Study on Skin-Irritating and Biological Properties of Monoalkyl Phosphate Anionic Surfactants

G. IMOKAWA, Tochigi Research Laboratories, Kao Soap Co. Ltd., 2606 Akabane, Ichkaimachi Hagagun Tochigi 321-34

ABSTRACT

Monoalkyl phosphate type of low irritating anionic surfactants were investigated with respect to their effect on various biological systems including proteins, enzymes, and lysosomes. Our results indicated that a remarkably weak membrane rupture effect at a concentration less than critical micellar concentration is important for low irritating feature. Mechanism by which irritation is caused by surfactants is discussed.

INTRODUCTION

In previous study (1), we have found that monoalkyl phosphate type of surfactants exhibit adequate surface active properties similar to commercially used anionic surfactants like sodium dodecyl sulfate, but possess a remarkably weak irritating effect on human skin. Many factors, such as protein denaturation (2,3), permeability (4,5), and inflammatory reaction (6) have been suggested which contribute to the skin-irritating effects of surfactants, and are primarily ascribed to their surface active potential, namely surface active properties and critical micellar concentration. Consequently, it has been assumed that surfactants with a high surface active property would also exhibit a marked capacity to irritate the skin. Contrary to such generally accepted notions, monoalkyl phosphate type of surfactants have good surface active power as well as low irritancy. Therefore, an attempt to analyze the causes responsible for low and high irritancy would give significant information on the mechanism by which irritation is caused and may lead to the further development of low irritating surfactants. In the present work, low irritating effect of monoaklyl phosphate type of surfactants in high concentrations is confirmed in an animal study, and its effect on various biological systems such as proteins, enzymes, and lysosomal membranes is analyzed.

MATERIALS AND METHODS

Surfactants

Alkyl sulfate (AS) ($C_nH_{2n+1}OSO_3Na$[C_nAS]), alkyl polyoxyethylene sulfate(ES) ($C_nH_{2n+1}(CH_2CH_2O)_pSO_3Na$[$C_n$-pEO]), alkyl benzene sulfonate (LAS) (C_nH_{2n+1}⬡SO_3Na[C_nLAS]), alfa-olefin sulfonate (AOS) ($C_{n-3}H_{2n-5}CH_2$-CH=$CHSO_3Na$ and $C_{n-3}H_{2n-5}CH(OH)$-$CH_2CH_2SO_3Na$[C_nAOS]), alkyl polyoxyethylene (EO) ($C_nH_{2n+1}O(CH_2CH_2O)_pH$[$C_n$-pEO]), monoalkyl acylglutamate (AGS) ($C_nH_{2n+1}CONHCH(COOH)CH_2CH_2COONa$ [C_nAGS]), alkyl trimethyl ammonium chloride (TAC) ($C_nH_{2n+1}N(CH_3)_3Cl$[C_nTAC]), and monoalkyl phosphate (MAP) ($C_nH_{2n+1}P$=O(OH) (OX), X= Na or Triethanolamine (TEA) [C_nMAP]) used are identical with those reported previously (1,7-10), with respect to alkyl distribution, ratios of the isomer of alkylbenzene sulfonate, and ratios of alkene and hydrox forms in alfa-olefin sulfonate. The list of surfactants is shown in Table I together with their alkyl distribution and purities.

Irritation Test

Twenty-four hour closed patch test on guinea pig skin (11). This method consists of a patch test technique on intact back skin of at least six albino guinea pigs (average weight 100 g) freed of hair 24 hr prior to surfactant application. The test material was introduced in 1.5 cm^2 patch, with 0.1 ml of the aqueous solution having various concentrations. The test animal was scored 2 hr and 24 hr after the patches were removed. The skin reaction was evaluated by adding the scores for erythema and edema, each being assigned values from 0 to 2 to indicate the severity of the reaction. A rating of 0,0.5, 1.0, and 2.0 was assigned to skin showing, nil, slight, moderate, and severe reactions,

TABLE I

The List of Employed Surfactants

Surfactant[a]		Alkyl distribution	Purities[b] (%)
AS	C_8 AS	C_8: 99.8%	97.72
	C_{10} AS	C_{10}: 99.9%, C_{12}: 0.1%	97.17
	C_{12} AS	C_{12}: 100%	100
	C_{14} AS	C_{14}: 99.8%	99.30
ES	C_{12}-3ES	C_{12}: 52.0%, C_{13}: 48.0%	99.8
LAS	C_{12}LAS	C_{12}: 100%	97.88
	LAS	C_{10}: 7.0%, C_{11}: 36.0%, C_{12}: 33.7 C_{13}: 23.4%	99.8
AOS	C_{12}AOS	C_{12}: 99.7%, C_{14}: 0.03%	96.0
	AOS	C_{16}: 57.6%, C_{18}: 40.8%	99.8
TAC	C_{12}TAC	C_{12}: 98.93, C_{14}: 0.06%, C_{16}: 0.8%	94.77
MAP	C_8 MAP	C_8: 100%	97.23
	C_{10}MAP	C_{10}: 100%	99.61
	C_{12}MAP	C_{12}: 100%	99.45
	C_{14}MAP	C_{14}: 100%	100
	C_{16}MAP	C_{16}:100%	99.83
	C_{18}MAP	C_{18}: 100%	98.40
	C_{18}MAP	C_{18}: 100%	97.60
AGS	C_{12}AGS	C_{10}: 2.5%, C_{12}: 95.0%, C_{14}: 2.5%	97.80

[a]AS = alkyl sulfate; LAS = alkyl benzene sulfonate; AOS = alfa-olefin sulfonate; ES = alkyl polyoxyethylene sulfate; TAC = alkyl trimethyl ammonium chloride; MAP: monoalkyl phosphate; AGS = monoalkyl acylglutamate.
[b]Determined by the data on elemental analysis.

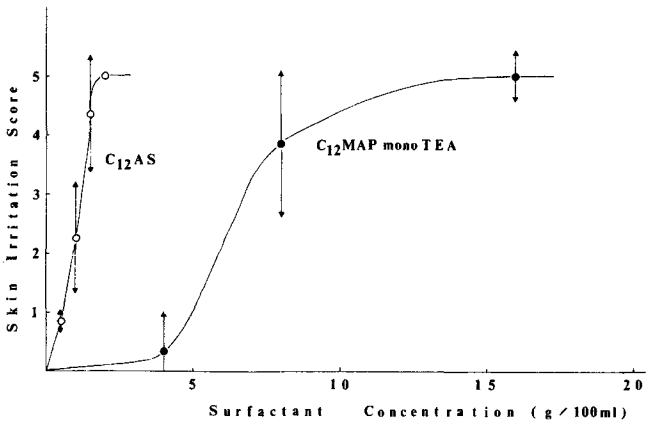

FIG. 1. Comparison of irritations following closed patch test using C_{12}MAP mono TEA and C_{12}AS. MAP monoalkyl phosphate, AS = alkyl sulfate.

respectively, for erythema and for edema. This permits a total score of 4.0. However, when crusts were formed, a score of 5.0 was given.

Cumulative open patch test on guinea pig skin (11). The hair on the back of guinea pig was cut off 7 hr before the initiation of this test. Varying concentrations of surfactant solution in 10 mm diameter tubes were applied for 2 seconds on the skin of guinea pigs. The treated skin was allowed to air dry. These procedures were carried out on the same area twice a day morning and evening, and continued for a total of 9 treatments. The skin reaction was evaluated immediately before every treatment with the same criteria used in the closed patch test.

Measurements of SH Amounts

This measurement was performed according to the method of Harrold (12). One g/100 ml of bovine serum albumin (BSA) and 0.1-1.5 g/100 ml of surfactant solutions were prepared in 0.1 M acetate buffer (pH 5.9) and mixed. To ensure complete interaction, the mixed solution was allowed to stand overnight at 30 C; 0.5 ml of this mixture was added to a centrifuge tube containing 1.0 ml of 0.1 M phosphate buffer (pH 7.0) and 5 ml of isoamyl acetate solution of mercury orange with 1.46 of absorbance at 470 nm and agitated with shaker for 30 min. At the end, the supernatant was separated by centrifuging at 2000 rpm for 15 min, and its absorbance at 470 nm was measured to estimate SH group and compared to the standard curve produced by using glutathione.

Twenty mg of bovine skin powder (BSP) was added into a 10 ml centrifuge tube, and after treating with 10 ml of acetate buffer (0.1 M pH 7.0) containing surfactants of various concentrations (0.1-1.0 g/100 ml), the mixture was agitated with a rotating plastic stirrer for 1.5 hr. Following centrifugation for 10 min at 2000 rpm, the resulting skin powder in the sediment was assayed for amounts of the SH group as described above.

Inhibitory Measurements

Acid phosphatase (AcPase) was assayed according to the method of Shibko (13) at pH 6.0 or 5.0 at 37 C for 20 min incubation using p-nitrophenyl phosphate as the substrate. Prior to enzymic assay, 1.0 ml of enzyme solution (1 mg/10 ml in 0.1 M acetate buffer) was incubated for 1 hr at 37 C with 1.0 ml of various concentration of surfactants solubilized in 0.1 M acetate buffer. Inhibition percent was represented as a ratio of the initial rates of released p-nitrophenol observed between surfactant-treated and non-treated enzymes.

Lysosome Labilizing Effect

Guinea pig epidermis was removed according to the stretching method (14). They were immediately chilled in ice cold 0.25 M sucrose solution and then homogenized for 1 min in Ultra Turrax homogenizer. After filtration through one or two layers of cheesecloth to remove gross tissue fragments, the homogenate was treated with Teflon homogenizer chilled with ice. The suspension obtained was first centrifuged at 700 g for 10 min to remove unruptured cells and nuclear debris. The residue was discarded. The supernatant was further centrifuged at 12000 g for 10 min to obtain a lysosome-containing fraction. The supernatant was discarded. The residue was resuspended in cold 0.25 M sucrose solution to adjust the suspension to 5-6 mg protein/ml concentration.

One ml of lysosome preparation was incubated at 37 C for 1 hr with 1.0 ml of surfactant solutions (pH 7.0) or sucrose solution only for control. After incubation, this mixture was centrifuged at 12000 g for 10 min, and to evaluate the labilizing effect, the activity of lysosomal enzymes released into the supernatant was estimated.

Enzymic Decompositability of MAP by Homogenates from L-Cell and Guinea Pig Epidermis

L-cells grown in Eagles' MEM medium supplemented with 10% new born calf serum were collected through the Petri dish by scrubbing, and after adding 0.25 M sucrose solution, homogenized with Teflon homogenizer. This homogenate was adjusted to ca. 5 mg protein/ml concentration by diluting with 0.25 M sucrose and then incubated at

TABLE II

Comparison of Irritancy of MAP Monosodium Salt with Various Kind of Surfactants at 2.0 g/100 ml

	24 hr Closed patch test 2 hr	24 hr	Cumulative open patch test
C_8 MAP monosodium salt	0	(0)	0
C_{10} MAP monosodium salt	0	(0.17)	0.33
C_{12} MAP monosodium salt	0	(0)	0.08
C_{14} MAP monosodium salt	0	(0)	0
C_{16} MAP monosodium salt	0	(0.17)	0
C_{18} MAP monosodium salt	0.08	(0.08)	0
C_{12} AGS	0.25	(0.17)	0.42
C_{12}-14EO	0.33	(0.33)	0.08
C_{12} ES	1.33	(1.00)	1.25
C_{12} AS	4.67	(4.67)	3.00
LAS	2.75	(2.75)	0.58
AOS	1.00	(0.67)	0.42
C_{12} LAS	3.00	(3.00)	1.42
C_{12} AOS	2.67	(2.50)	1.67
C_{12} TAC	3.08	(4.00)	4.00

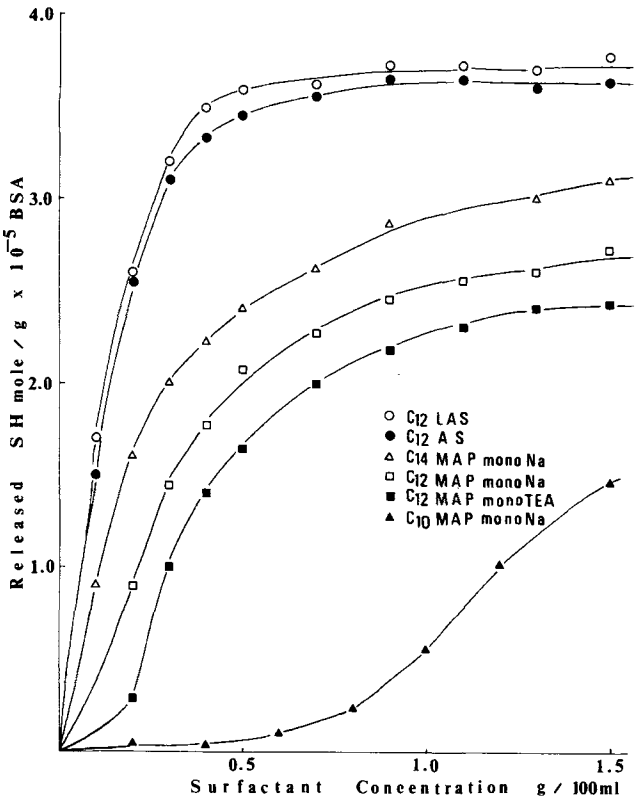

FIG. 2. Liberation of sulfhydryl groups from BSA following incubation with MAP and other surfactants. MAP = monoalkyl phosphate.

37 C with 5 mM C_{12} MAP mono TEA salts in three different buffers having a pH of 5.0, 6.8, and 8.5. In the case of epidermal homogenate, they were incubated in protein concentration of ca. 2 mg/ml with 5 mM of the MAP at pH of 5.0. Decompositiability of the MAP was estimated by measuring the released Pi according to the method of Chen (15).

RESULTS

Skin-Irritating Properties

Figure 1. shows irritancy of C_{12}MAP mono TEA salt, a typical type of MAP, as a function of the concentration in comparison with C_{12}AS. It is clear that elicitation of the irritation by this compound requires a concentration of more than 4 g/100 ml, whereas C_{12} AS produces an irritating response even at 0.05 g/100 ml. After occlusive application of 2.0g/100 ml of C_{12} AS, a marked irritation accompanyed by crusta was observed, whereas C_{12} MAP produced a similar effect at 16.0 g/ 100 ml. These findings indicate that this compound is at least 10 times less irritating than C_{12} AS.

In Table II, irritancy of MAP mono sodium salts with various alkyl chain lengths is compared with that of a variety of common anionic surfactants at the concentration of 2.0 g/100 ml. In closed patch test, all MAPs elicit a slight irritating response on guinea pig skin, while popularly known anionic surfactants elicit higher degrees of irritating reactions, the highest being C_{12}AS. Thus, the closed patch test carried out using guinea pig skin has revealed that this type of surfactant has a significantly weak irritating property as compared to other surfactants including even C_{12}-3ES and AOS, which are noted to be relatively less irritating. Comparison of cumulative irritancy by a homologenous series of MAP mono sodium salts indicates that although C_{10}-compound invokes a slightly weak erythematous reaction after nine applications, all other MAPs exhibit no irritating effect. In contrast, C_{12}AS and C_{12}-3ES show relatively marked cumulative irritation, and irritancy of C_{12}AGS, known to be relatively less irritating, is also significantly higher than that of MAP.

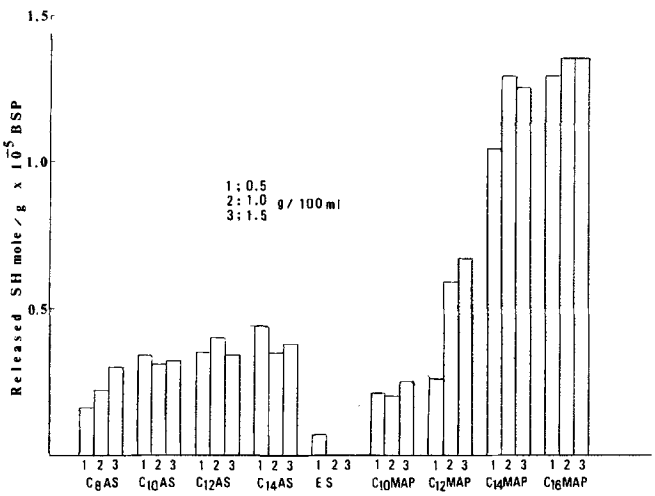

FIG. 3. Liberation of sulfhydryl groups from BSP following incubation for 1.5 hr with MAP and AS. BSP = bovine skin powder; MAP = monoalkyl phosphate; AS = alkyl sulfate.

Protein Denaturing Ability

Figure 2 shows the released amounts of the SH group from bovine serum albumin as a function of the MAP concentrations. All MAPs other than C_{10}-compound have a similar pattern of released SH amounts – concentration curve to that of C_{12}AS and C_{12}LAS, although their releasing effect is less than that of C_{12}AS and C_{12}LAS.

In the previous study (16), it was established, using bovine skin powder and human callus, that surfactants release the SH groups in comparable manner in both. Therefore, in order to clarify the effect of MAP on human horny layers, its SH group-releasing ability was measured for bovine skin powder (Fig. 3). From Figure 3, it is apparent that like common anionic surfactants, these compounds also show increased releases of the SH groups with increasing alkyl chain length. The magnitude is significantly higher in C_{12}, C_{14} and C_{16} MAP than in C_{12} and C_{14}AS. Based on the protein-denaturing effects observed for bovine serum albumin and bovine skin powder, it is suggested that in general these compounds have a strong capacity to denature

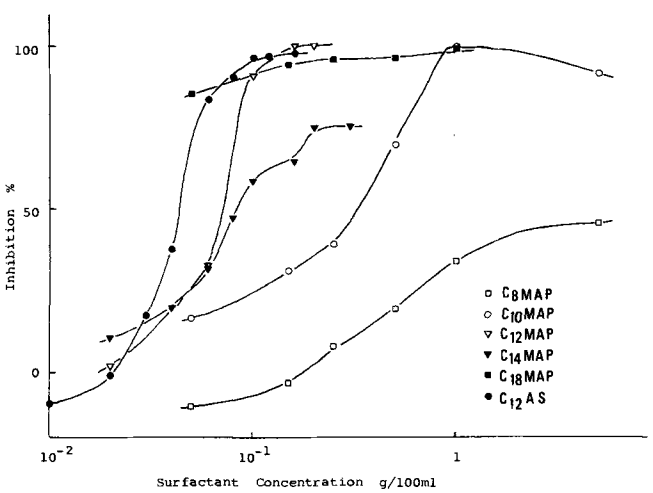

FIG. 4. Inhibitory effect of MAP on acid phosphatase as a function of the concentration. MAP = monoalkyl phosphate.

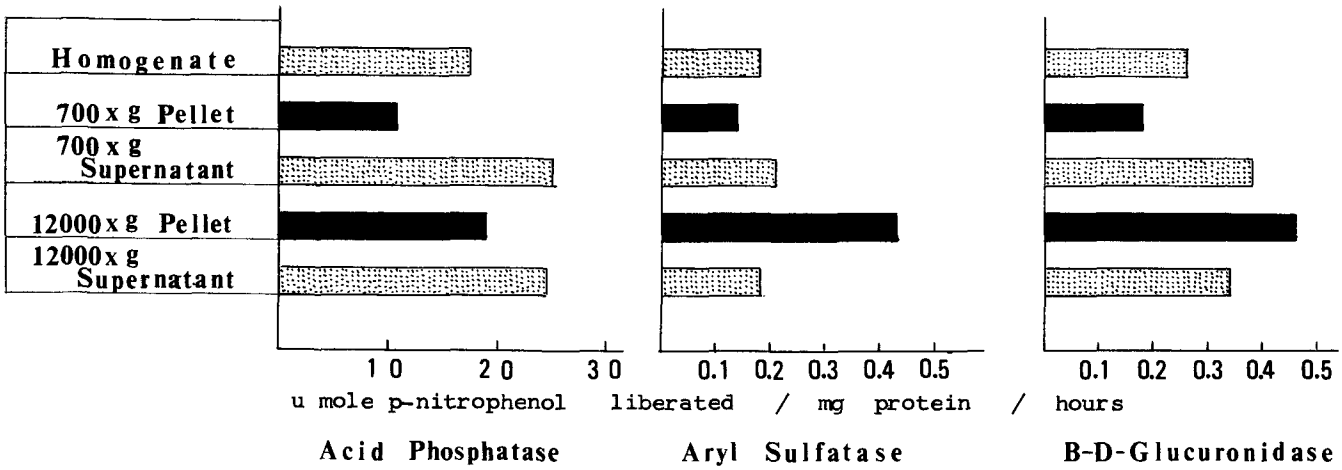

FIG. 5. Distribution of acid phosphatase, aryl sulfatase and β-glucuronidase in various epidermal fractions of guinea pig skin.

proteins.

Enzyme Inhibitory Ability

In previous study (16), we have reported an inhibitory effect of various common anionic surfactants with different alkyl chain lengths on acid phosphatase, one of lysosomal enzymes, and suggested that although inhibition and irritation is not related, surfactants with a certain level of irritating effect seem to have a marked inhibitory effect on acid phosphatase. Therefore, evaluation of the inhibitory effect by MAP type of surfactants is also required for the clarification of the causative factors responsible for low irritation. Figure 4, exhibiting % inhibition plotted against the additive surfactant concentrations, indicates that C_{18F} and C_{12}MAP have a similar inhibitory ability to C_{12}AS, whereas the effect of C_{14}-, C_{10} and C_8 MAP monosodium salts is at significantly lower level. Although the increased inhibitory potential appears to be in the order of C_8-, C_{10}, C_{14}-, C_{18F}- and C_{12}MAPs, consideration of the limited solubility of C_{14} MAP leads us to suggest that inhibitory effect corresponds with the alkyl chain length, a behavior which is in agreement with those (16) seen for a homologenous series of the other anionic surfactants such as AS, LAS, and AOS. These findings indicate that in general these compounds possess almost the same inhibitory potential on acid phosphatase as common anionic surfactants except soap, indicating a similar order to protein-denaturing effect described in SH release experiments.

Epidermal Lysosome Labilizing Ability

The distribution of acid phosphatase, β-glucuronidase and aryl sulfatase among the nuclear, lysosome and supernatant fractions are shown in Figure 5. The highest activity of the latter two enzymes is found to be present in the lysosome fraction obtained after centrifugation at 12000 g for 10 min. Comparison of enzymic activity within epidermal homogenate indicates that acid phosphatase is highest with a magnitude of 100 times more than that of aryl sulfatase and β-glucuronidase.

Table III shows a labilizing effect of MAP on the lysosome fraction compared to C_{12}AS and C_{12}-12EO. With C_{12}AS, a release of acid phosphatase beyond control level was first found at 5 x 10^{-5} g/ml concentration. However, when a concentration of 5 x 10^{-4} g/ml was employed, less acid phosphatase activity than that of control was observed, probably due to its inhibitory action. In the case of C_{12}-14EO, while the level of acid phosphatase activity released in the supernatant fraction does not differ from

TABLE III

Labilizing Effect of MAP on Epidermal Lysosome-Rich Fraction of Guinea Pig in Comparison with C_{12}AS and C_{12}-14EO

		% Release of acid phosphatase into the supernatant				
	g/ml	1	2	3	4	Mean ± SD
C_{12} AS	5 x 10^{-6}		---	101.85	---	101.85
	5 x 10^{-5}		126.27	142.02	131.1	133.13 ± 8.07
	5 x 10^{-4}		65.03	65.73	64.97	65.24 ± 0.42
	5 x 10^{-3}		---	---	0.57	0.57
C_{12} MAP	5 x 10^{-6}	102.14	---	101.24	---	101.69
	5 x 10^{-5}	95.62	97.14	100.28	100.00	98.26 ± 2.26
	5 x 10^{-4}	74.18	66.34	83.43	72.29	74.06 ± 7.08
	5 x 10^{-3}	---	---	---	0	0
C_{12}-14EO	5 x 10^{-6}		---	107.81	---	107.81
	5 x 10^{-5}		113.11	116.74	96.34	108.73 ± 10.88
	5 x 10^{-4}		118.53	161.35	176.01	151.96 ± 29.87
	5 x 10^{-3}		---	---	---	---
C_{14} MAP	5 x 10^{-6}	96.29				96.29
	5 x 10^{-5}	104.38				104.38
	5 x 10^{-4}	56.31				56.31
	5 x 10^{-3}	---				---
C_{10} MAP	5 x 10^{-6}	105.64				105.64
	5 x 10^{-5}	108.19				108.19
	5 x 10^{-4}	145.85				145.85
	5 x 10^{-3}	---				---

control at the concentration of 5 x 10^{-5} g/ml, release of acid phosphatase beyond control level occurs at 5 x 10^{-4} g/ml. The labilizing data for various MAPs indicate that the induction of labilization requires a surfactant concentration of more than 5 x 10^{-4} g/ml. When C_{12}- and C_{14} MAP are employed, a decrease in acid phosphatase activity was observed at 5 x 10^{-4} g/ml due to its inhibitory effect. These different concentrations required for the initiation of lysosome labilization suggest that the labilizing ability of MAP is similar to C_{12}-14EO and about 10 times as a potent as C_{12}AS.

Decomposition of MAP by L-Cell and Guinea Pig Epidermis Homogenates

Figure 6 shows decomposition characteristics of MAP by L-cell homogenate as revealed by released Pi. The decomposition curve demonstrates that this compound has a tendency to be easily degraded, especially at pH 8.5 and 5.8, suggesting that alkali or acid phosphatase-like enzymes can act on MAP molecules. Figure 7 also shows enzymic degradation by guinea pig epidermis homogenate.

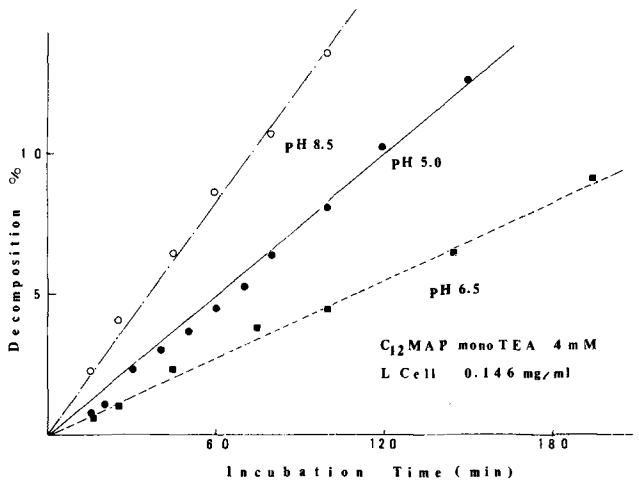

FIG. 6. Enzymic degradation of MAP in L-cell homogenate. MAP = monoalkyl phosphate.

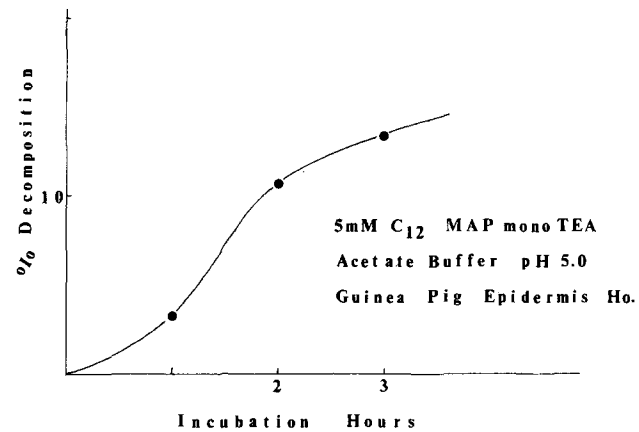

FIG. 7. Enzymic degradation of MAP in epidermal homogenate of guinea pig skin. MAP = monoalkyl phosphate.

DISCUSSION

Results of closed patch and cumulative open patch tests using guinea pig skin have revealed that even under exaggerated conditions, the irritancy of MAP type of surfactants is lower than other variety of common anionic surfactants, the magnitude being 4 to 10 times as mild as C_{12}AS.

In the case of most ionic surfactants excepting soap, it is accepted that at a concentration less than the cmc, keratin protein denaturation, which in turn probably leads to the breakdown of the barrier function of horny layers, is not produced when applied on the skin, and in addition to this, the binding ability of surfactant molecules with keratin protein may result in the abolishment of their penetration through horny layers. This view suggests that high surfactant concentrations to induce keratin protein denaturation are required for the elicitation of irritation. In this connection, Scala et al (4) demonstrated that penetration of ionic surfactants, such as LAS and TAC, through the epidermis does not show a linearity with the time of application, but is markedly enhanced, whereas nicotinate usually has a linear penetration curve. They suggested that this increased penetration is due to the decrease of epidermal permeability barrier function of horny layers resulting from keratin denaturation induced by these surfactants. In addition to protein denaturation, it is conceivable that to some degree inflammatory effects as represented by vasodilatation are also associated with damaging of cell membranes including lysosome rupture, a process in which inflammatory chemical mediators are released.

In order to analyze the low irritating effect of MAP, the above mentioned factors, namely protein denaturation and lysosome rupture, were investigated. Thus, SH-releasing ability, enzyme inhibition, and lysosome labilization were studied using bovine serum albumin (BSA), bovine skin powder, acid phosphatase, and epidermal lysosome fraction from guinea pig skin.

As expected from its good surface active properties, MAP was found to elicit relatively potent denaturing action on bovine serum albumin and bovine skin powder, with a magnitude similar to C_{12}AS for bovine serum albumin and in the case of bovine skin powder, greater than that. Comparison of these findings with those (16) of other varieties of anionic surfactants suggests that protein-denaturing potential of MAP is relatively high even in comparison with common anionic surfactants such as AOS and paraffin sulfonate. Investigation for enzyme effects also discloses a marked inhibition of acid phosphatase activity, especially by C_{12}- and C_{18F}MAP mono sodium salts, suggesting denaturing effect similar to bovine serum albumin and bovine skin powder. Thus, our comparative study on various kinds of proteins has revealed that MAP has more or less the same denaturing potential as other typical anionic surfactants including C_{12}AS, LAS, AOS and ES.

As mentioned above, the protein-denaturing property of surfactants is believed to be related to their skin-irritating effect. But it appears that low irritating effect of MAP is not due to this property, as protein denaturation does occur with bovine serum albumin, bovine skin powder, and acid phosphatase.

Results of lysosome labilization experiments indicated significant difference between C_{12} AS and MAP, while C_{12}AS, the most potent irritant, yielded a greater release of acid phosphatase at a concentration of 5 x 10^{-5} g/ml, in the case of MAP there was no induction of labilization at the concentration ranging from 5 x 10^{-6} to 5 x 10^{-5} g/ml. With C_{12}-14EO, a lesser irritant, the labilization is first invoked at 5 x 10^{-4} g/ml. Since increased concentrations were required for the onset of labilization, it is concluded that MAP has a labilizing feature in common with nonionic surfactants such as C_{12}-14EO.

Since MAP is a phosphate derivative, precise evaluation of its lysosome-labilizing effect must take into consideration the possibility of phosphatase-mediated decomposition before it can initiate labilization. The results using both L-cell and guinea pig epidermis homogenates indicate that only 2.8-8.6% of MAP is decomposed during 1 hr in-

cubation. Therefore, in experiments for labilization effect which were completed for 1 hr, their enzymic decomposition of MAP is insignificant, but in skin application where a long time is required for initiation of irritation, these factors are of considerable import.

It is possible that when surfactants penetrate the epidermis, actual contact concentration of surfactants with native epidermal cells is relatively low. At low concentrations less than cmc, there is a significant difference in the biological membrane-disrupting effect between MAP and other common anionic surfactants. This fact seems to account for the low irritating effect of MAP. Furthermore, the fact that in epidermal homogenates there is a 10-fold higher acid phosphatase activity than that of aryl sulfatase suggests that even if MAP can penetrate horny layers, it may be more easily decomposed than alkyl sulfate, probably leading to relatively low irritation.

ACKNOWLEDGMENTS

Dr. Nagase encouraged this work and permitted publication of this paper.

REFERENCES

1. Imokawa, G., H. Tsutsumi, and Y. Kurosaki, JAOCS 55:839 (1978).
2. Blohm, S., Acta. Derm. Venereo. 37:269 (1957).
3. Lansdown, A.B.G., and P. Grasso, Br. J. Dermatol. 86:361 (1972).
4. Scala, J., D.E. Mcosker, and H.H. Reller, J. Invest. Dermatol. 50:371 (1968).
5. Howes, D., J. Soc. Cosmet. Chem. 26:47 (1975).
6. Mezei, M., J. Invest. Dermatol. 54:510 (1970).
7. Imokawa, G., K. Sumura and M. Katsumi, JAOCS 52:479 (1975).
8. Imokawa, G., K. Sumura and M. Katsumi, Ibid. 52:490 (1975).
9. Imokawa, G., and Y. Mishima, Jpn. J. Dermatol. 86:473 (1976).
10. Imokawa, G., K. Sumura, and M. Katsumi, J. Jpn. Oil Chem. Soc. 23:719 (1974).
11. Brown, V.K.H., J. Soc. Cosmet. Chem. 22:411 (1971).
12. Harrold, S.P., J. Invest. Dermatol. 32:581 (1959).
13. Shibko, S., and A.L. Tappel, Biochem. J. 95:731 (1965).
14. Tabachnick, J., J. Invest. Dermatol. 32:56 (1959).
15. Chen, P.S., Jr., T.Y. Toribara and H. Warner, Anal. Chem. 28:1756 (1956).
16. Imokawa, G., and M. Katsumi, J. Jpn. Oil Chem. Soc. 25:24 (1976).

[Received August 28, 1978]