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

I. Inhibition of Cell Replication

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Mammalian hepatoma liver cells were grown as a suspension tissue culture to allow investigation of the effects of nonvolatile anesthetics on cell replication. Logarithmic growth of a rat hepatoma tissue culture was reversibly inhibited by thiopental, methohexital, amobarbital, phenobarbital, lidocaine, and prilocaine in a dose-dependent fashion. Barbituric acid also reversibly inhibited logarithmic growth, but only at drug concentrations greater than those necessary for inhibition by the anesthetic drugs. (Key words: Cell multiplication; Suspension tissue culture; Thiopental; Methohexital; Amobarbital; Phenobarbital; Lidocaine; Prilocaine; Barbituric acid; Drug resistance.)

MOST ANESTHETIC DRUGS, as well as some narcotics and tranquilizers, interfere with cell multiplication.[1] Neither the clinical significance nor the underlying mechanism(s) of this important property of anesthetics has been determined. The inhibition of cell replication by several barbiturates has recently been studied in tissue culture.[2-4] We have used a different tissue-culture system for our studies of the metabolic effects of anesthetics, and this paper describes the reversible inhibition of replication of mammalian hepatoma (liver) cells by local anesthetic drugs, barbituric acid, and several barbiturates.

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Methods

Rat hepatoma tissue culture (HTC) cells were used for study. The HTC cells were originally derived from the ascites form of a rat-carried Morris hepatoma, no. 7288C, which, in turn, had been derived from a solid hepatoma.[5] HTC cells are hypotetraploid and their morphologic appearance is that of epithelioid cells.[5, 6] They have a logarithmic (exponential) growth phase in either monolayer or suspension culture, with a doubling time in suspension culture of approximately a day at 37 C. *In vitro* HTC cells retain the biochemical ability to increase their rate of synthesis of tyrosine aminotransferase in the presence of a glucocorticoid hormone inducer.

The growth experiments reported in this paper were performed during a period of 21 months at two institutions. The HTC cells used at the two institutions unavoidably were derived from different clones.‡ In order to ascertain that the cells remained grossly similar in biochemical properties throughout the experimental period, they were intermittently tested and demonstrated to possess a glucocorticoid-mediated increase in tyrosine aminotransferase biosynthesis.[7, 8]

The basic growth substratum was a modified Swim's S-77 medium without sodium bicarbonate,§ to which N-tris (hydroxymethyl) methyl glycine,[9] heat-inactivated bovine sera and fetal bovine sera, cystine, glucose, penicillin, streptomycin, and freshly thawed gluta-

‡ Courtesy of Dr. Gordon Tomkins, Department of Biochemistry, University of California, San Francisco, California.

§ Grand Island Biological Company, Grand Island, New York.

mine were added. Cells were grown in suspension in volumes of 100–400 ml of media in 500-ml loosely capped, screw-top glass bottles. Teflon-covered 2-inch magnetic stirrers (50–60 r.p.m.) were used to maintain cellular suspension without inducing mechanical injury. Incubation temperature was maintained at 36–37 C.

Logarithmic growth of the culture was investigated at suspended cell densities in ranges from 40,000 to 550,000 cells/ml. Cells were counted in a hemocytometer or with an electronic Coulter counter. The coefficient of variation of sample counts was small (<2 per cent) and constant throughout the experimental range. Monthly cultures for bacteria, fungi, and pleuropneumonia-like organisms demonstrated the absence of nonviral infections.

The drugs investigated were barbituric acid, sodium amobarbital (Amytal, Lilly), sodium phenobarbital (Luminal, Winthrop), sodium thiopental (Pentothal, Abbott), sodium methohexital (Brevital, Lilly), lidocaine hydrochloride (Xylocaine, Astra), and prilocaine hydrochloride (Citanest, Astra). These drugs contained no organic preservative, but the sodium thiopental and sodium methohexital contained sodium carbonate. The *p*H of barbituric acid was adjusted with sodium hydroxide to 6.9. No significant change in *p*H or osmolality of the culture medium was found after addition of these drugs at concentrations ranging from 10^{-5} M to 10^{-2} M. Control cultures received equivalent volumes of water and, when appropriate, inorganic salts. Each drug concentration examined was tested on at least five separate occasions at each institution.

In a typical experiment, HTC cells in high-density exponential growth were initially divided into one control and three experimental samples of low cell density (*e.g.*, 50,000/ml) and placed in bottles. After 23 hours, continuous logarithmic growth was verified through cell count determination. At hour 24, drugs were added aseptically. Cell counts were again determined at 48, 72 and 96 hours, and the doubling time for the 48–72 hour interval was calculated. The equation describing exponential growth of HTC cells, which multiply in binary fashion, is [10]:

$$S(t_1) = S(t_o)\, 2^{\frac{t_1 - t_o}{D}} \qquad (1)$$

where

$S(t)$ = number of cells per ml at time t;
t_1 = time in hours the second day after drug administration;
t_o = time in hours the first day after drug administration;
D = doubling time, which is that period of time necessary for the cell population to double in number.

Solving (1) for D,

$$D = \frac{0.3\ (t_1 - t_o)}{\log S(t_1) - \log S(t_o)} \qquad (2)$$

The growth rate is conveniently expressed as the inverse of the doubling time in generations per hours. Drug-induced growth inhibition (I) was determined by expressing the growth rate of each drug-treated culture as a percentage of the growth rate of its control culture. A dose–response function could then be constructed for each drug, with the derived value of I as the response.

Reversibility of effect was tested for those drug concentrations effecting I values as low as 10 per cent by resuspending the 48-hour-treated cells in fresh growth medium and then following daily cell counts. Reversibility was defined as re-establishment of an I value of 100 per cent.

Results

Figure 1 depicts the results of a typical experiment. Growth rates in the presence of increasing concentrations of methohexital (5×10^{-5} M, 5×10^{-4} M, 9×10^{-4} M) were compared with control values. At hour 23, all four bottles showed continuing exponential growth, with doubling times of about 22 hours. Methohexital was added at hour 24. Cell counts at hours 48 and 72 indicated that the cell populations in all four bottles were increasing, but at different rates. The doubling times (48–72-hour interval) of the control and the bottle containing the lowest concentration of methohexital were essentially identical (23 hours), but the doubling times of the two higher concentrations of methohexital were in-

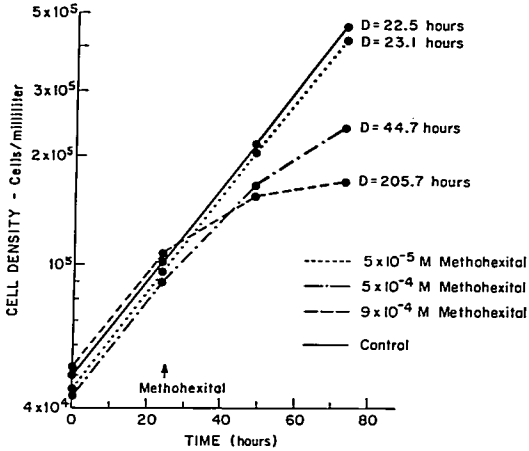

FIG. 1. Typical growth experiment. Doubling times (*D*) during the 48- to 72-hour time interval following drug administration increase with increasing concentrations of drug.

creased. As a result, *I* for the 5×10^{-4} M bottle was 50 per cent; for the 9×10^{-4} M bottle *I* was 11 per cent. The doubling times during the 72–96-hour period were similar to those during the 48–72-hour period.

At appropriate concentrations all drugs inhibited logarithmic growth. Figures 2 and 3 show *I* plotted as a function of drug dose. For comparative purposes, the dose necessary to cause an *I* of 50 per cent (I_{50}) was determined for each drug. These equipotent concentrations were: lidocaine, 1.2×10^{-3} M; prilocaine, 9.1×10^{-4} M; methohexital, 4.6×10^{-4} M; phenobarbital, 4.0×10^{-3} M. The Stanford HTC cell line was less susceptible than the NIH cell line to the growth-inhibitory effects of thiopental and amobarbital, so that the I_{50} drug concentration increased from 3.1×10^{-4} M (NIH) to 5.7×10^{-4} M (Stanford) for thiopental and from 5.0×10^{-4} M (NIH) to 8.0×10^{-4} M (Stanford) for amobarbital (fig. 3). At concentrations as high as 3.0×10^{-2} M, barbituric acid failed to inhibit growth to a degree sufficient to allow determination of an I_{50}.

With the exception of phenobarbital, the effects of drug concentrations producing *I* values of 10 per cent or greater were entirely reversible with a single cell washing. Phenobarbital-treated cells required several prolonged washings before complete reversal of growth inhibition could be obtained.

Discussion

Animal cells in tissue culture show the classical growth kinetics of bacteria, yeasts and protozoa.[11] When a sample of HTC cells is taken from a dense stationary culture and inoculated into fresh medium, the new culture's growth will follow a definite course. During the *lag phase*, cell multiplication is slow, but growth soon becomes apparent as the population enters the *logarithmic phase*, in which there is regular cell multiplication at maximal speed. Since there is a geometric progression in cell count, the logarithm of the count is a linear function of time. We chose to compare the effects of drugs on cell multiplication during this logarithmic growth phase only, because it is the most definable and reproducible physiologic state of a tissue culture. Vital-stain studies demonstrated that fewer than 2 per cent of our HTC cells in a logarithmic suspension were nonviable, and this indicated that our index of growth, the doubling time, was similar to the actual generation time. The logarithmic phase ends when the cells' environment is so altered by their own metabolic

activity that it no longer provides the milieu necessary to maintain their previous rate of growth; then the *stationary phase* begins.

The monolayer technique of tissue culture involves the growth of cells on a surface, usually under a stationary liquid environment. Within this monolayer system, cells may not be in equilibrium with their liquid environment, which, in turn, may not be in equilibrium with the gas phase above.[‡] The suspension technique of tissue culture, in which the cells are freely suspended in their liquid medium, is an attempt to approximate more closely such an equilibrium between the cells and their liquid and gaseous environment, particularly when the culture's surface-to-volume ratio is large. However, even in this system, although the reservoir gas above the cell suspension was in continuum with room air, carbon dioxide accumulated slowly ($P_{CO_2} < 10$ torr), and an air-to-medium oxygen gradient—greater with larger cell densities and smaller surface-to-volume ratios of the suspension—also developed slowly. Although true equilibration was not achieved, P_{O_2} (4–110 torr), P_{CO_2} (5–50 torr), and *p*H (6.75–7.50) always were adequate to support rapid logarithmic growth. Kenny also demonstrated a broad optimal range of P_{O_2} for growth of several cell strains in tissue culture.[12] However, he measured the oxygen tension of the gas above his monolayer cultures, and, as Nunn [13] suggested, the oxygen tension of the medium itself was almost certainly much lower.

Our results demonstrated a dose-related inhibition of cell-culture logarithmic growth by several barbiturate compounds and local anesthetics. Growth-inhibitory concentrations of several barbiturates for a murine mastocytoma culture [4] and of amobarbital for a mouse heteroploid culture [3] approximated those of our system. Fink demonstrated a dose-related depression of oxygen consumption of mouse heteroploid cultures with the same drugs used in our growth study.[14] The 50 per cent inhibitory drug concentrations determined for his system were two to 40 times greater than those of our growth system, and little or no depression of oxygen consumption occurred at the I_{50} drug doses determined by our growth system. Our effective growth-inhibitory drug concentrations do correspond more closely to those found in other *in vitro* investigations of oxygen uptake,[15-17] however, thereby underlining the variation in susceptibility of *in vitro* systems to growth-inhibitory drugs.

‡ The roller-tube system of monolayer culture may more closely approximate equilibrium than does the classical stationary monolayer system.

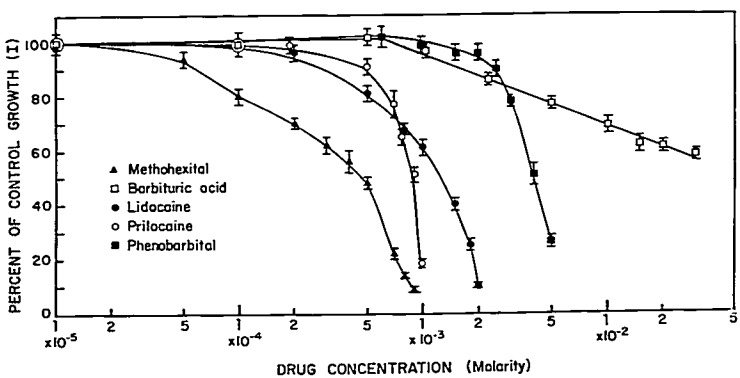

FIG. 2. Drug-induced inhibition of culture growth (cell multiplication). Growth inhibition (*I*) is determined by expressing the growth rate of a drug-treated culture as a percentage of the growth rate of its control culture. Each point represents the mean, and the accompanying bars represent the standard error.

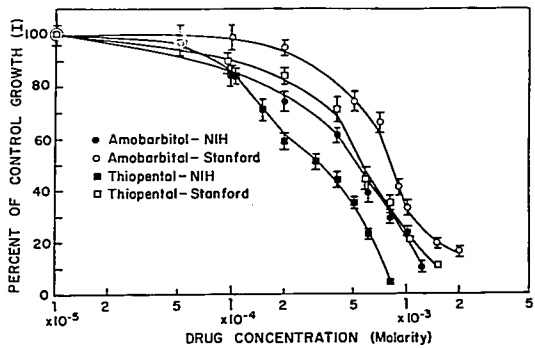

FIG. 3. Drug-induced inhibition of culture growth (cell multiplication). Growth inhibition (*I*) is determined by expressing the growth rate of a drug-treated culture as a percentage of the growth rate of its control culture. The Stanford HTC cell line is less susceptible than the NIH HTC cell line to the growth inhibitory effects of thiopental and amobarbital. Each point represents the mean, and the accompanying bars represent the standard error.

Barbituric acid (malonylurea), the unsubstituted parent compound from which all barbiturates are derived, is not a central nervous system depressant.[18] Its pharmacologic impotency is predictable from its pK_a (4.03[19]), from which it is calculated that only 0.1 per cent of barbituric acid exists in the nonionized form at physiologic *p*H. Indeed, 3×10^{-2} M barbituric acid effected an *I* value of 58 per cent. However, if only the concentration of nonionized drug were being considered, our results might be interpreted as indicating that barbituric acid is the most potent barbiturate inhibitor of cell replication.

The mechanism(s) underlying the inhibitory effects of anesthetics on cell replication is unknown, but presumably would be a result of either direct or indirect (primary depression of energy production) interference with one or more stages of the cell cycle. Excluding grossly toxic doses, barbiturate interference with oxidative metabolism has not been shown to cause inhibition or aberration of cell replication. Analysis of data from studies of the effects of amobarbital on both cell multiplication[3] and oxygen uptake[11] in the same culture system indicates that amobarbital inhibited both to similar degrees. Fink even showed that, within limits, in the presence of glucose in amounts sufficient to support a compensatory increase of anaerobic glycolysis amobarbital may decrease aerobic metabolism without affecting cell culture growth.[2] This suggests that biochemical processes more directly associated with replication should be investigated.

The demonstration of the reversibility of barbiturate-induced growth inhibition confirms recent work with amobarbital[3] and pentobarbital[4] and extends these observations to other barbiturate compounds and local anesthetics. Whereas we showed reversal after two days of drug treatment, Fink was able to reverse 28 days of amobarbital-mediated growth inhibition.[2] Two explanations for this reversibility are offered: 1) viability of the entire cell culture was maintained during the variable arrest of cell multiplication; 2) viability was retained by a genetically resistant segment of the cell culture. Fink's data generally supported the former explanation. Our unpublished observations of the time course of reversal also support the first explanation, as does the recent work of Whyatt, who cloned pentobarbital-treated cells and found that the entire drug-treated cell population remained viable.[20] The difficulty that we encountered in reversing phenobarbital-induced growth inhibition remains unexplained.

This investigation has provided further evidence that anesthetics interfere with cellular multiplication, and this characteristic of anesthetics might be related to their toxic properties. For instance, each step in embryogenesis depends on a previous one, and consequently, even a temporary delay in the replication of one group of differentially susceptible cells may place it out of phase with the remainder of the embryo and result in malformation. Indeed, many drugs which inhibit cellular replication have been shown to be teratogens,[21] and

increasing numbers of anesthetics have been found to possess teratogenic properties.[22-27]

The difference between sensitivities to thiopental and amobarbital found in our two lines of HTC cells was not unexpected and reflects a haunting problem for tissue culturists, namely, the difficulty in experimenting with genetically identical cells during a prolonged period of investigation. Our two cell lines were apparently identical in four characteristics: 1) histologic appearance; 2) control growth; 3) methohexital, phenobarbital, prilocaine, and lidocaine growth-inhibitory responses; 4) tyrosine aminotransferase induction. However, the two lines differed in their adhesive properties in monolayer culture, and the Stanford line was more resistant to growth inhibition by both thiopental and amobarbital. These results suggest that during tissue culture investigations it is essential to determine intermittently by several measures the nature of the cell line being used.

References

1. Andersen N: The effects of CNS depressants on mitosis. Acta Anaesth Scand suppl 22: 1–36, 1966
2. Fink BR, Kenny GE: Effects of halothane on cell culture metabolism, Toxicity of Anesthetics. Edited by BR Fink. Baltimore, Williams & Wilkins, 1968, pp 37–49
3. Fink BR, Kenny GE: Long-term reversible arrest of cell growth by amobarbital. ANESTHESIOLOGY 32:300–305, 1970
4. Whyatt P, Cramer J: Barbiturates in cell cultures as inhibitors of cell growth and macromolecular synthesis. Pharmacologist 12:298, 1970
5. Thompson EG, Tomkins GM, Curran JF: Induction of tyrosine α ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. Proc Nat Acad Sci 56:296–303, 1966
6. Tomkins GM, Thompson EB, Hayashi S, *et al.*: Tyrosine transaminase induction in mammalian cells in tissue culture. Cold Spring Harbor Symp Quant Biol 31:349–360, 1966
7. Hayashi S, Granner DK, Tomkins GM: Tyrosine aminotransferase purification and characterization. J Biol Chem 242:3998–4006, 1967
8. Diamondstone TI: Assay of tyrosine transaminase activity by conversion of p-hydroxyphenylpyruvate to p-hydroxybenzaldehyde. Anal Biochem 16:395–401, 1966
9. Gardner R: The use of tricine buffer in animal tissue culture. J Cell Biol. 42:320–321, 1969
10. Clifton C: Introduction to Bacterial Physiology. New York, McGraw-Hill, 1957, pp 293–295
11. Paul J: Cell and Tissue Culture. Third edition. Edinburgh, E. and S. Livingstone, 1965, pp 49–52
12. Kenny GE, Fink BR: The effect of oxygen tension on growth and glucose consumption of cells in culture, Toxicity of Anesthetics. Edited by BR Fink. Baltimore, Williams & Wilkins, 1968, pp 11–23
13. Nunn H: Discussion after presentation, Toxicity of Anesthesia. Edited by BR Fink. Baltimore, Williams & Wilkins, 1968, p 22
14. Fink BR, Kenny GE, Simpson WE III: Depression of oxygen uptake in cell culture by volatile, barbiturate and local anesthetics. ANESTHESIOLOGY 30:150–155, 1969
15. Webb JL, Elliott KAC: Effects of narcotics and convulsants on tissue glycolysis and respiration. J Pharmacol Exp Ther 103:24–34, 1951
16. Aldridge WN, Parker VH: Barbiturates and oxidative phosphorylation. Biochem J 76: 47–56, 1960
17. Chance B, Hess B: Metabolic control mechanisms. III. Kinetics of oxygen utilization in ascites tumor cells. J Biol Chem 234:2416–2420, 1959
18. Sharpless S: The barbiturates, The Pharmacological Basis of Therapeutics. Edited by LS Goodman and A Gilman. New York, Macmillan, 1965, pp 105–128
19. Mandel H, Riss M: Interference of barbiturates with pyrimidine incorporation. II. Structural specificity of the inhibition of orotate uptake in *Bacillus cereus*. Biochem Pharmacol 19: 1867–1872, 1970
20. Whyatt P: Personal communication
21. Goldstein A, Aronow L, Kalman S: Principles of Drug Action. Harper and Row, New York, 1969, pp 711–734
22. Andersen N: The toxic and teratogenic effect of cyclopropane in chicken embryos, Toxicity of Anesthetics. Edited by BR Fink. Baltimore, Williams & Wilkins, 1968, pp 294–307
23. Shepard T, Fink B: Teratogenic activity of nitrous oxide in rats, Toxicity of Anesthetics. Edited by BR Fink. Baltimore, Williams & Wilkins, 1968, pp 308–323
24. Basford A, Fink B: The teratogenicity of halothane in the rat. ANESTHESIOLOGY 29:1167–1173, 1968
25. Mellin G: Drugs in the first trimester of pregnancy and the fetal life of *Homo sapiens*. Amer J Obstet Gynec 90:1169–1180, 1964
26. Smith B, Gaub M, Lehrer S: Teratogenic effects of diethylether in the chicken embryo, Toxicity of Anesthetics. Edited by BR Fink. Baltimore, Williams & Wilkins, 1968, pp 269–278
27. Cahen R: Experimental and clinical chemoteratogenesis. Advances Pharmacol 4:263–349, 1966