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RELATIVE AFFINITIES OF THE ANIONS OF STRONG ACIDS FOR WOOL PROTEIN [1]

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ABSTRACT

Evidence presented in previous papers supported the view that wool immersed in solutions containing hydrochloric acid combines stoichiometrically not only with the hydrogen ions of the acid but with chloride ions as well. As a consequence it appeared that the specific affinities for wool of the anions of different acids might vary considerably, and that therefore the positions of the titration curves of this protein with respect to the pH axis might vary by correspondingly large amounts according to the acid used.

The present paper describes measurements of the combination of wool with 19 different acids, ranging in complexity from some of the mineral acids most commonly used through the simpler aromatic sulfonic, carboxylic, and phenolic acids to a soluble monoazo acid dye. It is shown that wide differences exist between the positions with respect to the pH axis of the titration curves of wool obtained with different strong acids, and that these differences may be ascribed to wide variations in the anion dissociation constants characterizing the corresponding protein-anion combinations. Equations previously derived to account for effects caused by variations in chloride concentration have been generalized for use in calculating these dissociation constants. A scale of relative affinities of anions for wool, based on these constants, and applicable to acid dyes, is proposed. Predictions as to the effects of variations of anion concentration and of temperature, based on the same generalized equations, have been tested and confirmed.

Measurements of the combination of a number of the same acids with a soluble protein, crystalline egg albumin, have also been made. Since qualitatively similar differences in the positions, with respect to the pH axis, of the titration curves obtained with different acids are found with both proteins, it is concluded that the property of combining with anions is not restricted to insoluble proteins. The affinity of anions for proteins of both classes appears to increase with the dimensions of the anion, and is higher in aromatic than in aliphatic ions of the same size. The bearing of these relationships on the well known specific effects of ions on proteins and on the nature of the forces involved in the binding of anions by proteins is considered.

CONTENTS

[1] Part of the material in the present paper was presented at the meeting of the American Society of Biological Chemists at New Orleans, La., in March 1940 [37].

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I. INTRODUCTION

It is well recognized that the fastness of wool dyes to washing, and their related properties of exhausting onto wool or other protein fibers from a dye bath, vary widely not only among different classes of dyes, but also among the members of a given class. These variations are frequently regarded as manifestations of differences in affinity of the various dyestuffs for the fibers. The existence of similar differences in the affinities of ions for proteins in general is suggested by the frequently reported and repeatedly investigated instances of specific differences in the effects of various ions on protein behavior.

A possible basis for understanding such specific effects in terms of the participation of the anions of acids and the cations of bases in the combination of wool protein with acid and base has already been described in two earlier communications [36, 38].[3] Evidence was presented in support of the view that the combination of wool with hydrochloric acid may be resolved into two partial reactions, one with hydrogen ions and one with chloride ions, and that each reaction is governed by its own equilibrium constant. On the basis of this assumption it proved possible to account for the large effects produced by varying the concentration of potassium chloride on the combination of wool with hydrochloric acid. Among other consequences of this view was a strong possibility that the specific affinities for wool of the anions of different acids might vary considerably, and that therefore the positions of the titration curves of this and other proteins with respect to the pH axis might vary by correspondingly large amounts according to the acid used. The existence of such a variation would constitute critical evidence in support of the hypothesis of anion association, since alternative analyses of the effect of salt, based upon the Donnan membrane-equilibrium equations [36], offer no obvious basis for the prediction of such an effect.

The present paper describes measurements of the combination of wool with 19 different acids, ranging in complexity from some of the mineral acids most commonly used in the laboratory through the simpler aromatic, sulfonic, carboxylic, and phenolic acids to a soluble monoazo acid dye. The acids employed were selected from among those readily available, with the purpose in view of obtaining data which could be simply calculated and interpreted in terms of molecular size, chemical structure, and the effects of specific substituents. For this reason most of the results reported in the present paper deal with acids which are monobasic and virtually totally dissociated in water. A limited number of weak acids which are analogues of some of the

[3] Figures in brackets indicate the literature references at the end of this paper.

strong acids used (for example, benzoic acid is analogous with benzenesulfonic acid in the sense used here) have been included for the specific purpose of examining the effects of this analogy. An effort has also been made to include organic acids with all of the more common dissociating groups. Since strong acids containing carboxylic or phenolic radicals as dissociating groups can be obtained only by loading with substituents such as halogens or nitro groups, other related acids have been included in order to make possible an independent appraisal of the effect of various substituent groups, and of more strictly physical factors, such as the size and shape of the anion produced.

In order to determine whether differences in affinity between the ions of different acids are restricted to wool, or to the insoluble class of proteins for which the theory of anion association was specifically proposed [36], measurements have also been made of the combination of a number of the same acids with a well-characterized *soluble* protein, crystalline egg albumin. Demonstration that the hypothesis of anion association is applicable to both would have an obvious bearing on the interpretation of the titration curves of both classes of proteins, and of the wide range of phenomena which depend on the combination of protein with acid or base.

II. EXPERIMENTAL PROCEDURE

Details of the purification of the wool and of the method used in measuring pH values and the amounts of acid combined have already been described [36, 38]. To improve the accuracy of estimation of quantities depending on pH differences, pH values are given to three decimal figures wherever the precision of the measurements, determined as previously described, is better than 0.003 unit. The absolute accuracy of the values given depends on the accuracy of the pH values assigned to the primary standards [36].

Additional details which concern the materials used in the present investigation and modifications of the earlier procedures are described in the following subsections.

1. MATERIALS

(a) WOOL

Except where otherwise noted, all of the measurements were made with wool from the batch used in a previous investigation [38]. Samples of this wool, on incineration at 700° C, yielded a residue of 0.26 percent by weight; the hydrogen-ion equivalence of their cation content, as shown by electrodialysis [32], was 0.036 millimole per gram. Determination by the methods previously described [36] showed that of this ash 0.032 millimole per gram was combined as base, and that therefore a correspondingly larger quantity of acid is neutralized by this wool than would be neutralized by ash-free fibers.

In calculating the results obtained with most of the acids used, this small excess of acid has been subtracted from the apparent amounts of acid bound at every pH. The validity of this procedure was self-evident in the measurements with hydrochloric acid, but it is somewhat open to question when anions of much higher affinity for wool than that of chloride are present. It is shown elsewhere in

this paper that the affinities of certain anions for wool are so high as to produce a net negative charge on the fibers even in acid solutions in which combination with hydrogen ions usually results in a net positive charge. Since the negative charge may be neutralized in part by cations other than hydrogen ion, including the cations naturally in the ash, not all of the alkaline ash is available for neutralization of the dissolved acid. No correction for ash has been applied therefore to the data obtained with Orange II, the anion of which has the highest affinity of those included in this paper. With picric and flavianic acids, next highest in order of affinity, the full correction has been applied; a slight overcorrection may result, especially in the range of pH in which only small amounts of acid are bound.

(b) PREPARATION OF EGG ALBUMIN

Crystalline egg albumin was prepared by the method of Kekwick and Cannan [22], recrystallized five times, and dialyzed free of sulfate. The resulting stock solution contained 7.02 mg of nitrogen per milliliter and gave a dry residue, on evaporation in a vacuum oven at 100° C, of 45.2 mg/ml. The closeness of the pH of the diluted stock solution (5.09 at 22° C) to the isoionic pH of this protein, indicates that the preparation was practically free of combined ions. The nitrogen content, 15.5 percent, is in good agreement with the values hitherto reported.

(c) SOURCE AND PURIFICATION OF ACIDS

Care was required in the removal of ionogenic impurities from all the acids used, especially when the affinity of the anion of the acid for the protein was much lower than the affinity of one or more of the anions contributed by the impurities. When only a small proportion of all the acid present is combined (as at low pH), the amount combined may be almost entirely the impurity and not the acid under investigation.

In every case the absence of sulfate or chloride was established by analysis. The possibility of the presence of appreciable amounts of salts was excluded by titrating weighed portions of the dry preparations.

The source and method of purification of the acids used are itemized below:

1. Hydrochloric, nitric, and trichloroacetic acids were commercial cp products used without further purification.
2. Hydrobromic acid was a commercial cp product, from which appreciable quantities of free bromine were removed under vacuum.
3. Ethylsulfuric acid was prepared by dissolving diethyl sulfate in a large excess of absolute ethanol, and heating the solution to 55° C for 24 hours, as described in Beilstein [I, 325]. The solution was then evaporated at room temperature under vacuum, and dilutions were made of the resulting approximately 1 *M* stock solution. The solutions of this acid thus contained varying but small quantities of ethyl alcohol. Analyses showed the presence of small amounts of sulfate ion, which were removed by the addition of an accurately determined equivalent quantity of barium hydroxide. The methods of preparation used for both this acid and the acid which follows excluded the possibility of the presence of salts.

4. Benzenesulfonic acid was prepared by treating redistilled benzenesulfonyl chloride with silver oxide and passing hydrogen sulfide through a solution of the resulting silver salt.

5. *o*-Nitrobenzenesulfonic acid, 2,4,6-trichlorophenol, 2,4-dinitrophenol, and 2,4,6-trinitroresorcinol were Eastman products purified by two or more recrystallizations from water.

6. *p*-Toluenesulfonic acid, 2,4-dinitrobenzenesulfonic acid, *o*-xylene-*p*-sulfonic acid, naphthalene-β-sulfonic acid, and flavianic acid (1-naphthol-2,4-dinitro-7-sulfonic acid) were Eastman products from which traces of impurities which were only slightly soluble in water were removed by filtration.

7. Picric acid (2,4,6-trinitrophenol) was Merck's U.S.P. grade containing 15 percent of added water, used without further purification.

8. Benzoic acid was a National Bureau of Standards Standard Sample (No. 39e).

9. 2,5-Dichlorobenzenesulfonic acid and 4-nitrochlorobenzene-2-sulfonic acid were Eastman products which contained appreciable amounts of sulfuric acid as an impurity. They were purified by precipitating the sulfate with an accurately determined equivalent quantity of barium hydroxide.

10. The sodium salt of Orange II (*p*-sulfobenzene-azo-β-napthol), obtained from the Eastman Kodak Co., was further purified by repeated salting out according to the method of Robinson and Mills [27]. The free acid was prepared by dissolving the salt in 2 *M* hydrochloric acid and washing the precipitated dye acid with several portions of the same hydrochloric acid solution. When appreciably higher or lower concentrations of the acid are used, the loss of dissolved acid dye in subsequent washing is very considerable. The precipitate of acid dye was air-dried, then oven-dried at 60° C, and finally dried to constant weight in a vacuum desiccator over KOH. Tests showed that hydrochloric acid had been completely removed in the drying process. The acid dye, as well as its sodium salt, is very soluble in water, but efforts to prepare concentrated solutions of the acid revealed the presence of a small quantity of a less soluble impurity. Most of the impurity was removed by decanting concentrated solutions from the insoluble residue.

2. METHODS

(a) MEASUREMENT OF ACID COMBINED WITH WOOL

Strong acids which gave colored solutions were titrated with bromthymol blue, instead of the bromcresol purple previously used, because its color change was more easily detected. With weak acids thymol blue or phenolphthalein was used. Densely colored solutions of Orange II were titrated to an electrometric end point at pH 7.

Although it proved possible in the experiments with hydrochloric acid to attain equilibrium within a period of 72 hours at 0° C, and in still shorter periods of time at 25° C, and 50° C [36, 38], some of the acids used in the present investigation required very much longer periods of time at all these temperatures. Thus, with flavianic acid a period of 23 days at 0° C proved insufficient for the combination of over 0.42 millimole per gram, while values approaching the usual

maximum of about 0.8 millimole per gram were obtained in a few days at 25° C. A period of 30 days at the latter temperature proved insufficient with Orange II, while at 50° C a period of 5 days sufficed. When the attainment of equilibrium requires long periods, the rate of uptake may become undetectably small when much less than equilibrium amounts have been combined. In general, the length of time which must be allowed for essentially complete attainment of equilibrium increases with the affinity for wool of the anion of the acid.

The reversibility of the combination with several of the acids used has been demonstrated by subjecting the fibers to prolonged washing and by replacement of combined anions on immersing the fibers in solutions of other anions having higher affinities.

(b) MEASUREMENT OF ACID COMBINED WITH EGG ALBUMIN

The acid combined by a dissolved protein obviously cannot be measured by the change in the titratable acidity of a solution, as was possible with wool. Methods of calculating this quantity for dissolved proteins are all based on differences in pH between solutions of the acid alone and of the acid plus protein. They have been described by Loeb [25], Cohn, Green and Blanchard [8], Kekwik and Cannan [22], and Hitchcock [20], among others, and are summarized in reviews by Hitchcock [21] and by Schmidt [30]. In the present investigation pH measurements were made of serial dilutions of each acid used in the absence of protein as well as in the presence of 0.113 percent by weight of dissolved egg albumin. The difference in pH caused by the protein gives directly the proportion of the total acid present which has been neutralized (combined by protein), if it can be assumed that the presence of this low concentration of protein is without significant effect on the activity of the uncombined acid. Differences in the activity coefficients of the acid due to its dilution by partial neutralization were taken into account when the initial concentration of acid was so high as to warrant application of the correction. However, owing to the low concentration of the protein, total concentrations of acid in excess of 0.01 *M* gave rise to such small pH differences that calculations of bound acid at pH values below 2 were subject to large uncertainties. In general, the effect of the variation of activity coefficients with concentration is considerably smaller than these uncertainties.

In calculating the results obtained with flavianic acid, it was assumed that every molecule of the acid contributed two hydrogen ions to the solution. A comparison of the pH values with the titers of the experimental solutions containing no protein indicated that this assumption is approximately valid in the pH range of the curve for this acid. Any error introduced by the assumption probably affects only the highest two points on the curve, which would be slightly lower if an exact calculation were made.

III. RESULTS AND DISCUSSION

1. RESULTS OBTAINED IN THE ABSENCE OF SALTS

The results obtained with 18 different acids in the absence of salt at 0° C are summarized in table 1. The data obtained with the 16 strong acids included are assembled in figure 1. In drawing the curves, an effort has been made to represent the measurements fairly

with smooth curves rather than to preserve the appearance of homogeneity of the data. The resulting tendency of certain neighboring curves to cross or converge is usually within the range of effects which may be ascribed to experimental error.

The most noticeable feature of the data in figure 1 is the wide range of positions with respect to the pH coordinates which the titration

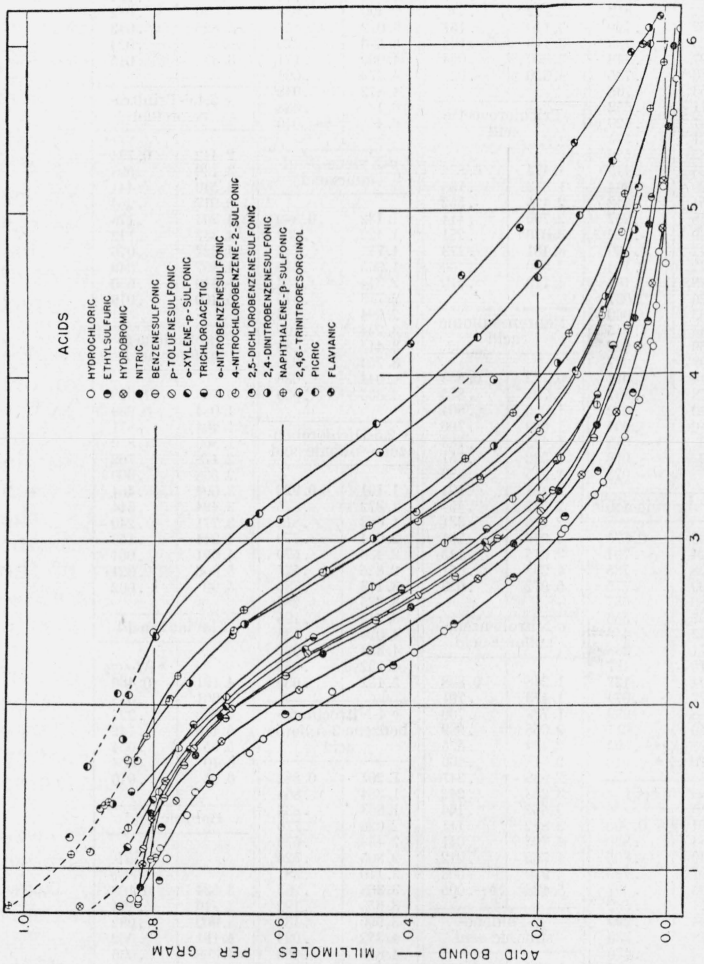

FIGURE 1.—*Combination of wool protein with 16 different strong acids as a function of* pH *at 0° C.*

The measurements with flavianic acid are represented in milliequivalents instead of millimoles. Points representing the combination of less than 0.14 millimole per gram of three acids (*p*-toluenesulfonic, *o*-xylene-*p*-sulfonic, and *o*-nitrobenzenesulfonic) would fall at lower pH values than are required for congruence with the curves for the other acids, and have been omitted to avoid confusing the figure. Data for the two dibasic acids, flavianic and trinitroresorcinol, are only partially represented, as explained elsewhere.

curves occupy. It is also apparent that except for minor deviations the curves form a coherent family, near neighbors following a fairly parallel course. The change in the slopes of the lower portions of the curves, from the more sharply inflected curve for hydrochloric and ethylsulfuric acids at one end of the pH range to the less sharply inflected curves for picric and flavianic acids at the other end, tends to be gradual and progressive. The appearance of the curves as a group is entirely consistent with the view that they all describe analogous phenomena.

TABLE 1.—*Combination of wool protein with various acids at 0° C, in the absence of added salt*

Hydrochloric acid	
pH	*Millimoles/g*
0.447	0.868
.635	.838
.813	.836
.822	.812
1.062	.779
1.125	.789
1.327	.748
1.470	.735
1.553	.708
1.711	.652
1.797	.629
1.931	.574
2.085	.522
2.228	.445
2.320	.394
2.451	.348
2.748	.247
3.039	.166
3.072	.160
3.272	.114
3.348	.101
3.620	.067
3.783	.060
3.844	.045
4.169	.022
4.198	.027
4.414	.017
4.668	.001
4.740	.012
5.140	.004
5.58	[a]−.012
5.79	[a]−.018
6.1	[a]−.023

Hydrobromic acid[b]	
0.783	0.875
1.024	.791
1.308	.785
1.539	.775
1.780	.687
2.087	.566
2.332	.456
2.771	.288
3.115	.187
3.394	.127
3.772	.074
4.168	.039
4.550	.011
4.878	[a]−.003
5.170	[a]−.010

Nitric acid	
0.601	0.900
.884	.820
1.340	.805
1.697	.735
1.936	.697
2.311	.532
2.594	.399
2.978	.239
3.296	.170
3.694	.111
4.339	.050
4.906	.027
5.50	[a]−.006
5.97	[a]−.012
6.6	[a]−.026

Ethylsulfuric acid	
0.928	0.790
1.252	.788
1.520	.713

Ethylsulfuric acid	
pH	*Millimoles/g*
1.949	0.595
2.242	.427
2.498	.332
2.792	.240
3.140	.151
3.418	.104
3.890	.054
4.320	.031

Trichloroacetic acid	
1.474	0.836
1.883	.738
2.415	.552
2.700	.414
3.070	.281
3.481	.173
3.890	.109
4.170	.079

Benzenesulfonic acid	
0.772	0.853
.791	.825
1.060	.804
1.069	.796
1.435	.783
1.632	.751
1.915	.689
2.181	.594
2.512	.454
2.786	.331
3.010	.265
3.615	.115
4.295	.061
5.072	.023

o-Nitrobenzenesulfonic acid	
1.248	0.838
1.473	.791
1.739	.760
2.058	.689
2.434	.575
2.718	.460
2.989	.347
3.253	.242
3.502	.166
3.812	.111
4.405	.041
4.752	.012
4.930	.003
5.428	[a]−.005

p-Toluenesulfonic acid	
1.124	0.817
1.411	.764
1.732	.738
1.984	.678
2.315	.560
2.667	.388
2.954	.280
3.178	.198
3.398	.131
3.638	.085
3.997	.036
4.280	.013

2,4-Dinitrobenzenesulfonic acid	
pH	*Millimoles/g*
1.399	0.863
1.787	.848
2.138	.746
2.490	.657
3.012	.457
3.570	.270
4.069	.171
4.470	.094
4.872	.048
6.1	.028
6.4	[a]−.010

o-Xylene-*p*-sulfonic acid	
1.132	0.840
1.422	.800
1.734	.743
1.995	.688
2.344	.564
2.716	.415
2.994	.284
3.254	.199
3.445	.130
3.754	.085
4.044	.036
4.335	.009

[b] 2,5-Dichlorobenzene-sulfonic acid	
1.191	0.926
1.372	.875
1.663	.819
2.044	.770
2.436	.670
2.825	.525
3.153	.385
3.385	.285
3.743	.195
4.020	.136
4.393	.092
4.732	.059
5.132	.036

[b] 4-Nitrochlorobenzene-2-sulfonic acid	
1.201	0.882
1.384	.868
1.663	.833
2.026	.770
2.453	.670
2.806	.526
3.110	.381
3.368	.285
3.653	.192
3.999	.132
4.352	.094
4.859	.063
5.001	.038

Picric acid	
1.624	0.904
2.059	.838
2.072	.857
2.414	.797
3.116	.654
3.165	.638
3.708	.453
4.229	.296

Picric acid	
pH	*Millimoles/g*
4.586	0.200
4.666	.200
4.964	.134
5.296	.082
5.826	.042
6.00	.023
6.35	.015

[b] 2,4,6-Trinitroresorcinol	
2.442	0.794
3.130	.598
3.540	.441
3.917	.264
4.204	.176
4.387	.112
4.627	.076
4.897	.049
5.310	.030
5.460	.016

Napthalene-β-sulfonic acid	
0.769	1.028
1.084	0.930
1.404	.871
1.806	.810
2.178	.762
2.598	.660
3.094	.464
3.494	.314
3.771	.240
4.244	.153
5.091	.064
5.606	.024
5.90	.002

Flavianic acid	
	[c] *M-eq/g*
4.191	0.400
4.601	.318
4.898	.227
5.410	.142
5.95	.054
6.10	.034
6.26	.010

Benzoic acid	
	Millimoles/g
3.534	0.168
3.710	.124
3.900	.082
4.191	.062
4.398	.036
4.842	.021
4.989	.005

2,4-Dinitrophenol	
4.83	0.085
5.27	.056
5.63	.031
5.90	.011
6.19	[a]−.004
6.33	[a]−.011

[a] Negative values indicate loss of bound acid or combination with hydroxyl ion.
[b] A different batch of wool having an exceptionally low ash content and a slightly higher maximum combining capacity was used in these measurements.
[c] Note that in this group milliequivalents rather than millimoles are given.

It is a familiar fact that the position on a pH grid of the titration curves of the common weak bases is determined by their own dissociation constants and not by the dissociation constant or by other chemical properties of the acid employed in the titration. Sets of titration curves obtained with any number of weak or strong acids would be expected to be practically superimposed on one another. Exceptions arise, as in the work of Cannan and Kibrick [7] and of Greenwald [16, 17] with carboxylic acids and phosphates, when the compound formed by the reaction of the base with certain acids is not typically salt-like and does not exist in solution predominantly in the dissociated form. These exceptions find their counterparts, in the case of ampholytes, in the formation of only partially dissociated stoichiometric complexes between the anions of the acids and the ampholyte which has combined with hydrogen ions. When these are formed, the positions of the titration curves and to a lesser extent their shapes, will differ according to the extent to which the anion is dissociated [36].

The wide range of the measurements serves to emphasize the very large differences which may be found when different acids are used. If the position of each curve is characterized by the pH value at which half the maximum amount of acid (about 0.4 millimole per gram) is taken up, there is a difference of almost 2 pH units between the curves shown at the extremes of the series. Between these extremes the results obtained with the other acids are distributed with a fair degree of uniformity, with no evident tendency to resemble closely the results obtained with hydrochloric acid, the acid which has hitherto been most widely used in measuring the acid-combining properties of proteins.[4]

Attention must also be directed to another feature of many of the data represented in figure 1. This is the combination of amounts of acid in excess of 0.82 millimole per gram, the amount earlier reported as the "maximum" obtained with hydrochloric acid. The maximum of 0.82 millimole per gram was sometimes exceeded by small amounts when high concentrations of acid (>0.2 *M*) were used. Measurements of acid combined are susceptible to a relatively large experimental error when such high concentrations of acid are used, because (a) only a small proportion of the acid initially present is combined, (b) some acid hydrolysis of the protein cannot be avoided, and (c) exchanges of water between the partially hydrated fibers and the solutions, difficult to evaluate exactly, have a relatively large influence on the results obtained. For these reasons little weight was placed on these measurements, although they were consistent with the results of similar determinations on bone and hide collagen [2], and might be explained by the presence in proteins of extremely feeble basic groups, such as the amide groups of glutamine and asparagine, and the large content of peptide nitrogen. Special interest, therefore, attaches to the finding that in every case in which sufficiently high concentrations of other acids are used, a second step in the curve of acid combination apparently begins at pH values more acid than those at which the curve that covers the usually investigated range of combination flattens. Since this fairly well-marked "maximum" of about 0.82 millimole per gram has been shown to correspond closely to the

[4] Comparable differences between the titration curves of wool with hydrochloric and with picric acids were found by Elöd [11].

primary amino content of the fibers, the additional uptake in more concentrated solutions must be accounted for by the more weakly basic groups or by combination by means of entirely different mechanisms. One such alternative mechanism is suggested by the titration curve of wool with Orange II at 50° C, shown in figure 6, in which the excess combination is even larger. The molecules of many dyes are known to be aggregated in aqueous solutions to a greater or lesser extent [40]. Although no evidence suggesting that Orange II is highly aggregated has ever been put forward, the failure of its solutions to obey Beer's law suggests that some degree of aggregation may occur in all but extremely dilute solutions of this dye. Combination of the fiber, at least in part, with aggregated dye anions might then account for the large amounts taken up, and for the anomalous relation to pH of the amounts combined at high concentrations.

The displacement of the pH range, in which the excess combination with most of the acids is found, removes much of the uncertainty attending the earlier observation of similar excess combination with hydrochloric acid. All three of the possible sources of experimental error mentioned above are reduced or eliminated when the excess combination occurs in the presence of low concentrations of acid. In addition, the excess found in concentrated solutions ($1.03-0.82=0.21$ in the case of naphthalenesulfonic acid) is far outside the limits of experimental error or of the possible range of effects on concentration due to exchange of water between fibers and solution.

Although the scatter of the points representing the combination of more than 0.8 millimole per gram is considerable, the portions of the curves which represent the second step of acid combination appear to run parallel to one another to about the same extent as do the main curves at higher pH values. The displacements of these parts of the curves thus appear to correspond, qualitatively at least, to the similar displacements of the parts of the curves which represent the combination of the first 0.82 millimole per gram. Should further work establish that the concepts applied in this paper to the lower section of the curves may also be employed in the analysis of the excess combination of acid, the possibility is opened of studying in an aqueous environment the combination of these weakly basic groups with acid, and thus determining the number of these groups and the extent of their dissociation.

2. CALCULATION OF THE AFFINITY OF ANIONS FOR WOOL

Any simple analysis of the experimental results in terms of the hypothesis of stoichiometric anion association depends on the assumption that the curve of acid combination is homogeneous, that is, the curve is determined by the combination of hydrogen ions and anions by sets of essentially similar groups in the fiber. If this is not the case, it is meaningless to ascribe shifts in the positions of the titration curves as a whole to variations in a single anion dissociation constant presumed to characterize each protein-anion combination. The assumption is empirically justified when the displacement in the positions of the curves affects all parts of the curve to the same extent. With the minor exceptions noted elsewhere, the data in figure 1 appear to justify a treatment based upon such homogeneity.

(a) RELATION OF THE POSITIONS OF THE TITRATION CURVES TO THE ANION AFFINITY

Since it has been assumed that differences in the positions of the curves with respect to the pH axis are determined by differences in K_A', the protein-anion dissociation constant, it is appropriate to attempt to evaluate K_A' for the anions of each of the acids used by means of the position of the corresponding titration curve. The reciprocal of K_A' is referred to hereafter as the anion affinity constant, or simply as the anion affinity.

It has already been shown [36] that changes in the positions of the titration curves obtained with a single acid, which are produced by adding different amounts of the anion of the acid in the form of neutral salts, can be described by the equation:

Fraction of wool combined

$$\equiv \frac{[WHA]+[WH^+]}{[WHA]+[WH^+]+[WA^-]+[W^\pm]} = \frac{1}{1+\frac{K_H'}{a_H}\left(\frac{a_A+K_A'}{a_A+K_A}\right)} \quad (1)$$

in which a_H and a_A represent the activities of the hydrogen ion and of the anion respectively, the terms containing W represent ionic states of wool, and the constants K'_H, K'_A, and K_A govern the following hypothetical dissociation equilibria:

$$K_H' \quad WHA \rightleftharpoons WA^- + H^+ \quad (2)$$
$$K_A' \quad WA^- \rightleftharpoons W^\pm + A^- \quad (3)$$
$$K_A \quad WHA \rightleftharpoons WH^+ + A^-. \quad (4)$$

The constant K_H governing a fourth possible equilibrium:

$$WH^+ \rightleftharpoons W^\pm + H^+ \quad (5)$$

does not appear in eq 1, since it is obvious that the four constants are interrelated by the relation $K_H' K_A' = K_H K_A$.

The assumptions entering into the definition set down in the left-hand part of eq 1 have already been discussed [36]. It has also been shown that each experimental curve is actually somewhat broader (covering a wider range of pH values) than the S-shaped curves described by eq 1, but that the displacement between curves obtained at different values of a_A are accurately described by the equation.[5] The difference between the curves obtained with and without a constant concentration of anions present is also accurately described by converting eq 1 into the equivalent form, valid when salt is absent:

Fraction combined

$$\equiv \frac{[WHA]+[WH^+]}{[WHA]+[WH^+]+[WA^-]+[W^\pm]} = \frac{1}{1+\frac{K_H'}{a_H}\left(\frac{a_H+K_A'}{a_H+K_A}\right)} \quad (6)$$

The derivation of eq 1 and 6 made clear that their validity is limited to conditions under which the term $[WH^+]$ is greater than the term

[5] Reasons have been given for attributing this broadening of the experimental curves to the high degree of polyvalency of the wool protein [36], which has the effect of introducing an empirical exponent on the hydrogen-ion term in the simple equation [23]. The simplified treatment also necessarily leaves out of account all effects due to the mutual interaction of dissociating groups in the molecule except for the effect implied broadly in the inequalities $K_H' < K_H$ and $K_A < K_A'$.

$[WA^-]$. These conditions are realized, except when much salt is present, when K_A' is greater than K_H. The latter constant has not been evaluated, but it must be considerably larger than K_H', which has been assigned the value 6.3×10^{-5} at 0°C [36]. Thus eq 6 cannot be used for anions having affinities which result in a displacement of the titration curve by more than 1 or 2 pH units to the right of the position of the curve obtained with hydrochloric acid.

It is possible to derive an equation, similar to eq 6, which has the complementary condition for validity, that is, $[WA^-]>[WH^+]$, or $K_A'<K_H$. This equation, applicable to acids with high affinity for wool, is obtained by equating the fraction combined to

$$\frac{[WHA]+[WA^-]}{[WHA]+[WA^-]+[WH^+]+[W^\pm]}$$

instead of to

$$\frac{[WHA]+[WH^+]}{[WHA]+[WH^+]+[WA^-]+[W^\pm]}$$

and leads to the general result:[6]

$$\text{Fraction combined}=\frac{1}{1+\frac{K_A}{a_A}\left(\frac{a_H+K_H}{a_H+K_H'}\right)} \tag{7}$$

or, in the absence of salt, to the equivalent form:

$$\text{Fraction combined}=\frac{1}{1+\frac{K_A}{a_H}\left(\frac{a_H+K_H}{a_H+K_H'}\right)} \tag{8}$$

The dependence of the pH coordinate of the midpoint of each curve on K_A' or K_A, in terms of either eq 6 or 8, may be described by equating the right-hand member of each equation to 0.5. When this is done with eq 6, the result is

$$K_A'=\frac{a_H(a_H+K_H)}{K_H'}-a_H=\frac{a_H^2-a_HK_H'}{K_H'}-\frac{K_H}{K_H-a_H}. \tag{6'}$$

With eq 8 the result is

$$K_A'=\frac{K_H}{K_H'}-a_H\left(\frac{a_H+K_H'}{a_H+K_H}\right). \tag{8'}$$

Equations 6′ and 8′ both contain constants which have not previously been evaluated (K_A and K_H), as well as the constant K_H' to which the value 6.3×10^{-5} has previously been assigned.[7] Because of electrostatic requirements, however, $K_H>K_H'$ and $K_A'>K_A$ [36]. The ratios between pairs of dissociation constants which differ mainly because of the electrostatic effect of a single charge (such as the first and second dissociation constants of symmetrical dicarboxylic acids) always lie between 10 and 1000. If this analogy is accepted, K_H probably lies between $10^{-3.2}$ and $10^{-1.2}$. Since the factor $K_H/(K_H-a_H)$

[6] When salt is present, this equation gives the amount of acid *plus salt* combined since the excess negative charge due to $[WA^-]$ may be neutralized in part by adsorbed cations other than hydrogen ions. The amount of salt combined is large only when anions of high affinity are present, or when high concentrations are used, so that the titration curve falls in a region of high pH values.

[7] In all the calculations that follow, the reasonable assumption is made that K_H' is an intrinsic property of the protein, and is not appreciably affected by the nature of the anion combined ([36], footnote 18).

in eq 6′ must be positive, the curves obtained with acids of lower affinity than hydrochloric acid (not reported in this paper) further restricts K_H to values greater than $10^{-2.1}$. The factor thus approaches unity for values of a_H at the midpoints of practically all the curves represented in figure 1. Thus, between limits represented by the midpoint of the hydrochloric acid curve and the midpoints of curves for which the condition of validity for eq 6′ ($K_A' > K_H$) no longer applies, the equation may be simplified to

$$K_A' \doteq \frac{a_H^2 - K_H' a_H}{K_H}. \tag{6''}$$

The corresponding simplification of eq 8′, valid when $a_H \ll K_H$ (throughout practically the entire range of pH values in which the midpoints of the curves occur), is

$$K_A' \doteq \frac{a_H^2 + K_H' a_H}{K_H'}. \tag{8''}$$

The calculation of K_A', the reciprocal of the affinity, thus depends in either case on an accurate knowledge of K_H', the intrinsic acidity constant of the carboxyl groups of the fiber. By assigning the previously determined approximate value at 0° C to this constant, the relation of K_A' to the pH of the midpoint has been calculated for this temperature according to both equations. The result of this calculation is plotted logarithmically in figure 2, in which the midpoint pH, and therefore the value of the affinity constant, according to both eq 6″ and eq 8″ has been indicated for each of the acids represented in figure 1.

It is apparent that in the region of low pH values both equations give practically identical results. The curves approach the same straight line having a slope of 2. In this region the ratio of affinities between two anions is given with a high degree of approximation by the antilogarithm of twice the difference in pH between the midpoints of the titration curves obtained with the acids corresponding to the anions.

At pH values above 3, however, the curves diverge appreciably, and at pH values above 4.2 (pK_H' at 0° C), no physically real values of K_A' are given by eq 6″. Obviously, however, the conditions under which eq 6 and 6″ are valid ($K_A' > K_H$) are no longer realized when the curves are displaced to such high ranges of pH. Since both equations lead to essentially the same relation between K_A' and midpoint pH in the range of pH in which eq 6″ is nearly exact, all calculations of affinity in this paper have been based on eq 8″, regardless of the range of pH in which the midpoint of a given titration curve may lie.

It should be noticed that as the affinity increases, the difference in the positions of the curves produced by a given increase in affinity becomes larger until ΔpH, which at one extreme is equal to $-\frac{1}{2}\Delta pK_A'$, at the other extreme approaches $-\Delta pK_A'$. As the latter limit is approached closely, the affinity constant becomes independent of the value assigned to K_H' and is equal to the midpoint a_H. It will be shown later that the affinities of commercial acid dyes probably fall in this range; thus, the comparison of their affinities relative to one another may be much simplified.

Included in figure 2 is a third curve, also representing eq 8″, in which the value 9.32×10^{-5} has been used for K_H' instead of the

value 6.30×10^{-5}. This curve shows the relation between midpoint pH and log K_A', at a temperature of 25° C [38], and also illustrates the relatively large effect of variations in K_H' on the estimation of K_A' when the latter is large, and the virtual absence of this effect when the latter is small.

By making use of the curve which represents eq 8″ at 0° C, values of K_A' which correspond to each of the acids represented in figure 1 have been determined. These values are given in the fifth column of table 2. The ion with least affinity, chloride, has a value of K_A',

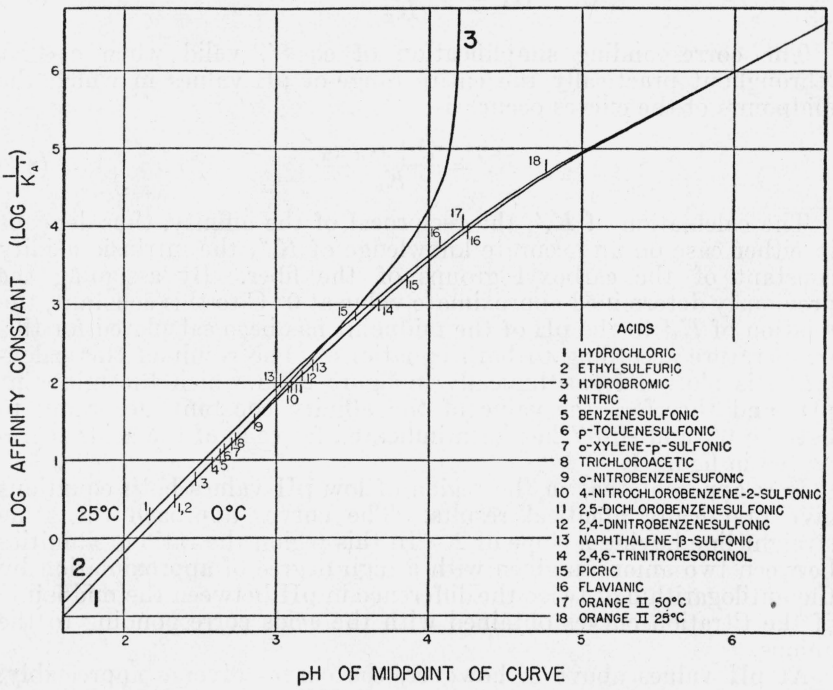

FIGURE 2.—*Relation between the position of the acid titration curve with respect to* pH *and the affinity of the anion of the acid for protein.*

Curves 1 and 2 represent eq 8″ with numerical values of K_H' at 0° and 25° C, respectively, inserted. Curve 3 represents eq 6″ with the numerical value of K_H' at 0° C. Curves 1 and 3 coincide at low pH values.

0.35; picrate, the most tightly bound monovalent ion studied at this temperature, has an affinity almost one thousand times as great as that of chloride, and the divalent flavianate ion has an affinity still higher.[8] It is apparent from the results summarized in table 2 that the affinity of the anion tends to rise as the molecular weight increases but the tendency is not regular and exceptions in the series occur.

Since the experimental curves are broader than those predicted by the much simplified eq 6 and 8, the use of these equations in analyzing the shifts in position of the curves would be rendered more significant if the progressive change in their slopes could also be shown to be a consequence of the same differences in K_A' which result in their displacements to progressively higher pH values. This demonstration

[8] For comparison, the affinity of the divalent flavianate ion is expressed in the same units as the affinities of the monovalent acids in the table.

has been made by substituting representative numerical values in eq 6 and 8. For the sake of definiteness, the value 0.01 has been assigned to the ratio K_H'/K_H, which has been shown to have a range of reasonable values of 0.01 to 0.001. The result of this calculation with six different values of K_A' is shown in figure 3. The calculated curves simulate the curves drawn through the experimental data not only in the relation between the magnitudes of their displacements from one another but also in the relation of their slopes. Thus, the curves representing low values of K_A' (high affinities) are everywhere less steep than those which correspond to high value of K_A' (low affinities). The progressive change in slope at the midpoints of the experimental curves is in the same direction. The qualitative agreement with experiment of these calculations would not be altered by the assignment of other reasonable numerical values to the ratio K_H'/K_H.

TABLE 2.—*Calculation of anion affinity constants* $(1/K_A')$ *at 0° C*

Acid	Molecular weight	pH of midpoint	$-\log K_A'$ from eq 8''	K_A'	Affinity relative to affinity of chloride ion	pH at which curve crosses HCl 0.1 molal curve	Affinity relative to affinity of chloride ion (method of crossing point)
Hydrochloric	36.5	2.33	0.45	0.35			
Ethylsulfuric	126.1						
Hydrobromic	80.9	2.47	.74	.18	1.9		
Nitric	63.0	2.58	.96	.11	3.2	1.71	3.9
Benzenesulfonic	158.2	2.63	1.05	.089	3.9	1.71	3.9
p-Toluenesulfonic	172.2	2.66	1.10	.080	4.4	1.73	4.1
o-Xylene-*p*-sulfonic	186.2	2.71	1.21	.062	5.6	1.83	5.2
Trichloroacetic	163.4	2.73	1.25	.056	6.2	1.93	6.6
o-Nitrobenzenesulfonic	203.2	2.86	1.50	.032	11.0	2.05	8.6
4-Nitrochlorobenzene-2-sulfonic	237.6	3.08	1.93	.0118	29.7	2.68	36.8
2,5-Dichlorobenzenesulfonic	227.1	3.09	1.95	.0112	31.2	2.68	36.8
2,4-Dinitrobenzenesulfonic	248.2	3.17	2.09	.0081	43	2.82	51
Naphthalene-β-sulfonic	208.2	3.24	2.23	.0059	59	3.05	86
2,4,6-Trinitroresorcinol	245.1	3.67	3.02	.00096	366		
Picric	229.1	3.86	3.34	.00046	758		
Flavianic	314.2	4.24	[a] 3.94	[a] .000115	[a] 3,020		

[a] Calculated, for comparison, as if the anion were monovalent. Since equilibrium was not attained in 23 days, the relative affinity reported is minimal.

(b) ALTERNATIVE METHOD OF CALCULATING RELATIVE ANION AFFINITIES

In column 6 of table 2 is given the ratio of the affinity of the anion of each acid to that of chloride ion. This has been tabulated because the same relative affinity factor may be calculated directly from the experimental results represented in figure 1 by combining them with the data for hydrochloric acid at constant chloride concentrations in an earlier paper [36]. The values so obtained thus do not depend on the equations just used.

This alternative method of calculation is made possible by the fact that curves obtained in the presence of constant anion concentrations are less steep than those obtained in the absence of salt and therefore cover a wider range of pH values. Thus, for example, a curve representing results obtained with hydrochloric acid at a constant concentration (0.1 *M*) of chloride ions would intersect all of the curves represented in figure 1 with the exception of the curve for flavianic

acid. The intersections with the curves for hydrobromic acid and picric acid would occur so near the extremes of the 0.1 *M* curve that they are not well suited to the present calculation, but all of the other intersections may be used. At the point of intersection of the 0.1 *M* hydrochloric acid curve with any curve in figure 1, the same amounts of two different acids are bound by the wool at the same pH. The same degree of combination with two different anions is thus brought about by the presence of different amounts of the respective anions by virtue of the difference of affinity for wool characterizing these

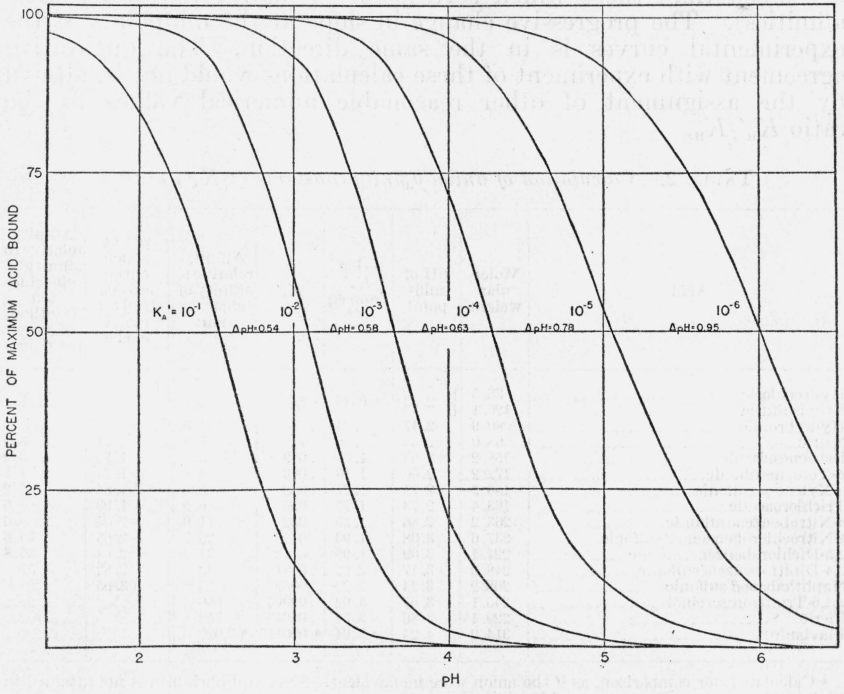

FIGURE 3.—*Dependence of the slope of theoretical titration curves on the protein-anion dissociation constant.*

The two leftmost curves are calculated from eq 6 and the others from eq 8. The change from eq 6 to eq 8 between $K_A'=0.01$ and $K_A'=0.001$ has been made in order to preserve consistency with the assumption $K_B'/K_B=0.01$.

anions. It follows from the range of ratios of K_H'/K_H which has been adopted elsewhere that the ratio of the activities of the two anions in the two different experiments is inversely proportional to the ratio of the respective anion affinity constants. The anion affinity ratios which are obtained should be nearly the same as those given in column 6 of table 2.

The activity of potassium chloride at 0.1 *M* at 0° C is 0.0768 [18]. The assumption has been made that the activity of the anion in each experiment made in the absence of salt is equal to the activity of the hydrogen ion. It is very unlikely that this assumption is true; but the experimental value, pH, is related to the activities of both ions, and methods of obtaining individual ion activities are at present unknown. By dividing 0.0768 by the antilogarithm of the negative

of the pH, the values given in the last column of table 2 have been obtained. In view of the fact that the intersecting curves were drawn freehand and that the two kinds of data were obtained with different lots of wool, the agreement of the values in the last column with those in column 6 is satisfactorily close.[9]

3. RESULTS OBTAINED IN THE PRESENCE OF SALT

It should be possible to predict the displacements that would be found if the same acids were used in the presence of constant concentrations of their anions, added as neutral salts. By starting with eq 1 and 7 instead of eq 6 and 8, and making the same simplification which was carried out in arriving at eq 6″ and 8″, formulas which give the relation between K_A' and the midpoint pH when the measurements are made in the presence of any constant concentration of anions are obtained:

$$K_A' = a_A \frac{a_H - K_H'}{K_H'} \qquad (1'')$$

$$K_A' = a_A \frac{a_H + K_H'}{K_H'} \qquad (7'')$$

In these equations the quadratic term a_H^2, found in eq 6″ and 8″, does not appear. In the range of relatively low affinities, therefore, a given change in K_A' will, in the presence of constant anion concentrations, produce twice the pH change which corresponds to this change in K_A' in the absence of salt. With anions having higher affinities, however, displacements in midpoint pH values obtained in the presence and absence of salt will differ by less than a factor of 2. These predictions have been tested by making measurements with a number of the acids included in table 1, in the presence of 0.1 *M* concentrations of their anions, added as the potassium salts. The results of these titrations are summarized in table 3 and are represented graphically in figure 4. As the figure shows clearly, the difference in the pH values of the midpoints of the hydrochloric acid and benzenesulfonic acid curves is twice as great for curves obtained in the presence of salt as for those obtained in its absence. The difference between the midpoints of the hydrochloric acid and naphthalenesulfonic acid curves is somewhat less than twice as great in the presence of salt as in its absence. Both differences thus agree with the predictions of the equations.

Similar experiments have also been made at 25° C with Orange II in the presence of a constant concentration of its colored anion, added as the sodium salt. The affinity of this anion is so great that it combines with the protein in considerable amounts at pH values at which only small amounts of hydrogen ion are normally taken up. The resulting negative charge on the fiber is neutralized, as in all the cases covered by eq 7 and 8, by adsorption of positive ions. In the presence of salt, appreciable quantities of sodium ions, as well as hydrogen ions, are bound. Thus, eq 7 no longer gives the amount of acid taken up alone, but the sum of the amounts of acid and of salt. Since

[9] A third method of calculating relative affinities has also been made use of. Mixtures of acids have been equilibrated with wool, and the relative amounts of the two acids combined have been determined. The relative affinities of the two anions calculated from this ratio agree closely with the results of the methods described in this paper. Since the method has thus far been applied to experiments with acids for which no data are given in the present paper, and since work with these acids is still in progress, no examples of these calculations are given here.

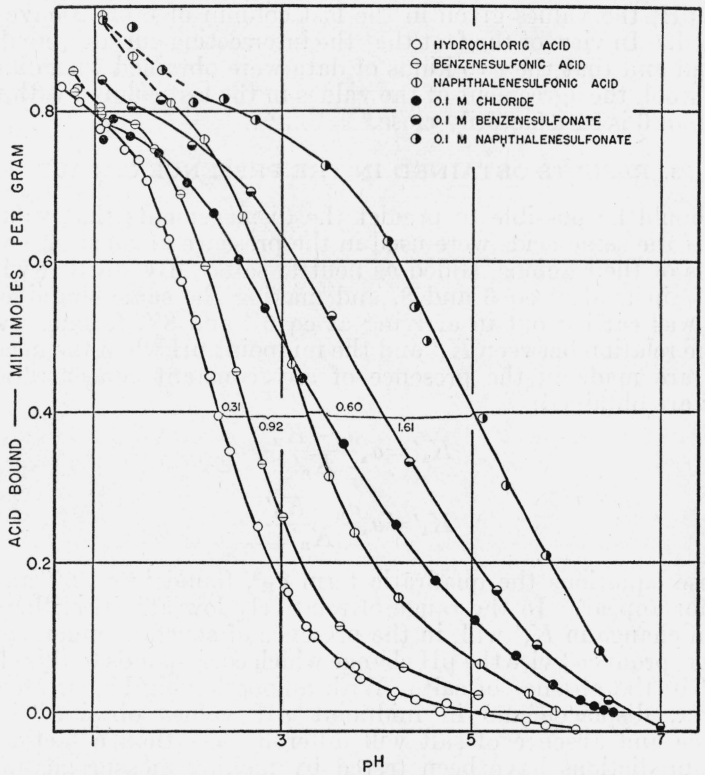

FIGURE 4.—*Comparison of the titration curve of wool protein obtained with three different acids in the absence of salt at 0° C, with the curves obtained at the same temperature in the presence of a constant concentration (0.1 M) of the anions of the acids.*

TABLE 3.—*Combination of wool protein with various acids, in the presence of a constant concentration of anions of the acids (0.1 M), at 0° C*

[a] Hydrochloric acid		Benzenesulfonic acid		Naphthalene-β-sulfonic acid	
pH	*Millimoles/g*	pH	*Millimoles/g*	pH	*Millimoles/g*
1.087	0.762	1.083	0.842	1.090	0.921
1.201	.789	1.391	.805	1.400	.912
1.380	.767	1.715	.814	1.721	.854
1.678	.745	2.038	.754	2.053	.810
1.988	.704	2.657	.693	2.378	.816
2.269	.664	3.515	.528	2.706	.787
2.535	.603	4.352	.334	3.451	.727
2.810	.538	5.276	.163	4.118	.627
3.206	.446	5.695	.090	4.400	.540
3.650	.357	6.45	.016	4.522	.495
4.210	.250	7.00	−.019	5.111	.392
4.617	.176	7.45	−.052	5.361	.303
5.032	.122			5.791	.209
5.473	.075			6.35	.083
5.867	.037				
6.15	.016				
6.3	.008				
6.4	.003				
6.5	.000				

[a] Data for hydrochloric acid at several other chloride concentrations have already been published.

the analysis of these data thus requires the development of a technique for the accurate estimation of this sum, the data obtained with Orange II at a constant concentration of its anion are not included in the present paper. The great steepness of the 0.1 *M* naphthalenesulfonate curve in figure 4 is due, in part, to combination with small amounts of salt at high pH values. These should have been added to the amounts of acid combined in order that the resulting curve should be described by eq 7.

4. EFFECT OF TEMPERATURE ON ANION AFFINITY

It has been shown [38] that a comparison of titration data obtained at two different temperatures permits an estimation of heat changes accompanying the dissociation of the protein-anion complex. Measurements at 25° C have therefore been made with four acids of widely different affinities. The results, summarized in table 4, also provide a basis of comparison for those acids which it was impractical to investigate at 0° C. Results obtained at 25° C with one of these acids, Orange II, are also included in the table. All of the data of table 4, combined with the data from table 1 for three of the acids at 0° C, are shown in figure 5. As the figure makes clear, the effect of temperature on the position with respect to pH of the curves obtained with picric acid (0.34 pH unit) is considerably greater than its effect on the curves for naphthalenesulfonic acid (0.21 unit), which in turn show a greater effect than the curves for hydrochloric acid (0.16 unit). No estimate is attempted of the effect of temperature on the position of curves for flavianic acid, since the data obtained at 0° C with this acid probably did not represent final equilibrium.*

TABLE 4.—*Combination of wool protein with various acids at 25° C, in the absence of added salt*

Hydrochloric acid		Naphthalene-β-sulfonic acid		Picric acid		Flavianic acid		Orange II [a]	
pH	*M-eq/g*	pH	*M-eq/g*	pH	*M-eq/g*	pH	*M-eq/g*	pH	*M-eq/g*
0.638	0.877	1.452	0.827	1.625	0.903	1.365	1.555	1.656	0.627
.826	.802	1.850	.757	1.776	.886	1.664	1.378	1.825	.624
1.448	.687	2.240	.734	2.055	.797	2.040	1.140	2.062	.627
1.710	.599	2.591	.608	2.359	.773	2.479	0.930	2.668	.592
1.915	.520	3.037	.401	2,402	.762	3.214	.710	4.308	.500
2.191	.402	3.415	.272	2.924	.622	3.830	.460	5.059	.334
2.406	.316	3.852	.167	3.414	.447	4.390	.328	5.934	.204
2.722	.225	4.422	.094	3.552	.431	4.775	.212	6.169	.133
3.093	.143			3.786	.304	5.090	.153	6.408	.0856
3.370	.087			4.094	.209	5.419	.075	6.421	.0530
3.962	.036			4.465	.140	5.800	.047	6.879	.0390
4.603	.014			4.868	.094	6.11	.022	6.926	.0280
5.257	.013			5.36	.054			6.72	.0175
6.375	.026			5.68	.033				
6.825	.035			6.604	.015				
7.275	.042								

[a] Equilibrium not attained. See text, p. 314.

*The curve at 25° C. for flavianic acid requires further explanation. When plotted, as in figure 5, as *milliequivalents* per gram against pH, the data run nearly parallel to those for picric acid at the same temperature up to values of acid combined of at least 0.65 millimole per g. This furnishes a very strong indication that in this range of pH values the flavianate ions combining with the wool are predominantly doubly ionized, and effectively neutralize two of the positively charged basic groups in the protein. At lower pH values where the data are represented by a broken line, the proportion of the total flavianic acid which is doubly dissociated is so small that a part of the ions which combine with the fiber are monovalent. In the most concentrated solutions, combination with the fiber takes place almost entirely with the monovalent ion, so that a plateau at 0.82 *millimole* rather than at 0.82 *milliequivalent* would presumably be found. Since the concentration of flavianic acid is extremely low in the range of pH in which all the ions are predominantly dibasic, it is clear that the affinity of the doubly charged anion is considerably higher than that of the monovalent ion. Trinitroresorcinol also combines with wool as a dibasic acid, when present in greater dilution. To avoid complicating figure 1, only the measurements representing the monobasic function of this acid were included in the figure. Similar effects appear to characterize sulfuric acid [34].

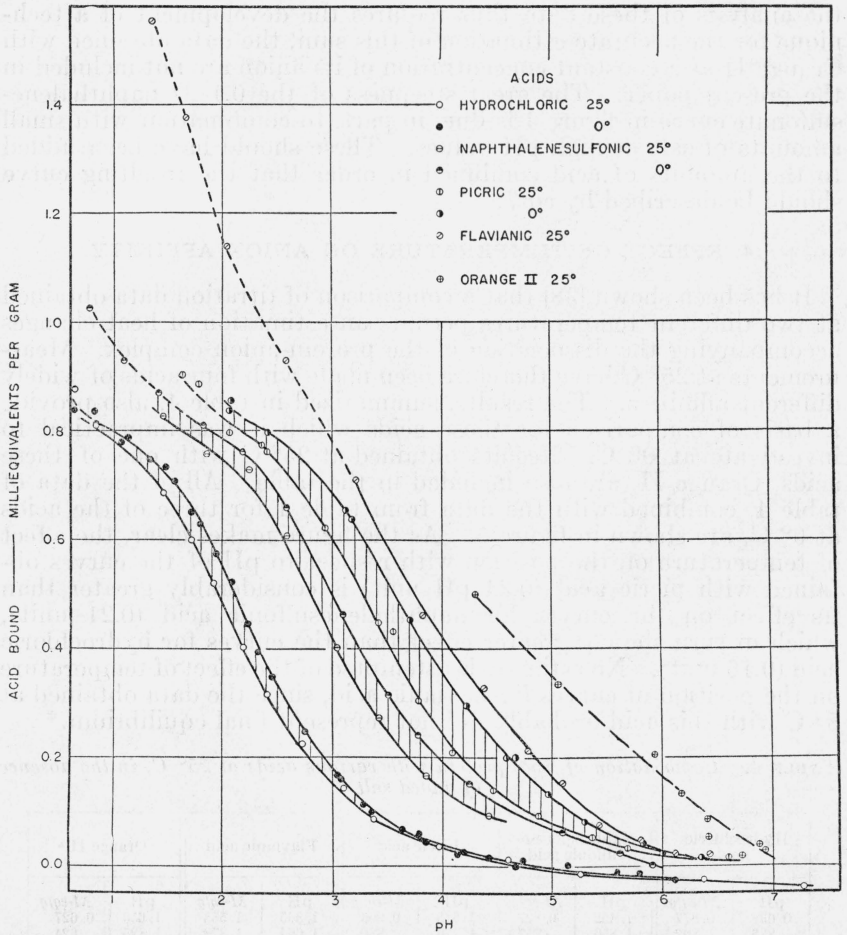

FIGURE 5.—*Comparison of the titration curves of wool with various acids at 0° and 25° C.*

TABLE 5.—*Calculation of anion affinity constants and heats of dissociation at 25° and 50° C*

Acid	pH of midpoint	$-\log K_A'$ from eq 8″	K_A'	Affinity relative to affinity of chloride	$\left(\frac{K_A'}{K_A'}\right)_{0°}^{25°}$	$\Delta H_{0°}^{25°}$ for anion dissociation
		25° C				*Calories*
Hydrochloric	2.16	0.31	0.49	----------	1.40	2,170
Naphthalene-β-sulfonic	3.03	1.97	.0107	46	1.81	3,850
Picric	3.52	2.89	.00129	380	2.80	6,670
Flavianic	4.07	[b] 3.77	.000170	2,880		
Orange II[a]	4.76	[c] 4.68	[c] .000021	[c] 23,400		
		50° C			$\left(\frac{K_A'}{K_A'}\right)_{25°}^{50°}$	$\Delta H_{25°}^{50°}$
Hydrochloric	2.21	0.38	0.42	----------	0.85	−1,630
Orange 11[a]	4.21	3.97	.000107	3,900	[c] 5.09	[c] 12,500

[a] Molecular weight, 328.3.
[b] Expressed for purposes of comparison as if the anion were monovalent. Refer to text.
[c] The Orange II data at 25° C do not represent equilibrium values. Thus the relative affinity for this temperature, and the value of $\Delta H_{25°}^{50°}$ are minimal.

The affinities at 25° C of the acids represented in figure 5, calculated from eq 8″, are given in the first part of table 5. The values of K_A' listed in column 4 were obtained graphically from the curve for 25° C in figure 2. Thus the effect of temperature on K_H' has been taken into account. Owing to the inclusion of data for Orange II, the differences of affinity comprised in this table cover almost eight times as wide a range as those listed in table 2.

The sixth column of the table gives the ratio of the protein-anion dissociation constants at 25° and 0° C. From this ratio, and the van't Hoff equation, the average heat of dissociation in this range of

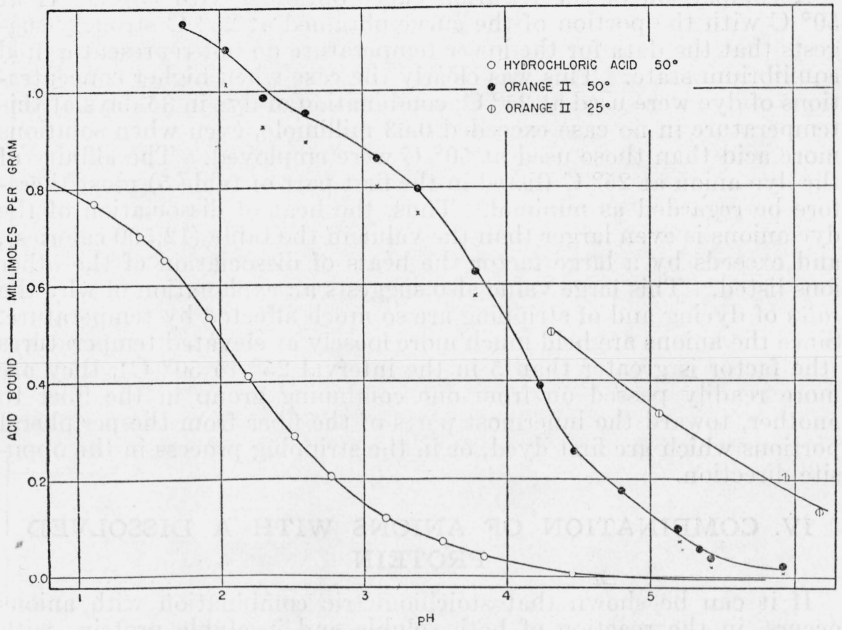

FIGURE 6.—*Combination of wool protein with the azodye acid Orange II, and with hydrochloric acid as a function of* pH *at 50° C.*

The significance of the crosses is explained in the text. For comparison, data obtained with Orange II at 25° C are included.

temperature has been calculated. The values so obtained are given in the last column. The heats of dissociation rise from some 2,000 calories with chloride ion to nearly 7,000 calories with picrate. As should be expected, more energy is required to dissociate the more tightly bound ions.[10]

In order to evaluate the effect of temperature on the affinity of Orange II, the amounts of this acid combined by wool were also measured at 50° C, after allowing a period of 5 days for the attainment of equilibrium. The results, together with data for hydrochloric acid at the same temperature are represented graphically in figure 6. The maximum amounts combined are in fair agreement with those reported for high concentrations of this dye by Ender and Müller [12]. Included in the figure for the purpose of comparison are the data

[10] The value of K_H' at 25° C was chosen on the basis of the earlier analysis of the effect of temperature on the combination of wool with hydrochloric acid [38], in which it was assumed that the heat of transfer of the ions from the solution to the fiber could be neglected. The heats of dissociation given in the table may therefore be subject to a small correction. Since this correction would be practically invariant, its application would not affect the relative order of the heats of dissociation, nor the great differences between them.

obtained with Orange II at 25° C. The measurements represented by crosses were obtained at 50° C by determining the increase in dry weight of the fibers as the result of combination with dye. The small discrepancies between the two sets of results (larger the more acid the solution) are accounted for by the fact that small amounts of amide nitrogen are split off, as a result of the prolonged exposure to dye at this temperature, and affect the apparent dye uptake estimated from the difference in the acid titer of the dye bath brought about by the wool. The amounts of ammonia produced by comparable exposure to the same concentrations of hydrochloric acid are smaller.

A comparison of the titration curve obtained with Orange II at 50° C with the portion of the curve obtained at 25° C strongly suggests that the data for the lower temperature do not represent a final equilibrium state. This was clearly the case when higher concentrations of dye were used at 25° C; combination of dye in 35 days at this temperature in no case exceeded 0.63 millimole, even when solutions more acid than those used at 50° C were employed. The affinity of the dye anion at 25° C (listed in the first part of table 5) must therefore be regarded as minimal. Thus, the heat of dissociation of the dye anions is even larger than the value in the table (12,500 calories), and exceeds by a large factor the heats of dissociation of the other ions listed. This large value also suggests an explanation of why the *rates* of dyeing and of stripping are so much affected by temperature. Since the anions are held much more loosely at elevated temperatures (the factor is greater than 5 in the interval 25° to 50° C), they are more readily passed on from one combining group in the fiber to another, toward the innermost parts of the fiber from the peripheral portions which are first dyed, or in the stripping process in the opposite direction.

IV. COMBINATION OF ANIONS WITH A DISSOLVED PROTEIN

If it can be shown that stoichiometric combination with anions occurs, in the reaction of both soluble and insoluble proteins with acids, there is a wide range of application in which it must be taken into account.[11] If it occurs only with wool or with fibrous proteins,[12] the distinction must be regarded as a clue to the nature of the specific structural differences between soluble and insoluble proteins.

To determine whether the marked differences in the titration curves of wool obtained with different acids are also to be found with dissolved proteins, determinations were made of titration curves of the soluble protein, egg albumin, with several of the same acids. The results of these measurements at 22° C are summarized in table 6 and shown in figure 7. The position of the broken line at the top of this figure, inserted for reference, shows the maximum acid-binding capacity, indicated by the work of Kekwick and Cannan with hydrochloric acid [22]. The crosses distinguish measurements in which incipient precipitation was indicated by a faint opalescence. In every case measurements with higher concentrations of the acid produced a visible precipitate, and have been omitted from both the

[11] Examples are (a) the calculation of Donnan membrane potentials or ion-distribution ratios, in which disagreement with experiment often appears [3, 4] unless combination with ions other than H^+ and OH^- is taken into account [1]; (b) the possibility of forming physiologically significant weakly dissociated protein complexes, such as with calcium [10].

[12] The amounts of acid or base combined with *silk* at any pH [13] have the same first-order dependence on the concentration of anions and cations, respectively, that characterizes wool.

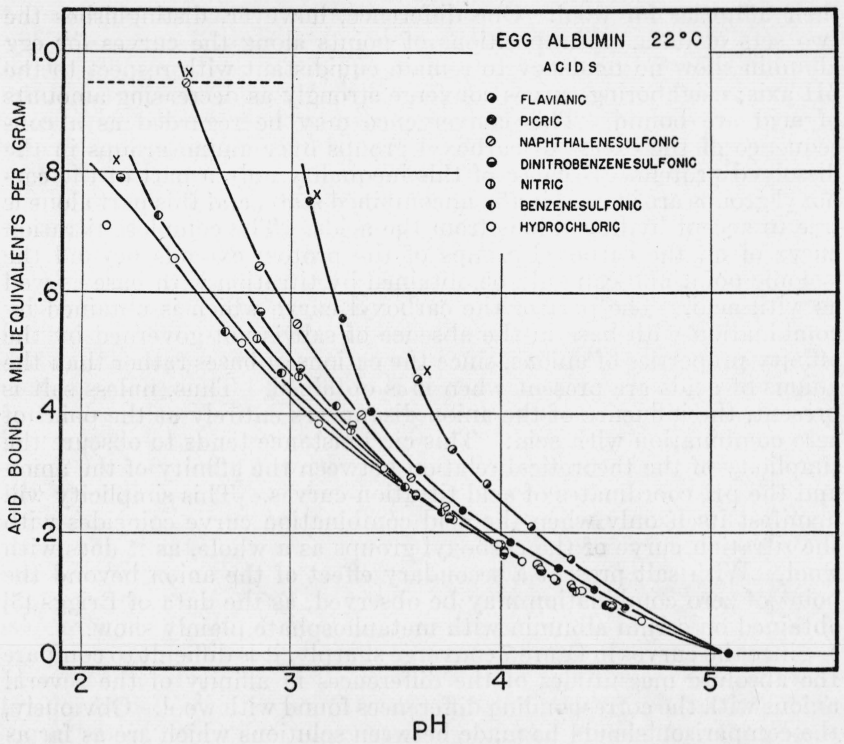

FIGURE 7.—*Combination of a dissolved protein, egg albumin, with various strong acids as a function of* pH.

The significance of the crosses is explained in the text.

TABLE 6.—*Combination of a dissolved protein, egg albumin, with various acids at 22° C, in the absence of added salt*

Hydrochloric acid		Benzenesulfonic acid		Nitric acid		2,4-Dinitrobenzenesulfonic acid		Naphthalenesulfonic acid		Picric acid		Flavianic acid	
pH	*M-eq/g*	pH	*M-eq/g*	pH	*M-eq/g*	pH	*M-eq/g*	pH	*M-eq/g*	pH	*M-eq/g*	pH	*M-eq/g*
2.109	0.710	2.362	0.728	2.840	0.525	[a] 2.181	0.790	[a] 2.488	0.942	[a] 3.086	0.762	[a] 3.608	0.456
2.438	.658	2.688	.523	3.041	.462	2.499	.621	2.848	.645	3.248	.564	3.772	.340
2.766	.524	2.959	.468	3.282	.371	2.859	.568	3.028	.550	3.385	.404	3.939	.282
3.140	.392	3.220	.411	3.598	.262	3.049	.474	3.340	.396	3.618	.303	4.150	.211
3.548	.277	3.450	.309	3.764	.221	3.298	.380	3.580	.292	3.825	.237	4.338	.155
3.777	.222	3.726	.236	4.028	.163	3.599	.262	3.740	.244	4.047	.185	5.092	.000
3.994	.181	4.020	.169	4.198	.135	3.760	.224	4.009	.180	4.282	.141		
4.105	.152	4.273	.120	4.360	.102	4.022	.166	4.181	.148	4.450	.105		
4.260	.123	4.515	.079	4.559	.074	4.196	.139	4.339	.116	4.600	.076		
4.393	.103	5.092	.000	5.092	.000	4.348	.109	4.537	.081	5.092	.000		
4.677	.055					4.535	.076	5.092	.000				
5.092	.000					5.092	.000						

[a] Solution opalescent. Higher concentrations of acid produced a visible precipitate. Measurements made at these higher concentrations are therefore omitted from the table.

table and the figure in order to avoid all possibility that the effects described may have been due, in whole or in part, to the existence of two macroscopic phases.

The differences between the curves shown in figure 7 obviously correspond to the differences found with wool. With one exception, the affinities of the anions for egg albumin are in the same order as

their affinities for wool. One difference, however, distinguishes the two sets of data. The positions of points along the curves for egg albumin show no tendency to remain equidistant with respect to the pH axis; neighboring curves converge strongly as decreasing amounts of acid are bound. This convergence may be regarded as a consequence of the excess of carboxyl groups over amino groups in the dissolved protein. Because of this inequality only a part of the carboxyl groups are ionized in the uncombined state, and this part alone is free to accept hydrogen ions from the acids. The complete titration curve of all the carboxyl groups of the protein extends beyond the isoionic point and can only be obtained by titration with base as well as with acid. The part of the carboxyl curve which is obtained by combination with base in the absence of salt is not governed by the affinity properties of anions, since the cations of bases rather than the anions of acids are present when it is obtained. Thus, unless salt is present, the influence of the anion disappears entirely at the point of zero combination with acid. This circumstance tends to obscure the simplicity of the theoretical relation between the affinity of the anion and the pH coordinates of acid titration curves. This simplicity will manifest itself only when the acid combination curve coincides with the titration curve of the carboxyl groups as a whole, as it does with wool. With salt present a secondary effect of the anion beyond the point of zero combination may be observed, as the data of Briggs [5] obtained on serum albumin with metaphosphate plainly show.

Since the curves in figure 7 converge sharply, it is difficult to compare the absolute magnitudes of the differences in affinity of the several anions with the corresponding differences found with wool. Obviously, the comparison should be made between solutions which are as far as possible from the isoionic point in order to minimize the effects of the convergence. The data do not extend to very acid solutions, but at the extreme of the range represented the differences in the pH coordinates of the curves approach values only slightly smaller than the differences between curves for the same acids in figure 1.[13]

Since the theory of stoichiometric anion combination was originally formulated to account for the large effects of the presence of salt on the hydrochloric acid combination curves of wool, the application of this theory to the much smaller salt effects described by Cannan for egg albumin [6] seemed at first uncalled for. The experiments just described, however, suggest that the difference between the titration curves of dissolved and undissolved proteins in the absence of salt [36] cannot be due to the failure of anions to combine with the dissolved protein. The contradiction between the results of the two kinds of experiments is only apparent, since the existence of a stoichiometric salt effect is not an inevitable consequence of every situation to which eq 1 may be applied. Such salt effects are found only when $K_A' \gg a_A \gg K_A$. When K_A' and K_A are both very large, the bracketed term in the denominator of eq 1 approaches a constant and is nearly independent of a_A. When both constants are very small, the bracketed term approaches unity and is again nearly independent of a_A. In

[13] Results of the kind described for both proteins may furnish a basis for explanations of the many reports of ion specificity in the literature of protein and enzyme chemistry. Attempts have been made to deal with some of these specific properties, such as differences in salting-out effectiveness, by treatment of the electrostatic interaction between ions having different sizes, shapes, and electric moments. Many phenomena of specificity remain, however, for which plausible explanations, based on simple electrostatic considerations alone, are not easily obtained. Among these are numerous instances of differences in the electrophoretic mobility of various proteins when different anions or different concentrations of anions are present in buffers [9, 14, 31, 39], and large-scale differences in the effectiveness of various anions in environments producing protein denaturation [15, 35].

either case (corresponding to extremely low or extremely high affinity of anions for the protein) the availability of hydrogen ions rather than of anions becomes the factor limiting the extent of combination with acid. Thus, as in the case of egg albumin, the titration curve obtained in the absence of salt will not differ greatly from those obtained at constant anion concentrations.

In view of the numerous indications that dissolved proteins do combine with such commonly employed anions as chloride, acetate, and phosphate, but not to an extent comparable to their combination with hydrogen ions in acid solutions [1, 3, 14, 39], the first of the two alternatives (K_A' and K_A large) probably applies in the case of these simpler ions. The difference in the absolute values of these constants characterizing the soluble and insoluble proteins may be related to the structural factors responsible for the differences in their respective states of dispersion.

It should be noted in figure 7 that precipitation occurred at concentrations which were in the inverse order of the affinities. Picrate and flavianate are ions having a high precipitating efficiency for proteins, but many others, such as tannate and metaphosphate, are equally well known [28, 29, 26, 5]. It would be of interest to determine to what extent protein precipitation by specific reagents, such as picrate or tannate, and the salting out of proteins with various salts may be regarded as manifestations of the same general protein-anion equilibrium. When the affinity is so great that the presence of only small amounts suffices to cause combination and precipitation, the anion or acid is naturally described as a specific protein precipitant. When the affinity is low and large excesses are required, the combination and precipitation may have many of the characteristics associated with salting out. With extremely low affinities, such as that of chloride, salting out is often not practicable.

V. THE RELATION OF ANION AFFINITY TO SIZE, STRUCTURE, AND BASICITY OF IONS

If the phenomena reported in the present paper are to be ascribed to the formation of partially dissociated protein-anion combinations, it is logical to inquire into the nature of the forces which confer on different ions so wide a range of affinities for protein. The existence in certain kinds of solutions of ion combinations which are only partially dissociated is now well established [7, 24]. Such combinations may be formed in two possible ways, involving either directed or undirected valence forces. The first alternative involves the formation of a bond having a partially covalent nature, or a hydrogen bond; the second comprises various kinds of polar forces, whether electrostatic (coulombic) in nature or otherwise and includes van der Waals forces. If the first type is involved, the principal relation found among the anions should be an inverse one of affinity to the intrinsic strength of the acid.[14] The relations found in cases to which the second alternative applies would be expected to be more complex.

Since most of the acids included in the present research are of approximately equal strengths in water, but differ widely in affinity, the first alternative seems to have little in its favor. Nevertheless, a *part* of the total binding energy between anion and protein might

[14] Here the possibility of the formation of a bond between the protein and the charged group of the anion (when the anion is polyatomic) is alone under consideration.

still be due to the formation of a directed bond, especially since the intrinsic strengths of the various acids, as distinguished from their apparent strengths in water, are not all equal.

In an effort to appraise more closely the influence of the basicity of the anion on the affinity, comparisons (fig. 8) have been made of the results obtained with pairs of closely similar acids, such as benzenesulfonic and benzoic acids, which differ widely in strength. The data shown are limited to ranges of pH in which the concentration of the undissociated form of the weak acid does not exceed 0.1 *M*, in order to avoid effects, due to the presence of larger amounts, which have been described elsewhere [37]. There is very little difference

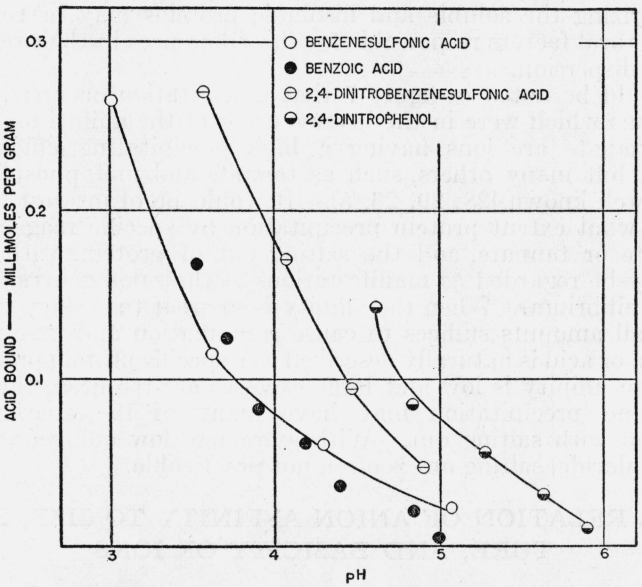

FIGURE 8.—*Comparison of portions of the acid titration curves of wool protein obtained with pairs of analogous acids which differ widely in strength.*

between the amounts of strong and of weak acid combined at a given pH in the case of the benzenesulfonic-benzoic acid comparison. On the other hand, the anion of dinitrophenol appears to have a definitely higher affinity for wool than the anion of its totally dissociated analogue, dinitrobenzenesulfonic acid. The opposite situation is found in experiments (still in progress) on a series of aliphatic carboxylic acids. These have even lower affinities for wool than has hydrochloric acid; in addition, the weaker acids have lower affinities than the relatively strong members of the series. The affinities of all of them are increased by the introduction of a halogen or of a hydroxyl group, although the derivatives thus formed are considerably stronger acids. Since there is no consistency in these results, it seems likely that the differences found are due to specific structural effects rather than to differences in acidity.

An indication of the nature of the *undirected* forces to which the principal recourse must therefore be had is to be found in the fairly regular correspondence between the order of affinities and of molecular

weights, shown in table 2. Such a dependence on the mass of the anion is consistent with the Fajans rule, which deals with the degree of ionic character of bonds and their consequent tendency to dissociate. However, a dependence on mass, or functions of ion size related to it, is also generally characteristic of similar phenomena which depend, at least in part, on van der Waals forces, or adsorption [19].

The several exceptions to a strict correspondence between the relative masses of the ions and their affinities provide further information as to the relation between structure and affinity. Thus the affinities of the aliphatic carboxylate ions already mentioned do not fall into a molecular weight order; likewise, ethylsulfate ion has as low an affinity as the much smaller chloride ion, and is lower in relative affinity than the smaller bromide and nitrate ions. The anion of trinitroresorcinol is slightly lower in affinity than picrate, although it is slightly heavier. Napthalenesulfonate is higher in affinity than the heavier dinitrobenzenesulfonate ion.

A possible way of resolving these discrepancies without introducing a host of special assumptions is suggested if due regard is had for the shapes of the various ions and the character of the surfaces which they present to the protein. Two-dimensional ions (those derived from simple benzenoid compounds) appear to possess higher affinities than either compact three-dimensional or chain-like particles of the same mass. The nature of the substituents added appears to be of less importance than their mass as long as there is no steric hindrance to their lying in the plane of the ring. Such a hindrance appears to operate in the case of the monovalent trinitroresorcinol ion, which has an appreciably lower affinity than picrate. It appears doubtful, from the study of models, whether the undissociated hydroxyl group in this ion can lie in the plane of the ring. When the flat configuration is imposed by the existence of conjugated double bonds, as in naphthalenesulfonic acid, the affinity relative to a disubstituted benzenoid compound of the same mass gains accordingly. Wide differences between the degree of substantivity of *o*- and *m*-substituted benzidine azo dyes cited by Robinson and Mills [27] furnish extreme examples which are entirely consistent with the present suggestion.

The fact that ion flatness appears to favor affinity for protein need not be interpreted as an exclusive dependence of affinity on the size of the surface presented by an ion. Saturated aliphatic hydrocarbon chains in an ion probably do not exercise any considerable attractive forces on the fragments of hydrocarbon chains which exist on the surface of the protein. A close approach between them is obstructed by the outer shell of hydrogen atoms common to both. Thus, the greater affinity of ions composed of aromatic hydrocarbons may be due to the absence of this obstructive shell on two of their largest surfaces. If, in addition, electronegative substituents, such as chlorine, or the oxygen of nitro groups, are presented by the ion to the protein, strong attractive forces may arise, replacing the relative indifference which determines the behavior toward protein of the unsubstituted hydrocarbon. Experiments designed to test these views, and the possibility of applying them in the deliberate control of affinity, are at present in progress.

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