combination with the increase in drainage water, led to net losses 9, 8, 3, and 20 times greater, respectively, than similar losses from five undisturbed ecosystems between June 1966 and June 1967. Concentrations of Al^{+++} rose about 1 month later than the initial rise in nitrate, while sulfate showed a sharp drop in concentration, coincident with the rise in nitrate (Fig. 1).

These results indicate that this ecosystem has limited capacity to retain nutrients when the bulk of the vegetation is removed. The accelerated rate of loss of nutrients is related to the cessation of uptake of nutrients by plants and to the larger quantities of drainage water passing through the system. Accelerated losses may also relate to increased mineralization resulting from changes in the physical environment, such as change in temperature or increase in available substrate.

However, the effect of the vegetation on the process of nitrification cannot be overlooked. In the cutover ecosystem the increased loss of cations correlates with the increased loss of nitrate; consequently, if the intact vegetation inhibits the process of nitrification (*13*) and if removal of the vegetation promotes nitrification, release from inhibition may account for major losses of nutrients from the cutover ecosystem.

These results suggest several conclusions important for environmental management:

1) Clear-cutting tends to deplete the nutrients of a forest ecosystem by (i) reducing transpiration and so increasing the amount of water passing through the system; (ii) simultaneously reducing root surfaces able to remove nutrients from the leaching waters; (iii) removal of nutrients in forest products; (iv) adding to the organic substrate available for immediate mineralization; and (v), in some instances, producing a microclimate more favorable to rapid mineralization. These effects may be important to other types of forest harvesting, depending on the proportion of the forest cut and removed. Loss of nutrients may be greatly accelerated in cutover forests where the soil microbiology leads to an increase of dissolved nitrate in leaching waters (*10*).

2) Management of forest ecosystems can significantly contribute to eutrophication of stream water. Nitrate concentrations in the small stream from the cutover ecosystem have exceeded established pollution levels (10 parts per million) (*14*) for more than 1 year, and algal blooms have appeared during the summer.

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References and Notes

1. W. H. Carmean, *Science* **153**, 695 (1966); E. S. Deevey, *ibid.* **154**, 68 (1966).
2. F. H. Bormann and G. E. Likens, *ibid.* **155**, 424 (1967).
3. Hubbard Brook Experimental Forest is part of the Northeastern Forest Experiment Station.
4. G. E. Likens, F. H. Bormann, N. M. Johnson, R. S. Pierce, *Ecology* **48**, 772 (1967).
5. W. E. Sopper and H. W. Lull, *Water Resources Res.* **1**, 115 (1965).
6. F. H. T. Juang and N. M. Johnson, *J. Geophys. Res.* **72**, 5641 (1967).
7. D. Fisher, A. Gambell, G. E. Likens, F. H. Bormann, in preparation.
8. H. J. Thome, personal communication, 1967.
9. F. E. Allison, *Advan. Agron.* **3**, 213 (1955).
10. R. H. Nye and D. J. Greenland, *Tech. Commun. 51* (Commonwealth Bureau of Soils, Harpenden, England, 1960).
11. G. Hart, R. E. Leonard, R. S. Pierce, *Forest Res. Note 131* (Northeast. Forest Exp. Sta., Upper Darby, Pa., 1962).
12. S. L. Jansson, *Kgl. Lantbrukshogskol. Ann.* **24**, 101 (1958).
13. E. L. Rice, *Ecology* **45**, 824 (1964).
14. F. H. Rainwater and L. L. Thatcher, *U.S. Geol. Surv. Water-Supply Paper 1454* (U.S. Government Printing Office, Washington, D.C., 1960).
15. Supported by NSF grants GB 1144, GB 4169, GB 6757, and GB 6742. We thank Noye Johnson, David Pramer, William Smith, and Garth Voigt for suggestions and for reviewing the manuscript. Contribution 4 of the Hubbard Brook Ecosystem Study; a contribution to the U.S. program for the International Hydrological Decade, and to the International Biological Program.

28 December 1967

Antigen Binding to Cells: Determination by Enzymic Fluorogenic Group Hydrolysis

Abstract. *A sensitive method for detecting cells containing antibody to β-galactosidase has been devised. The enzyme attached to the cells containing antibody can hydrolyze a fluorogenic substrate and yield fluorescent products which are measured microphotofluorometrically. This method of detecting a few molecules of antibody is applicable to other enzyme antigen systems.*

A method for measuring cellular antibody in very small amounts is necessary when only a few molecules of antibody may be present in a cell, such as very early in the antibody response, during states of immunologic unresponsiveness, or possibly at receptor sites on the membranes of cells bearing immunologic memory (*1*). Our method was designed to detect only a few molecules of cellular antibody.

Using the substrate fluorescein-di-β-galactopyranoside (FDβG), Rotman *et al.* measured the β-galactopyranoside activity of single molecules (*2*), individual bacterial cells (*3*), and ribosomes (*4*). This substrate is nonfluorescent and upon hydrolysis of its glycosidic bonds yields fluorescent products (fluorescein-mono-β-galactoside or fluorescein, depending on whether one or both glycosidic bonds are cleaved, respectively). Antibody to β-galactosidase does not impair the activity of the enzyme (*5*), and so the enzyme-antienzyme complex can hydrolyze the substrate also.

For the assay, the spleens of immunized or normal A/Jax mice were removed and macerated; the fragments were screened through a 60-gauge and then a 250-gauge stainless steel gauze in Hanks's balanced salt solution, with 50 μg of streptomycin, 60 units of penicillin, and 20 units of heparin per milliliter. The resultant single-cell suspension was washed once in the same solution and centrifuged. The cell pellet was treated with 95 percent ethanol for 10 minutes at room temperature, washed again, and suspended in medium to yield a 20-percent suspension of cells.

A portion of the suspension was removed for measurement of "intrinsic" β-galactosidase, including the activity of endogenous mouse-spleen β-galactosidase and activity of any possible residual antigen from the immunization. The rest of the cells were incubated with 15 μg of β-galactosidase per milliliter of cell suspension for 1 hour at 37°C and then overnight at 4°C (*6*). The cells were then washed four times with 40 ml of medium to remove the free enzyme, and suspend-

ed in assay buffer consisting of 0.1M sodium phosphate, pH 7.3, containing 0.1M 2-mercaptoethanol, 0.001M $MgSO_4$, and 0.002M $MnSO_4$. Cell dilutions were made in the assay buffer, and FDβG was added so that an appropriate number of cells were present in droplets of 0.2 μl containing 2.4 $\times$ $10^{-5}M$ FDβG.

With hyperimmunized mice, 100 cells per droplet gave sufficient activity in most droplets after several hours. For detection of early primary responses, droplets should contain many more than 100 cells to ensure activity after 24-hour incubation with substrate.

The droplets were placed in a silicone oil chamber, 3 mm deep, made from a glass slide (7.5 by 5 cm), similar to one reported (*2*). The slides were incubated at room temperature (22° to 24°C) for 20 hours. The amount of fluorescence was determined with a Zeiss fluorescence microscope equipped with a phase-contrast condenser attached to an Aminco photomultiplier microphotometer (model 10-213). A 12-volt tungsten lamp, with BG-12 filter, was used for excitation; the Zeiss 500-mμ barrier filter was the secondary filter. The droplets were viewed at $\times$ 125 magnification and measured at various intervals. Only intervals showing a linear increase in the appearance of product were used in the calculations. The activity in the droplets is the result of hydrolysis of the substrate by the enzyme bound to the cells. For convenience, the method of assay may be termed A-H, an acronym for *a*ntigen *b*inding to *c*ells *d*etermined by *e*nzymatic *f*luorogenic *g*roup *h*ydrolysis.

Table 1 shows two evident results:

1) Antigen-binding activity of the spleen cells was greater than that normally present as early as 12 hours after intraperitoneal injection of 100 μg of β-galactosidase. This result, confirmed in four experiments, contrasts with results by conventional techniques in which detectable antibody to protein antigens does not appear for at least several days (*7*); the peak of the response appears about the 6th day (*8*).

2) Mice injected with β-galactosidase and tested 6 months later have neither serum antibody nor cells containing antibody as measured by fluorescent antibody staining. By the A-H method, however, appreciable binding activity can be detected, perhaps on the part of the memory cells.

In theory, even one molecule of bound antibody should be detectable (*2*), but under current conditions our limit of detection is approximately 100 to 200 molecules. The unit of antibody is defined as the amount of antibody that will bind sufficient enzyme molecules to produce in 1 hour at room temperature an intensity of 1 $\times$ 10^{-8} ampere on the microphotometer, this being the smallest unit measurable by the instrument. Each unit corresponds to the activity of 225 molecules of enzyme under these conditions if one assumes a molecular weight of 540,000 for β-galactosidase (*9*).

Table 1. Values by A-H assay under various conditions. Values represent typical pools, each of three spleens from A/Jax mice immunized intraperitoneally once or more often with 100 μg of β-galactosidase.

Type of cells	Condition	Antibody units per cell
	Unimmunized	
Intrinsic	No enzyme added	140
Normal Cytophilic*	Mice not immunized	700
	Hyperimmune serum added to normal cells before assay	4,200
	Immunized	
Intrinsic	12 hours after immunization	410
Primary	12 hours after immunization	2,800
Primary	6 days after immunization	71,000
Memory	6 months after first injection	9,100

* A 20-percent cell suspension (0.25 ml) was incubated with 0.25 ml of undiluted pooled hyperimmune serum for 1 hour at 0°C, washed three times in 1 ml of Hanks's medium, and processed according to our standard procedure.

The number of enzyme molecules fixed to spleen cells of normal mice is greater than desired for detection of only a few molecules. The sensitivity can be increased at least two orders of magnitude by a combination of increased optical efficiency, reduction in the level of enzyme applied to the cell suspension, and smaller droplets. Studies with single cells (*10*) show that the number of antibody units equivalent to one positive cell are much greater than the average value per cell; thus it is probable that one or at most very few cells are active in our droplets, which contain several hundred cells.

By conventional methods, such as histochemistry with fluorescent antibody (*11*) or radioautographic staining with radioactive antigen (*12*), detection depends on ability to visualize a certain number of fixed, labeled molecules. Likewise, the formation of a hemolytic plaque in gels (*13*) requires the activity of many antibody molecules, as do antibody assays performed in microdroplets, which measure the output of single antibody-producing cells (*14*). Our method involves a time-amplification factor, since we exploit the enzymatic activity of a few molecules of antibody-bound β-galactosidase, which can continue to hydrolyze substrate until enough fluorescent molecules are produced for their detection. The A-H assay is specific; it does not detect antibody-containing cells directed against contaminating, non-cross-reacting materials in the immunogen.

Fluorogenic substrates for many enzymes have been synthesized (*15*) and are commercially available for β-galactosidase and phosphatases (*16*). Thus one may apply this type of assay to other enzyme-antigen systems in which the antibody does not inactivate the enzyme. If the antibody inactivates the enzyme, the A-H assay can be modified to detect residual enzymatic activity after the addition of a known amount of enzyme to the cell population.

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References and Notes

1. S. Sell and P. G. H. Gell, *J. Exp. Med.* **122**, 423 (1965).
2. B. Rotman, *Proc. Nat. Acad. Sci. U.S.* **47**, 1981 (1961).
3. H. R. Revel, S. E. Luria, B. Rotman, *ibid.*, p. 1956.
4. S. Lederberg, B. Rotman, V. Lederberg, *J. Biol. Chem.* **239**, 54 (1964).
5. M. Cohn and A. M. Torriani, *J. Immunol.* **69**, 471 (1952).
6. We thank I. Zabin for the β-galactosidase which was extracted from *Escherichia coli* ML 308 and purified by the method of U. Karlsson, S. Koorajian, I. Zabin, F. S. Sjostrand, A. Miller [*J. Ultrastruct. Res.* **10**, 457 (1964)].
7. W. O. Weigle, *Proc. Soc. Exp. Biol.* **94**, 306 (1957); T. L. Vischer and P. Stastny, *Immunology* **12**, 675 (1967).
8. F. Modabber and E. Sercarz, *Bacteriol. Proc.* **67**, 90 (1967).
9. G. Craven, E. Steers, Jr., C. B. Anfinsen, *J. Biol. Chem.* **240**, 684 (1965).
10. F. Modabber, T. McDowell, B. Kauffman, E. E. Sercarz, in preparation.
11. A. H. Coons, E. H. Leduc, J. M. Connolly, *J. Exp. Med.* **102**, 49 (1955).
12. M. C. Berenbaum, *J. Clin. Pathol.* **11**, 543 (1958).
13. N. K. Jerne and A. A. Nordin, *Science* **140**, 405 (1963); J. Ingraham, and A. Bussard, *J. Exp. Med.* **119**, 667 (1964).
14. O. Makela and G. J. V. Nossal, *J. Immunol.* **87**, 447 (1961); G. Attardi, M. Cohn, K. Horibata, E. S. Lennox, *ibid.* **92**, 335 (1964).
15. B. Rotman, J. Zderic, M. Edelstein, *Proc. Nat. Acad. Sci. U.S.* **50**, 1 (1963).
16. Mann Research Laboratories, Inc., New York.
17. Aided by grants from NSF (GB-1619) and the Academic Senate of the University of California, Los Angeles (2044). We thank Boris Rotman for a gift of substrate and for his helpful advice during the early phases.

5 January 1968