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The Metabolic Effects of Nonvolatile Anesthetics on Mammalian Hepatoma Cells in Vitro:

II. Inhibition of Macromolecular Precursor Incorporation

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Mammalian hepatoma cells in suspension culture were exposed to thiopental, methohexital, amobarbital, and lidocaine in doses that reversibly inhibit cell multiplication. The effects of these drugs on the incorporation of exogenously administered macromolecular precursors (thymidine, uridine, and leucine) into their respective macromolecules (deoxyribonucleic acid, ribonucleic acid, and protein) were determined. All of these anesthetics produced nonselective, dose-related inhibition of precursor incorporation into the acid-insoluble cell fraction. This parallels their inhibitory effect on the rate of cell multiplication. The methodology used does not permit a more precise localization of the block in the incorporation processes. (Key words: Deoxyribonucleic acid; Ribonucleic acid; Protein; Macromolecular synthesis; Thymidine; Uridine; Leucine; Precursor transport; Thiopental, Methohexital; Amobarbital; Lidocaine.)

MOST ANESTHETIC DRUGS—as well as some narcotics and tranquilizers—interfere with cell multiplication,[1] and numerous anesthetics possess teratogenic properties.[2-7] In an attempt to understand the mechanisms underlying these adverse cellular effects of anesthetics, we have studied the effects of anesthetics on mammalian hepatoma cells grown in tissue culture. Recently we described the dose-related inhibition of replication of these cells induced by several barbiturates and local anesthetics.[8] In this paper, we describe the inhibitory effects of thiopental, methohexital, amobarbital and lidocaine on the incorporation of exogenous precursors into deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein.

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Methods

Rat hepatoma (HTC) cells were grown in continuous logarithmic suspension culture, as previously described.[8] This investigation of HTC cell replication had defined the reversibly inhibitory concentrations of sodium thiopental (Pentothal sodium, Abbott), sodium methohexital (Brevital sodium, Lilly), sodium amobarbital (Amytal sodium, Lilly) and lidocaine hydrochloride (Xylocaine hydrochloride, Astra).

The method used to investigate the effects of these four anesthetics on the cellular biosynthesis of deoxyribonucleic acid, ribonucleic acid, and protein involved measurement of the incorporation of exogenous radioactive labelled nucleosides or amino acids into cold acid-insoluble cell material.† The precursors used in these incorporation studies were methyl-^{3}H-thymidine (0.2 c/mM), uridine-2 ^{14}C 1.7 mc/mM) and L-(4,5-^{3}H) leucine (40 c/mM).‡ Approximately 12 minutes prior to the administration of drug or vehicle (control), the labelled precursor—tritiated thymidine (7.5×10^{-2} μM), carbon-14-uridine (1.5×10^{-1} μM), or tritiated L-leucine (3.0×10^{-4} μM)—was added to a separate 500-ml culture bottle containing 100 ml of logarithmically multiplying HTC cells at a density of 2.0×10^{5} cells/ml. At zero time, the drug or the appropriate ve-

† The acid-insoluble fraction of a cell is composed of macromolecules, such as the nucleic acids and proteins, which are insoluble in solutions of 4 per cent trichloroacetic acid.

‡ Fresh culture medium contained 26 mg/ml L-leucine.

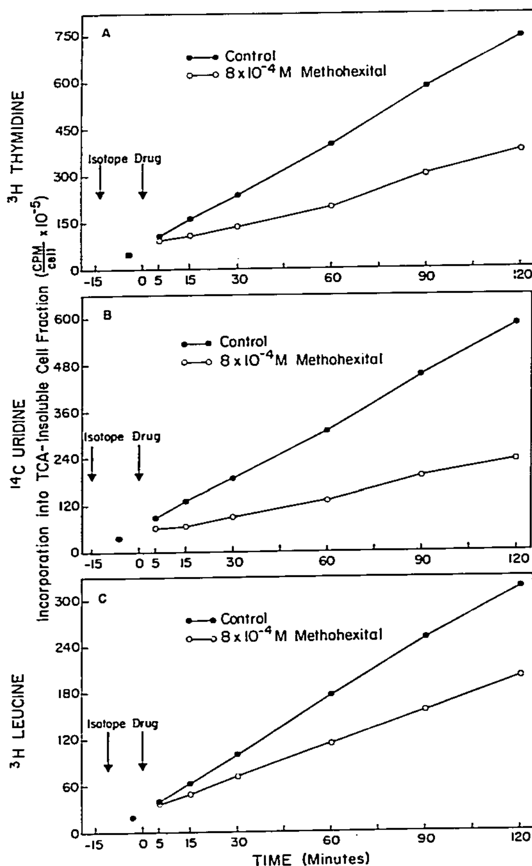

FIG. 1. A typical experiment examining the effects of nonvolatile anesthetics on the incorporation of exogenous macromolecular precursors into the TCA-insoluble fraction of rat hepatoma cells in suspension culture. The isotopes were added to the cell suspension about 12 minutes prior to drug administration (in this experiment, 8.0×10^{-4} M methohexital). Linear rates of incorporation of *a*) tritiated thymidine, *b*) carbon-14-uridine and *c*) tritiated L-leucine into the TCA-insoluble cell fraction were obtained for both control and treated cultures, the rates of incorporation of the methohexital-treated cultures being depressed.

hicle was added in a volume of 1 ml. Duplicate 5-ml volumes of the cell suspension were withdrawn 4 minutes before and 5, 15, 30, 60, 90, and 120 minutes after addition of the drug. The sample was centrifuged immediately at 2,500 rpm for one minute at 4 C. The supernatant was aspirated off and the resultant cell pellet was resuspended in cold 4 per cent trichloroacetic acid (TCA) containing 1 mg/ml of each of the unlabelled precursors, mixed vigorously, and allow to stand in ice for 20 minutes. The cold acid-precipitated material was collected by centrifugation at 2,500 rpm for two minutes, washed twice with the cold TCA solution, and collected by filtration with mild suction onto 2.4-cm Whatman glass-fiber filter discs. Each glass disc was then dried and placed in a liquid scintil-

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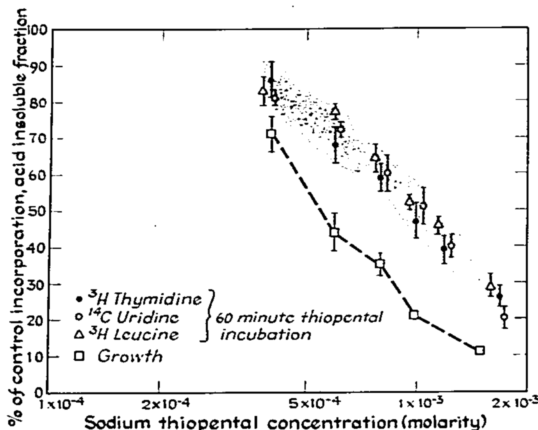

FIG. 2. The percentages of control incorporation of exogenous DNA, RNA and protein-precursors into their respective macromolecules by rat hepatoma cells in suspension culture treated 60 minutes with thiopental are plotted as a function of thiopental concentration. Because the points for the three precursor incorporations almost coincide, the general area representing these points is stippled. The broken line represents inhibition of cell multiplication (from data from our previous study[8]). Each point represents the mean, and the accompanying bars represent the standard error.

lation counting vial. Ten ml of scintillator solution (40 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene per liter of toluene) were added, and the radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

Results

The HTC cells incubated with labelled precursors were exposed to drug or vehicle and the incorporation of each labelled macromolecular precursor into the acid-insoluble cell fraction was plotted as a function of time for both control and drug-treated cultures. Figure 1 depicts a typical experiment for thymidine, uridine, and leucine incorporations; in this particular experiment, methohexital (8×10^{-4} M) was added to the experimental culture. Linear rates of incorporation of all three precursors with respect to time were obtained for both control and methohexital-treated cultures. However, the rate of incorporation for each of the drug-treated cultures was considerably less than that for its control. The maximum inhibitory effects of methohexital on the three precursor incorporations were achieved within 60 minutes. Similar patterns of inhibition were found for each of the four drugs studied.

The effect of drug dose on the inhibition of precursor incorporation into the acid-insoluble HTC cell fraction at 60 minutes was determined. Figure 2 is such a dose–response function for thiopental, and shows that the percentage inhibitions of the three precursor incorporations were similar at each thiopental concentration tested. These data suggest that thiopental produces nonselective, dose-related inhibition of precursor incorporation into the acid-insoluble cell fraction. The inhibition of incorporation parallels the inhibitory effect on the rate of cell replication (fig. 2). Table 1 summarizes the results of the same experiments for methohexital, amobarbital and lidocaine. With each, there was a dose-related inhibition of precursor incorporation into the acid-insoluble cell fraction, but there was no indication of any selective effect.

Discussion

Several sequential steps are involved in the biosynthesis of macromolecules from exogenous precursors. The extracellular precursor must first enter the cell. The nature of the transport mechanisms for nucleosides and amino acids into HTC cells has not been determined, but pyrimidine nucleosides and their analogues may be taken into mammalian

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cells by an uptake process other than simple diffusion, especially when they are at very low concentrations.[9-11] Likewise, the uptake of several amino acids into mammalian cells has been shown to be dependent upon active transport involving a cell membrane–carrier complex.[12] Intracellular nucleosides must be converted to triphosphorylated forms before they are incorporated into nucleic acids. Intracellular amino acids must be converted into phosphorylated amino acyl-activating enzyme complexes before their incorporation into protein.

The data in this paper demonstrate that thiopental, methohexital, amobarbital, and lidocaine effect dose-related inhibition of exogenous macromolecular precursor incorporation into the acid-insoluble cell fraction, but the techniques employed did not permit more precise localization of the site or sites at which this inhibition occurs. Only a few reports in the literature relate to the effects of anesthetics on macromolecular synthesis. From their experiments with Ehrlich ascites cells, Schmidt and Ryan concluded that lidocaine and procaine (4.0×10^{-3} M–4.0×10^{-2} M) depressed the syntheses of DNA (most sensitive), RNA, and protein[13]; however, their conclusions were achieved by equating inhibition of incorporation of precursors with inhibition of macromolecular synthesis, and this is unjustified. Other experiments with these cells *in vitro* have shown that pentobarbital (10^{-3} M) inhibits exogenous nucleoside incorporation into nucleic acids.[14,15] Mandel concluded that the inhibition was achieved by a block of the uptake of externally administered nucleic acid precursors,[15] and he strengthened this contention by showing in bacteria that barbiturates inhibited the transport system for the pyrimidine, orotate.[16,17] Using a murine mastocytoma cell culture system, Whyatt and Cramer demonstrated that barbiturates (10^{-3} M) depressed nucleic acid and protein syntheses, but because their cells did not require exogenous nucleic acid precursors for replication, they postulated that the underlying mechanism probably was not reduced transport of nucleosides.[18] We have found that barbiturates and lidocaine inhibit the HTC cellular uptake of nucleosides (unpublished data). The available evidence, therefore, suggests that nonvolatile anesthetics interfere with macromolecular precursor uptake processes and possibly also macromolecular syntheses. Anesthetic-induced interference with nucleoside or amino-acid uptake would be additional evidence for a significant effect of anesthetics on cellular uptake processes.[10] Although barbiturates impair aerobic metabolism, it is not known whether these effects on cellular energetics are related to depressed uptake of precursors or to their incorporation into macromolecules.

Drugs that inhibit protein synthesis prolong both barbiturate sleep time[20-23] and local an-

TABLE 1. Inhibition of Precursor Incorporation into Macromolecules

	Concentration	Per Cent of Control*			
		Cell Multiplication	Precursor Incorporation into DNA	Precursor Incorporation into RNA	Precursor Incorporation into Protein
Methohexital	2×10^{-4} M	70 ± 2	84 ± 4	80 ± 6	91 ± 4
	5×10^{-4} M	48 ± 2	67 ± 5	64 ± 6	83 ± 3
	8×10^{-4} M	14 ± 1	52 ± 4	42 ± 5	64 ± 5
Amobarbital	5×10^{-4} M	74 ± 4	80 ± 2	74 ± 4	87 ± 4
	8×10^{-4} M	50 ± 3	55 ± 6	60 ± 7	69 ± 5
	1.8×10^{-3} M	15 ± 2	42 ± 4	47 ± 3	52 + 5
Lidocaine	6×10^{-4} M	75 ± 3	98 ± 8	82 ± 7	84 ± 5
	1.1×10^{-3} M	54 ± 3	82 ± 3	64 ± 4	72 ± 6
	1.9×10^{-3} M	15 ± 2	68 ± 3	48 ± 3	61 ± 5

* Mean ± SE.

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esthetic blockade of neuronal tissue.[24] Our work has demonstrated that several barbiturates and lidocaine depress the incorporation of extracellular leucine into cellular protein, and raises the question whether, *if* barbiturates and local anesthetics were inhibitors of protein synthesis, they might necessarily prolong the rate of recovery from their anesthetic effects. In addition, in the clinical setting, patients undergoing anesthesia often receive other drugs (antibiotics, steroids, immunosuppressives, antimetabolites) known to inhibit protein synthesis which conceivably might act synergistically with anesthetics in this respect.

Each step in embryogenesis depends on a previous one, and because many tissues and organs are developing simultaneously, even a temporary delay in the development of one group of differentially more susceptible cells may place it out of phase with the remainder of the embryo and result in malformation. As would be expected, many drugs which inhibit cell division and/or macromolecular synthesis have been shown to be teratogens.[25] Several nonvolatile anesthetics now have been shown to inhibit cell multiplication and, possibly, events—still to be defined—which are associated with macromolecular synthesis.[8, 18, 26] These properties might be related to their suspected teratogenicity.[6, 7]

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Blood Transfusion

PLATELET STORAGE AT 22 C Recent studies indicate that for transfusion purposes platelet viability is maintained best at 22 C, rather than 4 C. The higher temperature is suitable when the platelets are stored for 3–4 days only. The present study has attempted to clarify metabolic, morphologic and functional changes which take place during platelet storage to uncover factors which contribute to deterioration, which occurs generally at the rate of 15–20 per cent of the platelet population per day even under optimal conditions.

Platelet-rich plasma (PRP) stored for 3–4 days and platelet concentrates (PC) stored for 24 hours were evaluated. Lactate accumulated continuously without significant change in platelet count, *p*H or plasma glucose. However, platelet glycogen, determined by chemical assay and by electron microscopy, decreased, while adenosine triphosphate (ATP), adenosine disphosphate (ADP), and intracellular potassium did not change. After storage, the capacity for glucose utilization by way of glyoclysis, the hexose monophosphate shunt, and the tricarboxylic acid cycle remained intact. Although platelet volume did not change during storage, disc-to-sphere transformation was observed by phase-contrast microscopy. Platelet aggregation with ADP was reduced after a day of storage. After transfusion of stored platelets to thrombocytopenic patients, recovery of platelet glycogen and capacity for aggregation occurred within 24 hours. The platelets remained intact during the intervals studied; defects which do develop are reversible after transfusion even in the circulation of a thrombocytopenic recipient whenever viability has been maintained. The authors were unable to identify a "storage lesion" responsible for the loss of viability.

The investigation underscores the basic principle that neither viability nor functional capacity of a stored transfusion product may be predicted according to its performance *in vitro* before transfusion. It would appear that once viability has been established only bleeding-time measurements and careful clinical evaluation following platelet transfusion will demonstrate the efficacy of these stored products. (*Murphy, S. and Gardner, F. H.: Platelet Storage at 22 C; Metabolic, Morphologic, and Functional Studies, J. Clin. Invest. 50: 370 (Feb.) 1971.*)

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