

The Metabolic Effects of Halothane on Mammalian Hepatoma Cells in Vitro:

II. Inhibition of DNA Synthesis

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Halothane inhibits cell replication and depresses the incorporation of ^3H -thymidine into trichloroacetic-acid (TCA)-insoluble material in a concentration-dependent manner. Three per cent halothane effects a time-dependent decrease of incorporation of ^3H -thymidine into both TCA-soluble and TCA-insoluble material. The inhibition of incorporation into TCA-soluble material is delayed and of smaller magnitude. This indicates that halothane inhibits the synthesis of DNA and does not affect thymidine transport. (Key words: Halothane; DNA synthesis; Thymidine transport; Thymidine incorporation; Transport.)

CELL MULTIPLICATION is inhibited by halothane^{1,2}; however, the stage(s) within the cell cycle at which the inhibition occurs has not been determined. Nunn and co-workers have suggested that halothane inhibits mitosis (M stage),³⁻⁵ whereas Bruce^{6,7} and Cullen⁸ have presented evidence that DNA synthesis (S stage) is inhibited. In the latter experiments DNA synthesis was measured by—and equated with—cellular incorporation of exogenously administered tritiated thymidine into trichloroacetic acid (TCA)-insoluble material. Whether the inhibition of extracellular thymidine incorporation into TCA-insoluble material by halothane represents actual inhibition of nucleic acid synthesis or inhibition of the transport of thymidine into the cell⁹ has not been previously investigated. The evidence presented in this paper suggests that halothane inhibits DNA synthesis without affecting thymidine transport.

Methods

The maintenance of HTC rat hepatoma cells and the system for delivery of halothane have been described.^{2,10}

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Accepted for publication March 29, 1973. Supported by Ayerst Laboratories Grant-in-Aid Support for Research in Pharmacology.

In experiments examining the effect of halothane dosage on the incorporation of tritiated thymidine into TCA-insoluble material, 10 ml of logarithmically growing HTC cells (7.5×10^5 cells/ml) were added to 90 ml of medium pre-equilibrated with carrier gas (98 per cent air, 2 per cent CO_2) or halothane (0.1–5 per cent) in carrier gas. Four bottles were employed for each control or treated group, and each halothane concentration was examined on three separate occasions. The cells were treated with halothane for 120 minutes prior to the addition of 2.2×10^{-5} M methyl- ^3H -thymidine (0.24 Ci/mM). Sixty minutes later, duplicate 10-ml aliquots were withdrawn and centrifuged immediately at $1,500 \times g$ for 1 minute at 4 C. The supernatant was aspirated, and the resultant cell pellet was resuspended in 5 ml of ice-cold 4 per cent TCA containing 1 mg/ml of unlabelled thymidine, mixed vigorously, and allowed to stand in ice for 20 minutes. The TCA-insoluble material⁹ was collected on 2.4-cm Whatman glass-fiber filter discs, which were dried and placed in scintillation vials to which 10 ml of scintillation fluid (40 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-[2(5-methyl-5-phenyloxazolyl)]-benzene per liter of toluene) were added. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

The effect of 3 per cent halothane on the time course of incorporation of ^3H -thymidine into the intracellular 1) TCA-soluble material (DNA precursor pool) and 2) TCA-insoluble material (DNA) was determined by the following two methods.

SINGLE LABEL

Sixty milliliters of culture medium were pre-equilibrated with carrier gas or 3 per cent halothane in carrier gas (four bottles in the control group, eight bottles in the treated

⁹ 94 per cent of the TCA-insoluble ^3H -thymidine is DNA in our HTC cells.

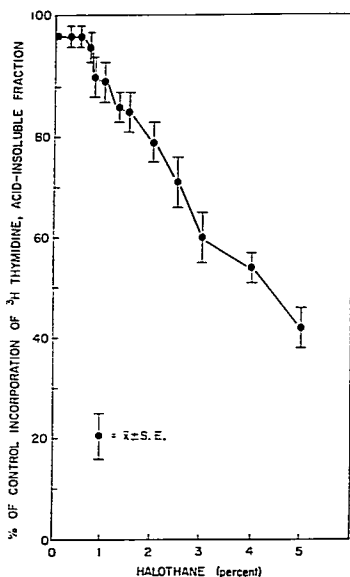


FIG. 1. Concentration-dependent inhibition of ³H-thymidine incorporation into TCA-insoluble material by halothane treatment for 180 minutes.

group, three experiments). At zero time, 7 ml of logarithmically growing HTC cells (7.5×10^5 cells/ml) were added to each bottle. Methyl-³H-thymidine, 1.6×10^{-5} M (0.2 Ci/mM), was added to one control and two halothane-treated cultures at zero time, 30, 60 and 90 minutes. Duplicate 10-ml aliquots were removed 30 minutes later and added to an equal volume of ice-cold N-tris (hydroxymethyl) methylglycine †-buffered balanced salt solution containing 2×10^{-6} M unlabelled thymidine. After centrifugation at $1,500 \times g$ for 1 minute, the supernatant was aspirated and the cell pellet was resuspended in 2 ml of water and sonicated. Of the sonicate (total uptake), 0.2 ml was added to liquid scintillation-counting vials containing 1 ml NCS ‡ tis-

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‡ Amersham/Searle Corporation, Arlington Heights, Illinois.

sue solubilizer and incubated at 37 C for one hour prior to addition of scintillation fluid. One milliliter of 8 per cent TCA was added to ml of the sonicate, which was mixed vigorously, allowed to stand in ice for 20 minutes and then assayed for TCA-insoluble material as described above, except that the filter discs were incubated with NCS solubilizer for one hour at 37 C. After correction for sonicate volumes, the difference between the values for total uptake and TCA-insoluble material represented the ³H-thymidine counts in the TCA-soluble material.

DOUBLE LABEL

¹⁴C-inulin was utilized as an extracellular fluid marker, allowing for measurement of extracellular ³H-thymidine contamination of the cell pellet.^{11, 12} This technique made it possible: 1) to investigate the early (10-30 minutes)

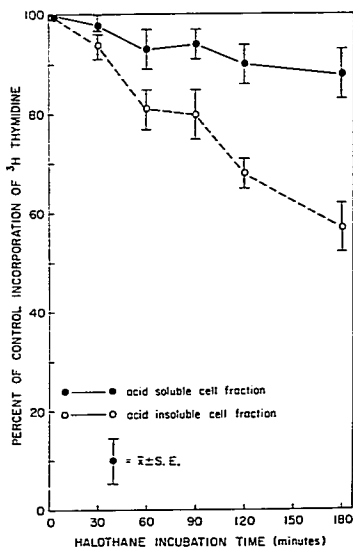


FIG. 2. Time-dependent inhibition of ³H-thymidine incorporation into both TCA-soluble and TCA-insoluble material by 3 per cent halothane. Single-label experiment.

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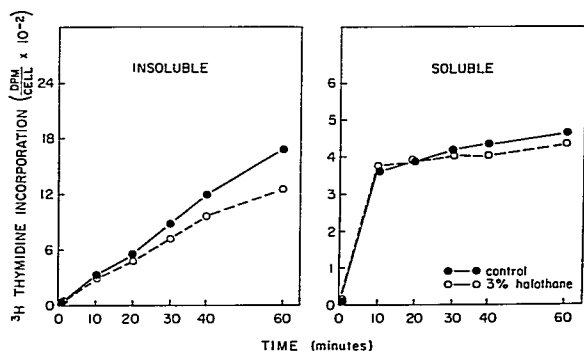


FIG. 3. Inhibition of ^3H -thymidine incorporation into both TCA-soluble and TCA-insoluble material by 3 per cent halothane. Early time course, one of two essentially identical double-label experiments employing larger amounts of ^3H -thymidine and ^{14}C -inulin.

time course for the incorporation of ^3H -thymidine into TCA-soluble and TCA-insoluble material, and 2) to compare the single- and double-label techniques following 30 and 60 minutes of 3 per cent halothane treatment. Three double-label experiments were performed. In two of these, 3×10^{-9} M ^3H -thymidine (0.09 Ci/mM) and $0.5 \mu\text{Ci } ^{14}\text{C}$ -inulin (2.2 mCi/g) were added to the pre-equilibrated medium one minute prior to addition of cells. A third such experiment employed smaller amounts of radioactivity. Cells were added at zero time, and 10-ml aliquots were removed at 0, 10, 20, 40 and 60 minutes and centri-

fuged immediately at $1,500 \times g$ for 1 minute. The supernatant was aspirated and 0.1 ml was added to a scintillation-counting vial containing 0.5 ml NCS solubilizer and incubated for 1 hour at 37°C prior to the addition of scintillation fluid. The cell pellet was assayed for radioactivity as described for the single-label experiment. The radioactivity was determined in a double-channel liquid scintillation counter in which the ^{14}C and ^3H windows were adjusted to allow for maximal efficiency of counting within a setting of minimal overlap. Cell uptake of ^3H -thymidine was calculated in disintegrations per minute (dpm), as follows:

$$^3\text{H-thymidine uptake}_{\text{dpm}} = \text{total pellet } ^3\text{H}_{\text{dpm}} - \frac{\text{total pellet } ^{14}\text{C}_{\text{dpm}}}{\text{supernatant } ^{14}\text{C}/^3\text{H}}$$

Extracellular ^3H -thymidine contamination of the TCA-insoluble material was negligible. TCA-soluble counts represented the difference between total uptake and TCA-insoluble values.

Results

The incorporation of ^3H -thymidine into TCA-insoluble material was inhibited by 180 minutes of halothane treatment in a concentration-dependent manner (fig. 1). Inhibition was first detectable with 0.7 per cent halo-

thane. Five per cent halothane (the highest concentration studied) produced the largest depression, 58 per cent.

The effects of length of incubation with 3 per cent halothane on the incorporation of thymidine (single-label experiment) into both TCA-soluble and TCA-insoluble material are shown in figure 2. The inhibition of incorporation into both TCA-insoluble and TCA-soluble material increased with time; however, the depression of incorporation into TCA-soluble

material was much smaller than that of the TCA-insoluble material. For example, at 120 minutes there was 33 per cent inhibition of TCA-insoluble incorporation and only 10 per cent inhibition of TCA-soluble incorporation.

Figure 3 depicts the results of a typical double-label (early time course) experiment. Depression of the TCA-insoluble fraction is apparent within 20 minutes, whereas there is no inhibition of the TCA-soluble fraction within the initial 30 minutes. The same amounts of inhibition were obtained with the single- and double-label techniques after both 30 and 60 minutes of halothane treatment.

Discussion

In 1966, Andersen concluded that volatile anesthetics specifically inhibit the metaphase portion of mitosis.¹² Recent studies by Nunn have indicated that this effect may be related to the reversible depolymerization of mitotic spindle microtubules.^{4, 5, 14, 15}

Bruce has presented evidence that suggests that halothane also inhibits DNA synthesis: 1) in the rat small bowel, halothane prolonged the time for epithelial cell DNA synthesis without affecting the mitotic phase,⁶ and 2) in human peripheral lymphocyte phytohemagglutinin-activated preparations, halothane inhibited the incorporation of exogenously administered thymidine into TCA-insoluble material.⁷ The findings of Cullen⁸ with a similar lymphocyte transformation system were in agreement with the results of Bruce. Both investigators noted that their TCA-insoluble thymidine incorporation data indicated only the depression of exogenous thymidine incorporation into DNA. Such an experimental approach does not take into account a possible effect on the transport of thymidine into the cell.¹⁶ The property of anesthetic drugs to alter both lipid and protein hydrophobic interactions and the structure of water at membrane surfaces suggest that such drugs might affect both physical diffusion and carrier-facilitated transport within cell membranes.¹⁷ Indeed, such an effect has been demonstrated in muscle and brain tissue and in human erythrocytes.¹⁸⁻²⁰

A small (<10 per cent) and non-concentration-related depression of incorporation of thymidine into the TCA-insoluble material of

HTC cells has been reported previously.⁷ We have demonstrated that halothane inhibits the incorporation of extracellular thymidine into DNA in a concentration-dependent and time-dependent manner. The earlier study⁷ inadequate control of administered dose of halothane (liquid halothane added directly to a variably open system) and the resultant high degree of cytotoxicity probably account for the difference in results. In addition, the kinetic data obtained from these single-label experiments with thymidine failed to differentiate between effects of halothane on transport or synthesis.

In order to study the effects of a drug on the transport of DNA precursors into cells, it is necessary to study the incorporation of a utilizable precursor into both the TCA-soluble and the TCA-insoluble fractions.¹⁶ We found that the incorporation of exogenous thymidine into the intracellular TCA-soluble material was only slightly depressed and did not follow the pattern of inhibition of thymidine incorporation into TCA-insoluble material. If the temporal patterns of thymidine incorporation into TCA-soluble and TCA-insoluble materials had been similar, then an effect on transport would have been suggested.¹⁶ Therefore, our data indicate that halothane does not affect thymidine transport, but does inhibit either the process of phosphorylation of intracellular thymidine or the synthetic incorporation of phosphorylated thymidine derivatives into DNA.

The author thanks Dr. Steven Mizek for intellectual stimulation, assistance in experimental design, and reviewing the manuscript, and Dr. Lewis Aronow for encouragement and extreme generosity.

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Drugs and Their Actions

NALOXONE IN METHADONE POISONING

Naloxone was used in four children and six young adults to reverse the effects of methadone poisoning. Naloxone (0.01 mg/kg) was given intravenously. A maximum response was seen in a few minutes: respiratory rate increased, the level of consciousness improved, arterial blood pressure rose, and pupils dilated. When improvement did not occur, the same dose (0.01 mg/kg) was repeated (iv) after 5 minutes. Two important points must be made in using naloxone: 1) The duration of action of naloxone is two to four hours, while narcotic poisoning may last one or two days. Patients in coma caused by narcotic poisoning when treated with naloxone may make an apparently complete, but transient, recovery; these patients must be watched con-

tinuously for several days. 2) Naloxone does not have any narcotic action of its own, unlike nalorphine and levallorphan. When used to treat non-narcotic poisoning, it will not cause respiratory depression. Finally, in treatment of any patient in coma with depressed respiration, the first problem is to ventilate; in a properly equipped emergency room, a mask with a self-inflating bag with added O₂ is a practical system to use. Potential aspiration of gastric contents must be prevented. This can be handled by tracheal intubation with a cuffed tube, which also facilitates mechanical respiration if needed. Only then should reversal of suspected narcotic poisoning be attempted. (Buchner, L. H., and others: *Naloxone Reversal of Methadone Poisoning*. *N. Y. State J. Med.* 72: 2305, 1972.)