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ORIGINAL PAPER

Enzyme stabilization by glass-derived silicates in glass-exposed aqueous solutions

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Objectives: **To analyze the solutes leaching from glass containers into aqueous solutions, and to show that these solutes have enzyme activity stabilizing effects in very dilute solutions.**
Methods: **Enzyme assays with acetylcholine esterase were used to analyze serially succussed and diluted (SSD) solutions prepared in glass and plastic containers. Aqueous SSD preparations starting with various solutes, or water alone, were prepared under several conditions, and tested for their solute content and their ability to affect enzyme stability in dilute solution.**
Results: **We confirm that water acts to dissolve constituents from glass vials, and show that the solutes derived from the glass have effects on enzymes in the resultant solutions. Enzyme assays demonstrated that enzyme stability in purified and deionized water was enhanced in SSD solutions that were prepared in glass containers, but not those prepared in plastic. The increased enzyme stability could be mimicked in a dose-dependent manner by the addition of silicates to the purified, deionized water that enzymes were dissolved in. Elemental analyses of SSD water preparations made in glass vials showed that boron, silicon, and sodium were present at micromolar concentrations.**
Conclusions: **These results show that silicates and other solutes are present at micromolar levels in all glass-exposed solutions, whether pharmaceutical or homeopathic in nature. Even though silicates are known to have biological activity at higher concentrations, the silicate concentrations we measured in homeopathic preparations were too low to account for any purported *in vivo* efficacy, but could potentially influence *in vitro* biological assays reporting homeopathic effects.** *Homeopathy* (2010) **99**, 15–24.

Keywords: Glass; Silica; Drug packaging; Enzyme activity; Homeopathy acetylcholine esterase

Introduction

Many pharmaceuticals, as well as homeopathic preparations, are stored and shipped in glass containers and it is often assumed by researchers that the glass containers are chemically inert and resistant to hydrolysis. However, this assumption is incorrect, and indeed there is a significant literature on the dissolution of glass materials into solutions contained in them.[1–3] In the case of pharmaceuticals, it is important to know of any possible contamination issues that might adversely affect efficacy. In the case of highly-diluted homeopathic preparations, claims are often made that the preparations are solute-free, despite

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a paucity of investigations into the actual contents of such preparations.

Pharmaceuticals such as insulin and many vaccines are distributed in glass vials rather than plastic vials, and the distribution process can involve significant agitation of the contents during shipment. It has been shown that heat sterilization of pharmaceuticals or parenteral nutrients packaged in glass containers causes glass constituents to leach from the glass surface, including high levels of aluminum[4] and silicates.[3] Further, the chemical nature of the compounds in solution can affect the level of solutes leaching from the glass, wherein compounds with an alkaline pH leach higher levels of aluminum and silicates. Aluminum oxides and boric oxide are added to many modern glass formulations to increase strength, and high pH conditions act to dissolve more glass constituents into solution.

Homeopathic preparations are typically produced by vigorously shaking and serially diluting various compounds in water or water–alcohol mixtures in glass vials. The serial dilution and vigorous agitation process involved in making homeopathic preparations is performed repetitively, until little or none of the starting solutes remain. An initial tincture of the starting material is put in a glass vial and shaken by rapidly and repeatedly impacting the vial on a solid, elastic surface, a process known as succussion. In typical homeopathic preparations, an aliquot usually ranging from 0.01% to 10% of the original volume is then pipetted into a fresh glass vial containing the same volume of water (or water/ethanol), and the succussion process is repeated. This serial succussion and dilution (SSD) process is carried out up to 200 times or more to generate the final, highly-diluted homeopathic preparation.

The process of SSD has been proposed to increase the potency of a homeopathic preparation,[5] even when carried out to a point calculated to be *"beyond the reciprocal of Avogadro's number"* (BRAN) for the starting solutes (approximately $1/(6.02 \times 10^{23})$ M or twelve 100-fold dilutions of a 1 M starting solution). Such a notion is contrary to existing pharmacological principles that link the efficacy of a bioactive agent to its activity as an agonist or antagonist at specific receptors, or its ability to block or enhance specific enzyme, channel, transduction or transport systems. Many theories have been proposed by proponents of homeopathy to account for how the efficacy of a remedy could increase with increased dilution and shaking. These theories include concepts such as bioactive clathrates formed in water.[6] Such theories are based on conjecture rather than experimental data, and for the most part do not provide testable hypotheses.

Previous studies have shown that BRAN-type SSD preparations can have biological effects,[7] [10] but such studies have not been consistently replicated elsewhere,[11] and no efficacious agents are known to be present in such preparations. Persistent water structures have been proposed as the active agents in homeopathic preparations.[12,13] Studies of BRAN-type homeopathic solutions with techniques including nuclear magnetic resonance (NMR) have suggested that SSD preparations have altered properties relative to pure water, but more recently, some of those results have been attributed to contamination of the samples by silicates leaching from the glass cuvettes used in the studies.[14]

Careful analyses of homeopathic preparations using NMR have shown that no stable H-bonding was present, and that no differences between experimental and control solutions were detectable.[15,16] Micromolar or lower concentrations of contaminants were found in commercial and non-commercial homeopathic preparations and control samples in one of these studies,[15] including acetate, formate, lactate, acetone, ethanol and methanol. Rarely in homeopathic studies are the micro-concentrations of minerals that leach into the water from the glass containers analyzed for their concentration or effects.[17]

During the course of work in our laboratory to develop enzyme-based bioassays to analyze homeopathic preparations and differentiate them, if possible, from control solutions, we found that enzyme activity was preserved significantly longer in glass-exposed than in plastic-exposed water. The stabilization of enzyme activity in water did not correlate in a consistent manner with starting materials in SSD preparations made in glass vials, and increased succussion cycles did not increase the effect.

In order to investigate this glass-exposure effect we used highly sensitive analytical techniques to determine the levels of solutes present in BRAN-type SSD solutions. We examined glass-exposed solutions and homeopathic preparations by a variety of techniques, including elemental analysis, colorimetric silicate assays, enzyme assays, and scanning electron microscopy (SEM) with elemental mapping in order to determine their solute content.

We show that SSD solutions made in glass vials contain micromolar levels of silicates, borate and sodium, and trace levels of other solutes. Enzyme assays were used to show that these solutions have stabilizing effects on enzyme activity in dilute solution, and that silicates are the most likely active agents in preserving enzymatic activity in dilute solutions.

Methods

Chemicals and acetylcholine esterase were from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO). All solutions were prepared with purified, deionized water (Milli-Q; Millipore, Billerica MA). Screw cap borosilicate glass tubes (24 mL) were from VWR Scientific (#66011-358), and soda-lime glass vials (30 mL) were from Wheaton Scientific Products (# W216976). Polypropylene centrifuge tubes (15 mL) were from Corning. Arsenicum album 30c and 200c preparations were obtained from Washington Homeopathic Products (Berkley Springs, WV).

Preparation of succussed solutions for enzyme assays

Aliquots of 100 μl of stock solutions, which had been prepared in plastic tubes, including 1 M L-glutamate and 10 mM acetylthiocholine were added to 10 mL of purified and deionized water in either a 23 mL borosilicate glass tube, a 30 mL soda-lime glass tube, or in a 15 mL polypropylene tube, and succussed 120 times (2/s) by impacting the tubes on a solid surface. Some SSD solutions were not prepared with a starting solute, and these are described as 5c and 30c water. A 100 μl aliquot of resulting solution was added to a fresh tube of the same type containing 10 mL of purified, deionized water and succussed again as described above. This succussion and dilution cycle was repeated 28 times more to obtain '30c' SSD solutions. Liquid handling was done with fixed volume disposable polystyrene pipettes or adjustable volume pipettors with disposable polypropylene tips. Control solutions of water consisted of purified, deionized water stored in polypropylene tubes, and were not succussed unless otherwise noted.

Preparation of acetylcholine esterase

Acetylcholine esterase was diluted in the respective substrate succussed water or in purified, deionized water in polypropylene Eppendorf tubes. The enzyme preparations were left at room temperature (23°C) and the enzyme activities were measured at different time points to determine enzyme stability in dilute solution.

Preparation of water glass solutions

Water glass (sodium silicate solution) containing ~27% SiO_2 and ~14% NaOH obtained from Sigma–Aldrich was used for making the SiO_2 solutions. Varying concentrations (2–100 μM) of SiO_2 were prepared in Milli-Q purified and deionized water in polypropylene tubes, and the pH was adjusted to 7.4 with HCl.

Assay of enzymes

Enzyme assays were done colorimetrically. All enzyme incubations and activity assays were done in polypropylene Eppendorf tubes. Acetylcholinesterase activity was determined as follows. Briefly, a 50 μl aliquot of acetylthiocholine iodide (4.5 mM in water) and 100 μl 5-dithiobisnitrobenzoic acid (1 mM in 0.1 M phosphate buffer, pH 7.0) were added to the wells in a 96 well plate and incubated at 37°C for 10 min. A 50 μl aliquot of the acetylcholine esterase (0.0317 units/mL) dissolved in 30c acetylthiocholine iodide was then added and the incubation continued for an additional 25 min. The product thiocholine, released from acetylthiocholine by the enzyme, converts 5-dithiobisnitrobenzoic acid to a yellow colored product, and the optical density was measured at 405 nm in a Dynex multi well plate reader.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) methods

Trace elemental analysis was performed on several SSD preparations by ICP-OES (Optima 3000, manufactured by Perkin Elmer, Norwalk, CT). Multi-element stock solutions for standard preparation were purchased from Spex Industries, Edison, NJ. Deionized and purified water from a Millipore ultra-pure water system was used for the preparation and dilution of all reagents, samples and standards. The B, Si and Na concentrations in the samples were determined by ICP-OES using an external calibration curve of 5, 50, 500 and 2500 mg/L. The samples and standards were introduced into the ICP-OES at a flow of 1 mL/min and were analyzed at 1200 W radio frequency (RF) power, 0.8 L/min nebulizer gas flow, 15 L/min plasma gas flow and 1.2 L/min auxiliary gas flow. The plasma emission lines were detected at 249.773 nm for B, 589.588 nm for Na and 251.611 nm for Si.

Molybdate assays

Silicate concentrations in some solutions were estimated by the colorimetric molybdate method described earlier.[2] Briefly, the test reagent was prepared by mixing 5 mL of water with 2 mL of 4.1% sulfuric acid (v/v) and 1 mL of a solution containing 10% ammonium molybdate (w/v) and 4.7% ammonium hydroxide (v/v) in water. Soluble silicate concentrations were measured by mixing 20 μl of the test solutions with 80 μl of test reagent, which were then vortex mixed and kept at room temperature for 10 min before reading the optical density at 410 nm.

SEM

Samples of SSD solutions made in borosilicate glass vials were prepared for SEM by lyophilization. Samples were dried onto aluminum stubs covered with carbon adhesive tabs. Spectra were collected on uncoated samples using an AMRAY 1000B scanning electron microscope equipped with an EDAX DX-Prime Energy Dispersive Spectroscope. SEM parameters were set as follows: 20 keV (accelerating voltage), 30 degree sample tilt, 15 mm working distance, and 34 mm detector distance. For imaging, samples were coated with gold using a Denton Desk II sputter coater. Micrographs were captured using an AMRAY 1000B scanning electron microscope operated at 20 keV and 8 mm working distance.

Results

We tested enzyme stability in SSD solutions as measured by colorimetric enzyme activity assays under various conditions at room temperature. In the course of developing enzyme assays to analyze the bioactivity of SSD preparations, it was observed that enzyme activity was slightly enhanced in SSD solutions immediately after dissolving them (see time zero in Figure 3). However, we observed that after 24 h of incubation in these solutions at room temperature, enzyme activity was substantially preserved relative to enzymes dissolved in purified, deionized water. The starting solutes (e.g., glutamate or acetylthiocholine) used to make the SSD preparations had no systematic effect on the final enzyme-stabilizing effect in the resulting solutions (data not shown). We investigated the enzyme-stabilizing effect of SSD preparations made in glass vials, and the efficacious agents involved, in the following series of experiments.

SSD production in glass and plastic containers

In order to ascertain the relationship between the types of containers used to make SSD preparations, and the resulting enzyme-stabilizing activity, we made SSD preparations in borosilicate glass tubes and polypropylene plastic tubes (Figure 1). No enzyme stabilization was observed in purified, deionized water control solutions, or in SSD solutions

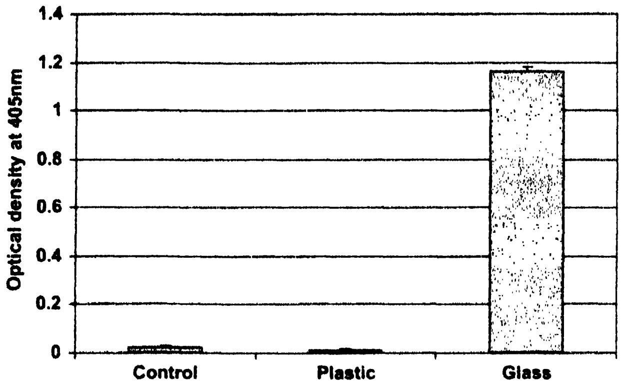

Figure 1 Acetylcholine esterase stabilization in dilute solutions prepared in plastic and glass, as compared with purified and deionized water (control). The solutions prepared in plastic (polypropylene) and glass (borosilicate) were succussed and diluted (120 succussions per dilution, thirty 100× dilutions, to yield 30c water preparations). Only glass-exposed solutions showed increased enzyme stabilization after 24 h incubation at room temperature. Values are means ± standard deviation (SD) (*n* = 6, from 6 independently prepared samples).

prepared in plastic (30c glutamate made in polypropylene plastic tubes). However, substantial enzyme activity remained after 24 h in SSD water prepared in glass vials (30c glutamate made in borosilicate glass vials).

SSD production in borosilicate and soda-lime glass containers

Acetylcholine esterase activity stabilization was analyzed in SSD solutions (15c glutamate) made in both soda-lime glass tubes, and in borosilicate glass tubes, as compared with purified, deionized water kept in plastic. Because the glass vials were of different size (24 mL borosilicate and 30 mL soda-lime) the volumes of water used was adjusted to keep relative volumes the same (10 mL water in borosilicate vials, 12.5 mL in soda-lime glass vials). Acetylcholine esterase activity was preserved significantly in SSD solutions made in either type of glass vial, whereas activity was mostly lost after 24 h in purified, deionized water kept in 15 mL polypropylene plastic tubes (Figure 2).

Stability of acetylcholine esterase in SSD solution for 7 days

We tested the stability of enzymatic activity of acetylcholine esterase in SSD and unsuccused control solutions at several time points over 7 days (Figure 3). At the initial time point immediately after diluting stock enzyme solutions in deionized, purified water or an SSD preparation, enzyme activity was already reduced by over 20% in the purified, deionized water ($p < 0.004$ by 2-tailed *t*-test). At 24 h only 13% of the enzyme activity remained in the purified, deionized water samples, whereas in the SSD solution prepared in glass, over 80% of the enzymatic activity remained. After 7 days there was no enzymatic activity as measured by optical density of the product when enzymes were dissolved in pure water. In contrast, when the acetylcholine esterase was stored for 1 week (in capped Eppendorf tubes) in an SSD solution prepared in glass vials approximately 50% of the enzymatic activity remained after 7 days at room temperature.

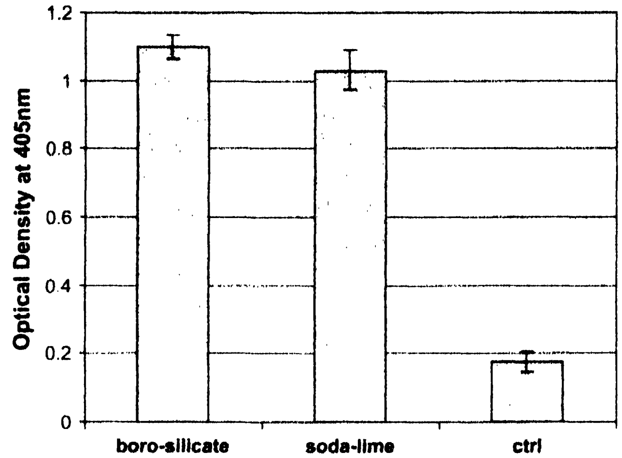

Figure 2 Stabilization of acetylcholine esterase 24 h after addition to SSD preparations (15c glutamate) made in borosilicate vs. soda-lime glass tubes, as compared with acetylcholine esterase dissolved in pure water kept in polypropylene tubes (ctrl). Both types of glass leached compounds into the water that acted to stabilize enzyme activity in solution relative to pure water. Values are means ± SD (*n* = 6, from 6 independently prepared samples).

Elemental analyses

ICP-OES was used to determine the levels of the most concentrated dissolved solids in the SSD preparations made in glass containers, and these were compared to solutions with known concentrations of sodium silicate (Figure 4). Silicon was the most concentrated element in SSD preparations made in borosilicate glass containers, with an approximate concentration of approximately 3 mg/L. Sodium and boron were present at approximately 1.5–2 mg/L.

Succussion *vs.* vortex mixing

Two similar SSD preparations were made in borosilicate glass tubes; one solution was vortex mixed for 1 min, and

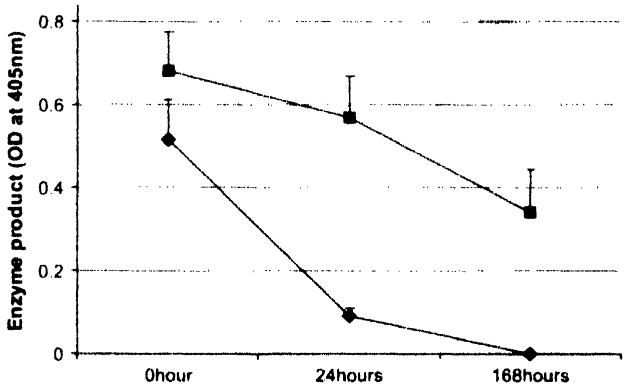

Figure 3 Acetylcholine esterase activity over time in pure water (not glass-exposed) and in SSD preparations (30c acetylcholine) made in borosilicate glass vials. Filled squares are acetylcholine esterase activity in SSD solutions made in borosilicate glass, filled diamonds depict activity in purified, deionized water. Values are means ± SD (*n* = 7 independently prepared samples, tested with 3 different stock enzyme preparations each, and each sample/enzyme prep mixture was assayed in triplicates at each time point).

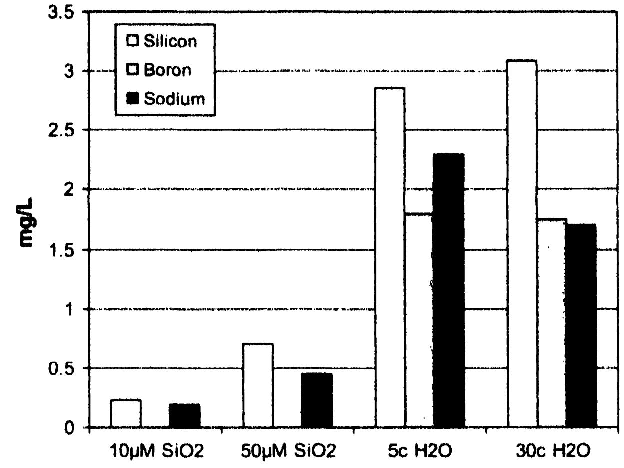

Figure 4 Elemental analyses (ICP-OES) of samples including water with soluble silicon dioxide added (10 and 50 μM sodium silicate), and two SSD solutions made by shaking water 120 times each in 5 successive borosilicate glass tubes (5c H_2O) and in 30 successive borosilicate glass tubes (30c H_2O). The sodium silicate solutions contained sodium and silicon, but had undetectable levels of boron. In contrast, the SSD preparations made with deionized water in glass vials contained sodium, silicon and boron, and the silicon levels were approximately 4 times those found in the 50 μM sodium silicate solution. Values are from individual samples. Experiments were repeated 3 times with similar results.

the other was prepared by SSD for one minute (2 Hz succussion) (Figure 5). The solution prepared by succussion was found to stabilize acetylcholine esterase activity approximately 20% more than the vortex-mixed solution after 24 h ($p < 0.001$), presumably due to higher levels of dissolved solutes including silicates and other buffering salts dissolving from the glass. It is likely that the more forceful mixing associated with impacting the vials repeatedly on a solid surface resulted in significantly more solutes dissolving from the vial walls.

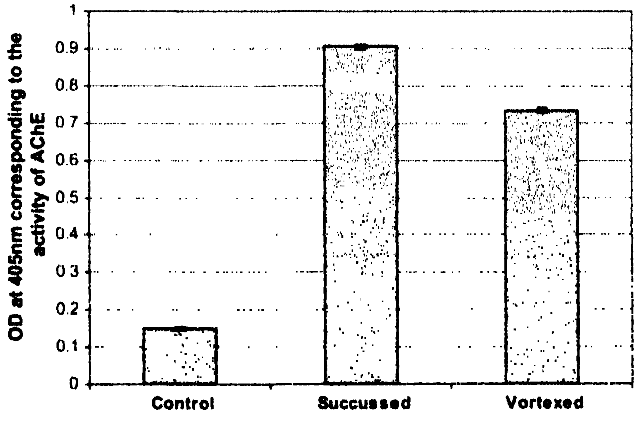

Figure 5 Succussion *vs.* vortex mixing. Acetylcholinesterase activity in pure water (control) and in water succussed in a borosilicate glass tube 120 times in 1 min, compared with water that had been vortex mixed in the same type of tube for 1 min. Enzyme activity was measured after 24 h in solution at room temperature. Values are means ± SD ($n = 6$, from 6 independently prepared samples). The vortex-mixed samples were significantly different from the SSD samples as shown by 2-tailed *t*-test ($p < 0.001$).

pH change of solutions with repeated succussion in glass

We determined how the solutes dissolving from glass affected the pH of water with repeated succussion cycles. Ten milliliters of purified, deionized water was added to a 24 mL borosilicate glass vial, and quickly vortex mixed. The pH was taken (Orion pH meter Model No. 350) and then the vial was capped and succussed twice per second for 1 min. The screw cap was removed, and pH was taken again, before replacing the cap and repeating the process.

Figure 6 shows that the initial pH of the solution after vortex mixing in the glass vial was approximately 8.5. This is due to the initial release of the most soluble components of borosilicate glass, including sodium or potassium oxides. The released metal oxides would form NaOH or KOH in solution, raising the pH to approximately 8.5. With each succussion cycle in the same glass vial, the pH value was reduced until a pH of approximately 7.0 was reached by the 8th succussion cycle. The reason for the drop in pH after the vial is opened, tested for pH, and then capped and succussed again is due to CO_2 dissolving into solution from the air. The carbonic acid formed from CO_2 in solution would counteract the metal hydroxides formed when sodium or potassium oxides dissolve from the glass surface. As the soluble oxides near the glass–water interface are exhausted, the pH of the solution eventually becomes buffered between NaOH/KOH and carbonic acid.

Phosphate buffer effects on enzyme stability

Enzyme stability was studied in SSD preparations made in glass with and without phosphate buffer added at various concentrations (Figure 7). The SSD preparation made in glass tubes stabilized acetylcholine esterase activity approximately as well as 100 μM phosphate buffer, and the effects of SSD glass preparations and phosphate buffer on enzyme activity stabilization were partially additive. Note that 100 μM phosphate buffer is 100 times more dilute than standard phosphate buffers used for enzyme assays (10 mM phosphate). It is noteworthy that even 1 μM of sodium phosphate added to an SSD preparation (made in glass

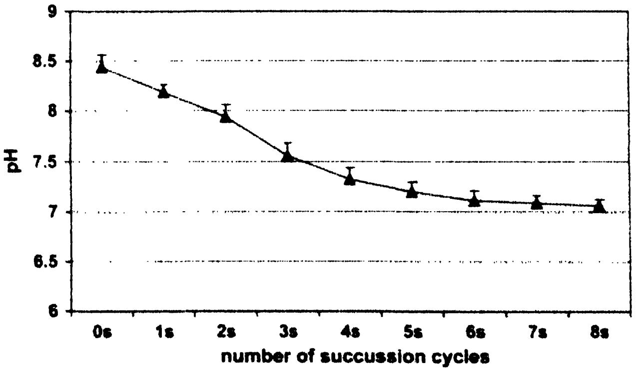

Figure 6 Effect of number of succussion cycles (s) on the pH of an aqueous solution made in a borosilicate glass tube. The same glass vials containing the same water were succussed and then tested for pH for 8 succussion cycles. The vials and the water in them were not changed between succussion cycles. Values are means ± SD ($n = 3$, from 3 independently prepared samples).

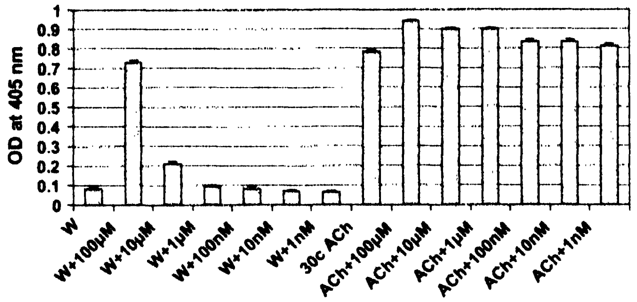

Figure 7 Phosphate buffer and SSD effects on enzyme stability after 24 h. The enzyme-stabilizing effect of an SSD preparation made with acetylthiocholine (30c ACh) was approximately the same as with 100 µM sodium phosphate buffer in water (W). Adding 100 µM sodium phosphate buffer to the 30c ACh solution (ACh + 100 µM) increased enzyme stability more than 15% as compare with 30c ACh alone ($n = 6$, ±SD, from 6 independently prepared samples).

vials) increased enzyme stability further than with the SSD preparation alone.

Dilution tests

Tests were carried out to see if dilution with pure water resulted in a linear diminution of the enzyme-stabilizing effect. Figure 8 shows that the enzyme-stabilizing effect was reduced by approximately 60% with a 5-fold dilution, and over 80% with a 20-fold dilution. The enzyme-stabilizing effect was completely lost at dilutions between 80- and 150-fold.

Silicate levels in homeopathic preparations

Because commercial homeopathic preparations are typically made and/or stored in glass vials, it would be anticipated that they would contain solutes such as silicates. We used molybdic acid assays for soluble silicates and found that the two common homeopathic preparations tested (arsenicum album 30c and 200c) contained silicates at levels near those found in an SSD preparation (30c glutamate) produced in our laboratory (Figure 9). The values for dissolved silicates observed using the molybdate assays were very similar to those obtained *via* ICP-OES (see Figure 4).

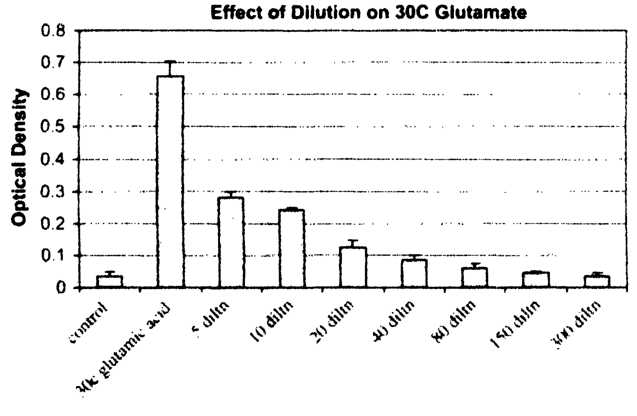

Figure 8 Acetylcholine esterase activity after 24 h incubation in 30c glutamate stock solution, and a number of dilutions made and vortex mixed in plastic tubes. The 30c stock was prepared as described in borosilicate glass tubes, and then diluted (5 diltn = 5-fold dilution, etc.) with purified, deionized water ($n = 6$, ±SD, from 6 independently prepared samples).

Sodium silicate *vs.* SSD preparations

Enzyme assays were used to determine the enzyme-stabilizing capacity of SSD preparations by comparison to a sodium silicate concentration range. A standard SSD preparation was found to stabilize acetylcholine esterase activity approximately as well as 50–100 µM sodium silicate in water (Figure 10).

SEM and silicon mapping of lyophilized SSD samples prepared in glass vials

Samples of SSD solutions prepared in glass vials were lyophilized and processed for SEM and energy dispersive spectroscopy. Under low power magnification the dried residue often formed a solid ring of amorphous material around the edge of the aluminum studs with additional areas of dried material scattered over the remainder of the surface (Figure 11A). Embedded in the amorphous material were small, micron-sized particles containing silicates (Figure 11B) that most likely represent colloidal particles from the silicate-saturated SSD solutions (Figures 11A and 12).

Discussion

Modern glasses, such as common borosilicate glass, are amorphous solids that are composed predominantly of silica (silicon oxides), boric oxides, sodium or potassium oxides, and often aluminum oxide. The metal oxides exist as inclusions in the silicate matrix of the glass. When exposed to water, the more soluble boric, sodium and potassium oxides form borate (BO_3^{3-}) and hydroxide (OH^-) in solution, with sodium or potassium as the counter ions. As these ions dissolve from the glass surface in contact with water, they initially tend to increase the pH of the solution (see Figure 6). Monosilicic acid will slowly dissolve from the glass surface, and a layer of silica gel forms at the glass–water interface.

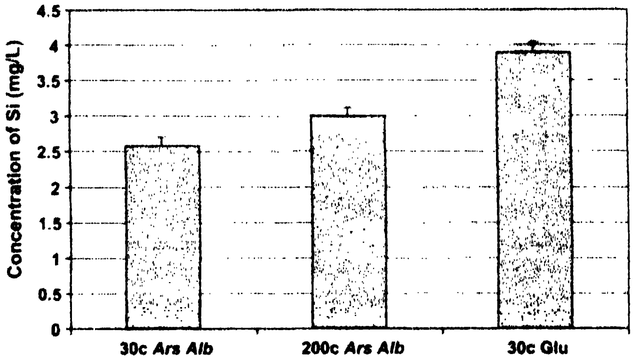

Figure 9 Molybdate assay for silicate content in 2 commercial homeopathic preparations (*Arsenicum album: Ars Alb*) and 30c glutamate (30c Glu) prepared in our laboratory ($n = 3$, ±SD, from 3 independently prepared 30c Glu samples and 3 different commercial arsenicum album vials).

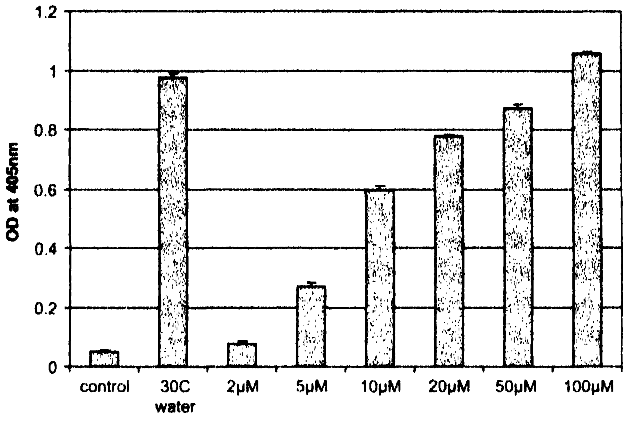

Figure 10 Effect of dissolved silicates (NaOH/silicate) on acetylcholine esterase enzyme activity in solution as compared with an SSD preparation made with purified, deionized water in borosilicate glass tubes (30c water). The silicate solutions (2–100 μM) were prepared in polypropylene tubes and were vortex mixed. A 100 μM sodium silicate solution had a similar enzyme-stabilizing capacity as the SSD preparation made in borosilicate glass tubes ($n = 6$, ±SD, from 6 independently prepared samples).

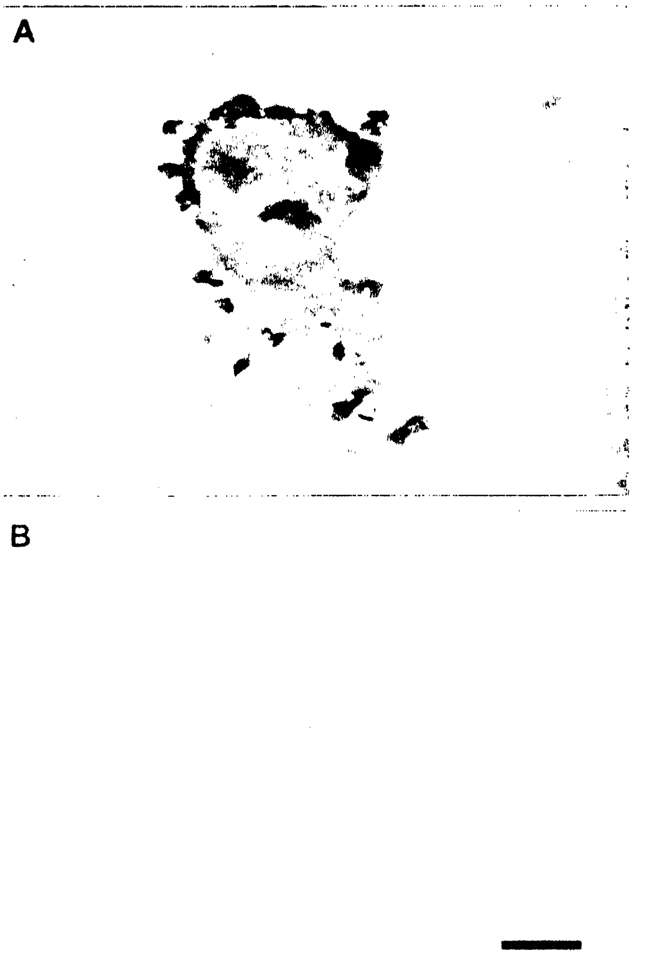

Figure 11 SEM image and silicon mapping of a patch of lyophilized residue from an SSD preparation. SEM image is shown in (A), and the corresponding silicon map is shown in (B). Colloidal silicate-containing particles can be seen around the edge of the dried residue. Bar = 50 μm.

Figure 12 Scanning electron micrograph of a colloidal silicate particle lyophilized from a sample of an SSD preparation made in a borosilicate glass vial. Proteins bind tightly to colloidal silica particles in solution, and this has been reported to enhance enzymatic activity.[23,24] Bar = 5 μm.

Carbon dioxide dissolving into solution as more succussion and dilution cycles are done will decrease the pH of the solution if the glass vial is not changed between succussion cycles. Glass vials vary greatly between manufacturers, and between lots, with some vials having few surface imperfections, and others exhibiting extensive surface imperfections. The level of surface imperfections has been found to correlate with increased delamination or dissolution of the glass surface in water.[18]

The solubility of glass has direct application to the pharmaceutical industry, because many protein or peptide-based pharmaceuticals such as vaccines and insulin are stored and transported in glass vials, and components leaching from the glass vial surface can interact with drugs, possibly influencing their effectiveness.[1] Likewise, some outcomes of *in vitro* laboratory experiments in homeopathy could be due to the interaction of dissolved glass components influencing the biological measure.[17,19,20]

Silicate chemistry is complex, and in many ways unique. Numerous types of silicon oxides occur naturally, including soluble silica (silicon dioxide and monosilicic acid; $Si[OH]_4$), oligomers of silica (polysilicic acids with molecular weights up to 100,000), and colloidal silica (particles of silica larger than 50 Å).[2] The predominant species of silicates present in SSD and traditional homeopathic preparations are unknown, but there is a significant literature related to the behavior of soluble and colloidal silicates forming in saturated silicate solutions.

All glasses dissolve slowly in water, initially producing a dilute solution containing dissolved silicates and other glass constituents. However, the solubility of monomeric silicates such as orthosilicic acid is very limited.[2] Once saturation is reached in aqueous solution at near-neutral pH values, monomers condense into nanometer sized colloidal particles suspended in solution.[21] As the monomers aggregate into particles, more silicates can leach from the glass surface, thus increasing the number of colloidal particles in solution.

When a solution is supersaturated with silicates and insufficient solid silica surface is available to permit rapid deposition, new smaller nuclei are formed by intercondensation of monomers and low polymers. Silica continues to deposit on these nuclei until supersaturation is relieved.[2] It has also been shown that adding amino acids to a dilute silicic acid solution favors polymerization into particles.[22]

Proteins have been shown to bind tightly to the surface of the nanometer scale silica particles,[23] and in the case of enzymes, binding to silica particles has been reported to enhance enzyme stability and activity.[24] Silicate nanoparticles and flakes are formed from saturated silicate solutions when interacting with proteins,[25] and silicates have been shown previously to immobilize and stabilize enzymes such as horseradish peroxidase and acetylcholine esterase.[26,27]

Our results suggest two possible mechanisms that may account for why enzyme activity is stabilized in dilute solution by the action of preparing SSD solutions in glass vials. One is the fact that dissolved silicates and colloidal silica particles have a strong negative surface charge that cause them to bind tightly to positively charged groups on the surface of proteins, thus stabilizing their 3D structure.

The other is the buffering capacity of SSD solutions prepared in glass vials which combine NaOH/KOH and borate (BO_3^{-3}) derived from the glass with carbonic acid derived from atmospheric CO_2 (see Figure 6). Enzymes dissolved in deionized water without salts or buffers, and kept in plastic containers will rapidly denature due to changes in their tertiary structure. Silicates and metal salts dissolving from the glass vials act to stabilize the tertiary structure of proteins in solution, preserving their enzymatic activity for longer than in pure water.

The biology of silicates in humans has been most extensively studied in the epidemiological literature in relation to occupational exposure to silicate particles. Inhalation of silica dust (predominantly silicon dioxide) induces lung inflammation, and after prolonged exposure lung fibrosis can occur, a condition known clinically as silicosis.[28] The mechanisms by which silica exposure leads to silicosis are uncertain. Evidence implicates the induction of tumor necrosis factor-alpha (TNF-α) release by macrophages and subsequent activation of nuclear factor kappa-B (NF-κB),[29] as well as induction of reactive oxygen and nitrogen species.[30,31] *In vitro* studies have shown that silica particles are phagocytized by macrophages, which release reactive oxygen and nitrogen species in response.[32]

In addition to the well documented connection between silica and the lung disease silicosis, prolonged, excessive silica exposure has also been linked to autoimmune diseases including systemic sclerosis, rheumatoid arthritis and systemic lupus erythematosus.[32] Again, the biological mechanisms involved are mostly unknown, but involve inflammation associated with silicate exposure as an early phase of the biological response. The type of silicate exposure has a profound influence on the onset and progress of disease. In particular, the surface properties of the silica particles are one of the critical properties involved in eliciting inflammation. Freshly fractured silicate particles contain surface radicals resulting from the breaking of silicon–oxygen bonds, and these newly exposed surfaces are significantly more potent at inducing free radical generation by macrophages than aged silica dust.[33]

Silica is taken up from soil into plants and polymerized within their tissues, especially in monocotyledonous plants, including grains. Silica content varies greatly between different plant species and different parts of the same plant. For example, the silica content in polished rice has been reported to be 0.5 g/kg, whereas in rice bran the level is 100 times higher at a level of 50 g/kg, and in rice hulls, the level is 230 g/kg.[34] Because grains and their associated bran are common constituents of human diets, there are ample sources of silica that greatly exceed the levels present in ground water, or in glass-exposed foods or pharmaceuticals. Because plant materials contain monosilicates, complex polysilicates and silicate aggregates, often at relatively high levels,[34] it seems unlikely that the low levels present in homeopathic preparations would have any specific biological effects when ingested.

Standard homeopathic preparations contain low concentrations of the constituent compounds from the mineral or biological starting materials. In contrast, BRAN-type homeopathic preparations contain only water impurities and glass-derived constituents. The concentrations of silicates in the homeopathic preparations we investigated are in the range of approximately 1–4 mg/L. However, very high concentrations of silicates are often necessary to elicit biological responses; hundreds or thousands of times higher than those found in homeopathic preparations.[28] As such, it is unlikely that silicates in homeopathic preparations are significantly more concentrated than in any other pharmaceutical or food product stored and shipped in glass containers, especially if those containers are heat sterilized with the contents in them.[4]

Such low concentrations would be negligible once introduced into a biological system with highly concentrated solutes. However, in much simpler *in vitro* assay systems it is possible that silicates and other solutes leaching from glass containers could affect certain measures. Enzyme stabilization or other effects of silicates binding to proteins in solution may explain in part some of the *in vitro* effects reported in the homeopathic literature, when the outcome measure could be influenced by small changes in protein surface charge or conformation.

Summary and conclusions

We have demonstrated the presence of silicates and other constituents leaching from glass containers in SSD preparations made in our lab and in commercially prepared homeopathic preparations. We have further shown that the dissolved silicates are present in the 1–4 mg/L concentration range. Finally, we have demonstrated that these silicates are able to stabilize enzymes in a functional state in dilute aqueous solution relative to enzymes dissolved in deionized, purified water.

The implications of these findings are distinct for the pharmaceutical industry and for hypotheses of homeopathic efficacy. The fact that silicates leaching from glass vials can act to stabilize enzyme activity in solution may have positive implications for pharmaceuticals. Protein or polypeptide-based pharmaceuticals including insulin and vaccines are invariably stored and shipped in glass vials. Our results suggest that silicates leaching from the glass vials may prolong the shelf life and potency of protein-based pharmaceuticals. Further research will be required to confirm this possibility.

Our data do not discount any hypothetical involvement of silicates as active ingredients in homeopathic preparations as has been proposed previously,[32–35] and provide experimental support for the idea that homeopathic preparations made in glass vials are saturated with silicates. However, as noted above, dietary sources of silicates far exceed the doses found in glass-exposed solutions. Further, our pH measurements in combination with what is currently known about silicate-water chemistry[2] suggest that complex silicates with specific biological actions would not persist in SSD preparations. This is due to the mildly alkaline pH (approximately 8.5) attained when each new glass vial is used to make a successive dilution.

In saturated silicate solutions stored in glass between pH 7 and 8.5 the dissolved silicates exist mainly as colloidal particles in equilibrium with monomeric $Si(OH)_4$ and there is constant exchange of soluble and insoluble silicates.[15] Lower levels of polysilicates would also exist in equilibrium with monosilicates, but due to their lower solubility and silicate saturation of the solution, silicate polymers would eventually bind to either the glass walls of the container, or to the colloidal silicate particles in suspension.

As homeopathic solutions age in the glass vials they are stored in, the constant dissolution of silicates from the glass surface and re-condensation onto surfaces and suspended particles would eventually eliminate any specific polysilicates that had formed during the initial preparation of the solutions. Nonetheless, future *in vitro* homeopathic experiments will need to take into account the fact that significant levels of dissolved solids exist in glass-exposed solutions, and that these can have functional effects on proteins dissolved therein.

Disclaimer

This manuscript has been reviewed in accordance with the policy and guidelines of the Armed Forces Institute of Pathology and the Department of Defense, and approved for publication. Approval should not be construed to reflect the views and policies of the Department of the Army, the Department of Defense, or the United States Government, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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