filtrate caused *A. conoides* to form traps and contained at least 100 but less than 200 dilution units of activity. Since uninoculated broth was inactive, the results provided unequivocal evidence that a metabolic product of the nematode was responsible for the formation of traps by the fungus. "Nemin" is proposed as the name of the substance or substances that cause trap formation by predaceous fungi. The morphogenic effect of nemin is illustrated in Fig. 1. The nemin activity of a second 9-month-old culture of *Neoaplectana glaseri,* which had supported 28×10^3 worms after 6 weeks', and 2×10^3 worms after 7-months', incubation, was more than 10 but less than 50 dilution units. The numbers of traps on plates treated with undiluted culture filtrates were consistently less than those on plates treated with low dilutions (1/5, 1/10) of the active broths (Table 1). This suggests that there was a nemin concentration optimal for inducing trap formation, or that the culture filtrates contained a nemin inhibitor which was removed by dilution.

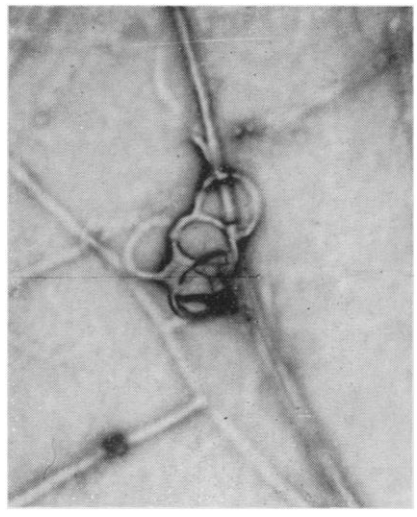

Fig. 1. Nematode-trapping hyphal loops produced by the predaceous fungus *A. conoides* in response to worm-free culture filtrates of the nematode *N. glasseri* (× 220).

To determine the time and stage of development at which nemin was elaborated by *N. glaseri,* a 100-worm inoculum was added to each of a series of tubes containing culture medium and the tubes were incubated at 22°C. Individual tubes were withdrawn after 4, 8, 12, 16, 21, 25, and 60 days of incubation. A portion of the contents of each tube was used to determine the number of worms present. The remaining broth was freed of nematodes by filtration and assayed for nemin activity. Following inoculation with third-stage larvae, young *N. glaseri* from the resulting adults first appeared on the fourth day, and the worm population then increased to a maximum in 15 days. The viable count decreased slightly after 21 days of incubation and then tended to remain con-