

ELECTROKINETIC PHENOMENA

XIII. A COMPARISON OF THE ISOELECTRIC POINTS OF DISSOLVED AND CRYSTALLINE AMINO ACIDS*

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INTRODUCTION

The electrokinetic properties of particles in a given medium are due entirely to a surface film which may or may not be chemically similar to the bulk of the particle. Many of the quantitative experiments on electrophoresis have dealt with systems designed to study the influence of the surface film on the bulk of the particle. In these systems the surface films have frequently been proteins and the bulks of the particles have been such diverse substances as quartz, air, and paraffin oil. With few exceptions a film of a given protein adsorbed on inert surfaces has electrokinetic properties quite similar to the electrokinetic properties of the protein when dissolved, or suspended as an amorphous, relatively insoluble particle. Inasmuch as protein films and protein particles have been extensively investigated, the electrokinetic properties of simpler ampholytes like amino acids merit analysis by the same methods used to investigate the electric mobilities and the isoelectric points.

The concept of the isoelectric point was first used to indicate a reference concentration at which the electric mobility of a particle of any sort is equal to zero. Although it was first employed by Hardy (1) to designate the point of reversal of sign of charge of *particles* of heat denatured albumin, hydrogen ions

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were not the first used to cause the reversal of the sign of surface charge by electrolytes. In 1880, about twenty years before the experiments of Hardy, Gore (2) investigated electroosmosis through clay diaphragms. He noticed that an alcoholic solution of barium bromide reversed the sign of surface charge. The term isoelectric point later became of importance in connection with dissolved ampholytes. Pauli in 1906 (3) investigated solutions of proteins; and since that time, the isoelectric point has been of importance not only in connection with the characterization of the electrical properties of *surfaces* but also with the conditions under which the net charge of dissolved ampholytes sums up to zero over a time average. Both on the basis of usage and experiment, therefore, a crystal of an amino acid suspended in a liquid may have an isoelectric point as well as the dissolved amino acid itself. Recent discussions frequently consider only the thermodynamic aspects of the isoelectric point of dissolved ampholytes. However, it does not seem, as some of these recent discussions imply, that the term should be restricted to dissolved substances or thermodynamic considerations alone, especially when it is considered that the isoelectric point of particles or of surfaces is usually obtained by a conductance method.

It seems desirable, therefore, to define the isoelectric point as suggested by one of the writers (4) so that the definition includes both dissolved and suspended materials. According to this point of view, the isoelectric point of an amino acid, whether dissolved or crystalline, may be defined as a reference concentration ($[H^+]$ or $[Th^{++++}]$, for example) at which

$$\frac{1}{T} \int_0^T dt \left\{ \sum_1^s n_i z_i(e) + \sum_1^s n_i z_i(-e) \right\} = 0,$$

($T \gg \tau$).

Here e is the electronic charge and n_i is the number of ions of the i -th type, of valence z_i , at the surface during the time dt . T represents the time of observation and τ the life period of an ion at the surface. That is, the time average of the total sum of all of the positive and negative ions of the ampholyte is zero in the case of the dissolved ampholyte. Similarly, in the case of any surface or of a large ion, the isoelectric state is that in which the sum of all of the positive and negative charges on the surface over a time average is equal to zero. The conditions relating to the life period of the ion τ may be fulfilled by observing a large number of fluctuations during the period of a measurement; *i.e.*, a statistical sample.

A case in point is the cholesterol surface. It has been observed recently by Moyer (5) that the point of reversal of the sign of charge of crystals of cholesterol and ergosterol occurs near pH 3.2. From the point of view of the organic chemist, these sterols can hardly be considered ampholytes. Yet these surfaces in contact with an aqueous medium show ampholytic properties similar to those of adsorbed protein films. They have, therefore, an isoelectric point and can be considered to have amphoteric surfaces which can hardly be described by thermodynamic considerations on the basis of their formulas.

In this communication, a comparison will be made of the isoelectric points of dissolved amino acids obtained by the conventional thermodynamic methods and the isoelectric points of amino acid crystals determined by measuring the electric mobilities of these crystals themselves. The significance of these measurements will be discussed in connection with the constitution of the limits of crystal lattices of amino acid surfaces in their own saturated solutions.

HISTORICAL

There is a surprisingly large number of substances which are usually negatively charged when suspended in their own saturated solutions and which show reversal of sign of charge when acids or polyvalent ions are added. Thus, it was shown by Perrin (6) for aqueous media, that crystals of phenyl salicylate, naphthalene, sulfur, and metallic oxides may be reversed in sign of charge by hydrogen ions. In addition, zinc sulfate, barium sulfate, chromic chloride as well as silicon carbide, showed similar properties. Since that time, a veritable host of surfaces, especially those composed of silver halides, have been investigated in connection with the effect of isomorphous and homeomorphous ions on the position of the isoelectric point. These crystals have had the added advantage of being studied by both thermodynamic and electrokinetic investigations. In connection with the present report may be mentioned the work of Mukherjee and Iyer (7) who studied certain organic acids and amino acid crystals in their own saturated solutions. These investigators found that phenyl glycine-*o*-carboxylic acid crystals reversed their sign of charge in dilute aqueous hydrochloric acid, whereas crystals of *m*-amino-benzoic acid were positive in their own saturated solutions and were reversed by alkali.

The work of Wintersteiner and Abramson (8) showed that surfaces of insulin crystals have a different isoelectric point than adsorbed films of insulin on quartz. By having a sufficient amount of insulin dissolved in the solutions, the crystals apparently adsorb the dissolved protein more rapidly than larger crystals can be built and, under these conditions, the crystal surface behaves like the surface of adsorbed insulin.

Methods

Two horizontal, flat electrophoresis cells, whose dimensions have been previously described (9), were used in the measurements of electric mobility. A detailed description of the electrophoretic methods has been given elsewhere (10-12). All measurements were performed at room temperature (23-27°C.). In difficult determinations, measurements were made at successive levels throughout the cell and the resulting parabola integrated (13), but in most cases data were obtained at the stationary levels.

The cystine preparations were obtained from three different laboratories. Tyrosine and aspartic acid were from Eastman Kodak Company and were studied in the form as purchased and after subsequent recrystallization. The melting points of these preparations were all high. Usually the amino acid crystals were finely ground and suspended in the acid or buffer solutions for 24 hours before measurements of pH and electric mobility were carried out. Many experiments were performed after $\frac{1}{2}$ hour with no observable differences.

The rapid settling of the larger particles to the floor of the electrophoresis cell and the consequent alterations of the surface properties of the glass floor were liable to produce asymmetry of the mobility-depth parabola. In addition, the high conductance of solutions below pH 3 required the use of currents which in some cases appeared to produce turbulence. When such effects were noted, the cells were always cleaned and the measurements repeated. Alternate use of two cells of different dimensions provided a check on the reliability of the data. Many of these difficulties might have been eliminated by the use of the vertical cell described by Abramson, Moyer, and Voet (14), had it been available at the time when these measurements were made.

EXPERIMENTAL

Analysis of the v -pH Curves.—Fig. 1 shows the electrophoretic mobility-pH curve of aspartic acid in solutions of hydrochloric acid. It will be noticed that the isoelectric point of the suspended crystals lies near pH 2.3. The arrow and the cusp in the titration curve indicate the position of the isoelectric point of dissolved aspartic acid at pH 2.9 as calculated from the dissociation constants (15).

Cystine crystals in hydrochloric acid solutions likewise are isoelectric near pH 2.4 (Fig. 2). The three sets of circles are data for the three different preparations. It was desirable to confirm the fact that the isoelectric point of cystine crystals differs in position from the isoelectric point calculated from dissociation constants. Data were therefore obtained in $M/200$ acetate buffers of constant ionic strength. A few points were also obtained in the same buffer to which

m/5 NaCl had also been added. Note in Fig. 2 that at the isoelectric point of dissolved cystine, as calculated by thermodynamic methods (15), the electric mobilities of the crystals of cystine are rather high and easily measured. The addition of salt to the solution lowers the electric mobility in a way similar to its effect on the mobilities of inert surfaces or of ionogenic surfaces. Although the data obtained in sodium acetate buffers are not sufficiently accurate or numerous for extrapolation to an isoelectric point, the general direction of the smooth curve drawn through the points in Fig. 2 is toward

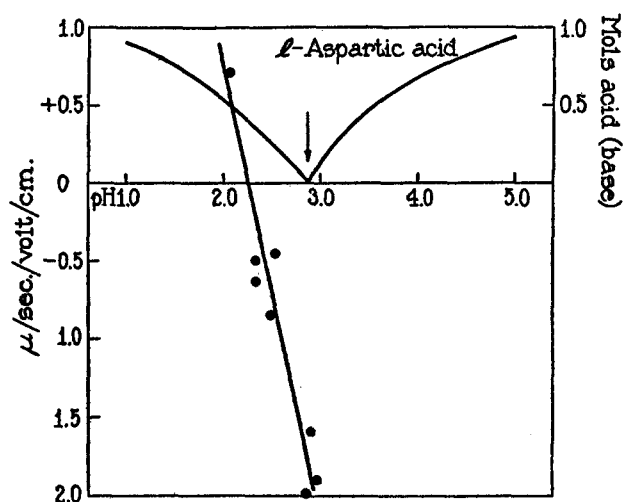


FIG. 1. A comparison of the electric mobilities of aspartic acid crystals in HCl solutions and the titration curve of dissolved aspartic acid. The arrow indicates the position of the isoelectric point of dissolved aspartic acid. ● = HCl. Smooth curve—titration.

the region designated as the isoelectric point in solutions of hydrochloric acid at pH 2.3. The smooth curve above the abscissa is drawn from the data given by Sano (16) for the solubility of cystine.

In Fig. 3 are presented results of measurements of the electrical mobilities of tyrosine. Here a similar comparison is made with the solubility of tyrosine (17) and with the isoelectric points determined for the dissolved tyrosine (15) and the crystals themselves. It is somewhat surprising to note that although the isoelectric point of dissolved tyrosine calculated in the usual way lies near pH 5.7, the

isoelectric point of the crystals falls very close to the isoelectric point noted in the foregoing for cystine and for aspartic acid. As mentioned previously for cystine, tyrosine crystals suspended in $M/200$ acetate buffers possess a negative charge, and the data indicate that an extrapolation of the smooth curve will pass near pH 2.4. As in the case of cystine, addition of salt, here $M/8$ NaCl, lowers the electrical mobilities. It is also of interest that in Figs. 1-3, there is no point of

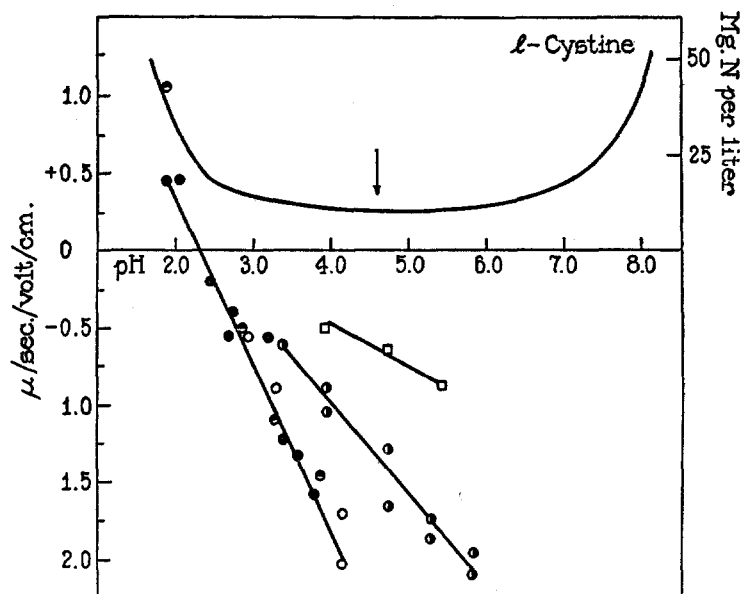


FIG. 2. The electric mobilities of cystine crystals suspended in hydrochloric acid and in sodium acetate buffers. The arrow points to the isoelectric point of dissolved cystine. ●○ = HCl; ● = $N/200$ NaAc; □ = $N/200$ NaAc + $N/5$ NaCl. Smooth curve—solubility (Sano).

inflection on the mobility-pH curves of the suspended crystals near the values of pH given for the isoelectric point of the dissolved amino acids.

In a few experiments with cystine the surface properties seem to be reversible, for dilution of the medium initially at a pH below the isoelectric point of the crystals to a value above their isoelectric point

resulted in reversal of sign of charge.¹ Data for the construction of smooth electric mobility-pH curves for these crystals in HCl solutions are presented in Table I.

Effects of Salts.—As mentioned previously, univalent salts depress the electric mobility with increase of the ionic strength analogous to that observed with other surfaces (18). An increase in the ionic strength of the medium has little if any effect on the position of the isoelectric point of the crystals studied (Figs. 2 and 3). It was of

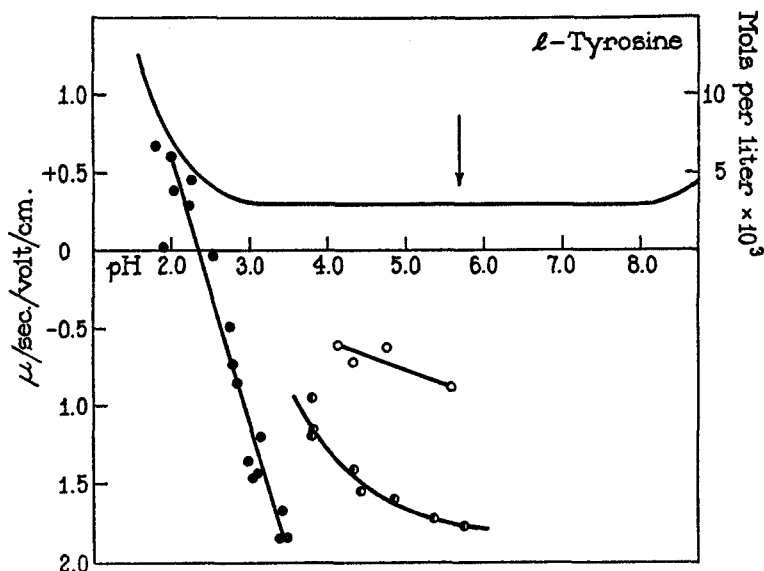


FIG. 3. The electric mobilities of tyrosine crystals suspended in hydrochloric acid and in sodium acetate buffers. The arrow points to the isoelectric point of dissolved tyrosine. ● = HCl; ● = N/200 NaAc; ○ = N/200 NaAc + N/8 NaCl. Smooth curve—solubility (Hitchcock).

¹ Some interesting but rather difficult experiments were performed with amino acid crystals while they were actively dissolving. A quantity of finely ground tyrosine crystals was added to sodium acetate buffer and electrophoresis measurements were performed immediately. Although sufficient quantitative measurements were not feasible at that time, the data obtained indicated that the electric mobilities of dissolving crystals were close to those of the crystals in their own saturated solutions, and did not markedly differ from those in Fig. 3.

interest to study the effect of a polyvalent ion. Fig. 4 shows the effect of adding low concentrations of AlCl_3 to a suspension of tyrosine crystals in hydrochloric acid. The concentration of aluminum ion is expressed as pAl to show that an isoelectric point may be reached

TABLE I

Data for the Construction of Smooth Electric Mobility-pH Curves for Amino Acid Crystals Suspended in Hydrochloric Acid Solutions

Aspartic acid		Tyrosine		Cystine	
pH	ν	pH	ν	pH	ν
2.30	0	2.33	0	2.37	0
2.75	-1.50	3.24	-1.50	3.75	-1.50

* $\mu/\text{sec.}/\text{volt}/\text{cm.}$

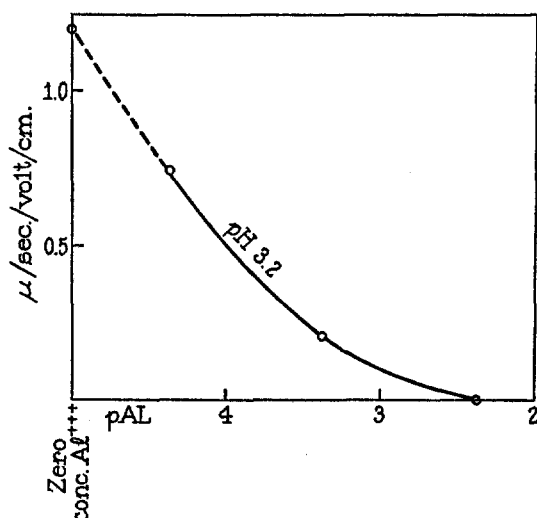


FIG. 4. The electric mobility of tyrosine crystals in dilute solutions of AlCl_3 at approximately constant pH. This curve demonstrates that the surfaces of tyrosine are affected by Al^{+++} ions in the same way as other surfaces.

by addition of Al^{+++} ions to a certain activity of aluminum ion just as by adjustment of the solution to a certain pH value an isoelectric point in terms of pH may be attained. It may be observed that aluminum ion acts in a conventional way by lowering the electrical

mobilities of the negatively charged crystal surfaces even in very high dilution. pH changes due to the addition of the AlCl_3 were of no importance for the pH rose as the aluminum concentration was increased. This would presumably have tended to increase mobilities rather than decrease them. Other surfaces, not usually considered amphoteric, such as cellulose (19), may be reversed by polyvalent ions although not by hydrogen ions.

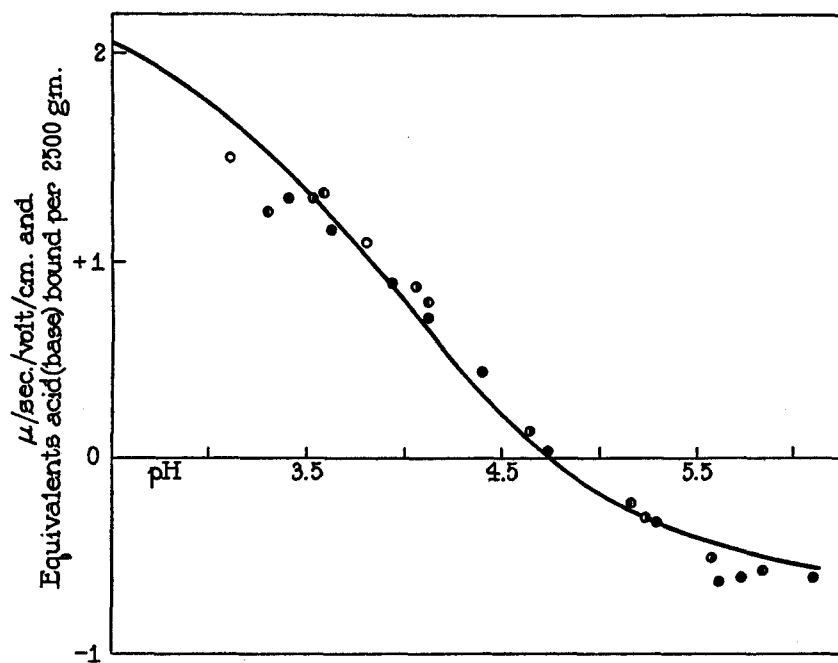


FIG. 5. These data demonstrate that amino acid crystals behave very much like inert surfaces in their ability to adsorb gelatin. Evidently the amphoteric properties of the surfaces of the amino acid crystals do not interfere with the adsorption of another ampholyte. ● = gelatin adsorbed by quartz; ○ = collodion (Loeb); ◐ = cystine; ◑ = tyrosine. Smooth curve—titration.

Effect of Added Ampholytes.—When crystals of tyrosine or cystine were suspended in solutions of Cooper's gelatin in $m/150$ acetate buffers, they yielded the same v -pH curve as particles of collodion or quartz when these too were suspended in gelatin (Fig. 5). In other words, the gelatin coats the surface of these amino acid crystals just

as it does an inert surface. This lack of specificity of the underlying surface has been observed with most, but not all, adsorbed proteins (20, 21). The smooth curve is the titration curve fitted to the same scale as the mobility data. Similar correspondence between mobility and titration curves has been noted and discussed previously (20, 22).

A series of qualitative experiments on the effect of a simple dissolved ampholyte, glycine, on the isoelectric point of cystine crystals showed no marked influence.

Comparison with Simple Alkyl Benzenes.—In Fig. 6 are the mobility-pH curves of droplets of *n*-propyl benzene and ethyl benzene in HCl solutions. Within the limits of error the data were the same for two substances. No reversal of sign of charge of the alkyl benzene droplets was noted. At the lowest pH values investigated, their mobility became zero within the limits of error. The surfaces of the amino acid crystals as determined by the slopes of their mobility-pH curves are not markedly different from those of the alkyl benzenes. However, they have one property which markedly distinguishes them from the alkyl benzenes. It is the property which results in reversal of sign of charge in their own saturated solutions at sufficiently low values of pH.

Effect of Size and Shape on Electric Mobility.—The long needle-like structure of the tyrosine crystals offered an excellent opportunity for the investigation of the effect of needle length and orientation on the electric mobility. Using Eastman Kodak Company tyrosine, it was found that (considering the difficulty of making measurements on particles of this type) crystals and fragments of crystals between 2–30 μ migrated independent of their length in the electric field. Particles less than 2 μ long gave a variable mobility, moving more slowly than the others.

Carefully recrystallized tyrosine was powdered and suspended in sugar solution to make a more careful analysis of the effect of particle length on electric mobility. Previous microscopic examination of the unpulverized material in the same medium had shown a uniform suspension of long needles with no very small particles. Examination of the electric mobility revealed that tyrosine needles or fragments of tyrosine crystals from 2–100 μ migrate independent of their length and

orientation. Particles of the order of 2μ or less show slightly decreased electric mobilities.

Two explanations might be offered for the fact that tyrosine particles 2μ in length show decreased mobilities. One of these is the diminution of mobility caused by the size of the particle approaching the thickness of the double layer, that is, a decrease in κr below a critical value.

An alternate explanation, which appears more plausible, is that which has to do with the nature of the crystal itself. In order to obtain small fragments of tyrosine needles, the crystals have to be finely ground. This results in a change from the natural surface of the crystal formed during crystallization: the area of the fractured surface approaches the area of the natural crystalline surface and therefore the electric mobility is changed.

DISCUSSION

Since x-ray diffraction data do not give very exact information in regard to the constitution of the limits of the crystal lattices of the substances investigated when suspended in aqueous media, it is difficult to offer precise mechanisms explaining the behavior of the surfaces of the amino acids under discussion. A point of view which is derived from the analysis of the crystal structure and the electrophoretic behavior of the silver halides may be of service in this connection (reviewed by Abramson (23) and Verwey (24)). The reversal of the sign of charge of silver chloride crystals may be visualized by considering that at the limits of the crystal lattice one of each of the six ions of the lattice is lacking for each ion of Ag^+ and Cl^- . This results in unsaturated valence forces being made available for reactions with ions opposite in sign of charge. Thus, an excess of silver ion reverses the negative sign of charge of silver chloride or silver iodide crystals. In a similar fashion, negatively charged barium sulfate crystalline surfaces may be reversed in sign of charge. We have seen that hydrogen ions alone do not reverse the sign of charge of simple alkyl benzenes. We must invoke, therefore, some mechanism essentially connected with the constitution of the crystal lattice of the amino acid crystal to explain the apparently simple reversal of the sign of surface charge. The only mechanism which has occurred to

us is one which would assume that reversal of the sign of charge is, in part, brought about by the deposition in the lattice of the isomorphous ions of the positively charged dissolved amino acids themselves. In this way, in the case of tyrosine, for example, if the pH is decreased and more tyrosine goes into solution, not only will there be an increasing number of ions available for adsorption, but also a markedly increased number of positive tyrosine ions. It is possible that these tyrosine ions may fit into the lattice and produce a reversal of sign of charge in the same way that silver ions may produce reversal of sign of surface charge of a crystal of silver chloride. In this connection, observe the slopes of the mobility-pH curves of the crystals of amino acids in Fig. 6 where all three are given for comparison. At first glance, it would seem that reversal of sign of surface charge occurs more rapidly in those instances where the increased solubility occurs most rapidly; that is, the slope of the tyrosine curve is greater than the slope of the cystine curve, and is less than that of the aspartic acid. However, the rapid change in the mobility of the alkyl benzenes with pH does not support this point of view. Not all crystalline organic surfaces, however, would fall into the category established for the silver halides or for the point of view given here for the amino acid crystals. Thus Moyer (5) has shown that the crystal surfaces of cholesterol appear to be simply reversed in sign of charge by hydrogen ions. From the point of view of structural chemistry, there are no groups which react with hydrogen ions to produce reversal of sign of charge, nor is cholesterol sufficiently acid in character to account for its isoelectric point at pH 3.2, the point given by Moyer for his purest preparations. One might suppose that the order of increasing slopes of the mobility-pH curves (Fig. 5) would follow the order of pH values of the corresponding isoelectric points for dissolved amino acid molecules. Thus an amino acid whose isoelectric point (in the dissolved state) was high on the pH scale would begin to have its mobilities reduced by its adsorbing positively charged ions at a pH value where crystals of another amino acid, with dissolved molecules isoelectric at a lower pH, would be scarcely affected. As seen from Fig. 6, however, the results do not bear out this simple picture for the order of the slopes is not the same as the order of the corresponding isoelectric points. It will be necessary to include in the development of

a complete theory of the effect of pH on the electric mobilities of crystalline amino acids, the effect of adsorbed hydrogen ions in addition to the entrance of dissolved amino acid molecules into the surface, even though the effects of hydrogen ions may be less important than isomorphous ions.

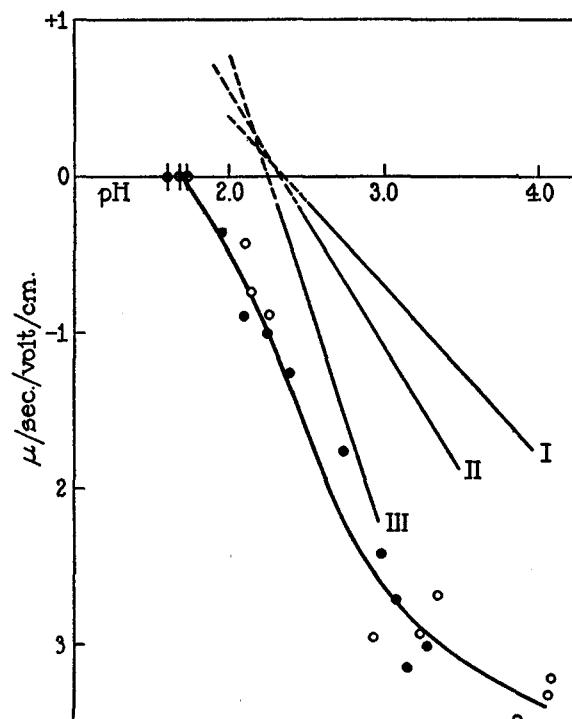


FIG. 6. A comparison of the electric mobilities of emulsion droplets of simple alkyl benzenes with those of the surfaces of crystalline amino acids. \circ = *n*-propyl benzene; \bullet = ethyl benzene; I = cystine (crystal); II = tyrosine (crystal); III = aspartic acid (crystal).

In the case of the surfaces of the keratins, such as wool (25), hair, skin, and finger nails (26), the electrokinetic properties are probably influenced more than adsorbed proteins by the constraints occurring at the surface of the crystal lattice. Since in the case of the keratins the elongated protein molecules are arranged side by side and held together by S—S linkages for the most part (27, 28), it seems likely that the constraints imposed on the reacting groups of the sur-

face are not so important as they would be in determining the electrokinetic properties of a truly crystalline amino acid and that the isoelectric point found by Harris represents the ionization of the protein molecules fairly closely. Goddard and Michaelis (28) have presented values for the flocculation maximum of wool brought into a dissolved state by thioglycolic acid and other reducing agents. Data on the electrokinetic properties of these dissolved keratins would be of interest. A simpler case is afforded by the experiments of Harris (29) on silk fibroin. In this protein the elongated molecules in the crystallite are not held together so tightly as in keratin since peptization may be readily produced. S—S linkages are also lacking. Harris has found that the electric mobilities of particles of ground-up silk fibroin are identical with the mobilities of silk fibroin adsorbed on quartz particles. The isoelectric point was the same for both crystallite surface and adsorbed protein surface, lying near pH 2.5.

Comparison should be made between these results and those of Wintersteiner and Abramson (8), discussed in the foregoing, for crystalline and amorphous insulin surfaces, an example of a more truly crystalline protein. It is probable that the rearrangement occasioned by the transformation of a spherical, dissolved protein such as egg albumin or insulin into the crystalline state would produce effects which serve to distinguish surfaces of this type from the keratin surface.

A better understanding of the nature of the surfaces of amino acid crystals and the surfaces of crystallites like keratin must await the investigation of the surfaces of long-chain amino acids and polypeptides.

SUMMARY

1. Although the isoelectric points of dissolved cystine, tyrosine, and aspartic acid molecules lie at widely differing pH values, the isoelectric points of the surfaces of these substances in the crystalline state are all near pH 2.3. This was found to be true in solutions of hydrochloric acid and in acetate buffers of approximately constant ionic strength.

2. When suspended in gelatin, tyrosine and cystine crystals adsorb

the protein and attain a surface identical in behavior with gelatin-coated quartz or collodion particles.

3. Aluminum ions at low concentrations reduce the electric mobilities of tyrosine crystals to zero in a manner analogous to their effect on other surfaces.

4. Alkyl benzene droplets also have their electric mobility reduced to zero at low pH values but, unlike the amino acids, a change in sign was never noticed.

5. The mobility of tyrosine crystals is independent of crystal length between 2-100 μ . Below this size the mobilities are decreased.

6. These results are discussed in connection with the concept of the general definition of the isoelectric point and the behavior of certain insoluble proteins such as wool and silk fibroin.

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