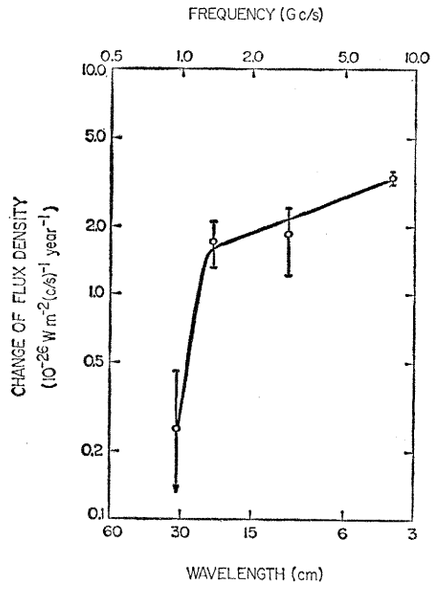

Fig. 2. Observed change in flux density per year for 3C 273 plotted against the wavelength (frequency).

with its cosmological distance of 470 megaparsecs. On the other hand, the source *could* be very much nearer to us; if it were, the dimensions of the variable component would be proportionately reduced (to maintain the same cutoff wavelength), and the time scale of the variation would be determined by some other property of the source.

In an effort to simulate the "change spectrum" of Fig. 2, we have investigated the effect of changing various physical conditions in several types of model sources. The best fit to the observations was obtained when we increased the density of relativistic electrons in a fairly simple spherical source region which becomes optically thick at 20 cm. The slope between 20 and 3 cm is well reproduced if we assume that there are moderate inhomogeneities in the source. There might, for instance, be regions of higher than average density or magnetic field strength occupying about a tenth of the total volume. These condensations would become optically thick in the 3- to 20-cm wavelength range.

A disadvantage of this model is that the energy density in relativistic particles is appreciably larger than the energy density of the magnetic field. Under such conditions the particles would not be confined but would explode outward at a velocity approaching that of light. Hoyle and Burbidge (*6*) have proposed a family of models with a strong radial dependence of the magnetic field. In such a model the imbalance of particle and field energy densities could be reduced. Hoyle and Burbidge's requirement that the source be optically thin at all wavelengths can be modified on the basis of our observations.

In any case it is necessary to account for the longer wavelength radiation from 3C 273B by adding another, larger-diameter component which has a constant intensity from about 20 cm to at least 2 m and which decreases in intensity at shorter wavelengths. At epoch 1965.0 the contribution from this latter component might be one-third to one-half of the total intensity observed at 3.75 cm.

The difference between our interpretation of the variations in 3C 273B and that of Dent comes from Dent's assumption that the source contains a single, homogeneous component. Under this assumption, Dent required that the source be optically thin to wavelengths of at least 2 m and yet be small enough to display a significant intensity change in a few years. Our measurement of the spectrum of the intensity variation indicates that 3C 273B is not homogeneous; the component which varies is optically thick at wavelengths greater than 20 cm. Calculations based on our model indicate that such a condition is physically plausible. The radiation from 3C 273B at longer wavelengths must, of course, come from another component which has a larger diameter.

The large red shifts recently reported for quasi-stellar objects (*7*), of which 3C 273 is the prototype, promise to give us unique information about the evolution of the universe—provided we can be certain that these red shifts are of cosmological origin. We conclude that the spectrum of the intensity variations in 3C 273B is entirely consistent with the cosmological distance and luminosity as derived from Hubble's law and the observed red shift.

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11. A detailed description of our observations and model calculations is in preparation. We thank E. B. Fomalont, D. H. Rogstad, J. B. Whiteoak, and J. D. Wyndham for permitting us to use their observations. Research supported by ONR contract Nonr 220(19). P.M. is on leave of absence from the Institute of Theoretical Astrophysics, Oslo, Norway; A.T.M. is an Alfred P. Sloan Research Fellow.

21 July 1965

Erythrocyte Membrane: Chemical Modification

Abstract. *Erythrocytes treated with 1-fluoro-2,4-dinitrobenzene become permeable to Na^+ and K^+, but not to small water-soluble nonelectrolytes or hemoglobin, and eventually lyse in isotonic buffer. Erythrocytes treated with 1,5-difluoro-2,4-dinitrobenzene become permeable to Na^+ and K^+ but do not lyse in buffer or in water, even after extraction with lipid solvents. The difluoro compound cross-links the membrane and increases its strength. Both reagents appear to remove the positive fixed charge responsible for the cation impermeability of the normal cell.*

This report concerns the reaction of human erythrocytes with 1-fluoro-2,4-dinitrobenzene (Sanger's reagent) and with 1,5-difluoro-2,4-dinitrobenzene. These reagents react readily by displacement of fluorine with free amino, sulfhydryl, tyrosyl, or histidyl groups to form stable dinitrophenyl derivatives. The monofluoro compound reacts with one such group, but the difluoro compound reacts with two groups, provided they are about 5 Å apart, to form a dinitrophenylene cross-link (*1*).

The starting point was the observation that cells treated with the difluoro reagent fail to lyse when suspended in distilled water. Cells obtained from fresh, oxalated, human venous blood were washed repeatedly at 23°C with 0.9 percent NaCl and then with a Krebs buffer (*2*). One milliliter of cells was added to 200 ml of buffer, and a control sample of 40 ml was withdrawn. To the remaining 160 ml, 0.9 ml of a 10-percent methanolic

solution of 1,5-difluoro-2,4-dinitrobenzene (2.8 m*M* in the reaction mixture) was added. At 30-minute intervals, 40-ml samples of the mixture were centrifuged, the supernatant was removed, and the cells were suspended in 5 ml of water. The suspension was centrifuged, and the extent of hemolysis was measured by comparing the optical density of the supernatant at 540 mμ with that of the hemolyzate of the normal cell control. About 10 percent of the cells in the first sample hemolyzed, but no hemolysis could be detected for cells treated with the reagent for 1 hour or more (*3*). Cells treated in this way and kept in distilled water or buffer at 5°C failed to lyse as long as observed (up to 2 weeks).

In contrast, the monofluoro reagent does not prevent lysis. When the experiment was repeated with 1-fluoro-2,4-dinitrobenzene (3.0 m*M* in the reaction mixture) all the cells lysed in distilled water. In addition, the treated cells eventually lysed in buffer; for example, lysis was 80 percent complete after a sample treated with the reagent for 2.5 hours was washed and suspended in buffer for 4.5 hours.

As a control against the possibility that these effects are due to fluoride ions liberated during the reaction, the experiment was repeated with sodium fluoride (5.0 m*M* in the reaction mixture). The cells were not lysed after several hours in the fluoride solution, but they did lyse in water. Hence neither the protection against lysis afforded by the difluoro reagent nor the lysis in buffer caused by the monofluoro reagent can be due to fluoride ions (*4, 5*).

We next established that cells treated with the monofluoro reagent eventually lyse in buffer because they become permeable to sodium and potassium. Cells were treated with the monofluoro reagent for 1 hour and then washed and suspended in buffer. At various times during the following 7 hours cell samples were centrifuged and weighed. The extent of hemolysis was determined from the optical density of the supernatant at 540 mμ. The cells were lysed completely in a known amount of water, the ghosts were discarded, and the Na^+ and K^+ concentrations of the lyzate were measured with a flame photometer. The Na^+ and K^+ concentrations of the cells were then computed by multiplying by the appropriate dilution factors.

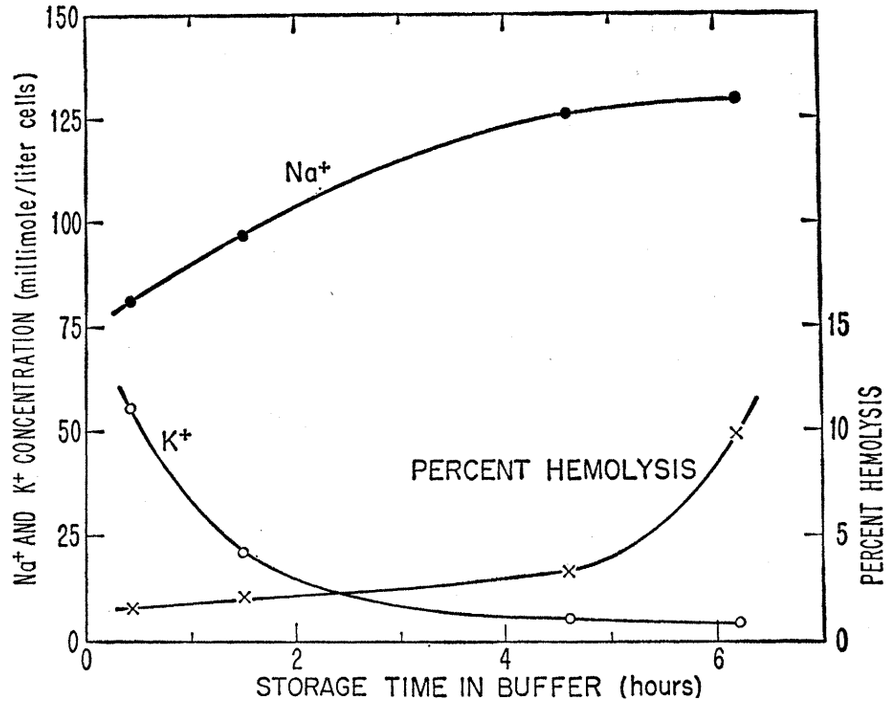

Fig. 1. Intracellular Na^+ and K^+ concentration and extent of hemolysis of cells reacted 1 hour with the monofluoro reagent and subsequently stored in buffer, as a function of storage time in buffer.

The treated cells steadily lost K^+, which was initially more concentrated in the cells than in the buffer, and gained Na^+, which was initially more concentrated in the buffer than in the cells (Fig. 1). They also increased in weight and finally hemolyzed. Even by the time of the first sampling (Fig. 1), the treated cells contained more Na^+ than K^+, whereas normal cells contain only 0.14 as much Na^+ as K^+. In normal cells the outward diffusion of K^+ and the inward diffusion of Na^+ is just balanced by metabolically driven ion "pumps," and if these pumps are blocked, K^+ is lost and Na^+ gained with a half-time of about 35 hours (*5*). The half-time for the treated cells is about 1 hour (Fig. 1). There must therefore be a large increase in the cation permeability of the treated cells, regardless of whether the cation pumps have been blocked (*6*). Once the cells lose their normal impermeability to salt, they lyse because of the osmotic imbalance caused by hemoglobin.

An analogous experiment shows that cells treated with the difluoro reagent become even more permeable to sodium and potassium. Cells were treated for 1.2 or 1.9 hours with the reagent and washed once with buffer. The cells were then weighed (0.44 g), suspended in 1.00 ml of distilled water, and centrifuged. Three minutes after the addition of water the supernatant was removed (*7*). The cells were suspended in another 1.00 ml of water and centrifuged. Three minutes after the second addition of water, 0.50 ml of the supernatant was removed, and the cells in the remaining mixture were broken with high-frequency sound (*8*). The ratios of the concentrations of Na^+ to K^+ in the sound-disrupted samples and in both sets of supernatants ranged from 31.2 to 39.3, in agreement with the ratio found for buffer (38.2) rather than with the value expected for normal cells (0.14). Calculation of the Gibbs-Donnan equilibrium (*9*) showed that the Na^+ and K^+ concentrations were those expected if the treated cells came into equilibrium with the buffer during the reaction and then equilibrated with the water during each 3-minute wash. Thus, the half-time for Na^+ gain and K^+ loss in cells treated with the difluoro reagent for 1.2 or 1.9 hours is small compared to 3 minutes, and the rates are at least 10^3 times larger than in normal cells.

Since cells treated with the difluoro reagent are permeable to Na^+ and

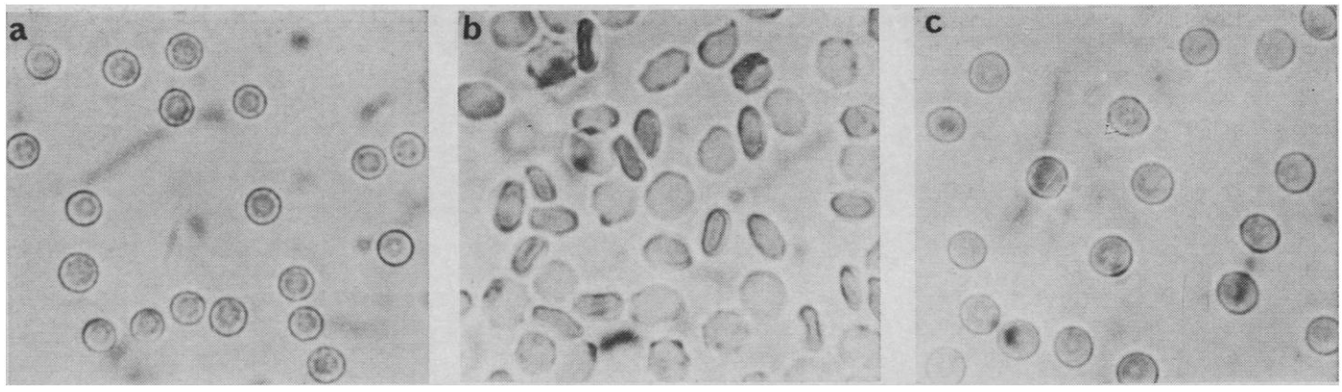

Fig. 2. (*a*) Normal cells in buffer (× 570). (*b*) Cells treated with the difluoro reagent for 1.5 hours and suspended in buffer for 3 hours (× 570). This suspension contains more cells, and the crowding causes some to be seen edgewise. (*c*) Cells treated with the monofluoro reagent for 1 hour and suspended in buffer for 3 hours (× 570).

K^+ (and presumably to Cl^- and HCO_3^-), the equilibrium osmotic pressure which they must withstand in water is due only to hemoglobin and its counterions. From analysis of the Gibbs-Donnan equilibrium (*9*) we estimate this pressure to be about 0.6 atm. Cross-linked cells are strong enough to withstand such a pressure, but cells treated with the monofluoro reagent are not.

Cells treated with the difluoro reagent also are surprisingly rigid. Some are crenated spheres which have diameters about 10 percent larger than the disc diameter of normal cells, while others are smooth biconcave discs with diameters about 30 percent larger than normal (Fig. 2, *a* and *b*). These increases in dimensions are in agreement with a 100 percent increase in volume, determined by weighing equal numbers of normal and treated cells. No change in shape or weight can be detected on transferring treated cells from buffer to water (weight change less than 0.2 percent). The cells do not shrink in solutions of hemoglobin. Some samples cannot be broken with our ultrasonic generator (*8*) or by grinding with powdered glass. The cells do not lyse when shaken with saponin, *n*-butanol, or ether. Cells treated for 2 hours have been lysed with ethanol-ether-water mixtures, but little lysis occurs if the cells have been treated as long as 8 hours.

Cells treated with the monofluoro reagent are not only more spherical than normal cells, but they also have disc diameters at least 10 percent larger than normal (Fig. 2*c*). If all dimensions increased by a factor 1.1, the volume would increase by a factor $(1.1)^3 = 1.33$, in agreement with a 40-percent increase determined by weighing.

Finally, the permeability of treated cells to small water-soluble nonelectrolytes was measured by weighing cells which had beeen suspended in hypertonic solutions containing erythritol, xylose, or sucrose. A suspension of normal cells was divided into three equal parts. The first was set aside, the second was treated 1.5 hours with the monofluoro reagent, and the third was treated 2.4 hours with the difluoro reagent.

Each mixture was subdivided into four equal parts, which were centrifuged for 20 minutes. The average mass of the packed cells of each type is indicated by the "first spin control" coordinate in Fig. 3. The cells of the first tube of each series were suspended in buffer, and the cells in the second, third, and fourth tubes were suspended in buffer containing in addition 200 m*M* erythritol, 200 m*M* xylose, and 200 m*M* sucrose, respectively. The tubes were centrifuged for 20 minutes, and the packed cells were weighed.

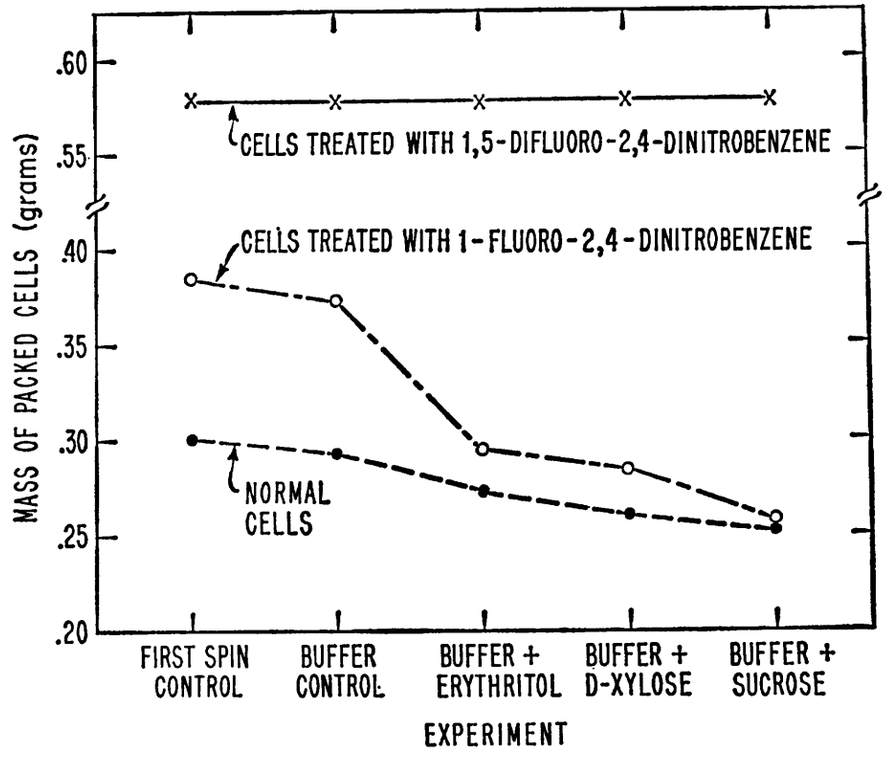

Fig. 3. Weights of equal numbers of normal and treated cells after suspension in buffer ("first spin control"), and after suspension for an additional 20 minutes in buffer ("buffer control") or in buffer containing 200 m*M* erythritol, xylose, or sucrose.

Both normal cells and cells treated with the monofluoro reagent shrank somewhat in erythritol, more in xylose, and still more in sucrose (Fig. 3). The shrinkage increases with the molecular weight of the solute because the smaller solutes are more permeable, and therefore diffuse more into the cells during the 20-minute centrifugation, reducing the effective concentration gradient. Since the cells treated with the monofluoro reagent do shrink, any increase in their permeability to these solutes must be small, even though the permeability to Na^+ and K^+ is increased by a factor of at least 35. In fact, the greater shrinkage of the treated cells suggests that the permeability to these nonelectrolytes may, if anything, decrease. The experiment does not allow us to conclude whether the difluoro reagent changes the nonelectrolyte permeability of the cells, since the observation that cells treated with this reagent fail to shrink (Fig. 3) can be attributed to their rigidity.

Normal erythrocytes are extremely permeable to small anions (such as Cl^-) but relatively impermeable to small cations (Na^+ and K^+). This difference is attributed to the presence of positive fixed charge in the wall of the aqueous channels which traverse the membrane (*5*). The increase in Na^+ and K^+ permeability could either be due to the removal of positive fixed charge or to an expansion of channel diameter to the extent that the charge is effectively shielded by free anions. The latter alternative is ruled out, at least in the case of the monofluoro reagent, because the permeability to water-soluble nonelectrolytes with molecular diameters as small as 7 Å remains low. We conclude that the changes in Na^+ and K^+ permeability are probably due to the removal of positive fixed charge. This is entirely reasonable from a chemical point of view, since the reagents react with free amino groups to form uncharged derivatives, removing charge by the reaction sequence

$$\text{R-NH}_3^+ \rightleftharpoons \text{R-NH}_2 + \text{H}^+ \xrightarrow{\text{fluorodinitrobenzene}}$$

$$\text{R-NH}\!\!<\!\!\!>\!\!\text{NO}_2 + 2\text{H}^+ + \text{F}^-$$

where R is a constituent of the membrane. The reaction product of 1-fluoro-2,4-dinitrobenzene with the ϵ-amino group of lysine has been identified in the acid hydrolyzate of hemoglobin-free stroma obtained from treated cells. Since the ϵ-amino group of lysine has a pK_a of about 10, it is predominantly charged at *p*H 7.8 and thus could contribute to the positive fixed charge of membrane channels.

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2. The buffer composition (millimolar) was: Na^+, 143.0; K^+, 3.6; Mg^{++}, 1.2; Ca^{++}, 1.2; Cl^-, 125.4; HCO_3^-, 22.6; $SO_4^=$, 1.2; $HPO_4^=$, 0.5; and glucose, 10. The solution was equilibrated with CO_2 and air at *p*H 7.8.
3. The reaction is *p*H-sensitive; more or less time is required to prevent lysis at lower or higher *p*H, respectively. All of the reactions were run at *p*H 7.8 with the procedure described for the difluoro reagent.
4. Fluoride inhibits glycolysis at 5 m*M*, but has no known direct effects on membrane permeability except at much higher concentrations (see *5*).
5. R. Whittam, *Transport and Diffusion in Red Blood Cells* (Williams and Wilkins, Baltimore, 1964).
6. Blockage is likely, since the reagent diffuses into the cells and reacts with protein.
7. The samples were centrifuged at top speed in Pyrex test tubes in a clinical centrifuge (International, Model CL). Cells reacted with the difluoro reagent pack tightly, and tubes can be inverted after being spun 2 minutes without loss of cells.
8. M.S.E. Ultrasonic disintegrator, 60 watts, 18 to 20 kc/sec.
9. The calculation is too long to be given here. The reagent changes the permeability of the membrane, the volume of the cell, and the net charge on the hemoglobin molecule, and all these effects must be considered.
10. Supported by NIH grant GM-08520. Two of us (H.C.B. and J.M.D.) carried out this work during tenure of Junior Fellowships from the Harvard Society of Fellows, Harvard University.

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Glucose-6-Phosphate Dehydrogenase Mosaicism: Utilization as a Cell Marker in the Study of Leiomyomas

Abstract. *The sex-linked electrophoretic variants A and B of glucose-6-phosphate dehydrogenase were studied in 86 samples of myometrium and 27 leiomyomas from five heterozygous women. All but one sample of myometrium had both A and B bands in equal or nearly equal amounts. In contrast to this, all of the leiomyomas had either an A band or a B band. Both A and B tumors were found in all uteri. These findings are consistent with the hypothesis that these tumors arose from single cells.*

Females heterozygous for the sex-linked glucose-6-phosphate dehydrogenase (G6PD) locus have two cell populations, each expressing the phenotype of one of the two alleles (*1, 2*). The two cell populations reproduce true to type throughout somatic growth (*2*) and can be used as tracers in various studies of development (*3, 4*). We now report on the application of this system to a study of multiple leiomyomas in individuals heterozygous for the G6PD electrophoretic variants A and B (*5, 6*). Our purpose was to ascertain whether these tumors arise from one or several cells. Tumors arising from single cells should exhibit only single G6PD phenotype, whereas, if the tumors originate from more than one cell, some tumors should show mixed phenotypes.

Twenty-seven leiomyomas and 86 normal samples of myometrium were obtained from the uteri of five adult females undergoing hysterectomies. Crude extracts (glass homogenates) of normal myometrium and the leiomyomas were analyzed by starch-gel electrophoresis (*6*) to determine their G6PD electrophoretic phenotypes: fast, A; slow, B; or both fast and slow, AB. Estimates of the relative amounts of A and B were made by visual comparison of the band intensities directly on the starch gel. Seven classes were distinguished, ranging from no detectable B (>95 percent A) to no detectable A (>95 percent B). The standards for classification were established from known mixtures of A and B cells. Thirteen of the leiomyomas were examined for a sex chromatin body. In all cases there was a single chromatin body, an indication of two X chromosomes per cell.

The presence of both A and B bands in nearly all samples of normal myometrium (Table 1) indicates that the average patch (group of like cells) size is smaller than the sample size.