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Protein Instability Following Transport or Storage on Dry Ice.

Murphy BM, Swarts S, Mueller BM, van der Geer P, Manning MC, Fitchmun MI.

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To the Editor: It is common practice to place protein solutions on dry ice for storage or transport, but this may lead to an unrecognized problem. A series of assay failures after short-term storage of antibody solutions on dry ice led to our observation that the pH of the thawed solutions was between 5.5 and 6.0 even though they had been formulated at pH 7.2. We hypothesized that exposure of the solutions to CO_2 caused the formation of carbonic acid, resulting in protein damage from the pH drop. Protein properties affected by pH include tertiary and quaternary structure, enzymatic rate constants, solubility, tendency to aggregate, susceptibility to chemical degradation and propensity to adsorb to surfaces[1]. We therefore examined possible interactions between dry ice and sealed frozen protein solutions.

We evaluated four types of cryogenic vials, three types of conical tubes, two types of glass vials and one type of microtube (**Supplementary Methods**). Vessels containing a buffered pH indicator solution were placed on dry ice or into a –70 °C freezer for 48 h. Upon thawing, most samples placed on dry ice experienced a substantial decrease in pH (**Supplementary Table 1**), and no container closure system consistently prevented acidification. pH changes were not observed in –70 °C freezer controls.

Sample acidification appears to result from two distinct events. First, CO_2 enters the container's headspace but is unreactive, having negligible solubility in ice. If we vented headspace before sample thawing, no acidification was observed (**Fig. 1a**). Also, placing samples into a –70 °C freezer for 96 h allowed the CO_2 to dissipate, after which no acidification was observed. The second event occurs if the sample is thawed while CO_2 is still in the headspace. Acidification was seen to originate at the liquid-gas interface and expand through the sample as it warmed (**Fig. 1b** and **Supplementary Video 1**).

We calculated pH as a function of headspace CO_2 for 1.5-ml microtubes containing Tris buffer (**Fig. 1c**). Predicted drops in pH ranged from 1.5 to 2.7 pH units depending on the starting pH and sample volume. Calculations for other buffer systems such as phosphate-buffered saline produced similar results.

Proteins generally exhibit low solubility near their isoelectric point (pI). Therefore, acidic proteins have an increased tendency to aggregate or precipitate as pH falls below physiological levels. As a model, we formulated β-lactoglobulin (pI 5.2) in citrate or phosphate buffers between pH 4.8 and 7.3. Aggregation index measurements (**Supplementary Methods**) were not substantially different at 2, 5 and 24 h post-formulation for solutions between pH 5.8 and 7.3, but aggregation index values increased with decreasing pH and increasing time below pH 5.8 (**Fig. 1d**). Stressing the samples by vortexing caused a marked increase in aggregation index values below, but not at or above pH 5.8.

We further examined the acidic protein carbonic anhydrase (pI 5.9) and the basic protein lysozyme (pI 9.3), each in 10 mM Tris, pH 7.3, or 10 mM phosphate, pH 7.3. Exposure to dry ice for 48 h before thawing resulted in substantial acidification of all solutions. Aggregation index values increased substantially for the acidic but not the basic proteins (**Supplementary Table 2**). Returning the samples to a –70 °C freezer for 96 h before thawing prevented the pH drop and increase in aggregation index value.

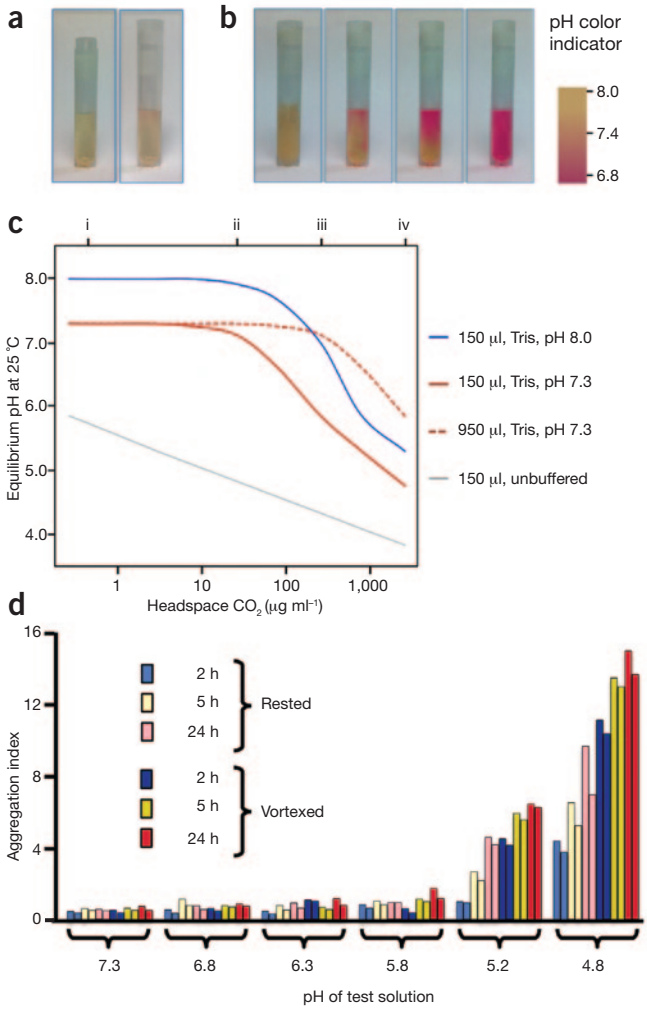

Figure 1 | Effect of dry ice on pH and aggregation index. (**a**,**b**) Cryogenic vials containing a Tris-buffered colorimetric pH indicator solution were placed into a −70 °C freezer for 48 h and then onto dry ice for 48 h. Removing the cap just before thawing (**a**; left) or, alternatively, returning the vials to a −70 °C freezer for 96 h (**a**; right) prevented acidification. Otherwise, upon thawing, acidification was seen as a color shift from yellow to red originating at the sample-headspace interface (**b**; **Supplementary Video 1**). (**c**) Theoretical predictions of pH versus headspace CO_2 for samples in 1.5-ml microtubes. Calculations were for unbuffered samples or for 10 mM Tris at indicated volumes and pH. Atmospheric CO_2 concentration, 0.5 μg ml^{-1}, and that inside a dry-ice shipper, 2,700 μg ml^{-1}, are indicated (i and iv, respectively). Between 1% and 10% of the CO_2 concentration inside a dry-ice shipper (ii and iii), samples are predicted to begin experiencing acidification. (**d**) Aggregation index value measured for β-lactoglobulin solutions formulated at pH values from 4.8 through 7.3. Rested samples were gently inverted immediately before measurement. Vortexed samples were vortexed twice for 3 s before the 2-h reading and gently inverted immediately before the 5- and 24-h readings.

Although changes in pH can affect a variety of protein properties, we examined only protein solution stability. The commonly used aggregation index does not discriminate between pH-mediated protein precipitation and pH-mediated aggregation resulting from protein damage. Often, protein aggregate growth does not proceed to precipitation[2,3]. Soluble (nonprecipitating) aggregates can behave differently from nonaggregated proteins with respect to specific activity, availability of epitopes and reactive sites, biodistribution, half-life and immunogenicity[4].

It is conceivable that CO_2 leaks through nonintegral seals or diffuses through container materials. However, even for the more CO_2-permeable materials (that is, polypropylene and silicone rubber), the diffusion constants and low temperatures would not result in diffusion rates high enough to cause the observations in this study. Therefore, poor seal integrity at ultralow temperatures is the likely explanation. Also, large variations observed with some containers (**Supplementary Table 1**) are consistent with seal failure but not diffusion.

We examined possible preventative measures (data not shown). Wrapping vials with Parafilm had no measurable benefit. Placing vials inside laminated aluminized Mylar zip seal bags provided some protection, but the results were inconsistent. Heat-sealing sample vials in laminated aluminized Mylar bags did appear to prevent acidification. Also, as shown above, if temperatures remained below −40 °C, CO_2 did not interact with the sample. Thus, decreased pH and increased aggregation index values are most reliably prevented by venting the headspace or allowing samples to sit in a −70 °C freezer for 96 h before thawing.

However frequent, damage to proteins placed on dry ice may be overlooked because it is impractical to measure the aggregation or pH of the small-volume samples common in biochemical research. Moreover, the factors described above—container size, sample volume, buffer type, protein pI and time elapsed since transport—combine to create sporadic and inconsistent shifts in sample pH. It is important to keep this phenomenon in mind and take simple precautionary steps when using dry ice so as to ensure consistent protein quality across experiments.

Note: Supplementary information is available at http://dx.doi.org/10.1038/nmeth.2409.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available at http://dx.doi.org/10.1038/nmeth.2409.

Brian M Murphy[1,2], Spencer Swarts[3], Barbara M Mueller[4], Peter van der Geer[3], Mark C Manning[1,2] & Mark I Fitchmun[5,6]

[1]Legacy BioDesign LLC, Johnstown, Colorado, USA. [2]Department of Chemistry, Colorado State University, Fort Collins, Colorado, USA. [3]Department of Chemistry and Biochemistry, San Diego State University, San Diego, California, USA. [4]Torrey Pines Institute for Molecular Studies, San Diego, California, USA. [5]Somatek Inc., San Diego, California, USA. [6]Accelagen Inc., San Diego, California, USA.
e-mail: fitchmun@somatek.com

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