Cell Cycle Phase Specificity of Antitumor Agents

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SUMMARY

The sensitivity to drugs of synchronous and asynchronous populations of DON cells was studied. Agents that were cytotoxic at a specific phase of the cell cycle gave dose-survival curves that decreased to a constant saturation value. DNA synthesis inhibitors such as 1-β-D-arabinofuranosylcytosine, 5-azacytidine, 5-hydroxy-2-formylpyrimidinethiosemicarbazone (NSC 107392), sodium camptothecin (NSC 100880), 5-fluorodeoxyuridine, and pseudouracil (NSC 56054) were most cytotoxic to cells in the S phase. However, the DNA synthesis inhibitor, actinomycin D and nocamamide, was most cytotoxic to cells in the G1 phase. The protein synthesis inhibitors, pactamycin and sparsomycin, were also most cytotoxic to cells in the S phase. Cells in the G1-S border region were most sensitive to the RNA synthesis inhibitors, actinomycin D and nogalamin, and to the alkylating agents, 1,3-bis(2-chloroethyl)-1-nitrosourea, and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea. Streptozotocin and tubercidin, which markedly inhibit the synthesis of DNA, RNA, and protein, were cytotoxic to cells in all phases of the cell cycle. 5-Fluorouracil was also not phase specific. Cells in mitosis and in the G1 phase were most sensitive to chlorambucil, L-phenylalanine mustard, and ellipticine.

INTRODUCTION

Bruce et al. (12) have classified a number of chemotherapeutic agents into 3 groups. The 1st group, consisting of γ-radiation and nitrogen mustard, killed both proliferative and resting cells in all portions of the generation cycle. In contrast, the agents in the 3rd group killed only proliferative cells in all phases of the cell cycle. Agents belonging to both of these groups gave exponential curves, when survival was measured at different drug concentrations. The 2nd group of agents gave dose-survival curves that decreased to a constant saturation value at high doses, indicating that they killed cells in 1 portion of the cell cycle; i.e., these agents were phase specific.

The variation in the sensitivity of cultured mammalian cells through the division cycle to different agents has been reported. The recent paper by Mauro and Madoc-Jones (33) summarizes the results obtained with a large number of agents. Such studies are useful for 2 reasons: (a) They may enable us better to understand the parameters of the cell cycle by correlating the biochemical effect of the drug to its lethal effect. For example, many drugs are maximally toxic to cells at the G1-S boundary, and a correlation between the lethal and biochemical effects of the drug may help to delineate the biochemical reaction that triggers the cell across the G1-S boundary. (b) They may help in designing combinations of drugs on a rational basis. Thus, 2 drugs affecting the same phase of the cell cycle would not give additive effects, if combined.

Our studies were intended to determine the phase specificity of several clinically active agents and of agents with known biochemical activity. Parts of this study were reported previously (7).

MATERIALS AND METHODS

Cell Culture. DON cells, from a Chinese hamster fibroblast line (ATCC CCL16), were grown at 37°C in McCoy's 5A medium modified by the addition of lactalbumin hydrolysate (0.8 g/liter) and fetal calf serum (200 ml/liter). The medium was obtained from the Grand Island Biological Company, Grand Island, N.Y. The cells were grown in 8-oz prescription bottles planted with about 2 X 10⁶ cells in 25 ml of medium and were maintained in logarithmic growth by subculture every 2 days. With renewal of the medium every 2 days, an 8-oz bottle could support logarithmic cell growth, with a generation time of 10 to 12 hr, until there were about 10⁸ cells/bottle. For subculturing, the cell monolayer was detached from glass by treatment with a 0.1% trypsin solution; the cells were then dispersed in medium, and an aliquot was planted in bottles.

Synchronous DON Cells. A modification of the method of Stubblefield et al. (42) was used to obtain a population of mitotic cells. Logarithmically growing cells were planted in roller bottles (11 x 28.5 cm; 840-sq cm growth surface; Bélico Glass, Inc., Vineland, N. J.) at about 10⁷ cells in 200 ml of medium. The bottles were gassed with a 5% CO₂:95% air mixture and were rotated for 2 days on a roller apparatus (Belco) at 0.6 rpm. After 2 days of incubation, Colcemid (demecolcine; Ciba Pharmaceutical Company, Summit, N. J.) was added to the medium to give 0.06 µg/ml, and the bottles were incubated for 3 more hr. The medium was then poured off, and we selectively removed the mitotic cells by rolling the bottles with 40 ml of trypsin (at 4°C; 0.125 mg/ml) and 1 ml of NaHCO₃ (7.5%). The mitotic cells were centrifuged and resuspended in medium at 4°C, and about 10⁶ cells were planted in 3-oz bottles containing medium at 37°C. The mitotic cells constituted between 85 and 95% of the harvested cells.

Determination of Percentage of Cell Survival after Exposure of DON Cells to Drug. Cells growing in synchrony were...
exposed to drug for 1 hr at different times after the planting of mitotic cells to expose cells in different parts of the cell cycle. Asynchronous cells were also exposed to the drug for 1 hr.

After exposure to drug, the supernatant medium was poured off, and the cells were detached with trypsin and resuspended in medium at 37°. When it was suspected that the drug might also detach cells, the supernatant medium was also centrifuged, and these cells were added to the total cell pool. The cells were diluted in medium at 37°, and 2 ml of cells were planted in plastic Petri plates (Linbro Chemical Co., New Haven, Conn.) to give 10 to 100 colonies per plate. Twelve plates were planted for each sample. After incubation for 7 to 8 days in an atmosphere of 8% CO₂:92% air at 37°, the medium was poured off, and the colonies were stained with 0.2% methylene blue in 70% ethanol. The colonies were counted with a Quebec colony counter (Spencer Lens Co., Buffalo, N. Y.). The plating efficiencies were around 50% for synchronous cells and 70% for asynchronous cells. For calculation of the percentage of survivals, the control (no drug treatment) samples were normalized to 100% survival. In these experiments, the coefficient of variation in determining cell survival was about 15%, the coefficient of variation being the standard deviation expressed as a percentage of the mean.

Thus, if the percentages of survival of 2 different samples were 50 and 30%, respectively, then they would be statistically significantly different at the 95% confidence level. Almost all experiments were repeated, and the results were found to be reproducible; in duplicate determinations, the individual values were within 10% of the mean value.

**Determination of Macromolecule Synthesis.** Asynchronous DON cells were planted at 10⁶ cells/3-oz bottle in 10 ml of medium. After overnight incubation, the cells were refed with fresh medium, and drug and labeled precursors were added for 1 hr. To stop uptake of radioactivity, cells were detached with 0.1% trypsin containing unlabeled precursors (100 µg/ml), and the cells were suspended in 0.9% NaCl solution. One aliquot was counted in the Coulter counter to give the number of cells, while another (1-ml) aliquot of cells was filtered through 0.45-µ Millipore filters. The filter was washed 4 times with cold 10% trichloroacetic acid and once with ethanol. The filter was then incubated with 0.5 ml of 0.5 N perchloric acid at 70° for 20 min. Diotol (15 ml) was added, and the filter was counted in a scintillation counter.

**Autoradiography and Mitotic Index Determination.** The slides were prepared for autoradiography and mitotic index determinations, as previously described (28).

**Drug Samples.** The drugs with the NSC numbers were obtained from Chemotherpy, National Cancer Institute, Bethesda, Md. The other drugs (ara-C,² 5-azaCR, Tu, streptozotocin, nogalamycin, pactamycin, sparsomycin, and cycloheximide) were developed by The Upjohn Company, Kalamazoo, Mich. The solubilities and structures of the drugs are as follows. FUdR, neocarzinostatin (NSC 69856; Ref. 32), thio-TEPA (NSC 6396), cycloheximide, ara-C, 5-azaCR, 5-fluorouracil; ara-C, 5-azaCR, thio-TEPA, tris(l-aziridinyl)phosphine sulfide, hydrochloride dihydrate; TdR, thymidine.

²The abbreviations used are: ara-C, L-β-D-arabinofuranosylcytosine (Cytosar); 5-azaCR, 5-azacytidine; Tu, tubercidin; FUdR, 5-fluorodeoxyuridine; thio-TEPA, tris(1-aziridinyl)phosphine sulfide; FU, 5-fluorouracil; NSC 107392, 5-hydroxy-2-formylpyridinemethosulfonamide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; pseudourae, 2',2'-(-9,10-anthrylatedimethylene)bis(2-thiopseudourae)dihydrochloride dihydrate; TdR, thymidine.
when mitotic DON cells were planted as a monolayer are < 0.05. Cell division was not complete until 2 hr after mitotic pulse), and they remained in S for 7 hr. After 7 hr, cells shown by decrease in mitotic index from about 0.9 to ranged from 0.85 to 0.95. Mitosis was completed within 1 hr, shown in Chart 1. The mitotic index in different experiments commencing about 9 hr from the time of planting of mitotic cells. Therefore, under these conditions, the approximate time started entering G2 with some loss of synchrony. Cell division cells were planted, and the relative cell number increased from 1 to 1.7 during this period. Within 3 hr after planting, about 80% of the cells had entered S phase (labeled by TdR-3H radioactivity precursor for 1 hr. TdR-3H, uridine-5-3H, and DL-valine-14C were added to give 5 µCi/2 µg/ml, 5 µCi/5 µg/ml, and 0.1 µCi/23 µg/ml, respectively. The specific activities of control (no drug) samples of DNA, RNA, and protein were (per 10^6 cells): 13.5 X 10^3, 20 X 10^3, and 16.1 X 10^3 cpm, respectively. All samples were in duplicate.

RESULTS

Cell Cycle of Synchronous DON Cells. The results obtained when mitotic DON cells were planted as a monolayer are shown in Chart 1. The mitotic index in different experiments ranged from 0.85 to 0.95. Mitosis was completed within 1 hr, as shown by decrease in mitotic index from about 0.9 to < 0.05. Cell division was not complete until 2 hr after mitotic cells were planted, and the relative cell number increased from 1 to 1.7 during this period. Within 3 hr after planting, about 80% of the cells had entered S phase (labeled by TdR-3H pulse), and they remained in S for 7 hr. After 7 hr, cells started entering G2 with some loss of synchrony. Cell division commenced about 9 hr from the time of planting of mitotic cells. Therefore, under these conditions, the approximate time taken for the different phases (after planting of mitotic cells) is from 0 to 2 hr to complete mitosis and G1, from 2 to 8 hr for S, and from 8 to 11 hr for G2 and mitosis.

Inhibition of Macromolecule Synthesis by Several of the Agents. For convenience in presentation, the agents have been subdivided into classes on the basis of their inhibition of macromolecule synthesis and known sites of action. This division does not, however, imply that a given agent has only 1 biochemical mode of action. The subdivision merely indicates that the agent in question markedly inhibits the synthesis of a particular macromolecule. Thus both ara-C and Tu inhibit DNA synthesis (Table 1) and are classified as DNA synthesis inhibitors. However, only ara-C specifically inhibits DNA synthesis, while Tu inhibits almost equally the synthesis of DNA, RNA, and protein. The sites of action of many of these agents have recently been compiled by Livingston and Carter (29). Other pertinent publications on their sites of action are listed below after each compound.

The inhibition of macromolecule synthesis by several of the agents is shown in Table 1. ara-C (14, 19), 5-azaCR (28), NSC 107392 (11), sodium camptothecin (23), Tu (1), streptozotocin (5), FU (21, 31), FuD (21), and neocarzinostatin (27, 34) have been classified in the following section as DNA synthesis inhibitors. Among them only ara-C, NSC 107392, and neocarzinostatin markedly inhibited DNA synthesis without simultaneous marked inhibition of RNA and protein synthesis. The other agents in this group markedly inhibited DNA, RNA, and/or protein synthesis.

Actinomycin D (38) and nogalamycin (8) have been classified as RNA synthesis inhibitors. Actinomycin D was a much more specific inhibitor of RNA synthesis than was nogalamycin.

Pactamycin (Ref. 18, pp. 169–173) and sparsomycin (Ref.

Table 1
Inhibition of macromolecule synthesis in DON cells by various agents

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (µg/ml)</th>
<th>Inhibitiona (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ara-Cb</td>
<td>100</td>
<td>92 10 24</td>
</tr>
<tr>
<td>ara-C</td>
<td>1000</td>
<td>96 24 17</td>
</tr>
<tr>
<td>5-AzaCRc</td>
<td>100</td>
<td>91 87 95</td>
</tr>
<tr>
<td>NSC 107392b</td>
<td>2</td>
<td>85 14 1</td>
</tr>
<tr>
<td>Sodium camptothecinb</td>
<td>1.85</td>
<td>60 53 0</td>
</tr>
<tr>
<td>Sodium camptothecin</td>
<td>9.2 77 64</td>
<td></td>
</tr>
<tr>
<td>Tu</td>
<td>2</td>
<td>84 86 88</td>
</tr>
<tr>
<td>Streptozotocin</td>
<td>1000</td>
<td>37 0 19</td>
</tr>
<tr>
<td>Streptozotocin</td>
<td>5000</td>
<td>75 68 79</td>
</tr>
<tr>
<td>Fu</td>
<td>100</td>
<td>37 84 0</td>
</tr>
<tr>
<td>FuDRc</td>
<td>100</td>
<td>40 64 41</td>
</tr>
<tr>
<td>Neocarzinostatin</td>
<td>5</td>
<td>44 −20 −9</td>
</tr>
<tr>
<td>Neocarzinostatin</td>
<td>50</td>
<td>60 −30 −31</td>
</tr>
<tr>
<td>Actinomycinb</td>
<td>1</td>
<td>7 87 0</td>
</tr>
<tr>
<td>Nogalamycinb</td>
<td>2</td>
<td>40 74 11</td>
</tr>
<tr>
<td>Pactamycinb</td>
<td>1</td>
<td>79 46 98</td>
</tr>
<tr>
<td>Pactamycin</td>
<td>100</td>
<td>80 91 99</td>
</tr>
<tr>
<td>Sparsomycinb</td>
<td>100</td>
<td>80 35 98</td>
</tr>
<tr>
<td>Pseudourea</td>
<td>1.1</td>
<td>52.6 35.1 25</td>
</tr>
<tr>
<td>Pseudourea</td>
<td>10</td>
<td>81 90 40.4</td>
</tr>
</tbody>
</table>

a In all cases, the DON cells were incubated with radioactive precursors for 1 hr. TdR-3H, uridine-5-3H, and DL-valine-14C were added to give 5 µCi/2 µg/ml, 5 µCi/5 µg/ml, and 0.1 µCi/23 µg/ml, respectively. The specific activities of control (no drug) samples of DNA, RNA, and protein were (per 10^6 cells): 13.5 X 10^3, 20 X 10^3, and 16.1 X 10^3 cpm, respectively. All samples were in duplicate.
b DON cells were exposed to agent and radioactive precursor for 1 hr.
c Cells were exposed to agent for 5 hr prior to addition of radioactive precursor for 1 hr.
d Cells were exposed to Tu for 2 hr prior to addition of labeled precursor for 1 hr.
e Cells were exposed to agent for 1 hr prior to addition of labeled metabolites. The agents in Group b rapidly inhibited macromolecule synthesis, while the other agents needed a longer period of contact with cells. Neocarzinostatin stimulated the incorporation of precursors into RNA and protein.
Cell Cycle Phase Specificity

100E

Chart 3. Sensitivity of asynchronous (a) and synchronous (b) DON cells to NSC 107392 and sodium camptothecin (NSC 100880). Protocol same as Chart 2, except drug exposure was for 1 hr.

Increased to a constant saturation value at high doses, indicating that ara-C is cytotoxic to a specific phase or phases of the cell cycle. Results obtained with synchronous cells (Chart 2b) showed that, at low levels, both ara-C and 5-azaCR were specifically cytotoxic to S-phase cells. High levels of 5-azaCR (100 µg/ml) were lethal to cells in G1, S, G2, and M, although cells in S phase were still most sensitive. However, ara-C at high level (1 mg/ml) was still lethal only to cells in S.

The dose-survival curve for NSC 107392 (Chart 3a) decreased to a constant saturation value, indicating that this

Chart 4. Sensitivity of asynchronous (a) and synchronous (b) DON cells to streptozotocin and Tu. Protocol same as in Chart 3.

18, pp. 410–415) almost completely inhibited protein synthesis, although DNA and RNA synthesis were also markedly inhibited.

BCNU (Ref. 29, pp. 360–365), CCNU, chlorambucil (Ref. 29, pp. 81–98), thio-TEPA, and phenylalanine mustard (Ref. 29, pp. 99–111), have been classified as alkylating agents.

Response to DNA Synthesis Inhibitors. The dose-survival curves of asynchronous cells exposed to ara-C (Chart 2a)

Chart 5. Sensitivity of asynchronous (a) and synchronous (b) DON cells to FU and FUdR. Protocol same as in Chart 3.

Chart 6. Sensitivity of asynchronous (a) and synchronous (b) DON cells to neocarzinostatin. Protocol same as in Chart 3. The results of 2 separate experiments are shown.
drug is cytotoxic to a specific phase or phases of the cell cycle. The dose-survival curve for sodium camptothecin (Chart 3a) suggests the presence of cell populations with varying sensitivities to the drug. The results with synchronous cells (Chart 3b) show that both of these agents are most cytotoxic to cells in the S phase. The percentage of cell kill obtained in different phases with sodium camptothecin, 10 µg/ml, was approximately the same as the percentage of cell kill at 2 µg/ml. Although both DNA and RNA synthesis were markedly inhibited by sodium camptothecin, 10 µg/ml (Table 1), the drug was still maximally lethal to cells in S. However, unlike NSC 107392, cells in M, G1, and G2 were killed by sodium camptothecin.

The results with asynchronous cells exposed to streptozotocin are shown in Chart 4a. The results with synchronous cultures (Chart 4b) showed that cells in all phases of the cell cycle were equally sensitive to the drug. This becomes particularly evident at the higher concentration (0.5 mg/ml) of streptozotocin.

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (µg/ml)</th>
<th>Cell kill (%)</th>
<th>Inhibition of protein synthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pactamycin</td>
<td>0.1</td>
<td>17.2</td>
<td>99.9, 19, 52.5</td>
</tr>
<tr>
<td>Pactamycin</td>
<td>1.0</td>
<td>24</td>
<td>99.97, 54.5, 62</td>
</tr>
<tr>
<td>Pactamycin</td>
<td>10</td>
<td>27.5</td>
<td>99.9, 91.9, 91.2</td>
</tr>
<tr>
<td>Puromycin</td>
<td>100</td>
<td>23.2</td>
<td>99.6, 38.5</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>100</td>
<td>21.2</td>
<td>99.9, 78</td>
</tr>
</tbody>
</table>

*a* After 1- or 4-hr exposure to the drug, the cells were washed, diluted, and planted to determine the percentage of surviving cells.

*b* The cells were exposed to the drugs for 1 hr. Immediately after drug exposure, the cells were given a 20-min pulse with DL-valine-1-14C (1 µCi/23 µg/ml medium) to determine the percentage of inhibition of protein synthesis before washing. A similar percentage of inhibition of protein synthesis (before washing) was obtained when cells were exposed to the drug for 4 hr.

*c* For determination of the percentage of inhibition of protein synthesis after washing, the cells were washed after 1 hr of drug exposure, were allowed a 1-hr recovery period, and then given a 20-min pulse of DL-valine-1-14C. Control cells incorporated 10^4 cpm/2 X 10^4 cells into the acid-insoluble fraction.

*d* Same as above, except cells were exposed to drug for 4 hr.
In synchronous cultures, Tu at low levels (0.2 µg/ml) was approximately equally cytotoxic to cells in all phases of the cell cycle (Chart 4b). However, at high levels (0.4 µg/ml), cells at the G1-S boundary region seemed to be more sensitive to Tu than were the cells in G1 or S.

FU was equally cytotoxic to cells in all phases of the cell cycle (Chart 5b). With asynchronous cells, the dose-survival curve for FUdR had a shoulder preceding the exponential component of the curve, since there was no cell kill with FUdR at 5 µg/ml or less. The exponential component of the dose-survival curve had 2 different slopes, indicating cell populations with different sensitivities to the drug (Chart 5a). This was reflected in the greater sensitivity of cells in the S phase to FUdR.

The results obtained in 2 different experiments with neocarzinostatin are shown in Chart 6. The difference between the 2 experiments with neocarzinostatin could be due to the variation in the neocarzinostatin preparation. With asynchronous cells, both experiments (in spite of the difference in percentage of survival values) show that the drug was maximally cytotoxic to cells in M and G1 phases. The sensitivity decreased as the cells progressed through S and increased again as the cells entered G2 and M.

RNA Synthesis Inhibitors. The dose-survival curves with asynchronous cells (Chart 7a) showed that actinomycin D, 0.1 µg/ml, and nogalamycin, 1 µg/ml, did not kill any cells, indicating the presence of a shoulder region preceding the exponential component. The results with synchronous cells (Chart 7b) indicated that cells in the G1-S boundary region were most sensitive to the agent. These results confirm those of Elkind et al. (15) and Mauro and Madoc-Jones (33).

Protein Synthesis Inhibitors. The dose-survival curves of asynchronous cells exposed to pactamycin, sparsomycin, and streptovitacin A are shown in Chart 8a. Only sparsomycin showed a shoulder region prior to the exponential component of the curve. Streptovitacin A and sparsomycin curves reached saturation values for percentage of survival at high doses, indicating that these agents are phase specific. The results obtained with synchronous cells are given in Chart 8b. Cells in S phase were most sensitive to pactamycin and sparsomycin. Cells in S phase have been shown to be sensitive to streptovitacin A by Mauro and Madoc-Jones (33).

The dose (in µmoles/ml) for 50% cell kill after 1 hr of exposure to several protein synthesis inhibitors was: streptovitacin A, 0.0018; pactamycin, 0.025; sparsomycin, 0.095; puromycin, >0.2; and cycloheximide, >0.7. These results indicate that, except for streptovitacin A, the protein synthesis inhibitors tested (pactamycin, puromycin, cycloheximide, and sparsomycin) were relatively ineffective in killing cells after 1 hr of exposure. The results in Table 2 may indicate a possible explanation of this effect. Cells exposed to protein synthesis inhibitors for 1 hr have greater than 99% of their protein synthesis inhibited, and yet only 25% of the cells were killed. This was due to the fact that when the agents were removed by washing after 1 hr of exposure, the cells recovered their protein synthesizing ability. This was not true when high levels of pactamycin were used, possibly indicating that all of the pactamycin was not removed by washing. However, after 4
had been severely damaged so that the cell did not completely recover its synthetic capacity. Therefore, the percentage of inhibition of protein synthesis after the drug had been washed off compared favorably with the percentage of cell kill.

Alkylating Agents. BCNU and CCNU (Chart 9b) were most cytotoxic to cells in G1-S border or early S. With both BCNU and CCNU, the sensitivity of cells decreased as the cells progressed into S. However, with CCNU and to a lesser extent with BCNU, the cell sensitivity increased again as the cells entered late S or G2.

Thio-TEPA, phenylalanine mustard, and chlorambucil were most cytotoxic to cells in the M and G1 phase (Chart 10b). At the higher concentration of phenylalanine mustard and with thio-TEPA, cells in G2 were as sensitive as the G1 cells.

Miscellaneous Agents. The results with pseudourea and ellipticine, 2 agents with unknown sites of action, are shown in Chart 11. Pseudourea was most cytotoxic to cells in S, while ellipticine was most cytotoxic to cells in M and G1. As the cells progressed through S, their sensitivity to ellipticine decreased, but ellipticine toxicity increased again as the cells passed through late S or early G2. The greater sensitivity to ellipticine of cells in M and G1 as compared with cells in S or G2 was seen at drug doses ranging from 0.36 µg/ml (not shown in chart) to 10 µg/ml.

The inhibition of macromolecule synthesis by pseudourea is shown in Table 1. Pseudourea inhibited DNA synthesis more than RNA or protein synthesis at the lower concentrations, while at 10 µg/ml of the drug, DNA and RNA syntheses were equally inhibited.

DISCUSSION

The selective detachment method of Terasima and Tolmach (43) for obtaining mitotic cells does not subject the cells to any perturbations and is therefore the ideal method for synchronizing cells. However, the low yield of mitotic cells forced us to use the present method of selectively removing mitotic cells from a culture treated with Colcemid. Stubblefield et al. (42) reported that the cells suffered no lasting effects from a 2-hr Colcemid (0.06 µg/ml) treatment. However, Kato and Yosida (24) have shown that chromosomal nondisjunction occurred frequently after Colcemid treatment and that the plating efficiency of synchronized cells was lower than that of untreated control cells. We found that synchronized cells had about 70% of the plating efficiency of asynchronous cells. Also, when an asynchronous population of DON cells was exposed to Colcemid (0.06 µg/ml) for 4 hr, only about 80% of the cells survived. These results indicate that our synchronized population may contain a certain percentage of dead or dying cells. However, the measurement of reproductive cell survival (or colony-forming ability) after treatment with cytotoxic agents is not affected by the presence of dead cells in the culture at the time of drug treatment. Although 20% of the cells had lost their reproductive potential, they still were able to synthesize DNA, since almost 90% of the cells were labeled with TdR-3H pulse (Chart 1).

The index of synchrony (F) was determined by the method of Blumenthal and Zahler (9) to be 0.59. This compared favorably with the F values of 0.6 of Kim and Stambuck (26) and the calculated F value of 0.65 for the synchronized cultures of Pfeifer and Tolmach (35).

During the 1st cell division, the relative cell number increased from 1 to 1.7 (instead of the expected 2) during a 2-hr period. During the 1st hr, mitotic division was completed, and the mitotic index decreased from 0.9 to <0.05. This result might indicate that some of the cells completed mitotic division but either did not complete cell division or did not separate after cytokinesis and were counted as 1 cell in the Coulter counter. Pfeifer and Tolmach (35) found that their relative cell number increased from 1 to 1.8.

The cells stayed in S till about 7 hr, and then they began entering G2 with some loss of synchrony. The loss of synchrony increased as the cells progressed through the cell cycle from G1 to S to G2. This is indicated by the steeper slope of the percentage-labeled cell curve as the cells go from G1 to S, compared with the slope of the curve as the cells go from S to G2. Such loss of synchrony is due to the variation in the intermitotic times of individual cells, as shown by Sisken (40).

In our experiments, the cells were exposed to drug usually for 1 hr. Such a short period of exposure was chosen for 2 reasons: (a) the G1 and G2 phases of DON cells are of less than 2-hr duration. Therefore, in order to expose cells in these phases of the cell cycle, it was necessary to use a short exposure period. (b) Many drugs have a short plasma half-life, such that usually the cells in vivo are exposed to drug for a short time. For example, ara-C (10), pactamycin (6), 5-azaCR (37), actinomycin D (36), and streptozotocin (39) have plasma half-lives of about 20 min, <5 min, <5 min, <5 min, and 5

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**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>DNA Synthesis</th>
<th>RNA Synthesis</th>
<th>Protein Synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudourea</td>
<td>0.36 µg/ml</td>
<td>0.36 µg/ml</td>
<td>0.36 µg/ml</td>
</tr>
<tr>
<td>Ellipticine</td>
<td>0.36 µg/ml</td>
<td>0.36 µg/ml</td>
<td>0.36 µg/ml</td>
</tr>
</tbody>
</table>
min, respectively. Therefore, cytotoxicity of a drug determined after 1 hr of exposure might correlate better with the in vivo effects of the drug than might the usual method of cytotoxicity determination, wherein the cells are exposed to the drug for 2 to 3 days.

Mauro and Madoc-Jones (33) suggested that most of the drugs studied gave dose-survival curves in which a shoulder region preceded the exponential portion of the curve. The presence of a shoulder region suggests that the cells accumulate damage which ultimately leads to a lethal effect and that survivors have sublethal damage which is repaired (16). Of all the agents reported here, only FuDR, nogalamycin, actinomycin D, and sparsomycin gave dose-survival curves in which a shoulder region was obviously present.

At the present state of our knowledge, it may not be possible to predict the most susceptible phase from a knowledge of the biochemical site of action. However, attempts have been made to explain the sensitivity of different phases on the basis of the site of action of the drug; this is discussed below.

(a) For a valid correlation, exposure to a given drug should be completely terminated by washing the cells to remove the drug. However, nucleosides such as Tu, 5-azaCR, FU, etc., are converted to their respective nucleotides inside the cell and may be held in the intracellular pool. For example, the lethal action of FU can be expressed even 80 hr after the exposure of cells to FU (31). Therefore even if FU killed only S-phase cells, G1 and G2 cells exposed to the drug will accumulate the nucleotide and be killed when the cells enter S.

(b) It would be logical to suppose that agents that specifically inhibit DNA synthesis will be lethal to cells in S phase only. ara-C, NSC 107392, and neocarzinostatin are 3 such agents. The first 2 compounds are specifically lethal to cells in S, while neocarzinostatin is more cytotoxic to cells in M and G1 than in S. The strange behavior of neocarzinostatin might be explained by the observation (27) that cells in G1, exposed to the drug, were unable to synthesize DNA on reaching S, which probably resulted in cell death. Drug added to cells in S did not inhibit DNA synthesis.

(c) Sodium camptothecin inhibits equally both DNA and RNA synthesis and kills cells in all phases of the cycle, although the cells in S were always more sensitive to the drug. In contrast, streptozotocin, Tu, FU, and FuDR at high concentration (30) all inhibited DNA and RNA and/or protein synthesis but were toxic to cells in all phases.

(d) Actinomycin D (38) and nogalamycin (8) bind to the DNA template and inhibit RNA synthesis more than DNA synthesis (Table 1). Cells in G1-S or early S are most sensitive to both these drugs (this paper and Refs. 15 and 33). Fujiwara (17) found that, when actinomycin D was added in G1, the synthesis of early replicating DNA was inhibited. However, actinomycin D added in the middle of the S phase had little effect on the late replicating DNA. The results indicate that inhibition of RNA synthesis in G1 led to the inhibition of early replicating DNA, which probably led to cell death.

(e) Cells in the S phase were most sensitive to protein synthesis inhibitors, such as pactamycin and sparsomycin. Spalding et al. (41) showed that, during replication of chromosomes, S-phase cells also synthesize a new complement of histones. The process of DNA and histone synthesis is tightly coupled so that interruption of histone synthesis results in the inhibition of DNA synthesis. This would account for the sensitivity of cells in S to protein synthesis inhibitors.

(f) Our results, together with those of Mauro and Madoc-Jones (33), show that alkylating agents are characterized by toxicity to cells in M, G1, or G1-S transition. Whether this common site for the alkylating agents indicates vulnerability of the mitotic chromosomes or of DNA before replication is not known.

In contrast to our results with FuDR, Lozzio (30) reported that FuDR was equally cytotoxic to cells in all phases of the cell cycle except the mitotic stage. However, he did find that 10−5 M FuDR killed 97% of the cells in early S, compared to only 70% of the cells killed in G1 or late S. However, at higher levels of the drug, cells in all phases were equally sensitive.

Our results with BCNU differ from those of Barranco and Humphrey (3), who found that Chinese hamster ovary cells were most sensitive in mid-S phase. Their method differed from ours in 2 aspects:

(a) Our DON cells were about 14 times more sensitive than were the ovary cells. Thus, 90% cell kill of asynchronous cells after 1-hr exposure required BCNU, in amounts of 85 and 6 μg/ml for the ovary cells and DON cells, respectively. Therefore, in our phase specificity experiments, DON cells were exposed to BCNU, 2 μg/ml, compared with 100 μg/ml for the ovary cells (3).

(b) Their method for synchronizing cells consisted of a double treatment with excess TdR and so differed from ours. Both of us found that cells in the G1-S border were highly sensitive to BCNU. The cells decreased in sensitivity as they progressed through S, with another zone of great sensitivity in mid-S (3). Here, our results differ from theirs. Whether the difference in the method of synchrony and the levels of BCNU used would account for the difference in our results needs investigation.

Bruce et al. (12) differentiated between certain (phase-nonspecific) agents, such as nitrogen mustard and γ-rays, that killed cells in all phases of the cell cycle, and phase-specific agents such as ara-C, that killed cells in a specific phase of the cell cycle. The studies reported here and those of Mauro and Madoc-Jones (33) and others (15, 16) have shown that there is a marked difference in the sensitivity of cells in different phases of the cell cycle toward "phase-nonspecific" (12) agents. Thus, cells at G1-S transition are 50 times more sensitive to BCNU than the cells in G2 (Chart 9b). The cells in M and G1 were 250 times more sensitive to ellipticine (Chart 11b) than cells in G2. By proper manipulation, this differential sensitivity may be utilized in cancer chemotherapy.

We suggest that knowledge of the phase specificity of agents may enable us to devise rational combinations of drugs. Thus, 2 drugs inhibiting in the same phase, if given together, will not result in improved cell kill, although they may delay the development of resistance to either drug. However, it is essential that we also know the effect of the drug on progression of the cells through the cell cycle. Thus, it has been suggested that ara-C and HU block cells at the G1-S interface (2, 19, 25). Our studies (B. K. Bhuyan, W. N. Vorhof, etc.)
and T. J. Fraser, paper in preparation) also indicate that NSC 107392 blocks at the G1-S interface. Thus, it is possible that these drugs (ara-C, HU, and NSC 107392) could be beneficially combined with agents that acted either in M and G1 (e.g., neocarzinostatin) or at the G1-S interface (e.g., actinomycin D). Such studies are in progress.

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