These results demonstrate the practicality of performing in vivo ^{31}P NMR spectral imaging. By using an imaging technique in which spatial information is encoded as the phase of an echo, it is possible to retain the spectral information needed for interpreting the phosphate metabolic information. The data presented here show that discrete spectra can be obtained in vivo from slices of tissue a few millimeters thick. No attempt was made to resolve different areas of each plane, but useful spectral signals must have originated from the animal tissue within the homogeneous region of the magnet, which in our case approximates a sphere of diameter 25 mm. Thus, for our resolution elements, the effective tissue volume of each slice is approximately 1 ml, for which we obtained a signal-to-noise ratio of about 20:1 in less than 1 hour.

We can use these results to predict the performance of metabolite imaging systems for larger (human) objects. We assume that we are able to scale up the linear dimensions of our present system fivefold in order to image human limbs. If the noise in our spectra arises predominantly from the RF coil, then the signal-to-noise ratio of a spectrum recorded from a volume V of tissue will be proportional to V/R, where R is the radius of the RF coil. Then if we wish to resolve a human limb to the same fractional resolution obtained for the rat, we may expect a volume element of approximately 125 ml. So for a 1-minute scan with a single 15-echo sequence, we may expect a signal-to-noise ratio of 100. Thus extension to two-dimensional slice information from human limbs and heads in times well under 1 hour seems readily possible.

There is no reason why this technique of spatially resolving metabolite spectra cannot be extended to three dimensions in vivo and to other nuclei such as ^{13}C and ^{23}Na.

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Hemoglobin Aggregation in Single Red Blood Cells of Sickle Cell Anemia

Abstract. *A laser light scattering technique was used to observe the extent of hemoglobin aggregation in solitary red blood cells of sickle cell anemia. Hemoglobin aggregation was confirmed in deoxygenated cells. The light scattering technique can also be applied to cytoplasmic studies of any biological cell.*

Polymerization of hemoglobin into fibers is a key phenomenon in sickle cell anemia (*1*). The red blood cells undergo reversible sickling and unsickling according to whether the hemoglobin is polymerized (deoxygenated) or depolymerized (oxygenated). The polymerization of hemoglobin in sickle cell anemia has been widely investigated, but in most cases cell-free solutions have been used (*1*). It is highly desirable to study the aggregation, nucleation, and polymerization of hemoglobin in intact red blood cells.

To make such observations, we have developed a laser light scattering apparatus for photon correlation spectroscopy under an optical microscope. Using this apparatus, we measured the Brownian motion of hemoglobin molecules inside a single red blood cell. The rate of the Brownian motion is directly proportional to the rate of intensity fluctuations of laser light scattered from the randomly moving hemoglobin molecules. The photon correlation technique is very sensitive to the slowing down of Brownian motion associated with dimerization, trimerization, and further aggregation of the hemoglobin molecules.

The experimental apparatus is shown in Fig. 1 (*2*). The beam of a He-Ne laser is expanded to a parallel beam 6 mm in diameter and then sharply focused into a

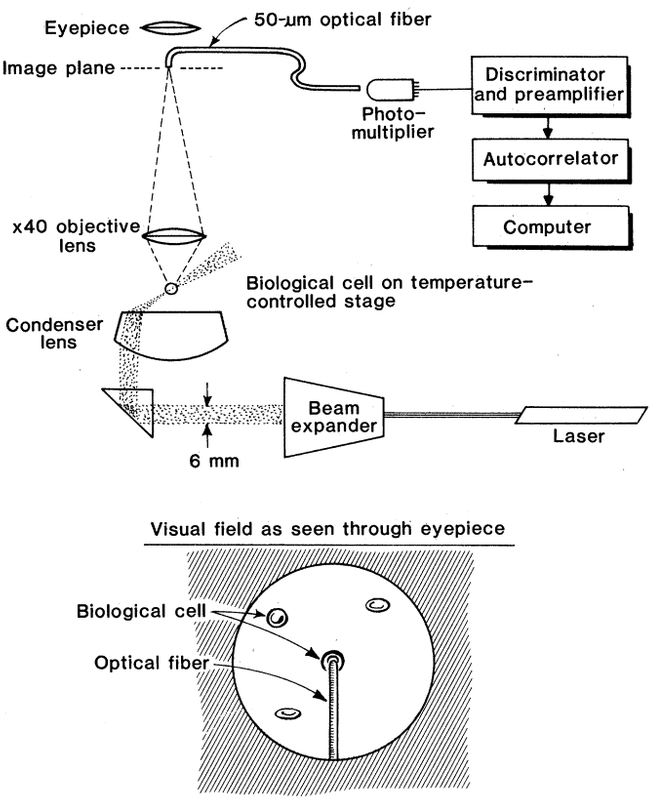

Fig. 1. Schematic diagram of the laser light scattering apparatus used to study the Brownian motion of a local region of cytoplasm in a single red blood cell. An optical fiber embedded in the eyepiece of the microscope collects only light scattered from a volume of 2 μm^3, allowing characterization of the extent of hemoglobin aggregation in the cell.

red blood cell by the condenser lens of a microscope. The beam enters the cell at an angle 35° from the vertical and has a diameter of approximately 2 μm at the focus. Light scattered from the cell is collected by a long-working-distance objective lens (×40), forming a magnified real image on the primary plane of the eyepiece. An optical fiber embedded in the center of the eyepiece collects light from the primary plane. The diameter of the fiber is 50 μm, thus it collects light from a region 1.25 μm in diameter from the focal plane in the blood cell. The scattered light passes through the optical fiber onto a photomultiplier tube. Thus, light is collected from a scattering volume of less than 2 μm^3. Light scattered from other regions, including the cell membrane, is avoided. This arrangement enables investigation of a local region of cytoplasm (*3*).

In the case of a red blood cell, the temporal fluctuations of the scattered light intensity, $I(t)$, directly reflect fluctuations in the concentration of hemoglobin due to Brownian motion in the cell. The fluctuations are recorded in the form of a correlation function,

$$C(t) = \langle I(t')I(t + t')\rangle_{t'} \quad (1)$$

where the brackets indicate the time average over t'. The correlation function can be expressed in terms of the diffusion coefficient D of the hemoglobin molecules (*4*),

$$C(t) = An^2\exp(-2Dk^2t) + B \quad (2)$$

where n is the hemoglobin concentration, $k = (2\pi/\lambda)\sin(\Theta/2)$ is the scattering wave number, λ is the wavelength of the laser light in water, Θ is the scattering angle (35° in this experiment), and A and B are constants. Equation 2 shows that the decay rate of the correlation function is proportional to the diffusion rate of the hemoglobin molecules. The diffusion coefficient is further related, through the Stokes-Einstein-Kawasaki-Ferrel formula, to the correlation length, ξ, of the hemoglobin molecules,

$$D = kT/6\pi\eta\xi \quad (3)$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of water (*5*). The correlation length is the average distance beyond which the motion of neighboring molecules of hemoglobin becomes independent. When the interaction among the molecules is negligible, the correlation length is simply the hydrodynamic radius of the hemoglobin molecule (*5*).

When there are hemoglobin aggregates of different sizes and therefore different diffusion coefficients, the correlation function is given by the sum of the correlation functions corresponding to each size aggregate. By precise analysis of the correlation function, it is, in principle, possible to determine the size distribution of the hemoglobin aggregates.

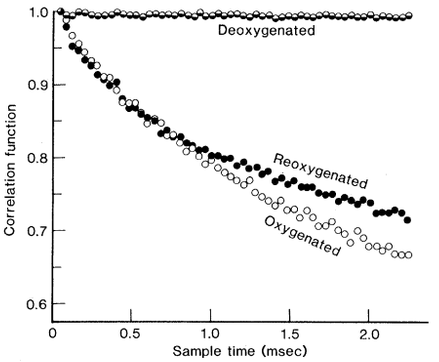

Fig. 2. Correlation functions for the light scattered from the center of individual oxygenated, deoxygenated, and reoxygenated red blood cells from a sickle cell anemia patient. Oxygenated hemoglobin undergoes rapid Brownian motion, and its correlation function decays quickly. The correlation function of deoxygenated hemoglobin is flat, indicating that Brownian motion is essentially stopped by extensive intracellular aggregation. The similarity between the correlation functions of the original oxygenated hemoglobin and the reoxygenated hemoglobin shows that the aggregation was reversed.

We examined the Brownian motion of hemoglobin in a single red blood cell from a patient with sickle cell anemia. Blood taken from the patient was washed and centrifuged three times with buffered saline. The red blood cells were then dispersed in saline or in a reducing medium consisting of buffered saline and 2 percent $Na_2S_2O_5$. The latter solution deoxygenates hemoglobin molecules (*6*). For the light scattering measurements a small amount of the suspension was placed on a thin glass slide and sealed with a cover glass and high-vacuum grease. To reoxygenate the red blood cells we washed and centrifuged them several times with buffered saline. Deoxygenation and reoxygenation were confirmed by changes in the color of the solutions. Measurements of the correlation function of light scattered from hemoglobin at the center of each cell were carried out under oxygenated, deoxygenated, and reoxygenated conditions.

Brownian motion of oxygenated hemoglobin was rapid, as indicated by the high decay rate of the correlation function (Fig. 2). The correlation length of oxygenated hemoglobin molecules was approximately 300 Å. On deoxygenation most of the cells became sickled. The correlation functions for both sickled and round cells were flat, showing that Brownian motion of the hemoglobin was essentially stopped by extensive aggregation in each cell. When the deoxygenated cells were reoxygenated with saline, the correlation function regained its original form, indicating that polymerization of the hemoglobin molecules was reversed.

We also studied oxygenated and deoxygenated normal human red blood cells. We found no significant change in the correlation function of the scattered light, indicating no hemoglobin aggregation in the normal deoxygenated cell.

In conclusion, we successfully observed Brownian motion and determined the diffusion coefficients of hemoglobin molecules in single red blood cells from a sickle cell anemia patient. We found that the hemoglobin molecules aggregate on deoxygenation and dissociate on reoxygenation. This finding is consistent with the results of (i) the experiments with cell-free solutions and (ii) the photolysis experiments on single red blood cells by Coletta *et al.* (*3*). The technique presented in this report should prove valuable in future studies of sickled red blood cells. It is quantitative, noninvasive, and can be applied to single live cells. Although we used the technique on fully aggregated hemoglobin molecules, it is also suited to the study of the entire process of hemoglobin aggregation. Such information is vital not only in promoting our understanding of the mechanism of hemoglobin polymerization and depolymerization but also in testing the effect of antisickling drugs. The technique may also be applied as a diagnostic tool in other red blood cell abnormalities. Finally, this application of laser light scattering spectroscopy should be invaluable for the cytoplasmic study of any biological cell.

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Mutation Affecting the 12th Amino Acid of the c-Ha-*ras* Oncogene Product Occurs Infrequently in Human Cancer

Abstract. *A point mutation alters the 12th amino acid of the c-Ha-*ras *oncogene product p21 in a human bladder cancer cell line. This is, at present, the only mutation known to result in a human transforming gene. This mutation may therefore represent a possible target for mutagenesis leading to carcinogenesis in humans. By means of restriction enzyme analysis, 29 human cancers, including 20 primary tumor tissues, derived from organs commonly exposed to environmental carcinogens, were tested for the presence of this mutation. None of ten primary bladder carcinomas exhibited the mutation; nor did nine colon carcinomas or ten carcinomas of the lung. Thus the point mutation affecting the 12th amino acid of the c-Ha-*ras *gene product, while a valuable model for carcinogenesis, does not appear to play a role in the development of most human epithelial cancers of the bladder, colon, or lung.*

It was recently observed that a point mutation in the cellular oncogene c-Ha-*ras* occurs in a cell line derived from a human bladder carcinoma. The c-Ha-*ras* gene product is a protein of 21,000 daltons (p21). The mutation alters this protein by converting its 12th amino acid (normally glycine) to valine. The altered p21 is responsible for the ability of DNA from this bladder tumor cell line to transform NIH 3T3 cells upon DNA transfection (*1*, *2*). Altered p21 proteins of related *ras* genes also seem to be responsible for the transforming activities of lung and colon cancer cell lines, although the genetic change resulting in this alteration has not yet been defined at the molecular level (*3*). It is interesting that all transforming *ras* oncogenes that have been sequenced so far, be they of viral or cellular origin, exhibit a mutation at the 12th amino acid of p21 (*1*, *2*, *4*). On the basis of computer models of the protein, a change in the 12th amino acid is expected to result in a dramatic change in the structure of the protein, this structural change presumably resulting in transforming capacity (*1*).

One of the most important implications of this observed mutation is that the codon for the 12th amino acid of the c-Ha-*ras* gene product may represent a precisely defined target for mutagenesis leading to carcinogenesis. This implication has obvious relevance to the large body of data correlating mutagenicity with carcinogenicity in laboratory animals and man (*5*). It also raises the question of how frequently this mutation occurs in human cancers, especially those that may arise as a result of exposure to environmental carcinogens. To answer this question, we analyzed 29 human cancers for the presence of a mutation of the codon for the 12th amino acid of the c-Ha-*ras* gene product. We

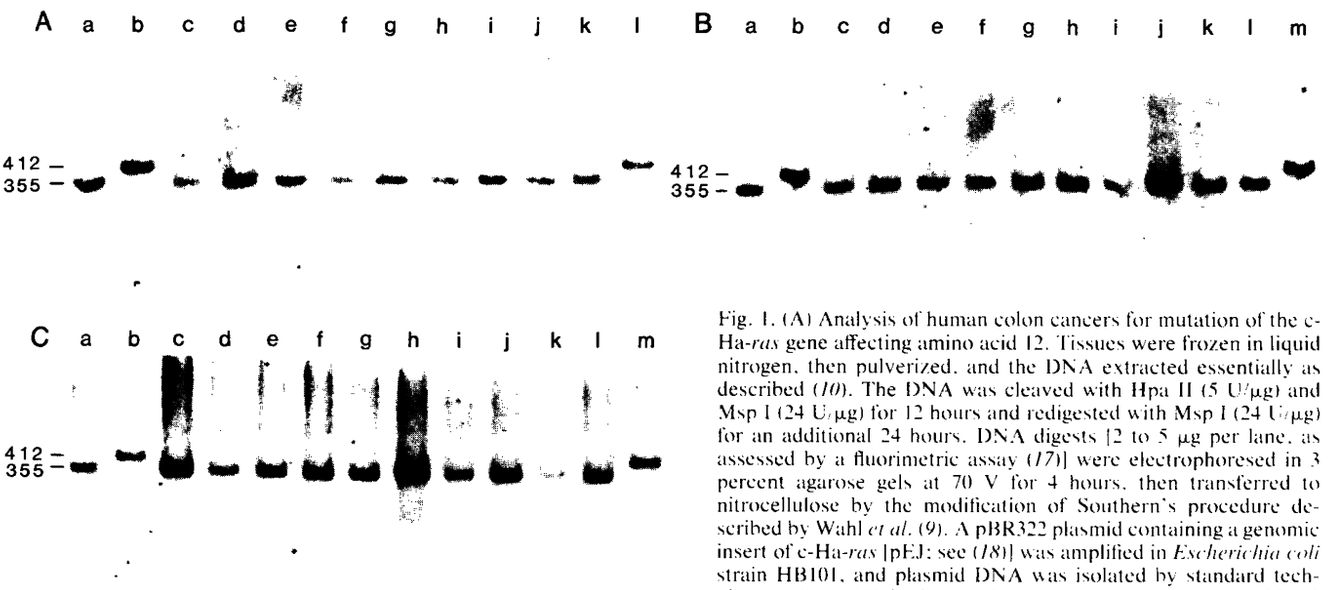

Fig. 1. (A) Analysis of human colon cancers for mutation of the c-Ha-*ras* gene affecting amino acid 12. Tissues were frozen in liquid nitrogen, then pulverized, and the DNA extracted essentially as described (*10*). The DNA was cleaved with Hpa II (5 U/μg) and Msp I (24 U/μg) for 12 hours and redigested with Msp I (24 U/μg) for an additional 24 hours. DNA digests [2 to 5 μg per lane, as assessed by a fluorimetric assay (*17*)] were electrophoresed in 3 percent agarose gels at 70 V for 4 hours, then transferred to nitrocellulose by the modification of Southern's procedure described by Wahl *et al.* (*9*). A pBR322 plasmid containing a genomic insert of c-Ha-*ras* [pEJ; see (*18*)] was amplified in *Escherichia coli* strain HB101, and plasmid DNA was isolated by standard techniques (*19*). A 600-bp insert of pEJ containing the relevant Msp I fragments was obtained by digesting pEJ DNA with Sma I (*20*). The Sma I fragment was purified and labeled to 10^9 dpm/μg with ^{32}P-labeled deoxycytidine triphosphate by a technique described elsewhere (*21*). Hybridization and autoradiography were performed as described (*21–23*). Molecular weight standards consisted of Hinf I–digested pBR322 and Hae III–digested ΦX174 DNA. Lane a, normal placenta; lanes b and l, T24 DNA (the bladder cancer cell line with the c-Ha-*ras* gene mutation); lanes c to k, colon carcinomas from patients 1 to 9, respectively. (B) Analysis of human lung carcinomas for the c-Ha-*ras* mutation. The experiment was performed as described in the legend to (A). Lane a, normal placenta; lanes b and m, T24 DNA; lanes c to l, lung carcinomas from patients 10 to 19, respectively. (C) Analysis of ten human bladder carcinomas for the c-Ha-*ras* mutation. The experiment was performed as described in the legend to (A). Lane a, normal placenta; lanes b and m, T24 DNA; lanes c to l, bladder carcinomas from patients 20 to 29, respectively.