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The Metabolism of Oat Leaves during Senescence

I. RESPIRATION, CARBOHYDRATE METABOLISM, AND THE ACTION OF CYTOKININS[1,2]

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ABSTRACT

When the detached first leaves of green or etiolated oat (*Avena sativa* cv. Victory) seedlings senesce in the dark, their oxygen consumption shows a large increase, beginning after 24 hours and reaching a peak of up to 2.5 times the initial rate by the 3rd day. This effect takes place while the chlorophyll of green leaves, or the carotenoid of etiolated leaves, is steadily decreasing. Kinetin, at a concentration which inhibits the decrease in pigment, completely prevents the respiratory rise; instead, the oxygen consumption drifts downwards. Lower kinetin concentrations have a proportional effect, 50% reduction of respiration being given by about 0.1 mg/l. About one-fifth of the respiratory rise may be attributed to the free amino acids which are liberated during senescence; several amino acids are shown to cause increases of almost 50% in the oxygen consumption when supplied at the concentrations of total amino acid present during senescence. A smaller part of the rise may also be due to soluble sugars liberated during senescence, largely coming from the hydrolysis of a presumptive fructosan. The remainder, and the largest part, of the increase is ascribed to a natural uncoupling of respiration from phosphorylation. This is deduced from the fact that dinitrophenol causes a similar large rise in the oxygen consumption of the fresh leaves or of leaf segments kept green with kinetin, but causes only a very small rise when the oxygen consumption is near its peak in senescent controls. The respiration of these leaves is resistant to cyanide, and 10 mM KCN even increases it by some 30%; in contrast, etiolated leaves of the same age, which undergo a similar rise in oxygen consumption over the same time period, show normal sensitivity to cyanide. The respiratory quotient during senescence goes down as low as 0.7, both with and without kinetin, though it is somewhat increased by supplying sugars or amino acids; glucose or alanine at 0.3 M bring it up to 1.0 and 0.87, respectively.

N^6-Benzylaminopurine and Δ-2-isopentenylaminopurine act similarly to kinetin in repressing the respiratory rise, the former being five times as active as kinetin, while the latter has only 1% of the activity of kinetin. Zeatin also powerfully prevents senescence. Because the repression of the respiratory rise is shown by each cytokinin at the concentration at which it inhibits senescence, the action is ascribed in both cases to the maintenance of a tight coupling between respiration and phosphorylation. It is pointed out that such an effect would explain many features of cytokinin action.

A change in the methodology of the senescence experiments is described and compared with the method previously used, and the influence of temperature and age of the plants on the course of leaf senescence are presented in detail.

When the detached first leaves of oat seedlings (*Avena sativa* cv. Victory) are placed in the dark, proteolysis begins within 6 hr and the first evidence of Chl destruction follows about 18 hr later (19, 27). Along with the proteolysis, however, protein synthesis is still continuing actively (19), as Chibnall and Wiltshire (4) and Yemm (38) found much earlier with leaves of *Phaseolus* and *Hordeum*, respectively. In the oat leaf, this synthesis is evidently a prerequisite for the senescence process, because inhibition of protein synthesis by cycloheximide, anaerobiosis, etc., effectively prevents senescence. As in other material, cytokinins also prevent senescence, but in the oat leaf this action of cytokinin was found to be strongly antagonized by L-serine, and to a lesser extent by L-cysteine, both of which markedly accelerated senescence (20, 27). Glycine and L-alanine also had small effects. The hypothesis was advanced, therefore, that senescence is dependent on the *de novo* synthesis of one or more proteolytic enzymes, having serine or cysteine, or both, at their active centers. Extraction of proteolytic enzymes showed appropriate sensitivity to reagents for both —OH and —SH groups (19, 20). Such reagents also inhibited senescence *in vivo*.

In these processes proteolysis appeared to play such a dominant role that the participation of carbohydrate metabolism was not studied; indeed Moyse (22) had concluded earlier with several species that carbohydrates exert little major effect on leaf senescence. However, Marré *et al.* (18) found with potato leaves that the application of sugar solution did delay senescence, at least for the 48 hr of their experiments. The need to clarify this aspect, as well as our unexpected observation that the leaf respiration greatly increases during senescence, led to the studies of changes in carbohydrate metabolism and oxygen consumption which are reported here.

MATERIALS, METHODS, AND LIMITING CONDITIONS

Methodological changes, new procedures, and much more extensive experimental background since the earlier publications (19, 20, 27) require a full description here.

Method of Application. In view of the many biochemical determinations required in this work, the previously used mode of application of test solutions to the leaf was, in most ex-

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[2] This paper is dedicated to the memory of Solon A. Gordon because of his continued interest in plant metabolism and his many contributions to it, and in recognition of a long standing friendship.

periments, changed. Instead of applying a single 10-μl droplet, 5 to 20 leaf segments (the 3-cm apical portion of the 7-day-old first leaf of light-grown *Avena* cv. Victory) were floated on 10 to 20 ml of solution in a Petri dish. The leaf segments remained floating throughout the 3 to 5 days of the experiment and did not sink below the surface. In a few cases, washed cotton gauze was laid under the segments to keep them from moving.

After the usual 3 days in darkness, instead of a green zone surrounding the droplet of cytokinin solution, the green color was evenly distributed throughout the leaf segment. As a result, much lower cytokinin concentrations can be used in this procedure. The concentration of kinetin needed to preserve 90% of the Chl after 3 days in the dark, which for the droplet method was 3 mg/l, was only about 0.2 mg/l for floated segments (Fig. 1). The amino nitrogen values (closed symbols in Fig. 1) showed similar or even greater ratios. Thus the floating method is around 15 times more sensitive (in terms of concentration) than that with droplets (although requiring a much larger volume of solution). However, in order to obtain the maximum effect, Figure 1 shows that 3 mg/l or close to it, is needed and, therefore, 3 mg/l was used in most of the experiments described here.

In the experiment described in Figure 8, the droplet method was used—2 droplets being placed at one-third and two-thirds the distance from the apex, respectively. This produced a kinetin response similar to that resulting from flotation.

Effect of Temperature. Because these experiments were to be spread over nearly 2 years with consequent changes in room temperature, it was felt to be essential to determine the temperature sensitivity of the senescence process. This information is essential, in any event, if the results of numerous experiments are to be directly compared. The variations with temperature of the Chl, protein, and amino nitrogen of the floated leaf segments after 3 days in darkness are shown in Figure 2. Clearly, senescence proceeds at maximum rate at 30 C, as judged by Chl degradation, or at 26 to 30 C, as judged by α-amino nitrogen. At higher temperatures, the rate of senescence falls off very steeply, and at 37 C the Chl content in controls remains actually at 94% of the initial value. At above 37 C, there was obvious damage, as shown by leakage of colored material into the medium, and a fall in the α-amino nitrogen content to less than half of the initial value, while

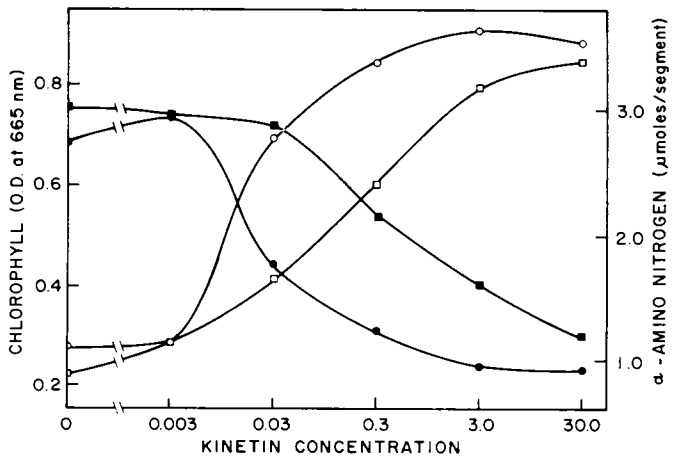

FIG. 1. Comparison of the flotation method with the droplet method of application of kinetin to leaf segments. Leaves from 7-day-old plants allowed to senesce 3 days in darkness at 26 C. Open symbols: Chl; closed symbols: α-amino nitrogen; ○: flotation method; □: droplet method (10 μl). Kinetin concentration in mg/l.

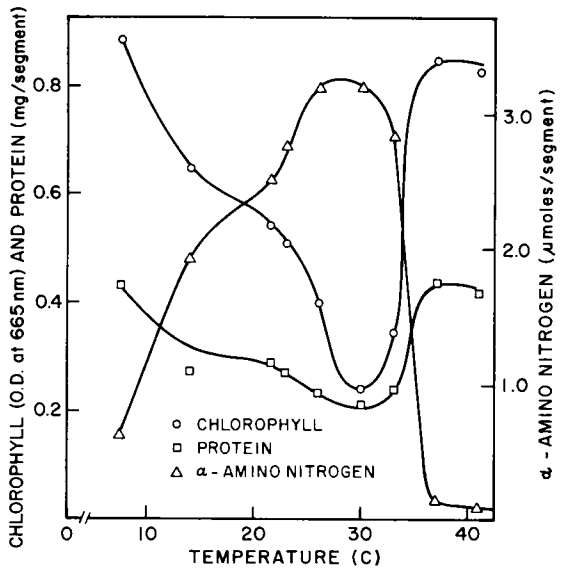

FIG. 2. Effects of temperature on senescence after 3 days in darkness.

above 41 C, thermal effects begin to cause secondary yellowing. These data support the concept previously presented that senescence is an active metabolic process.

The temperature for the experiments to be reported here was 26 C, which is close to the optimum, unless otherwise noted.

Influence of the Age of the Plant. The plants were grown in vermiculite in a growth chamber under 200 ft-c (or in a few cases noted, 400 or 600 ft-c) cool white fluorescent lights at 26 C. Previous work has always made use of 7-day-old plants, since at this stage the first leaf has just finished elongating, but during this work it was felt desirable to follow the effect of the age of the plants over a wide range. Leaves were therefore taken almost daily from plants 5 days old (when the unrolled leaf blade just exceeds 3 cm in length) up to 14 days old, when they are already beginning to senesce while still on the plant. Figure 3A shows the Chl contents of the freshly cut 3-cm apical segments of the first leaf each day, together with the time courses of senescence of each of these segments when detached and placed in the dark. The initial Chl contents appear to pass through a flat maximum about the 9th day, but the slopes of the senescence curves shown are very similar from the 5th to the 11th day; the most consistent behavior is shown from the 7th to the 9th day.

Figure 3B shows the initial contents, in the same segments of leaves taken from the 5th to the 14th day, of reducing sugar, invertase-liberated reducing sugars ("sucrose"), protein, and α-amino nitrogen. The protein curve, like the Chl curve in Figure 3A, shows that some senescence has clearly begun in the attached first leaf after about the 9th day. The other curves reflect the general changes that are occurring simultaneously, namely growth, senescence, and transport of material out of the leaf. They provide useful initial points for many of the senescent changes here studied. These data confirm that days 7, 8, or 9 are optimal for the study of senescence, and leaves of these ages have been used throughout, unless otherwise noted.

Sugar Determinations. Duplicate aliquots of boiling 80% ethanol extracts (50–500 μl, three–six replicates) were evaporated to dryness in a cool air stream. To one of the tubes, 1 ml of 25 mM acetate buffer, pH 4.5, was added, and reducing values were directly determined by the Somogyi (30) modification of the Nelson method; to the other tube, 1 ml of the same acetate buffer containing 5 μg of crystalline yeast in-

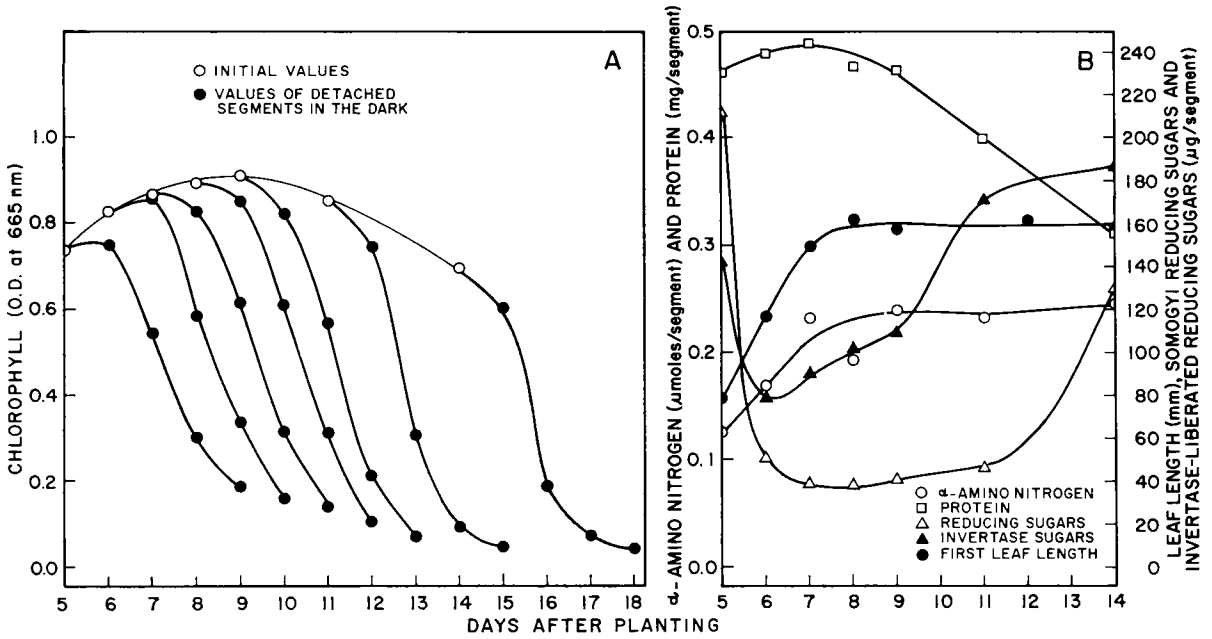

FIG. 3. A: Effect of age of the leaf on the progress of senescence; B: time course of the variation in initial values of length and of the constituents of the leaves shown as open circles in A.

vertase (L. Light and Co., Ltd., Colnbrook, England) was added, the reaction mixture was incubated at 37 C for 4 hr, and the reducing sugar again was determined. This invertase concentration was sufficient to hydrolyze 100 μg of sucrose completely in less than 30 min. Invertase-liberated reducing sugars were calculated from the difference between the two determinations.

Infusion. Infusion of oat leaf segments was accomplished by placing the segments between the bottoms of two small nested beakers in order to restrict their movement. The solution to be infiltrated was introduced between the beakers until all segments were covered. The beakers were then placed in a vacuum desiccator and evacuated to approximately 30 mm Hg for about 5 min. Air was allowed to re-enter the desiccator slowly to effect gentle infusion of the leaves. The segments were blotted to remove surface water before respiratory studies were carried out. The volume of solution entering by infusion was determined to be 6.5 to 7.5 μl per 3-cm leaf segment.

Respiration. Oxygen consumption was determined in the dark on samples of 10 leaf segments in Warburg vessels at 27 C. Respiration rates were repeatedly observed to be linear for over 4 hr, sometimes after minor fluctuations in the 1st hour or so. They were therefore measured for 1 to 3 hr, and the rates were averaged; the standard errors, using three to four replicates, did not exceed 5%.

Pigments. The Chl content of green leaves was measured at 665 nm, and the carotenoid content of etiolated (dark-incubated) leaves at 443 nm.

RESULTS

Time Course of Respiration. Because in some experiments it was desirable to infuse the leaves with test solutions, the effect of such infusion on the oxygen consumption was first determined. Figure 4A shows that infusion with water decreased the observed respiratory rate by some 10%; within the 3-hr period both rates are seen to be fully linear. Figure 4B supplies a control for infusion with inorganic ions; it will be seen that, while the effects can be quite large, significant stimulations of oxygen uptake are not seen below about 30 mM, or 60 milliosmolar. It should be noted that any reported increases in respiration due to salts of organic acids at concentrations above 30 mM have to be regarded with caution.

Typical time course curves for the respiration of both green and etiolated senescing leaf segments are shown in Figure 5. By the end of the first 24 hr the respiration rate normally showed only a small increase from that at the outset, but during the second 24 hr it rose steeply, to reach a maximum usually on the 3rd day. Thereafter the decline was almost as steep as the rise. In the presence of 3 mg/l of kinetin, however, the rise was totally suppressed, and instead the respiration rate steadily declined. The same general behavior, reaching a peak on the 3rd (or occasionally the 2nd) day, in controls, with total repression by cytokinins, has been seen in all of our experiments. The etiolated leaves show the same behavior (Fig. 5, C and D), and it is of interest that the carotenoids in these leaves disappear during senescence, much as Chl does in the green tissues. In this respect, the senescence of etiolated leaves is like that of green leaves.

The above data refer to 3 mg/l kinetin (Fig. 1), which is maximally effective for the flotation method. Over the whole range, however, Table I shows that those concentrations effective in preserving Chl are also effective in suppressing the increase in respiration; a maximum effect is observed at 3 mg/l for each effect. A more detailed comparison is seen below in Figure 11.

The 24-hr lag before the respiratory rise occurs parallels the lag before Chl disappearance (Figs. 5 and 10; *cf.* ref. 27). Figure 6A shows that at 24 hr the senescent changes are largely reversible on transfer to kinetin, but at 48 hr they are not. Leaf segments floated on water for 24 hr and then infused with kinetin correspondingly show a rapid effect on oxygen consumption (Fig. 6B), with no rise, but even a modest decrease, easily detectable after 3 hr. The lag is therefore not due to slow entry of kinetin into the cells from the infused solution. It is noteworthy that the effect of kinetin on the incorporation of leucine into protein from applied droplets was found earlier to be detectable in 6 hr after the same water treatment (27).

Most of the subsequent experiments were devoted to

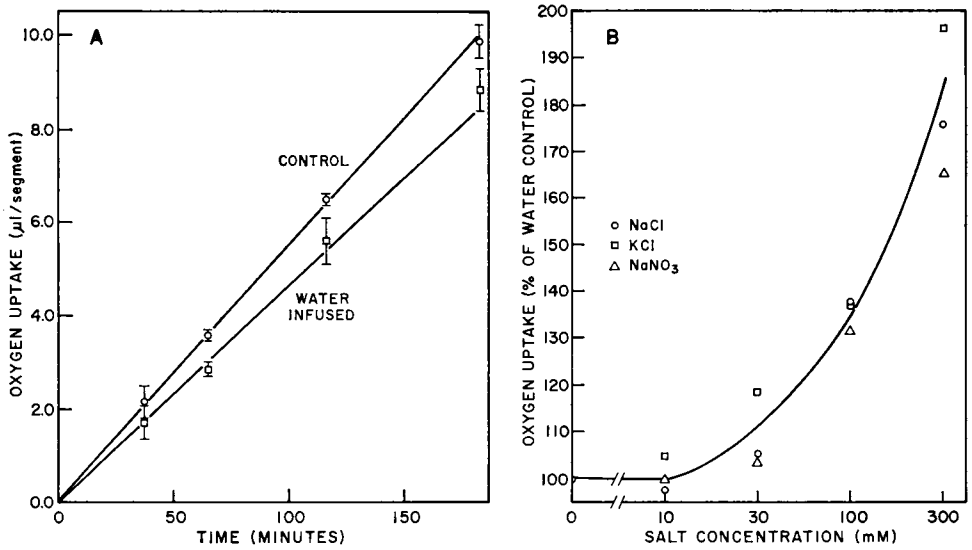

FIG. 4. Effects of infusion with water and salts on the oxygen consumption of 3-cm leaf segments. A: Water infusion, 9-day-old leaves; bars represent 90% confidence limits. B: Infusion with salts; NaCl, 8-day-old leaves, KCl, 9-day-old leaves, $NaNO_3$, 10-day-old leaves. Each point the value from 10 segments.

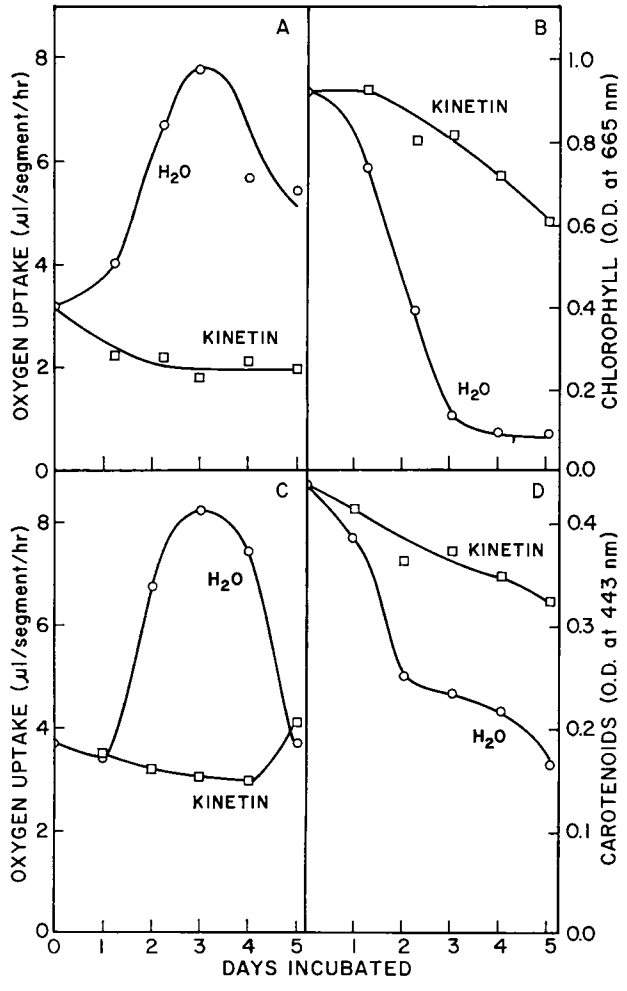

FIG. 5. Time course of the oxygen consumption and pigment loss during senescence. ○: water; □: 3 mg/l kinetin; A and B: green leaves; C and D: etiolated leaves; both 7 days old.

Table I. *Comparison of Effects of Kinetin on Chlorophyll and on Oxygen Uptake*

Three-cm apical leaf segments were floated on kinetin solutions for 3 days in darkness at 27 C. Results are the average of three to four replicates.

Kinetin Concn	Values after 3 Days in Darkness	
	Chl content	O_2 Uptake
mg/l	*% of initial*	
0	19.7	206
0.03	53.6	176
0.3	86.0	85.5
3.0	92.7	70.7
30.0	89.4	87.3

attempting to understand these respiratory changes. They could be due to changes in substrate, changes in the oxidase, or changes in a control mechanism. With respect to the possible role of substrates, both amino acids and carbohydrates were studied.

Amino Acids as Respiratory Substrate. It has been shown several times (*e.g.* Fig. 1 of ref. 19) that in 72 hr in the dark the free amino nitrogen rises to nine times the initial value. Since the initial content is 0.31 μmole per segment, weighing 16 mg each, the initial concentration in "leucine equivalents" is about 19.4 mM, and the 72-hr concentration would be about 175 mM. New determinations have given a 72-hr value of 2.9 μmoles per leaf segment or 181 mM, which agrees closely.

Accordingly, the influence of exogenous amino acids on the respiration of fresh leaf segments was investigated, using concentrations comparable to these values. Figure 7A presents results of infusing the leaves with L-alanine, L-serine, and glycine. With L-alanine, the respiration rate is increased 29% at 0.1 M and 52% at 0.3 M. In two other experiments, the increases at 0.3 M were 37 and 50%, respectively. The serine also shows maximum response at about 0.3 M, and glycine gives nearly maximum response at 0.1 M (Fig. 7A). Higher con-

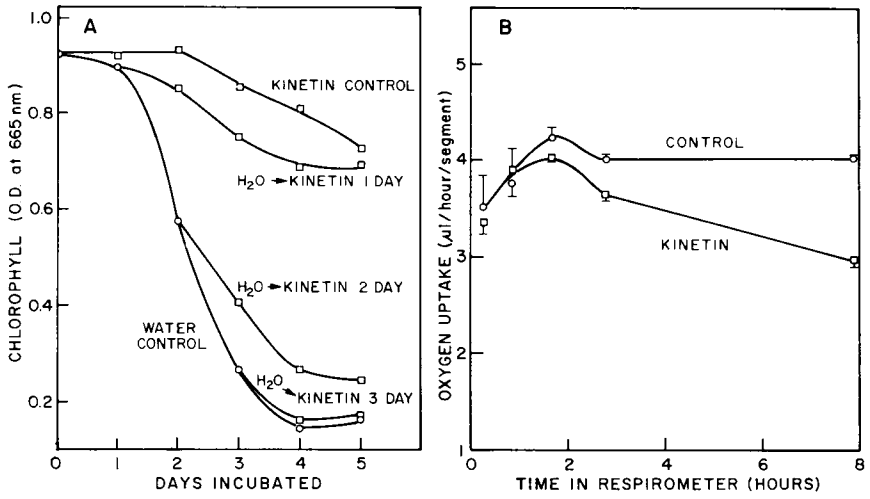

FIG. 6. A: Escape of leaf segments from the action of kinetin. Segments transferred from water to 3 mg/l kinetin on the 1st, 2nd, and 3rd day after detachment and darkening. B: Record of the respiration of leaf segments which had been 1 day in water and were then transferred to water or 3 mg/l kinetin, (second curve in A) showing rapid onset of the depression of respiration by the kinetin. Bars represent standard errors, 4 replicates of 10 segments each.

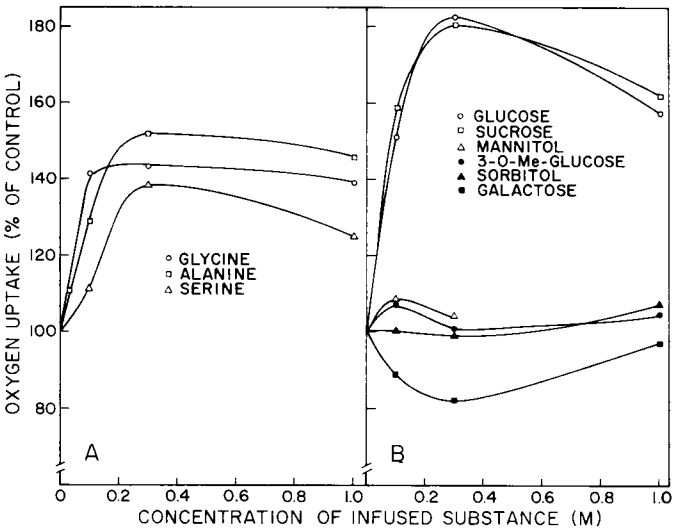

FIG. 7. Effect of infusion with amino acids and sugars on the respiration of leaf segments in the dark. A: amino acids; B: sugars and sugar derivatives. Oxygen uptake measured for 1.5 to 2.5 hr beginning 1 hr after infusion.

Table II. *Effect of Alanine Isomers on Oxygen Uptake of Fresh, Infused Leaf Segments*

Each datum is the mean of three replicates ± SE. Plants were grown at 200 ft-c, 26 C.

Infusion Treatment	Respiration	
	Uptake	Increase
	µl O_2/segment·hr	%
H_2O	2.67 ± 0.05	
β-Alanine, 0.3 M	3.61 ± 0.11	35.5
D-Alanine, 0.3 M	3.82 ± 0.11	43.1
L-Alanine, 0.3 M	4.12 ± 0.04	50.4

centrations give no greater effect. Even assuming rapid cellular entry, the solution applied will inevitably be diluted up to 2-fold by the cell contents. Evidently, a respiratory rise approaching 50% might be ascribed to the liberated amino acids, but there must be other and larger contributions if the whole respiratory rise of around 250% is to be accounted for.

Table II shows that the isomers of L-alanine are also effective in increasing oxygen uptake, though somewhat less than L-alanine.

Changes in Carbohydrate during Senescence. Figure 8 (open and filled circles) shows the time course of changes in the reducing sugars and the invertase-liberated reducing sugars ("sucrose") soluble in hot 80% alcohol. The decrease in sucrose is about enough to account for the increase in reducing sugars, but the time courses (Fig. 8) do not match, and since

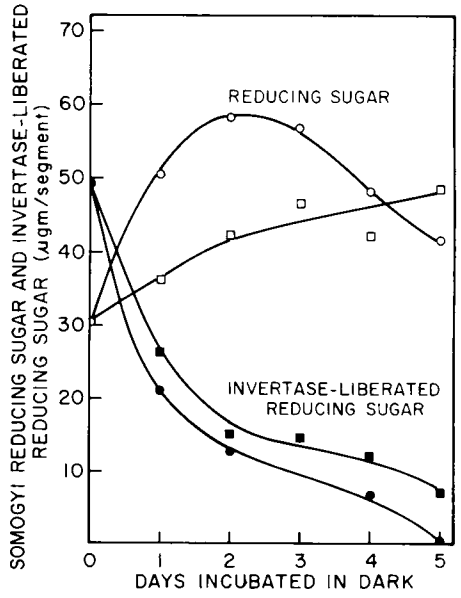

FIG. 8. Time course of the sugar content of leaf segments after detachment and darkening. ○: water controls; □: 3 mg/l kinetin, applied as two 10-µl droplets.

both values decrease after the 3rd day, it is likely both that polysaccharide is being hydrolyzed and that metabolic consumption is taking place at the same time.

The effect of kinetin on these changes is also seen in Figure 8 (open and filled squares). For the first 4 days, kinetin strongly retards the rise in reducing sugar, and at least for 2 days it slightly retards the fall in "sucrose." But by the 4th day and 5th days of senescence, the reducing sugars are becoming depleted so rapidly in the controls that the curves cross. (By the 5th day, the control leaves have lost 90% of their Chl, the leaves in kinetin only about 20%.) If we add the two curves to get the total monosaccharide equivalent, we find that the values in kinetin, which are lower than the controls for the first 3 days, finish somewhat higher on the 5th day. In general, kinetin retards all the changes in carbohydrate—decrease of disaccharide, increase of monosaccharide, and consumption of both.

It was noted above that some polysaccharide hydrolysis is probably occurring. This is supported by another experiment in which the reducing sugars rose sharply on the 2nd and 3rd day with no further decrease in the "sucrose," so that the total monosaccharide equivalent was above the initial level. The nature of the polysaccharide involved is of interest since no iodine-staining starch is seen in the fresh leaves.

The present experiments have indicated that the materials attacked by invertase include not only sucrose but also a series of over eight other oligomers (extractable in 80% ethanol at 80–85 C) which apparently correspond to a polyfructosan series, probably of the levulin type. Chromatography has shown that these fructosans, whose description will not be given here, are completely hydrolyzed by the invertase treatment, which yields glucose and fructose as sole detectable products. For the present, it is assumed that the invertase-labile substances found in freshly cut or in dark-incubated sections are similar in nature to those found in light-incubated leaves.

Carbohydrates as Respiratory Substrates. For this study both the fresh and the senescent leaf segments were infused with carbohydrate solutions as for the amino acids above. As expected, the oxygen uptake of the fresh leaves was greatly increased by the soluble sugars. Figure 7B compares the effects of glucose and sucrose with those of galactose, mannitol, sorbitol, and 3-O-methylglucose, which are known not to be generally available as respiratory substrates. These provide controls for a nonspecific effect on respiration like those given by salts in Figure 4B, and make it clear that the increase caused by glucose and sucrose is due to the provision of respiratory substrate. The curves show a clear optimum for glucose and sucrose at about 0.3 M; fructose at 0.3 M has about the same effect.

The fact that both the amino acids and the soluble sugars are maintained at a low level in presence of kinetin should make such leaves more responsive to additions of substrate than leaves which have senesced normally and hence are rich in the hydrolysates. To test this deduction, leaf segments were allowed to senesce in the dark with and without kinetin and then after 3 days infused with glucose, or with L-alanine, and oxygen uptake was measured. The results (Table III) show a striking difference between the respiratory effects of control and kinetin-treated segments. At this time, the leaves whose senescence has been prevented by kinetin are using oxygen at a rate less than half that of controls. Neither L-alanine nor glucose further increases the oxygen uptake of the senescent controls, but both exert large promotive effects on leaf segments that have been maintained on kinetin.

Unfortunately, for an explanation of the rise in respiration during senescence, the data above show that the sugars liberated during senescence cannot be making a major contribution to the respiratory rise. Thus the maximum sugar level reached, after 2 to 3 days in darkness, is 71 μg (monosaccharide equivalent) of total sugar per leaf segment. Since the segment weighs 16 mg, this corresponds to a concentration of 24 mM. From Figure 7B, such a concentration could produce a respiratory rise of no more than 12 to 15%. While this would represent a small contribution, the main causes of the respiratory rise therefore have been sought elsewhere and, in particular, in the coupling between respiration and synthetic processes.

Table III. *Stimulation of Respiration by Glucose and by Alanine With and Without Kinetin*

Leaf segments infused with 300 mM glucose or L-alanine solution or water, after 3 days floating in darkness at 26 C on water or kinetin 3 mg/l.

Pretreatment, 3 Days in Dark	Chl	α-NH_2N	Infused with	Respiration	
	A_{665}	μmoles/segment		$\mu l\ O_2$/segment·hr	change, %
Water	0.339	2.98	Water	6.82 ± 0.09	
Water	0.339	2.98	Glucose	6.62 ± 0.19	−2.9
Kinetin	0.888	0.87	Water	2.37 ± 0.06	
Kinetin	0.888	0.87	Glucose	5.05 ± 0.09	+113
Water	0.142	3.132	Water	6.62 ± 0.10	
Water	0.142	3.132	Alanine	6.16 ± 0.11	−6.9
Kinetin	0.838	1.148	Water	2.38 ± 0.13	
Kinetin	0.838	1.148	Alanine	2.97 ± 0.04	+25.1

Table IV. *Effects of Glucose and Mannitol on the Retention of Chl in the Dark*

Leaf segments from 7-day- or 8-day-old plants floated on test solutions. For experiment A, 250 mg/l chloramphenicol was added; for experiments B and C 0.1 mM crystalline penicillin G was used.

	Chl Content (A_{665})
Experiment A	
Initial value	0.944 ± 0.021
After 3 days	
On water	0.297 ± 0.012
On glucose 300 mM	0.616 ± 0.029
Experiment B	
Initial value	1.009 ± 0.014
After 3 days	
On water	0.262 ± 0.009
On mannitol 300 mM	0.183 ± 0.009
Experiment C	
Initial value	1.004 ± 0.017
After 3 days	
On water	0.172 ± 0.010
On glucose 300 mM	0.533 ± 0.012
On kinetin 3 mg/l	0.873 ± 0.017
On glucose plus kinetin	0.953 ± 0.018

Influence of Carbohydrates on Senescence. Since reducing sugars are thus both produced and oxidized during senescence, it is natural to inquire whether applied glucose can modify the course of senescence. Leaf segments were therefore floated on glucose solutions, with and without 3 mg/l kinetin, and on mannitol as a nonrespirable control substance. Antibiotics were added, and the segments were left to senesce for 3 days in the usual way. Table IV presents three such experiments. It is

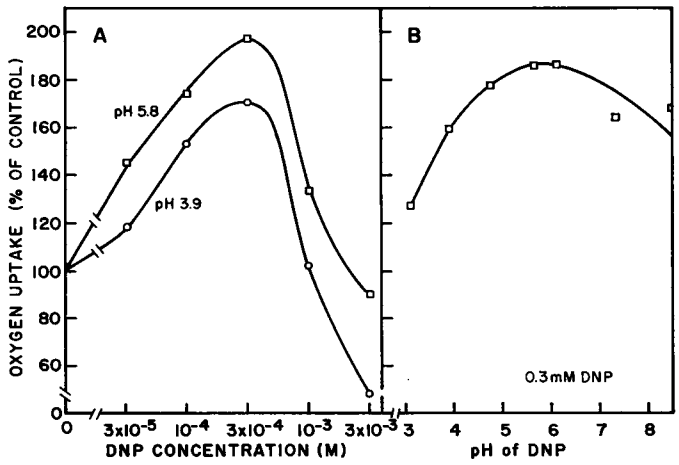

FIG. 9. Determination of the conditions for optimal action of 2,4-dinitrophenol on the respiration of freshly cut leaf segments. A: Effect of concentration at two pH values; B: effect of pH at 3×10^{-4} M.

Table V. *Action of 2,4-Dinitrophenol on Respiration of Senescent Oat Leaf Segments in Presence and Absence of Kinetin*

Experiment	Pretreatment	Chl	DNP (0.3 mM, pH 6)	Respiration	
				O_2 uptake	Increase
		A_{665}		μl O_2/segment·hr	%
I. 3 days in dark[1]	water	0.155	0	5.88	3.6
			+	6.09	
	kinetin 3 mg/l	0.783	0	3.59	53.5
			+	5.51	
II. 3.5 days in dark[2]	water		0	6.87	15.4
			+	7.93	
	kinetin 3 mg/l		0	3.62	71.3
			+	6.20	

[1] Values are means of 3 groups of 10 segments each.
[2] Values are means of 2 groups of 10 segments each.

evident that glucose has a considerable effect in preserving Chl in the dark, although at its optimal level (100 or 300 mM), it is only about half as effective as 10 μM kinetin. There is a slight additive effect with kinetin. Mannitol is inactive. A more detailed study of the action of sugars will be presented in a later paper, in which their action will be compared with that of light.

Action of Dinitrophenol on Respiration. Preliminary experiments with 2,4-dinitrophenol (Fig. 9, A and B) established that its optimum effect in increasing respiration was exerted at 3×10^{-4} M, and pH 5.5 to 6.3. Table V shows the results of two experiments with this treatment. Leaves were floated over gauze, on water or 3 mg/l kinetin, for 3 days (1st experiment) or 3.5 days (2nd experiment), then infused with water or DNP[3] briefly, and the oxygen uptake was determined about 30 min later and followed for 4 hr, using the average of 20 leaf segments run in duplicate. The data show that the lowered respiratory rate in kinetin can be brought up by DNP to over 90% of the control rate (5.51 against 5.88; 6.20 against 6.87). The DNP, however, further raises the control rate by a small amount.

It thus appears that the senescent increase in respiratory rate renders this rate relatively insensitive to further increase by DNP, while, on the other hand, the repression of the increase by kinetin makes the rate highly DNP-sensitive. To study this effect further, the sensitivity to DNP was followed daily during senescence (without kinetin). Figure 10 shows that the increment due to DNP, which is almost 100% at first, steadily diminishes (though it does not fully disappear) with the rise in the respiratory rate and reaches a minimum on day 3. The kinetin treatment results in a persistence of DNP sensitivity (Table V). The obvious implication is that the fraction of the rise which is not accounted for by increase in substrate is due to a natural uncoupling, which, when it has reached its maximum, can be only slightly further increased by DNP. Since kinetin maintains the DNP sensitivity (Table V), it follows as a corollary that the repression of the respiratory rise by kinetin could be ascribed to its maintenance of a tight coupling.

Comparative Effectiveness of Other Cytokinins on Respiration. Since there was always the possibility that the striking respiratory effects exerted by kinetin might be due to some secondary properties of that specific molecule, a small group of other cytokinins was tested in a similar way. In the Chl retention test, benzyladenine is about five times as effective as kinetin, and zeatin is about equal to kinetin, while we have found Δ^2-isopentenyladenine (2*ip*), known to be of very high activity on tissue cultures, surprisingly ineffective, averaging less than 1% of the activity of kinetin. Using *Raphanus* leaf discs, Hamzi and Skoog (9) placed kinetin, benzyladenine, and 2*ip* in the same order as here, while Latham (14), using *Nicotiana* and *Brassica* leaf discs, also found isopentenyladenine to have very low activity. Figure 11 compares the effectiveness of these four cytokinins and two related compounds in retaining

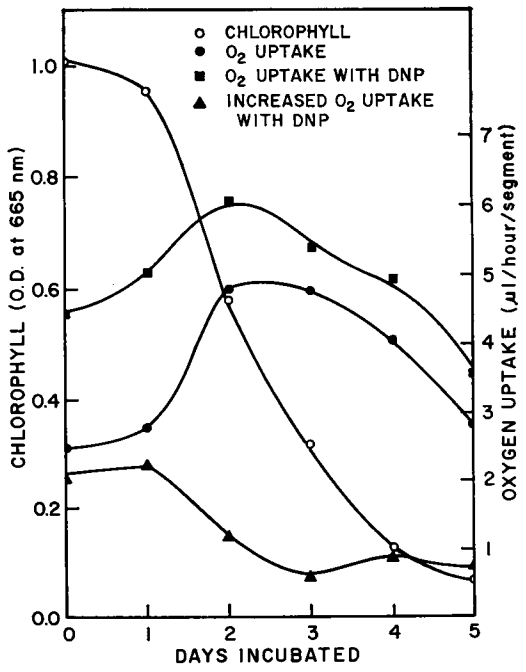

FIG. 10. Time course of the DNP effect during senescence of detached leaf segments, showing that the increase in oxygen uptake due to DNP reaches a minimum when respiration is near its peak. Oxygen uptake measured for 1.5 to 2.5 hr.

[3] Abbreviations: DNP: 2,4-dinitrophenol.

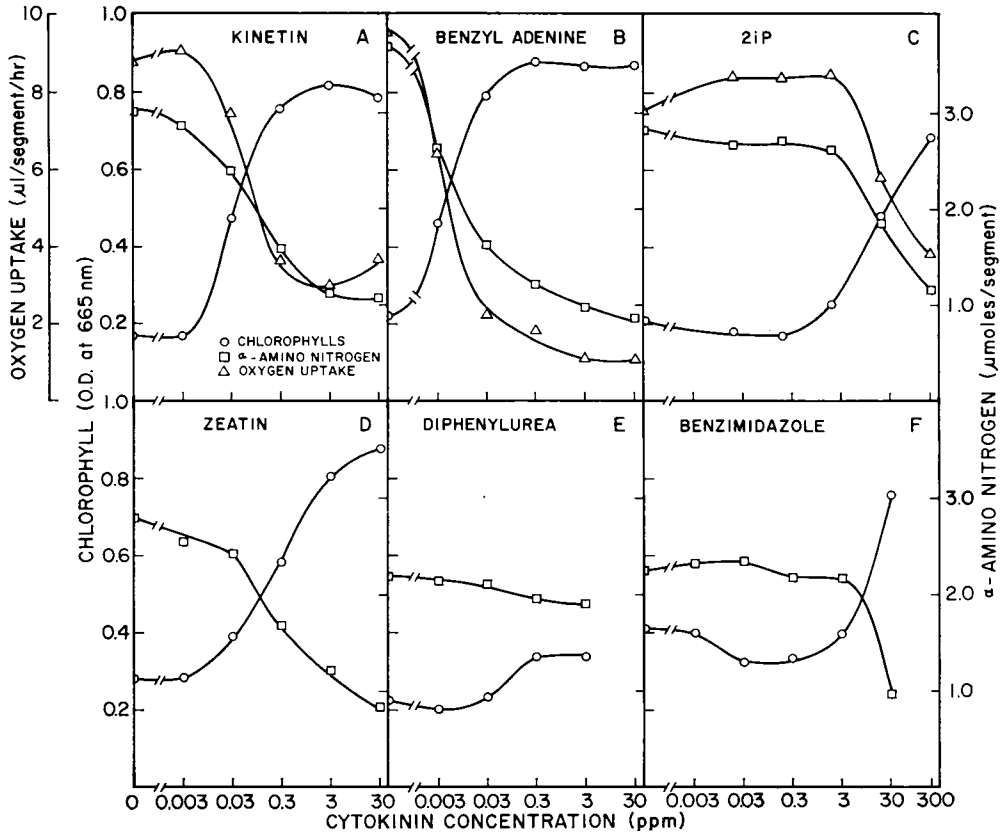

FIG. 11. Comparison of the effects of four cytokinins and two related compounds on the different criteria of senescence. The Chl and α-amino nitrogen are the values after 3 days in the dark; the oxygen uptake values (△) were measured for 1.5 to 2.5 hr.

Chl and preventing proteolysis after 72 hr in darkness, and also (for three of them) with their effectiveness in repressing the respiratory rise. While the Chl and α-amino nitrogen curves are, as expected, mirror images of one another, the curves for lowering of the respiratory rate follow those for lowering the free amino nitrogen closely. Whichever effect is used as criterion, the relative activities of the four compounds (benzyladenine, kinetin, zeatin, and 2*ip*) are essentially unchanged, namely about 5, 1, 1, and 0.01. It is probable, therefore, that the action on respiration is an essential part of the cytokinin syndrome.

PROPERTIES OF THE RESPIRATORY SYSTEM

Opportunity was taken, during these experiments, to make a more general study of the respiratory system of oat leaves. This brought to light two interesting properties, which will be reported briefly here.

1. Respiratory Quotient. In several experiments the RQ of the leaf segment was determined by the usual method, *i.e.* with and without KOH in the well of the Warburg vessel. The values were all unexpectedly low. Typically, the RQ of the fresh leaves was 0.81 to 0.84; it fell to 0.7 for the first 2 days and later returned to about its initial value. In kinetin the RQ slowly dropped to 0.70 and remained there. Only the infusion of glucose at 300 mM gave the 1.0 value, but this concentration, as shown above, is many times the physiological concentration reached in the aged leaves. Infusion of 300 mM L-alanine gave an RQ of 0.87. Since the theoretical value for the total oxidation of lipids is 0.71 and those for other substrates, known to be present, are higher, it is deduced that some system for dark retention of CO_2 is operating in these leaves, such as the organic acid accumulation which has often been found to occur in senescence.

2. Cyanide Sensitivity of the Respiration. As is well known, many plant tissues show respiration which is insensitive to cyanide or azide, and often also to carbon monoxide (see Tables XVI and XVII collected by Beevers [1]). In *Atropa* leaves, cyanide insensitivity develops with increasing age, and finally becomes a slight promotion (16). It was therefore of interest to determine the cyanide sensitivity of the respiration of the oat leaves here used, especially of older leaves. Figure 12A presents the response to cyanide of 13-day-old light-grown leaf segments, still fully dark green, and of 10-day-old etiolated leaf segments, grown in the dark room with very occasional exposure to weak green light. The 63% inhibition of the respiration of the etiolated leaves by 10 mM KCN is quite typical; carrot slices, young carrot leaves, wheat, and barley roots have given very similar figures (1). However, the older green leaves actually show an 18% increase in respiration at 1 mM KCN; at 10 mM the increase becomes as high as 29%. Only at 100 mM KCN is there inhibition and this was accompanied by evident toxicity.

Further experiments have shown, however, that the onset of cyanide insensitivity is not primarily a function of age but is a function of greening. Figure 12B shows how cyanide insensitivity develops in leaf segments of etiolated oat plants when they are illuminated; to a striking degree (except for an initial lag) it parallels Chl development. A second experiment gave similar results.

In view of the fact that etiolated leaves show a respiratory increase during senescence very similar to that of the green leaves (Fig. 5), it is certainly remarkable that their respiratory systems behave so differently in regard to cyanide sensitivity.

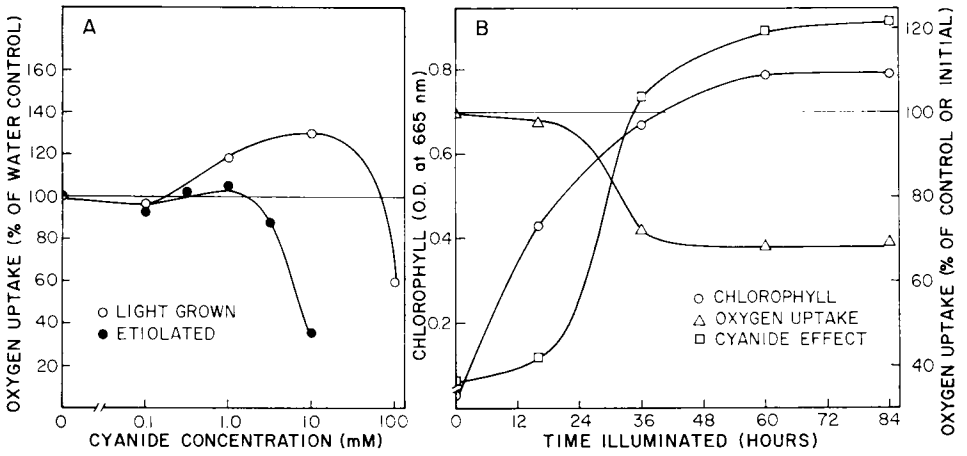

FIG. 12. Response of leaf segments to cyanide. A: Effect of cyanide on the respiration of green and etiolated leaf segments in darkness; B: change in cyanide sensitivity of the leaves when etiolated oat plants (7 days old) were brought into the light. △: oxygen uptake as percentage of the initial value; □: effect of KCN as percentage of the oxygen uptake of control leaves given the same illumination. Respiration measured for 1.5 to 2 hr; each point the mean of 2 replicates of 10 segments each.

Apparently the system which resists, and is even stimulated by, cyanide is associated with development of the photosynthetic apparatus. Thus the development of cyanide resistance, though interesting for its own sake, appears to bear only indirectly on the senescence problem.

DISCUSSION

The large rise in respiration after the 1st day of senescence (Fig. 5, A and C) calls for special comment. The reason it was not observed by many of the older workers on senescence may be that such a rise was superimposed on a continuous decrease, due to the use of attached leaves, or mature leaves already approaching senescence; such usage contrasts with our use of seedling leaves near the peak of activity (Fig. 3) but induced to senesce rapidly by detachment and darkening. Many of the early workers, also, made use of leaves which senesce very slowly. Yemm (37) did note respiration "drifts," but they were not similar to those found here. Wood *et al.* (36) figured a marked rise superimposed on a downward drift, and referred to it as a "climacteric hump." Indeed, we had noted the similarity to the "climacteric" rise in maturing fruit, and for this reason have studied the possible role of ethylene in leaf senescence. However, we have found no evidence that ethylene plays any causative role in the process (unpublished results). Krotkov (13) has reported a large increase in CO_2 production of isolated wheat leaves, and it peaks, as in Figures 5 and 6, on the 3rd day, thus closely resembling our present results. That the RQ in senescence tends to be below 1.0 has occasionally been noticed before (*e.g.* in ref. 38).

The reports in the literature of respiratory effects of cytokinins have also not been as clear cut as in this system. A variety of results has been reported in different tissues. In general, kinetin has been found to inhibit oxygen uptake somewhat in excised leaves, stems and flowers (5, 6, 10, 12, 17, 26, 31, 33, 35) with a few exceptions (8, 15) but the effects are often only small. In tissue cultures, however, both inhibitions (2, 3, 23, 24, 28) and small stimulations (7, 21) have been reported. Apparently cytokinins promote the respiration in those systems, and at those concentrations, where growth is promoted, while they inhibit respiration in tissue cultures growing slowly at high cytokinin levels, or in senescing or mature tissues generally. For example, 1 mM benzylaminopurine decreased the oxygen uptake of carnation flowers about 11% in 4 days (10). Where respiration was increased by low cytokinin concentrations, higher concentrations often caused inhibition; thus in soybean callus where 5 μM kinetin (about 1 mg/l) increased respiration optimally by about 12%, 50 μM (about 10 mg/l) decreased it by 16% (21). The large inhibitions observed here, brought about by cytokinin concentrations which maximally prevent senescence, or even those 10 times lower, can best be viewed as the prevention of a rise rather than as an inhibition *per se*.

The pattern of carbohydrate change shown in Figure 8 is similar to that observed in "starved" wheat leaves (13) and in darkened barley leaf segments (37). In both cases the reducing sugars first increased and then declined, while sucrose showed a steady decline from the start.

There is a striking contrast between the cyanide inhibition of the respiration of etiolated leaves, and the cyanide promotion of the respiration of green leaves (Fig. 12, A and B). A similar change, parallel to greening, occurs in the development of cyanide resistance in the germinating zygospores of *Chlamydomonas* (11). In that case, Chl content reached its full value in 14 hr of light, while resistance to 1 mM cyanide (and stimulation by it) became maximal almost at the same time—about 16 hr. In *Chlamydomonas,* however, stimulation of oxygen uptake by cyanide was not more than 10 to 15%, as against 29% (to 3.20 from 2.48) in our oat leaves in 10 mM cyanide. This promotion by KCN is not a salt effect since 10 mM is below the minimum value for that (Fig. 4B). Resistance to cyanide is commonly ascribed to mediation of an autoxidizable *b*-type cytochrome (*e.g.* see ref. 34), but while this could provide a bypass, it would not seem to allow for actual promotion. A more probable explanation would be that cyanide is to some extent an uncoupling agent for green tissues and for a few others; indeed, Slater (29) demonstrated partial uncoupling with heart muscle sarcosomes in which 1 mM cyanide lowered the P:O ratio by half.

Lastly, the proposed action of cytokinins in tightening of coupling or maintenance of tight coupling between respiration and synthetic reactions could have interesting implications. In the first place, it has a suggestive parallel with auxins. In chloroplasts, Tamás *et al.* (32) found that IAA produces a 20% increase in phosphorylation, and they propose that "IAA increases the degree of coupling." Although in general auxins increase respiration in a wide variety of tissues, they have occasionally been reported to delay or prevent senescence (25)

thus acting like cytokinins in this respect; however, oat leaves under our conditions have shown little response to physiological auxin concentrations. Dinitrophenol has long been known to prevent many auxin effects, especially growth. It is notable too that Yemm (38) actually suggested that nitrophenols cause "changes in metabolism similar to those associated with senescence."

In the second place, the action of cytokinins in preserving such macromolecules as proteins and polysaccharides from breakdown would have many ramifications. The reported inhibition of ethylene formation, for instance, falls well into line, since by preventing proteolysis cytokinin would, *inter alia*, deprive the tissue of free methionine, a major ethylene precursor. The more general effects of cytokinins in promoting growth of tissue cultures, development of buds, and cell division all would fit well with a maintenance of the energy supply to the cells through maintenance of coupling, although more specific effects would doubtless be operating as well.

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