alcohol in the presence of sulfuric acid.[13] Fractional distillation under vacuum yielded the pure ester. Preparation of the monoethyl ester of ADCA could not be found in the literature; the method used, which has been mentioned, involved partial hydrolysis of the diester. The usual procedure of preparing diethyl acetylenedicarboxylate was employed. After refluxing the potassium salt of ADCA in alcohol, the resulting solution was decanted, diluted with an equal volume of water, cooled and made slightly basic to phenolphthalein with cold 15% NaOH solution. Saponification of the first ethyl group is extremely rapid and, as soon as a lasting red color appears, the hydrolysis is stopped by making the solution acidic again with sulfuric acid. The saponification can be followed polarographically (see data on ADCA and its esters). Ether extraction removes the ester from water and inorganic materials; the ether solution is washed with water, dried and evaporated on a steam-bath. The residue can be distilled in vacuum; the ester distils at 134–135° at 6 mm.

Dibromofumaric acid, prepared by the controlled addition of bromine to ADCA,[14] was recrystallized twice from water; its purity was shown by melting point and polarographic analysis. Its diethyl ester was prepared by treating the silver salt with excess ethyl bromide[15]; fractional freezing followed by recrystallization gave the pure solid ester. The anhydride of dibromomaleic acid (Metro Chemical Co.) was purified by sublimation; the diethyl ester, prepared by treating this anhydride with absolute alcohol containing dry HCl,[16] was purified by vacuum distillation.

The anhydride of α,α'-dimethylmaleic acid was prepared[17] and purified by sublimation. The *trans*-dimethylfumaric acid, prepared by heating the *cis*-acid with HCl, was purified by ether extraction and water recrystallization.

Buffers were prepared as previously described[1]: HCl–KCl, *p*H 0.4 to 2.0; Na_2HPO_4–citric acid, *p*H 2.0 to 7.0; NH_3–NH_4Cl, *p*H 8.2 to 9.7.

Apparatus.—A Sargent Model XXI polarograph was used in conjunction with a potentiometer. A Beckman Model G *p*H meter was used for *p*H measurement. The coulometric apparatus has been described.[1]

Procedures.—The polarographic and coulometric procedures were the same as those previously reported.[1] The acids were studied at 25 ± 0.1°. The esters were studied at 2 ± 0.5°; the test solutions had 4 volume % of alcohol to ensure solubility of the esters. This concentration of alcohol seemed to have no effect on the polarographic results.

Acknowledgment.—The work described was supported by the U. S. Atomic Energy Commission, to whom the authors wish to express their thanks.

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Electrokinetic Changes in the Starch Medium During Zone Electrophoresis

BY ILSE DOROTHEA RAACKE

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The present investigation was undertaken in order to determine the factors influencing the determination of mobilities on starch. It was found that marked changes took place in the conductivity as well as the *p*H of the buffer in the starch medium, which in turn caused changes in the electrical quantities. These changes can be accounted for by the ion-exchange properties of starch and can for the most part be avoided by washing the starch with buffer before the experiment. It was found that the most reproducible mobility measurements were obtained when the field strength was taken as V/l, and it is suggested that corrections for the higher ionic strength in the solid medium be made if comparison with mobilities in free solution is desired.

The introduction of a supporting medium in zone electrophoresis has created many electrokinetic problems which are not encountered with electrophoresis in free solution. Some of these problems have been discussed in connection with paper electrophoresis[1–4] but have received only scant attention in connection with supporting media other than paper.[5–8] It is the purpose of this report to present data on some of the changes that occur during zone electrophoresis when starch is used as the supporting medium[9] and to elucidate some of the observed effects in terms of the ion-exchange properties of starch.

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(6) H. G. Kunkel in D. Glick, ed., "Methods of Biochemical Analysis," Vol. I, Interscience Publishers, New York, N. Y., 1954, pp. 141–170.
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Apparatus and Materials

The electrode vessels are made of plastic (Perspex) and separated into several compartments by means of baffles. Each vessel measures 17 × 17 × 17.5 cm. and holds 2 l. of buffer. The rear compartment contains a well filled with KCl into which silver–silver chloride electrodes are dipped. In the front compartment electrical contact is made by pressing the ends of the trough against a thick wad of filter paper saturated with buffer. A connection of rubber tubing between the vessels ensures hydrostatic equilibrium. A more complete description of the apparatus can be found elsewhere.[15]

Commercial potato starch, trademark "Swan," was used for all reported experiments. This starch contained 15.7% moisture; when dissolved in distilled water it gave a *p*H of 7.00. The starch was packed into semicylindrical glass troughs with open ends, previously described by Fønss-Bech and Li.[10] Three sizes of troughs were used, measuring 3.8 × 1.8 × 40 cm., 3.6 × 1.7 × 60 cm. and 1.5 × 0.7 × 40 cm., respectively.

The compositions of the buffers most commonly used are given in Table I.

Procedure

The starch is mixed with enough buffer to give a viscous paste which is then poured into the troughs. The escape

(10) P. Fønss-Bech and C. H. Li, *J. Biol. Chem.*, **207**, 175 (1954).

TABLE 1

COMPOSITION OF BUFFERS

No.	Buffer Type	Composition	Ionic strength	*p*H
1	Acetate	0.19 *M* HAc .037 *M* NaOH	0.037	3.7
2	Acetate	.17 *M* HAc .0325 *M* NaOH .0675 *M* KCl	.1	4.0
3	Acetate	.025 *M* HAc .075 *M* NaAc	.075	5.2
4	Acetate	.025 *M* HAc .075 *M* NaAc .025 *M* KCl	.1	5.1
5	Cacodylate	.055 *M* MeAsH .04 *M* NaOH .06 *M* NaCl	.1	6.5
6	Cacodylate	.045 *M* MeAsH .04 *M* NaOH .06 *M* NaCl	.1	6.9
7	Phosphate	.035 *M* K_2HPO_4 .065 *M* KH_2PO_4	.15	6.6
8	Veronal	.01 *M* HV .04 *M* NaV .06 *M* NaCl	.1	8.4
9	Borate	.05 *M* HB .012 *M* NaOH .05 *M* KCl	.1	8.55
10	Glycine	.06 *M* Gly .04 *M* NaOH .06 *M* NaCl	.1	9.85
11	Carbonate	.1 *M* Na_2CO_3	.3	11.25

of trapped air bubbles is facilitated by passing a spatula along the length of the trough until no more bubbles are visible. The excess buffer is then absorbed by blotting it off with filter paper or by adding more dry starch; finally the slab is shaped with a spatula to make it completely even with the trough.

A strip of *parafilm* is pressed onto the starch and the ends of the trough are fitted with 1.5–4 cm. wide strips of filter paper folded to at least three thicknesses. One end of these strips is held down by the glass cover of the trough (kept in place by means of tight rubber bands) and the other is allowed to hang over the open ends of the trough. The troughs are then put in place on the electrode vessels by pressing the ends against the paper wicks, and equilibration is allowed to take place for a few hours or overnight, according to the size of the trough.

To apply the protein sample, it is dissolved in a small amount of buffer and mixed with starch to form a solid paste; the trough is opened, and a section of starch 0.5–1.0 cm. wide is cut out at a previously marked position and replaced by the starch paste containing the sample. The location of this section depends upon the expected direction of migration, but the initial 6 to 10 cm. at either end of the trough are avoided whenever possible.

After the rubber tubing between the two vessels is closed off, the electrodes are connected to a constant voltage power supply. The voltage across the ends of the trough and the current are measured periodically. All experiments were performed in the cold room at 3°; their duration was from 16 to 90 hr.

The temperature in the troughs was not checked separately. Several colored proteins, however, were found to migrate in a straight narrow band, indicating that no temperature gradient sufficient to affect the mobilities beyond the limits of accuracy of the method was present. When, on the other hand, higher tensions than those used in the present study were tried, the protein bands obtained were highly curved or even completely disrupted, effects that may be due to an increase in temperature in the center of the trough.

After completion of the run, the starch in the troughs was cut into 1 cm.-sections and extracted with water (2–5 ml.) by means of stirring with a pointed spatula and subsequent gentle shaking. The tubes were centrifuged, or, alternatively, they were allowed to stand in the refrigerator until all the starch had settled. An aliquot of the supernatant liquid was used for the estimation of protein by the method of Lowry, *et al.*,[11] and another was used for *p*H measurements. The *p*H was measured in a Beckman *p*H meter with a glass electrode at 25°.

Ratio of Starch of Buffer.—The relative amounts of starch and buffer normally present in a trough were determined in the following manner: A trough with a capacity of 87 cc. was packed in the usual manner with a mixture of starch and distilled water, equilibrated overnight with water and weighed. It was then dried to constant weight at 105°; the average loss of weight was 58 g.

Thus, each cc. of total volume consists of 0.67 ml. of water and 0.33 ml. of starch or 0.495 g. of dry starch (sp. gr. 1.5). However, the starch as used contains 15.7% moisture; hence there is actually 0.585 g. of wet starch and 0.58 ml. of water or, in an actual experiment, of buffer. This buffer is, however, diluted by the water contained in the starch to the extent of 11.7%.

Determination of Electrical Constants.—The field strength, F, was calculated by the expression

$$F = \frac{V}{l} \qquad (1)$$

where V is the average voltage across the ends of the trough and l the geometrical length in cm. of the trough.

The electroösmotic flow, u_{el}, was determined by comparing the ratios of the mobilities of two proteins in free solution and on starch, according to the method previously described.[12] The values for u_{el} were determined for each buffer and used as constants throughout the investigation.

The mobility was calculated according to the expression

$$u = \frac{d}{Ft} - u_{el} \qquad (2)$$

where d is the observed distance of migration in cm. on starch, and t is the time in seconds.

The conductivity of the buffer in the trough was determined before and after electrophoresis. For this purpose a number of starch sections along the trough were extracted with water before and after a run and the conductivities were measured at 0° in a standard cell connected to a Leeds and Northrup conductivity bridge. Corrections were applied for the dilution of the buffer during extraction as well as for the dilution of the buffer due to the moisture contained in the starch.

Titration Curve.—For the determination of the titration curve, 100 mg. of untreated starch was suspended in 1 ml. of 0.1 *N* KCl and stirred overnight in order to allow the starch to swell; acid or base was added and the *p*H was measured, by means of a Radiometer (No. GK2021-B) glass electrode adapted to a Beckman *p*H meter, after an equilibration period of 5 minutes. Equilibration was practically complete in this time, since there was no significant discrepancy between the forward and reversed titration curves.

Results

Ion-exchange Properties of Starch.—The titration curve of starch, reproduced in Fig. 1, is complex and indicates the presence of several ionizable groups. Between *p*H's 2 and 9 the curve can be described by the titration of two main ionizable groups with a pK of 5.7 and 8.0, respectively. Above *p*H 9 other ionizable groups with a very high pK begin to appear. The maximal exchange capacity of the starch between *p*H's 2 and 9 is very low, being 1 milliequivalent per 50 g. of starch.

Changes in Voltage, Current and Resistance of the Trough.—A change in voltage across the ends of the trough was always observed during the run

(11) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1953).

(12) I. D. Raacke and C. H. Li, *ibid.*, **215**, 277 (1955).

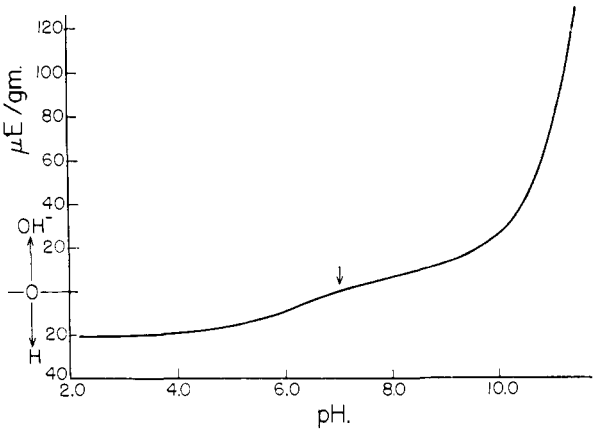

Fig. 1.—Titration curve of potato starch "Swan." The arrow in center of curve indicates the starting point.

even though the voltage across the electrodes was constant. This change varied with the buffer; it was negligible with borate and veronal buffers, for example, (not more than 10 volts) and was the most extensive with carbonate and bicarbonate buffers, where in the course of a 48-hour run a drop in potential of as much as 60 to 80 volts was frequently observed. There also occur changes in current, more marked than the corresponding changes in voltage and not as reproducible. It can be seen from Table II, where data from a few typical experiments are reproduced, that the change in current is greatest at the beginning of the run, with an accompanying large initial drop in resistance, calculated according to Ohm's law. When the starch is thoroughly washed with buffer before the experiment, this initial change in resistance is not observed, the voltage and current then remaining constant within the limits of the experimental error.

TABLE II

VARIATION OF POTENTIAL AND CURRENT DURING RUNS OF ZONE ELECTROPHORESIS ON STARCH

Buffer (Type-No.)[a]	Time, hr.	Potential, v.	Current, ma.	Resistance, ohms
Acetate-3	0	190	10	19,000
	29	175	11	15,900
	72	165	11	15,000
Cacodylate-5	0	200	32	6,250
	49	190	42	4,500
	72	200	44	4,500
Veronal-8	0	185	19	9,700
	17	175	21	8,300
	71	175	22	8,000
Borate-9	0	190	11	17,300
	4	197	13	15,100
	16	190	12	15,800
	20	190	12	15,800
	28	195	13	15,000
	38	200	12	16,700
Borate-9	0	105	10	10,500
	14	102	11	9,300
	28	110	12	9,200
	38	105	12	8,700
Carbonate-11	0	195	20	9,800
	2	185	28	6,600
	13	185	28	6,600
	24	180	26	6,900
	28	155	24	6,500
	48	130	18	7,200

[a] See Table I.

Changes in the Conductivity of Buffer in the Trough.—The sharp initial increase in current noted above indicates that either the conductivity of the buffer or that of the supporting medium is increased. It was found, in fact, that the conductivity of the buffer in the starch medium was different from that of the buffer in bulk.

When the conductivity of the buffer in the trough before electrophoresis was calculated from the known volume of buffer in the starch paste and from the dilutions applied, the value obtained was higher than that of the starting buffer for acid buffers, and lower than expected for alkaline buffers. This is in line with the fact that at *p*H's below 7 the starch gives up Na^+ and hence increases the con-

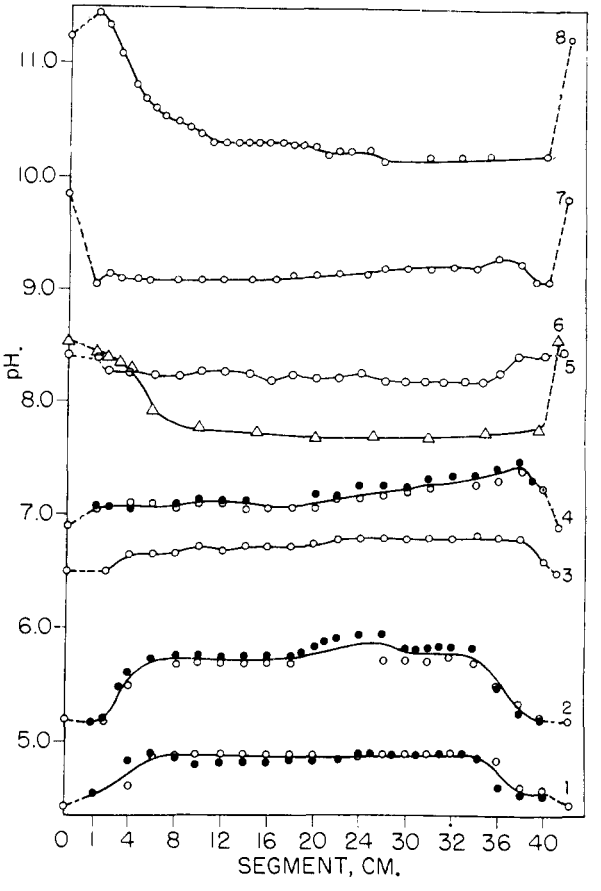

Fig. 2.—Variation of *p*H along starch troughs after electrophoretic runs at an average of 3.5×10^5 volt. sec./cm., with reversible electrodes and in different buffers. The *p*H in the electrode vessels (which remained constant during the run) is indicated by the ends of the broken lines on either side of the *p*H curves: 1, acetate, *p*H 4.0 (No. 2); 2, acetate, *p*H 5.15 (No. 4); 3, cacodylate, *p*H 6.5 (No. 5); 4, cacodylate, *p*H 6.9 (No. 6); 5, Veronal, *p*H 8.4 (No. 8); 6, borate, *p*H 8.55 (No. 9); 7, glycine, *p*H 9.85 (No. 10); 8, carbonate, *p*H 11.2 (No. 11). The numbers in parentheses refer to the number of the buffers in Table I. Different points on the same curve represent duplicate experiments.

TABLE III

CONDUCTIVITY OF BUFFER IN THE STARCH TROUGH BEFORE AND AFTER ELECTROPHORESIS

Buffer Type	No.	$K_b \times 10^3$, ohm^{-1}	K_s obs./K_s calc. Before electrophoresis: Without ion exchange	With ion exchange	Washed starch	After electrophoresis: Without ion exchange	With ion exchange	$Ft \times 10^{-5}$, v. sec./cm.
Acetate	4	4.79	1.22	0.98	1.00	1.44	1.20	3.59
						1.97	1.79	8.3
Cacodylate	6	4.79	..	..	..	1.24	1.20	3.69
Veronal	8	5.10	..	..	..	0.89	1.05	3.6
		5.15	0.87	0.99	..	1.10	1.28	6.5
Borate	9	3.84	0.89	1.03	..	0.64	0.70[a]	8.5
						1.47	1.54[b]	

[a] Cathode half of trough. [b] Anode side of trough. K_b is conductivity of buffer in bulk. K_s is conductivity of buffer in starch.

centration of salt in the buffer, whereas at *p*H's above 7, it picks up Na^+ and hence decreases the concentration of the buffer ion (see titration curve, Fig. 2). When the conductivities were calculated on the basis of the buffer composition expected from the amount of Na^+ exchanged by the starch at the *p*H of the trough (see below), they did, in fact, coincide with the experimentally determined values, as can be seen from Table III. Complete agreement between the expected and experimentally determined values also was obtained when the starch was prewashed with the buffer. After the trough had been electrolysed, there was, however, a further change in the conductivity of the buffer in the starch medium, which could not be accounted for by the base exchange capacity of the starch. The average conductivity was always higher than expected (Table III), indicating an accumulation of salt that was not, however, uniform throughout the trough. The only exception was found in the case of borate buffer where, after electrophoresis, the conductivity in the cathode side of the trough was lower than that of the starting buffer.

***p*H in the Trough.**---After the passage of current, there are marked changes in the *p*H of the buffer in the starch trough. When a plot of *p*H along the trough is made, more or less complex curves are obtained which are very reproducible for the same kind of buffer but which vary in shape from one buffer to the other. In Fig. 2 are reproduced typical *p*H curves for a number of buffers. From an inspection of these curves it becomes immediately apparent that all acid buffers show an increase in *p*H, whereas all alkaline buffers show a decrease. Again, this is to be expected from the ion-exchange properties of starch.

When two buffers of different *p*H are mixed, the more alkaline one will give up base to the more acid one until the *p*H's of both buffers are equal. The amount of base exchanged (x) can be calculated from the expression

$$pK_1 + \log \frac{\text{salt} + x}{\text{acid} - x} = pK_2 + \log \frac{\text{salt} - x}{\text{acid} + x}$$

where pK_1 is the pK of the buffer and pK_2 is the pK of the starch.

However, this value can also be determined with good approximation by the following method. The amount of base given or taken up by 1 g. of starch at an arbitrarily chosen *p*H is read directly from the titration curve; then, from the known ratio of starch to buffer, the absolute amount of base exchanged is calculated. From this the new composition of the buffer is ascertained and its *p*H is then calculated from the Henderson–Hasselbalch equation. If this *p*H is not close to the assumed one, the procedure is repeated until good agreement between the initially assumed and the calculated *p*H is obtained. As can be seen from Table IV, good agreement is obtained between the observed *p*H and the calculated one, indicating that the change in *p*H is, in fact, due primarily to the ion-exchange properties of the starch. This is further substantiated by the fact that when the starch is pre-washed with the buffer, the average *p*H in the trough is not significantly different from that of the buffer in bulk. However, small changes of *p*H near the ends of the trough, qualitatively similar to those observed with unwashed starch (Fig. 2) are not obviated by pre-washing of the starch and hence must be attributed to causes other than ion exchange.

TABLE IV

COMPARISON BETWEEN OBSERVED AND CALCULATED *p*H's OF SEVERAL BUFFERS IN THE STARCH TROUGH

Type	Initial buffer No.[a]	*p*H	*p*H of buffer in starch: Pre-washed	Unwashed: Calcd.	Unwashed: Obsd.[b]
Acetate	2	4.0	4.0	4.47	4.55
Acetate	4	5.2	5.3	5.59	5.70
Cacodylate	5	6.5	6.5	6.62	6.57
Cacodylate	6	6.9	6.9	7.00	7.10
Veronal	8	8.4	8.3	8.12	8.25
Borate	9	8.55	8.7	7.96	7.90
Carbonate	11	11.3	11.0	10.4	10.2

[a] See Table I. [b] Average of *p*H's in the trough from a minimum of 5 experiments.

Electroösmotic Flow.—Starch is a substance composed of small granules surrounded by a semi-permeable membrane, which permits the passage of water, electrolytes and other low-molecular weight compounds, but not of larger molecules such as polysaccharides or proteins. It seems likely that the negative charge carried by the starch is located on the outside of these membranes and consequently that the electroösmotic flow also takes place in the liquid between the granules. One would then expect that low-molecular weight substances inside the membrane would be relatively unaffected by electroösmosis, and it has been found, in fact, that such neutral flow indicators as caffeine,[13] dinitrophenyl (DNP)-arginine[13] and DNP-

(13) A. J. Parcells, private communication.

serine amide were not displaced at all or only to an insignificant extent under conditions where considerable flow was known to occur. It is therefore necessary to use dextran[9,14] or even proteins as flow indicators.

The present indirect method for determination of the electroösmotic flow from a comparison of the ratios of the mobilities of two proteins in free solution and on starch[12] has the added advantage of relating the results obtained with starch to those obtained with classical electrophoresis by permitting a direct comparison of isoelectric points obtained by the two methods. The validity of the method has been confirmed previously[12] since essentially the same results were obtained with two different pairs of proteins.

The quantity determined by the present method was found to have all the attributes of true electroösmotic flow. It is independent of the diameter of the trough and directly proportional to the field strength; hence it can be expressed in terms of mobility. The flow was found to be inversely proportional to the ionic strength as can be seen from Fig. 3 where u_{el} is plotted against ionic strength for acetate buffers at two different pH's.

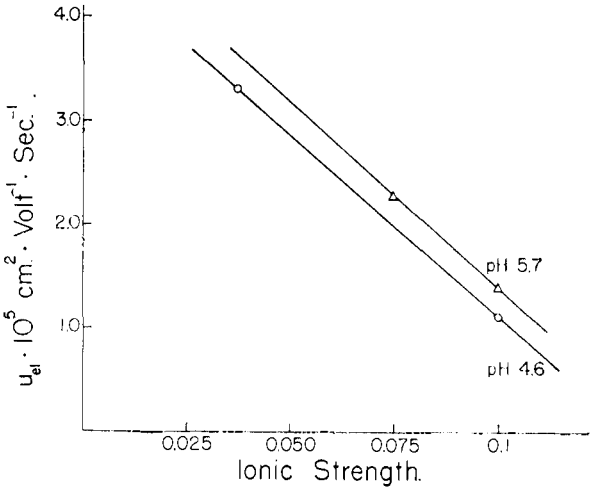

Fig. 3.—Electroösmotic flow of two acetate buffers as a function of ionic strength.

For monovalent buffers of equal ionic strength u_{el} should be proportional to the negative charge on the starch; *i.e.*, a plot of u_{el} *versus* pH should follow the titration curve. This condition also is satisfied, as can be seen from Fig. 4. Below pH 3 the starch is completely in the acid form, and the value for u_{el} is negligibly small. Because of the flatness of the titration curve, one would expect pre-washing of the starch with buffer to effect practically no change in the value for u_{el}, since the slight difference in pH would mean a negligible change in the net charge of the starch. This also has been borne out by experiment. The values for u_{el} obtained with washed starch, namely, 1.4×10^{-5} cm.^{2} volt^{-1}. sec.$^{-1}$ for acetate buffer of pH 5.3; 2.02×10^{-5} for veronal at pH 8.1 and also the value of 2.5×10^{-5} obtained by Kunkel and Slater[9] for veronal buffer at pH 8.6 on the same curve as the values for unwashed starch.

(14) H. G. Kunkel and A. Tiselius, *J. Gen. Physiol.*, **35**, 89 (1951).

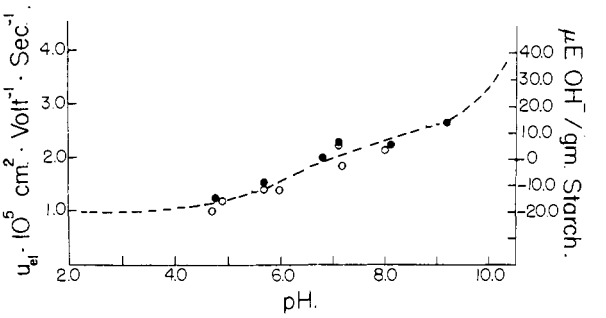

Fig. 4.—Electroösmotic flow of a series of monovalent buffers of 0.1 ionic strength as a function of pH. The values were obtained from the ratio of mobilities of ribonuclease and bovine serum albumin (open circles) and of chymotrypsinogen and bovine serum albumin (closed circles); the broken line represents the titration curve of starch.

As expected, a simple relationship between u_{el} and the titration curve does not hold when polyvalent buffer ions are present; the values of $u_{el} \times 10^5$ for a few such buffers are: 0.8 for phosphate (No. 7), 3.45 for borate (No. 9) and 2.0 for 0.1 M Na_2CO_3.

Electrophoretic Mobilities.—Extensive mobility studies on several standard proteins (bovine serum albumin, ribonuclease, chymotrypsinogen, chymotrypsin and lysozyme) have been made, but the data are not reproduced here. Mobility results for some of these proteins have been published elsewhere.[12,15,16]

The mobilities showed good reproducibilities when the field strength F was determined by direct measurement of the voltage per cm. As expected, they were lower than the corresponding mobilities in free solution; the ratio of the mobilities in the two media was, however, strongly dependent on the nature and ionic strength of the buffer used and relatively independent of the nature of the protein.

Discussion

The data presented show that the electrokinetic changes in the starch system observed soon after the current is turned on can be accounted for by a change in the composition of the buffers due to the ion-exchange properties of the supporting medium. As soon as equilibration has taken place, no further gross changes in the properties of the system occur. Similarly there are no marked changes if the buffer is thoroughly equilibrated with the starch, by washing the starch with buffer, before the start of the experiment.

Abnormal electrical behavior due to ion exchange has been described for collodion membranes by Sollner and co-workers[17] and for synthetic ion-exchange membranes by Albrink and Fuoss.[18]

There occur, in addition, gradual changes in the starch system throughout the duration of the experiment, changes which cannot be accounted for by ion exchange. For example, the observed

(15) I. D. Raacke, Ph.D. Thesis, University of California, Berkeley, 1954.

(16) I. D. Raacke, *Arch. Biochem. Biophys.*, **62**, 184 (1956).

(17) K. Sollner, I. Abrams and C. W. Carr, *J. Gen. Physiol.*, **25**, 411 (1942).

(18) W. S. Albrink and R. M. Fuoss, *ibid.*, **32**, 453 (1949).

change in *p*H is always slightly larger than would be expected on the basis of ion exchange, and the conductivity of the buffer in the trough increases slowly throughout the run, indicating that salt is accumulated in the starch medium. This accumulation of salt probably is due to swelling of the starch granules and consequent accumulation of salt inside the granule membrane due to the Donnan effect, although a decrease in the rate of migration of the buffer ions in the starch medium might also play a role. Evaporation seems to be excluded as a factor of any importance, since the effect is strongly dependent upon the type of buffer, being in general larger for buffers with a *p*H below 7 than for those with a *p*H above 7 (Table III).

Finally, it should be pointed out that since the mobility of a charged molecule varies in an inverse manner with the ionic strength of the medium, the relatively higher concentration of buffer ions in the starch might well, at least in part, be a contributing factor in the lowering of the mobility of proteins in this medium as compared with that of proteins in free solution at the same *p* H.

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[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF NORTHWESTERN UNIVERSITY]

The Stereochemistry of Proton Transfer Reactions. VII[1]

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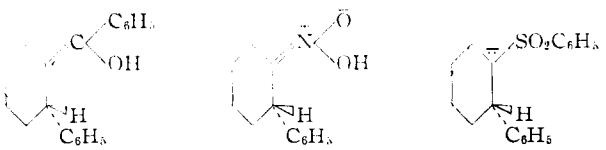
The stereochemistry of protonation of the conjugate base of 1-benzenesulfonyl-2-phenylcyclohexane has been investigated. This has been found to lead, in sharp contrast to the behavior of the carbonyl and nitro analogs, preferentially to the more stable of two stereoisomeric products. This antithetical situation is considered to result from the difference in the energetic importance of electron delocalization in carbanions stabilized by the sulfone group as opposed to the carbonyl and nitro groups.

In previous studies of ketonization of enols[2] and tautomerism of *aci*-nitro to nitro compounds[3] it has been concluded that the proton transfer process occurs *via* an essentially sp^2 hybridized transition state with steric hindrance to protopic attack controlling the stereochemistry of the reaction under kinetically controlled conditions. This interpretation derives first from the observation that under non-equilibrating reaction conditions the less stable of two possible stereoisomers frequently results and, secondly, from a consideration of possible transition states leading to these products.

Thus, ketonization of the unstable enol I led stereoselectively to *cis*-1-benzoyl-2-phenylcyclohexane.[4] Similarly tautomerism of the *aci*-nitro compound II was found[3] to proceed by way of its conjugate base with formation of *cis*-1-nitro-2-phenylcyclohexane.



I II V

Of special interest was the stereochemistry of protonation of the related sulfone system V, not only as an integral part of our investigations into the mechanism of proton transfer reactions but also as a source of information which might bear on the nature of carbanion stabilization by the sulfone group. The particular system V was chosen for this study since considerable information was available from investigations[5] of the carbonyl and nitro analogs.

Needed for the study were: first, syntheses of *cis*- and *trans*-1-benzenesulfonyl-2-phenylcyclohexane and proof of their configurations; secondly, procedures for unambiguously preparing the conjugate base (V) of these; and finally, investigation of the stereochemistry of protonation of this conjugate base. As part of the first objective, *cis*-1-benzenesulfonyl-2-phenylcyclohexane (IVa) was conveniently prepared by free radical addition of thiophenol to 1-phenylcyclohexene followed by peracetic acid oxidation without purification of the intermediate phenyl 2-phenylcyclohexyl sulfide. The crude oxidation product was found by quantitative infrared analysis to consist of 92% *cis*-1-benzenesulfonyl-2-phenylcyclohexane (IVa) and only 8% of the *trans* isomer IVb. This stereochemical result represents support for the assigned configurations, since it has been shown by Bordwell[6] and Goering[7] that the free radical addition of thiols to 1-substituted cyclohexenes preferentially yields the *cis* product. Recrystallization of the crude sulfone product afforded pure *cis*-sulfone IVa, m.p. 120°, without difficulty.

As further evidence for the assigned configurations and as a convenient preparation of the

(1) Presented in part at the Organic Division, A.C.S. Meeting, New York, September, 1957.

(2) Paper VI of this series, Howard E. Zimmerman and Theodore W. Cutshall, THIS JOURNAL, **80**, 2893 (1958).

(3) Howard E. Zimmerman and Thomas E. Nevins, *ibid.*, **79**, 6559 (1957).

(4) H. E. Zimmerman, *J. Org. Chem.*, **20**, 549 (1955).

(5) See ref. 2 and earlier papers cited therein.

(6) F. G. Bordwell and W. A. Hewett, THIS JOURNAL, **79**, 3493 (1957).

(7) H. L. Goering, D. I. Relyea and D. W. Larson, *ibid.*, **78**, 348 (1956).