

# STRATIGRAPHIC DISTRIBUTION OF CARBOHYDRATE, HEXOSAMINE, AND HYDROXYPROLINE IN CATTLEHIDES\*

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## ABSTRACT

A precision slicing technique was employed to cut consecutive slices of equal thickness parallel to the grain surface of cattlehides. Quantitative determinations of carbohydrate, hexosamine, and hydroxyproline were made on the slices. The actual weight of all three components was lower in the grain slices than in the corium. However, when calculated on a dry-weight or nitrogen-content basis, the carbohydrate and hexosamine appear more concentrated in the grain region. Hydroxyproline follows the distribution of dry weight and nitrogen content more closely but indicates that a large part of the nitrogen content of the grain is not due to collagen. A steerhide and a cowhide were studied.



## INTRODUCTION

The stratigraphic study of the chemical composition of cattlehides makes possible the quantitative measurement of variations in concentration of constituents through the thickness of a hide. Therefore, it is possible to amplify the qualitative or semiquantitative observations obtained by histological studies and extend studies to reactions not possible by histological techniques. Roddy (1) recently reviewed the known structure of animal hides which was obtained chiefly through histological studies. Comparatively few stratigraphic studies have been made, but they have added considerably to the knowledge of the structure and reactivity of animal hides.

Stubbings and Theis (2) demonstrated through a stratigraphic study of swelling phenomena that the grain protein was less dense than the corium

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protein and this variation in density resulted in differences in swelling under various conditions. A similar difference in apparent density between the grain and the center of the corium was obtained by Mellon *et al.* (3) in stratigraphic studies of the distribution of dry matter and nitrogen in a steerhide. These studies indicated that the center of the corium was almost twice as dense as the grain region.

The stratigraphic studies by McLaughlin and Theis (4), Strandine *et al.* (5), and Kritzinger and Van Zyl (6) show a higher concentration of moisture in the grain region than in the center of the corium. Whether this additional water is free and can be squeezed out or is bound in some rigid structure such as a gel has not been determined. Two possible gel systems are the mucoid materials and the nonfibrous proteins. Reed (7) discussed the histological distribution of mucoids and showed that, although they existed through the entire thickness of the hide, there were higher concentrations in the grain region. Thus the presence of mucoid gels could explain not only the high water content of the grain region but also the high water content throughout the hide. Stubbings (8) showed a similar distribution for the nonfibrous proteins which could be solubilized by 10 percent sodium chloride solutions. Therefore, the nonfibrous proteins as well as the mucoids appear to be distributed similarly to the water content of a hide.

The present study was undertaken to measure the distribution of two components of mucoid materials—carbohydrate and hexosamine—through the thickness of a hide and to compare their concentration with the content of dry matter, nitrogen, and collagen.

#### EXPERIMENTAL PROCEDURE

*Preparation of Samples*—The steerhide samples were taken from the same hide used in the previous study of dry matter and nitrogen content (3). The cowhide samples were from the bend portion of a hide from a ten-year-old cow. Details of the treatment, which was the same for both hides, are given in the previous study. The fresh hides were washed, fleshed, and frozen for storage. For each run a small area of hide was thawed, shaved, soaked overnight, then cut into pieces approximately one and a half centimeters square. Approximately eight of these pieces were sliced to obtain the analytical samples. The flesh side of each piece was frozen to the microtome stage and sliced parallel to the grain surface into slices which were 0.1 mm. thick. If a cut across the entire piece was not obtained by the fourth slice, the entire piece was rejected. Five consecutive slices were pooled and called a layer, and each layer was analyzed separately. The layers are numbered from the hair side.

*Analytical Methods*—The slices representing each layer were dehydrated with several changes of acetone and dried to constant weight at 45° C. in a

vacuum oven through which a stream of air, dried over magnesium perchlorate, was circulated. Details of this procedure and of the Kjeldahl nitrogen determination are given in reference 3. It is unfortunate that the hydrolysis conditions required to obtain maximum values for carbohydrate, hexosamine, and hydroxyproline are considerably different. This necessitated separate runs for each material and increased the number of dry weight and nitrogen values which had to be made.

Most satisfactory results for carbohydrate were obtained by hydrolyzing the sample for four hours in 4 normal hydrochloric acid (9). Carbohydrate was determined on this hydrolyzate by the anthrone method of Trevelyan and Harrison (10). The data are expressed as milligrams of glucose by calculation from a standard curve for glucose, although other sugars may also be present.

For the hexosamine determination by the method of Boas (11), the slices were hydrolyzed for sixteen hours in 2 normal hydrochloric acid. Glucosamine was used as the standard, although other hexosamines may also be present in the hydrolyzate.

For the hydroxyproline determination by the Bowes (12) modification of the Neuman and Logan method, the slices were hydrolyzed for sixteen hours in 6 normal hydrochloric acid. A standard curve was run with each determination, although the variations in the curves from determination to determination were slight.

## RESULTS AND DISCUSSION

Previous studies (3) on the stratigraphic distribution of dry matter and nitrogen in steerhides showed that the concentration (weight per unit volume) of dry matter and nitrogen is twice as great in the corium region as it is in the grain region. Because of this difference in concentration of dry matter, comparisons made on a dry-weight basis may differ considerably from comparisons made on a unit-volume basis. Since each layer analyzed for a given run has the same volume, the actual weights within any single run which are given in Tables I, II, and III reflect the variations in concentration for that run. The weights of carbohydrate, hexosamine, and hydroxyproline divided by the weights of either the dry matter or nitrogen produce a ratio which is also useful for comparison.

Table I contains the experimental values obtained in the carbohydrate study. Runs 1 and 2 are on the steerhide, and run 3 is on the cowhide. Figure 1 graphically reproduces the actual weight data for runs 1 and 3 so that a visual comparison can be made with subsequent figures. The concentration of carbohydrate in the grain region is less than in the corium region. In this manuscript the boundary between the grain region and the corium region, which occurs at about the base of the deepest hair follicles, comes between

TABLE I  
THE DRY WEIGHT (Wt), NITROGEN (N), AND CARBOHYDRATE (C)  
CONTENT OF STRATIGRAPHIC LAYERS OF ACETONE  
EXTRACTED CATTLEHIDES

Layers*	Run 1			Run 2			Run 3		
	Wt. mg.	N mg.	C mg.	Wt. mg.	N mg.	C mg.	Wt. mg.	N mg.	C mg.
1	112	18	0.49	132	22	0.69	97	16	0.55
2	162	27	.68	168	28	.82	143	23	.66
3	184	32	.69	193	32	.70	136	22	.56
4	224	38	.68	269	45	.90	198	34	.54
5	275	48	.75	322	56	1.02	249	43	.63
6	288	51	.79	319	56	1.02	305	54	.82
7	310	54	.80	331	58	1.12	331	57	.96
8	301	53	1.04	348	61	1.16	317	56	.83
9	320	56	.98	350	62	1.29	318	56	1.01
10	317	55	.91	322	57	1.25	301	53	.91
11	316	55	1.03	316	56	1.18	243	42	.84
12	335	58	.98	300	54	1.06	†	†	†
13	336	58	.95	200	35	.88	†	†	†
R‡	1022	179	3.68	429	76	2.05	254	44	1.13

\*Numbered from hair surface.  
 †This specimen was several layers thinner.  
 ‡The residue which contains the last full layer plus the remaining partial layer.

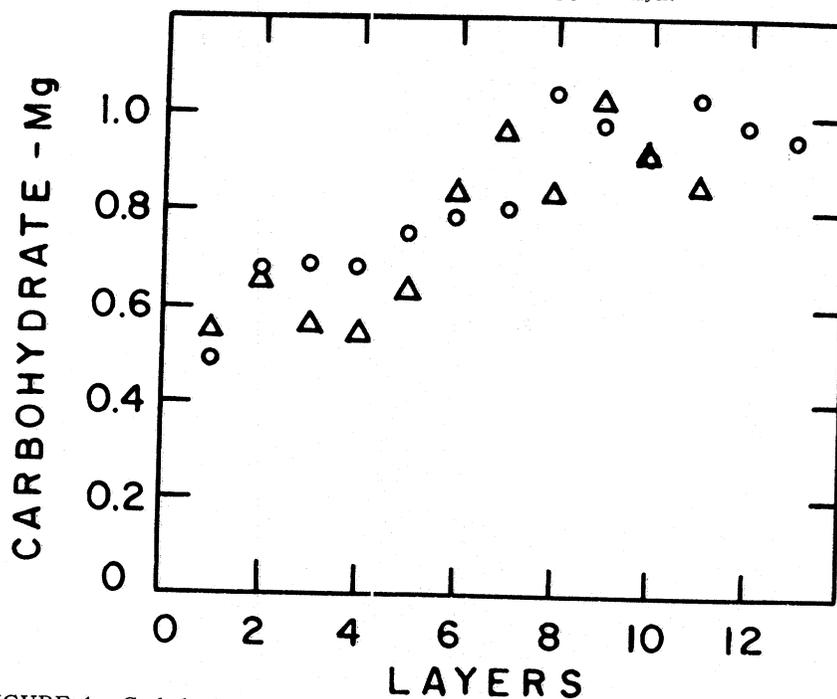


FIGURE 1.—Carbohydrate content of serial layers of cattlehides numbered from the hair surface: O a steer, Δ a 10-year-old cow.

**TABLE II**  
**THE DRY WEIGHT (Wt), NITROGEN (N), AND HEXOSAMINE (H)**  
**CONTENT OF STRATIGRAPHIC LAYERS OF ACETONE**  
**EXTRACTED CATTLEHIDES**

Lay- ers*	Run 4			Run 5			Run 6		
	Wt. mg.	N mg.	H mg.	Wt. mg.	N mg.	H mg.	Wt. mg.	N mg.	H mg.
1	242	37	1.11	153	25	1.12	125	21	.74
2	333	51	1.63	225	38	1.28	174	29	.85
3	452	71	1.04	305	48	1.23	167	29	.85
4	547	89	1.11	352	57	1.05	197	35	.68
5	648	104	1.20	429	74	1.36	362	56	.82
6	643	98	1.24	395	69	1.24	344	61	.82
7	654	101	1.20	443	78	1.33	375	67	.98
8	621	98	1.29	426	74	1.30	413	74	1.16
9	643	104	1.37	443	78	1.39	364	65	.94
10	585	95	1.42	408	72	1.35	371	66	1.06
11	560	90	1.44	404	71	1.36	†	†	†
12	528	84	1.33	368	65	1.43	†	†	†
R†	836	141	2.17	833	143	3.38	672	117	2.39

\*Numbered from hair surface.

†This specimen was several layers thinner.

‡The residue which contains the last full layer plus the remaining partial layer.

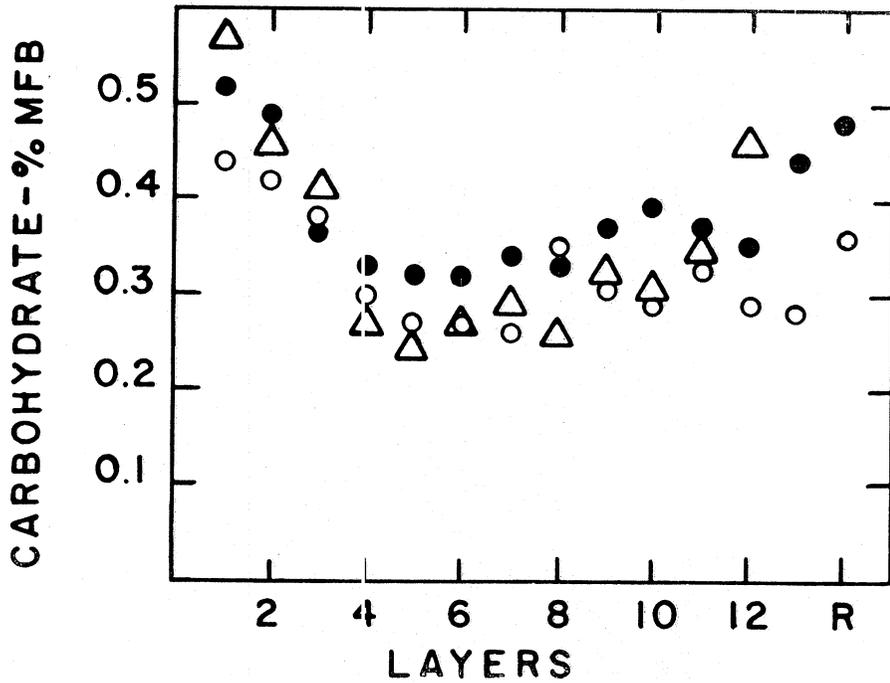


FIGURE 2.—Carbohydrate content on a dry-weight basis for serial layers of cattlehides: ● and ○ a steer, △ a 10-year-old cow. R is the flesh side residue.

**TABLE III**  
**THE DRY WEIGHT (Wt), NITROGEN (N), AND HYDROXYPROLINE (HP)**  
**CONTENT OF STRATIGRAPHIC LAYERS OF ACETONE**  
**EXTRACTED CATTLEHIDES**

Layers*	Run 7			Run 8			Run 9		
	Wt. mg.	N mg.	HP mg.	Wt. mg.	N mg.	HP mg.	Wt. mg.	N mg.	HP mg.
1	88	15	7	92	15	7	72	12	5
2	166	28	14	120	20	11	102	17	9
3	207	35	19	160	28	15	118	20	10
4	280	48	34	189	33	22	145	25	17
5	323	56	39	230	40	27	171	29	21
6	379	66	43	202	36	24	179	32	20
7	413	72	48	225	40	29	187	33	22
8	376	66	49	275	49	35	202	36	26
9	387	67	45	228	40	23	212	38	28
10	422	73	50	239	42	25	184	33	23
11	377	66	42	250	44	27	199	35	25
12	225	39	24	251	44	31	194	34	24
13	145	25	16	89	16	10	157	28	16
R†	524	88	56	399	70	45	359	62	39

\*Numbered from hair surface.

†The residue which contains the last full layer plus the remaining partial layer.

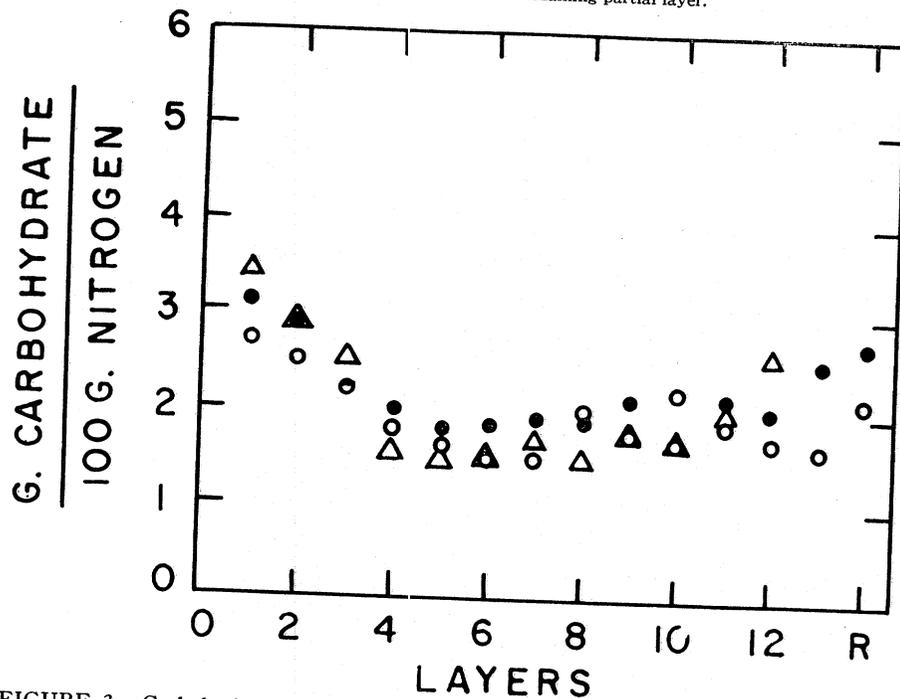


FIGURE 3.—Carbohydrate content related to the nitrogen content of serial layers of cattlehides: ● and ○ a steer, ▲ a 10-year-old cow. R is the flesh side residue.

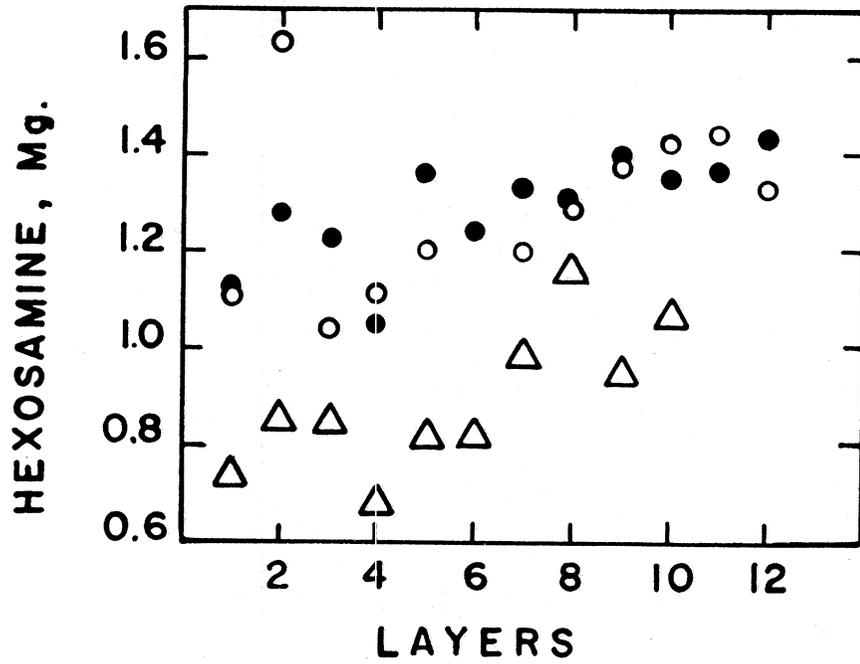


FIGURE 4.—Hexosamine content of serial layers of cattlehides numbered from the hair surface: ● and ○ a steer, △ a 10-year-old cow.

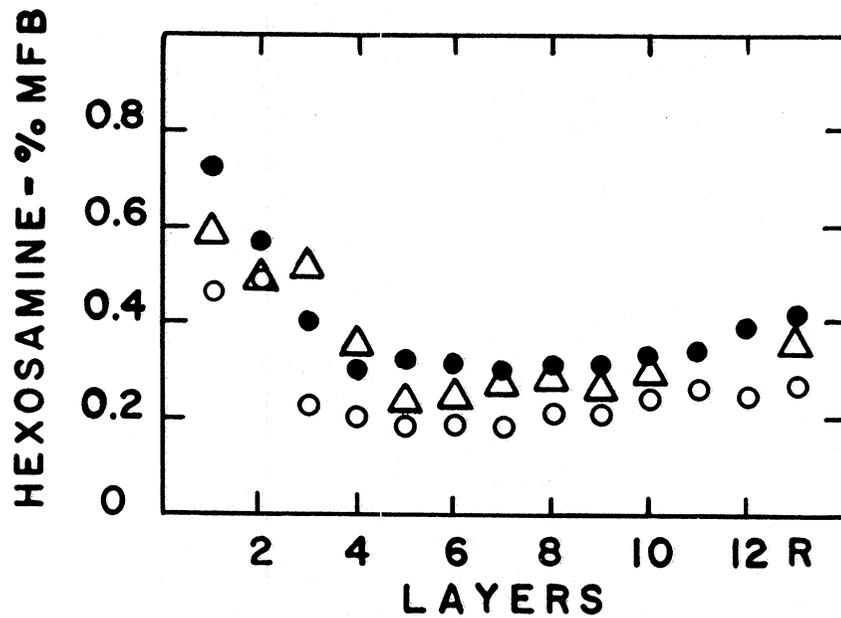


FIGURE 5.—Hexosamine content on a dry-weight basis of serial layers of cattlehides: ● and ○ a steer, △ a 10-year-old cow. R is the flesh side residue.

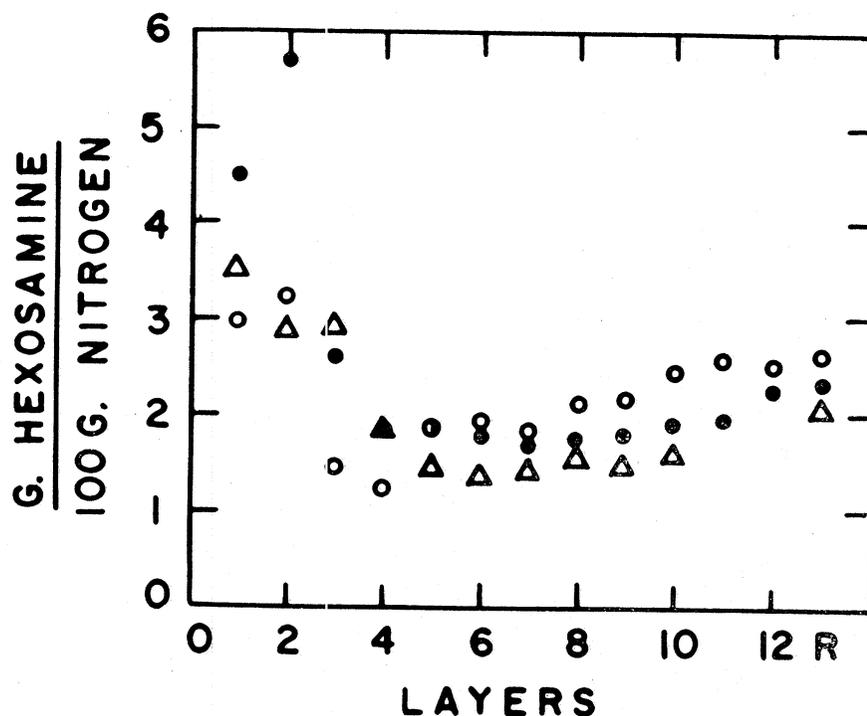


FIGURE 6.—Hexosamine content related to the nitrogen content of serial layers of cattlehides: ● and ○ a steer, △ a 10-year-old cow. R is the flesh side residue.

the third and fourth layers. Since the steer was about three years old and the cow was ten years old at slaughter, the sex or the age of the animal does not appear to alter the pattern. The carbohydrate content as a percent of the dry weight, which is plotted in Figure 2, gives a contrasting curve with high values in the grain region and lower values in the corium region. A similar curve, Figure 3, is obtained when the carbohydrate content associated with 100 grams of total nitrogen is plotted. We must bear in mind that Figures 2 and 3 do not show a higher concentration of carbohydrate in the grain region. The values are high only because the dry weight and nitrogen are low in the grain region. If any carbohydrate constituents are responsible for the high water content of the grain layers, these constituents must be different or react differently in the grain region than in the corium.

The results of the hexosamine study are given in Table II. The actual weight of hexosamine found in each slice is plotted in Figure 4. The content of hexosamine is slightly lower in the grain region than it is in the corium region. The triangular points representing the data on the 10-year-old cow are low only because fewer pieces were sliced for this experiment. In Figure 5,

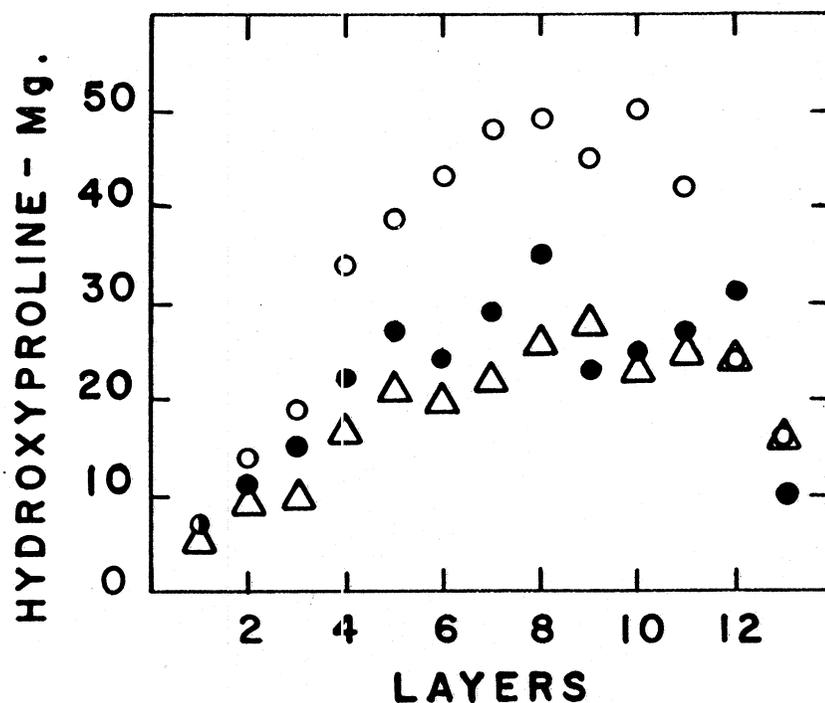


FIGURE 7.—Hydroxyproline content of serial layers of a steerhide numbered from the hair surface: ●, ○, and △ are from closely related areas near the center of the hide.

which shows the variation of the hexosamine content as a percent of the dry weight, the value is high for the grain region and practically constant throughout the corium region. Similar results are obtained when the grams of hexosamine associated with 100 grams of nitrogen content are plotted as shown in Figure 6. Again we must bear in mind that Figures 5 and 6 do not show a higher concentration of hexosamine in the grain region. The values are high only because the dry weight and nitrogen are low in the grain region. Therefore, if any hexosamine-containing materials are responsible for the high water content of the grain layers, these materials must be different or react differently in the grain region than in the corium.

These findings on the distribution of carbohydrate and hexosamine containing materials appear to be contradictory with histological findings. Nevertheless, the facts obtained by both methods can be reconciled by recognizing the difficulties involved in making quantitative estimations from histological observations. It is true that staining reveals localized high concentrations of mucoid materials in the papillary and dermal-epidermal regions, and these can give the impression that the grain layer is exceptionally rich in these materials.

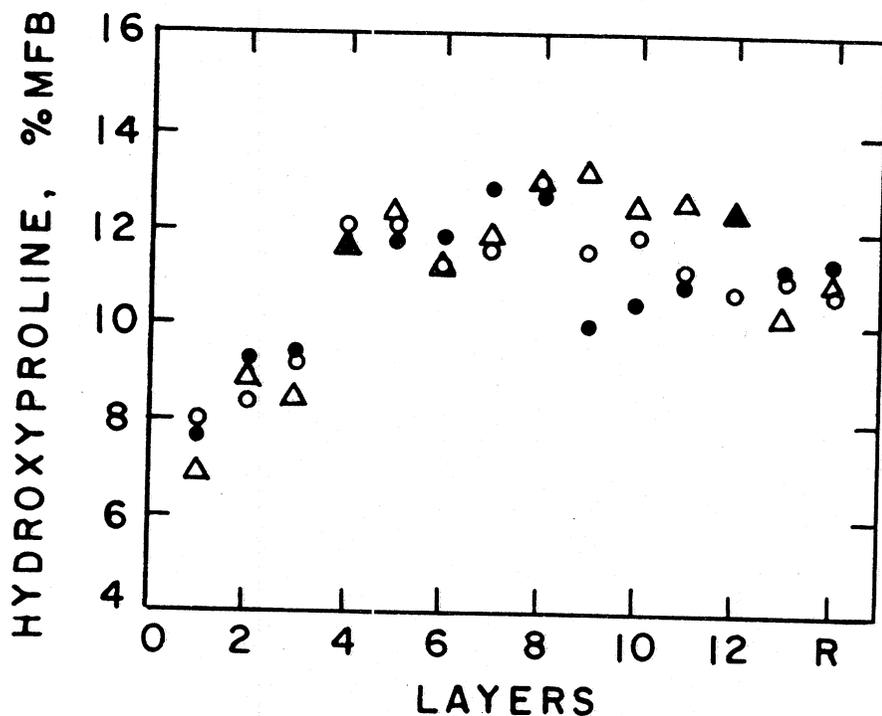


FIGURE 8.—Hydroxyproline content on a dry-weight basis for serial layers of a steerhide: ●, ○, and △ are from closely related areas near the center of the hide, R is the flesh side residue.

The data for the hydroxyproline study are presented in Table III. Only the steerhide was used in this study. Figure 7 shows that the actual weight of hydroxyproline content is very much less in the grain region than in the corium. Even when the hydroxyproline is considered as a percent of the dry matter, Figure 8, the values are lower in the grain region than in the corium. This is also true when grams of hydroxyproline associated with 100 grams of nitrogen content are plotted, Figure 9. The maximum value reached about the center of the corium is just slightly below the value of 75 found in many samples of purified collagen. This is a very good indication that the protein in the center of the corium is mostly collagen. The very low value in the grain region emphasizes that much of the protein in this region is not collagen. The concentration of these noncollagenous proteins changes rapidly in the region between the grain and the corium. This change is similar to the change in the water content, and therefore it appears that the noncollagenous proteins could be the components responsible for retaining the high proportion of water in the grain region.

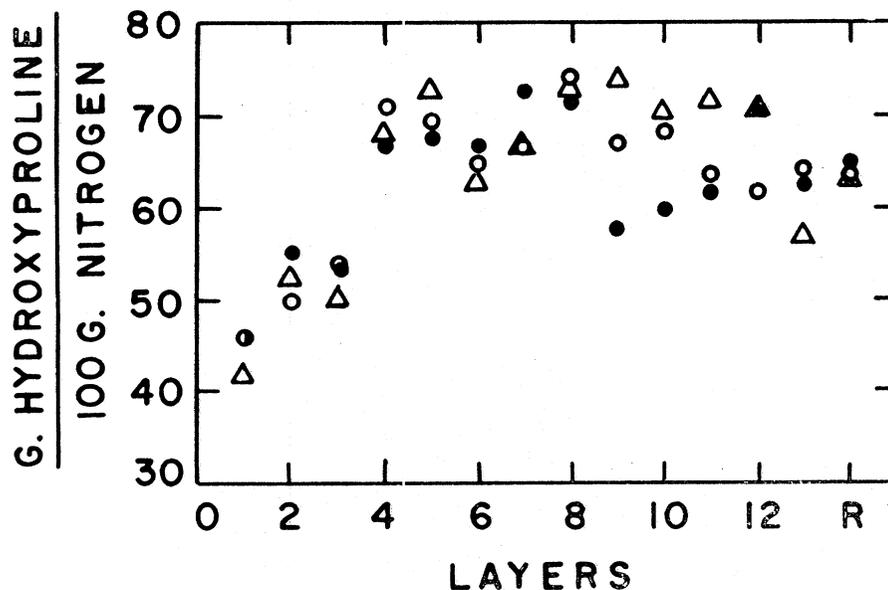


FIGURE 9.—Hydroxyproline content related to the nitrogen content of serial layers of a steerhide: ●, ○, and △ are from closely related areas near the center of the hide. R is the flesh side residue.

#### CONCLUSIONS

The concentration of both carbohydrate and hexosamine on a volume basis of fresh hide is less in the grain region of cattlehides than in the corium region. This was found for the hides of both a steer and a ten-year-old cow. Therefore, unless there is a difference between the carbohydrates found in the grain region and the corium, it is unlikely that these materials are the cause of the high hydration of the grain region.

The concentration of hydroxyproline is very low in the grain region. The ratio of hydroxyproline to nitrogen content is also low. Therefore, there is less collagen and more noncollagenous proteins in the grain region than in the corium. The change in concentration of these noncollagenous proteins follows the change in moisture content which occurs through the thickness of a hide. The hydration of these proteins may explain the high moisture content of the grain region of fresh cattlehides.

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## DISCUSSION

DR. ROBERT M. LOLLAR (Armour Leather Company): Again we see a presentation of one of these carefully prepared papers full of theoretical knowledge which we have come to associate with the Eastern Regional Research Laboratory. As one who is today quite interested in the practical aspects of the development of leather chemistry, I should like also to record my appreciation of the fact that today we are becoming more and more dependent upon laboratories such as this for this theoretical work which we need as a basis for our practical developments.

I think it is quite important that we continue to have that base line of theoretical information on which we can make our practical developments of the future.

Turning now to the specific paper and going back to some of the work that I was conducting in the Tanners' Council Laboratories in Cincinnati (before I left there a couple of years ago), there are a couple of points in the paper that we might comment on first.

The first one specifically concerns itself with the question of the hydrolysis conditions. Dr. Mellon said that it was impossible to use the same conditions for all analyses. I would like him to comment on the criteria which he used to select the hydrolysis conditions in each case.

DR. MELLON: In the analysis for carbohydrates and hexosamine, we had to make a compromise between two facts. One is that these materials are bound in certain combinations in the material and we have to try to hydrolyze the material to free these compounds. On the other hand, the acids and the temperature which are used for the hydrolysis cause a decomposition of these materials and convert them into other materials which would not be measured with the method of analysis. Since we have no alternative, we work out the conditions which will give us a maximum value. The amounts of acid and times of hydrolysis which we have used give us a maximum value.

This would bear some relationship to the actual concentration of the material in the hide, but it is not an excellently proved value because there is a very good possibility that we have not completely freed these materials from the combinations they are in; and also, in the length of time that we have tried to separate them from these combinations, some material has become decomposed. So the values we have are somewhat less, perhaps, than the total amount present in the hide.

If we could find a method of analyzing for these materials without hydrolyzing the hide, I am pretty sure the values would be higher, but we feel the values that we have presented are proportionate to the content in the hide.

DR. LOLLAR: Did you, in the course of your work to determine the optimum conditions, consider ion exchange resin hydrolysis?

DR. MELLON: We did not consider it. We used the resin column to purify the hexosamine before we determined it, but we did not consider using the resin in place of the acid for the hydrolyzing medium.

DR. LOLLAR: Of course, you are well aware that these resins in their acid form have been used as hydrolysis media. In some of the work that we were doing at Cincinnati when I left, we did find some evidence on the basis of paper chromatography of the hydrolysates, and of recovery values, that it might be possible that better recoveries would be obtained if resin hydrolysis were used in place of straight acid hydrolysis.

I think perhaps we might need to consider this approach if we were attempting to get absolute recoveries. This may perhaps be another lead to this point that you alluded to in your discussion—that we may not have maximum recovery.

DR. JOHN H. HIGHBERGER (United Shoe Machinery Corporation): Was the method you used for hexosamine the Boas Method?

DR. MELLON: Yes.

DR. PETER R. BUECHLER (Rohm & Haas Co.): A lot of this work Dr. Mellon is doing is dependent on the distribution of water in the steerhides and indicates a large amount in the grain area.

Since, in his analytical work, he and Mr. Viola had to determine the moisture content of the samples, I wonder if it would not be possible for him to discuss with us in these very same hides the water distribution on the same basis that he has presented the distribution of hexosamine, collagen, and carbohydrate.

DR. MELLON: The main reason we have not attempted to do this is because we use a fairly large area of tissue on the microtome stage, and in order to freeze this tissue on the stage so that we can slice it through the entire thickness of the hide, we have to freeze a little water on to the stage first so that our slicing blade will not hit the metal part of the freezing component. We usually slice 12 to 13 layers of tissue at 5 slices to a layer, which makes somewhere around 60 slices to a piece. And if we get two-thirds of the way through and then knock the piece off the stage, we have to throw away all of those slices. In order to keep our sample on the stage until we have completely sliced it, we build a ring of water or ice up around the sample to add this additional prop to the sample.

Each slice, as we slice it off, has this little ring of ice around the outside. And since the slices are quite thin, it would be quite difficult to remove this ring of ice and therefore obtain a sample which contained only the tissue. We feel, therefore, that we would be introducing as much error if we tried to take these slices, melt the ice, and then blot them to obtain only a tissue specimen, as we would by basing all our studies on the dry weight or the actual volume of tissue which we are cutting.

DR. BUECHLER: This point is very well taken. However, I wonder if, on the same hide, you could not take a very small section, such as we occasionally did, of the raw stock, and freeze it just using the moisture of the tissue itself? Of course, you have washed it and I suppose this would introduce another problem.

DR. MELLON: Since we do soak this hide in water overnight to make sure the water is uniformly distributed through the hide, we have to consider these moisture relationships as being maximum values that the hide could obtain. I feel, myself, that the hide on the animal's back does not contain quite as much moisture as the samples that we have been analyzing.

JOSEPH JANY (Ontario Agricultural College): Dr. Mellon, your hydroxyproline and hexosamine determinations were influenced to a certain extent by the severe conditions of the strong hydrolysis to which these substances were subjected. As this circumstance is a rather disturbing factor, I wonder whether or not you ever tested solutions of chemically pure hydroxyproline or hexosamine under the same severe conditions, in order to determine to what extent they may be attacked and to what extent your results may be influenced by this fact.

DR. MELLON: All we have done is to determine the values on material already in the hide by varying the condition of hydrolysis to obtain the maximum value. We ran the purified hexosamine to establish what our colori-

metric base line should be, but we did not run those materials through the hydrolysis procedure.

MR. JANY: Then I want to ask about the moisture determination. It is quite a problem generally, because if the substance is not disintegrated, then it is very hard to get a stable moisture content. If it is disintegrated, then in disintegration we lose some moisture. So I wonder if you used the xylene method where you don't have this problem?

DR. MELLON: We have developed a moisture determination which we have used for a number of years in the protein group of our Laboratory. It involves a vacuum oven. The one innovation is that instead of just sealing the oven and pumping on it with a vacuum pump, we pass through the oven a stream of air which has been dried by passing through magnesium perchlorate, so that the air stream flowing through the oven has practically zero relative humidity. In the samples which we have been studying—and this holds for a large number of proteins—we can get a constant value which holds for temperatures anywhere from 40° to 70° or 80°. We can put the sample in and obtain a value at 40°, and we can raise the temperature to 70°, and we will not have any difference in this value. If you go to much higher temperatures with some proteins, you will obtain some sort of a decomposition which will give you different readings. So we limit our dry weight determinations to the temperature between 40° and 70°, using the flowing stream of dry air to carry away any moisture which would be in the sample. Since we can get a reproducible value over this temperature range, we feel that our moisture values are about as constant as anyone can determine. We feel they are much more reproducible than if you determine moisture in an oven at 105° or higher.