MOLECULAR PHARMACOLOGY, 6, 273–280

Fluorinated Pyrimidines

XXXVII. Effects of 5-Trifluoromethyl-2′-deoxyuridine on the Synthesis of Deoxyribonucleic Acid of Mammalian Cells in Culture

YOSHISADA FUJIWARA,[1] TOSHIKAZU OKI, AND CHARLES HEIDELBERGER[2]

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706

(Received December 12, 1969)

SUMMARY

5-Trifluoromethyl-2′-deoxyuridine (F_3TdR), a thymidine analogue, is incorporated into the DNA of HeLa and leukemia L5178Y cells in culture. Under conditions of synchrony induced by the production of thymidine deficiency and rescue by thymidine or a thymidine analogue, the addition of F_3TdR does not permit any cell division, in contrast to thymidine, 5-bromo-2′-deoxyuridine, or 5-iodo-2′-deoxyuridine, which support cell division. However, F_3TdR is incorporated into the cellular DNA, but to only about one-fourth the extent of thymidine. Alkaline sucrose gradient sedimentation revealed that the molecules of DNA containing F_3TdR are smaller than normal DNA, suggesting that the rate of growth or joining of chains of DNA is inhibited as a result of the incorporation of the analogue.

INTRODUCTION

5-Trifluoromethyl-2′-deoxyuridine (trifluorothymidine) was first synthesized in this laboratory (1) as an analogue of thymidine. As expected, it is incorporated into DNA of bacteriophage T4 (2), mammalian cells in culture (3), and to a small extent into mouse tumors *in vivo* (4). Moreover, the nucleotide F_3TdRP[3] inhibits thymidylate synthetase irreversibly (5). F_3TdR is an active inhibitor of the growth of mouse tumors (6), is currently undergoing initial clinical evaluation in patients with advanced cancer, and is very effective against herpes simplex keratitis in rabbit (7, 8) and human eyes.[4] In our laboratory, we have shown that F_3TdR inhibits the replication of vaccinia virus in HeLa cells (9). This inhibition is prevented by the simultaneous, but not by the delayed, addition of thymidine, suggesting that an irreversible event occurred (9), which we have found to be the incorporation of the analogue into the vaccinia viral DNA (10, 11).

It has been shown by many others that the extensive incorporation of 5-bromo-2′-deoxyuridine and 5-iodo-2′-deoxyuridine into the DNA of synchronized mammalian cells permits them to divide once (12–18). We report here that the incorporation of a small amount of F_3TdR into the DNA of synchronized HeLa and L5178Y cells prevents

This work was supported in part by Grant CA 7175 from the National Cancer Institute, National Institutes of Health.

[1] Present address, Laboratory of Medical Biophysics, Kobe University School of Medicine, Kobe, Japan.

[2] American Cancer Society Professor of Oncology, to whom requests for reprints should be sent.

[3] The abbreviations used are: F_3TdRP, 5-trifluoromethyl-2′-deoxyuridine 5′-monophosphate; F_3TdR, 5-trifluoromethyl-2′-deoxyuridine; FUdR, 5-fluoro-2′-deoxyuridine; TdR, thymidine; BUdR, 5-bromo-2′-deoxyuridine; IUdR, 5-iodo-2′-deoxyuridine.

[4] H. E. Kaufman, personal communication.

even the first division, probably because of incomplete replication of the DNA.

MATERIALS AND METHODS

Cells. HeLa S3 and leukemia 5178Y cells, mycoplasm-free by monthly tests using a modified Hayflick method (19), were cultured in suspension as previously described (20).

Synchronization. Exponential cultures (8–10 × 10^4 cells/ml) were treated with amethopterin and adenosine or with 5-fluoro-2′-deoxyuridine and uridine to produce a thymidine-deficient state (21). In the case of HeLa cells, 10^{-6} M amethopterin and 5×10^{-5} M adenosine were added for 16 hr. L5178Y cells were treated with $1–5 \times 10^{-9}$ M FUdR and $1–5 \times 10^{-7}$ M uridine for 10–12 hr (the approximate generation time) (20). The thymidine-deficient cells were then rescued by 10^{-6} M thymidine, or equimolar concentrations of analogues, to produce partial synchrony.

Determination of cell number and mitoses. Cell numbers were determined with a Coulter counter with appropriate settings (20). The accumulation of cells in mitosis was determined by scoring 500 or more acetic orcein-stained cells that had been arrested in metaphase with 0.05 μg/ml of colchicine.

Labeling of cells. To the thymidine-deficient cells either TdR-methyl-^{3}H ($1.0–7.7 \times 10^{-3}$ μCi/ml; specific activity, 11.7 Ci/mmole; Schwarz BioResearch, Inc.), BUdR-6-^{3}H (0.1 μCi/ml; specific activity, 2.3 Ci/mmole; Amersham-Searle), or F_3TdR-2-^{14}C (7.7×10^{-3} μCi/ml; specific activity, 7.7×10^{-3} μCi/mmole) was added for various lengths of time. With the exception of F_3TdR, the labeled nucleosides were diluted with the corresponding nonradioactive compound to give a final concentration of 10^{-6} M, unless otherwise stated.

Isolation and quantitation of DNA. DNA was isolated from 2×10^6 to 10^8 cells by the method of Kirby and Cook (22), or by the following modification of the method of Haut and Taylor (23), especially suitable for small cell mass. The cells were lysed in 2% sodium dodecyl sulfate in 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0, and 1.5 M EDTA at 60° for 10 min. To this mixture 5 M $NaClO_4$ was added to give a final concentration of 1 M, and this material was deproteinized several times with chloroform–isoamyl alcohol (24:1). The nucleic acids were precipitated with 2 volumes of cold ethanol and redissolved in 0.15 M NaCl plus 0.015 M sodium citrate, and the RNA was hydrolyzed with heat-treated RNase (Worthington). Further deproteinizations were done until no more material was visible at the interface between the water and organic phases. The DNA concentration was determined by the methods of either Burton (24) or Kissane and Robins (25), using calf thymus DNA as a standard.

Alkaline sucrose gradient sedimentation of DNA. L5178Y cells were lysed in 0.05 M Tris-HCl, pH 8.0, 5 mg/ml of Chelex (Bio-Rad), 0.2% sodium dodecyl sulfate, and 3 mg/ml of Pronase (Calbiochem) according to the method of Schandl and Taylor (26). The enzyme was heated for 90 min at 37° and for 2 min at 80° before addition. The cell lysate was incubated with Pronase for 6 hr at 37°. The DNA was then denatured by dropwise addition of 0.4 M NaOH until the solution reached pH 12. Centrifugation was carried out at 22,000 rpm (40,000 × *g*) for 6 hr at 15°. A 5–20% linear gradient of sucrose containing 0.8 M NaCl, 0.2 M NaOH, and 1 mM EDTA, pH 12.5–13.0, was prepared according to McGrath and Williams (27), and 0.2–0.5 ml of the denatured DNA solution was layered on top. With the Spinco SW 39L rotor, 4 ml of gradient were used; for the International SB405 rotor, the volume of the gradient was 3.0 ml. After centrifugation, the bottoms of the tubes were pierced, 25–30-drop fractions were collected directly into 1 ml of water in liquid scintillation vials, and 10 ml of Scintisol (Isolab, Inc.) were added for counting.

For neutral sucrose sedimentation of native DNA, the NaOH was not added after Pronase treatment, and a 5–20% sucrose gradient containing 0.15 M NaCl, 0.015 M sodium citrate, and 1 mM EDTA, pH 7.0, was used (28).

Preparative CsCl density gradient equilibrium centrifugation. The preparative CsCl centrifugation at both neutral and alkaline pH was carried out according to Szybalski

(29). The initial density of the CsCl solution, containing 1 mM Tris and 1 mM EDTA, was adjusted to a density of 1.72 g/ml for normal cellular DNA (for BUdR-containing DNA, the density was 1.76 g/ml), and the pH was adjusted to 12.5 with 2 N NaOH. The density equilibration was attained by centrifugation at 38,000 rpm in an SW 50 head of the Spinco model L ultracentrifuge for 48 hr at 20–25°. After centrifugation, 5-drop fractions were collected from the bottom, the densities of the individual fractions were measured both by refractometry and by pycnometry at 25°, the fractions were diluted to 1 ml with distilled water, and the radioactivity and absorbance at 260 mμ were determined.

Measurement of radioactivity. DNA was dissolved in 88% formic acid, which was then evaporated in a vacuum over KOH, and the hydrolyzed DNA was dissolved in water for counting. Alternatively, DNA was digested with DNase (Worthington) directly in the counting vial. The radioactivity of DNA precipitated on filters was measured in 2,5-diphenyloxazole, 1,4-bis [2-(5-phenyloxazolyl)]benzene, and toluene, and the DNA hydrolysates were counted in "ANPO" mixture (738 g of naphthalene, 46 g of 2,5-diphenyloxazole, 0.46 g of α-naphthylphenyloxazole, 3500 ml of xylene, 3500 ml of dioxane, and 2100 ml of absolute ethanol) in a Packard Tri-Carb liquid scintillation spectrometer. More recently, Scintisol (Isolab, Inc.) was used instead of "ANPO."

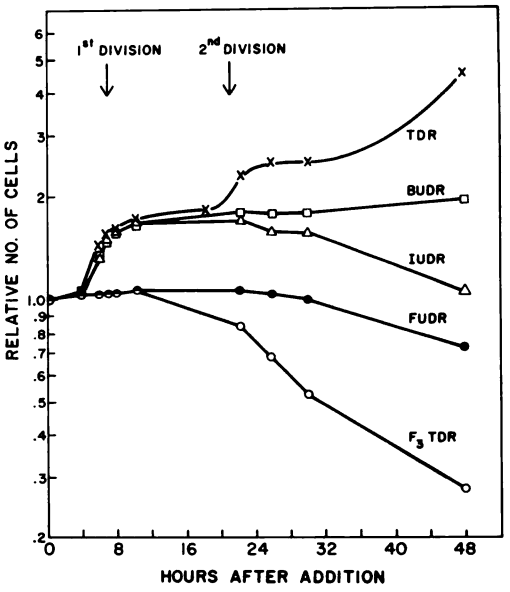

FIG. 1. *Effects of F_3TdR, BUdR, IUdR, and TdR on cell division in partially synchronized populations of L5178Y cells*

Exponentially growing L5178Y cells were subjected to the inhibition of DNA synthesis by exposure to 10^{-9} M FUdR plus 10^{-7} M uridine for 12 hr. Thymidine-deficient cells (9.75×10^4 cells/ml) at time zero were rescued with 10^{-6} M TdR and equimolar amounts of analogues in the continued presence of FUdR plus uridine. The increase in cell number, expressed as relative number of cells, was determined up to 48 hr after synchronization. ●——●, FUdR plus uridine; ○——○, 10^{-6} M F_3TdR; □——□, 10^{-6} M BUdR; △——△, 10^{-6} M IUdR; ×——×, 10^{-6} M TdR.

RESULTS

Effect of FUdR, BUdR, IUdR, TdR, and F_3TdR on the growth of thymidine-deficient cells. Exponentially growing L5178Y cells were treated with 10^{-9} M FUdR plus 10^{-7} M uridine for 12 hr to inhibit DNA synthesis by causing a thymidine deficiency. At zero time in Fig. 1, TdR or its analogues were added, and cells were counted periodically. When 10^{-6} M TdR was added in the continued presence of FUdR plus uridine, the cells began to increase in number at 4 hr, and the first wave of cell division was complete by 10 hr. A second wave of mitosis occurred from 20 to 26 hr. In the case of those cells maintained on FUdR plus uridine without thymidine addition, there was no change in cell number for 32 hr, followed by a decrease. The addition of 10^{-6} M BUdR (with continuing FUdR plus uridine) caused a first division of cells equivalent to that produced by TdR. However, no second division occurred, and the cell number remained approximately constant thereafter. Under the same circumstances, 10^{-6} M IUdR also caused the first division, but not the second; these cells, in contrast to those treated with BUdR, subsequently died. These results with BUdR and IUdR are in agreement with those of many other investigators (12–18). However, as shown in Fig. 1, the addition of 10^{-6} M F_3TdR, although it is capable of being incorporated into DNA, did

not allow any cell division in this system, and after about 16 hr there was considerable cell death. Similar findings were made in comparable experiments with HeLa cells.

The same phenomenon was confirmed in L5178Y cells by measuring the accumulation of cells in metaphase induced by colchicine. Since TdR caused the initiation of cell division at 4 hr (Fig. 1), 0.05 μg/ml of colchicine was added at that time. As shown in Fig. 2, TdR and BUdR addition led to the accumulation of metaphase cells at identical rates and without delay. By contrast, F_3TdR addition did not give rise to any mitoses, although the cells appeared to be healthy by microscopic examination up to 10 hr. Later, the type of morphology of cell death induced by F_3TdR resembled that due to unbalanced growth (30).

These results suggest that (a) F_3TdR behaved identically with FUdR to inhibit DNA synthesis, and was not incorporated into DNA, or (b) F_3TdR was incorporated into DNA, but the resulting DNA was nonfunctional for some reason.

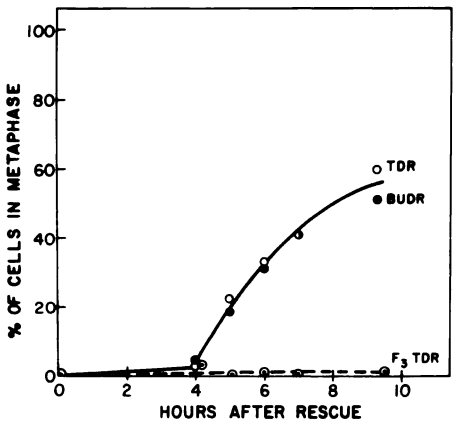

FIG. 2. *Effect of 10^{-6} M F_3TdR on accumulation of metaphases in L5178Y cells*

Thymidine-deficient cells at a density of 1.04×10^5 cells/ml, produced by the conditions described in the legend for Fig. 1, were rescued with TdR, BUdR, and F_3TdR at concentrations of 10^{-6} M in the continuous presence of FUdR plus uridine. Colchicine (0.05 μg/ml) was added to the cultures 4 hr after rescue. The cells arrested in metaphase were counted on acetic orcein-stained preparations by scoring about 500 cells. ○——○, 10^{-6} M TdR; ●——●, 10^{-6} M BUdR; ⊙——⊙, 10^{-6} M F_3TdR.

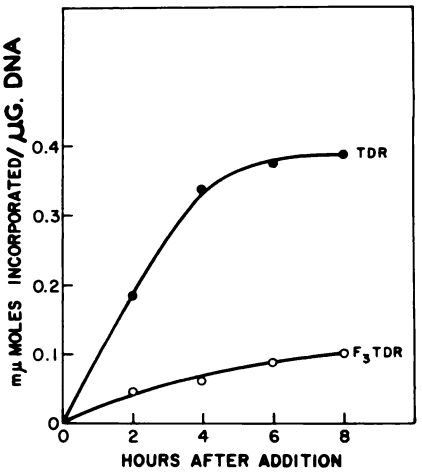

FIG. 3. *Incorporation of TdR-^{3}H (10^{-6} M, 1 μCi/ml) and F_3TdR-2-^{14}C (10^{-6} M, 7.7×10^{-3} μCi/ml) into the DNA of partially synchronized HeLa cells*

Thymidine-deficient cells (8×10^4 cells/ml), which had been treated with 10^{-6} M amethopterin plus 5×10^{-5} M adenosine for 16 hr, were labeled with 10^{-6} M TdR-^{3}H (1 μCi/ml) or F_3TdR-2-^{14}C (7.7×10^{-3} μCi/ml) for various times up to 8 hr. DNA was isolated from about 6×10^6 cells as described under MATERIALS AND METHODS, and determined by the modified Kissane and Robins method (25). The radioactivity of DNA was measured after hydrolysis in 88% formic acid, desiccation, and dissolution in distilled water. The incorporation is expressed as nanomoles incorporated per microgram of DNA as a function of time after reversal and was calculated as described in the text. ●——●, TdR-^{3}H; ○——○, F_3TdR-2-^{14}C.

Incorporation of F_3TdR into cellular DNA. A comparison was made of the incorporation of 10^{-6} M TdR and 10^{-6} M F_3TdR into the DNA of HeLa cells that had been arrested and made thymidine-deficient by treatment with 10^{-6} M amethopterin plus 10^{-5} M adenosine for 16 hr. It was found (Fig. 3) that the kinetics of the uptake of TdR into DNA in the first replication cycle reached a plateau at 6 hr, and the total amount of substitution of new TdR in the DNA amounted to 45% of the total DNA TdR, which is equivalent to 90% synchrony. This corresponds to the amount of substitution obtained with BUdR (see below). The thymine content of the HeLa cell DNA (micrograms of dTMP per microgram of DNA) was

calculated from the equation of Schildkraut *et al.* (31):

$$\% \text{ GC} = \frac{\rho - 1.660}{0.098}$$

where ρ = 1.700 g/ml for HeLa cell DNA, and the molar percentage of thymine is thus 30%. If DNA were a statistical tetranucleotide, the weight percentage of dTMP would be 23.9%. Therefore, the micrograms of dTMP per microgram of DNA = 0.239 × 0.30/0.25 = 0.287. Furthermore, the nanomoles of dTMP in 1 μg of DNA = 0.287 μg/322 (molecular weight of dTMP) = 0.864 mμmole. Therefore, an incorporation of 0.385 mμmole of dTMP at 8 hr (Fig. 3) equals a replacement of 0.385/0.864 = 45%.

On the other hand, the rate of incorporation of F_3TdR-2-^{14}C into the cellular DNA was considerably slower than that of the TdR, and at 8 hr the replacement of TdR in the DNA amounted to 12%. Thus, although there was some synthesis of cellular DNA in the presence of F_3TdR, which was incorporated, there was no cell division (Fig. 1). Similar results were obtained with L5178Y cells.

Incorporation of BUdR into cellular DNA. Thymidine-deficient HeLa cells were rescued with 10^{-5} M BUdR-6-^{3}H, and the DNA was isolated 10 hr later and subjected to preparative neutral and alkaline cesium chloride density gradient centrifugation. As shown in Fig. 4, the buoyant density of the hybrid DNA was 1.725 g/ml. As mentioned above, HeLa cell DNA has a thymine content of 30%. According to Erikson and Szybalski (32), the complete replacement of the 30% TdR in the DNA by BUdR would give a density increment of 0.062 g/ml. Since (Fig. 4) the buoyant density in neutral CsCl of the BUdR-containing DNA is 1.725 g/ml, this represents a replacement of 40% of the dTMP in the DNA. In addition, the alkaline CsCl centrifugation profile revealed that in the heavy strand (ρ = 1.831 g/ml) BUdR replaced 90% of the TdR, based on the calculations of Baldwin and Shooter (33). Thus, the replacement of 40–45% in double-stranded HeLa cell DNA with either TdR or BUdR appears to be maximal for the replication of the first cycle

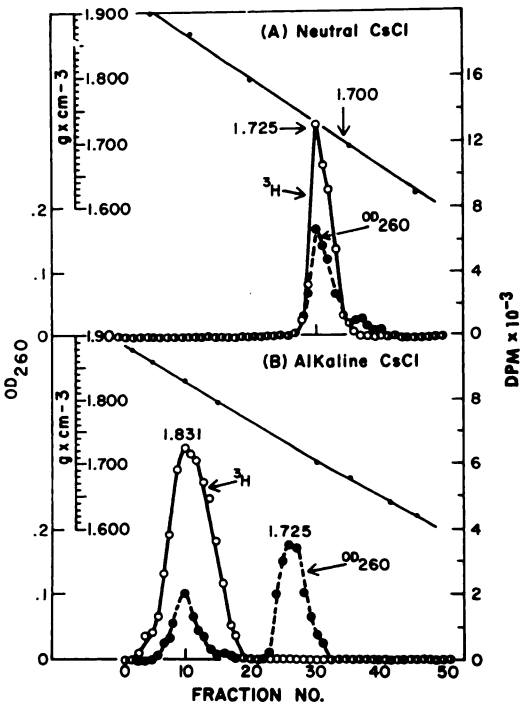

FIG. 4. *Preparative alkaline and neutral CsCl density equilibrium centrifugation of BUdR-containing hybrid DNA from partially synchronized HeLa cells*

Thymidine-deficient HeLa cells (2.70 × 10^5 cells/ml) were incubated with 10^{-5} M BUdR-6-^{3}H (0.05 μCi/ml) for 10 hr after rescue. DNA was isolated by the method of Kirby and Cook (22). The DNA (100 μg) was applied to each tube, containing neutral or alkaline CsCl solution. The density and pH were adjusted and centrifugation was carried out as described under MATERIALS AND METHODS. After densitometry, each 5-drop fraction was diluted to 1 ml for measuring the absorbance at 260 mμ and radioactivity.

in chemically synchronized cells. The fact (Fig. 4) that all the ^{3}H from the labeled BUdR was contained in the denser strand demonstrates the effectiveness of the synchronization.

Alkaline sucrose sedimentation pattern of DNA. It is now known that during DNA biosynthesis in bacteria (28), in whole mammalian cells (26), and in isolated HeLa cell nuclei (34) the DNA is initially formed in rather small fragments that become assembled into the high molecular weight DNA found normally in the organism. This se-

quence of events can be studied by alkaline sucrose gradient sedimentation of the DNA labeled after various time periods.

We have done so in L5178Y cells that had been labeled with either ^{3}H-TdR or ^{14}C-TdR for 48 hr before partial synchronization so that both strands of the DNA were uniformly labeled. Then the block of DNA synthesis was released by ^{3}H-TdR (with the ^{14}C-TdR-labeled cells) or by ^{14}C-F$_3$TdR (with the ^{3}H-TdR-labeled cells) for various periods of time from 5 min to 7.5 hr (at which time DNA synthesis is complete). The results of alkaline sucrose density gradient sedimentation of the DNA are shown in Fig. 5. Under the conditions used, involving Pronase treatment (26), the previously labeled DNA sedimented with approximate sedimentation coefficients (27) ranging from 33 to 38, which are within experimental error. In the sequence of experiments with thymidine (Fig. 5), it is clear that with the short pulses, small pieces of DNA are synthesized with somewhat variable sedimentation coefficients. By 2 hr the DNA is almost completely assembled, and at 7.5 hr the pre- and post-label sedimentation patterns coincide exactly. Examination of the sequence of experiments with F$_3$TdR reveals that the DNA was assembled more slowly when it contained the analogue than under normal conditions. Even after 7.5 hr, when DNA synthesis has been completed, the newly synthesized strands are still considerably smaller than those of the normal previously labeled DNA, corresponding to one or two breaks per strand.

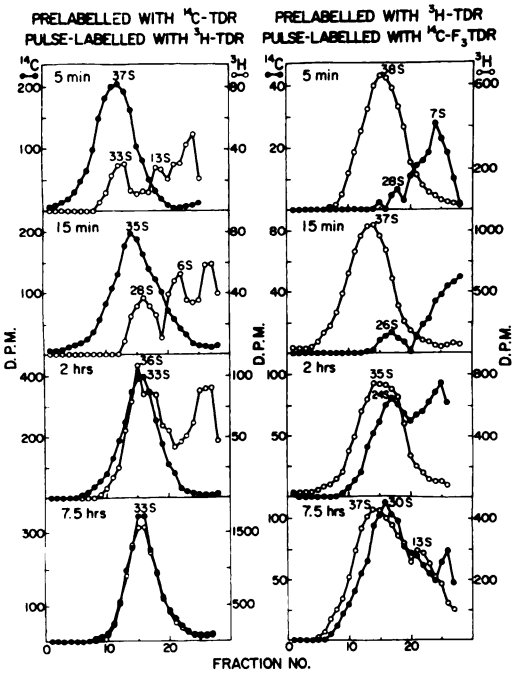

FIG. 5. *Alkaline sucrose gradient centrifugation of DNA of L5178Y cells*

Cells were first labeled either with ^{14}C- (0.01 μCi/ml) or ^{3}H- (0.1 μCi/ml) thymidine for 48 hr. They were then harvested and partially synchronized with FUdR as described in the text. They were then released from the block and pulse-labeled for the times indicated with 10^{-6} M ^{3}H-TdR (7.7×10^{-3} μCi/ml; Schwarz BioResearch) or 10^{-6} M F$_3$TdR-2-^{14}C (7.7×10^{-3} μCi/ml). The cells were worked up and centrifuged as described in MATERIALS AND METHODS. The *S*-values were calculated from the equation of McGrath and Williams (27),

$$S = \frac{7.1 \times 10^{10} d}{(\text{rpm})^2 t}$$

where t = time of centrifugation and d = distance of sedimentation in centimeters.

As shown in Fig. 6, neutral sucrose gradient sedimentation of DNA samples mixed after 7.5 hr of labeling following release of the FUdR block with ^{3}H-TdR and ^{14}C-F$_3$TdR demonstrates that there is an exact coincidence of the main DNA peaks, and that there is an additional small peak at 10 S from the F$_3$TdR-treated cells.

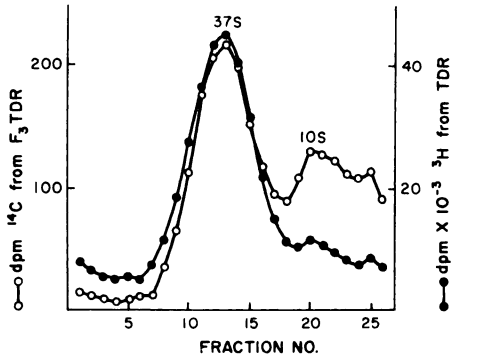

FIG. 6. *Neutral sucrose gradient centrifugation of DNA of L5178Y cells*

Cells were blocked as described with FUdR and released with 10^{-6} M ^{3}H-TdR or 10^{-6} M F$_3$TdR-2-^{14}C and incubated for 7.5 hr at 37°. The procedure is described in MATERIALS AND METHODS.

DISCUSSION

The pyrimidine nucleoside analogues BUdR and IUdR are very extensively incorporated into the DNA of various cells. As a characteristic consequence of such incorporation, cells containing the hybrid DNA synthesized in the presence of the analogue following the release of a thymidine-deficient state and under the inhibition of dTMP synthesis *de novo* can divide only once in the presence of FUdR (12–16) (Fig. 1). Under comparable conditions, Morris and Cramer have shown that one-half of IUdR-treated P815Y cells lose their ability to synthesize DNA (17). Moreover, Berkovitz *et al.* (18) have recently demonstrated that the combination of BUdR plus FUdR specifically inhibits HeLa cell division but permits DNA synthesis to continue, which leads to the production of giant, multinucleated cells.

By contrast, F_3TdR, as we have shown here, completely prevents even the first cell division after synchronization of HeLa and L5178Y cells. It was found under these conditions that there was incorporation of F_3TdR into the DNA, but the rate was slower and the saturation level lower than was found with TdR. The inhibition of DNA synthesis produced by F_3TdR under conditions of rescue from the thymidine-deficient state may result from impaired phosphorylation of the analogue or the inhibition of DNA polymerase. It has been shown by Bresnick and Williams (35) and in this laboratory[5] that F_3TdR is phosphorylated to the monophosphate by thymidine kinase at approximately the same rate as are TdR, BUdR, and IUdR. However, the further phosphorylation of F_3TdRP to the di- and triphosphates takes place at a greatly reduced rate.[6] It is further possible that the F_3TdR triphosphate (which has not yet been synthesized) might be an inhibitor of dTMP kinase and either an inhibitor of, or a poor substrate for, DNA polymerase.

We have demonstrated by means of the BUdR–cesium chloride density gradient experiment (Fig. 4) that in the release from the blockage of DNA synthesis that leads to synchronous division, only one strand of DNA is synthesized. We have then investigated the assembly of small pieces of DNA in the new strand by alkaline sucrose gradient centrifugation and have found that the rate and extent of assembly are greatly diminished when F_3TdR is used for the release from the block and is incorporated into DNA (Fig. 5). The fact that in neutral sucrose sedimentation there is no difference between the main peaks obtained by TdR and F_3TdR release from the FUdR block shows that the incompletely joined pieces of DNA in the new strand are hydrogen-bonded to the other strand in the double helix such that the sedimentary behavior of the native DNA with and without the analogue is the same. It is not clear at the present time why the presence of F_3TdR in the DNA of mammalian cells or vaccinia virus (11) causes the production of smaller fragments of DNA that do not become joined, or become joined much more slowly, to form the larger molecules of DNA found in mammalian cells or the virus. Whether this effect is caused by partial chain termination resulting from F_3TdR incorporation, by inhibition of ligase activity, or by some other mechanism remains to be determined.

In our alkaline sucrose gradient sedimentation experiments it was necessary to treat the lysed cells with Pronase according to the method of Schandl and Taylor (26) in order to obtain reproducible values of completely formed DNA in the range of 33–38 S. These are in good agreement with the results of the above authors (26), who obtained values of about 40 S. Using the same technique for the isolation of DNA obtained from the incubation of HeLa cell nuclei, Kidwell and Mueller obtained a sedimentation of 24 S (34); however, their incubation periods were shorter than ours. In preliminary experiments in which we lysed the cells in the presence of sodium dodecyl sulfate, all the previously labeled DNA quickly sedimented to the bottom of the tube, with sedimentation coefficients greater than 900 with TdR, but much lower with F_3TdR. Such aggregation has been reported by Lett *et al.* (36), using detergent methods, and

[5] R. J. Kent and C. Heidelberger, manuscript in preparation.

[6] R. J. Kent and C. Heidelberger, unpublished observations.

sedimentation coefficients of 450 were described by Terasima and Tsuboi (37) under similar conditions. Although the latter authors imply that this very large size is correct for mammalian DNA, we believe that the consistent results that we and others have obtained when the lysate is also treated with Pronase suggest that with the use of detergents alone, either the DNA is incompletely removed from protein or else segments of DNA are connected by protein residues. To add to the complications, Fox[7] has obtained consistent values of 104 S for mammalian cell DNA in alkaline sucrose centrifugation following lysis with a modified detergent procedure, and Humphrey *et al.* (38), also with a modified detergent method, found *s*-values of 64 in alkaline sucrose.

From all the above-mentioned experiences it is evident that the method of treatment of the cells determines the size of mammalian cell DNA found in centrifugation; consequently its true size and molecular weight remain unknown. Nevertheless, we believe the decrease that we have consistently found in the rate of assembly of a DNA strand containing F_3TdR is valid, regardless of the size that mammalian cell DNA eventually turns out to have.

ACKNOWLEDGMENT

We are grateful to Mrs. Barbara Lindsey for skilled technical assistance.

REFERENCES

1. C. Heidelberger, D. G. Parsons and D. C. Remy, *J. Med. Chem.* **7**, 1 (1964).
2. H. Gottschling and C. Heidelberger, *J. Mol. Biol.* **7**, 541 (1963).
3. W. Szybalski, N. K. Cohn and C. Heidelberger, *Fed. Proc.* **22**, 532 (1963).
4. C. Heidelberger, J. Boohar and B. Kampschroer, *Cancer Res.* **25**, 377 (1965).
5. P. Reyes and C. Heidelberger, *Mol. Pharmacol.* **1**, 14 (1965).
6. C. Heidelberger and S. W. Anderson, *Cancer Res.* **24**, 1979 (1964).
7. H. E. Kaufman and C. Heidelberger, *Science* **145**, 585 (1964).
8. H. E. Kaufman, *Ann. N. Y. Acad. Sci.* **130**, 168 (1965).
9. M. Umeda and C. Heidelberger, *Proc. Soc. Exp. Biol. Med.* **130**, 24 (1969).
10. Y. Fujiwara and C. Heidelberger, *Fed. Proc.* **27**, 797 (1968).
11. Y. Fujiwara and C. Heidelberger, *Mol. Pharmacol.* **6**, 281 (1970).
12. M. T. Hakala, *J. Biol. Chem.* **234**, 3072 (1959).
13. J. W. Littlefield and E. A. Gould, *J. Biol. Chem.* **235**, 1129 (1960).
14. E. H. Simon, *J. Mol. Biol.* **3**, 101 (1961).
15. K. Kajiwara and G. C. Mueller, *Biochim. Biophys. Acta* **91**, 486 (1964).
16. J. W. Cramer and N. R. Morris, *Mol. Pharmacol.* **2**, 363 (1966).
17. N. R. Morris and J. W. Cramer, *Exp. Cell Res.* **51**, 555 (1968).
18. A. Berkovitz, E. H. Simon and A. Tolliver, *Exp. Cell Res.* **53**, 497 (1968).
19. L. Hayflick, *Tex. Rep. Biol. Med.* **23**, Suppl. 1, 285 (1965).
20. M. Umeda and C. Heidelberger, *Cancer Res.* **28**, 2529 (1968).
21. R. R. Rueckert and G. C. Mueller, *Cancer Res.* **20**, 1584 (1960).
22. K. S. Kirby and E. A. Cook, *Biochem. J.* **104**, 254 (1967).
23. W. F. Haut and J. H. Taylor, *J. Mol. Biol.* **26**, 389 (1967).
24. K. Burton, *Methods Enzymol.* **12**, Pt. B, 163 (1968).
25. J. M. Kissane and E. Robins, *J. Biol. Chem.* **233**, 184 (1958).
26. E. K. Schandl and J. H. Taylor, *Biochem. Biophys. Res. Commun.* **34**, 291 (1969).
27. R. A. McGrath and R. W. Williams, *Nature* **212**, 534 (1966).
28. R. Okazaki, T. Okazaki, K. Sakabe, K. Sugimoto and A. Sugino, *Proc. Nat. Acad. Sci. U. S. A.* **59**, 598 (1968).
29. W. Szybalski, *Methods Enzymol.* **12**, Pt. B, 330 (1968).
30. A. Lindner, *Cancer Res.* **19**, 189 (1959).
31. C. L. Schildkraut, J. Marmur and P. Doty, *J. Mol. Biol.* **4**, 430 (1962).
32. R. L. Erikson and W. Szybalski, *Radiat. Res.* **20**, 252 (1963).
33. R. L. Baldwin and E. M. Shooter, *J. Mol. Biol.* **7**, 511 (1963).
34. W. R. Kidwell and G. C. Mueller, *Biochem. Biophys. Res. Commun.* **36**, 756 (1969).
35. E. Bresnick and S. S. Williams, *Biochem. Pharmacol.* **16**, 503 (1967).
36. J. T. Lett, I. Caldwell, C. J. Dean and P. Alexander, *Nature* **214**, 790 (1967).
37. T. Terasima and A. Tsuboi, *Biochim. Biophys. Acta* **174**, 309 (1969).
38. R. M. Humphrey, D. L. Steward, and B. A. Sedita, *Mutat. Res.* **6**, 459 (1968).

[7] B. Fox, Christie Hospital, Manchester, England, personal communication.