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# *The Effects of Anesthetic Agents on Serum Proline Hydroxylase*

*H. David Stein, M.D.,* Harry R. Keiser, M.D.,† Albert Sjoerdsma, M.D., Ph.D.‡*

**The effects of exposure to either 0.3 per cent methoxyflurane or 80 per cent nitrous oxide ($N_2O$) on levels of proline hydroxylase (PPH) in the serum and liver were studied in rabbits. Serum PPH levels rose fourfold and twelvefold after two and four hours of exposure to methoxyflurane, respectively. After two hours of exposure to methoxyflurane there were also highly significant increases in serum SGOT, SGPT and alkaline phosphatase in all animals tested, while after two hours of exposure to $N_2O$ only SGOT was altered significantly. The increases in enzymic activity were not prevented by administering the methoxyflurane in 99 per cent oxygen or by inhibition of protein synthesis with cycloheximide. Levels of PPH in liver were not affected by the various treatments. These changes in serum enzymes after exposure to methoxyflurane are similar to those in patients, and appear to reflect liver-cell injury, not enzyme induction. (Key words: Proline hydroxylase; Methoxyflurane; Anesthetic agents; Cycloheximide; Hepatotoxicity.)**

PROTOCOLLAGEN PROLINE HYDROXYLASE (PPH) is an enzyme which catalyzes the hydroxylation of peptide-bound proline to hydroxyproline during the biosynthesis of collagen.[1] In a recent study, we demonstrated the presence of this enzyme in serum and found marked elevations in enzyme levels in patients following anesthesia with methoxyflurane.[2] Although PPH activity in tissues correlates well with the rate of collagen synthesis,[3-5] our clinical data suggested that increased PPH activity in blood was a reflection of hepatic injury; however, the possibility of enzyme induction could not be excluded. The present studies in rabbits were undertaken to determine: 1) whether PPH and other serum enzymes in animals respond to methoxyflurane like the same enzymes in man; 2) whether enzyme induction might account for the changes in serum PPH.

## Materials and Methods

Sixty-six male New Zealand rabbits weighing 2.5 to 3.5 kg were used in these studies. They were placed in a special cage and exposed to 0.3 per cent methoxyflurane (Abbott Laboratories) or 80 per cent nitrous oxide (Ohio Medical Products). The anesthetic agents were delivered mixed with compressed air unless otherwise indicated, and flow was monitored using a Foregger anesthesia machine equipped with a Copper Kettle, set up as a nonrebreathing system. The total gas flow (9 l/min) was fed into a large (2 × 2 × 4-foot) cage completely enclosed in a plastic bag. Gas escaped from the bag by a one-way valve. As many as four rabbits could be accommodated at one time.

Three protocols were followed. In the first, the effects of various durations of exposure to methoxyflurane on serum PPH measured 24 hours later were studied. Twelve rabbits divided into three equal groups were exposed to methoxyflurane for period of 30 minutes, two hours, and four hours.

In the second protocol, the time courses of changes in PPH and other serum enzymes were studied after two hours of exposure of ten rabbits to methoxyflurane and six rabbits to nitrous oxide. In order to eliminate the effects of anoxia, two additional rabbits were exposed to methoxyflurane in 99.7 per cent $O_2$ for two hours.

In the third protocol, the effects of the protein-synthesis inhibitor, cycloheximide (Calbiochem), on PPH activity in serum and liver were studied after exposure to methoxyflurane.

* Resident in General Surgery, Albert Einstein College of Medicine, New York, New York.
† Senior Investigator, Experimental Therapeutics Branch, National Heart and Lung Institute.
‡ Chief, Experimental Therapeutics Branch, National Heart and Lung Institute.
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TABLE 1. Serum Proline Hydroxylase Activity in Rabbits before and after Various Durations of Exposure to Methoxyflurane

| Duration of Exposure (Hours) | Number of Rabbits | Proline Hydroxylase (dpm/ml/Hour) (mean ± SE)* |
|---|---|---|
| 0 | 12 | 256 ± 36 |
| ½ | 4 | 222 ± 36 |
| 2 | 4 | 1,084 ± 196 |
| 4 | 4 | 3,034 ± 508 |

*Serum samples collected 24 hours after 0 time.

Thirty-two rabbits were divided into four equal groups. One group was exposed to methoxyflurane for two hours. A second group received cycloheximide, 1 mg/kg body weight, in physiologic saline solution by intraperitoneal injection at the start of the experiment and again six hours later. The third group received both cycloheximide and exposure to methoxyflurane, and the fourth (control) group received comparable ip injections of physiologic saline solution.

Blood samples were obtained from ear veins at various times, allowed to clot at room temperature, centrifuged in the cold, and serum harvested and frozen until analyzed. Livers were removed and frozen until assayed for PPH activity. Serum PPH was measured as cited by Stein *et al.*[2] in 0.5-ml samples of serum to which dithiothreitol (Calbiochem) had been added to a final concentration of $10^{-4}$ M before freezing. Results are expressed as disintegrations per minute (dpm) of tritium released from a L-3,4-T-proline-labelled protocollagen substrate per ml of serum per hour of incubation. While all rabbits were of the same strain, age, and sex, and were obtained from the same supplier, there were significant but unexplained differences among control levels of serum PPH activity in the groups of animals used in different experiments. Using the same method, PPH in the liver was measured in 20-$\mu$l samples of 16,000 × g supernatants of 1:20 homogenates made at 0 C in a mixture of 0.05 M Tris (pH 7.5), $5 \times 10^{-5}$ M EDTA, and $10^{-4}$ M dithiothreitol. Protein content of the supernatants was determined according to the method of Lowry.[6] Serum glutamic-oxaloacetic transaminase (SGOT), glutamic-pyruvic transaminase (SGPT), and alkaline phosphatase (AP) were analyzed on a Technicon AutoAnalyzer using standard technique.

## Results

Exposure to methoxyflurane for 30 minutes produced no changes in serum PPH measured 24 hours later, compared with control values (table 1). After two hours of exposure,

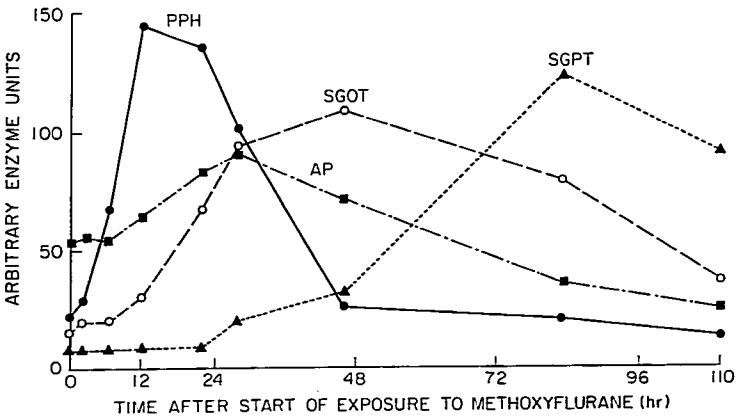


FIG. 1. Serum enzyme levels in one rabbit after two hours of exposure to methoxyflurane. One arbitrary enzyme unit is equal to 80 dpm/ml/hour of PPH, 2 IU of SGOT, 4 IU of SGPT, or 1 IU of AP.

TABLE 2. Maximum Serum Enzyme Levels in Rabbits after Two Hours of Exposure to Either Methoxyflurane or Nitrous Oxide*

| Group | Proline Hydroxylase (dpm/ml/Hour) | SGOT (IU) | SGPT (IU) | Alkaline Phosphatase (IU) |
|---|---|---|---|---|
| Control (16 rabbits) | 977 ± 269 | 38 ± 3 | 56 ± 5 | 26 ± 4 |
| Methoxyflurane (10 rabbits) | 7,612 ± 1,164‡ | 402 ± 124‡ | 352 ± 108† | 55 ± 7‡ |
| Nitrous oxide (6 rabbits) | 1,136 ± 364 | 76 ± 12‡ | 74 ± 15 | 31 ± 6 |

* Values shown are means ± SE. Significant differences compared with controls were as follows: † $P < 0.02$; ‡ $P < 0.01$.

TABLE 3. Enzyme Levels in Sera and Livers of Rabbits 24 Hours after Exposure to Methoxyflurane or Cycloheximide Alone, or to Both*

| Group | Serum | | | | Liver |
|---|---|---|---|---|---|
| | PPH (dpm/ml/Hour) | SGOT (IU) | SGPT (IU) | AP (IU) | PPH (dpm/mg/Hour) |
| Control (8 rabbits) | 1,250 ± 330 | 61 ± 13 | 43 ± 3 | 40 ± 17 | 13,734 ± 3,834 |
| Methoxyflurane (8 rabbits) | 8,296 ± 2,746† | 65 ± 9 | 56 ± 8 | 39 ± 8 | 16,604 ± 2,890 |
| Cycloheximide (8 rabbits) | 4,432 ± 1,946 | 72 ± 14 | 54 ± 4† | 29 ± 8 | 14,574 ± 1,386 |
| Methoxyflurane + cycloheximide (8 rabbits) | 14,992 ± 2,058‡ | 114 ± 27 | 57 ± 6 | 40 ± 8 | 12,190 ± 1,312 |

* Values shown are means ± SE. Significant differences compared with controls were as follows: † $P < 0.05$; ‡ $P < 0.01$.

serum PPH levels were nearly four times greater than controls, and after four hours of exposure, serum PPH levels were nearly 12 times greater than controls. The time course of the response after a two-hour exposure is shown in figure 1, along with changes in other enzymes.

PPH activity rose to a peak nearly eight times the control level after 12 to 28 hours (fig. 1, table 2) and returned to the control level after about 48 hours. Serum SGOT rose more slowly, but significantly, to a peak nearly 11 times the control level after 48 to 96 hours. Serum SGPT and alkaline phosphatase rose to peaks six and two times the control levels, respectively. Rabbits exposed to $N_2O$ for two hours showed no significant changes in serum PPH, SGPT, or alkaline phosphatase. Only serum SGOT was elevated significantly, to a peak two times the control level. In an attempt to rule out anoxia as the cause of the changes, two rabbits were exposed to 0.3 per cent methoxyflurane in 99.7 per cent oxygen for two hours. These rabbits had the same increases in serum PPH, SGOT, and AP as animals exposed to methoxyflurane in compressed air.

When cycloheximide was given alone to prevent protein synthesis, it also elevated serum PPH activity, as well as serum SGPT (table 3). When animals received both cycloheximide and methoxyflurane, the effects on serum PPH and SGOT were additive. Levels of PPH activity in the livers of these rabbits were not significantly different from control values whether the animals received methoxyflurane, cycloheximide, or both (table 3).

## Discussion

These results demonstrate that rabbits develop a marked increase in serum PPH activity in the 48-hour period following exposure to inhaled methoxyflurane. This is identical to the response found previously in patients undergoing general anesthesia with methoxyflurane.[2] We have referred to "exposure to anesthetic agents" rather than to "anesthesia" because of possible misinterpretation. While the inhaled concentrations of both methoxyflurane (0.3 per cent) and $N_2O$ (80 per cent) used are within the normal anesthesia maintenance levels for man, the rabbits were not really anesthetized. At the end of four hours of exposure they were only lethargic, not asleep. The advantage of this is the avoidance of the deleterious effects of profound anesthesia. The mixture of 80 per cent nitrous oxide in air is hypoxic. It was specifically

chosen as a rigorous contrast to 0.3 per cent methoxyflurane in air, which is not hypoxic. It is of interest that the hypoxic mixture produced such minimal changes in serum enzyme levels.

The increased PPH activity in serum after exposure to methoxyflurane was not evident immediately at the end of exposure, but rose gradually thereafter to a peak 12 to 24 hours later. This indicates that the changes in serum PPH activity are not merely the result of high concentrations of the drug itself in the blood, since peak PPH activity occurred when there should have been little anesthetic left in the blood. Addition of methoxyflurane to incubation mixtures containing PPH from blood or liver has no effect on measured PPH activity.

The liver has one of the highest concentrations of PPH and the largest amount of PPH activity in the body.[4] Data on serum PPH activity in patients indicate that the liver is the major source of abnormally elevated PPH levels.[2] Therefore, our data strongly indicate that the liver is the source of abnormal elevations in serum PPH activity. Rabbits exposed to methoxyflurane had marked increases in serum PPH, SGOT, and SGPT, while animals exposed to $N_2O$ did not. This suggests hepatic cell dysfunction or toxicity related to a particular anesthetic agent, and not to anesthesia in general. The combination of light anesthesia and inability to prevent the serum enzyme changes by administering methoxyflurane in 99.7 per cent oxygen strongly suggests that anoxia is not the cause. The data from animals given cycloheximide indicate that the changes in serum PPH activity are not the result of enzyme induction. Thus, all of our data point to leakage of PPH from injured hepatic cells under the influence of exposure to methoxyflurane or its metabolites.

Methoxyflurane anesthesia has been associated with serious hepatic injury in a very small number of patients.[7-9] The hepatic injury appears to be similar to that reported in association with halothane anesthesia, also in a very small number of patients.[10] Several incomplete studies have shown significant elevations (not necessarily to abnormal values) in SGOT, SGPT, and BSP retention in many subjects anesthesized with methoxyflurane.[11-13] Our data have little, if any, bearing on the rare cases of serious hepatic damage associated with methoxyflurane anesthesia, but they agree with the mild elevations of hepatic enzymes seen in many patients receiving methoxyflurane anesthesia. However, PPH activity in serum rises significantly more in essentially all people and rabbits exposed to methoxyflurane. Thus, measurement of serum PPH activity appears to provide a sensitive new tool for studying effects of anesthetic agents and their possible hepatotoxicity in man and animals.

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