Biochemical Pharmacology, Vol. 21, pp. 1977–1988. Pergamon Press, 1972. Printed in Great Britain.

ANTINEOPLASTIC DRUG ACTIVITY IN THE MITOTIC CYCLE—EFFECTS OF SIX AGENTS ON MACROMOLECULAR SYNTHESIS IN SYNCHRONOUS MAMMALIAN LEUKEMIC CELLS*

H. BRUCE BOSMANN†

Department of Pharmacology and Toxicology, University of Rochester School of Medicine and Dentistry, Rochester, N.Y. 14642, U.S.A.

(*Received* 14 *December* 1971; *accepted* 4 *February* 1972)

Abstract—The direct effects of six antineoplastic agents on DNA, RNA, protein and glycoprotein synthesis were measured in synchronized populations of mouse leukemic cells L5178Y. L5178Y cells were synchronized by excess thymidine treatment (S block) followed by colcemid treatment (M block). After release from the M block, aliquots of synchronized cells were exposed to the antineoplastic agents at two concentrations, and the drug effects on macromolecular synthesis were measured. Camptothecin was mitotic cycle specific, the most sensitive portion of the mitotic cycle being the S phase; DNA and RNA synthesis was inhibited maximally in the S period of the L5178Y mitotic cycle. Ethidium bromide inhibited macromolecular synthesis throughout the mitotic cycle with primary inhibition occurring at early time periods after mitosis. Hydroxyurea was cell cycle specific with primary effects in the G_1 period and the early and mid S periods of the mitotic cycle. Azaleucine inhibited DNA synthesis primarily in the early to mid S time period, RNA synthesis throughout the mitotic cycle, and protein synthesis in time periods exclusive of the G_1 period. D-Glucosamine inhibition of macromolecular synthesis occurred primarily in the G_1 and early S time periods. L-Asparaginase inhibited protein and glycoprotein synthesis throughout the mitotic cycle; primary inhibition occurred in the S time period of the L5178Y mitotic cycle.

MANY DRUGS are mitotic cycle specific, that is, they exert their action primarily at a distinct period in the cell mitotic cycle. This is particularly true with drugs affecting DNA synthesis, since, by definition, in mammalian cells DNA synthesis occurs at a discrete time in the cell cycle—the S phase. Recently, this laboratory has completed studies on the molecular toxicity of six antineoplastic agents: camptothecin,[1,2] L-asparaginase,[3] hydroxyurea,[4] D-glucosamine,[5] azaleucine[6] and ethidium bromide.[7] Because each of these drugs is a potent inhibitor of the synthesis of macromolecules in mammalian cells,[1–7] it seemed of interest to determine whether inhibition of macromolecular synthesis occurred throughout the cell cycle or whether inhibition was limited to certain specific periods in the cell cycle. The importance of such work is illustrated by reference to recent work in which indirect methods were utilized to show that camptothecin not only inhibited DNA synthesis but also caused a G_2 "lesion"[8] and hydroxyurea primarily caused cell death in the S phase of mouse spleen cell cultures immunized with sheep red cells.[9] The work should also be important for chemotherapeutic reasons; i.e., knowledge of the mitotic cycle specificity of antineoplastic drugs should be of value

* Supported in part by Grant P529 from the American Cancer Society, and Grants CA13220 and GM15190 from the National Institute of Health.

† Research Career Development Awardee from the National Institute of General Medical Sciences.

in administration of these drugs in the treatment of neoplasia. It should be noted, however, that periods of maximum inhibition of a given macromolecule by a given drug may or may not correspond to the period of maximum lethality of that drug. Indeed the period of maximum lethality of the drug may be the result of inhibitions of macromolecular synthesis occurring many hours previous to the actual time of "cell death". Furthermore, the failure of a cell to proceed through mitosis due to lack of a critical macromolecule can be important in chemotherapy even though cell death does not occur. However, since in treatment many non-neoplastic populations would be in the G_0 or resting state whereas the neoplastic population would be rapidly dividing, the selective toxicity of these agents might be able to be exploited, if their activity in the mitotic cycle were known so that correct dosing regimens could be worked out.

The present study utilizes direct methods to determine when in the mitotic cycle each of the six antineoplastic drugs inhibits mammalian macromolecular synthesis. The procedure utilized was to synchronize mouse leukemic cell L5178Y and at various times in the mitotic cycle to expose an aliquot of the synchronous population to a pulse of the given drug and measure macromolecular synthesis in this interval. For each drug two concentrations were utilized. The first concentration was a low drug concentration which inhibited 10–20 per cent of macromolecular synthesis; at this concentration any inhibition of macromolecular synthesis that occurred would be expected to occur at the most sensitive portion of the cell cycle. The other concentration of drug utilized was a very high concentration which inhibited macromolecular synthesis (when applicable) by 80–90 per cent. At this drug concentration any synthesis that occurred would be expected to occur at the least sensitive portion of the cell cycle. Because it was deemed important to monitor small differences between the control and drug-treated cells, the experiments were repeated 6–11 times to ensure low standard errors of the means.

MATERIALS AND METHODS

Cell culture. L5178Y cells (mouse lymphoma cell line) were grown in suspension culture in sealed containers in Fischer's medium[10] plus 10% horse serum and were used in the exponential growth phase. Fischer's medium in dry form and horse serum were supplied by Grand Island Biological Co., Buffalo, N.Y.; to the supplied medium, penicillin (500 units/ml) and streptomycin (0·05 mg/ml) were added. Cell numbers were determined by counting in a Coulter counter, research model B.

Synchronization of cells. Cells were synchronized by the method of Doida and Okada,[11] by applying one treatment with excess thymidine followed by one treatment with colcemid and deoxycytidine as previously described.[12–15] Details of generation time, protein content, and synchrony of L5178Y cells are described in previous publications.[12–15] Per cent of synchrony determined by per cent labeled cells,[11] cell number, or cell volume as measured with the Coulter volume plotter for 12 experiments was 99, 99, 95, 93, 89, 89, 89, 89, 89, 88, 39 and 27 per cent. Data from the latter two experiments were discarded. These data are indicative of this laboratory's experience with the synchrony method, i.e., for reasons unknown, in between 10 and 20 per cent of the synchrony attempts synchrony is not obtained. This observation is also

made in the original paper.[11] For all experiments reported herein, the per cent of synchrony was 85 or higher.

Experimental design. L5178Y cells were synchronized and, after the colcemid and deoxycytidine were removed, the cultures were brought up to a final volume of 400 ml. At 1-hr intervals after release from the colcemid block, 30 ml (10^6 cells) of the synchronous cell suspension was removed and the amount of drug necessary to give the desired final concentration was added (controls were always performed simultaneously with no added drug). The suspension was then incubated for 30 min at 37° with 5·0 μc/ml of Fischer's medium with [^{3}H]leucine, [^{3}H]glucosamine (or [^{3}H]fucose), [^{3}H]uridine or [^{3}H]thymidine. After the incubation, macromolecular-bound radioactivity was determined as given below.

Determination of macromolecular-bound radioactivity. After the incubation, the labeled cells were centrifuged and washed twice in 0·9% NaCl and the cellular pellet was precipitated with 10% trichloroacetic acid. Two more washes with 10% trichloroacetic acid were followed by an ethanol–diethyl ether wash (2:1, v/v) and the precipitate was resuspended in 0·4 ml of 1 N NaOH; 0·2 ml was used for protein determinations and 0·2 ml was plated on a glass fiber filter disc for counting in a 2,5-diphenyloxazole (PPO)–1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP)–toluene counting fluid in a Nuclear Chicago liquid scintillation counter. The data are expressed as counts per min per milligram of protein.

Radioactively labeled compounds. L-[^{3}H]leucine (specific activity, 25 c/m-mole), [^{3}H]glucosamine (10 c/mole), [^{3}H]fucose (10 c/mole), [methyl-^{3}H]thymidine (15 c/m-mole), and uridine-5-^{3}H (10 c/m-mole) were all purchased from New England Nuclear. Each compound was utilized at this specific activity in all experiments herein. Experiments indicated that utilizing the radioactively labeled compounds at these specific activities and in the amounts given above, linear incorporation into trichloroacetic acid-insoluble material occurred for up to 1 hr. Experiments utilizing DNase and RNase indicated, under the experimental conditions given above, that 96 per cent of the uridine-5-^{3}H product was RNA.

Drugs. Camptothecin and L-asparaginase (360 U/mg) were kindly supplied by Drug Development Branch, National Cancer Institute. Azaserine, D-glucosamine, hydroxyurea, and ethidium bromide were purchased from Sigma Chemical Co, St. Louis, Mo.

Protein. Protein determinations were made by the procedure of Lowry *et al.*[16] Samples were prepared by precipitation with 1% phosphotungstic acid in 0·5 N HCl, washing three times with 5% trichloroacetic acid, and washing once with diethyl ether–ethanol (1:2, v/v). The resulting residue was dissolved in 1 ml of 0·2 N NaOH and analyzed. Crystalline bovine serum albumin was used as standard.

RESULTS

Camptothecin. Camptothecin is a plant alkaloid originally isolated from the oriental plant *Camptotheca acuminata*[17] which has a potent antineoplastic action.[1,2,18–20] The antibiotic is thought to act principally by inhibiting synthesis of ribosomal precursor RNA[21] and DNA,[2,20] and this inhibition is in some respects reversible.[2,20,21] Gallo *et al.*[8] have observed that camptothecin acts as a G_2 blocking agent and Kessel *et al.*[2] have demonstrated that camptothecin primarily inhibits cell division by acting in the S phase. Recently Horowitz and Horowitz[22] have shown that camptothecin

affects DNA degradation. The present work records the direct effects of camptothecin on macromolecular synthesis in synchronized L5178Y cells. At 1 mg/ml of camptothecin, DNA synthesis in asynchronous L5178Y cells was inhibited 50 per cent, RNA synthesis was inhibited 75 per cent, protein synthesis 25 per cent and glycoprotein synthesis 0 per cent.[1] At 1 μg/ml, camptothecin inhibited asynchronous L5178Y cell DNA synthesis 20 per cent, RNA synthesis 10 per cent, protein synthesis 0 per cent and glycoprotein synthesis 0 per cent.[1] These were the two dose levels of camptothecin chosen for the present work on mitotic cycle effects of camptothecin.

DNA synthesis. At 1mg camptothecin/ml, DNA synthesis was inhibited principally in the S period, with maximum inhibition occurring 5 hr post-mitosis (Fig. 1). At 1 μg/ml, camptothecin inhibited DNA synthesis at 3–6 hr post-mitosis; there was no inhibition at other time periods.

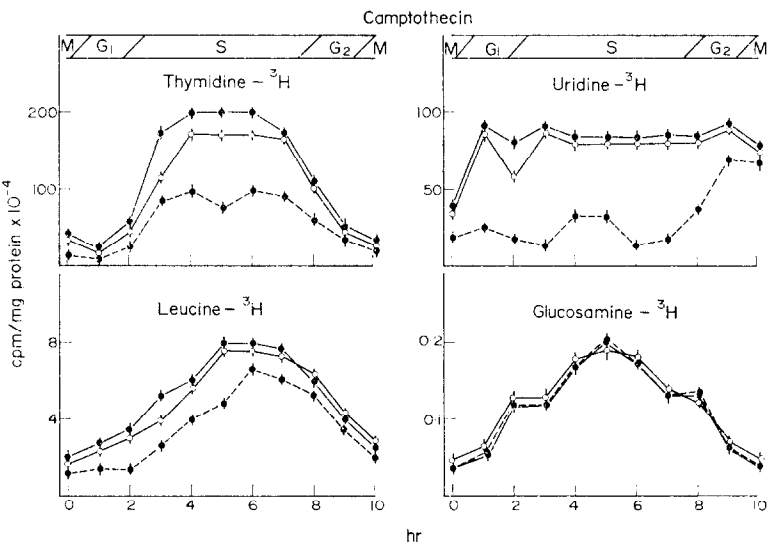

FIG. 1. Effect of camptothecin on macromolecular synthesis in a synchronous population of L5178Y cells. Experiments were performed as given in Materials and Methods. At zero hour, cells were released from the "M block". A representation of the cell cycle is given above the panels. Means ± 1 S. D. from 6–11 separate determinations. ●——●, Control; ●- - -●, 1 mg/ml of camptothecin; and ○——○, 1 μg/ml of camptothecin. The drug was present for 30 min at each interval.

RNA synthesis. RNA synthesis was inhibited throughout the mitotic cycle at 1 mg camptothecin/ml, periods 0, 9 and 10 hr post-mitosis being the least affected. At 1 mg camptothecin/ml, RNA synthesis was inhibited maximally at 3 and 6 hr post-mitosis. At the low dose of camptothecin of 1 μg/ml, RNA synthesis was inhibited only at the G_1–S interface, at 2 hr post-mitosis. RNA synthesis was thus most sensitive to camptothecin in the S period, particularly in the early and late S phases.

Protein synthesis. Camptothecin does not inhibit protein synthesis except at high concentrations.[1] At 1 mg/ml of camptothecin, protein synthesis was inhibited at 1–7 hr post-mitosis; maximum inhibition occurred at 2 and 5 hr post-mitosis.

Glycoprotein synthesis. Camptothecin at either dose level had no effect on glycoprotein synthesis, as shown in Fig. 1.

Implications. Camptothecin in the present work is mitotic cycle specific with its principal action in the S period of the synchronized L5178Y cells. This is consistent with the postulated modes of action of the alkaloid on nucleic acid polymerases,[2] DNA degradation[22] or ribosomal precursor RNA synthesis.[21] It should be noted that the preponderance of nucleic acid synthesis occurring with the high level of camptothecin present may be mitochondrial nucleic acid synthesis,[15] since the antibiotic does not seem to inhibit mitochondrial or bacterial macromolecular synthesis.[1] The fact that nucleic acid synthesis is inhibited primarily in the S phase is in agreement with the fact that camptothecin causes an S or G_2 block;[2,8] i.e., cells in exponential growth would not be expected to pass through G_2, since considerable inhibition of RNA and DNA occurs in S.

L-*Asparaginase.* L-Asparaginase (L-asparagine aminohydrolase; EC 3.5.1.1), which hydrolyzes the amide group of asparagine, is valuable clinically in the treatment of certain acute leukemias, principally by adversely affecting leukemic cell nutrition.[23] Recently it has been demonstrated[1,24,25] that asparaginase inhibited glycoprotein synthesis in L5178Y mouse leukemic cells and in isolated mitochondria. At 1 U/ml of L-asparaginase in asynchronous L5178Y cells, DNA and RNA synthesis was unaffected by the drug, while protein synthesis was inhibited 55 per cent and glycoprotein synthesis 60 per cent.[24] At 0·01 U/ml of L-asparaginase in asynchronous L5178Y cells, DNA and RNA synthesis was unaffected by the drug, whereas protein synthesis was inhibited 30 per cent and glycoprotein synthesis was inhibited 30 per cent. These were the two doses of L-asparaginase utilized in the cell-synchrony experiments described here (Fig. 2).

DNA synthesis. Under the conditions employed in these experiments there was no effect of L-asparaginase on DNA synthesis in the synchronous L5178Y cell.

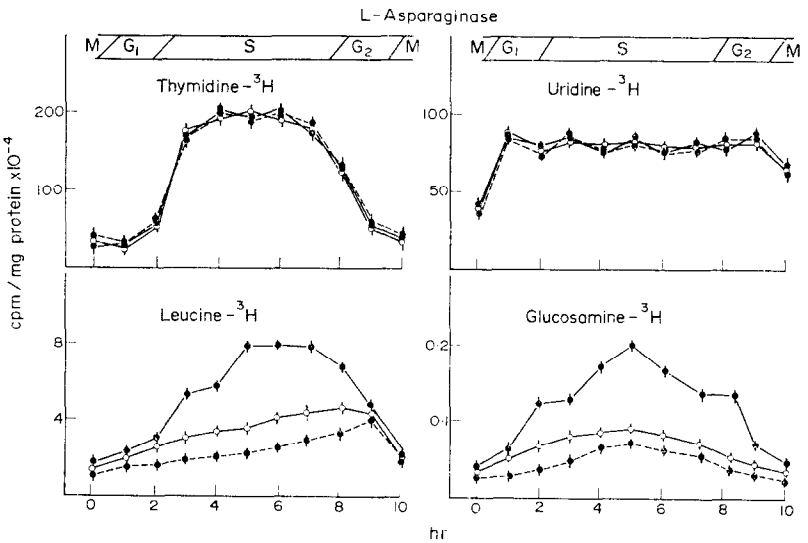

FIG. 2. Effect of L-asparaginase on macromolecular synthesis in a synchronous population of L5178Y cells. Experiments were performed as given in Materials and Methods. At zero hour, cells were released from the "M block". A representation of the cell cycle is given above the panels. Means ±1 S. D. from 6–11 separate determinations. ●——●, Control; ●- - -●, 1 U/ml of L-asparaginase; and ○——○, 0·01 U/ml of L-asparaginase. The drug was present for 30 min at each interval.

RNA synthesis. Under the conditions employed in these experiments there was no effect of L-asparaginase on RNA synthesis in the synchronous L5178Y cell.

Protein synthesis. At 1 U of L-asparaginase/ml, protein synthesis was inhibited primarily in the G_1 and S periods; maximal inhibition occurred in mid S at 5 hr post-mitosis. Little inhibition of protein synthesis by L-asparaginase at 1 U/ml occurred in late G_2 or M. At 0·01 U/ml, L-asparaginase inhibited protein synthesis primarily in the S period; maximal inhibition occurred 5 hr post-mitosis. From the data, it can be concluded that the major inhibitory effect of L-asparaginase on protein synthesis occurs in the S period of the cell cycle.

Glycoprotein synthesis. The 1 U/ml dose of L-asparaginase inhibited glycoprotein synthesis throughout the cell cycle with primary inhibition in the S phase. Similarly the 0·01 U/ml dose of L-asparaginase inhibited glycoprotein synthesis throughout the cell cycle, except that essentially no inhibition occurred in M or early G_1 (1 hr post-mitosis).

Implications. The data indicate that the cell is sensitive to L-asparaginase throughout the cell cycle with primary senstivity in the S period when protein and glycoprotein synthesis is maximal.[13] L-Asparaginase inhibits protein synthesis by deprivation of L-asparagine for synthesis of proteins containing asparagine and inhibits glycoprotein synthesis by elimination of the amide nitrogen of asparagine necessary for the asparagine: *N*-acetylglucosamine linkage which initiates many carbohydrate sequences of glycoproteins.[3] The present work suggests that such synthesis occurs throughout the cell cycle, and thus the L-asparagine is required for the cell during the entire cell cycle, and that the requirement is greatest in the S period of the cell cycle.

Ethidium bromide. The trypanocidal drug ethidium bromide (3,8-diamino-6-phenyl-5-ethylphenanthridinium bromide) has been shown to inhibit growth and nucleic acid synthesis in HeLa cells[26] and Ehrlich ascites carcinoma cells.[27] In asynchronous mouse leukemic cell L5178Y,[7] 500 μM ethidium bromide inhibits RNA, DNA and protein synthesis 95 per cent; glycoprotein synthesis is inhibited 50 per cent. Similarly,[7] at 100 μM ethidium bromide, RNA synthesis is inhibited 30 per cent, protein synthesis 5 per cent, DNA synthesis 5 per cent and glycoprotein synthesis 5 per cent. These were the levels of drug utilized to determine the ethidium bromide sensitivity of macromolecular synthesis in synchronous L5178Y cells, as shown in Fig. 3.

DNA synthesis. DNA synthesis was severely inhibited by 500 μM ethidium bromide; the only periods in which total inhibition of DNA synthesis did not occur were 6, 7, 8 and 10 hr post-mitosis. At 100 μM ethidium bromide, the only significantly sensitive period of ethidium inhibition of DNA synthesis was 3 hr post-mitosis. It can be concluded that DNA synthesis is very sensitive to inhibition by ethidium bromide and that the early periods after mitosis, i.e. G_1, early and mid S, are the most sensitive.

RNA synthesis. At 500 μM ethidium bromide, RNA synthesis was also severely inhibited; except at 6 and 7 hr post-mitosis, total inhibition of RNA synthesis occurred. At 100 μM ethidium bromide, statistically significant inhibition occurs only at 0, 1, 2 and 3 hr post-mitosis; hr 1 was most affected. It can be concluded that RNA synthesis, like DNA synthesis (see above), is inhibited by ethidium bromide, primarily in the early periods after mitosis in G_1 and early S.

Protein synthesis. The synthesis of protein was inhibited completely by 500 μM ethidium bromide, except at 6 and 7 hr post-mitosis. The only time period in which

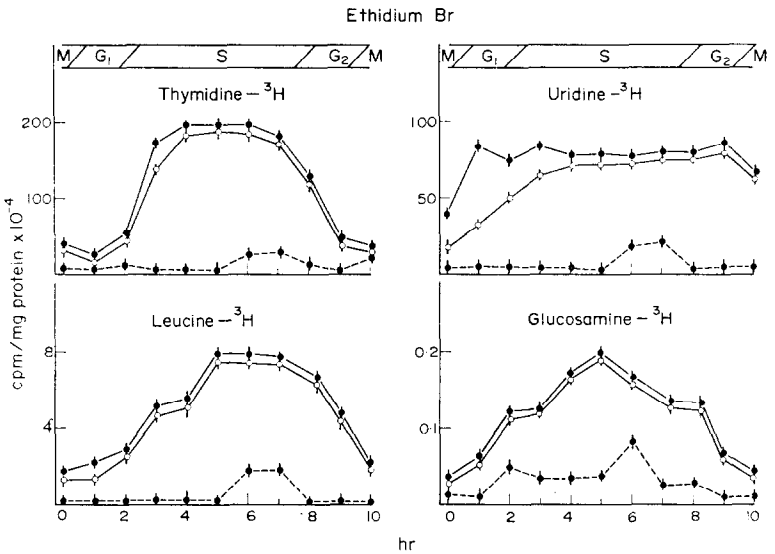

FIG. 3. Effect of ethidium bromide on macromolecular synthesis in a synchronous population of L5178Y cells. Experiments were performed as given in Materials and Methods. At zero hour, cells were released from the "M block". A representation of the cell cycle is given above the panels. Means ±1 S. D. from 6–11 separate determinations. ●——●, Control; ●– – –●, 500 μM ethidium; and ○——○, 100 μM ethidium. The drug was present for 30 min at each interval.

inhibition of protein synthesis occurred at 100 μM ethidium bromide was 1 hr post-mitosis. The inhibition of protein synthesis follows closely the inhibition of RNA synthesis and the former inhibition may be the result of the latter.

Glycoprotein synthesis. At 500 μM ethidium bromide, glycoprotein synthesis was inhibited on a percentage basis essentially the same amount throughout the cell cycle; the largest amount of synthesis occurred 6 hr post-mitosis in the presence of 500 μM ethidium bromide. At 100 μM ethidium bromide, essentially no significant inhibition of glycoprotein synthesis occurred.

Implications. The potent inhibitory effects of ethidium bromide at high concentrations seem to manifest themselves throughout the cell cycle, with the early time periods being most sensitive to the drug. Ethidium bromide intercalates between DNA bases[28] and this is thought to inhibit DNA-dependent RNA synthesis and RNA-dependent protein synthesis through this primary mechanism. The present data are consistent with this mode of action; that is, DNA, RNA and protein synthesis are inhibited in essentially the same time periods with essentially similar degrees of severity. The data show that inhibition of glycoprotein synthesis probably occurs by an unrelated mechanism.

Hydroxyurea. Hydroxyurea, which has antileukemic activity in mice[29] and humans,[30] but is not as active against solid tumors,[31] is a potent inhibitor of DNA synthesis.[32,33] At 2 mg hydroxyurea/ml, DNA synthesis in asynchronous L5178Y cells is inhibited 95 per cent, RNA synthesis 30 per cent, protein synthesis 60 per cent and glycoprotein synthesis 50 per cent.[4] At 20 μg hydroxyurea/ml, DNA synthesis in asynchronous L5178Y cells was inhibited 55 per cent, RNA synthesis 5 per cent,

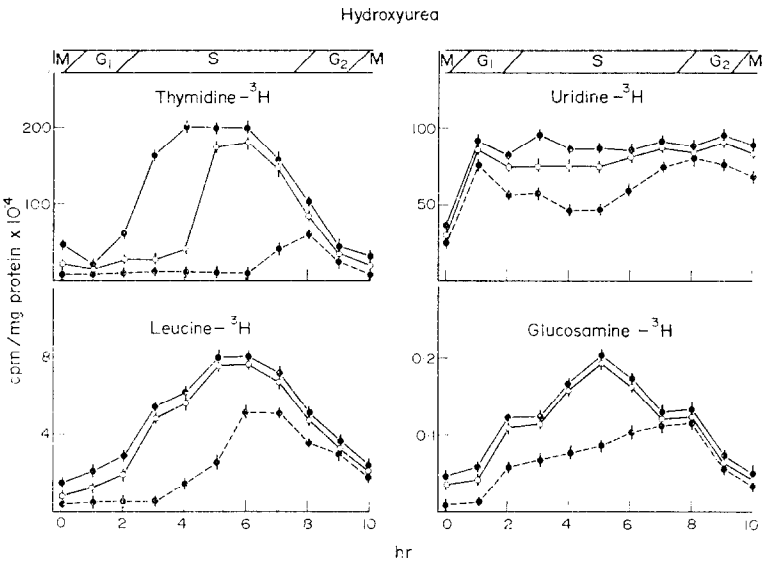

FIG. 4. Effect of hydroxyurea on macromolecular synthesis in a synchronous population of L5178Y cells. Experiments were performed as given in Materials and Methods. At zero hour, cells were released from the "M block". A representation of the cell cycle is given above the panels. Means ±1 S. D. from 6–11 separate determinations. ●——●, Control; ●– –●, 2 mg hydroxyurea/ml; and ○——○, 20 μg hydroxyurea/ml. The drug was present for 30 min at each interval.

protein synthesis 5 per cent and glycoprotein synthesis 5 per cent.[4] These were the hydroxyurea concentrations utilized in the present experiments (Fig. 4).

DNA synthesis. DNA synthesis was inhibited essentially 100 per cent at all periods except 7, 8 and 9 hr post-mitosis in the presence of 2 mg/ml of hydroxyurea. At 20 μg hydroxyurea/ml, DNA synthesis was severely inhibited at 2, 3 and 4 hr post-mitosis; little inhibition occurred at 6–10 hr post-mitosis. It can therefore be concluded that hydroxyurea has its maximal inhibitory effect in the late G_1 and early to mid S periods. The species of DNA being synthesized in the mid to late S periods (6–8 hr post-mitosis) must not be hydroxyurea sensitive. Such insensitivity to hydroxyurea could occur by changes in the plasma or nuclear membrane permeability to the drug.

RNA synthesis. RNA synthesis was inhibited primarily in the late G_1 and early and mid S in the presence of 2 mg/ml of hydroxyurea. At 20 μg hydroxyurea/ml, RNA synthesis was inhibited only in S at 3, 4 and 5 hr post-mitosis. It is evident that RNA synthesis is inhibited primarily in the late G_1 and early and mid S, perhaps as a consequence of the inhibition of DNA synthesis.

Protein synthesis. In the presence of 2 mg hydroxyurea/ml, protein synthesis was inhibited maximally at 0–6 hr post-mitosis; relatively little inhibition occurred in late S or G_2. Little inhibition of protein synthesis occurred with 2 μg hydroxyurea/ml; only slight inhibition occurred at 0–3 hr post-mitosis. Therefore protein synthesis is inhibited by hydroxyurea primarily in G_1 and early and mid S in the L5178Y cells.

Glycoprotein synthesis. Glycoprotein synthesis was inhibited at 0–6 hr post-mitosis in the presence of 2 mg/ml of hydroxyurea. In the presence of 20 μg/ml of hydroxyurea, essentially no inhibition of glycoprotein synthesis occurred.

Implications. The present results demonstrate conclusively: (1) that hydroxyurea is cell cycle specific with its primary effects in G_1 and early and mid S, and (2) that hydroxyurea may inhibit macromolecular synthesis in the sequence DNA → RNA → protein → glycoprotein with the primary effect on DNA synthesis.

Azaleucine. Azaleucine and azaserine have been utilized as antineoplastic agents.[6] Azaleucine at 100 mM concentration inhibited asynchronous L5178Y DNA synthesis 95 per cent, RNA synthesis 95 per cent, protein synthesis 80 per cent and had no effect on glycoprotein synthesis.[6] At 0·1 mM, azaleucine inhibited DNA synthesis 30 per cent, RNA synthesis 10 per cent, protein synthesis 5 per cent and had no effect on glycoprotein synthesis.[6] These were the concentrations of azaleucine utilized in the cell-synchrony experiments given in Fig. 5.

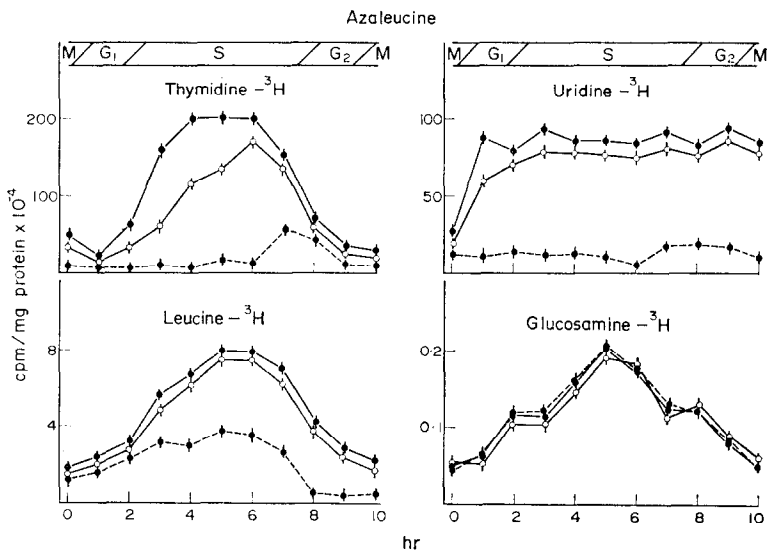

FIG. 5. Effect of azaleucine on macromolecular synthesis in a synchronous population of L5178Y cells. Experiments were performed as given in Materials and Methods. At zero hour, cells were released from the "M block". A representation of the cell cycle is given above the panels. Means ±1 S. D. from 6–11 separate determinations. ●——●, Control; ●– –●, 100 mM azaleucine; and ○——○, 0·1 mM azaleucine. The drug was present for 30 min at each interval.

DNA synthesis. At 100 mM azaleucine, DNA synthesis was inhibited essentially 100 per cent at all time periods except 7 and 8 hr post-mitosis. At 0·1 mM azaleucine, DNA synthesis was inhibited primarily at 2–6 hr post-mitosis, maximal inhibition occurring at 3 and 4 hr post-mitosis. It can be concluded that the early and mid S phase are the primary periods of azaleucine inhibition of L5178Y DNA synthesis.

RNA synthesis. Azaleucine at 100 mM inhibited RNA synthesis throughout the cell cycle with maximal inhibition occurring 6 hr post-mitosis. At 0·1 mM, azaleucine inhibited RNA synthesis to a slight degree at each of the time periods studied. Thus RNA synthesis in synchronous L5178Y cells is inhibited throughout the cell cycle by azaleucine.

Protein synthesis. At 100 mM azaleucine, protein synthesis was inhibited at 3–10 hr post-mitosis; there was no synthesis of protein occurring in the presence of azaleucine

at 8, 9 and 10 hr post-mitosis. At 0·1 mM azaleucine, there was essentially no inhibition of protein synthesis. Protein synthesis in synchronous L5178Y cells, therefore, is inhibited at periods exclusive of G_1 by azalcucine.

Glycoprotein synthesis. There was no inhibition of glycoprotein synthesis at either dose of azaleucine employed in the synchronous L5178Y cells.

Implications. The present results indicate that the effects of azaleucine on DNA synthesis are cell cycle specific, occurring in early to mid S; the effects on RNA synthesis are not cell cycle specific, occurring throughout the cycle; and the effects of protein synthesis are specific, occurring in periods exclusive of G_1. These results, therefore, indicate that the inhibition of macromolecular synthesis by azaleucine is not related to cellular membrane permeability changes.

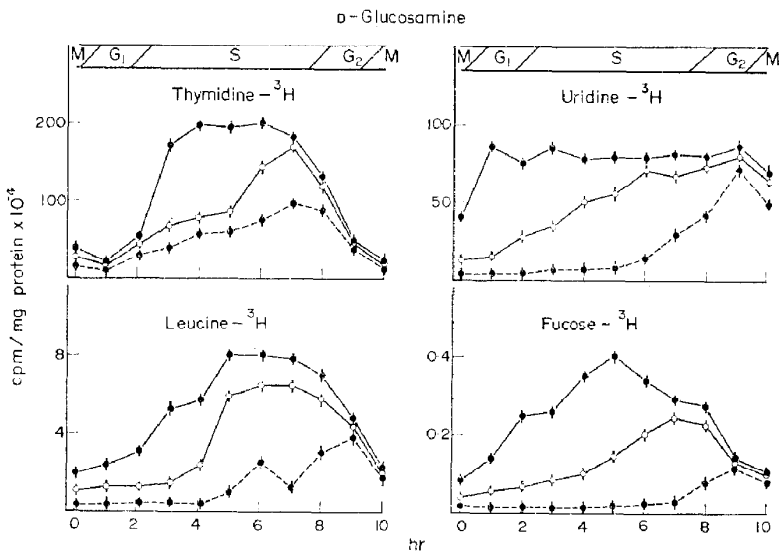

FIG. 6. Effect of D-glucosamine on macromolecular synthesis in a synchronous population of L5178Y cells. Experiments were performed as given in Materials and Methods. At zero hour, cells were released from the "M block". A representation of the cell cycle is given above the panels. Means ±1 S. D. from 6–11 separate determinations. ●——●, Control; ●——●, 0·1 M D-glucosamine; and ○——○, 0·01 M D-glucosamine.

D-Glucosamine. The amino sugar D-glucosamine is an interesting molecule with low molecular weight which seems to selectively inhibit neoplastic cell growth and macromolecular synthesis (as opposed to normal cell growth and macromolecular synthesis), possibly by depleting the neoplastic cell of endogenous nucleotide pools[34–50] At 0·1 M, D-glucosamine inhibits, in asynchronous L5178Y cells, DNA synthesis 65 per cent, RNA synthesis 80 per cent, protein synthesis 75 per cent, and glycoprotein synthesis 85 per cent.[5] At 0·01 M, D-glucosamine inhibited DNA synthesis 35 per cent, RNA synthesis 30 per cent, protein synthesis 30 per cent and glycoprotein synthesis 50 per cent.[5] These were the levels of D-glucosamine utilized in the present experiments with synchronized L5178Y cells (Fig. 6).

DNA synthesis. At 0·1 M D-glucosamine, DNA synthesis was inhibited at 2–8 hr post-mitosis; maximal inhibition occurred at 3 and 4 hr post-mitosis. With the 0·01 M

level of D-glucosamine, DNA synthesis was inhibited only at 3–6 hr post-mitosis. D-Glucosamine, therefore, inhibits DNA synthesis in L5178Y cells primarily in the early and mid S periods.

RNA synthesis. With 0·1 M D-glucosamine, RNA synthesis was severely inhibited in 0–8 hr post-mitosis; little inhibition occurred in 9 and 10 hr post-mitosis. At 0·01 M, D-glucosamine inhibited RNA synthesis during 0–6 hr post-mitosis. It can be concluded that D-glucosamine inhibits RNA synthesis primarily in G_1 and S and not in G_2.

Protein synthesis. With the 0·1 M D-glucosamine, protein synthesis was inhibited 100 per cent at 0–4 hr post-mitosis and inhibited severely at 5–8 hr post-mitosis; no inhibition occurred at 9 or 10 hr post-mitosis. At the 0·01 M level, D-glucosamine inhibited protein synthesis at 0–8 hr post-mitosis, the inhibition being greatest during the period 0–4 hr post-mitosis. Protein synthesis then is inhibited primarily in G_1 and early S and not in G_2.

Glycoprotein synthesis. At the 0·1 M level of D-glucosamine, glycoprotein synthesis was inhibited 100 per cent at 0–7 hr post-mitosis; no inhibition occurred at 9 and 10 hr post-mitosis. With the 0·1 M dose of D-glucosamine, glycoprotein synthesis was inhibited primarily at 0–6 hr post-mitosis. Glycoprotein synthesis, then, like protein synthesis, is inhibited in the L5178Y cells primarily in G_1 and S and not in G_2.

Implications. The data clearly demonstrate that D-glucosamine toxicity is primarily a G_1 and early S phenomenon and that inhibitory effects of D-glucosamine on macromolecular synthesis do not occur in G_2.

DISCUSSION

The diversity of the molecules utilized in these studies does not allow generalizations to be drawn. However, this very diversity and the observed effects seem to indicate that the early time periods of the cycle, particularly early S, are relatively sensitive to inhibition of macromolecular synthesis, whereas the late time periods, particularly G_2, are insensitive to inhibition. This may in itself reflect a cellular protective mechanism; i.e., the cell is most vulnerable to inhibition when it is "young" and less vulnerable when its membrane etc., have "matured" biochemically and it prepares for division.

Even though the L5178Y cell is "abnormal" even for a neoplastic cell because of its large nucleus and very long S period (relative to the generation time), the present data may be applicable to other neoplastic cells. Furthermore, because of the number of cells needed and the timing of the present experiments, a chemical synchrony method was employed as opposed to a mechanical or physical synchrony method even though the present method probably results in unbalanced growth. However, the results demonstrate that even if some of the effects are the result of the thymidine or colcemid utilized to synchronize the cells and the resultant unbalanced growth, the drugs in any event evidence specificity (that is, for each drug on each type of macromolecular synthesis, a discrete pattern was obtained). Finally, it should be noted that the present results demonstrate where in the mitotic cycle each of the neoplastic drugs inhibits DNA, RNA, protein and glycoprotein synthesis. Further work will have to be performed to determine the mechanisms of such inhibitions.

Acknowledgements—I thank Mr. Kenneth R. Case, Mrs. Gerilyn Z. Pike and Miss Melinda B. Shea for valuable technical assistance.

REFERENCES

1. H. B. BOSMANN, *Biochem. biophys. Res. Commun.* **41,** 1412 (1970).
2. D. KESSEL, H. B. BOSMANN and F. LOHR, *Biochim. biophys. Acta.* **269,** 210 (1972).
3. H. B. BOSMANN and D. KESSEL, *Nature, Lond.* **226,** 850 (1970).
4. H. B. BOSMANN, *Chem. Biol. Interact.* **3,** 429 (1971).
5. H. B. BOSMANN, *Biochim. biophys. Acta* **241,** 74 (1971).
6. H. B. BOSMANN, *Res. Commun. Chem. Path. Pharmac.* **2,** 271 (1971).
7. H. B. BOSMANN and J. T. MACGREGOR, *Biochem. Pharmac.* **20,** 2921 (1971).
8. R. C. GALLO, J. WHANG-PENG and R. H. ADAMSON, *J. natn. Cancer Inst.* **46,** 789 (1971).
9. B. N. JAROSLOW and L. ORTIZ-ORTIZ, *Cell. Immun.* **2,** 164 (1971).
10. G. A. FISCHER and A. C. SARTORELLI, *Meth. med. Res.* **10,** 247 (1964).
11. Y. DOIDA and S. OKADA, *Expl Cell Res.* **48,** 540 (1967).
12. H. B. BOSMANN, *Biochim. biophys. Acta* **203,** 256 (1970).
13. H. B. BOSMANN and R. A. WINSTON, *J. Cell Biol.* **45,** 23 (1970).
14. H. B. BOSMANN and R. J. BERNACKI, *Expl Cell Res.* **61,** 379 (1970).
15. H. B. BOSMANN, *J. biol. Chem.* **246,** 3817 (1971).
16. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
17. M. E. WALL, M. C. WANI, C. E. COOK, K. H. PALMER, A. T. MCPHAILY and G. A. SIM, *J. Am. chem. Soc.* **88,** 3888 (1966).
18. L. G. HART, J. B. COLL and V. T. OLIVERIO, *Cancer Chemother. Rep.* **53,** 211 (1969).
19. A. M. GUARINO, L. G. HART, J. B. COLL and V. T. OLIVERIO, *Proc. Am. Ass. Cancer Res.* **10,** 33 (1969).
20. S. B. HORWITZ, C. CHANG and A. P. GROLLMAN, *Pharmacologist* **12,** 283 (1970).
21. D. KESSEL, *Biochim. biophys. Acta* **246,** 225 (1971).
22. M. S. HOROWITZ and S. B. HOROWITZ, *Biochem. biophys. Res. Commun.* **45,** 723 (1971).
23. R. H. ADAMSON and S. FABRO, *Cancer Chemother. Rep.* **52,** 617 (1968).
24. D. KESSEL and H. B. BOSMANN, *Fedn Eur. Biochem. Soc. Lett.* **10,** 85 (1970).
25. H. B. BOSMANN, *Life Sci.* **9,** 851 (1970).
26. E. ZYBLER, E. C. VESCO and S. PENMAN, *J. molec. Biol.* **44,** 195 (1969).
27. J. F. HENDERSON, *Cancer Res.* **23,** 491 (1963).
28. L. V. CRAWFORD and M. J. WARING, *J. gen. Virol.* **1,** 387 (1967).
29. L. J. LERNER, A. BIANCHI, E. YIACAS and A. BORMAN, *Cancer Res.* **26,** 82 (1966).
30. W. G. THURMAN, *Cancer Chemother. Rep.* **40,** 1 (1964).
31. L. J. LERNER, A. BIANCHI, M. CZELZKALNS and M. DEPHILLIPO, *Proc. Am. Ass. Cancer Rep.* **4,** 37 (1963).
32. J. W. YARBRO, B. J. KENNEDY and C. P. BARNUM, *Proc. natn. Acad. Sci. U.S.A.* **53,** 1033 (1965).
33. H. S. ROSENKRANTZ and J. A. LEVY, *Biochim. biophys. Acta* **96,** 181 (1965).
34. J. H. QUASTEL and A. CANTERO, *Nature, Lond.* **171,** 252 (1953).
35. A. FJELDE, A. SORKIN and J. M. RHODES, *Expl Cell Res.* **10,** 88 (1956).
36. J. LINDER, H. A. VON SCHWEINTZ and K. BECHER, *Klin. Wschr.* **38,** 763 (1960).
37. J. G. BEKESI, E. BEKESI and R. J. WINZLER, *J. biol. Chem.* **244,** 3766 (1969).
38. R. G. SPIRO, *J. biol. Chem.* **233,** 547 (1958).
39. A. N. WICK, D. K. DRURY, H. I. NAKADA, H. N. BARNET and T. N. MORITA, *J. biol. Chem.* **213,** 907 (1955).
40. H. I. NAKADO, T. N. MORITA and A. N. WICK, *J. biol. Chem.* **215,** 803 (1955).
41. G. F. CAHILL, J. ASHMORE, A. S. EARLE and S. YOTHER, *Am. J. Physiol.* **192,** 491 (1958).
42. J. M. MARTIN, *Can. J. Physiol. Pharmac.* **45,** 577 (1967).
43. M. KONO and J. H. QUASTEL, *Biochem. J.* **85,** 24 (1962).
44. S. KORNFELD and V. GINSBURG, *Expl Cell Res.* **41,** 592 (1966).
45. S. KORNFELD, R. KORNFELD, E. F. O'BRIEN and P. O'BRIEN, *Proc. natn. Acad. Sci. U.S.A.* **52,** 371 (1964).
46. T. GUNTHER and H. GREILING, *Hoppe–Seyler's Z. physiol. Chem.* **318,** 234 (1960).
47. T. GUNTHER, M. WENZEL and H. GREILING, *Hoppe–Seyler's Z. physiol. Chem.* **326,** 212 (1961).
48. T. GUNTHER, M. WENZEL and T. Z. EBERHARD, *Klin. Chem.* **3,** 76 (1965).
49. J. G. BEKESI and R. J. WINZLER, *J. biol. Chem.* **244,** 5663 (1969).
50. H. B. BOSMANN, *Microbios.* **4,** 57 (1971).