Anesthetic Gases and Water Structure

The effect of xenon on tritiated water flux across the gut

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ABSTRACT Pauling and Miller have independently proposed that the presence of an anesthetic gas in tissue induces a cage-like arrangement of hydrogen-bonded water molecules. The theories recognize that most gas-hydrate crystals would not form at the temperature and pressure that exist during anesthesia and propose that other components of tissue such as protein should have a stabilizing effect. Measurements of the behavior of water, rather than the anesthetic agent, would provide alternative information about the likelihood of hydrate crystal formation and this information could be such as to be applicable to body temperature and to pressures used for anesthesia. If the number of hydrogen-bonded water molecules in tissue is increased, then the movement of an average water molecule should be hindered. Movement of water through the tissue may be measured by tagging it with tritium and the anesthetic gas should then slow the movement of tritiated water through the tissue. The flux of tritiated water through rat cecum is indeed slowed when the cecum is exposed to the anesthetic gas, xenon, which can participate biochemically only by virtue of its van der Waals interaction. The decrement in water flux is in reasonable agreement with what could be expected theoretically from calculations based on the activation energy for the self-diffusion of water and the degree of hypothermia necessary to produce narcosis.

In 1961 Pauling (16) and Miller (15) independently suggested that the mechanism of action of gaseous anesthetics is the result of an interaction of the anesthetic agent with the water in the brain, rather than with the lipid in the brain, leading to the formation of minute hydrate crystals of the clathrate type (16) or minute gas-filled "icebergs" (15). The theories recognize that most gas-hydrate crystals would not form at the temperature and pressure that exist

during anesthesia and propose that other components of tissue such as protein would have a stabilizing effect.

The evidence for the theories of Pauling (16, 18) and of Miller (15) is based on measurements of the anesthetic agent, in particular the inverse relationship between the partial pressure required for anesthesia and the mole refraction representing the energy of the van der Waals interaction between the water cage and the enclosed anesthetic molecule. To seek evidence about the possible stabilization of a cage of water molecules by an anesthetic gas in living tissue, it is proposed that measurements of the behavior of the water, rather than the anesthetic agent, would provide alternative information about the likelihood of hydrate crystal formation and, further, this information could be such as to be applicable to the temperature and pressure used for anesthesia. The question then becomes: What is the effect of the anesthetic agent on the water in the tissue? If the number of hydrogen-bonded water molecules in the tissue at any given instant is increased, then the movement of an average water molecule should be hindered. The movement of water through the tissue may be measured by tagging it with tritium, and the anesthetic agent should then slow the movement of tritiated water through the tissue.

Among the anesthetic agents, a noble gas was chosen for study (6, 9, 17) because these gases cannot participate in biochemical reaction except by virtue of their van der Waals interactions, and among the noble gases xenon was chosen because it was likely to produce the greatest decrease in water flux in view of the fact that its mole refraction is the largest. The intestine was chosen for study because we had been studying tritiated water flux across it and found a barely minimal response to pitressin in vitro, a contrary response in vivo (water flux decreased), and also that the activation energy for the diffusion of water was plainly lower than the activation energy for the diffusion of water across the toad bladder (11, 13). This led to the impression that for the intestine the rate-limiting barrier to tritiated water flux is not a highly structured hydrogen-bonded water when compared, for example, to the toad bladder (11, 13).

METHODS AND PROCEDURES

Tritiated water flux was measured across the full thickness of the intestine. The mucosal surface of the intestine is lined with epithelial cells, beneath which is a loose connective tissue containing lymphatics and blood vessels, a muscle layer follows and then a thin serosal coat. The epithelial cells, including those of the rat cecum, evidence a "brush border" on the luminal surface. The brush border proves to be microvilli when examined by the electron microscope. Adjacent epithelial cells interdigitate at their lateral walls forming a potential lateral. intercellular space The space is sealed at the luminal end by a terminal bar, and the space is open at the end facing the basement membrane. Under certain conditions when a net transfer of water

occurs, for example, through the rabbit gallbladder, these intercellular spaces appear distended suggesting an intercellular pathway for water. The observations of tritiated water flux across the rat cecum are presented here without prejudice as to the site or nature of the rate-limiting barrier (2, 7).

The first experiments used the rat small intestine in vitro in a flow-through system, but this was later abandoned because of the possibility that changes observed with xenon might be a consequence of changes in the state of contraction of the intestinal smooth muscle which could alter the area available for tritiated water flux. The rat cecum was later selected for study because with the relaxed cecum, spread as a membrane between two chambers, the problem of changes in surface area were minimized by observing it grossly. (If the contracted cecum is so mounted it eventually relaxes and bulges into the serosal chamber.) The full thickness of the rat cecum was mounted vertically as a sheet between two warmed Lucite chambers (3) and the chambers immersed in a 37°C water bath. 3 ml of isotonic Krebs-Ringer bicarbonate containing 1 mM per liter of calcium and 0.2% glucose prewarmed to 37°C was added on each side of the cecum. The chambers were covered with a porous cup and each chamber was mixed by two gas lines, one line delivering either 95% oxygen—5% carbon dioxide or 100% xenon near the tissue (3) and a second line carrying 95% oxygen—5% carbon dioxide into the bath continuously. (The second line was a fine polyethylene tube which entered through a hole near the top of the chamber.) Each gas line was monitored continuously with a flowmeter employing a suspended float. Tritiated water, 50 μc in 0.1 ml of Krebs-Ringer, was added to either the mucosal or serosal side of the cecum and at 10 min intervals thereafter a 10 μl sample was removed from each side, and the tritium was assayed by liquid scintillation analysis.

In experiments to establish stability, tritiated water flux was observed for 90 min without intervention. The flux during the first 40 min was compared to the flux during the subsequent 50–90 min period. In experiments in which the tissue was exposed to xenon, each tissue served as its own control. In these experiments oxygenation was maintained throughout via the polyethylene tube, while the gas line near the tissue was changed after the first 40 min from 95% oxygen–5% carbon dioxide to 100% xenon (or if xenon was used first, from xenon to 95% oxygen–5% carbon dioxide) and five more paired samples (one from the mucosal bath and one from the serosal bath) were removed at 10 min intervals during the subsequent 50–90 min period.

In the experiments in which the tissue was exposed to xenon the rates of gas flow were adjusted so that xenon would comprise either 50 or 80% of the total gas flow to a single chamber. (In man consciousness is lost when xenon concentration in the inspired gas reaches 50% and surgical anesthesia ensues with an inspired concentration of 80% [6, 17].) Exposure of the bath to concentrations of 50 and 80% xenon represents a maximum concentration to which the tissue could be exposed, which assumes immediate and complete equilibration of xenon dependent on the lipid solubility and protein binding in addition to the solubility in the aqueous phase (23). The exponential decline of the difference in the specific activity of tritiated water between the chambers indicated that a steady state existed during xenon exposure, at least within the errors of the method. Concentrations of 50 and 80% xenon represent conservative estimates of what exists in the tissue, for if the tissue were not fully equilibrated the

observed decrement in flux would represent a minimum value, and if the tissue were completely equilibrated the decrement in flux would be still larger than that observed.

The net displacement of water in this system was a small fraction of the flux of water from mucosa to serosa and its simultaneous counterpart, the flux from serosa to mucosa. It was assumed that these fluxes are equal, and the flux was calculated from the equation.

$$\alpha_{ms} = \beta_{sm} = \frac{\ln(\theta_m^o - \theta_s^o) - \ln(\theta_m - \theta_s)}{\left[\frac{1}{M_o} + \frac{1}{S_o}\right] t} \quad (1)$$

where α_{ms} is the flux from mucosa to serosa and β_{sm} its counterpart from serosa to mucosa, $(\theta_m^o - \theta_s^o)$ is the difference in specific activity between the two baths at one time and $(\theta_m - \theta_s)$ this difference at a subsequent time, M_o and S_o are the volumes of the mucosal and serosal baths respectively, and t is the time interval between $(\theta_m^o - \theta_s^o)$ and $(\theta_m - \theta_s)$ (4). The quantity $[\ln(\theta_m^o - \theta_s^o) - \ln(\theta_m - \theta_s)]/t$ for the 40 min interval of interest was determined from a least squares solution of the relationship of the natural log of the difference in specific activity between mucosal and serosal bath to time.

OBSERVATIONS

To Establish a Stable Water Flux

The major difficulty in devising a means of testing the effect of xenon on water flux proved to be the ability to insure a sufficiently stable water flux for a period long enough to permit a test of the effect of xenon, an effect which was anticipated to be relatively small (see below). Some of these problems are described herewith. For surgical excision of the cecum, inhalation anesthetics obviously could not be used. Killing or stunning the animal yields a bloodless intestine which does not resemble the intestine obtained from an anesthetized animal, and also the flux of water across this cecum progressively increases with time. Pentobarbital in dosage of 30-40 mg per kg, administered subcutaneously 30-90 min prior to surgery proved to be the most suitable anesthetic among the alternatives tested, yet even here a word of caution is needed. If additional pentobarbital is administered shortly before surgery, water flux increases with time in control observations. (The addition of pentobarbital to the fluid bathing the cecum to achieve a concentration of 60 mg per liter induces a 25% increase in water flux.)

Exposure of the cecum to ambient temperature had to be kept as brief as possible to prevent cooling of the tissue. The cecum was transferred rapidly after removal from the rat to an oxygenated preparatory bath of Krebs-Ringer glucose solution at 37°C where it was kept immersed while it was opened and cleaned prior to mounting between chambers. If the cecum was

deliberately cooled by immersing it in an oxygenated preparatory bath at ambient temperature and then brought to 37°C, the subsequent exposure to xenon failed to slow water flux across the tissue. Anoxia per se (measurement of water flux with 95% oxygen–5% carbon dioxide and then substituting

TABLE I
STABILITY OF TRITIATED WATER FLUX ACROSS RAT CECUM

Date	Water flux		
	0–40 min	50–90 min	
	$\mu l/cm^2/hr$	$\mu l/cm^2/hr$	*% Change*
9-14-65	315	309	−1.9
9-20-65	182	179	−1.6
9-21-65	287	282	−1.7
9-23-65	285	300	+5.3
9-27-65	402	386	−4.0
10-4-65	206	207	+0.5
10-8-65	382	414	+8.4
12-10-65	241	240	−0.4
12-14-65	314	330	+5.1
12-16-65	369	329	−10.8
12-17-65	245	283	+15.5
12-20-65	632	648	+2.5
1-11-66	370	376	+1.6
1-13-66	311	342	+10.0
1-14-66	260	254	−2.3
1-19-66	353	373	+5.7
1-20-66	233	255	+9.4
1-25-66	307	309	+0.6
1-27-66	220	220	0.0
2-1-66	394	359	−8.9
7-20-67	353	335	−5.1
7-24-67	258	257	−0.4
7-25-67 a	309	338	+9.4
7-25-67 b	305	320	+4.9
7-27-67	281	318	+13.2
		Mean ± SE	+2.2±1.3

95% nitrogen-5% carbon dioxide) had no effect on water flux as long as the mucosa did not slough in which case loss of mucosa leads to an increase in water flux.

Evaporative losses as well as the net transfer of water were estimated by incorporating inulin-carboxyl-^{14}C as a volume indicator in the bath. In experiments in which a Visking membrane was substituted for the cecum, the concentration of inulin-carboxyl-^{14}C in successive samples of fluid from the chamber indicated that the evaporative losses were at most 1 or 2% over a

90 min period. Inulin-carboxyl-^{14}C was also used to establish net water transfer in independent experiments with the rat cecum. These observations are the basis of the assumption that the change in volume of the bath was negligible in comparison to the magnitude of the unidirectional fluxes. What

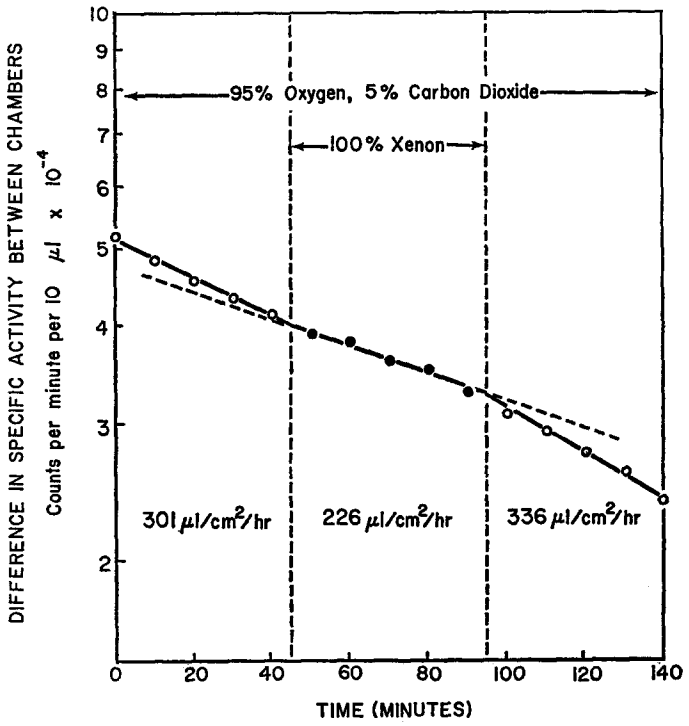

FIGURE 1. Sample experiment. In this particular experiment water flux was measured before, during, and after exposure of the rat cecum to 80% xenon in the gas flow. The difference in specific activity between chambers is plotted against time, the slope representing the rate of water flux across the cecum. When the cecum was exposed to xenon water flux slowed, to recover again when xenon was withdrawn. Flux is calculated from equation (1) in text. For the first control period, the difference in specific activity between chambers was 51,450 cpm per 10 μl at the start and 40 min later, 40,970; the volume of the serosal bath was 3.0 ml, the mucosal bath, 3.1 ml; the surface of the cecum between chambers was 1.73 cm^2.

error there is in the assumption tends to overestimate the water flux as the experiment proceeds; therefore any decrement due to xenon tends to be underestimated. The removal of 10 μl samples also affects the calculated flux in the same direction.

Damage to the tissue was ascertained by microscopic examination of any floating particles in the serosal or mucosal bath at the end of the experiment. Experiments were discarded if shreds of tissue were found. Saturating xenon with water vapor before it reached the bath seemed to mitigate tissue damage.

The majority of the experiments reported here were conducted in the fall and winter of 1965–66. In the spring of 1967 they were repeated, and again there were difficulties in establishing a stable water flux. Water flux now decreased with time in control experiments. The cause was eventually traced

TABLE II

EFFECT OF XENON ON TRITIATED WATER FLUX ACROSS RAT CECUM

Date	Water flux		Xenon effect	p*
	Control	Xenon		
	$\mu l/cm^2/hr$	$\mu l/cm^2/hr$	% Change	
Experiments in which xenon comprised 50% of total gas flow				
12-14-65	284	252	−11.3	
12-16-65	228	197	−13.6	
12-17-65	259	247	−4.6	
1-13-66	278	256	−7.9	
1-14-66	391	384	−1.8	
		Mean ± SE	−7.8±2.2	0.018
Experiments in which xenon comprised 80% of total gas flow				
1-19-66	301	226	−24.9	
1-27-66	313	218	−30.4	
2-1-67	305	292	−4.3	
2-3-67	508	399	−21.5	
2-10-67	288	280	−2.8	
2-11-67	236	227	−3.8	
2-14-67	208	205	−1.4	
2-17-67	236	218	−7.6	
7-20-67	439	341	−22.3	
7-24-67	311	305	−1.9	
7-27-67	346	272	−21.4	
		Mean ± SE	−12.9±3.3	0.002

* p calculated by comparison to +2.2 ± 1.3 (Table I).

to a contaminant in the gas washing bottle. The contaminant was characterized by its absorbance of ultraviolet light which became evident at 250 mμ and increased to a maximum at 210 mμ. The source of the contaminant was later found to be Tygon tubing which had been used for gas lines.

The stability of water flux eventually achieved is demonstrated by 25 experiments in which the flux was measured continuously without exposure of the cecum to xenon (Table I). The flux measured from 50 to 90 min was no different from that measured during the first 40 min, the average change being + 2.2 ± 1.3%.

Xenon Slows Water Flux across the Rat Cecum

To evaluate the effect of exposure to xenon on tritiated water flux, each cecum served as its own control. In a sample experiment (1-19-66) water flux slowed 24.9% from 301 to 226 $\mu l/cm^2/hr$ when xenon comprised 80% of the total gas flow to the tissue (Fig. 1). In this particular experiment water flux was measured beyond 90 min to observe a recovery when xenon was discontinued. With the tissue exposed to 50% xenon in the gas flow, 5 such technically satisfactory experiments (though not necessarily including a recovery period) were completed, and 11 such experiments were completed with the tissue exposed to 80% xenon (Table II).

When xenon comprised 50% of the total gas flow, tritiated water flux across the cecum slowed by 7.8 ± 2.2% (mean ± standard error). When xenon comprised 80% of total gas flow, water flux slowed by 12.9 ± 3.3% (Table II). The probability values are 0.018 for 50% xenon and 0.002 for 80% xenon compared to a +2.2 ± 1.3% change representing the stability of water flux across the cecum unexposed to xenon (Table I).

The anesthesia required for surgery and damage to the tissue lead to increasing water flux with time while evaporative and sample losses lead to overestimation of the water flux as the experiment proceeds. In all but the 12-14-65 experiment the order of the observations was control followed by xenon, thus in 15 of 16 experiments the xenon-associated decrement in water flux was observed despite these experimental difficulties which tend to reduce the decrement.

DISCUSSION

The observation that xenon decreases water flux across the rat cecum is consistent with the theories of Pauling and Miller. If these theories are pursued the mechanism for the delay is related to an increase in hydrogen bonded water molecules in the tissue when xenon is present. The water molecules would be those associated with a surface, such as that presented by a protein. When water is structured around protein by noncovalent interactions, potential cavities are possible (free volumes) which may accommodate a molecule of the gaseous anesthetic. The presence of xenon in such a cavity gives stability by its van der Waals force to this cloud of water molecules or "ice cover" around the protein. The average span of time any individual water molecule spends in any one position associated with a protein molecule is probably a matter of a fraction of a second so that while the structure of the cloud around the protein is preserved, there is a continuous split second shuffling of the individual water molecules somewhat similar to the flickering clusters of water molecules proposed by Frank and Wen (10). Xenon could

create a delay in the shuffling of some of these molecules. As two possibilities, the observed decrease in water flux may be due to the water molecule lingering near a xenon atom or to a "stiffening" of the membrane because of formation of hydrate microcrystals.

On the other hand xenon may effect a structural change in protein with an attendant interference with its function which could lead to a delay in water flux. The solubility of xenon in aqueous solution is increased by the presence of protein (23) and more pointedly, x-ray diffraction studies have located xenon associated with sperm whale myoglobin buried inside the protein molecule (20) while xenon associated with horse hemoglobin is close to the surface (19). Balasubramanian and Wetlaufer (1) have reported that passing xenon over a gently stirred protein solution induces a 1.8% increase in the optical rotation of β-lactoglobulin and a 0.9% increase in the optical rotation of bovine plasma albumin, all of which changes are reversible when the xenon is swept out with moist nitrogen. Balasubramanian and Wetlaufer interpret this observation as representative of a structural change in the protein; however, Kauzmann (12) has indicated that optical rotation "is influenced by changes in the immediate vicinity of individual groups in the molecule, whether such changes alter the general external shape of the molecule or not."

Xenon is quite soluble in lipid and the delay in water flux may somehow be related to this property. Thus there are possibilities for the mechanism of the delay in water flux other than an increase in the number of hydrogen-bonded molecules present in the tissue at any instant. It must be remembered that these mechanisms in tissue are not mutally exclusive and it is perfectly possible to have xenon offer stability to a cloud of water molecules surrounding one protein molecule while it might be buried inside the structure of another.

How much of a decrease in water flux could be anticipated when a tissue is exposed to an anesthetic gas? The theory implies that at a given instant there are more hydrogen-bonded water molecules when an anesthetic gas such as xenon is present, that is, a more ice-like state exists in the tissue. The same would occur if the temperature of the tissue were lowered. If brain temperature is lowered, narcosis ensues. The precise cerebral temperature at which narcosis ensues is an elusive number in a vast literature. The value is elusive because of the need to use anesthetic agents in homothermic beings to avoid the uncomfortable period of shivering and a precipitous fall in temperature when cooling is effected, because esophageal or rectal temperature is not the temperature of the brain, etc. From the available information, 5–10°C would be a fair estimate for the decrement in brain temperature necessary for narcosis in man (5, 8, 14, 21). The Arrhenius equation,

$$\ln \frac{k_2}{k_1} = \frac{E}{R}\left[\frac{1}{T_1} - \frac{1}{T_2}\right],$$

defines the relation between reaction velocity and temperature where k_1 and k_2 are reaction velocities at absolute temperatures T_1 and T_2, E is the activation energy, and R is the gas constant, 1.986 cal per degree mole. The activation energy for the self-diffusion of water is 4.6 kcal per mole at 25°C and changes but slightly between 10° and 50°C (22). If a 5 or 10°C fall in brain temperature is necessary for narcosis, then k_2/k_1 may be estimated; for example, if narcosis ensues when brain temperature falls 5°C from 37° to 32°C, then T_1 is 310° and T_2 is 305° and if E is 4.6 kcal per mole, then k_2/k_1 is 0.8847 which indicates a 11.5% decrease in velocity. Assuming an activation energy of 4.6 kcal per mole and a change in temperature from 310°K to 305°K or 300°K, the Arrhenius equation predicts a decrease in the reaction velocity of 11.5–22.0%. The unidirectional flux of water through rat cecum exposed to partial pressures of xenon anesthetic for man appears in our experiments to decrease to about the same extent. Thus, if the present agreement between (*a*) the experimentally found decrease in unidirectional water flux in tissue subjected to anesthetic concentrations of xenon, and (*b*) the theoretically estimated decrease in unidirectional water flux in water resulting from a 5–10° temperature drop, is not fortuitous, it may be inferred that the decrease of unidirectional flux of water in tissue is at least a correlate and perhaps a cause of the anesthetic state in general. If, further, the decrease of unidirectional water flux produced by xenon is regarded as due to formation of Pauling or Miller type structures, then the present experiment links these structures to the anesthetic state.

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