REFERENCES

1. Cox, M.F., and T.P. Matson, *J. Am. Oil Chem. Soc. 60:*1170 (1983).
2. Matson, T.P., and M. Berretz, *Soap Cosmet. Chem. Spec. 56:*41 (1980).
3. Kravetz, L., D.H. Scharer and H. Stupel, *Tr.-Mezhdunar. Kongr. Poverkhn. - Akt. Veshchestvam, 7th, 3:*192 (1978).
4. Dauben, D.L., and H.R. Froning, *J. Petrol. Technol. 23:*614 (1971).
5. Novosad, J., B. Maini and J. Batycky, *J. Am. Oil Chem. Soc. 59:*833 (1982).
6. Lunkenheimer, K., and K.D. Wantke, *Colloid Polym. Sci. 259:*354 (1981).
7. Scamehorn, J.F., R.S. Schechter and W.H. Wade, *J. Dispersion Sci. Technol. 3:*261 (1982).
8. Rubingh, D.N., in *Solution Chemistry of Surfactants,* edited by K.L. Mittal, Plenum Press, New York, 1979, p. 337.
9. Rosen, M.J., *J. Am. Oil Chem. Soc. 59:*582 (1982).
10. Zhu, B.Y., and M.J. Rosen, *J. Colloid Interface Sci. 99:*435 (1984).
11. Rosen, M.J., and B.Y. Zhu, *Ibid. 99:*427 (1984).
12. Holland, P.M., and D.N. Rubingh, *J. Phys. Chem. 87:*1984 (1983).
13. Holland, P.M., *Adv. Colloid Interface Sci.,* in press.
14. Kamrath, R.F., and E.I. Franses, *Ind. Eng. Chem. Fundam. 22:*230 (1983).
15. Shinoda, K., in *Colloidal Surfactants,* edited by K. Shinoda, B. Tamamushi, T. Nakagawa and T. Isemura, Academic Press, New York, 1963, p. 58.
16. Rathman, J.F., and J.F. Scamehorn, *J. Phys. Chem. 88:*5807 (1984).
17. Rathman, J.F., and J.F. Scamehorn, "Counterion Binding on Mixed Micelles," presented at 58th ACS Colloid and Surface Science Symposium, Pittsburgh, PA (June, 1984).
18. Mitchell, D.J., G.J.T. Tiddy, L. Waring, T. Bostock and M.P. McDonald, *J. Chem. Soc. Faraday Trans. I 79:*975 (1983).
19. Burchfield, T.E., and E.M. Woolley, *J. Phys. Chem. 88:*2149 (1984).
20. Tsujii, K., N. Saito and T. Takeuchi, *J. Phys. Chem. 84:*2287 (1980).
21. Hato, M., and K. Shinoda, *J. Phys. Chem. 77:*378 (1973).
22. Peacock, J.M., and E. Matijevic, *J. Colloid Interface Sci. 77:*548 (1980).
23. Somasundaran, P., K.P. Ananthapadmanabhan, M.S. Celik and E.D. Manev, *Soc. Pet. Eng. J. 24:*667 (1984).
24. Bozic, J., I. Krznaric and N. Kallay, *Colloid Polym. Sci. 257:*201 (1979).
25. O'Connell, J.P., and R.D. Walker, "Surfactant Precipitation and Pressure Induced Phase Behavior in Micellar/Polymer Systems," presented at AICHE National Meeting, Houston, (April, 1981).
26. Gerbacia, W.E.F., *J. Colloid Interface Sci. 93:*556 (1983).
27. Nishikido, N., H. Akisada and R. Matuura, *Mem. Fac. Sci., Kyushu Univ., Ser. C 10:*91 (1977).

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Effect of Protein Cationicity on Inhibition of in vitro Epidermis Curling by Alkylbenzene Sulfonate

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Dilute solutions of linear alkylbenzene sulfonate (LAS), an anionic detergent, caused strips of epidermis to twist and curl. Four commercially available protein hydrolysate mixtures and a synthetic peptide, when added to the LAS solution, countered this to varying degrees, from the epidermis being as distorted as the LAS control to as flat as the water control. A study to determine the contribution of these materials' positive charge (isoionic point) to in vitro epidermis flatness demonstrated a direct linear relationship, i.e., the more positive the charge the flatter the epidermis. This effect was even discernible in a 1 to 30 ratio of a highly cationic protein to detergent. One of the protein mixtures, which was then fractionated according to charge, showed a linear regression correlation coefficient of 0.86 for this relationship. Because the twisting and curling of epidermis has been demonstrated to be related to human skin irritation, these results suggest that positively charged proteins might increase the mildness of solutions containing anionic detergents.

Surface active agents have been employed in household formulations in order to obtain efficient cleansing. Some of these agents have the potential to cause skin irritation (1-4), but progress has been made to overcome this (5,6). The study by Eigen and Weiss (5) showed that addition of partially degraded protein mixtures to a dishwashing liquid composition containing anionic surfactants protects the skin from irritation. The nature of the active components in these protein mixtures, however, was not known. It was the purpose of the present study to determine their nature.

This research focused on investigating the contribution of protein charge on ameliorating the skin damaging effect of linear alkylbenzene sulfonate (LAS). An in vitro test method in which detergents caused human epidermis to twist and curl served as the model for in vivo human skin irritation (7). The results demonstrated a sizable effect of protein charge.

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EXPERIMENTAL

Materials. Spectrapor 6 dialysis bags, (Spectrum Medical Industries, Los Angeles, California) AG-1-X2 acetate ion exchange resin, (Bio-Rad, Rockville Center, New York) Amberlite MB-1AR mixed bed ion exchange resin, (Rohm and Haas, Philadelphia, Pennsylvania) Glycylarginine, (Vega Biochemicals, Tucson, Arizona) Protein mixtures I, II, III, IV; Linear alkylbenzene sulfonate (LAS), Continental Oil Co., Houston, Texas.

Determination of isoionic point. Determination of the isoionic point of protein mixtures was accomplished by batch phase ion exchange treatment (V. Johnson, Inolex Corp., Chicago, Illinois, private communication), a modification of Janus' (8) column method. A micromethod of Johnson's procedure was developed because of the small quantities of material available, sometimes less than 1 g. Five ml of a 5% protein solution was prepared and 1 ml pipetted into each of three 10 × 75 mm test tubes equipped with stirring bars. Fifty mg Amberlite MB-1AR was added to the second tube, and 100 mg to the third tube. The tubes were stirred for 30 min and the pH measured. Another 150 mg resin was added to each tube, providing 150, 200 and 250 mg total resin in each, after which the mixtures were again stirred for 30 min and the pH measured. Addition of resin, stirring, and measurement of pH were repeated until the pH became constant in at least two consecutive tubes. This pH is the isoionic point.

Dialysis and lyophilization of protein mixture III. The protein mixture was diluted to make a 5% solution with distilled deionized water. Seventy-five ml was put into each dialysis bag (Spectrapor 6, 1000 molecular weight cutoff) and placed into an 18-l battery jar containing distilled, deionized water. The solutions underwent equilibrium dialysis in a refrigerator for approximately 24 hr and were then lyophilized.

Fractionation of protein mixture III by ion exchange treatment (9). Six 400-g quantities of AG-1-X2 (acetate) were washed with water, adjusted to pH 2,4,6,8,10 and 12 with dilute HCl or dilute NaOH, and filtered. Six 1000-ml quantities of 2% dialyzed lyophilized protein solutions were adjusted to these pH's. The wet resins were added to the corresponding protein solutions and stirred for one hr while maintaining the pH. The mixtures were then filtered and the pH of the filtrates adjusted to 7. The resins were washed with a small amount of water and the washings combined with the filtrates. The pH's of the combined solutions were adjusted to 7, the fractions dialyzed overnight under equilibrium conditions, and the contents of the bag lyophilized. The resins were washed with 2M NaCl at the treatment pH until protein no longer came off (The ninhydrin test was used to minitor this.), and the residue washings were collected and adjusted to pH 7. These washings were dialyzed for one hr periods under equilibrium conditions until there was no meaningful change in refractive index of the dialysate and all salts had been removed.

Separation of epidermis from skin. The "moderately elevated temperature" procedure of Kligman and Christophers' (10) technique was used. The enzymatic treatment and removal of the mushy epidermis were bypassed.

Cutting and mounting epidermis. This procedure was developed by Fernee and Robbins (11). Strips of epidermis were cut crosswise to the longitudinal axis of the cadaver into pieces 0.5 cm wide, and cut into 4- to 5-cm lengths. Plastic tabs were glued with Duco Cement onto both ends of the strip so as to encase the ends of the epidermis, providing final dimensions of 2.5 × 0.5 cm for the exposed strip.

Testing solutions by degree of curling of epidermis. Bent copper wires or partially untwisted paper clips were inserted through the tabs, and the strips were suspended in water overnight in a refrigerator (Fig. 1A). Equal volumes of 0.2% protein and 0.3% LAS were mixed, equilibrated at ambient temperature overnight, and then adjusted to pH 5.3, the approximate pH of human skin (12). Four strips were removed from the water and suspended in the protein-LAS solution until a meaningful change was observed. (See Fig. 1B for effect of LAS.) This usually was an overnight period, but varied from epidermis sheet to epidermis sheet. While immersed, the widths of the strips were measured at the narrowest point, where the degree of curling was most pronounced, or a correction for possible variations in the initial widths of the skin slices could be made by dividing the narrow width by

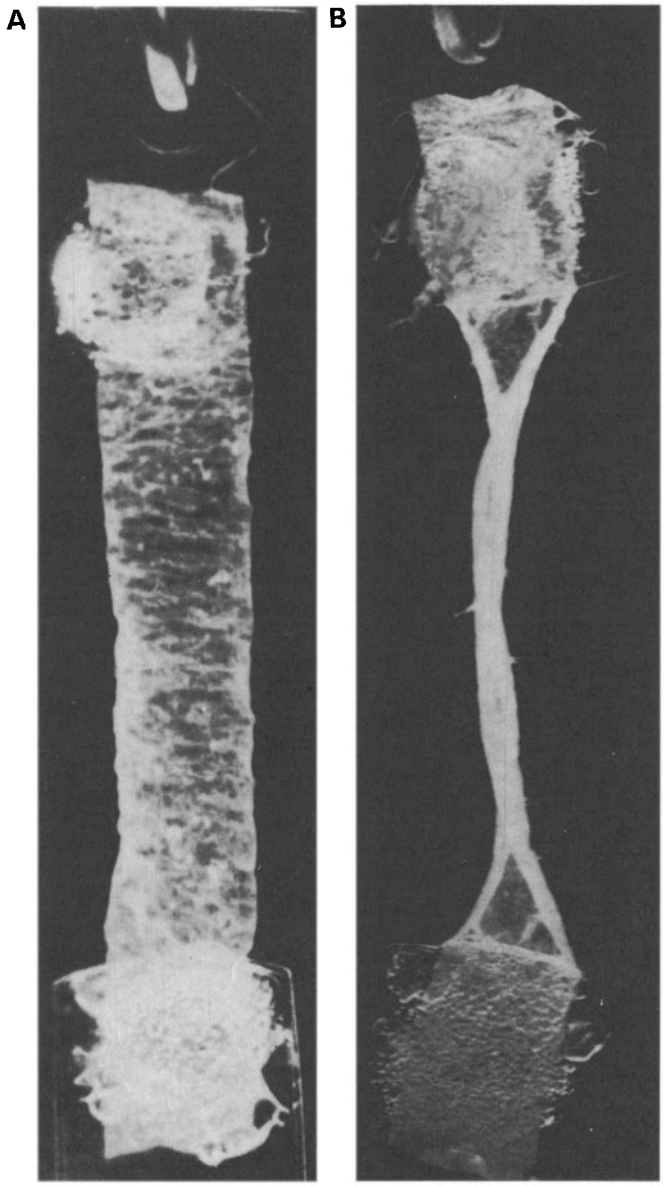

FIG. 1. Epidermis strip. A, before treatment (Soaked in Water), and B, after treatment (Soaked in LAS).

the end width after treatment. Measurements were made with a ruler on an enlarged photographic image.

RESULTS

As shown in Figure 1, LAS, an anionic surfactant, caused strips of epidermis to twist and curl (7). Four commercially available protein hydrolysate mixtures and glycylarginine, a small synthetic peptide, were investigated for their ability to counter this effect. The protein mixtures are referred to as I, II, III and IV, the names omitted because of proprietary confidentiality. They are quite different from each other, varying in source, molecular weight, charge, and process of hydrolysis. The proteins were mixed with LAS and tested for degree of curling of epidermis. LAS, itself, and water served as controls. The effect on the epidermis strips varied considerably among the test agents, some resulting in much curling and twisting, similar to the LAS control, and others resulting in fairly flat strips of epidermis, similar to the water control. The proteins were analyzed for degree of charge by determination of isoionic points (Table 1). The isoionic point of glycylarginine was estimated to be approximately the same as arginine. The data showed a meaningful direct relationship between protein isoionic point and flatness of in vitro epidermis (Fig. 2), demonstrating that the greater the degree of positive charge on protein, the greater its ability to counteract the curling caused by LAS.

One of these protein mixtures, number III, was separated into fractions according to charge by ion exchange treatment. The degree of charge was determined by isoionic point measurement (Table 2). The protein fractions and LAS were then mixed and studied with the epidermis curling test. The results of the experiment are plotted in Figure 3. Linear regression analysis showed that the coefficient for the correlation (r) between isoionic point of proteins and reduction in curling is 0.86. This indicates a high degree of confidence that as the positive charge on a protein increases, so does its ability to counter the harsh effect of LAS.

The effects on epidermis of mixtures of protein fractions of various charge, with LAS, can be seen in Figure 4. LAS alone caused severe twisting and curling of the epidermis (Fig. 4A), while water did not (Fig. 4B). When a negatively charged protein fraction was added to LAS, the epidermis was still twisted and curled (Fig. 4C). However, when a positively charged protein fraction was added to the LAS (Fig. 4D), the epidermis remained flat, similar to the water control, demonstrating the marked importance of protein charge.

The solutions described above had a detergent to protein ratio of 30:20. An active cationic protein mixture, mixture I, was tested at a detergent to protein ratio of 30:1. The results showed the solution with protein to be milder than without protein at $\alpha = .001$ (Sign Test) and $\alpha = .005$ (Analysis of Covariance).

The cationic proteins also reduced the damaging effect of LAS in an vivo skin patch test performed on guinea pigs and primary dermal and eye irritation studies on rabbits (data not shown).

Although the cationic proteins neutralized the effect that LAS had on epidermis, no difference in foam height or number of dishes cleaned was observed (data not shown). Furthermore, the cationic proteins actually stabilized the foam height.

TABLE 1

Isoionic Points of a Peptide and Commercially Available Protein Mixtures

Glycylarginine	11.0
Protein Mixture I	10.0
Protein Mixture II	7.4
Protein Mixture III	4.8
Protein Mixture IV	4.3

TABLE 2

Isoionic Points of Fractions of Protein Mixture III

Fraction	pI	pH of Mixture	Source
D	3.5	4	resin
B	3.7	2	resin
F	4.1	6	resin
I	4.2	8	resin
G	4.4	6	filtrate + resin
K	4.4	10	resin
M	4.5	12	resin
A	4.8	2	filtrate
C	5.0	4	filtrate
E	7.1	6	filtrate
H	7.7	8	filtrate
J	8.3	10	filtrate
L	8.7	12	filtrate

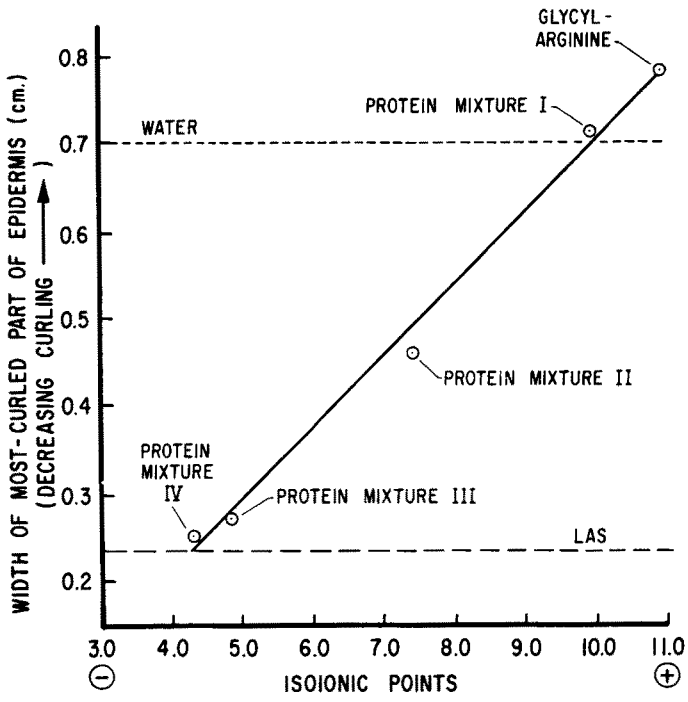

FIG. 2. Effect of protein charge on LAS-induced curling of epidermis.

DISCUSSION

Our data showed that the more cationic the protein the more it countered LAS-induced curling and twisting of the epidermis. A literature search revealed that when proteins are mixed with anionic detergents, complexes of the two are formed. The existence of these complexes has been proven in many ways including equilibrium dialysis (13-21), electrophoresis (13,14,21, 22), viscometry (15,17,19,21,23) and others (15-18, 21-29).

The effect of charge on proteins was investigated as a contributor toward complexing with anionic detergents. The amount of charge was determined by acid binding capacity (19,23), reaction with an acidic dye (30), determination of isoionic point (8), determination of bound metaphosphate (31), and amino acid analysis (30).

Much evidence demonstrates the requirement of cationic sites on proteins for complexing with anionic detergents. Putnam and Neurath (27) showed that only cationic proteins are precipitated by anionic detergents. Klotz et al. (20) showed that the maximum number of bound anionic dye molecules, 22, corresponds roughly to the number of positively charged arginine residues, 25, in bovine serum albumin (BSA). Neurath and Putnam (32), working with horse serum albumin and sodium dodecyl sulfate (SDS), isolated two complexes. There are 62 strongly basic groups (positively charged) on horse serum albumin, and 55 of them are combined with SDS in the first complex. The number of moles of SDS in the second complex is related to the total potential positive charge of the protein, as determined by its acid combining capacity. Putnam (21) reported that the number of moles of another anionic surfactant, alkylbenzene sulfonate, strongly bound in neutral solution by egg albumin, corresponded to the sum of the cationic groups in that protein. The study by Harris et al. (28) provided similar results. They showed that the moles of dodecyl

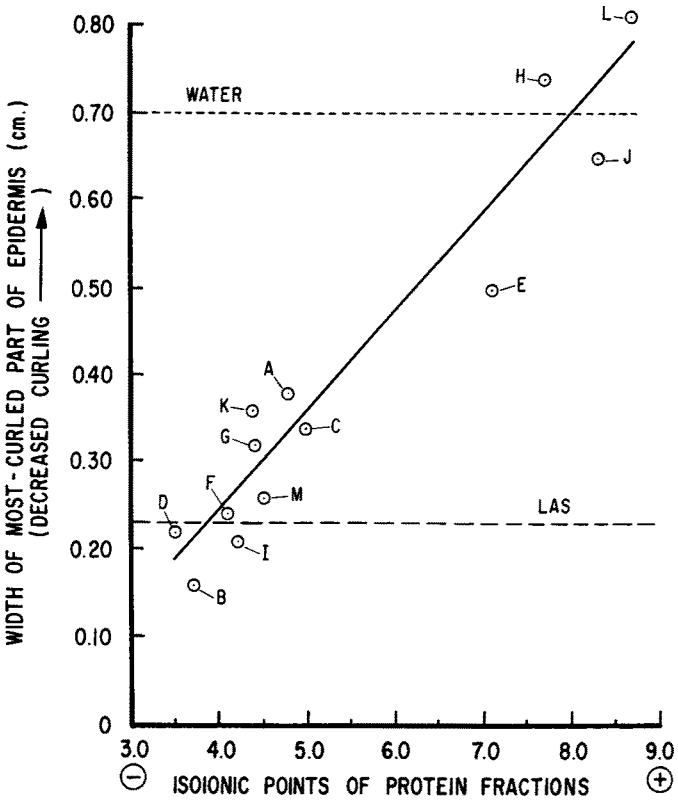

FIG. 3. Effect of charge of protein mixture III fractions on LAS-induced curling of epidermis.

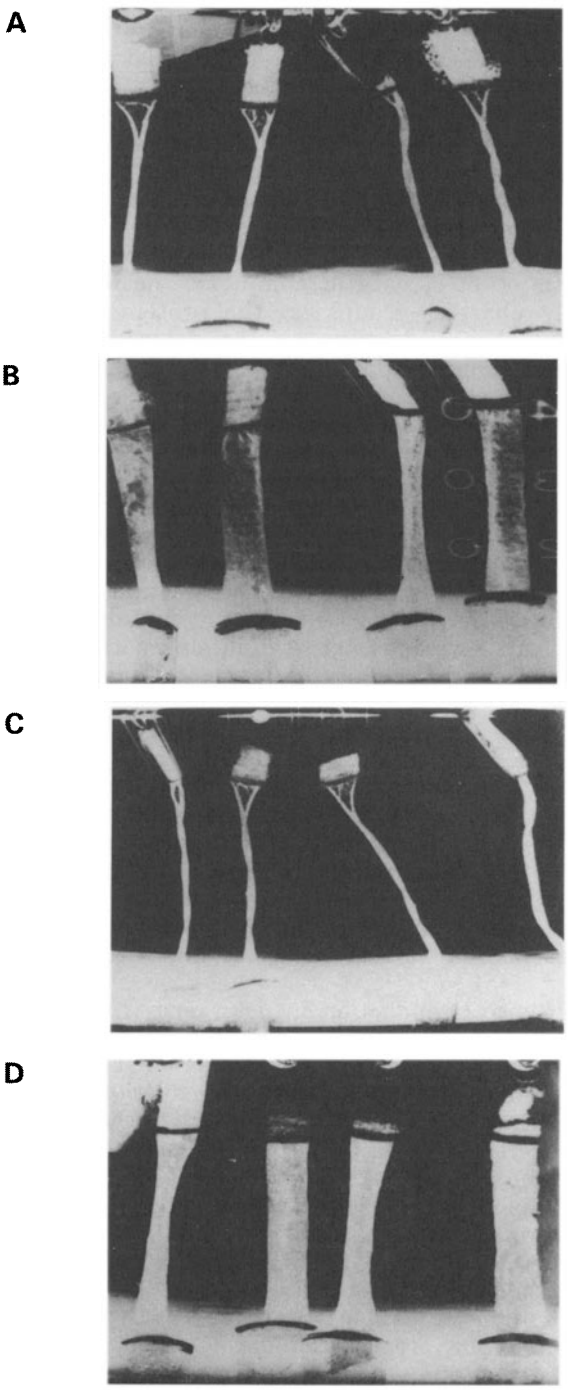

FIG. 4. Effect of charged protein mixture III fractions on LAS-induced curling of epidermis. A, LAS; B, water; C, most negatively charged fraction + LAS; and D, most positively charged fraction + LAS.

sulfate anions bound to gelatin are equivalent to 93% of the total nitrogen atoms of the protein. Complexes were formed by adsorption of detergent at both the positively charged groups of the basic side chains and at the positively charged backbone imide nitrogens, the link here being of the ion-dipole type made possible by resonance of the keto-imide groups. Swaney and Klotz (33) showed that one of the strong sites to which SDS binds to albumin contains a cationic peptide, Lys-Ala-Trp-Ala-Val-Ala-Arg.

Researchers also have studied the importance of cationic sites by modifying them. The study by Harris et al. (28) with SDS and gelatin showed that when the positively-charged groups of the basic side chains were removed (deaminated), the moles of detergent complexed were reduced by 7/8. Nelson (34) showed that succinylating, glyoxylating or carbamylating the amino groups on BSA decreased the complexing.

Altogether, these results are convincing evidence that anionic detergents interact with proteins by formation of electrostatic complexes between the positively charged groups on the protein and the negatively charged groups on the detergents. The amount of complexing might be quite large. For example, somatostatin, a cationic peptide, complexes with SDS to the extent of about 17 g of bound anionic detergent per g of peptide (35).

A reasonable mechanism for cationic protein induced decrease in epidermis curling involves the effect of complexing on the concentration of the LAS monomer. L. Rhein (Colgate Palmolive Research Center, Piscataway, New Jersey, private communication) showed that addition of one part of protein mixture I to 3 parts of LAS lowered the critical micelle concentration (CMC) of LAS from 2.2 mM to 0.32 mM. A decrease in the CMC of the LAS means a decrease in the concentration of LAS monomer in solution. Since the monomer is responsible for effects related to skin irritancy (29, 36), reduction in monomer concentration would be expected to cause reduction in epidermis twisting and curling, which was observed. One would not expect anionic detergent micelles or the detergent protein complex to be an irritant because they are probably too large to penetrate the interstitial spaces of the epidermis, as well as being too high in negative charge.

The absence of reduction in detergency properties and foaming by the protein LAS mixture, as well as the increase in foam stability, deserves comment. Rhein's finding of protein induced reduction of the LAS CMC indicates that the complexing of the protein with the detergent results in more or larger micelles. Because the soil removed from dishes enters micelles, one might predict at least equivalency in dish cleaning ability. Perhaps other factors such as the change in character of the micelle might explain why the dish cleaning ability did not actually improve. The absence of change in initial foam height, as well as the increase in foam stability, are very similar to the findings of Goddard and Hannan (37). They reported that in an aqueous solution of a water-soluble cationic polymer and an anionic detergent there was little effect on initial foaming, but a marked increase in foam stability. Their research showed that the cationic polymer caused a pronounced reduction in the surface tension of the detergent. This surface tension lowering was ascribed to the polymer being modified and rendered highly surface active by adsorption onto it of detergent anions. At higher concentrations the detergent surface tension curves, with and without polymer, merged. In the case of our present study, this would be translatable into equal initial foam height, which was observed. Our demonstrated increase in foam stability would be expected from a complex of a charged detergent and the polyelectrolytic protein. This complexing should result in net charge reduction which should be manifested in increased association between molecules of detergent polymer complex; i.e., increased viscoelasticity. An increase in viscoelastic property would be expected to result in strengthened foam bubbles. Goddard's studies showed that the addition of a cationic polymer to an anionic detergent resulted in increased surface viscosity. The polymer imparted to the foam a definite resistance to deformation, a direct consequence of the increased surface viscosity.

Taken altogether, the above results suggest that addition of a minor amount of a water-soluble, positively charged protein hydrolysate mixture to a commercial liquid detergent product containing an anionic detergent would increase the mildness of the detergent product without reducing its foaming and detergency properties.

ACKNOWLEDGMENT

S. Vratsanos of Columbia University demonstrated his ion exchange procedure.

REFERENCES

1. Polano, M.K., *J. Soc. Cosmet. Chem. 19*:3 (1968).
2. Brown, V.K.H., *Ibid. 22*:411 (1971).
3. Kligman, A.M., and W.M. Wooding, *J. Invest. Dermatol. 49*:78 (1967).
4. Imokawa, G., K. Sumura and M. Katsumi, *J. Am. Oil Chem. Soc. 52*:479 (1975).
5. Eigen, E., and S. Weiss, U.S. Patent 3,548,056 (1970).
6. Schubert, W.R., U.S. Patent 4,148,743 (1979).
7. Tavss, E.A., E. Eigen and A.M. Kligman, *J. Soc. Cosmet. Chem. 36*:251 (1985).
8. Janus, J.W., A.W. Kenchington and A.G. Ward, *Research* (London) *4*:247 (1951).
9. Vratsanos, S.M., and I.D. Mandel, *J. Dent. Res. 56*:B109, Special Issue B (1977).
10. Kligman, A.M., and E. Christophers, *Arch. Dermatol. 88*:702 (1963).
11. Fernee, K., and C.R. Robbins, *J. Soc. Cosmet. Chem. 32*:53 (1981).
12. Arbenz, H., *Dermatologica 105*:333 (1952).
13. Yang, J.T., and J.F. Foster, *J. Am. Chem. Soc. 75*:5560 (1953).
14. Pallansch, M.J., and D.R. Briggs, *Ibid. 76*:1396 (1954).
15. Reynolds, J.A., S. Herbert, H. Polet and J. Steinhardt, *Biochemistry 6*:937 (1967).
16. Steinhardt, J., and J.A. Reynolds, in *Multiple Equilibria in Proteins*, Academic Press, New York (1969) pp. 234-300.
17. Steinhardt, J., J. Krijn and J.G. Leidy, *Biochemistry 10*:4005 (1971).
18. Steinhardt, J., N. Stocker, D. Carroll and K.S. Birdi, *Ibid. 13*:4461 (1974).
19. Malik, W.U., and V.P. Saxena, *A.C.S. Symp. Ser. 9*:299 (1975).

20. Klotz, I.M., F.M. Walker and R.B. Pivan, *J. Am. Chem. Soc. 68*:1486 (1946).
21. Putnam, F.W., *Advances in Protein Chemistry 4*:79 (1948).
22. Putnam, F.W., and H. Neurath, *J. Biol. Chem. 159*:195 (1945).
23. Polet, H., and J. Steinhardt, *Biochemistry 7*:1348 (1968).
24. Goddard, E.D., and B.A. Pethica, *J. Chem. Soc.* 2659 (1951).
25. Cockbain, E.G., *Trans. Faraday Soc. 49*:104 (1953).
26. Rosenberg, R.M., and I.M. Klotz, *J. Am. Chem. Soc. 77*:2590 (1955).
27. Putnam, F.W., and H. Neurath, *Ibid. 66*:692 (1944).
28. Harris, I.I., K.G.A. Pankhurst and R.C.M. Smith, *Trans. Faraday Soc. 43*:506 (1947).
29. Miyazawa, K., M. Ogawa and T. Mitsui, *Int. J. Cosmet. Sci. 6*:33 (1984).
30. Fraenkel-Conrat, H.L., and M. Cooper, *J. Biol. Chem. 154*:239 (1944).
31. Perlmann, G., *Ibid. 137*:707 (1941).
32. Neurath, H., and F.W. Putnam, *Ibid., 160*:397 (1945).
33. Swaney, J.B., and I.M. Klotz, *Biochemistry 9*:2570 (1970).
34. Nelson, C.A., *J. Biol. Chem. 246*:3895 (1971).
35. Holladay, L.A., and P. Wilder, *Biochim. Biophys. Acta 629*:156 (1980).
36. Faucher, J.A., and E.D. Goddard, *J. Soc. Cosmet. Chem. 29*:323 (1978).
37. Goddard, E.D., and R.B. Hannan, *J. Colloid Interface Sci. 55*:73 (1975).

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