BUOYANT DENSITY STUDIES OF BOVINE MERCAPTALBUMIN RELATED TO THE PROBLEM OF GENERAL ANESTHESIA

Chemistry 80 Thesis

by

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There are at present two major schools of thought concerning the method of chemical action of general anesthetics in producing anesthesia. Though the anesthetic effect seems to be in evidence in many parts of living things, explanations of the effect are usually postulated with respect to the nervous system - that system being more responsive to anesthesia than are others. One theory postulates various modes of direct physical-chemical action by the anesthetic agent on the lipid and protein components of the nerve cells and synaptic regions. The second theory suggests the formation of labile hydrate microcrystals in the synaptic fluid. These "microcrystals" involving anesthetic molecules, small ions, charged protein side chains, and water are thought to cause an impedance change in the synapse by slowing down the ionic species involved in them. Thus they have an anesthetizing effect on nerve impulse transmission. This is the Pauling theory of anesthesia (1).

Pauling points out that the trend in pressure required for anesthesia of various gases indicates that the effect of general anesthetics results from a van der Waals interaction with the encephalonic fluid or membrane of the synapse. Hydrates similar to the type Pauling proposes are known to exist in water solutions at lower temperatures in the absence of proteins - for example the $Xe \cdot 5\frac{3}{4}\ H_2O$ hydrate @ 0°C (2), and his calculations show that their enthalpy of formation may be readily understood in terms of van der Waals stabilization of the hydrate by Xe.

Experimentally it is quite difficult to distinguish between the lipid and hydrate theories. As will be seen below, the work presented here attempts to make use of the different sizes of cavities in Pauling's proposed clathrate structure for this purpose.

Most previous work on this problem of general anesthesia has been performed on biological systems. These experiments suffer severely from the variability in response of biological systems, and the errors in this data are therefore so great as to make thermodynamic conclusions difficult. Also the compexity of such systems confuses interpretation of their response. Some studies of solubility of anesthetic agents in protein solutions and the viscosities of such solutions have been begun (3), but so far no adequately quantitative measure of a property change in a living or model system under the influence of anesthetic agents has been reported.

The purpose of these experiments was to find a quantitatively measurable system in which to gain evidence pertaining to the existence of Pauling's suggested microcrystals. We also hoped to demonstrate the impedance increase which he has proposed will accompany their formation in anethetized systems. The work was part of a group project under the direction of Professor L. Pauling and Dr. W. F. Dove during the summer of 1962. Other individuals on the project were Lawrence Hall, John Aupers, Peter Hall, and Lawrence Oliver.

Studies were undertaken using a model system of 10% by weight bovine serum albumin (BSA) and .15 F NaCl in water. Qualitatively, the anesthetic effects on BSA side chain pK's, anesthetic solubilities, anesthetic entropies of solution, anesthetic effects on solution and membrane conductivity, and anesthetic effects on the buoyant density of bovine mercaptalbumin (BMA) were investigated by the group. Of these, the solubility work by Lawrence Hall and the buoyant density experiments which I conducted are especially

interesting. Hall investigated the solubility of CHI_3 and H_2S in the BSA system and in polyglutamic acid, polylysine, and various model salt solutions. This work is continuing.

The object of the density gradient experiments was to investigate the effect of anesthetic agents on the buoyant density of BMA. If the Pauling theory is correct, a co-operative anesthetic effect might be expected when two anesthetics with different sized molecules are applied to the system. If a big moleculed anesthetic which is the size of the larger hole in the proposed structure is added, a certain amount of anesthetic effect should be observed. If likewise a small molecule is added, an effect should be observed. Then, if both the large and the small are added at the same time, the increased stabilization from filling all the cavities in the hydrate would cause a more than additive effect. The effect observed in these experiments was the change in buoyant density of BMA as it became associated with the anesthetic molecules which were chosen for their size and high density. It will be seen that, although such a co-operative effect was observed, the changes in density involved were so small that no final conclusions should be drawn from the data as it stands.

Pure BMA which had been prepared by Ifft (4) was used. The salt concentration was, of course, dictated by the gradient method rather than our physiological model.

First Ifft's experiment of determining the buoyant density of BMA in CsCl with a NaAc-HAc buffer at 25°C was reproduced (4). His value was 1.282, mine 1.284. This run was done in a single, double sector cell. Further runs were done in twin double sector cells.

The first run being satisfactory, I chose CHI_3 for the first

"anesthetic" experiment. Theoretically this molecule has a poor size for the structures which Pauling has proposed, since it is a little larger than the largest cavity in the postulated clathrate. However, it was chosen because Hall's work had demonstrated a marked dependence of CHI_3 dissolved on BSA concentration and because it has a useful density, 4.0 g/ml. The first attempt showed that BMA was denatured by stirring with CHI_3 (a solid) if high salt concentrations were present. Therefore, the BMA and buffer solutions were saturated with CHI_3 and the CsCl added thereafter. A buoyant density of 1.291 d.u. or an increase of .007 d.u. over untreated BMA was found. The approximate concentration of CHI_3 was determined directly in the centrifuge cells in a Cary spectrophotometer. Using the extinction coefficient determined by Hall, I calculate that approximately 3 CHI_3 were present for each BMA molecule. It was not determined how much of this was bound to the protein.

Next I investigated an anesthetic agent of more ideal size (size of the large cavity) and less ideal density (1.5 d.u.), $CHCl_3$. When I used anesthetics which were liquid, the experiments were run with an excess of liquid in the bottom of the cell. There was some denaturation with $CHCl_3$, but most of the BMA banded at a density of 1.288 or an increase of .004 d.u. Though this shift is significant, I hoped to have a larger effect to work with in the co-operative effect experiment; so I chose another agent.

$CHBr_3$, a liquid of high density (2.9 d.u.) was tried. This run produced a very large increase of around .070 d.u. However, the stock solution of $CHBr_3$ was observed to have a brown tinge which might have been Br_2. To test the effect of Br_2 on my system, I prepared a run

using $CHCl_3$ treated with Br_2 until its color matched that of the $CHBr_3$. This run produced a density shift of .030 to .040; and, when several attempts to purify the $CHBr_3$ of Br_2 failed, I abandoned $CHBr_3$.

With little time remaining, we decided to try CBr_4, a solid of questionable anesthetic properties but a high density (3.4 d.u.). A preliminary run showed a significant increase in density, so I prepared two twin double sector runs to test for a possible co-operative effect with Xe. CBr_4 is about the size of the large cavity and Xe is about the size of the small cavity. In the first run, one sector contained BMA and the other BMA and Xe. In the second run, one sector contained BMA and CBr_4; while the other contained BMA, CBr_4, and Xe. The samples were saturated by diffusion by letting them stand for 12 hours over solid CBr_4 and under approximately one atmosphere of Xe. The resulting buoyant densities were as follows:

	Sector Contents	d.u.
Run #1	BMA Xe	1.292
	BMA	1.291
Run #2	BMA Xe CBr_4	1.312
	BMA CBr_4	1.308

Keeping in mind the possible error of .002 d.u., the effect seems to be more than additive. Also, it is interesting that Xe gives no increase by itself. Midway through the summer I visited the lab of C. A. Muehlbaecher at the University of California School of Medicine in San Francisco. She is working on anesthetic solubilities in protein solutions using a gas chromatograph for analysis, and she had preliminarily found no increase in Xe solubility in H_2O with the addition of BSA. If we are correct, it would appear that BSA does not bind Xe. Yet, there may be a co-operative effect as indicated by run #2 above.

However, why does BMA here show a density of 1.291? This run was done in a different centrifuge which was not in so good a condition as and had entirely different temperature calibrations than that used in my initial runs. Though certainly comparable with their twins in the same run, it is understandable how these densities could vary some from the initial runs. An additional danger may be bromination, although I don't know how this could be affected by Xe.

Unfortunately, the series of experiments is incomplete. The interesting ones need to be reproduced and further clarifying experiments based upon them.

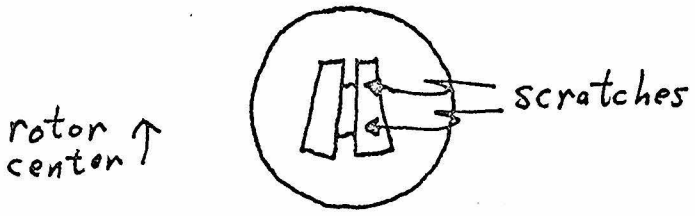
The CBr_4-Xe experiment at 25°C and the CHI_3 experiment at 25°C need to be repeated. I suggest also that the method of saturating with Xe be improved by placing the entire centrifuge cell in a Xe filled chamber rather than running tubes to the filling holes of the cell.

Though the effects so far observed are small, with careful work they may prove quantitative enough to be of value. Perhaps by lowering the temperature to 20°C the effect will be increased a significant amount in the CBr_4-Xe system. Also, a similar series using $CHCl_3$ in place of CBr_4 might prove quite interesting in view of the known existence of $CHCl_3 \cdot 2Xe \cdot 17H_2O$ clathrates at lower temperatures. I hope to be able to perform these experiments during the coming summer.

Experimental

The experiments were all done using a sample of the BMA purified

by Ifft (4). All were buffered to pH 5.5 with .01 M NaAc-HAc buffer; all were run at 25°C; all were spun at 56,000 rpm in the centrifuges indicated on the accompanying sheet. Run #1 was performed using one double-sector, epoxy-aluminum centerpiece and the calculated gradient for this system. Two of these centerpieces were employed in experiments #2 - #12. By arranging the mean solution densities in the two on either side of the protein density according to the method developed by Ifft and Vinograd (5), the gradients in these runs were determined by experiment. In experiments #13 and #14 the cells contained again different solutions as indicated in the tabulation. Densities were determined from refractive index measurements corrected for protein content using the formulae given by Ifft (4). The double sector centerpieces were scratched in the following way to allow equilibrium of meniscus between the experiment and the blank.



He was introduced through brass tubes fitted into the filling holes of the cells; $CHCl_3$ and $CHBr_3$ were present as liquid layers in the cell bottoms during the runs, and the solutions were saturated with CHI_3 and CBr_4 before introduction into the cells.

I would like to thank Dr. Jerome Vinograd and Dr. J. H. Fessler for instructing me in the use of the Centrifuge and advising me relative to the technique used in these experiments.

Tabulation of Gradient Runs

Run #	Component	ρ of Component	Comments
1	BMA	1.284	one cell, calculated gradient
2	BMA + CHI_3	-----	plugged scratch denat. dep. on salt conc..
3	BMA + CHI_3	1.291	~3 CHI_3/BMA No denat.
4	BMA + $CHCl_3$	1.288 (band) 1.296 (ppt)	Some denaturation
5	BMA + $CHBr_3$	-----	Drive failure
6	BMA + $CHBr_3$	-----	Off Field
7	BMA + $CHBr_3$	~1.35	
8	BMA + $CHCl_3$ + Br_2	~1.32	
9	BMA + Xe	-----	Leak
10	BMA	-----	No leak } Optics out of adjustment.
11	BMA + Xe	-----	No leak } Cell bottom not in photo.
12	BMA + CBr_4	↓↓	
13	BMA + Xe	1.292	
	BMA	1.291	
14	BMA + Xe + CBr_4	1.312	
	BMA + CBr_4	1.308	

Centrifuges Used

Run #	Centrifuge	Spinco #
1	Dr. Hodge's	443
2	Dr. Hodge's	443
3	Dr. Hodge's	443
4	Dr. Hodge's	443
5	Dr. Bonner's	521
6	Dr. Vinograd's - Antony	186
7	Dr, Sinsheimer's - 1578	264
8	Dr. Sinsheimer's - 1579	264
9	Dr. Delbruck's	285
10	Dr. Delbruck's	285
11	Dr. Delbruck's	285
12	Dr. Delbruck's	264
13	Dr. Sinsheimer's - 1582	285
14	Dr. Vinograd's - Antony	186

References

1) Linus Pauling, Science 134, 15-21 (1961)

2) M. von Stackelberg et al, Fortschr. Mineral 26, 122 (1947); -----, Naturwissenschaften 36, 322, 359 (1949); -----, ibid. 38, 456 (1951); -----, ibid. 39, 20 (1952); -----, J. Chem. Phys. 19, 1319 (1951); -----, Z. Electrochem. 58, 25, 40, 99, 104, 162 (1954).

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3) R. M. Featherstone's Group, Univ. of California School of Medicine, San Francisco, California.

4) J. B. Ifft, Phd. Thesis, California Institute of Technology (1962)

5) Jerome Vinograd & John E. Hearst, Progress in the Chemistry of Organic Natural Products 20, 373 - 422 (1962).