The Association of Xenon with Subcellular Components of Rat Cerebral Cortex

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SUMMARY

Subcellular fractions isolated from rat cerebral cortex by differential and density gradient centrifugation were resuspended in mannitol and saturated with xenon at various temperatures and pressures. The association of the gas with subcellular components, and also with water, oil, and brain lipid extracts, was measured by gas chromatography. The affinity of xenon for particulate fractions exceeds that predicted from estimations of solubility in aqueous and lipid constituents. The association of the gas with non-lipid components is not influenced by pH but is affected to a small extent by changes in temperature and is dependent on gas pressure, particularly in the range of 0.6–1.5 atm.

INTRODUCTION

Xenon, an inert monatomic gas, has the well-established capability of producing anesthesia in man and certain experimental animals (1–3). During studies of the anesthetic action and distribution of xenon, it became apparent that more xenon is transported by the blood than would be anticipated from its solubility in plasma. Solubility studies (4–6) subsequently indicated that xenon and other inert anesthetic gases, including cyclopropane and nitrous oxide, bind to certain proteins, including myoglobin and hemoglobin. Detailed structural analysis of these proteins utilizing X-ray diffraction techniques has made it possible to examine the nature of the forces involved in rare gas-protein associations (7–9), with the conclusion that the energy of binding originates mainly in London interactions. These studies have been of great value in considerations of drug-"receptor molecule" association at the molecular level and may have direct relevance to the mode of gas transport in the blood. In terms of the mechanisms by which such agents exert anesthetic action, however, these studies would remain "model systems" until such interactions could be demonstrated at the level of molecular components of neural tissue. We now report on an association of xenon with subcellular particulate fractions derived from rat cerebral cortex in excess of that predicted on the basis of gas solubility in aqueous and lipid components of the tissue. This interaction is not influenced greatly by variations in pH and temperature but is dependent on gas pressure in the range of 0.6–1.5 atm.

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METHODS

Preparation of subcellular fractions. Cerebral cortex tissue was separated from the brains of 12–16 male Sprague Dawley rats (250–300 g) and cleared of white matter and capillaries. A 10% dispersion of the tissue was prepared in a medium containing 0.3 M mannitol and 5 mM EDTA (Tris salt) at pH 7.2, using a glass homogenizer with a Teflon pestle (A. H. Thomas

& Company). The homogenate was centrifuged at 900 × *g* (avg) for 10 min in a refrigerated centrifuge, and the pellet (nuclei and undisrupted tissue) was discarded. The supernatant fluid was centrifuged at 10,000 × *g* for 20 min, and the particulate fraction obtained (P-1) was washed twice by resuspension in 0.3 M mannitol and centrifugation. In some experiments fraction P-1 was resuspended in mannitol to give a dispersion equivalent to 400 mg of tissue (wet weight) per ml, which was used for gas-binding studies. Other preparations of P-1 were subjected to the density gradient fractionation procedure of Gray and Whittaker (10) to separate more homogeneous fractions containing mitochondria and synaptosomes, which, following sedimentation, were also resuspended in mannitol. Microsomal fractions were prepared by centrifugation of the 10,000 × *g* supernatant at $10^5 \times g$ for 70 min in the No. 40 head of a Spinco model L ultracentrifuge. The resulting supernatant (soluble) fraction was concentrated by rotary evaporation at 15° in a vacuum. Samples of all subcellular fractions were analyzed for protein, total lipid, and succinic dehydrogenase activity both before and after gas saturation experiments.

Measurement of xenon association with solution and tissue fractions. Solutions or tissue dispersions to be saturated with xenon were placed in small flasks (similar in size and appearance to Warburg vessels) fitted with side arms which could be sealed with "wrap-over" rubber serum caps. The flasks were attached to a vacuum line by O-ring connectors (Viton), and the contents were carefully degassed using a vacuum pump (Hyvac 7, Central Scientific Company) until the gas pressure approximated the vapor pressure of water at 25°. Xenon was then introduced into the system, which was equilibrated for at least 60 min at any given temperature and pressure. Equilibration was facilitated by magnetic stirring and was judged complete when gas pressure remained constant at a steady temperature. It is important that the maintenance of a gas-tight system is critical and requires careful assembly of O-ring connections and optimal lubrication of glass stopcocks in the vacuum line, especially for studies with gas pressures above 1 atm.

Aliquots (100–200 μl) of the saturated solutions of dispersions were removed anaerobically through the rubber serum caps on the side arms of the vessels, using Hamilton syringes, and were analyzed by gas chromatography (9). A Beckman GC-2A gas chromatograph was used together with a Fisher clinical gas partitioner, permitting direct analysis of liquid samples. Analysis was carried out on a 12-inch molecular sieve 13X (42/60 mesh) column maintained at 220°, with a 250-mamp current and a helium carrier gas pressure of 29 psi. Under these conditions the retention time of xenon was 2.7 min. The instrument was calibrated with known volumes of pure xenon gas, and the gas content of the sample was calculated from the integral of the area under the xenon peak on the chromatograph recorder. A minimum of four separate samples were taken for analysis at each temperature and pressure in each experiment. Control flasks containing dispersion media were run concurrently with vessels containing dispersions of the brain subcellular fractions.

Extraction of cerebral lipids. Gray matter from rat cerebral cortex was extracted with either acetone, ethanol, or a mixture of chloroform and methanol (2:1 by volume) following extraction of the tissue with 10% trichloracetic acid (11). The solvent extracts were concentrated by rotary evaporation in a vacuum and then used for estimations of xenon solubility by the method detailed above.

Chemical and enzymatic analyses. Protein was determined by the biuret procedure of Gornall, Bardawill, and David (12); cholesterol, by the method detailed by McIlwain and Rodnight (11); and succinic dehydrogenase, according to Aldridge and Johnson (13). The water content of tissue samples was estimated by drying to constant weight at 105°. Total lipid was measured by weighing the dry residue obtained following evaporation of a chloroform–methanol (2:1) extract subsequent to tissue extraction with trichloracetic acid.

TABLE 1

Chemical and enzymatic composition of subcellular fractions derived from rat cerebral cortex tissue

The preparation of subcellular fractions and the chemical and enzymatic determinations were as detailed in METHODS. Figures represent mean values derived from five distinct determinations.

Fraction	Protein	Cholesterol	Total lipid	Succinic dehydrogenase
	mg/g tissue	*μg/mg protein*	*mg/mg protein*	*μg CO_2/mg protein/hr*
Dispersion	102	128	0.64	142
Particulate (P-1)	48.5	171	0.96	192
Soluble	35.2	32	0.20	20
Mitochondrial	13.7	92	1.10	318
Synaptosomal	9.4	122	0.80	109
Microsomal	9.5	235	1.05	36

Statistical methods. Statistical analysis was performed by Student's *t*-test, analysis of variance, and the Schaffé test, where appropriate.

RESULTS

Composition of subcellular fractions. The protein, cholesterol, and total lipid contents of subcellular fractions used in the xenon association studies are given in Table 1. The distribution of succinic dehydrogenase activity, accepted generally as a mitochondrial marker, is indicative of the heterogeneity of the fractions.

Xenon solubility in oil, water, and cerebral lipid fractions. The influence of temperature on xenon solubility in olive oil and water is shown in Table 2, where it is expressed as milliliters of gas dissolved in 1 g of solution after saturation at atmospheric pressure and a given temperature. The values of xenon solubility in water are close to those previously reported (14). The oil-to-water solubility ratio is shown to increase with increase in temperature, reflecting the greater dependency on temperature of the gas-water solution process. This ratio at 37° and 1 atm pressure is lower than the figure of 20 reported by others (15) and quoted extensively in the literature.

The solubility of xenon in oil and water at 27° and at varying gas pressures is shown in Table 3. The values for solubility at a pressure of 40 psi are approximately 15% less than those predicted from Henry's law, based on a calculation of a constant determined at 10 psi. The ratio of gas solubility in oil to that in water is not sig-

TABLE 2

Effect of temperature on xenon solubility in oil and water

Solutions were saturated with xenon at atmospheric pressure in the apparatus described in METHODS. Aliquots were removed for xenon estimation by gas chromatography. The figures represent mean values from three replicate experiments.

Temperature	Xenon solubility in Oil (A)	Xenon solubility in Water (B)	A/B
	ml/g	*ml/g*	
10°	1.828	0.151	12.1
20°	1.676	0.112	14.9
30°	1.560	0.091	17.1
37°	1.488	0.081	18.4

TABLE 3

Effect of pressure on xenon solubility in oil and water

Solutions were saturated with xenon at 27° and at varying gas pressures in the apparatus described in METHODS. Aliquots were removed for xenon estimation by gas chromatography. The figures represent mean values from three replicate experiments.

Pressure	Xenon solubility in Oil (A)	Xenon solubility in Water (B)	A/B
psi	*ml/g*	*ml/g*	
10	0.998	0.068	15.4
20	1.951	0.129	16.0
30	2.684	0.170	15.8
40	3.388	0.212	16.0

TABLE 4

Xenon solubility in oil and lipid fractions extracted from rat cerebral cortex

Lipid extracts were prepared as described in METHODS. Solutions were saturated with xenon at a pressure of 20 psi and 27°. Aliquots were removed for xenon estimation by gas chromatography. Figures represent mean values from three distinct experiments.

Lipid	Xenon solubility
	ml/g
Olive oil	1.93
Acetone extract	1.46
Ethanol extract	1.81
Chloroform–methanol extract	1.54

nificantly influenced by gas pressures over the range studied.

The solubility of xenon in lipid fractions extracted from rat cerebral cortex and saturated with the gas at a pressure of 20 psi and 27° is expressed as milliliters of gas per gram of lipid in Table 4. The concentrated extracts contained the following lipid components. The acetone extract contained cholesterol and neutral lipids; the ethanol extract contained lecithins, cephalins, and some sphingolipids; and the chloroform–methanol extract contained most cerebral lipids, including proteolipids and gangliosides. Xenon solubility in the various lipid fractions was not uniform and in no case exceeded the value obtained in olive oil.

Association of xenon with subcellular fractions of rat cerebral cortex. Subcellular fractions suspended in 0.3 M mannitol were saturated with xenon at a pressure of 20 psi at 27° (Table 5). The figures in column A represent the amounts of the gas (microliters per milligram, dry weight) associated with the tissue components, corrected for xenon solubility in the aqueous phase of each subcellular fraction. Column B represents computations of xenon association with lipid components of each fraction, based on a lipid solubility equivalent to that of the gas in olive oil at the given pressure and temperature. It is important to emphasize that such computations of gas-lipid associations are derived with the unproven, but not improbable, assumption that xenon solubility in lipid components of the subcellular fractions does not exceed that in olive oil (Table 4). The subtraction of the figures for lipid solubility from those given in column A permits an estimation of xenon association with "protein" components (column C). Although lipid and protein together comprise over 80% of the dry weight of cerebral tissue (16), it is appreciated that the term "protein" is not exclusive of carbohydrates and nucleic acid moieties and is used merely for convenience

TABLE 5

Association of xenon with subcellular fractions derived from rat cerebral cortex tissue

Subcellular fractions, prepared as described in METHODS, were saturated with xenon at a pressure of 20 psi and 27°. Aliquots were removed for xenon estimation by gas chromatography. Figures in column A represent mean values and standard deviations derived from 20–30 replicate determinations. Column B lists the calculated association of xenon with lipid components present in a 1-mg sample (dry weight) of particulate fraction. The subtraction of B from A gives an estimation of xenon association with "protein" components of each fraction, expressed on a milligram basis, in column C.

Fraction	Xenon solubility (A)	Calculated association with lipid of fraction (B)	A − B	Xenon to "protein" ratio (C)
		μl/mg, dry wt		*μl/mg*
Particulate	1.19 ± 0.03	0.95	0.24	0.48
Mitochondrial	1.28 ± 0.06	1.06	0.22	0.48
Synaptosomal	1.07 ± 0.04	0.87	0.20	0.36
Microsomal	1.31 ± 0.06	0.99	0.32	0.66
Soluble	0.41 ± 0.09	0.33	0.08	0.09

to denote non-lipid components other than tissue water.

The association of xenon with such components of a microsomal fraction was significantly greater ($p < 0.05$) than with other subcellular fractions. No significant differences were found between gas association with crude particulate, mitochondrial, or synaptosomal fractions. Association of xenon with non-lipid components of the soluble fraction was significantly lower ($p < 0.01$) than that with all of the other fractions.

Effect of temperature and pH on xenon association with a particulate fraction of rat cerebral cortex. The results of studies in which the temperature and pH were altered independently are shown in Tables 6 and 7, respectively. The association of xenon with a crude particulate fraction following equilibration at a gas pressure of 20 psi decreased with increase in temperature. However, the percentage decrease in succinic dehydrogenase activity following incubation at different temperatures indicates that the observed effects on xenon interaction with non-lipid components may have been due partly to denaturation.

TABLE 6

Effect of temperature on xenon association with a particulate fraction derived from rat cerebral cortex

Particulate fractions were saturated with xenon at a pressure of 20 psi as described in METHODS. Aliquots were removed for xenon estimation by gas chromatography, and the association of xenon with "protein" components was computed as described for Table 5.

Temperature	Xenon to "protein" ratio	Decrease in succinic dehydrogenase activity following incubation
	μl/mg	%
7°	0.52	4
17°	0.48	12
27°	0.48	16
37°	0.42	28

Changes in pH between 5.5 and 8.9 had no effect on gas association with the particulate fraction, suggesting that the molecular components involved in the interaction are insensitive to charge differences within the pH range studied, as far as xenon binding is concerned.

TABLE 7

Effect of pH on xenon association with a particulate fraction derived from rat cerebral cortex

Particulate fractions were saturated with xenon at a pressure of 20 psi and 27° as described in METHODS. Aliquots were removed for xenon estimation by gas chromatography, and the association of xenon with "protein" components was computed as described for Table 5.

pH	Xenon to "protein" ratio	Decrease in succinic dehydrogenase activity following incubation
	μl/mg	%
5.5	0.47	15
7.2	0.48	18
8.9	0.48	12

Effect of pressure on xenon association with subcellular fractions of rat cerebral cortex. Subcellular fractions suspended in 0.3 M mannitol were saturated with xenon at varying gas pressures and a temperature of 27°. The effect of gas pressure on the association of the gas with the non-lipid components of the crude particulate and soluble fractions is shown in Fig. 1. Interaction of xenon with the soluble fraction was not significantly different at pressures ranging from 10 to 40 psi. The degree of association of the gas with the particulate fraction increased with increase in pressure most significantly over the pressure range from 10 to 20 psi. Above a gas pressure of 25 psi, no increase in xenon association was observed, suggesting a saturation phenomenon. The increase in the association of xenon with the non-lipid components of the particulate fraction over the 10–20 psi range is approximately twice that predicted for a gas-solvent system between these pressures according to Henry's law. The more homogeneous subcellular fractions (mitochondrial, synaptosomal, and microsomal) also exhibited this effect (Fig. 2). In each case, the increased gas interaction between pressures of 10 and 20 psi is greater than that predicted by Henry's law for simple gas-solvent systems.

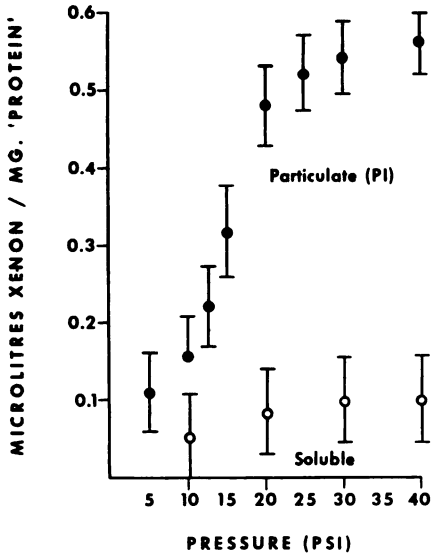

FIG. 1. *Effects of gas pressure on xenon association with components of brain particulate and soluble fractions*

Subcellular fractions, prepared as described in METHODS, were saturated with xenon at varying gas pressures and 27°. Aliquots were removed for xenon estimation by gas chromatography. Xenon association is expressed as microliters of gas per milligram of "protein" (nonaqueous and non-lipid components; see the text). Each point on the figure represents the mean value of 24 determinations. Vertical bars represent one standard deviation.

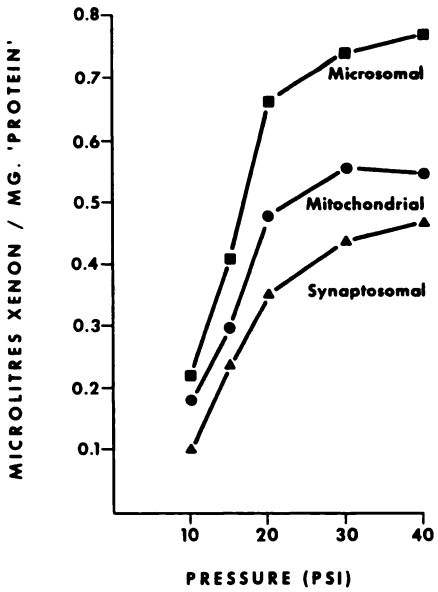

FIG. 2. *Effects of gas pressure on xenon association with components of subcellular fractions derived from rat brain*

Subcellular fractions, prepared as described in METHODS, were saturated with xenon at varying gas pressures and 27°. Aliquots were removed for xenon estimation by gas chromatography. Xenon association is expressed as microliters of gas per milligram of "protein" (nonaqueous and non-lipid components; see the text). Each point on the figure represents the mean value of 10 determinations.

DISCUSSION

Although the phenomenon of anesthesia has been studied extensively, biochemical approaches have not defined unequivocally the mechanisms by which anesthetics act. Indeed, evaluation of experimental results with *fixed* anesthetic agent leads to the conclusion that they may act at more than one locus (17, 18). In the case of gaseous anesthetics in general, and the inert gases in particular, there is little evidence that alterations in any biochemical property of nervous tissue underlie their specific action. The inert gases are of special interest because they are incapable of participation in ionic, covalent, or hydrogen bonding under physiological conditions (19); thus considerations of the mechanisms by which they cause anesthesia must be restricted to the physical level of molecular interaction. The existence and nature of such interactions involving London forces have been clearly documented in studies of the association of inert gases with proteins of well-established structure (7–9). That such interactions may result in functional sequelae is suggested by the observations that xenon binding to myoglobin affects the affinity of carbon monoxide for myoglobin (20) and that helium-group gases can inhibit active sodium transport systems (21). Similarly, nitrogen and other inert gases at high pressures will inhibit certain oxygenases (22), and cyclopropane is capable of reversibly inhibiting a membrane-bound Na^+- and K^+-activated ATPase preparation derived from rat brain (23).

At the present time, detailed knowledge of macromolecular structure is limited to

relatively few proteins, none of which is of neural origin. For this reason, any investigation of inert gas association with macromolecular components of cerebral tissue must follow an indirect approach. The results of the present study suggest that the association of xenon with particulate fractions of cerebral cortex is in excess of that predicted on the basis of solubility in lipid and aqueous components of such fractions. This apparent association with non-lipid components is not influenced by conditions expected to alter charge on protein molecules, and in this respect behaves similarly to the interactions of cyclopropane (6) and hydrocarbons (24) with proteins in solution. Increases in temperature may decrease this association, perhaps by denaturation of the components involved. The pressure dependence of such associations is of interest, since an increase in pressure from 10 to 20 psi results in an increase in gas binding at least twice that expected from considerations of Henry's law. Such an effect is analogous to the association of oxygen with hemoglobin as a function of pressure (25), and in this respect might be termed "facilitative." At pressures above 25 psi, no further association of xenon occurs, suggesting a saturation of available binding sites. The minimal binding of xenon to non-lipid components of the soluble fraction suggests that structural requirements peculiar to particulate material may be essential for these inert gas associations.

The present experiments throw little light on the molecular nature of the component(s) involved. It is tempting to suggest that they are of protein nature, since this molecular species represents the major non-lipid component of cerebral tissue other than water (16) and xenon associations with certain (but not all) proteins is established (26). It is reasonable, however, to conclude that considerations of anesthetic action purely in terms of solubility in lipid materials of neural tissue are unnecessarily restrictive.

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