References and Notes

1. J. Mendecki, S. Y. Lees, G. Brawerman, *Biochemistry* **11**, 792 (1972); R. Sheldon, C. Jurale, J. Kates, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 417 (1972).
2. N. Sullivan and W. K. Roberts, *Biochemistry* **12**, 2395 (1973).
3. K. N. Prasad, B. Mandal, J. C. Waymire, G. A. Lees, A. Vernadakis, N. Weiner, *Nat. New Biol.* **241**, 117 (1973).
4. K. N. Prasad and A. W. Hsie, *ibid.* **233**, 141 (1971); K. N. Prasad and A. Vernadakis, *Exp. Cell Res.* **70**, 27 (1972); K. N. Prasad and S. Kumar, in *Control of Proliferation in Animal Cells* (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1974), p. 581.
5. H. Sheppard, G. Wiggan, W. H. Tsiem, *Adv. Cyc. Nucleotide Res.* **1**, 102 (1972).
6. A 10 percent homogenate of chick brain in 0.32*M* sucrose was centrifuged (27,000*g* for 7 minutes). The supernatant was adjusted to contain 0.02*M* KCl, 0.004*M* $MgCl_2$, and 0.016*M* tris · HCl (*p*H 7.8) and incubated with puromycin (125 μg/ml) for 20 minutes at 37°C. It was then brought to 1 percent sodium deoxycholate and centrifuged at 100,000*g* for 90 minutes. This procedure causes complete release of messenger RNA from ribosomes (S. C. Bondy and J. L. Purdy, in preparation). The ribosomal pellet was suspended in 0.1*M* KCl, 0.1*M* glycine, and 0.01*M* EDTA, *p*H 9.5, and RNA was prepared as described in the text.
7. P. K. Sarkar, B. Goldman, A. A. Moscona, *Biochem. Biophys. Res. Commun.* **50**, 308 (1973).

8. Supported by PHS grants NS 09603 and NS 09230 and by Foundations' Fund for Research in Psychiatry grant 70-487. We thank Dr. H. Sheppard of Hoffmann–La Roche for supplying RO20-1724 compound.

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Deuterium Micromapping of Biological Samples by Using the D(T,n)^{4}He Reaction and Plastic Track Detectors

Abstract. *A technique has been developed to micromap deuterium by using the D(T,n)^{4}He reaction and plastic track detectors. Labeling of cells with subpicogram quantities of deuterium was demonstrated. The technique was used to localize human lymphocytes transformed in vitro.*

The use of stable isotopes as tracers in biomedical and other fields is now of interest (*1*). In particular, the detection and localization of deuterium, the stable isotope of hydrogen, would be useful. Since nonexchangeable hydrogen is contained in most organic molecules, deuterated organic compounds could be used as tracers in vivo. We show here that the D(T,*n*)^{4}He reaction can be a sensitive indicator for the presence of deuterium and that plastic track detectors can be used to reveal the microscopic position of the deuterium (*2*). A general scheme for the localization of stable isotopes using nuclear reactions was proposed theoretically by Malmon (*3*).

Conventional autoradiography (ARG) of radioactive nuclides is one of the most valuable microlocalization techniques available for studying the incorporation and transport of molecules in living cell systems. The concomitant biological effect of the radiation on the living system under study may occasionally restrict the scope of information available from animal experiments, and certainly inhibits conventional ARG studies in human clinical investigation (*4*). For example, if one wished to trace autologous lymphocytes during biomedical studies of adoptive immunotherapy (*5*), it would be preferable to use a stable isotope rather than tritium to label the cells in vitro before reinjection. Tritiated lymphocytes could also be traced in the body after reinjection, but they might be damaged by chronic low-level beta radiation.

The deuterium is detected by bombarding the sample with a triton beam to produce the D(T,*n*)^{4}He reaction. The unique feature (*6*) of this reaction is a strong resonance with a peak total cross section of 5 barns at a low bombarding energy of 160 kev and a high total energy released in the nuclear reaction (*Q* value) of 17.5 Mev. The full width at half maximum of this resonance corresponds to 2.5 μm in tissue. The bombarding energy is so low that the yield of background alpha particles produced by triton bombardment of elements heavier than deuterium is negligible. An alpha particle emitted in the forward direction has an energy of 4.7 Mev. This means that samples up to several micrometers thick can be used with an absorber to stop the incident triton beam. The alpha particles, after passage through a 6-μm-thick Teflon (*7*) absorber, have an energy of about 2 Mev, which is suitable for detection in plastic track detectors.

In our experiments, an 8-μm-thick cellulose nitrate plastic track detector on a 100-μm Mylar backing for mechanical support (*8*) is mounted on an aluminum slide (2.54 cm by 7.62 cm). A 6-μm Teflon film is fastened, wrinkle-free, to the surface of the track detector. A drop of cells in suspension is evaporated to dryness on the Teflon. To minimize clumping of the cell specimens due to surface tension, the Teflon is coated with a layer of carbon 50 to 100 Å thick by vacuum evaporation to complete the detector assembly (see Fig. 1). The slide is mounted in thermal contact with a metal block cooled with air circulating through a Dry Ice–alcohol mixture during bombardment by a beam of 400-kev diatomic tritons (TT^+) at the Research Van de Graaff accelerator of Brookhaven National Laboratory. After bombardment, the detector is removed from the slide and etched in 6.25*N* NaOH at 40.0°C for 45 minutes to develop the alpha particle tracks as characteristic etched cones. The specimens and track detector can be examined and photomicrographed before and after bombardment.

Plastic track detectors (*9*) are very insensitive to other radiations, including photons, electrons, and neutrons produced during bombardment. The position and direction of the etched alpha particle tracks locate the spatial origin of the nuclear reaction to about 2 to 10 μm. The alpha particles which enter the track detector are detected with nearly 100 percent efficiency under these physical conditions.

The effective reaction cross section, $\bar{\sigma}$, taking into account the target thickness and efficiency of the track detector, is given by

$$\bar{\sigma} = \frac{1}{X_1} \int_0^{X_1} \sigma(\eta) G(\eta, X_1, X_2, X_3) d\eta \quad (1)$$

where X_1 and X_2 are the thicknesses of the sample and of the absorber, respectively, X_3 is the minimum detectable depth of alpha penetration into the detector, G is the geometrical efficiency, σ is the nuclear reaction cross section, and the integration is carried out over the particle penetration distance η (see Fig. 1).

In the materials used and for the energies encountered, the range of an alpha particle is approximately proportional to energy. With this approximation, the geometrical efficiency is (*10*)

$$G(\eta, X_1, X_2, X_3) = \frac{1}{2}\left[1 - \frac{X_1 - \eta}{R_1} - \frac{X_2}{R_2} - \frac{X_3}{R_3}\right] \quad (2)$$

where R_1, R_2, and R_3 are the calculated ranges (*11*) of reaction alpha particles in the sample, absorber, and detection plastic materials, respectively. The biological samples analyzed were typically 1 to 3 μm thick, the absorber was 6-μm Teflon, and the minimum detectable

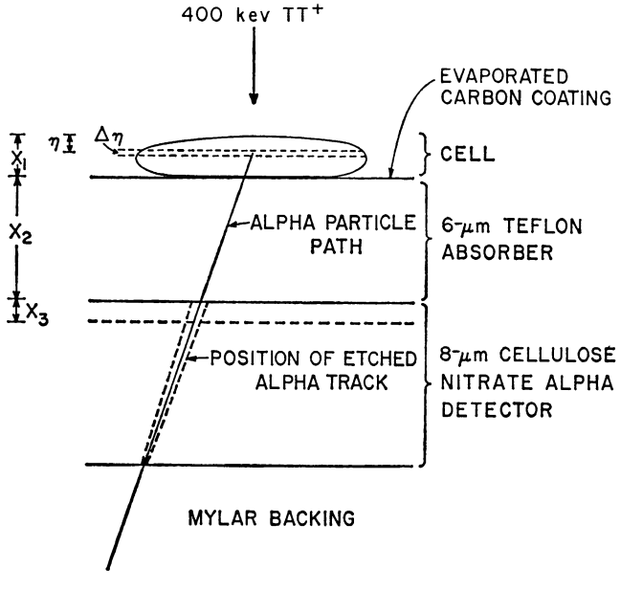
depth of a track in a cellulose nitrate plastic detector was 1 μm. The theoretical effective cross section for the samples analyzed, $\bar{\sigma}$, was obtained by inserting Eq. 2 into Eq. 1, and numerical integration was performed, assuming an isotropic spatial distribution of monoenergetic alpha particles (*6*). The calculated effective average cross section is 1.1 barns. It varies only $\pm$ 10 percent over a sample thickness of 1 to 3 μm when the energy of the incident tritons is 200 kev and the beam is normal to the surface of the absorber.

An estimate of the reaction rate for a typical bombardment can be made by using the 1.1-barn effective cross section. We have estimated the natural deuterium content of a dehydrated and defatted mammalian cell nucleus 5 μm in diameter to be 1.2×10^8 atoms (*12*). This corresponds to a density of 2.0×10^{-9} g/cm^2. Beam currents of about 1.8×10^{11} tritons per second per square centimeter were used throughout, which resulted in an induced reaction rate of 0.1 track per hour per normal nucleus. The average power dissipated is 6 mw/cm^2 on the sample. Thus, the deuterium content of the cell nucleus would need to be enhanced by a factor of 10 to 100 to make it convenient to observe. The use of higher beam currents would also increase the sensitivity.

Three types of samples were analyzed to demonstrate the feasibility of this method: *Chlorella vulgaris* algae (98 percent deuterated), human erythrocytes fixed with deuterated formaldehyde, and human lymphocytes labeled with deuterated thymidine. The algae (*13*) remained microscopically intact after 16 hours of irradiation and produced a dense, radial cluster of tracks (*12*).

Human erythrocytes were fixed with deuterated formaldehyde (*12*, *14*). Single erythrocytes were identified and matched with their radial track patterns (Fig. 2, a and b). For 26 cells, the average number of tracks observed per cell was 36. A strip of Kimfol (*15*) plastic 3.5 μm thick was irradiated simultaneously as a deuterium standard. By comparing the track densities from the cells and the Kimfol, the deuterium content of a cell was estimated to be $(300 \pm 75) \times 10^{-15}$ g and the enhancement of deuterium over its natural abundance was estimated to be 640 ± 160.

Finally, human lymphocytes were labeled in vitro with deuterated thymidine. The labeled precursor was prepared by modifying a synthesis of thymidine from 5-bromodeoxyuridine (*16*), using deuterated methyl iodide (*14*) as a reactant. Ultraviolet spectrophotometry, thin-layer chromatography, and mass spectrometry ($m/e = 245$) of the product indicated that methyl-deuterated thymidine was formed. The deuterated thymidine (5 μM) was added to 5-ml cultures (*17*) of fresh human leukocytes 24 hours after initiation of lymphocyte transformation by phytohemagglutinin. After 72 hours of culture, the erythrocytes were lysed and the lymphocytes were centrifuged through 95 percent ethanol and then through absolute ethanol. Microdrops of ethanol containing lymphocytes in suspension were evaporated on the surface of the detector assembly. Control cells similarly prepared with nondeuterated thymidine were deposited on another portion of the same surface. The cells were observed and photographed with incident-light microscopy and were unstained throughout the experiment. In this experiment, the slide was mounted at 45° to the beam, rather than at 90° as in the initial studies. Thus, the beam was effectively increased in width without significant loss of detector efficiency. The cooled slide was bombarded for 56 hours. The bombarded cells remained generally in-



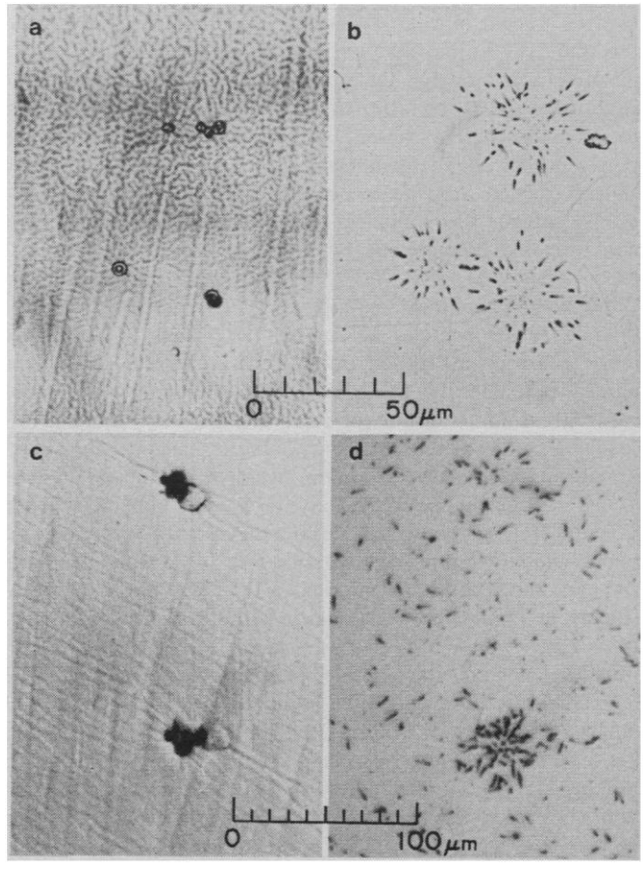


Fig. 1 (left). Diagram of detector assembly. See text for explanation. Fig. 2 (right). (a) Human erythrocytes fixed in deuterated formaldehyde and photographed after bombardment by tritons. (b) Alpha particle tracks in cellulose nitrate detection film, corresponding to (a). (c) Transformed human lymphocytes labeled in vitro with deuterated thymidine. Note distortion of cells due to triton bombardment. (d) Alpha particle tracks in cellulose nitrate detection film, corresponding to (c). Note that only the cell in the lower half of (c) was heavily labeled with deuterium.

tact, but suffered considerable distortion. The nuclei seemed to undergo partial homogenization, losing their normal chromatin pattern, while the nuclear membrane appeared to become thicker.

Further work will be necessary to determine whether these changes are caused by thermal or radiation damage from the incident triton beam. The cell damage, if caused by heating, may be ameliorated in future experiments by cooling the block with liquid nitrogen and eliminating the thermal insulation formed by the 100-μm Mylar supporting the cellulose nitrate film. Alternatively, cooling might be accomplished by conduction to a cold gas contained above the sample.

Although all erythrocytes fixed in deuterated formaldehyde produced radial track patterns, some phytohemagglutinin-treated lymphocytes exposed to deuterated thymidine did not (Fig. 2, c and d). Sparse tracks were noted over all nondeuterated control lymphocytes. It is inferred that the labeled cells were transformed by the phytohemagglutinin, while the cells associated with few tracks in excess of background were not transformed. The number of alpha particle tracks in individual clusters corresponding to lymphocytes were counted. The greatest deuterium content of a lymphocyte was estimated to be $(6 \pm 3) \times 10^9$ atoms. A lymphocyte fully labeled with methyl-deuterated thymidine is expected to incorporate 5×10^9 deuterium atoms during one cell division (*12*). Unexpectedly, a randomly oriented background track pattern was observed. We suspect that this was due to contamination of the Teflon film by detergent during the dispersion and fusion of the 0.2-μm tetrafluoroethylene particles which were used during the casting of the film. Any contaminant of tetrafluoroethylene that contains hydrogen should introduce deuterium in the absorber and result in randomly oriented background tracks. The radial track pattern associated with cells was readily distinguishable from the random background pattern, especially at low magnification. This pattern was not observed in the erythrocyte samples because the triton fluence—and hence the sensitivity—was lower by a factor of 50.

The system of deuterium micromapping described in this report is less sensitive than conventional tritium ARG, but it may prove useful for certain clinical studies of patients to whom it may be considered inappropriate to administer tritiated metabolic precursors or tritiated cells. As a particular example, the feasibility of labeling human lymphocytes with deuterated thymidine is demonstrated.

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References and Notes

1. P. D. Klein and S. V. Peterson, Eds., *Proceedings of the First International Conference on Stable Isotopes in Chemistry, Biology and Medicine* (USAEC CONF-730525, Atomic Energy Commission, Washington, D.C., 1973).
2. F. H. Geisler, K. W. Jones, H. W. Kraner, D. N. Slatkin, A. P. Wolf, J. S. Fowler, E. P. Cronkite, *Bull. Am. Phys. Soc.* **18**, 410 (1973); F. H. Geisler, K. W. Jones, J. S. Fowler, H. W. Kraner, D. N. Slatkin, *ibid.* **19**, 373 (1974).
3. A. G. Malmon, *J. Theor. Biol.* **9**, 77 (1965).
4. H. A. Johnson, in *Medical Radionuclides: Radiation Dose and Effects*, R. J. Cloutier, C. L. Edwards, W. S. Snyder, Eds. (USAEC CONF-691212, Atomic Energy Commission, Washington, D.C., 1969).
5. J. H. Frenster and W. M. Rogoway, in *Proceedings of the Fifth Leukocyte Culture Conference*, J. E. Harris, Ed. (Academic Press, New York, 1971), pp. 359–373.
6. C. P. Baker, M. G. Holloway, L. D. P. King, R. E. Schreiber, *Report AECD-2226* (U.S. Atomic Energy Commission, declassified 10 August 1948); H. Liskien and A. Paulsen, *Nucl. Data* **A11**, 569 (1973).
7. Tetrafluoroethylene film, Dilectrix, Farmingdale, N.Y.
8. Kodak Pathe film LR-115, Eastman Kodak, Rochester, N.Y.
9. Plastic track detectors are discussed in R. L. Fleischer, H. W. Alter, S. C. Furman, R. M. Walker, P. B. Price, *Science* **178**, 255 (1972).
10. F. H. Geisler, thesis, Washington University, St. Louis (1972).
11. P. G. Steward, *Report UCRL-18127* (1968).
12. D. N. Slatkin, K. W. Jones, F. H. Geisler, A. P. Wolf, J. S. Fowler, H. W. Kraner, E. P. Cronkite, in (*1*), pp. 410–420.
13. Merck Sharp & Dohme of Canada Ltd., Pointe-Claire, Quebec.
14. Stohler Isotope Chemicals, Waltham, Mass.
15. Peter J. Schweitzer Division, Kimberly-Clark, Lee, Mass.
16. T. L. V. Ulbricht, *Tetrahedron* **6**, 225 (1959).
17. Leukocytes (10^6 per milliliter) were cultured in Eagle's minimum essential medium with glutamine (1 percent), fetal calf serum (15 percent), streptomycin (0.1 mg/ml), and penicillin (100 unit/ml) at 37°C. Phytochemagglutinin was added as 0.02 ml of Bacto-Phytochemagglutinin M (Difco Laboratories, Detroit, Mich.) per milliliter of culture medium.
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Singlet Excited Oxygen as a Mediator of the Antibacterial Action of Leukocytes

Abstract. *Human polymorphonuclear leukocytes kill a colorless mutant strain of* Sarcina lutea *much more readily than a carotenoid-containing strain. A similar protective effect has been reported in the organism during photodynamic inactivation, where it is attributable to the quenching of singlet excited oxygen by carotenoids. The findings with leukocytes support the suggestion that singlet excited oxygen acts as one of the mediators of their bactericidal action.*

Although oxidation has been considered one of the primary means whereby polymorphonuclear (PMN) leukocytes destroy ingested bacteria, the actual bactericidal cellular reactions have not been well characterized. The respiratory burst which accompanies phagocytosis in leukocytes was assumed by Iyer *et al.* (*1*) to be due to the formation of H_2O_2, and Paul and Sbarra (*2*) subsequently made direct measurements of an increase in H_2O_2 production by leukocytes during phagocytosis. Klebanoff and co-workers (*3*) extended these observations and suggested that a three-component system including myeloperoxidase, H_2O_2, and halide (Cl^- or I^-) is responsible for the bactericidal activity of PMN leukocytes. The role of these compounds as antibacterial agents in PMN leukocytes has been studied in patients suffering from chronic granulomatous disease, whose PMN leukocytes are unable to produce H_2O_2 when stimulated (*4*), and in patients with myeloperoxidase deficiency (*5*). In both of these cases, bactericidal activity is impaired.

Another hypothesis has been advanced to explain bacterial killing within leukocytes. Allen *et al.* (*6*) reported the appearance of chemiluminescence from human PMN leukocytes after stimulation with either bacteria or latex particles. They proposed that the chemiluminescence reflects the generation of singlet excited oxygen ($^1O_2$*), which acts as the bactericidal agent. The $^1O_2$* could be formed, according to Allen *et al.*, from the decomposition of an oxygen intermediate, such as the superoxide radical ($\cdot O_2^-$), formed during pyridine nucleotide oxidation. Several workers (*7*) have suggested that the spontaneous dismutation of $\cdot O_2^-$ produces $^1O_2$*, as follows

$$\cdot O_2^- + \cdot O_2^- \xrightarrow{+2H^+} H_2O_2 + {}^1O_2{}^*$$