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

$$R\text{-}NH_3^+ \rightleftharpoons R\text{-}NH_2 + H^+ \xrightarrow{\text{fluorodinitrobenzene}}$$

$$R\text{-}NH\text{-}\langle\rangle\text{-}NO_2 + 2H^+ + F^-$$

(NO_2 substituents on the benzene ring)

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*).
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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.
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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.

Table 1. Electrophoresis of myometrium and leiomyomas from AB heterozygotes.

Case No.	Size of uterine sample analyzed (10^3 mm^3)	No. of samples of each phenotype			Tumor size (10^3 mm^3)	No. of tumors of each phenotype		
		A	B	AB*		A	B	AB
3	0.001 to 0.100	0	0	7	0.100 to 92.0	2	3	0
4	0.001 to 0.100	0	1	6	0.004 to 269.0	4	1	0
5	0.001 to 0.100	0	0	14	3.300 to 180.0	5	2	0
6	0.001 to 0.100	0	0	18	0.003 to 14.2	3	1	0
11	0.001 to 0.100	0	0	40	0.036 to 150.0	3	3	0

* Only equal classes of A : B observed ($0.50 \pm .10$).

The great majority of our samples measured approximately 1 mm^3. We have had a similar experience with a variety of normal tissue samples (many less than 1 mm^3) from ten other heterozygous individuals (*3*). Of 80 samples analyzed in these normal subjects, 78 had both bands present, while only 2 (both from the cervical mucosa of one individual) had single bands.

In contrast to these results from normal tissues, all of the leiomyomas analyzed in the present study were either A or B, never AB, with both A and B tumors being found in each individual (Table 1). The presence of both A and B tumors in an individual indicates an independent origin of the multiple tumors, which is expected in a benign tumor such as the leiomyoma. In the case of malignancy, the opposite might be expected, and it seems clear that this methodology should be useful in analyzing the pattern of cellular proliferation in metastasis.

The apparent homogeneity of these tumors (from 38 mm^3 to 2.7×10^5 mm^3 in size) may seem surprising, since one might expect endothelial cells, lymphocytes, fibrocytes, and histiocytes within the tumors to produce contaminating cells. We cannot, however, detect less than 5 percent of one component, and in these tumor samples, nontumor cells within and adherent to the tumor must contribute considerably less than 5 percent of the total G6PD activity. In very small tumors these factors may play a more important role, and at present we are inclined to ascribe to such contamination the presence of a trace of a second band in a 27-mm^3 leiomyoma reported earlier (*3*).

The finding of only single phenotype tumors is consistent with the concept that each tumor arose from a single cell. It has been shown, however, in individuals with multiple myeloma who are also heterozygous at the autosomal Gm locus, that only one of the two alleles at each locus is functional within a tumor (*7*). There is no evidence of mosaicism at the Gm locus normally. Therefore these results in cases of multiple myeloma raise the possibility of disturbances of regulatory control in tumor tissue. Since there is normally mosaicism in the G6PD system, it does not seem likely that an upset in regulatory control is the basis of the single phenotype leiomyomas observed in G6PD heterozygotes. However, in studying 18 leiomyomas from five subjects heterozygous at the autosomal phosphoglucomutase locus, we have found that all of the tumors exhibited the heterozygous phenotype.

Alternatively, tumors of single phenotype might arise from multiple cells by selective overgrowth of one cell type. Selective differences have been observed in normal cell cultures between the cell types in mosaic populations of heterozygotes for both the quantitative Mediterranean variant of G6PD deficiency (*3*) and those for the A:B heterozygotes reported in this study (for example, one culture changed from 60B:40A to 20B:80A over a period of about 25 cell generations). However, the presence of both A and B tumors in each case, and the absence of heterogeneity within the tumors, make it unlikely that selection is the basis of the single phenotype tumors in heterozygotes.

It is also possible that single phenotype tumors could arise from multiple adjacent cells which by chance were of the same type. The chance that adjacent cells will be alike or different depends on how large a role clonal growth plays in the development of the tissue. If there is clonal growth, daughter cells remain next to one another. If growth were mainly nonclonal (extensive migration of cells), then groups of like cells (clones or patches) would be small and irregular in shape, and the chance that adjacent cells will be alike is relatively low. With clonal growth, patches of like cells would be relatively large and the chance that adjacent cells will be alike is relatively high. We have previously considered the statistical problem of estimating the patch or clone size in normal tissues by looking for heterogeneity in the A:B composition of very small tissue samples (*3*). This estimate is based upon the fact that the degree of heterogeneity in A : B composition between samples is inversely related to the number of clones or patches per sample. Sample measurements are converted to cell number estimates by DNA determinations of the sample and known numbers of euploid fibroblast controls. Applying this methodology to our present material, we estimate that the patch size in myometrium

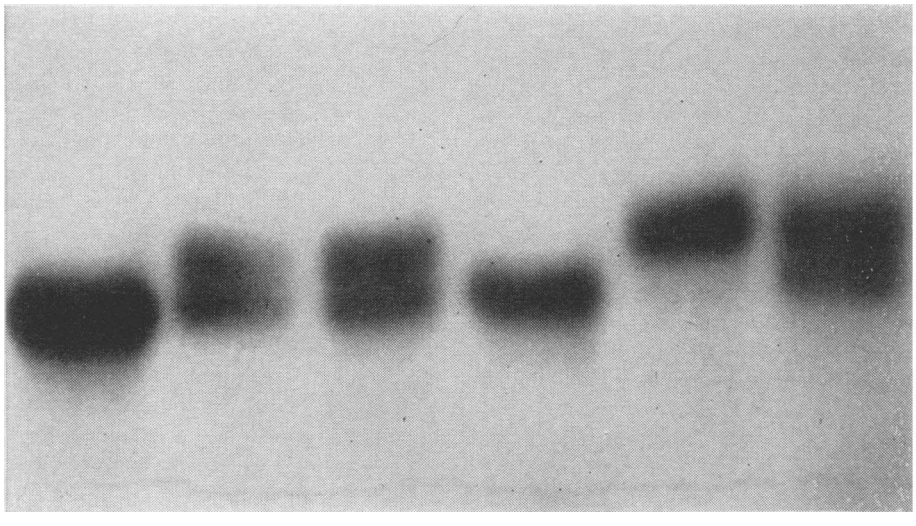

Fig. 1. Electrophoresis of tissue samples from normal myometrium and leiomyomas in one individual. Slots from left to right: 1st, B tumor; 2nd and 3rd, AB normal myometrium samples; 4th, B tumor; 5th, A tumor; 6th, AB normal myometrium sample.

is of the order of several thousand cells, possibly as high as 10,000 cells.

Assuming a patch size of 10,000 cells and a regular patch shape (cubic), we can estimate the chance that a single phenotype tumor will arise from a given number of adjacent cells. We calculate this probability only for the case of two cells, since the probability for any higher number of adjacent cells will necessarily be lower. The probability that a single phenotype tumor can arise from two cells is equal to the ratio of like pairs of adjacent cells to the total pairs of adjacent cells within and surrounding a cube of 10,000 cells. This turns out to be 0.96, and the corresponding probability for our run of 27 tumors is about 33 percent. While our data are insufficient to exclude a two-cell origin of these tumors, and therefore statistically prove single cell origin, they do indicate that the tumors arise from a small number of cells. For example, for a starting number of seven cells, the probability of our run of 27 single phenotype tumors is less than 5 percent.

Whether the leiomyomas begin from one or a few cells, the single G6PD phenotypes of these tumors (some containing more than 10^{11} cells) in heterozygous individuals constitute exceptionally strong evidence for the permanence of X-chromosome inactivation.

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Hormone-Induced Stabilization of Soluble RNA in Pea-Stem Tissue

Abstract. *When indoleacetic acid labeled with carbon-14 in the carboxyl group is fed to excised green pea-stem segments, growth is initiated, and there is a parallel progressive labeling of the RNA extracted by cold phenol. The bulk of the label is found in the 4S fraction. This fraction is more resistant to degradation by ribonuclease than a similar fraction obtained from tissue not treated with indoleacetic acid.*

There is evidence (*1*) that hormone action is intimately associated with RNA synthesis. Both in animals (*2*) and in plants (*3*) the amount of RNA rises in the target tissue shortly after hormone application, and this rise can be blocked by actinomycin D. Such a rise in RNA could be due to (i) an increased rate of synthesis, (ii) a decreased rate of degradation, or (iii) both. In green pea-stem tissue stimulated to growth by the plant hormone indole-3-acetic acid (IAA), both mechanisms seem to be operative. In this report we shall describe only the decreased susceptibility to degradation by ribonuclease.

Subapical stem sections, 10 mm in length, cut from 14- to 15-day-old pea seedlings grown in light, were excised and induced to rapid growth by the application of $10^{-4}M$ IAA in 1 percent sucrose and 0.02*M* potassium phosphate, *p*H 6.1 (*4*). The IAA was labeled with C^{14} in the carboxyl group (*5*) and had a specific activity of 16.9 mc/mmole. RNA was extracted by homogenizing the frozen tissue with cold, freshly redistilled phenol in the presence of an equal volume of 0.01*M* tris-HCl buffer, *p*H 8.0 (*6*). The homogenate was allowed to stand at room temperature for 1 hour, and then it was centrifuged for 20 minutes at 3500*g* and the aqueous layer removed. To this layer were added 2 percent (final concentration) potassium acetate and 2.5 to 3.0 volumes of cold 95 percent ethanol. After standing overnight in a cold room, this mixture had deposited a flocculent white precipitate which was harvested by centrifugation at 20,000*g* for 20 minutes. The precipitate was redissolved in the tris-HCl buffer and reprecipitated with potassium acetate–ethanol. This procedure was repeated three additional times and it yielded a product with a characteristic RNA spectrum and constant specific activity (*7*). The longer the incubation of the tissue in labeled IAA and the higher the concentration of labeled IAA in the incubation medium, the higher was the specific activity of this purified RNA (Fig. 1).

RNA from both control and IAA-treated tissue was incubated with crystalline pancreatic ribonuclease. The reaction was stopped and unhydrolyzed RNA was precipitated by the addition of a mixture of uranyl acetate and perchloric acid (*8*). Then the reaction mixture was passed through a 0.45-μ Millipore filter. The optical density (O.D.) of the filtrate at 260 mμ, as measured in a Perkin-Elmer recording spectrophotometer, model 350, was taken as a measure of hydrolysis of RNA by enzyme. Radioactivity was measured in a liquid scintillation counter (ANSitron).

The longer the incubation period of the tissue in $10^{-4}M$ IAA and the higher the specific activity of the extracted

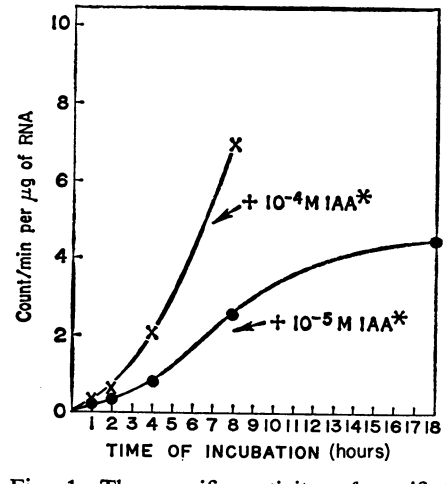

Fig. 1. The specific activity of purified RNA of pea-stem sections incubated in the presence of indoleacetic acid labeled with C^{14} in the carboxyl group for varying periods of time. At the indicated times, the sections were harvested, homogenized in phenol, and the RNA was purified as described in the text. Samples were then counted for radioactivity and measured for optical density, and this provided the data for calculation of the specific activity.