Stress-Relaxation Studies on Hydrated Callus Strips II

Effects of Some Electrolytes and Nonelectrolytes in Aqueous Solutions

By WILLIAM J. TILLMAN* and TAKERU HIGUCHI

Mechanical responses of stretched, hydrated callus strips to several electrolytes and nonelectrolytes in dilute aqueous solutions have been determined. At equal concentrations of cations (of the order of 0.1 *M*), sodium chloride, potassium chloride, lithium chloride, and sodium sulfate produced marked decreases in tension essentially indistinguishable from each other. Magnesium chloride elicited a similar response but at much lower concentrations. The limiting values at higher concentration were, however, roughly the same as those produced by the monovalent cations. The astringency of aluminum ion was clearly reflected in the behavior of the cornified strip to its salts. Responses to urea and dextrose solutions have also been studied.

RESULTS OF further investigations on the mechanoelastic behavior of cornified epithelium elicited by changing solution environments are presented. In an earlier publication (1) the solvating and softening effects of several hydroxylic solvents were reported. The present communication is concerned with the results of similar experiments designed to determine the influence of certain nonelectrolytes and electrolytes on the tendency of stretched callus strips in aqueous media to shrink or relax. Information of this nature is presumed to be of value in the rational formulation of preparations applied to the skin for either cosmetic or medicinal purposes. The degree of solvation and hydration of the cornified layer, the primary factor in these measurements, is becoming increasingly recognized as a major concern in maintenance of the health and appearance of the outer epidermal layer.

This report is concerned specifically with results obtained on hydrated callus strips when exposed to aqueous solutions of sodium chloride, potassium chloride, lithium chloride, magnesium chloride, aluminum chloride, several sulfates, dextrose, and urea.

EXPERIMENTAL

The technique and strain-gauge apparatus employed in this study have been previously described by Tillman and Higuchi (1). The only significant modification for this study was an insertion of a preamplifier in the circuit before the recorder to increase the sensitivity of the method.

Materials.—The chemicals used in this study were reagent grade unless otherwise noted. The callus strips used in this study were prepared as previously described (1).

Received March 23, 1964, from the School of Pharmacy, University of Wisconsin, Madison.
Accepted for publication April 27, 1964.
* Present address: Smith Kline and French Laboratories, Philadelphia, Pa.

General Procedure for Obtaining Stress Relaxation and Modulus Data.—Six to 12 hours prior to a run a dehydrated callus strip was removed from a desiccator. The strip was extracted twice for 30-minute periods in fresh anhydrous ether and stored in distilled water at 5°. Just prior to use, the strip was wiped dry, measured, and quickly weighed. The strip was then clamped between the two stainless steel clips of the apparatus (with the ridges and grooves of the callus running parallel to the applied force) and immersed in distilled water at 24 ± 1°.

The continuity of the strip was tested by applying a tension of approximately 20 Gm. (10×10^6 dynes/cm^2). If no breaks, tears, or holes appeared, the strip was considered suitable for further experimentation. A load of approximately 2.5 Gm. (12.5×10^5 dynes/cm.^{2}) as indicated by the recorder next was applied sharply to the strip by extending it, and the relaxation of this force at constant extension was followed on the recorder. After equilibration (about 45 minutes), the additional force required to extend the fiber 1% of its length (the 1% index) was determined; the length of the strip between the two clips was measured with a cathetometer. The fiber was stretched and immediately returned to its original length, the force response being automatically followed on the recorder chart.

After allowing a short time for the reattainment of equilibrium, the distilled water in the chamber was replaced by the solution under study, the force reading being recorded continuously. After a 15-minute exposure to this solution, the above readings were repeated. In most cases the strip was then removed from the solution and again immersed in distilled water to observe whether the effect was reversible. Each callus strip was used once.

RESULTS AND DISCUSSION

Effects of Extraction on Callus Strips.—When stress-relaxation studies were carried out in aqueous solutions on several unextracted hydrated callus strips, responses were variable. However, successive extraction with ether and water yielded specimens which behaved reproducibly. This phenomenon may be explicable at least in part on the basis of a study by Blank (2), who reported that the extraction of strips of cornified epithelium with lipid solvents, *e.g.*, ether, was necessary for the subsequent extraction with distilled water of hydro-

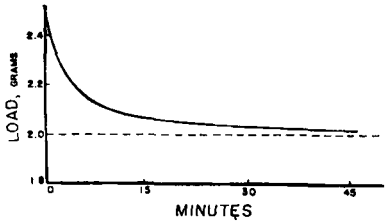

Fig. 1.—Typical stress-relaxation phenomenon observed when a hydrated, extracted callus strip was placed under an initial load while immersed in distilled water.

philic material normally present in the strips. Varying concentrations of this component in the unextracted callus strips may be responsible for the differences observed.

Before ether extraction, a dehydrated strip appeared translucent and straw-yellow in color. After ether extraction, the strip became opaque-white, similar in appearance to a hydrated unextracted strip. The ether-extracted strip was less flexible than the unextracted strip and appeared to imbibe significantly less water on rehydration. Extraction with water, followed by dehydration, left the strip translucent and off-white in color. Upon hydration, the ether-water extracted sample appeared opaque along the groove areas and translucent to slightly cloudy in the ridge areas. The unextracted strips, on the other hand, became completely opaque-white following hydration.

Stress-Relaxation Behavior of Extracted Callus Strips in Distilled Water.—Figure 1 depicts a typical stress-relaxation phenomenon observed when a hydrated callus strip, subjected to ether-water extraction as described, was immersed in distilled water and placed sharply under a 2.5-Gm. load. The relaxation under constant extension appeared to involve two parallel processes. The relatively fast exponential decrease in tension observed during the early phase may be attributed to changes in the amount of water held by the protein structure. This initial rapid decrease, which seemed to be completed within about 10 minutes, appeared to be followed by a very slow continuing decrease in tension as indicated in the figure. A similar relaxation effect noted with wool fibers has been ascribed to the slipping of polypeptide chains over one another, a result of scission of side-chain bonds (3).

A typical chart recording obtained during the course of these studies is presented in Fig. 2. This particular record depicts the response elicited by a 0.1 *M* magnesium chloride solution. The modulus (1% index) measurements are recorded as perpendicular lines and are labeled 1%. All subsequent figures depicting load *versus* time curves are tracings of the actual recordings.

Effect of Dextrose.—The response of extracted callus strips, initially equilibrated with distilled water and under load, to an isotonic solution of dextrose (0.30 molal) is shown in Fig. 3. The behavior depicted in the recorded plot and those given in the succeeding sections for other systems were at least qualitatively reproducible even when using strips from various sources. Quantitative reproducibility was unusually good in experiments in which the strips used were from a single callus source and best when the strips were from a single layer. Relative reproducibility of such systems is discussed in greater detail under *Effect of Sodium and Magnesium Chloride.*

As can be seen in the plot, the switch from distilled water to dextrose solution resulted in a slight increase in tension, followed by a relaxation of equal magnitude. The sharp initial rise in stress can probably be ascribed to a change in the degree of hydration of the strip (osmotic effect); the subsequent relaxation may be the result of either a minor molecular reorientation or, more probably, a diffusion of dextrose into the interior of the hydrated strip thereby restoring the system to its initial osmotic state.

The reversibility of the observed phenomenon was demonstrated by reimmersing the callus strip in distilled water. As can be seen in Fig. 3, a slight decrease in tension, followed by a shrinkage of equal magnitude, was recorded. The callus strip apparently had returned to its original state. These observations were further substantiated by 1% index measurements which showed no apparent change in magnitude both before and 15 minutes after immersion of the strip in the dextrose solution and distilled water.

Effect of Urea.—Small decreases in tension and a small decrease in the 1% index were observed when a stretched callus strip was immersed in an isotonic (0.30 molal) solution of urea. These responses were reversed by replacing the strip in distilled water. No significant change in the appearance of the strip was noticeable.

With a more concentrated 6 *M* urea solution, a very large relaxation was recorded, even though the strip became completely translucent upon immersion in the solution. There was also a very large decrease, amounting to approximately 60%, in the 1% index measurements. The loss in opacity of the strip

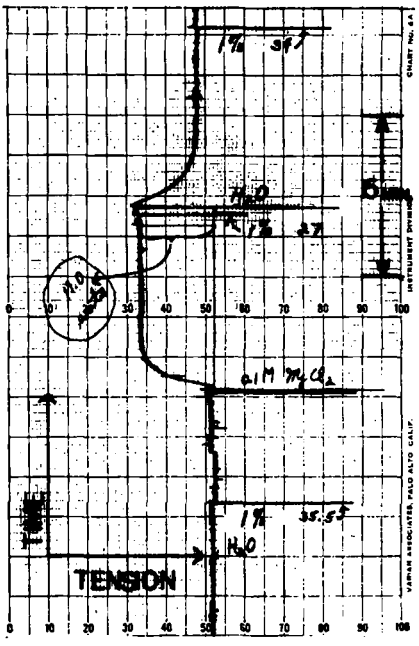

Fig. 2.—Photograph of actual chart recording obtained during the study of the effect of 0.1 *M* magnesium chloride on a hydrated, extracted callus strip. 1% ≈ 1% index (in chart units).

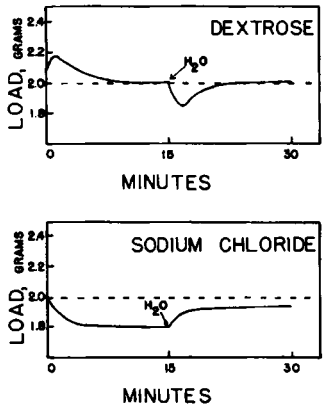

Fig. 3.—Change in load maintaining hydrated, extracted callus strips at a fixed extension upon the immersion of the strips into isotonic dextrose and isotonic sodium chloride solutions. Response obtained upon reimmersion of the treated strip into distilled water is also presented.

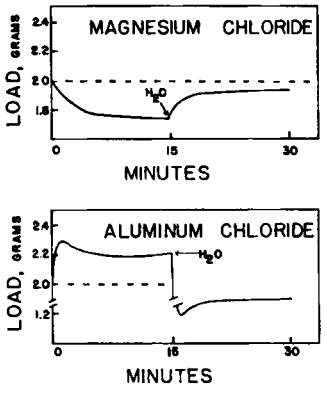

Fig. 4.—Same as Fig. 3, but for isotonic magnesium chloride and isotonic aluminum chloride.

was most probably due to the dehydration of the strip caused by the osmotic properties of the highly concentrated urea solution. This effect, if it were the only thing happening in the system, would have caused a significant increase in tension. But this response was probably obscured by the effect of the urea on the protein, resulting in relaxation of the forces maintaining this strip at constant length.

It is obvious from these results that urea does not behave as a simple nonelectrolyte (like dextrose) but does, in some manner, affect the structural components of the system. Possibly, the reported ability of urea in high concentrations to alter protein hydrogen bonding may be involved. Contrary to these observations were those reported by Matoltsy and Balsamo (4), who reported that epidermal keratin was not visibly affected by neutral solutions of urea (10 to 50%), whereas it was readily dissociated in alkaline urea, such as 50% urea in 0.02 *N* sodium hydroxide. Their conclusions were based on double refraction data.

Effect of Sodium and Magnesium Chloride.—Immersion of a hydrated extracted callus strip, under load, into either an isotonic solution of sodium chloride (0.163 molal) or magnesium chloride (0.113 molal) usually produced an immediate and lasting decrease in tension as shown in Figs. 3 and 4; the extent of relaxation was always somewhat greater for magnesium chloride. In some instances a very slight initial increase in tension was noted before the relaxation occurred; this was thought to be an osmotic effect. This effect was small and usually obscured by the tension decrease caused by the salt. The effect of the salt ions on the ionic linkages existing between the protein chains of the callus may be responsible for the relaxation effect. The forces between the ionized parts of the protein system would be expected to be substantially reduced by any increase in the ionic strength of the environment. One per cent index measurements made before and after immersion of the strip in the salt solution also decreased in magnitude.

Reimmersion of the treated strips in distilled water did not appear to reverse the effect of sodium or magnesium chloride completely. This failure to return, within 15 minutes, to the initial equilibium tension was reflected in the 1% index measurements. They, too, did not return to their original values. It is possible, however, that a more extended soaking period might have reversed completely the effect of these salts.

Additional experiments performed with sodium and magnesium chloride indicated that the absolute change in force was dependent upon thickness of the strip and the concentrations of the salt solution employed. As is evident in Fig. 5 a curious sort of a straight-line relationship seems to exist between the change in the residual tension and the thickness of the specimens. The initial load applied to each strip in these measurements was approximately 2 Gm. Because of the variation in the thickness of the strips (ranging from 40 to 100 μ), force per unit area (tension) varied from about 10 to 25 $\times$ 10^5 dynes/cm.^{2}.

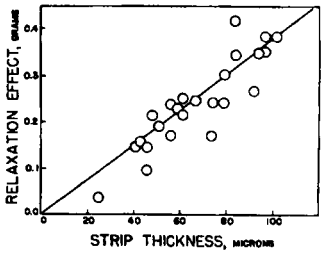

Fig. 5.—Dependency of the relaxation response elicited by isotonic sodium chloride solution on the thickness of the hydrated, extracted callus strips.

TABLE I.—AVERAGE REDUCTION IN FORCE PER UNIT AREA PRODUCED BY SODIUM CHLORIDE SOLUTION ON HYDRATED CALLUS STRIPS CUT FROM VARIOUS SPECIMENS

Specimen	N[a]	$\bar{x}$,[b] (dynes/cm.^{2}) $\times 10^{-5}$	S.E.,[d] (dynes/cm.^{2}) $\times 10^{-5}$
1	13	1.99	0.06
2	10	1.91	0.08
3	4	1.34	0.31
4	4	1.65	0.11
5	2	1.96	0.15
		Av.[c] = 1.77	0.14

[a] N = Number of runs per sample. [b] $\bar{x} = \Sigma x/N$, where x equals the reduction in tension for each run per sample. [c] Av. = $\Sigma\bar{x}/5$. [d] S.E. = Standard error = standard deviation/$\sqrt{N}$.

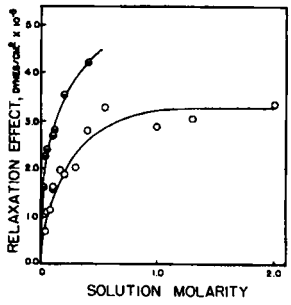

Fig. 6.—Dependency of the relaxation response on the concentrations of sodium chloride and magnesium chloride. Key: Upper curve $MgCl_2$; lower curve NaCl.

The reduction in tension at constant extension produced by the salt solutions was quite reproducible when expressed as changes in force per unit area (see Table I). An average decrease in tension of 1.77×10^5 dynes/cm.^{2} was elicited by the sodium chloride solution on 33 individual strips from five different callus sources (the mean of the average values from each source). The average decrease in tension obtained for isotonic magnesium chloride was of the order of 2.8×10^5 dynes/cm.^{2}, which appears to be significantly larger than the value obtained for sodium chloride. The changes in modulus (1% index measurements) produced by the salt solutions appeared to parallel the reduction in tension.

The relationship observed between the concentration of salt present and the decrease in tension elicited is evident in the results presented in Fig. 6. It can be seen from the plot that for both the sodium and magnesium chloride systems the response was fairly proportional to concentration in the low concentration range; but at higher concentrations it appears to level off, suggesting a limiting effect. An equation representing such a situation is: $1/\text{response} = (1/k_1C) + (k_2/k_1)$. Plots of the reciprocal response *versus* reciprocal concentration as shown in Fig. 7 appear to yield straight lines for both salt systems. The near identity of the intercept values strongly suggest that the limiting effects of the two cationic species are the same. It would appear that the response is produced by a process similar to an ion exchange process and that the full effect is produced when the system is saturated, irrespective of the nature of the particular cation.

Effect of Aluminum Chloride.—Transfer of a hydrated extracted strip from distilled water to isotonic (0.098 molal) aluminum chloride solution resulted in a rather large initial increase in tension (astringency) which reached a maximum within approximately 1 minute. This increase was followed by a momentary relaxation, then by a very gradual increase in tension. At the end of 15 minutes, a very slight decrease in the 1% index was recorded. The reimmersion of the strip in distilled water resulted in a large relaxation as shown in Fig. 4. This large change was considered to be due to the alteration in protein structure caused by both the effect of the aluminum ion and the acidic pH of its solution.

A significant factor in the effect of aluminum chloride solution was the pH of the solution. An isotonic aluminum chloride solution had a pH of 3.17, while isotonic concentrations of sodium and magnesium chlorides did not alter the pH of distilled water significantly. A more detailed study of the effect of pH on the effect of the various salt ions will be described in a subsequent report.

The binding of aluminum ion to powdered callus has been reported by Lyon and Klotz (5). Their investigation also included studies of Al^{3+} binding by human skin (surface scrapings) and hair. They found that aluminum ion was bound by each and in the following decreasing order: callus > skin > hair cuticle. It was suggested that the primary sites of Al^{3+} [and $Al(OH)^{2+}$ and $Al(OH)_2^+$] sorption were the carboxylate groups of the protein. The extent of binding was found to be dependent upon pH.

The reason for the initial increase in tension observed with aluminum chloride, as opposed to the decrease noted with sodium and magnesium chlorides, is probably the ability of the tripositive aluminum ion to react with more than one carboxylate group. The Al^{3+} could possibly bind with carboxylate groups from more than one chain causing a tightening of the structure.

Effect of Other Inorganic Ions.—Studies performed with lithium chloride and potassium chloride indicated that the lithium and potassium ions produced effects essentially the same as those of the sodium ion. No differences could be detected within the sensitivity of the method employed.

Runs were also made in which the sulfate salts of sodium, magnesium, and aluminum were employed. In unbuffered aqueous solutions the concentration of the cation was the important variable. Equivalent relaxation factors were obtained with solutions containing 0.1 *M* sodium chloride and 0.05 *M* sodium sulfate and similarly with 0.1 *M* magnesium chloride and 0.1 *M* magnesium sulfate. In the case of aluminum chloride and aluminum sulfate no quantitative data were collected because of the type of response elicited by these salts, but qualitatively these substances yielded similar responses. The major difference between the two salts was that the rather pronounced relaxation following the initial increase in tension obtained with aluminum chloride was almost completely missing in the aluminum sulfate response. It was noted also that in the case of aluminum sulfate the change in the 1% index reading was greater than 100% of the original value.

GENERAL DISCUSSION AND CONCLUSIONS

Data presented in the preceding section suggest that stress-relaxation measurements of this type can yield information of significant interest to those

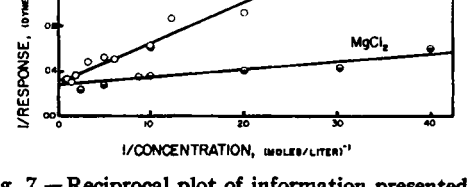

Fig. 7.—Reciprocal plot of information presented in Fig. 6 on solution molarity.

concerned with influences of various solutes and vehicles on the physical states of cornified epithelial tissues. The presence of a natural hydrating factor which can be removed by the ether-water treatment appears to be clearly established by these studies. It is further evident that very dilute aqueous solutions of various salts and nonelectrolytes have pronounced effects on the configuration of the protein structure.

Some of these observations may be pertinent in formulation of skin preparations. Although the astringency of aluminum ion is well known, observation of the effect of sodium, potassium, lithium, and magnesium ion in producing relaxation of stressed tissue, even in dilute solutions, appears to be new. This behavior seems to be in opposition to the dehydrating effect normally produced by the presence of solutes in water.

REFERENCES

(1) Tillman, W. J., and Higuchi, T., *J. Invest. Dermatol.*, 37, 87(1961).
(2) Blank, I. H., *ibid.*, 21, 259(1953).
(3) Meredith, R., "The Mechanical Properties of Textile Fibers," Interscience Publishers, Inc., New York, N. Y., 1956, p. 13.
(4) Matoltsy, A. G., and Balsamo, C. A., *J. Biophys. Biochem. Cytol.*, 1, 339(1955).
(5) Lyon, I., and Klotz, I. M., THIS JOURNAL, 47, 509 (1958).

Procedure for Assay and Stability Determination of Idoxuridine

By CHARLES A. SIMPSON and ALFRED F. ZAPPALA

In acid solution, idoxuridine (5-iodo-2′-deoxyuridine) is decomposed by light to form 2′-deoxyuridine and material whose U.V. absorption has been destroyed. Idoxuridine is hydrolyzed by heat to form 5-iodouracil and 2-deoxyribose in nearly quantitative yield; a small amount of 2′-deoxyuridine is also formed. A procedure for assay of these mixtures is given. The method involves a chromatographic separation on a partition column with a standing phase of 0.1 *N* HCl and a moving phase of chloroform plus *n*-butanol (5 : 1 by volume). That portion of the eluate containing the idoxuridine is analyzed by its U.V. absorption. Data are presented to show the accuracy and precision of the method.

IDOXURIDINE (5-iodo-2′-deoxyuridine) was first synthesized in 1959 by Prusoff (1). Following its synthesis, several papers were published in connection with its effect on transplantable neoplasms (1–3). In addition, it was studied in its role as a bacterial inhibitor (1, 4). More recently, Kaufman has shown that it is an agent of proven value in treatment of herpetic keratitis in man (5). This has been hailed as the first chemotherapeutic agent effective against a specific virus. Because of this growing biochemical and medical interest in idoxuridine, we undertook to study its decomposition and find a method for stability determination. This report describes the results of that effort.

EXPERIMENTAL

Study of Light-Initiated Decomposition.—Unbuffered aqueous solutions of idoxuridine (1 mg./ml., pH = 5–6) were placed in quartz containers and irradiated with a mercury vapor arc lamp. Figure 1 shows that the U.V. absorption spectra of these solutions decrease in intensity with an increase in time of exposure. The nature of this decomposition product was not studied, since it appeared to offer no interference with an ultraviolet method of determination. Moreover, the photolytic decomposition of related compounds such as uridine and uracil has been studied by Sinsheimer and Hastings and many others (6). These studies showed that the product formed is the 4-hydroxyhydro derivative.

Another considerably less severe irradiation experiment was conducted with a Sylvania type RS sunlamp, where the spectrum includes portions of the I.R. and visible as well as the U.V. ranges. As previously, the solutions irradiated were unbuffered and aqueous (1 mg./ml., pH = 5–6). After 3 months of exposure, the solution was spotted on paper along with a known solution of some suspected decomposition products, and a chromatogram was developed in a butanol–3 *N* ammonia system. The results of this study are shown in Table I.

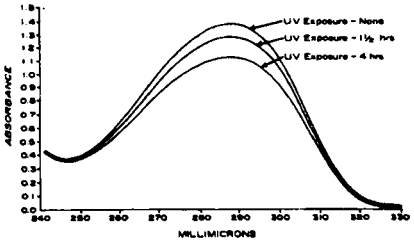

Fig. 1.—U.V. absorption spectra of aqueous idoxuridine exposed to U.V. light.

Received February 11, 1964, from the Research and Development Division, Smith Kline and French Laboratories, Philadelphia, Pa.
Accepted for publication April 27, 1964.
The authors thank Mr. Alex Post of our laboratories for his paper chromatographic work which assisted greatly in this investigation. Recognition is also given to Dr. Herbert Winicov, under whose direction the pure idoxuridine standard was prepared.