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THE DIFFERENT EFFECTS OF THE SORPTION OF CALCIUM AND OF SODIUM IONS ON THE SWELLING OF HIDE COLLAGEN*

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ABSTRACT

Swelling of hide collagen and the absorption of calcium and sodium ions by hide collagen reach equilibrium levels in very short time intervals. Therefore, measurements of these phenomena can be made before secondary changes occur in the collagen. Quantitative measurements of the amount of calcium and sodium absorbed indicate that calcium ions are more firmly bound than sodium ions and that calcium ions have a limited absorption, while sodium ions appear to be absorbed in the multilayer water of hydration. The concentration of sodium ions in this bound water appears to be closely related to the concentration of the sodium ions in the surrounding solution. Increasing the salts present in the multilayer bound water increases the degree of swelling. The calcium ions bound on specific sites do not have much effect on swelling in these solutions containing sodium ions.

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INTRODUCTION

The importance of lime treatments to the leather and gelatin industries has stimulated a vast amount of research on the effect of lime and lime with various additives on hides and purified collagen. Many of these studies were reviewed recently by Lollar (1). The swelling phenomena of collagen were also critically reviewed by Gustavson (2). Some of the most elaborate and interesting studies have been reported by Bowes and Kenten (3-7). These workers have studied the swelling of collagen under both acid and alkaline conditions, employing different acids and bases, and including the influence of added salts on the swelling produced by these reagents.

There appears to be a considerable difference in the degrees of swelling produced by sodium and calcium hydroxides at comparable alkalinities.

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The swelling in lime solutions is considerably less than in sodium hydroxide solutions even when allowance is made for the bivalent nature of the calcium ions (7). The effect of neutral salts added to lime solutions is also different from the effect when the same salts are added to sodium hydroxide solutions. When sodium chloride is added to sodium hydroxide solutions, the swelling effect on collagen is decreased (6). In contrast to this the addition of sodium chloride to lime solutions produces an increase in the swelling action (4).

These observations indicate that there must be some difference in the mode of action of calcium and sodium ions on the swelling phenomena of collagen. The previous studies have been concerned mostly with the relationship of the composition of the solution to the swelling phenomena. The present study is concerned chiefly with the relationship of the ions absorbed to the swelling phenomena. Because of the apparent difference in action between calcium and sodium ions, some experiments were arranged to study their behavior when they were in a competitive situation, and the change in the ratio between the ions in the solution and the ions absorbed in the collagen was determined.

EXPERIMENTAL

Preparation of collagen.—Fresh hides were washed with water and 8% salt solution, fleshed, clipped, and split to obtain the center layer. The center layer was then extracted three times each with 8% salt, acetone, water, half-saturated lime water, water, dilute acetic acid, water and acetone. The air-dried pieces were cut into thin shreds with a paper cutter. They contained 18.1% nitrogen, 0.65% amide nitrogen (3.6% of the total nitrogen), and 13.6% moisture. There was 0.045 millimoles of calcium and 0.046 millimoles of sodium in each gram of dry collagen.

Swelling studies.—Two grams of the air-dried shreds were weighed in a covered Petri dish and then put into a covered glass dish containing 50 ml. of the lime or sodium hydroxide solutions, or 100 ml. of the salt-containing solutions. At completion of the swelling period the shreds were removed from the solution, blotted twice on clean cotton towels, placed in the covered Petri dish in which they were originally weighed, and weighed to determine the increase in weight. The increase in weight plus the weight of water originally present in the air-dried shreds was used to express the moisture of the swollen sample. The swelling data are, therefore, expressed as grams of water per grams of moisture-free collagen.

The swelling studies in salt solutions were all of one-hour duration, and 100 ml. of the salt solution was used. Where the studies were performed at pH 12.0 the pH was adjusted continuously throughout the run by adding 1*N* sodium hydroxide from a burette. The pH was determined with a high-alkalinity glass electrode calibrated at pH 12.5 with a suspension of calcium hydroxide.

For the few solutions containing only calcium salts the adjustment to pH 12.0 was made using lime water in place of the 1*N* sodium hydroxide so that these solutions would be free of sodium ions.

Analysis of lime and sodium hydroxide solutions.—Ten ml. of the solution remaining after the removal of the shreds was titrated with 0.1*N* hydrochloric acid to determine the concentration of lime or sodium hydroxide remaining. The original volume of solution minus the volume absorbed (measured by the increase in weight of the shreds) times the concentration of the final solution was used as a measure of the lime or sodium hydroxide not absorbed by the shreds. The difference between this quantity and the quantity added was considered a measure of the amount absorbed by the collagen.

Twenty ml. of the residual solution was analyzed for nitrogen by a semi-micro Kjeldahl procedure. The total amount of nitrogen extracted was calculated from the result of this analysis and the calculated volume of the residual solution.

Analysis of the salt solutions.—Calcium was determined by a Versene titration method (8). Ten ml. of the solution remaining after the shreds had been removed was transferred to a 3-inch casserole and adjusted to approximately 100 ml. Two ml. of 10% potassium cyanide was added, and the solution was adjusted to pH 12.5 with 10% potassium hydroxide. Then 200 mg. of Cal Ver II indicator was added, and the calcium was titrated with a standard solution of disodium dihydrogen ethylenediamine tetraacetate.

Sodium was determined by the uranyl zinc acetate method (9). One ml. of the solution to be analyzed was added to 15 ml. of the zinc uranyl acetate reagent and, after standing, the precipitate was filtered off in a sintered glass crucible, washed according to the method, dried, and weighed.

Analysis of treated collagens.—The blotted and weighed shreds from the swelling experiments were put into large platinum dishes and ashed at 600°C. for four hours. The ash was dissolved in a small amount of hydrochloric acid and made to 25 ml. volume, and aliquots of this solution were used for the calcium and sodium determinations previously described. The recoveries of both calcium and sodium varied between 98% and 102% of the amounts added.

RESULTS AND DISCUSSION

Previous studies of the swelling phenomena of lime and sodium hydroxide have tried to simulate actual tannery conditions as to time and composition of the solutions. No studies have been reported where the treatment was less than three days. The data of Table I show that the swelling of collagen produced by both dilute sodium hydroxide and lime water solutions reaches

TABLE I
LIME- AND ALKALI-SWELLING OF COLLAGEN

Solution	°C.	Grams Water/Grams Dry Collagen				
		1 hr.	2 hr.	after 4 hr.	17 hr.	24 hr.
Water	25	1.59	1.60	1.59		
0.1% NaOH	25	2.62	2.80	2.81	2.83	
Half-saturated Ca(OH) ₂	25	1.92	1.90	1.94		1.86
Saturated Ca(OH) ₂	25	1.98	2.04	2.04		2.05
0.1% NaOH	8	2.56	2.77	2.63	3.04	
Half-saturated Ca(OH) ₂	8	2.12	2.01	2.06		2.18
Saturated Ca(OH) ₂	8	2.12	2.12	2.19		2.08

an equilibrium value within one or two hours. The equilibrium value is maintained for at least 24 hours. It is also quite apparent that the lime solutions do not cause as much swelling as the sodium hydroxide solution. This agrees with the observations of Bowes and Kenten (4, 6) for experiments of longer duration. Furthermore, while the swelling produced by sodium hydroxide is decreased slightly by lowering the temperature, the swelling produced by lime solutions is increased slightly by lowering the temperature.

The half-saturated lime solution was originally almost equivalent to the sodium hydroxide solution in basicity, while the saturated lime solution contained almost the same concentration of calcium ions as the sodium hydroxide solution contained sodium ions, that is, the saturated lime solution had almost twice the basicity of the sodium hydroxide solution. However, due to the absorption of the reagents from the solution, reported in Table II, the

TABLE II
ABSORPTION OF ALKALI AND LIME BY COLLAGEN

Solution	°C.	Milliequivalents/Gram Dry Collagen				
		1 hr.	2 hr.	after 4 hr.	17 hr.	24 hr.
0.1% NaOH	25	0.27	0.25	0.23	0.24	
Half-saturated Ca(OH) ₂	25	0.37	0.42	0.45		0.90
Saturated Ca(OH) ₂	25	0.63	0.57	0.61		0.95
0.1% NaOH	8	0.26	0.27	0.27	0.28	
Half-saturated Ca(OH) ₂	8	0.35	0.39	0.39		0.39
Saturated Ca(OH) ₂	8	0.51	0.49	0.51		0.52

equilibrium concentrations of these solutions were not directly comparable. The concentrations of the equilibrium solutions and of the original solutions at zero time are given in Table III. The 24-hour calcium absorption figures

TABLE III
CONCENTRATION OF REMAINING SOLUTION

Solution	°C.	Milliequivalents per Liter after					
		0 hr.	1 hr.	2 hr.	4 hr.	17 hr.	24 hr.
0.1% NaOH	25	25	17	18	19	19	
Half-saturated Ca(OH) ₂	25	22	10	8	7		7
Saturated Ca(OH) ₂	25	44	24	27	25		12
0.1% NaOH	8	25	18	17	17	17	
Half-saturated Ca(OH) ₂	8	22	12	9	9		9
Saturated Ca(OH) ₂	8	44	29	30	29		28

for the lime solutions at 25° are high because of precipitation of some of the calcium by atmospheric carbon dioxide. The data of these two tables show emphatically that calcium is bound to collagen much more abundantly than sodium even from solutions of considerably lower concentration.

The nitrogen solubilized by these solutions in the short times allotted are shown in Table IV. There appears to be a slight amount of nitrogenous

TABLE IV
NITROGEN SOLUBILIZED

Solution	°C.	Milligrams Nitrogen/Grams Dry Collagen after				
		1 hr.	2 hr.	4 hr.	17 hr.	24 hr.
0.1% NaOH	25	0.21	0.34	0.86	1.15	
Half-saturated Ca(OH) ₂	25	0.28	0.33	0.45		0.75
Saturated Ca(OH) ₂	25	0.37	0.45	0.52		0.87
0.1% NaOH	8	0.20	0.19	0.23	0.36	
Half-saturated Ca(OH) ₂	8	0.10	0.13	0.21		0.37
Saturated Ca(OH) ₂	8	0.09	0.22	0.28		0.45

material which is extracted very rapidly and may represent noncollagenous materials still remaining in the purified collagen. There is also a continuous increase in the amount of nitrogenous material solubilized as time proceeds. This probably indicates that changes are occurring in the collagen, although they have not proceeded to the extent that they influence the absorption of water or metals from the solution.

The differences in the absorption of calcium and sodium ions make it difficult to obtain their effects under comparable conditions, for if the solution concentrations are kept equal, the amounts absorbed are different; and

if the amounts absorbed are made equal, then the solutions must be of different concentrations. Neither of these situations could give comparable results for phenomena such as swelling where the result might depend both upon the solution and the materials absorbed. Swelling phenomena have usually been studied in relation to the concentration of the solution in equilibrium with the collagen, and the relationship of the materials absorbed has been studied only in incidental fashion.

TABLE V
EFFECT OF SALTS ON SWELLING OF COLLAGEN AT pH 7.0

Ionic Strength	Swelling*	Solution Analysis			Solids Analysis		
		Calcium†	Sodium‡	Ratio**	Calcium‡	Sodium‡	Ratio**
0.20	1.68	0.036	0.088	0.41	0.113	0.109	1.04
0.21	1.75	0.027	0.126	0.21	0.096	0.169	.57
0.22	1.81	0.018	0.169	0.11	0.084	0.239	.35
0.17	1.89	0.049	0.026	1.88	0.137	0.020	7.00
0.19	1.81	0.041	0.064	0.65	0.124	0.056	2.22
0.20	1.79	0.033	0.096	0.35	0.109	0.102	1.07
0.35	1.80	0.099	0.053	1.86	0.209	0.048	4.36
0.38	1.82	0.083	0.129	0.65	0.201	0.161	1.25
0.40	1.90	0.067	0.199	0.34	0.180	0.271	0.66
0.39	1.75	0.072	0.172	0.42	0.191	0.244	0.78
0.41	1.83	0.054	0.252	0.21	0.160	0.332	0.48
0.44	1.81	0.036	0.335	0.11	0.124	0.495	0.25

*Grams of water per gram of dry collagen

†Moles per liter of solution

‡Millimoles per gram of dry collagen

**Calcium to sodium ratio

The data of Tables V and VI were obtained from experiments designed to determine the effects of calcium and sodium ions on the swelling of collagen under competitive conditions using solutions of approximately equal ionic strength so that the electrical properties of the systems would be as constant as possible. The ionic strength for the solutions used here is one-half the sum of each ion concentration times the square of the valence of the ion.

$$\mu = \frac{1}{2} [4 (\text{Ca}^{++}) + (\text{Na}^+) + (\text{Cl}^-)]$$

This adjusts the solution to account for the stronger electrical effects caused by the divalent calcium ions. The ionic strengths reported were calculated using the concentrations found by the analysis of the solutions after equilibration. Two levels of ionic strength, 0.2 and 0.4, were used for the experiments at neutrality, and a 0.25 level was used for the experiments at pH 7.0

TABLE VI
EFFECT OF SALTS ON SWELLING OF COLLAGEN AT pH 12.0

Ionic Strength	Swelling*	Solution Analysis			Solids Analysis		
		Calcium†	Sodium‡	Ratio**	Calcium‡	Sodium‡	Ratio**
0.01	2.52		0.009			0.116	
0.13	1.93	0.042			0.278		
0.17	1.94	0.056			0.305		
0.24	1.99	0.080			0.342		
0.24	2.05	0.066	0.046	1.43	0.339	0.096	3.53
0.25	2.05	0.055	0.084	0.65	0.298	0.162	1.84
0.26	2.33	0.026	0.177	0.15	0.219	0.413	0.53
0.26	2.61		0.260			0.850	
0.29	2.79		0.287			0.914	
0.51	2.62		0.513			1.387	
0.07	2.67		0.067			0.400	
0.12	2.64		0.116			0.507	
0.17	2.59		0.168			0.623	

*Grams of water per gram of dry collagen

†Moles per liter of solution

‡Millimoles per gram of dry collagen

**Calcium to sodium ratio

Tables V and VI show that the ratio of calcium to sodium is always greater in the collagen than it is in the solution, indicating that calcium is bound more abundantly than sodium. Figure 1 shows that the amount of calcium

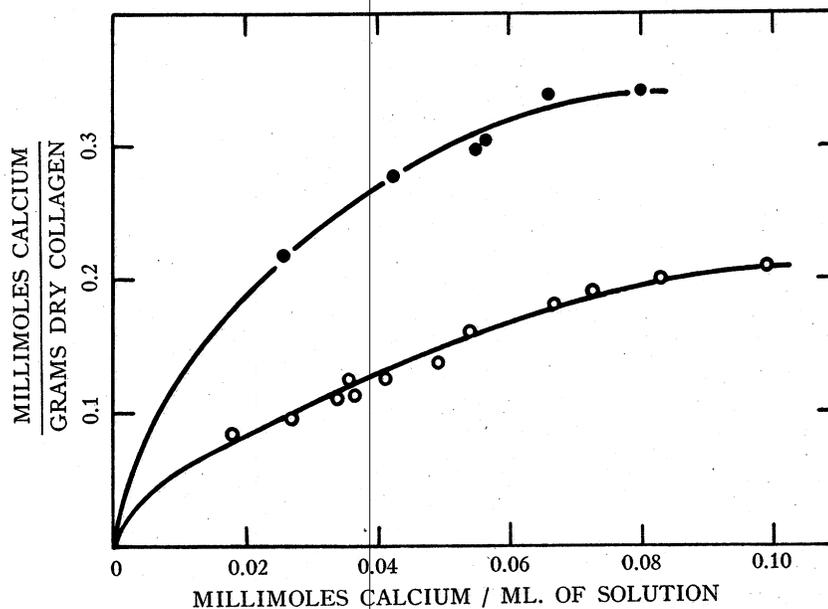


FIGURE 1.—Absorption of calcium by collagen in the presence of sodium chloride. ○, at neutrality; ●, at pH 12.0.

absorbed from these solutions increases with increasing calcium content of the solution except at the higher calcium concentrations, where there appears to be a limitation of the amount of calcium bound by the collagen. The amount of calcium bound at pH 12.0 is considerably greater than the amount bound at pH 7.0, but here again there appears to be a limitation to the amount of calcium bound at high solution concentrations.

The binding of sodium ions by collagen in the neutral solutions does not give the typical parabolic absorption but rather gives a straight line relationship (Fig. 2) of all the points extending from very low concentrations to

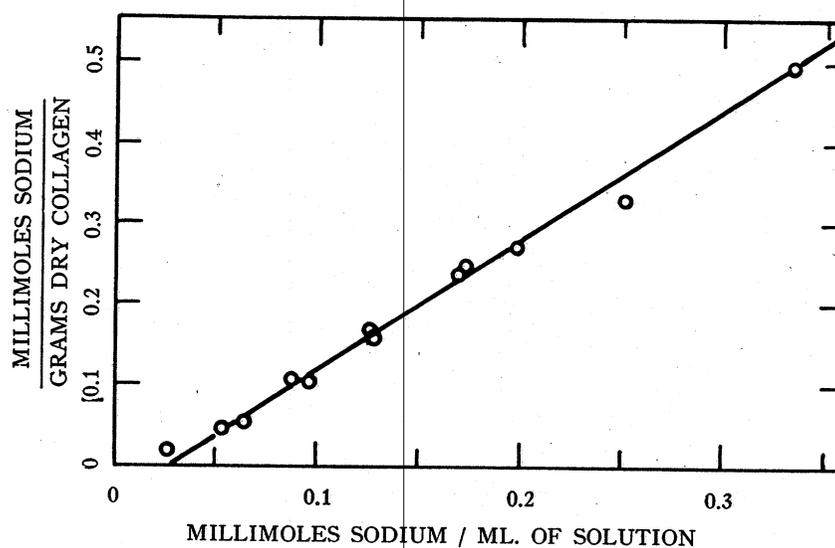


FIGURE 2.—Absorption of sodium by collagen at neutrality in the presence of calcium chloride.

very high concentrations. The slope of this line indicates that the millimoles of sodium absorbed per gram of collagen is 1.5 times the millimoles in one ml. of the solution, or in other words, the amount absorbed is the amount in 1.5 ml. of the solution. One gram of the collagen absorbs an average of 1.8 g. of these solutions. Therefore, it appears that possibly only about 0.3 g. of the absorbed water is bound to the collagen by primary linkages and does not contain dissolved salts. The remainder absorbs sodium ions approximately in equilibrium with the surrounding solution. The 0.3 g. of bound water is approximately equal to the amount found by Rougvie and Bear (10) at the second inflection point of the water vapor-absorption isotherm for collagen. This is the region of the curve where multilayer absorption of water begins to have its effect.

The present data do not indicate whether any of the calcium absorbed is located in the multilayers of sorbed water, although its presence there might be expected. If the amount of calcium present in 1.5 ml. of the neutral solutions is deducted from the amount absorbed, the remainder, which varies between 0.06 and 0.08 millimoles of calcium per gram of moisture-free collagen, could be considered the specifically bound calcium. This treatment of the data, however, cannot explain the limitation of the calcium binding at high calcium concentrations.

Ionic strength is not an important factor in controlling the sorption of calcium or sodium ions from neutral solutions, for both the 0.2 and 0.4 ionic strength points lie on a single smooth curve. The nearness of these solutions to the isoelectric point, 8.3, of this collagen preparation may account for this lack of effect. Here it must be remembered that although the net charge is zero at the isoelectric point, the total charge on the collagen is close to its maximum, and ionic interactions can exist.

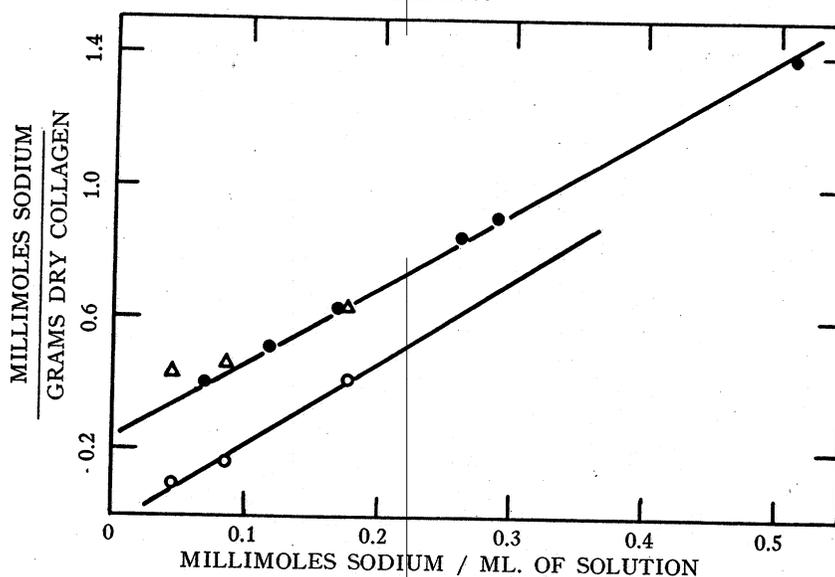


FIGURE 3.—Absorption of sodium by collagen at pH 12.0 in the presence of calcium chloride. ○, sodium values for mixed solutions; ●, sodium values for solutions containing only sodium cations; Δ, sodium plus calcium values for mixed solutions.

The plot (Fig. 3) of the sodium concentration in the solids versus the sodium concentration in the solutions at pH 12.0 shows two straight lines. The upper line traces points obtained from solutions containing only sodium cations. The lower line traces points obtained from solutions containing both calcium and sodium ions. The points on the upper curve designated with triangles were obtained by adding the millimoles of calcium absorbed to the millimoles of sodium absorbed which are shown on the lower curve.

These data have been interpreted as showing that there is a competition between calcium and sodium ions for certain specific sites on the collagen molecule. Calcium ions are more strongly bound than sodium ions, and if sufficient calcium ions are present, very few sodium ions will be specifically bound. The sodium concentration of the mixed solutions multiplied by the volume of the solution absorbed gives almost identically the total amount of sodium ions absorbed from these solutions. Therefore, it appears that most of the sodium ions absorbed from these mixed solutions are in the multilayers of absorbed water and that very few sodium ions are specifically adsorbed on the collagen.

Similar calculations with the calcium ion contents show that the amount of solution absorbed can contain no more than about a third of the calcium absorbed. Most of the calcium ions are, therefore, specifically adsorbed on the collagen.

In the absence of calcium ions more sodium ions are absorbed at the same sodium ion content of the solution. Therefore, the presence of adsorbed calcium ion prevents the specific adsorption of sodium ions. The triangular points of Fig. 3 show that the number of sodium ions prevented from adsorbing is almost numerically equal to the number of calcium ions adsorbed. It has already been shown that most of the calcium ions are specifically adsorbed. Therefore, most of the calcium ions are replacing sodium ions, ion for ion, and consequently only one of the two charges on the calcium ion is necessary for the binding of calcium ions by collagen. This would establish two significant points. The negative charges of collagen to which the positive ions are adsorbed are too widely separated to enable one calcium ion to satisfy two of them, and the binding of calcium ions by the negatively charged groups of collagen introduces positive charges in these areas which should change the electrical properties of the molecule.

The concentration of calcium ions in saturated lime solutions is not sufficient to supply the maximum amount of calcium that can be bound by collagen. Therefore, the addition of calcium chloride to lime solutions could produce the increase in swelling described by Vowes (4) through the introduction of additional bound positive charges which by repulsion of the existing positive charges could open up the protein to the penetration of more water.

The present experiments indicate that this effect is probably counteracted by the presence of sodium salts or the lower pH used, for in Table VI there appears to be little variation in the swelling of collagen for those solutions containing the varying concentrations of calcium. Figure 4 shows graphically that while there is little dependence of swelling upon the calcium content, there is a gradual increase in swelling as the concentration of sodium chloride is increased. However, there appears to be a limitation even on the sodium salt effect at moderate salt concentrations.

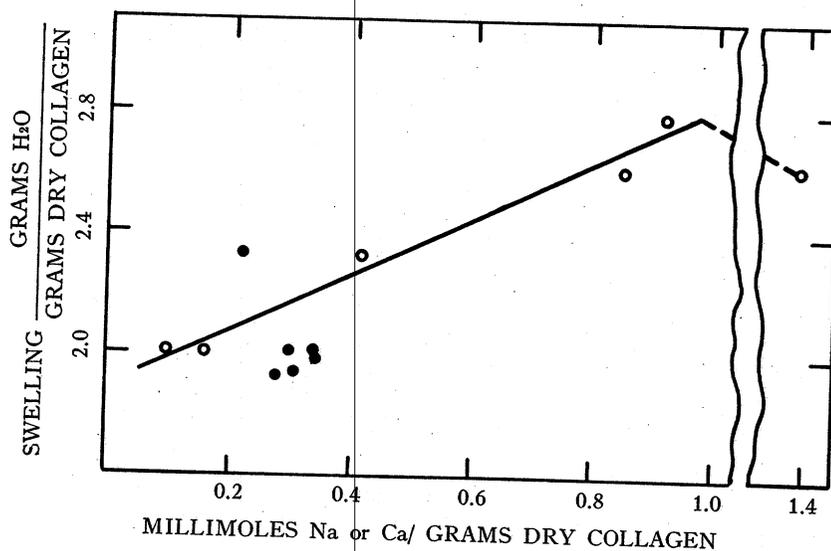


FIGURE 4.—Effect of absorbed salts on swelling of collagen at pH 12.0. O, sodium values; ●, calcium values.

CONCLUSIONS

1. At neutrality and pH 12.0 there is a limited specific adsorption of calcium ions by purified hide collagen.
2. The specific adsorption of calcium ions is competitive with the specific adsorption of sodium ions on a molar basis.
3. The specific adsorption of calcium ions is stronger than the specific adsorption of sodium ions, and therefore, the calcium ions monopolize most of the sites for the specific adsorption.
4. In the presence of an adequate number of calcium ions the sodium ions appear to be chiefly in the multilayers of sorbed water which are at equilibrium with the surrounding solution.
5. In solutions containing both sodium chloride and calcium chloride at pH 12.0 the swelling appears to be independent of the bound calcium but increases directly with increases in the amount of bound sodium.
6. Since calcium and sodium ions compete on a molar basis, only one charge from the calcium ion can be involved in the adsorption process; therefore, the negative binding sites of collagen must be separated sufficiently that two of them cannot bind the same calcium ion to form a bridge type of linkage.
7. The adsorption of calcium ions on the negative binding sites of collagen will transform these sites into positive ones and thus change the electrical properties of the collagen.

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DISCUSSION

DR. ROBERT M. LOLLAR (Armour Leather Co., Chicago): There are a couple of points that I wish to emphasize.

First, we should recall, as Dr. Mellon said, that this work was done on shreds of collagen under ideal conditions, and we should not, therefore, allow those ideal conditions to confuse our thinking with reference to the rapidity with which the attainment of equilibrium was found to occur in this work in comparison with our experience in the processing of skins and hides in the intact form.

However, I was rather interested in your observation, Dr. Mellon, that the calcium functions monovalently. It makes me think of some of the practical problems which one encounters—for instance, the case of the heavy neck area of skins and hides, where, because of slightly short bating and pickling, one may find streaks of lime present in the stock as it goes into tannage, and the resistance which these areas have to tannage.

From your observation that the calcium functions monovalently, these areas would contain positively charged protein carboxy groups which would give you the same charge as would be present in the chromium complex in many of your liquors. This idea furnishes a new interpretation for some of the difficulties one sometimes encounters in tannage.

Two questions occur to me: On the basis of the total nitrogen and amide-nitrogen analyses that were presented it is rather evident that the collagen used is a fairly pure collagen. Were analyses performed on these collagens to find the content of amino sugar that was present?

Second, observations were made in the paper on the solubilization of the protein and you mentioned in your discussion the effect of longer times in

the solutions on the deterioration of the collagen. Would you care to hazard a guess as to whether any hydroxyproline, for example, might be solubilized during this time?

DR. MELLON: We did not determine any of the amino sugars on this preparation. We have done it on similar preparations, however, and there are very slight traces in them. I don't think they are great enough to affect the values which we have presented.

In the paper we are presenting some additional data which show the amount of nitrogen which is solubilized in these solutions of lime and sodium hydroxide. The amount of nitrogen liberated seems to increase gradually with time. We believe this is a measure, somewhat, of deterioration or solubilization phenomena occurring in these solutions.

There is quite a rapid initial increase in the soluble nitrogen. You get a curve which comes up rapidly and then has a gradual slope, the sharp rise occurring within the one-hour period. We have a feeling that that is the removal of some nitrogenous material—not necessarily non-collagenous material. It could be collagen which has been partially degraded to gelatin as a result of our purification process. We rather feel that the longer you keep collagen in water, the more nearly you transform the collagen toward gelatin.

We regret that we did not perform hydroxyproline determinations on these solutions, so we cannot confirm whether this actually is solubilization of collagenous material or extraction of materials which we did not remove in the purification process, although the purification process did include extractions with salt solutions and half-saturated lime solutions.

In this respect the product we were working with did have a slight content of calcium and sodium ions, and these amounts have been included in the values which we have calculated as absorption.

Now, on continuous washing of these absorbed materials, all the sodium and calcium ions will be removed. It takes a considerably long washing period to remove the last residual traces, but I feel quite confident that most of it could be washed out if a sufficient length of time were given and a sufficient number of changes of solution. So we do feel this absorption is completely at equilibrium.

JAMES M. CASSEL (National Bureau of Standards): I wonder about this suggestion that only one of the valences of calcium was being tied up with the carboxyl groups in the collagen. Has something of this same nature been verified with resins, where calcium has been shown to have only one of the valences tied to the ion exchange resins?

DR. LOLLAR: You are asking whether, if there are ion exchange resins with the carboxyl groups, calcium functions only monovalently. Dr. Mellon?

DR. MELLON: I have no experience with it, but I have not been looking for anything of that nature.

MR. CASSEL: It seems to me that there is an exact comparison.

DR. MELLON: It might be quite similar, yes.

DR. PETER R. BUECHLER (Rohm & Haas Co.): It was quite apparent that at neutrality relatively little calcium is absorbed in comparison with the calcium which is taken on at high pH's. However, the material used—if I recall correctly, having prepared similar materials at one time—has an isoelectric point of somewhere around 7.8. Therefore, at neutrality you are working on the acid side of the isoelectric point and you have repression of the carboxyl group ionization. Consequently, this would very definitely, I think, affect the absorption of calcium. I wonder if you are in agreement concerning this explanation.

DR. MELLON: I am not quite sure that it is the full explanation. By the time you have reached a pH of 7, most of the carboxyl groups should be fully ionized. Only a few of them would not be. We cannot deny that there is a difference due to pH, but I don't think our data are sufficient to show what it is at the present time.

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