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ACUTE EFFECTS OF CYTOTOXIC COMPOUNDS ON INCORPORATION OF PRECURSORS INTO DNA, RNA, AND PROTEIN OF HeLa MONOLAYERS*

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Abstract—Acute effects of drugs on incorporation of radioactive precursors into DNA, RNA, and protein of HeLa cells were assayed by means of a sequential isotope technique. The technique involves a 15- to 30-min control preincubation of the cells without drugs, followed by a 30-min experimental incubation with drugs. During the control incubation the medium contains a precursor labeled with ^{14}C; during the experimental period the same precursor is present, now labeled with tritium. The precursors used are ^{14}C- and ^{3}H-thymidine for DNA, ^{14}C- and ^{3}H-uridine for RNA, and ^{14}C- and ^{3}H-leucine for protein. The $^{3}H/^{14}C$ ratios for groups of monolayers are determined by liquid scintillation techniques. Drugs which change the $^{3}H/^{14}C$ ratio from that obtained in control monolayers are assumed to have an acute effect. Twenty cytotoxic compounds and two normal nucleosides were tested. Maximal drug concentrations of 10 to 1,000 times the reported IC_{50} (that level inhibiting growth of cells *in vitro* by 50%) usually were employed before excluding an acute effect. Deoxyadenosine, 5-iodo-2-deoxyuridine, cytosine arabinoside, hydroxyurea, and hydroxyurethan inhibited incorporation of thymidine. Puromycin, cycloheximide, and acetoxycycloheximide inhibited incorporation of both leucine and thymidine. Actinomycin D inhibited incorporation of uridine and thymidine. Streptonigrin inhibited incorporation of all three precursors. 6-Mercaptopurine, 6-diazo-5-oxo-L-norleucine, urethan, vinblastin, deoxyguanosine, triethylene melamine, and mitomycin C had no significant effect.

THE usefulness of dual-isotope techniques in studies of the synthesis of hemoglobin[1] and the metabolism of cholesterol[2] suggested that similar techniques could be used to measure effects of drugs on cells. In the method to be described, monlayers of HeLa cells are briefly exposed to medium containing a ^{14}C-labeled precursor of DNA, RNA, or protein and then removed to medium containing the same precursor labeled with ^{3}H. Drugs under study are present in the medium during the second incubation. The ^{14}C content of a monolayer is related to the metabolic activity of its cells during the period before exposure to drugs. Similarly, ^{3}H content is related to cellular metabolic activity in the presence of drugs. If the cell monolayers are exposed to identical concentrations of labeled precursors for an identical period of time, and if the pattern of cellular metabolism does not alter significantly over the study period, they should contain ^{3}H and ^{14}C in a relatively constant ratio. Significant departure from this constant ratio in association with exposure to a drug can be interpreted as

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an effect of the agent. The sequential isotope technique is a simple method for assaying actions of drugs on cultures of mammalian cells. Some of the observations detailed herein have been reported previously in abstract form. [3, 4]

MATERIALS AND METHODS

Cell line and incubation techniques. HeLa cells were obtained from Microbiological Associates and maintained in monolayer culture by weekly subdivision. Eagle's minimal essential medium, supplemented with calf serum, penicillin, and streptomycin was used for maintenance of cells.

Chemicals. ^{3}H- and ^{14}C-labeled thymidine, uridine, and leucine were obtained from the New England Nuclear Corp. Deoxyadenosine (AdR), deoxyguanosine (GdR), and 6-azauridine (AZUR) were purchased from the California Corp. for Biochemical Research and 2, 4-dinitrophenol from the Fisher Scientific Co., New York, N.Y.

The following were generously provided: 5-fluoro-2-deoxyuridine (FUdR); 5-fluorouridine (FUR); 5-iodo-2-deoxyuridine (IUdR, Hoffman-LaRoche Inc., Nutley, N.J.); 6-mercaptopurine (6-MP, Wellcome Research Labs, Tuckahoe, N.Y.); amethopterin and triethylene melamine (TEM, Lederle Labs. Div., American Cyanamid Co., Pearl River, N.Y.); cytosine arabinoside, cycloheximide (Upjohn Co., Kalamazoo, Mich.); acetoxycycloheximide, streptonigrin (Charles Pfizer Inc., New York, N.Y.); urethan, actinomycin D (Merck & Co., Rahway, N.J.); 6-diazo-5-oxo-L-norleucine (DON, Parke-Davis, Detroit, Mich.); vinblastin (Eli Lilly, Indianapolis, Ind.); hydroxyurea (E. R. Squibb & Sons, New York, N.Y.); hydroxyurethan (Dr. A. Bendich, Sloan-Kettering Institute); and puromycin (Cancer Chemotherapy National Service Center, Bethesda, Md.).

Isotope incorporation studies. HeLa cells (3×10^5/ml) were subcultured on glass cover slips (22-mm diameter) in 60-mm petri dishes (Falcon Plastics, B-D Laboratories, Rutherford, N.J.). After cell attachment occurred, the cover slips were transferred to sterile porcelain racks (A. H. Thomas, Phildelphia, Pa.) and incubated for 48 to 72 hr in a closed glass jar. Under these conditions the cells had a doubling time of approximately 24 hr. At the time of isotope incorporation studies, cells were in the log phase of growth. For incorporation studies monolayers were first incubated in medium containing a carbon-14 precursor (thymidine-2-^{14}C, 0·03 μc/ml, $1{\cdot}2 \times 10^{-6}$ M; uridine-2-^{14}C, 0·02 μc/ml, $0{\cdot}7 \times 10^{-6}$ M; or L-leucine-1-^{14}C, 0·4 μc/ml, $1{\cdot}6 \times 10^{-5}$ M) for 15 to 30 min at 37°. After a rinse with warmed medium, racks of monolayers were placed in nonradioactive medium for 10 to 30 min;* monolayers were then removed from the racks and transferred in groups of three to Columbia staining jars (A.H. Thomas, Philadelphia, Pa.). These jars contained the same precursor labeled with tritium (thymidine-[methyl-^{3}H], 0·56 μc/ml, $0{\cdot}8 \times 10^{-7}$ M; ^{3}H-uridine, 0·8 μc/ml, $2{\cdot}3 \times 10^{-7}$ M; or DL-leucine-[4-5-^{3}H], 0·8 μc/ml, $1{\cdot}5 \times 10^{-7}$ M). Drugs were present in the tritium-containing medium at zero time. After a 30-min incubation at 37° the monolayers were rinsed with iced saline, fixed with 5% TCA, extracted with ethanol

* A 10-min period was sufficient to exhaust unincorporated ^{14}C when thymidine or leucine was used as the precursor. However, after removal of the monolayers from ^{14}C-uridine to unlabeled medium, incorporation of ^{14}C continued beyond a 10-min period. This observation is consistent with the reported results of others[5, 6]. In view of this, monolayers were exposed to ^{14}C-uridine-containing medium for only 15 min and placed in nonradioactive medium for 30 min prior to exposure to ^{3}H-uridine and drugs. In spite of this precaution, the true extent of the inhibition of incorporation of ^{3}H-uridine was probably slightly underestimated in severely inhibited samples.

and ether, and air dried. In studies with leucine, nucleic acids were removed by exposing cells on the cover slips to 5% TCA at 95° for 15 min prior to lipid extraction. The cover slips were fragmented and placed in glass liquid scintillation counting jars. Cells were solubilized with Hyamine (Rohm and Haas), 1 M in methanol, and heat (65° for 1 to 2 hr then 37° overnight). Tritium and ^{14}C content in each sample was determined by the method of Kabara *et al.*[7] in a dual-channel liquid scintillation counter (Packard Instrument Corp., La Grange, Ill.).

In experiments with labeled thymidine and uridine, precursors were added to the medium in which the monolayers had been growing for the previous 48 hr. However, when we measured effects on incorporation of leucine, the use of fresh, leucine-free medium with 1% calf serum increased the radioactivity per sample and the consistency of experimental results.

RESULTS AND DISCUSSION

Precursor specificity. Thymidine has been reported to be a specific precursor of cellular DNA in the rat,[8] mouse,[9] chick embryo,[10] and in cultures of HeLa cells.[11] Uridine is incorporated into both RNA and DNA. Specificity of labeling was studied in DNA, RNA, and protein fractions isolated from HeLa monolayers which had incorporated labeled thymidine or uridine over incubation periods of from 5 to 240 min. A procedure modified from that of Schmidt, Thannhauser, and Schneider was used for these extractions.[12] The results (Table 1) confirmed the specificity of the

TABLE 1. INCORPORATION SPECIFICITY OF THYMIDINE AND URIDINE IN HELA MONOLAYERS

Time of incorporation (min):		5	30	120	240
		Total acid-insoluble radioactivity (%)			
2-^{14}C-Thymidine	RNA	nd*	nd	nd	nd
	DNA	100	99·0	98·0	98·5
	Protein	nd	1·0	2·0	1·5
Methyl-^{3}H-thymidine	RNA	1·3	0·4	0·4	0·2
	DNA	98·5	99·3	97·5	99·4
	Protein	0·2	0·3	2·1	0·4
2-^{14}C-Uridine	RNA	100	95·0	90·0	88·1
	DNA	nd	4·5	8·5	11·0
	Protein	nd	0·5	1·5	0·9
^{3}H-Uridine	RNA	100	97·5	94·6	92·7
	DNA	nd	2·5	5·4	7·2
	Protein	nd	nd	nd	0·1

* Radioactivity not detectable.

thymidine incorporation. Radioactivity from uridine appeared initially in RNA, and by 2 hr was seen in DNA in significant quantity. Because of this, incorporation periods with uridine were limited to 30 min. Over this short period the specificity of uridine for RNA is adequate for use in the described metabolic screening technique.

Reproducibility of tritium/^{14}C ratios in replicate samples. The coefficients of variation "C" (standard deviation from the mean expressed as a per cent of the mean) of control

group $^3H/^{14}C$ ratios are shown in Fig. 1. All experiments in which we followed the described technique are included. The control "C" was 9% or less in 29 of 37 experiments and 11% or less in 35 of 37 experiments. In two experiments variation in controls was 14% and 19%.

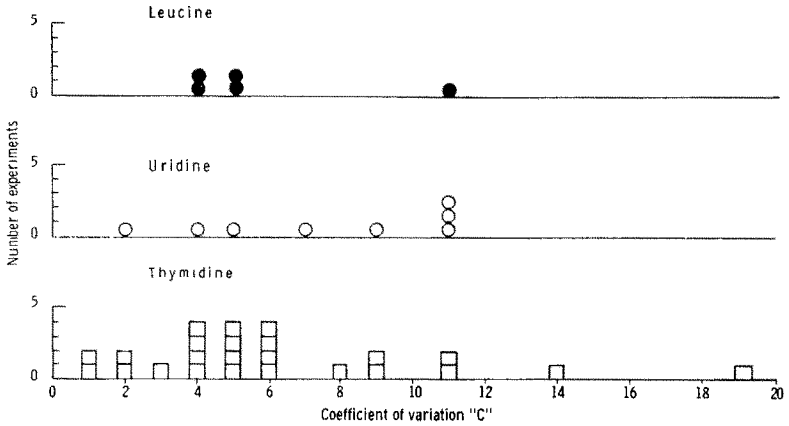

FIG. 1. Coefficient of variation "C" of $^3H/^{14}C$ ratio in control groups. Sequential isotope technique.

TABLE 2. ACUTE EFFECTS OF DRUGS ON PRECURSOR INCORPORATION INTO HELA MONOLAYERS*

Compound	Reported IC_{50} (μM)†	Maximal conc. tested (μM)	Precursor Thymidine	Uridine	Leucine
5-Iododeoxyuridine	30 (HEp 1)[13]	28	—	0	0
Amethopterin	0·02[14]	18·3	—	0	0
5-Fluorodeoxyuridine	0·003 (KB)[15]	40·6	—	0	0
5-Fluorouridine	0·003 (KB)[15]	38·2	—	—	0
Deoxyadenosine	< 2,000 (Ehrlich)[16]	100	—	0	0
Deoxyguanosine	< 1,000 (Ehrlich)[16]	1,000	0	0	0
Cytosine arabinoside	0·1 (L5178Y)[17]	100	—	0	0
Hydroxyurea	66[18]	1,320	—	0	0
Hydroxyurethan		1,320	—	0	0
Actinomycin D	0·0056[14]	7·8	—	—	0
Puromycin	0·11[14]	210	—	—	—
Cycloheximide	0·35 (KB)[19]	35	—	0	—
Acetoxycycloheximide	0·15 (HEp 1)‡	29	—	0	—
2, 4-Dinitrophenol	5·4–54 (KB)[20]	3,000	0	0	—
Streptonigrin	0·04 (cytotoxic)[21]	4	—	—	—
6-Mercaptopurine	2·4[14]	66	0	0	0
6-Diazo-5-oxo-L-norleucine	73 (cytotoxic)[22]	2,920	0	0	0
6-Azauridine	2·4 (KB)[19]	410	0	—	0
Triethylene melamine	1·0[14]	100	0	0	0
Mitomycin C	0·75 (KB)[19]	30	0	0	0
Vinblastin	1·3 × 10^{-6} (KB)[19]	12·7	0	0	0
Urethan	113–1,130 (KB)[20]	1,320	0	0	0

* Incorporation was measured after 30-min incubation; drugs and labeled compounds were present at zero time. Code: + = enhancement ($\bar{R} > 1{\cdot}5$); — = inhibition ($\bar{R} < 0{\cdot}6$); 0 = no significant effect ($0{\cdot}8 < \bar{R} < 1{\cdot}2$) where $\bar{R}$ is the mean of ($^3H/^{14}C$ experimental)/($^3H/^{14}C$ control) in at least three experiments.

† That concentration which inhibited growth of cells in culture by 50%, HeLa unless otherwise indicated. In some instances only a "cytotoxic" concentration is reported.

‡ C. W. Young, unpublished data.

Agent effects. The effects of a number of cytotoxic compounds and normal cell metabolites are shown in Table 2. The maximal concentrations tested and the reported growth-inhibitory concentrations for HeLa or comparable tissue culture lines[13–22] are listed for purposes of comparison.

Effects of compounds that interfere with synthesis of deoxyribonucleotides were readily detected in this system. IUdR inhibited incorporation of thymidine. This inhibitory effect has been extensively studied; IUdR and/or its phosphorylated derivatives decrease utilization of thymidine by competition for thymidine kinase, thymidylate kinase, and DNA polymerase. The specific site of inhibition varied with different cell types.[23] IUdMP is also incorporated into DNA in place of thymidylate.[24] Amethopterin, FUR, and FUdR enhanced incorporation of thymidine (Fig. 2). The FUdR

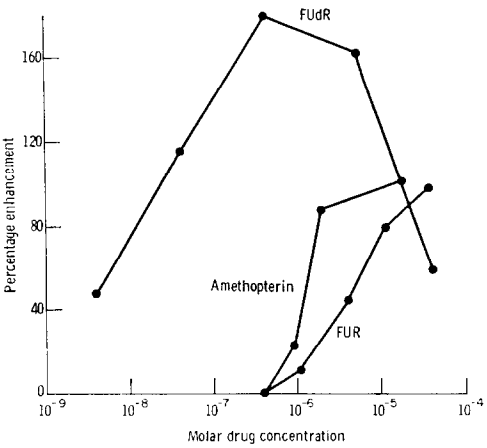

FIG. 2. Enhancement of incorporation of ^{3}H-thymidine into monolayers of HeLa cells by FUR, FUdR, and amethopterin. Per cent enhancement = ($^{3}H/^{14}C$ experimental)/($^{3}H/^{14}C$ control) − 1 × 100. Drugs and ^{3}H-thymidine were present at zero time; the incorporation period was 30 min.

effect was detectable at the concentration that inhibits cell growth by 50%. Other workers have observed enhanced cellular incorporation of thymidine in the presence of fluorinated pyrimidines.[25, 26] Presumably such agents lower cellular concentrations of nonradioactive deoxythymidylate (dTMP) and deoxythymidylate triphosphate (dTTP). Since dTTP exerts an inhibitory effect on thymidine kinase[2,7] a lowering of dTTP levels may enhance exogeneous thymidine uptake. Decreased isotope dilution from *de novo* formation and enhanced thymidine kinase activity would lead to an increased specific activity of DNA formed in the presence of labeled thymidine.

Deoxyadenosine (10^{-4} M) and cytosine arabinoside (10^{-6} M) inhibited incorporation of thymidine into DNA, whereas deoxyguanosine (10^{-3} M) had no significant inhibitory effect. These results are in agreement with the observations of Klenow[28] and Prusoff[29]. Anabolites of deoxyadenosine, deoxyguanosine, and cytosine arabinoside are reported to inhibit the reduction of ribonucleotides to deoxyribonucleotides.[17, 30–32] Inhibition of this reduction step would cause depletion of one or more of the deoxyribonucleotide triphosphates required for the synthesis of DNA. Hydroxyurea and hydroxyurethan inhibited incorporation of thymidine but left incorporation

of uridine and leucine unaffected. The inhibitory patterns of these two structurally related compounds were quite similar to those obtained with cytosine arabinoside and deoxyadenosine. More detailed study has suggested that hydroxyurea may interfere with deoxyribonucleotide formation from ribonucleotides.[33]

Predictably, actinomycin D inhibited incorporation of both thymidine and uridine. The concentration–effect relationships for effects on the synthesis of RNA and DNA (Fig. 3) are similar to those reported by other workers.[34–36]

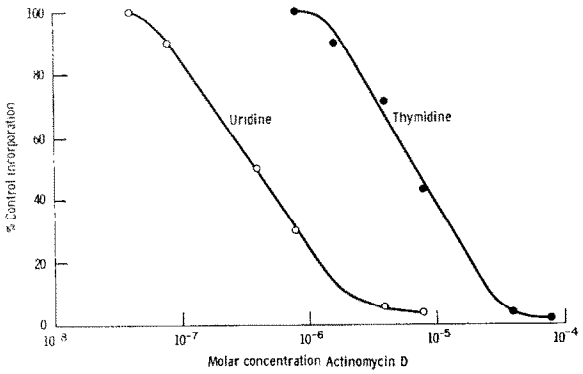

FIG. 3. Inhibition of incorporation of ^{3}H-thymidine and ^{3}H-uridine into monolayers of the HeLa cells by actinomycin D. Per cent control incorporation = (^{3}H/^{14}C experimental)/(^{3}H/^{14}C control) × 100. Drug, labeled precursor, and time sequence as in Fig. 2.

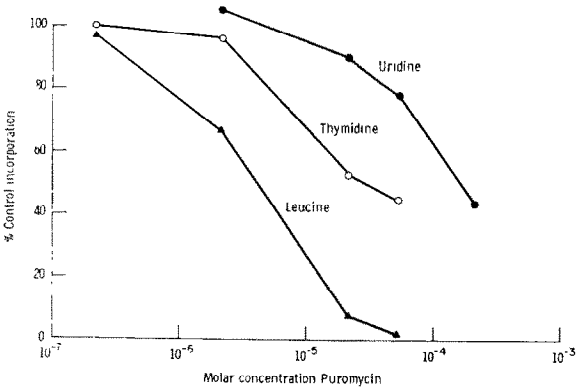

FIG. 4. Effects of puromycin on ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine incorporation into monolayers of HeLa cells. Drug, labeled precursor, and time sequence as in Fig. 2.

Puromycin, cycloheximide, and acetoxycycloheximide are known to be inhibitors of protein synthesis.[37, 38] They had acute inhibitory effects on cellular incorporation of precursors of both protein and nucleic acid (Fig. 4, 5). These results are consistent with the observations of other workers.[11, 39, 40] It is interesting that 2, 4-dinitrophenol inhibited incorporation of leucine but did not alter incorporation of thymidine and uridine (Table 2).

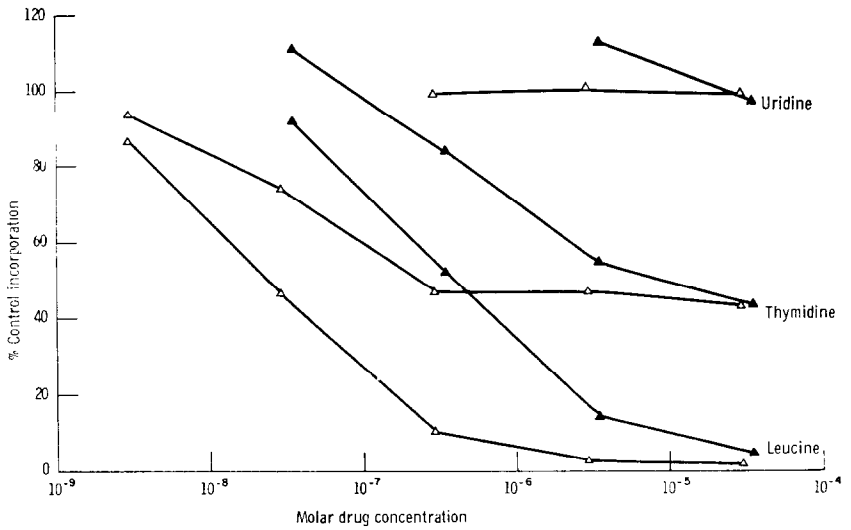

FIG. 5. Effects of acetoxycycloheximide (△——△) and cycloheximide (▲——▲) on ^{3}H-uridine, ^{3}H-thymidine, and ^{3}H-leucine incorporation into monolayers of HeLa cells. Drug, labeled precursor and time sequence as in Fig. 2.

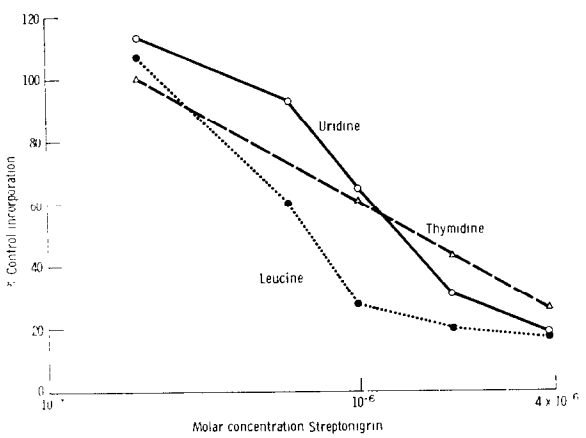

FIG. 6. Effects of streptonigrin on ^{3}H-uridine, ^{3}H-thymidine and ^{3}H-leucine incorporation into monolayers of HeLa cells. Drug, labeled precursor, and time sequence as in Fig. 2.

Streptonigrin, an antibiotic of known structure but with an unknown mechanism of action,[41] inhibited incorporation of all three precursors to an equal degree (Fig. 6).

Three inhibitors of *de novo* synthesis of purines were tested. Amethopterin enhanced incorporation of exogenous thymidine but had no effect on the incorporation of uridine; 6-MP and DON had no effect upon incorporation of any precursor. In view of these observations it is probable that this technique does not detect a block of *de novo* purine synthesis.

AZUR decreases synthesis of pyrimidines *de novo* through the inhibitory effect of azauridylic acid (AZUMP) upon the enzyme orotidylic decarboxylase.[42, 43] Pasternak and Handschumacher observed that incorporation of uridine into nucleic acids of mouse liver, intestine, and tumor was not inhibited by doses of AZUR that markedly depressed incorporation of orotic acid.[42] In the currect studies very high concentrations of AZUR inhibited cellular incorporation of uridine, but inhibitory effects were not significant at lower concentrations (4×10^{-5} M) which are still inhibitory to growth of cells *in vitro*. Since uridine kinase is known to phosphorylate AZUR in addition to uridine,[44] it is somewhat surprising that such high concentrations were required for unequivocal inhibition of incorporation of uridine. Subcellular data suggest that uridine enjoys at least a slight advantage over AZUR as a substrate for the kinase.[42] In addition, AZUR may induce an alteration in feedback suppression of synthesis of pyrimidine nucleotides. Inhibition of orotidylic decarboxylase induced by AZUMP decreases intracellular UTP and CTP concentrations. Since UTP and CTP are reported to be effective inhibitors of the uridine kinase reaction,[45] a decrease in their intracellular concentration would release the enzyme from normally existing feedback suppression. This release may partially offset the inhibitory effect of competition between AZUR and uridine as substrate for the enzyme. FUR also inhibited incorporation of uridine; the concentration that induced 50% inhibition was $3{\cdot}8 \times 10^{-5}$ M. Since inhibition of growth is seen at a FUR concentration of 3×10^{-9} M, the recorded effect on incorporation of uridine is probably of little significance.

Alkylating agents (TEM, mitomycin C), a plant alkaloid (vinblastin) and urethan had no significant effects at the concentrations tested (Table 2). Cytotoxic alkylating agents inhibit incorporation of precursors into cellular protein and nucleic acids when these drugs are present in very high concentrations or when exposure to the agent occurs some hours before the labeled precursor is given. The inactivity of TEM and mitomycin C in the current studies may have been related to the concentrations used and the brief period of incorporation employed. Creasey and Markiw have reported on effects of vinblastin on sarcoma 180 cells *in vitro*.[46] In their studies vinblastin did not alter incorporation of uridine into total RNA; incorporation of thymidine was inhibited at concentrations of vinblastin that were six to sixty times greater than the maximum employed by us. Where their experimental conditions were comparable to those employed in this study the results are in agreement.

It is often difficult to delineate the sequence in which metabolic changes occur after cellular exposure to a toxic agent. In the studies described above we have approached this problem by limiting the period of observation to the initial 30 min of drug exposure. By testing the study system with a number of extensively investigated agents we have attempted to learn which type of toxic effect would be detectable acutely, and which type would not. Effects were seen in tests with cytotoxic agents that are known to produce the following: inhibition of synthesis of deoxythymidylate from deoxyuridylate, inhibition of deoxyribonucleotide formation from ribonucleotides, inhibition of DNA-primed synthesis of RNA, and inhibition of synthesis of protein. Drugs with inhibitory effects limited to steps in *de novo* synthesis of purines were inactive in this system. It seems probable, therefore, that the existing intracellular purine nucleotide pool is large enough to permit synthesis of nucleic acids at a normal rate for at least 30 min after inhibition of the *de novo* pathway. Theoretically, inhibition of

de novo synthesis of pyrimidines might be manifested acutely by enhanced incorporation of uridine. This effect was not demonstrated in studies with AZUR, perhaps because of competition for uridine kinase between labeled uridine and AZUR. This sequential isotope technique serves as a rapid metabolic screen that can detect inhibitory effects of some cytotoxic agents. It may prove useful in studies of mechanisms of drug action and in studies of structure–activity relationships.

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