b. The ring protons, c, are further removed from the site of interaction and would not be expected to respond to the same degree.

Transmitted changes in the electronic environment resulting from this type of interaction would account for the changes in chemical shift of the lecithin protons. The more pronounced changes in the chemical shift of the *N*-methyl protons could result from their proximity to the interacting molecule. Normally one would expect the phosphorylcholine moiety to assume a conformation allowing interaction of the quaternary nitrogen and the negative charge of the phosphate.

The effect of the DDT on the resonance peak of the protons of the associated water molecule depends on the initial concentration of the lecithin. At concentrations higher than 0.018 molal of lecithin, a low-field change in the chemical shift of lecithin is observed by the addition of DDT. In more dilute solutions of lecithin, however, DDT produced significant line broadening with only small changes in chemical shift.

The potential for such an interaction in biological systems will depend on the nature of the environment of the lecithin molecule in a membrane, and this situation has not yet been defined. However, a rather strong association can occur between lecithin and DDT, and further studies in more complex systems should provide some indication of the significance of this interaction. The involvement of the benzylic proton of the DDT is of significance, and this observation would substantiate observations of Ross and Biros (3).

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Xeroderma Pigmentosum: A Rapid Sensitive Method for Prenatal Diagnosis

Abstract. *When normal human cells, capable of repairing ultraviolet-induced lesions in their DNA, are incubated in the thymidine analog 5-bromodeoxyuridine after ultraviolet irradiation, the analog is incorporated into the repaired regions. When such repaired cells are subsequently irradiated with 313-nanometer radiation and placed in alkali, breaks appear in the DNA at sites of incorporation of 5-bromodeoxyuridine, inducing a dramatic downward shift in the sedimentation constant of the DNA. Cells from patients with the disease xeroderma pigmentosum, which causes sensitivity to ultraviolet, are incapable or only minimally capable of repair; such cells incorporate little 5-bromodeoxyuridine into their DNA under these conditions and, upon 313-nanometer irradiation and sedimentation in alkali, exhibit only minor shifts in DNA sedimentation constants. When fibroblasts developed from biopsies of normal skin and of skin from patients with xeroderma pigmentosum, as well as cells cultured from midtrimester amniotic fluid, were assayed in this fashion unequivocal differences between normal and xeroderma pigmentosum cells were shown. Xeroderma pigmentosum heterozygotes are clearly distinguishable from homozygous mutants, and results are available 12 hours after irradiation.*

A method for prenatal diagnosis for a genetic defect should be unequivocal and reasonably rapid so that a therapeutic abortion, if indicated, can be performed as soon as possible. In the case of the rare autosomal recessive human disease xeroderma pigmentosum (XP), no prenatal diagnostic test having these characteristics has previously been described. Any decision to perform an abortion is best made after consideration of the results of the method outlined below, other analyses of the repair capabilities of amniocentetic cells, and medical and genetic analysis of the family.

Xeroderma pigmentosum is characterized by extreme sensitivity to sunlight resulting in changes in skin cells which eventually lead to multiple actinic carcinomas (1). When XP cells are examined for DNA repair after ultraviolet irradiation they show a decrease or absence of unscheduled synthesis (2), decrease or absence of repair replication (2), and no excision of pyrimidine dimers induced by ultraviolet (3). These phenomena reflect the apparent molecular basis of the disease —lack of a functional form of the ultraviolet endonuclease (3, 4). However, the three phenomena are less satisfactory as tests for normal or abnormal repair in fetal cells derived from transab-
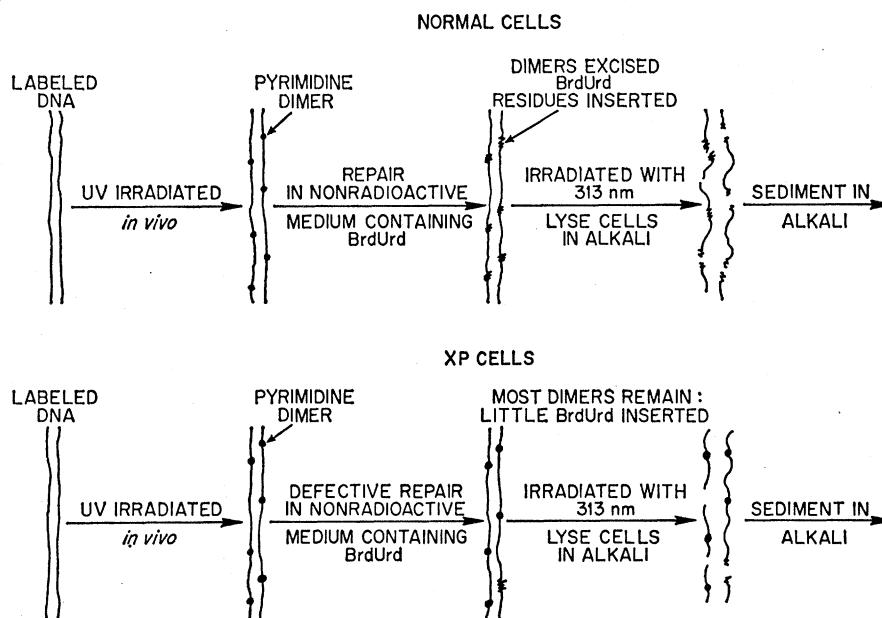

Fig. 1. Scheme for the detection of normal or defective repair in normal or XP cells. For control purposes, both cell types are incubated in nonradioactive medium containing thymidine rather than BrdUrd.

dominal amniocentesis if therapeutic abortion is to be achieved, where indicated, within a reasonable time (5). Unscheduled synthesis may require a week or more for autoradiographs to develop; repair replication requires a long centrifugation to equilibrium in cesium chloride, and the differences between normal and XP cells are not large; dimer excision measurements are difficult and, since normal cells excise only about 50 percent of the ultraviolet-induced dimers from their DNA (6), differences between normal and XP cells may not be impressive.

We have developed an assay for repair of ultraviolet damage in which approximately threefold differences in the molecular weight of the cellular DNA of normal cells (repairing ultraviolet damage) and XP (not repairing such damage) are observable after ultraviolet irradiation. The data can be made available 12 hours after irradiation. The method employs centrifugation in alkaline sucrose of the DNA of cells that have been allowed to repair-replicate in the presence of the thymidine analog 5-bromodeoxyuridine (BrdUrd). The repair-replicated regions containing BrdUrd are rendered alkaline-labile by irradiation with 313-nm light

(7) (see Fig. 1). We have applied this assay to (i) three cell cultures derived from cells obtained by midtrimester transabdominal amniocentesis (from patients with no history or familial incidence of XP, (ii) three fetal skin fibroblast cultures (one from the same pregnancy as the source of an amniocentesis culture), (iii) two skin fibroblast cultures from two unrelated patients with the uncomplicated form of XP, (iv) one skin culture from a patient with XP and neurological complications [the DeSanctis-Cacchione syndrome (8)], and (v) a skin fibroblast culture of a presumed XP heterozygote (the mother of the patient from whose cells we derived one of our XP cultures). The normal cells, that is, the three amniocentesis cultures and the normal skin fibroblasts, all exhibit large shifts in the molecular weight of the DNA (as observed on alkaline sucrose gradients) after the BrdUrd protocol of Fig. 1. The two XP cultures and the DeSanctis-Cacchione culture do not exhibit such large changes in molecular weight. Although the normal fibroblasts used in this study were all of fetal origin, we have performed numerous experiments (unpublished) that demonstrate the excision of pyrimidine dimers from a variety of cell lines (except for XP cells) of postnatal and adult origin. These results indicate that excision repair persists through neonatal and adult life. Since normal skin and amniocentetic cells repair in a similar manner and XP skin cells show reduced repair, it is reasonable to expect that XP amniocentetic cells will behave like XP skin cells. However, we have had no opportunity to examine any potential XP amniocentetic cells. In any prenatal assay it is important that the heterozygote not be confused with mutant homozygote. The heterozygote cells in our assay exhibited decreases in molecular weight indistinguishable from the other normal cells examined.

Procedures for deriving cell cultures from skin biopsies and their subsequent routine handling have been described (3), as have procedures for securing fetal cells by transabdominal amniocentesis and the subsequent culture of such cells (9). Fifty thousand cells were plated in 50-mm plastic petri dishes, and the DNA was labeled by incubating the cells in Eagle's medium with 10 percent calf serum and 1 to 5 μc of [^{3}H]thymidine per milliliter (Schwarz BioResearch) for 18 to 24 hours. The labeled medium was replaced with nonradioactive medium for 4 hours to deplete the radioactive thymidine pool.
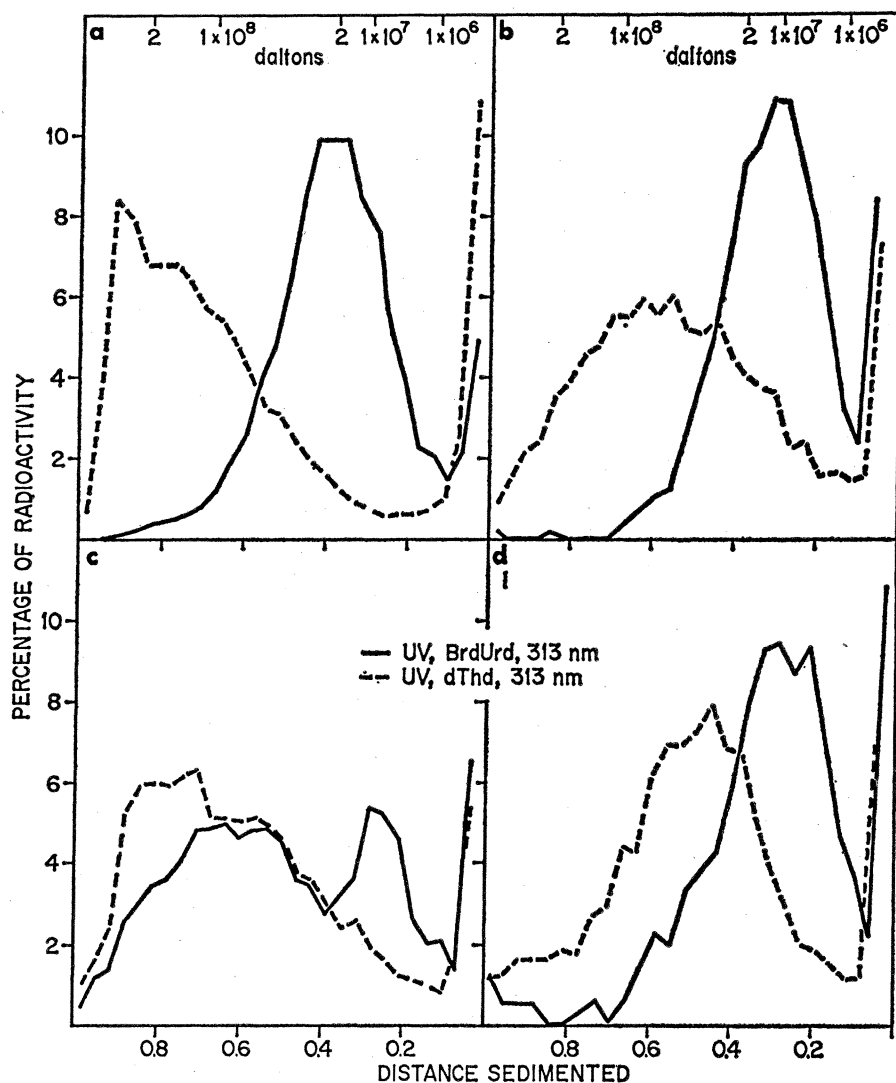

Fig. 2. Sedimentation patterns in alkaline sucrose of the radioactivity from cells labeled with [^{3}H]thymidine which have been irradiated with 200 ergs of 254-nm radiation per square millimeter, incubated for 16 to 20 hours in bromodeoxyuridine (BrdUrd) (———) or thymidine (dThd) (- - - -), and then exposed to 313-nm radiation before being lysed on top of the gradients. (a) Normal fibroblasts 1; (b) heterozygote fibroblasts; (c) XP fibroblasts 1; and (d) normal amniocentetic cells (AC-1). Sedimentation conditions are described in the text. The curves shown are tracings of the output of a computer that subtracted background and calculated the percentage of the total radioactivity in each fraction, and plotted these values against the distance sedimented. The molecular weight scale for single-stranded DNA, by reference to separately sedimented phage T4 DNA, λ DNA, and ϕX174 DNA, is shown at the top of the figure. Doses of 313-nm light were: (a, c, and d) 4.5×10^5 erg/mm^2; (b) 7×10^5 erg/mm^2.

The medium was removed briefly, the cells were irradiated with 200 ergs of ultraviolet per square millimeter from a germicidal lamp emitting principally 254-nm radiation, and the medium was immediately replaced and made $10^{-4}M$ with either BrdUrd or thymidine. Hydroxyurea ($2 \times 10^{-3}M$) was also present to eliminate competition between semiconservative replication and repair replication for the added nucleosides (10). After 16 to 18 hours the cells were removed from the petri dishes by scraping into a solution of ethylenediaminetetraacetate (EDTA) and saline (3). The cells were counted, resuspended at 2×10^5 cells per milliliter, and irradiated, if desired, in microcuvettes having a light path of 1 cm, with 313-nm light obtained from a large quartz-prism Hilger monochromator. The fluence through a thin piece of Mylar, to filter out any shorter wavelengths, was 0.9×10^5 erg/(mm² × min). For sedimentation analysis approximately 10^4 cells were lysed by being gently pipetted onto a mixture of 0.2 ml of $1M$ NaOH and $0.01M$ EDTA, on top of 3.6 ml of an alkaline ($0.3M$ NaOH), 5 to 20 percent sucrose gradient containing $2M$ NaCl. After the samples had remained for 1 hour at room temperature, they were centrifuged at 20°C in a Beckman SW-56 rotor for 3 hours at 30,000 rev/min. Between 27 and 30 fractions (eight drops each) of the gradients were collected from the bottom of the tubes onto filter paper. The acid-insoluble radioactivity was counted in a scintillation counter, and the distribution of radioactivity and the weight-average and number-average molecular weights were obtained by computer analysis (7). All the radioactivity (± 15 percent) applied to the gradient was collected from it.

The DNA of cells not irradiated with 313-nm light sedimented to positions corresponding to weight-average molecular weights of 140 (± 20) $\times 10^6$ (data not shown), and similar values were found for cells incubated in either thymidine or BrdUrd irrespective of whether they had received 200 ergs of 254-nm light per square millimeter (7).

Irradiation by 313-nm light alters the sedimentation pattern by amounts dependent on the history of the cells, that is, the presence or absence of previous ultraviolet irradiation and whether the cells were incubated in thymidine or BrdUrd. Typical comparisons for 254-nm-irradiated cells incubated in either thymidine or BrdUrd are shown in Fig. 2. Large differences are apparent in the

Table 1. The single-strand weight-average molecular weights (12) of DNA of cells exposed to 4.5 × 10⁵ ergs of 313-nm light per square millimeter after irradiation with 254-nm light and incubation in thymidine or bromodeoxyuridine (BrdUrd) for 16 to 20 hours. [In the absence of 313-nm irradiation the weight-average molecular weights of all samples were 140 (± 20) × 10⁶.] AC, amniocentetic cells.

Cell type	Molecular weights × 10⁻⁶				Calculated excess breaks per 10⁸ daltons* (BrdUrd compared with thymidine)
	No 254 nm		254 nm		
	Thymidine added	BrdUrd added	Thymidine added	BrdUrd added	
Normal skin 1		120	140	29	2.7
Normal skin 2	77	72	77	24	2.9
Heterozygote skin†	56		83	16	4.9
AC-1		37	62	20	3.4
AC-2		83	110	22	3.7
AC-3		39	62	16	4.5
XP skin 1	110	91	110	77	0.4
XP skin 2	77	72	56	50	0.2
XP skin 3‡	110	91	110	67	0.6

* If molecular weights are in units of 10⁸, the excess breaks per 10⁸ is given by excess breaks = 1/(M : BrdUrd) − 1/(M : thymidine). For example, for AC-1, excess breaks = 1/0.20 − 1/0.62 = 3.4; excess breaks = 3.4 per 10⁸ molecular weight. † 313 nm, 7 × 10⁵ erg/mm². ‡ DeSanctis-Cacchione syndrome.

sedimentation patterns of the DNA of cells incubated in thymidine after ultraviolet irradiation (and exposed to 313-nm light). We have not investigated this aspect of the problem, except to note that the decrease in sedimentation constant is smaller in the more vigorously growing cultures (11). Despite these changes in the sedimentation patterns of cells incubated in thymidine—what we might call the background noise of this experiment—it is important to note that, even in Fig. 2d, most of the radioactivity sediments to positions corresponding to sizes greater than 2×10^7 daltons of single-stranded DNA. On the other hand, the sedimentation pattern of irradiated cells incubated in BrdUrd is markedly lower for normal and heterozygous fibroblasts and for amniocentetic cells (Fig. 2, a, b, and d). We estimated earlier (7) that an exposure of 7×10^5 ergs of 313-nm light per square millimeter would cause a break in 30 to 50 percent of the repaired regions in normal fibroblasts. The exposure used in most of our experiments, 4.5×10^5 erg/mm², would result in a break in 20 to 30 percent of the repaired regions. Although the DNA from XP fibroblasts incubated in BrdUrd also sediments slower than the thymidine control (Fig. 2c), the degree of change is obviously much less than that observed in the other cell lines.

The typical experiments, shown in Fig. 2, and numerous others are summarized in Table 1. Clearly, the smallest weight-average molecular weights (12) are observed in ultraviolet-irradiated normal, heterozygote, and amniocentetic cells incubated in BrdUrd. The difference between cells incubated in BrdUrd and those incubated in thymi-

dine is well indicated by the last column in the table. There are two categories of cells—the normal cells, heterozygotes, and amniocentetic cells which display large amounts of repair in this type of assay; and the xeroderma pigmentosum cells which do not. The number of breaks in the nonrepairing cells —the three xeroderma pigmentosum fibroblasts—are almost an order of magnitude smaller than those in the other three cell types. The nature of the small changes observed for XP cells is not known but may be associated with a low level of excision repair or some other type of repair system (13).

The experiments indicated in Fig. 2 and summarized in Table 1 represent the effects of 313-nm light on cells incubated for 16 to 20 hours after an initial exposure to 200 ergs of 254-nm light per square millimeter. We have chosen these parameters because the effects are maximum with 200 erg/mm² and incubation for 12 hours. However, it is easy to detect the changes in normal fibroblasts after only 2 hours of incubation (unpublished experiments). Bigger changes can be observed with higher doses of 313-nm light, but such exposures also affect cells incubated in thymidine and hence do not improve markedly the signal-to-noise ratio. Thus, in the employment of this assay for the prenatal diagnosis of XP we may expect (i) large, unequivocal (approximately threefold or more) differences between normal and XP cells; (ii) no confusion of heterozygote with homozygous mutant; and (iii) rapid availability of the results. These are the essential requirements of an adequate prenatal diagnostic procedure. This test can be applied to subse-

quent pregnancies in families where there is already one offspring with XP. To minimize any familial idiosyncracies in nucleoside metabolism, amniocentetic cells should be compared to fibroblasts from parents and prior offspring.

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11. Cells derived from amniocentesis may be in a much later stage of cellular aging than cells taken directly from biopsies of fetal or neonatal skin [see (5)]. The greater sensitivity of amniocentetic cells to 313-nm light after ultraviolet and incubation in thymidine compared to fetal or other skin culture cells may be a reflection of differential cellular age.
12. We have tabulated weight-average rather than number-average molecular weights. For practical reasons all the samples were sedimented for the same length of time and the range of molecular weights in any particular gradient may be from 10^8 to 10^9. Since the molecular weights corresponding to fractions near the top of the gradient approach zero, the value of the number-average molecular weight is exquisitely dependent on the number of fractions at the top of the gradient which are excluded from the estimation of this average. (If all fractions were included, the number-average molecular weight would be zero.) The weight-average molecular weight is much less dependent upon this arbitrary decision. The use of the weight-average molecular weight obviously does not give the proper average number of breaks, but it does yield a reproducible set of numbers.
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Amino Acid Composition of Proteins as a Product of Molecular Evolution

Abstract. *The average amino acid composition of proteins is determined by the genetic code and by random base changes in evolution. Small but significant deviations from expected composition can be explained by selective constraint on amino acid substitutions. In particular, the deficiency of arginine in proteins has been caused by constraint, during evolution, on fixation of mutations substituting arginine for other amino acids.*

The average amino acid composition of proteins can be predicted from the genetic code, if there is random arrangement of nucleotide bases within the genes (cistrons) (*1–3*). The predicted amino acid frequencies are accurate except for that of arginine, whose observed frequency is only half as large as that expected from random arrangement of bases (*2, 3*). For other amino acids, the difference between observed and expected frequency is much smaller, and sometimes is negligible. According to King (*4*), the same amino acids occur more frequently than expected, and others less frequently than expected, whether proteins from mammals or from bacteria are sampled.

Amino acid composition is a product of molecular evolution. The overall agreement between observed and expected amino acid compositions suggests that amino acid substitutions in evolution were produced by random fixation of selectively neutral or nearly neutral mutations (*2, 5, 6*). Deviations from the expected frequency, such as the deficiency of arginine, must have been caused either by nonrandom mutations or, more likely, by selective constraints. In fact, many mutations appear to be deleterious, and are eliminated from the population by selection, although neutral mutations predominate among those mutants that contribute to molecular evolution and enzyme polymorphism (*7*). Some evidence suggests that functionally similar amino acids are substituted more frequently than less similar ones (*8–10*). We now report our efforts at clarifying the nature of the nonrandomness in the evolution of the amino acid composition in proteins.

Probably the simplest way of treating the process of evolutionary change in amino acid composition is to use the method of Markov chains. We use a 20 by 20 matrix giving transition probabilities for any one of the 20 amino acids to any other during a unit of time. Starting from a given amino acid composition, we can then compute the expected composition after *n* units

of time by taking the *n*th power of the matrix. From an estimation of the ancestral sequences and with the use of comparative data on amino acid sequences in homologous proteins, a transition probability matrix could be constructed. However, for such a matrix, a large body of data would have to be compiled. To avoid these difficulties, we have tentatively used the "mutation probability matrix" of Dayhoff *et al.* (*9*). These workers counted 814 "accepted point mutations" among closely related sequences from cytochrome c, globins, virus coat proteins, chymotrypsinogen, glyceraldehyde-3-phosphate dehydrogenase, clupeine, insulin, and ferredoxin. From these mutations, they constructed an "accumulated matrix of accepted point mutations" (*9*, figure 9-3).

They multiplied this matrix by the overall "mutability" of individual amino acids per unit time to obtain the mutation probability matrix. This matrix (M) is reproduced as Fig. 1.

In contrast, if we assume that mutant base substitutions are completely random, we can construct a corresponding transition probability matrix from the genetic code (assuming only one-step mutations). This matrix (R) is shown in Fig. 2.

There are several differences between M and R. Particularly noteworthy is the fact that the transition to Arg (*11*) from a number of amino acids, such as Cys, Gly, Ile, Leu, Pro, Ser, Thr, and Trp, is much lower in M than in R. This indicates that these single-step mutations were seldom accepted by natural selection. The reverse transitions are also somewhat restricted, but not so severely. These transition rates can account for the significantly lower frequency of Arg than is expected from random base arrangement. The biochemical explanation for such selective constraint is not known, but it is conceivable that, because Arg is unusually large and contains three amine groups, its insertion might disturb normal configuration of proteins.