Paper Electrophoresis with Superimposed pH Gradient

Kolin (*1*) has recently reported a new technique of free-boundary electrophoresis in which a *p*H gradient is superimposed on the potential gradient. The components of a mixture separated by this technique assume an order according to their isoelectric points. In the present paper, the use of *p*H gradients in paper electrophoresis is described (*2*).

Figure 1 shows protein patterns after electrophoresis by the conventional technique and in a *p*H gradient; Whatman No. 3 filter paper mounted horizontally and bromophenol blue stain (*3*) were used. Patterns *a* and *b* were obtained with Veronal buffer of ionic strength 0.05 and *p*H 8.6 after 3.5 hr at 200 v across the electrodes (exposed length of paper strip 21 to 23 cm, sample applied near the end of the strip). In experiments *c* and *d*, a *p*H gradient was established by wetting the paper strip from the side of the positive electrode with 0.05*M* NaH_2PO_4, which has a *p*H of about 4.3, and from the other side with 0.01*M* phosphate buffer solution of *p*H 7.4. The sample was then applied as a streak along the line where the two phosphate solutions met, and 200 v was applied for 6.7 hr.

Staining with Sudan Black B (*4*) showed that the largest amounts of lipid, usually associated with beta-lipoprotein, were located at or behind the beta-globulin protein band in Veronal, but in the patterns obtained with the *p*H gradient they were located at the front edge of the component (marked 2), which was provisionally identified with beta-globulin. In both techniques, the lipid of the chylomicrons remained at the site of application of the sample, and small amounts of lipid were found in the albumin region on the side toward the globulins (alpha$_1$-lipoprotein).

Although the two methods should not be expected to yield exactly the same distribution of the components, analysis of the eluates (0.01N NaOH) from sections of the paper strip (Table 1) gave fair agreement when it was assumed that component 1 corresponds to alpha$_2$-globulins, component 2 to beta-globulins, and component 4 to gamma-globulins. Fibrinogen was contained in component 3. The discrepancies in the values for albumin plus alpha$_1$-globulin may be attributed to the trail observed in Veronal at *p*H 8.6 between the beta-globulin and the origin, if it is assumed that all of this trail is caused by albumin. In electrophoresis in Veronal, the trail on the gamma-globulin may be obviated by placing the sample at the center of a longer paper strip, whereupon the gamma-globulin moves backward. The example in Table 1 is quoted to demonstrate the extent of possible error as a result of trailing. The band of component 1 in the plasma pattern analyzed included the material that was irreversibly adsorbed at the site of application, and this may have been the cause of the low value for plasma component 3. In the patterns shown in Fig. 1*c*, *d* the material at the site of application was included in component 2.

The movement of a band depends on the mobility of the protein and also on the rate of flow of the buffer solution, which in turn depends on the rate of evaporation of solvent and the position on the strip. The flow is greatest at the ends of the paper strip that dip into the buffer solutions and is zero approximately in the center. The flow pattern can be regulated by varying the concentrations of the two buffer solutions used.

Whenever the electrophoretic migration velocity of a material is equal and opposite to the flow of the buffer solution, the band will remain stationary (*3*). Macheboeuf (*5*) has realized that this property can be used, among other applications, for the electrophoretic study of very dilute solutions without previous concentration, since all the material applied collects in narrow bands at the appropriate positions of zero velocity. Macheboeuf regulated the evaporation of the solvent by means of holes in the hood that covered the filter paper strip. In both methods employed by us the strips were kept under closed hoods. Nevertheless the rate of evaporation of solvent from the paper was large enough to aid in sharpening the bands.

Figure 1*e* shows the pattern of the plasma of *b* and *d* analyzed with the *p*H gradient after 1-to-20 dilution with 0.01*M* phosphate of *p*H 7.4. The colorimetric values of the components of a 1-to-20 diluted serum agreed within 1 percent of the total optical density with those obtained with undiluted serum, except for the band No. 2. This band contained the site of application in the case of the undiluted serum, and the percentage value for this component was 2.0 higher than that for diluted serum.

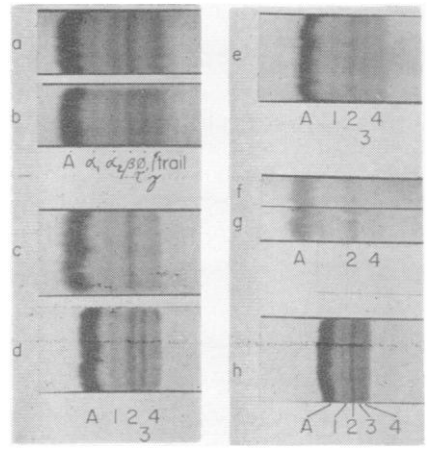

Fig. 1. Electrophoretic patterns of human serum (*a, c*), plasma (*b, d, e, h*), and cerebrospinal fluid (*f, g*); *a* and *b* obtained by conventional technique, all others in a *p*H gradient.

Still more attenuated conditions with regard to the concentrating effect were present in two experiments with unconcentrated cerebrospinal fluid that contained 0.01 (Fig. 1*f*) and 0.02 (Fig. 1*g*) grams of protein per 100 ml, respectively. On the narrower strip 0.8 ml cerebrospinal fluid was used.

The sharpness of the bands can be improved at the expense of the extent of the pattern by increasing the buffer salt concentration (Fig. 1*h*; 0.08*M* NaH_2PO_4 and 0.01*M* phosphate at *p*H 8; 6.7 hr at 200 v). Since the salt concentration on the paper strip increases continually, the pattern of the separated proteins narrows slowly on prolonged electrophoresis. There is, therefore, an optimum time for the greatest separation of the components.

The separation of proteins whose isoelectric points differ little—for example, hemoglobins A and F—could not be achieved by *p*H-gradient paper electrophoresis. The bands overlapped to a similar extent whether the *p*H gradient was steep or flat. It is possible to separate such components by electrophoresis at a single *p*H if the operation is carried on

Table 1. Analysis of serum and plasma proteins by the *p*H-gradient method (in percentage of total optical density at 595 mμ).

Method of analysis	Serum		Plasma	
	*p*H gradient	Veronal, *p*H 8.6	*p*H gradient	Veronal, *p*H 8.6
Albumin	71.8	60.1	67.5	56.6
alpha$_1$-		4.8		5.3
"1" or alpha$_2$-	7.3	7.8	10.5*	6.9
"2" or beta-	11.7†	11.0	10.3	9.9
"3" or tau (+ fibrinogen)	1.9	3.2	5.7	12.5
"4" or gamma-	7.3	11.7	6.0	8.1
Trail at rear of gamma-		1.4		0.7

* Includes some particulate matter staining at site of application.
† Includes little particulate matter staining at site of application.

for a sufficient length of time. The *p*H-gradient method is particularly useful for the analysis of very dilute solutions and appears to offer advantages over Macheboeuf's electrorheophoresis (*5*) because of its flexibility in directing the displacements of the bands and because of its avoiding excessive concentration of buffer salt on the paper.

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References and Notes

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Direction of Ionic Addition to Olefinic Double Bonds

When the addition of an unsymmetrical molecule to an olefinic double bond begins with addition of a cation to the pi electrons (*1*), the direction of the addition can be simply explained, without the concept of hyperconjugation (*2*) or the assumption of polarity in the double bond (*3*). The cation tends to become finally joined to that one of the two unsaturated atoms from which an electron is less readily removed, except where this effect is overcome by electrostatic forces. As a general principle, two factors appear to control the ease of withdrawing an electron from an atom. One is the partial charge on the atom: the less positive and more negative the charge, the greater the ease of electron withdrawal. The other is the nature of attached atoms and groups: the more readily these atoms can supply charge to compensate for the withdrawal, the greater is the ease of withdrawal.

The fundamental basis for the proposed explanation is information on charge distribution obtained by methods recently described (*4*). It is possible to estimate what the partial charge on each atom of a molecule would be, if the only factor were the initial electronegativity differences and the process of equalization during combination (*5*). Similarly, the effect of adding a cation to a double bond may be estimated, assuming that the principle of electronegativity equalization is valid here. The detailed application of such partial charge information can best be illustrated by examples.

Consider an olefinic bond in an alkene. According to the theory, such a bond must always be electrically symmetrical (nonpolar), because an alkyl group contributes exactly the same partial charge to an unsaturated carbon as does hydrogen. However, alkyl groups are potentially more capable of contributing negative charge (are better reservoirs). When a cation—for example, a proton from CHl—adds to the pi electrons of an unsymmetrical olefin like propene, the charge may be considered as becoming distributed throughout the resultant carbonium ion by an adjustment of bond polarities, causing in effect an electron flow toward the adding proton. The partial charges on carbon and hydrogen in propene are -0.040 and 0.020 electron. In the carbonium ion they would be 0.055 and 0.119. The charge of 0.881 contributed to the proton would come 0.293 from the CH_2 side of the double bond and 0.586 from the CH_3CH side.

In order that a cation become finally attached by a covalent bond to one of the originally unsaturated carbon atoms, one of the electrons for the bond must be removed entirely from the other carbon. This removal quite logically can occur more readily from the carbon that has the greater reservoir of charge. As is indicated by the afore-mentioned relative charge contributions, this carbon is the center one of propene. The proton, accordingly, becomes permanently attached to the more hydrogenated carbon of the double bond. This leaves the central carbon with an unoccupied orbital, a ready acceptor of an electron pair of the chloride ion. Markownikoff addition to other unsymmetrical olefins may be explained similarly.

The opposite mode of addition may occur when a strongly electron-withdrawing group is attached to the side of the double bond that otherwise would be expected to serve better as an electron reservoir. The inductive effect of this group makes the removal of an electron from the unsaturated carbon nearer it more difficult. Hence, the cation becomes attached to this carbon, and the anion adds farther from the electronegative group. For example, in the α,β-unsaturated acid, CH_2=CHCOOH, each hydrogen bears a charge of 0.102 and each carbon, 0.042. Addition of a proton to the pi electrons would result in a carbonium ion in which a contribution of 0.803 electron would be made to the adding proton. The electron-withholding action of the oxygen prevents the central carbon from contributing more than 0.278, leaving 0.524 to be contributed by the CH_2 group. Accordingly, the proton here becomes attached to the less hydrogenated center carbon, and the adding anion joins the unsaturated carbon farther from the carboxyl group.

On the other hand, an electron-releasing atom such as silicon has the effect in an olefin that the adding cation becomes attached to the carbon farther from this atom. This occurs, for example, in hydrogen halide addition to $(CH_3)_3Si$—CH_2—CH=CH_2 (*6*).

Major exceptions to addition as explained in the foregoing paragraphs result when an atom attached directly to an olefinic carbon bears a relatively high partial charge. Then the electrostatic effect on the pi electrons appears dominant. One illustrative example is addition to vinyl chloride, CH_2=CHCl. Electrons should be more available from the CH_2 than from the CHCl. Explained on this basis, an adding cation should become attached to the chlorinated carbon. However, the partial charge of -0.238 on the chlorine appears sufficient to repel the pi electrons, making them actually more available at the opposite carbon. The double bond is thus permanently polarized, and it is the anion that becomes attached to the chlorinated carbon. An opposite illustration is the example of HCl addition to $(CH_3)_3Si$—CH=CH_2. Despite the electron-releasing action of the silicon, the addition is non-Markownikoff (*7*) and the proton becomes attached to carbon next to silicon. Evidently the dominant effect is the attraction of the pi electrons by the silicon, with its partial charge of 0.273.

None of the afore-mentioned factors is likely to be completely dominant except in extreme instances. The explanations offered are therefore intended to account for principal trends only.

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Antiparasitic Activity of Substituted Carbanilide Complexes

As part of an investigation of the therapeutic potentialities of aryl ureas, we have observed that molecular complexes of certain substituted carbanilides possess antiparasitic activity. In particular, several of these complexes have shown significant activity in avian coccidiosis. The complex (I) of 4,4′-dinitro-