Biochemical Pharmacology, Vol. 22, pp. 85–93. Pergamon Press, 1973. Printed in Great Britain.

5-ETHYL-2′-DEOXYURIDINE: EVIDENCE FOR INCORPORATION INTO DNA AND EVALUATION OF BIOLOGICAL PROPERTIES IN LYMPHOCYTE CULTURES GROWN UNDER CONDITIONS OF AMETHOPTERINE-IMPOSED THYMIDINE DEFICIENCY

KRYSTYNA M. ŚWIERKOWSKA, JANINA K. JASIŃSKA and JAN A. STEFFEN

Department of Radiobiology, Maria Skłodowska–Curie Institute of Oncology, Wawelska 15, Warszawa 22, Poland

(*Received* 2 *May* 1972; *accepted* 31 *July* 1972)

Abstract—It was demonstrated that 5-ethyl-2′-deoxyuridine (EtUdR), an analogue of thymidine, can restore cell proliferation in human phytohemagglutinin (PHA)-stimulated lymphocyte cultures grown under condition of DNA synthesis block due to amethopterine induced thymidine deficiency. Under similar conditions, ^{14}C-EtUdR was found to be incorporated into nuclei of lymphocytes entering the S (DNA synthesis) phase of the cell cycle, following release of a G_1/S cell cycle block by the analogue. The lower efficiency of EtUdR, as compared with thymidine, for reversal of amethopterine imposed DNA synthesis inhibition was shown to be due to the slowdown of the passage of cells through the proliferation cycle, when thymidine was substituted by the analogue. High excess of EtUdR blocks the passage of cells through the cycle, similarly or even more efficiently than thymidine, but does not affect metabolic processes preceding DNA synthesis.

No changes in chromosome structure were found in cultures grown in media containing amethopterine and EtUdR, which implies that the analogue does not induce chromosome aberrations at least during the first generation following incorporation into DNA of somatic cells.

5-ETHYL-2′-DEOXYURIDINE (EtUdR), an analogue of thymidine (5-methyl-deoxyuridine)[1] was shown to be incorporated into phage DNA.[2,3] It was also found that 5-ethyluracil may undergo incorporation into bacterial DNA in T^- strains of *Escherichia coli*.[4] It has been reported that EtUdR exerts an antiviral effect against vaccinia virus in HeLa cells[1] and against herpes simplex virus in experimental keratitis.[5] On the other hand the analogue was found to be nonmutagenic in T_4 phage in an assay system as developed by Freeze.[6] One may speculate as to the chemotherapeutic potential of EtUdR, but nothing is known about uptake of the analogue by, and its effect on, mammalian cells.

When phytohemagglutinin (PHA)-stimulated lymphocytes are grown in the presence of amethopterine (methotrexate-MTX), the activated cells accumulate at the G1/S point or early in the S phase of the first generation cycle. The DNA synthesis block can be reversed within minutes by exogenous thymidine, which results in the restoration of the passage of cells through the cycle and initiation of mitotic activity some hours later.[7] Since the block following incubation with amethopterine results from thymidine

deficiency, the system is particularly suitable for tests of biological properties of thymidine analogues. The system, as outlined above, was employed in the present work for studies of some biological effects of EtUdR upon mammalian cells. In particular: (1) Experiments were carried out to test if EtUdR can restore the passage of cells through the cycle in cultures grown in presence of MTX. The ability of EtdUR, as compared to TdR, to reverse the MTX-imposed block was subsequently tested over a wide concentration range. (2) Using a ^{14}C-labelled analogue and autoradiography, evidence of EtUdR incorporation into the nuclei of lymphocytes entering the S phase of the cell cycle under conditions of thymidine deficiency was obtained. (3) Experiments were designed to test possible effects of EtUdR on metabolic processes preceding DNA synthesis in culture. (4) The relative rates of passage of cells through the proliferation cycle in cultures in which the MTX imposed block was reversed by EtUdR and TdR, respectively, were compared. (5) The mutagenicity of EtUdR was tested by a search for chromosome abnormalities under conditions of reversal of the MTX imposed DNA synthesis block by the analogue.

MATERIALS AND METHODS

The technique of leucocyte separation and cultivation is described in detail elsewhere.[7]

Blood for all experiments was obtained from blood donors and mixed immediately with Hanks solution (1 part of Hanks per 6 parts of blood) containing heparin and kanamycin. Following 2 hr incubation at 37°, the plasma layer containing leucocytes was separated. Lymphocytes contributed usually about 70 per cent of the total leucocyte count. No attempts were made to further separate lymphocytes from polymorphonuclear cells.

The culture medium consisted of 75 per cent of Parker's TC 199 solution freshly supplemented with glutamine and 25 per cent of autologous plasma. Phytohemagglutinin (PHA-purified A from Wellcome) was added to this medium to a concentration of 5 μg/ml. This concentration was found to induce, in several preliminary experiments, optimum growth stimulation as defined by the mitotic index, following 3 hr incubation with colcemide at the end of day 3 of cultivation. The lymphocyte count in the final medium was adjusted to 0·75–1·0 $\times$ 10^6 per ml.

In the majority of experiments, amethopterine (methotrexate–MTX, from Lederle) and adenosine (Ad, from Reanal) were added to the culture media from the onset of incubation at concentrations of 3 $\times$ 10^{-7} and 1·5 $\times$ 10^{-3} M respectively.

The pH of the medium was always corrected to about 6·9 with 1·0 N HCl. Lymphocyte suspensions in medium were cultivated in 4 ml aliquots in rubber-stoppered culture vials at 37°. The medium was routinely replaced by a fresh one, without PHA, at the end of day 2 of incubation. In some experiments, the following compounds were also used: (a) 5-ethyl-2′-deoxyuridine (EtUdR), was dissolved in Parker's TC 199 solution and added to a culture to a concentration of 2 $\times$ 10^{-4} M, unless otherwise stated. 5-Ethyl-[^{14}C]-2′-deoxyuridine (sp. act. 27 mCi/mM) was a kind gift of Dr L. Pichat (Service des Molecules marquées, C.E.N. Saclay, France) to Professor D. Shugar. (b) Bromodeoxyuridine (BUdR) from Sigma, in Parker's TC 199 solution was added to cultures at a concentration of 2 $\times$ 10^{-4} M. (c) Thymidine (TdR) from

Sigma, in Parker's solution, if not otherwise stated, was added to cultures at a concentration of 2×10^{-4} M. ^{14}C-2-Thymidine, from Amersham (sp. act. 80·5 mCi/mM was diluted with "cold" thymidine to reduce the specific activity to 27 mCi/mM).

Cultures were usually harvested at the end of the third, or during day 4 of cultivation. Colcemide, $5{\cdot}4 \times 10^{-7}$ M, was always added 3 hr prior to harvest. Several air-dried preparations were made from the contents of each culture vial according to the method of Moorhead *et al.*,[8] that is after hypotonic treatment and acetic acid–methyl alcohol fixation. The preparations were stained either with aceto-orcein or Giemsa. The mitotic index was calculated after scoring 2000 cells per culture. To evaluate the number of cells incorporating 5-ethyl-[^{14}C]-2′-deoxyuridine or ^{14}C-2-thymidine, air-dried slides were covered with Kodak AR stripping film, exposed in darkness at 0–4° for 7–10 days and, following development according to standard procedures, stained with Harris's hematoxylin. At least 1000 cells were scored for the label on each slide.

In experiments where chromosome aberrations were scored, the scoring criteria, essentially as defined by Sharpe *et al.*,[9] were adopted. Chromatid breaks were recognized when there was a discontinuity in one chromatid without dislocation of the peripheral fragment. In cultures which had been irradiated with X-rays, only asymmetric exchanges (dicentric and rings) and acentric chromosome fragments (terminal and intercalary in one class) were scored. Metaphases with dicentrics and rings, without accompanying fragments, were particularly carefully analysed to rule out possible bias.

RESULTS AND DISCUSSION

Restoration of cell proliferation by EtUdR in lymphocyte cultures grown in the presence of MTX. Several experiments were carried out to compare the ability of EtUdR and TdR to restore cell proliferation in cultures grown in the presence of amethopterine.

In eight experiments EtUdR or TdR were added at a concentration of 2×10^{-4}M at the onset of incubation to replicate cultures suspended in a medium containing MTX and Ad. The cultures were harvested at the end of day 3 of cultivation and mitotic indexes in corresponding EtUdR and TdR cultures were compared.

It is apparent from the results obtained (Table 1) that the analogue can restore in part the passage of cells through the cycle, though, at the concentration tested, less efficient than TdR. It is also evident that the ratio of mitotic indexes in both types of cultures is subject to considerable variability.

In 3 subsequent similar experiments EtUdR and TdR, respectively, were added to parallel, replicate cultures at the onset of cultivation at concentrations ranging from 2×10^{-8} to 8×10^{-4} M.

It appears from these experiments (Table 2) that the efficiency of EtUdR vs. TdR in restoring cell proliferation in MTX cultures is lower over the entire concentration range tested. The plateau of mitotic activity, corresponding to the maximum reversal of the MTX block, is reached with TdR concentrations at least one order of magnitude lower as compared with EtUdR.

Initiation of DNA synthesis in lymphocyte cultures was demonstrated to vary considerably from donor to donor.[10] Hence, if an assumption is made that EtUdR can replace TdR in DNA under conditions of MTX-imposed thymidine deficiency, but simultaneously slows down the passage of cells through the S and/or G2 phase,

TABLE 1. CELL PROLIFERATION IN LYMPHOCYTE CULTURES GROWN IN MEDIA CONTAINING MTX AND TdR OR EtUdR ADDED AT THE ONSET OF CULTIVATION

Exp. No.	Mitotic indexes (%) in cultures: Control without EtUdR or TdR	EtUdR	TdR	M.I. $\frac{\text{EtUdR}}{\text{TdR}^*}$
1	0·00	0·50	2·30	21·5
		0·20	1·80	11·0
2	0·00	1·50	1·95	79·9
		0·70	2·15	32·5
3	0·00	2·15	2·55	84·3
		2·30	2·80	82·1
4		1·80	3·15	57·0
5	0·00	1·15	1·85	59·8
6		0·80	2·80	28·5
7	0·00	1·20	2·70	44·4
		2·25	3·65	56·1
8	0·25	1·75	5·20	33·6
		1·80	5·95	30·2

Lymphocyte cultures were cultivated 70 hr in a medium containing MTX (3×10^{-7} M) and adenosine ($1{\cdot}5 \times 10^{-3}$ M). TdR or EtUdR (2×10^{-4} M) were added at the onset of incubation. The cultures were harvested following 3 hr incubation with colcemide. Mitotic indexes were evaluated by scoring 2000 cells per culture.

* $\frac{\text{Mitotic index (\%) in cultures with EtUdR}}{\text{Mitotic index (\%) in cultures with TdR}} \times 100$

This ratio, expressed in percent, shows the ability of EtUdR to replace TdR in reversing of DNA synthesis block.

the variability of comparative efficiencies of EtUdR vs. Tdr in restoring cell proliferation can be well explained.

Autoradiographic proof of substitution of TdR by 5-EtUdR under conditions of MTX-induced DNA synthesis block. To furnish a direct proof that 5-ethyl-deoxyuridine can replace thymidine in the DNA of proliferating cells under conditions of thymidine deficiency, two types of experiments were carried out (Table 3): (1) 0·5 μCi of ^{14}C-5-EtUdR or ^{14}C-TdR per 1 ml of culture were added respectively to parallel replicate cultures after 69 hr of cultivation in a medium containing MTX. The cultures were harvested 3 hr later and, following autoradiography, the percentage of labelled cells was evaluated. Since in both experiments only a fraction of cells incorporated labelled EtUdR or TdR, and since the size of this fraction was similar when either was employed, it may be inferred that: (a) EtUdR, like TdR, is incorporated only into cells ready to enter the S phase, (b) all cells which display the ability to incorporate TdR into the nuclei following prolonged growth in presence of MTX can, under condition of thymidine deficiency, incorporate EtUdR instead.

(2) In a parallel experiment 1·0 μCi of ^{14}C-EtUdR and 12·5 μg of "cold" EtUdR or 1·0 μCi of ^{14}C-TdR and 12·5 μg of "cold" thymidine respectively were added to 4-ml cultures, together with MTX at the initiation of cultivation. The cultures were

TABLE 2. CELL PROLIFERATION IN REPLICATE LYMPHOCYTE CULTURES GROWN IN MEDIA CONTAINING MTX AND DIFFERENT CONCENTRATIONS OF EtUdR OR TdR

Exp. No.	Concentration of TdR or EtUdR (Mole)	Mitotic indexes (M.I.) in culture with		M.I. $\frac{\text{EtUdR}^*}{\text{TdR}}$
		TdR	EtUdR	
1	2×10^{-8}	0·20	0·15	
	4×10^{-8}	0·10	0·25	
	1×10^{-7}	0·15	0·20	
	2×10^{-7}	0·80	0·27	
	2×10^{-6}	2·70	0·42	15·5
	2×10^{-5}	3·60	0·15	4·1
2	None	0·00		
	5×10^{-6}	3·40	0·50	14·4
	1×10^{-5}	3·40	0·85	23·0
	2×10^{-5}	3·25	0·65	20·0
	5×10^{-5}	3·55	0·90	25·3
	1×10^{-4}	4·40	1·30	29·5
	2×10^{-4}	3·15	1·80	51·1
3	None	0·25		
	3×10^{-4}	4·85	1·55	31·8
	4×10^{-4}	3·65	1·35	36·9
	5×10^{-4}	4·75	1·25	26·3
	6×10^{-4}	3·00	0·45	
	8×10^{-4}	3·35	1·05	31·3

Lymphocyte cultures were cultivated 70 hr in a medium containing MTX (3×10^{-7} M), adenosine ($1{\cdot}5 \times 10^{-3}$ M) and TdR or EtUdR in different concentrations. The cultures were harvested following 3 hr incubation with colcemide. Mitotic indexes were evaluated by scoring 2000 cells per culture.

* $\frac{\text{Mitotic index (\%) in cultures with EtUdR}}{\text{Mitotic index (\%) in cultures with TdR}} \times 100$

This ratio, expressed in per cent, shows the ability of EtUdR to replace TdR in reversing of DNA synthesis block.

TABLE 3. PERCENTAGE OF CELLS INCORPORATING ^{14}C-TdR AND ^{14}C-EtUdR IN LYMPHOCYTE CULTURES GROWN IN A MEDIUM CONTAINING MTX

Conditions of labelling	% of labelled cells	
	^{14}C-TdR	^{14}C-EtUdR
A* ^{14}C-TdR or ^{14}C-EtUdR added 3 hr prior to harvest	40·5	41·8
	52·3	43·8
B† ^{14}C-TdR or ^{14}C-EtUdR added at the onset of incubation	53·5	39·1
	49·8	38·5

* 0·5 μCi of ^{14}C-EtUdR or ^{14}C-TdR per 1 ml of culture were added respectively to parallel replicate lymphocyte cultures after 69 hr of cultivation (3 hr prior to harvest). Following autoradiography, the per cent of labelled cells was evaluated.

† 1·0 μCi of ^{14}C-EtUdR and 12·5 μg of "cold" EtUdR or 1·0 μCi of ^{14}C-TdR and 12·5 μg of "cold" TdR respectively were added to replicate cultures, together with MTX at the onset of incubation. The cultures were terminated after 72 hr of cultivation and the per cent of labelled cells was scored following autoradiography.

terminated after 72 hr of incubation and, following autoradiography, both the percentage of labelled interphase nuclei and metaphases were evaluated. The percentage of labelled interphase nuclei was somewhat lower in cultures incubated with EtUdR. It was assumed that the difference in per cent labelled cells in both types of cultures may depend upon the slow-down of passage of cells through the successive generations under conditions of EtUdR incorporation. The assumption is strengthened, as in previous experiments, by the differences in the respective mitotic indexes (1·3 and 1 % in EtUdR vs. 3·8 and 3·6 % in TdR cultures). In EtUdR and TdR cultures, 200 and 138 metaphases, respectively, were scored for the presence of label. The finding that all metaphases were labelled both in TdR and EtUdR cultures, implies that substitution of thymidine by the analogue in newly synthesized DNA is compatible with the progression of cells through the cycle and, moreover, that under conditions of thymidine deficiency the incorporation of EtUdR is a prerequisite for the passage of cells into the DNA synthesis period, and subsequently, mitosis.

Reversibility of proliferation block imposed by high concentrations of EtUdR. It has been demonstrated previously that thymidine, when added to lymphocyte cultures in high concentrations can block the passage of the cells through the cycle presumably through inhibition of DNA synthesis and that this effect is entirely reversible following the removal of excess of nucleoside from the medium.[7] A similar effect was sought for EtUdR in an experiment which was designed as follows: Replicate lymphocyte cultures were grown, respectively: (1) in the presence of MTX and Ad in standard concentrations, (2) TdR (concentration $7{\cdot}5 \times 10^{-3}$ M) and (3) EtUdR (concentration $7{\cdot}5 \times 10^{-3}$ M), added at the onset of cultivation. Part of these cultures were harvested at the end of day 3 of cultivation. The respective mitotic indexes from these cultures were: 0·0, 0·8 and 0·05 per cent. In a second set of duplicate cultures, the medium was exchanged for a fresh one in the case of (1) MTX cultures containing TdR in a concentration of 2×10^{-4} M, and in (2) TdR and (3) EtUdR cultures without the respective compound. Following 18 hours of incubation colcemide was added and the cultures were harvested 3 hr later. Mitotic indexes of these cultures were respectively 4·5, 6·5 and 5·25 per cent. It is apparent from this experiment that high excess of EtUdR blocks the passage of cells through the cycle, similarly to, or even more efficiently than, TdR. The effect is entirely reversible following the wash-out of excess of analogue. It may therefore be inferred that EtUdR, even in high concentration, does not affect irreversibly the course of metabolic events leading to the initiation of DNA synthesis in the culture.

Evaluation of the kinetics of passage of cells through the cycle following reversal of the MTX imposed DNA synthesis block by EtUdR. To explain the relatively lower efficiency in the reversal of MTX imposed proliferation block by EtUdR as compared with TdR, the assumption was put forward that the analogue slows down the passage of cells through the cycle when added to cultures in which TdR incorporation is blocked. Two types of experiments were carried out to test this assumption:

(a) Two parallel culture series were grown in the presence of MTX and Ad until the end of day 3 of incubation, when TdR or EtUdR, respectively, were added in standard concentrations to restore cell proliferation. During the 2 subsequent days of cultivation, parallel cultures were harvested at 3, and later at 6, hr intervals following incubation with colcemide.

Mitotic curves following block reversal in these cultures are plotted in Fig. 1. It is

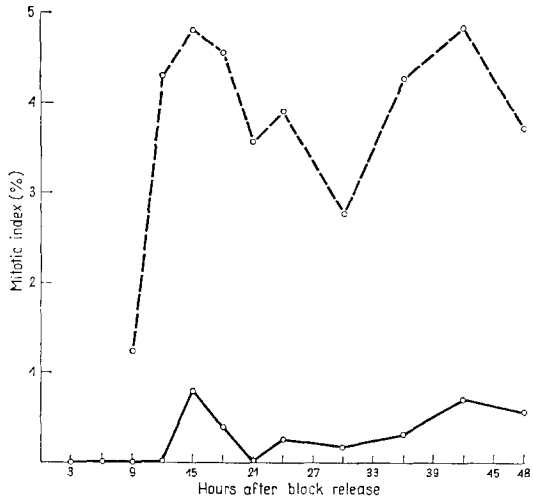

FIG. 1. Mitotic curves following reversal of MTX imposed DNA synthesis block by TdR (○- - -○) and EtUdR (○——○). Two series of replicate lymphocyte cultures were grown in a media containing MTX and adenosine. After 70 hr, EtUdR and TdR, respectively, at concentration of 2×10^{-4} M, were added to these cultures which were subsequently harvested at 3 and later 6 hr intervals, following 3 or 6 hr incubation with colcemide.

apparent that the mitotic wave following MTX block reversal by EtUdR is somewhat delayed in time and considerably lower than in the TdR control. This result is compatible with the assumed delay of passage of cells through the cycle following reversal of MTX block by EtUdR.

(b) Two parallel cultures series with MTX and Ad in the medium, and TdR and EtUdR respectively added at the onset of cultivation, were irradiated with a total dose of 212 rads of hard X-rays (250 kV, 1·5 mm Cu filtration, dose rate 48·6 rads/min) immediately before PHA was added. Duplicate parallel cultures were then harvested at the end of days 3, 4 and 5 of incubation. One-hundred well spread metaphases were analysed for the presence of asymmetric chromosome aberrations (dicentrics and rings) and acentric fragments. It is apparent from Fig. 2, in which the data from this experiment are assembled, that although at day 3 of cultivation the frequency of acentric fragments was comparable in both types of cultures, the rate of the elimination of aberrations during the 2 subsequent days of incubation was slower by a factor close to 5 in EtUdR cultures. On the other hand, 2·5 times more asymmetric chromosome aberrations in TdR, as compared with EtUdR cultures, were not accompanied by an acentric fragment. Since it is well established that acentric chromosome fragments are virtually completely eliminated at the first postirradiation division, whereas the elimination of asymmetric aberrations due to secondary rearrangements is somewhat slower,[11] the results clearly imply that many more cells are at their first postirradiation division in EtUdR cultures following 5 days of incubation then in the parallel TdR controls. The rate of elimination of acentric fragments, and the relatively slower increase of the fraction of cells carrying asymmetric chromosome exchanges without corresponding fragments, indicates therefore that this is due to the slowdown of the passage of cells through the proliferation cycle in EtUdR cultures.

Search for chromosome abnormalities in cultures in which cell proliferation was restored by EtUdR. It is known that the incorporation of some nucleoside analogues into DNA of cells in culture results in the induction of certain types of chromosome abnormalities.[12] It was demonstrated in several preliminary experiments that in cultures grown in presence of MTX (without adenosine), following block reversal by thymidine, essentially no chromosome aberrations are found. Although, exceptionally, some chromatid breaks are encountered, their frequency is always below 10 per 100 metaphases. This value corresponds to the background in asynchronous lymphocyte cultures.

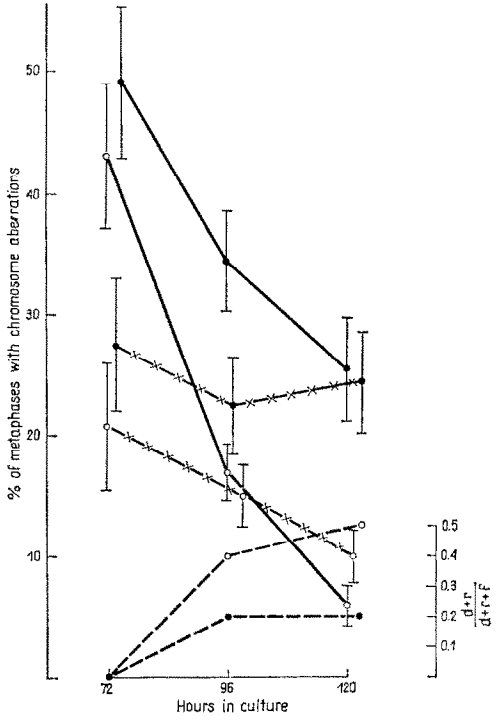

FIG. 2. Frequency of cell carrying X-ray-induced chromosome aberrations in relation to the time of culture in lymphocyte cultures grown in media containing MTX and TdR or EtUdR. Replicate lymphocyte cultures grown in media containing MTX and TdR or EtUdR (2×10^{-4} M) were irradiated at the onset of cultivation with 212 rads of hard X-rays (250 kV, 15 mA, 1·5 mm Cu filtration, dose rate 46·8 rev./min) and harvested after different time intervals. The frequency of cells carrying acentric chromosome fragments in EtUdR (●——●) and TdR (○——○) cultures, and the frequency of cell carrying asymmetric chromosome aberrations with accompanying fragment(s) in EtUdR (●–×–×–●) and TdR (○—×—×—○) cultures were estimated. The ratio of cells carrying asymmetric chromosome aberrations (dicentrics and/or rings) without accompanying fragment to all cells carrying asymmetric chromosome aberrations in EtUdR (●– – –●) and TdR (○– – –○) cultures was calculated.

To explore the possible mutagenic effects of EtUdR, an experiment was carried out in which three replicate parallel cultures established in a medium containing MTX were incubated from the onset of cultivation with EtUdR, TdR and BUdR respectively at concentrations of 2×10^{-4} M. The cultures were harvested after 70 hr of incubation

and 204–223 metaphases were analysed for chromosome aberrations. The results of the experiments are summarized in Table 4: with the exception of chromatid breaks, no other chromosomal abberations were encountered in all 3 types of cultures. The frequency of chromatid breaks in TdR and EtUdR cultures corresponded to background levels. On the other hand chromatid breaks were encountered quite frequently in BUdR cultures, in line with previous reports.[12] The results of this experiment

TABLE 4. FREQUENCY OF CHROMATID ABERRATIONS IN METAPHASES FROM STIMULATED LYMPHOCYTES CULTURES GROWN IN MEDIA CONTAINING MTX AND TdR, BUdR OR EtUdR, RESPECTIVELY

MTX block reversed by	No. of metaphases scored	No. of chromatid breaks per cell
TdR	204	0·02
EtUdR	223	0·01
BUdR	220	0·10

Replicate lymphocyte cultures were grown in media containing MTX and TdR, BUdR or EtUdR (2×10^{-4} M) from the onset of incubation. All cultures were harvested after 72 hr of cultivation. Colcemide was added 3 hr prior to harvest.

prove that EtUdR, while restoring proliferation in MTX blocked cultures, does not induce noticeable aberrations of chromosome structure, at least during the first generation following incorporation into DNA. This can however not be accepted as final evidence for non-mutagenicity of the analogue incorporated into DNA of somatic mammalian cells, since the possibility of point mutations must still be considered.

Acknowledgements—We wish to express our thanks to Professor David Shugar for critical reading of the manuscript. We acknowledge the skilful technical assistance of Mrs. Eugenia Kling and Mrs. Barbara Zygry-Traczyk. This work was supported in part by a WHO research Grant No. R00145 to J.S. 5-ethyl-[^{14}C]-2′-deoxyuridine was a kind gift of Dr. L. Pichat (Service des Molecules marquées, C.E.N. Saclay, France) to Professor D. Shugar. Purified phytohemagglutinin was a kind gift of the Wellcome Research Laboratories.

REFERENCES

1. M. SWIERKOWSKI and D. SHUGAR, *J. Med. Chem.* **12,** 535 (1969).
2. I. PIETRZYKOWSKA and D. SHUGAR, *Biochem. biophys. Res. Commun.* **25,** 567 (1966).
3. I. PIETRZYKOWSKA and D. SHUGAR, *Acta Biochim. Pol.* **14,** 169 (1967).
4. M. PIECHOWSKA and D. SHUGAR, *Biochem. biophys. Res. Commun.* **20,** 768 (1965).
5. K. K. GAURI and G. MALORNY, *Naunyn-Schmiedebergs Arch. Pharmak. exp. Pathol.* **257,** 21 (1967).
6. E. FREESE, *Proc. natn. Acad. Sci. U.S.A.* **45,** 622 (1969).
7. J. A. STEFFEN and W. M. STOLZMAN, *Exp. Cell Res.* **56,** 453 (1969).
8. P. S. MOORHEAD, P. C. NOWELL, W. J. MELLMAN and D. M. BATIPPS, *Exp. Cell. Res.* **20,** 613 (1960).
9. H. B. A. SHARPE, D. SCOTT and G. W. DOLPHIN, *Mutation Res.* **7,** 453 (1969).
10. J. JASIŃSKA, J. STEFFEN and A. MICHALOWSKI, *Exp. Cell Res.* **61,** 333 (1970).
11. M. A. BENDER, in *Advances in Radiation Biology* (Eds. L. G. AUGUNSTEIN, R. MASON and M. ZELLE) Vol. 3, p. 215, Academic Press, New York (1969).
12. T. C. HSU, *Proc. natn. Acad. Sci. U.S.A.* **47,** 396 (1961).