# *HYBRIDIZATION OF TWO BIOCHEMICALLY MARKED HUMAN CELL LINES**, †*

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*Abstract.*—A hybrid cell line of clonal origin has been obtained by cocultivation of two biochemically marked human cell strains. One parental line is diploid and derived from a male infant with orotic aciduria, a rare autosomal recessive disease. This line has deficient activity for the final two enzymes in the biosynthetic pathway leading to uridylic acid and possesses the B electrophoretic type of glucose-6-phosphate dehydrogenase. The other parental line (D98/AH-2) is heteroploid, is resistant to 8-azahypoxanthine, and has deficient inosinic acid pyrophosphorylase activity. It displays the $A^+$ variant of glucose-6-phosphate dehydrogenase. The $A^+$ and B types of this dehydrogenase are known to be determined by allelic, sex-linked, Mendelian genes. The cloned hybrid cells exhibit genetic traits of both parents: (1) Their modal chromosome number is approximately the sum of those of the two parental lines; (2) they have levels of activity for both enzymes affected by the gene for orotic aciduria which are intermediate between those of the two parental lines; (3) they have higher activity than the D98/AH parent for inosinic acid pyrophosphorylase; (4) they have both $A^+$ and B isozyme bands of glucose-6-phosphate dehydrogenase. These hybrid cells represent the first known example of a cloned line of mammalian origin in which two X-linked allelic genes function.

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In 1960, Barski, Sorieul, and Cornefert[1] reported the formation of a hybrid cell line which was obtained from a mixed culture of two heteroploid mouse lines. Since that time, many somatic cell crosses, both within and between species, have been described. A human cell line was employed as one of the parental cell types in the cross reported by Weiss and Green.[2] The purpose of this paper is to describe a hybrid cell line in which both parental cell lines are of human origin, and in which marker genes on X chromosomes derived from each parent are active.

One parental line is a diploid strain which was obtained from a patient with orotic aciduria,[3] a rare autosomal recessive disease. Cells of this line have greatly reduced quantities of the final two enzymes in the biosynthetic pathway leading to uridylic acid.[4, 5] The affected enzymes are orotidine-5′-monophosphate (OMP)‡ decarboxylase (E.C. 4.1.1.23) and OMP pyrophosphorylase (E.C. 2.4.2.10).

The other parental cell line, D98/AH,[6] is resistant to 8-azahypoxanthine and has deficient levels of activity for inosinic acid pyrophosphorylase (or hypoxanthine:xanthine:guanine phosphoribosyltransferase (E.C. 2.4.2.8)).[7, 8] This enzyme catalyzes the conversion of hypoxanthine to inosinic acid and is

also believed to be responsible for the conversion of the 8-aza analogue of hypoxanthine to its cytotoxic ribotide.

*Materials and Methods.—Cell strains:* The orotic aciduric (AUC) strain[4] is a diploid line developed from a male infant.[9] The heteroploid human line (D98/AH-2)[6] is probably descended from the HeLa line.[10] It was obtained from the American Type Culture Collection (CCL 18.3).

*Culture media:* The medium used to select against the D98/AH parent was modified HAT medium[6, 11, 12] and contained Eagle's[13] minimal essential medium (MEM) supplemented with 10% fetal calf serum and $10^{-6}$ *M* amethopterin (Lederle, Pearl River, New York), and $1.6 \times 10^{-5}$ *M* thymidine, $10^{-4}$ *M* hypoxanthine, and $6 \times 10^{-6}$ *M* glycine (Nutritional Biochem. Co., Cleveland, Ohio). The hybrid line and the D98/AH line were maintained in MEM supplemented with 10% fetal calf serum; the AUC strain was maintained in MEM supplemented with 5% NCTC 109, 1 mM Na pyruvate, 0.1 mM nonessential amino acids,[13] 15 $\mu$g/ml uridine, and 12% fetal calf serum (all from Grand Island Biol. Co., Grand Island, New York). We shall designate this medium as medium A. In a number of experiments, the three lines were grown in nucleomedium.[4] In these experiments, nucleomedium contained 12% (v/v) pooled fetal calf sera, rather than 12% pooled human sera as previously described.[4] Cloning medium (medium B) contained one part of fresh MEM with 10% fetal calf serum and one part of the same kind of medium in which a monolayer of uncloned hybrid cells had grown for 3 days and which was subsequently filtered.

*Hybridization:* $10^7$ AUC cells were mixed with $2 \times 10^6$ D98/AH cells in 30 ml of medium A. After incubation for 2½ hr at 37°C, with additional mixing at 15-min intervals, the cell suspension was evenly distributed among eight Falcon flasks (30-ml) and the volume of medium in each flask brought up to 5 ml. On day 2, the medium in all flasks was replaced by selective HAT medium. D98/AH cells die in HAT,[12] presumably because of their deficient activity for inosinic acid pyrophosphorylase.[6] The AUC cells and the hybrid cells survive and grow in HAT medium. The hybrid cells were maintained with one subculture, and frequent renewals of selective medium, for 28 days. Thereafter, the cells were propagated in either MEM with 10% fetal calf serum or nucleomedium.

*Chromosome studies:* Chromosome preparations were made as previously described.[12]

*Cell cloning:* Hybrid cell clones were obtained by a method modified from that of Schenck.[14] Falcon Petri dishes (60-mm) containing broken pieces of cover slips were plated with low inocula of cells (25, 50, 100 cells/dish) in medium B. After 24 hr, each cover-slip piece on which a single cell had attached was transferred to a separate 35-mm Falcon Petri dish containing medium B.

*Enzyme assays:* The assays for OMP decarboxylase and OMP pyrophosphorylase,[5, 15] for dihydroorotase,[5] for dihydroorotic acid dehydrogenase,[16] and for inosinic acid pyrophosphorylase[7, 8] have all been previously described. The conditions of the OMP decarboxylase assay have been somewhat modified from those originally used.[17]

*Starch gel electrophoresis:* Cells harvested for electrophoretic studies were sedimented by centrifugation and resuspended in a solution containing one part Triton-X (Rohm and Haas Company, Philadelphia) and nine parts triply distilled water. The lysate was then sedimented by centrifugation at 17,000 *g* for 15 min. An equal part of a 40% sucrose solution was added to the supernatant solution which was applied to the starch gel.

The electrophoretic mobility of glucose-6-phosphate dehydrogenase,[19] phosphoglucomutase,[20] lactate dehydrogenase,[21] 6-phosphogluconate dehydrogenase,[21] and malate dehydrogenase[21] was measured according to the protocols of the authors cited.

*Results.—Isolation of the DAU hybrid:* During the four weeks of growth in selective medium, small numbers of diffuse colonies of large epithelial cells were observed among the degenerating D98/AH epithelial cells and the slowly growing AUC fibroblasts. Chromosome preparations made on day 25 revealed

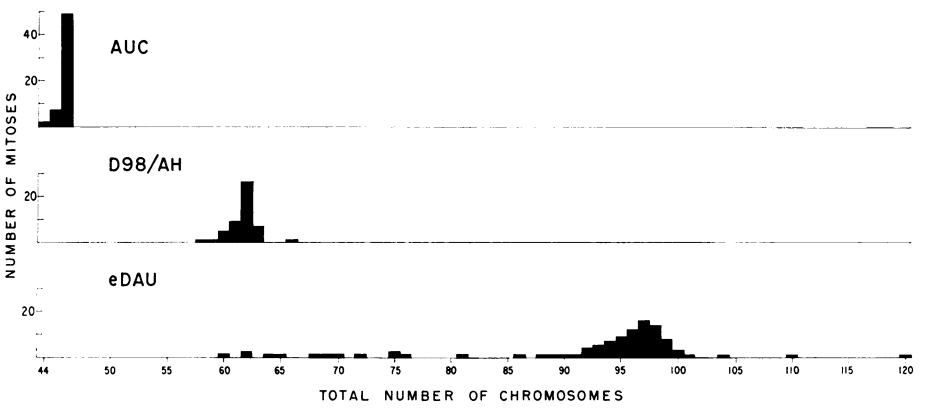


FIG. 1.—Histogram showing the chromosome number of the parental (AUC and D98/AH) and the hybrid (eDAU) cell lines.

a significant number of hypertetraploid cells, suggesting that hybridization may have occurred; these putative hybrid cells were designated as DAU. The culture was then cloned, and the studies reported here have been carried out on one such clone, eDAU. These studies were performed from two to ten months after isolation of the eDAU clone. The mean plating efficiency in medium B of DAU at the time of cloning was 84 per cent.

*Karyotypic studies:* Figure 1 shows the range of chromosome numbers in the three cell lines. The D98/AH cell line has a modal chromosome number of 62. The diploid AUC strain has a chromosome number of 46, with a normal male complement. The hybrid cell line (eDAU) has a modal number of 97 chromosomes with 16 per cent of the cells at the mode; 84 per cent had 90 or more chromosomes. There is clustering about the mode such that cells having 96, 97, or 98 chromosomes comprised 42 per cent of the total number of cells counted. The mode has remained unchanged through 250 cell doublings after the initial count was made. Figure 2 shows a typical metaphase preparation of an eDAU cell.

*Cellular morphology and growth characteristics:* In monolayer culture, the D98/AH cells have an epithelioid shape and are easily distinguished from the fibroblastic AUC cells (Fig. 3*A*, *D*). The hybrid eDAU cells are epithelioid like the D98/AH parent, but the nuclei appear larger and appear to have a greater number of nucleoli (Fig. 3*C*, *F*). The D98/AH cells form colonies that are more compact and have sharper margins than those of

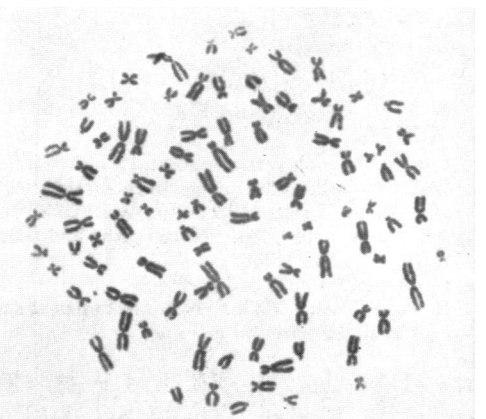

FIG. 2.—A metaphase of a hybrid eDAU cell with 97 chromosomes. ×725.

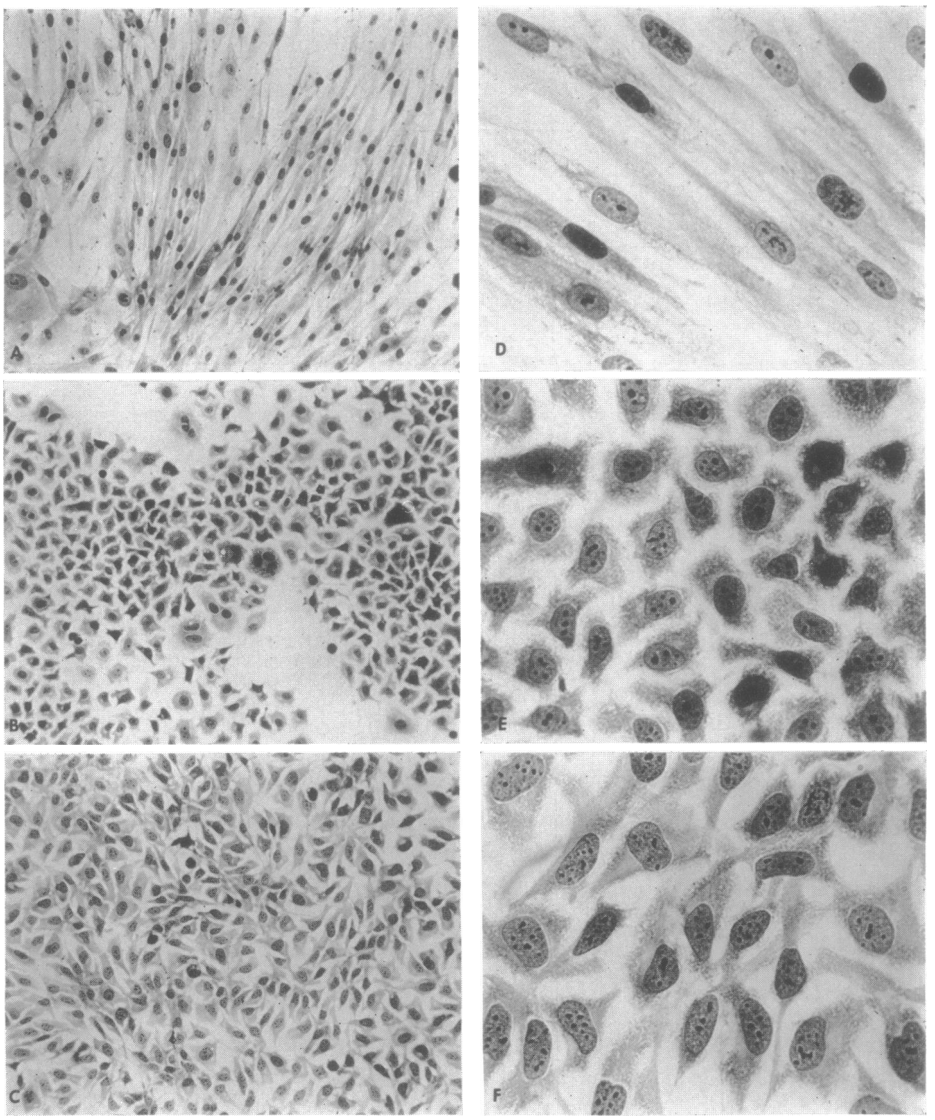


FIG. 3.—Monolayers of parental and hybrid cells. May-Grunwald-Giemsa.
(*A*) AUC cells ×86. Note fibroblastic growth.
(*B*) D98/AH cells ×86. Note epithelioid shape and space between colonies.
(*C*) eDAU cells ×86. Note epithelioid shape and spread-out growth pattern.
(*D*) AUC cells ×336.
(*E*) D98/AH cells ×336.
(*F*) eDAU cells ×336. Note that nuclear size is larger and number of nucleoli greater than in either parental cell line.

the eDAU line (Fig. 3*B*, *E*; Fig. 4). The fibroblastic AUC cells form diffuse, cometlike colonies. The hybrid eDAU cells appear to be capable of indefinite growth *in vitro*, and have now been serially propagated for more than one year with no detectable prolongation of generation time.

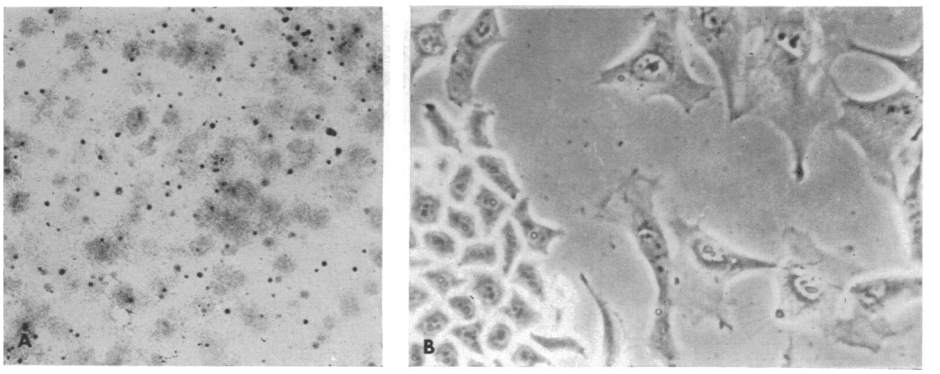

FIG. 4.—(*A*) Portion of Petri dish containing compact D98/AH and more diffuse DAU colonies. May-Grunwald-Giemsa. ×1.6.
(*B*) Living D98/AH cells are growing compactly at left as contrasted with DAU growing diffusely at right. Phase contrast ×211.

*OMP decarboxylase and OMP pyrophosphorylase activities:* Table 1 shows the specific activities of four enzymes of the biosynthetic pathway leading to uridine-5′-monophosphate. It can be seen that the eDAU hybrid line has intermediate specific activities for both OMP decarboxylase and OMP pyrophosphorylase. The other two enzymes, dihydroorotic acid dehydrogenase and dihydroorotase, are not affected by the gene for orotic aciduria.[5, 16] The specific activities of these two enzymes differed somewhat among the three cell lines. The heteroploid lines, D98/AH and eDAU, have consistently shown higher activities than the diploid line has shown for dihydroorotic acid dehydrogenase and dihydroorotase.

*Inosinic acid pyrophosphorylase activity:* The survival of the eDAU hybrid cells in HAT medium suggests that they possess inosinic acid pyrophosphorylase activity, whereas the death of the D98/AH cells suggests a deficiency for this enzyme. This possibility is supported by determination of specific enzyme activity in all three lines. In the one experiment thus far performed, both AUC and eDAU possessed considerably higher levels of inosinic acid pyrophosphorylase activity than D98/AH.

*Starch-gel electrophoretic phenotype:* Figure 5 illustrates the electrophoretic mobility of the glucose-6-phosphate dehydrogenase activity from each of the three cell lines (AUC, D98/AH, eDAU) and also from a mixture of AUC and D98/AH extracts. The D98/AH line has the $A^+$ phenotype[10] for glucose-6-phosphate dehydrogenase and the AUC line the B phenotype. In the gel used

TABLE 1. *Specific activities for four of the enzymes of pyrimidine biosynthesis.*

| Enzyme | Cell Line AUC | D98/AH | eDAU |
|---|---|---|---|
| OMP decarboxylase* | 0.011 | 5.999 | 2.687 |
| OMP pyrophosphorylase* | 0.065 | 38.346 | 14.674 |
| Dihydroorotic acid dehydrogenase* | 10.6 | 40.98 | 50.00 |
| Dihydroorotase* | 24.91 | 55.58 | 59.13 |

* Specific activity is expressed in mμmoles product formed/hour incubation/mg cell protein.

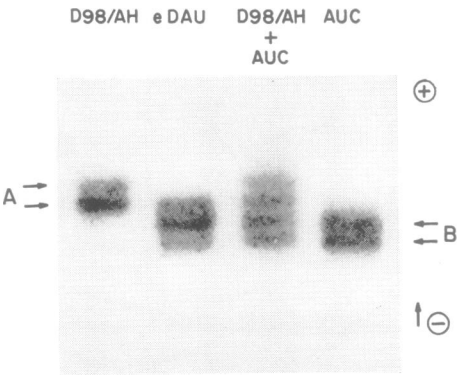


FIG. 5. — Glucose-6-phosphate dehydrogenase electrophoretic isozyme patterns of AUC, eDAU, D98/AH, and a mixture of D98/AH and AUC cell extracts.

for Figure 5, the *A* and the *B* electrophoretic patterns were each composed of two bands. The presence of two bands for each phenotype has also been reported for leukocyte extracts.[22] The mixture of the parental extracts showed two pairs of bands, corresponding respectively to the *A* and *B* patterns. In this, as in most preparations, the eDAU clone had three discernible bands, and the middle band was the most intensely stained. The slowest band of the eDAU cells migrated to the position of the slower band of the *B* phenotype. The middle, intense band of eDAU was at the position of the faster band of the *B* phenotype, and the fastest band of eDAU was at the position of the slower band of the *A* phenotype.

Many electrophoretic experiments were done with conditions varied in order to get the best possible resolution of the bands. A band similar to the faster band of the *A* phenotype was usually not seen, except in one early experiment when a faintly staining fourth band was seen. We are not sure if this fourth band, present in only one experiment, reflects the presence of its component in the cell or if it is an artifact. In two experiments, the mixture of the *A* and the *B* parents formed only two bands, while the eDAU cells continued to show the three-band pattern depicted in Figure 5. One possible interpretation of the eDAU pattern is that it reveals an *A* band and a *B* band, that the middle band represents a molecule which contains both *A* and *B* subunits, and that this heteropolymer migrates to the same position as that of the faster of the two *B* bands. The other possible interpretation is that the eDAU clone reveals two bands derived from the AUC parent and one from the D98/AH parent. In any case, the isozyme pattern of eDAU is different from that of either parental extract and is usually different from that of a mixture of the two parental extracts.

The electrophoretic mobility of four other enzymes (phosphoglucomutase, lactate dehydrogenase, 6-phosphogluconate dehydrogenase, and malate dehydrogenase) was also measured in each of the three cell lines, but no variation was found.

*Discussion.*—A hybrid cell strain (eDAU) of clonal origin has been formed by cellular and nuclear fusion between two human cell lines (AUC and D98/AH). Several lines of evidence support this conclusion (Table 2). (1) The eDAU clone has a modal chromosome number (97) that is almost the sum of those of the two parental lines (46 + 62). (2) The eDAU clone possesses a level of specific activity for OMP decarboxylase and OMP pyrophosphorylase that is nearly 200 times that found in the AUC line, indicating that the D98/AH parent contributed at least one normal locus for these enzymes. (3) eDAU cells have higher activity than D98/AH for inosinic acid pyrophosphorylase,

which they presumably inherited from the AUC parent. (4) The glucose-6-phosphate dehydrogenase activity of the eDAU clone migrates to the positions of both the *A* and the *B* bands, indicating a contribution from the AUC as well as the D98/AH strain. (5) The colonial and cellular morphology of eDAU cells is significantly different from that of either parental line.

TABLE 2. *Characteristics of parental and hybrid lines.*

| Characteristic | Line AUC | Line eDAU | Line D98/AH |
|---|---|---|---|
| Cellular morphology | Fibroblastlike | Epithelioid | Epithelioid |
| Colonial morphology | Cometlike | Diffuse | Compact |
| Total chromosome number | 46 | 97 (modal) | 62 (modal) |
| Growth in HAT medium | + | + | − |
| Inosinic acid pyrophosphorylase activity | + | + | − |
| OMP decarboxylase activity | − | + | + |
| OMP pyrophosphorylase activity | − | + | + |
| Glucose-6-phosphate dehydrogenase electrophoretic phenotype | *B* | *AB* | *A* |

The alleles responsible for the *A* and *B* types of glucose-6-phosphate dehydrogenase are at a locus carried on the X chromosome.[23] The presence of *A and B* bands in the cloned hybrid line suggests that there are at least two active X chromosomes present in the eDAU clone.

Human diploid cell strains, developed from the tissues of females who are *AB* heterozygotes for the gene affecting glucose-6-phosphate dehydrogenase, have the *AB* phenotype in mass culture. However, when such cultures are cloned, each clone has *either* the *A or B* phenotype,[23, 24] suggesting that only the maternal *or* paternal X chromosome of the donor functions in each clone. Other human sex-linked mutations that have been studied in this way behave similarly.[25] These results are consistent with the single active X theory.[26]

There are at least two possible explanations for the presence of more than one genetically active X chromosome in the eDAU clone. One is that X chromosome inactivation can occur only in embryonic cells. Since the hybrid cell was formed from two strains derived from nonembryonic donors, it may no longer be capable of X inactivation. Another possibility is that the cell somehow regulated X inactivation, so that the ratio of active X chromosomes to each diploid set of autosomes is approximately 1:1. In the case of a hypertetraploid cell such as the eDAU hybrid, the 1:1 ratio would require that two X's be active.

Human-human hybrids have been sought in the hope that segregation or chromosome loss could be used to locate known genetic markers in linkage groups or even on cytologically distinguishable chromosomes. The fact that many chromosomes are retained in the hybrid line makes such studies more complicated than in the mouse-human hybrid, which conveniently loses most of its human chromosomes soon after hybridization.[2] Human-human hybrid cells are, however, likely to be of particular value as a means for studying complementation, and perhaps recombination, between mutant genes which have been recovered from different families.

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‡ Abbreviations used: OMP, orotidine-5′-monophosphate; AUC, orotic aciduric cell strain; D98/AH-2, heteroploid parental line; DAU, hybrid cell line; eDAU, clone of hybrid cell line; MEM, Eagle's minimal essential medium; HAT, selective medium containing hypoxanthine, aminopterin or amethopterin, and thymidine.

[1] Barski, G., S. Sorieul, and F. Cornefert, *Compt. Rend.*, **251**, 1825 (1960).

[2] Weiss, M. D., and H. Green, these PROCEEDINGS, **58**, 1104 (1967).

[3] Smith, L. H., C. M. Huguley, and J. A. Bain, in *The Metabolic Basis of Inherited Disease*, ed. J. B. Stanbury, J. B. Wyngaarden, and D. S. Frederickson (New York: McGraw-Hill Book Co., Inc., 1966), 2nd ed., p. 739.

[4] Krooth, R. S., in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 29 (1964), p. 189.

[5] Howell, R. R., J. R. Klinenberg, and R. S. Krooth, *Johns Hopkins Med. J.*, **120**, 81 (1967).

[6] Szybalski, W., and E. H. Szybalska, in *Approaches to the Genetic Analysis of Mammalian Cells*, ed. D. J. Merchant and J. V. Neel (Ann Arbor: The University of Michigan Press, 1962), p. 11.

[7] Kelley, W. N., F. M. Rosenbloom, J. F. Henderson, and J. E. Seegmiller, *Biochem. Biophys. Res. Commun.*, **28**, 340 (1967).

[8] Seegmiller, J. E., F. M. Rosenbloom, and W. N. Kelley, *Science*, **155**, 1682 (1967).

[9] Becroft, D. M. O., and L. I. Phillips, *Brit. Med. J.*, **1**, 547 (1965).

[10] Gartler, S. M., *Nature*, **217**, 750 (1968).

[11] Littlefield, J. W., *Science*, **145**, 709 (1964).

[12] Silagi, S., *Cancer Res.*, **27**, 1953 (1967).

[13] Eagle, H., *Science*, **130**, 432 (1959).

[14] Schenck, D. M., and M. Moskowitz, *Proc. Soc. Exptl. Biol. Med.*, **99**, 30 (1958).

[15] Pinsky, L., and R. S. Krooth, these PROCEEDINGS, **57**, 925 (1967).

[16] Wuu, K. D., and R. S. Krooth, *Science*, **160**, 539 (1968).

[17] Henderson, J. F., L. W. Brox, W. N. Kelley, F. M. Rosenbloom, and J. E. Seegmiller, *J. Biol. Chem.*, **243**, 2514 (1968).

[18] Krooth, R. S., Y. L. Pan, and L. Pinsky, in preparation.

[19] Shows, T. B., R. E. Tashian, G. Brewer, and R. Dern, *Science*, **145**, 1056 (1964).

[20] Spencer, N., D. A. Hopkinson, and H. Harris, *Nature*, **204**, 742 (1964).

[21] Weitkamp, L., personal communication.

[22] Boyer, S. H., I. H. Porter, and R. G. Weilbacher, these PROCEEDINGS, **48**, 1868 (1962).

[23] Davidson, R. G., H. M. Nitowsky, and B. Childs, these PROCEEDINGS, **50**, 481 (1963).

[24] DeMars, R., *Natl. Cancer Inst. Monograph*, **13**, 181 (1964).

[25] Krooth, R. S., G. A. Darlington, and A. A. Velazquez, in *Annual Reviews of Genetics*, ed. H. L. Roman (Palo Alto: Annual Reviews, Inc., 1968), vol. 2, p. 141.

[26] Lyon, M., in *Annual Reviews of Genetics*, ed. H. L. Roman (Palo Alto: Annual Reviews, Inc., 1968), vol. 2, p. 31.