GENETICS OF HUMAN CELL LINES

III. Incorporation of 5-Bromo- and 5-Iododeoxyuridine into the Deoxyribonucleic Acid of Human Cells and Its Effect on Radiation Sensitivity*

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Strain D98S, a single cell isolate derived from human sternal marrow (1), can be cultivated indefinitely in the presence of 5-bromodeoxyuridine (BUDR)[1] at concentrations below 10 μg. per ml. Under these conditions BUDR is incorporated into the deoxyribonucleic acid (DNA) component of the cells, partially replacing thymidine. A comparison of the properties of these chemically modified cells with those of the parent cells revealed that a striking increase in radiation sensitivity is associated with BUDR incorporation (2–4). This finding in mammalian cells is analogous to that reported for BUDR-containing bacteriophages (5) and bacteria (6).

Several aspects of the incorporation of BUDR and another thymidine analog, 5-iododeoxyuridine (IUDR), including its effect on radiosensitivity, were the subjects of this study. The opportunities afforded by BUDR labeling of DNA synthesized under various conditions were exploited, and the physicochemical and biological properties of such modified DNA were evaluated.

Materials and Methods

Strain D98S, culture media, cultivation, plating, staining, colony-counting procedures, and the bromophenol blue method for protein determination were described earlier (1). DNA was determined by a micro modification of the Burton (7) procedure, using an extract (90°C., 15 minutes, 5 per cent $HClO_4$) from defatted, cold 5 per cent perchloric acid–washed cells.

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[1] DNA, deoxyribonucleic acid; RNA, ribonucleic acid; BUDR, 5-bromodeoxyuridine; IUDR, 5-iododeoxyuridine; FUDR, 5-fluorodeoxyuridine; BU, 5-bromouracil; CM, chloramphenicol; FA, *p*-fluorophenylalanine; TCA, trichloracetic acid.

BUDR and IUDR were purchased from the California Corporation for Biochemical Research, Los Angeles.

For quantitative determination of the purine and pyrimidine bases in DNA, a simplification of the procedure described by Hershey *et al.* (8) was followed. In brief, 2×10^7 cells were treated overnight (37°C.) with N NaOH (to remove RNA), followed by precipitation of DNA and protein with cold 0.3 M trichloracetic acid (TCA). DNA was extracted from the washed precipitate with hot (90°C.) TCA, and, after evaporation of the latter, hydrolyzed with 6 N HCl in sealed ampules (100°C., 3 hours). The HCl-free DNA hydrolysate was chromatographed, using acidified isopropanol (65 ml. concentration HCl, 210 ml. isopropanol, 65 ml. water) for the first dimension and alkaline isopropanol (2.5 ml. 15 N NH_4OH, 170 ml. isopropanol, 30 ml. water) for the second dimension development. For these, R_f values were respectively: 0.35, 0.40 (adenine); 0.24, 0.13 (guanine); 0.48, 0.29 (cytosine); 0.69, 0.38 (uracil); 0.79, 0.56 (thymine); and 0.78, 0.28 (5-bromouracil), the last two becoming separated only after the second dimension development. A uracil spot appeared during chromatography of the BUDR-labeled DNA, most probably as a consequence of the hydrolytic debromination of the latter during alkaline hydrolysis (9). For quantitative determination of 5-bromouracil (BU) incorporation, the millimolar extinction coefficients of 0.1 N HCl eluates from both the BU (7.01 at 276 mμ) and uracil (8.20 at 259 mμ) spots were compared with the thymine extinction coefficient (7.89 at 264 mμ.). In some experiments, when only the BU to thymine molar ratio was to be assessed, the cells were washed with cold TCA, but RNA removal by 1 N NaOH hydrolysis was omitted, and only the molar absorbances of eluates from the BU and thymine spots were compared.

For ultraviolet (UV) sensitivity determination, 5,000 to 10,000 cells were plated per 60 mm. Petri dish containing 3 ml. of E_{90} medium (1) (Eagle's basal medium (10) with 10 per cent horse serum; Wyeth Inc. Marietta, Pennsylvania). After 2 to 3 hours of incubation at 37°C. in an atmosphere of 5 to 10 per cent CO_2, during which the cells became attached firmly to the glass, the medium was replaced by 1 ml. of balanced salt solution (8.0 gm. NaCl; 0.4 gm. KCl; 0.35 gm. $NaHCO_3$; 1000 ml. H_2O; equilibrated in an atmosphere of 10 per cent CO_2). The plates were irradiated with a 15 watt Westinghouse sterilamp (hot cathodes) placed at a distance of 59 cm. from the plates (irradiation intensity: 11.1 ergs/sec. mm.^{2}). In some experiments the cells were irradiated just after complete removal of the medium, without addition of balanced salt solution. Following irradiation, E_{90} medium was immediately added (5 ml.) and the culture was incubated for 7 to 14 days until countable colonies developed. Since the cells growing near the edges of the Petri dish were partially screened from the UV effect, all peripheral colonies were eliminated from the total count in the irradiation experiments by the use of special masks on the dishes during the colony count.

In another variant of the irradiation procedure ("irradiation in suspension"), a suspension of trypsinized cells was diluted in balanced salt solution to a concentration of 5,000 cells per ml. One ml. of this diluted suspension was irradiated in 60 mm. Petri dishes as previously described, but with simultaneous agitation of the plate contents by means of a mechanical rotator. Immediately after irradiation, 4 ml. of E_{90} medium was added, followed by the routine incubation and colony count.

For the determination of the density distribution of BUDR-labeled DNA, the equilibrium sedimentation technique devised by Meselson *et al.* (11, 12) was employed. Silicon-coated lusteroid tubes (Spinco No. 5050) were each filled with 2.5 ml. of purified cesium chloride solution (1.820 gm./cm.^{3}, 25°C.) mixed with 0.5 ml. of a solution of the DNA in 1 M NaCl (50 to 400 μg. DNA/ml.). An overlayer of 1.5 ml. of paraffin oil was added last. Centrifugation was carried out for 72 hours at 35,000 R.P.M. in the SW-39 swinging bucket rotor of the Spinco model L ultracentrifuge at 20°C. During centrifugation, an equilibrium density gradient of CsCl solution was established, and at the same time the DNA fractions formed thin layers

at the characteristic neutral buoyancy levels corresponding to their individual densities. To determine the position of these layers, consecutive 0.1 ml. fractions were collected from the bottom of the gradient by means of a special apparatus described by Szybalski (13). The density of each sample was determined refractometrically, while the DNA content was calculated from optical density determinations at a wavelength of 260 mμ (Beckman, model DU spectrophotometer; 0.1 ml. "pyrocell" micro cuvettes).

Preparation of the DNA samples for the centrifugation procedure involved the following steps (14). Approximately 5×10^6 cells were harvested by trypsinization, centrifuged (2000 R.P.M.), and resuspended in nine volumes of 1 M NaCl plus 0.02 M sodium citrate solution (per volume of packed cells). This suspension was homogenized in a Teflon tissue homogenizer (Tri-R Instruments Co., Jamaica, New York), and extracted three times by gentle shaking with equal volumes of water-saturated phenol. All operations were performed at 0–4°C. High speed centrifugation was employed to separate the aqueous phase after each extraction, while the phenol phase was discarded. Residual phenol was removed from the nucleic acid solution with ether, and the remaining traces of ether were blown off with nitrogen. Overnight storage of this solution, at 0°C., after adjustment of the NaCl concentration to 1 M, resulted in precipitation of a considerable proportion of the RNA, which was centrifuged off. The remaining RNA was removed by 30 minutes of incubation (37°C.) with approximately 50 μg. per ml. of heated RNase solution (3 times crystallized; Worthington Co.), followed by overnight dialysis at 0–4°C., and alcohol precipitation of the DNA on a glass rod ("spooling out"). The DNA precipitate was rinsed with alcohol and immediately dissolved in 1 M NaCl at concentrations of 200 to 1000 μg. per ml. Human DNA prepared in this manner was in native form (no change in the UV absorption in the range of 25–83.5°C.), with the molecular weight in the neighborhood of 10 million (sedimentation constant, for 0.002 per cent DNA solution in 0.15 M NaCl plus 0.015 M sodium citrate, $s_{w,20} = 26.5$ to 28.0 s).

RESULTS

Effect of BUDR and IUDR on Cell Multiplication.—Although unimpaired cell multiplication could be attained with the D98S line only at concentrations below 10 μg. BUDR per ml., the cells were able to grow for a limited period and to remain viable at concentrations up to 100 μg. BUDR per ml.

To determine the growth rate, plating efficiency, and viability of BUDR-exposed cells, 60 mm. Petri dishes containing E_{90} medium supplemented with various concentrations of BUDR (each concentration in quadruplicate) were inoculated with 5000 cells each, and incubated. After 2 to 4 hours of incubation, 20 glass-attached cells were chosen at random and their positions were marked. The number of progeny in each of the colonies derived from these cells was determined every day with the aid of an inverted microscope. On the 9th day of incubation, half of the cultures were fixed and stained, while the cells in the remaining identical set of dishes were harvested by trypsinization (1), counted, and plated again at various BUDR concentrations. This procedure was repeated several times, leading to the selection of strain D98/BUDR, characterized by higher resistance to BUDR inhibition.

Table I and Fig. 1 summarize the results obtained. It is apparent that in the presence of BUDR in concentrations as high as 10 μg. per ml. the wild-type strain D98S plated and formed colonies with an efficiency equal to that of the BUDR-free control (63 to 64 per cent). At these concentrations, however, the cells multiplied more slowly, with the average generation time increased by a

factor of 1.37 (for 2.5 μg. BUDR per ml.) to 2.21 (for 10 μg. BUDR per ml.) compared with that in BUDR-free medium. A large proportion of the cells cultivated at 2.5 to 10 μg. BUDR per ml. remained viable, as determined by trypsinization and replating of the 8-day-old, BUDR-grown cells in BUDR-free and in BUDR-containing medium. However, the plating efficiency fell below the usual average of 60 per cent characteristic for D98S cells (Table I).

TABLE I

Multiplication Rate and Plating Efficiency of Strains D98S and D98/BUDR during Long Term Cultivation in the Presence of Various Concentrations of BUDR

Strain	Growth cycle*	BUDR concentration upon replating	BUDR concentration during first growth cycle, μg./ml.....	0	2.5	5	10	20	50
		μg./ml.							
D98S	1st		Average generation time, hrs.§	28.5	40.5	46.1	65.5		
	1st		Plating efficiency (per cent colony forming cells)	63.1	64.0	63.6	63.0	12.0	0.0
	2nd	0			45.0	32.1	21.5		
	2nd	5			25.0	14.7			
	4th	0				35.0‡			
	4th	5				35.0‡			
D98/BUDR	1st		Average generation time, hrs.§	29.0		36.5	52.5		123.0
	1st		Plating efficiency, per cent	41.1		42.6	34.5		24.3

* Each growth cycle consisted of 7 to 8 days of incubation (approximately 5000 cell inoculum per 60 mm. Petri dish, 37°C., 5 per cent CO_2, daily changes of E_{90} medium supplemented with various concentrations of BUDR indicated in the top line of the table), followed by trypsinization and replating at BUDR concentration indicated in the 3rd column of the table.

‡ Cultivated for the previous three growth cycles in the presence of 5μg. BUDR/ml.

§ Duration of the growth period (in hours) divided by the $\log_2$ of the average number of cells per colony after 7 to 8 days of incubation.

At concentrations above 10 μg. BUDR per ml., D98S cells could undergo only a limited number of divisions, associated with a high mortality rate. Viability was retained for only 2 to 3 days in the presence of 50 to 100 μg. BUDR per ml.

The concentration of 5 μg. BUDR per ml. was selected for continuous cultivation of the D98S cell line. A characteristic drop in the division rate was observed during the first growth cycle, but only upon completion of the first division in the presence of BUDR. The multiplication rate slowly increased during four growth cycles (8 days each) reaching the rate of the BUDR-free control (4th cycle, as shown in Fig. 1). After approximately 40 cell generations in the

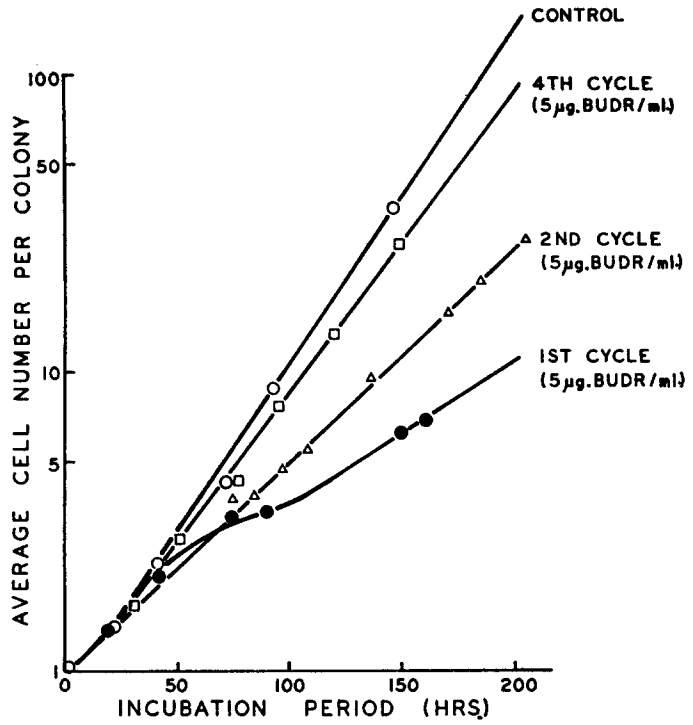

FIG. 1. Rate of multiplication of D98S cells, as assessed by daily microscope determination of clone size (cell number per colony), in the absence (○) and in the presence of BUDR. Curves 1 (●), 2 (△), and 4 (□) represent the rates of multiplication during the 1st, 2nd, and 4th successive cell transfer (8-day growth cycle each) in the presence of 5 μg. BUDR/ml. The average inoculum was 5000 cells per 60 mm. Petri dish.

TABLE II

Multiplication Rate and Plating Efficiency of Strain D98S in the Presence of Various Concentrations of IUDR

Strain	Growth cycle*	IUDR concentration upon replating	IUDR concentration during first growth cycle μg./ml.....	0	5	10	20	40
D98S	1st		Average generation time‡	29.0	47.8	65.0	81.4	95.1
	1st		Plating efficiency (per cent colony-forming cells)	72.3	72.5	52.6	51.6	41.1
	2nd	0		60.1	0.0	0.0	0.0	0.0
	2nd	5		58.3	0.0	0.0	0.0	0.0

* A growth cycle consisted of 7 and 8 days of incubation (approximately 5000 cell inoculum per 60 mm. Petri dish, 37°C., 5 per cent CO_2 atmosphere, daily changes of E_{90} medium supplemented with IUDR concentration indicated in the top line of the table), followed by trypsinization and replating at IUDR concentrations indicated in the 3rd column.

‡ *cf.* Table I, footnote§.

presence of 5 μg. BUDR per ml., a pure line of cells was isolated by cloning (15). This line, D98/BUDR, was less susceptible to the inhibitory effects of BUDR than the parental strain D98S, as can be inferred from Table I.

The effect of IUDR on D98S cells was determined in a manner similar to that

TABLE III

Chromatographic Determination of Per Cent Thymidine Replacement by BUDR during Growth in the Presence of Various Concentrations of BUDR and FUDR

Strain	BUDR concentration	FUDR concentration	Per cent thymidine replacement by BUDR*			
			Days of incubation with BUDR and FUDR:			
			2	4	6	>70‡
	μg./ml.	μg./ml.				
D98S	10	0	14.0	27.0	35.0	
	10	0.004	20.0	45.5		
	50	0	39.3			
D98/BUDR	5	0				17.0
	5	0.004				45.4§

* The molar ratio of 5-bromouracil (BU) to the sum of BU and thymine, as determined by chromatographic analysis of the hydrolyzed DNA.

‡ Transferred once a week.

§ FUDR present only during the final 6 days of incubation.

TABLE IV

Reversal of FUDR Inhibition by BUDR and Its Effect on BUDR Incorporation by Cell Line D98/BUDR

BUDR concentration, μg./ml FUDR concentration (μg./ml.)	0 0	5 0	0 0.004	5 0.004
Plating efficiency	41.1	42.6	0	42.3
Average generation time, *hrs.*	29.0	36.5	∞	38.6
Molar ratio: BUDR/(BUDR + thymidine)	0	17.0	0	45.4
Protein content per cell, $\mu g. \times 10^{-4}$	3.4	5.5	—	7.5
DNA content per cell, $\mu g. \times 10^{-6}$	10.0	10.6	—	8.9

employed for BUDR, although to a more limited extent. Cultivation for 7 days in the presence of IUDR (5 to 40 μg. per ml.) resulted in complete loss of viability, as assayed by replating in IUDR-free and in IUDR-containing medium (Table II). The effects of long-term growth in the presence of IUDR were therefore not studied. A separate series of experiments indicated that in the presence of 5 to 50 μg. IUDR per ml. the cells retained their viability for a period of 2 to 4 days only. The rate of cell multiplication was affected by IUDR to an extent similar to that observed for BUDR.

BUDR Incorporation into DNA.—It has been demonstrated by Weygand and Wacker (16) that 5-bromouracil is incorporated into the nucleic acid fraction of bacterial cells. That it replaces thymidine in the DNA is evidenced by the studies of Zamenhof and Griboff (17), Dunn and Smith (18), and Wacker *et al.* (19) with bacteria, and Eidinoff *et al.* (20), Hakala (21), and Szybalski and Djordjevic (3, 4) with mammalian cells.

Table III summarizes the results of chromatographic analysis of the extent of thymidine replacement by BUDR under the present experimental conditions; *i.e.*, for *fully viable* cells (capable of forming colonies when replated in E_{90} medium).

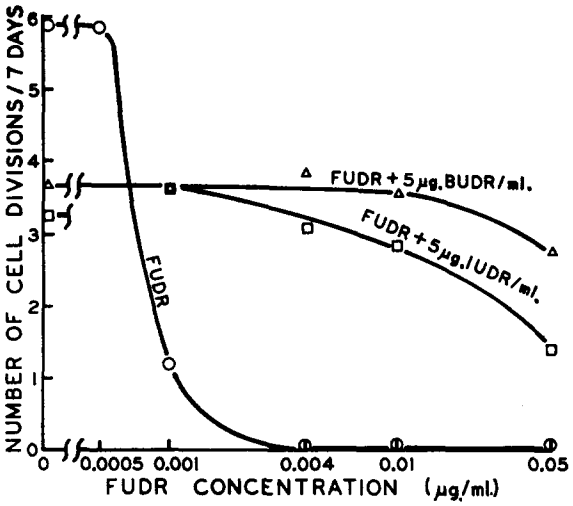

FIG. 2. Effect of various concentrations of FUDR on the multiplication of D98S cells in the absence (○) or in the presence of 5 μg. BUDR/ml (△), or of 5 μg. IUDR/ml. (□). The number of generations was calculated as the $\log_2$ of the average number of cells per colony after 7 days of incubation.

In some experiments referred to in Table III, 5-fluorodeoxyuridine (FUDR) was employed. This analog was shown to be a powerful inhibitor of intracellular synthesis of thymidylic acid from deoxyuridylic acid, while not interfering with the kinase-mediated phosphorylation of extracellularly supplied thymidine (22–23). As may be seen in Fig. 2 and Table IV, BUDR reversed the inhibitory effect of FUDR. In the presence of FUDR, BUDR incorporation was more extensive,[2] especially with strain D98/BUDR (Table III). IUDR also reversed the inhibitory effect of FUDR (Fig. 2).

Chromatographic determination of the extent of thymidine replacement by

[2] This method was developed by Lorkiewicz and Szybalski (38), in their studies with *Escherichia coli* strain Sd4.

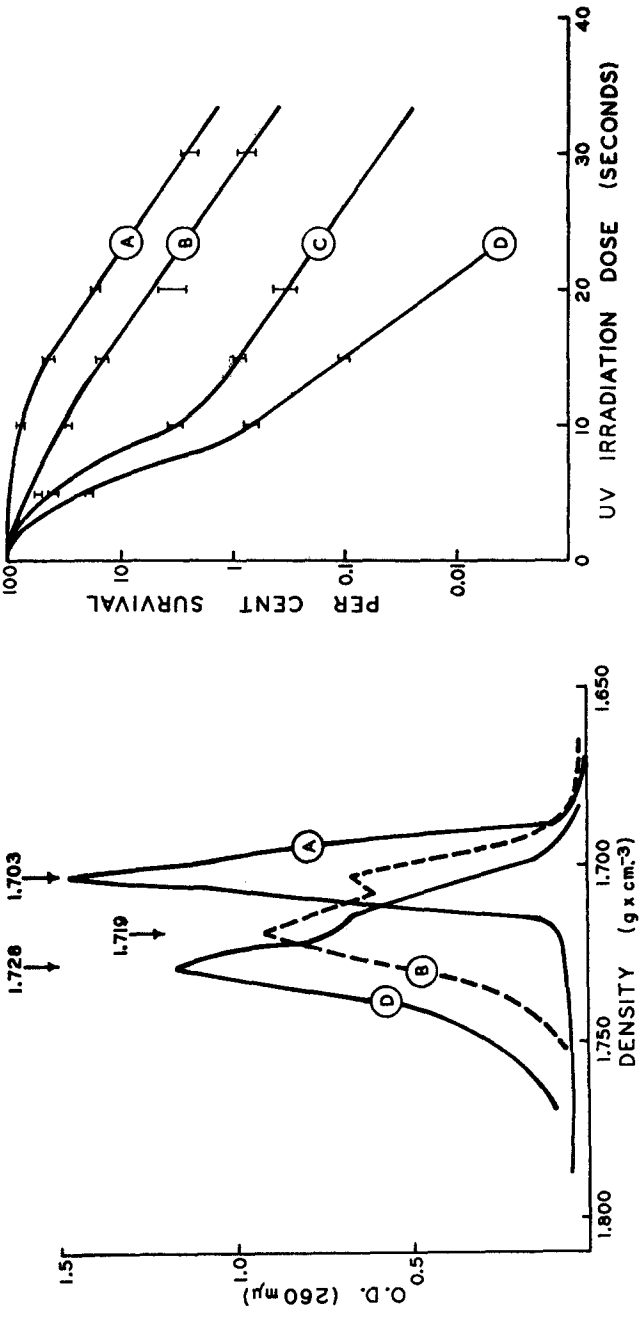

FIG. 3 *a*

FIG. 3 *b*

FIG. 3. Buoyant density distribution of extracted DNA (Fig. 3 *a*) and UV sensitivity (Fig. 3 *b*) of D98S cells grown for 96 hours in E_{90} medium, either BUDR-free (curve A) or supplemented for the last 24 hours (B), the last 48 hours (C), or the whole 96 hour period (D) with 10 μg. BUDR/ml. Each of the four cultures was harvested and divided into two portions: (1) The major part served as a source of DNA, for extraction and centrifugation in the CsCl gradient, according to the procedure described in Materials and Methods. Fig. 3 *a* represents the plot of the refractometrically determined density (gm./cm.^{3}) against the optical density (260 mμ) of each of the 30 samples collected after equilibrium centrifugation. (2) The remaining trypsinized cells were immediately replaced (5,000 to 10,000 cells per 60 mm. dish), and 3 hours later the glass-attached cells were irradiated with different UV doses. Fig. 3 *b* shows the colony-forming capacity of the cells (assessed after 10 days of incubation) at various UV doses.

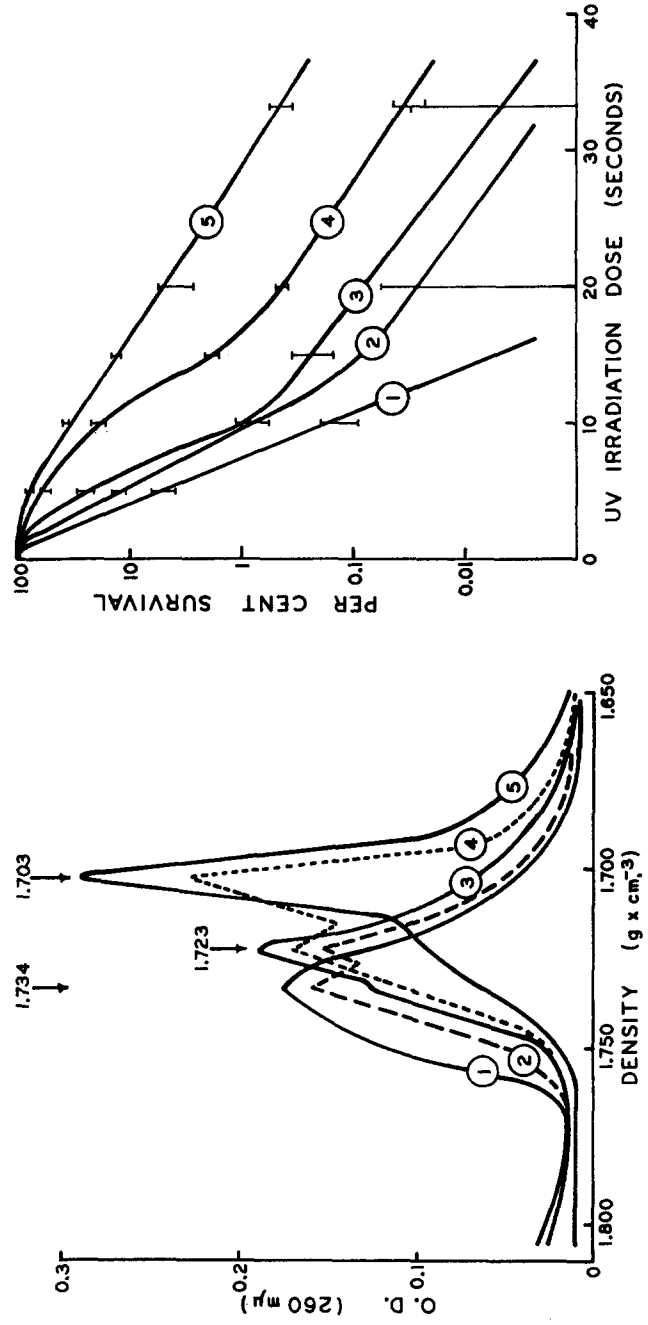

FIG. 4 *b*

FIG. 4 *a*

FIG. 4. Buoyant density distribution of extracted DNA (Fig. 4 *a*) and UV sensitivity (Fig. 4 *b*) of D98/BUDR cells cultivated through over 70 generations in E_{10} medium supplemented with 0.004 μg. FUDR/ml. and 5 μg. BUDR/ml. (curve 1), and then transferred to BUDR-free medium (0.004 μg. FUDR/ml. and 5 μg. thymidine/ml.) for the last 13 hour (curve 2), 24 hour (3), 65 hour (4), or 112 hour (5) period, prior to harvesting, replating, and irradiation of all five diversely treated cultures, in parallel with DNA density determination by the CsCl gradient centrifugation procedure (*cf.* Fig. 3).

BUDR does not give any clue as to the pattern of distribution of the label throughout the DNA molecule. According to Watson and Crick (24), each DNA molecule is composed of two complementary polynucleotide strands. The question therefore arises as to whether BUDR is present in only one or in both strands. This problem bears on the mode of DNA replication in human cells, and can be approached in a manner analogous to that employed by Meselson and Stahl (12) for bacteria, substituting BUDR for the N^{15} label, as in the studies of Kozinski and Szybalski (5).

Two types of experiments were devised to evaluate the manner of BUDR distribution in the newly synthesized DNA. In the first (Fig. 3 *a*), cultures derived from the same inoculum of D98S cells were grown in E_{90} medium and exposed to 10 μg. BUDR per ml. for periods of 0, 24, 48, and 96 hours. The other type of experiment (Fig. 4 *a*) employed D98/BUDR cells, grown for many generations in the presence of 5 μg. BUDR and 0.004 μg. FUDR per ml. of E_{90} medium, and subsequently transferred to medium containing 0.004 μg. FUDR and 5 μg. thymidine per ml. and incubated for 13, 24, 65, and 112 hours. The cells were homogenized, the DNA was extracted by the phenol procedure, RNA was removed by RNase treatment with simultaneous dialysis, and the density distribution of the isolated DNA was determined by CsCl gradient centrifugation (11–13).

Figs. 3 *a* and 4 *a* were prepared by determining the absorbance at 260 mμ and the density of all the individual 0.1 ml. fractions collected after equilibrium centrifugation in the manner described in Materials and Methods. In both experiments, the DNA banded predominantly in three layers, corresponding to densities of 1.703, 1.719, and 1.728 in the first experiment and to densities of 1.703, 1.723, and 1.734 in the second. When correlated with the rates of division under the experimental conditions (Table I), these data (Fig. 3 *a*) indicate that during the first round of DNA replication BUDR was incorporated into only one strand of each newly synthesized DNA double helix ("unifilar" labeling), and consequently these "hybrid" molecules of intermediate density (1.719 gm./cm.^{3}) were replacing the parental DNA of density 1.703. Upon subsequent replication, new DNA molecules of still higher density (1.728) ("heavy") appeared, presumably with BUDR incorporated into both strands ("bifilar" labeling). The analogous situation arose (Fig. 4 *a*) when BUDR-labeled cells were transferred to BUDR-free medium. DNA banded at only three discrete densities corresponding to: (*a*) the parental "heavy" molecule (1.734) with both DNA strands BUDR-labeled, (*b*) the "hybrid" molecule (1.723) with one new BUDR-free strand and one parental BUDR-labeled strand, and (*c*) the "light" (1.703) BUDR-free DNA double helix.

These data pertain not only to the manner of BUDR incorporation into DNA but also to the mode of DNA replication in human cells. They suggest very strongly that human DNA replicates in a semiconservative manner, similar to that observed for microbial DNA (12), and in agreement with the semiconservative mode of chromosomal replication postulated by Taylor *et al.* (25). Fig. 5

depicts in schematic fashion the semiconservative mode of replication of the double stranded DNA molecule.

Since the purine and pyrimidine content of the DNA and the extent of BUDR substitution were known, the theoretical densities of the "heavy" and "hybrid" DNA's (Fig. 4 *a*, curves 1 and 3 respectively) could be calculated, assuming that the molecular volume of the DNA did not change as a result of BUDR incorporation. As can be seen in Table V, the experimentally determined densities were smaller than the calculated densities. Moreover, the density differential between the "heavy" and "hybrid" DNA's was less than that between the

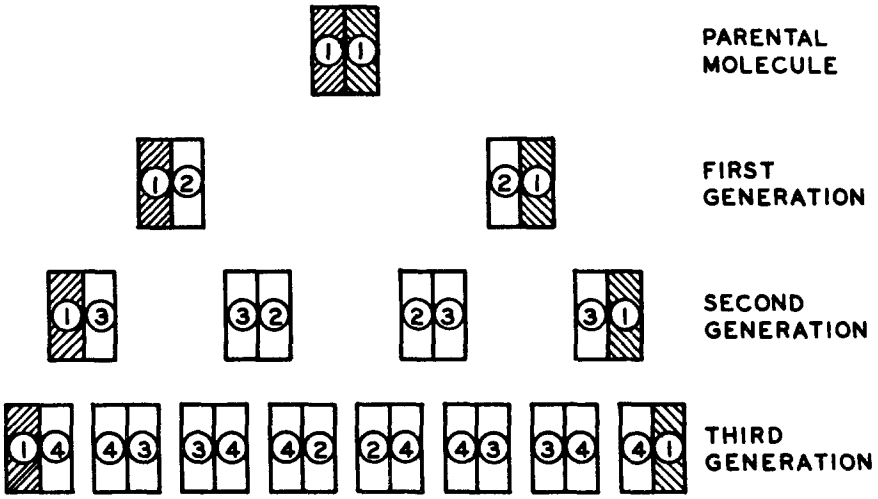

FIG. 5. Diagrammatic representation of the semiconservative mode of DNA replication, compatible with the results obtained in the experiments illustrated in Figs. 3 and 4. Each strand of the original parental molecule (1) is conserved through successive replications, remaining BUDR-free (Fig. 3) or BUDR-labeled (Fig. 4), but associated with a labeled (Fig. 3) or unlabeled (Fig. 4) strand newly synthesized during each replication. The proportion of "hybrid" ("unifilarly" labeled) molecules, 100 per cent in the first generation, decreases by a factor of two in each successive generation.

"hybrid" and "light" DNA's (*cf.* Figs. 3 *a* and 4 *a*). These data suggest that the molecular volume of DNA increases as a result of "bifilar" BUDR incorporation (Table V).

BUDR-labeled DNA exhibited a broader density distribution than normal "light" DNA, probably reflecting uneven labeling of DNA molecules caused by small fluctuations in the BUDR concentration between medium renewals. An unsuccessful preliminary attempt was made to "split" the "hybrid" DNA molecule into "light" and "heavy" single strands by heating the appropriate fraction (density 1.719, curve B, Fig. 3) to 98°C. for 10 min. Subsequent centrifugation

in a CsCl gradient revealed only one peak, of maximum density 1.733 gm./cm.[3] (an increment of 0.014 density units), indicating collapse of the heat-denatured DNA molecules but no separation into "light" and "heavy" components as observed by Meselson and Stahl (12) for *Escherichia coli* DNA.

Effect of BUDR and IUDR incorporation on radiation sensitivity. To test the effect of BUDR and IUDR incorporation on the radiation sensitivity of D98S cells, the UV and x-ray[3] dose-survival curves for normal cells and for analog-labeled cells were compared.

Initially, the labeled cells were prepared by cultivation for the last 3 days of

TABLE V

Effect of BUDR Incorporation into One or Both Polynucleotide Strands on DNA Density

	DNA		
	"Light"	"Hybrid"	"Heavy"
BUDR substitution, *per cent*	0	22.7	45.4
Calculated density, *gm./cm.*[3]	1.703*	1.727‡	1.751‡
Determined density, *gm./cm.*[3]*	1.703	1.723	1.734
Increase in specific volume, *per cent*	0	0.17	0.88

* *cf.* Fig. 4 *a*.

‡ Calculated for 45.4 per cent BUDR substitution, and for thymine to thymine + cytosine molar ratio of 0.58, assuming a linear relationship between increase in molecular weight (M.W. of Br = 80; M.W. of CH_3 = 15) and DNA (acid form) density. For sodium or cesium salts of DNA (45.4 per cent BUDR substitution) the calculated densities would amount to 1.747 or 1.737 ("heavy") and 1.725 or 1.719 ("hybrid"), respectively. The 100 per cent bifilar BUDR substitution should correspond to buoyant densities of 1.808 (acid form), 1.801 (sodium salt), and 1.776 (cesium salt of DNA).

the 6-day growth period in the presence of various concentrations of BUDR or IUDR. After trypsinization, the cells were irradiated "in suspension" with UV light (*cf.* Materials and Methods). Scoring of the survivors by plating and colony count (Fig. 6) revealed a pronounced effect of analog incorporation on the UV sensitivity of the cells. Dose reduction factors of the order of 3.8- to 16-fold for IUDR and 22-fold for BUDR can be calculated from the final slopes of the dose-survival curves for normal and analog-labeled cells.

Neither further increase in the BUDR concentration from 10 to 50 μg. per ml. nor prolongation of the exposure period from 3 to 6 days (10 μg. BUDR per ml.) had any pronounced additional effect on the final slope of the survival curve of BUDR-containing cells. In other experiments it was found that cells grown

[3] The x-ray irradiation was kindly performed by Dr. J. R. Helff at Middlesex General Hospital, New Brunswick, New Jersey. The cells were irradiated with the Picker x-ray tube operating at 140 kv. and 24 ma., filtered with 1 mm. of aluminum and 0.25 mm. of copper. Dose rates of 20 to 30 r per minute were employed.

for the 6-day period in the presence of 2.5 or 5 μg. BUDR per ml. show a degree of radiosensitization almost equivalent to that observed with 10 μg. per ml. In the case of IUDR, exposure for a period of more than 3 days resulted in a drop in plating efficiency to almost zero. This made further evaluation of UV effects impractical.

Monolayers of glass-attached cells grown in 60 mm. Petri dishes, washed, and covered with 1 ml. of balanced salt solution (*cf.* Materials and Methods) were more UV-sensitive than cells "irradiated in suspension" (Fig. 6). Since the glass-

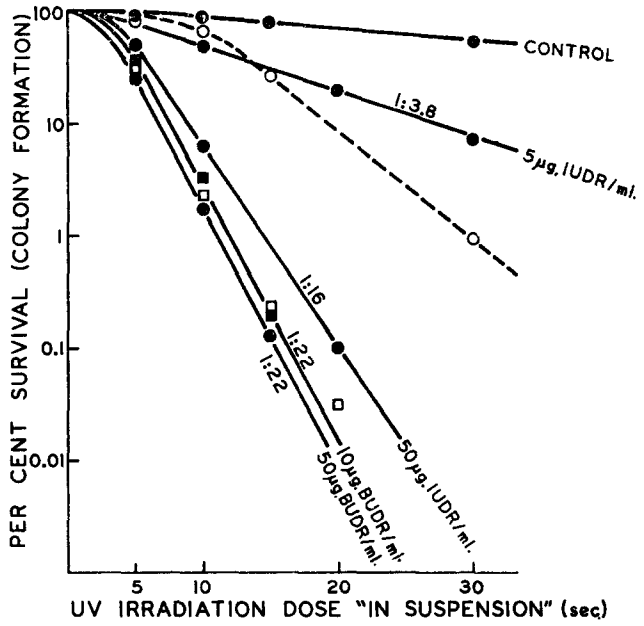

FIG. 6. The UV sensitivity of D98S cells cultivated for the last 3 days (or 6 days: ■) in the absence ("control") or the presence of BUDR (10 or 50 μg./ml.) or IUDR (5 or 50 μg./ml.), trypsinized, and irradiated "in suspension" (see Materials and Methods). The broken line (O---O) represents the UV sensitivity of an aliquot of control cells, never exposed to BUDR, irradiated 6 hours after replating (as glass-attached cells) while covered with 1 ml. of balanced salt solution per 60 mm. Petri plate.

attached cells were actually more effectively screened by the medium than the suspended cells, their greater sensitivity could be attributed rather to flattening, with resultant enlargement of the target area and lessening of the screening effect of the protoplasmic layer on the nuclei. Most of the subsequent experiments were performed on glass-attached cells, although it later became apparent that variation in the "flatness" of the cells might have affected somewhat the reproducibility of the results.

Preliminary experiments indicated that BUDR incorporation also significantly increases the sensitivity of D98S cells to x-rays. The "one-hit" dose (ap-

proximately 37 per cent survival) of approximately 270 r for the control cells was reduced to 125 or 76 r for cells cultivated for 2 days in the presence of 10 or 50 μg. BUDR per ml. respectively.

Cultivation of D98S cells in the presence of 5-bromouracil or 5-iodouracil, at concentrations up to 100 μg. per ml., had no effect on either the rate of multiplication or on sensitivity to UV light or x-rays, most probably reflecting lack of the enzymatic mechanism (permease, or phosphorylase) necessary for effective incorporation of these free pyrimidines into the DNA precursor pool.

Since two types of BUDR-labeled DNA, "hybrid" and "heavy," had been demonstrated, the question arose as to whether the radiosensitization effect was associated with BUDR incorporation into one DNA strand only ("unifilar" labeling) or into both strands ("bifilar" labeling). To answer this question, the UV sensitivity of cells grown for various periods of time in the presence (Fig. 3*b*) and absence (Fig. 4 *b*) of BUDR was evaluated in parallel with determination of the pattern of BUDR distribution in the DNA by CsCl gradient centrifugation. The density determinations have already been described and the data are illustrated in Figs. 3 *a* and 4 *a*. The UV survival data are presented in the corresponding Figs. 3 *b* and 4 *b*. As far as data obtained with poorly synchronized cultures permit (the cells are 50 to 70 per cent synchronized during the first division after the addition of BUDR), the following conclusions may be drawn: Cells containing only BUDR-free DNA (curve A) or a predominance of BUDR-free DNA molecules (curve 5) are represented by UV survival curves with slopes approximately $\frac{1}{4}$ as steep as the slopes of the corresponding curves for cells containing only (curve 1) or predominantly (middle section of curve D) "bifilarly" labeled DNA molecules. Cell populations with intermediate degrees of labeling are represented by survival curves (2 to 4 and B, C) composed mainly of two parts with slopes characteristic for cells containing either unlabeled or "bifilarly" labeled DNA. These data seem to indicate that "unifilar" BUDR labeling of DNA does not confer upon the cells a radiation sensitivity much greater than that characteristic for BUDR-free DNA, while "bifilar" BUDR labeling results in a precipitous drop in radioresistance. Thus the large increase in radiosensitivity is manifested only after the second cell division in the presence of BUDR. Due to the independent reassortment of chromosomes during mitosis, almost every daughter cell would acquire some "bifilarly" BUDR-labeled DNA as a component of its chromosomal complement.

It might be speculated that the UV sensitivity of the cells continues to increase slightly for a few cell divisions beyond the second replication, since this would result in a larger proportion of "bifilarly" BUDR-labeled chromosomal material in each cell. The experimental findings would seem to corroborate this hypothesis. On the other hand there should be little difference between the slopes of the UV survival curves for cells containing a predominance of "bifilarly" labeled DNA molecules, after the BUDR-to-thymidine replacement ratio

had reached some critical value. As discussed earlier, this critical replacement can be attained by growing the cells for 6 days at BUDR concentrations even as low as 2.5 μg. per ml.

Since FUDR was used in conjunction with BUDR or thymidine in the experiments presented in Figs. 4 *a* and 4 *b*, the effect of this analog (0.01 μg. FUDR per ml., 2-day exposure) on radiation sensitivity of the cells was evaluated. FUDR alone causes only a slight increase in the UV sensitivity of the cells, which effect is abolished by the simultaneous presence of thymidine. Thus the pronounced radiosensitivity of the BUDR + FUDR-grown cells could be ascribed to the radiosensitizing effect of BUDR only.

Biological Functionality of DNA Synthesized during Inhibition of Protein Synthesis.—Chloramphenicol (CM) and *p*-fluorophenylalanine (FA) were found to

TABLE VI

Colony Formation by D98S Cells in the Presence of Various Concentrations of Chloramphenicol or p-Fluorophenylalanine (10-Day Incubation Period)

	Plating efficiency (per cent colony-forming cells) at concentrations (μg./ml.) of CM or FA of:						
	0	10	20	40	80	100	160
p-Fluorophenylalanine (FA)*	55		56	18.5	0.65	0.06	
Chloramphenicol (CM)	55	35	0.86	0.01	0.03		<0.01

* FA present during the initial 48 hour incubation period only.

inhibit the multiplication of D98S cells at concentrations above 10 or 40 μg. per ml., respectively (Table VI), while 5-methyltryptophan did not affect the cells at concentrations up to 100 μg. per ml. Since the DNA produced during CM inhibition of protein synthesis had been shown to be functional in bacteria (26, 27) and bacteriophages (28–30), a similar problem was posed for mammalian cells (31). A criterion for the biological functionality of human DNA was available in the pronounced change in the UV sensitivity of D98S cells associated with the synthesis of new BUDR-containing (Figs. 3 *a*, *b*) or BUDR-free (Figs. 4 *a*, *b*) DNA. Should DNA produced during inhibition of protein synthesis confer upon the cells the novel biological property of radiosensitivity or radioresistance, such DNA might be considered functional.

The cells were grown for 5 to 6 days in the presence of 40 or 80 μg. CM per ml., since at these concentrations protein synthesis is depressed by more than 65 per cent (determined for the period between the 4th and 6th day of incubation, during which time half the cultures were exposed to 10 μg. BUDR per ml.), without appreciable cell loss due to detachment from the glass or cell death (Table VII). The cells were trypsinized on the 6th day and replated; and the UV sensitivity of the glass-attached cells was determined (*cf.* Materials and Methods).

As can be seen in Fig. 7, the cells grown for the 5-day period in the presence of CM became slightly more radiation-resistant. The ratio of the final slopes of curves 1 (control) and 2 (CM-grown cells) is approximately equal to 1.5. The slight break in curve 2 may indicate some inhomogeneity in the cell population grown in the presence of CM. Essentially identical results were obtained with CM concentrations of 40 and 80 μg./ml. The slight increase in the radioresistance of CM-grown cells may be interpreted as the result of a selective increase in the DNA (and RNA) content per cell, with associated self-screening against UV light (32, 33).

TABLE VII

Protein Synthesis by D98S Cells in the Presence of Various Concentrations of Chloramphenicol (CM) and BUDR

CM concentration (1st to 6th day), *μg./ml.*	0	40	80	0	40	80
BUDR concentration (4th to 6th day), *μg./ml.*	0	0	0	10	10	10
Protein, 4th day, *μg. per plate*	14	14	13	14	14	13
6th day, *μg. per plate*	40	23	21	36	23	22
Net synthesis (4th to 6th day), *μg. protein/plate*	26	9	8	22	9	9
Inhibition of net protein synthesis, *per cent**	0	65.5	69.3	15.4	65.5	65.5
DNA content per cell (6th day), *μg.* × 10^{-6}	10.0			8.1		

* Per cent decrease in net protein synthesis between 4th and 6th day of incubation, in relation to net protein synthesis in CM- and BUDR-free culture. Cells were harvested on the 6th day, and their UV sensitivity was determined (Fig. 7).

The cells grown in the BUDR-containing media, both in the presence or in the absence of CM, became highly radiation-sensitive, although CM inhibition again had a small radio-desensitizing effect (curve 4). The slope ratio for UV survival curves 1 and 3 (CM–non-inhibited cells) is approximately equal to 1/5.2, while the ratio of the final slopes of curves 2 and 4 (CM-inhibited cells), is equal to 1/3.7. These data indicate that human DNA produced during partial inhibition of protein synthesis confers upon the cells the new biological property of radiosensitivity; *i.e.*, such DNA is biologically functional.

The reciprocal experiments, based on the design presented in Fig. 4, were performed with BUDR + FUDR–grown D98/BUDR cells. Both in the presence and in the absence of CM or FA inhibition of protein synthesis, the cells transferred from BUDR-containing to thymidine-containing medium produced new BUDR-free DNA, which conferred upon the cells a high increase in radioresistance. This observation testifies again, and in an even more convincing manner, to the biological functionality of DNA produced by human cells during inhibition of protein synthesis.

Discussion and Evaluation of the Theoretical and Practical Implications of the Results

These experiments have clearly demonstrated that the human cell line D98S can multiply indefinitely in the presence of BUDR, under conditions which permit replacement of up to 45 per cent of the thymidine in the DNA by BUDR. Although the growth rate of such chemically modified cells is initially slower, almost complete viability is retained, as evidenced by unimpaired plating efficiency.

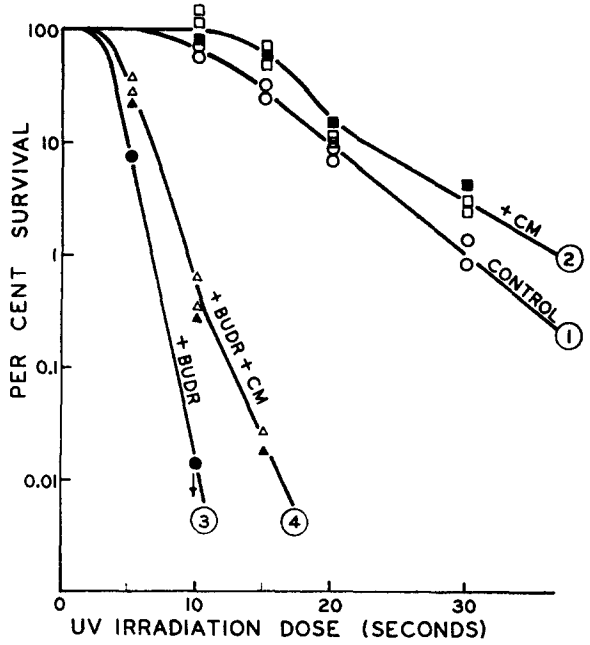

FIG. 7. Effect of BUDR incorporation, in the presence and absence of inhibitory concentrations of chloramphenicol (CM), on the UV sensitivity of D98S cells. The cells were cultured for 6 days in the presence of CM, while BUDR was added only during the last 2 days of this period. Three hours after replating, the glass-attached cells were irradiated. Curve 1: control, without CM or BUDR (○); Curve 2: only CM at 40 μg./ml. (□) or at 80 μg./ml. (■); Curve 3: only BUDR at 10 μg./ml. (●); Curve 4: BUDR at 10 μg./ml. plus CM at 40 μg./ml. (△) or 80 μg./ml. (▲). (For protein synthesis during the last 2 day period, *cf.* Table VII.)

Cursory examination of the chromosomal complement of the BUDR-labeled cells (up to 45 per cent thymidine replacement) and comparison with that of normal cells, conducted in cooperation with Dr. A. Levan of the University of Lund, revealed essentially no differences in chromosome number or morphology and only very occasional chromosome breaks in BUDR-labeled cells. Only at

high BUDR concentrations (above 10 μg./ml. for D98S cells or much higher concentrations for the D98/BUDR line), which noticeably affect cell viability (Table I), did the chromosomal morphology become significantly distorted.

Since the DNA of human cells seems to replicate in a semiconservative fashion, as indicated by the results of this study, BUDR is incorporated only into newly synthesized polynucleotide strands during the first DNA duplication (Fig. 5). Expressed otherwise, after a single replication in the presence of BUDR, only "hybrid" DNA molecules are present, each composed of one BUDR-free and one BUDR-labeled strand. Only after the second replication do "heavy" DNA molecules, with both strands BUDR-labeled, appear. According to the scheme depicted in Fig. 5, the proportion of "hybrid" molecules quickly diminishes during successive cell divisions.

Thus the maximum degree of BUDR incorporation achieved (about 45 per cent thymidine substitution) (Table III) results from a fortuitous balance between the intracellular availabilities of 5-bromodeoxyuridylic and thymidylic acids for DNA synthesis, rather than from the incorporation of BUDR into only one of the two strands of each DNA molecule, as has been postulated by others.

Comparative studies on the radiation sensitivity of normal D98S cells and cells grown in the presence of BUDR revealed the association of a sharp increase in UV light and x-ray sensitivity with BUDR incorporation into the DNA. Whether this phenomenon can be attributed directly to modification of the DNA structure or to some other BUDR effect cannot be determined at this time. However, the following observations would seem to favor the direct effect:

(*a*) There is a definite correlation between the manner of BUDR distribution in the DNA and the radiation sensitivity of the cells; *i.e.* normal cells and those believed to contain exclusively "hybrid" DNA molecules exhibit similar dose-survival curves, with reduction in the multi-hit character for the latter cells (Fig. 3 *b*). Only with the formation of "heavy" DNA molecules is radiosensitivity profoundly altered. Since this is achieved after only two cell divisions in the presence of BUDR, "bifilar" labeling of approximately half the DNA molecules is apparently sufficient (Fig. 5). Moreover, at each mitotic division these "heavy" molecules must be approximately equally distributed between the daughter nuclei through random chromosome reassortment.

(*b*) BUDR incorporation is known to increase the radiosensitivity of a variety of organisms, including mammalian cells, certain bacteria (6), and the bacteriophage ΦX-174 (5). In the latter case, a direct role of modification of DNA structure in the radiosensitization phenomenon is particularly suggestive, since extracellular phage contains only DNA and protein components. It is rather improbable that the protein coat of phage and proteins of diverse unicellular "organisms" would have a common function in BUDR-initiated radiosensitization.

The nature of the BUDR-effected increase in UV and x-ray sensitivity has

not yet been elucidated. The apparent increase in the specific volume of the DNA molecule as the result of BUDR incorporation into *both* strands, indicates either (*a*) modification of the hydrogen bonding between adenine and BU, or (*b*) a higher degree of hydration of the substituted DNA molecule during centrifugation in the CsCl gradient. The results of preliminary studies by Dr. Z. Lorkiewicz, Z. Opara-Kubinska, and one of the present authors on the heat denaturation (melting temperature) (34) of the normal and BUDR-labeled DNA (dissolved in 0.15 M NaCl + 0.015 M sodium citrate) would not seem to favor the first interpretation, since the "melting" temperature is hardly affected by BUDR incorporation (85.8°C. for normal and 85.4°C. for BUDR-labeled DNA, with the shape of the "melting" curve indicating the marked inhomogeneity of human DNA). Similarly, the marked radiosensitization effect observed with phage ΦX-174 (5), each particle of which is known to contain only one DNA molecule in *single stranded* form (35), seems also to argue against any role of hydrogen bonding between adenine and BU.

It might be pertinent to recall that the comparative spectrophotometric studies of Moore and Thomson (36) indicated a higher susceptibility of BU to irreversible UV damage than of thymine.

All these data suggest that UV damage is localized largely in the thymine or BU moieties of the DNA strands and takes place when the DNA is in a transiently (dividing cells) or permanently (extracellular phage ΦX-174) single stranded state. It is also probable that BUDR incorporation either potentiates irreversible radiation damage and consequently destroys the function of the DNA as a primer (template), or prevents the (enzymatic?) repair of reversible radiation damage.[4]

IUDR also exhibits a radiosensitizing effect, but the low viability of IUDR-treated cells makes more extensive study impractical. Bromine, with its atomic radius (1.95 A) very close to that of the methyl group (2.0 A) of thymine, seems to be a much better substitute for the latter than the iodine atom, which has a substantially larger radius (2.16 A).

The radiosensitizing effect of BUDR incorporation opens new possibilities for selective potentiation of the radiotherapy of localized tumors. The rationale for this new approach would be based on: (*a*) the selectivity of the radiosensitizing action for *dividing* cells, *i.e.* primarily for neoplastic cells, since BUDR is incorporated only during the synthesis of new DNA; and (*b*) the practicality of strictly *localized* irradiation, a normal practice in radiation therapy. To achieve substantial BUDR incorporation into *both* strands of the tumor DNA would

[4] L. Okun and F. Stahl (personal communication) have observed that UV-inactivated, BU-labeled phage T2 is not reactivable by visible light, and postulated that BUDR substitution renders DNA incapable of entering into the repair process. Impairment of photoreactivation of BU-containing bacteria was reported by Greer (*J. Gen. Microbiol.*, in press), and by Lorkiewicz and Szybalski (38).

require long term preliminary administration of BUDR, in such a manner as to maintain a level of greater than 2 μg. BUDR per ml. in the tumor cell environment during the course of at least two cell divisions. Local administration of BUDR might be the most effective means of achieving this. The results obtained *in vitro* suggest that low concentrations of FUDR or other thymidylic acid–synthetase inhibitors might increase the extent of BUDR incorporation *in vivo*. It is obvious that studies of the distribution of BUDR in normal and tumor tissue in the body are needed. Preliminary tests indicate that BUDR has very low toxicity for mice. This observation and the *in vitro* data suggest that BUDR would not significantly affect the viability of normal dividing cells of the body, such as bone marrow or intestinal epithelium, into which it might be incorporated. Although the latter might also become radiosensitized, the selectivity of localized irradiation practices should prevent overexposure of these tissues.

The localization of BUDR and IUDR in tumor tissue by virtue of preferential incorporation into proliferating (DNA-synthesizing) cells, in conjunction with the existence of gamma-emitting isotopes of bromine and iodine, suggest two further potential applications of these analogs in the therapy of cancer: (*a*) diagnostic localization of tumors by gamma ray scanning after administration of radioactive BUDR or IUDR, and (*b*) internal irradiation of tumors by means of incorporated BUDR or IUDR, either injected as radioactive compounds or made radioactive *in situ* by neutron irradiation (neutron capture).

Since BUDR-labeled DNA seems to function normally in the cells, BUDR represents a very useful experimental tool. The new biological property (radiosensitivity) of BUDR-labeled DNA and its increased density (as measured by CsCl gradient centrifugation) permit it to be conveniently traced. In the present study, BUDR labeling was utilized as a tool for elucidating the mode of replication of human DNA, and also gave some clues as to the normal biological nature of the DNA produced during partial inhibition of protein synthesis.

It might also be of some practical interest that the antibiotic chloramphenicol inhibits human bone marrow–derived cells *in vitro* at rather low concentrations, and long term exposure results in irreversible damage. A similar effect may account for the *in vivo* toxicity of chloramphenicol for the hematopoietic system (37).

Further studies on the biological and physicochemical properties of substituted DNA are being actively pursued in this laboratory.[5]

SUMMARY

The human cell line D98S can be cultivated indefinitely in the presence of up to 3×10^{-5} M 5-bromodeoxyuridine (BUDR), without loss of cell viability. During this time, BUDR is incorporated into both strands of the DNA mole-

[5] McArdle Memorial Laboratory, The University of Wisconsin Medical School, Madison.

cules, replacing up to 45 per cent of the thymidine and thereby rendering the cells highly sensitive to UV light and to x-rays. Cells grown for a limited period of time in the presence of 5-iododeoxyuridine (IUDR) become UV-sensitized, while prolonged cultivation with IUDR results in the loss of cell viability. The properties of the BUDR label permitted the demonstration that: (*a*) human DNA replicates in a "semiconservative" manner; (*b*) the degree of radiosensitization of BUDR-treated cells depends on whether the DNA has been substituted in one strand only ("unifilarly") or in both strands ("bifilarly"); (c) functional human DNA is produced during partial inhibition of protein synthesis. The potential applicability of this new rational principle of radiosensitization to the radiotherapy of neoplastic diseases is discussed.

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