It will thus be seen that the action of enzymes in the drench is an indirect one.

TAN LIQUORS

In the tan liquors changes go on which are brought about by enzymes; some of these are secreted by molds and yeasts and others are present in the plants from which the tanning materials are extracted. Unfortunately very little is known about these enzymes, but attention should be called to the work of Fernbach¹ in France, who prepared an enzyme which he called tannase from the mold *Aspergillus niger*, obtained from gall nuts. He cultivated the mold in Raulin's liquid, using tannin in place of the sugar. The product was macerated in water, the maceration concentrated at a low temperature *in vacuo*, the liquid precipitated by alcohol, and the precipitate treated exactly as in Lintner's method for the preparation of amylase. The gray powder thus obtained, when dissolved in water, acted rapidly on tannin at 50°,

converting it into gallic acid. The solution of tannase filtered through a Chamberland filter into a sterilized solution of tannin acted just as effectively, proving that the action was due to an enzyme and not to a fermentation through the agency of organized cells.

H. Poltevin¹ prepared a solution of tannase by a process similar to that of Fernbach, but independently of him. Along with the gallic acid formed there is also a variable amount of glucose in the case of commercial tannins, but he considers these commercial tannins as mixtures. From a purified tannin he obtained gallic acid corresponding to 98.5 per cent of the tannin. The fact that tannase also hydrolyzes phenyl and methyl salicylates lends support to Schiff's formula for tannin, $C_6H_2(OH)_3.COO.C_6H_2(OH)_2.COOH$. In nature tannin is often accompanied by gallic acid. No doubt the latter is formed by the action of tannase, which Poltevin found to exist in sumac leaves, and which no doubt occurs in other substances containing tannin.

A Critical Study of Bating^{1,2}

By John Arthur Wilson and Guido Daub

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In an earlier paper⁴ it was shown that the mechanism of bating limed skins in the tannery by means of pancreatin and ammonium chloride consists of two distinct parts: reducing the skins to a condition of minimum swelling, and digesting the elastin fibers which are present only in the outer layers of the skin. The progress of this digestion was shown by means of photomicrographs of cross sections of the skin taken at intervals during bating. The examination of more than 200 sections under the microscope failed to reveal any other function of the bating process, even when a great variety of stains was employed on the sections. It therefore would appear that the primary function of bating is the removal of elastin, since the skins may be brought to a condition of minimum swelling simply by regulating the hydrogen-ion concentration of the solution with which they are in contact and, furthermore, this condition is only a temporary one, while the removal of elastin is permanent. The work described in this paper was confined to a study of the removal of elastin from calfskin and its effects.

PREPARATION OF SKIN FOR EXAMINATION

The most reliable method we know for studying the progress of bating is by means of the microscope. The per cent of elastin removed by any treatment is estimated by observing under the microscope cross sections of the samples taken before and after the treatment. For this purpose the sections must be suitably prepared, and the following procedure has been found quite satisfactory. Strips of the sample, about 2 in. $\times$ 0.5 in., are dehydrated with alcohol and soaked successively in (1) a mixture of equal volumes of alcohol and xylene, (2) a mixture of 1 volume of melted phenol to 3 of xylene, (3) pure xylene, and (4) melted paraffin, after which they are imbedded in paraffin and sectioned at 40 μ . The sections are then stained either by means of Weigert's stain as described by Gage⁵ or by the following method which the writers have found convenient: The section is washed on a slide with xylene, then with the alcohol-xylene mixture, and finally with pure alcohol. The whole slide is covered with alcohol saturated with Bismarck brown and

kept over night in an airtight jar containing alcohol. Next day the section is rinsed with alcohol, alcohol-xylene, phenol-xylene, and finally with pure xylene. A drop of Canada balsam and a cover glass are then put over the section, which is now ready for examination and may be kept indefinitely for record.

FIG. 1.—CROSS SECTION OF SKIN OF YOUNG HEIFER CALF. MAGNIFICATION, 25 DIAMETERS

Fig. 1 is a photomicrograph of a cross section of the skin of a healthy young heifer calf. A strip from the butt was taken an hour after the death of the animal and fixed in Erlicki's fluid. A section was stained first with Weigert's stain and then with an alcoholic solution of picro-red. It was photo-

¹ *Compt. rend.*, 26 (1848), 1214; *J. Soc. Chem. Ind.*, 20 (1901), 137.

² Presented before the Section of Leather Chemistry at the 62nd Meeting of the American Chemical Society, New York, N. Y., September 6 to 10, 1921.

³ Received August 3, 1921.

⁴ *THIS JOURNAL*, 12 (1920), 1087.

⁵ "The Microscope," 1917, 355.

¹ *Compt. rend.*, 26 (1848), 1215; *J. Soc. Chem. Ind.*, 20 (1901), 137.

FIG. 2—CROSS SECTION OF CALFSKIN TAKEN AFTER FLESHING, LIMING, UNHAIRING, AND WASHING, BEFORE BATING. MAGNIFICATION, 25 DIAMETERS

graphed through a combination of Wratten filters B-2 (green) and E-2 (orange). The elastin fibers show as a dark layer about 0.4 mm. deep between the epidermis and hair roots. Other layers of elastin fibers are visible in the flesh, supporting the nerve bundles and fat cells.

Fig. 2 represents a section from the butt of a calfskin taken after fleshing, liming, unhairing, and washing, but before bating. The flesh, epidermis, and hair have been entirely removed, but the elastin fibers are still intact and show as dark layers near the upper and lower surfaces of the skin. The main body of the skin contains no elastin.

Fig. 3 represents a section from the same skin as that shown in Fig. 2, but was taken after bating. The elastin fibers have been completely digested by pancreatin. The sections shown in Figs. 2 and 3 were prepared and stained with alcoholic Bismarck brown exactly as described above. They were photographed through a Wratten filter H (blue-green).

Estimates of the per cent of elastin removed were made on the basis of removal from the grain layer only. In some cases all of the elastin was removed from the grain layer before half of it was removed from the flesh layer. Since the shaving operation removes practically all of the flesh elastin, its removal in bating is of little importance.

MATERIALS USED

The chemicals employed were the ordinary C. P. variety. The enzyme was a commercial sample of U. S. P. pancreatin which showed by analysis: water, 6.3 per cent; insoluble matter, 40.0 per cent; ash, 6.8 per cent; N, 11.0 per cent; Cl, 1.7 per cent; SO_3 , none; and P_2O_5 , 3.5 per cent. By the method for determining tryptic activity described by Thomas,¹ 100 mg. of the sample acting upon 1 g. of casein in 100 cc. of solution for 1 hr. at 40° digested 64 mg. of nitrogen.

GENERAL PROCEDURE

For each series of experiments a piece of limed and unhaird calfskin was cut into strips about 2 in. $\times$ 0.5 in. and thoroughly washed. There is a small, but appreciable, difference in time required for complete removal of elastin from skins of different thickness and for this reason great care was exercised in selecting all strips for any one series from the same part of the same skin, so as to have them all as nearly identical as possible. Each strip was put into 500 cc. of liquor, a volume large enough to prevent the skin

from seriously altering the concentration of the liquor. The liquors were put into dark brown bottles to shield them from the light and were kept in a large Freas thermostat for the stated lengths of time at 40° $\pm$ 0.01°, the optimum temperature for most enzyme actions.¹

Every liquor contained 0.02 mole per liter of added phosphoric acid to act as a buffer in addition to the enzyme and the KOH required to give the desired hydrogen-ion concentration. The hydrogen-ion concentration of each liquor was determined both before and after the digestion period by means of Hildebrand electrodes and a Leeds and Northrup potentiometer, excepting where it was proved by previous test that the results obtained by the Clark and Lubs series of indicators were sufficiently accurate. Except for the more strongly acid and alkaline solutions, the change in pH value during digestion was practically negligible.

As a rule, a preliminary series covering a very wide range was run, followed by a second series covering only the active range of the enzyme. A third series was usually run as a check.

EFFECT OF HYDROGEN-ION CONCENTRATION

It is well known that the hydrogen-ion concentration is an important factor in determining the rate of digestion by enzymes. Using 0.1 g. of pancreatin per liter and digesting for 24 hrs., the writers obtained complete removal of elastin from the skin only between the pH values 7.5 and 8.5. A portion of the pancreatin was put into a collodion sac and dialyzed against running tap water in a dark room for 16 hrs. and used in a duplicate series in such quantity as to represent 0.1 g. per liter of the original pancreatin. The results were the same as those obtained with undialyzed enzyme. A series was then run in which the concentration of pancreatin was increased to 1.0 g. per liter. Complete removal of elastin was obtained between the pH values 5.5 and 8.5. The results of the two series, which are shown in Fig. 4, were carefully checked to insure their accuracy. The per cent of total elastin removed is plotted against the pH value of the solution taken after digestion and cooling to 25°.

The peculiar relation of the curves to each other is significant. They nearly coincide at all pH values above 7.5, but at 6.0 the stronger solution is still at its optimum activity, while the weaker one has apparently entirely lost

FIG. 3—CROSS SECTION OF CALFSKIN TAKEN AFTER BATING. MAGNIFICATION, 25 DIAMETERS

¹ J. Am. Leather Chem. Assoc., 15 (1920), 221.

² Falk, "The Chemistry of Enzyme Actions," 1921, 77.

FIG. 4—REMOVAL OF ELASTIN FIBERS FROM LIMED CALFSKIN AS A FUNCTION OF HYDROGEN-ION CONCENTRATION. TIME OF DIGESTION 24 HRS.; TEMPERATURE 40° C.

its elastin-digesting powers. When an enzyme has been found to have different optimum pH values with different substrates, it has been supposed that the effect of the hydrogen-ion concentration upon the substrate has been the determining factor. But here we have the same enzyme and the same substrate, with a change in the optimum range due merely to a change in concentration of the enzyme.

An explanation is suggested by the recent work of Northrop,¹ who has shown that the activity of an enzyme solution is not necessarily a function of the apparent total enzyme concentration, but that a portion of the enzyme may be inactivated by combining with peptone or other foreign matter. He has pointed out further that the extent of the formation of addition compounds between protein and enzyme depends upon the concentration of protein ion, which is in turn a function of the hydrogen-ion concentration. If some protein other than elastin is responsible for the inactivation of a portion of the enzyme, we should expect such action to be a minimum at the isoelectric point of this protein.

After bating, the strips of calfskin were all carefully examined for the "bated feel," which apparently bears no relation to elastin removal, but corresponds to a condition of minimum swelling of the skin. The only strips passing this test were those from liquors having pH values between 6.10 and 9.80, suggesting that the isoelectric point of at least one of the skin proteins lies between these points. The average of these is 8, which is also the midpoint of the optimum range for the more dilute enzyme solution.

The following explanation is therefore not an improbable one: At a pH value of about 8, practically all of the enzyme is left free to attack the elastin, but as the pH value is decreased and the concentration of protein ion correspondingly increased, more and more enzyme is removed from the field of action by combining with it. In the weaker solution at pH = 6.0 practically all of the enzyme is in combination with this protein, whereas in the stronger solution the excess of enzyme is still sufficient to digest elastin. In dealing with the effect of hydrogen-ion concentration upon enzyme action, it is evidently important to know the effect

FIG. 5—REMOVAL OF ELASTIN FIBERS FROM LIMED CALFSKIN AS A FUNCTION OF TIME OF DIGESTION. TEMPERATURE 40°; pH VALUE 7.6

of the hydrogen-ion concentration upon each substance in contact with the solution.

It is interesting to compare the optimum pH values for tryptic digestion found by other investigators:¹ for albumose Michaelis and Davidsohn² found 7.7, for casein Sherman and Neun³ found 8.3, while Long and Hull⁴ found 5.5 to 6.3, and for fibrin Long and Hull⁴ found 7.5 to 8.3. The total range of 5.5 to 8.3 corresponds closely to the range found by the present authors, 5.5 to 8.5, for complete removal of elastin by the more concentrated enzyme solution.

At pH values less than 3.0 there was a marked destruction of the collagen fibers, possibly due to acid hydrolysis, and the strips were much swollen and rubbery, but no removal of elastin could be detected.

EFFECT OF TIME OF DIGESTION

Two series of solutions were prepared, one in which the concentration of enzyme was 0.1 g. per liter and the other in which it was 1.0. All members of both series were otherwise identical. The pH value of each liquor was brought to 7.6 and this did not change during digestion. Each strip of calfskin was kept in a separate bottle. The bottles were removed from the thermostat at fixed intervals during 24 hrs.

Complete removal of the elastin was effected by the stronger enzyme solution in 6 to 8 hrs., but in the weaker solution 24 hrs. were required. The progress of the digestion is shown in Fig. 5. The time required to start digestion, 2 hrs. for the stronger and 5 hrs. for the weaker solution, was apparently the time required for the enzyme to diffuse into the region of the skin containing the elastin fibers. As will be seen from Fig. 2, these begin about 0.1 mm. below the grain surface.

EFFECT OF CONCENTRATION OF ENZYME

Two identical series of solutions were prepared in which the individual members differed only in concentration of pancreatin. The pH value was fixed at 7.6. One series was kept in the thermostat for 5 hrs. and the other for 24 hrs. The results are shown in Fig. 6 and furnish a study in economy.

¹ Cf. Falk, *Loc. cit.*, p. 66.

² *Biochem. Z.*, **36** (1911), 280.

³ *J. Am. Chem. Soc.*, **38** (1916), 2203; **40** (1918), 1138.

⁴ *Ibid.*, **39** (1917), 1051.

¹ *J. Gen. Physiol.*, **2** (1920), 471; **3**, 211.

FIG. 6—REMOVAL OF ELASTIN FIBERS FROM LIMED CALFSKIN AS A FUNCTION OF CONCENTRATION OF ENZYME. TEMPERATURE 40°; pH VALUE 7.6

Complete removal of elastin is effected by 0.1 g. of pancreatin in 24 hrs. or by 1.1 g. in 5 hrs.

EFFECT OF CONCENTRATION OF AMMONIUM CHLORIDE

A study of bating would not be complete if it did not include the effect of ammonium chloride, one of the most abundant constituents of commercial bating materials. Aside from its use as a filler, it has been assumed to be beneficial in removing lime from the skins and tending to maintain a slight alkalinity favorable to tryptic digestion.

In this experiment two series of solutions were prepared in which the concentration of enzyme was 0.1 and 1.0 g. per liter, respectively. To each successive member of each series increasing amounts of ammonium chloride were added and the pH values of all members brought to 7.6. The time of digestion was 24 hrs. (Fig. 7).

In working with very dilute enzyme solutions, a distinct activating effect was noted upon the addition of 0.5 g. per liter of ammonium chloride, while larger amounts showed an inhibitory effect. With thin calfskin the activating effect was not detectable with the solution containing 0.1 g. per liter of enzyme after a 24-hr. digestion period, because all of the elastin was removed without adding any ammonium chloride. In order to show the activating effect in these experiments, strips from heavier skins were used, which require a somewhat longer time for complete removal of elastin under fixed conditions. The activating effect of 0.5 g. of ammonium chloride per liter and the inhibitory effect of greater concentrations are very marked. It is also interesting to note that the effect of ammonium chloride can be entirely overcome by a sufficient excess of enzyme.

This behavior of ammonium chloride is interesting in view of the finding of Thomas¹ that potassium bromide in concentrations of 0.0 to 0.1 mole per liter has an inhibitory effect upon the action of malt amylase, but in greater concentration has an activating effect.

At concentrations greater than 50 g. per liter the ammonium chloride exerted a destructive action upon the collagen fibers, possibly due to the formation of ammonia.

It is not our purpose to discuss the merits of commercial

bates, but the question naturally arises as to what is the effect of the large amount of ammonium chloride which they contain. A sample which we believe contained pancreatin was found to have no elastin-digesting power at any concentration. It was our first thought that the trouble was due to too high a ratio of ammonium chloride to enzyme, but the preparation was still quite without action after the salt had been removed by dialysis. The real cause may have been the presence of much woody fiber used as a filler. If protein can remove the enzyme from the field of action, it is possible that woody fibers can also and, if these have an isoelectric point much removed from that of the protein, it is possible that at any concentration or at any pH value, a greater proportion of enzyme to woody fiber would be required than the sample contained.

EFFECT OF ELASTIN REMOVAL

A limed calfskin was cut into halves along the line of the backbone, the elastin was completely removed from one half by means of pancreatin, while the other half was simply treated with dilute ammonium chloride solution having a pH value of 8 in order to reduce its swelling to a minimum. Both halves were then thoroughly washed. It was recognized that an exact comparison of the two halves during tanning could not be made, if the pH values of the absorbed water were very different. Every effort was made to have the pieces identical, excepting for elastin content.

The most noticeable difference was observed during the early stage of vegetable tanning. The surface layers of the skin naturally tan more rapidly than the fibers in the interior and there is a tendency for the grain surface to expand temporarily to a greater extent than the rest of the skin. The elastin fibers in the unbated half evidently tended to prevent this expansion and the result of the tension produced was a slightly harsh feel, although the grain appeared tight and smooth to the eye. The grain of the completely bated half, however, actually expanded, giving the skin temporarily a wrinkled appearance, although the grain felt very soft and silky. When both halves had become completely tanned, this difference had almost disappeared. In the finished leather, the only difference in appearance was a slightly lighter color in the bated half. Photomicrographs

FIG. 7—REMOVAL OF ELASTIN FIBERS FROM LIMED CALFSKIN AS A FUNCTION OF THE CONCENTRATION OF AMMONIUM CHLORIDE. TIME OF DIGESTION 24 HRS.; TEMPERATURE 40°; pH VALUE 7.6

¹ J. Am. Chem. Soc., 39 (1917), 1501.

FIG. 8—GRAIN SURFACE OF FINISHED CALF LEATHER WITH NONE OF ITS ELASTIN REMOVED. MAGNIFICATION, 15 DIAMETERS

of exactly corresponding points on the grain surface of the two halves are shown in Figs. 8 and 9.

While bated and unbated finished leathers appear much alike to the eye, there are perceptible physical differences such as one might expect to find in view of the fact that the elastin fibers have been removed from under the grain of the bated leather. The desirability of completely, or even partially, removing elastin from skin depends upon the use to which the leather is to be put.

SUMMARY

A critical study of bating has been made which tends to elevate a heretofore mysterious process to a scientific plane.

The primary function of bating is to remove elastin fibers from the skin prior to tanning.

When a dilute solution of pancreatin was employed, complete digestion of the elastin was effected only when the pH value of the solution lay between 7.5 and 8.5, but when a more concentrated solution was used, the range was extended to 5.5 and 8.5. An explanation of this is given on the assumption that an addition compound between the enzyme and protein other than elastin is formed in increasing amounts as the pH value is reduced from 8.

The rate of removal of elastin from calfskin is shown as a function of the concentration of enzyme and of the time of digestion.

FIG. 9—GRAIN SURFACE OF FINISHED CALF LEATHER FROM WHICH ELASTIN HAS BEEN ENTIRELY REMOVED. MAGNIFICATION, 15 DIAMETERS

Ammonium chloride shows an activating effect in concentrations up to 0.5 g. per liter and a marked inhibitory effect in higher concentrations.

The failure of commercial bates to remove elastin from calfskin is attributed to the presence of woody fibers.

A comparison of bated and unbated leathers is made.

NOTE: Since submitting this paper for publication, we have received an interesting communication from L. Krall of Geneva, Switzerland, bearing upon the subject. Having just seen the German translation of our first paper, he has written to claim priority in discovering, by means of the microscope, that the chief function of bating is the removal of elastin fibers from the skin. His experiments, performed at the University of Geneva from 1914 to 1916, proved that the elastin fibers of skin can be entirely removed by digestion in an infusion of dog dung at 40°. His photomicrographs show that the action of dung is practically identical with the action we found for pancreatin, thus furnishing further evidence of the soundness of Wood's conclusion that pancreatin is the active constituent of dung in bating. We did not experimenting with dung. Krall's important paper was unfortunately buried in a private bulletin published by the *Société Anonyme, anc. B. Siegfried*, of Zofingue, Switzerland, in June 1918, and we were not aware of its existence until his communication was received.

Cider Preservatives¹

By R. D. Scott and E. G. Will

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The alcoholic content of cider having become of some importance with the advent of the Eighteenth Amendment, the need exists for an efficient preservative, both to prevent fermentation of fresh cider and for court work, to hold the alcoholic percentage of seized samples unchanged pending analysis.

Sodium sulfite, sodium benzoate, and salicylic acid are known to be used commercially for preserving cider, but no literature concerning any except sodium benzoate could be found. Accordingly, the following experimental work was undertaken.

EXPERIMENTS ON FRESH CIDER

The cider used was prepared by the writers from reasonably sound, unwashed, and unpared apples, of which ten varieties were used. From this stock, 400-cc. portions were trans-

ferred to 500-cc. flasks which were loosely stoppered after the addition of the preservatives, and were placed in a dark cupboard. Both alcoholic and acetic fermentation would probably be more rapid under such conditions than if barrel lots had been used, thus subjecting the preservatives to more rigorous tests than might be the case in practice. Alcohol (per cent by volume) and acidity (g. per 100 cc. as acetic) were determined at intervals of 2 wks., 2 mo., and 6 mo. The room temperature during this period ranged from 18° to 25° C. Results after 6 mo. only are recorded in Table I, in which Samples 1 to 11 and Control A represent one lot of juice; Samples 12 to 18 and Control B, a different lot of juice, expressed 1 wk. later from the same lot of apples.

RESULTS—Saccharin, formaldehyde, sodium hypochlorite, and β -naphthol were not effective in preventing alcoholic fermentation when used in amounts up to 0.1 per cent.

¹ Received August 1, 1921.