Vol 25 No 9 September 1974

Journal of the Society of Cosmetic Chemists

Contents

Page

ORIGINAL PAPERS

The simultaneous determination of cysteinyl and S-sulfocysteinyl residues in keratin
Phillip E. Sokol, Francis H. Girard, D. Y. Hsiung, and Carolyn Pictor 461

Testing antiacne agents in Mexican hairless dogs
J. J. Loux, P. D. DePalma, and S. L. Yankell 473

A study on the differential thresholds of sensory "firmness" and "viscousness" of cream base substances
Keiji Morosawa, Chiyoko Ohtake, Motoji Takahashi, Takeo Mitsui, and Seiichi Ishikawa 481

Kinetics of degradation of the parabens
Seymour M. Blaug and Donald E. Grant 495

GENERAL PAPER

NMR—A new instrumental tool for the analysis of cosmetic ingredients
Richard Kaplan and Stephen F. Laczynski 507

DEPARTMENTS

Synopses for card indexes xvii
Letter to the editor 515
Book reviews 517
Index to advertisers xxxiv

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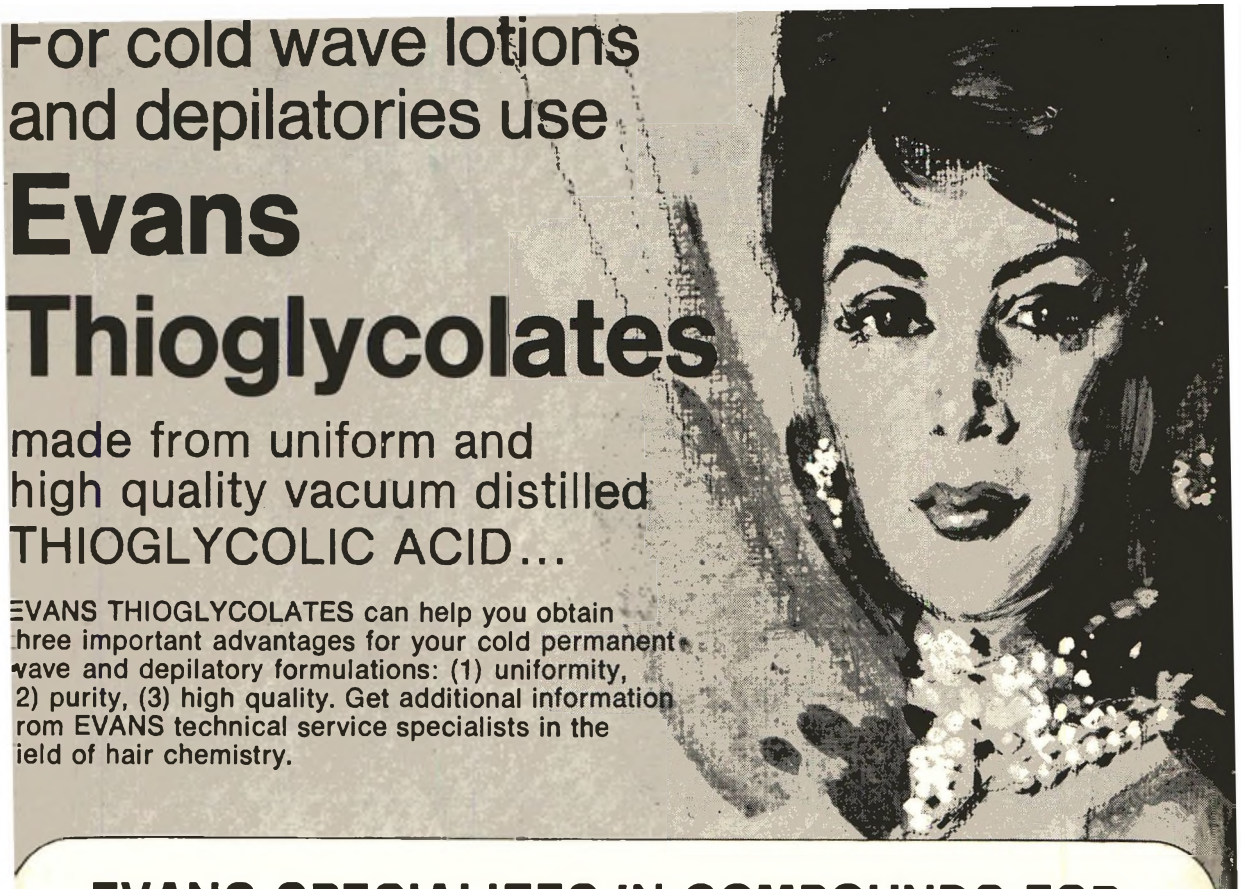

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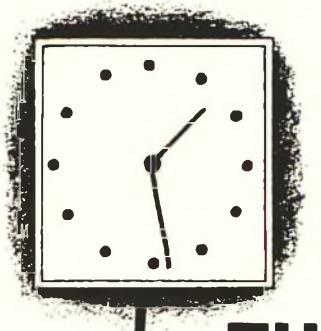

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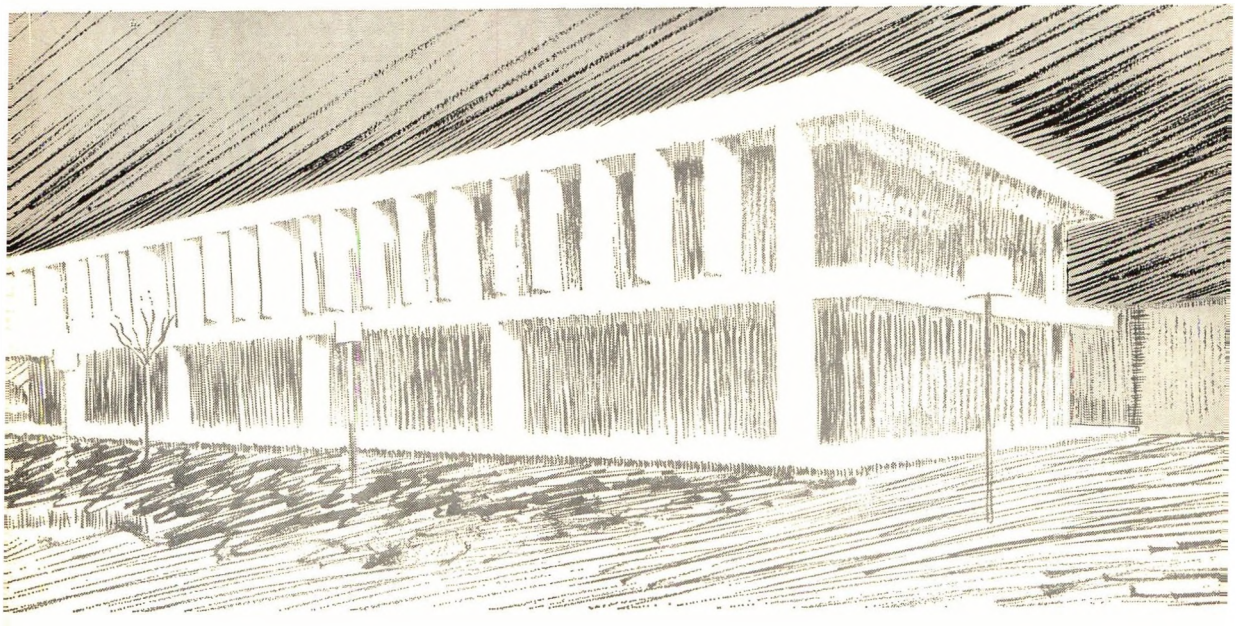

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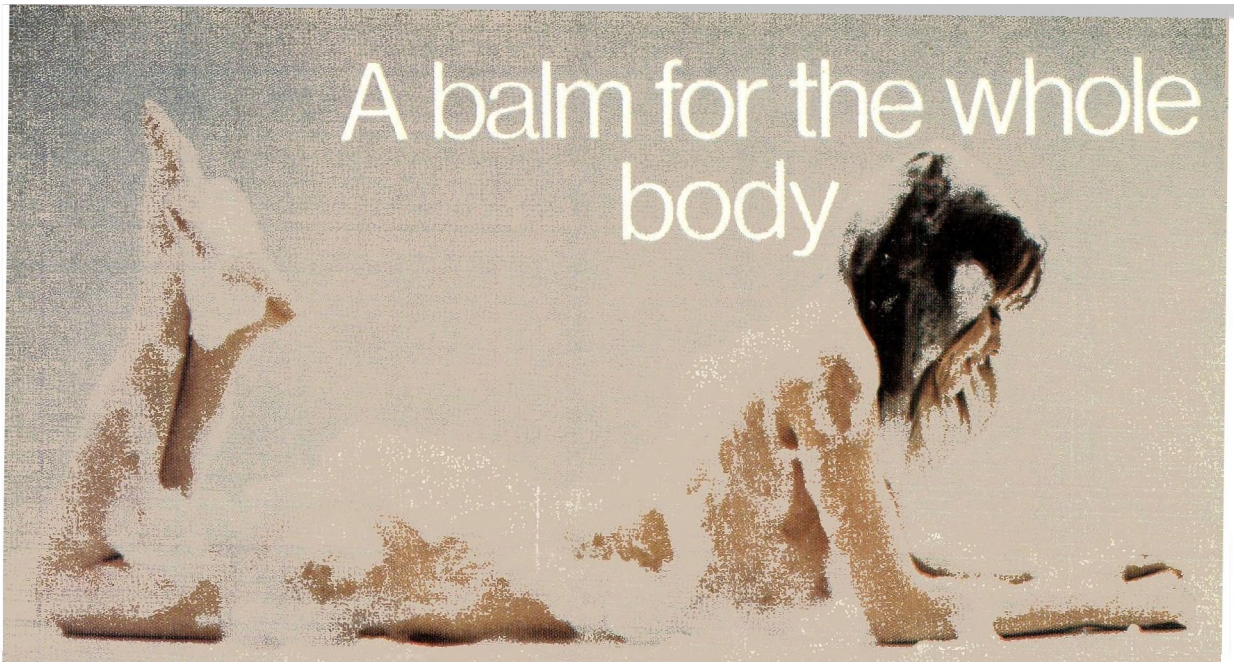

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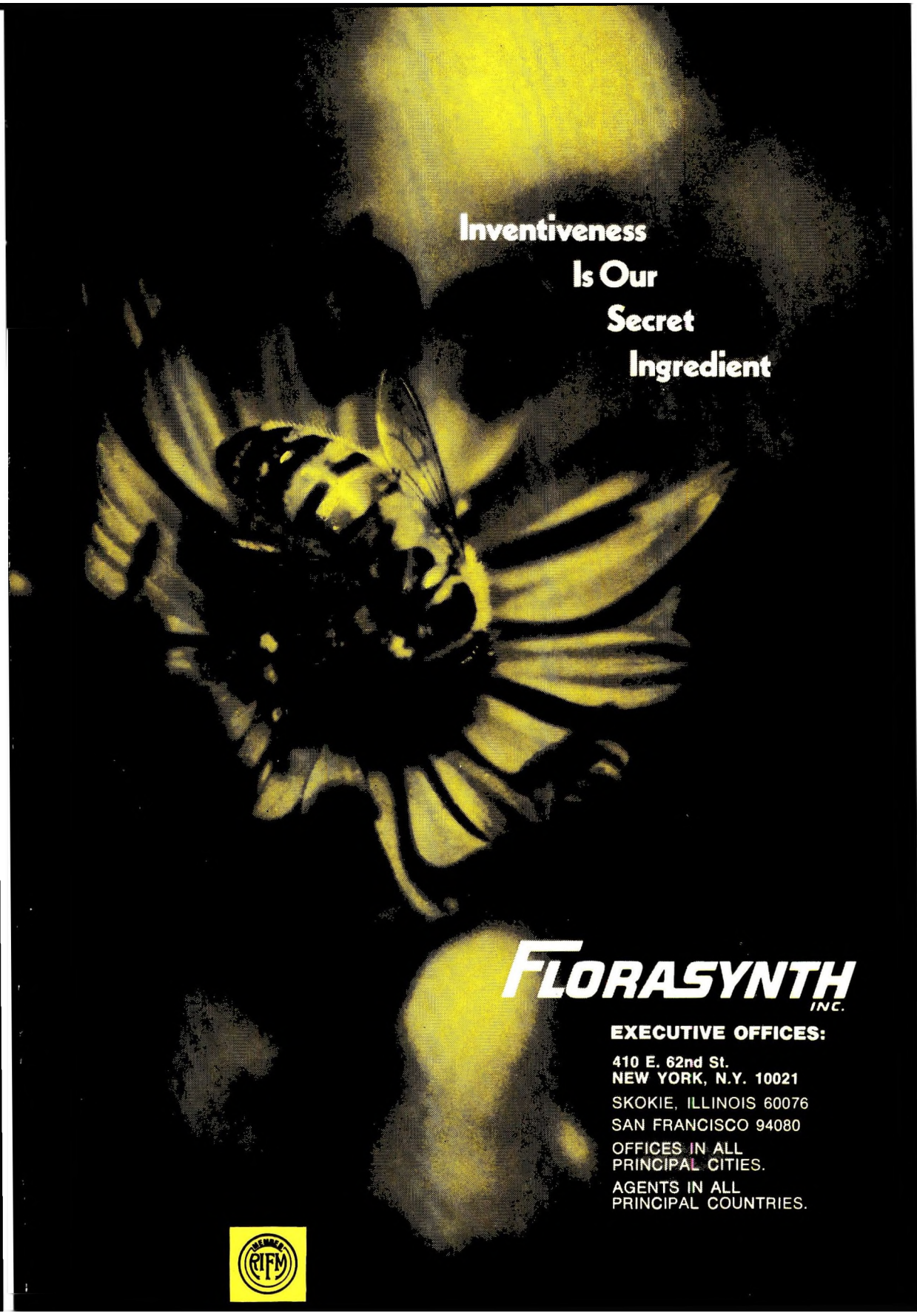
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Lead	20 ppm max.	20 ppm max.	20 ppm max.	20 ppm max.
Arsenic	3 ppm max.	3 ppm max.	3 ppm max.	3 ppm max.
Microbial analysis	Total count: 100 colonies per gram maximum Pathogens: negative	Same	Same	Same

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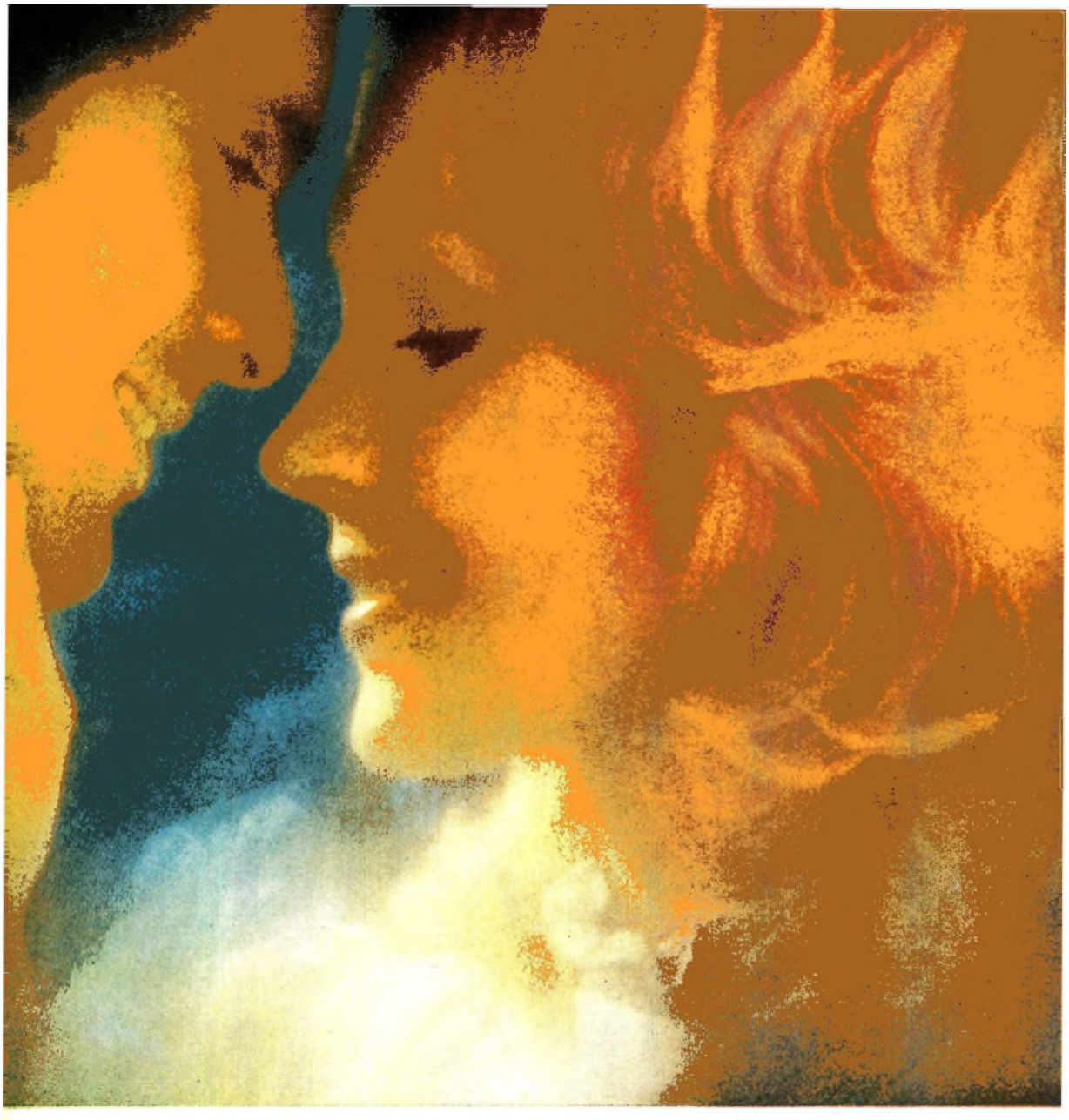

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SYNOPSES FOR CARD INDEXES

The following synopses can be cut out and mounted on 3 x 5 in. index cards for reference, without mutilating the pages of the Journal.

The simultaneous determination of cysteinyl and S-sulfocysteinyl residues in keratin: Phillip E. Sokol, Francis H. Girard, D. Y. Hsiung, and Carolyn Pictor. *Journal of the Society of Cosmetic Chemists* **25,** 461 (September 1974)

Synopsis—An analytical method is described for the simultaneous determination of S-sulfocysteinyl ($CySSO_3^-$) and crysteinyl (CySH) residues in keratin. This new method, a modification of Valk and Gerthsen's procedure, consists of the salyrganic acid mercurial titration of acid hydrolysates of treated keratin in which CySH residues are differentiated from $CySSO_3^-$ residues by a simple, but controlled, blocking of the CySH with acrylonitrile prior to keratin hydrolysis.

Publication of this method was, in part, stimulated by Valk and Gerthsen's report that the CySH/$CySSO_3^-$ ratio in sulfite-treated keratin is dependent upon the pH of the treatment medium, deviating largely from 1.0 outside the pH range of 3–6. The method reported here indicates that the stoichiometry of the reaction Ker-CySSCy-Ker + HSO_3^- $\leftrightharpoons$ Ker-CySH + Ker-$CySSO_3^-$ is obeyed at all pH's examined (3.5–8.5). The method is precise, accurate, and rapid. In addition, the method is valid for keratin samples containing a wide range of CySH/$CySSO_3^-$ ratios as well as samples containing one of these groups exclusively.

Testing antiacne agents in Mexican hairless dogs: J. J. Loux, P. D. DePalma, and S. L. Yankell. *Journal of the Society of Cosmetic Chemists* **25,** 473 (September 1974)

Synopsis—The plugged follicles on the backs and flanks of Mexican hairless dogs share clinical and histologic similarities to the comedones seen in man. Soap treatments cleaned follicle-containing areas but did not remove the follicular plugs. Salicylic acid preparations were similarly ineffective. The use of benzoyl peroxide produced slight extrusion of the follicular keratin plus mild irritation, and vitamin A acid was markedly effective with associated dermal irritation.

A study on the differential thresholds of sensory "firmness" and "viscousness" of cream base substances: Keiji Morosawa, Chiyoko Ohtake, Motoji Takahashi, Takeo Mitsui, and Seiichi Ishikawa. *Journal of the Society of Cosmetic Chemists* **25,** 481 (September 1974)

Synopsis—The differential thresholds of sensory "firmness" and "viscousness" were studied. It was noted that these properties changed continuously depending upon changes in hardness and viscosity which were measured instrumentally. The minimum values of differential thresholds of both firmness and viscousness were found to be about 10% at 80% level of confidence.

Kinetics of degradation of the parabens: Seymour M. Blaug and Donald E. Grant. *Journal of the Society of Cosmetic Chemists* **25,** 495 (September 1974)

Synopsis—The effect of pH and temperature on the hydrolysis of methyl, ethyl, propyl, and *n*-butyl paraben was studied at 70°C in 0.1*M* phosphate buffer solutions at ionic strength 0.3 from pH 2.75 to 9.16 and at 40° and 50°C at pH 9.16. The reaction was first order with respect to paraben. Energies of activation were determined from Arrhenius plots. Rate constants and half-lives of each paraben at 25°C were obtained by extrapolation of the Arrhenius plots.

The half-life of each paraben at 70°C and pH 8.24 was essentially independent of the initial concentration of paraben. Increasing ionic strength resulted in a slight increase in the rate of hydrolysis of each paraben. Increasing the phosphate concentration in buffer solutions at pH 8.24 at 70°C produced an increase in the rate of hydrolysis of each paraben. This indicated that the parabens undergo general base catalysis and that hydroxyl ion is not the only species that can catalyze their hydrolysis.

NMR—A new instrumental tool for the analysis of cosmetic ingredients: Richard Kaplan and Stephen F. Laczynski. *Journal of the Society of Cosmetic Chemists* **25,** 507 (September 1974)

Synopsis—A basic introduction into the theory and quality control applications of a 60-Megahertz NMR Spectrophotometer is given. Quantitative methods for evaluation of iodine number, ester value, hydroxyl number, and moles of ethoxylation on cosmetic raw ingredients are presented. Determination of alcohol-water ratios on finished ingredients are shown to comply with a time-consuming distillation method. Determinates affecting accuracy and precision in compliance with classical wet chemistry tests for the above are also discussed.

J. Soc. Cosmet. Chem., 25, 461–472 (September 1974)

The Simultaneous Determination of Cysteinyl and *S*-Sulfocysteinyl Residues in Keratin

PHILLIP E. SOKOL, Ph.D.,* FRANCIS H. GIRARD, Ph.D.,†
D. Y. HSIUNG, Ph.D.,† and CAROLYN PICTOR, B.S.†

Presented October 9, 1973, Joint Symposium of the Society of Cosmetic Chemists and the Association of Official Analytical Chemists, Washington, D.C

Synopsis—An analytical method is described for the simultaneous determination of S–SULFOCYSTEINYL ($CySSO_3^-$) and CYSTEINYL (CySH) residues in KERATIN. This new method, a modification of Valk and Gerthsen's procedure, consists of the SALYRGANIC ACID MERCURIAL TITRATION of acid hydrolysates of treated keratin in which CySH residues are differentiated from $CySSO_3^-$ residues by a simple, but controlled, blocking of the CySH with acrylonitrile prior to keratin HYDROLYSIS.

Publication of this method was, in part, stimu'ated by Valk and Gerthsen's report that the $CySH/CySSO_3^-$ ratio in sulfite-treated keratin is dependent upon the pH of the treatment medium, deviating largely from 1.0 outside the pH range 3–6. The method reported here indicates that the stoichiometry of the reaction Ker-CySSCy-Ker + HSO_3^- ⇌ Ker-CySH + Ker-$CySSO_3^-$ is obeyed at all pH's examined (3.5–8.5). The method is precise, accurate, and rapid. In addition, the method is valid for keratin samples containing a wide range of $CySH/CySSO_3^-$ ratios as well as samples containing one of these groups exclusively.

Introduction

For a number of years we have been interested in the chemistry of the keratin-bisulfite reaction. The reaction, Ker-CySSCy-Ker + HSO_3^- ⇋ Ker-CySH + Ker-$CySSO_3^-$,‡ is the basis of several commercial products. We were interested in determining the exact extent of this reaction as well as

* Gillette Research Institute, Rockville, Md. 20850.
† Gillette Co. Personal Care Division, Boston, Mass. 02106.
‡ Ker- represents the noncysteinyl and noncystinyl containing portions of the keratin protein.

in determining the relative amounts of cysteinyl (CySH) and S-sulfocysteinyl ($CySSO_3^-$) residues produced in the reaction since, under certain reaction conditions, one might not be expected to obtain equal amounts of each group. The objective of this work was, therefore, to develop an analytical method which quantitatively determines the amounts of $CySSO_3^-$ Bunte salt and CySH thiol groups in chemically modified keratin.

The analytical determination of these residues presents several difficult problems. For example, the method of Elsworth and Phillips (1), which measures the sulfur dioxide evolution after treatment of the modified keratin sample with acid, is very laborious and requires replicate samples which contain identical amounts of sorbed bisulfite. Further, the procedure determines only $CySSO_3^-$ residues and cannot be used to determine CySH residues. The iodoacetamide procedure (2) and polarographic methods (3) are not useful for the determination of both $CySSO_3^-$ and CySH residues because at the high pH's needed, reversal of the CySSCy + HSO_3^- reaction occurs rapidly and leads to false results.

To overcome these difficulties, Valk and Gerthsen (4) expanded the mercurial titration procedure for CySH residues to determine $CySSO_3^-$ residues also according to the following scheme:

$$\text{Ker-CySSCy-Ker} \xrightarrow{HSO_3^-} a\ \text{Ker-CySH} + b\ \text{Ker-CySSO}_3^- \qquad (1)$$

$$a\ \text{Ker-CySH} + b\ \text{Ker-CySSO}_3^- \xrightarrow[\Delta]{H_2SO_4} (a+b)\ \text{CySH} + b\ SO_4^= \qquad (2)$$

$$a\ \text{Ker-CySH} + b\ \text{Ker-CySSO}_3^- \xrightarrow[(OH^-)]{ICH_2CO_2H} \xrightarrow[\Delta]{H_2SO_4} a\ \text{CySCH}_2\text{CO}_2\text{H} + b\ \text{CySH} + b\ SO_4^= \qquad (3)$$

From eq 2 the combined CySH and $CySSO_3^-$ contents are determined. From eq 3 the CySH residue is blocked with iodoacetate and, therefore, the CySH content resulting from the acid hydrolysis of the $CySSO_3^-$ groups is determined. Using this procedure, Valk and Gerthsen found $CySSO_3^-$/CySH ratios of unity for wool reduced with bisulfite over the pH range from 3 to 6. Outside of this pH range, the ratios were significantly different from unity which, they suggested, was due to a change in the keratin-bisulfite reaction mechanism. We had reason to doubt this was the case and were able to show the ratio to be unity at all pH's studied—from 3.5 to 8.5. We also had need for an independent determination of CySH and $CySSO_3^-$ residues in keratin fibers for product development studies.

The procedure developed for this determination consists of the mercurial titration of acid hydrolysates of chemically treated keratin in which CySH residues are differentiated from $CySSO_3^-$ residues by a simple, but con-

trolled, blocking of the Ker-CySH prior to keratin hydrolysis. Nitroprusside is used as the indicator in the reaction. This mercurial-nitroprusside titration procedure is abbreviated as MNP

Derivation of the Method

The new method reported here is essentially a variation of that of Valk and Gerthsen but with some changes introduced to avoid losing $CySSO_3^-$ residues when the pH is raised in an uncontrolled manner. Additionally, the alkylation of CySH residues is carried out in a more quantitative fashion. For this new method, three separate subsamples are required for the simultaneous determination of CySH and $CySSO_3^-$ content.

Subsample 1 is hydrolyzed in acid and the cysteinyl content is determined by mercurial titration. This step, which is identical to Valk and Gerthsen's initial step, yields the MNP_1 value and is a measure of the total cleavage level of the keratin sample assuming that the CySSCy is cleaved in a nucleophilic process (e.g., by bisulfite, thioglycolate, etc).

$$x\,\text{Ker-CySH} + y\,\text{Ker-CySSO}_3^- \xrightarrow[\Delta]{6N\ H_2SO_4} (x+y)\,\text{CySH}$$

Subsample 2 is extensively water-rinsed and treated with alkali to reverse the cystine-bisulfite reaction in a controlled manner. The sample is hydrolyzed in acid and the results obtained are expressed as the MNP_2 value.

$$x\,\text{Ker-CySH} + y\,\text{Ker-CySSO}_3^- \xrightarrow{(OH^-)}$$

$$z\,\text{Ker-CySSCy-Ker} + (x-z)\ \text{Ker-CySH} + (y-z)\ \text{Ker-CySSO}_3^-$$

$$\xrightarrow[\Delta]{6N\ H_2SO_4} (x+y-2z)\,\text{CySH}$$

Clearly, one-half of the value of MNP_1 minus MNP_2 is a measure of the amount of $CySSO_3^-$ residues consumed during the reversal step.

Subsample 3 is water-rinsed and treated with alkali in a manner identical to subsample 2, and is then alkylated with acrylonitrile. This treatment converts remaining CySH groups to nontitratable β-cyanoethylsulfide residues ($CySCH_2CH_2CN$). Because the alkylation is performed after alkali reversal, no further reversal during alkylation is likely. The sample is then hydrolyzed in acid and the MNP_3 titer is determined.

$$x\,\text{Ker-CySH} + y\,\text{Ker-CySSO}_3^- \xrightarrow{(OH^-)}$$

$$z\,\text{Ker-CySSCy-Ker} + (x-z)\,\text{Ker-CySH} + (y-z)\,\text{Ker-CySSO}_3^- \xrightarrow{CH_2=CHCN}$$

$$z\ \text{Ker-CySSCy-Ker} + (x - z)\ \text{Ker-CySCH}_2\text{CH}_2\text{CN} + (y - z)\ \text{Ker-CySSO}_3^- \xrightarrow[\Delta]{6N\ \text{H}_2\text{SO}_4} (y - z)\ \text{CySH}$$

This titer is then a measure of the $CySSO_3^-$ content which has survived the reversal reaction.

The MNP_1, MNP_2, and MNP_3 determinations are summarized below.

MNP_1 = sum of combined Ker-CySH and Ker-$CySSO_3^-$

$MNP_3 + \frac{1}{2}(MNP_1 - MNP_2)$ = Ker-$CySSO_3^-$ content of original sample

$MNP_1 - [MNP_3 + \frac{1}{2}(MNP_1 - MNP_2)]$ = Ker-CySH content of original sample

MNP_1 is the sum of the combined CySH and $CySSO_3^-$ content. The $CySSO_3^-$ content of the original sample is then the MNP_3 titer, that is, the $CySSO_3^-$ content which survived reversal and alkylation, plus one-half the value of MNP_1 minus MNP_2, the $CySSO_3^-$ content of the original sample. The CySH content of the original sample is obviously then MNP_1 minus the $CySSO_3^-$ content of the original sample.

Experimental

Materials

Brown, European, human hair* was cleaned by treatment with an aqueous solution of anionic detergent and then used throughout this investigation. The analytical results are expressed on the basis of vacuum oven-dried (one hour, 105°C, ~1 mm Hg pressure) hair weight.

All reagents employed were the best grade available and were used without further purification. Salyrganic acid† (Fig. 1) was used as the organic mercurial titrant. The mercurial was made up to approximately 3×10^{-3} molar concentration in 3×10^{-2} molar aqueous sodium chloride. Klotz and Carv-

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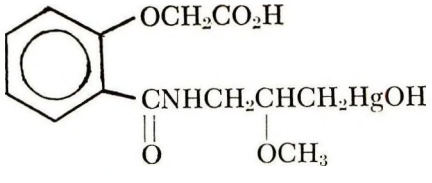

Figure 1. Salyrganic acid

er (5) used this mercurial previously and had found it to be more soluble than many of the other mercurials. The salyrganic acid solution was standardized against a sample of cysteine of known purity. A 1% aqueous solution of sodium nitroprusside was used as the indicator for the mercurial-cysteine reaction, the color change at the endpoint being pink to colorless.

Methods

Each analysis requires three preweighed subsamples of approximately 0.1 to 0.2 g taken from the treated keratin specimen to be analyzed. Where not specified, liquor-to-keratin ratios in the analytical procedure are 125:1 or greater. After treatment, each keratin sample is stripped of excess treatment liquor by blotting with filter paper and then washed by immersion in three portions of 6*N* H_2SO_4.

Subsample 1 is hydrolyzed for MNP_1 titer. MNP hydrolyses are carried out in about 85 ml of 6*N* sulfuric acid, for 17 hours at 95°C. The effects of varying the time and temperatures of hydrolysis were briefly studied. Comparable results could be obtained with shorter hydrolysis times at higher temperatures in an evacuated bomb, for example 3½ hours at 160°C. We prefer, however, the hydrolysis at 95°C because of its relative safety and simplicity. In all cases we found that the hydrolysate contains a small amount of fibrillar material, less than 3% by weight of the hair sample, which does not appear to interfere with the analysis.

Subsample 2 is blotted with filter paper and washed by immersion in two portions of deaerated distilled water under nitrogen to prevent oxidation of sulfhydryl groups. The sample is blotted and alkali-reversed by successive immersion in two portions of 0.2*M* sodium sesquicarbonate under nitrogen. The sample is next washed by a 1-min immersion in the deaerated distilled water under nitrogen and then hydrolyzed to obtain the MNP_2 value.

Subsample 3 is treated as was subsample 2 prior to the hydrolysis step. The sample is then blotted and immersed in a 5% solution of acrylonitrile in 0.1*M*, pH 9.2 borate buffer at 32°C for 30 min. The sample is then rinsed in running water for 1 min and then hydrolyzed to obtain the MNP_3 titer.

After hydrolysis, the samples are cooled to room temperature and diluted to 100 ml with distilled water. It was found convenient to carry out the hydrolysis in a 100-ml volumetric flask. A 5- or 10-ml aliquot is removed and added rapidly with stirring to four times its volume of saturated sodium carbonate solution. This produces a blanket of carbon dioxide which inhibits aerial oxidation of mercaptide. Salyrganic acid solution is added from a buret, and 10–15 drops of nitroprusside are added just before the endpoint. One or two titrations are necessary to determine the approximate endpoint. Care should be taken to avoid early addition of nitroprusside since it decomposes to an orange-colored compound in the alkaline medium which obscures the endpoint.

Table I
Comparison of MNP_1 and Elsworth-Phillips
Determinations of Ker-$CySSO_3^-$ in Human Hair[a]

Analytical Method	(Ker-CySH + Ker-$CySSO_3^-$) (meq/g)	(Ker-$CySSO_3^-$) (meq/g)
MNP_1	0.60[b]	...
Elsworth-Phillips	...	0.30

[a] Immersion for 60 min in 1.0*M* NH_4HSO_3–3.0M urea, pH 5.8, 32°C, > 50:1 bath ratio followed by centrifugation.
[b] Corrected for Ker-CySH content of untreated hair.

Results and Discussion

The first phases of experimental work were directed at establishing the validity of this method which could not be established by using standard keratin samples and controls since none were available. The Elsworth-Phillips method (1) was found, in one instance (Table I), to give a $CySSO_3^-$ content equivalent to one-half the MNP_1 value on bisulfite-treated hair. This was, however, laborious and required close to three days of work.

We did, however, demonstrate the validity of the assumptions on which our procedure is based and then showed that the analytical results obtained in several experiments were consistent with expectations for samples containing predictable ratios of $CySSO_3^-$ and CySH.

The three groups of assumptions on which this method is based are: (a) rinsing treated (bisulfite, mercaptan) keratin with 6*N* sulfuric acid quenches the keratin-bisulfite reaction, removes soluble mercaptans, and quenches the keratin-mercaptan reactions; (b) treatment of hair containing $CySSO_3^-$ and CySH residues with alkali causes rebuilding of the disulfide bonds and that these residues are not consumed by other chemical reactions under reversal conditions; and (c) treatment of alkali-reversed keratin with acrylonitrile causes a quantitative conversion of cysteinyl sulfhydryl groups to β-cyanoethylsulfide groups in a manner that does not change the $CySSO_3^-$ content.

In the case of the first assumption, the quenching of the keratin-bisulfite reaction, bisulfite-treated keratin samples which were quenched and rinsed in 6*N* sulfuric acid were analyzed by the MNP_1 procedure after storage in 6*N* sulfuric acid for from 10 min to 7 days. The data are reported in Table II. Clearly one sees no change in the MNP_1 titer over the time studied. These results also indicate that large numbers of samples can be stored for long periods of time which expedites sample handling. Thus, we see that the keratin-bisulfite reaction is quenched and is not reversed in this acidic medium. The one question which remains unanswered in this and others' work is: does the 6*N* sulfuric acid change the instantaneous cleavage level

Table II
MNP_1 Titer of Sulfite-Treated Hair
Samples After Storage in 6*N* H_2SO_4[a]

Time of Storage (days)	Ker-CySH + Ker-$CySSO_3^-$ meq/g hair (MNP_1)
0 (control)	0.81
2	0.81
5	0.82
7	0.82

[a] 1.0*M* NH_4HSO_3–3*M* urea, pH 5.8, 32°C, > 50:1 bath ratio for 30 min.

Table III
Effect of 6*N* H_2SO_4 Rinsing on
Ker-CySH Level of Thioglycolate-Reduced
Hair as Determined by the Iodoacetamide Procedure[a]

Reduction Time[b] (minutes)	meq SH/g	
	Before Rinsing	After Rinsing
5	0.25	0.23
10	0.41	0.41

[a] Two 1-min and one 3-min rinses with isopropanol followed by two 1-min and one 13-min rinses with 6*N* H_2SO_4.
[b] 0.30*N* ammonium thioglycolate, pH 9.2, 32°C, 50:1 bath ratio.

achieved in the keratin-bisulfite mixture? We have assumed not in the absence of any clear experimental data to the contrary.

That the acid rinse does not change the reduction level of keratin treated with mercaptan is verified in the experiment in which thioglycolate-treated hair was analyzed by the iodoacetamide procedure (2) before and after acid rinsing, as shown in Table III.

The remainder of assumption (a), that acid rinsing desorbs soluble mercaptan from keratin, was demonstrated on thioglycolate-treated hair. Using only the acid rinse sequence described in Table III, no sulfhydryl content, as determined by nitroprusside, could be found in the second and third acid rinses.

Pertinent to assumption (b), the alkaline reversibility of the cystine-bisulfite reaction, is the fact that rinsing bisulfite-treated keratin with an alkaline buffer causes a lowering of the MNP_1 titer of the fiber as shown in Fig. 2. Our method is based on the assumption that exactly one-half of the titratable groups lost during alkaline reversal are $CySSO_3^-$ groups and the other half are CySH groups. This assumption is supported by Wolfram's

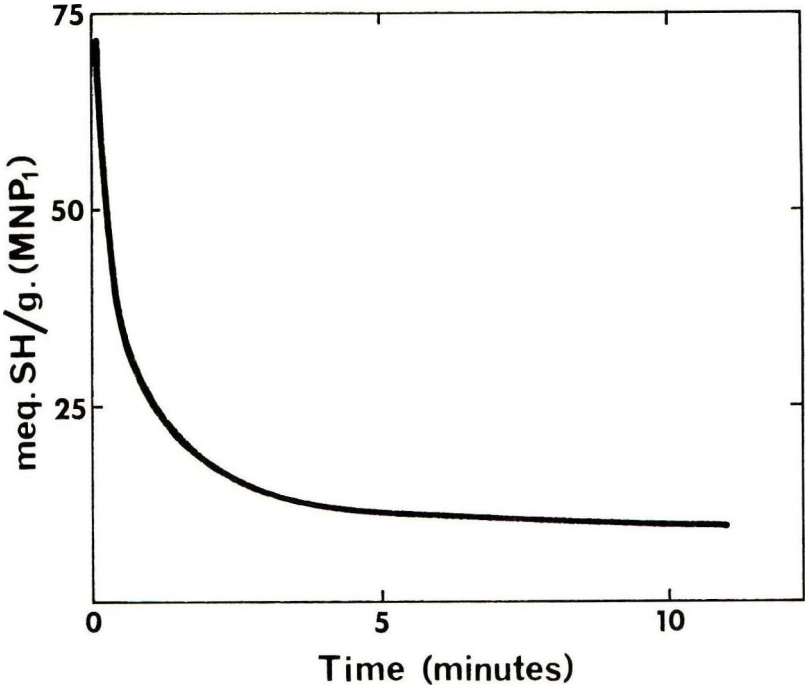

Figure 2. MNP_1 titer of bisulfite-treated hair as a function of pH 10 rinse time. Treatment conditions: 1.0 *M* NH_4HSO_3–3M urea, pH 6.6, 32°C, 3:1 bath ratio for 30 min

finding that the decrease of MNP_1 titer in an alkaline reversal process is balanced by an increase in the fiber cystine content (6).

The data presented in Table IV show that CySH groups alone are not lost during alkaline reversal; that is, MNP_1 is equal to MNP_2 for hair containing only cysteinyl residues.

To examine this point further, we prepared hair containing only $CySSO_3^-$ residues using the oxidative sulfitolysis procedure with bisulfite-tetrathionate (7). The data presented in Table V also show that $CySSO_3^-$ groups alone are not lost during reversal for hair containing only these residues; that is, $MNP_1 = MNP_2 = MNP_3$. Since, under the conditions of alkaline reversal, the disappearance of either $CySSO_3^-$ or CySH residues does not occur unless both species are present (and when it does occur it is balanced by an increase in the fiber cystine content), it is difficult to envision a mechanism of reversal other than that described for the classical cystine-bisulfite reaction. That is, one fiber $CySSO_3^-$ residue combining with one fiber CySH residue to yield one fiber cystinyl group.

The third assumption on which our method is based is that acrylonitrile alkylation of alkali reversed keratin quantitatively converts cysteinyl mecaptan to β-cyanoethylsulfide without loss of $CySSO_3^-$ residues. The validity

Table IV
MNP Analysis of Thioglycolate-Reduced Hair[a]

	meq SH/g
Total cleavage (MNP_1)	0.34
After alkaline reversal (MNP_2)	0.33
After alkylation (MNP_3)	0.00

[a] 0.30*N* ammonium thioglycolate, pH 9.2, 32°C, 50:1 bath ratio for 30 min.

Table V
Analysis of Hair Treated with Bisulfite-Tetrathionate[a]

	meq SH/g
MNP_1	0.87
MNP_2	0.87
MNP_3	0.86
Ker-CySH	0.01
Ker-$CySSO_3^-$	0.86

[a] 30-min immersion in 1.0*M* NH_4HSO_3–4.0*M* urea–0.4*M* tetrathionate, pH 8.0, 50:1 bath ratio, 32°C.

of this assumption is confirmed by two experiments. The data in Table IV show that for hair reduced with thioglycolate, the alkylation with acrylonitrile is quantitative; that is, $MNP_3 = 0$. The fact that alkylation does not destroy $CySSO_3^-$ groups is demonstrated in Table V for the analysis of hair containing only $CySSO_3^-$ groups. MNP_2 and MNP_3 are essentially the same, indicating that $CySSO_3^-$ groups are not consumed during the alkylation reaction.

In the application of the method to hair containing predictable $CySSO_3^-$ to CySH ratios, the utility of this method depends on whether or not the method can quantitatively distinguish $CySSO_3^-$ from CySH in keratin containing these residues in all proportions. There is ample evidence that this is so. A test of the validity of the method was obtained by performing the analyses on hair samples containing predictable ratios of $CySSO_3^-$ and CySH.

Hair was modified as described in Table VI. Treatment with bisulfite afforded hair with equal amounts of cysteinyl and S-sulfocysteinyl residues. Reduction with thioglycolate followed by bisulfite yielded hair in which there were more CySH residues than $CySSO_3^-$ residues. Treatment with bisulfite and tetrathionate followed by treatment with bisulfite provided hair where the number of $CySSO_3^-$ residues exceeded the CySH residues. As discussed previously, treatment with bisulfite-tetrathionate, that is, oxidative sulfitolysis, yielded hair with $CySSO_3^-$ residues only, while reduction with thioglycolate afforded hair with CySH residues only.

Table VI
Preparation of Hair Containing
Unequal Amounts of Ker-CySH and Ker-$CySSO_3^-$

Condition	Treatment
Ker-CySH = Ker-$CySSO_3^-$	Bisulfite
Ker-CySH > Ker-$CySSO_3^-$	Thioglycolate followed by bisulfite
Ker-CySH < Ker-$CySSO_3^-$	Bisulfite-tetrathionate followed by bisulfite
Ker-$CySSO_3^-$ only	Bisulfite-tetrathionate
Ker-CySH only	Thioglycolate

Table VII
Analysis of Hair Treated with Sulfite Systems[a]

pH	Immersion Time (hours)	meq SH/g			Ker-$CySSO_3^-$ meq/g		Ker-CySH meq/g	
		MNP_1	MNP_2	MNP_3	Theor[b]	Calc[c]	Theor[b]	Calc[d]
3.5	1.25	0.70	0.32	0.15	0.35	0.35	0.35	0.36
4.5	1.25	0.88	0.20	0.10	0.44	0.44	0.44	0.44
5.5	1.25	0.94	0.10	0.08	0.47	0.50	0.47	0.44
6.5	1.00	0.81	0.01	0.00	0.40	0.40	0.40	0.41
7.5	1.00	0.50	0.19	0.09	0.25	0.25	0.25	0.26
8.5	2.00	0.36	0.19	0.09	0.18	0.18	0.18	0.18

[a] 1.0*M* NH_4HSO_3–3.0*M* urea, 32°C, 50:1 bath ratio.
[b] $\frac{1}{2}MNP_1$.
[c] $MNP_3 + \frac{1}{2}(MNP_1\text{-}MNP_2)$.
[d] $MNP_1\text{-}[MNP_3 + \frac{1}{2}(MNP_1\text{-}MNP_2)]$.

The first analysis was performed on the bisulfite-treated hair where the $CySSO_3^-$ to CySH ratio would be expected to be one. These results are summarized in Table VII for hair treated with bisulfite at pH's ranging from 3.5 to 8.5. The data clearly show that the $CySSO_3^-$ to CySH ratio for hair treated in this broad pH range is one. These results are totally consistent with the general cystine-bisulfite reaction mechanism and reflect the pH dependence of the cystine-bisulfite equilibrium (8). It also shows that the analytical method is applicable to hair treated with bisulfite at higher pH's than previously observed.

The method was next applied to hair containing excess cysteinyl residues. The results are described in Table VIII. For hair treated with thioglycolate followed by bisulfite, there is obtained a total $CySSO_3^-$ content of 0.17 meq/g and a CySH content of 0.34 meq/g.

For hair containing excess $CySSO_3^-$ residues, the treatment of hair with bisulfite-tetrathionate followed by bisulfite was considered. The data are

Table VIII
Analysis of Hair Treated with Thioglycolate (TGA)[a]
Followed by Bisulfite[b]

	meq SH/g
MNP_1 of TGA treatment	0.19
MNP (after TGA and bisulfite treatment)	
MNP_1	0.53
MNP_2	0.26
MNP_3	0.06
Ker-$CySSO_3^-$ (calculated[c])	0.17
Ker-$CySSO_3^-$ (found)	0.19
Ker-CySH (found[d])	0.35

[a] 1.0-hour immersion in 0.15*N* ammonium thioglycolate, pH 9.2, 32°C, 50:1 bath ratio, 30-min water rinse under N_2.

[b] 15-min immersion in 0.5*M* NH_4HSO_3–1.0*M* urea, pH 6.5, 32°C, 50:1 bath ratio.

[c] Ker-$CySSO_3^-$ (calculated) $= \left[\frac{MNP_1\text{-}MNP_1 \text{ of TGA treatment}}{2}\right]$.

[d] Ker-CySH (found) = [MNP_1 (after TGA and bisulfite treatment)–Ker-$CySSO_3^-$ (found)].

Table IX
Analysis of Hair Treated with
Bisulfite-Tetrathionate[a] Followed by Bisulfite[b]

	meq SH/g
MNP_1 of bisulfite-tetrathionate treatment	0.26
MNP (after bisulfite-tetrathionate treatment and bisulfite treatment)	
MNP_1	0.61
MNP_2	0.32
MNP_3	0.26
Ker-$CySSO_3^-$ (calculated[c])	0.44
Ker-$CySSO_3^-$ (found)	0.40
Ker-CySH (found[d])	0.21

[a] 10-min immersion in 1.0*M* NH_4HSO_3–4.0*M* urea–4.0*M* tetrathionate, pH 8.0, 50:1 bath ratio, 32°C, followed by 30-min immersion in water.

[b] 15-min immersion in 0.5*M* NH_4HSO_3–1.5*M* urea, pH 6.5, 50:1 bath ratio, 32°C.

[c] Ker-$CySSO_3^-$ (calculated) = ½[MNP_1 + MNP_1 of bisulfite-tetrathionate].

[d] Ker-CySH (found) = [MNP_1-Ker-$CySSO_3^-$ (found)].

reported in Table IX. Again there is seen consistent behavior. The $CySSO_3^-$ content is 0.44 meq/g while the CySH content was found to be 0.21 meq/g.

For hair containing S-sulfocysteinyl residues only as previously discussed (Table V), evidence of only $CySSO_3^-$ residues is observed; no CySH residues can be determined. Similarly, for hair treated with thioglycolate only (Table IV) cysteinyl residues are observed.

A final point to be made in these studies is the very small amount of cysteine lost, less than 0.05 meq, when either reduced or untreated hair is hydrolyzed in acid in the presence or absence of added cysteine. In the absence of hair, cysteine can be recovered from the hydrolysis and titration procedure in quantitative amounts. In the presence of hair, however, the very small amount, 0.05 meq, is lost. The reason for this loss is not well understood but the magnitude of the loss clearly does not interfere with the utility of the procedure.

Conclusions

A method has been developed for the quantitative differentiation of $CySSO_3^-$ and CySH residues in chemically treated keratin. The procedure was validated by verifying the assumptions on which it is based and showing that keratin, in this case human hair, containing predictable $CySSO_3^-$ to CySH ratios gave analytical results consistent with expectations. The method was shown to be applicable to analyzing keratin treated under a variety of conditions including acidic and alkaline bisulfite. The method was also shown to be useful on hair containing a wide range of $CySSO_3^-$ to CySH ratios as well as hair containing only $CySSO_3^-$ or CySH groups.

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Testing Antiacne Agents in Mexican Hairless Dogs

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Synopsis—The plugged follicles on the backs and flanks of MEXICAN HAIRLESS DOGS share clinical and histologic similarities to the comedones seen in man. SOAP treatments cleaned follicle-containing areas but did not remove the follicular plugs. SALICYLIC ACID preparations were similarly ineffective. The use of BENZOYL PEROXIDE produced slight extrusion of the follicular keratin plus mild irritation, and VITAMIN A ACID was markedly effective with associated dermal irritation.

Introduction

Several authors have reviewed the literature for information relating animal and clinical skin research (1, 2). A symposium presented at the 52nd annual meeting of the Federation of American Societies for Experimental Biology discussed the choice of animal models for the study of disease processes in man (3). Although there are many mammalian and avian models for clinical skin diseases, no references were cited in the above papers on any form of spontaneous animal acne.

Currently, laboratory evaluations of acne treatments rely on chemically induced comedones in the ears of rabbits. The hyperkeratinization is similar to that causing follicular hyperkeratosis in occupational chloracne (4). Human sebum lipids (5) and fatty acids (6, 7) which have relevance to the etiopathogenesis of acne also have been employed as acnegens in the rabbit.

Van Scott reported at the 1970 symposium on the Biology of the Skin that the rhino mouse was a natural model of keratin invagination (8). By studying the skin histology of this animal in relation to the animal's age, a reproducible method for measuring progressive cyst formation was developed. Experimental materials used to treat clinical acne were applied to the skin to test their efficacy in preventing or alleviating this keratin invagination. Sulfur and resorcinol had no activity; however, retinoic acid proved very effective.

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In our laboratory, the flanks and back of the Mexican hairless dog have been observed to contain pigmented keratin follicular plugs which share some or certain clinical and histologic similarities to the comedones seen in man (9). In an endeavor to determine the suitability of these animals for routine acne experiments, a study was conducted to evaluate preparations currently employed to treat clinical acne.

Materials and Methods

Four test sites, approximately 2.5 cm in diameter, with grossly evident follicular plugs, were selected on the back of each dog. Areas were carefully photographed prior to treatment to record control appearance and again at the conclusion of the study. Efficacy and irritation potential were evaluated from the resulting slides. Before and after treatment, biopsies were obtained with a 6-mm Keyes cutaneous punch for histological evaluations. Following fixation of the tissues in 10% formalin, slides were prepared by routine histologic means, stained with hematoxylin and eosin, and examined utilizing standard dermatopathologic criteria (10, 11) employed in recognizing inflammatory, degenerative, or proliferative changes.

Test or control materials in solution or suspension were applied with a cotton-tipped applicator, Q-tip,* gently rubbed over the test site for 5 sec per treatment. Sites were treated at least once a day for 14–21 days.

Materials tested included sulfur, hexachlorophene, salicylic acid alone or in various combinations; benzoyl peroxide, and vitamin A acid (retinoic acid). Appropriate vehicles used for each test material were also evaluated on control sites. The influence of a bland soap, Ivory,† or an abrasive soap, Amo-Derm,‡ on site cleaning and comedone removal was also evaluated. The method used to test these soaps was as follows: a paper towel, moistened with water and rubbed with soap to produce a lather, was rubbed on the assigned test area on the dog's back for 5 sec. The lather was removed from the site with a clean towel moistened with water, and the treated area was then dried with a fresh dry towel. Photographs were taken prior to and upon completion of these experiments; however, no histological evaluations were performed.

Results

The results of this study are given in Table I. Several vehicles used in the attempt to aid or enhance the penetration or efficacy of the test agent were tested without active agents as controls. A propylene glycol vehicle was de-

*Chesebrough-Pond's, New York, N.Y.
†Procter & Gamble, Cincinnati, Ohio.
‡High Chemical Co., Philadelphia, Pa.

Table I
Effects of Materials Applied on the Skin of Mexican Hairless Dogs

Preparation Tested	Conc. (%)	Vehicle	Conc. (%)	Description[a]			
				Cleaner	Drying, Flaking	Comedone Extrusion Removal	Irritation
Salicylic acid	3	Propylene glycol	100	+/−	+	−	−
...	...	Propylene glycol	...	−	−	−	−
Salicylic acid	2	Alcohol	70	+	+	+/−	−
Hexachlorophene	1	Water	30				
...	...	Above vehicle	...	+	+	−	−
Benzoyl peroxide	10	Alcohol[b]	40				
		Cellosolve	40				
		Propylene glycol	20	+	+/−	+	+
...	...	Above vehicle	...	+	−	−	−
Vitamin A acid	0.1	Alcohol	70				
		Propylene glycol	30	+	++	+++	++
...	...	Above vehicle	...	+/−	+	−	−

[a]Code: −, none; +/−, questionable; +, slight; ++, moderate; +++, marked.
[b]Suggested by Fulton (12).

void of activity. Alcohol-containing vehicles produced a slight cleansing and also resulted in drying or flaking of the treated areas. Cleansing of the treated sites, when observed, was attributed to the activity of these vehicles.

Tissue sections of these control sites yielded no evidence of irritation. Superficial and deep follicular structures were consistently filled with keratin which confirmed the inability of the vehicle treatment to cause comedone loss.

Salicylic acid, alone or with hexachlorophene, produced drying of the treated areas. No irritation was produced by these preparations. Minimal overt evidence of keratin extrusions from follicular sites was observed and was confirmed histologically.

Benzoyl peroxide treatment resulted in slight keratin extrusion from follicular sites which was accompanied by slight erythema as observed grossly. Histologically, evidence of minimal to moderate epidermal parakeratosis was seen.

Figure 1. Appearance of plugged follicles (site to be treated is within four marked spots)

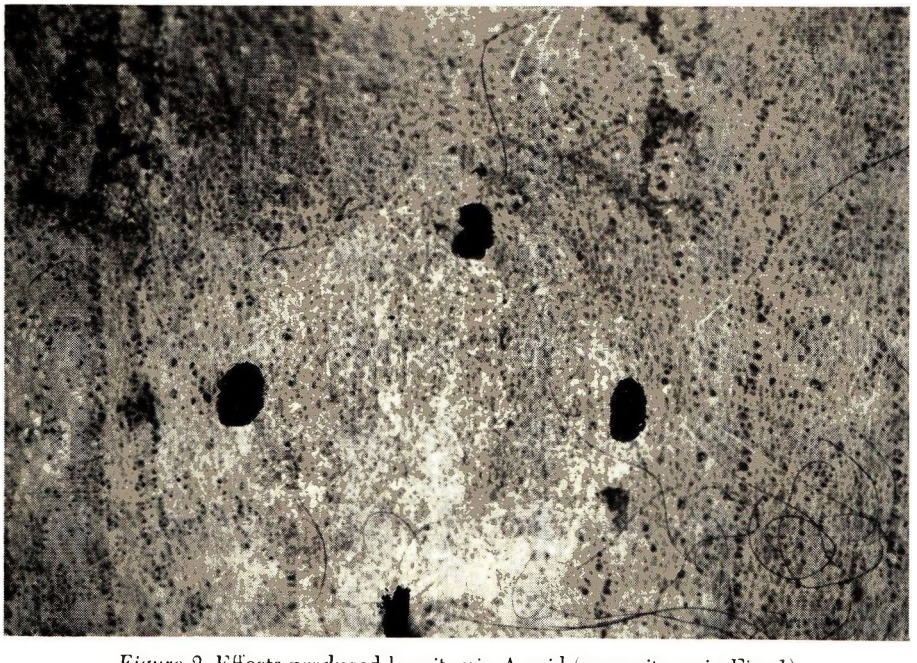

Figure 2. Effects produced by vitamin A acid (same site as in Fig. 1)

Vitamin A acid was the most effective material tested. In addition to marked extrusion of the plugged follicles (Figs. 1 and 2), vitamin A acid treatment caused exfoliation of epidermal keratin layers and irritation as evidenced by moderate erythema on all sites. Slight edema also was observed on several treated sites. Histologically, sites treated with vitamin A acid showed hyperplastic and parakeratotic epidermal changes. Minimal subacute inflammatory reactions were also evident in the upper dermis. There was no evidence of keratin plugging on microscopic examination of the vitamin A acid treated sites.

The influence of soap and washing was studied in the last series of tests. Both the bland and abrasive soaps had a marked cleansing effect on the topical surface layers; however, neither of the preparations removed any of the deeper-seated keratogenous material from follicular sites. The use of an abrasive cleanser showed no overt benefits over the conventional soap preparation.

Discussion

The skin of the Mexican hairless contains follicular structures filled with black keratogenous material, much like the "blackheads" observed in human acne. Their number varies widely. In certain areas, they are too numerous and closely congregated to be accurately counted. Other areas are devoid of these follicular plugs. The dorsal aspects of the trunk and the lateral aspects of the hind quarters usually contain rows of these follicules distributed so that identification and counting can be performed (Fig. 1). The lipid content of expressed comedones is currently being compared with normal surface lipids. Preliminary results (13) indicate a higher concentration of free fatty acids and ester waxes from these follicles than from surface lipids. These lipid fractions have been implicated as comedogenic agents in both animals (5, 7) and man (14). Histologically, these plugged follicles appear to be invaginations of the epidermis; they are large cystic structures with sebaceous glands and clusters of nevus-like cells appearing at their base (Fig. 3).

Our results indicate that two distinct types of effects occurred as a result of topical therapy: (a) cleansing, which was evident by the gross removal of surface lipids with entrapped dirt, and (b) extrusion of keratin plugs from follicular sites.

The use of vehicles or soaps to wash or cleanse the skin are beneficial in that they remove excess surface lipids and dirt particles (15) which may help in the formation of follicular plugs. Thus, this may be more properly considered a preventative measure than actual acne therapy as these preparations do little, if anything, to existing follicular plugs.

In the search for an active agent to enhance the extrusion of follicular plugs, vitamin A acid has been shown to be a very effective agent (16), a fact con-

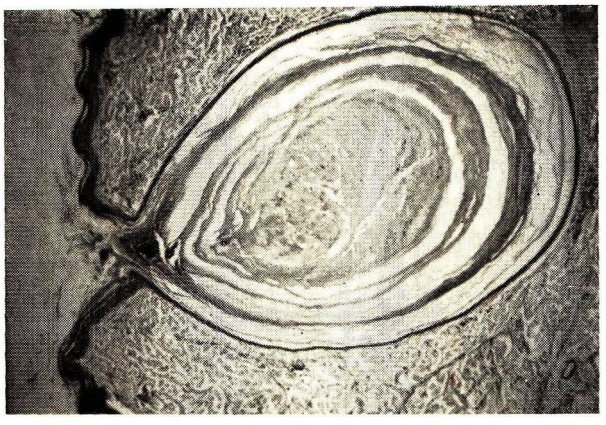

Figure 3. Histologic section through plugged follicle (H. and E. X 40)

firmed in our studies. While other agents, such as salicylic acid, etc., have been reported to have some beneficial effect on various forms of acne, it is generally agreed that such agents are not consistently effective. Our animal model shows this to be the case. While individual animals may have responded to such agents, the group, as a whole, showed no significant or uniform activity. Benzoyl peroxide produced slight but consistent effects on comedome removal. Histologically, acceleration of the keratinization process in hairless dog skin is similar to reactions observed with this agent in man (17).

While it would be tempting to try to explain the mechanism of follicular clearing as observed in these experiments, it is beyond the scope of this initial investigation. These experiments were performed primarily to test the predictive capabilities of the Mexican hairless dog as a nonclinical model for screening antiacne preparations. We have noted that vitamin A acid was the most effective agent tested. In addition to comedone extrusion, vitamin A acid treatment produced exfoliation of keratin layers and a moderate irritation similar to that observed clinically (16). Also, as in human acne, questionable efficacy with the other agents tested was observed in the Mexican hairless. These dermatological reactions in Mexican hairless dogs appear to have sufficient similarities to clinically observed acne responses to be employed as a valid preclinical antiacne assay method.

Conclusions

The flanks and back of the Mexican hairless dog often contain large numbers of plugged follicles which are grossly and histologically similar to those observed in man. These sites were treated with materials used in clinical acne therapy. Color slides were taken prior to and at the conclusion of treatment in each study to record and help evaluate site cleaning, comedone re-

moval and/or irritation. In addition, biopsies were taken from selected sites to evaluate comedone extrusion and irritation-induced changes. Preparations containing salicylic acid with or without hexachlorophene and several soap products produced no significant improvement of the comedone condition. No consistent irritation was observed. Treatment with 10% benzoyl peroxide resulted in slight improvement accompanied by some irritation. Vitamin A acid (retinoic acid) at 0.1% in an ethanol/propylene glycol vehicle caused marked comedone extrusion and irritation. Since similar results have been reported in clinical studies, the Mexican hairless dog appears to be a predictive model for assaying both efficacy and potential irritation of agents proposed for acne therapy.

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A Study on the Differential Thresholds of Sensory "Firmness" and "Viscousness" of Cream Base Substances

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Presented September 14, 1972, 7th IFSCC Congress, Hamburg, Germany

Synopsis—The DIFFERENTIAL THRESHOLDS of sensory "FIRMNESS" and "VISCOUSNESS" were studied. It was noted that these properties changed continuously depending upon changes in HARDNESS and VISCOSITY which were measured instrumentally. The minimum values of differential thresholds of both firmness and viscousness were found to be about 10% and 80% level of confidence.

Introduction

The differential thresholds of sensory "firmness" and "viscousness" have been used to describe some of the properties of cosmetic creams which are related to their application to skin.

Weber's law, which is applicable to sense properties, states that when two stimuli, S and $S + \Delta S$ (ΔS is the differential threshold in stimulus S), are given, $\Delta S/S$ (Weber's ratio) represents the region in which $\Delta S/S$ is constant with variance of the given stimulus. This observation has been reported for visual sensitivity (1), hearing sensitivity (2), and taste sensitivity (3). However, up until the present, there have been few reports as to touch sensitivity. Scott-Blair (4) investigated the threshold of firmness using vulcanized rubber (elasticity, $1–2 \times 10^7$ dyne/cm^2) and that of viscousness using bitumen (viscosity, $10^6–10^7$ cps) and found that the differential threshold of firmness was ⅓ that of viscousness indicating that firmness was three times easier to

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distinguish than viscousness. In this case, the study was carried out using materials having a greater hardness and viscosity than cosmetics.

It was difficult to prepare cosmetic creams for use in this study which would have a small difference in hardness and viscosity. This difference was necessary in order to determine the differential thresholds of firmness and viscousness.

Therefore, the cream bases developed for this study were prepared in the same ranges of hardness and viscosity as those of cosmetic creams by using oil and waxes which are commonly used constituents of cosmetics. The differential thresholds of sensory firmness and viscousness for the respective changes in hardness and viscosity of these samples were obtained and compared to the values obtained using a standard consisting of a mechanical spring and silicone oil which had the ideal elastic property and viscous property, respectively. Moreover, several experiments were carried out by using samples varying in both hardness and viscosity, and the effect of viscosity on the evaluation of firmness and the effect of hardness on the evaluation of viscousness. In this paper, the terms "firmness" or "viscousness" will be used to express the sensory touch when the samples are applied to the skin, whereas the terms hardness or viscosity will be used to define the instrumentally measured values of the samples.

Experimental

Preparation of Samples

Cream Base of Given Viscoelasticity

Liquid paraffin (Saybolt viscosity 70 sec) was used as the oil and solid paraffin, ceresin, and microcrystalline wax were employed as the waxes. Cream base substances with a wide range in both hardness and viscosity were prepared by changing the quantity of ceresin, microcrystalline wax, and liquid paraffin in the formulations.

The samples were prepared in groups of five (indicated by A,B,C,D, and E) ranging in hardness between 10^1 and 10^4 g/cm^2, and keeping the viscosity almost constant. In a similar manner, additional samples in groups of five were prepared (indicated by a,b,c,d, and e) ranging in viscosity between 10^2 and 10^4 cps, and keeping the hardness almost constant. The formulation for c is given in Table I, and Fig. 1 illustrates the hardness and viscosity of each of these samples. Each sample was prepared at least 6 or 7 times and good reproducibility of results was obtained as can be seen in Table I.

Cosmetic creams generally have a hardness from 10 to 10^3 g/cm^2 and a viscosity of from 10^2 to 10^4 cps, as shown in Fig. 2. Compared to these figures, the cream bases used in this study were judged as having the same range of hardness and viscosity as those of commonly used cosmetic creams.

Table I
Sample Group (c) in which Viscosity
Was Varied, with Constant Hardness at 25°C

Sample No.	Formulation				Measurement Results	
	Solid Paraffin (%)	Micro-crystalline Wax (%)	Ceresin (%)	Liquid Paraffin (%)	Hardness (g/cm^2) x 10^2	Viscosity (cps) x 10^2
1	10.0	8.0	4.0	78.0	1.3	7.2
2	10.0	7.5	3.7	78.8	1.3	6.6
3	10.0	6.75	3.1	80.15	1.2	6.0
4	10.0	6.0	2.6	81.4	1.2	5.3
5	10.0	5.3	2.0	82.7	1.2	4.7
6	10.0	4.6	1.25	84.15	1.2	3.7

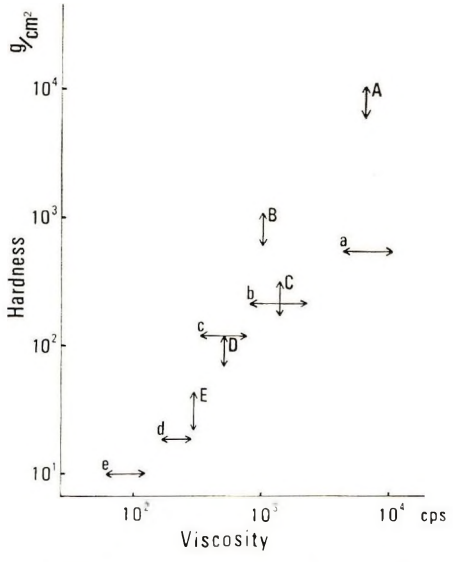

Figure 1. Hardness and viscosity of prepared samples

Standard Substances having Ideal Elasticity and Viscosity

Mechanical springs in groups of 4 grades (F, G, H, and I) and in the hardness range between 70 and 3200 g/cm^2 were used as the ideal elastic substance, and silicone oils in groups of 5 grades (J, K, L, M, and N) in the viscosity range between 110 and 20000 cps served as the ideal viscous substance.

The mechanical spring was made from a cylindrical spiral spring 3 cm in diameter and 3 cm in height and two sheets of cardboard, about 5 cm in diameter. The cardboard was fixed to the top and bottom of the spring so that they were parallel.

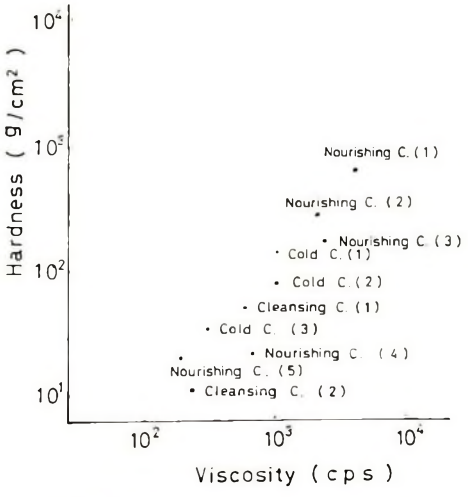

Figure 2. Hardness and viscosity of cosmetic creams

Apparatus For Measuring Hardness and Viscosity

Hardness was measured using a curd tension meter. In this instrument, a load is applied to the penetration rod which is fitted with a round disk (1.5–10.0 mm in diameter) at the bottom. The hardness, expressed as grams per cm^2, is the value of the load just as the disk begins to penetrate into the sample.

Viscosity, expressed as cps, was measured with a Ferranti-Shirley Cone and Plate Viscometer at a shear rate of 1700 sec^{-1} and a sweep time of 10 sec.

Sensory Evaluation

Sensory evaluation was carried out by trained panels of 10 subjects (5 males and 5 females each). The panel was instructed to arrange each series of samples in their order according to their firmness or viscousness. This was repeated 3 times by each individual.

Evaluation of firmness was made by pressing the surface of the samples in the container with a finger, and viscousness was evaluated by taking out a small amount of the sample with the forefinger and applying it to the forearm.

In the case of the standard, the spring was pressed down by pushing with a finger at the center of the cardboard and the resistance which is sensed when the spring is compressed 1 cm by a finger pressure was evaluated as "firmness," and "viscousness" of silicone oil was evaluated in a similar manner as in the case of the cream base substances.

Table II
Correlation Coefficients

Sensory Firmness and Measured Hardness			Sensory Viscousness and Measured Viscosity		
Sample Group	Average Hardness (g/cm^2)	Correlation Coefficient (r)	Sample Group	Average Viscosity (cps)	Correlation Coefficient (r)
A	8.8×10^3	0.812	a	7.9×10^3	0.660
B	8.5×10^2	0.893	b	1.5×10^3	0.860
C	2.5×10^2	0.934	c	5.5×10^2	0.873
D	9.5×10^1	0.748	d	2.2×10^2	0.831
E	3.4×10^1	0.580	e	9.0×10^1	0.905

Results

Experiments on Cream Base Substances

Correlation Coefficient

Through Spearman's rank method, the correlation coefficient between sensory firmness and measured hardness were given, and the same calculations were also made between sensory viscousness and measured viscosity and are shown in Table II.

It was found that the correspondence of sensory firmness to measured hardness was shown, on the whole, in a comparatively high hardness range; however, in the extremely high hardness range the correlation between the two was shown to be small. On the other hand, the correspondence of sensory viscousness to measured viscosity was shown to be essentially in a low viscosity range.

Discrimination Threshold

Differential threshold is usually obtained by using the paired comparison method, but the number of samples was not sufficient for carrying out the paired test in this experiment. Therefore, another method has been designed and employed. First, the panel members were asked to rank and arrange one series of samples composed of 6 or 7 samples according to the order of sensory firmness or viscousness. Next, every possible pair from the previously ranked data according to the sensory firmness or viscousness were compared in each pair, and was regarded as the correct judgment if it corresponded to the measured order. Accordingly, the percentage of correct judgments was calculated.

All data were plotted with the percentage of correct judgments as the ordinate and the relative difference as the abscissa. The relative difference (Δ) is the proportion of the difference to a mean value of two samples obtained by instrumental measurement and is shown as percentage.

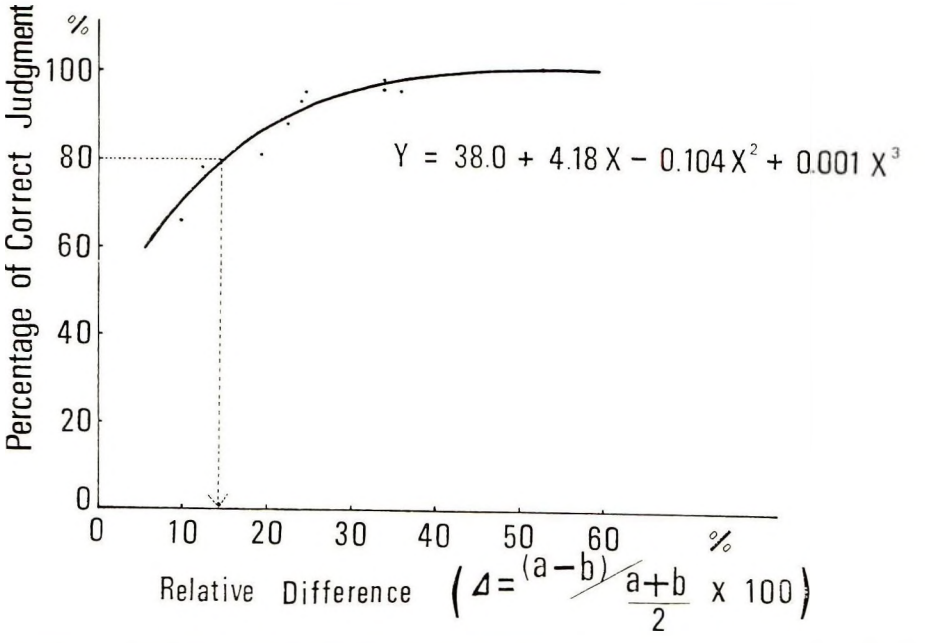

Figure 3. Examples of approximation curves obtained by least squares method (sample c)

$$\Delta = \frac{a-b}{\frac{a+b}{2}} \times 100$$

The approximation curve was calculated from these points, using the least squares method, and is shown in Fig. 3. The value of the relative difference where 80 or 90% of the panels were able to give a correct judgment ($p = 80$, or $p = 90$) was obtained from this curve. In this paper, this value is referred to as the (relative) discrimination threshold of sensory firmness and viscousness, and it indicates that the difference of hardness or viscosity in the two samples is judged correctly by 80 or 90% of the panel members.

The discrimination thresholds of 80 and 90% correct judgments are shown in Table III. This is shown graphically in Figs. 4 and 5.

Experiments on Standard Substances

The discrimination thresholds for firmness of the standard elastic substance and viscousness of the standard viscous substance were studied.

The discrimination thresholds of 80 and 90% correct judgments for firmness and viscousness were obtained for the mechanical spring and silicone oil in the same manner as previously described for the cream base substances. These results are shown in Table IV and Figs. 6 and 7.

Table III
Discrimination Thresholds of Firmness and Viscousness of 80% and 90% Correct Judgements (Cream Base Substances)

Firmness			Viscousness		
Sample Group	Discrimination Threshold (%)		Sample Group	Discrimination Threshold (%)	
	$P = 80\%$	$P = 90\%$		$P = 80\%$	$P = 90\%$
A	9.5	18.5	a	44.0	59.0
B	9.0	14.0	b	25.0	32.5
C	11.0	18.0	c	14.5	23.0
D	16.0	24.0	d	9.5	17.0
E	28.0	44.0	e	15.5	27.5

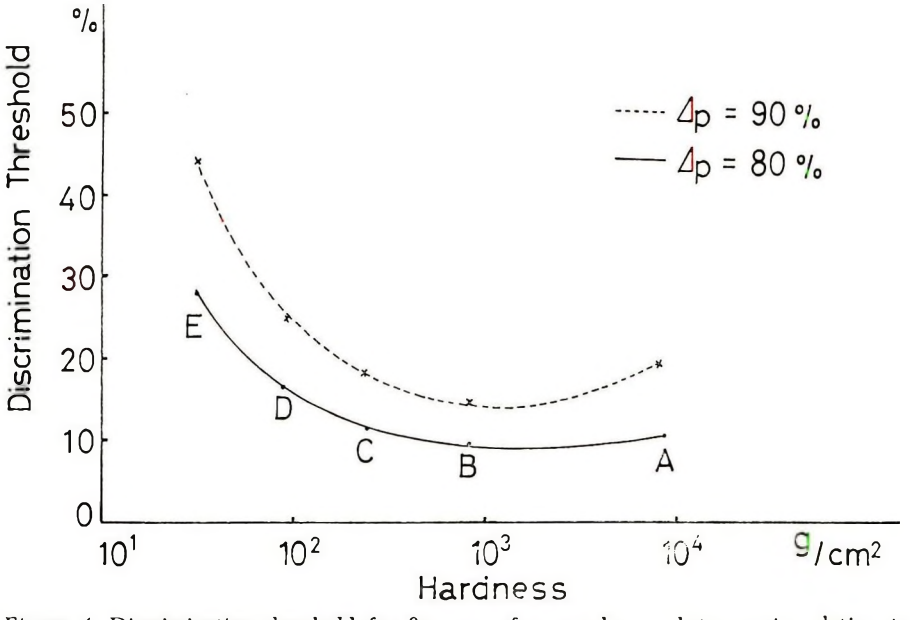

Figure 4. Discrimination threshold for firmness of cream base substances in relation to hardness

Discussion and Conclusions

In the case of firmness, as is shown in Fig. 4, the tendency of becoming easy to discriminate as the hardness increased was noted; however, extreme increase of hardness, such as seen in sample A, showed the reverse effect. Sample group B was the easiest to discriminate, that is, 80% of the panel

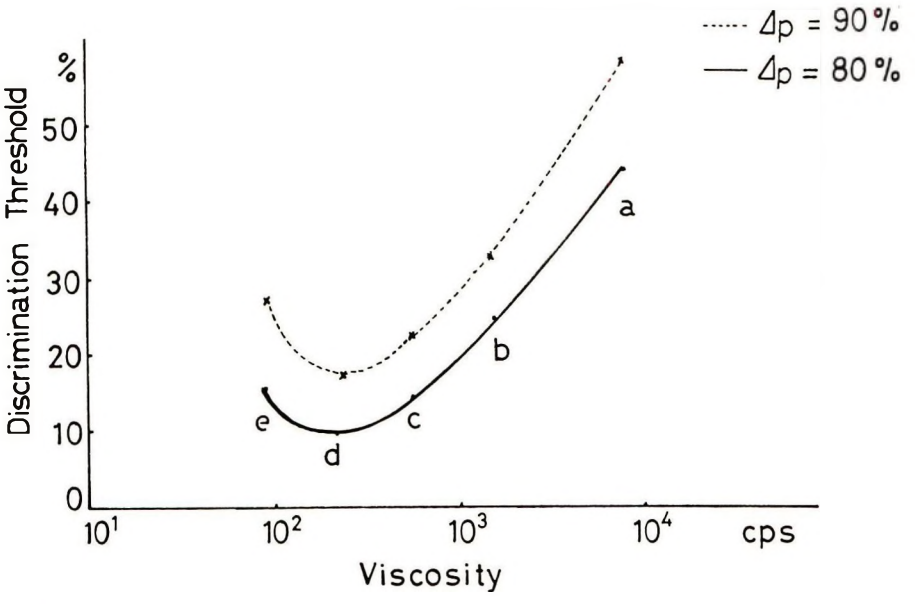

Figure 5. Discrimination threshold for viscousness of cream base substances in relation to viscosity

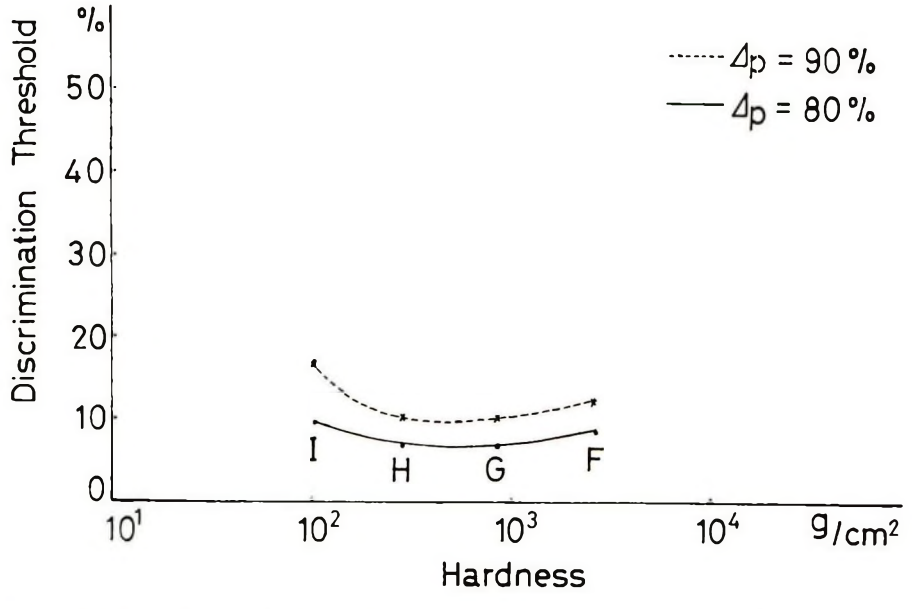

Figure 6. Discrimination threshold for firmness of mechanical spring in relation to hardness

Table IV
Discrimination Thresholds of Firmness and Viscousness of 80% and 90% Correct Judgements (Standard Substances)

Firmness (Spring)				Viscousness (Silicone Oil)			
Sample Group	Average Hardness (g/cm^2)	Discrimination Threshold (%)		Sample Group	Average Viscosity (cps)	Discrimination Threshold (%)	
		$P = 80\%$	$P = 90\%$			$P = 80\%$	$P = 90\%$
F	2.54×10^3	8.0	11.5	J	1.49×10^4	15.5	27.5
G	8.47×10^2	9.5	16.0	K	6.43×10^3	16.5	27.0
H	2.77×10^2	6.0	10.0	L	1.98×10^3	12.5	17.5
I	1.06×10^2	9.0	17.0	M	3.19×10^2	22.5	37.5
				N	1.72×10^2	30.0	47.5

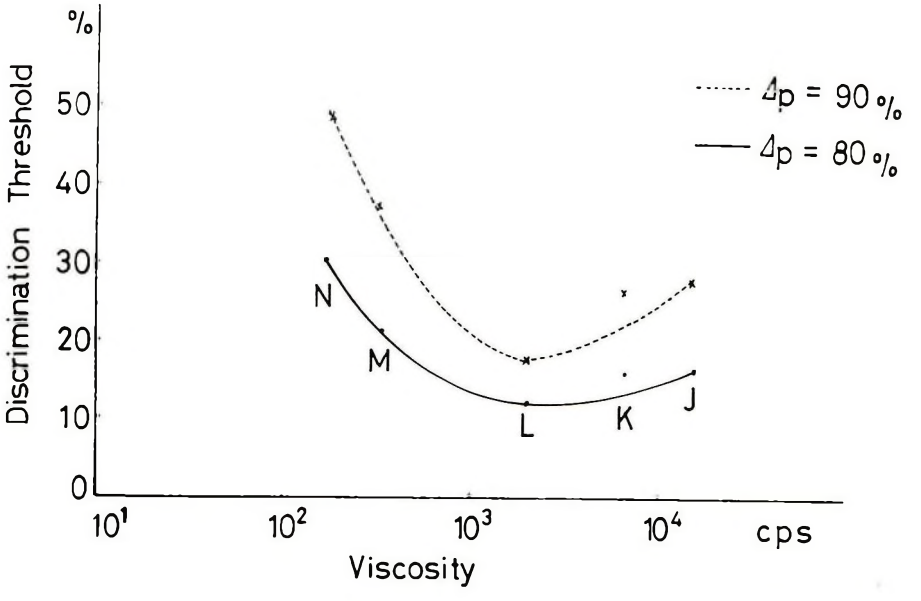

Figure 7. Discrimination threshold for viscousness of silicone oil in relation to viscosity

members were able to discriminate the samples if the relative difference of sample's hardness was about 10%.

In the case of viscousness, as is shown in Fig. 5, there was the tendency of becoming easy to discriminate as the viscosity decreased; however, the extreme decrease of viscosity, as in sample e, showed a reverse effect. Sample group d showed the easiest discrimination.

Based upon these results, it was found that the discrimination threshold of sensory firmness and viscousness changed continuously depending upon the changes of the instrumentally measured hardness and viscosity of the

samples having the same ranges of hardness and viscosity as cosmetic creams. There was a minimum discrimination threshold for both sensory firmness and viscousness, and this value was found to be about 10% at 80% correct judgment. Similar results were obtained using the standard substance prepared from the mechanical spring and the silicone oil.

Since hardness and viscosity are two physically different properties, it is difficult to equate in principle one property with the other. However, as can be seen in Fig. 1, the relation between hardness and viscosity of the cream base substances which were prepared in this study was found to be linear. For example, the sample having the hardness of 10^2 g/cm^2 showed 10^3 cps in viscosity, and that with 10^3 g/cm^2 hardness showed 10^4 cps in viscosity. In view of this, both hardness and viscosity of the cream base substances were plotted on the abscissa at the same time, respectively, and it was found that the points of 10^2 g/cm^2 hardness and 10^3 cps viscosity overlapped.

Discrimination thresholds of 80% correct judgments of firmness and viscousness were plotted on the ordinate, and the results are shown in Fig. 8. From this figure, the differential threshold curves of firmness and viscousness were shown to cross each other at the middle of those ranges. That is, in the range at a hardness of above 9 x 10 g/cm^2 and a viscosity of above 9 x 10^2 cps, it was easier to discriminate firmness than viscousness, but this was reversed at lower values as it was easier to discriminate viscousness than firmness.

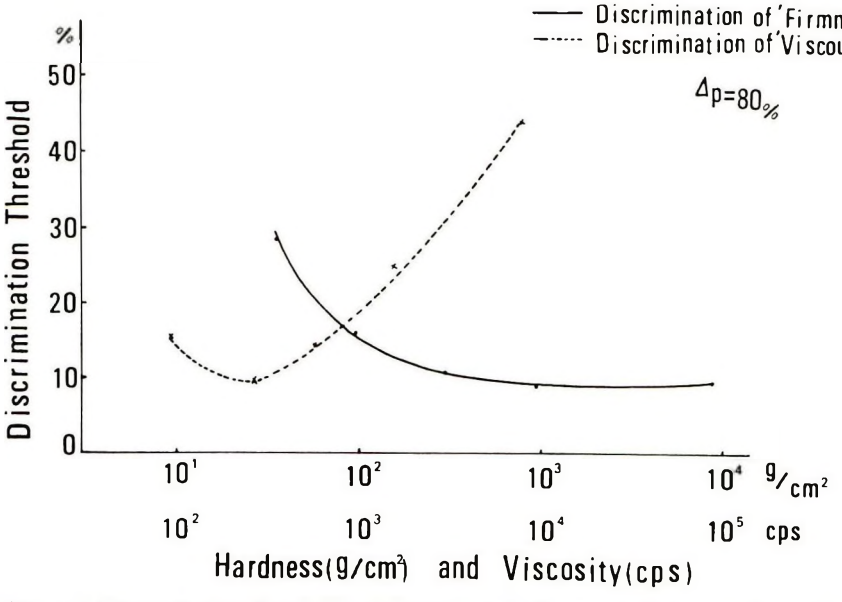

Figure 8. Discrimination thresholds of firmness and viscousness for cream base substances in relation to hardness and viscosity

Scott-Blair and Coppen (4, 5) reported that the firmness was considered to be easier to distinguish than viscousness. From our study it was noted that two areas for the discrimination thresholds of cosmetic creams were present, one where firmness was easier to discriminate than viscousness in the range of high hardness and viscosity (which corresponded well to the result obtained by Scott-Blair and Coppen) and the other, where the relationship was *vice versa* in the range of low hardness and viscosity.

In order to compare the hardness with the elasticity, the elasticity of the same samples used in this study was also measured with a parallel plate plastometer. The correlation coefficient between the hardness and the elasticity was found to be very high ($r = 0.997$) as seen in Fig. 9. Therefore, it is considered that the hardness of the samples used in this study did correspond to the elasticity.

As indicated above, our discrimination thresholds were calculated from the data obtained by judging correctness or incorrectness for all possible combinations of the two obtained from the data of the ranking method, which differed apparently from the ordinary differential threshold. Accordingly, an analysis was carried out by applying the constant method for converting our data to ordinary differential threshold (6). The calculated data are shown in

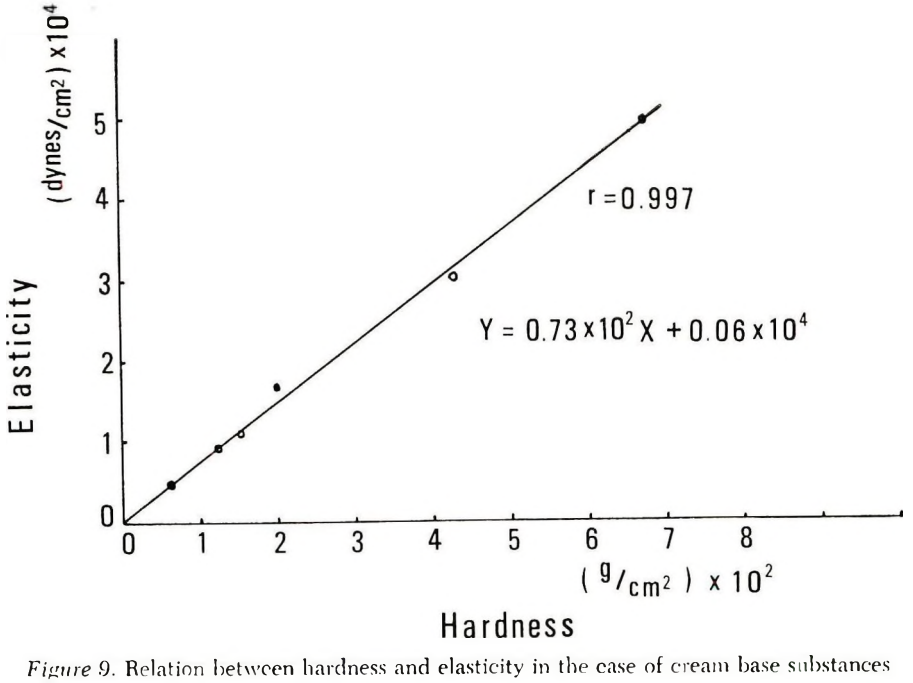

Figure 9. Relation between hardness and elasticity in the case of cream base substances

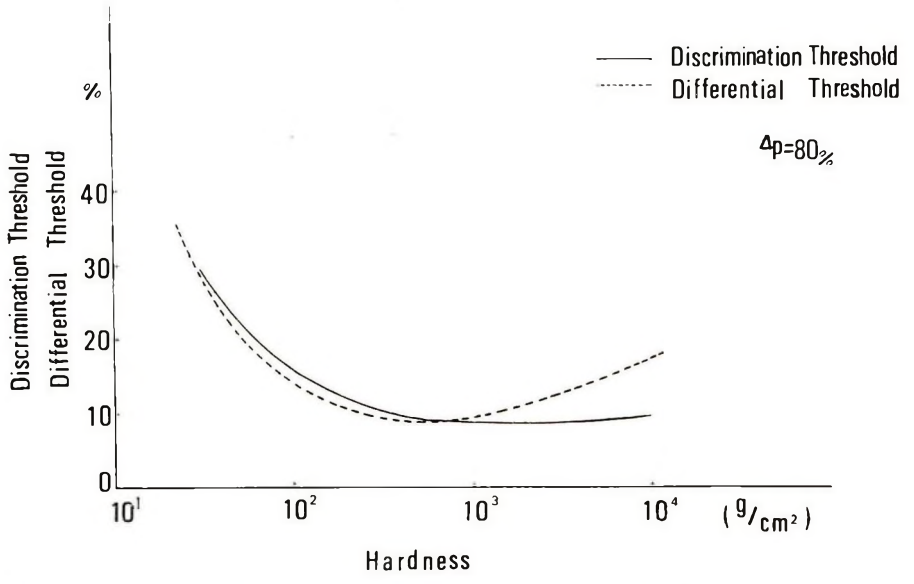

Figure 10. Comparison between discrimination threshold and differential threshold for firmness of cream base substances in relation to hardness

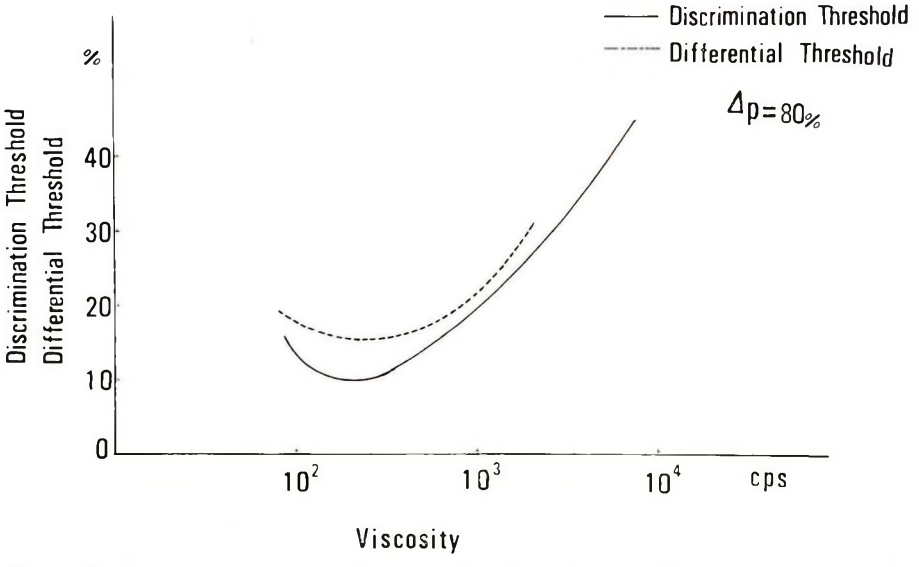

Figure 11. Comparison between discrimination threshold and differential threshold for viscousness of cream base substances in relation to viscosity

Figs. 10 and 11, and these curves show similar behavior with those of differential threshold obtained previously, although slight differences were observed. Therefore, the discrimination thresholds obtained by these authors were considered to correspond well to the ordinary differential threshold.

In this study, the differential thresholds of sensory firmness and viscousness were determined by preparing cream base substances having a constant viscosity and variable hardness and *vice versa*. Additional samples were prepared which varied both in hardness and viscosity simultaneously, and the effect of viscosity on the judgment of firmness and the effect of hardness on the judgment of viscousness were investigated.

Samples with a hardness of about 2×10^2 g/cm^2 and a viscosity of about 2×10^3 cps were selected within the range where discrimination threshold of firmness was smaller than that of viscousness. Viscousness was evaluated with 3 sample groups (O, P, and Q) in the range where viscosity variance was kept almost constant, while that of the hardness variance was given in the 3 stages as shown in Fig. 12. In sample group Q, the viscosity was varied while the hardness was almost constant.

In the case of P and O, both hardness and viscosity were made variable, where the rate of variance of hardness in group O is higher than that of group P, and the range of viscosity in each group was kept about the same.

The results, shown in Table V, show that the correlation coefficient is highest in the case of sample group O, when the hardness variance is the maximum, and lowest in the case of samples Q without hardness variance. It was

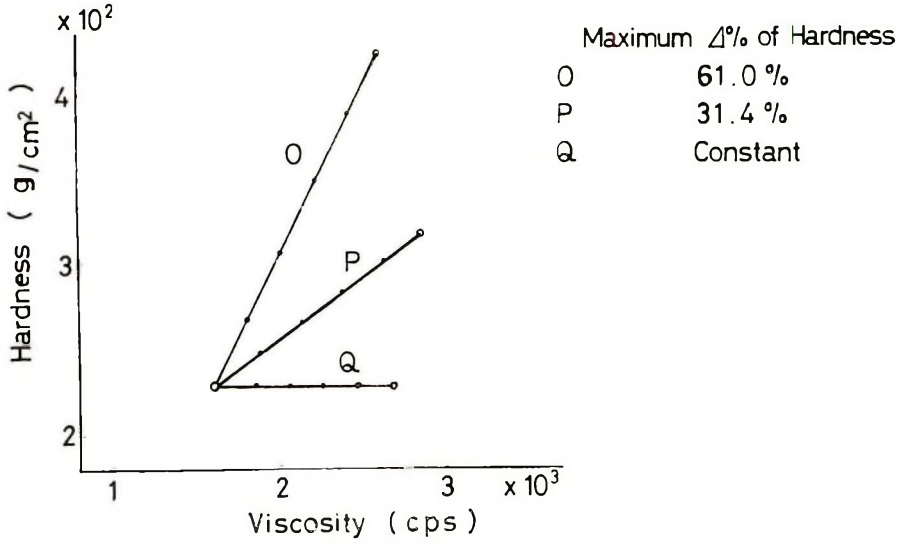

Figure 12. Prepared samples to investigate the effect of hardness on evaluation of viscousness

Table V
Effect of Variance Range in Hardness of Prepared Samples on Evaluation of Viscousness

Sample Group	Hardness		Viscosity		Correlation Coefficient between Viscousness and Viscosity (r)
	Range of Variance ($g/cm^2 \times 10^2$)	Maximum (%)	Range of Variance ($cps \times 10^3$)	Maximum (%)	
O	2.26–4.25	61.0	1.69–2.56	40.5	0.917
P	2.33–3.20	31.4	1.64–2.69	48.4	0.886
Q	2.40	Constant	1.62–2.56	45.0	0.826

concluded from these results that the judgment of sensory viscousness was affected considerably by the variance in hardness of prepared samples.

Although the experimental data were omitted, it was found that the judgment of sensory firmness was not affected by the variance in viscosity of prepared samples. Moreover, in the range where discrimination threshold of sensory firmness was larger than that of sensory viscousness, the judgments of both sensory firmness and sensory viscousness were not affected by the hardness and viscosity.

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Kinetics of Degradation of the Parabens

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Synopsis—The effect of pH and TEMPERATURE on the HYDROLYSIS of methyl, ethyl, propyl, and *n*-butyl PARABEN was studied at 70°C in 0.1*M* phosphate buffer solutions at ionic strength 0.3 from pH 2.75 to 9.16 and at 40° and 50°C at pH 9.16. The REACTION was first order with respect to paraben. ENERGIES OF ACTIVATION were determined from Arrhenius plots. RATE CONSTANTS and HALF-LIVES of each paraben at 25°C were obtained by extrapolation of the Arrhenius plots.

The half-life of each paraben at 70°C and pH 8.24 was essentially independent of the initial concentration of paraben. Increasing ionic strength resulted in a slight increase in the rate of hydrolysis of each paraben. Increasing the phosphate concentration in buffer solutions at pH 8.24 at 70°C produced an increase in the rate of hydrolysis of each paraben. This indicated that the parabens undergo general base catalysis and that hydroxyl ion is not the only species that can catalyze their hydrolysis.

Introduction

The parabens have been reported to be stable, and to show no hydrolysis under conditions of heat sterilization (2 hours at 100°C or 0.5 hour at 120°C) at a pH range of 3–8 (1). Pekkarinen and Tommila (2) studied the alkaline hydrolysis of ethyl esters of para and meta hydroxybenzoates in potassium hydroxide solution, and in various organic solvents. They concluded that the hydrolysis takes place solely as a reaction between the ester ion and the hydroxide ion. Other authors found that the methyl ester of *p*-hydroxybenzoic acid is hydrolyzed at high temperature and in strongly acid solution (3). Ravel and Parrott (4) studied the hydrolysis of methyl paraben in aqueous solutions at temperatures of 70–85°C in a pH range of 6–9.

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Methyl, ethyl, propyl, and butyl paraben are widely used in many types of pharmaceutical preparations such as ophthalmic, oral, and topical dosage forms and in cosmetic creams and lotions. They are used singly or in combination with one another. Mixtures of the parabens have been shown to be more effective as preservatives than the individual parabens (5, 6). Their hydrolysis yields *p*-hydroxybenzoic acid, which possesses little preservative activity. It was the purpose of this investigation to study the stability of the esters of *p*-hydroxybenzoic acid over pH range of 3–9 and various temperatures.

Experimental

Reagents and Apparatus

Reagents used were *p*-hydroxybenzoic acid (mp 213–214°C), methyl paraben (mp 126–128°C), ethyl paraben (mp 116–117°C), propyl paraben (mp 95–97°C), and *n*-butyl paraben (mp 73°C), all recrystallized from hydroalcoholic solution; also, sodium hydroxide, monobasic potassium phosphate, potassium chloride, phosphoric acid, and chloroform, all AR grade.

All pH's were measured using a Beckman model H-2 pH meter* and spectra were obtained with a Beckman DU spectrophotometer* with 1-cm quartz cells. A constant temperature bath with a Haake thermoregulator, series ED,† with a thermometer calibrated to 0.1°C was used for all the studies.

Procedure

Preparation of Buffers

The buffers used in this investigation were prepared from monobasic potassium phosphate solutions of desired molarity which were adjusted to the desired pH with sodium hydroxide pellets and phosphoric acid. Buffers were adjusted to constant ionic strength of either 0.3, 0.6, 0.9, or 1.2 by the addition of potassium chloride. All buffers were prepared at 25°C. Since the studies were conducted at elevated temperatures, the pH of the buffers at those temperatures would be different from the pH determined at 25°C. The pH values of the buffers were calculated using the ion product of water at the various temperatures of the study, 40°, 50°, and 70°C. For example, a buffer of pH 9 at 25°C would have a calculated pH of 8.24 at 70°C. The pK_w of water at 70°C is 12.82 (7, 8).

Calibration Curves for the Parabens

For each of the parabens studied, calibration curves were prepared using chloroform as the solvent. The optical density of each paraben dilution was

*Beckman Instruments, Fullerton, Calif.
†Gebrüder Haake, Berlin, Germany.

determined at 253 nm, the wavelength of maximum absorbance, using chloroform as the blank. A Beer's law relationship was obtained for methyl, ethyl, propyl, and *n*-butyl paraben in chloroform at 253 nm.

Method of Analysis

The following general procedure was developed and used for studying the effects of various factors, i.e., pH, temperature, ionic strength, on the rate of hydrolysis of the parabens.

An accurately weighed quantity of paraben was added to a 100-ml volumetric flask and dissolved in a small volume of appropriate buffer solution with the aid of heat. The solution was diluted to volume with the buffer solution which had been preheated to the temperature at which the particular analysis was made. The flask was placed in a constant temperature circulator bath that had been previously adjusted to the desired temperature (±0.1°C). The solution was allowed to thermally equilibrate for 10 min, then a 5-ml aliquot, representing the zero hour sample, was withdrawn and transferred to a separatory funnel which contained 20 ml of chloroform. At pH 9.0, *p*-hydroxybenzoic acid (pK_a 4.48) exists as a salt which remained in the aqueous phase when the solution was extracted with chloroform. Complete extraction of the undecomposed paraben was accomplished using 3 x 20 ml of chloroform extractions. The combined chloroform extracts were filtered into a 100-ml volumetric flask through filter paper that had been previously wetted with chloroform. The solution was diluted to volume with chloroform, and the absorbance was determined at 253 nm using chloroform as the blank. Samples were removed at definite time intervals and analyzed by this procedure.

Since *p*-hydroxybenzoic acid is the major degradation product of hydrolysis of the parabens (1, 9), mixtures containing known concentrations of methyl, ethyl, propyl, and *n*-butyl paraben and *p*-hydroxybenzoic acid were prepared. The ester concentration in each mixture was determined using the extraction procedure previously described. The per cent recovery of the parabens alone and in combination with varying concentrations of *p*-hydroxybenzoic acid is shown in Table I. The initial concentration of each paraben in all subsequent studies was 5 mg/l.

pH and Temperature Effects

In order to study the effect of pH and temperature on the rate of hydrolysis of methyl, ethyl, propyl, and *n*-butyl paraben, the reactions were carried out at three temperatures in buffer solution as follows: 70°C—pH 2.75, 3.66, 4.58, 5.49, 6.41, 7.33, 8.24, and 9.16; 50°C—pH 9.16; 40°C—pH 9.16. Reactions were carried out for a maximum of 160 hours, and all aliquots were analyzed using the extraction procedure described above.

Table I
Recovery of Paraben from a Mixture of Paraben and *p*-Hydroxybenzoic Acid

Composition of Mixture (mg/l.)		Amount of Paraben Found (mg/l.)	% Recovery
Paraben	Acid		
Methyl Paraben			
503	0.00	4.95	98.4
4.02	1.03	4.05	100.7
3.02	2.06	3.00	99.3
2.01	2.58	2.03	100.9
1.01	5.16	0.98	97.0
Ethyl Paraben			
4.66	0.00	4.70	100.9
3.73	1.03	3.70	99.2
2.80	2.06	2.77	98.9
1.86	2.58	1.85	99.5
0.93	5.16	0.95	102.2
Propyl Paraben			
5.31	0.00	5.40	101.7
4.25	1.03	4.22	99.3
3.19	2.06	3.20	100.3
2.12	2.58	2.10	99.1
1.06	5.16	1.07	100.9
n-Butyl Paraben			
5.29	0.00	5.30	100.2
4.23	1.03	4.12	97.4
3.17	2.06	3.17	100.0
2.12	2.58	2.05	96.7
1.06	5.16	1.10	103.8

Effect of Paraben Concentration

Solutions of methyl, ethyl, propyl, and *n*-butyl paraben were prepared at at 70°C in 0.0005–0.20*M* phosphate buffer at pH 8.24 and constant ionic strength of 0.3, and the hydrolysis was followed at 70°C in the manner previously described.

Effect of Ionic Strength

The effect of ionic strength on the hydrolysis of the parabens was investigated by studying the hydrolytic rate, at 70°C, of paraben solutions in 0.1*M* phosphate buffer at pH 8.24. The buffers were adjusted to ionic strengths of 0.3, 0.6, 0.9, and 1.2 by the addition of potassium chloride. The effect of buffer concentration on hydrolytic rate was also studied by following the hydrolysis at 70°C in 0.005–0.20*M* phosphate buffer at pH 8.24 and constant ionic strength of 0.6.

Results and Discussion

All of the rate constants were calculated from the first-order rate equation. The slopes of the lines were calculated by regression analysis using an electronic calculator.*

The effect of pH and temperature on the hydrolysis of the parabens was studied at 3 temperatures and at pH values ranging from 2.75–9.16. The calculated values of the rate constants and half-lives at different pH values and temperatures are summarized in Tables II and III. Typical plots showing the hydrolysis of ethyl paraben at 70°C and various pH values and at various temperatures at pH 9.16 are shown in Figs. 1 and 2. These plots indicate that the reaction was first order with respect to ethyl paraben since straight lines were obtained when the log of concentration was plotted against time, which was true for each paraben studied. The reaction overall would be pseudo-first order since the concentration of water can be regarded as constant.

As shown in Tables II and III and in Figs. 1 and 2, as the pH and temperature increase, the rate of hydrolysis of the parabens increases. One would expect *n*-butyl paraben to have a lower rate constant than propyl paraben, yet the data shown in Tables II and III show an increase in the rate constants for

*Wang model 360K/362K, Wang Laboratories, Inc., Tewksbury, Mass.

Table II
Specific Rate Constants and Half-life Periods for the Hydrolysis of Parabens at 70°C in 0.1*M* Phosphate Buffer, Ionic Strength 0.3, at Various pH Values

Paraben	pH	$k \times 10^3$ (hours^{-1})	$t^{1/2}$ (hours)
Methyl	9.16	30.63	22.6
	8.24	17.80	38.9
	7.33	10.24	67.6
	6.41	2.710	255.0
	5.49	0.414	1673.0
Ethyl	9.16	8.877	78.1
	8.24	7.667	90.4
	7.33	6.540	106.0
	6.41	1.203	576.0
Propyl	9.16	7.163	96.7
	8.24	5.982	120.0
	7.33	4.238	164.0
	6.41	0.568	1218.0
n-Butyl	9.16	9.391	73.8
	8.24	6.387	109.0
	7.83	4.580	151.0
	6.41	1.153	601.0

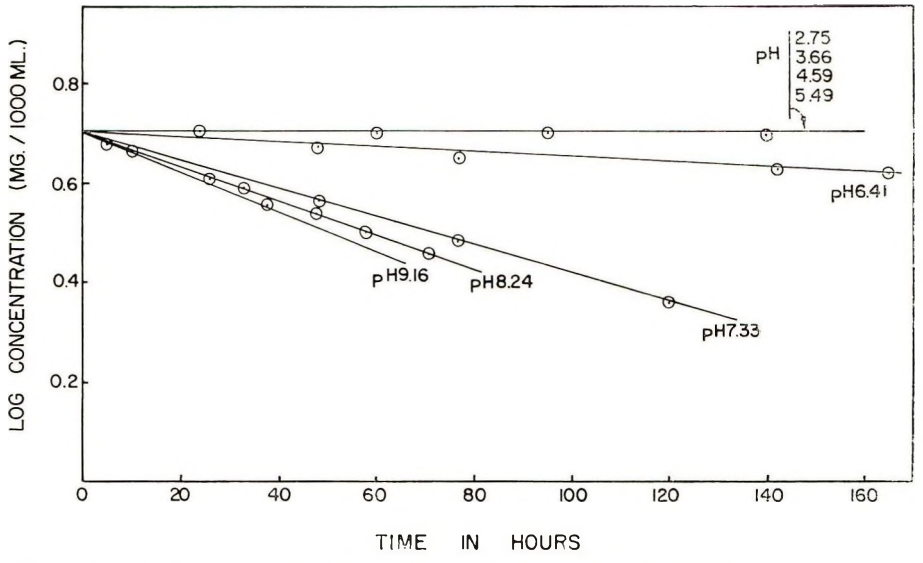

Figure 1. Log of concentration of ethyl paraben against time at 70°C in 0.1*M* phosphate buffer, ionic strength 0.3, at various pH values

Table III
Specific Rate Constants and Half-life Periods for the Hydrolysis of Parabens at Various Temperatures in 0.1*M* Phosphate Buffer, pH 9.16, and Ionic Strength 0.3

Paraben	Temperature (°C)	$k \times 10^4$ (hours^{-1})	$t_{1/2}$ (hours)
Methyl	40	1.941	356.0
Ethyl	40	0.735	943.0
Propyl	40	0.529	1310.0
n-Butyl	40	0.536	1292.0
Methyl	50	5.776	120.0
Ethyl	50	1.752	396.0
Propyl	50	1.205	575.0
n-Butyl	50	1.347	515.0
Methyl	70	30.63	22.6
Ethyl	70	8.877	78.1
Propyl	70	7.163	96.7
n-Butyl	70	9.391	73.8

n-butyl paraben, at the pH's and temperatures used in this study, when compared to the values obtained for propyl paraben. At 70°C in 0.1*M* phosphate buffer at pH 9.16, *n*-butyl paraben hydrolyzed faster than ethyl and propyl parabens. This phenomenon is difficult to explain since there is little difference between the activation energies of the parabens at pH 9.16, as shown in

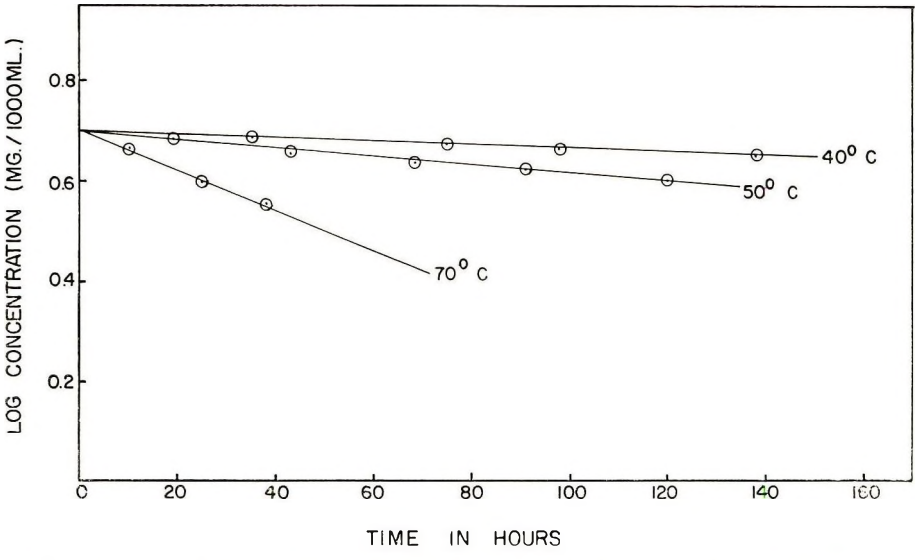

Figure 2. Log of concentration of ethyl paraben against time at various temperatures in 0.1*M* phosphate buffer, pH 9.16, and ionic strength 0.3

Table IV
Energies of Activation of the Parabens as Determined from the Slopes of the Arrhenius Plots at pH 9.16 in 0.1*M* Phosphate Buffer and Ionic Strength 0.3

Paraben	Energy of Activation (kcal/mole)
Methyl	20.8
Ethyl	18.7
Propyl	19.3
n-Butyl	21.0

Table IV. It is difficult to rationalize a change in the mechanism of hydrolysis for a simple ester of *p*-hydroxybenzoic acid.

The effect of temperature on the reaction rate can be expressed using the Arrhenius equation (10). Plots of log k *versus* $1/T$ yielded a straight-line relationship with a negative slope for each of the parabens studied, as shown in Fig. 3 for ethyl paraben. This indicates that the mechanism for the degradation of the parabens does not change with temperature at pH 9.16. Energies of activation for each paraben are shown in Table IV. These values are in good agreement with literature values for the hydrolysis of simples esters such as methyl acetate and ethyl benzoate as reported by Newling and Hinshelwood (11). By extrapolating the Arrhenius plots of each paraben to 23°C, rate constants and half-lives were predicted, as shown in Table V. It is valid to fol-

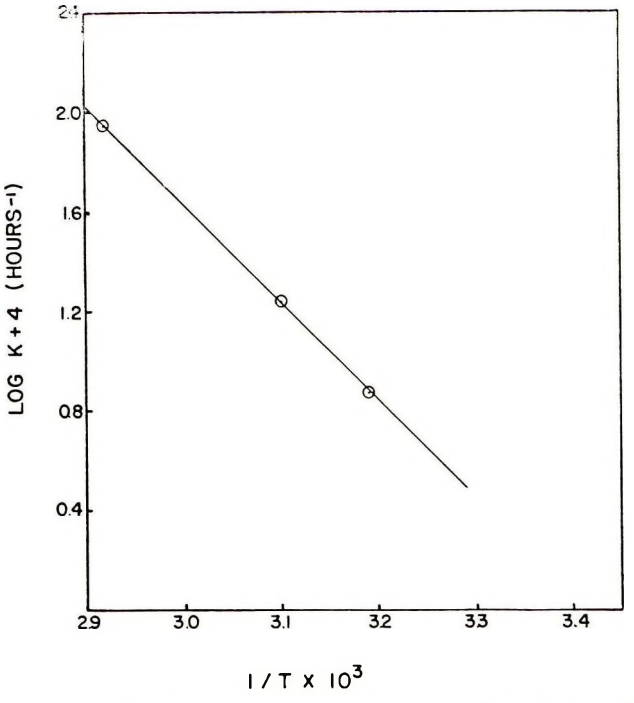

Figure 3. Arrhenius plot showing temperature dependence of the ethyl paraben hydrolysis at pH 9.16 in 0.1*M* phosphate buffer and ionic strength 0.3

Table V
Specific Rate Constants and Half-life Periods for the Hydrolysis of Parabens at 25°C in 0.1*M* Phosphate Buffer, pH 9.16 and Ionic Strength 0.3, as Calculated from Arrhenius Plots

Paraben	$k \times 10^4$ (hours^{-1})	$t^{1/2}$ (hours)
Methyl	4.015	1726
Ethyl	1.514	4577
Propyl	1.122	6176
n-Butyl	1.096	6323

low the extrapolated value for the rate constant if the experimental activation energy falls within a range of 10–30 kcal/mole (12, 13).

Although data already obtained showed that the reaction of the parabens at constant hydrogen ion concentration was first order with respect to paraben, this first-order dependency was further verified by a study of the effect of initial paraben concentration on the hydrolysis rate. The results shown in Table VI indicate that for each paraben studied between concentrations of 4–7 mg/l, the rate constants were essentially independent of the initial concentration of paraben.

Table VI
Effect of Paraben Concentration on the Specific Rate Constants at 70°C in 0.1*M* Phosphate Buffer, pH 8.24, and Ionic Strength 0.3

Paraben	Initial Concentration (mg/l.)	Rate x 10^3 (hours^{-1})
Methyl	6.90	16.86
	5.00	17.81
	3.62	17.48
Ethyl	7.00	7.946
	5.00	7.667
	4.10	8.096
Propyl	5.95	6.063
	5.00	5.982
	4.00	6.229
n-Butyl	6.96	6.449
	5.00	6.387
	3.77	6.295

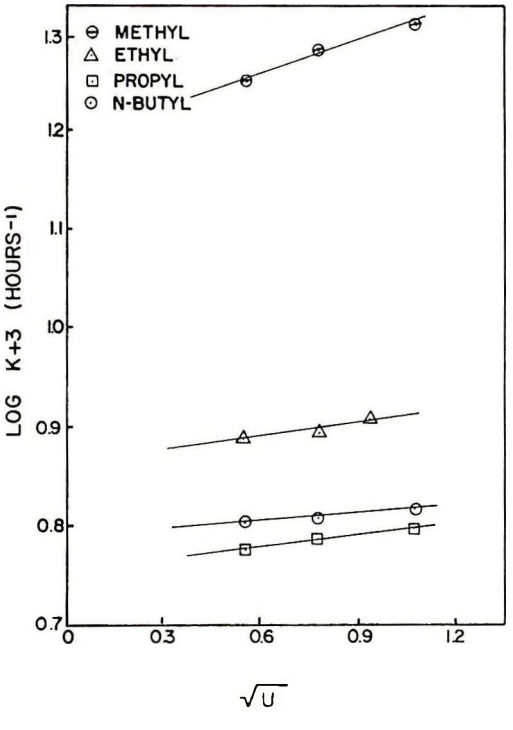

Figure 4. Effect of ionic strength on the hydrolysis of the parabens at 70°C in 0.1*M* phosphate buffer, pH 8.24

Table VII
Effect of Phosphate Concentration on the Specific Rate Constants for the Hydrolysis at 70°C, pH 8.24, and Ionic Strength 0.6

Paraben	Moles of Phosphate	$k \times 10^5$ (hours^{-1})	$t^{1/2}$ (hours)
Methyl	0.02	11.46	60.3
	0.04	12.84	53.9
	0.08	16.42	42.2
	0.10	19.05	36.4
	0.20	26.87	25.8
Ethyl	0.02	5.175	133.0
	0.08	6.920	100.0
	0.10	7.862	88.1
	0.20	10.45	66.3
Propyl	0.005	2.638	262.0
	0.02	3.470	199.0
	0.04	4.747	146.0
	0.10	6.145	113.0
	0.20	7.947	87.2
n-Butyl	0.005	2.486	278.0
	0.02	3.836	181.0
	0.04	4.759	146.0
	0.08	5.634	123.0
	0.10	6.418	108.0
	0.20	8.008	86.5

Figure 4 shows the effect of ionic strength on the hydrolysis rate of the parabens. A positive slope was obtained for each paraben, which indicates a reaction of ions of like sign, i.e., a reaction between the hydroxyl ion and the negatively charged *p*-hydroxybenzoate. Also, the rate of hydrolysis of each paraben was influenced slightly by the presence of potassium chloride as noted by an increase in the rate constants with an increase in ionic strength.

The effect of buffer concentration on hydrolytic rate is summarized in Table VII. The rate constants were obtained from log concentration *versus* time plots for the parabens at each buffer concentration. Each plot yielded a straight-line relationship indicating that the reaction was first order with respect to paraben at all of the phosphate concentrations. Figure 5 shows the relationship between phosphate concentrations and rate constants for ethyl paraben. A linear relationship was also obtained for methyl paraben but the propyl and butyl esters produced a distinct break in the curve which may have been due to the catalytic effect of other species in the phosphate buffer (mainly monohydrogen phosphate ion at pH 8.24). As shown in Fig. 5 and Table VII, the rate constants and the half-lives decreased with increasing phosphate concentration for all of the parabens. This would indicate that the

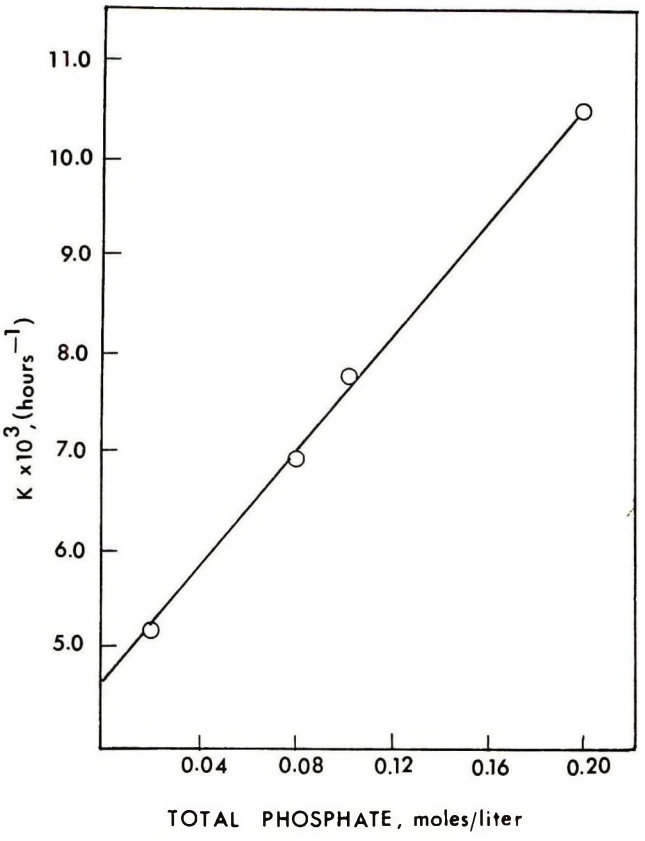

Figure 5. Effect of phosphate concentration on the rate of hydrolysis of ethyl paraben at 70°C, pH 8.24, and ionic strength 0.6

parabens undergo general base catalysis, and that hydroxyl ion is not the only species which can catalyze their hydrolysis.

(Received October 25, 1973)

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J. Soc. Cosmet. Chem., **25**, 507–514 (September 1974)

NMR—A New Instrumental Tool for the Analysis of Cosmetic Ingredients

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Presented October 9, 1973, Joint Symposium of the Society of Cosmetic Chemists and the Association of Official Analytical Chemists, Washington, D.C.

Synopsis—A basic introduction into the theory and quality control applications of a 60 Megahertz NMR Spectrophotometer is given. Quantitative methods for evaluation of IODINE NUMBER, ESTER VALUE, HYDROXYL NUMBER, and MOLES OF ETHOXYLATION on cosmetic raw ingredients are presented. Determination of ALCOHOL-WATER RATIOS on finished ingredients are shown to comply with a time-consuming distillation method. Determinates affecting accuracy and precision in compliance with classical wet chemistry tests for the above are also discussed.

Introduction

Basic NMR Theory

Many atomic nuclei, in addition to carrying a positive charge, spin about an axis of rotation. These particles because of their charge and movement behave as tiny bar magnets. One such nucleus (the one with which we are most concerned) is the proton, the nucleus of ordinary hydrogen.

The hydrogen protons exist in a ground state consisting of two or more indistinguishable energy levels in the absence of an applied magnetic field. If the nuclei are placed in an external magnetic field, alignment with and against this field occurs. It would appear plausible that the majority would align with the applied magnetic field. Actually, quantum mechanics theory states that only an extremely small portion aligning with the field are in excess. Protons aligning with the field have lower energy and those aligning against the field are said to have higher energy.

The Boltzman distribution equation states that in a field of 10,000 Gauss only an excess of 7 out of 1 million protons are in the lower energy state. This

*Avon Products, Inc., Suffern, N.Y. 10901.

is due to the thermal motions of the nuclei. It is these 7 which make the NMR phenomenon possible.

Energy is required to change their orientation. The energy difference between the two alignments under an applied field of H_o is expressed by eq 1:

$$E = \gamma \times H_0 \frac{h}{2\pi} \tag{1}$$

where γ = gyromagnetic ratio (a constant for each isotope)

h = Planck's constant

H_0 = applied magnetic field

To elevate a nucleus from the ground state to the excited state, energy of E is required. In NMR, this energy is in the radio frequency region or mathematically:

$$E = h\nu \tag{2}$$

Substituting ΔE in eq 1 and solving for ν we obtain the fundamental NMR equation:

$$\nu = \gamma \times H_0 \times \frac{1}{2\pi} \tag{3}$$

This equation states that there is some frequency of electromagnetic radiation that will cause the nuclei to change their orientation in a field of strength H_o.

Under an applied magnetic field of H_o = 14,092 Gauss, all protons will undergo the NMR transition requiring 60 Megahertz as the frequency necessary to cause the realignment discussed.

We have explained the NMR phenomenon in reference to protons only. The orbiting electrons for each nucleus create a magnetic field of their own opposing H_o. Thus, the nucleus is shielded to some extent from H_o and the proton experiences a true magnetic field of H_o (1-σ), where σ is the shielding or screening constant for that nucleus (1).

One must instrumentally increase or decrease the applied magnetic field, depending upon the type of electronic environment surrounding the nucleus (shielding) in order to allow the proton to experience approximately 14,092 Gauss and absorb a quantized energy of 60 Megahertz.

The different magnetic field strengths required give rise to unique spectra for each substance. In the case of acetic acid (Fig. 1), the proton from the hydroxyl group requires less of a magnetic field in undergoing an NMR transition because it is deshielded by the presence of the two electron withdrawing oxygens. This proton appears downfield from TMS (tetramethylsilane), a highly shielded compound generally used as a reference.

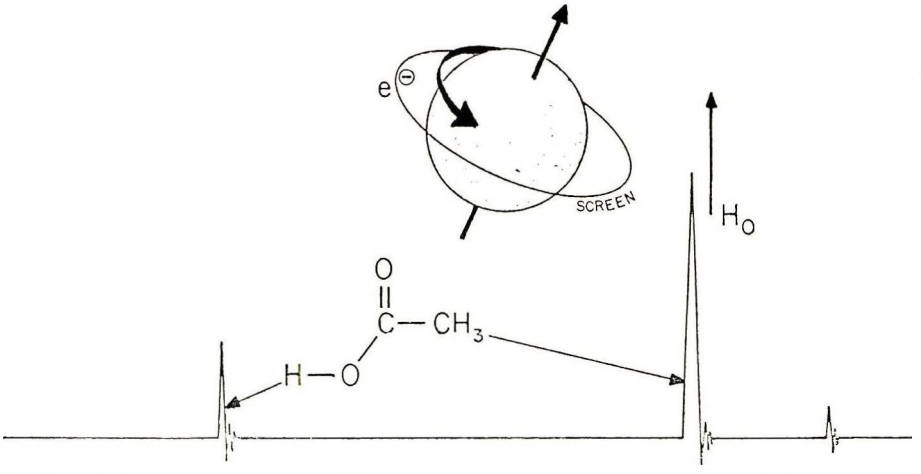

Figure 1. Spectrum of acetic acid (the separation between hydroxyl and methyl protons is caused by deshielding)

The three hydrogens on the methyl groups are more shielded and require a higher magnetic field in order for absorption and resonance to occur.

Instrumentation

A high resolution NMR spectrophotometer* is composed of five major units (2):

1. A stable magnet strong enough to generate a homogeneous magnetic field of 14,092 Gauss between its pole faces.
2. A means of varying the magnetic field over a narrow milligauss range. This is achieved by passing a direct current through coils surrounding the sample.
3. A radio frequency oscillator—supplying the radio frequency signal necessary to elevate the nuclei from the ground state energy level.
4. A radio frequency receiver to detect energy absorption by the sample.
5. A recorder and integrator.

Quantitative Analysis

The spectrum resulting from the absorption of electromagnetic energy can be used to determine the nature of the chemical environment, i.e., the number and location of protons in a molecule. The area under each absorption band is directly proportional to the number of protons which absorb at that frequency. Hydroxyl, olefinic, carboxyl, aldehydic, ester, and ethoxy protons, because of their proximity to an electron-withdrawing substituent, oxygen,

*The instrument used in this work was a Model R-24, Perkin-Elmer Corp., Norwalk, Conn. 06852.

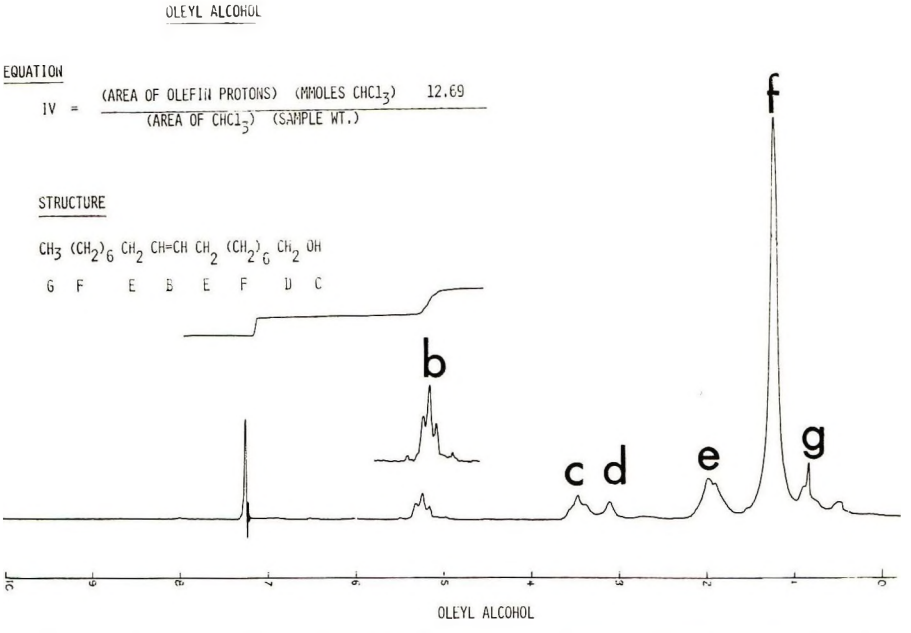

Figure 2. Spectrum of oleyl alcohol and calculation of iodine value (CCl_4 as solvent)

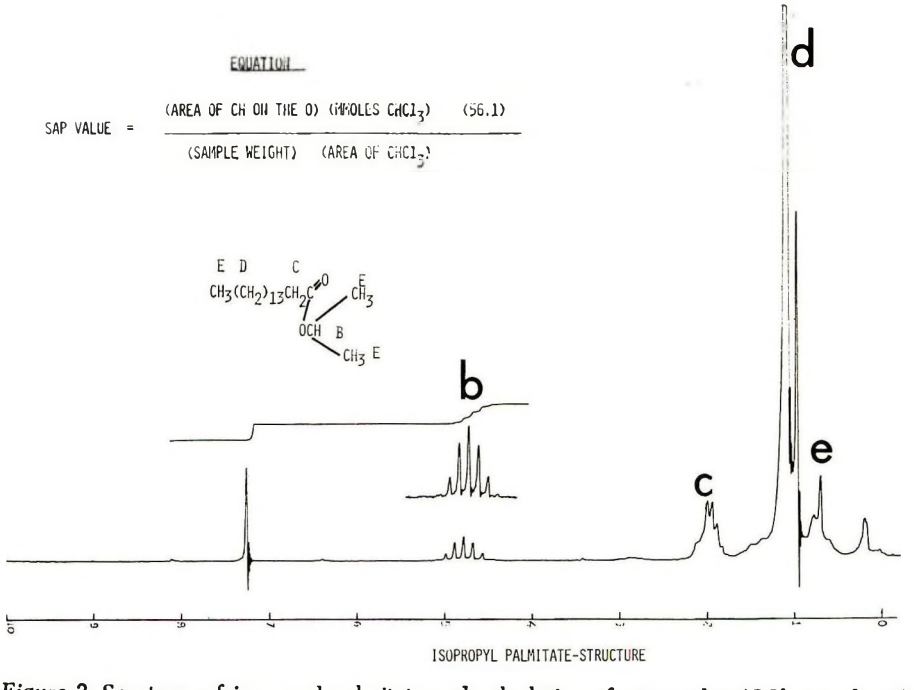

Figure 3. Spectrum of isopropyl palmitate and calculation of ester value (CCl_4 as solvent)

all appear downfield from normal methyl, methylene, and methine protons. The peaks caused by the resonance of these deshielded protons are usually well resolved from other peaks and can therefore be easily quantitated. We have calculated iodine value, hydroxyl value, ester value, and moles of ethoxylation on raw ingredients employing the internal standard method.

Internal Standard Method

This method utilizes a standard compound with a known amount of protons as a quantitative reference peak. This compound preferably should resonate as a single band and be well resolved from any other peaks. Chloroform, benzene, and TMS are examples of some commonly used internal standards. The sample, internal reference, and solvent (usually CCl_4, deuterated chloroform, or deuterated acetone) are accurately weighed directly into the NMR tube, scanned, and the resulting data are calculated. Figure 2 is a spectrum of oleyl alcohol. The equation used to calculate the iodine number using chloroform as an internal standard is:

$$\text{Iodine value} = \frac{(\text{olefin "b" area})\ (\text{Mmoles } CHCl_3)\ (12.69)}{(\text{sample wt})\ (CHCl_3 \text{ "a" area})} \quad (4)$$

Figure 3 shows an NMR application for calculating the ester value on isopropyl palmitate using the equation:

$$\text{Ester value} = \frac{(\text{area of CH on oxygen})\ (\text{Mmoles of } CHCl_3)\ (56.1)}{(\text{sample wt})\ (\text{area of } CHCl_3)} \quad (5)$$

Figure 4 depicts the ease with which an estimate of the moles of ethoxylation for a compound such as polyoxylthylene stearyl ether can be calculated. The terminal CH_3 peak is used as a reference, and because it is not well resolved only an estimate can be provided. Its area is divided by three to obtain the area per proton. Three protons must be subtracted from the ethoxylate peak, because the hydroxyl proton and the chain methylene group bonded to the ethoxylate oxygen resonate at approximately the same chemical shift. The unit structure of an ethoxylate $[(CH_2)_2O]$ reveals four protons per each mole of ethoxylation. The area per proton times four yields the area responsible for each mole of ethoxylation. Thus, dividing the total area due to the ethoxylation (corrected for the hydroxyl and methylene protons) by the area per mole of ethoxylation will result in the moles of ethoxylation in the compound under investigation.

Chain branching in ethoxylated materials disallows the use of CH_3 as a reference peak and is therefore a possible pitfall in this calculation.

The alcohol-water ratio for colognes, after-shave lotions, and many other finished products containing high percentages of these two substituents can be calculated by NMR. Figure 5 shows the spectrum for a mixture of ethyl

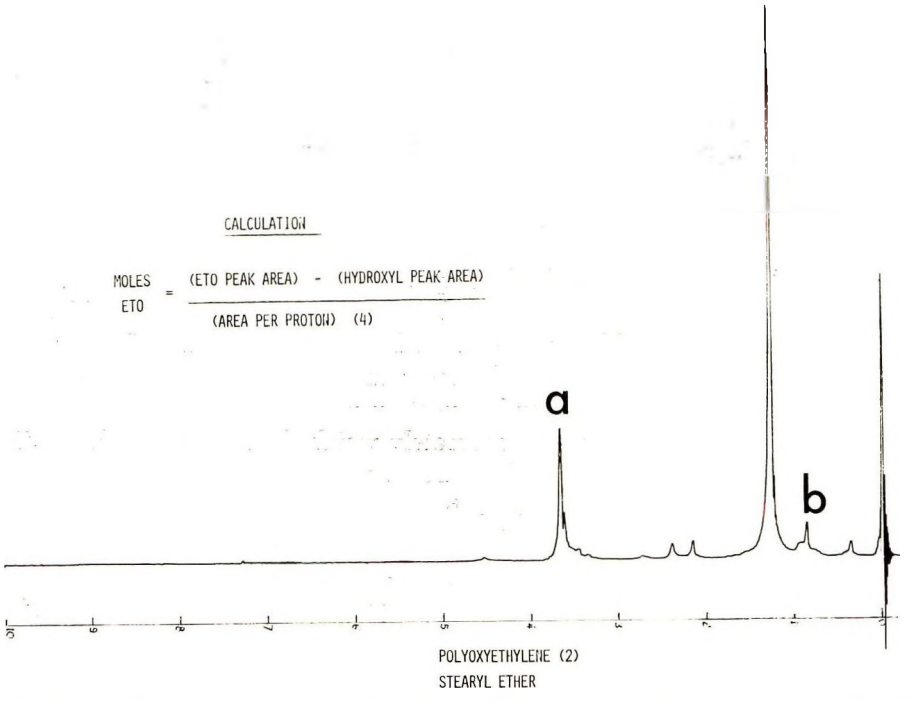

Figure 4. Spectrum of polyoxyethylene stearyl ether and calculation of average moles of ethoxylation ($CDCl_3$ as solvent)

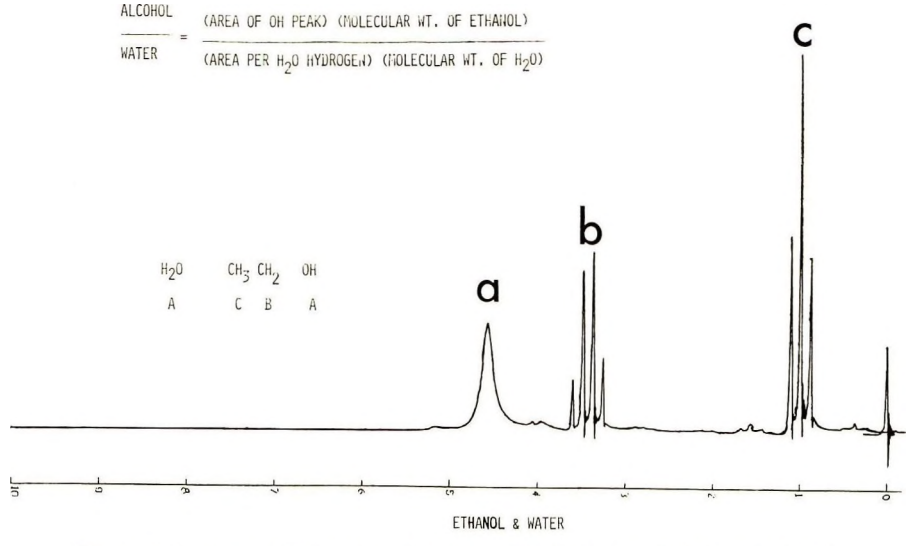

Figure 5. Spectrum of ethanol and water with calculation of their ratio (neat)

alcohol and water. The triplet at 1.0 ppm and the quartet at 3.45 ppm represent the methyl and methylene groups, respectively, of ethyl alcohol. The hydroxyl hydrogen rapidly exchanges with the water hydrogens producing a single peak which shifts with concentration. In this spectrum the broad peak appears at 4.55 ppm. Again, by using, the methyl group as a reference, we can calculate the area per proton by dividing the triplet area by three. The area produced by the OH of ethanol, which is equal to the area per proton, can then be subtracted from the OH–H_2O peak, leaving the area of the 2 water hydrogens. This area is then divided by two, producing the area for each water hydrogen. The following equation is then used to calculate the alcohol-water ratio:

$$\frac{\text{Alcohol} = (\text{area of OH peak})\ (\text{molecular wt of ethanol})}{\text{Water} = (\text{area per } H_2O \text{ hydrogen})\ (\text{molecular wt of } H_2O)} \quad (6)$$

In evaluating uncomplicated spectra, accuracy and precision of the results are largely dependent upon two variables, namely, the accuracy of the integrator and the ability of the operator to determine the various inflections relating to each peak in the spectrum.

Instrumental conditions are set so that maximum integrated inflection points are obtained and the operator's interpretation of where the peak begins (inflection from the baseline) and where the peak ends is thus minimized.

The reproducibility of the integrator is generally ±5% (i.e., areas obtained on the same sample run consecutively).

The integrator reproducibility became a problem in our analysis for ester value. The factor of 56.1 included in the calculation causes minimal errors in the integration to produce unacceptable deviations in the final values.

In order to correct this deviation, we are now undertaking raw data studies preliminary to programming an 1800 IBM Computer for NMR peak area analysis.

Table I
Comparison of NMR *vs.* Wet Analysis
Iodine Value

Compound	Specs.	Wet	NMR (3 runs)
Oleyl alcohol	89.0 – 93.0	94.6	94.0 ± 0.2
Sesame oil	103.0 – 116.0	105.0	105.6 ± 1.1
Refined avocado oil	65.0 – 95.0	83.0	83.4 ± 0.8
Isodecyl oleate	54.0 – 62.0	57.8	57.6 ± 1.3
Acetylated ethoxylated lanolin alcohol	18.0 – 22.0	...	21.7 ± 0.4
Oleyl alcohol (ETO)	30.0 – 40.0	33.2	34.1 ± 1.2
Oleyl alcohol (ETO)	19.0 – 25.0	21.0	21.6 ± 1.4

Table II
Comparison of NMR *vs.* Wet Analysis
Ethoxylation (Moles)

Compound	Suppliers Spec.	NMR (3 runs)
Oleyl alcohol (ETO)	10	10 ± 0.7
Acetylated ethoxylated lanolin alcohol	10	9.7 ± 0.9
Polyoxyethylene (2) stearyl ether	2	2.2 ± 0.3
Polyoxyethylene (2) cetyl ether	2	2.3 ± 0.1
Oleyl alcohol (ETO)	20	19.8 ± 0.6

In the analysis for iodine value, concurrent wet chemistry tests were carried out to see the comparisons of NMR results *versus* the more classical methods. Table I lists these results. Table II lists the values of moles of ethoxylation compared to the approved supplier's specification.

Summary.

The NMR spectrophotometer is no longer solely the qualitative tool of a research chemist. The cost is not prohibitive and its data can be applied to quality control work to replace time consuming wet analysis tests such as ester value, iodine value, average moles of ethoxylation, and alcohol-water ratios.

Acknowledgment

The authors would like to express their appreciation to the Communication Skills Corp. for the use of their demonstration figures.

(Received January 31, 1974)

References

(1) Flockhart, B. O., and Pink, R. C., Applications of nuclear and electron magnetic resonance in analytical chemistry, *Talanta*, **12**, 529–57 (1965).

(2) Parfitt, R. T., Nuclear magnetic resonance spectroscopy, *Pharm. J.*, **203**, 300–3 (1969).

Letter to the Editor

Sir:

We were interested to read the paper by Kligman *et al.* (1) in a recent issue of your Journal. Having been concerned with the clinical testing of antidandruff compositions for many years, we should like to comment on some of the views expressed in this paper.

The authors made the surprising assertion that dandruff is often confused with seborrheic dermatitis and that the fluctuations reported by us (2) pertained to the latter condition. We look forward to the forthcoming publication in which Kligman and co-workers promise to elaborate their views on the distinction between the two conditions. In our studies, volunteers are included only if scaling is the sole recognizable manifestation. Microscopic examination, as we and others have shown, indicates that dandruff scales usually show layers of parakeratotic and of normal corneum. This suggests that parakeratosis in a limited region of the scalp is a transient phenomenon and helps to explain our macroscopic observation that areas of scaling are not static on the scalp; the scaling is undoubtedly patchy in distribution, though often symmetrically distributed. Taking the scalp as a whole, we frequently see in untreated subjects that the overall level of scaling varies, presumably according to which areas are parakeratotic at a particular time.

There are obviously many different ways in which a clinical assessment of scaling may be attempted. Kligman *et al.* refer to our segment measuring systems as if they are unbearably tedious, but we can assure these authors that we have routinely operated them for at least 10 years quite successfully. We have felt it necessary to operate in this way partly because of our strong objection to using any procedure which itself interferes with the state of scaling on the scalp; for this reason, we do not favor Kligman *et al.'s* method of using a tongue depressor to "throw up some scurf," which could well have an unpredictable effect on subsequent scaling.

A possible explanation for the differences between our views and those of Kligman and co-workers is that they prefer to study the course of dandruff on institutionalized subjects and that they do not consider it feasible to have simultaneous test and control groups (although they sometimes apparently use such groups—as reported on page 86 of their paper). In the absence of clear information on etiology, e.g., with respect to the significance

of infection, the use of institutionalized subjects may well lead to unrecognized bias in the findings; the absence of simultaneous controls could likewise be expected to limit the validity of any conclusions drawn, partly because there would be no possibility of evaluating treatments on a double-blind basis.

Apropos the disputed periodicity of dandruff scaling, the connection between number and frequency of observations and the ability to register fluctuations should be mentioned. Obviously the spacing of observations could easily influence conclusions reached on the extent of fluctuation; we have simply reported what we have seen with frequent observations in prolonged studies. The fluctuating scaling thus recorded is in accordance with the experience of many lay volunteers, who claim that their scaling does indeed fluctuate considerably.

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P. M. Dean
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Great Britain

References

(1) Kligman, A. M., Marples, R. R., Lantis, L. R., and McGinley, K. J., Appraisal of efficacy of antidandruff formations, *J. Soc. Cosmet. Chem.*, **25**, 73 (1974).
(2) Van Abbé, N. J., and Dean, P. M., The clinical evaluation of antidandruff shampoos, *Ibid.*, **18**, 439 (1967).

Book Reviews

McCutcheon's Detergents and Emulsifiers, 1973 Annual, North American Edition. McCutcheon's Division, Allured Publishing Corp., New Jersey, 1973. 224 pages, indexed. Price $10.00. Also, 1973 Annual, International Edition. 80 pages, indexed. Price $6.00.

This year brings a two-volume publication which, as in the previous editions, very thoroughly lists the detergents and emulsifiers available. As in previous editions, classification is by trade name, manufacturer, class and formula, forms available, and recommended use applications.

The North American Edition is classified into five sections. A new section includes "experimental surfactant materials" but unfortunately consists of only two pages with a minimum of information for those products listed. Perhaps this new section will be expanded in future editions with the inclusion of results from irritation and toxicity tests performed on these experimental surfactants. The HLB Index has been expanded to include approximately 1150 compounds which range from a low value of 0.5 to a high value of 80.0. The product listing section includes such terms as "toxicologically suitable for cosmetic use" (page 51), "non-irritating foam booster" (page 109), "non-toxic characteristic" (page 129) which are vague and incomplete. A separate heading should be provided for irritancy and toxicity information for those manufacturers who deem it essential in the product description.

The International Edition is a mini-version of its older brother and contains two sections, namely (1) Product Listing and (2) Company Index by Country. Hopefully this edition will be expanded in the next edition.

For the chemist or formulator who needs a handy reference for detergent and emulsifier materials, this book will serve as a ready reference. —A. Iannacone—New York, N.Y.

Allyl Compounds and Their Polymers (including Polyolefins) by C. E. Schildknecht, John Wiley and Sons, Inc., New York, 1973. IX + 736 Pages, indexed. Price $29.95.

This book by Dr. Schildknecht, consultant and professor at Gettysburg College, Pa., surveys for the first time the preparation and the properties of allyl compounds and their polymers together with their applications in plastics, fibers, synthetic rubber, and adhesives. As is well known, numerous monomeric allyl and related compounds are

found to be useful in flavors, perfumes, pharmaceuticals, and antimicrobials. Some of the allyl flavor compounds are those of onions and mustard. The author points out that allyl compounds exhibit an amazing range of pungent odors, flavors, toxicity, and biological activity. In the 31 chapters of this book, the author covers basically all the major allyl derivative classifications, such as allyl alcohols, esters, ketones, aryl ethers, phenols, amines, phthalates, etc. The book contains little theory and only the most significant references. The introductory chapters define the allyl compounds and discuss their reactivity in comparison to typical vinyl compounds such as styrene, acrylic monomers, vinyl halides, and vinyl esters.

It is a book that is recommended for reading by anyone in the cosmetic and pharmaceutical industries who deals with flavors and perfumes. Recently, some patents have been issued on the use of some of the allyl polymers in cosmetics. The book is written in a language which is simple to read and comprehend, and will offer no problem to either a beginner or expert in the field.—H. Y. Saad—Avon Products, Inc.

Correction

The correct title of the book reviewed on page 109 of the February 1974 issue of this Journal should be Kosmetikum Feinseife, Abriss seiner Technologie, by Dieter Osteroth; in Abhandlungen aus den Interessengebieten der Kosmetik, edited by Hans Freytag. Dr. Alfred Huthig Verlag GmbH, Heidelberg, 1972. x + 152 pages, illustrated and indexed.

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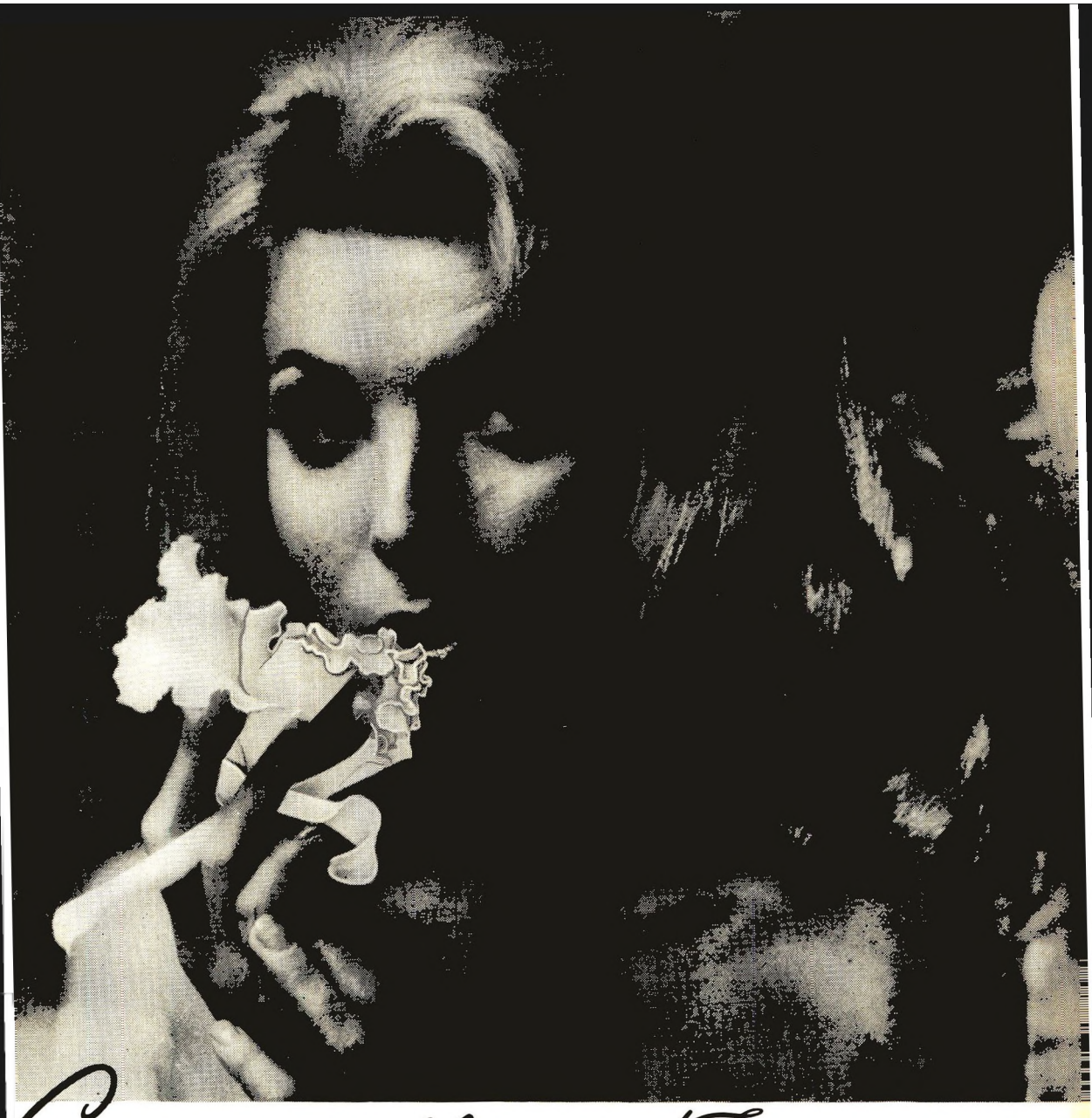

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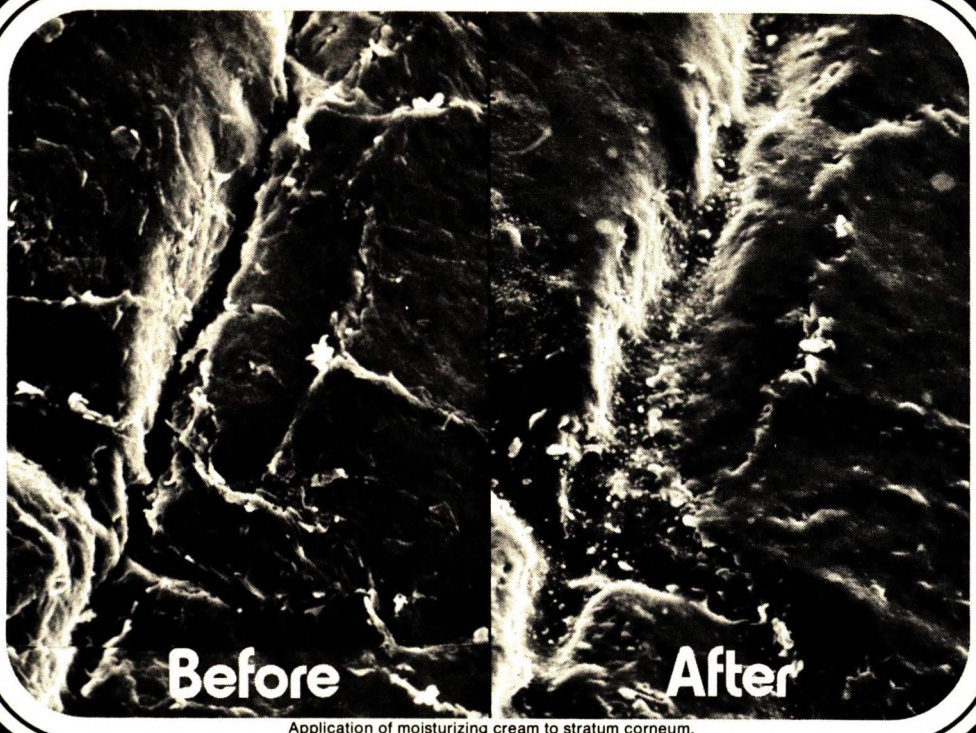

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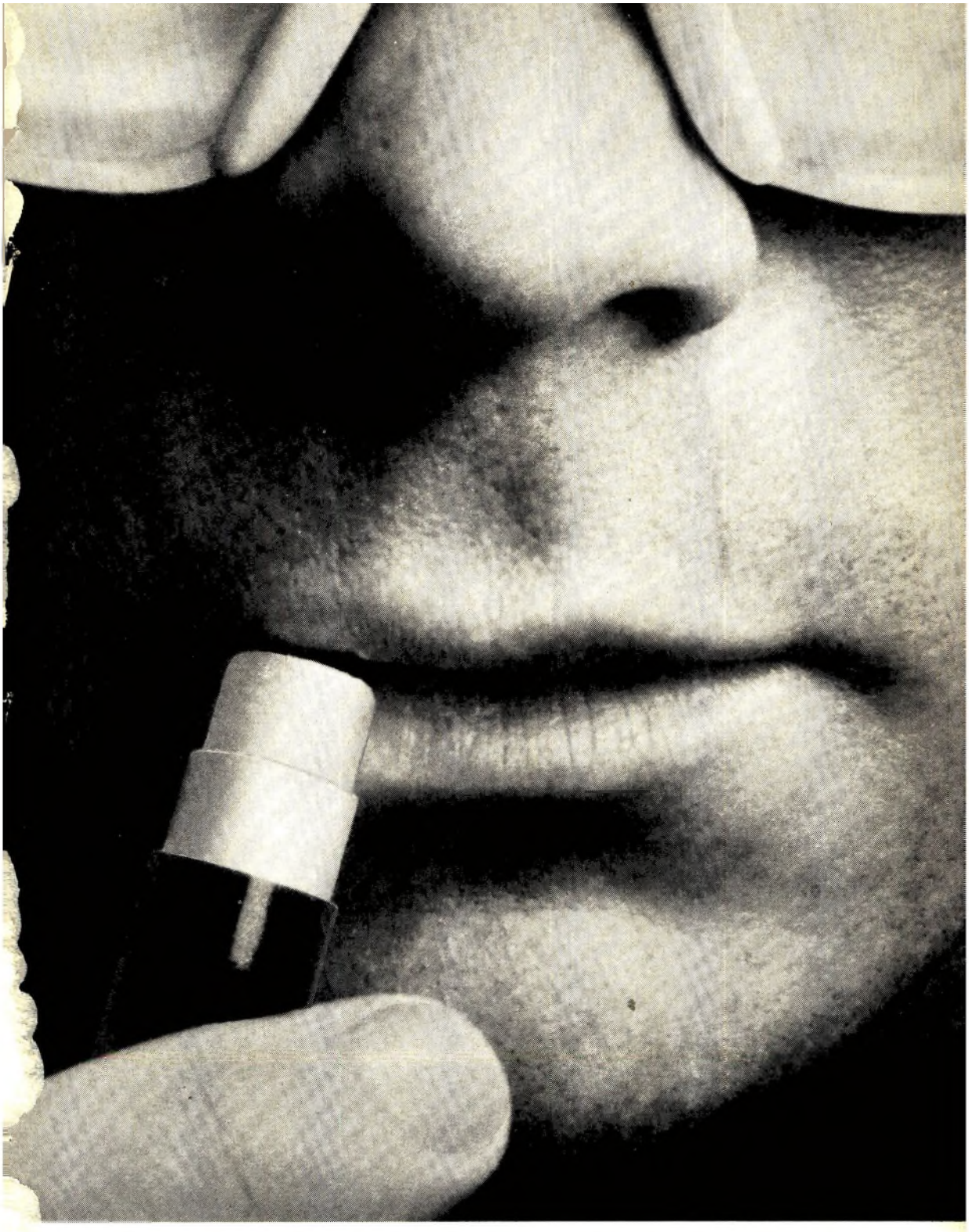
Amerchol ..... Outside back cover
Croda, Inc. XXXVIII
Dragoco Inc. IV
Elias and Company XXIV
Evans Chemetics, Inc. I
Felton International, Inc. XIII
Florasynth, Inc. IX
Fritzsche, Dodge & Olcott, Inc. ..... Inside back cover
Givaudan Corp. ..... Inside front cover
Halby Div. Argus Chem. Corp. III
ICI United States, Inc. XV
International Flavors & Fragrances XXXII
Knapp Products, Inc. XXX
Lonza, Inc. XXVI
Lonza, Inc. XXXVI
Mallinckrodt Chem. Works/Washine Div. XXXVII
Mallinckrodt Cosmetic Chem. XIV
J. Manheimer, Inc. XXXIII
Norda XXII
Noville Essential Oil Co. XXVI
Parento, Compagnie, Inc. XXVII
Penreco, Inc. X
Perry Bros., Fragrances XXIX
Reheis Chemical Co. XVI
REWO Chemical Co. ..... VI-VII
R.I.T.A. Chemical Corp. VIII
Robeco Chemical Co. XII
Robinson Wagner Co. XXVIII
Shaw Mudge & Co. XXX
Structure Probe, Inc. XXXI
Ungerer & Co. XXI
Van Dyk & Co. XXIII
Albert Verley & Co. XI
Whittaker, Clark & Daniels, Inc. XXV
Witco Chemical Co. XXXV



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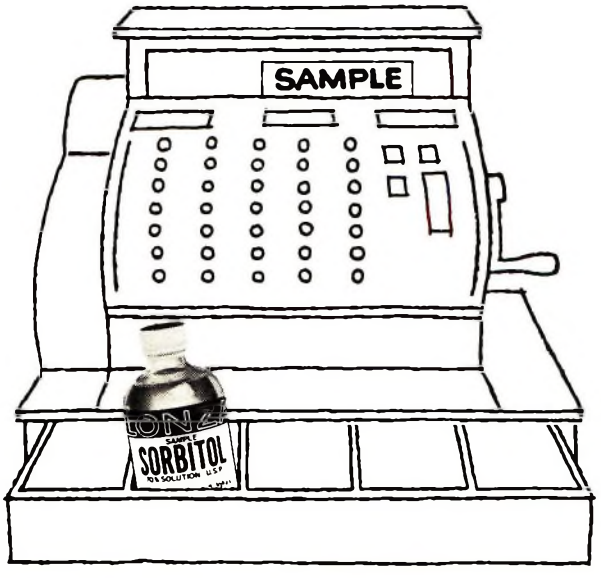

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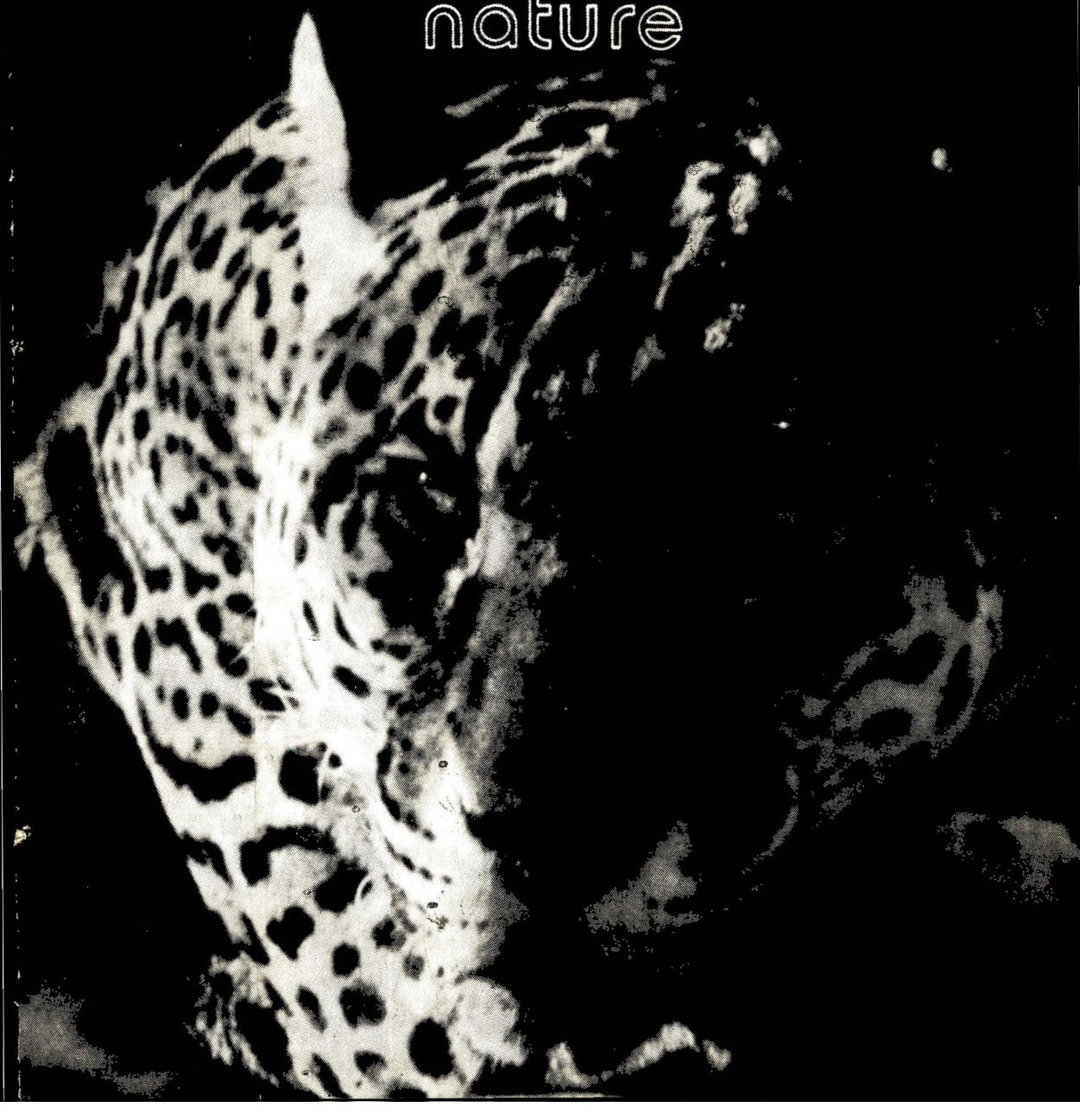

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