| Compound | Formula | Sb Calcd., % | Sb Found, % |
|---|---|---|---|
| I | $C_{12}H_9O_4N_2SbNa_2$ | 29.5 | 31.0 |
| II | $C_{12}H_8O_4N_2ClSbNa_2$ | 27.2 | 26.9 |
| III | $C_{12}H_8O_6N_3SbNa_2$ | 26.6 | 26.4 |
| IV | $C_{12}H_{10}O_4N_3SbNa_2$ | 28.3 | 27.9 |
| V | $C_{13}H_{11}O_4N_2SbNa_2$ | 28.5 | 28.0 |
| VI | $C_{13}H_9O_6N_2SbNa_2$ | 26.6 | 26.9 |

The analyses of these compounds occasioned great difficulty and many methods were tried with varying results.[7]

For the estimation of antimony in the acid dyes, a modification of the method of Schmidt was used. After the sample had been digested with 5 g. of sodium bisulfate and 10 cc. of concd. sulfuric acid until colorless, aqueous solutions of sulfur dioxide and tartaric acid were added to reduce pentavalent antimony. The sulfur dioxide was removed by boiling while carbon dioxide was passed in, the solution cooled, neutralized with sodium bicarbonate and titrated with 0.1 *N* iodine solution.

The method of Macallum[7c] works very well for the water-soluble sodium salts.

## Summary

Stibanilic acid has been diazotized and coupled with phenol, *o*-chlorophenol, *o*-nitrophenol, *o*-aminophenol, *o*-cresol and salicylic acid to give compounds of the type $HO(R)C_6H_3N{:}NC_6H_4SbO_3H_2$, in which R is H, —Cl, —$NO_2$, —$CH_3$, —$NH_2$ or —COOH. These acids form disodium salts which are stable in water solution.

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[CONTRIBUTION FROM THE LABORATORY OF WIDEN-LORD TANNING COMPANY]

# A MAXIMUM REACTIVITY OF THE HIDE PROTEIN IN ITS ISO-ELECTRIC ZONE[1]

BY K. H. GUSTAVSON

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The concept of protein as ampholyte and the application of physico-chemical laws to protein behavior have marked a great advance in this field and have also made possible quantitative treatment of a number of protein reactions as, for example, the combination of acids and alkalies with gelatin, and tannin with collagen. The ionic protein chemistry postulates the existence of protein cations on the acid side and protein anions on the alkaline side of the iso-electric point of the protein. In the former range only the combination between cations of the protein and anions of the

[7] (a) Rohmer, *Ber.*, **34**, 1565 (1901). (b) Fargher and Gray, *J. Pharmacol.*, **18**, 356 (1921). (c) Macallum, *J. Soc. Chem. Ind.*, **42**, 468T (1923). (d) Ref. 4 d, p. 244.

[1] Read before the Division of Leather and Gelatin Chemistry, 72nd meeting of the American Chemical Society, September 6–11, 1926, Philadelphia, Pennsylvania.

electrolyte is possible where, on the other hand, the reverse applies to reactions in the alkaline range with reference to the iso-electric point. As at the latter the acidic and basic ionization is of equal magnitude and further possesses a minimum, it is evident that the protein must exist chiefly in a non-ionized state in the vicinity of the iso-electric point. Accordingly, the formation of protein salts, of primary valence nature is as a rule not possible and the protein must under all circumstances possess a minimum chemical reactivity in its iso-electric state. Loeb[2] has shown that a number of physicochemical properties of gelatin exhibit very pronounced minima in this point, and the same holds for the rate of combination of tannins with hide protein according to Thomas and Kelly.[3]

The ionic concept of protein behavior has, however, in its simplification of very complex reactions only taken into consideration the primary valence factor. The residual valence, forces of specific surface nature and those controlling the degree of aggregation of the protein have been completely ignored. This paper deals with a new type of protein reactions which cannot be explained on the basis of a salt formation by means of primary valence. The fixation of anionic chrome complexes from oxalato-chromiate is shown to take place on both sides of the iso-electric point of the hide protein and, further, a pronounced maximum is located in the region of hydrogen-ion concentration corresponding to the iso-electric state of the protein. The latter finding is evidently contrary to the requirements of a true salt formation as formulated by Loeb.

## Material and Technique

The salt studied, the *cis* form of sodium dioxalato-diaquo-chromiate, $Na[(H_2O)_2Cr(C_2O_4)_2]$, is formed according to the equation $Na_2Cr_2O_7 + 7H_2C_2O_4 = 2Na[(H_2O)_2Cr(C_2O_4)_2] + 3H_2O + 6CO_2$.

Sodium dichromate and oxalic acid, of chemically pure grades in proportional quantities required by the above reaction, were employed. A hot, saturated solution of oxalic acid was added to a highly concentrated, hot solution of sodium dichromate during continuous stirring. After reduction had become complete the solution was boiled for one-half hour to expel the carbon dioxide formed. From portions of the stock solution a number of basic solutions were prepared by the addition of increasing amounts of $N$ sodium hydroxide solution. Series of solutions with the concentrations of chromic oxide as noted in Table I were prepared. Addition of alkali leads to the formation of the hydroxo compounds. The general type of this reaction is represented by the equation[4]

[2] Loeb, "Proteins and the Theory of Colloidal Behavior," McGraw-Hill Book Co., New York, **1925.**

[3] Thomas and Kelly, *Ind. Eng. Chem.*, **15,** 1148 (1923).

[4] Werner, *Ann.*, **406,** 261 (1914). See also Weinland, "Einführung in die Chemie der Komplex-Verbindungen," Enke, Stuttgart, **1924,** p. 128.

$$Na[(H_2O)_2Cr(C_2O_4)_2] + NaOH = Na_2\left[\begin{matrix}H_2O\\HO\end{matrix}\cdot Cr\cdot(C_2O_4)_2\right] + H_2O$$

The *cis* form further gives the di-ol compound, resulting from secondary valence reactions between the hydroxo groups. The solutions were aged for more than half a year. Hydrolysis equilibria of the solutions were established within this time. Instability of the chromium complexes was indicated at Sörensen ($P_H$) values from 8 to 8.5.

Amounts of American Standard Hide Powder, equal to 2.00 g. of hide protein, were treated during continuous shaking with 100cc. portions of these solutions for 48 hours. The treated specimens of hide powder were separated from the respective solutions by filtering through hardened filter papers in Büchner funnels. They were then washed free from adhering chromium. The Sörensen values of the filtrate were immediately ascertained electrometrically.

The treated specimens of hide powder, after being dried first at 40° and finally at 100°, were analyzed for chromium and protein. Portions equal to 0.5000 g. were simultaneously weighed out. Chromium was determined iodimetrically after fusion of the ash in platinum crucibles with a mixture of alkali carbonate and borax glass. Protein was determined by the Kjeldahl-Arnold-Gunning method, employing Winkler's modification for collecting the liberated ammonia. The concentrations of solutions were 5.0, 10.0, 24.0 and 55.0 g. of chromic oxide per liter.

The data are presented in Table I. A graphical representation of the

TABLE I

RESULTS

FIXATION OF CHROMIUM BY HIDE POWDER FROM ANIONIC OXALATO-CHROMIATE

CONCENTRATION: 5.0 G. OF $Cr_2O_3$ PER LITER

| No. | $P_H$ of orig. soln. | $P_H$ of filtrate | Anal. of treated hide powder: $Cr_2O_3$, % | Anal. of treated hide powder: Collagen, % ($N \times 5.62$) | G. of $Cr_2O_3$ combined with 100 g. of collagen |
|---|---|---|---|---|---|
| 1 | 4.37 | 4.81 | 2.01 | 80.1 | 2.51 |
| 2 | 4.70 | 5.11 | 2.44 | 79.2 | 3.08 |
| 3 | 4.97 | 5.31 | 2.76 | 78.5 | 3.52 |
| 4 | 5.26 | 5.42 | 3.74 | 76.9 | 4.86 |
| 5 | 5.43 | 5.70 | 3.62 | 76.8 | 4.72 |
| 6 | 5.72 | 5.91 | 3.12 | 76.6 | 4.07 |
| 7 | 6.01 | 6.17 | 3.12 | 77.7 | 4.02 |
| 8 | 6.24 | 6.41 | 2.86 | 79.6 | 3.59 |
| 9 | 6.80 | 7.02 | 2.29 | 81.0 | 2.83 |
| 10 | 7.31 | 7.38 | 2.08 | 80.2 | 2.60 |
| 11 | 7.68 | 7.73 | 1.61 | 81.5 | 1.98 |
| 12 | 7.87 | 7.90 | 1.77 | 82.6 | 2.14 |
| 13 | 8.02 | 8.05 | 1.66 | 83.4 | 2.00 |
| 14 | 8.15 | [a] | 1.98 | 83.4 | 2.39 |
| 15 | 8.24 | [a] | 1.98 | 83.4 | 2.39 |
| 16 | 8.37 | [a] | 1.93 | 84.2 | 2.30 |

TABLE I (*Concluded*)

CONCENTRATION: 10.0 G. OF $Cr_2O_3$ PER LITER

| No. | *P*H of filtrate | Anal. of treated hide powder: $Cr_2O_3$, % | Anal. of treated hide powder: Collagen, % ($N \times 5.62$) | G. of $Cr_2O_3$ combined with 100 g. of collagen |
|---|---|---|---|---|
| 1 | 2.47 | 1.74 | 82.8 | 2.10 |
| 2 | 3.27 | 2.46 | 78.7 | 3.12 |
| 3 | 4.18 | 3.42 | 77.4 | 4.42 |
| 4 | 4.52 | 4.01 | 77.8 | 5.15 |
| 5 | 4.81 | 4.31 | 77.1 | 5.59 |
| 6 | 5.09 | 4.58 | 76.3 | 6.00 |
| 7 | 5.40 | 4.52 | 76.2 | 5.93 |
| 8 | 5.87 | 2.94 | 79.3 | 3.71 |
| 9 | 6.82 | 1.28 | 83.8 | 1.53 |
| 10 | 7.28 | 0.72 | 84.9 | 0.84 |
| 11 | 6.52 | .64 | 85.1 | .75 |
| 12 | 7.68 | .56 | 85.7 | .65 |
| 13 | 8.11 | .48 | 86.1 | .56 |
| 14 | 8.27 | .59 | 85.5 | .69 |

CONCENTRATION: 24.0 G. OF $Cr_2O_3$ PER LITER

| No. | *P*H of filtrate | G. of $Cr_2O_3$ combined with 100 g. of collagen |
|---|---|---|
| 1 | 2.14 | 2.82 |
| 2 | 3.08 | 5.90 |
| 3 | 4.13 | 9.77 |
| 4 | 4.45 | 10.39 |
| 5 | 4.52 | 11.04 |
| 6 | 4.68 | 11.40 |
| 7 | 4.80 | 11.78 |
| 8 | 4.97 | 11.39 |
| 9 | 5.12 | 10.50 |
| 10 | 5.43 | 8.62 |
| 11 | 6.62 | 3.46 |
| 12 | 7.80 | 0.81 |
| 13 | 8.14 | .31 |
| 14 | 8.37 | .32 |

CONCENTRATION: 55.0 G. OF $Cr_2O_3$ PER LITER

| No. | *P*H of filtrate | G. of $Cr_2O_3$ combined with 100 g. of collagen |
|---|---|---|
| 1 | 1.27 | 2.02 |
| 2 | 2.50 | 6.40 |
| 3 | 3.64 | 9.75 |
| 4 | 4.01 | 10.29 |
| 5 | 4.37 | 9.70 |
| 6 | 4.79 | 8.32 |
| 7 | 5.36 | 6.02 |
| 8 | 5.84 | 3.90 |
| 9[a] | 6.31 | 2.83 |
| 10 | 7.43 | 0.92 |
| 11 | 8.09 | .38 |

[a] Reproducible potentials could not be obtained, probably due to the considerable hydrolysis of collagen at these high *P*H values.

fixation of anionic chromium as a function of the Sörensen values of solution is shown in Fig. 1.

## Discussion of Results and Theory

The oxalato-chromium-collagen compounds formed in the optimal range of hydrogen-ion concentration possessed nearly the same degree of stability as the corresponding collagen compounds with cationic chromium (sulfates and chlorides) as measured by their resistance to boiling water. The amounts of non-hydrolyzable substance based on the ash-free, dry substance of the treated hide powders were 76 and 90% for the specimens

tanned at the maximum chrome-fixation zone with solutions containing 10.0 and 24.0 g. of chromic oxide per liter, respectively.

The rate of chrome fixation as a function of hydrogen-ion concentration of solutions increases greatly with increase in Sörensen value until a pronounced maximum zone is established in the range from $P_H$ 4 to 5.6, beyond which a rapid decline occurs. The location of this maximum is evidently largely determined by the concentration of the solution and with decrease of the latter it is shifted toward the alkaline side of the isoelectric point of the hide powder. In the maximum chrome-fixation zones of the different solutions the ratio of the amount of sodium hydroxide to chromic oxide in the original solutions is not constant. The data further

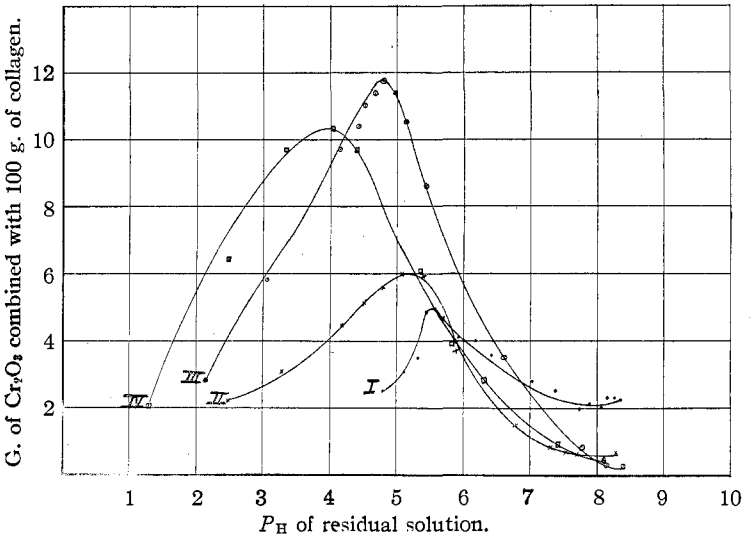


Fig. 1.—Fixation of chromium by hide powder from solutions of anionic oxalato-chromiate as a function of their Sörensen values. Concentration of solution, g. of $Cr_2O_3$ per liter: *I*, 5.0; *II*, 10.0; *III*, 24.0; *IV*, 55.0.

show that the chrome fixation is not controlled by the ionization tendency of the protein. If this was the case, the maximum would be located at the same Sörensen value, independent of the concentration of solutions. The series with deaminized hide powder further substantiate this finding as will be shown later. The degree of colloidality of the chromium compound is evidently only of secondary importance as it was found that the degree of dispersity is considerably lowered first at $P_H > 6$, in which region only a slight fixation of chromium takes place.

The formation of molecular compounds by means of secondary valence forces from the basic protein groups to the central atom in the anionic complex seems to be the most reasonable explanation of this reaction and at the present the only one possible. A number of facts, later to be dis-

cussed, support this view. The dependence of the location of maximum chrome-fixation zone upon the concentration of the liquor is thus explained by constitutional changes in the chrome complex. A similar constitutional factor is also evident in the fixation of cationic chromium salts of strong acids by hide protein.[5] The maximum chrome fixation coincides with the maximum secondary valence potency of the anionic complex. The view of this reaction as a regular ionic salt formation between cationic collagen and anionic chromium complexes assuming the existence of a second iso-electric point of collagen at about $P_H$ 8, is inadequate to explain the pronounced maxima in the neighborhood of the iso-electric point reported in this paper. The possibility of changes in the residual valence capacity of the hide protein taking place with variation of Sörensen values has also to be considered. From the standpoint of the "internal salt" hypothesis of the structure of ampholytes of amino acid type as advanced by Bjerrum,[6] it would be expected that the residual valence activity of the protein would be greatest in its iso-electric state.

In Table II the data from comparative experiments with the same oxalato solutions employing regular hide powder and the same with the amino groups removed are given. The deaminized hide powder was prepared according to the method of Thomas and Foster.[7] The alcoholic dehydrated specimens of the two kinds of hide powder in portions corresponding to 3.00 g. of collagen were first hydrated with 50 cc. of water for 12 hours and then treated for 48 hours with oxalato-chromiate solutions of different Sörensen values. The final concentration was 12.0 g. of chromic oxide per liter.

TABLE II

THE FIXATION OF CHROMIUM FROM SODIUM-OXALATO-CHROMIATE BY HIDE POWDER AND DEAMINIZED HIDE POWDER

| | Regular hide powder | | Deaminized hide powder | |
|---|---|---|---|---|
| No. | $P_H$ of filtrate | G. of $Cr_2O_3$ combined with 100 g. of collagen | $P_H$ of filtrate | G. of $Cr_2O_3$ combined with 100 g. of collagen |
| 1 | 4.50 | 4.96 | 4.31 | 3.93 |
| 2 | 4.68 | 5.94 | 4.58 | 4.50 |
| 3 | 4.97 | 6.73 | 4.75 | 5.17 |
| 4 | 5.16 | 7.09 | 5.01 | 5.48 |
| 5 | 5.45 | 6.78 | 5.28 | 5.02 |
| 6 | 5.70 | 5.45 | 5.52 | 4.55 |

The removal of amino groups from the protein tends to inhibit the chrome fixation but evidently the reaction is practically independent of the extent of ionization of the hide protein. Only a slight shift of the maximum zone of chrome fixation toward the acid side results where, on the other

[5] Gustavson, *Collegium*, **1926**, p. 153. Gustavson and Widen, *Ind. Eng. Chem.*, **17**, 577 (1925).

[6] Bjerrum, *Z. physik. Chem.*, **104**, 147 (1923).

[7] Thomas and Foster, THIS JOURNAL, **48**, 489 (1926).

hand, a reaction governed by the ionic state of the hide protein would be expected to show a shift to a $P_H$ between 3 and 4 as the iso-electric zone of deaminized hide powder falls in the range of $P_H$ 3.5 to 4.0.[7]

The retardation of chrome fixation observed in the series with deaminized hide powder indicates the probability of the basic protein groups being chiefly involved in the anionic chrome fixation. In this connection, the fact is worth noticing also that the fixation of cationic chromium from solutions of sulfates and chlorides is decreased by the deamination,[8] which finding is taken by these authors as an indication that nitrogen groups play a significant role in this mode of chrome fixation. Chemical inactivation of basic protein groups seems as a rule to lead to a decrease in the combining capacity of such treated hide protein toward cationic chromium. A number of facts indicate, if they do not prove, that the acidic protein groups form the principal vehicle for this reaction. The finding of Thomas and Foster is probably explained by the diminished fixation of the free, hydrolyzed acid in equilibrium with the basic chromic salt. The result is a depression in the acid hydrolysis of the salt. The fixations of the "basic" and "acidic" constituents of the chromic salt are two mutually influenced reactions. Accordingly, the extent of combination with cationic chromium complexes is bound to be decreased.

It was thought that further information in regard to protein reactions in general and the mechanism of chrome tanning in particular would be obtained by investigations of hide powder on the one hand and structurally altered hide powder on the other toward other agents. Detailed accounts of these researches will be presented in separate articles. In this connection only the findings of direct applicability to the problem under discussion will be given.

Hide powder treated in the iso-electric state with neutral salts shows practically identical chromic oxide values after interaction with basic sulfates and chlorides of chromium of cathodic migration and of crystalloid nature. The oxalato compound, in its rate of fixation by the neutral salt-treated, hide-powder specimens manifests, on the other hand, a definite dependence on the previous history of the protein, the recorded fixation, being in accordance with the Hofmeister series.

Hide powder which has been treated with acids and alkalies and thereafter brought back to its iso-electric state in its behavior toward the two classes of chromium salts gives data which support the view of the dual nature of the chrome fixation. Acid treatment of the protein leads, also, in the instances where colloidal side reactions are excluded, to an increased rate of anionic chrome fixation, but the cationic salts are not influenced in their reactions with the treated stock. An activation of amino and other basic groups—besides changes in degree of aggregation of the protein, a

[8] Thomas and Foster, THIS JOURNAL, **48**, 1312 (1926).

function not here concerned—probably occurs by the formation of the protein acid salt. This formation would indicate that the basic groups play the principal role in reactions with anionic chromium. Alkali-treated hide powder has greater affinity for both classes of compounds compared with the regular hide powder.

Cationic chrome-tanned hide powder possesses the same tendency as hide protein proper, or slightly greater, to combine with vegetable tannins under the same experimental conditions and with correction applied for the decrease in Sörensen value caused by displacement of part of the acid combined with the protein by the tannins. Hide powder tanned with oxalato-chromiate shows in all instances a slight but definite decrease in tannin-fixative capacity compared to untreated hide powder.

Vegetable tanned hide powder possesses less affinity for both cationic and anionic chromium. The retardation of the cationic chromium fixation is not unexpected in light of the mutual dependence of the cationic chromium and the hydrolyzed acid, which latter has a less number of basic protein groups available for its combination with hide protein.

The fixation of dyes at identical Sörensen values of the dye solutions and with other experimental conditions the same indicates a marked difference in the nature of cationic and anionic chrome-tanned hide powder. An acid dye, for example, Acid Blue Black, shows very great affinity for the former compound but it is considerably less taken up by the anionic chrome collagen. The dyeing process in this instance is, by its nature, a reaction between the colored anion of the dye and the protein cation, and therefore the basic protein groups are involved. Accordingly, the data confirm the hypothesis of the dual nature of chrome fixation and, further, they demonstrate the partial inactivation of the basic protein groups caused by the treatment with anionic chromium salts. The dyeing with basic dyes, for example, Methylene Blue, results in the reverse behavior, as would be expected in view of the fact that the anionic chrome-tanned stock possesses the original amount of reactive acidic groups which partake in this mode of dyeing whereas, on the other hand, in the hide powder tanned with basic chromic sulfates of cationic nature part of these groups have reacted with the electropositive chrome complexes and thus are not available for further reactions.

The foregoing considerations favor the view that different radicals of the protein are involved in reactions with cationic and anionic chromium compounds. The present status of protein chemistry in regard to structural details and the problem of valence distribution does not permit its use as conclusive proof. A very simple and, in the writer's opinion, conclusive demonstration of the advanced concept is afforded by the data from the fixation of cationic and anionic chromium by cationic chrome-tanned hide powder and the reverse procedure. Hide protein containing as great

an amount as about 20% of chromium oxide on the basis of collagen gives, upon treatment with solutions of oxalato-chromiate, the same additional chrome fixation as that recorded for regular hide powder, with due correction applied for the decrease in Sörensen value caused by removal of the acid sulfate by the anionic complex. A cationic chromium salt of more than twice the affinity toward collagen as that of the oxalato compound used gives no further increase in content of chromic oxide on the basis of collagen upon interaction with the cationic chrome-tanned stock. The anionic chrome complexes and the vegetable tannins thus show similarity in their behavior toward hide protein in combination with cationic chromium. The mineral acidity of the latter compound, very likely existing in part as collagen sulfate, is considerably reduced by both of these reactions. The view of the vegetable tannage as partly taking place by means of the basic protein groups has received substantial experimental support by the fundamental investigations of Thomas and his collaborators.[8,7,9] The cationic chrome fixation is considerably decreased by pretreatment with anionic chrome salts; thus, again, a parallelism between the action of oxalato anions and tannins is evident.

The type of protein reactions exemplified by the hydroxo-oxalato-chromiate cannot be explained from the standpoint of proteins as ampholytes and their reactivity due to their ionization potential, a primary valence compound being the final result. Application of the "Zwitterion" hypothesis of Bjerrum upon protein behavior may make possible an explanation of the maximum reactivity in the iso-electric zone of the protein reported in this paper. The ionic concept of protein reaction is evidently not applicable to all types of protein reactions.

## Summary

The fixation of chromium by hide protein from sodium-oxalato-chromiate in the range from $P_H$ 2 to 8.5 shows a maximum in the neighborhood of the iso-electric point of the protein. The view of the formation of molecular compounds between the anionic chrome complexes and the hide protein by means of its basic groups as the mechanism of this reaction has received experimental substantiation from investigations employing structurally changed hide protein. The ionic concept of protein behavior fails in elucidation of this type of reaction.

DANVERS, MASSACHUSETTS

[9] Thomas and Kelly, *Ind. Eng. Chem.*, **16**, 800, 925 (1924).