Effect of 1-β-D-Arabinofuranosylcytosine Triphosphate on DNA Synthesis in Isolated HeLa Cell Nuclei†

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ABSTRACT: 1-β-D-Arabinofuranosylcytosine 5′-triphosphate (ara-CTP) was found to be a competitive inhibitor of DNA synthesis in nuclei isolated from synchronized S-phase HeLa S_3 cells. The inhibition was reversed by dCTP. The main inhibitory effect was to reduce the rate of elongation of the primary DNA pieces. The size of the primary pieces from ara-CTP-treated nuclei after a 60-s pulse was about 115–120 nucleotides vs. 215–220 in control nuclei. A reduction in the frequency of initiation of primary DNA pieces probably also occurred. A slight inhibition of the transfer of labeled material from the primary pieces into high-molecular-weight DNA was noted and probably arose from the fact that the reduced rate of growth of the primary pieces caused the critical size for ligation to be reached later. Synchronized HeLa S_3 cells in suspension were treated with 1-β-D-arabinofuranosylcytosine for 2 h in S-phase to give a 99% inhibition of DNA synthesis prior to isolation of nuclei. Neither the rate nor the extent of [^{3}H]TTP incorporation into DNA decreased when such nuclei were incubated in the absence of ara-CTP, but the rate of ligation of primary pieces was reduced.

DNA synthesis in HeLa cell nuclei isolated from synchronized S-phase cells has been shown to proceed through small 2–4S DNA pieces (primary DNA pieces) and is apparently discontinuous on both strands within each replicon (Krokan et al., 1975a,b). These primary pieces are subsequently ligated into DNA pieces of higher molecular weight. In the isolated nuclei the size of the labeled primary pieces increases up to about 12–14 S with increasing pulse length. While the ligation is a feature of normal DNA replication, the increase in size of the primary pieces with increasing pulse length may not be so (Krokan et al., 1975b).

We have now studied the effect of 1-β-D-arabinofuranosylcytosine triphosphate (ara-CTP)[1] on isolated HeLa cell nuclei. The corresponding nucleoside (ara-C)[1] inhibits semiconservative DNA synthesis (Masker and Hanawalt, 1974; Stenstrom et al., 1974) in both prokaryotes and eukaryotes. Ara-C (in whole cells) and ara-CTP (in isolated nuclei) strongly inhibit DNA synthesis in several systems including HeLa cells (Mattocia and Roberti, 1974), L-cells (Silagi, 1965; Graham and Whitmore, 1970a), primary rabbit kidney cells (Kaplan et al., 1968), hepatocytes (Stenstrom et al., 1974), human lymphocytes (Fridlender et al., 1974), normal and transformed hamster embryo fibroblasts (Hawtrey et al., 1974), L-1210 murine leukemia cells (Kessel, 1974), African Green Monkey kidney cells (Brander and Mueller, 1974), SV 40-infected CV_1 cells (Manteuil and Girard, 1974), and polyoma-infected 3T3 cells (Hunter and Francke, 1974; Magnusson et al., 1974). Neither repair synthesis (Stenstrom et al., 1974) nor mitochondrial DNA synthesis (Mattocia and Roberti, 1974) is inhibited.

There is apparently no complete agreement on the mode of action of ara-CTP (or of ara-C). The three main hypotheses have been: (1) Ara-C acts via inhibition of the ribonucleotide reduction system. This was suggested by Chu and Fischer (1962) but later found to be unlikely (Moore and Cohen, 1967; Skoog and Nordenskjöld, 1971). It is now gererally agreed that when inhibition of ribonucleotide reduction does occur it is a secondary effect arising from the deoxyribonucleotide pool size. (2) Ara-C is incorporated into DNA and may exert its lethal effect by way of either configurational changes in the helical DNA (Silagi, 1965) or the blocking of further addition of nucleotides, i.e., by being incorporated mainly or exclusively into the 3′-hydroxyl end of the DNA chains (Momparler, 1969, 1972; Waqar et al., 1971). Burgoyne (1974) later reported that the rate of extension of chains that have received one or more ara-CMP residues is low, but not abolished. Graham and Whitmore (1970a,b), however, found that more than 70% of the incorporated ara-C was released as a 3′-phosphate and therefore had been incorporated internally in the DNA chain rather than at the 3′-hydroxyl end. They also emphasized that the inhibition must be reversible, since cells may recover and form colonies even after very severe inhibition of DNA synthesis. Evidence for the incorporation of ara-C into internucleotide linkages has also been obtained using synchronized L 5178 mouse lymphoma cells (Zahn et al., 1972) and polyoma virus synthesizing systems (Magnusson et al., 1974; Hunter and Francke, 1975). (3) Ara-C (ara-CTP) acts by being a competitive inhibitor of DNA polymerases (Furth and Cohen, 1968; Müller et al., 1972). The inhibition is reversed by dCTP, and calculations show that the apparent K_m for dCTP is of a magnitude similar to that of the K_i for ara-CTP (Müller et al., 1972; Furth and Cohen, 1968; Graham and Whitmore, 1970b). There is no effect of ara-CTP on the synthesis of poly(dAT), i.e., there is no effect on the enzyme itself (Furlong and Gresham, 1971). Thus, of the three proposed mechanisms, this latter remains the most likely and most consistent with the existing data (Graham and Whitmore, 1970b).

We report here on the effect of ara-CTP on the synthesis and subsequent ligation of primary DNA pieces in isolated HeLa cell nuclei and also include some data on the effect of ara-C on intact HeLa cells.

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[1] Abbrevations used are: ara-CTP, 1-β-D-arabinofuranosylcytosine triphosphate; ara-C, 1-β-D-arabinofuranosylcytosine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene.

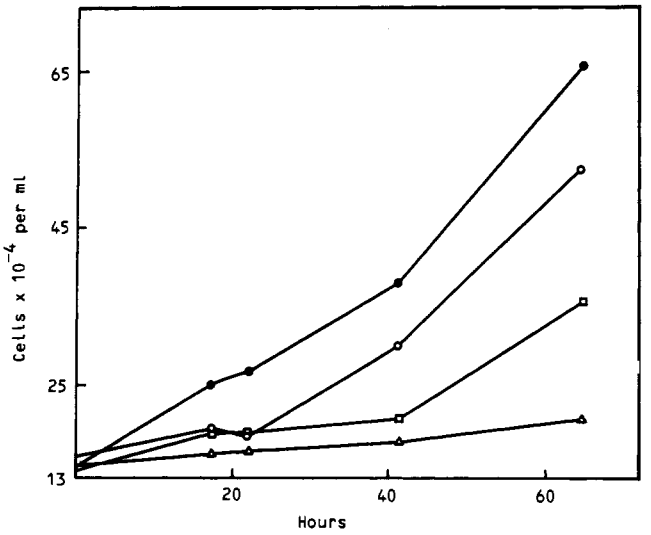

FIGURE 1: Effect of ara-C on growth of HeLa cells in suspension culture. (-●-) Control without ara-C; (-○-) 10^{-7} M ara-C; (-□-) 10^{-6} M ara-C; (-△-) 10^{-5} M ara-C.

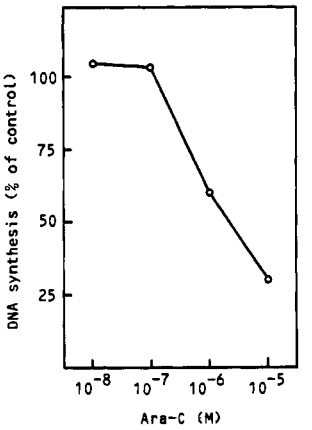

FIGURE 2: Effect of ara-C on incorporation of [^{3}H]TdR into acid-insoluble material in intact HeLa cells. Exponentially growing HeLa cells in suspension culture were incubated for 10 min at 37 °C with ara-C in various concentrations and [^{3}H]TdR (final concentration 0.05 μCi/ml). The reaction was stopped with ice-cold medium. The cells were centrifuged, washed, and lysed in 0.1 M NaOH, and the lysate was precipitated with 10% Cl_3CCOOH containing 20 mM sodium pyrophosphate. Acid-insoluble material was collected on Whatman GF/C filters and counted.

Materials and Methods

Deoxyribonucleoside 5′-triphosphates, ribonucleoside 5′-triphosphates, and 1-β-D-arabinofuranosylcytosine and its triphosphate were obtained from Sigma. The Radiochemical Centre, Amersham, U.K., provided [^{3}H]TdR, [^{3}H]TTP, [^{14}C]TdR, and [^{14}C]TTP.

HeLa S_3 cells were cultured, maintained, and synchronized as described (Krokan et al., 1975a). Regularly, 3 μg of thymidine/10^6 cells was added for reversal of the amethopterin/adenosine block, and the cells were harvested 3 h later. In all experiments, nuclei freshly isolated from synchronized cells, as described earlier (Krokan et al., 1975a), were used. The regular incubation solution contained 65 mM Tris, 65 mM NH_4Cl, 10% glycerol, 50 mM glucose, 1 mM EGTA,[1] 0.05 mM each of CTP, GTP, and UTP, 0.1 mM each of dATP, dGTP, and dCTP, 10 mM ATP, 10.7 mM $MgCl_2$, and 5 μM [^{3}H]TTP (final specific activity 0.05–30 mCi/μmol) and was titrated to a final pH 8.0 (20 °C) with 1 N HCl. Other incubation conditions are given in the legends to the figures.

Alkaline lysis of nuclei and alkaline sucrose gradient centrifugation, and fractionation were carried out as reported earlier (Krokan et al., 1975b), except that in some cases an SW 40 rotor was used with a gradient volume of 11 ml. The specific conditions for centrifugation are given in the figure legends. [^{32}P]Poly(dT) of chain length 160 or 200 nucleotides (kindly provided by Dr. K. Kleppe, University of Bergen, Norway) was used as marker and to monitor the trapping of primary DNA pieces in the high-molecular-weight DNA sedimenting to the bottom of the gradients.

The assay for buoyant density (cesium chloride centrifugation), [^{3}H]TTP incorporation, and DNase-sensitivity have been described (Krokan et al., 1975a).

Diluene (Packard Instrument International, Zürich, Switzerland) or a scintillation fluid containing 5 g of PPO and 50 mg of dimethyl-POPOP/l. of toluene (Koch-Light, Colnbrooke, Buckinghamshire, U.K.) was used for radioactivity counting.

DNA was determined by Burton's method (Burton, 1956) and extracted as described (Krokan et al., 1975a).

Isolation of Primary DNA Pieces. The relevant fractions (fractions 2–7 under standard alkaline sucrose gradient conditions) were pooled and precipitated with an equal volume of 20% trichloroacetic acid containing 20 mM sodium pyrophosphate at 4 °C for 30 min. After centrifugation at 7700*g* and 4 °C for 1 h, the pellets were extracted with water-saturated ether and recentrifuged. The pellets were then resuspended in 1 ml of a buffer of pH 8.1 containing 0.3 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and digested for 1 h at 37 °C with Pronase (final concentration 250 μg/ml) that had previously been autodigested for 30 min at 37 °C.

The final mixtures were deproteinized by three extractions with chloroform–isoamyl alcohol (24:1) equilibrated with 10 mM Tris-HCl and 10 mM EDTA, pH 7.5. The aqueous phase was filtered through a column of Sephadex G-50 (14 × 1.5 cm) equilibrated and eluted with 20 mM Tris-HCl (pH 8.1) containing 0.3 M NaCl, 1 mM EDTA, and 0.1% sodium dodecyl sulfate. Fractions of 32 drops were collected, and 100 μl of each fraction was counted in diluene.

The primary DNA pieces eluted after the void volume. The appropriate fractions were pooled and precipitated with 2 volumes of ethanol at −20 °C overnight, centrifuged at 7700*g* and 4 °C for 100 min, and the pellet was dried at 50 °C for 20 min. The recovery of primary DNA pieces was about 80%.

Results

The Effect of Ara-C on HeLa Cells. The growth of the HeLa cell strain used here was inhibited by concentrations of ara-C down to 10^{-7} M (Figure 1). The effect was only temporary when low concentrations were used. Essentially complete inhibition of the increase in cell number lasted for about 24 h when the ara-C concentration was 10^{-7} M and for about 42 h with 10^{-6} M ara-C. Concentrations of 10^{-5} M of ara-C led to cell death after about 60 h. The incorporation of [^{3}H]TdR into DNA was also markedly inhibited during a 10-min treatment of intact cells with ara-C (Figure 2).

The Effect of Ara-CTP on Isolated Nuclei. Since ara-CTP is a competitive inhibitor of various DNA polymerases and the inhibition is reversed by dCTP, it was important to determine the minimum concentration of dCTP that gave maximum [^{3}H]TTP incorporation. Absence of dCTP reduced the incorporation to 8% of the control. The apparent K_m for dCTP was 1.4 μM. A concentration of 12.5 μM dCTP gave 100%

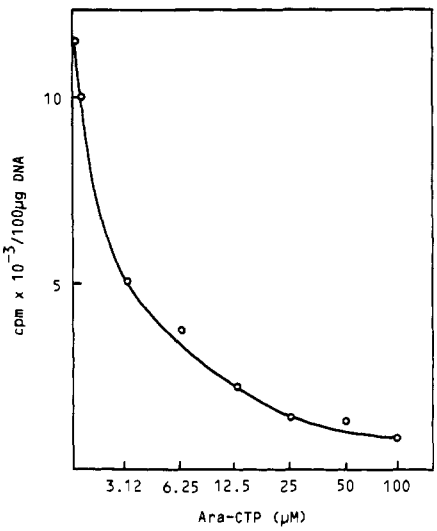

FIGURE 3: Effect of ara-CTP on incorporation of [^{3}H]TTP into DNA in isolated S-phase nuclei. The cells were prelabeled with [^{14}C]TdR (final specific activity 80 pCi/ml) for two generations before synchronization and isolation of nuclei. The nuclei were incubated for 10 min at 37 °C in the solution described under Methods, except that dCTP was at 12.5 μM concentration and [^{3}H]TTP at 50 μM. Final specific activity of [^{3}H]TTP was 50 μCi/μmol. The reaction was stopped with 5% Cl_3CCOOH containing 20 mM sodium pyrophosphate and [^{3}H]TTP incorporation was assayed as described (Krokan et al., 1975a).

incorporation. Ara-CTP had a marked inhibitory effect on the incorporation of [^{3}H]TTP into DNA (Figure 3) in the presence of 12.5 μM dCTP. The inhibition was reversed by increasing the concentration of dCTP and was competitive with a K_i = 0.5 μM. The shape of the inhibition curve (Figure 3) corresponds closely to that of the curve obtained with decreasing concentrations of dCTP in the system.

Synthesis of Primary DNA Pieces in the Presence of Ara-CTP. In nuclei incubated at 37 °C for 60 s with 0.1 mM ara-CTP in the incubation mixture, the overall rate of DNA synthesis was 60–70% inhibited. However, alkaline sucrose gradient analyses of lysates from such nuclei showed that low-molecular-weight primary DNA pieces were also synthesized in the presence of ara-CTP (Figure 4). A slightly greater proportion of the total incorporated radioactivity was present in the top eight fractions of the gradient from the system incubated with ara-CTP (33 vs. 26% in the same fractions from control nuclei).

The primary pieces were isolated from the appropriate sucrose gradient fractions as described under Methods. When recentrifuged under identical conditions, an aliquot of the isolated material sedimented as a sharp peak in the same fractions. In CsCl gradients the primary pieces from both ara-CTP and control nuclei banded at the same density of about 1.710 g/ml. The resultant peak was relatively broad, but no significant amount of radioactivity was found outside this peak (Figure 5). The isolated product was sensitive to DNase treatment with more than 90% of the radioactivity being rendered acid soluble.

The primary DNA pieces synthesized in the presence of ara-CTP seemed to be smaller than those in the control incubations after equal pulse lengths (Figure 4A). Further experiments (Figure 4B,C) confirmed this. After a 60-s pulse at 37 °C, the average size of the primary DNA pieces from the ara-CTP incubations was about 115–120 nucleotides compared to about 215–220 in the controls (Figure 4B,C). The size of the primary pieces increased when either a 90- or a 60-s pulse

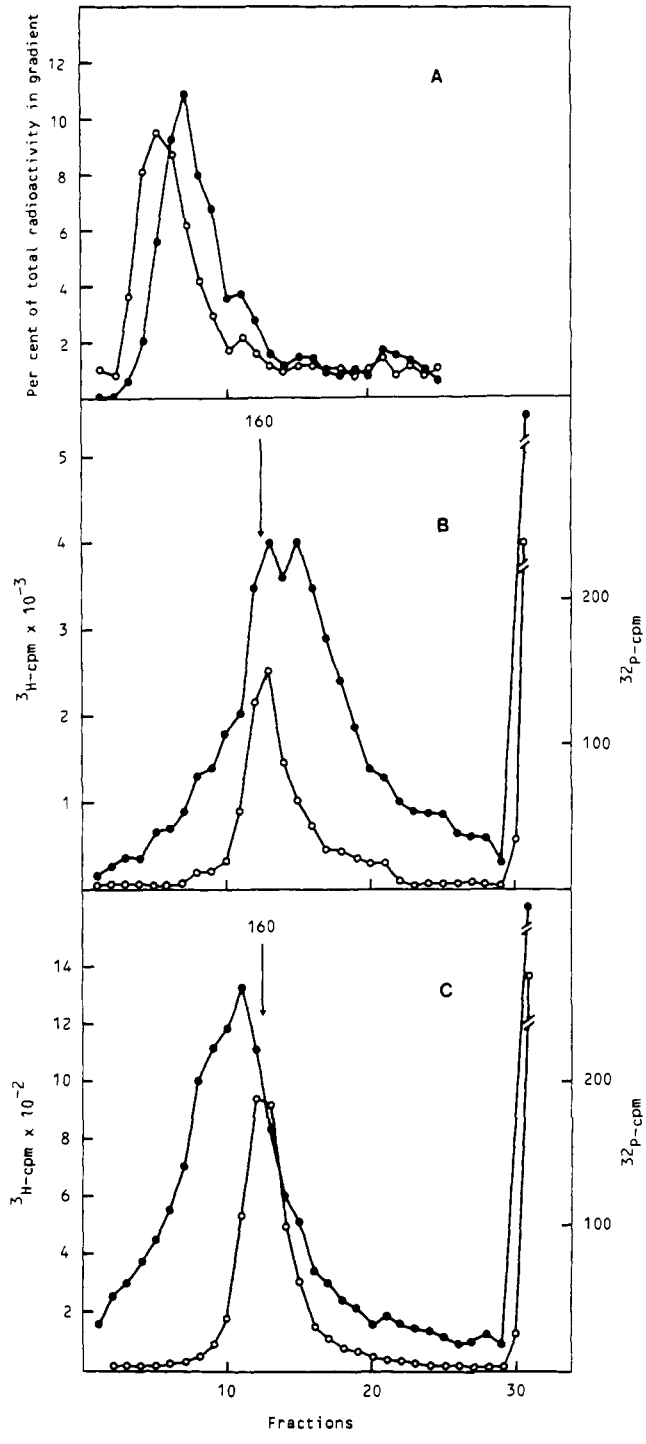

FIGURE 4: Effect of ara-CTP on size of primary DNA pieces. (A) Nuclei were incubated at 37 °C for 60 s with or without ara-CTP (0.1 mM). [^{3}H]TTP (5 μM) (final specific activity 30 mCi/μmol) was added to the ara-CTP incubation. [^{14}C]TTP (5 μM) (final specific activity 60 μCi/μmol) was added to the control incubation. The lysates from both incubations were gently mixed and submitted to alkaline sucrose gradient centrifugation at 40 000 rpm for 20 h at 5 °C in the SW 40 rotor. Fractions of 12 drops were collected. The total radioactivity incorporated in the ara-CTP sample was 30 000 cpm of which 27% sedimented to the bottom and in the control sample 495 cpm (26% in the bottom fraction). (-●-) Control; (-○-) ara-CTP. Nuclei were incubated at 37 °C for 60 s without (B) and with (C) ara-CTP (0.1 mM) in the incubation mixture. Final specific activity of [^{3}H]TTP was 30 mCi/μmol. The marker is [^{32}P]-poly(dT) (chain length 160 nucleotides). The lysates were submitted to alkaline sucrose gradient centrifugation at 40 000 rpm for 40 h at 5 °C in an SW 40 rotor. Fractions of 12 drops were collected. (-●-) Nuclear DNA; (-○-) marker DNA.

TABLE I: Effect of Pretreatment of Whole Cells with Ara-C on DNA Synthesis in Isolated Nuclei.[a]

Experiment	Total cpm Incorporated[b]		% of Total cpm in: Primary pieces		% of Total cpm in: Bottom fraction	
	Pulse	Pulse/Chase	Pulse	Pulse/Chase	Pulse	Pulse/Chase
Ara-C I	224 800	250 700	28	28	30	39
Control I	178 300	198 300	27	9	41	60
Ara-C II	144 600	131 100	51	30	30	43
Control II	146 600	132 200	25	7	52	73

[a] Intact cells were treated for 2 h with ara-C, harvested, and their nuclei were isolated. Suspensions of nuclei were incubated at 37 °C for 60 s (pulse) or 60 + 90 s (pulse/chase). The final specific activity of [^{3}H]TTP for the pulse was 30 mCi/μmol and an 188-fold excess of unlabeled TTP was added to start the chase. I and II are separate experiments. [b] Normalized to same DNA content.

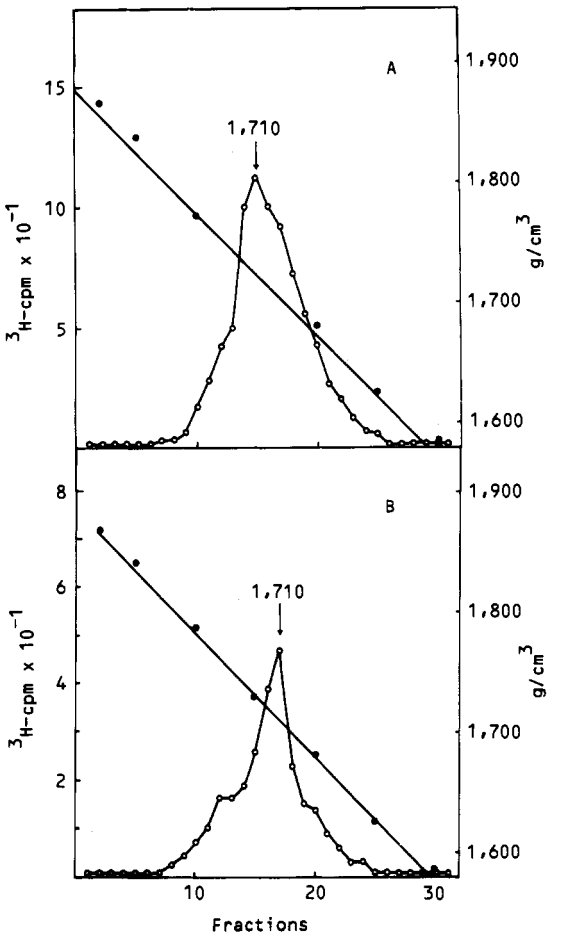

FIGURE 5: Isopycnic centrifugation of primary DNA pieces. (-O-) Radioactivity; (-●-) density. (A) Control nuclei; (B) nuclei incubated with ara-CTP (0.1 mM).

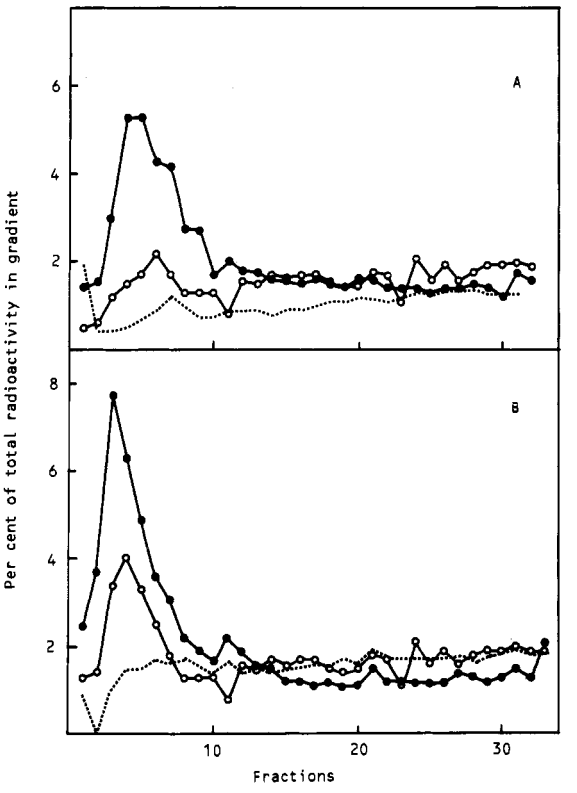

FIGURE 6: Transfer of label from primary DNA pieces into high-molecular-weight DNA in pulse-chase experiments. Nuclei were incubated at 37 °C for 60 s in incubation mixture without (A) and with (B) ara-CTP (0.1 mM) and with [^{3}H]TTP (5 μM) (final specific activity 30 mCi/μmol), followed by a chase of 30 or 90 s in 188-fold excess of unlabeled TTP. The nuclear lysates were submitted to alkaline sucrose gradient centrifugation at 27 000 rpm for 17 h at 5 °C. Fractions of 32 drops were collected from the top of the gradient. The total amounts of radioactivity incorporated in the ara-CTP samples were 86 900 cpm (pulse), 76 700 (pulse + 30 s chase), and 75 500 (pulse + 90 s chase). The corresponding control figures were 251 400 (pulse), 203 100 (pulse + 30 s chase) and 288 300 (pulse + 90 s chase). The bottom fractions contained 19.5, 35.7, and 45.0% of the total cpm in the ara-CTP experiments and 33.9, 37.8, and 63.7% in the controls. (-●-) Pulse 60 s; (-O-) pulse 60 s, chase 30 s; (- - -) pulse 60 s, chase 90 s.

followed by a 30-s chase were given. However, the size never reached that of the controls. In longer chases not enough of the radioactivity remained in the primary pieces for accurate determination and in longer pulses the size of the primary pieces increased continuously in the controls also (Krokan et al., 1975b). Hershey and Taylor (1975) suggested that a substantial amount of material lost from the primary DNA pieces during chasing was not recovered in the high-molecular-weight fraction. We found no evidence for such loss during chasing (Table I). The trapping of poly(dT) by high-molecular-weight DNA in these experiments was 15–45%.

Joining of Primary DNA Pieces. Ligation of primary DNA pieces into high-molecular-weight DNA takes place in isolated nuclei (Krokan et al., 1975b). Similar pulse–chase experiments were carried out to study the ligation of the primary pieces synthesized in the presence of ara-CTP. Nuclei were labeled for 60 s followed by a chase (188-fold excess of unlabeled TTP) of varying length (Figure 6). In the ara-CTP incubations 42.5 and 70.9% of the label were transferred from the primary

pieces into a high-molecular-weight DNA after chases of 30 and 90 s, respectively. In the control incubations the corresponding figures were 61.2 and 77.1%. The primary DNA pieces were thus ligated to secondary pieces larger than 30 S even when ara-CTP was present. However, the percent of label transferred from the primary pieces to high-molecular-weight DNA was always somewhat lower in the ara-CTP incubations for labeling up to 60 s (Figures 4 and 6).

Effect of Pretreatment with Ara-C in Vivo. HeLa cells were prelabeled for 48 h with a low dose of [^{14}C]TdR and treated with ara-C at a final concentration of 0.1 mM from 1 h after reversal of the amethopterin/adenosine block until the cells were harvested 2 h later. Nuclei were isolated and incubated without ara-CTP for 60 s at 37 °C. In some experiments the pulse was followed by a 90-s chase. Concomitantly, a 30-min [^{3}H]TdR pulse was given to aliquots of the ara-C-treated and the control cell suspensions to monitor the effect on DNA synthesis. A 98–99% inhibition was found. There was, however, no significant effect of the prior ara-C treatment on the total incorporation of [^{3}H]TTP into DNA in the isolated nuclei, showing that the inhibition of DNA synthesis by ara-C is rapidly and essentially completely reversible when ara-C is removed (Table I). There was no evidence for a smaller size of the primary DNA pieces synthesized by nuclei from ara-C treated cells. On the other hand, a markedly reduced rate of ligation of primary pieces into high-molecular-weight DNA was observed (Table I).

Discussion

Ara-C and ara-CTP seem to have at least two separate effects in the present DNA synthesizing system. In accordance with results from other systems (Furth and Cohen, 1968; Müller et al., 1972; Graham and Whitmore, 1970b; Furlong and Gresham, 1971) we find clear evidence for a competitive inhibition of DNA synthesis by ara-CTP. The inhibition is completely reversible by dCTP, and the K_m for dCTP and the K_i for ara-CTP are of similar magnitude (1.4 and 0.5 μM). These values agree quite well with those reported by Furth and Cohen (1968) for calf thymus DNA polymerase. Treatment of intact cells with ara-C prior to isolation of nuclei also shows that the inhibition is easily reversible (from 98–99% inhibition in ara-C-treated cells to no inhibition in nuclei isolated from such cells) when ara-C is removed and dCTP is added. There is, thus, no indication in our results of any inhibitory effect on total DNA synthesis caused by the incorporation of ara-CMP into DNA. The alternative explanation that chain-terminating ara-CMP is removed by exonuclease action during isolation and incubation of nuclei, thus allowing further DNA synthesis, cannot be completely excluded. However, the demonstration of the incorporation of ara-C into internal positions and the absence of any preferential 3′-terminal localization of ara-C (Graham and Whitmore, 1970a,b; Zahn et al., 1972; Magnusson et al., 1974; Hunter and Francke, 1975) make it less likely that ara-C functions mainly as a chain terminator.

The three processes involved in the synthesis of DNA, initiation, elongation, and ligation, have all been demonstrated in isolated HeLa nuclei (Krokan et al., 1975a,b). In the presence of ara-CTP the size of the primary DNA pieces at a given (short) pulse length is about half (54% average) that of primary pieces in control nuclei (Figure 4). At the same concentration of ara-CTP overall DNA synthesis was regularly inhibited by about 65%. Thus, the reduced length of the primary pieces accounts for about 45% of the total 65% reduction in DNA synthesis. The remaining 20% may be accounted for by reduced frequency of initiation of new primary DNA pieces. We are inclined to draw this conclusion in spite of the varying degree of trapping of primary pieces in the bottom fractions of the gradients, since the difference of about 20% has been observed in several experiments. In addition, our data do not suggest any selectivity in this reduced frequency of initiation, since the primary pieces from ara-C-treated and control nuclei have the same buoyant density of 1.710 g/ml. This is higher than the density of HeLa cell DNA of 1.699 g/ml described earlier (Krokan et al., 1975a), probably because the primary pieces are at least partially single stranded after the alkaline sucrose gradient centrifugation.

The reduced size of the primary pieces in ara-CTP-treated nuclei clearly demonstrates that the reduced rate of elongation is an important component of the total inhibitory effect of ara-CTP in the present system. Similar results have been reported with Okazaki fragments in a nuclear polyoma synthesizing system (Magnusson et al., 1974).

Finally, when cells were pretreated with ara-C and the nuclei subsequently isolated, the transfer of primary DNA pieces into high-molecular-weight material was somewhat inhibited (Table I). A slight but reproducible inhibition of this transfer was also observed after brief pulses (<90 s) in isolated nuclei treated with ara-CTP. It is probably necessary for the primary DNA pieces to reach a certain length before ligation can take place. Due to the lower rate of elongation in ara-CTP-treated nuclei, this minimum length is reached more slowly. After longer chase periods (90 s) the percent of labeled material transferred from the primary pieces to high-molecular-weight DNA is similar in treated and control nuclei. It is therefore possible to conclude that the slightly lower rate of transfer in isolated ara-CTP-treated nuclei is caused by a slower growth of the primary pieces.

This cannot, however, explain the reduced transfer seen when nuclei are isolated from ara-C-pretreated cells and incubated without ara-CTP. Since the inhibition of overall DNA synthesis by ara-C is rapidly and completely reversible, reduced transfer may be caused by another effect. One possibility is the decay during the period of blocked DNA synthesis in the whole cells of some factor(s) necessary for the ligation process.

In conclusion, ara-CTP seems to exert its main effect in the present system by inhibiting the growth of the primary DNA pieces, in agreement with the results of Magnusson et al. (1974). These workers suggested that the addition of the next deoxyribonucleotide to a growing DNA chain containing ara-CMP at the 3′ end was slowed down and that this formed the basis for the inhibition of DNA synthesis. This might create the impression of chain termination in brief incubations and is also consistent with the finding that most of ara-C is incorporated into internucleotide linkages.

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