

Anesthetics May Act by Collapsing pH Gradients

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This study was aimed at providing a possible, testable explanation for the state of deep unconsciousness that ensues in an animal when anesthetic molecules reach a concentration of 3-5 moles per 100 moles of membrane lipid. Initially using liposomes as models for synaptic vesicles, it was confirmed that they accumulate and retain catecholamines provided their interior aqueous compartment is more acid than the exterior. This is because bimolecular phospholipid membranes are very much more permeable to nonionized than to ionized solutes, including also H^+ and OH^- . Thus, so long as a low intraliposomal pH is maintained, catecholamine bases remained charged and trapped. It is shown that anesthetics (butanol, hexanol, chloroform, and halothane) at activities (c/c_0 , where c and c_0 are the concentrations of the test and aqueous-saturated solutions, respectively) appropriate to general anesthesia (0.01-0.10) increase the proton permeability of liposomes, thus promoting the collapse of any pH gradient and indirectly facilitating the rapid outward permeation of the now uncharged, membrane-permeable transmitter substances. Synaptic vesicles from rat brain also accumulate catecholamines in response to a pH gradient, and anesthetics over a range of general and local concentrations release them. On the basis of these results, it is suggested that one mode of anesthetic action may be the release of catecholamines from synaptic storage vesicles, initially into the nerve terminal cytosol, leading to depressed neurotransmission, perhaps involving both inhibitory and excitatory pathways. (Key words: Brain: synaptic vesicles. Membrane: liposomes. Neurotransmitters: catecholamines. Theories of anesthesia: pH gradients.)

TWO RECENT ARTICLES^{1,2} have conjectured as to how and where, at a molecular level, general anesthetics might function. This paper puts forward a mechanism based on a number of properties well established for a model membrane system, the liposome. It is proposed that one mode of anesthetic action may be the depletion of basic and acidic neurotransmitter storage levels of synaptic vesicles of the central nervous system, by partially collapsing, or allowing decay of, transmembrane pH gradients.

The relevant properties of liposomes are:

1) They are exceedingly impermeable to cations and anions³ but substantially more permeable to protons and hydroxyls.⁴⁻⁷ Because the enthalpy of activation, $\Delta H^* = 10-15$ kcal, of the exceedingly infrequent permeating event is low,⁸ it has been suggested that it is an event restricted to very limited areas of the membrane, *e.g.*, to occasional random line dislocations taking place in the quasi-crystalline bilayer.⁹

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Furthermore, the channels are likely to be aqueous, with a high proportion of ordered water.

2) Liposomes are 10^5 times more permeable to protons than to Na^+ or K^+ ,^{6,7} consistent with the relative mobilities of these ions in an aqueous channel in which a high proportion of water molecules, and particularly those adjacent to the hydrocarbon chains of the dislocated phospholipids, would be ordered as in ice.

3) In clinically effective concentrations, anesthetics increase the permeability of liposomes to cations by a small factor, without significantly altering ΔV^* (the volume of activation) or ΔH^* .^{8,10} Thus, from the equation

$$\Delta G^* = \Delta H^* + P\Delta V^* - T\Delta S^*$$

where ΔG^* is the overall Gibbs free energy of activation and P , T and V are pressure, temperature and volume, it is evident that anesthetic molecules must change the entropy (ΔS) of the membrane^{11,12} in a manner associated with one of increasing disorder, *i.e.*, more frequent line dislocations.⁹ Predictable results from the above equation have been obtained for variations in temperature and pressure.^{8,10}

4) Small uncharged solutes, *e.g.*, water, amides, ureas and certain amino acids,^{9,13,14} are relatively permeable by virtue of their freedom to break the small number of hydrogen bonds they make to solvent water and thereafter to diffuse anhydrously across any part of the membrane.⁹ This permeating mechanism is relatively unaffected by the presence of anesthetics but is sensitive to membrane composition and phase state.

5) Liposomes may be prepared with an acidic buffer and then dialyzed against an isotonic solution of different composition and pH. Their impermeability to cations ensures that the interior of the liposome remains acidic for hours.⁵ If low concentrations of a weak base, *e.g.*, 9-aminoacridine, methylamine, or catecholamines, are now added to the external alkaline solution, they freely enter the liposomes as uncharged molecules, but find themselves unable to escape as they become protonated,^{15,16} and as such, they approximate a model of a transmitter vesicle.

Thus, basic or acidic neurotransmitters, assuming they are trapped in, respectively, acidic or basic compartments, may be expected to leak out of liposomes at least as rapidly as protons, or hydroxyls, as the pH of the environment in which they are trapped changes and the uncharged species becomes more abundant.

TABLE 1. Summary of Experiments Relating pH,

	Inside Liposome		Outside Liposome	
	Composition	pH	Composition	pH
Experiment 1	Na ₂ HPO ₄ or K ₂ HPO ₄ + monoamine transmitter*	8.0	K ₂ HPO ₄	8.0
Experiment 2	Na ₂ HPO ₄ or K ₂ HPO ₄ + monoamine transmitter*	4.8	NaH ₂ PO ₄ or KH ₂ PO ₄	4.8
Experiment 3	NaH ₂ PO ₄ + monoamine transmitter	4.8	K ₂ HPO ₄ or degassed KCl	8.0
Experiment 4	NaH ₂ PO ₄ + monoamine transmitter	4.8	K ₂ HPO ₄ or degassed KCl	7.0
Experiment 5	NaH ₂ PO ₄ + monoamine transmitter	4.8	K ₂ HPO ₄ or degassed KCl	7.0
Experiment 6	NaH ₂ PO ₄ + monoamine transmitter	4.8	K ₂ HPO ₄ or degassed KCl	7.0
Experiment 7	NaH ₂ PO ₄ + monoamine transmitter	4.8	Na ₂ HPO ₄ or degassed NaCl, Na ₂ SO ₄	8.0
Experiment 8	NaH ₂ PO ₄ + monoamine transmitter	4.8	Na ₂ HPO ₄ or degassed NaCl, Na ₂ SO ₄	7.0
Experiment 9	Na ₂ H ₂ PO ₄ + monoamine transmitter	4.8	K ₂ HPO ₄ or degassed KCl	8.0
Experiment 10	Na ₂ H ₂ PO ₄ + monoamine transmitter	4.8	K ₂ HPO ₄ or degassed KCl	7.0
Experiment 11	Na ₂ H ₂ PO ₄ + monoamine transmitter	4.8	K ₂ HPO ₄ or degassed KCl	7.0

* E.g., dopamine, epinephrine, norepinephrine.

Earlier work³ showed that anesthetics increase cation permeability of membranes in a nonspecific fashion, by a factor of 2 or more, so that if the proton is now considered to be the rate-determining ion, progressive collapse of a pH gradient would be accompanied by a concomitant depletion of neurotransmitter. Observations that support this hypothesis are presented here, for liposomes in the first instance, and for synaptic vesicles isolated from the rat central nervous system.

Methods

Liposomes were prepared from a 9:1 mixture (mole/mole) of egg phosphatidyl choline:dicetylphosphoric acid. The lipid was dried from chloroform under vacuum, then allowed to swell in 100 mM NaH₂PO₄, pH 4.8. The lipid suspension was then sonicated in a bath sonicator for 30 min to produce unilamellar liposomes.

In the first group of experiments, liposomes were passed over a Sephadex[®] G-50 column into 100 mM KCl. Further manipulations were carried out in a N₂ atmosphere using nearly CO₂-free solutions. The N₂ equilibration was allowed to set the pH, usually to around pH 6.8–7.0. A 0.5-ml volume of the liposome mixture (containing 6.5 μmol lipid) was added to a stirred beaker containing a final volume of 3.0 ml of 100 mM KCl, to which various volumes of a half-saturated ($a_1 = c/c_0 = 0.5$) anesthetic solution (with chloroform, halothane, butanol, or benzyl alcohol) in KCl had been added. The pH was measured continuously with a Pye[®] H⁺-electrode. A stable and flat baseline was recorded on a Servoscribe[®] pen recorder. Following this, 1.0 μl (1.0 mg/ml in ethanol) of valino-

mycin, the K⁺-specific ionophore, was added, and the leak of H⁺ measured as a function of increasing acidity in the external solution. Occasionally, CCCP (the uncoupling agent carbonyl cyanide m-chlorophenyl hydrazone, 0.5–1.0 μl of a 1.0 mg/ml solution in ethanol) was added after the valinomycin, resulting in a nearly total release of H⁺ trapped by the liposomes, as calculated from the trapped volume and known acid content.

In a second group of experiments, sonicated liposomes (pH 4.8 inside) were passed over a Sephadex G-50 column into 90 mM KCl, 10 mM K₂HPO₄, pH 8.0. To the external solution was added ³H-dopamine (0.5–1.5 μCi) at a final concentration of 5 × 10⁻⁷ M. The liposomes were allowed to accumulate ³H-dopamine for 30 min, at which time a stable loading was achieved. The liposomes were then divided into aliquots containing 26 μmol lipid in 2.0 ml, and half-saturated butanol in an isotonic buffer added to give the required concentration of anesthetic. As the time course of catecholamine release was found to occur on a time scale much slower than that required for mixing, the addition of a half-saturated alcohol solution was deemed not a disadvantage, and preferable to addition of bulk alcohol to the liposome suspension. Alcohol concentrations were determined by gas-liquid chromatography. At various times a 0.5-ml sample was taken over a Sephadex G-50 column to remove external ³H-dopamine. The retained dopamine was calculated relative to control samples without anesthetic. In addition, a further sample to which NH₄Cl (20 mM) or valinomycin + CCCP (1.0 μl of each from a 1.0-mg/ml stock solution in ethanol) had been added was found to have

Cation and Catecholamine Movements on Liposomes

Reagent(s) Added	Rate of Transmitter Depletion	Comments
	Too fast to measure Too slow to measure	Abundance of nonionized transmitters permitting rapid equilibration Transmitter retained inside liposome by acidic buffer
Valinomycin alone CCCP alone Valinomycin/CCCP	Very slow Slow Slow Very fast	Transmitter trapped in acidic interior of liposome K ⁺ for H ⁺ exchange not allowed H ⁺ for K ⁺ exchange not allowed Intraliposomal H ⁺ free to exchange with external K ⁺ , collapse of pH gradient, and release of monoamine ensues
Valinomycin/CCCP	Very slow Fast, partial	H ⁺ for Na ⁺ exchange not allowed A small pH change observed; valinomycin is a poor Na ⁺ carrier
Anesthetic alone Valinomycin alone Anesthetic/valinomycin	Very slow Slow Fast	Transmitter trapped Small increase in K ⁺ for H ⁺ exchange brought about by anesthetic Anesthetics provide extra pathway for H ⁺ leak, valinomycin stimulates K ⁺ leak, leading to large K ⁺ for H ⁺ exchange and collapse of pH gradient

retained less than 5 per cent of its accumulated ³H-dopamine at 10 min. All experiments were done at 20–22 C.

Synaptic vesicles were prepared from whole rat brain by a modification of the method of Kuriyama *et al.*¹⁷ The final pellet of vesicles was suspended in an incubation buffer containing 100 mM KCl, 10 mM NaCl, 2 mM MgCl₂ and 10 mM KH₂PO₄, pH 7.4. ³H-dopamine was added to a final concentration of 5 × 10⁻⁷ M at zero time. In these experiments the pH was increased from 7.4 to 8.4, in the absence of ATP, at 10 min. At various intervals, 120-μl samples were taken, neutralized with 0.01 N HCl, pH 7.4, and combined with 10 μl of dextran blue in incubation buffer (20 mg/ml). A 100-μl sample was passed over a G-25 Sephadex column into incubation buffer at pH 7.4. The excluded volume was collected and counted and the absorbance of dextran blue used to correct for recovery. Absorption of dextran blue to the column material was negligible (<5 per cent) in these experiments. At a later time of incubation in this experiment, the pH was returned to 7.4, a control retention of ³H-dopamine established, and anesthetic or ionophore added to various incubation pots. Further samples were taken as described above with the exception that HCl was not added to change the pH. ³H-dopamine accumulated by the synaptic vesicles was assayed chromatographically following the experiment to ensure that degradation of the dopamine had not occurred over the experimental time course. At least 95 per cent of the radioactivity cochromatographed with dopamine. In a second series of studies, identical procedures were followed, except that the pH was maintained at 7.4, and ATP added. Anes-

thetics and ionophores were added when a steady state had been reached. In both experiments, a further sample was examined, namely, one that had not been loaded with a H⁺ gradient or exposed to ATP. Temperature was 37 C in both experiments.

Results

Table 1 summarizes the all-or-none characteristics of liposomes set up to resemble catecholamine-loaded vesicles *in vivo* and subsequently exposed to various reagents, including anesthetics. Experiments 1 and 2 showed that the depletion rate for catecholamines initially sequestered inside liposomes was related to the abundance of the uncharged species and was unrelated to any pH gradient. Experiment 3 confirmed that catecholamines in an acidic environment were impermeable and did not equilibrate despite the existence of a pH gradient. Experiments 4, 5 and 6 showed that a pH gradient, set up in a liposome system, could be collapsed when carriers for protons and charge exchange cations were present and able to permeate. The potent uncoupler CCCP together with valinomycin and K⁺ outside provided these conditions, but only when they were present together. When Na⁺ was substituted for K⁺ outside (Experiment 7) there was only a partial equilibration of pH and depletion of transmitter. Finally, in Experiments 9, 10, and 11, it was shown that anesthetics themselves facilitated proton permeability, and when a K⁺ pathway was also present, pH gradients collapsed without the addition of a specific proton uncoupler.

Figure 1A illustrates the effects of general anesthetics (halothane, chloroform, benzyl alcohol, and

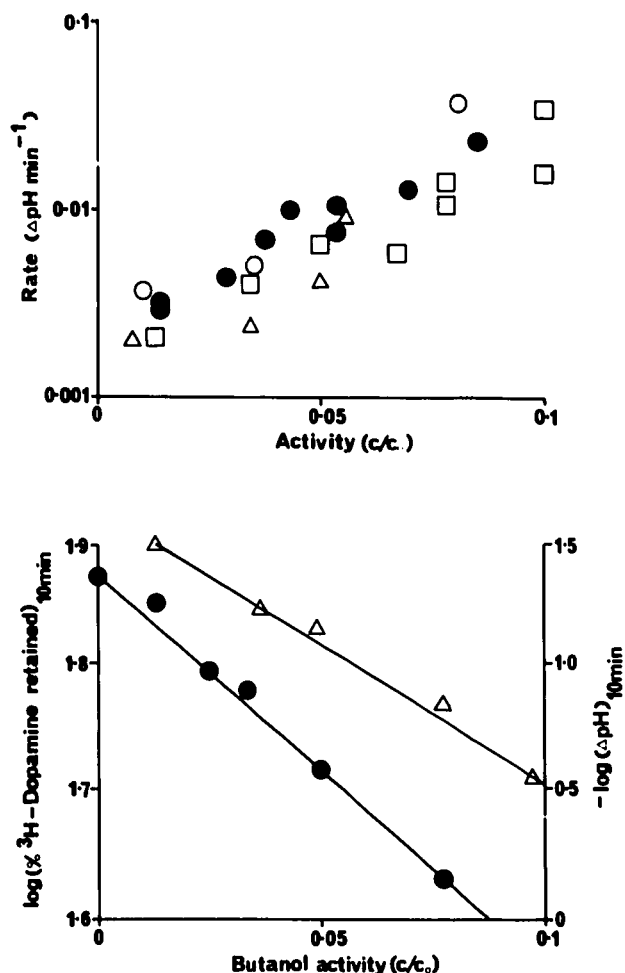


FIG. 1. The actions of general anesthetics on (A, above) proton permeability of liposomes and (B, below) proton and dopamine losses from unilamellar liposomes. Liposomes for both experiments were prepared as described in the text, from a 9:1 mixture of phosphatidylcholine:dicetylphosphoric acid, with the internal content being 100 mM NaH_2PO_4 , pH 4.8. Liposomes were sonicated until they were ≥ 99 per cent unilamellar. The external medium was then changed by gel filtration to 100 mM KCl, pH 6.8–7.0, buffer-free as a solution equilibrated in N_2 (CO_2 -free). Further experimental procedures are given in the text. In figure 1A, the rate of pH change is plotted against the activity of anesthetic for a variety of anesthetic compounds within the range of general anesthetic use. Under the conditions used, anesthetic activity was unaffected by the lipid concentration in the suspension. The rate of pH change prior to the start of each experiment was effectively 0, *i.e.*, the baseline was completely stable and drift-free. The loss of protons in the presence of general anesthetics was measured with approximately $1 \mu\text{M}$ valinomycin in the external solution, to provide a pathway for K^+ loss, thus facilitating K^+ – H^+ exchange. At this concentration, valinomycin had no effect on pH. However, under these conditions, when valinomycin was added in the presence of CCCP (also $1 \mu\text{M}$), a rate of pH change of 0.2–0.5 units min^{-1} was observed, representing a nearly maximal rate of proton permeation across the liposome membrane. Δ , chloroform, \square , butanol, \circ , halothane, and \bullet , benzyl alcohol.

In figure 1B, liposomes were loaded with ^3H -dopamine as described in the text. One sample was assayed as described in the text for retained dopamine as a function of increasing activity of

butyl alcohol) on proton release from a liposome system containing acidic buffer (100 mM NaH_2PO_4 , pH 4.8). Liposomes similarly placed in a NaCl or a Na_2SO_4 solution by Sephadex G-50 gel filtration showed only a small response to valinomycin + CCCP. The rate of H^+ release in KCl brought on by valinomycin + CCCP was 0.2–0.5 pH unit $\cdot \text{min}^{-1}$. Control liposomes used in all these experiments showed no detectable loss of pH gradient (as measured by the magnitude of ΔpH upon addition of valinomycin + CCCP) over eight hours.

Figure 1B illustrates the effect of butyl alcohol on a liposome model of a transmitter vesicle, *i.e.*, containing acidic buffer and ^3H -dopamine, in this instance. This figure further demonstrates the correlation between loss of H^+ from the liposome and loss of ^3H -dopamine as assayed 10 min after addition of the anesthetic. Results similar to those in figure 1B were also obtained with similar activities (c/c.) of the anesthetics halothane, chloroform, and benzyl alcohol, but are not shown. The results shown in figure 1 encompass a region of anesthetic activities including both local and general ranges. One significant feature of these results is their linearity with anesthetic activity/concentration. Thus, whereas the rate of H^+ or catecholamine leakage may be slower at low anesthetic concentrations, the ultimate effect of net leakage of H^+ or catecholamine will still be present even at concentrations below those necessary for general anesthesia.

In figure 2 are illustrated results obtained in synaptic vesicles prepared from whole rat brain, which had previously been loaded with ^3H -dopamine either by application of a pH gradient (fig. 2A) or by the conventional technique of loading with ATP and Mg^{++} at pH 7.4 (fig. 2B). The final amount of ^3H -dopamine retained by the vesicles varied between the two techniques: in the case of the pH gradient the amount of ^3H -dopamine retained increased exponentially with increasing pH, *i.e.*, linearly with increasing external abundance of uncharged species. Addition of either CCCP alone, or valinomycin and CCCP together, to deplete the H^+ concentration gradient substantially depleted the ^3H -dopamine content of the vesicles, as found in previous work on these vesicles.²¹ However, the addition of anesthetic compounds, including the

butanol, this being the test anesthetic for this experiment. Included in the lipid suspension was $1 \mu\text{M}$ valinomycin to provide a pathway for K^+ leakage. In a second experiment on an identical preparation, the loss of protons from the liposome suspension was assayed as in figure 1A, and the value given in the figure expressed as the total H^+ loss at 10 min. In this figure, the closed circles represent dopamine loss and the open triangles, pH change. Temperature was 20–22 C in all experiments.

n-alkyl alcohols, chloroform, ether, and benzyl alcohol, at clinically effective concentrations (for these experiments, anesthetic activities of 0.01–0.05 were used) below those required for block of nerve conduction also brought about depletion of ³H-dopamine. Similar results were obtained for the other ³H-catecholamines examined, norepinephrine and epinephrine. This effect was only slightly enhanced in the presence of valinomycin, a result that perhaps indicates that isolated vesicles, unlike liposomes, have some permeability to K⁺.

Discussion

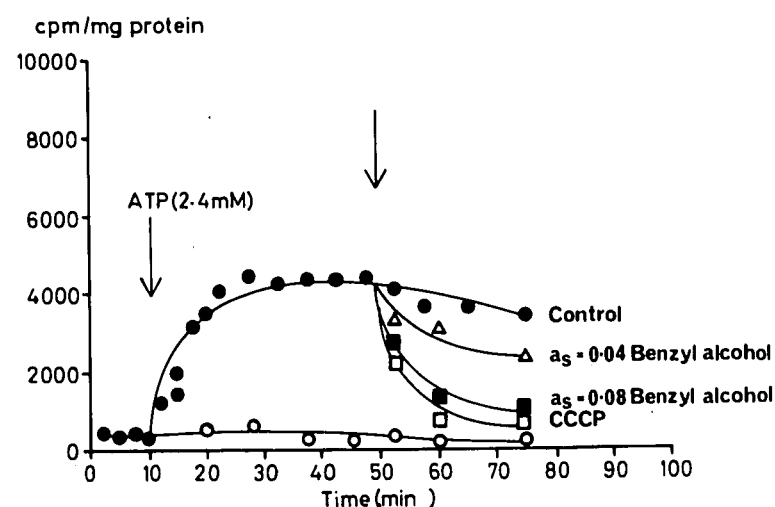
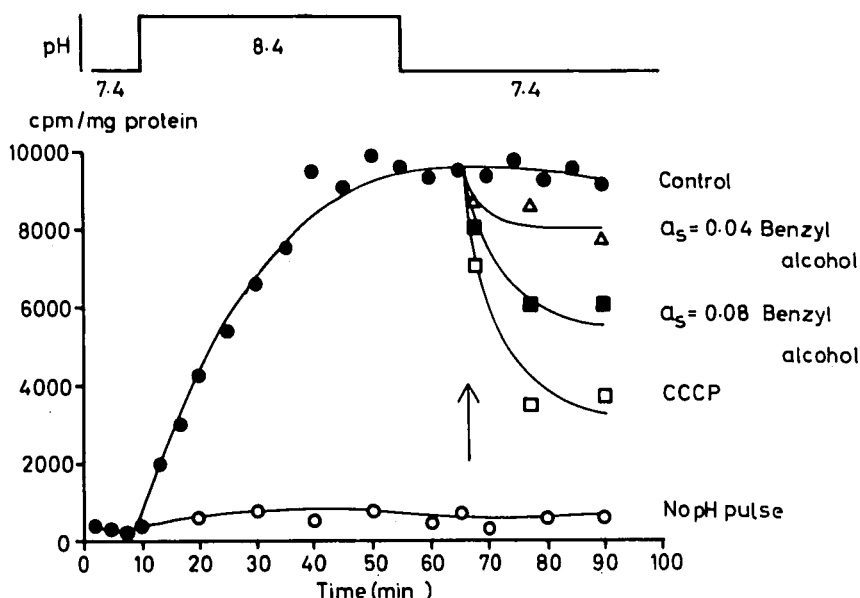
Synaptic vesicles and other biological cells and subcellular membranes appear to maintain a transmem-

brane pH gradient and to accumulate catecholamines.^{12–26} Consistent with these findings, it was observed that synaptic vesicles from rat brain may be loaded with catecholamines such as dopamine, epinephrine, and norepinephrine either when the external pH is made alkaline (pH 7.5–9.0) in the absence of ATP and Mg⁺⁺, or when they are supplied with ATP and Mg⁺⁺ at pH 6.5–7.4. Both mechanisms presumably rely on the development of a pH gradient, as uncouplers of these gradients such as valinomycin–CCCP, NH₄Cl, or nigericin (all of which allow the exchange of K⁺ for H⁺) discharge the previously accumulated ³H-catecholamine contents of the synaptic vesicles.

The results presented in this report suggest that general anesthetics may act on both catecholamine

FIG. 2. The actions of pH gradients and general anesthetics on ³H-dopamine content of synaptic vesicles from whole rat brain. Vesicles for both experiments were prepared as described in the text, according to the technique of Kuriyama *et al.*¹⁷ In figure 2A (*above*), the pH was raised as indicated, with ³H-dopamine present outside of the vesicles. The rise in pH brought about substantial retention of catecholamine. When the pH outside was returned to normal, little if any leakage occurred initially, although some slight leakage was observed over a longer period. ³H-dopamine was not accumulated under control conditions, where the vesicle suspension was not exposed to a pH pulse (*open circles*). At the arrow, benzyl alcohol was added at the indicated activities, or in the lower curve, CCCP was added to bring about substantial depletion of catecholamine. A range of anesthetic compounds, including halothane, chloroform, and butanol, gave similar results for equivalent activities.

In figure 2B (*below*), synaptic vesicles were loaded somewhat more conventionally, using 2.4 mM ATP (*closed circles*). A control preparation (*open circles*) was not exposed to ATP, and showed little retention of catecholamine. At the second arrow, the synaptic vesicle suspensions were exposed, as in figure 2A, to the given activity of benzyl alcohol or CCCP, with results similar to those in 2A, *i.e.*, depletion of catecholamine by the benzyl alcohol, graded with anesthetic activity, and release virtually to the control level brought about by CCCP. Temperature in all experiments was regulated at 37 C, and all vessels were closed to prevent evaporation.



release from and catecholamine uptake into liposomes and synaptic vesicles. This could result ultimately in a decreased catecholamine content as assayed biochemically, but in the short term, in a decreased quantal content of neural transmission because of the release of catecholamines from vesicles into the nerve terminal cytosol. This suggestion is consistent with observed depression of synaptic transmission by general anesthetics,²⁷⁻³³ although in many cases the specific neurotransmitter acted upon by anesthetics has not been identified. It is clear that our suggestion is not obviously relevant to the case of cholinergic or most peptidergic transmitters, which are probably never uncharged, and would not therefore be vulnerable to this mechanism.

It might also be predicted from these results that a similar mechanism could act in the case of liposomes or synaptic vesicles containing alkaline buffers—they should sequester acidic transmitters such as glutamic and aspartic acids, and relaxation of the *pH* gradient should likewise cause their depletion. Indeed, a decane-water partition coefficient profile against *pH* suggests that the greatest abundance of the uncharged species of glutamic acid occurs at *pH* 6–8. Experimentally, modest trapping of glutamic and aspartic acids could be demonstrated with intraliposomal *pH*s below this value.

Similar movements of catecholamine across the synaptosomal membrane have also been found, although this mechanism, while not necessarily bearing relevance to general anesthesia, serves to underline the nonspecific action of general anesthetics on H⁺ permeability of membranes.

Whereas the present experiments have dealt with an isolated and heterogeneous population of synaptic vesicles and with the model liposomal membrane, the picture gained from these experiments is probably decidedly oversimplified when their interpretation is applied to the whole animal.

Because anesthetics may have both pre- and post-synaptic effects which may be either facilitatory or inhibitory, and because the mechanism suggested here is not restricted to any particular stage of neurotransmission, depression of catecholaminergic transmission may have inhibitory or facilitatory effects on cholinergic and peptidergic transmission, albeit indirectly.

Although a body of evidence exists which bears on acidic and basic neurotransmitter release, the interaction of anesthetics with these and other transmitters is not clear-cut, perhaps because of the variation and multiplicity in their sites of action (pre- vs. postsynaptic). It is this wide difference in modes of action that leads us to predict that although the basic principle of interaction of general anesthetics with *pH* gradients

controlling amine uptake and release may be straightforward, the ultimate neurophysiologic manifestations may prove complex, but undoubtedly will be significant.

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