THE ISO-ELECTRIC POINT OF COLLAGEN.[1]

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In 1910, Michaelis and Mostynski[2] published their theory of the iso-electric point of a protein, defining it as that reaction where the relation of the concentration of the hydrogen ions to hydroxyl ions in the solution is the same as the relation of the acid dissociation constant (k_a) of the protein to its basic dissociation constant (k_b). At the iso-electric point the number of protein anions is equal to the number of protein cations present and the sum of the protein ions in relation to non-ionized protein is at its minimum.

Previous to 1910, Michaelis tried to determine the sign of the electrical charge of proteins in solution by U-tube electrophoresis, and noted that the migration of a particular protein was cathodic or anodic depending entirely upon the reaction of the solution. These experiments led to the derivation of the theory of the iso-electric point, and several papers since, by Michaelis and co-workers, give values for the iso-electric points of several proteins.

Prior to Michaelis' researches, proteins were considered merely as colloids, and explanation of their behavior in aqueous solutions, in the presence of electrolytes, was influenced by the supposed fundamental effect of the Hofmeister ion series, selective adsorption, etc. The tenacity with which this point of view has held even up to date ignoring the amphoteric properties of proteins has been an obstacle in the progress of the physical chemistry of the proteins.

The recent brilliant work by Jacques Loeb[3] has most strikingly shown the great importance of a knowledge of the iso-electric points of proteins. Loeb clearly demonstrated in the case of gelatin, albumin, and casein that at the iso-electric points these proteins are in the most inert condition, while on the acid side they exist and react as cations, and on the alkaline side they are anions.

The making of leather consists fundamentally in converting the scleroproteins, or albuminoids of hide substance into an insoluble and imputrescible substance. This consists generally in combining the protein with the complex weakly acidic substances known as tannins, or in converting it to a chromium compound. Obviously, a better understanding of these reactions requires a knowledge of the iso-electric point of hide protein, or collagen.

[1] Presented before the Leather Chemistry Section at the 62nd Meeting of the American Chemical Society, New York City, Sept. 6–10, 1921.

[2] Michaelis and Mostynski, *Biochem. Z.*, **24**, 79 (1910).

[3] Series of papers in *J. Gen. Physiol.*, **1918-21**.

This investigation was undertaken with the purpose of establishing the iso-electric point of hide substance, $i.$ $e.$, that hydrogen-ion concentration of the solution in which hide substance protein is in its minimum ionized state, on the alkaline side of which it exists as an anion, and on the acid side as a cation.

Since it has been most clearly proved by Loeb that a protein is at its minimum degree of swelling at the iso-electric point, we decided to try to locate the point by swelling measurements.

Swelling Method.

In all swelling experiments, 1920 American Standard Hide Powder[4] was used as source of hide substance. Since hide substance is insoluble in water, the simple swelling technique as used by Loeb was not applicable to this substance. Some method had to be devised whereby the interstices between the particles could be reduced to a minimum. This was accomplished by sifting the hide powder through a 20-mesh sieve, and centrifuging the fine siftings in contact with solutions of various hydrogen-ion concentrations as described below.

Portions of 0.3 g. each of fine siftings were placed in each of a series of eight 100cc. "oil tubes" (graduated tubes drawn out to a conical end), in which there had been previously placed 50 cc. of solution, and carefully shaken. When the hide powder had become thoroughly wetted, another 50 cc. of solution was added and the tubes were allowed to stand

EXPERIMENT 8

	Approx. molar conc. of solution		Log. C_{H^+}	Volume of hide powder after standing in contact with solution for the stated time				
			Hours	24 Cc.	48 Cc.	72 Cc.	96 Cc.	384 Cc.
1	0.0002	HCl	. . . .	2.7	2.8	2.7	2.5	2.7
2	0.0001	HCl	− 3.75	2.3	2.5	2.4	2.3	2.4
3	Dist. H_2O		− 5.58	2.1	2.3	2.2	2.1	2.3
4	Dist. H_2O		− 5.58	$\overline{2.1}$	$\overline{2.1}$	$\overline{2.3}$	$\overline{2.1}$	$\overline{2.3}$
5	0.0001	NaOH	− 9.18	$\overline{2.1}$	$\overline{2.4}$	$\overline{2.5}$	$\overline{2.8}$	$\overline{3.0}$
6	0.0001	NaOH	− 9.18	$\overline{2.1}$	2.3	2.6	2.7	2.8
7	0.00025	NaOH	−10.00	$\overline{2.4}$	3.0	3.2	3.4	3.5
8	0.0005	NaOH	. . . .	3.0	3.4	3.5	3.5	3.7

HYDROGEN-ION CONCENTRATION OF WASHINGS

Solution	Washings after the stated time		
	Hours 24	72	96
3	−5.25	−5.58	−5.88
4	−5.43	−5.88	−5.88
5	−5.88	−6.45	−6.64
6	−5.88	−6.45	−6.64

[4] Manufactured by the Standard Mfg. Co., Ridgway, Pa.

for 24 hours. They were then centrifuged altogether for 5 minutes at about 2000 r. p. m., and the volumes occupied by the various hide-powder specimens recorded. The supernatant liquid was poured off, and an additional 100 cc. of solution added, the contents thoroughly mixed, allowed to stand for an additional 24 hours, and the centrifuging, etc., repeated. An example of such an experiment precedes.

It will be seen that upon 24 hours contact with the solutions, the minimum swelling is indicated in distilled water and in 0.0001 M sodium hydroxide, but upon repeated treatment, the 0.0001 M sodium hydroxide solutions show a slightly greater swelling than distilled water, thus according to this technique, setting the distilled water as the solution nearest the hydrogen-ion concentration of the iso-electric condition of the hide substance, namely log $C_{H+} = -5.58$. It is noted that after 24 hours contact with hide substance, the logarithm of the hydrogen-ion concentration of the distilled water washings became lower, from -5.58 to -5.88, which may be explained as due to removal of carbonic acid by the hide protein. The 384-hour tubes were discarded on account of putrefaction having taken place to a small extent.

In a subsequent experiment a few drops of toluol were added to each tube. Instead of letting the tubes containing the hide powder stand for 24 hours in contact with the solutions, they were rotated in a tumbling machine for 1 hour to insure thorough mixing, and then centrifuged for 5 minutes, the volumes read, washings decanted, and the operation repeated several times, but it is evident that differences between the tubes of minimum swelling and tubes containing solutions beyond the minimum swelling point were not of any significant magnitude. There was a large error involved in reading the volumes of the hide powders because of uneven settling, and although the technique indicated that the hide powder in contact with distilled water was nearest the iso-electric point, this point was by no means definitely established. The addition of toluol made the readings more doubtful because some of the powder was adsorbed by the toluol layer and thus removed from the bulk of the deposit in the conical bottom of the tube, and in addition a small part of the powder stuck to the side of the tube.

The swelling method for this particular protein is, therefore, applicable only for the purpose of locating the approximate iso-electric region when solutions of widely differing hydrogen-ion concentrations are employed and consequently large swelling differences are obtained. The method, even for approximate results, must involve continued washing or treatment with the solutions until the hydrogen-ion concentration of the washings is identical with that of the original solutions, thereby insuring that equilibrium has been reached and all impurities removed in each case.

The method is slow and laborious, and since the addition of toluol is detrimental, it is not easy to reach equilibrium before decomposition sets in.

For these reasons it was decided to employ the dye technique. As Loeb has shown that protein can combine with cations only on the alkaline side of the iso-electric point, and with anions only on the acid side, collagen should combine with the colored cation of a basic dye when on the alkaline side and with the colored anion of an acid dye on the acid side of its iso-electric point.

Dye Method.

In these experiments, 4 different batches of hide powder were employed, —1921, 1920 and 1919 (kindly furnished by Dr. L. E. Levi), and 1918 (kindly furnished by Mr. G. W. Schultz).

The acid dye used throughout was Martius yellow, and fuchsin served as basic dye, except in one case where neutral red was employed.

First Technique.—Six 1g. portions of hide-powder siftings were placed in 250cc. beakers, and 100 cc. of solutions of various concentrations of hydrogen ion were added. The mixtures were frequently stirred, and at the end of 18 hours were filtered through small pads of cotton on Gooch funnels and washed several times with a new portion of the original solutions. Finally they were treated with distilled water 2 or 3 times. A 20cc. portion of a 0.001% solution of the basic dye, or a 0.002% solution of the acid dye was poured on the residue on each funnel and allowed to drain through. The solutions of fuchsin were completely decolorized where the hide powder was decidedly on the alkaline side of its iso-electric point, and the Martius yellow solutions decolorized by the hide powder which was definitely acid. In such instances more dye was poured through. In our early experiments the residues were then washed with distilled water until all uncombined dye had been removed, but later were washed with solutions of the same hydrogen-ion concentration as those in which the hide powders had been soaked in order to preclude any chances of hydrolysis of the protein-dye compound.

The residues were then examined for color and those two samples of hide powder (both soaked in solutions of the same hydrogen-ion concentration) which showed minimum fixation of color were judged to be in the iso-electric condition.

Possibly an explanation is required at this point because of the popular assumption that at the iso-electric point a protein is absolutely inert and in an un-ionized state. Relatively speaking this is correct, but in an absolute sense it is not. Actually a protein is at its minimum degree of ionization at the iso-electric point and is capable of combining with either a cation or an anion to a slight degree. This point has been discussed by Sörensen.[5]

This technique indicated that the iso-electric point is in the region of log C_{H^+} −5.25 to −6.45; but it was found faulty as pointed out in the swelling experiments, since the hide powder was treated only once with the contact solutions.

Second Technique.—The portions of hide powder and solutions were placed in 400cc. bottles fitted with rubber stoppers and rotated for one hour in a tumbling machine.

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[5] Sörensen, *Compt. rend. trav. lab. Carlsberg*, **12**, (1915–17).

In the first experiments (a) with this technique no test for equilibrium was applied but later (b) the supernatant solutions were tested for hydrogen-ion concentration, new portions of solutions placed on the protein in the bottles and tumbled for another hour, etc., until the decanted solutions showed the same hydrogen-ion concentration as the originals. Twenty cc. portions of dye solutions were added and the contents tumbled for 10 minutes when the contents were poured through glass-wool pads in Gooch funnels. They were then washed with solutions of the same hydrogen-ion concentration as those in which they had been soaked.

Third Technique.—The whole operation was carried out in "oil tubes" instead of using bottles. The advantages of this operation were that at the end of each washing, the powder was packed down by centrifuging before the liquid was poured off, and instead of a diffuse layer of treated hide powder in Gooch funnels, the color or lack of color was more easily distinguishable in the columns of powder in the apices of the tubes.

The results of these experiments are summarized below. For economy of space the details of each experiment are omitted, and only Nos. 10, 19 and 20 are given as typical.

EXPERIMENT 10

Portions of 0.5 g. of 1920 hide-powder siftings treated in bottles successively with 200, 100, 100 and 100 cc. of solution (2nd technique (a))

Approx. molar conc. of solution		Color after washing		Log. $C_{H}+$ of solution
		Fuchsin	Martius yellow	
0.0001		faint pink[a]	yellow	−3.8
0.000075		faint pink	yellow	−4.0
0.00005	HCl	pale pink	pale yellow	−4.6
0.000025		pale pink	colorless	−4.9
Dist. H₂O		deep pink	colorless	−5.6
0.000025	NaOH	deep pink	colorless	−6.1

[a] These hide powders always retained a faint trace of the fuchsin and for this reason neutral red was substituted in Expt. No. 20.

The iso-electric point is indicated at −4.6, or possibly between −4.6 and −4.9.

EXPERIMENT 19

Each bottle containing 0.5 g. of siftings from 1918 hide powder treated as described in Technique 2 (b)

Approx. molar conc. of solution		Color after washing		Log. $C_{H}+$ of solution
		Fuchsin	Martius yellow	
0.00006				−4.1
0.00004				−4.2
0.00003	HCl	faint pink	yellow	−4.6
0.00002				−4.8
0.00001		pale pink	colorless	−5.4
Dist. H₂O		pink	colorless	−5.8

The above experiment would indicate that the iso-electric point is −4.8 to −5.4, or −5.1.

EXPERIMENT 20

Each bottle containing 0.5 g. of siftings from 1918 hide powder, treated by Technique 2(b). Instead of adding 20 cc. of very dilute dye solution, 8 drops of 0.1%

neutral red were added to one set of samples during the last treatment with solution, and 16 drops of 0.1% Martius yellow to a corresponding set of samples.

Approx. molar concn. of solution		Color after washing		Log C_H+ of solution
		Neutral red	Martius yellow	
0.0001			. . . .	−3.8
0.00006			. . . .	−4.0
0.00004	HCl	colorless	. . . .	−4.2
0.00003			yellow	−4.6
0.00002			pale yellow	−4.8
0.00001			colorless	−5.2
Dist. H₂O		pale pink	colorless	−5.4
0.0001 NaOH		pale pink	. . . .	−6.0

The iso-electric point is indicated at −4.8 to −5.4, or −5.1.

SUMMARY OF DYE TECHNIQUE EXPERIMENTS

Expt.	Material	Method	Iso-electric C_H+	Remarks
10	0.5 g. of 1920 H. P. siftings in 400cc. bottles	2nd tech. (b) 4 treatments	−4.6	
11	1921 siftings as in No. 10		−4.5	
12	1920 siftings as in No. 10	2nd tech. (b) 6 treatments	−4.5 to −4.6	
13	1921 siftings as in No. 10		−4.1?	
14	1920 siftings, 0.15 g. in 15 cc. oil tubes	3rd technique 16 treatments	−4.2 to −5.0	Equilibrium reached as shown by C_H+ of washings.
15	0.3 g. of 1921 siftings in 100cc. tubes	3rd technique 4 treatments	−4.2 to −5.0	
16	0.3 g. of 1920 siftings	3rd technique 9 treatments	−4.2 to −4.8	
17	0.3 g. of 1919 siftings	3rd technique 11 treatments	−4.6 to −5.3	
18	0.5 g. of 1919 siftings		−4.7 to −5.6	
19	0.5 g. of 1918 siftings	2nd tech. (b)	−4.8 to −5.4	
20	0.5 g. of 1921 siftings		−4.8 to −5.4	

Inspection of these results reveals a failure to obtain a sharp iso-electric point for hide substance after many experiments. We conclude, therefore, that hide substance, generally referred to as collagen, is a mixture of proteins rather than one simple protein and possibly the differences in properties noticed in the tanning of skins from different animals may be due to the different distribution of the proteins in the hide substance.

The iso-electric point of the proteins of hide substance is at hydrogen-ion concentration 10^{-5}, as determined by means of American Standard Hide Powder.

At various times the iso-electric points of several proteins have been reported in the literature, and since they are not available in any one place, we append them hereto.

		Reference
Casein (cow)	2.4–2.5×10^{-5}	6
	1.8×10^{-5}	7
	2×10^{-5}	8
Gelatin	2.5×10^{-5}	9
	2×10^{-5}	10
Serum albumin	2×10^{-5}	11
Serum globulin	3.6×10^{-5}	7
Egg albumin (hen)	1.5×10^{-5}	12
Denatured serum albumin	0.4×10^{-5}	11
Oxyhemoglobin	1.8×10^{-7}	14
Carbon monoxide hemoglobin	1.7×10^{-7}	15
Reduced hemoglobin	1.7×10^{-7}	15
Stroma globulins of blood corpuscles	1×10^{-5}	13, 14
Red blood cells	2.5×10^{-5}	16
Yeast extract protein (globulin)	2.5×10^{-5}	21
Gliadin	6.0×10^{-10}	7
Edestin	1.3×10^{-7}	7
Tuberin (potato)	approx. 10^{-4}	17
Carrot protein	approx. 10^{-4}	17
Tomato protein	approx. 10^{-5}	17
Trypsin	1.35–2.6×10^{-4}	18
Pepsin	5.5×10^{-3}	19
Nucleic acid	approx. 2×10^{-1}	20

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[6] Michaelis and Pechstein, *Biochem. Z.*, **47**, 260 (1914).

[7] Rona and Michaelis, *ibid.*, **28**, 193 (1910).

[8] Loeb, *J. Gen. Physiol.*, **2**, 577 (1920).

[9] Michaelis and Grineff, *Biochem. Z.*, **41**, 373 (1912).

[10] Loeb, *J. Gen. Physiol.*, **1**, 39 (1918).

[11] Michaelis and Davidsohn, *Biochem. Z.*, **33**, 456 (1911).

[12] Sörensen, Ref. 5.

[13] Michaelis and Davidsohn, *Biochem. Z.*, **41**, 102 (1912).

[14] Michaelis and Takahashi, *ibid.*, **29**, 439 (1910).

[15] Michaelis and Bien, *ibid.*, **67**, 198 (1914).

[16] Coulter, *J. Gen. Physiol.*, **3**, 309 (1921).

[17] Cohn, Gross and Johnson, *ibid.*, **2**, 145 (1919).

[18] Michaelis and Davidsohn, *Biochem. Z.*, **30**, 481 (1911).

[19] Michaelis and Davidsohn, *ibid.*, **28**, 1 (1910).

[20] Michaelis and Davidsohn, *ibid.*, **39**, 496 (1912).

[21] Fodor, *Kolloid. Z.*, **27**, 58 (1920).