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Additive Effects of Pentobarbital and Halothane to Inhibit Synthesis of Lung Proteins

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The effect of pentobarbital on synthesis of lung proteins was investigated, both when administered alone and in combination with halothane. When rat lungs perfused *in situ* with Krebs-Henseleit bicarbonate buffer containing plasma levels of 19 amino acids, 690 μM phenylalanine, 5.6 mM glucose, and 4.5 per cent fraction V bovine serum albumin were exposed to pentobarbital, a dose-related inhibition of [^{14}C]phenylalanine incorporation into protein was observed, with a maximal inhibition (74 per cent) at a pentobarbital concentration of 324 μg/ml. Halothane (1–4 per cent equilibrated with $O_2/N_2/CO_2$, 4:15:1) also rapidly inhibited synthesis of lung proteins in a dose-dependent manner. At the maximally effective concentration of pentobarbital, exposure of the lungs to halothane enhanced the inhibition of protein synthesis; halothane concentrations ranging from 1 to 4 per cent were equally effective. Furthermore, when lungs were exposed to a combination of pentobarbital (100 μg/ml) and halothane (1 per cent) at doses which had no effect when given alone, protein synthesis was inhibited 35 per cent ($P < 0.001$). Thus, the metabolic effects of the anesthetics were potentiated when the drugs were administered in combination. The inhibition of protein synthesis by pentobarbital (324 μg/ml), with or without 4 per cent halothane, was fully reversible. A similar inhibitory effect of pentobarbital was observed in perfused rat hearts. (Key words: Anesthetics, intravenous: pentobarbital. Anesthetics, volatile: halothane. Heart: metabolism. Lung: metabolism; perfusion. Protein: synthesis.)

EFFECTS OF VOLATILE ANESTHETICS, including halothane, on cellular metabolism have been reported by a number of authors.[1] However, little is known of the effects of these or other anesthetic agents on metabolism of the lung. The uptake of circulating norepinephrine by perfused rabbit lungs[2] and by the lungs of intact dogs[3] was inhibited by halothane, as was the uptake of 5-hydroxytryptamine by perfused rat lungs.[4] The effect of halothane on norepinephrine uptake was enhanced when nitrous oxide was also present.[2] Several authors reported inhibitory effects of anesthetic agents on the incorporation of radioactive amino acids into protein[5,6]§; recent studies from this laboratory[7] showed that synthesis of proteins by rat lungs perfused *in situ* was rapidly inhibited by halothane in a dose-dependent and reversible manner. Direct studies of cultured lung cells[7] and pulmonary macrophages[8] indicated that the inhibition of protein synthesis in lung tissue by halothane was not secondary to changes in perfusion parameters, but was exerted at the cellular level.

During the course of these previous studies,[7] control experiments showed that the pentobarbital used to anesthetize the animals prior to lung perfusion did not affect the rate of protein synthesis *in vitro* (CA Watkins, DE Rannels, unpublished observations). However, when the anesthetizing dose of pentobarbital was added directly to the perfusion buffer, rates of protein synthesis declined significantly. The present studies were carried out to define further this inhibitory effect of pentobarbital and to determine whether the drug interacted with halothane. The results indicated that the extent of inhibition of protein synthesis by either agent alone was potentiated when the lungs were exposed to combinations of both drugs.

Materials and Methods

Male Sprague-Dawley rats (175–200 g) obtained from Charles River Laboratories, were provided Agway RMH 3000® chow and water *ad libitum*. Following intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight), the animals were weighed and surgically prepared for lung perfusion as described earlier.[9] As discussed below, direct measurements showed little carry over of the injected dose of pentobarbital into the perfusion buffer. Ventilation of the *in situ* perfusion preparation was accomplished through a tracheostomy using a Harvard® small animal respirator (Model 680). Tidal volume was 10.0 ml/kg body weight (72 cycles/min); the ventilatory gas was warmed (37° C), humidified $O_2/N_2/CO_2$ (4:15:1). Perfusion pressure was held at 20 cmH_2O; a positive end-expiratory pressure of 2 cmH_2O was provided. Briefly, the surgical procedures were as follows: The abdomen was opened and the diaphragm was carefully trimmed from the ribs. The incision was continued anteriorly along the sternum. The cut edges of the thoracic cage were clamped with hemostats and the ribs were reflected to allow the surgical field to be trimmed free of membranous elements, remnants of the diaphragm, and the thymus. Sodium heparin (10.0 mg/kg in 0.15 M NaCl) was injected into the right ventricle.

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§ Chvapil M, Hameroff SR: Lidocaine effects on collagen synthesis. American Society of Anesthesiologists, 1978 Abstracts, pp 139–140.

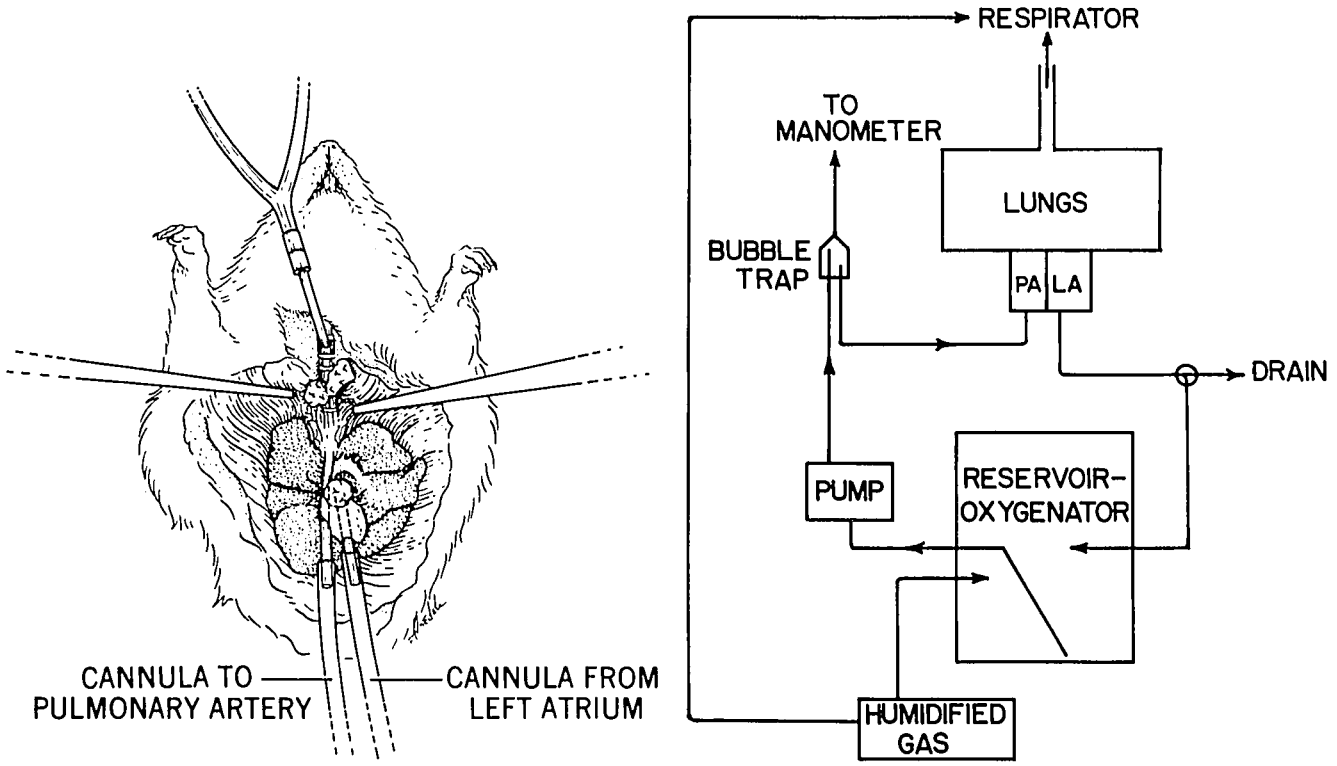

FIG. 1. Perfusion of rat lungs *in situ*. Panel A shows the anatomic relationship of the arterial and venous cannulae and the heart and lungs in the *in situ* perfusion preparation. Panel B is a schematic diagram of the perfusion apparatus. Both panels are described in the text. PA = pulmonary artery; LA = left atrium.

A loose double ligature was placed around the aorta and pulmonary artery; one loop was rotated around the heart to surround the pulmonary veins and left atrium. In all procedures, special care was taken not to contact the lungs. Circulation was interrupted by cutting the vena cava; next, incisions were made in both ventricles. A grooved stainless steel cannula was inserted through the mitral valve and tied at the base of the left atrium (fig. 1, panel A). An arterial cannula, filled with perfusate, was inserted through the right heart and secured in the pulmonary artery. Perfusion was begun immediately. Under these conditions, the total duration of interruption of the pulmonary circulation was about 30 s. The preparation was rinsed with warm 0.15 M NaCl, the hemostats retracting the rib cage were removed, and the thoracic cage was covered with plastic film. The lung perfusion preparation was enclosed within a temperature-controlled (37° C) Plexiglas® box. A rotating reservoir drum served as an oxygenating chamber for the perfusate, which was supplied to the arterial cannula through a bubble trap (fig. 1, panel B) using a peristaltic pump (Gilson Minipuls 2®). Arterial pressure was monitored using a manometer. A variety of physiologic and biochemical criteria[9,10] showed that this preparation was stable for a minimum of 240 min of perfusion.

The perfusion medium was a modified Krebs-Henseleit bicarbonate buffer containing 4.5 per cent (w:v) bovine serum albumin (Pentex, Fraction V; Miles Laboratories), 5.6 mM glucose, and 19 amino acids at levels measured in rat plasma.[10] Perfusate phenylalanine was 690 μM, ten times the plasma level (specific radioactivity, 0.15 μCi/μmol). Under these conditions, [^{14}C]phenylalanine added to the perfusate rapidly equilibrated with the tissue phenylalanine pool ($t_{1/2}$ = 81 s) and was incorporated into lung proteins at a linear rate.[10] Furthermore, when extracellular phenylalanine was present at this concentration, the specific radioactivity of phenylalanine remained constant with time and the specific radioactivity of phenylalanyl-tRNA was equal to that of phenylalanine in the perfusate.[10] Thus, rates of protein synthesis could be calculated directly based on the radioactivity in lung protein and the specific radioactivity of extracellular phenylalanine. The perfusate was equilibrated with warmed (37° C) humidified O_2/N_2/CO_2 (4:15:1); halothane was mixed with this gas mixture as indicated, using a Fluotec 3 vaporizer (Fraser Sweatman, Inc., Lancaster, New York). The concentration of anesthetic in the perfusate was verified using a Hewlett-Packard 5840-A® gas chromatograph standardized against halothane solutions of known concentration.[8]

Solutions of pentobarbital sodium (Fort Dodge Laboratories, Fort Dodge, Iowa) were lyophilized before addition to the perfusate.

The first 30 ml of perfusate to pass through the lungs was discarded; as recirculation of the remaining buffer (100 ml) was begun, radioactive phenylalanine was added to the buffer reservoir. In some experiments, the entire left lung was removed from the preparation to determine the rate of phenylalanine incorporation over the initial interval of perfusion; previous studies showed that this procedure did not affect the rate of protein synthesis in the remaining tissue.[7] Tidal volume was reduced 40 per cent when the left lung was removed. Incorporation of radioactive phenylalanine into lung protein was determined and rates of protein synthesis were calculated as described previously.[10]

Hearts from rats similar to those above were perfused by a modified Langendorff technique as detailed previously.[11] The perfusion buffer contained plasma levels of 19 amino acids,[10] 690 μM phenylalanine, and 16.8 mM glucose; it was equilibrated with humidified O_2/CO_2 (19:1). Perfusion pressure was 60 mmHg. Following a preliminary perfusion of 10 min, during which the buffer passed through the heart a single time, 10 ml of buffer containing radioactive phenylalanine (690 μM) was washed through the heart, and recirculation of 25 ml of the same perfusate was begun. Rates of protein synthesis were estimated as described earlier.[12]

For measurements of ATP and creatine phosphate, perfused tissues were frozen rapidly by clamping them between blocks of aluminum cooled to the temperature of liquid nitrogen. The tissues were pulverized at the same temperature and extracted with perchloric acid for enzymatic measurements of these intermediates, as detailed previously.[9] Per cent dry weight was determined by drying an aliquot of powdered tissue in an oven, to constant weight.

Statistical significance was determined with Student's t test using a two-tailed analysis; values with $P < 0.05$ were considered to be significantly different.

Results

Linear rates of incorporation of [^{14}C]phenylalanine into protein were observed in rat lungs perfused under control conditions for 60 minutes (fig. 2). Previous studies showed that the same synthetic rate continued for at least four hours *in vitro*.[9] Addition of 324 μg/ml pentobarbital¶ to the perfusion buffer resulted in a rapid inhibition (70 per cent) of phenylalanine incorporation,

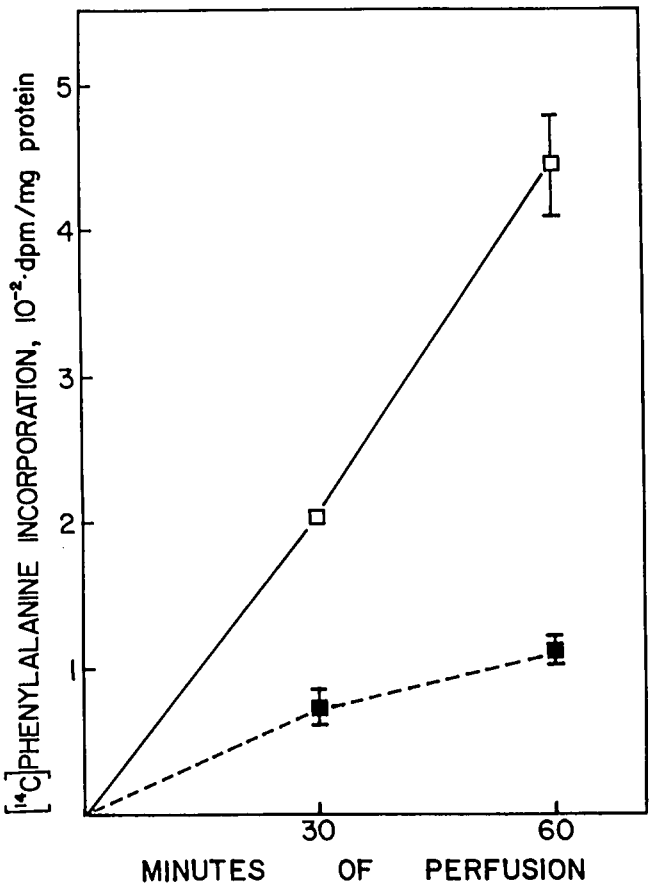

FIG. 2. Effect of pentobarbital on [^{14}C]phenylalanine incorporation in rat lungs perfused *in situ*. Lungs were perfused 60 min as described in the Materials and Methods section. Data from control and pentobarbital (324 μg/ml)-exposed lungs are shown by the open and closed symbols, respectively. Incorporation values after 30 min were determined by removing the single lobe of the left lung from the preparation.[8] [U-^{14}C]Phenylalanine (Amersham/Searle) was added to the perfusion buffer at a specific radioactivity of 0.15 μCi/μmol (690 μM). Values are the means ± SEM of 4–6 observations; where SEM is not shown, it did not extend beyond the symbol.

which was evident after 30 or 60 min of perfusion (fig. 2). The extent of inhibition of phenylalanine incorporation by pentobarbital was dose-related (fig. 3). In 60-min experiments, pentobarbital concentrations of 50 or 100 μg/ml had no effect, while phenylalanine incorporation was inhibited 45 per cent and 75 per cent at doses of 200 and 324 μg/ml, respectively. No additional inhibition was observed when levels of the drug were increased to 500 μg/ml.

Previous studies from this laboratory showed a similar dose-related inhibitory effect of halothane on protein synthesis in intact lungs perfused *in situ* and in cultured lung cells and pulmonary macrophages.[7,8] The data in figure 4 show that for each per cent halothane to which the lungs were exposed, protein synthesis was inhibited

¶ This concentration of pentobarbital represented a total dose equal to that routinely administered to anesthetize rats by intraperitoneal injection.

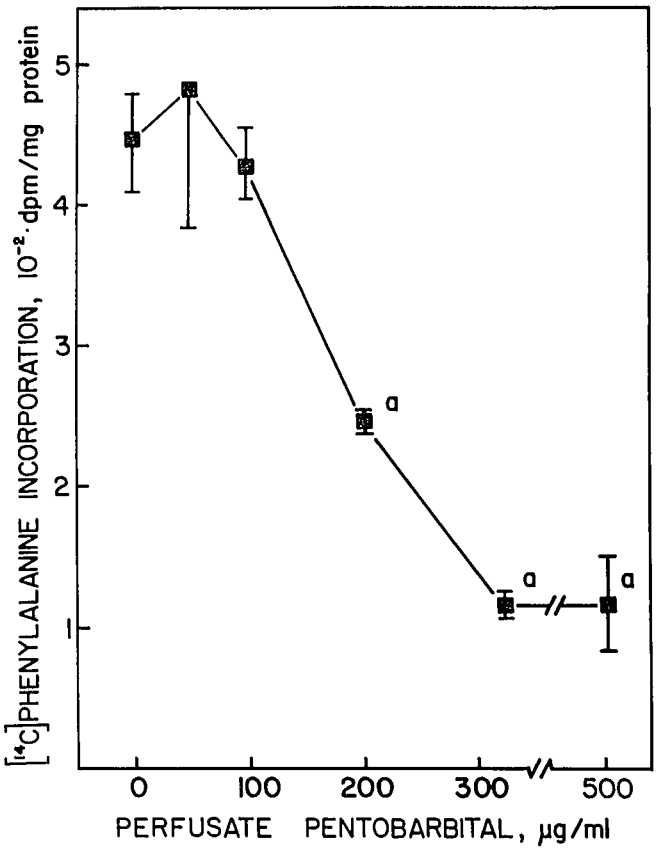

FIG. 3. Dose-response relationship of the inhibition of phenylalanine incorporation by pentobarbital. Lungs were perfused 60 min, as described in figure 2, with buffers containing pentobarbital at the concentrations indicated. Data represent the means ± SEM of at least three observations. a = $P < 0.001$ *vs.* control, without pentobarbital.

about 10 per cent. Because barbiturates and volatile anesthetics are frequently used in combination, the potential of additive effects of pentobarbital and halothane was investigated. Barbiturate concentrations which had no effect alone (100 µg/ml) and which were highly inhibitory (324 µg/ml) were tested over a range of halothane levels (fig. 4). When lungs perfused with buffer containing 324 µg/ml pentobarbital were exposed to halothane, protein synthesis was reduced further to only 11 per cent of the rate observed in control lungs. Under these conditions, the dose-response relationship for halothane exposure was abolished; 1 per cent and 4 per cent halothane were equally inhibitory. When 100 µg/ml pentobarbital was present, exposure of the lungs to 1 per cent halothane reduced protein synthesis 35 per cent during one hour of perfusion, even though at these doses, neither pentobarbital (fig. 3) nor halothane[7] alone were inhibitory. Under similar conditions, the effects of 4 per cent halothane were doubled, resulting in an 80 per cent inhibition of the synthetic rate.

The inhibition of protein synthesis by high doses of pentobarbital or halothane may have been associated with extensive damage to the preparation resulting in cell death. Thus, the reversibility of the inhibitory effect of these drugs was investigated directly (fig. 5). Rates of phenylalanine incorporation were measured in the same tissues both during and after exposure to 324 µg/ml pentobarbital, with or without 4 per cent halothane present. In these studies, the extent of inhibition of phenylalanine incorporation in 30 min experiments was similar to that shown earlier (fig. 4, left bars). However, the effects of pentobarbital alone and of both drugs in combination were fully reversed within 30 min after anesthetic exposure was stopped (fig. 5, right bars). These observations showed that the inhibitory effects of the drugs did not reflect irreversible damage to the perfused lung preparation.

It was of interest to determine whether the effect of pentobarbital on protein synthesis was limited to lung tissue, or whether it reflected a general depression of protein metabolism which might be observed in other organs. Thus, perfused rat hearts were exposed to buffer containing pentobarbital (table 1). At 200 µg/ml, pentobarbital rapidly reduced the heart rate 50 per cent;

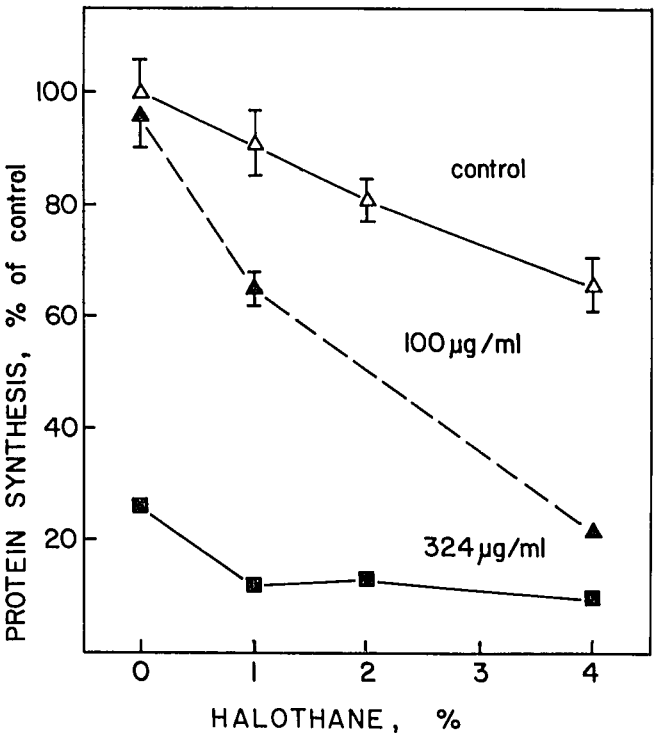

FIG. 4. Effects of pentobarbital and halothane on protein synthesis in lung. Lungs perfused 60 min as described in figure 2 were exposed to halothane levels ranging from 0 to 4 per cent, as indicated. Perfusion buffers contained 0 (open triangles), 100 µg/ml (closed triangles), or 324 µg/ml (closed squares) pentobarbital, as indicated. Data represent the means ± SEM of at least three observations; where SEM is not shown, it did not extend beyond the symbol.

beating in hearts perfused under this condition was somewhat irregular. Higher doses of the drug (324 μg/ml) completely abolished contractile activity. Coronary flow remained relatively unaffected under the same conditions. When hearts were returned to drug-free perfusate after 60 min of perfusion, contractile rates returned to the control value within several minutes, indicating that the myocardium had not been irreversibly damaged (data not shown). Pentobarbital inhibited synthesis of heart proteins in a dose-dependent manner, although at a given concentration of the drug, the extent of inhibition was about half that observed in lung.

Exposure of perfused hearts to similar concentrations of pentobarbital was shown previously to be associated with increased reduction of pyridine nucleotides and with decreased oxygen consumption in isolated rat heart mitochondria.[13] Thus, tissue levels of high-energy phosphate compounds were measured (table 1). Pentobarbital did not severely reduce myocardial ATP; however, creatine phosphate decreased 50 per cent in the presence of the drug. These observations suggested that the inhibition of protein synthesis in heart muscle by pentobarbital might result from energy depletion. Accordingly, an additional set of experiments were performed to examine the effect of pentobarbital and/or halothane exposure on ATP levels in the lung.

As the perfusate pentobarbital concentration was increased from 0 to 200 μg/ml (without halothane present), a significant inhibition of synthesis of lung proteins was observed with no ATP depletion (table 2). At a maximally effective dose of the drug (324 μg/ml), ATP was reduced to a small, but significant, extent (12 per cent). As reported previously,[7] the inhibition of protein synthesis in lungs exposed to a high concentration of halothane (4 per cent) was not accompanied by ATP depletion. Similarly, at given concentrations of halothane, significant inhibition of protein synthesis by pentobarbital was observed without a reduction in tissue levels of ATP (table 2). However, under conditions where pentobarbital inhibited protein synthesis most severely (*e.g.*, in the presence of 4 per cent halothane), ATP was lowered.

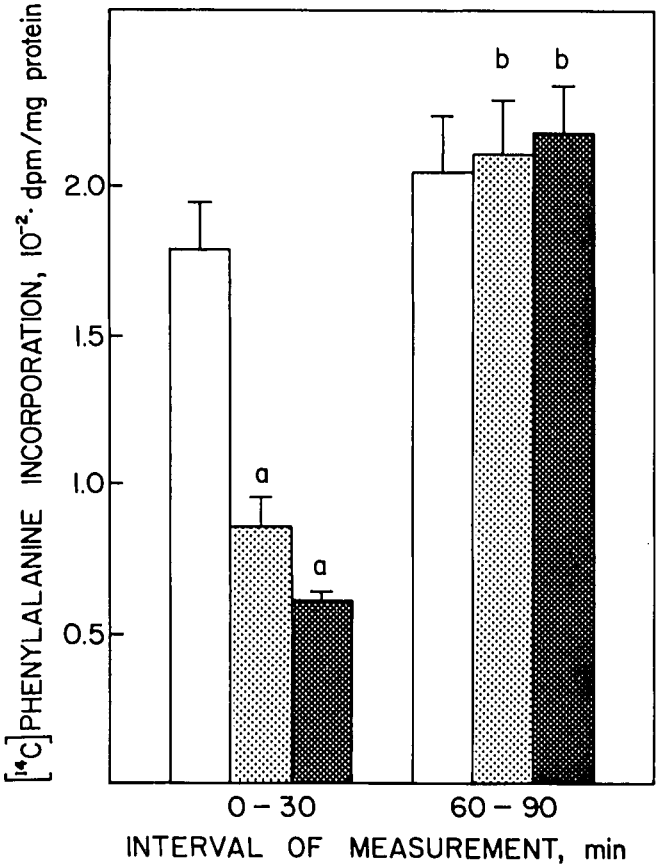

FIG. 5. Reversibility of the effects of pentobarbital and halothane on the synthesis of lung proteins. Lungs were perfused as described in figure 2. Incorporation of [ring 2,6-^{3}H(N)]phenylalanine into protein during the first 30 min *in vitro* (left bars) was estimated in control tissues (open bars), and in lungs exposed to 324 μg/ml pentobarbital alone (shaded bars) or in combination with 4 per cent halothane (dark bars), by removal and analysis of the entire left lung. At this time, halothane delivery was stopped and 100 ml of fresh perfusate without pentobarbital was washed through the lungs and discarded. Recirculation of similar perfusate was continued and at 60 min, [U-^{14}C]phenylalanine was added to estimate the rate of protein synthesis over the final 30 minute interval of perfusion (right bars). Values represent the means ± SEM of three observations. a = $P < 0.001$ *vs.* control; b = $P < 0.001$ *vs.* 0–30 min, same condition of perfusion.

Discussion

The importance of nonventilatory functions of the lung has been recognized only relatively recently. Consequently, little is known of the effects of anesthetic agents or other drugs on lung metabolism. Nevertheless, such effects may be of considerable importance in view of the fact that the lung parenchyma is exposed to high concentrations of volatile anesthetics and that endothelial cells lining the pulmonary capillaries are subject to the entire plasma content of circulating agents. Modifications of the metabolic functions of the pulmonary endothelium upon exposure to halothane and nitrous oxide[2-4] provide an example of the type of alteration in lung integrity which may result from anesthetic exposure.

Pentobarbital and related compounds are commonly used clinically, as well as to anesthetize animals prior to perfusion of organs for *in vitro* metabolic studies. In the latter case, if the drug were carried over into the perfusion medium, its effects on cellular metabolism could affect experimental observations. Doses of pentobarbital administered to animals used in this laboratory resulted in circulating plasma levels of the drug of 50–100 μg/ml, but in negligible carryover to the recircu-

TABLE 1. Effect of Pentobarbital on Perfused Rat Hearts

Variable	Perfusate Pentobarbital (μg/ml)		
	0	200	324
Heart rate (beats/min)	198 ± 7 (7)	92 ± 36 (4)*	0 (9)†
Coronary flow (ml/min)	15.1 ± 1.1 (9)	18.7 ± 1.0 (4)*	12.4 ± 1.2 (9)
Phenylalanine incorporation (nmol · mg protein^{-1} · h^{-1})	0.93 ± 0.2 (9)	0.76 ± .03 (4)†	0.56 ± .03 (9)†
ATP (μmol/g dry weight)	23.6 ± 1.8 (4)	19.1 ± 0.5 (4)*	19.8 ± 1.0 (4)
Creatine phosphate (μmol/g dry weight)	20.7 ± 1.3 (4)	8.8 ± 1.3 (4)†	10.4 ± 1.6 (4)†

Hearts were perfused for 60 min as described in the Materials and Methods section. [^{14}C]Phenylalanine (690 μM) was added to the perfusate at a specific radioactivity of 0.15 μCi/μmol; pentobarbital was present as indicated. Data represent the means ± SEM of the number of observations shown in parenthesis.

* $P < 0.05$ *vs.* control.

† $P < 0.005$ *vs.* control.

lating perfusate (0.49 ± 0.07 μg/ml; six observations). *In vitro* measurements showed that at either of these concentrations, the drug had no detectable effect on the metabolism of lung proteins. However, if perfused lungs were exposed to a total dose of pentobarbital similar to that administered to the rats *in vivo* (final perfusate concentration, 324 μg/ml), extensive inhibition of protein synthesis resulted. Similarly, pentobarbital inhibited the synthesis of myocardial proteins in a dose-dependent fashion, although the sensitivity of the heart to the drug was somewhat less than that of lung. As reported by others,[13] exposure of the myocardium to doses of pentobarbital in the range tested sharply reduced contractile activity, perhaps by a mechanism involving altered availability of calcium for the contractile process.[14] This change appeared, however, to be rapidly reversible, as determined by the ability of drug-exposed hearts to regain normal rates of contraction following pentobarbital removal. Similarly, effects of the drug on synthesis of lung proteins could be quickly reversed.

TABLE 2. Relationship between Protein Synthesis and Tissue ATP in Anesthetic-exposed Lungs

Condition of Perfusion		Parameter Measured	
Pentobarbital (μg/ml)	Halothane (Per Cent)	Phenylalanine Incorporation, (nmol · mg protein^{-1} · h^{-1})	ATP (μmol/g dry)
0	0	1.78 ± 0.08 (29)	7.52 ± 0.29 (22)
50	0	1.87 ± 0.14 (11)	8.04 ± 0.34 (8)
100	0	1.84 ± 0.08 (12)	8.25 ± 0.68 (9)
200	0	1.04 ± 0.03 (3)*	7.51 ± 0.17 (3)
324	0	0.66 ± 0.12 (12)*	6.59 ± 0.21 (10)†
0	1	1.63 ± 0.10 (3)	6.68 ± 0.33 (8)
50	1	1.84 ± 0.18 (8)	8.59 ± 0.75 (8)‡
100	1	1.28 ± 0.09 (13)*,‡,§	7.79 ± 0.84 (9)
0	4	0.99 ± 0.11 (7)*	6.45 ± 0.92 (5)
50	4	0.81 ± 0.07 (8)*,§	6.70 ± 0.42 (8)¶
324	4	0.20 ± 0.02 (10)*,‡,§	0.85 ± 0.35 (6)*,‡,§

Lungs were perfused for 60 minutes as described in the Materials and Methods section with buffer containing 690 μM [^{14}C]phenylalanine (specific radioactivity, 0.15 μCi/μmol). Pentobarbital and/or halothane were present, as indicated. Data represent the means ± SEM of the number of observations shown in parenthesis.

* $P < 0.001$ *vs.* control without pentobarbital or halothane.

† $P < 0.02$ *vs.* control without pentobarbital or halothane.

‡ $P < 0.01$ *vs.* no pentobarbital, same per cent halothane.

§ $P < 0.01$ *vs.* no halothane, same pentobarbital.

¶ $P < 0.05$ *vs.* no halothane, same pentobarbital.

Previous studies which indicated that halothane inhibited the synthesis of proteins, both in lung[7,8] and other tissues,[6] were confirmed by the present experiments. Although the mechanism of this effect is not known, the observation that halothane further reduced protein synthesis in the presence of a maximally effective dose of pentobarbital suggested that the drugs inhibit the synthetic pathway by different mechanisms. In heart muscle, pentobarbital had little or no effect on tissue ATP, but reduced creatine phosphate levels 50 per cent. Energy depletion associated with hypoxia inhibited synthesis of both heart[15] and lung[9] proteins and, in perfused hearts, a graded dose-response relationship was observed as the oxygen supply was reduced progressively,[16] reflecting the numerous energy-requiring steps in the synthetic pathway.[17]

In perfused lungs, halothane exposure did not lower tissue levels of ATP. Furthermore, inhibition of protein synthesis by pentobarbital did not appear to require ATP depletion. Although a small reduction in ATP was observed in lungs exposed to 324 μg/ml pentobarbital, a significant inhibition of synthesis was measured in tissues where ATP levels were normal (*e.g.*, 200 μg/ml pentobarbital alone; 50 μg/ml pentobarbital plus 4 per cent halothane). These observations did not define the mechanism of action of pentobarbital in lung tissue, but they suggested that more than ATP depletion was involved. In contrast, high doses of halothane (4 per cent) and pentobarbital (324 μg/ml) in combination led to extensive depletion of high energy phosphates, reflected in a 90 per cent reduction in lung ATP. Nevertheless, this condition was reversible. Separate studies showed that return of the rate of protein synthesis to normal following removal of the drugs was accompanied by an increase in lung ATP to the control level (data not shown).

The role of creatine phosphate depletion in accounting

for the anesthetic-induced inhibition of synthesis of lung proteins is less certain. Concentrations of creatine phosphate in control lungs were low as compared to heart muscle, amounting to about 20 per cent of ATP (unpublished observations). This placed them near the limit of detection in the assay employed. In anesthetic-exposed lungs, creatine phosphate could not be measured reliably, suggesting that, as in heart muscle, the inhibition of protein synthesis may be associated with some energy depletion. This possibility requires further investigation using more sensitive assays of high-energy phosphate compounds.

A variety of experimental and clinical procedures involve administration of barbiturates and volatile anesthetics in combination. The present studies showed that at sufficient doses, either type of drug may be inhibitory to the pathway of protein synthesis and that these effects are not limited to lung tissue. Of greater significance is the observation that when the drugs were present in combination at concentrations of clinical relevance, where either compound had no effect alone, a rapid and significant depression of protein synthesis resulted. Although these changes appeared to be reversible, they emphasize the need for more extensive investigations of the metabolic effects of anesthetic agents, both when administered alone or in combination. Furthermore, these observations underline the necessity to take such effects into account in the interpretation of metabolic studies using anesthetized animals.

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