It is hoped that the current and voltage data given above will be of considerable assistance to others in building furnaces of somewhat modified design. Ease of repacking, heavy electrode connections, positive pressure on the electrodes, and solid construction in general are emphasized.

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Action of Trypsin upon Diverse Leathers[1,2]

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It is shown that trypsin is capable of hydrolyzing collagen which has been treated with various agents, such as gallotannin, quinone, formaldehyde, and copper sulfate. Where the tanning agent combines only with the carboxyl groups of the collagen, as with copper, hydrolysis is as great as with untanned collagen, and does not depend on the amount of tanning agent present. Where the tanning agent, such as formaldehyde and quinone, combines with the amino groups of the collagen, the amount of hydrolysis depends on (a) the nature of the linkage—i. e., the type of tannage, and (b) the amount of tanning agent combined with the collagen. Chrome collagen is not hydrolyzed by trypsin.

IT HAS been shown that the proteolytic enzyme, trypsin, catalyzes the hydrolysis of collagen[1,*] at a hydrogen-ion concentration pH = 5.9, and at the temperature of 40° C. Since the chemistry of the mechanism by which collagen combines with various substances to form leather is still almost entirely speculative, it seemed possible that interesting results might be obtained by subjecting collagen, tanned in various ways, to the action of trypsin. Not only would this help in elucidating the theory of tanning, but it might also give some idea of the mechanism of tryptic hydrolysis.

The primary difficulty in the study of the hydrolysis by trypsin of collagen which has been treated with various tanning agents lies in the fact that most of the substances used for tanning—e. g., heavy metal salts and formaldehyde—are very definite enzyme poisons. Nevertheless, it seemed at least possible that by thorough washing of the treated hide powder all soluble and ionized matter could be removed, leaving merely the insoluble combination of collagen and tanning agent. There then seemed no reason why a more complex compound, such as that of the collagen tanning agent, should not be hydrolyzed by trypsin, just as collagen alone is hydrolyzed. It is to be remembered that these various tanned collagen compounds are in general characterized by the fact that, unlike collagen alone, they are not attacked by boiling water to yield gelatin, although some—e. g., vegetable-tanned collagen—are not entirely unchanged by hot water. There is further the possibility that the trypsin might hydrolyze the tanned collagen sufficiently to liberate enough of the tanning agent to inhibit the further action of the trypsin by "poisoning" it—i. e., presumably by combining with or precipitating it.

Yet the possibilities of throwing light on (*a*) the point of attack of the trypsin in the collagen molecule, and (*b*) the nature of the combination in each tannage, seemed such that they warranted the employment of this method of attack.

MATERIALS USED

Standard hide powder was chosen as the source of collagen. The trypsin was a high-grade commercial product which was tested on a casein substrate, according to the condition laid down by Sherman and Neun[2]—namely, 2 mg. of trypsin acting on a casein substrate at pH = 8 at 40.00° C. for half an hour. Under these conditions 2 mg. of the enzyme gave 17.7 mg. of soluble nitrogen. This represents a trypsin strength approximately one-third that of the strongest high-grade commercial preparation used by Sherman and Neun. As much as 20 mg. of the enzyme tested under the conditions requisite for pepsin activity gave no soluble nitrogen whatever.

The acidity was controlled by a buffer solution, the hydrogen-ion concentration being determined electrometrically.

METHOD

Fine siftings (100 mesh) of hide powder were tanned in solutions of basic chromium sulfate, quinone, formaldehyde, copper sulfate, and gallotannin, washed and dried as described in detail below. About 0.5 gram of tanned hide powder was placed in a 10-cc. centrifuge tube with a conical bottom, graduated in tenths of a cubic centimeter. Ten cubic centimeters of the buffer solution at pH = 5.9 containing 0.5 per cent trypsin were added. The tubes were then corked and fastened to a shake machine, rotating at 8 r. p. m. in a water thermostat at 40.00° C. After rotation for 20 minutes, the tubes were removed and centrifuged for 20 minutes at 1200 "times gravity." Control tubes in which the trypsin was omitted were run parallel with the digestions and the percentage of hydrolysis determined by comparison of the volumes.

The accuracy obtained in this method of measurement is limited entirely by that in reading the level in the tubes. With the centrifuge and the fine sifted hide powder it was possible to obtain a well-defined boundary, and the percentage digestions so obtained are accurate to ±2 per cent. Considering the insoluble nature of the substrate, the method is probably the most accurate available, while being reasonably rapid. It is distinctly preferable to filtering off the undigested hide powder and determining that dissolved by an estimation of nitrogen in the filtrate, a procedure which is objectionable and inaccurate for several reasons. Only small quantities of liquid are available. It is difficult to filter off the undigested hide powder satisfactorily and to obtain a clear filtrate; the degradation products of hydrolysis are in part molecularly and in part colloidally dispersed, and any filtration will merely effect an arbitrary separation dependent upon the size of the filter pores. More particu-

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[2] From a part of the dissertation submitted by Mr. Seymour-Jones in partial fulfilment of the requirements for the degree of doctor of philosophy in the Faculty of Pure Science, Columbia University, June, 1923. Contribution No. 431 from Columbia University.

* Numbers in text refer to bibliography at end of article.

larly, part of the nitrogenous matter in solution will be absorbed by the undigested hide powder, and a true aliquot will be impossible.

Chrome-Tanned Hide Powder

A chrome liquor of approximate composition $Cr(OH)SO_4$ was made up from chromium sulfate and sodium hydroxide to give 1.04 per cent chromium. Ten grams of 100-mesh hide powder were tanned in 200 cc. of this liquor (*A*), and 10 grams further in 200 cc. of the stock liquor diluted 1:4 with water—i. e., 0.21 per cent chromium (*B*), for 22 hours on the shake machine. The tanned powders were thoroughly washed with distilled water until no test for Cr^{+++} could be obtained, then were dried at 75° C.

Hydrolysis was then carried out as described above, the results being given in Table I.

Table I—Digestion of Chrome Leathers

Chromed Hide Powder	Per cent Hydrolyzed—Trypsin	Control
A	3.6	0.0
B	6.9	0.0

Further sets of hide powder were tanned in basic chromium sulfate solutions of various concentrations, but when the tanned powder was subsequently treated at pH = 5.0, some of the chromium was stripped from the leather. Even with this, only the lightest tanned powder showed any digestion with trypsin. It was not found possible to prepare chrome liquors for tannage at pH = 5.9, since the chromium began to precipitate out at this hydrogen-ion concentration.

It appears from this that where hide powder is completely chrome-tanned, it is not hydrolyzed by trypsin. Whether this is due to the poisoning action of the chromium on the trypsin, or to the chromium masking the linkage which is attacked by trypsin, is not definitely established. In any case, the theory of chrome tannage[3] is at present in so uncertain a condition that no light can yet be thrown on the action of trypsin in this particular reaction.

Quinone-Tanned Hide Powder

About 10 grams of 100-mesh hide powder were drummed up for 24 hours in each of three solutions of 1.0 (*a*), 0.5 (*b*) and 0.25 per cent (*c*) quinone, respectively, at pH = 5.9, then allowed to stand for 48 hours, thoroughly washed to remove all uncombined quinone, and dried out at 40° C. Hide powder tanned in a 0.1 per cent quinone solution was discarded because it was obviously undertanned. These tanned powders were analyzed, giving the results shown in Table II*a*.

Table II*a*—Composition of Quinone-Tanned Leathers

Quinone Collagen	Water	Hide Substance ($N \times 5.62$)	Quinone (by Difference)
a	8.6	84.1	7.3
b	8.8	86.7	4.5
c	9.2	89.6	1.2

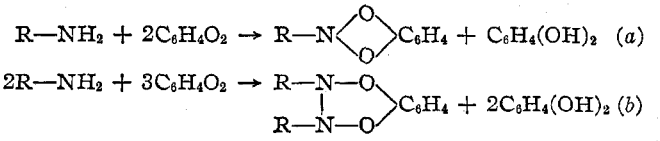
Half-gram lots of these three tanned hide powders together with some untanned hide powder for comparison were digested with trypsin as described before. Subsequently, the residual liquors were poured off, fresh trypsin and buffer solution added, and a further digestion period of 20 minutes was given.

Table II*b*—Digestion of Quinone Leathers

Quinone Collagen	Per cent Hydrolysis—1st 20 Minutes Trypsin	Control	2nd 20 Minutes Trypsin	Control
a	34	0	61	0
b	50	0	69	0
c	68	0	79	0
Raw collagen	86	13	91	23

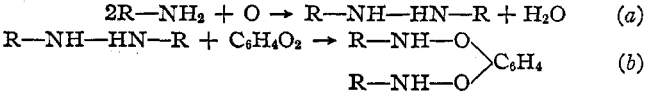
Considerable quantities of quinone and hydroquinone were liberated during hydrolysis, showing that the quinone collagen was decomposed. Neither quinone nor hydroquinone appears to destroy the trypsin. The group at which the collagen normally hydrolyzes with water appears to have been so changed by the tannage that hydrolysis no longer occurs. With trypsin some masking of the attacked group is shown by the lessened hydrolysis, which degree of hydrolysis varies with the amount of quinone used in tanning—i. e., in combination with the collagen.

Quinone tannage was discovered by Meunier[4] in 1908. His view[3] of the process is that it takes place by oxidation of the collagen and reduction of the quinone. Representing the collagen as R—NH_2, we may have either



These reactions are exactly like those between quinone and aromatic amines. Support is lent to this view by the fact that hydroquinone is found in the solution after tannage.

Fahrion,[5] on the other hand, presumes a preliminary oxidation of the collagen, followed by combination with the quinone.



This view does not explain the presence of hydroquinone in the residual liquor, unless it be supposed that the oxygen necessary for the first reaction is furnished by reduction of the quinone.

Whichever view of the mechanism of the tannage is accepted, it is the amino group which is linked through oxygen to the benzene ring. The quinone-tanned collagen is readily hydrolyzable by trypsin, although not quite so rapidly as the untanned hide powder. The combination therefore exerts only a slight retarding effect.

Formaldehyde-Tanned Hide Powder

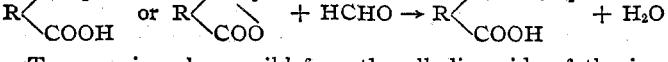
About 10 grams of 100-mesh hide powder were drummed for 24 hours in each of four solutions of (*a*) 1.90, (*b*) 0.95, (*c*) 0.38, (*d*) 0.19 per cent formaldehyde at pH = 5.9, then allowed to stand for 21 days, filtered, thoroughly washed, dried at 40° C., and powdered. Half-gram lots of these tanned hide powders were digested with trypsin, as described before.

Table III—Composition and Digestion of Formaldehyde Leathers

Sample	Water Per cent	Hide Substance Per cent	Formaldehyde (by Difference) Per cent	Per cent Hydrolysis Trypsin	Control
a	7.1	87.8	5.1	6	5
b	7.0	88.8	4.2	8	14
c	6.4	90.3	3.3	18	3
d	8.2	89.0	2.8	35	0
Raw collagen	..	..	..	86	13

The results shown in Table III, particularly in the controls, are somewhat irregular. Formaldehyde was found to be present in all the residual liquors after digestion, and its destructive action on the trypsin may well account for the comparatively small hydrolysis in the more heavily tanned powders.

Tannage with formaldehyde is of the type most generally acknowledged to be purely chemical in nature. It is usually represented as



Tannage is only possible[6] on the alkaline side of the isoelectric point of collagen. It may be taken that the formaldehyde masks the amino groups while freeing the carboxyl

groups in the collagen molecule. The formaldehyde-tanned collagen is hydrolyzable by trypsin, although to a lower degree than the untanned hide powder. This particular combination therefore exerts a fairly considerable retarding effect.

Copper Collagenate

Ten-gram lots of 100-mesh hide powder were drummed for 24 hours in 200 cc. of (*a*) 2.5 and (*b*, *c*) 5 per cent copper sulfate solution made up in a buffer of pH = 5.9, then allowed to stand for 24 hours, filtered, thoroughly washed, dried, and powdered. Powdering was not very satisfactory, since the tanned powder dried in hard fibrous lumps, not at all friable. Hide powders treated in more dilute copper sulfate solutions were discarded, since they gelatinized on drying. Tryptic digestion was carried out as before, the results being given in Table IV with the composition of the copper collagenate samples.

Table IV—Composition and Digestion of Copper-Collagen Compounds

Sample	Hide Substance Water Per cent	Hide Substance Per cent	Copper Per cent	Per cent Hydrolysis Trypsin	Per cent Hydrolysis Control
a	9.8	89.5	0.13	87	21
b	9.6	89.5	0.61	89	19
*c**	7.6	91.2	0.67	79	26
Raw collagen	..	..	..	86	13

* Sample *c* was tanned at a different time and was not so finely powdered as Sample *b*.

Copper was present in the residual liquors, but almost entirely in an un-ionized form. It is remarkable to note that hydrolysis is as great with the treated as with the untreated powder, and in the controls the copper treatment actually seems to activate the hydrolysis.

No work appears to have been done on copper tanning, and it is indeed very doubtful whether this treatment can be spoken of as a true tannage. The treated hide powder, when wet, feels like wet raw hide powder, and not like wet tanned hide powder, whether the tannage be vegetable, chrome, quinone, or formaldehyde. It may be supposed that we have here merely the formation of a salt between an amphoteric electrolyte, on the alkaline side of its isoelectric point, and a metallic cation, in accord with Loeb's work.[7]

Representing the collagen, as before, as $R\langle^{NH_2}_{COOH}$, the combinations would be:

$$2H_2N{-}R{-}COOH + CuSO_4 \rightarrow (H_2N{-}R{-}COO)_2\,Cu + H_2SO_4$$

Here it is to be noted that the copper combines with the carboxyl group, leaving the amino group free. The copper-tanned hide powder is hydrolyzable by trypsin or water to a degree as great as the untanned hide powder. This combination therefore appears to exert no retarding effect whatever on the hydrolysis.

Vegetable-Tanned Hide Powder

For purposes of comparison, 10 grams of 100-mesh hide powder were tanned in 200 cc. of 5 per cent gallotannin solution at pH = 5.9 for 48 hours, then thoroughly washed, dried out at 40° C., and powdered. Analysis of the product gave 5.44 per cent water, 72.23 per cent hide substance, and 22.33 per cent tannin (by difference). This was digested with trypsin as before. The tanned hide powder gave 48 per cent hydrolysis with trypsin and 19 per cent in the blank. The residual liquors from the trypsin gave a very faint positive gelatin-salt test for tannin.

There are innumerable theories of vegetable tanning, chemical, physical, and colloidal, but until more is known of the chemical nature of tannins and collagen it would be idle to speculate on exactly what type of combination occurs. Accepting Fischer's view of gallotannin as of the nature of a pentadigalloylglucose, it would seem reasonable to suppose that combination is largely with the basic groups of the collagen molecule. Considering the foregoing experiment in conjunction with those on the other types of tannage, this view would seem to be upheld.

Conclusion

In the preceding experiments two instances were found where tannage is believed to consist in combination with the amino group—e. g., with quinone and formaldehyde, and one instance where tannage probably consists in combination with the carboxyl group—e. g., with copper sulfate—where the tanned collagen was hydrolyzable by trypsin. In yet another instance, that of chrome tannage, which is sometimes believed to consist in a union of chromium with the carboxyl group, the product was not hydrolyzable by trypsin. In view of the first three instances cited, it would follow that chrome tannage must consist of some other kind of combination, and is obviously a more complicated process in its nature.

It appears also that where combination of the tanning agent occurs with the carboxyl group of the collagen, both tryptic and ordinary hydrolysis are as great as with untanned hide powder, such being the case with the copper tannage. Where combination of the tanning agent occurs with the amino group, it appears to depend on the nature of the linkage what degree of hydrolysis will be obtained. Naturally, the greater the amount of tanning agent combined, the less the hydrolysis.

These results of the action of trypsin on tanned collagen suggest a fertile field for further investigation on the mode of action of trypsin and possibly also on the nature of tanning. The possibility of combinations of the tanning agent with some of the polypeptide (—NH.CO—) linkage has not been considered, as the theory of tannage has not yet reached the stage where this would be profitable. Nevertheless, it is a possibility to be borne in mind in future work, particularly as it is at these linkages where hydrolysis probably occurs.

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