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Comparison of the Effects of British Anti-Lewisite (BAL) and Beta Mercapto Ethanol on the Reduction and Cleavage of Disulfide Bonds in IgG and Human Keratinocyte Proteins

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ABSTRACT: British anti-lewisite (BAL) has been proposed as the active component for an ophthalmic antidote to lewisite. BAL was proposed because of data regarding its efficacy, and it has been cleared for therapeutic use in humans. Because it contains 2 thiol groups, BAL can be expected to reduce disulfide linkages and denature enzymes and other proteins. Thus two protein populations containing numerous inter- and intrachain disulfide bridges were exposed to BAL, and the effects were examined using polyacrylamide gel electrophoresis. The first population was a purified preparation of IgG, and the second consisted of human keratinocyte proteins. The effects of BAL on these proteins were compared to those of a widely used disulfide-reducing agent, beta mercapto ethanol. The results indicate that BAL is capable of reducing both inter- and intrachain disulfide bridges in proteins, thus denaturing them and possibly causing a loss of function. British anti-lewisite and beta mercapto ethanol both caused alterations in the electrophoretic migration patterns of each protein population, but it was not possible to determine which compound was the more active with regard to denaturing human cellular proteins.



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INTRODUCTION:

The U.S. Army Medical Research and Development Command is involved in efforts to field an ophthalmic antidote to lewisite (1). The active component of one proposed antidote is the compound 2,3-dithio propanol (more commonly known as British anti-lewisite, or BAL) (2). As part of the evaluation process, a battery of assays needs to be performed on BAL. These should include metabolic fate studies, genetic toxicology tests, morphologic determinations of irritancy, and selected chemical interaction studies. This laboratory note addresses the last category.

British anti-lewisite contains 2 thiol groups, and can undergo thiol-disulfide exchange in the presence of disulfides (3). Thus BAL can cleave disulfide bonds in proteins by reducing them to their respective sulfhydryl derivatives. Earlier work performed on the interaction of BAL with insulin and other enzymes confirmed that this was true (4). Such effects are a matter of concern since introducing a disulfide reducer into the eye is likely to denature ocular proteins. We decided, therefore, to evaluate the disulfide-reducing capabilities of BAL, and to compare those capabilities to those of a widely used disulfide reducer, beta mercapto ethanol (BME). IgG was chosen as the initial test protein since it is known to have 4 interchain disulfide linkages (5, 6). Human keratinocytes were chosen as the second test preparation since a large supply was readily available, and keratinocytes share many properties with corneal epithelium cells, to include common embryology (7). Comparisons were made using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on the IgG preparation, and diagonal two-dimensional SDS-PAGE on the keratinocytes.

METHODS:

IgG - Four hundred and fifty micrograms (ug) of goat IgG was split into 9 equal fractions of 50 ug each. Fractions 1-3 were brought to a final concentration of 1% SDS and 25% glycerol. Fractions 4-6 were brought to a final concentration of 1% SDS, 2% BME, and 25% glycerol. Fractions 7-9 were brought to a final concentration of 1% SDS, 2% BAL, and 25% glycerol. The aliquots were loaded into separate lanes on a 5-15% polyacrylamide gradient gel and electrophoretically separated at 35 milliamps (ma) constant current with cooling until the tracking dye began to run off the gel (8). The gel was then fixed and stained with Coomassie Brilliant Blue R-250 (8, 9). This procedure was replicated once, for a total of 6 samples per condition.

Keratinocytes - The procedure carried out on the keratinocytes is commonly referred to as diagonal two-dimensional electrophoresis (see explanation, Figures 1 and 2). This method was chosen for the keratinocytes because it is a more powerful technique, and is better capable of resolving the greater number of components contained in the keratinocyte samples.

Human keratinocytes were grown in serum-free medium (10), desalted by rinsing with isotonic ethanol, and lysed/solubilized in a solution of 1% SDS, 25% glycerol. Fifty ug total protein was loaded onto each of 9 SDS-PAGE gels that had been poured into 1 mm diameter glass tubes (Figure 1A). Each gel consisted of 7% polyacrylamide, was 14 cm long, and had a 4% polyacrylamide, 1 cm long, discontinuous pH stacking gel (8). These preparations were electrophoretically separated vertically at 250 volts, constant voltage, with cooling until the tracking dye began to run out of the tubes. At the end of the run, the gels were extruded from the glass tubes (Figure 1B). Gels 1-3 were each soaked in 1 ml of 2% ethanol (ETOH) for 30 min. Gels 4-6 were each soaked in 1 ml of 2% ETOH, 2% BME for 30 min. Gels 7-9 were each soaked in 1 ml of 2% ETOH, 2% BAL for 30 min. After soaking, each cylindrical gel was laid horizontally on top of a SDS-PAGE slab gel (i.e., poured between 2 glass plates) and sealed into place with molten agarose (Figure 1C). Each slab gel consisted of 10% polyacrylamide, 14 cm long, with a 2 cm, 4% polyacrylamide, discontinuous pH stacking gel (8, 9). Electrophoresis was carried out at 35 ma per gel with cooling until the tracking dye began to run off the bottom of the gel (Figure 1C). Each gel was fixed and stained with Coomassie Brilliant Blue, as previously described.

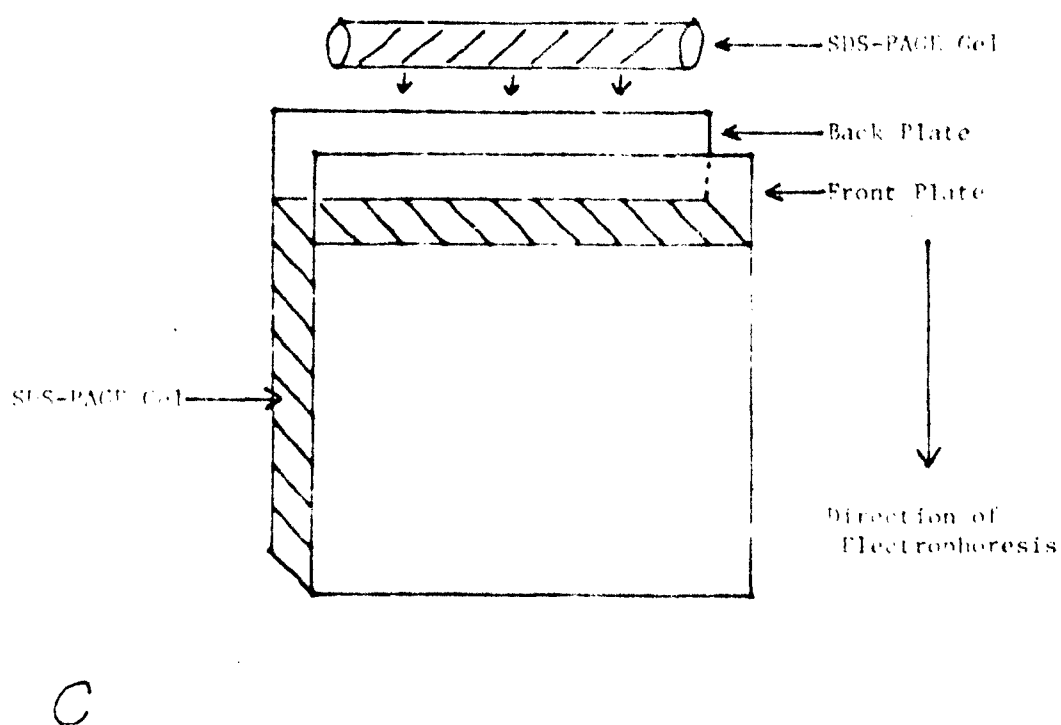
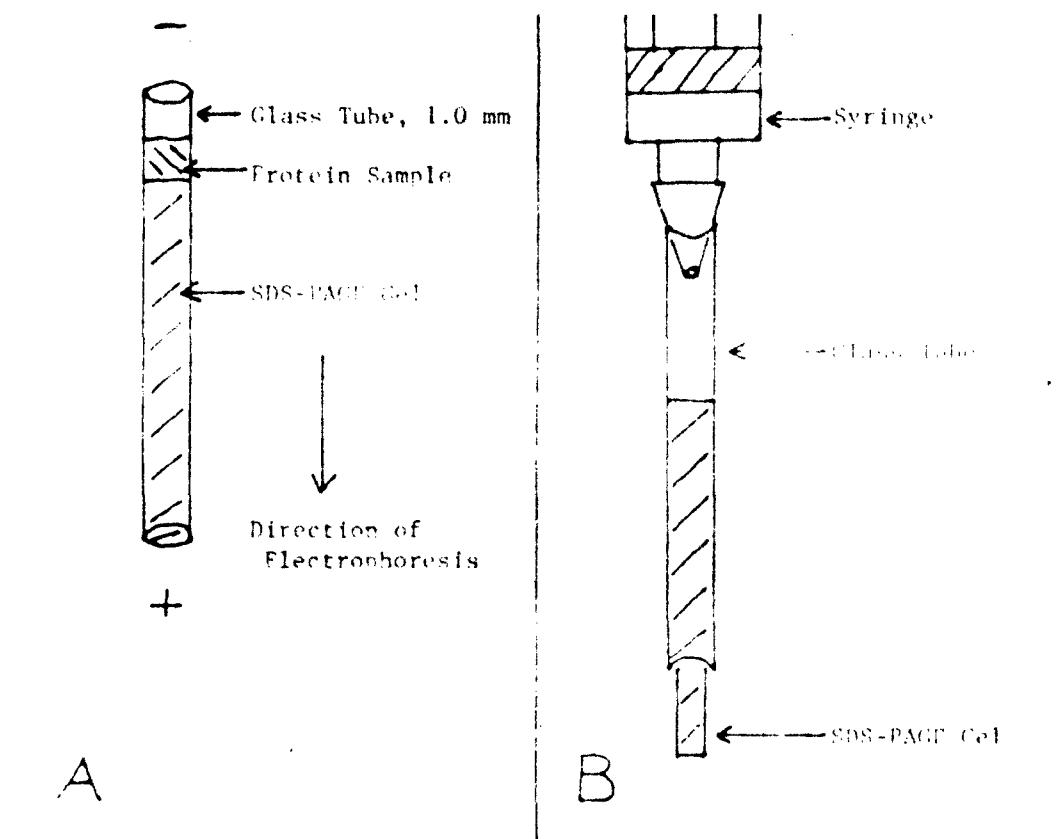


Figure 1. Methodology, diagonal two-dimensional electrophoresis. A, SDS-PAGE gels are poured into glass tubes with inner diameters of 1.0 mm, and the gels are allowed to polymerize. Sample solution containing the proteins of interest and bromophenol blue tracking dye is then placed on top of the gel. An electric field is then applied across the gel until the tracking dye begins to migrate off the bottom. At this point, the proteins are separated on the basis of molecular size along the length of the cylindrical gel. B, the gel is next extruded from the glass tube by applying pressure to the top of the gel. For gels of small diameter (2 mm or less), this can be accomplished by fitting a microliter pipet tip onto the end of a syringe. The syringe is then filled with distilled water, and the tip is inserted into the top end of the glass tube. The gel is then extruded by applying pressure to the plunger of the syringe. C, SDS-PAGE gels are poured in between 2 glass plates, and the gels are allowed to polymerize, resulting in what is commonly referred to as a sandwich gel or a slab gel. The cylindrical gel is then laid horizontally across the top of the gel sandwich, and care is taken to insure that the cylindrical gel makes full and complete contact with the polyacrylamide slab, i.e., all air bubbles are eliminated. The cylindrical gel is then sealed into place by overlaying it with molten agarose, which is then allowed to cool and harden. Since bromophenol blue tracking dye is required to monitor the progress of the electrophoresis, it is helpful to dissolve a small amount of tracking dye into the molten agarose. An electric field is then applied across the gel sandwich until the tracking dye begins to migrate off the bottom of the slab. At this point, the proteins which were contained in the cylindrical gel are separated, again on the basis of molecular size, along the length (top to bottom) of the gel sandwich. The proteins are then made visible by impregnating the slab gel with an appropriate dye or stain. The type of results that can be expected of this technique is graphically shown in Figure 2A.

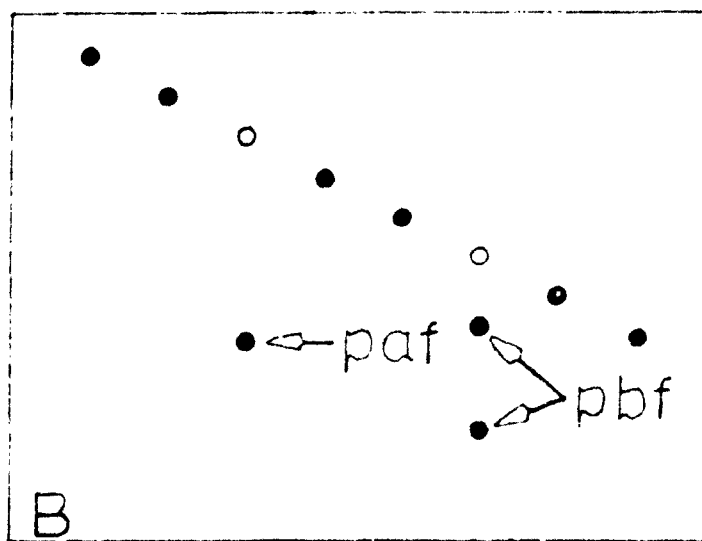
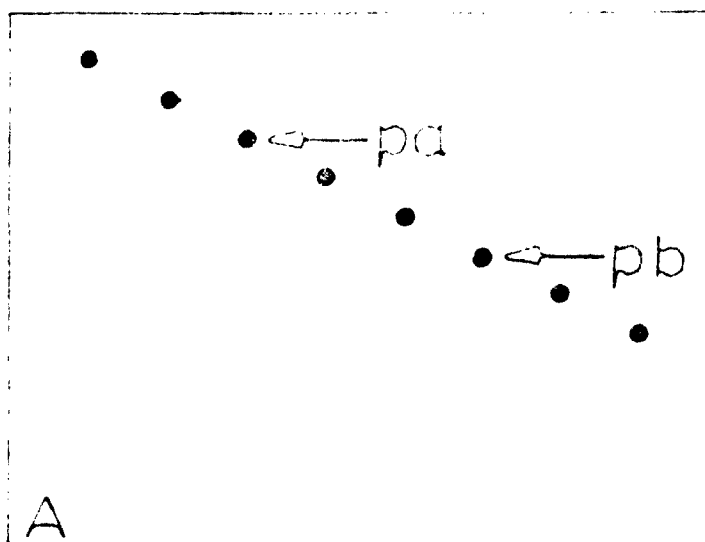


Figure 2. Diagrammatic representation of anticipated results from diagonal two-dimensional electrophoresis when samples are treated with a disulfide reducer. A, no reducing agent applied. The first and second dimension separations are both based on the same parameter, molecular size. Thus, the proteins appear on a diagonal across the final slab gel preparation. If, as in B, a disulfide reducer is applied to the proteins between the first and second dimension separations, those proteins containing interchain disulfide linkages will be cleaved. This results in the production of lower molecular weight fragments which resolve below (i.e., fall off) the diagonal shown in A. Two possible results are then anticipated. First, the fragments generated can be of equivalent molecular weight, as for protein a (pa). In this case, the resulting fragment (paf) will appear directly below the position of the uncleaved parent protein, will stain as heavily as the parent, and will have an apparent molecular weight half as great as the parent. Second, the fragments can be of unequal molecular weight, as for protein b (pb), causing at least 2 spots (pbf) to appear directly below the parent protein's position on the diagonal. (Note: this may not hold true if one or more of the fragments is so small that it migrates with the ion front on the gel, in which case it would disappear from the separation. This, however, is usually an exceptional event.) The staining intensity of each individual fragment will not be as great as that of the parent.

RESULTS:

IgG - In all of the IgG samples, the major component was a 68 kilodalton (KD) band, as expected (11, 12). Samples of IgG that were not treated with either BME or BAL displayed virtually no staining below the 68 KD band, as can be seen in lanes A-C, Figure 3. As expected, BME caused lower molecular weight (MW) bands to appear due to cleavage of interchain disulfides. The most prominent ones had MW's of approximately 50 and 35 KD, as shown in lanes D-F. Treatment with BAL caused approximately 5 low MW bands to appear, but the staining was very weak (lanes G-I), indicating that very little of the parent protein underwent disulfide cleavage. Given that the staining sensitivity of Coomassie R-250 is no better than 10 ng total protein per band (13), and that 50 ug of IgG was loaded onto each gel, it is likely that as little as 1% of the IgG was affected by the BAL. The low MW bands that appeared had MW's of approximately 60, 50, 30, 21, and 17 KD.

It can also be seen in Figure 3 that the BME-treated samples contain at least one more high MW band (i.e., greater than 68 KD) than the BAL-treated samples. Additionally, all of the high MW bands in the BME samples are more darkly stained than in the BAL samples.



Figure 3. Effects of beta mercapto ethanol (BME) and British anti-lewisite (BAL) on the SDS-PAGE electrophoretic profiles of IgG. Lanes A-C, control preparations; D-F, IgG treated with 2% BME prior to electrophoresis; G-I, IgG treated with 2% BAL prior to electrophoresis. std indicates lanes containing molecular weight standards. Standards were 92, 68, 45, 31, 21.5, and 14.4 KD in molecular weight.

Keratinocytes - The keratinocyte proteins underwent two electrophoretic separations, but both were based on the same parameter, molecular size. In the absence of additional treatments, this procedure ordinarily causes the separated proteins to distribute along an almost perfect diagonal line across the final slab gel. Such results are graphically outlined in Figure 2A, and can be seen in Figure 4A. In this paradigm, however, 3 of the first dimension gels (i.e., those run in the 1 mm glass tubes) were treated with BME, and 3 others were treated with BAL. The remaining 3, Figure 4A, were ethanol-treated controls. If cleavage of interchain disulfide bridges occurs, one or more resulting lower MW fragments should appear well below, i.e. fall off, the diagonal directly beneath the location of the uncleaved parent protein, as diagrammed in Figure 2B. As can be seen in Figures 4B (BME-treated) and 4C (BAL-treated), this did not occur. In the case of the BME-treated sample, Figure 4B, one protein (P1) separated such that it appeared approximately 2 mm below the diagonal. In the sample treated with BAL, Figure 4C, this same protein plus one other (P2) each fell approximately 2 mm off the diagonal. The 2 affected proteins, P1 and P2, have molecular weights in the 70-90 KD range. Neither has yet been identified, but are likely to be cytokeratins.

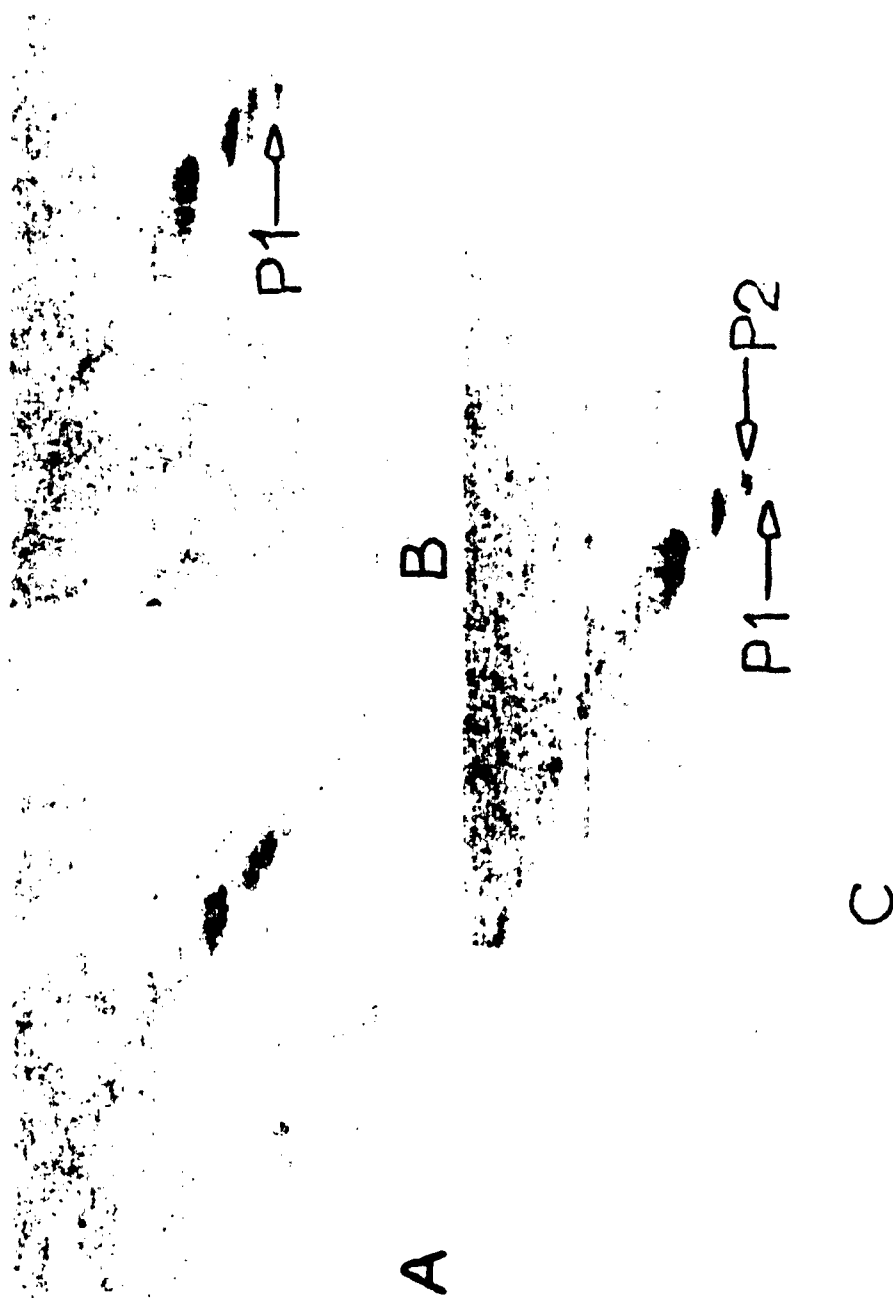


Figure 4. Effects of beta mercapto ethanol (BME) and British anti-lewisite (BAL) on the diagonal two-dimensional electrophoresis profiles of human keratinocyte proteins. A, control. The first dimension gel was soaked for 30 min in 2% ethanol prior to second dimension separation. B, first dimension gel soaked for 30 min in 2% ethanol and 2% BME prior to second dimension separation. C, first dimension gel soaked for 30 min in 2% ethanol and 2% BAL prior to second dimension separation. BME caused one protein, P1, to fall off the diagonal by a distance of approximately 2 mm.

BAL caused two proteins, P1 and P2, to fall off the diagonal, each a distance of approximately 2 mm. Because the displacement distances were so small, these results indicate that no interchain disulfide linkages were cleaved in either B or C. The minor alterations in second dimension migration, however, do suggest that the conformations of P1 (BME and BAL) and P2 (BAL only) could have been altered due to the cleavage of intrachain disulfide linkages.

DISCUSSION:

IgG - The results of the IgG portion of this study suggest that BAL is not as effective a disulfide reducer as BME, and, in that respect, might not be as noxious/irritating in human applications. While BAL produced a greater number of low MW fragments than BME did, the fragments generated by BAL were barely perceptible upon Coomassie staining, indicating that their total amounts were very small. As stated in the RESULTS section, the low MW fragments generated by BAL account for only about 1% of the parent protein.

The electrophoretic patterns found in the high MW portions of the IgG gels also indicate that BME is the stronger disulfide reducer. Some of the IgG moieties in the controls were probably too large to enter the gel matrix, and remained in the stacking gel (8, 9, 12, 13). Given that this occurred, then it would be reasonable for the BME-treated samples to contain a greater number of fragments in higher concentrations in the high MW region of the gel than in the BAL-treated samples.

That BAL generated fragments of different MW than BME is probably related to the relative efficacies of the 2 compounds as disulfide reducers. BAL is the larger of the 2 molecules, and is much less soluble in an aqueous environment (14, 15). Thus it is probably sterically hindered from affecting some of the disulfide linkages that BME has access to. Likewise, because of its larger size and lesser solubility, BAL might have more difficulty affecting readily accessible disulfides than BME.

Keratinocytes - Curiously, the results from the keratinocytes seem to contradict some of the conclusions drawn from the IgG data. In the BME-treated keratinocyte samples, one protein, P1, fell off the diagonal. No smaller fragments appeared directly beneath P1, however, and P1 fell only approximately 2 mm. This is not consistent with the results one would expect from interchain disulfide bridge cleavage, as outlined in Figure 2. One possible explanation for this result is that BME cleaved an intrachain disulfide linkage in P1. It is further possible that when the intrachain linkage was broken, it changed the conformation sufficiently to allow more sodium dodecyl sulfate (SDS) to bind to the protein. It is conceivable that with an increase in the amount of anionic SDS bound, the protein was pulled more strongly by the electric field. This, in turn, could have caused the protein to have a lower apparent molecular

weight, i.e. migrate farther, due to the increase in field effects. The same explanation applies to the migration of P2 in the samples treated with BAL. Thus, it appears that the changes seen in the electrophoretic patterns of the keratinocyte proteins can be explained by BME and BAL effects on intrachain disulfide linkages.

Beta mercapto ethanol affected only one of the keratinocyte proteins, P1, whereas BAL altered two, P1 and P2. Thus, it seems that for keratinocyte proteins, BAL is the more effective disulfide reducer. This, seemingly, is in contradiction to the conclusions drawn from the IgG portion of the study in that BAL appears to be the more damaging of the two compounds. The results, however, need not be seen as dichotomous. Keratinocyte proteins, particularly P2, may well be more sensitive to BAL than to BME due to structural properties, whereas the properties of IgG make it more sensitive to BME.

This study does confirm that BAL alters the electrophoretic patterns of some proteins. These alterations are likely due to cleavage of disulfide linkages. Thus application of BAL to any protein population, including that of the corneal epithelium, is likely to cause denaturation of proteins containing disulfide bridges. This could well result in loss of function of these proteins, as previously suspected (4). Whether or not BAL is as damaging to cells as strong disulfide reducers such as BME has yet to be determined. It is apparent, however, that more work remains to be performed before we can declare BAL the antidote of choice for treating ophthalmic lewisite casualties.

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