

## Normal Thymidine Entry into Halothane-treated Lymphocytes

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In human lymphocyte cultures containing phytohemagglutinin but studied prior to the onset of DNA synthesis, the uptake of tritiated thymidine ( $^3\text{HT}$ ) in whole cells and nuclei was unaffected by a concentration of halothane previously shown to inhibit PHA-stimulated DNA synthesis. The partition coefficient between culture medium and gas phase at 37 C was  $1.096 \pm 0.202$ . (Key words: Biotransformation; DNA synthesis; Halothane; Lymphocytes; Thymidine.)

Two independent investigations have shown an inhibitory effect of halothane on the stimulation of human lymphocyte cultures by phytohemagglutinin (PHA).<sup>1,2</sup> The evidence of anesthetic inhibition of the mitogenic action of PHA on these cells consisted of a dose-related impairment of the incorporation of tritiated thymidine ( $^3\text{HT}$ ), a DNA precursor. In interpreting these results, an important requirement relates to the use of the thymidine tracer. It is obvious that measurement of its content in extracts of the cells being studied, and relating this content to the intensity of DNA synthesis at the time the tracer was added, assume that the tracer enters the cell freely and in proportion to its intracellular incorporation into DNA. In the two studies cited, the investigators assumed that halothane did not prevent the free entry of thymidine into the cell cultures being studied. The present study was designed to test this assumption, since any subsequent work on DNA synthesis using tritiated thymidine incorporation as an end point will require that this assumption be correct.

A secondary purpose of the present study was to determine the partition coefficient of halothane between culture medium and gas phase in the cell incubator system in use, so

that the results of studies where a gas-phase concentration was reported<sup>2</sup> could be compared with those obtained when halothane was measured in terms of mg/100 ml in the culture liquid.<sup>1</sup>

### Methods

Healthy, fasting volunteer male subjects donated blood, obtained by venipuncture, at a time calculated to allow addition of  $^3\text{HT}$  to lymphocyte cultures at 7 AM the following day. Cell culture preparation was altered from our previously reported method<sup>1</sup> to accord generally with the better method reported by Cullen *et al.*<sup>2</sup> Exceptions to their method were our substitution of 4 per cent dextran (mol wt 200,000) for methylcellulose in the initial sedimentation step, and our single centrifugation following the first removal of iron-containing phagocytes by application of magnetic force. Our final cell preparation contained only one neutrophil per 150 to 200 lymphocytes. Using autologous plasma, a final concentration of 10<sup>6</sup> lymphocytes per ml of plasma was obtained. One-milliliter portions of this were added to 2 ml of minimum essential Eagle's medium (MEM) containing penicillin, streptomycin, glutamine, and 0.05 ml reconstituted PHA-M (Difco).

Cell cultures were maintained in sterile plastic tubes which were loosely capped and kept at 37 C in an incubator containing 5 per cent  $\text{CO}_2$  in air. One incubator was used for control cultures, and an identical incubator was perfused with halothane vaporized by a low flow of 95 per cent  $\text{O}_2$ -5 per cent  $\text{CO}_2$  passing through a Copper Kettle vaporizer and from it into the incubator. These low flow rates did not significantly alter the  $\text{O}_2$  content of the incubator air. Air from the incubator was taken from a sampling port for gas chromatographic analysis of halothane and  $\text{CO}_2$  content. After 24 hours' incubation, triplicate cultures prepared from blood from 12 different donors were taken from the halothane in-

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Accepted for publication June 8, 1972. Supported in part by USPHS GM 15420.

cubator. The liquid samples were injected directly into the gas chromatograph for flame-ionization detection of halothane content compared with an 18.6 mg/100 ml standard (1.0  $\mu$ l distilled halothane at 22 C in 10.0 ml saline solution). From the resulting data the partition coefficient,  $C_L/C_G$ , at 37 C was calculated.

For determination of halothane effect on  $^3\text{HT}$  transport, cultures were studied 17 hours after addition of PHA. This period precedes the onset of DNA synthesis, a factor necessary in such a study. Forty cultures were prepared from each of four donors; 20 of each 40 were incubated in air and 20 in 2 per cent halothane in air. At 17 hours post-PHA, 2  $\mu$ C  $^3\text{HT}$  (specific activity 5.0 Ci/mM) were added to each tube. One hour later, all tubes were harvested.

Harvest procedures to isolate whole cells or intact whole nuclei were then followed. It was assumed that the  $^3\text{HT}$  in these cells would to a great extent be in nucleoside or nucleotide form and, thus, exchangeable with wash fluids. The thymidine content of the culture medium was calculated after 2  $\mu$ C of the  $^3\text{HT}$  had been added. This calculated concentration was tripled, and nonradioactive ("cold") thymidine was added to all wash solutions in this increased amount. Thus, a concentration gradient from outside to inside the cell prevailed during the washing of the cells during their harvest.

To harvest whole cells, the contents of each tube were transferred quantitatively with saline washes to glass-fiber filters held in a multi-channel suction device. The cells remained on these filters and washes of saline solution, 15 ml, followed by methanol, 10 ml, were passed over them. The filters were then dried and placed in counting vials.

To isolate intact nuclei, a method based on that of Stewart and Ingram<sup>2</sup> was used. At the end of the one-hour exposure to  $^3\text{HT}$ , the cells were spun down into a pellet at 2 C, and rinsed with the saline wash solution. They were then resuspended to a volume of 0.5 ml, and 0.5 ml of a solution containing unlabelled thymidine and 0.85 mg Pronase (nuclease-free, Calbiochem) was added. The tube was then incubated 20 minutes at 37 C so that the proteolytic enzyme would digest dead cells and

debris which might bind  $^3\text{HT}$ . The tube contents were then transferred onto glass-fiber filters with washes of cetyltrimethylammonium bromide (Cetrimide, Eastman) which had been prepared by the method of Hatch and Balazs<sup>4</sup> and to which excess "cold" thymidine had been added. Following a 15-ml wash with Cetrimide solution, saline and methanol washes were used, as with the whole-cell separation. We verified microscopically that this procedure did in fact produce a suspension of cell nuclei.

Ten tubes were used for each of the following assays: whole-cell  $^3\text{HT}$ , air; whole-cell  $^3\text{HT}$ , 2 per cent halothane; nuclear  $^3\text{HT}$ , air; nuclear  $^3\text{HT}$ , 2 per cent halothane. To be sure that  $^3\text{HT}$  did not bind to proteins in the culture liquid or the filters themselves, tubes identical to those described, but containing no cells, were prepared with serum and MEM,  $^3\text{HT}$  added, and the harvest procedure carried out using all combinations of wash solutions. Only insignificant counts were obtained from the filters following these tests.

The filters were placed in a toluene scintillation "cocktail" and counted in a Nuclear Chicago Mark I liquid scintillation counter. Counts were corrected for quench and converted to disintegrations per minute (dpm). Data were analyzed statistically by the *t* test for nonpaired data.

## Results

The partition coefficient for halothane between culture liquid and incubator gas phases at 37 C was computed from measurements made after 24 hours of equilibration. In previous studies we had found that the cultures were equilibrated within 6 hours so that the time of testing was unquestionably ample. In converting liquid concentrations from mg/100 ml to volumes per cent, the following formula was used:

$$\frac{\text{ml halothane}}{100 \text{ ml culture}} = \frac{\text{mg halothane}}{100 \text{ ml culture}} \times \frac{22.4 \text{ ml/mm}}{197.4 \text{ mg/mm}} \times \frac{310 \text{ K}}{273 \text{ K}} \times \frac{760 \text{ torr}}{745 \text{ torr}}$$

Thus, vol % = mg/100 ml  $\times$  0.13

TABLE 1. Effect of 2 Per Cent Halothane on Lymphocyte  $^3\text{HT}$  Uptake 17 Hours Post-PHA

	Whole Cells, dpm				Nuclei, dpm			
	Air		Halothane		Air		Halothane	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Donor 1	1,638	251	2,200	585	453	19	418	21
Donor 2	2,314	606	2,174	351	649	34	803*	57
Donor 3	1,114	96	847	160	388	20	337	18
Donor 4	2,837	589	1,722	406	288	8	225	19

\* Counts significantly higher than those in nuclei exposed to air ( $P < 0.05$ ).

There was no need to include a specific-gravity factor, as done by Larson *et al.*,<sup>5</sup> since our standard and all subsequently measured liquid concentrations were in mg/100 ml, not mg/100 g. Using these measurements and calculations, the mean partition coefficient for measurement in the cultures from 12 donors was 1.096, with a standard deviation of 0.202.

Table 1 contains the whole-cell and nuclear dpm data following an hour of exposure to  $^3\text{HT}$  in lymphocyte cultures 18 hours after addition of PHA. The counts were low, and there was no statistically significant difference between those cultures exposed to air and those exposed to 2 per cent halothane, except in the nuclei of halothane-treated lymphocytes obtained from Donor 2, which showed a small but significant ( $P < 0.05$ ) increase in counts. These data give no evidence that halothane interferes with the entry of  $^3\text{HT}$  into either the whole cell or, subsequently, the nucleus where DNA synthesis occurs. An additional ten tubes were prepared from the blood of Donor 4, exposed to air, and whole cells harvested but then washed with trichloroacetic acid to isolate only the acid-insoluble nucleoprotein. These gave a mean dpm of 526, compared with 2,837 for intact whole cells. Clearly, the major portion of activity was in nucleoside or nucleotide form, and only a trace amount of DNA synthesis had occurred by this time. Furthermore, some of the activity was extranuclear, since the nucleoprotein count of 526 exceeded the nuclear count of 288.

It is well documented that DNA synthesis begins between 20 and 25 hours after addition of PHA. Two more experiments, in which

the  $^3\text{HT}$  was added at 21 and 24 hours, were done, and in these cases 2 per cent halothane treatment did result in lower counts in both whole cells and nuclei. The nuclei from cells exposed to halothane had counts 58 per cent of controls at 21 hours and 27 per cent at 24 hours. Halothane did not actually lower the counts, which were similar to those at 18 hours. Rather, these cells did not show the rapid increase in  $^3\text{HT}$  incorporation that the control cultures did at the later times.

### Discussion

Considering the vast literature on cell kinetics, produced principally with the use of tritiated thymidine incorporation to detect DNA synthesis, relatively little has been written concerning the entrance of this nucleoside into the cell. Thymidine enters the cell readily, then is phosphorylated to thymidine mono-, di-, and finally, triphosphate. The thymidine kinases facilitate these phosphorylations, which produce the thymine nucleotides. Finally, DNA polymerase acts upon thymidine triphosphate to assist its assembly into DNA. In most published work, the focus of attention has been these enzyme-associated intracellular events.

Thymidine appears rapidly in the cell, equilibrating with the external medium in a matter of minutes.<sup>6,7</sup> Its actual cellular entrance is thought to be by simple diffusion or facilitated transport, and "the concentrations

of nucleosides and free amino acids in the extracellular space tend to equilibrate through the cell membrane with the soluble fraction in the cell.<sup>8</sup> In the present study, we have seen that halothane does not interfere with this process.

Future studies of DNA synthesis may be done with the knowledge that decreased <sup>3</sup>H incorporation is not due to a block of its uptake by the exposure of the cell to halothane but rather to a more distal event in the sequence of steps in DNA synthesis.

Finally, the partition coefficient of about 1.1 indicates that our gas concentration of 2 per cent produced a liquid concentration of about 17 mg/100 ml. In our previous study this concentration caused about 60 per cent inhibition of <sup>3</sup>H incorporation, so we may be assured that the present results refute any idea of a block in precursor uptake at the membrane level.

Miss Louise Owen gave expert technical assistance. Halothane was donated as Fluothane by Ayerst Laboratories, Inc.

## References

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## Drugs and Their Actions

**BRETYLIUM TOSYLATE AND PULMONARY HEMODYNAMICS** The effects of bretylium tosylate on pulmonary and systemic circulations were studied in four patients recovering from open-heart surgery for mitral-valve disease. Hemodynamic measurements were made at 30-minute intervals for two hours after administration of bretylium tosylate (5 mg/kg). The elevated pulmonary arterial pressure and vascular resistance increased progressively in three patients. Systemic arterial pressure, systemic vascular resistance, and left atrial pressure decreased in all patients; one sustained a continued decline in arterial pressure and cardiac arrest, successfully treated by closed-chest massage and large doses of norepinephrine. Cardiac output decreased in two patients, rose slightly in one, and was unchanged in the fourth. Caution is recommended when bretylium tosylate is needed for the patient with increased pulmonary arterial pressure and vascular resistance. (Cotter, S., and others: *Effect of Bretylium on the Pulmonary and Systemic Circulation in Patients with Mitral Valve Disease after Cardiopulmonary Bypass*. *J. Clin. Pharmacol.* 11: 409-416, 1971.) **ABSTRACTER'S COMMENT:** This paper confirms the known pulmonary vasopressor response to bretylium tosylate; the number of observations was small and the control of variables questionable (e.g., marginal pH PaO<sub>2</sub> and PaCO<sub>2</sub>). Bretylium, formerly used as an antihypertensive agent and currently being investigated to treat ventricular arrhythmias, should be reserved for refractory, life-threatening arrhythmias, especially when pulmonary hypertension is present. Bretylium blocks norepinephrine release in response to adrenergic nerve stimulation, but does not impair the response to exogenous catecholamines. Therefore, norepinephrine is the preferred treatment when systemic hypotension ensues.