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BARBITURATE INHIBITION OF GROWTH AND OF THE SYNTHESIS OF DEOXYRIBONUCLEIC ACID, RIBONUCLEIC ACID AND PROTEIN IN CULTURED MAMMALIAN CELLS*

PHILIP L. WHYATT† and JOHN W. CRAMER‡

Department of Pharmacology and Therapeutics, University of Florida, College of Medicine, Gainesville, Fla. 32601, U.S.A.

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Abstract—Pentobarbital (PB) inhibited growth and the synthesis of nucleic acids and protein in murine, mastocytoma cells (P815Y) grown in culture. The inhibition increased with an increase in the concentration of drug and was also time-dependent with a high level of drug. For example, 0·5 mM PB (ID_{50}) reduced both the rate of division of the cells and the synthesis of DNA, RNA and protein by about one-half, compared with the control, over a 12-hr period. In contrast, treatment with a 2-fold higher concentration of PB (1 mM; ID_{100}) blocked both cell division and the synthesis of protein promptly. It also reduced the synthesis of DNA and RNA by about one-half, compared with the control, during the first 4 hr of treatment. After this time, however, the synthesis of both DNA and RNA stopped abruptly. It is concluded that the inhibition of DNA synthesis caused by barbiturate in the latter case may have been secondary to the inhibition of protein synthesis. Transfer of the inhibited (1 mM PB; 12 hr) cells to drug-free medium caused the synthesis of protein and RNA to begin without apparent delay. In contrast, the synthesis of DNA proceeded slowly for about 8 hr; then the amount of DNA increased parasynchronously. Cell division, which also proceeded slowly during the first 10 hr, occurred as a parasynchronous wave some 2 hr after DNA synthesis began. The inhibitory effects of PB were also studied in murine, lymphoblastic cells (L5178Y) synchronized by sequential treatment with thymidine (5 hr) and deoxycytidine plus Colcemid (5 hr). When the mitotically inhibited cells were transferred to normal medium containing PB (1·5 mM; ID_{100}), slightly more than one-half of the cells failed to complete mitosis and the synthesis of DNA, RNA and protein was blocked by the drug. The synthesis of DNA, RNA and protein was also blocked by PB addition in the middle of the S-phase. Its addition at the onset of the second wave of mitosis prevented mitosis as before and blocked the initiation of DNA synthesis.

THE OBJECT of the present paper was to investigate the inhibitory actions of barbiturates, especially pentobarbital (PB),§ upon the kinetics of cell growth and upon the synthesis of nucleic acids and proteins in mammalian tumor cells[1–5] grown in suspension culture. Direct colorimetric assays of DNA, RNA and protein provided

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† Present address: College of Pharmacy, University of Tennessee Medical Units, Memphis, Tenn. 38103.

‡ To whom reprint requests should be sent.

§ Abbreviations used: PB, pentobarbital sodium; PCA, perchloric acid; ^{3}H-TdR, tritiated thymidine; ^{3}H-UR, tritiated uridine; ^{3}H-leu, tritiated leucine; TdR, thymidine; dTTP, thymidine triphosphate; CdR, deoxycytidine; and ID, inhibitory dose.

information on the inhibition of the synthesis of these macromolecules by PB without the exclusive use of labeled precursors, as done by others.[1–5] The inhibitory actions of PB within the cell cycle were defined for the first time by the use of synchronized cultures of murine, leukemic lymphoblastic cells (L5178Y). The use of tumor cells provided conditions that presumably minimized any untoward effects of the barbiturate to uncouple oxidative phosphorylation.[6] The cell lines used were virtually anaerobic, requiring little or no oxygen for rapid growth;[7] thus the ATP required for cell division and metabolism was presumed to be derived mainly from glycolysis.[8]

It was found that 1 mM PB inhibited the synthesis of protein before it inhibited the synthesis of DNA or RNA in P815Y cells in logarithmic growth and that transfer of the inhibited cells to drug-free medium caused the synthesis of protein and RNA to begin before the synthesis of DNA. The data suggested that the inhibition of DNA synthesis by PB in this cell line might have been secondary to the inhibition of protein synthesis.

METHODS AND MATERIALS

Cell lines, medium and growth conditions. Two cell lines were used: a murine, mastocytoma cell (P815Y) and a murine, leukemic lymphoblastic cell (L5178Y). Both cell lines grew well in suspension culture with doubling times of about 10·5 and 9 hr respectively.[9]

The culture medium was prepared from a commercial powdered concentrate ("Fischer's Medium for Leukemic Cells of Mice," Grand Island Biological Company.[9]) It was supplemented before use with 10% horse serum (Grand Island Biological Company). Unless otherwise stated, reference to "medium" within the text refers to the growth medium plus the added horse serum.

Cells in logarithmic growth were used unless otherwise noted. Cultures were serially passaged weekly to maintain the cells in logarithmic division. Cultures required for the experiments were prepared by dilution of the stock cell cultures with fresh medium.

Cells were grown either in 5-ml portions of medium in 16 × 125 mm screw-capped culture tubes or in Erlenmeyer flasks, in which case a ratio of 1:6 for the volume of medium to the flask volume was required for optimal growth. Shaking of the culture was not required. The cells were grown at 37° in a Wedco Controlled Environment Incubator. Cell numbers were obtained by means of a Coulter Electronic Particle Counter (model A or model ZBI). The settings for the model A were: amplification, 4; aperture current setting gain, 4; gain trim, 9. For the model ZBI the settings were: amplification, 1; aperture current, 1 milliampere; matching switch, 20K; and gain trim, 5. For both the model A and the model ZBI the following settings were used: threshold range, 50–100; aperture diameter, 100 microns; mercury path volume, 0·5 ml; and for the sample dilution each 0·5 ml of growth medium plus cells was diluted with 9·5 ml of 0·9% NaCl.

Chemicals. Pentobarbital sodium (U.S.P.) was purchased from the S. B. Penick & Company, N.Y. Diphenylhydantoin and ethosuximide were gifts from Parke, Davis & Company, Detroit, Mich. All other drugs were U.S.P. quality. (Methyl-^{3}H)-thymidine (sp. act. 6 Ci/m-mole), (5-^{3}H)-uridine (sp. act. 4 Ci/m-mole) and L-(4,5-^{3}H)-leucine (sp. act. 2 Ci/m-mole) were purchased from Schwarz Bioresearch, Inc., Orangeburg, N.Y. All other biochemicals used were supplied by either CalBiochem (Los Angeles, Calif.)

or Sigma Chemical Corp. (St. Louis, Mo.) unless otherwise indicated. All inorganic chemicals were Reagent (A.C.S.) grade.

Determination of radioactivity. Liquid scintillation counting in a model 1650 Beckman Scintillation system was employed to determine tritium. Tritium was counted with an error of $\pm$ 2 per cent. The counting fluid consisted of 34 mg of *p-bis* [2-(5-phenyloxazolyl)] benzene (New England Nuclear), 2·7 g of 2,5-diphenyloxazole (New England Nuclear), 333 ml of absolute ethanol and sufficient toluene to bring the total volume to 1 liter. Samples were prepared for counting by placing either 0·1 or 0·5 ml of liquid in 20 ml of the scintillation fluid. Unless otherwise noted, when radioactive precursors were used in cell experiments, they were added to the medium together with unlabeled precursors in the following amounts: (methyl-^{3}H)-thymidine (Schwarz, 12·5 μCi/μmole, 10^{-5} M final medium concentration), (5-^{3}H)-uridine (Schwarz, 12·5 μCi/μmole, 10^{-5} M final medium concentration) and L-(4,5-^{3}H)-leucine (Schwarz, 1·7 μCi/μmole, $2{\cdot}3 \times 10^{-4}$ M final medium concentration).

Analysis of DNA and RNA. The inoculum for all experiments was 100,000 cells/ml. All appropriate operations were done at ice temperature. Two mg of crystalline bovine serum albumin (Nutritional Biochemicals Corp., Cleveland, Ohio) were added to the cell pack as a carrier and the pack was extracted three times with ice-cold 0·2 N perchloric acid (PCA). The washed, acid-insoluble precipitate was dissolved in 0·3 N NaOH, and the solution was incubated for 1 hr at 37° to hydrolyze the RNA.[10] The solution was then chilled and acidified with sufficient PCA to precipitate DNA and protein. The precipitate was sedimented by centrifugation and washed three times as before. Duplicate 1-ml aliquots of the combined supernatant fraction (6 ml) from the latter operation were analyzed for RNA colorimetrically with the orcinol reagent[10] or by u.v. absorbance.[11] Radioactivity was determined with duplicate 0·5-ml aliquots. The acid-insoluble pellet was treated further with 1 ml of 1·5 N PCA for 20 min at 70° to hydrolyze the DNA. The solution was chilled in ice, and the precipitate was sedimented by centrifugation and washed once with 0·5 ml of 1·5 N PCA. A 1-ml aliquot of the combined supernatant (1·5 ml) was analyzed for DNA colorimetrically by the method of Dische.[12] The remainder (0·5 ml) was used to determine radioactivity. In some experiments in which only the content of the DNA of the acid-insoluble pellet was determined, the step for the hydrolysis of RNA was omitted.

For the analysis of DNA from synchronized cultures of L5178Y cells, the modifications below were used because it was necessary to analyze smaller amounts of cells. The DNA of the acid-insoluble pellet derived from 8 ml of medium was analyzed by the Giles and Myers' modification of the diphenylamine reaction.[13] The procedures for the assay of RNA and protein in the synchronized L5178Y cells remained the same as described, except that the final volume before assay was reduced to 3 ml (RNA) or 6 ml (protein).

Analysis of protein. In some experiments in which the content of protein of the acid-insoluble pellet was determined, the addition of albumin as a carrier was omitted. The washed, acid-insoluble precipitate was treated with 1 N NaOH at 37° for 1 hr to solubilize the protein and diluted to 9 ml. A 0·5-ml aliquot of the resulting solution was analyzed colorimetrically for protein by the method of Lowry *et al.*[14] and a 0·1-ml aliquot was taken to determine radioactivity.

Incorporation of radioactive precursors. The effect of PB on the synthesis of DNA, RNA and protein was measured by the incorporation of radioactive precursors into

these macromolecules. Labeled precursors were added at the time of PB addition. Aliquots were taken for the analysis of radioactivity at various intervals of time as described above. The effect of PB on the synthesis of DNA was also measured by means of a pulse-incorporation experiment. (Results are discussed later but data are not presented herein.) In this case, both control and drug-treated cultures were treated for 30 min with ^{3}H-TdR at 0, 4, 8 and 12 hr after drug addition. At the end of the 30-min interval, 80-ml aliquots of the cells were harvested, washed and the radioactivity was determined in the same manner as described above.

Determination of cloning efficiency. Cells treated with drugs were sedimented by centrifugation, washed, and resuspended in fresh, warm, drug-free medium prior to the cloning procedures. Growth of single cells into colonies was carried out in a gel obtained by coagulation of a fibrinogen solution, as described by Schindler.[7] A suspension of cells (100,000/ml) was diluted serially with culture medium to obtain a final cell density of approximately 6 cells/ml. The suspension of diluted cells was treated with bovine thrombin (Parke, Davis & Company, Detroit, Mich.), 12 ml was mixed in a culture tube (capacity 50 ml) with 3 ml of a solution containing sodium citrate, salts and bovine fibrinogen (Pentex Biochemicals, Kankakee, Ill.) and the mixture was incubated at 37°. Within a few minutes, a transparent clot formed which kept the suspended cells in place. The colonies were counted 8–10 days later, in which time the individual cells had grown into visible colonies. The colonies in the gel were counted by eye and then checked with a low power microscope.

Synchronization of L5178Y cells. Synchronized L5178Y cells were used in some experiments rather than P815Y cells because they produced sharper synchrony. The method is outlined below and is essentially the method of Doida and Okada.[15] Exponentially growing cells (100,000/ml) were treated with thymidine ($2{\cdot}5 \times 10^{-3}$ M) for 5 hr to arrest cells in the DNA synthetic period of growth. During the 5 hr of thymidine treatment, the G_2-, M- and G_1-stage cells entered and remained in the S-period which occupies about 5 hr of the total generation time of about 8–9 hr. To reverse the S-phase block, deoxycytidine (1×10^{-5} M) was added at the end of the 5-hr period. To compress the cell population into a more narrow region of the life cycle, Colcemid (Ciba Pharmaceutical Company, Summit, N.J.) was added at the same time as deoxycytidine to the cultures at a concentration of 0·025 μg/ml for 5 hr to arrest mitotic cells in metaphase. At the end of this time, the cells were sedimented by centrifugation, washed and resuspended in fresh warm medium. Values for the degree of synchrony varied from 75 to 90 per cent in a series of eight experiments.

RESULTS

Effects of barbiturates on cells in logarithmic growth

Inhibition of cell growth. The inhibition of growth of P815Y cells with time against graded doses of PB was dose related; thus, for each of the observed time intervals, the rate of growth of the drug-treated cells decreased regularly as the concentration of PB in the medium was increased (Fig. 1). The concentration of PB (ID_{50}) that produced 50 per cent inhibition of cell growth was about 0·4–0·5 mM for both the P815Y and the L5178Y cells; the ID_{100} values were approximately 1 and 1·5 mM respectively. The progressive inhibition of growth by the graded doses of PB (Fig. 1) suggested the titration of an essential factor(s) for cell division. For example, a similar kinetic pattern

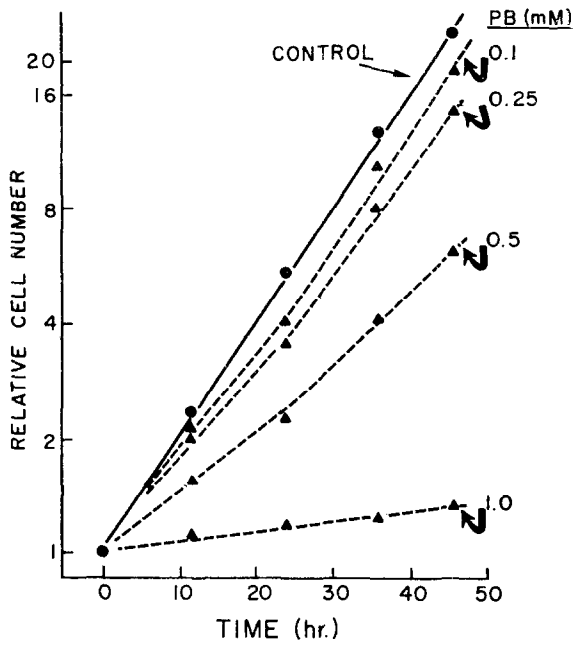


FIG. 1. Kinetics of inhibition of growth of the P815Y cell by graded doses of PB. Appropriate aliquots (0·5–2 ml) of a stock solution ($2{\cdot}5 \times 10^{-2}$ M) of PB in water or of a 1:10 dilution of the stock solution were added to 50-ml portions of medium with P815Y cells (44,000/ml). The control medium received 0·5–2 ml of sterile water. Duplicate 5-ml aliquots of the drug-treated or control medium were added to 125 × 16 mm, screw-capped, glass culture tubes, and these were incubated for various times up to 46 hr at 37°. At each time point the number of cells was determined by use of the Coulter Counter. Relative cell number is the ratio of the cell number at a given time to that of the inoculum at time zero. The symbols are: ●, control; ▲, drug-treated.

of growth inhibition of cells in culture has been noted for 5-fluoro-2′-deoxyuridine, an inhibitor that causes non-competitive inhibition of thymidylate synthetase and prevents the *de novo* synthesis of thymidylic acid.[16]

The inhibition of growth caused by PB could be reversed by transfer of the cells to medium without drug. (Results are not shown here.) However, a lag in growth occurred which was proportional to the prior length of drug treatment. For example, P815Y cells pretreated with drug (1 mM; ID_{100}) for 6 hr did not begin division until about 3 hr after transfer to drug-free medium. By the eighth hr, 40 per cent of the cells had divided. The number of cells then remained constant until the twelfth hr when a parasynchronous, near-doubling in the cell number began. Cells pretreated with PB for 12 hr showed a similar outgrowth pattern except that the yields were lower at both the first plateau and at the end of the parasynchronous wave. Cell cultures pretreated with PB for 24 hr showed no increase in cell number until about 8–10 hr after transfer to drug-free medium. After that time the outgrowth pattern was logarithmic.

The question arose whether the lag in growth of PB-treated cells in drug-free medium might be due to the presence of non-viable cells. Cells treated with or without the drug (1 mM) for 12 hr were harvested, washed and taken for cloning studies. The high viability of the 12-hr, PB-treated cells (99 and 89 per cent in two trials, Table 1) indicated that the lag in growth was not the result of any significant cell death. The lag

TABLE 1. EFFECT ON CLONING EFFICIENCY OF P815Y CELLS PRETREATED WITH PB FOR 12 HR*

Experiment	Pretreatment of culture	No. of cells/tube	Average No. of clones/tube†	Cloning efficiency (%)	Normalized cloning efficiency‡ (%)
1	Control	72	47	64	100
	1 mM PB	72	46	63	99
2	Control	72	50	69	100
	1 mM PB	72	44	61	89

* P815Y cells (100,000/ml) were grown for 12 hr in medium without or with PB (1 mM; near 100 per cent growth inhibition; Fig. 1), then sedimented and washed well before use in the cloning studies.
† Averages from 6 to 8 tubes.
‡ Normalized to a control value of 100 per cent.

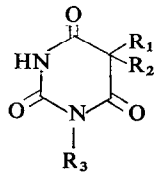
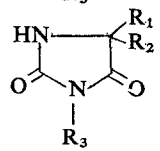
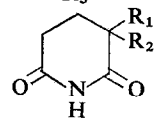
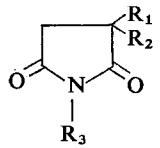
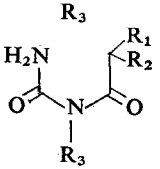
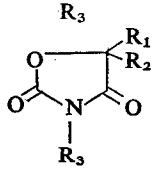
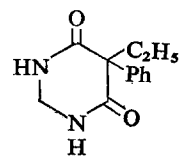
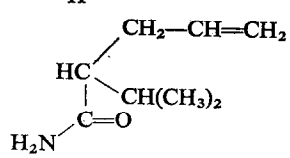
phase probably represented the time required for the diffusion of the drug away from critical inhibitory sites. It may also have been related to the time required for the resynthesis of some vital growth regulatory molecule(s) depleted during the inhibition of nucleic acid and protein synthesis by PB (see later results).

The inhibition of growth by other barbiturates was studied in an attempt to detect derivatives more active than PB in this respect. In these studies, thiamylal (0·1 mM), secobarbital (0·1 mM), amobarbital (0·2 mM), pentobarbital (0·4 mM), thiopental (0·4 mM), and dimethylethylbutylbarbiturate (0·5 mM) inhibited the growth of P815Y cells about equally well. (The ID_{50} values are shown in parentheses.) In contrast, phenobarbital showed no inhibition at 0·5 mM and only slight inhibition at 1 mM; barbital and barbituric acid failed to inhibit in concentrations as high as 1 mM. The inhibitory concentrations for the barbiturates described above corresponded well with those reported to inhibit the growth of a mammalian hepatoma cell in culture[5] and a cultured mouse heteroploid cell.[17]

Certain compounds bearing a structural resemblance to the barbiturates were tested to determine if structure-activity correlations could be established with respect to the inhibition of growth. In general, it was found that any departure from the barbiturate ring structure resulted in a loss of activity (Table 2). An exception was diphenylhydantoin (Dilantin) which caused growth inhibition comparable to that of PB. Parenthetically, this agent has been reported to have contrasting effects against cells in culture. In one study, it markedly stimulated the growth of human gingival cells at a concentration of 1 mM.[18] In another study the growth of L cells was inhibited at a final medium concentration of 0·1–0·5 mM.[19]

Inhibition of DNA, RNA and protein synthesis. The synthesis of DNA, RNA and protein, as determined by colorimetric analysis, was inhibited strongly by growth-inhibitory concentrations of PB. The effect was dose-related. Treatment of P815Y cells with 0·5 mM PB (ID_{50}) reduced the synthesis of DNA, RNA, and protein by roughly one-half compared with the control throughout a 12-hr period of measurement (Fig. 2). When the dose of PB was doubled (1 mM; ID_{100}), the synthesis of DNA and RNA during the first 4 hr in treated cells was again reduced and by an amount comparable with that of the 0·5 mM PB group. But after the fourth hr, the synthesis of these macromolecules appeared to be essentially blocked by 1 mM PB. The higher dose

TABLE 2. INHIBITION OF GROWTH OF THE P815Y CELL BY COMPOUNDS BEARING A STRUCTURAL RESEMBLANCE TO BARBITURATES*

Drug	Class structure	Compound(s) tested	Observed effect
Barbiturate		Pentobarbital	ID_{50} = 0·4 mM
Hydantoinate		Diphenylhydantoin	ID_{50} = 0·3 mM
Glutarimide		Glutethimide Thalidomide	30% inhibition at 1 mM No effect at 0·1 mM
Succinimide		Ethosuximide	15% inhibition at 1 mM
Acetylurea		Phenacemide Sedormid (R)	15% inhibition at 1 mM 10% inhibition at 1 mM
Oxazolidone		Trimethadione	No effect at 1 mM
Miscellaneous		Primidone	No effect at 1 mM
		Allylisopropylacetylurea	No effect at 1 mM

* Aliquots (0·05–0·2 ml) of a stock solution of the drugs were added as described in the legend of Table 1. Because of limited water solubility, glutethimide, thalidomide, phenacemide and Sedormid were first dissolved directly in the growth medium which was sterilely filtered before use.

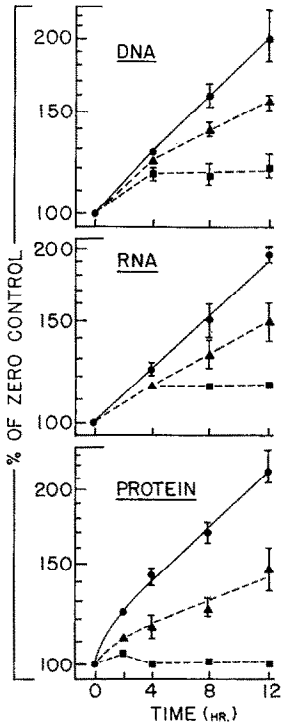


FIG. 2. Kinetics of inhibition of synthesis of DNA, RNA and protein in P815Y cells by PB. Cells (100,000/ml) were grown in 85-ml portions in 500-ml Erlenmeyer flasks in medium without or with PB (0·5 or 1 mM) for the periods indicated. At each of these times, 5 ml was withdrawn for the determination of the cell number and the remaining solution was sedimented by centrifugation and the cell pack was analyzed as described under Methods and Materials. The values are expressed as percentages of the value of the appropriate control at time zero. The respective zero control values for DNA, RNA and protein/80 ml of culture were as follows: 70, 475 and 1080 μg. The data points are the averages derived from the following numbers of experiments: DNA, five; RNA, two and protein, three. The ranges of the average values are indicated by the vertical bars. Vertical bars are not shown in those cases in which the range did not significantly exceed the width of the symbol. The range values for DNA at 4 hr were deleted from the figure for reasons of clarity. These were: control, 120–140 per cent; and 0·5 mM PB, 117–130 per cent. The symbols are: ●, control; ▲, 0·5 mM PB and ■, 1 mM PB.

of PB also appeared to block protein synthesis completely throughout the 12-hr period of study.

The incorporation of ^{3}H-TdR, ^{3}H-UR and ^{3}H-leu into an acid-insoluble form was used additionally to measure the capacity of the control and drug-treated cells to carry out the synthesis of DNA, RNA and protein respectively. By the end of 30 min the PB-treated cultures showed a marked reduction compared with the controls in the incorporation of all three of these precursors (Fig. 3). Indeed throughout the 12-hr period, the cumulative incorporation of these precursors was always much lower in the drug-treated cells than in the controls. The decreases were more marked with 1 than with 0·5 mM PB, in line with the above results obtained by direct colorimetric analysis. The DNA results were confirmed further in experiments not reported herein by showing that PB (0·5 or 1 mM) greatly reduced the incorporation into DNA of a pulse-dose (30 min) of ^{3}H-TdR measured after 4, 8 and 12 hr of drug treatment.

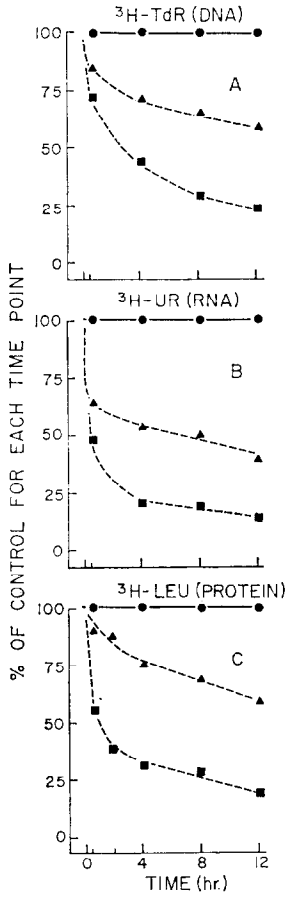


FIG. 3. Effect of PB on the cumulative incorporation of radioactive precursors of DNA, RNA and protein. The cells were prepared for analysis as described under Fig. 2. The appropriate radioactive precursors were added at time zero in the amounts indicated under Methods and Materials. The values are expressed as percentages of the control value (normalized to 100 per cent) for each time point. Radioactivity of the control in each case was: ^{3}H-TdR (30 min, 5448 counts/min/80 ml; 4 hr, 22,583 counts/min/80 ml; 8 hr, 50,770 counts/min/80 ml; 12 hr, 77,649 counts/min/80 ml); ^{3}H-UR (30 min, 6020 counts/min/80 ml; 4 hr, 81,020 counts/min/80 ml; 8 hr, 191,310 counts/min/80 ml; 12 hr, 370,830 counts/min/80 ml); and ^{3}H-leu (30 min, 7200 counts/min/80 ml; 2 hr, 19,584 counts/min/80 ml; 4 hr, 40,554 counts/min/80 ml; 8 hr, 84,096 counts/min/80 ml; 12 hr, 133,290 counts/min/80 ml). The data points are the averages from duplicate 80-ml aliquots. The symbols are the same as in Fig. 2.

In conjunction with the studies on reversal of growth inhibition by PB cited above, an experiment was carried out to determine how rapidly PB-treated cells, after transfer to drug-free medium, could achieve normal rates of synthesis of DNA, RNA and protein. P815Y cells were incubated for 12 hr in medium with 1 mM PB (ID_{100}); then they were sedimented and resuspended in drug-free medium. Portions of the culture were taken for the colorimetric analysis of DNA, RNA and protein every 4 hr over a 16-hr period. Initially, protein appeared to increase more rapidly than RNA or DNA (Fig. 4). It increased at a near-normal rate during the first 4 hr; then at a reduced rate from the fourth to the twelfth hr; and finally at a normal rate again from the twelfth to

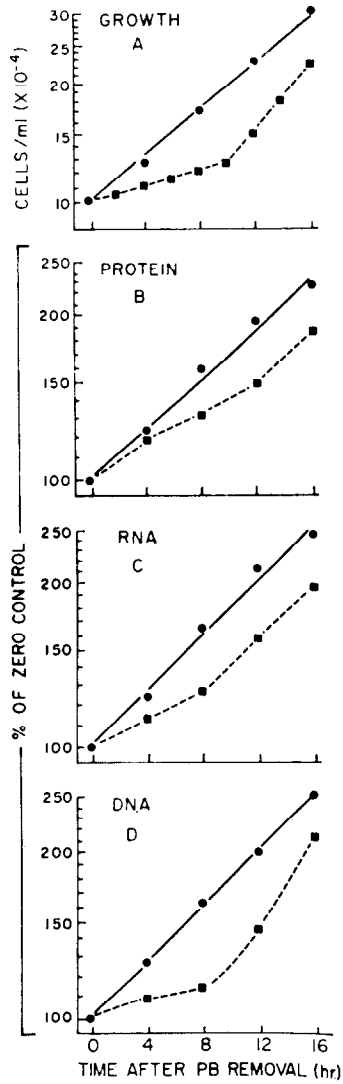


FIG. 4. Time course of the restoration of growth, and of the synthesis of DNA, RNA and protein in control or PB-pretreated P815Y cultures after resuspension of the cells in drug-free medium. P815Y cells (100,000/ml) were exposed to 1 mM PB for 12 hr. See Fig. 1 for the degree of growth inhibition in this case. At the end of this time, the cells were sedimented by centrifugation, washed and resuspended in fresh, warm medium and 85-ml aliquots were placed in 500-ml Erlenmeyer flasks and incubated at 37°. At the time intervals indicated, the flasks were removed from the incubator, the cell number was determined on a 5-ml aliquot and the DNA, RNA or protein content of the remainder was determined as described under Methods and Materials. The data points shown are the averages of duplicate 80-ml aliquots. The ranges of individual values for the various data points were not plotted using vertical bars, since these did not significantly exceed the widths of the symbols in any of the cases. The zero control values were: DNA, 80 μg/80 ml; RNA, 457 μg/80 ml and protein, 1440 μg/80 ml. The symbols are: ●, control; ■, PB-pretreated.

the sixteenth hr. RNA increased at a reduced rate for the first 8 hr, then at a normal rate from 8 to 16 hr. On the other hand, DNA increased relatively slowly during the first 8 hr. Over the next 8 hr, however, it increased at an above-normal rate. The kinetics were indicative of a parasynchronous mode of replication. In line with the results discussed earlier (cf Results, paragraph 2), the growth rate remained low initially before a parasynchronous pattern was established; thus active cell division did not begin until about 10 hr after transfer of the cells to drug-free medium.

Effect of PB on cells synchronized in division

Inhibition of cell growth. PB (1·5 mM; ID_{100}) was added at selected points within the cell cycle of L5178Y cells to determine the point of maximum sensitivity for the inhibition of cell division. When PB was added immediately after the cells were released from the mitotic block, only a limited fraction of the cells, ranging from 30 to 50 per cent in five different experiments, was able to complete mitosis (Fig. 5, zero hr). When PB was added after completion of the first mitotic wave (at 3 or 4 hr: late G_1 to early S), the subsequent division of the cells was totally blocked. When PB was added at the beginning of the second mitotic wave (at 8·5 hr), the yield of dividing cells was reduced by an amount comparable to that caused by PB addition at the zero hr.

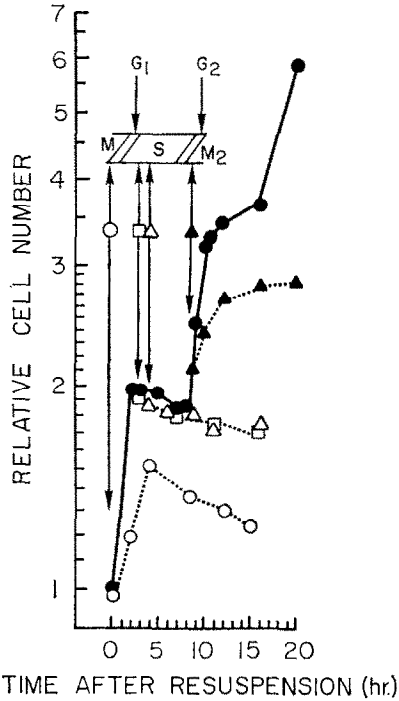


FIG. 5. Effect of PB on cell division of L5178Y cells synchronized in their growth cycle. PB was added at a final concentration of 1·5 mM at the points in the cell cycle indicated by the arrows. Control: ●, no addition of PB. Plus PB: ○, zero hr, first mitosis; □, 3 hr, late G_1-early S; △, 4 hr, middle S; ▲, 8·5 hr, second mitosis. The data shown are from a single experiment. The procedure was repeated with similar results in four other experiments.

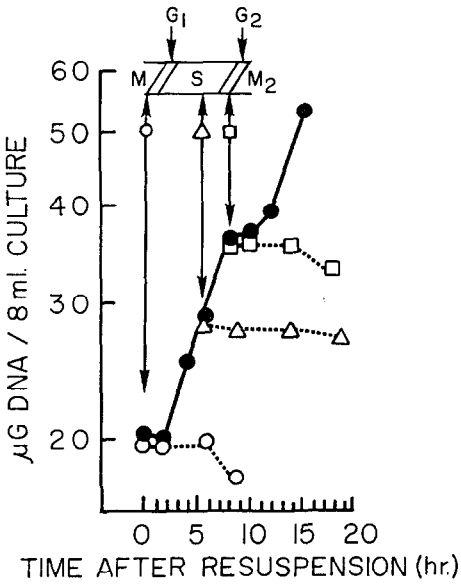


FIG. 6. Effect of PB on the content of DNA cultures of synchronized L5178Y cells. PB was added at a final concentration of 1·5 mM at the points indicated by the arrows. Control: ●, no addition of PB. Plus PB: ○, zero hr, first mitosis; △, 5·5 hr, middle S; □, 8 hr, second mitosis. Refer to Fig. 5 for representative growth curves.

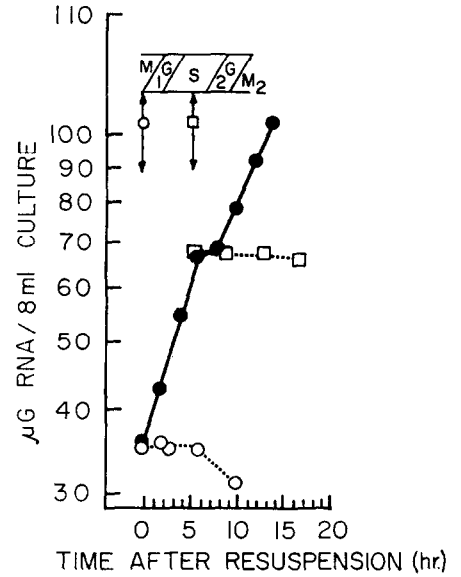


FIG. 7. Effect of PB on the content of RNA of cultures of synchronized L5178Y cells. PB was added at a final concentration of 1·5 mM at the points indicated by arrows. Control: ●, no addition of PB. Plus PB: ○, zero hr, first mitosis; □, 5·5 hr, middle S. Representative cell growth data appear in Fig. 5.

The division cycle of the L5178Y cell has been described by Bosmann:[20] the G_1-period is 1–1·5 hr; the S-period is 5–6 hr; the G_2-period is 1 hr; and the mitotic interval is 30 min.

Inhibition of DNA, RNA and protein synthesis. When PB was added immediately after release of the cells from the mitotic block, initiation of DNA synthesis was completely blocked (Fig. 6, zero hr). When the drug was added in mid-S (at 5·5 hr), there was also an immediate block on DNA synthesis. When it was added at the onset of the second mitotic wave (at 8·5 hr), initiation of DNA synthesis was blocked in a fashion similar to that caused by the addition of PB at the zero hr.

RNA and protein synthesis were also blocked in cultures treated with PB. The addition of the drug at either the onset of the first mitosis (at the zero hr) or in mid-S (at 5·5 hr) immediately blocked the production of these macromolecules (Figs. 7 and 8). The synthesis of RNA and protein in control cells released from the mitotic block

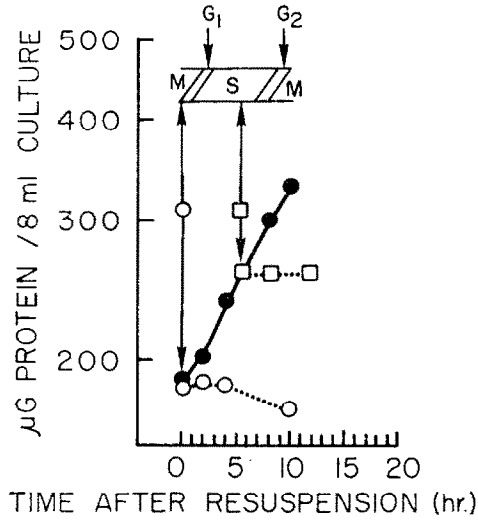


FIG. 8. Effect of PB on the content of protein of cultures of synchronized L5178Y cells. PB was added at a final concentration of 1·5 mM at the points indicated by the arrows. Control: ●, no addition of PB. Plus PB: ○, zero hr, first mitosis; □, 5·5 hr, middle S. Representative cell growth data appear in Fig. 5.

began immediately and continued throughout the cycle, in agreement with the pattern for the synthesis of these macromolecules in other synchronized cell systems.[21,22]

DISCUSSION

The barbiturates cause a wide range of inhibitory effects in biological systems: depression of the activity of nerve tissue, skeletal muscle, smooth muscle and cardiac muscle; inhibition of bioluminescence in bacteria; reduction in the oxygen consumption of mammalian tissues; and inhibition of oxidative phosphorylation in cell-free preparations of liver or brain mitochondria.[23] The barbiturates have also been shown to inhibit cell division and to inhibit the synthesis of nucleic acids and protein in the following systems: plants;[24–26] bacteria;[27–31] isolated partially purified enzyme

systems and particulate fractions of mammalian and bacterial origin;[32–35] animal tumors *in vivo*;[1,36] rapidly dividing tissues *in vivo*, namely, bone marrow,[37] jejunum,[1] and spleen;[1] and in normal[17] and neoplastic[1,5,38,39] mammalian cells grown in culture. In some of these systems, the inhibitory actions of barbiturates have been shown to be reversible. The barbiturates also stimulate the activity of the liver microsomal enzymes.[40] Despite these diverse findings, the precise mechanisms at the molecular level for the actions of barbiturates on cell metabolism and on the inhibition of growth and nucleic acid and protein synthesis remain unknown.

It was possible here to establish with improved resolution over previous investigations both the dose and time-course relationships for the inhibition by barbiturates of growth and of the synthesis of nucleic acids and proteins in cultured mammalian cells. The inhibition was manifest with barbiturate concentrations in the range of about 0·1–0·5 mM. This range is comparable to the range of concentration *in vivo* for certain therapeutic and toxic effects of barbiturates. For example, the plasma concentrations of PB or amobarbital required to maintain surgical anesthesia in the rabbit fall within a range of about 0·1–0·3 mM.[41] It is also known that administration of a dose of 20 mg/kg PB, which is sufficient to cause light anesthesia in rats and mice, depresses the synthesis of DNA in bone marrow,[37] jejunum and spleen.[1] Ancillary to these studies is the question of whether sufficient recognition has been given to the potential damage of administration of chronic, high doses of barbiturates in man, especially of damage to mitotically active cells.

We have observed, as have others,[1,5] that the inhibition of growth and of the synthesis of nucleic acids and proteins by barbiturates can be reversed after transfer of the cells to drug-free medium. In our studies two different outgrowth populations were discerned in cells treated for at least 12 hr with PB: one fraction which divided early, e.g. within 3–7 hr after treatment; and a second fraction which began division later, e.g. 10–12 hr after treatment. The early-dividing fraction could have resulted from cells blocked by the drug in the late S-, G_2- or M-stages of cell division. The low, progressive increase in the DNA content of the culture which began just after cessation of drug treatment is in accord with a late S-phase block (Fig. 4). Additionally, when PB was added to the synchronized cells either during release from the first mitotic block or after the second mitosis, only about one-half of the cell population was able to form daughter cells (Fig. 5). Thus, PB prevented a significant fraction of these cells from completing mitosis. A few isolated references in the literature indicate that barbiturates block cells in mitosis. Thus, a mitostatic action of PB on human lymphocytes has been reported.[42] PB was also shown to be a rapidly acting mitotic poison in a system in which mouse epidermis was studied.[43] The reasons for the possible mitostatic action of PB are unknown.

One question raised by our data is whether the inhibition of DNA synthesis by PB might be secondary to a primary inhibition of protein synthesis by the drug. It is well-documented in bacteria that the initiation of DNA synthesis, but not chain elongation, depends upon the continuation of protein synthesis.[44–46] In mammalian cells both initiation of DNA synthesis and chain elongation are dependent upon the continued synthesis of protein.[47–49] Thus, the early block in protein synthesis by PB (Fig. 2) might have been the cause, and not the result, of an inhibition of DNA synthesis. In line with this concept was the fact that both RNA and protein were synthesized well before DNA in PB-inhibited cells transferred to drug-free medium (Fig. 4). A similar

time sequence for the restoration of DNA, RNA and protein synthesis has been observed in other mammalian cell lines synchronized by the method of selective detachment of mitotic cells.[50,51]

Certain aspects of the physical and chemical interaction between barbiturates and cellular constituents should be considered in relation to the inhibitory action of the barbiturates studied here. Of these, the partition coefficient and ionization of barbiturates are important factors in determining the effects of the drug *in vivo*; and these undoubtedly play a role *in vitro*. The inability of barbital and phenobarbital to inhibit growth (cf. Results, paragraph 4) can probably be explained best in terms of their extremely low partition coefficients.[52] Barbituric acid with a pK_a of about 4 would exist totally in the dissociated (ionized) form at the pH of the medium (7·2) and this, coupled with its low partition coefficient, might explain its inability to inhibit the cells in culture.

Intracellular protein binding is probably a factor also governing barbiturate toxicity *in vitro*. In this regard the toxicity of the various barbiturates tested here (cf. Results, paragraph 4) correlates well with the ability of these agents to bind to plasma proteins.[53] Also in this regard, it has been proposed that PB inhibits the polymerization of deoxyribonucleoside triphosphates by interfering with DNA polymerase.[1] This concept was based upon the finding of increased levels of dTTP in PB-treated Ehrlich ascites cells *in vivo*. However, in our studies this effect was not confirmed, i.e. the percentage of dTTP was not increased relative to the control, both at 4 and at 12 hr of treatment with PB. (Results are not given here.) The inactivation of serum growth factors by PB binding was also apparently not a factor in our studies. An increase of the serum content of the medium of from 10 to 20 per cent did not reduce the inhibition of cell growth by PB nor did a reduction of the serum level of from 10 to 1 per cent increase the toxicity to PB. (Results are not given here.) However, so little is known concerning the nature of the specific proteins required for progression of mammalian cells through the cell cycle[54] that much more work would be needed before consideration could be given to possible inhibitory interactions between barbiturates and potential regulatory proteins of the cell.

Interaction between barbiturates and adenine-containing macromolecules in the cell might conceivably explain some of the inhibitory actions of the drug upon the synthesis of nucleic acids and protein. Recent work has shown that barbiturates form highly specific, hydrogen-bonded complexes with adenine[55–57] or with the adenine moieties of FAD and NAD.[58] Thus far these results apply only to observations made in aqueous solution with chemical derivatives of adenine. However, a logical extension of these findings might be the inactivation or alteration of messenger RNA through the binding of barbiturate to the recently discovered poly A-rich tracts of this class of polyribonucleotides.[59–63] Whether barbiturates are able to interact directly with adenine in nucleosides, nucleotides or even macromolecules within the cell and whether such interactions could cause some of the effects observed here is unknown, particularly when the complexities of the mammalian chromosomal structure are considered.[64] In our studies the addition to the medium of adenine, deoxyadenosine or dibutyryl cyclic AMP did not, however, reverse or oppose growth inhibition caused by PB. (Results are not included here.)

Finally, it was recently proposed by Beck and Mandel[4] that the transport of nucleosides is reduced in PB-treated cells. These workers concluded that the block by PB on

the synthesis of DNA in Ehrlich ascites cells and HeLa cells reported by Baserga and Weiss[1] was the result of a block in the transport of the labeled DNA nucleoside precursors rather than a block on their polymerization. We do not believe this criticism is germane here since we demonstrated by direct colorimetric analysis that PB blocked the increase in DNA *content* of the culture (Figs. 2 and 6). Moreover, the growth and nucleic acid metabolism of the P815Y and L5178Y cells does not depend upon the uptake of exogenous purines and pyrimidines from the medium, since these are not added, but are synthesized *de novo*.[9] Additionally, it was demonstrated by Rovera and Baserga[2] that PB directly inhibited RNA synthesis, as measured by the incorporation of ^{14}C-formate and ^{32}P-phosphate into RNA. In that study an indirect effect of PB on the uptake of radioactive precursor was ruled out.

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