

The Use of Reducing Agents

The presence of a reducing agent in experimental solutions is often necessary for some proteins to maintain activity and stability and/or prevent it from forming aggregates. Currently, the most popular reducing agent for proteins is dithiothreitol (DTT). However, DTT is unstable and tends to undergo oxidation in the solution in the presence of air. MicroCal research and customer experiences have showed that even at 0.5 mM concentration of DTT could have an adverse effect on the DSC and ITC baselines even though the solution was degassed under the mild vacuum (27 mm Hg) immediately prior to the experiment. Although we found that purging air from the solution with either nitrogen or argon could minimize the effect of DTT on the calorimetry baseline (see figure 1) This method appears to be impractical for a solution containing detergent or protein, as it will foam upon purging with nitrogen or argon.

There are commercially available alternatives to DTT that are as effective. One of them is β -mercaptoethanol, which was widely used in prior to 1970 before being replaced by DTT. DTT became popular quickly due to the foul odor of β -mercaptoethanol. More recently, another reducing agent, TCEP (tris(2-carboxyl)phosphine), has become increasingly popular not only due to its lack of odor, but also because of a stronger reducing capacity and decreased probability of oxidation in air^{1,2}. MicroCal has carried out studies to evaluate the effects of these two reducing agents on our calorimeter's baseline. It was found that these two reducing agents produced less DSC & ITC baselines artifacts than DTT (see Figures 1 & 2). We should emphasize that our conclusions are based on the limited experiments in a single buffer solution only (i.e. 40 mM Hepes, pH 7.5). The reducing agent solutions for our experiments were freshly prepared, and were used for the testing within a week. We also frequently found that the baseline artifacts caused by these reducing agents were not reproducible. The magnitude of artifacts usually decreased as the solution aged. The inconsistencies most likely depend on the air content of the solution, its pH, the state of oxidation, and temperature. The follows are some suggestions we think may be useful for calorimetry users:

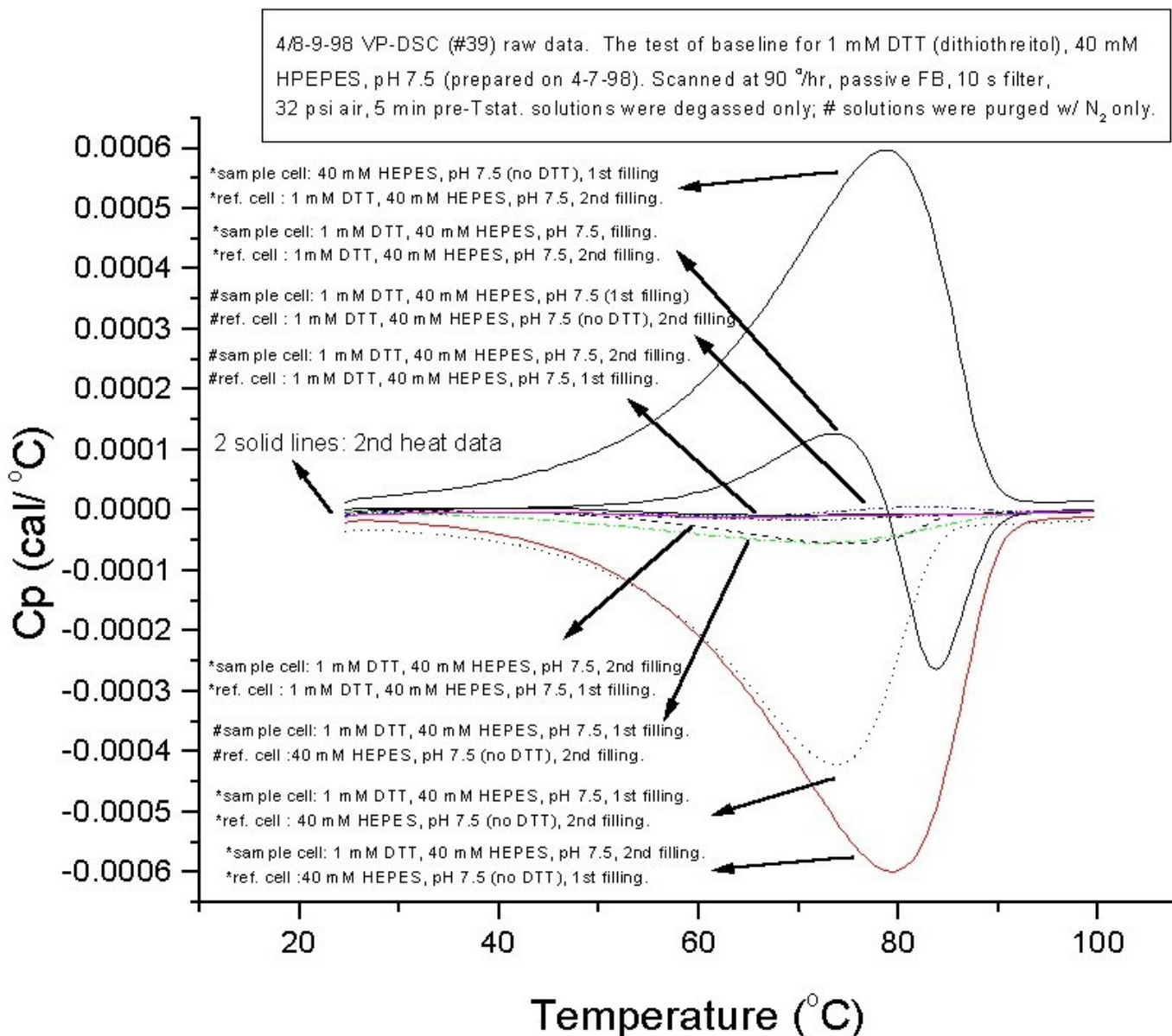
Reducing agents, particular DTT, should be avoided in the solutions when its presence is not absolutely necessary. If a reducing agent is needed, we suggest that users try β -mercaptoethanol first and then TCEP. However, if DTT is indispensable, we suggest trying a buffer vs. buffer (containing DTT) experiment while minimizing concentration. If a baseline abnormality is observed, try to purge the solution with argon or nitrogen to remove oxygen (de-aeration under the moderate vacuum doesn't work). This method may not work for solutions containing detergents or proteins that will induce foaming upon purging with gas. For labs equipped with a dry box, air-free DTT containing solutions should be prepared, and the calorimeter cells should be purged with nitrogen or argon immediately before the solution is loaded to prevent the solutions from contacting air.

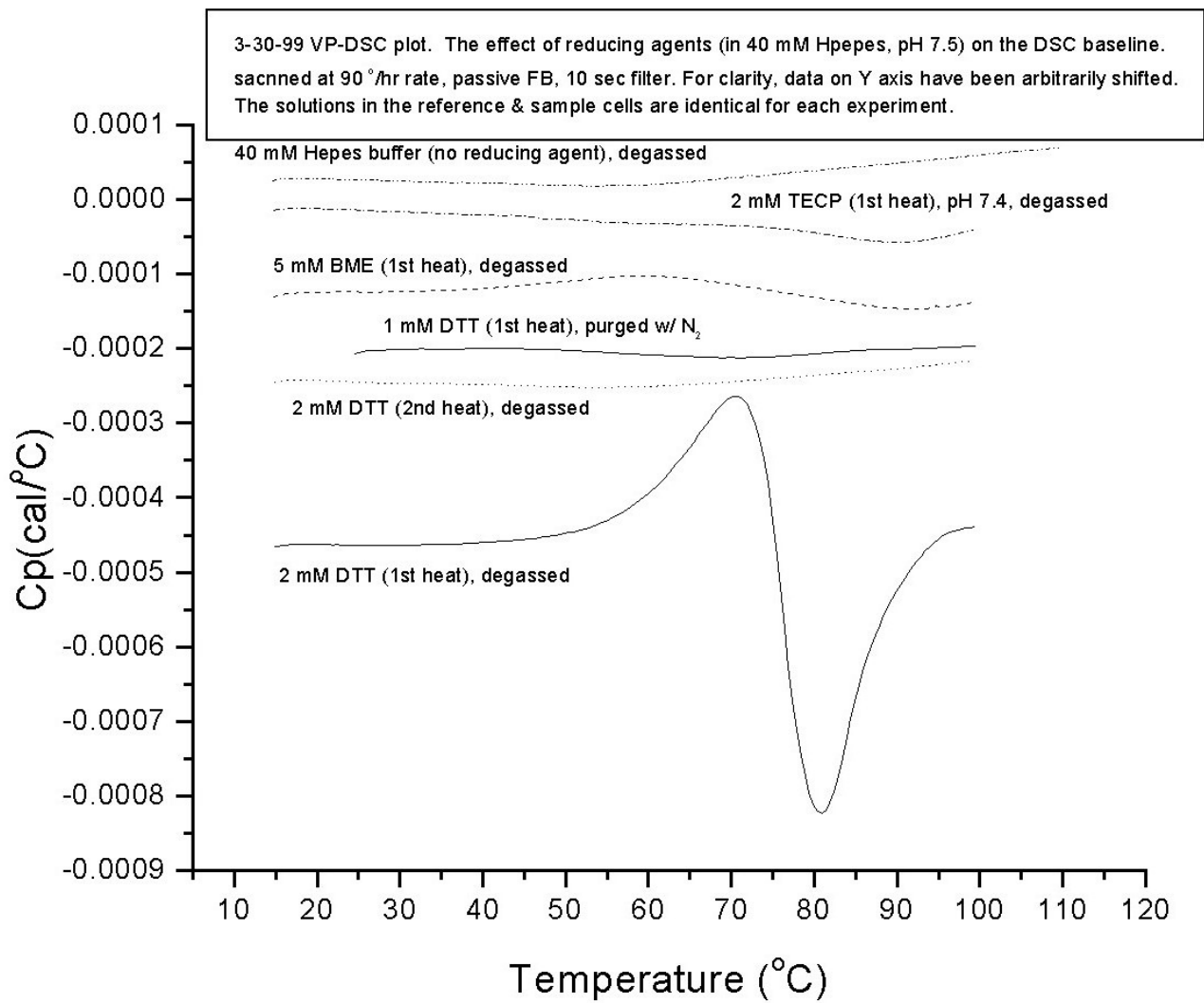
We have tried both β -mercaptoethanol and TCEP. We found that both reducing agents gave fewer baseline artifacts than DTT (see Figures 1 & 2). Our experiments have shown that a concentration of β -mercaptoethanol up to 5 mM in the solution has minimal adverse effects on the ITC baseline. The ITC baseline showed a slope only in the presence of 5 mM β -mercaptoethanol (Figure 2). However, β -mercaptoethanol did cause some abnormalities in the DSC baseline. The size of the artifact for 5 mM β -mercaptoethanol is rather small compared to that of DTT at 2 mM (Figure 1). We also found that ITC baseline shows a slope only in the presence of 2 mM TCEP. But, it caused some artifacts on the DSC baseline (Figure 2). The size of artifacts is small compared to that of DTT. Since no extensive investigation has been done on both β -mercaptoethanol and TCEP, we can only advise our customers to try them to see if they

work for their buffers and samples. It has been reported that TCEP is not stable in phosphate buffer¹. It is prudent for a user to do their control experiment first to make sure that the baseline artifacts are minimum.

We have noticed that some published literature has not reported any anomalies in the DSC or ITC baselines even though DTT was used in their calorimetric experiments (for examples: Arold et al., *Biochemistry* 37, 14683 1998; Popovic et al., in *Biocalorimetry*, Ladbury, J. E. & Chowdhry, B. Z., editors, John Wiley, 1998, p 277). This seems to suggest the possibility that under certain conditions DTT could have no adverse effect on ITC & DSC data.

¹Han, J. C. & Han, G. Y. (1994) *Anal. Biochem.* 220, 5-10; ²Preview (1999) volume 2, issue 4, page 16, a technical publication by Pierce Chemical Co.





From: VP-DSC MicroCalorimeter User's Manual, MicroCal, LLC