base searched included 145,581 amino acid residues comprising 684 individual sequences in the Newat list and 121,098 residues from 1081 sequences in the 1978 Dayhoff collection [*Protein Segment Dictionary 78*, M. O. Dayhoff, L. T. Hunt, W. C. Barker, R. M. Schwartz, B. C. Orcutt, Eds. (National Biomedical Research Foundation, Silver Spring, Md., 1978)].
20. PDGF-A (18,000 daltons) was prepared by reduction, alkylation, and gel electrophoresis of active PDGF (*18*). It consists of roughly equal amounts of PDGF-1 and -2. Treatment of this preparation with cyanogen bromide followed by gel electrophoresis yielded two protein bands (H. N. Antoniades and M. W. Hunkapiller, unpublished data). The larger of these, 18,000 daltons, contained only PDGF-1 sequence, indicating that PDGF-1 was not cleaved by the cyanogen bromide treatment, but that PDGF-2 was. The smaller band, 14,000 daltons, contained a single sequence that must be derived from PDGF-2 by cleavage at two sites near the amino terminus of PDGF-2, one of which is presumably at Met^{12} (methionine) of the PDGF-2 sequence. Amino acid sequence analysis was performed as described (*18*) with the Caltech gas phase protein sequenator.
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30. A preliminary announcement of the results reported here was made at a symposium on "The Role of Oncogenes in Carcinogenesis" at the 74th annual meeting of the American Society of Biological Chemists, San Francisco, Calif., 5 to 9 June 1983.
31. We thank T. Hunkapiller for preparation of Fig. 1, and the American Red Cross Blood Services, Northeast Section, for the supply of clinically outdated human platelets. Supported in part by NIH research grants RR00757 (R.F.D.) and CA30101 (H.N.A.) and by grants from the Council for Tobacco Research, USA, Inc. (H.N.A.) and the Weingart Foundation (L.E.H.).

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Rapid Changes in Tree Leaf Chemistry Induced by Damage: Evidence for Communication Between Plants

Abstract. *Potted poplar ramets showed increased concentrations and rates of synthesis of phenolic compounds within 52 hours of having 7 percent of their leaf area removed by tearing, as did undamaged plants sharing the same enclosure. Damaged sugar maple seedlings responded in a manner similar to that of the damaged poplars. Nearby undamaged maples had increased levels of phenolics and hydrolyzable and condensed tannin within 36 hours, but exhibited no change in rates of synthesis. An airborne cue originating in damaged tissues may stimulate biochemical changes in neighboring plants that could influence the feeding and growth of phytophagous insects.*

Attacks by herbivores on plants may reduce the quality of the plant tissues for subsequent feeding (*1*, *2*). It has not been known whether such changes occur quickly enough to reduce ongoing defoliation. We report here that poplar (*Populus* × *euroamericana*) ramets and sugar maple (*Acer saccharum* Marsh) seedlings exhibit elevated concentrations of phenols and increased protein binding of phenolic compounds in leaf extracts within 75 hours of being mechanically damaged. Synthesis of these compounds apparently increases in response to damage. Recently, Rhoades (*3*) presented bioassay results suggesting that a factor released by damaged trees stimulates changes in the leaf quality of undamaged neighbors. We also report chemical evidence of such communication between trees: leaves of undamaged individuals in the same enclosure as damaged plants exhibit similar chemical changes.

In two separate experiments 45 1.5-month-old poplar ramets and 45 4-month-old sugar maple seedlings grown under controlled conditions (*4*) were placed in two gas-tight Plexiglas enclosures (*5*) inside a growth chamber. One enclosure housed 15 true control plants, while the other housed 15 experimental and 15 "communication control" plants. Two leaves on each experimental plant were damaged (*6*), while the leaves of both control groups were untouched. At the time of damage, $^{14}CO_2$ was introduced into both enclosures and then scrubbed from the air (*7*) before the first of three sampling intervals (*8*). At each interval five plants from each group were removed from the chamber for analyses of four chemical leaf traits known to influence insect feeding and to be affected by herbivore damage in other tree species (*1*, *2*). Four leaves from each seedling (*9*) were extracted and analyzed for total phenolics, hydrolyzable and condensed tannins, and tanning activity (*10*). Carbon-14 was assayed in leaf extracts and in phenolics precipitated by aqueous lead acetate (*11*).

At the first sampling interval all seedlings had elevated chemical measures, probably because of metabolic stimulation by elevated CO_2 (*12*). Hence the effect of damage must be considered against the dynamic background of the true controls. Damaged plants and communication controls exhibited chemical changes that were significantly different from patterns seen in the true controls.

Total phenolics in leaf extracts from poplar ramets suffering damage to approximately 7 percent of their leaf area increased 123.3 percent within 52 hours ($P = .001$) and decreased to control levels by 100 hours (Fig. 1A). Communication controls produced leaf extracts with phenolic contents elevated 57.6 percent ($P = .001$) in less than 52 hours (Fig. 1A). Damaged poplars incorporated more photosynthate into phenolics than did controls; ^{14}C in phenolic precipitate increased 150 percent by 52 hours (Fig. 1B). Undamaged communication controls also showed increased phenolic synthesis; ^{14}C in phenolics increased 128 percent ($P = .01$) after neighboring trees were damaged (Fig. 1B).

Damaged sugar maple seedlings had a significantly higher total phenolic content at 75 hours than did true controls (Fig. 1C). The protein binding capacity of leaf extracts from damaged seedlings was 33 percent higher (equivalent to 10 percent tannic acid by dry weight) than that of leaves from true controls at 75 hours (Fig. 1D). The concentration of hydrolyzable tannin increased in all plants by 36 hours (Fig. 1E), perhaps because of elevated photosynthetic rates under conditions of enriched CO_2 (*12*). At 75 hours hydrolyzable tannin remained elevated in leaves from damaged trees, while it was reduced in leaves from controls. No changes in condensed tannins were evident in leaves from damaged trees or true controls.

Phenolic and hydrolyzable tannin contents in leaf extracts from undamaged communication controls kept in the same air space as damaged trees were significantly higher than those in true controls by 36 hours and remained significantly higher at 75 hours (Fig. 1, C and D). These extracts also had significantly higher concentrations of condensed tannin than extracts from true controls, increasing 21.4 percent after 36 hours to the dry weight equivalent of 0.04 percent red oak tannin (*10*). The level of condensed tannin declined to that of the true controls after 75 hours.

As in the damaged poplar seedlings, ^{14}C was actively incorporated into phenolic compounds by damaged maple seedlings: ^{14}C counts were 2.5 times greater in precipitated phenolics than in true controls at 75 hours ($P = .03$). However, the number of ^{14}C counts in phenolics from communication control leaves did not differ significantly from

that in true controls throughout the experiments, despite increases in total phenolics (Fig. 1C) and condensed tannin. Apparently the sugar maple and poplar communication controls differed in that new photosynthate was not incorporated into leaf phenolics in the former.

The apparent synthesis of new phenolics by poplars but not maples in response to nearby leaf damage may be due to differences in the age of the plants used as well as to species differences. Our results lead us to speculate that other metabolic activities were stimulated in the undamaged maple seedlings, including translocation of phenolic precursors or other processes normally found in damaged leaf tissue (*13*), such as oxidative polymerization of phenolics.

As in red oaks (*2*), the increase in phenolic contents and tanning coefficients in damaged maple and poplar trees seems great enough to affect foraging by herbivores. The increases we observed in sugar maple leaves are 10 to 20 times larger than the increases necessary to reduce the growth rate of some lepidopteran larvae eating artificial diets (*14*). The tree response occurs rapidly enough to alter food quality and thus to influence the foraging behavior of larvae sensitive to these chemical traits (*15*). Moreover, the trees' response to damage is sensitive; none of our experimental plants had more than 10 percent of its leaf area removed. However, all responses waned after 75 hours, suggesting that more continuous or intense stimulation is necessary to prolong them. The response to damage may be gas-mediated. Ethylene, which is produced by wounded tissues of many plant species and which can influence the biosynthesis of the compounds we assayed (*16*), is a likely candidate. We have sampled enclosed air (*17*) but have not completed analyses of potential chemical cues.

The finding that undamaged trees can respond like nearby damaged trees is of potential ecological importance. The amount of change in some constituents (such as hydrolyzable tannins) was similar to that for damaged trees; the effects on insects or pathogens could be as great. Other traits (such as tanning coefficients) changed less dramatically or not at all. Our experiments lasted only 3 days, and the communication controls may have exhibited preliminary stages of the responses apparent in damaged plants. Although wind may create complex patterns or dilute the impact of cues, undamaged trees in nature may deter oviposition by adult insects or the spread of larvae from damaged neighboring trees. We suggest that airborne cues in single tree canopies may stimulate and coordinate systemic induction responses (*1–3*) and could generate substantial chemical heterogeneity in individuals (*9, 15, 16*). Finally, such communication between trees may explain synchronous phenomena ranging from mast fruiting to the decline of herbivore outbreaks (*3*).

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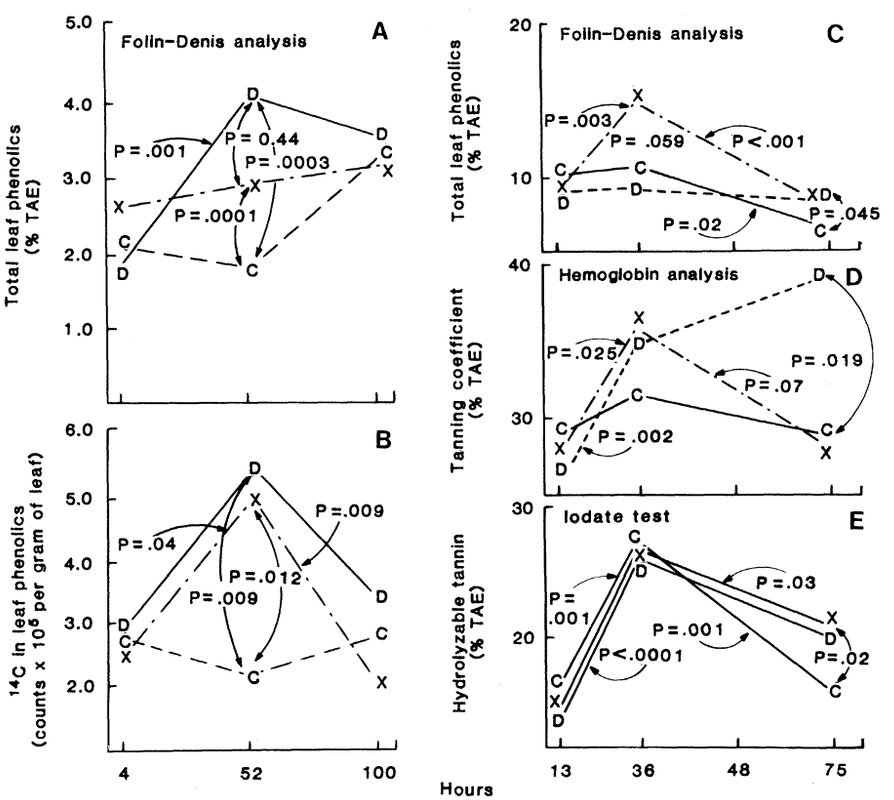

Fig. 1. Total phenolics (A) and ^{14}C (B) in poplar ramet leaf extracts. Total phenolic contents (C), tanning coefficient (ability to precipitate hemoglobin from solution) (D), and hydrolyzable tannin contents (E) in leaf extracts from sugar maple seedlings. Arrows denote significant changes between sampling intervals and significant differences between means for damaged (*D*), communication control (*X*), and true control (*C*) plants, determined with a modification of Student's *t*-test (*9*); *TAE*, tannic acid equivalents (*2, 10*).

References and Notes

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4. Maple seedlings, 30 to 40 cm high with a mean (± standard deviation) of 14 ± 4 fully expanded leaves, and poplar ramets (clone DN-34), 26 to 45 cm high with 20 ± 5 expanded leaves, were grown individually in soilless potting medium under a 16:8 hour dark:light cycle and 18.0° ± 0.5°C.
5. Each chamber (volume, 36 to 40 liters per seedling) had fittings for the introduction, removal, and circulation of gases.
6. The second and seventh leaves from the apex of maple seedlings and the third and seventh leaves from the apex of poplar ramets were torn in half with plastic gloves built into the enclosure walls.
7. Addition of $^{14}CO_2$ (36.7 μCi per seedling) raised CO_2 concentrations from 330 to 600 parts per million. The labeled CO_2 was removed by drawing it through an ethanolamine solution of ethylene glycol monomethyl ether.
8. Maple seedlings were removed 13, 36, and 74 hours after damage and poplar ramets were removed 4, 52, and 100 hours after damage.
9. In maples the fourth, fifth, sixth, and eighth leaves from the apex were removed and in poplars the first, fourth, eighth, and twelfth leaves were removed. These leaf positions are statistically independent samples and hence meet the requirements of Student's *t*-test. Sugar maple leaves show greater variances in tannin content within trees than between trees [J. C. Schultz, P. J. Nothnagle, I. T. Baldwin, *Am. J. Bot.* **69**, 753 (1982)]. All statistical comparisons were made with Student's *t*-test modified for unequal variances.
10. Leaves were extracted under N_2 in aqueous methanol and analyzed for tanning activity with hemoglobin as a binding substrate and for total phenolics by the Folin-Denis method [J. C. Schultz, I. T. Baldwin, P. J. Nothnagle, *J. Agric. Food Chem.* **29**, 823 (1981)]. Condensed tannins were measured as proanthocyanidins and hydrolyzable tannins were measured with an iodate technique (*2*). Condensed tannin values are expressed as purified red oak tannin equivalents per gram (dry weight) of leaf; the three other measures are expressed as tannic acid equivalents per gram of leaf (*2*) drawn from standard curves.
11. A 1-ml extract was acidified with 1 ml of 2*N* HCl and counted in 15 ml of hydrofluor (National Diagnostics) and quench-corrected with an internal standard. Carbon-14 in newly synthesized phenolics was determined in precipitation with

aqueous lead acetate [M. K. Siekel, in *Biochemistry of Phenolic Compounds*, J. B. Harborne, Ed. (Academic Press, New York, 1964), p. 37], which may selectively precipitate orthodihydroxy phenolics. Values are expressed as counts per minute per gram (dry weight) of leaf material.
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17. Constant-flow air samplers fitted with charcoal tubes approved by the National Institute for Occupational Safety and Health were used to sample air in the enclosure of the treatment and true control groups.
18. We thank C. Carl and M. DeMerritt for supplying the sugar maples and poplars, M. E. Montgomery for permission to cite unpublished work, and especially D. Rhoades for support, encouragement, and good ideas. R. T. Holmes, Cap'n K, and three anonymous reviewers helped improve the manuscript. D. Archambault, K. Hoy, T. Morrill, D. Munson, and D. Ward helped with chemical analysis. M. J. Richards drafted Fig. 1. Supported by grant DEB-8022174 from the National Science Foundation (J.C.S. and R. T. Holmes) as part of our continuing studies of herbivory at the Hubbard Brook Experimental Forest.

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Cooperative Immunoassays: Ultrasensitive Assays with Mixed Monoclonal Antibodies

Abstract. *Mixtures of certain monoclonal antibodies appear to bind human chorionic gonadotropin in a "cooperative" fashion because they form circular complexes with the hormone. Experiments illustrate how this property might be exploited to develop very sensitive immunoassays for human chorionic gonadotropin or any other antigen. Since the assays are not based on competitive inhibition between radiolabeled and unlabeled antigen, they are much more sensitive than a traditional radioimmunoassay in which either one of the same antibodies is used alone.*

The development of hybridoma technology facilitated preparation of antibodies that bind to a single antigenic determinant and thereby led to the development of valuable analytical reagents (*1*). Recent data show that combinations of selected monoclonal antibodies may be more useful for particular applications (*2–6*). We have shown that some mixtures of monoclonal antibodies to human chorionic gonadotropin (hCG) have a higher affinity than each individual antibody for the antigen (*2*). Since this effect was observed when $F(ab')_2$ fragments but not F(ab) fragments were substituted for the intact antibody, we concluded that the increase in affinity was a general phenomenon of bivalent antibodies, not unique to a peculiar feature of antibody-hCG interaction. Computer simulations of antibody-antigen binding suggested that the increase in affinity was caused by formation of a circular complex composed of two antigen molecules and one of each type of antibody (*7*). Biochemical evidence of a circular complex has also been obtained (*8*). The computer simulations also predicted that the binding of hCG to antibody mixtures would be cooperative and led us to perform the studies described here.

Monoclonal antibodies A102, B101, and B102 have been described previously (*2, 9*). A102 binds the α subunit of hCG with an affinity for the intact hormone of 0.2 nM^{-1}. B101 and B102 bind the β subunit with affinities for the hormone of 0.7 and 0.03 nM^{-1}, respectively. Computer simulations in which we used the binding constants of A102 and B102 illustrate the binding of radiolabeled hCG as a function of hCG concentration and K', the relative probability of forming a circular complex (Fig. 1). The value of K' is similar to K_2 defined by Crothers and Metzger (*10*) for use in describing multivalent binding of antibodies to polyvalent antigens. The curves produced were biphasic and had an ascending limb at very low hCG concentrations. Estimates of the amount of radiolabeled antigen bound, based on ratios of bound to free hCG in Fig. 1A, can be obtained by using the relationship

$$\text{Radioactivity bound} = Cb/H_o = Cb/f(1+b/f)$$

where C is the total radioactivity (in counts per minute) added, b is the mass bound, f is the mass of tracer remaining free, and H_o is the sum of b and f. Use of this equation indicates that the amount of radiolabel bound increases approximately threefold over a wide range of K'. The shape of the curve is due to several components. At infinitesimal hCG concentrations, the ratio of bound to free hormone is equal to $2K_a[A102] + 2K_b[B102] + 4K_aK_b[A102][B102]$, where K_a is the equilibrium affinity constant of A102 for hCG and K_b is the affinity constant of B102 for hCG. As the concentration of hCG is increased from very low levels, the fraction of hormone bound as tetrameric circular complex increases. Since this is the most stable complex (*7*), the ratio of bound to free hormone increases. When approximately 50 to 75 percent of A102 is bound to hCG, it is nearly all in the form of the circular complex. Although the amount of complex increases further at higher hCG concentrations at the expense of free A102, the increase in amount of complex is less than that of total hCG. When this occurs, the ratio of bound to free hormone declines.

We prepared computer simulations using several different amounts of antibody, different antibody ratios, and antibodies with different affinities to learn which factors would increase the assay sensitivity. The sensitivity varied dramatically with the affinity of the antibodies for hCG. For example, a twofold increase in the affinity of each monoclonal antibody for hCG would result in a 32-fold increase in the sensitivity of a cooperative assay. Although, in principle, one can assay nearly any concentration of hCG with antibodies having only moderate affinity (as the case with A102 and B102), the ultimate limiting value for assay sensitivity depends on tracer-specific activity.

The cooperative immunoassay, which we designate CIA, can also be performed with radiolabeled antibody (Fig. 1B). In this example, we assumed that A102 was radiolabeled and that we had a second antibody specific for B102 and B102 complexes. Binding of radiolabeled A102 to B102 (measured as bound) would occur only through an hCG bridge. When K' was large, the maximum amount of A102 that could bind to B102 was equal to one half of the total hCG present (Fig. 1B, broken line). Although A102 would not bind to B102 through a circular complex when K' was zero, the binding that would occur through formation of linear complexes cannot be ignored (Fig. 1B, dotted line). The binding expected using experimentally observed values of K_a and K_b at several values of K' is illustrated by the solid lines. Since the circular complex composed of two molecules of hormone and one each of antibody accounts for greater than 90 percent of the bound hormone, measurements made with radiolabled A102 or radiolabeled hCG should have the same sensitivity. The position of the maximum in the binding curve is considerably different for both types of assay (compare A and B in Fig. 1). In the case when the anti-