(wet). About 10 percent of the alkaline earths inside the leaves were leached out by 10 percent HCl (1 minute), mainly from the cut ends. The calcium/strontium and calcium/barium ratios in the washings were the same as those found inside the leaves but different from those in soil or in dust collected on plastic, an indication that these metals came from inside, so they were added by calculation to those found on the inside. The situation was different for lead; 83 percent of that metal was found on leaf surfaces and was collected by aerosol deposition as shown above by comparison with instrument collection data. The average concentration of lead in acid-washed sedge leaves was 0.06 ppm (wet).

Vole (*Microtus montanus*) muscle and bone from a single 30-g adult were analyzed, and metal values in these tissues were used to estimate total body burdens according to known distributions of lead and alkaline earths in mammals (*8*); the proportions of these tissues in rats (*9*) were scaled according to the body measurements of our vole specimen. The body burdens were as follows: calcium, 7800 ppm; strontium, 34 ppm; barium, 9 ppm; and lead, 0.06 ppm. The concentration of lead in vole muscle (biceps femoris) was found to be 0.0015 ppm (wet), whereas that in bone (femur) was 1.1 ppm (wet). These values were determined on an animal that had been dissected and chemically processed under strict clean-laboratory conditions which provided very low contamination levels, and whose parts had been analyzed by stable isotope dilution in a high-resolution thermal emission mass spectrometer which provided uniquely accurate concentration data. These factors, together with the nature of the collecting site, account for the difference between the above data and much higher lead concentration data reported by others in similar animals.

Of all grossly abundant nutritive metals, calcium is most useful to compare with nonnutritive lead in various components of a food chain because, like lead, the bulk of it is contained not in cell fluids in plants and animals but in and near membranes where it is relatively immobile. Calcium and lead have similar migration tendencies during senescence in plants; they are absorbed through the mucosal membrane of the gut and are excreted by the kidney in similar fashion in animals. Calcium might be expected to become purified of lead in nutritive steps involving active transport across membranes, because such transport mechanisms work specifically for certain nutritive metals but only inadvertently and less efficiently for other metals.

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References and Notes

1. J. J. Huntzicker and S. K. Friedlander, in *Background Papers for a Research Conference on California Ocean Pollution*, N. Brooks and E. Goldberg, Eds. (California Institute of Technology, Pasadena, 1973), p. 43.
2. T. Hinkley, *Geol. Soc. Am. Bull.*, in press.
3. M. Murozumi, T. J. Chow, C. Patterson, *Geochim. Cosmochim. Acta* **33**, 1247 (1969).
4. G. R. Bradford, F. L. Bair, V. Hunsaker, *Limnol. Oceanogr.* **13**, 526 (1968).
5. T. J. Chow and M. S. Johnstone, *Science* **147**, 502 (1965).
6. T. Hinkley and C. Patterson, *Nat. Phys. Sci.* **246**, 123 (1973).
7. *Soil Taxonomy of the National Cooperative Soil Survey* (Soil Conservation Service, U.S. Department of Agriculture, Washington, D.C., in press).
8. *Health Phys.* **3**, 1 (1960); H. P. Schroeder and I. W. Tipton, *Arch. Environ. Health* **17**, 965 (1968).
9. N. H. Donald, *The Rat* (No. 6, Memoirs of the Wistar Institute of Anatomy and Biology, Philadelphia, 1924).
10. We thank T. Hinkley and D. Settle (California Institute of Technology) for field and laboratory assistance; S. Friedlander and J. Huntziker (Caltech) and T. J. Chow (University of California, San Diego) for help on aerosol deposition studies; B. Brattstrom and J. D. Smith (California State University, Fullerton), C. Sharsmith (California State University, San Jose), and J. L. Patton (University of California, Berkeley) for assistance in animal and plant collections and identifications; A. J. Brown (California Department of Water Resources for use of snow survey samplers; and S. Wood (Caltech) and W. D. Nettleton (University of California, Riverside) for assistance and advice in soil analysis. Details of analytical procedures and clean-room techniques can be obtained from C.C.P. This work was supported by National Science Foundation grant GB-31038 (Division of Biology and Medicine, General Ecology) and by Rockefeller Foundation grant RF 72049.

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Enzyme Measurements on Single Cells

Abstract. *A simple and sensitive procedure has been developed for enzymatic assays in single cells and applied to the measurement of β-glucuronidase in single parenchymal cells of liver. Cells deposited in microdroplets under oil were ruptured by freezing and thawing and allowed to react with a fluorogenic substrate. The fluorescence is developed by diffusing an organic base into the droplets and measured in a fluorescence microscope equipped with a photomultiplier.*

We have developed a micro fluorimetric assay technique for measuring β-glucuronidase in single mammalian cells. The amount of fluorescent product formed is measured by means of a fluorescence microscope equipped with a photomultiplier microphotometer. The technique is derived from the method originally introduced by Rotman for the study of single molecules of β-galactosidase in *Escherichia coli* (*1*).

Single cell measurements of enzymes may prove useful in prenatal diagnosis after amniocentesis, and they are well suited to fundamental studies in cell biology.

Rotman's conception of using micro droplets to minimize background fluorescence and thus increase the signal-to-noise ratio embodies an important principle in developing a sensitive microassay for a fluorescent product. We found optimal those droplets whose volumes were 1 to 10 nl. Smaller droplets do not have an appreciably lower background in our system and are much harder to work with; larger droplets do have a higher background and present no compensating advantages. The micro droplets were deposited under oil on a microscope slide (25 by 75 mm). The slide was 1 mm (uniform) in thickness and was modified to contain oil by glueing glass rods 2 mm in diameter to the edges with silicone rubber. After trials with other oils, we chose Amoco white oil 35 (American Oil Company). White oil has a higher viscosity than commercial mineral oils and a lower water solubility than silicone oil. The oil (1 ml) was spread evenly over the slide just before the droplets were made.

The droplet "dispenser" was a piece of polyethylene tubing (0.3 cm, inside diameter, with a 0.15-cm wall) that was heated slowly over a small flame until transparent and then drawn to an outside diameter of 0.1 to 0.2 mm. The tubing was allowed to cool and then cut so that one end was at least 0.1 mm in diameter and the other end wide enough for attachment to a pump or aspirator. Uniformly spaced and sized droplets were deposited in rows under oil on the microscope slide by drawing

the assay mixture into the tubing by aspiration; then, while applying a constant, light, positive pressure, the tubing was moved slowly across the surface of the glass slide. Since the oil and polyethylene are both hydrophobic, the aqueous solution did not build up on the end of the tubing, but instead adhered to the glass in the form of micro droplets. The size of the droplets depended to some extent on the size of the tubing. Droplets of less than 1 nl can be made with minor alterations in the above method.

The assay mixture can be varied according to the enzyme being assayed and its source. However, since many organic compounds fluoresce, additives, such as solubilizing agents, should be tested before being used in the assay.

We have assayed β-glucuronidase in single liver cells. Suspensions of separated mouse parenchymal cells were obtained by the method of Howard *et al.* (*2*). A few drops of the suspension were placed in a shallow dish and, while being observed through a dissection microscope, individual cells were picked up with a micropipette and transferred to a second dish containing assay buffer and substrate. The buffer consisted of 0.1*M* acetate, 0.15*M* NaCl, and 0.1 percent bovine serum albumin, *p*H 6.0 (*3*). The substrate was 4-methylumbelliferyl-β-D-glucuronic acid (Sigma) diluted to $3 \times 10^{-4}M$ in assay buffer. The cells were transferred a second time and then taken up in the polyethylene tubing. If many cells were to be assayed at one time, they could be taken up in the same tubing with buffer interspaced between them. Cells and buffer were then dispensed in micro droplets on the microscope slides as described above. Droplets on the slide not containing cells served as controls.

In order to release enzyme activity and make the cells permeable to substrate the cells were frozen and thawed by placing the slides under an atmosphere of N_2 in an airtight container whose bottom was an aluminum plate. The container was placed directly onto a block of Dry Ice until all the droplets were frozen, then placed on a warming plate until the droplets thawed. This procedure was repeated five times. The dry atmosphere prevented water condensation on the surface of the oil.

After the freezing and thawing, the slides were placed in a humidor (a desiccator with a reservoir of buffer)

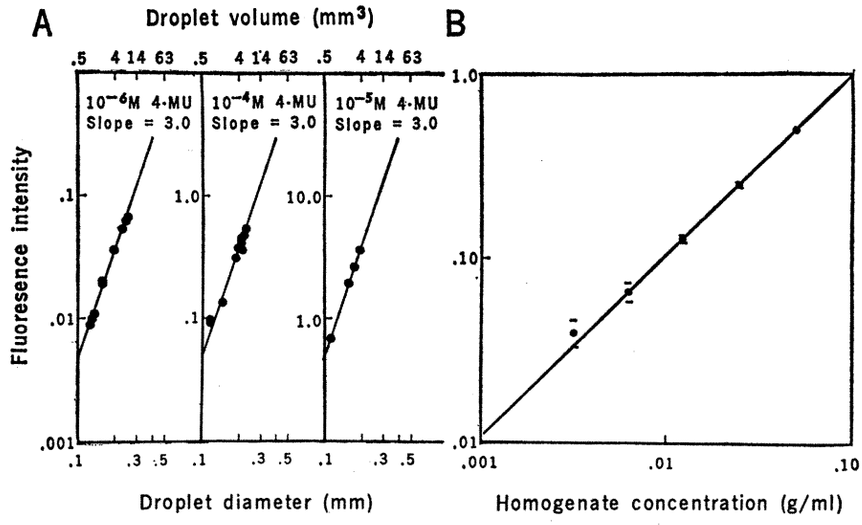

Fig. 1. (A) Fluorescence intensity as a function of droplet diameter at three concentrations of 4-methylumbelliferone (*4-MU*) plotted on a log-log scale. The slope for each of the curves is 3.0. (B) Fluorescence intensity as a function of enzyme concentration with liver homogenate. The reaction time was 1 hour. At each concentration, a standard curve similar to those in (A) was constructed. The fluorescence per unit volume of droplet was plotted against the concentration (grams of liver per milliliter) of C57BL/6J mouse liver homogenate. The data are plotted on a log-log scale.

and incubated for 1 to 2 hours at 37°C. To stop the enzyme reaction and develop the fluorescence, the slides were placed in a second sealed chamber with a reservoir of triethylamine for 1 hour. During this time the organic base diffused through the oil and dissolved in the aqueous droplets; the *p*H of the droplets was then approximately 10.

The apparatus for measuring fluorescence was constructed from a Nikon model S-U microscope with a type FL fluorescence microscope attachment equipped with an ultra dark-field condenser and a × 20 objective with a long working distance. The photomultiplier tube housing of an Aminco photomultiplier microphotometer was connected to the monocular sleeve of the trinocular eyepiece head. An Osram HBO 200-watt mercury arc lamp was used for illumination of the droplets. The photomultiplier tube was an RCA 931V-A. Interference filters (Baird Atomic) were used to obtain monochromatic light at the optimum wavelengths for excitation (365 nm) and emission (455 nm) of 4-methylumbelliferone (*4*). After the microscope was focused on a single droplet and the diameter was measured with an ocular micrometer under white light, the interference filters were positioned and the light path was directed to the photomultiplier tube for a fluorescence measurement.

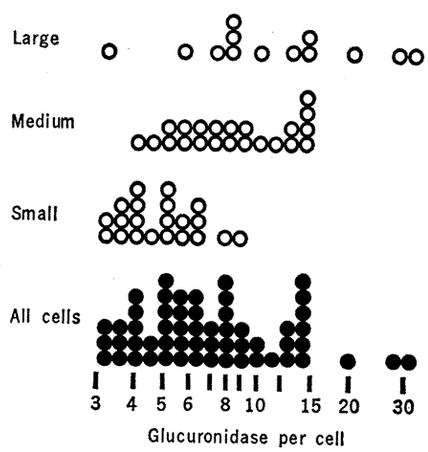

Fig. 2. Relative enzyme activities of β-glucuronidase in individual cells obtained from the liver of a C57BL/6J mouse. The cells were sorted into small, medium, and large categories. The lower distribution is a composite of all cells. Each symbol represents one cell. Activity is expressed in units of 10^{-14} mole of product per hour.

To validate the assay, we performed control experiments (Fig. 1). First, if a given concentration of 4-methylumbelliferone is deposited in droplets of varying sizes, the total fluorescence measured is a function of the third power of droplet diameter (Fig. 1A). Second, the amount of fluorescence at any given volume is directly proportional to the concentration of 4-methylumbelliferone present (Fig. 1A) over the range of approximately 10^{-4} to $10^{-6}M$. Thus, we can say that fluorescence is directly proportional to the total number of product molecules in the droplet over the range

of approximately 5×10^{-12} to 5×10^{-16} mole. Therefore, droplet diameter need not be considered in calculating the specific activity of β-glucuronidase in a single cell since the number of product molecules formed depends only on the amount of enzyme in the cell. However, if impurities are present in the assay mixture and contribute to background fluorescence the correction for this contribution does depend on droplet size since the amount of background fluorescence is proportional to droplet volume.

In the micro droplet assay the amount of product formed is directly proportional to the amount of enzyme present in the droplet (Fig. 1B). If the enzyme is first assayed by conventional means and then distributed in micro droplets, the same specific activity is obtained in both assays. This being so, we have calculated the sensitivity of our instrumentation. The minimum detectable amount of reaction product is 5×10^{-16} mole. The turnover number (that is, the number of product molecules formed per enzyme molecule per minute) for purified murine β-glucuronidase is approximately 6×10^3 at 37°C with the nitrophenyl substrate (*5*). We found the 4-methylumbelliferone substrate approximately as active. From these data we calculate that approximately 800 enzyme molecules are detected in a 1-hour incubation.

The range and distribution of β-glucuronidase activity in single liver cells is shown in Fig. 2. If the cells are separated into categories of small, medium, and large cells, we find that enzyme activity generally increased with cell size. This observation and the fact that cell size increases with the ploidy of the cell in liver (*6*) suggests that β-glucuronidase activity is a function of the number of gene copies for that enzyme.

The micro droplet assay is versatile. Almost any enzyme that can form a fluorescent product directly or through a coupled reaction can be assayed by this procedure. The sensitivity depends largely on the instrumentation used to measure fluorescence. Our method combines sensitivity with simplicity, making it possible to assay β-glucuronidase in as many as 100 individual cells in a few hours easily and accurately.

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References and Notes

1. P. C. Maloney and B. Rotman, *J. Mol. Biol.* **73**, 77 (1973); B. Rotman, *Proc. Natl. Acad. Sci. U.S.A.* **41**, 1981 (1961).
2. We thank Janice Peterson for her assistance in preparing the cells according to the method of R. B. Howard, J. C. Lee, and L. A. Pesch [*J. Cell Biol.* **57**, 642 (1973)].
3. If bovine serum albumin is used it should be exhaustively dialyzed to remove fluorescent impurities.
4. It is important that the primary filter does not fluoresce itself; otherwise the measured background will be too high.
5. S. Tomino, personal communication.
6. C. J. Epstein, *Proc. Natl. Acad. Sci. U.S.A.* **57**, 327 (1967)
7. Supported in part by NSF research grant GB 3049 and PHS grant GM 19521.
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Angiotensin II– and Angiotensin III–Induced Aldosterone Release in vivo in the Rat

Abstract. *The potential role of angiotensin II and its heptapeptide metabolite, des-aspartyl-angiotensin II, was studied in the conscious unanesthetized rat. Aldosterone release was induced by both peptides at physiologic doses (0.72 nanogram per minute). [1-Sarcosyl-8-alanyl]angiotensin II (P-113 inhibited angiotensin II more effectively than des-aspartyl-angiotensin II (101 percent as compared to 82 percent). These results indicate that angiotensin controls aldosterone release in the rat and that des-aspartyl-angiotensin II (that is, angiotensin III) may be important in this sequence.*

There are proponents (*1–4*) and opponents (*5*) to angiotensin's role in mediating aldosterone release in the rat. This controversy centers around studies with rats stressed by surgery and anesthesia. Since anesthesia inevitably induces renin release in the intact rat (*6*), we questioned the state of adrenal cortical sensitivity of the previous reports. We have found a remarkable responsiveness of aldosterone release to angiotensin infusion in the unanesthetized rat, suggesting that anesthesia introduced an important artifact in the previous studies. We now report our comparison of the effects of the heptapeptide des-aspartyl-angiotensin II (angiotensin III), and of the octapeptide angiotensin II on the adrenal receptor, using the new selective angiotensin antagonist, P-113 ([1-sarcosyl-8-alanyl]angiotensin II).

Male Wistar rats (300 to 350 g) were housed individually and exposed to light by an automated system from 6 a.m. to 6 p.m. The animals were given free access to tap water and Purina rat chow containing 152 meq of sodium per kilogram of food.

Experiments were done 24 hours after placement of a polyethylene catheter in the jugular vein, which was exteriorized posteriorly. Control rats were infused with 0.1*M* tris buffer, *p*H 7.5, in 0.2 percent lysozyme, and the experimental rats were infused with the same buffer containing des-aspartyl-angiotensin II (purchased from Schwarz-Mann; labeled heptapeptide) or angiotensin II amide (Ciba). All infusions were administered at a rate of 25 μl/min for 30 minutes. At the end of the infusion, the rats were decapitated, and blood was collected from the aorta in siliconized glass tubes kept on ice. The samples, after being allowed to clot, were centrifuged at 12,000*g* at 4°C for 20 minutes, and the serum was frozen (− 20°C) in capped plastic tubes until they were assayed.

Another group of identically prepared rats were given preliminary treatment with P-113 in the following manner. The P-113 (0.6 mg/kg) was injected subcutaneously (time zero). Ten minutes later, P-113 (10 mg/kg) was administered subcutaneously in 15 percent Pharmagel A in saline to ensure prolonged action (*7*). At 20 minutes, the 30-minute infusion was initiated as before with tris (control), angiotensin II, or the heptapeptide. The peptides were infused at a dose of 100 ng/min. At 50 minutes, the animals were decapitated and blood was collected for radioimmunoassay of aldosterone.

All samples were assayed for aldosterone according to the immunopurification method of Gomez-Sanchez *et al.* (*8*) with the modification of a 1 : 1,000,000 dilution of antibody to aldosterone (obtained from the National Institutes of Health) being used for the radioimmunoassay.

The results of the peptide infusions are shown in Fig. 1. Both peptides elicited dose-related aldosterone release over a wide range. The response of aldosterone release to the infusion at 0.72 ng/min was significant in both