Our findings support the conclusion that some biochemical feature of NADPH-d neurons makes them resistant to the degenerative process in HD. Diaphorase activity itself could be the responsible factor. NADPH-d participates in the detoxification of quinone derivatives (*21*). Excitotoxins have been used to model HD in animals, and one such neurotoxin, quinolinic acid (*22*), is present in the human brain (*23*) and produces selective neuronal lesions in rats (*22*). It is conceivable that the gene defect in HD could lead to accumulation of quinolinic acid or a similar compound. It has been shown in organotypic cultures that quinolinic acid toxicity depends on excitatory corticostriatal input (*24*). NADPH-d neurons might lack excitatory amino acid receptors, thus accounting for their resistance.

It is also possible that another as yet undiscovered property of NADPH-d neurons in the CN accounts for their viability in HD. Other unique properties of these neurons include their content of somatostatin and neuropeptide Y (*9*, *10*). Selective sparing of aspiny striatal neurons containing NADPH-d, somatostatin, and neuropeptide Y indicates that the neuronal loss in HD does not comprise a genetically programmed destruction of all cellular elements. These findings suggest that the gene defect is not expressed equally in all striatal neurons or is modified by the local biochemical environment.

Note added in proof. A recent report (*25*) indicates that both neuropeptide Y–like immunoreactivity and the number of neuropeptide Y–positive neurons are greater in the caudate nucleus and putamen in HD than in controls.

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13. One 10-μm paraffin section stained with cresyl violet and two 100-μm Vibratome sections, one stained for NADPH-d alone and the other for both NADPH-d and cresyl violet, were used in the cell counting.
14. All neurons with distinct nuclei and nucleoli were counted in 10-μm sections of the CN with a ruled-graticule eyepiece at 250×.
15. Due to the large number of total neurons, NADPH-d cells and total cells were counted along a 50-μm-wide track in the middle of the CN. The track was perpendicular to the ependymal surface and extended to the medial border of the internal capsule.
16. The area of the CN was measured from the ependyma to the medial border of the internal capsule at the level of the caudato-putaminal junction, excluding the nucleus accumbens.
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26. The secretarial assistance of K. Sullivan is gratefully acknowledged. We also thank J. Stukas for help in obtaining control specimens and R. Williams for reviewing the manuscript. This study was supported in part by the Huntington's Disease Center Without Walls; (PHS grants NS 16367 and MN1NS-3187); the Brain Tissue Resource Center, McLean Hospital; and the Julieanne Dorn Fund. N.W.K. was supported by a fellowship from the Medical Research Council of Canada. M.F.B. is supported by a Young Investigator Award (IR 23ns 19867-1).

20 May 1985; accepted 27 August 1985

The 36-Kilodalton Substrate of pp60$^{v\text{-}src}$ Is Myristylated in a Transformation-Sensitive Manner

Abstract. *A primary intracellular substrate for pp60$^{v\text{-}src}$ kinase in a variety of avian and mammalian cells is a protein of 34 to 39 kilodaltons (kD). After incubation of chicken embryo fibroblasts (CEF) with [^{3}H]myristic acid for 4 hours, the 36-kD protein contained covalently bound myristic acid by several criteria: (i) the radioactively labeled material comigrated with the 36-kD protein on sodium dodecyl sulfate–polyacrylamide gels in one and two dimensions, (ii) the labeled material was insoluble in chloroform-methanol, and (iii) radioactively labeled myristate could be recovered from the purified 36-kD protein. The resistance of the acyl fatty acid moiety to hydrolysis by hydroxylamine suggested that the covalent linkage to the 36-kD protein may be through an amide linkage. The [^{3}H]myristic-acid labeling of the 36-kD protein in Rous sarcoma virus–transformed CEF showed a reduction of up to 45 percent when compared to an identical amount of 36-kD protein derived from normal cells; this reduction was not due to general changes in myristic acid metabolism in transformed cells.*

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Transforming proteins of several oncogenic viruses have tyrosine-specific protein kinase activity (*1*, *2*), and much effort has been expended to identify physiological substrates of this activity (*3*). One putative substrate is a protein of 36 kilodaltons (kD); in cultured cells this protein is phosphorylated on tyrosine in a transformation-dependent manner (*4–6*) and it can be phosphorylated in vitro on the same sites by pp60$^{v\text{-}src}$, the transforming protein of Rous sarcoma virus (RSV) (*5*). Recent reports have suggested that the 36-kD protein may have a structural (*7*, *8*) or signaling function (*9*) in epithelial cells. In eukaryotic cells some membrane-associated proteins, including the transforming protein of RSV (*10–14*), have covalently bound fatty acid (*15–18*).

To determine whether the 36-kD protein contains covalently bound fatty acid, both normal and RSV-transformed chicken embryo fibroblasts (CEF) were labeled in culture with [^{3}H]myristic acid for 4 hours and cellular extracts were immunoprecipitated with antiserum to the 36-kD protein. The specificity of the antiserum (*19*) was confirmed by comparison of the partial proteolytic maps of the immunoprecipitated 36-kD protein with published patterns and by the migration of the immunoprecipitated protein in a two-dimensional gel system (*5*). We found that cells incubated with [^{3}H]myristic acid contained a tritium-labeled 36-kD protein that was specifically immunoprecipitated by the antiserum (Fig. 1A). The [^{3}H]myristate in the 36-kD protein could be detected after an incubation period as short as 1 hour, suggesting that the labeled myristate may have remained intact. This tritium-labeled protein comigrated on two-dimensional polyacrylamide gels predominately with the more basic ($pI \approx 7.9$) and unphosphorylated form of the 36-kD protein in transformed cells (Fig. 1B). In normal cells, the 36-kD protein isolated by immunoprecipitation was not phosphorylated and the [^{3}H]myristate label comigrated with the entire [^{35}S]methio-

nine-labeled protein. The amount of [^{3}H]myristate in the 36-kD protein was always reduced in transformed cells (a reduction of 20 to 45 percent after 4 hours of incubation in a series of experiments) when compared to normal cells (Figs. 1A and 2), whereas the amount of [^{35}S]methionine-labeled 36-kD protein in normal and transformed cells as derived from the immunoprecipitations was identical (±2 percent). Myristic acid, or a derivative, appears to be covalently linked to the 36-kD protein from both normal and wild-type RSV–transformed cells as (i) the immunoprecipitates were extensively washed in the presence of sodium dodecyl sulfate (SDS), deoxycholate, and NP-40; (ii) the radiolabel comigrated with the 36-kD protein after SDS-polyacrylamide gel electrophoresis (SDS-PAGE); and (iii) the radiolabel in the 36-kD protein was resistant to extraction from the gel by lipid solvents such as chloroform-methanol (Figs. 1A and 2).

To ascertain whether the label actually remained in a fatty acid covalently attached to the 36-kD protein, we isolated the ^{3}H-labeled 36-kD protein from an SDS–polyacrylamide gel and subjected it to hydrolysis in methanolic HCl. The hydrolyzate was extracted with petroleum ether and the organic phase was analyzed by thin-layer chromatography; the radiolabel migrated as a single major species with the mobility of myristic acid methyl ester (Fig. 3). These data support the idea that the 36-kD protein in CEF did contain a covalently bound fatty acid. Since the migration of palmitate methyl ester nearly coincides with the myristate methyl ester under the conditions shown (Fig. 3), the two fatty acids were separated by reversed-phase thin-layer chromatography (RP-18F, E. Merck) that was developed with acetic acid : acetonitrile (1:1). The tritium label in trichloroacetic acid (TCA)–precipitated, –washed, and –hydrolyzed protein was recovered as myristic acid and no conversion to palmitic acid was detected. Furthermore, the addition of 5 m*M* sodium pyruvate and nonessential amino acids to the cell culture medium did not alter the total incorporation, pattern of protein labeling, or recovery of [^{3}H]myristate when compared to nonsupplemented complete cell culture medium. Hydroxylamine (*p*H 9.8) treatment hydrolyzes fatty acids that are esterified to proteins through an oxygen linkage (*20*); however, the ^{3}H-labeled 36-kD protein was unaffected by such treatment (Fig. 2). Thus, the fatty acid may be bound to the 36-kD protein through a linkage other than the more common ester bond (*21*). We do not yet have direct evidence for an amide linkage between the fatty acid acyl group and, for example, the α-amino group of NH_2-terminal amino acid as has been reported for pp60$^{v\text{-}src}$ (*10–14*), bovine cyclic AMP–dependent protein kinase (*22*), calcineurin B (*23*), and some membrane-associated proteins encoded by the retrovirus (*24*). The myristate linkage to the 56-kD protein from LSTRA cells is unknown (*25*).

There remained the possibility that the reduction in 36-kD protein myristylation in transformed as compared to normal cells reflected a general alteration in myristate metabolism due to infection of the cells with virus. In order to eliminate this possibility, we analyzed the myristylation of the 36-kD protein in cells infected with a temperature-sensitive (ts) transformation mutant of RSV, tsNY68. Cells infected with this mutant are transformed at the permissive temperature of 36°C, but have a normal morphology at the nonpermissive temperature of 41°C. No consistent differences in [^{3}H]myristate labeling of proteins in whole cell lysates were evident when tsNY68-infected cells were compared at the permissive and nonpermissive temperatures (Fig. 4). In three separate experiments,

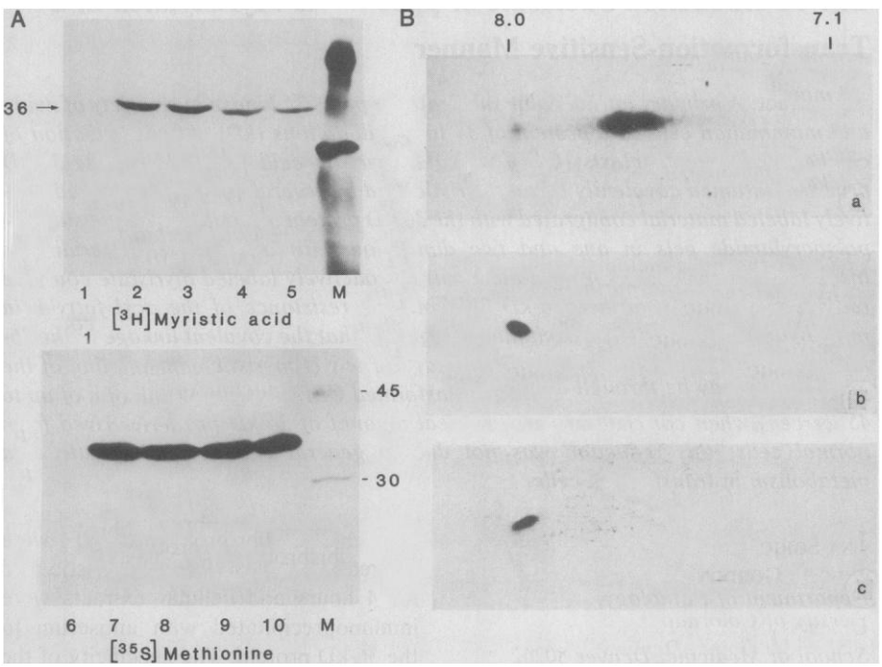

Fig. 1. (A) The 36-kD protein isolated from normal CEF may contain bound fatty acid which is a transformation-dependent phenomenon. Normal or RSV-transformed CEF were labeled with [^{35}S]methionine (100 μCi/ml, New England Nuclear) or 9,10[^{3}H]myristic acid (500 μCi/ml, New England Nuclear) for 4 hours. The [^{3}H]myristic acid (500 μCi) in the ethanol solvent was dried under minimum vacuum and a small volume of Dulbecco's minimum essential medium supplemented with 2 percent dialyzed calf serum was added to resuspend it. The solution was sonicated and was then added to fresh medium containing 2 percent dialyzed calf serum. The addition of [^{35}S]methionine was carried out in a similar fashion, except that the medium was methionine-free. After a 4-hour incubation, the cells, normalized for total lysate protein, were lysed in 0.25 ml of radioimmune precipitation buffer (RIPA) (1 percent NP-40, 1 percent sodium deoxycholate, 0.1 percent SDS, 0.15*M* NaCl, and 0.01*M* sodium phosphate at *p*H 7.2) per 35-mm dish (*32*). Immunoprecipitation with the use of *Staphylococcus aureus* (Cowan strain I) generally utilized 50 μl of cell lysate and 3 μl of antiserum to the 36-kD protein (*19*). The immunoprecipitated proteins were resolved by SDS-PAGE (*33*). After fluorography (*34*), the dried 2,5-diphenyloxazole (PPO)-pretreated gels were exposed to Eastman Kodak X-Omat preflashed film for 24 hours after labeling with [^{35}S]methionine and for 19 days after labeling with [^{3}H]myristic acid. (Lanes 1 to 3 and 7 and 8) Normal cells; (lanes 4 to 6 and 9 and 10) transformed cells; (lanes 1 and 6) nonimmune serum; (lanes 2 to 5 and 7 to 10) antiserum to the 36-kD protein. Molecular sizes are shown in kD. (B) Two-dimensional gel electrophoresis patterns of immunoprecipitated 36-kD protein from transformed CEF. The cells were labeled with [^{35}S]methionine (100 μCi/ml), [^{3}H]myristic acid (500 μCi/ml), and [^{32}P]orthophosphate (500 μCi/ml) for 4 hours as in (A). Washed immunoprecipitates of 36-kD protein were resuspended in 25 μl of two-dimensional solubilizing buffer (*5*) containing 2 percent Ampholiones (*p*H 5 to 8) and then diluted 1:2 with sample dilution buffer. Isoelectrophoresis was carried out with a *p*H gradient of 5 to 8 at 400 V for 16 hours. Gels were equilibrated with the second-dimension buffer (*5*) and placed on a 10 percent polyacrylamide SDS-containing gel. The 36-kD regions of the gels are shown. (a) [^{32}P]Orthophosphate-labeled 36-kD protein; exposure time, 2 days; (b) [^{35}S]methionine-labeled 36-kD protein; exposure time, 3 days; (c) [^{3}H]myristic acid–labeled 36-kD protein; exposure time, 21 days.

the incorporation of [^{3}H]myristate or [^{35}S]methionine into whole cell lysates (intracellular-free plus protein-bound label) or TCA precipitates (protein-bound label) was nearly identical at both temperatures. These data suggest that general metabolic incorporation and transport of myristate do not differ at 36° and 41°C. However, incorporation of [^{3}H]myristate into the isolated 36-kD protein was always reduced at 36°C (to 63 percent) compared to 41°C while incorporation of [^{35}S]methionine was somewhat higher at 41°C (109 percent) as determined by liquid scintillation counting of bands that had been immunoprecipitated and isolated by one-dimensional SDS-PAGE. Our finding of no general difference in protein myristylation plus the recent observation that the myristylation of pp60$^{v\text{-}src}$ is unaffected by a temperature shift in ts-mutant–infected cells (*13*) supports the conclusion that transformation of CEF by RSV leads to a relatively specific reduction in myristylation of the 36-kD protein (*26*).

Evidence suggests that the 36-kD protein is associated with the plasma membrane (*27, 28*), probably on the cytoplasmic surface, and that the extent of the association is not significantly altered by transformation of cells or even by phosphorylation of the 36-kD protein after transformation (*3*). Approximately half of the 36-kD protein recoverable in lysates behaves like a peripheral membrane protein, while the remainder behaves as if it may be integral to the membrane (*29*). The protein forms dimers (*5, 7, 30*), and the suggestion has been made that one-half of the dimer may be more tightly bound to the membrane (*29*). The function of the 36-kD protein is presently unknown although a structural role is possible in epithelial cells (*7, 8*). The significance of our finding that a fatty acid is covalently linked to the 36-kD protein is not yet clear, nor is the significance or function of fatty acid acylation of other proteins (*21*). It has been suggested that the covalently bound fatty acid links the protein to the plasma membrane or signals the transport of the protein to the membrane (*21*). But, if the addition of fatty acid to the 36-kD protein acts as a signal for membrane insertion, and previous studies have indicated no difference in cellular distribution of this protein between normal and transformed cells (*27–29*), we might have expected the 36-kD protein isolated from transformed cells to have the same fatty acid content as normal cells. This relationship is further obscured by the present belief that phosphorylation of the

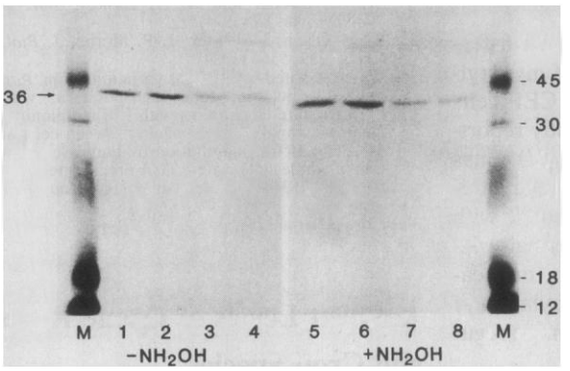

Fig. 2. The [^{3}H]myristic acid may be covalently linked to the 36-kD protein by a non-oxygen ester-type bond, as demonstrated by its resistance to hydroxylamine. After SDS-PAGE of the 36-kD protein isolated by immunoprecipitation as described in Fig. 1A, half of the gel was directly processed for fluorography ($-NH_2OH$). The other half of the gel was immersed for 4 hours at 23°C in 250 ml of a 1*M* solution of hydroxylamine titrated to *p*H 9.8 with sodium hydroxide ($+NH_2OH$) (*35*). Both gels then were directly impregnated with PPO and subjected to autoradiography. Molecular sizes are shown in kilodaltons. Hydroxylamine treatment did not change the intensity of the 36-kD protein isolated from either normal (lanes 1, 2, 5, and 6) or transformed (lanes 3, 4, 7, and 8) cells when compared to the untreated gel.

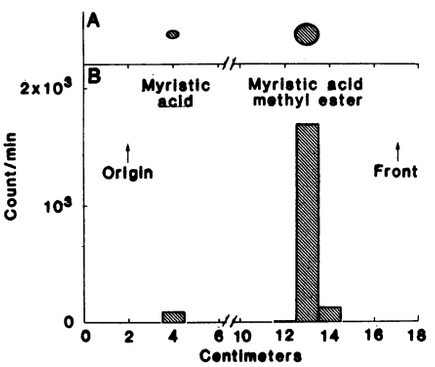

Fig. 3. Hydrolysis of ^{3}H-labeled 36-kD protein in methanolic HCl leads to the recovery of the radiolabel in a fatty acid compatible with myristic acid. The 36-kD protein was isolated from cells radiolabeled with [^{3}H]myristic acid for 2 hours as described in Figs. 1A and 2, and hydrolyzed in methanolic HCl for 16 hours at 90°C (*35*). The released material was analyzed by thin-layer chromatography on silica gel G in parallel with authentic myristic acid and methyl myristate. The scales of the two panels are identical. (A) A tracing of the myristic acid and the methyl myristate markers; (B) radiolabel released from the 36-kD protein. Background (60 count/min) has been subtracted from all values.

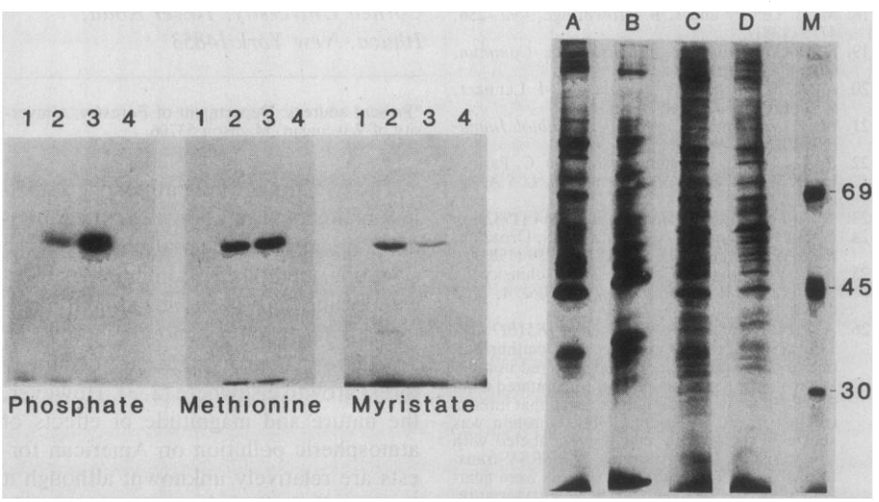

Fig. 4. A relative specific reduction in myristylation of the 36-kD protein isolated from CEF infected with the tsNY68 mutant of RSV. CEF were infected with tsNY68 and maintained at the nonpermissive (41°C) or permissive (36°C) temperature. Duplicate plates (60 mm) were labeled with [^{32}P]orthophosphate (0.5 mCi/ml), [^{35}S]methionine (100 μCi/ml), or [^{3}H]myristic acid (0.5 mCi/ml). After 4 hours labeling the cells were harvested into RIPA buffer and immunoprecipitated with antiserum to the 36-kD protein as described in Fig. 1A. The proteins were resolved on a 10 percent polyacrylamide gel and exposed for fluorography. Lanes 1 and 2 were from lysates of cells maintained at the nonpermissive temperature, whereas lanes 3 and 4 were from lysates of duplicate plates maintained at the permissive temperature. Lanes 1 and 4 display immunoprecipitates of these lysates with nonimmune rabbit serum and lanes 2 and 3 with antiserum to the 36-kD protein. The reduction of phosphorylation at 41°C (lane 2) is expected. Cells labeled with [^{3}H]myristate for 4 hours and maintained at the nonpermissive temperature (lane C) can be compared to cells at the permissive (lane D) temperature (5 μl of lysate). Cells labeled with [^{35}S]methionine are shown in lane A (41°C) and lane B (36°C) (*36*).

36-kD protein by pp60$^{v\text{-}src}$ occurs exclusively in the membrane (*29*).

Finally, in addition to being transformation-sensitive, the extent of myristylation of the 36-kD protein in CEF cells may be far less than that recently reported for pp60$^{v\text{-}src}$ protein kinase (*31*). Buss and Sefton (*31*) suggested a near stoichiometric modification of pp60$^{v\text{-}src}$ with myristate that may not differentially influence the structure, function, or location of pp60$^{v\text{-}src}$. The partial acylation of the 36-kD protein may have a more regulatory role in this regard.

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37. Supported by grant CA-35378 (J.A.G.) and indirectly by grant CA-15823 from the National Institutes of Health. We thank S. Decker (Rockefeller University) for a generous supply of antiserum to the 36-kD protein and E. Erikson for help and encouragement.

7 June 1985; accepted 10 July 1985

Ambient Levels of Ozone Reduce Net Photosynthesis in Tree and Crop Species

Abstract. *Experiments were conducted to measure the photosynthetic response of three crop and four tree species to realistic concentrations of ozone and (for tree species only) simulated acidic rain. The ozone concentrations were representative of those found in clean ambient air, in mildly to moderately polluted air such as occurs in much of the United States during the summer, and in more heavily polluted air. However, the highest concentrations of ozone used were lower than those found regularly in the Los Angeles area. The mean pH of the simulated acid rain treatments ranged from more alkaline to much more acidic than the mean pH of precipitation in the United States. Exposure to any increase in ozone reduced net photosynthesis in all species tested. In contrast, acidic rain had no negative effect on photosynthesis in tree species, and no interaction between ozone and acidic rain was observed. Ozone-induced reductions in photosynthesis were related to declines in growth or yield. Species with higher stomatal conductances and thus higher potential for pollutant uptake exhibited greater negative responses to similar ozone treatments. Since exposure to ozone concentrations typical of levels of the pollutant observed in the eastern half of the United States reduced the rates of net photosynthesis of all species tested, reductions in net photosynthesis may be occurring over much of the eastern United States.*

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The question of how atmospheric pollution affects vegetation in North America is receiving much attention in the scientific and political communities. Forest decline has been observed on extreme sites such as mountaintops (*1*), and there is some evidence for decline in forest growth elsewhere (*2, 3*). However, the nature and magnitude of effects of atmospheric pollution on American forests are relatively unknown, although it is generally believed that the situation in the United States is not yet as dire as that in Central Europe (*4, 5*).

The agents responsible for the observed dieback of forest trees in the United States or for potential decline in forest growth have not been identified, but many candidates have been proposed. In addition to natural agents such as insects, drought, and disease, atmospheric pollution has been considered a prime factor (*4, 5*). Acidic rain is the form of pollution currently receiving the most attention. Rainfall that is considered unnaturally acidic has been monitored across much of the United States, and its widespread occurrence has led to the suggestion of potentially large-scale impacts. However, in spite of considerable data indicating that acidic rain might be responsible for damage to lakes and aquatic systems (*6*), similar effects on terrestrial systems have not yet been documented.

In contrast, ozone (O_3) is considered to cause the greatest amount of damage to vegetation of any gaseous pollutant (*7*). In much of North America during the summer, vegetation is exposed frequently, if not daily, to low or moderate concentrations of O_3 of anthropogenic origin. For instance, mean daily 7-hour (1000 to 1700 hours) concentrations of O_3 in major agricultural areas of the United States are estimated to be between 0.04 and 0.07 ppm during the growing season (*8*). Such concentrations have reduced yields of crops and growth of tree species without visibly injuring foliage (*8–10*). It is still not clear how O_3 causes such effects or why certain species or cultivars are more sensitive than others to this pollutant.

The net photosynthetic assimilation of