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THE METABOLISM OF THYMIDINE BY MURINE LEUKEMIC LYMPHOBLASTS (L5178Y)*

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Abstract—A study has been made of the toxicity of a number of compounds for L5178Y cells in culture; these compounds included thymidine, 5-fluoro-, 5-bromo- and 5-iodo-2′-deoxyuridine. Under certain conditions the cells underwent only one division in the presence of 5-iodo-2′-deoxyuridine and 5-bromo-2′-deoxyuridine before death of the cells occurred. This phenomenon was shown to be associated with the extensive incorporation of the iodo-compound into DNA, as 5-iodo-2′-deoxyuridine 5′-phosphate, in place of thymidine 5′-phosphate.

Various attempts to induce synchronous division of culture of L5178 have been described.

THYMIDINE (or its 5′-phosphate) has been regarded as unique amongst the purine and pyrimidine derivatives found in mammalian cells, since it appeared to occur solely as a precursor of or as a component of DNA‡§; however, recent reports indicate that thymidine-containing coenzymes also may occur, at least in *E. coli*.[27] Nevertheless, deprivation of cells of thymidine or its metabolites affords a possible means of specific interference with the synthesis of DNA without causing profound disturbances of the other anabolic functions of the cell.

Several compounds have been developed which inhibit the reproduction of various mammalian cells, the toxicity of which, under certain circumstances, can be prevented by thymidine. Amongst these are amethopterin and 5-fluoro-2′-deoxyuridine, both of which inhibit the biosynthesis of thymidine 5′-phosphate; also, 5-iodo-2′-deoxyuridine and 5-bromo-2′-deoxyuridine not only inhibit the utilization of thymidine and its phosphorylated derivatives, but are also incorporated into DNA in place of thymidylic acid.

In this investigation, these inhibitory compounds have been used in the study of the metabolism of thymidine in murine malignant lymphoblasts, L5178Y, which can be grown indefinitely in suspension culture and retain their capacity to produce a fatal neoplasia in appropriate strains of mice.[11] Preliminary reports of these findings have been made.[18, 19]

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‡ G. A. Fischer, unpublished results.

§ Abbreviations used are: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; FUDR, 5-fluoro-2′-deoxyuridine; BUDR, 5-bromo-2′-deoxyuridine; IUDR, 5-iodo-2′-deoxyuridine; dAMP, deoxyadenylic acid; dCMP, deoxycytidylic acid; dGMP, deoxyguanylic acid; dTMP, thymidylic acid; Tris, tris-hydroxymethyl-aminomethane.

MATERIALS AND METHODS

Culture techniques have been described previously.[10] The medium[14] used in these experiments supports the clonal reproduction of the leukemic cells.* The normal plan of the synchronization experiments involved the incubation of the cells (1 or 2×10^5 per ml) with a specific antimetabolite at the minimal concentration necessary to inhibit the reproduction of the cells. Aliquots of the cells were removed at regular intervals; after harvesting by centrifugation, and washing, the cells were resuspended in the same volume of fresh medium containing thymidine (2 μg/ml; 8·3 μM), but no antimetabolite. After incubation, the growth responses were determined by hemocytometer counts at frequent intervals, 200–500 cells being counted for each determination. Amethopterin was a gift of the American Cyanamid Corporation. BUDR was purchased from the California Corporation for Biochemical Research. IUDR and ^{131}I-IUDR were supplied by Dr. W. H. Prusoff of this department and FUDR was a gift of Hoffmann-La Roche, Inc.

RESULTS AND DISCUSSION

Initially, a preliminary study was made of the toxicity of thymidine for L5178Y; the results are summarized in Fig. 1. In concentrations up to 2 μg/ml (8·3 μM), thymidine caused no observable toxicity. The long lag-period in the growth of the cultures incubated with thymidine at 15 μg/ml (62 μM) may represent the gradual

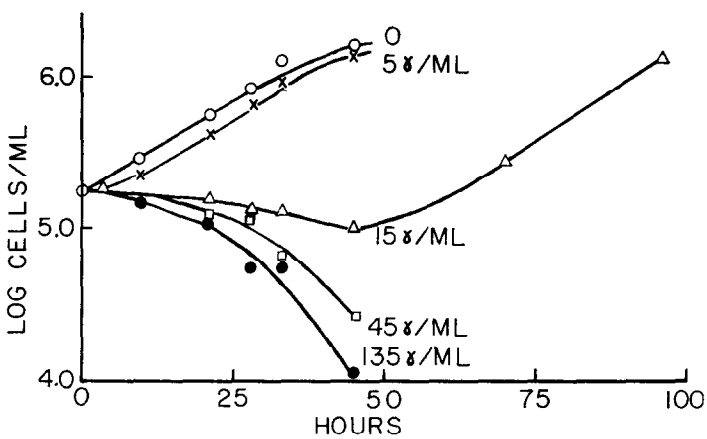

FIG. 1. Effects of thymidine on L5178Y cells grown in culture. The compound was added to the medium to give the final concentrations shown.

cleavage of thymine from the excess nucleoside; even at levels as high as 540 μM, the free pyrimidine, thymine, did not inhibit cellular reproduction and was not utilized by the cells for the synthesis of DNA. Cells which underwent several successive reproductions in thymidine at a concentration of 62 μM showed no evidence of adaptation and, indeed, when subcultured, exhibited exactly the same pattern of inhibition by thymidine. Inhibition of reproduction of L5178Y cells by a phosphorylated derivative of thymidine is attributable, in large part, to inhibition of the enzymes which reduce cytidine 5′-phosphate to 2′-deoxycytidine 5′-phosphate.[21, 22]

* G. A. Fischer, unpublished results.

Normally, L5178Y cells do not require exogenous thymidine for growth, but such a requirement can be induced in the presence of amethopterin. Thus, in media supplemented with both serine (15 μg/ml) and hypoxanthine (4 μg/ml), growth was completely prevented by amethopterin at a concentration of 0·025 μM, an inhibition which was nullified by the addition of thymidine at a final concentration of 8·3 μM.* Half-maximal reversal required thymidine at a level of 0·24 μg/ml (1·0 μM). With other cell-lines, similar results have been obtained by Hakala and Taylor;[12] however, these workers observed no requirement for serine, although a requirement for glycine was observed. Thymine, 2′-deoxyuridine, or 2′-deoxycytidine, even at concentrations of 100 μM, did not prevent the inhibition by amethopterin, although thymidylic acid, in a molar equivalent concentration, was as effective as thymidine.

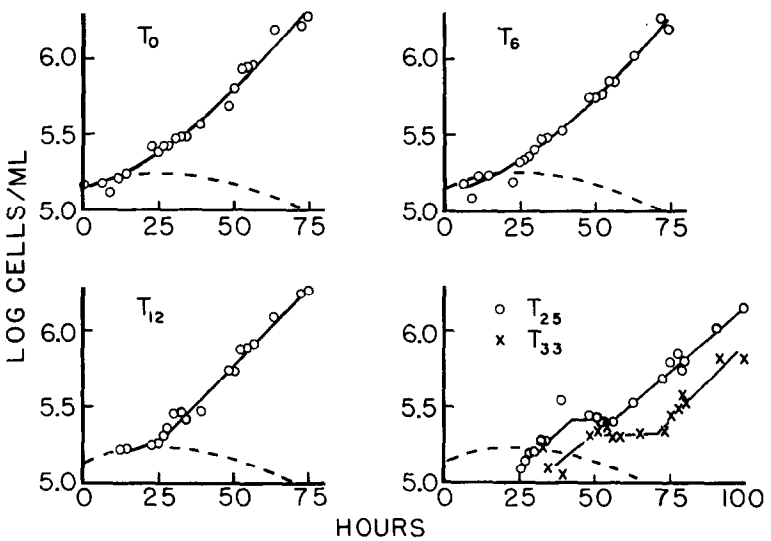

FIG. 2. Attempted synchronization of growth of L5178Y cells, using a thymidine-dependency obtained by the addition of amethopterin.
– – – – – Growth of the culture in the presence of amethopterin (0·025 μM).
——— Growth of samples removed to a medium containing thymidine and free of drug. T_0, T_6, T_{12}, T_{25} and T_{35} refer to samples removed at 0, 6. 12, 25, and 35 hr, respectively, following the initiation of incubation of the cells at 37°.

Barnes and Cohen[2] have reported synchronization of a thymine-requiring mutant of *E. coli*. The organism was incubated for a limited period in the absence of thymine, during which time the cells synthesized both protein and RNA. On the addition of thymine, the synthesis of DNA and cellular reproduction occurred in a stepwise fashion. An attempt was made to obtain synchronized reproduction of the lymphoblasts by the specific inhibition of DNA-synthesis, through the use of amethopterin and conditions which limited only the supply of thymidine. L5178Y cells ($1·5 \times 10^5$/ml) were incubated in a medium containing no thymidine, but supplemented with glycine, serine, hypoxanthine, and amethopterin (0·025 μM). Growth responses were determined at frequent intervals of time with aliquots of the inhibited cultures which had been washed free of amethopterin with medium, and then resuspended in medium containing no amethopterin, but supplemented with thymidine (8·3 μM);

* G. A. Fischer, unpublished result.

typical results are shown in Fig. 2. During the first 15 hr most of the cells removed from the inhibited cultures were able to divide when resuspended in the medium containing thymidine. Indeed, in those samples in which the medium was changed after 6–12 hr in the presence of the drug, a partial synchronization was obtained. However, the effect was small and the partial synchrony was rapidly lost. Cells that were resuspended after 26 hr in the presence of the drug roughly doubled in number, thereafter the count remained constant for up to 20 hr before increasing in a logarithmic fashion. This period of apparently suspended reproduction may represent a balance between the continuing division of some cells and the death of others. It is evident, however, that even after 36 hr in contact with amethopterin (0·025 μM), a significant portion of the tumor cells were viable.

The toxicity of FUDR for *E. coli* depends on its conversion to 5-fluoro-2′-deoxyuridine 5′-phosphate, a compound which inhibits thymidylic acid synthetase.[4] The growth of L5178Y cells in the presence of different concentrations of FUDR is illustrated in Fig. 3. At 0·001 μM the cell count remained constant for the first 40 hr

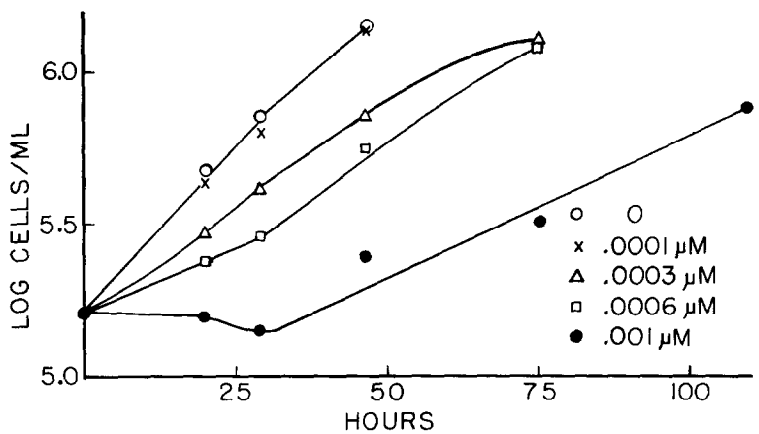

FIG. 3. Inhibition of growth of L5178Y cells by 5-fluoro-2′-deoxyuridine (FUDR).

and the cells became greatly enlarged. Thymidine at 8·3 μM completely prevented the toxicity of FUDR at 0·001 μM. It is of interest to compare the 50 per cent-inhibition points for a series of derivatives of 5-fluorouracil; with L5178Y cells, these were 0·5 μM for 5-fluorouracil, 0·01 μM for 5-fluorouridine, and 0·0006 μM for 5-fluoro-2′-deoxyuridine. Attempts to obtain synchronization by the method previously outlined were made using 0·001 μM FUDR in place of amethopterin; the results are shown in Fig. 4. Cells that had been in contact with the drug for a period of up to 10 hr recovered when transferred to fresh medium containing thymidine, and grew immediately at a normal rate indicating that a majority of the inhibited cells had retained viability. After 10 hr, however, a lag period was exhibited when the cells were transferred to the thymidine medium and growth did not occur for 12–24 hr.

Prusoff has shown that IUDR is a competitive antagonist of thymidine at the triphosphate level in the synthesis of DNA in mouse Ehrlich ascites carcinoma cells *in vitro*.[6, 25] The effect of this analog of thymidine on the leukemic lymphoblasts

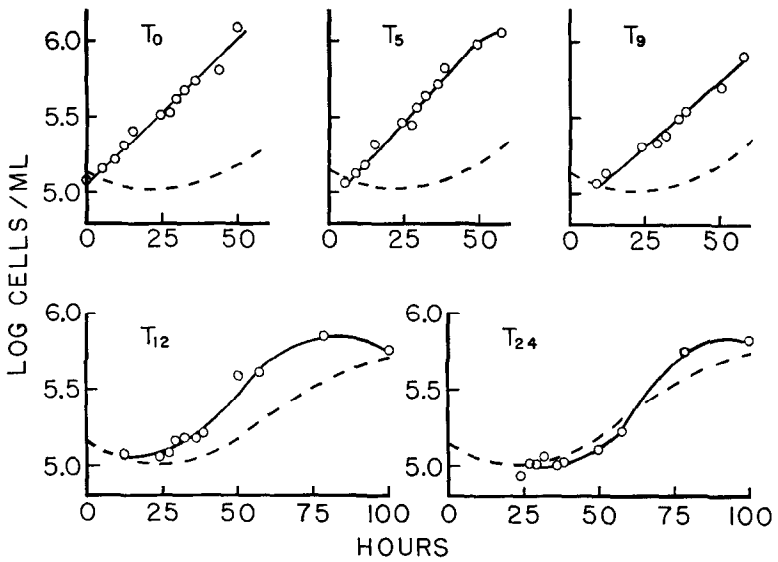

FIG. 4. Attempted synchronization of growth of L5178Y cells, using 5-fluoro-2′-deoxyuridine (FUDR).

– – – – – Growth of the culture in the presence of FUDR (0·001 μM).

——— Growth of samples removed to a medium containing thymidine (8·3 μM) and free of drug.

The subscripts indicate the time in hours at which the samples were taken.

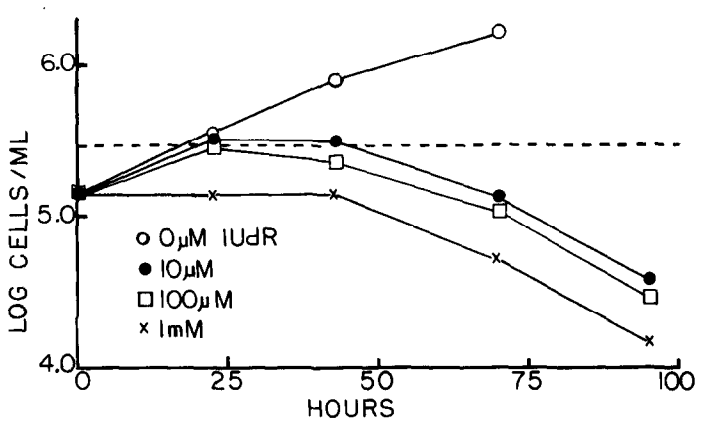

FIG. 5. Toxicity of 5-iodo-2′-deoxyuridine (IUDR) for L5178Y cells in culture. The dashed line represents a 2-fold increase in cell number.

has been investigated (Figs. 5 and 6). At the highest concentration of IUDR used (1000 μM), the cell count remained constant for 48 hr, and then rapidly fell; at a concentration of 330 μM a slight increase in cell number was followed by a progressive lysis. With concentrations of IUDR between 100 μM and 30 μM, the population doubled and subsequently diminished; this decline continued until no live cells could be detected. With IUDR at a level of 10 μM, the cultures behaved in the same

way, i.e., the count roughly doubled, after which many of the cells died, and, within 96 hr after the addition of the analog, the count fell to 6–12 $\times$ 10^4/ml. The inhibitory effect of the agent at 10 μM was completely prevented by the addition of thymidine to the medium at a concentration of 8·3 μM; however, when the IUDR-concentration

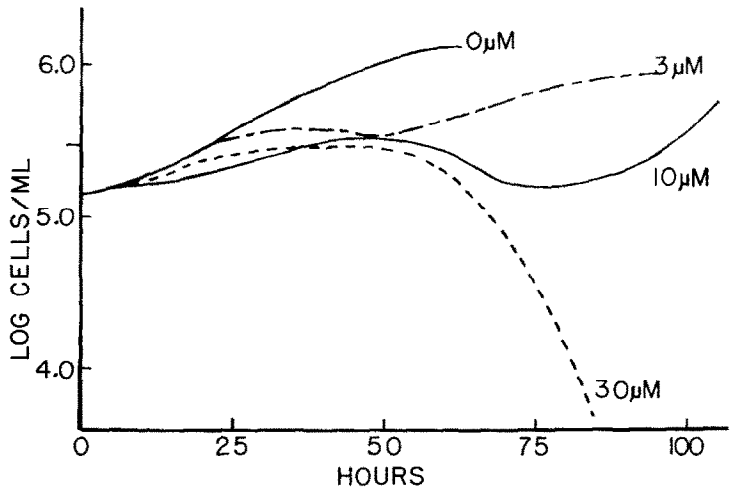

FIG. 6. Toxicity of 5-iodo-2′-deoxyuridine (IUDR) for L5178Y cells in culture. The bar on the ordinate is equal to double the original cell number.

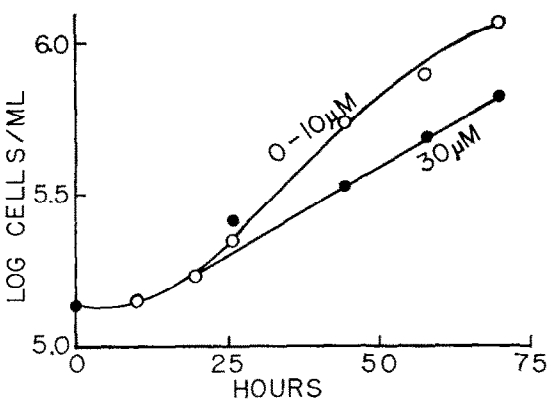

FIG. 7. Prevention by thymidine (2 μg/ml; 8·3 μM) of the toxic effects of 5-iodo-2′-deoxyuridine (IUDR) on the growth of L5178Y cells in culture. ○ Controls without IUDR, or in the presence of IUDR at 10 μM, with thymidine, 8·3 μM. ● Cultures containing IUDR, 30 μM, and thymidine, 8·3 μM.

was increased to 30 μM the simultaneous addition of thymidine (8 μM) resulted in growth at a slightly lower rate than that of the controls (Fig. 7). The phenomena of division followed by cell death recalls the behavior of *E. coli* grown in the presence of 5-bromouracil.[5] The toxicity of BUDR for L5178Y cell is shown in Fig. 8.

It should be noted that at 1 and 10 μM IUDR exerted no apparent effect upon the growth rate of L5178Y cells until after incubation for 48 hr, when progressive death of cells was observed, while at a concentration of 100 μM, the cell-number increased by a factor of 2·0–2·3 before the cell-count declined. With IUDR at 1000 μM, the cell-number did not increase and lysis commenced after about 24 hr.

It is of interest to contrast the toxicity curves obtained with IUDR and BUDR on the one hand with that of FUDR on the other. Although the former agents exerted scarcely any effect on the initial growth rate until the cell number had increased by a factor of 2·0–2·3, such populations were then essentially non-viable. However, the curve depicting the response to FUDR shows that with this compound there is a rapid and maintained inhibition of rate of reproduction which is proportional to the concentration of antimetabolite, and that such inhibited populations were largely viable.

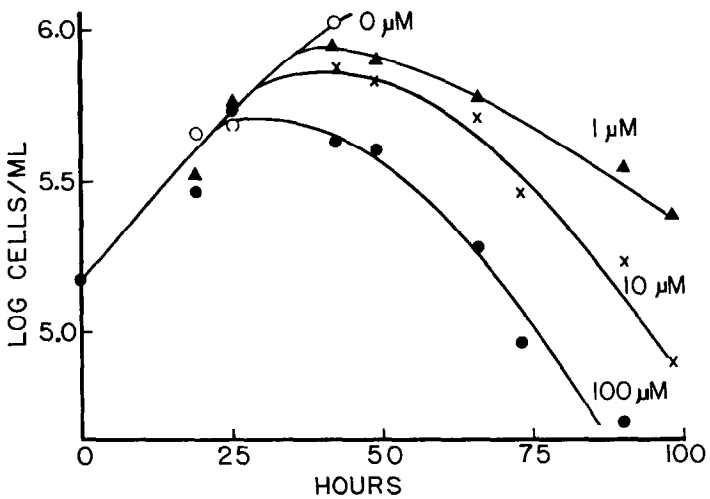

FIG. 8. Toxicity of 5-bromo-2′-deoxyuridine (BUDR) for L5178Y cells grown in culture.

The iodo- and bromo-derivatives have been shown to be incorporated into DNA,[9, 13, 25] while the fluoro-compound does not enter the DNA-molecule[4] and this may explain the differences observed in the response to these compounds. Not only do growth studies with L5178Y cells indicate that IUDR acts as an analog of thymidine which substitutes for it in the biosynthesis of DNA, but we also have demonstrated this with ^{131}I-IUDR, as has previously been done with other cell-lines.[25] Thus, an inoculum of 4·7 $\times$ 10^8 L5178Y cells was incubated for 18 hr with ^{131}I-IUDR (40 μC, 26 μM) in 1300 ml of medium. The cells were harvested by centrifugation and washed, first with fresh medium and then with 0·25 M sucrose containing 0·002 M $CaCl_2$; the yield was 520 mg of wet cells. DNA was extracted from these cells and purified as described in the accompanying paper,[20] except that, after the deproteinization stage,

the fibers of DNA were dissolved in 6 ml of a molar solution of sodium chloride. The solution was stirred with washed activated charcoal (0·2 g) at 4° for 2 hr to remove RNA.[29] The charcoal was filtered off and the activity of the various fractions was determined in a scintillation well-counter. The results are shown in Table 1.

The DNA fraction had a u.v.-absorption spectrum typical of that of DNA and only 0·2 per cent of the radioactivity was removed by dialysis for 16 hr against a solution of sodium chloride (3 per cent). To show that IUDR was actually incorporated into the DNA, the dialysed solution was diluted with water (4 ml), and 150 μg of crystalline pancreatic deoxyribonuclease (Worthington) was added in 1·5 ml of 0·4 M sodium acetate/acetic acid buffer of pH 6·0, together with 0·5 ml of 0·06 M magnesium acetate and a drop of toluene. The mixture was incubated at 37° for 16 hr. The pH

TABLE 1. RADIOACTIVITY OF THE MEDIUM AND FRACTIONS OF L5178Y CELLS GROWN IN THE PRESENCE OF RADIOACTIVE 5-IODO-2′-DEOXYURIDINE (^{131}I-IUDR)*

Fraction	Volume	cpm above background of 5-ml sample	Total cmp
Cytoplasmic†	17	$1{\cdot}58 \times 10^5$	$5{\cdot}4 \times 10^5$
DNA	6	$1{\cdot}04 \times 10^6$	$1{\cdot}3 \times 10^6$
Medium‡	1300	$1{\cdot}8 \times 10^3$	$4{\cdot}7 \times 10^7$

* The cells, $4{\cdot}7 \times 10^8$, were incubated with ^{131}I-IUDR (26 μM, 40 μC) for a period of 18 hr. The fractions were prepared and radioactivity was determined in a gasflow counter.

† The supernatant fraction from homogenization of the cells combined with the washings of the "nuclear" material.

‡ The medium in which the cells had been grown combined with the washings of the cells during their preparation for homogenization.

was adjusted to 8·8 with sodium hydroxide, and 3 ml of 2 M-Tris/HCl buffer, pH 8·8, and 3 ml of a preparation of snake venom phosphodiesterase were added. After incubation for 5 hr at 37°, the solution was diluted to 100 ml with water, and a samples was removed; this was dialysed against a molar solution of sodium chloride overnight at 4°. At the end of this time, there was approximately the same number of counts per ml, in the dialysate and the dialysand, a finding which showed that the radioactivity had become freely dialysable. The pH of the remainder of the dialysate was adjusted to 9·0 and the solution was run through a column of Dowex-1 × 4 (15 × 1 cm) in the formate form, followed by water 50 ml. The deoxyribonucleotides were removed from the column using a gradient of 1·0 M-ammonium formate, pH 4·25, running into a mixing vessel containing 500 ml of water.[3]

The eluate was collected in fractions of 7·5 ml. The radioactivity of each fraction was determined using a scintillation well-counter, and the optical density at 260 mμ was determined. The elution diagram is shown in Fig. 9. The fractions corresponding to each of the four main u.v.-absorbing peaks were combined, and the identity of the base present was established by an examination of the u.v.-absorption spectrum in acid and alkali. The amount of each deoxyribonucleotide was calculated from the

optical density at the maximum in acid. The results are summarized in Table 2. It will be seen that the amount of dTMP is significantly less than that of the other three nucleotides, about two-thirds the amount of dAMP. There were two radioactive peaks. The first (fractions 7–12) emerged from the column in the position normally occupied by IUDR. The fractions containing the main radioactive peak (30–40)

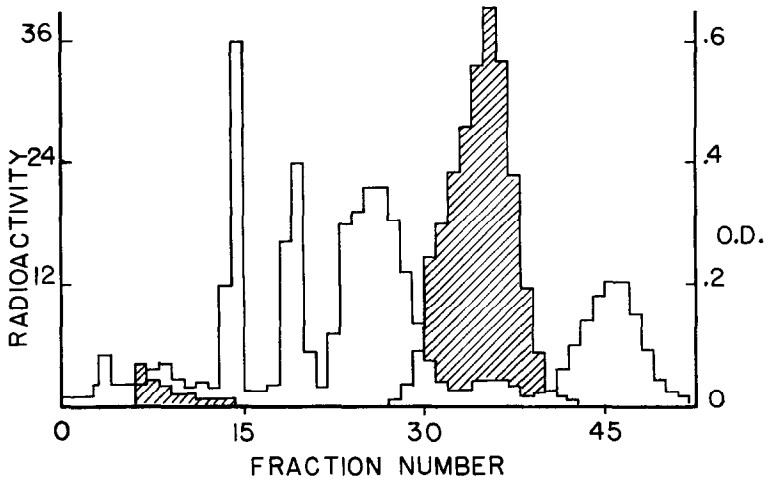

FIG. 9. Elution diagram of deoxyribonucleotides derived from DNA of L5178Y cells grown in the presence of 5-iodo-2′-deoxyuridine labeled with 131-iodine. Left hand ordinates: cpm (above background) $\times 10^{-3}$ per tube containing each fraction (cross-hatched areas). Right hand ordinates: O.D. at 260 mμ (unshaded areas).

TABLE 2. DEOXYRIBONUCLEOTIDE COMPOSITION OF DNA FROM L5178Y CELLS* GROWN IN THE PRESENCE OF RADIOACTIVE 5-IODO-2′-DEOXYURIDINE (^{131}I-IUDR)

Compound	μmoles
2′-Deoxyadenosine 5′-phosphate	0·94
2′-Deoxyguanosine 5′-phosphate	0·99
2′-Deoxycytidine 5′-phosphate	0·92
Thymidine 5′-phosphate	0·65
5-Iodo-2′-deoxyuridine 5′-phosphate	0·38

* Wet weight of cells, 520 mg.

The DNA was enzymically converted to the deoxyribonucleotides and resolved on a Dowex-1 × 4 column and the amount of each component was determined from the optical density at the maximum value, in acid, of each deoxyribonucleotide.

were combined and evaporated under reduced pressure to 10 ml and the radioactivity was measured in a gas-flow counter, and compared to that of a known dilution of the ^{131}I-IUDR used for the experiment ($7{\cdot}67 \times 10^5$ cpm/μmole). Since fractions 30–40 contained a total of $2{\cdot}53 \times 10^5$ cpm it can be calculated that these fractions contained 0·33 μmoles of 5-iodo-2′-deoxyuridine 5′-phosphate (IUDR-5′-P). The uv-absorption spectra in acid and alkali correspond closely to those of IUDR and the amount of the mono-phosphate, calculated from the optical density at 280 mμ in acid, was 0·38 μmoles

(based upon an E_{max} of 7700). The identification of the compound as 5-iodo-2′-deoxyuridine 5′-phosphate was confirmed by hydrolysis to IUDR using prostatic phosphatase. The concentrated fractions 30–40 were diluted to 50 ml with water and the pH was adjusted to 9. The IUDR-5′-P was adsorbed on a small column of Dowex-1 × 4 (formate) and eluted with a normal solution of formic acid. The fractions containing the single radioactive peak were combined and freeze-dried; the residue was dissolved in water (5 ml). The pH of a portion of this solution (2·5 ml) was adjusted to 5·2 and incubated with a solution of prostatic phosphatase (0·1 ml; provided by Dr. C. E. Carter) for 1 hr at room temperature. Non-radioactive IUDR (1 μmole) was added. Part of the digest was subjected to ion-exchange chromatography, as described for the DNA-hydrolysate. A single radioactive peak corresponding exactly to the peak of material absorbing in alkali at 290 mμ was obtained. The remainder of the enzymic digest was examined by paper chromatography in *iso*-propanol-HCl[28] and water-saturated butanol. In both solvents there was a single radioactive spot, exactly coincidental with the only u.v.-absorbing area. This experiment demonstrated that IUDR was extensively incorporated into the DNA of L5178Y. Experiments carried out with Dr. W. H. Prusoff on this department indicated that the growth medium (see Table 1) contained both 5-iodouracil, IUDR, and a large amount of radioactive iodide. It is assumed that an increase of cell number by the factor 2·0 results from a single division of the majority of cells in the population and that these progeny contain approximately the same amount of DNA per cell as do the normal cells. Further, assuming that the DNA from the normal cells contains roughly equivalent amounts of the four nucleotides, dCMP, dTMP, dAMP and dGMP, then IUDR and thymidine are incorporated in a ratio of approximately 2:1 in that DNA which was synthesized during the time that the cells were incubated with ^{131}I-IUDR and one mitotic cleavage occurred. The cells that contained this high proportion of IUDR evidently were no longer viable. This may be attributed to the biochemical incompetence of the "fraudulent" DNA or to the interference by IUDR with some other vital reaction in the cell. However, it has been shown that L5178Y cells incubated with IUDR in the presence of ^{3}H-thymidine or ^{14}C-formate showed a primary blockade of thymidine kinase and thymidylic acid kinase, respectively; the results concerning thymidine kinase were confirmed in a cell-free system.[6]

It is generally accepted that, in mammalian cells, DNA-synthesis occurs during a definite phase of the life cycle.[15, 16, 26] A culture of L5178Y growing logarithmically would contain cells at all stages, before, during and after the period of DNA-synthesis. Accordingly, if the exposure to IUDR were for a limited time, a proportion of the cells would incorporate the analog into their DNA and would die. Attempts were made to establish conditions such that many of the cells would be killed and the remainder, presumably at the same stage of growth, on transfer to fresh medium containing thymidine would divide in a nearly synchronized manner. Fig. 10 shows the results of such an experiment, using a concentration of IUDR of 20 μM. After incubation for 4 hr with IUDR the cells grew in a normal manner when transferred to fresh medium supplemented with thymidine (8·3 μM). Cells removed at 6 hr double in number, the cell count then remains constant for 48 hr, after which the cells go through one further division but further reproduction does not occur. Cells left in contact with the drug for more than 12 hr slowly die and the count falls to 4×10^4. Two reasons may be advanced in partial explanation of these observations. The synthesis

of DNA may not be restricted to a section of the life span of the cell.[1, 23] Alternatively, IUDR or its metabolites, once within the cell, are probably not removed simply by transferring the cells to a drug-free medium, as it would appear from the study of the growth curve of the T6 sample in Fig. 10. In contrast to amethopterin and FUDR, incubation for a period of 10 hr with IUDR is sufficient to kill the majority of treated cells in the population.

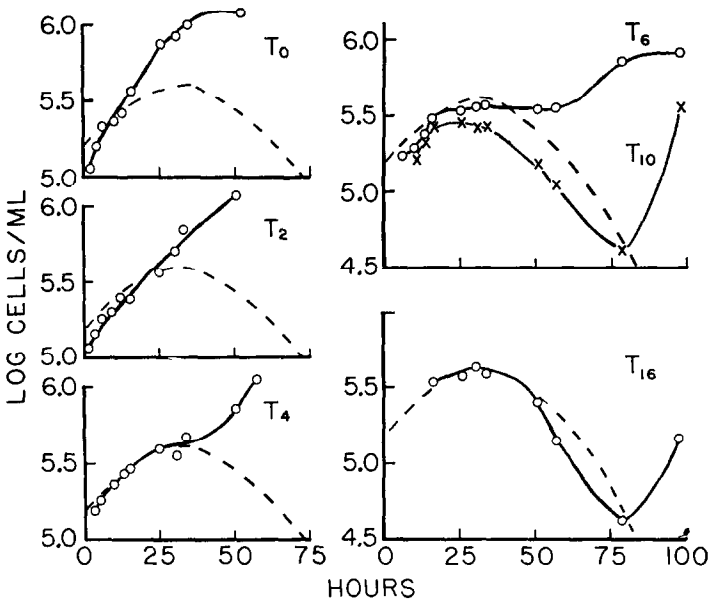

FIG. 10. Attempted synchronization of growth of L5178Y cells, using 5-iodo-2′-deoxyuridine (IUDR).
– – – – – Growth of the culture in the presence of IUDR, 20 μM.
——— Growth of samples removed to a medium containing thymidine (8·3 μM) and free of drug.
The subscripts indicate the time in hours at which the samples were taken.

The preliminary nature of the studies described does not permit a comparison with data obtained using other cell lines.[7–9, 13, 17] However, it may be suggested that with these near-diploid tumor cells, L5178Y, exposure to a proper concentration of IUDR or BUDR for a period corresponding to one average generation-time is sufficient to render the population non-viable, whereas amethopterin and FUDR, even after exposure for a period of time corresponding to three generations was insufficient to produce an equivalent lethal effect.

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