to methyladenine is observed (Fig. 2) (*9*). Our major finding is that the methyladenine sampled in the pyrolyzate is clearly 1-MeAde (*10*). Crude attempts at quantification by comparing MIKE peak heights indicated that 1-MeAde was less than 1 percent adenine. Treatment of both native and denatured salmon sperm DNA with ribonuclease A does not eliminate or noticeably attenutate the signal from 1-MeAde. This lessens the possibility that an RNA impurity is serving as the source of this modified base. Also, RNA should give N^6-MeAde as well (*11*). This is not observed.

In conclusion, a powerful new method is presented for the identification of modified nucleosides in intact DNA. Used in conjunction, CI and MIKE spectra provide the maximum advantages for pyrolysis studies on intact DNA. The MIKE spectrum of a single characteristic ion (for example, base $+H^+$) is used to supply structural information on ions formed from the pyrolyzate and to indicate the presence of particular nucleosides in the native DNA. Isomeric bases can be distinguished, and other ions having the same mass does not invalidate the procedure. The characterization is more secure than earlier procedures based on recognition of sets of characteristic ions or gas chromatography retention times (*12*). The detection of 1-methyldeoxyadenosine is noteworthy since it has not been detected in DNA previously (*13*).

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Premature Senescence in Cultured Skin Fibroblasts from Subjects with Cystic Fibrosis

Abstract. *Cultured skin fibroblasts from subjects with cystic fibrosis exhibited normal population doubling times in early passages. After about 13 cumulative population doublings, cystic fibrosis lines doubled more slowly than controls and ceased doubling after about 19 weekly passages. Control lines continued doubling for 27 passages. The premature senescence noted in cells from subjects with cystic fibrosis reconciles controversial observations of cell doubling reported in the literature. Data presented here demonstrate that experiments with cystic fibrosis cells in late passage may generate misleading results since differences from control lines may be ascribed to generalized senile changes rather than to specific results of the cystic fibrosis genotype.*

Cystic fibrosis (CF) is a lethal exocrinopathy (*1*) and is transmitted within families as an autosomal recessive condition. The primary gene product responsible for CF is not known. Although fibroblasts are not exocrine cells they are regarded typically as secretory cells. Because of their accessibility and retention of the donor's genetic properties over many generations in vitro, skin fibroblasts are a good model system for CF investigations. Reports from this and other laboratories suggest that skin fibroblasts in culture from subjects with CF do manifest abnormalities (*2*). As with much of the CF literature, however, frequent controversy and failure to confirm reported findings with these cells have occurred. A particular area of disagreement concerns cell population kinetics. Several laboratories reported that skin fibroblasts in culture from CF subjects divide more slowly than those from control subjects (*3*). Other investigations, including our own, failed to detect any differences in population doubling time (PDT) between lines from controls and from subjects with CF (*4*, *5*).

In experiments that we report here the PDT was dramatically increased in monolayers derived from CF subjects in comparison with control lines matched for sex, age, and passage number. In an attempt to determine the basis for these divergent conclusions we analyzed weekly mean population doublings (MPD) in terms of passage number in lines that had been maintained routinely. We found that in early passages (until approximately seven weekly subcultures had been made) no difference between CF and control lines occurred. With further passages, however, cell lines derived from subjects with CF doubled more slowly than matched control lines. Additional experiments confirmed that the replicative life-span (cumulative population doublings) and the number of subcultures before the cessation of cell renewal is significantly less in cells derived from subjects with CF. That is, in comparison with controls, cells derived from patients with CF manifest premature senescence. We propose that the subculture passage number or cell population generation at which PDT is determined is a crucial factor when CF cells are compared with controls. These findings appear to reconcile the opposing points of view concerning PDT in CF fibroblast monolayers and have major implications for interpretation of metabolic data derived from CF fibroblasts.

Skin fibroblast cultures were obtained, maintained, frozen, and thawed as described (*5*). Cell lines from eight subjects with CF (five males and three females; mean age 11 years) and five control subjects (two males and three females; mean age 13.2 years) at passages 9 and 10 were

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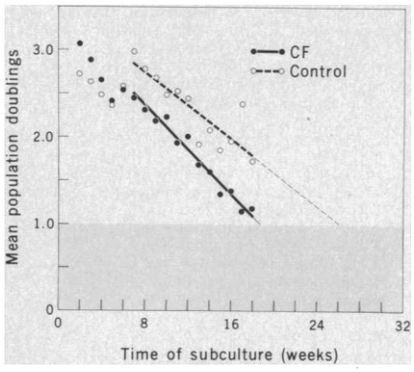

Fig. 1. Mean population doublings each week of subculture. Symbols: ●, the mean of four subjects with CF; ○, the mean of six control subjects at each passage. No difference between CF and controls occurred until passage 7, after which time the MPD of CF cells was less than controls (as determined by Student's t-tests each week). The least-squares regression lines were calculated for each group. Regression lines were calculated from peak cell population doubling, which occurred at 7 weeks, until the end of the experiment at 18 weeks. Extension of the regression lines to the estimated last week of population doubling (1.0) predicts that CF cell lines would cease doubling at about 19 passages and control lines at about 26 passages (*6*). [CF ($N = 4$), $b = -0.13$, $a = 3.39$, $r = -.98$, $s_{y \cdot x} = 0.09$; control ($N = 6$); $b = -0.09$, $a = 3.52$, $r = -.85$, $s_{y \cdot x} = 0.20$.]

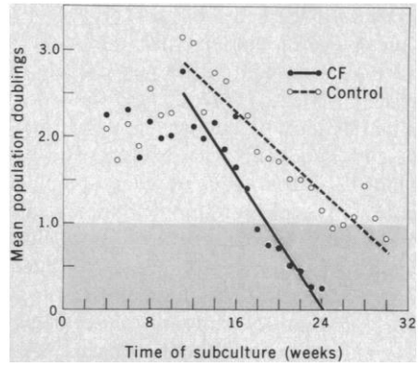

Fig. 2. Mean population doubling each week of subculture. Symbols: ●, six cell lines, two each from three CF subjects; ○, four cell lines, two each from two control subjects. No difference in PDT was observed during the first seven subcultures. After this time CF lines doubled more slowly (as determined by Student's t-tests each week). Regression lines were calculated from peak MPD (*6*) at passage 11 until the end of the experiment. The regression lines intersect the 1.0 population doubling at 18.9 weeks (CF) and 27.5 weeks (control). [CF ($N = 6$): $b = -0.19$, $a = 4.63$, $r = -.98$, $s_{y \cdot x} = 0.17$; control ($N = 4$): $b = -0.11$, $a = 4.12$, $r = -.94$, $s_{y \cdot x} = 0.24$.]

grown in 75-cm² plastic cell culture flasks (Falcon) in 25 ml of Eagle's minimum essential medium with L-glutamine, 10 percent fetal calf serum, and 2 percent (by volume) of penicillin-streptomycin (penicillin, 5000 U/ml; streptomycin, 5000 μg/ml) at 37°C, in an atmosphere of 95 percent air and 5 percent CO_2. Monolayers reached confluence in 6 to 8 days, and the cells were harvested, suspended, and counted. The cells (5×10^4 per milliliter of medium) were seeded in plastic disposable multiwell (24, 1.6-cm wells) trays (Linbro). The trays with cells in subculture Nos. 10, 11, or 12 were incubated at 37°C in 95 percent air and 5 percent CO_2 until the cells were sampled. At each sampling time after seeding (10, 24, 48, 72, and 96 hours) the medium in each well was removed, 1 ml of warm (37°C) trypsin-EDTA (Gibco) was added to each well and the cells incubated for 10 minutes at 37°C. The cells were suspended in the trypsin-EDTA solution by gentle pipetting. Portions were removed for cell counting in a ZBI Coulter Counter.

The mean cell number was obtained for each cell line at each sampling time and plotted against time on semilog paper. The PDT was determined graphically (least-squares analysis) for each cell line. Three separate experiments were performed three different weeks. Monolayer cells derived from subjects with CF doubled more slowly than those from controls [PDT (CF), 70.5 ± 3.0 hours; PDT (controls), 40.9 ± 1.2 hours; Student's t-test, 9.105, $P < .0005$]. These findings were at variance with our previous results (*5*) in which no PDT differences between CF and control lines were observed. Since these data were based on cells in passages 10 to 12 and our previous study was with cells in passages 2 to 7, we wondered whether there was some differential relationship between subculture number and PDT. This notion was tested.

Monolayers were generated from upper arm skin biopsies. After approximately 4 weeks the initial monolayer was obtained in 25-cm² flasks. Each flask was subcultured (passage 1) by seeding 6.5×10^5 cells in 75-cm² tissue culture flasks. Monolayers became confluent in about 1 week and the cells were counted. Each week thereafter until the end of the experiment, monolayers were harvested, portions counted, and the lines split 1:4 into 75-cm² flasks. Cells were reseeded each week at 6.5×10^5 cells per flask. Until subculture 7 (about 13 cumulative population doublings for all cell lines) no difference in MPD between CF and control cell lines occurred. However, with continued passage the number of cell doublings per week was less in the CF lines. The cell lines were terminated after 18 weekly subcultures. Regression analyses were performed on these data (Fig. 1). From the calculated least-squares regression lines we estimated that CF cell lines would cease their population doubling after about 19 weeks of weekly subculture and that the control lines would cease doubling at about 26 weeks of subculture. An additional study was designed to test these predictions.

Three CF and three control lines that had been stored in liquid nitrogen at passage 4 were thawed. Cells (1.6×10^5) from each line were seeded in two 25-cm² flasks, labeled A and B for each line. One control line (from a 25-year-old female) did not survive thawing. Accordingly, the MPD of three CF cell lines (10-year-old male, 16-year-old male, and 24-year-old female) and two control lines (13-year-old male and 12-year-old male) was determined. The cells in flasks A and B from each cell line were harvested on successive days once a week for the duration of the study and the cells counted each week. We were able to calculate from these experiments the MPD each week, the cumulative population doublings (replicative life-span), and the number of passages before senescence.

Using as a criterion of senescence in a cell line its failure to double in cell number in 1 week for four successive weeks, we found that none of the CF lines continued doubling after 22 weekly subcultures (mean, 18.8 ± 1.1 weeks). Control line 59A failed to double after subculture 23. At this writing, subculture B of control line 59 and both A and B of control line 60 are still doubling after 31 passages (mean, 29 ± 2.0) ($t = 4.89$, $P < .005$). The average cumulative population doublings were: CF, 28.9 ± 2.2; controls, 44.8 ± 2.1 ($t = 4.92$, $P < .005$) (*7*). Regression lines were calculated (Fig. 2). Population size determinations of cells in culture are subject to numerous experimental variables. We were surprised, therefore, at the unusual precision of predicted onset of senescence based on the first study (CF, 18.8, and controls, 26.3 weekly passages) and the data obtained in the second study [CF, 18.9, and controls (not yet senescent), 27.4 weekly passages]. Time of onset of senescence and cumulative doublings in control lines were consistent with other estimates (*8, 9*).

Other data in diverse systems also suggest that a premature aging process may occur in CF. (i) Increased sweat electrolytes provide the most secure diagnostic sign for CF (*10*). Sweat electrolytes increase with age; in fact there is overlap of sweat electrolytes between patients with CF and "normal" non-

carrier adults (*11*). (ii) Parotid saliva from subjects with CF and their heterozygous parents contains a greater number of "fast isoamylases" than do controls (*12*). It is thought that these isozymes of amylase migrate faster in electrophoretic fields because of deamidation of asparagine and glutamine residues (*13*). Deamidation of proteins in general (*14*) and parotid salivary amylase specifically (*15*) have been related to aging. (iii) Eosinophilic plugs were shown to occur frequently in the acini of the labial mucous salivary glands of children with CF. These plugs were frequently found in minor salivary glands of normal adults but were rarely observed in normal children less than 19 years old (*16*). It appears, therefore, that premature senescence in cultured skin fibroblasts from subjects with CF is not an isolated aging phenomenon in this disease. The abnormal gene product or products responsible for CF may have a specific effect on one or more factors involved in aging. It is of some interest that accumulation of cellular calcium has been discussed as a factor in aging (*17*). Intracellular calcium pool size is greater by about 30 percent in skin fibroblasts from CF and CF carriers in comparison with their respective controls (*5*, *18*). The reported calcium differences and the premature senescence in cultured skin fibroblasts from CF subjects may have implications for aging studies as well as for CF.

Our findings suggest that CF be added to that group of human diseases in which skin fibroblasts in culture express premature aging (*19*). Furthermore, our data reconcile controversial PDT findings by showing that at early passages no PDT differences exist between CF and controls and that at late passages CF cells do indeed replicate more slowly. Also, experiments with late-passage fibroblasts may generate misleading results in that observed differences between CF and control cell lines may be ascribed to generalized senile changes rather than to specific results of the CF genotype. Skin fibroblasts are a useful model for the study of CF. In view of the data presented here, however, we caution that interesting findings concerned with fibroblasts in CF be repeated with early-passage cells to avoid potentially misleading conclusions.

Note added in proof: Since this report was accepted, we have found that incorporation of tritiated thymidine into DNA and plating efficiency of cells support the cell population kinetic data. Incorporation of tritiated thymidine into DNA was significantly decreased in cells from CF subjects in comparison with controls at passages 10 to 12; no differences occurred at passages 4 to 7. Plating efficiency was no different at passages 5 to 7; but at passages 11 to 13, significantly fewer clones were formed from CF strains in comparison with controls.

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Methane Efflux from Lake Sediments Through Water Lilies

Abstract. *During winter, when water lilies have no surface leaves, the gases in the rhizome lacunae approach equilibrium with the gases of the sediment water. The resulting increase of internal pressure is manifested by the sustained streams of bubbles (up to 37 percent methane and 6 percent carbon dioxide) that escape when emerging leaves are torn in the spring. Methane continues to enter the roots and rhizome during summer, rapidly moves up the petioles, and passes out through the emergent leaves into the atmosphere.*

The importance of methane to the carbon cycle of lake ecosystems is beginning to be understood. It has been estimated that nearly half the organic carbon reaching the sediment of eutrophic lakes leaves the sediment as CH_4, either in dissolved form or as bubbles (*1*-*3*). In establishing the CH_4 budgets for lakes, investigators have thought that the only escape route from the lake is across the air-water interface, either as bubbles originating in the sediment or through limited surface exchange (*1*-*4*). We report here on another interface of potential significance in the loss of CH_4 to the atmosphere, the interface between the sediment water and the internal gas phase of rooted aquatic plants. We have found that a high percentage of the CH_4 leaving the littoral zone of eutrophic Duck Lake (*5*) during August escapes through the gas passages of the water lily *Nuphar luteum*.

Nuphar luteum, a yellow water lily, perennates in lakes by means of a horizontal creeping rhizome at or just below the surface of the lake sediment. The rhizome may be up to 10 cm in diameter and

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