EFFECT OF SPECIFIC INHIBITORS ON MITOCHONDRIAL DNA REPLICATION IN HeLa CELLS

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SUMMARY

The effect of different inhibitors of DNA synthesis on the labeling of mitochondrial DNA, isolated by CsCl-ethidium bromide density gradient and sedimentation velocity, has been studied in HeLa cells. Thymidine and deoxyadenosine excess, hydroxyurea and fluorodeoxyuridine inhibit the labeling of mitochondrial DNA less than that of the nuclear DNA (with a maximum difference of 22%) while cytosine arabinoside, which strongly inhibits nuclear DNA synthesis, has a very limited action on mitochondrial DNA, in analogy to what happens in the bacterial system. Ethidium bromide inhibits, as expected, only mitochondrial DNA replication.

INTRODUCTION

The study of the sensitivity to different drugs of the nuclear and mitochondrial replicative systems can help to understand the extent of the autonomy of mitochondrial biogenesis.

Vesco and Penman (1), in HeLa cells, found that the labeling of circular cytoplasmic DNA is relatively resistant to inhibition by hydroxyurea; Tershegget and Borst (2), in isolated chick-liver mitochondria, found that the incorporation of dATP into acid insoluble material is not inhibited by phenethyl alcohol and only to a limited extent by high concentrations of nalidixic acid. The possibility that thymidine excess does not block mitochondrial DNA (m.DNA) replication has been suggested by Pica Mattoccia and Attardi (3) to interpret the lack of synchronization of m. DNA synthesis after the release of HeLa cells from a double thymidine block. An alternative hypothesis was that the excess of thymidine might indeed block m. DNA synthesis but also cause an arrest of

Eth. br = ethidium bromide; HU = hydroxyurea; CA = cytosine arabinoside; d. Ade = deoxyadenosine; Thy = thymidine; FUdR = fluorodeoxyuridine

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mitochondria development at different stages of their life cycle, so that upon removal of the excess thymidine, mitochondria would resume their development and reach the stage of DNA replication asinchronously.

In the present paper we investigated the inhibition of labeling of m. DNA of HeLa cells grown in the presence of excess thymidine, excess deoxyadenosine, fluorodeoxyuridine, hydroxyurea, cytosine arabinoside and ethidium bromide. That was compared to the effect on total DNA labeling.

MATERIALS AND METHODS

a) Cells cultures.

HeLa cells (S_3 clonal strain) were grown in suspension in Eagle's modified medium (Joklik, Grand Island) supplemented with 5% calf serum at 37°C at a concentration ranging between 4.10^4/ml and 5.10^5/ml.

b) Drug treatment and labeling conditions.

i) Samples of exponentially growing cultures were centrifuged and resuspended in fresh medium at the concentration of 5.10^5/ml to 4.10^6/ml. Aliquots of 10 to 25 ml were incubated for two hours with [methyl-^{3}H] thymidine (5 or 10 μC/ml; 8 C/mM, Amersham) or with [^{3}H-G] deoxyadenosine (10 μC/ml; 7, 5 C/mM, Amersham) in the presence of the specific inhibitor added one hour before the radioactive isotope. The following drugs were used as DNA synthesis inhibitors: thymidine (Merck), hydroxyurea (Sigma), deoxyadenosine (Schwarz/Mann), fluorodeoxyuridine, cytosine arabinoside (cytosine 1-β-D arabino-furanosyl HCl, Sigma) ethidium bromide (Sigma).

ii) Drug-untreated HeLa cells (1 or 2.10^7 cells in 50 ml), to be used as an internal control for the recovery of m. DNA, were labeled with [2-^{14}C] thymidine (0, 1 μC/ml; 50, 5 mC/mM, New England Nuclear Corp.) for about 24 hours and mixed with the tritium labeled samples at the end of the incubation period.

iii) The incorporation of [methyl-^{3}H] thymidine or [^{3}H-G] deoxyadenosine and [2-^{14}C] thymidine into total cellular DNA was analyzed by measuring the radioactivity in samples of the total cell homogenate precipitated with 5% trichloacetic acid (TCA) in the cold and collected on glass fiber filters (Whatman GF/C). Samples labeled with tritiated deoxyadenosine were treated

with NaCH 0.5 N for 15 min at 37°C before precipitation with TCA, to hydrolize the RNA.

iiii) Cytosine-arabinoside incorporation into nuclear and m. DNA was measured in cell samples (70 ml, 1.10^6/ml) labeled for two hours with [5-^{3}H]cytosine β-D-arabinoside (2.5 or 5 μC/ml, 14 C/mM, Amersham); m. DNA was then isolated as described below.

c) Isolation of mitochondrial DNA.

m. DNA was isolated according to two different methods.

i) In the first method a sodium dodecyl sulfate lysate of the mitochondrial fraction was prepared and then centrifuged to equilibrium in a CsCl-ethidium bromide gradient as previously described (3).

ii) The second method, described below, is a modification of the Storrie and Attardi's procedure (4). The mitochondrial fraction, obtained as in the first method, was resuspended in 0.25 M sucrose, 0.0015 M $MgCl_2$, 0.05 M NaCl, 0.005 M Tris buffer (pH 7.2) (4 ml buffer/ml packed cells) and digested with DNase I and RNase A (10 or 100 μg/ml and 100 μg/ml respectively) for 15 min at 37°C. After the enzimatic treatment the mitochondrial fraction was centrifuged at 9600 rev/min in the Sorvall SS 34 rotor at 4°C for 10 min, the pellet was then resuspended in 0.25 M sucrose, 0.01 M Tris buffer (pH 7.2) (4 ml buffer/ml packed cells) and recentrifuged under the same conditions. The mitochondrial pellet was lysed in 3 ml of 1% sodium dodecyl sulfate (SDS), 0.01 M EDTA, 0.01 M Tris buffer (pH 7.2) and pronase (self-digested for two hours at 37°C) was added to a concentration of 75 μg/ml. After 30 min at 37°C, CsCl (Merck) was added to a final concentration of 1 M, the samples were chilled for 30 min and centrifuged at 12.000 rev/min in the Sorvall SS 34 rotor at 4°C for 15 min. The supernatant fraction was recentrifuged under the same conditions and two volumes of cold 95° ethyl alcohol were added in order to precipitate the nucleic acids. After at least two hours at -20°C the samples were centrifuged in the SS 34 Sorvall rotor at 14.000 rev/min for 20 min and the pelleted nucleic acids were resuspended in 1 ml 0.01 M Tris buffer pH 7.2, 0.01 M EDTA and sedimented through a two-step CsCl-ethidium bromide gradient (1 ml CsCl, ϱ=1.70, 3ml CsCl, ϱ = 1.40 in 0.01 M Tris buffer pH 7.2, 0.01 M EDTA, with 200 μg/ml ethidium bromide) in the Spinco SW 50.1 rotor at 33,000 rev/min for 5 hours

Table 1

Effect of different DNA synthesis inhibitors on the labeling of total cell DNA and m.DNA isolated by CsCl-ethidium bromide density gradient.

Expt. no	INHIBITOR	CONC.	PERCENTAGE OF INHIBITION	
			TOTAL DNA	m. DNA
1	Ethidium bromide	1µg/ml	0	100
2	Hydroxyurea	$1 \cdot 10^{-3}$M	95	82
3	"	"	84	59
4	"	$5 \cdot 10^{-4}$M	70	53
5	Cytosine arabinoside	$1 \cdot 10^{-6}$M	88	0
6	"	"	87	6
7	Deoxyadenosine	$2 \cdot 10^{-3}$M	96	84
8	"	"	87	71
9	"	"	70	79
10	Thymidine	$2 \cdot 10^{-3}$M	65	67
11	"	"	89	83
12	"	"	77	63
13	Fluorodeoxyuridine	1µg/ml	47	74
14	"	"	77	54
15	"	"	75	70

Table 1 Effect of different DNA synthesis inhibitors on the labeling of total cell DNA and m. DNA isolated by CsCl-ethidium bromide equilibrium density gradient.

m. DNA was isolated from HeLa cells labeled with tritiated thymidine (expts. 1-9) or deoxyadenosine (expts. 10-15) in the presence of specific inhibitors as explained in Materials and Methods section b (i) and in the legend of Fig. 1. The labeling of total cell DNA was calculated as ^{3}H cts/min present in the samples of the cell homogenate precipitated with TCA (Materials and Methods b (iii)) while the labeling of m. DNA was calculated as ^{3}H cts/min associated with the heavier band of the gradient (pertaining to closed circular DNA). The percentages of inhibition of radioactivity incorporation in total cell DNA and m. DNA were calculated relatively to the incorporation in controls.

at 20°C. After the run, fractions of about 120 µl were collected from the bottom of the tube, precipitated with TCA. collected on glass fiber filters and counted in a liquid scintillation counter. Samples labeled with [^{3}H-G] deoxyadenosine were treated with NaOH 0.5 N (15 min at 37°C) and neutralized before TCA precipitation.

RESULTS AND DISCUSSION

Effect of different inhibitors of DNA synthesis on m. DNA labeling.

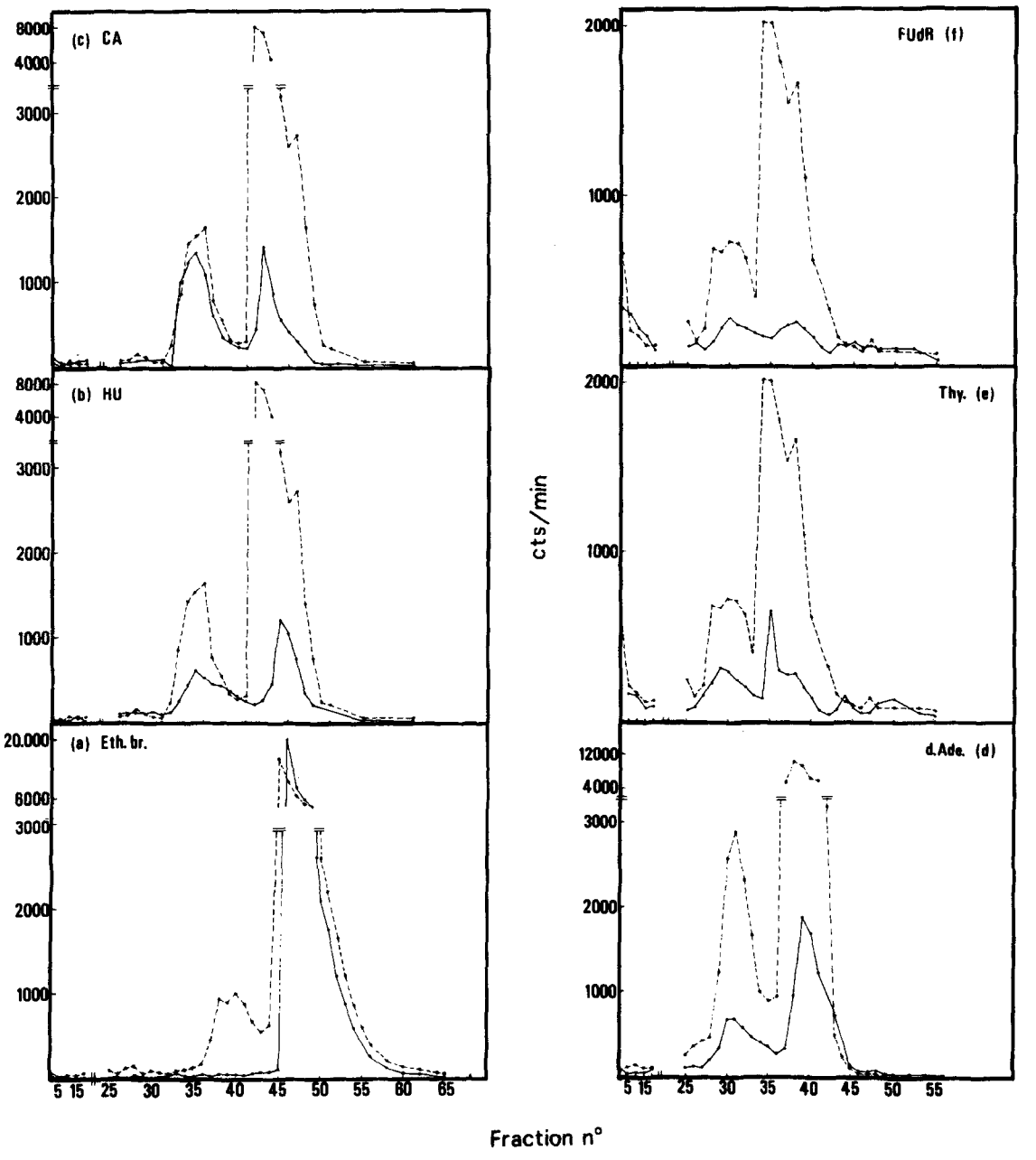
a) m. DNA isolated by CsCl-ethidium bromide density gradient.

Table 1 shows the results of a set of experiments in which cells were exposed to different inhibitors and the labeled m. DNA was isolated according to the method reported in Materials and Methods, section c(i). By this procedure only the supercoiled form I of m. DNA can be quantitated. It can be seen that ethidium bromide at a concentration of 1 μg/ml, completely inhibits m. DNA labeling, while it doesn't affect at all the labeling of total cell DNA. Hydroxyurea (1.10^{-3}M, 5.10^{-4}M), deoxyadenosine (2.10^{-3}M), thymidine (2.10^{-3}M) and fluorodeoxyuridine (1 μg/ml) inhibit, as expected, total cell DNA and to a lesser extent m. DNA labeling. Hydroxyurea, among this group of inhibitors, is consistently less active on m. DNA labeling with an average 20% difference in the inhibition of this verus that of total cell DNA. Cytosine arabinoside, finally, shows the expected strong effect on total cell DNA but it is very poorly active on m. DNA labeling. The analysis by CsCl-ethidium bromide equilibrium density gradients of the m. DNA labeled in the presence of these inhibitors is shown in Fig. 1. For each pattern the heavier band corresponds to closed circular m. DNA molecules and the major lighter band pertains to linear or open circular DNA molecules (mostly nuclear contaminants).

Fig. 1 Analysis by CsCl-ethidium bromide equilibrium density gradient of m. DNA labeled in the presence of different inhibitors.

HeLa cells were labeled with tritiated thymidine (a-d) or deoxyadenosine (e, f) as explained in Materials and Methods section b(i). DNA extraction from the mitochondrial fraction and banding in CsCl-ethidium bromide density gradients were carried out as previously described (3). Gradients were centrifuged for 48 hours at 35. K rpm in the SW41 Spinco rotor at 20°C, fractions of about 70 μl were collected from the bottom of the tube and aliquots of 20 μl (a), 30 μl (b, c) or 50 μl (d, e, f) were precipited with TCA, collected on glass fibre filters (Whatman GF/C) and counted in a scintillation counter. Aliquots from gradients e and f were treated with NaOH (0.5 N, 15 min at 37°C) and neutralized before being TCA precipitated.

a: ethidium bromide 1 μl/ml; b: hydroxyurea 1.10^{-3}M; c: cytosine arabinoside 1.10^{-5}M; d: deoxyadenosine 2.10^{-3}M; e: thymidine 2.10^{-3}M; f: fluorodeoxyuridine 1 μl/ml. .---.---. control; .——.—— inhibitor.



b) m. DNA isolated by sedimentation velocity through a CsCl-ethidium bromide two-step gradient.

It cannot be ignored that the precision of the m. DNA determinations may be significantly affected by the isolation procedure used, in view not only of the small proportion of m. DNA (relative to total DNA) in the cell, but also of its

Table 2

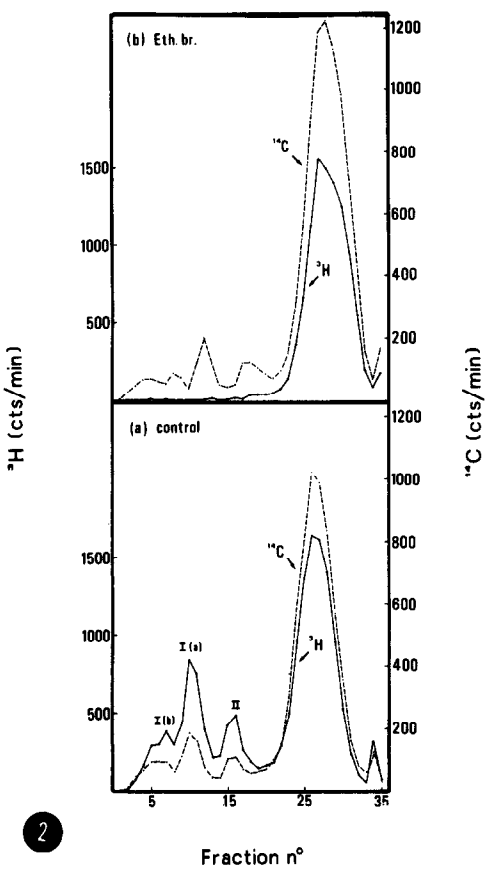
Effect of different DNA synthesis inhibitors on the labeling of total cell DNA and m.DNA isolated by sedimentation through a CsCl-ethidium bromide two step-gradient.

Expt. no	INHIBITOR	CONC.	PERCENTAGE OF INHIBITION	
			TOTAL DNA	m. DNA
1	Ethidium bromide	1µg/ml	0	100
2	"	"	20	100
3	Hydroxyurea	$1 \cdot 10^{-3}$M	82	65
4	"	"	98	89
5	"	"	96	75
6	Cytosine arabinoside	$1 \cdot 10^{-5}$M	83	47
7	"	"	96	34
8	"	"	95	24
9	"	"	94	28
10	"	"	96	20
11	"	"	97	15
12	"	$5 \cdot 10^{-6}$M	97	35
13	"	$1 \cdot 10^{-4}$M	96	37
14	"	"	96	14
15	Deoxyadenosine	$2 \cdot 10^{-3}$M	90	60
16	"	"	89	75
17	"	"	91	82
18	Thymidine	$2 \cdot 10^{-3}$M	70	57

Table 2 Effect of different DNA synthesis inhibitors on the labeling of total cell DNA and m. DNA isolated by sedimentation velocity through a CsCl-ethidium bromide two-step gradient.

Cells were labeled with tritiated thymidine (expts. 1-17) or deoxyadenosine (expt.18) as explained in Materials and Methods section b (i) and m. DNA was isolated as specified in section c(ii).
The labeling of total cell DNA was calculated as described in legend Table 1 while the radioactivity incorporated into m. DNA was calculated as ^{3}H cts/min associated with m. DNA form I (a and b) and form II as separated by sedimentation velocity (Fig. 2-4). Normalization for differences in recovery of m. DNA was done on the basis of the ^{14}C cts/min present in the same bands. The percentages of inhibition of incorporation in total cell DNA and m. DNA were calculated relatively to control samples. Each value of inhibition of m. DNA labeling represents the average of the separate values found for m. DNA form I and II.

presence in multiple forms with different physico-chemical properties. An accurate assessment of the differential effect of the inhibitors used here, which appeared to vary over a broad range of activities, required then to be



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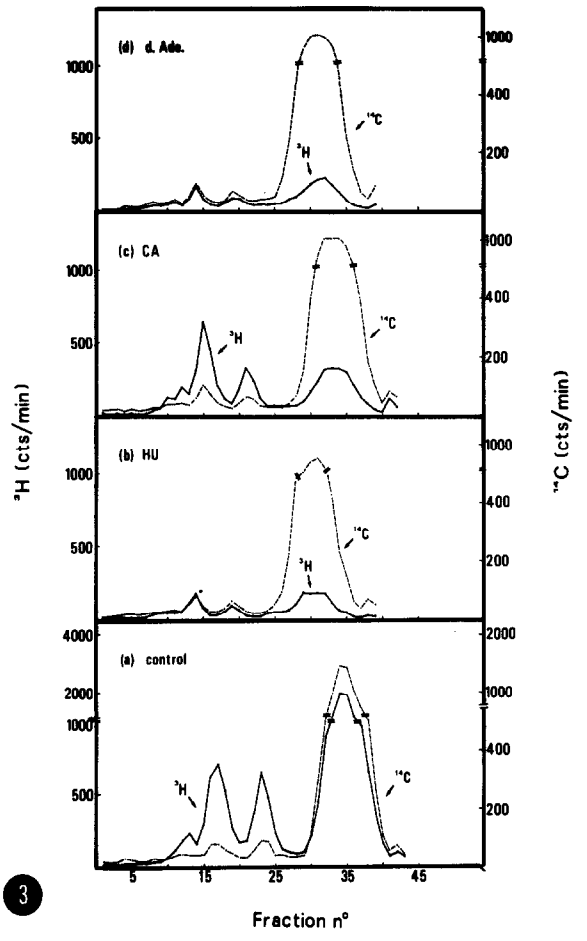

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Fig. 2 Analysis by sedimentation velocity in CsCl-ethidium bromide two-step gradient of m. DNA labeled in the presence of ethidium bromide.

HeLa cells (8.10^7 cells in 20 ml) were labeled with [methyl-^{3}H] thymidine (5 µl/ml, 8 C/mM) in the absence (a) or in the presence of ethidium bromide 1 µl/ml (b). Long-term [2-^{14}C] thymidine labeled cells were used as in internal standard for the recovery of m. DNA (Materials and Methods b (ii)). Isolation of m. DNA, conditions of the run and collection of fraction from the gradient are described in Materials and Methods c(ii). The numbered bands are referred to in the text.

. ——. ——. ^{3}H cts/min; . ------. ------. ^{14}C cts/min

Fig. 3 Analysis by sedimentation velocity in CsCl-ethidium bromide two-step gradient of m. DNA labeled in the presence of different inhibitors.

HeLa cells ($2.4.10^7$ cells in 20 ml) were labeled with [methyl-^{3}H] thymidine (5 µl/ml, 8 C/mM) in the absence (a) and in the presence of: hydroxyurea 1.10^{-3}M (b), cytosine arabinoside 1.10^{-5}M (c), deoxyadenosine 2.10^{-3}M (d) See also legend Fig. 2.

. ——. ——. ^{3}H cts/min . ------. ------. ^{14}C cts/min

verified by another method, independent from, and actually more sensitive than the classical method employed above. This procedure, described in Materials and Methods c (ii), separates m. DNA form I and form II from the nuclear contaminant band by sedimentation velocity. In this series of experiments we also introduced and internal standard for the recovery of m. DNA obtained by mixing with the experimental samples a constant amount of long-term [2-^{14}C] thymidine labeled cells. Table 2 shows the results of such experiments which should be compared to the data reported in Table 1 (except for fluorodeoxyuridine which was not re-examined). Figures 2-4 illustrate the analytical patterns obtained by sedimenting m. DNA through a two-step CsCl-ethidium bromide gradient. One can see, starting from the bottom of the gradient, a band (Ia) corresponding to monomeric closed circular m. DNA with a faster sedimenting shoulder (Ib) which presumably consists of oligomeric forms of m. DNA; a second band (II) corresponding to the open circular m. DNA. The slowest band corresponds to degraded nuclear DNA.

From the comparison of the two tables it appears that the differential effects on nuclear and m. DNA labeling caused by exposure to ethidium bromide, hydroxyurea, deoxyadenosine and thymidine are fairly well reproduced. In the experiments where cytosine arabinoside was used, a considerable differential effect was still found but smaller than in the first type of experiments. The possibility that the labeling of m. DNA form II (not resolved in the first method of separation of m. DNA) could be responsible for the difference of the results of Table 1 and 2 was discarded since the calculation of the inhibition values of m. DNA form I and II individually didn't show any significant difference. In addition, even within a series of experiments carried out by the same analytical procedure (Table 2) the effect of cytosine arabinoside on m. DNA labeling appeared to fluctuate widely (although remaining in all cases considerably smaller than that on total DNA). These variations could not be easily attributed to obvious artefacts such as: a) nicking of closed circles during extraction, since that could perhaps alter the results obtained with the first procedure used, but not with the second, where nicked molecules are separated and taken into account, b) variable recovery of materials, because the presence of an internal standard labeled with a different isotope eliminates this source of error, c) contamination of m. DNA bands by nuclear DNA, be-

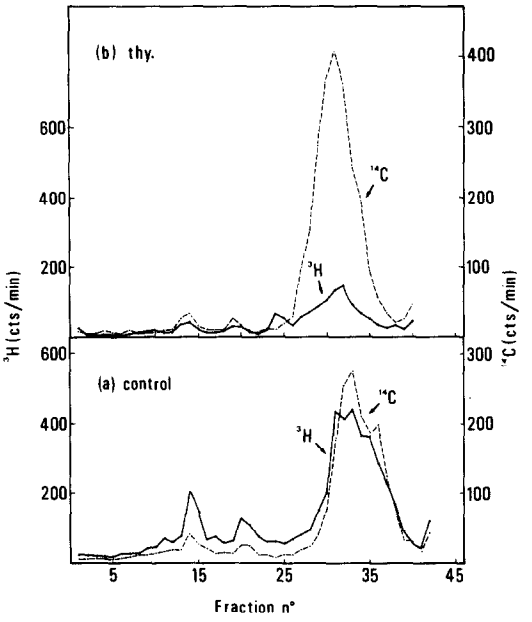

Fig. 4 Analysis by sedimentation velocity in CsCl-ethidium bromide two-step gradient of m. DNA labeled in the presence of thymidine.
HeLa cells (2, 2. 10^7 cells in 15 ml) were labeled with [^{3}H-G] deoxyadenosine (10 µl/ml, 7, 5 C/mM) in the absence (a) and in the presence (b) of thymidine 2.10^{-3}M. See also legend Fig. 2
. ——. ——. ^{3}H cts/min, .------.------. ^{14}C cts/min

cause all the experiments carried out in the presence of ethidium bromide (one of which is illustrated in Fig. 2) show a complete absence of ethidium--resistant radioactivity in the mitochondrial bands. It was also found that a ten-fold increase in the amount of DNase employed to purify mitochondria did not significantly change the results. We believe then that these variations might be at least in part dependent on the characteristics of action of the drug itself. It is quite likely that the relative insensitivity of m. DNA synthesis to cytosine arabinoside inhibition depends on mitochondrial inability to phosphorylate it to its triphosphate derivative (ara-CTP), in analogy to what happens in bacteria (5) where DNA replication is also not affected by the inhibitor, unless used at very high concentrations (6). Indeed, in two experiments where cells were incubated with radioactive cytosine arabinoside (Materials and Methods c(iiii)), no incorporation was found into m. DNA (Fig. 5). In HeLa cells, the effect of cytosine arabinoside on m. DNA does not seem to depend on

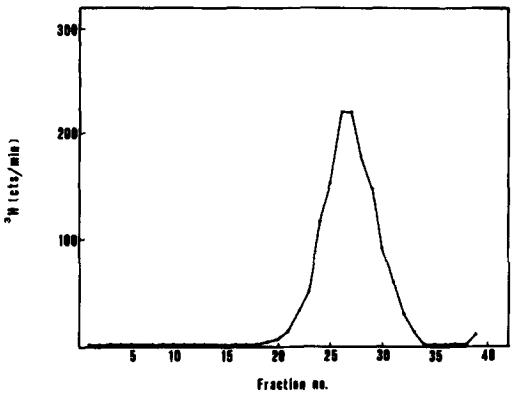

Fig. 5 Analysis by sedimentation velocity in CsCl-ethidium bromide two-step gradient of m. DNA isolated from HeLa cells (7.10^7 cells in 70 ml) labeled for two hours with [5-^{3}H] cytosine β-D-arabinoside (2, 5 μC/ml, 14 C/mM) See also legend Fig. 2.

the concentration (Table 2), on the other hand, since a variable inhibition does occur, we believe that permeability might play a role (depending in turn on uncontrolled factors in the cell cultures): the inhibitory effect would then be caused by variable leaking into the mitochondria of cytosine arabinoside phosphorylated by the nuclear system. It has been shown that in E. coli cells, permeabilized by toluene , ara-CTP can in fact suppress DNA synthesis (7). The concept of two distinct systems of nucleoside phosphorylation in animal cells is supported by the results obtained by Robberson and Clayton (8) in mouse L Thymidine Kinase cells where radioactive thymidine is incorporated only into m. DNA.

Our results, finally are in favour of the second hypothesis made by Pica Mattocia and Attardi to interpret the lack of synchronization of m. DNA synthesis after release of HeLa cells from double thymidine block.

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