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An Application of Electrophoresis to the Identification of Biological Activity with Characteristic Particles[1]

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By means of electrophoresis, five independent parameters of the infectious entity of Southern bean mosaic virus have been evaluated and have been shown to be identical with the comparable parameters for the characteristic particle of Southern bean mosaic virus. Four of these parameters were determined from the relationship between electrophoretic mobility and *p*H at 0.02 ionic strength. They can be expressed as four selected mobility values covering the mobility range from about 4×10^{-5} to about -6×10^{-5} cm.^{2}/volt sec. The standard error for the coincidences is about $\pm 0.1 \times 10^{-5}$ mobility unit. The isoelectric point in phosphate buffer at 0.10 ionic strength constitutes the fifth coincidence. It occurs at *p*H 5.10 and the standard error of the coincidence is about ± 0.06 *p*H unit.

Introduction

It has been emphasized recently[2] that conventional physico-chemical criteria of purity, when applied to preparations exhibiting biological activity, are not sufficient to prove that the single component so demonstrated in the "purified" preparation is the bearer of the biological activity, since the activity may be carried by another component present in amounts undetectable by these methods. It follows, therefore, that, until substantial evidence to the contrary is provided, such a preparation must be considered to contain both a "biological entity," which is the bearer of the biological activity, and an associated "physical entity" or characteristic particle, for which chemical and physical properties are directly demonstrable. In order to decide whether the biological entity and the physical entity are identical, methods must be devised to determine chemical and physical properties of the biological entity.[2] If these properties are found to coincide with those of the physical entity, such coincidences constitute evidence favoring the assumption that the two entities are identical. As the number of such coincidences is increased, the probability that such a series could occur by chance is reduced to the point where the assumption that the biological entity is a trace contaminant is excluded beyond reasonable doubt. It must be pointed out that success in such a series of investigations can only show that the characteristic particle is very probably the bearer of the biological activity and not that it is the smallest active unit.

The presence of the biological entity can be shown only by demonstrating its biological activity. It follows that the determination of its properties requires fractionation of the parent mixture followed by activity measurements on the resulting fractions. An understanding of the mechanism used for fractionation, together with the results of the activity measurements, allows the deduction of properties of the entity. Such determinations may lead to the evaluation of parameters defining a physical model or to empirical constants. The acceptance of an empirical coincidence as valid evidence favoring identity of the entities implies a sufficient understanding of the system employed to guarantee that it is independent of those coincidences already obtained. One case where coincidence does not occur is, of course, sufficient to prove lack of identity.

Previous studies in this Laboratory[3] have demonstrated the use of the ultracentrifuge in the identification of the infectious entity of Southern bean mosaic virus with its characteristic particle. The method employed has the advantage that the precision of the determination of the sedimentation rate of the infectious entity depends primarily upon the physical measurement of the position of the infectious entity boundary obtained in such experiments. Infectivity measurements were required only to demonstrate the presence or absence of infectivity in the fractions.

Electrophoresis presents a similar situation, and, therefore, allows the determination of electrophoretic properties in a similar manner, the precision of the determination likewise depending on the precision of a physical measurement and not on the precision of the biological measurement. The investigation here described was undertaken to determine the use that can be made of electrophoresis in the identification of a biological entity with its characteristic particle. Southern bean mosaic virus was chosen as the vehicle for the investigation and thus incidentally additional evidence was obtained for the identity of the physical and biological entities of this virus.

Theory

The determination of the electrophoretic properties of biological entities can be achieved by performing a conventional electrophoresis determination by the moving boundary method and then taking samples from definite positions in the electrophoresis cells. These samples are then tested for the presence or lack of activity. These data then serve to define bounds on the mobility of the biological entity. The use of indicator particles as suggested by Epstein and Lauffer[3] to prevent convection effects is desirable. In the special case considered here, the identification of an infectious entity with its characteristic particle, the characteristic particle itself can be used as the indicator particle. If, upon completion of the electrophoresis run, samples are taken just above each of the characteristic particle boundaries, and if no significant activity is found in either sample, an upper bound of mobility is established by the sample

(1) Condensed from a thesis submitted by Richard E. Hartman to the Graduate School of the University of Pittsburgh in partial fulfillment of the requirements for the Ph.D. degree.

(2) M. A. Lauffer, *Sci. Monthly*, **75**, 79 (1952).

(3) H. T. Epstein and M. A. Lauffer, *Arch. Biochem. Biophys.*, **36**, 371 (1952).

from above the ascending boundary and a lower bound is established by the sample from above the descending boundary. Together these results serve to show identity of the entities within the experimental limits. This is a decided advantage over the ultracentrifugal procedure, for, in that case, the characteristic particle can serve to establish only a lower bound on the sedimentation constant, other materials being required as indicator particles to establish upper bounds.

The electrophoretic mobility can be represented by the general expression[4]

$$v = \sigma/\eta\,(1/\kappa + r_i)\chi \qquad (1)$$

where σ is the charge density, η the viscosity, χ the function of the size and shape of the particles and of the properties of the suspending medium, κ the Debye–Hückel constant, and r_i the mean radius of ions in the ion atmosphere.

The charge density, σ, is dependent on the chemical properties of the entities and their interactions with the suspending medium; the shape factor, χ, depends on the size and shape of the entities and the nature of the ionic atmosphere surrounding them. It is therefore convenient to consider them separately.

Solutions for the shape factor, χ, have been obtained by Henry, for a spherical particle[5,6] and by Gorin, for a long cylinder.[7] Both solutions require that the Debye–Hückel approximations hold for the suspending medium and that the protein concentrations be sufficiently small so that they have insignificant influence on the Debye–Hückel constant. Gorin's solution is also limited by the condition, $\kappa l \gg 1$, where κ is the Debye–Hückel constant, and l the length of the cylinder.

Miller and Price[8] have shown that the characteristic particle is a sphere having a radius of 15.6 mμ. The ratio of the volume to the friction factor can therefore be computed for the characteristic particle, the friction factor of a sphere being defined by Stokes' law. From the ultracentrifugal coincidence previously demonstrated,[3] it then follows that this is also the ratio of volume to friction factor for the infectious entity. The dimensions of a prolate ellipsoid of revolution having the same ratio of volume to friction factor and any assumed axial ratio can be computed using the Herzog equation.[9] If it is then assumed that the equivalent ellipsoid can be approximated by a cylinder having length and diameter equal to the major and minor axes of the ellipsoid, values for χ can be computed using Gorin's solution. The results of these computations are given in Table I. Inspection of these values shows that, under widely different assumptions regarding size and shape of the entities, the values of the functions, χ, for a particular ionic strength s, are approximately equal. It must therefore be concluded that, on the basis of present understanding of electrophoretic mechanisms, coincidences found in studies primarily concerned with variations of ionic strength would be of doubtful significance and, therefore, that such studies are not indicated.

TABLE I

VALUES FOR χ FOR VARIOUS EQUIVALENT CYLINDERS

ba	s = 0.1	0.05	0.01	0.005	0.001	0.0005
1	0.823	0.775	0.656	0.583	0.440	0.235
10	.90	.81	.64	.58	.45	.29
100	.82	.74	.59	.54	.42	.26
1000	.78	.70	.57	.51	.37	.22

Changes in charge density, σ, arise as a result of the interaction of the ampholytes with the ions in the supporting medium. The most extensively studied of these reactions is the effect of hydrogen ion concentration on the net charge. The fact that the relationship between the electrophoretic mobility and the pH in well-defined buffer systems is characteristic of the ampholyte studied has been well established.[10] This relationship can be made independent of the effects of size and shape by conducting the studies under conditions such that the function χ remains constant. This can be achieved by using buffer systems at constant ionic strength and at concentrations for which the Debye–Hückel approximations hold. If coincidence of mobilities is found in all cases in such a study, the number of independent coincidences defined is equal to the number of parameters required to define the empirical pH *vs.* mobility relationship.

The effect on mobility of charging due to the interaction of ions other than hydrogen ions with the ampholytes is more difficult to separate from the shape factor, since, in general, these other ions contribute materially to the ionic strength of the supporting medium. However, the change in isoelectric point produced by varying the ionic strength of a given buffer system should be essentially independent of the shape factor. Smith[11] has shown with microelectrophoresis of ovalbumin adsorbed on collodion that this function is essentially linear for ionic strengths between 0.02 and 0.10 for a variety of buffers. Davis and Cohn,[12] in a study using moving boundary methods with carboxy hemoglobin in citrate and phosphate buffers, obtained similar results. Although their data depart somewhat from linearity, the first degree regression coefficients describe the data reasonably well within the limits 0.02 to 0.10 ionic strength. By generalizing from these observations, it follows that additional coincidences can be obtained by determining the slope of the isoelectric point *vs.* ionic strength relationship for various buffer systems. It is certain that such studies will soon reach a point of diminishing returns as the number of systems studied is increased, the independence of the additional coincidences becoming more and more unlikely.

Methods and Materials

The Virus Preparation.—The stock virus preparation was prepared from infected Bountiful bush bean plants, which

(4) H. A. Abramson, L. S. Moyer and M. H. Gorin, "Electrophoresis of Proteins and the Chemistry of Cell Surfaces," Reinhold Publishing Corp., New York, N. Y., 1942.

(5) D. C. Henry, *Proc. Roy. Soc. London*, **A133**, 106 (1931).

(6) M. H. Gorin, *J. Phys. Chem.*, **45**, 371 (1941).

(7) M. H. Gorin and L. S. Moyer, *J. Gen. Physiol.*, **25**, 785 (1942).

(8) G. L. Miller and W. C. Price, *Arch. Biochem.*, **10**, 467 (1946).

(9) R. D. Herzog, R. Illig and H. Z. Kudar *Z. physik. Chem.*, **A167**, 329 (1933).

(10) H. A. Abramson, *et al.*, ref. 4, chapter 1.

(11) E. R. B. Smith, *J. Biol. Chem.*, **113**, 473 (1936).

(12) B. D. Davis and E. J. Cohn, THIS JOURNAL, **61**, 2092 (1939).

had been ground and frozen at the time of harvesting. The final suspension of virus was a pool which contained material from nine different crops harvested in the period 1946–1951. The preparation was purified by the centrifugation procedure of Price[13] followed by the electrophoresis procedure of Lauffer and Price.[14]

Electrophoresis Procedure.—Samples having a protein concentration of approximately 0.5% were dialyzed for at least 24 hours in the rapid dialyzer with two changes of buffer. Subsequent conductivity measurements showed no significant difference between sample and dialyzate for any of the samples. Conductivity measurements were made at 0°. The water-bath was maintained at 2.5°, and the mobility values were corrected to 0°.[15] The *p*H determinations were made at room temperature. In order to conserve the sample, the *p*H measurements were made on the dialyzate.

The electrophoretic mobility of the characteristic particle was determined by conventional procedures in an Aminco–Stern electrophoresis apparatus. Two runs were made simultaneously. The field strength used was never greater than 10 v./cm.

Upon completion of the mobility determination, back compensation was started, using an electrolytic compensator.[16] The rate of compensation was such as to maintain the position of the boundary constant. During this portion of the run the cylindrical lens of the Philpot–Svensson optical system was removed and a knife edge was inserted at a point just below the undeviated beam. This made it possible to follow the boundaries in both limbs of the electrophoresis cell simultaneously and also to detect any disturbance in an early stage. The electrode vessels used were not designed for exceptionally long runs and there were occasional disturbances which were presumed to be due to the migration of KCl into the observation cell. If any disturbance occurred, the run was immediately terminated. Although an absolute mobility determination under such circumstances would be questionable, it seemed reasonable to assume that the aforementioned method of determining the coincidence, or lack of coincidence, of the mobilities would still be valid provided the boundary formed by the characteristic particles remained undisturbed. If no disturbance occurred, the runs were terminated in 10–12 hours.

Upon termination of the electrophoresis, four 0.1-ml. samples were taken, one several mm. above and one several mm. below each of the characteristic particle boundaries, just in the regions where the refractive index gradients approach zero. The samples were removed with a long, sterile syringe needle attached by a length of capillary tubing to a rubber bulb which was collapsed by a screw clamp. This assembly was attached to a microscope frame with which the needle could be slowly racked into position in the cell while following the simple knife-edge pattern to determine both the position of the needle and to guarantee that the boundaries were not disturbed. The sample was slowly withdrawn by loosening the clamp on the rubber bulb. The volume of the needle was great enough so that the sample was contained within the needle, thus avoiding contamination of the sampling device. The volume of the sample so obtained was approximately 0.1 ml.

Infectivity Measurements.—The 0.1-ml. samples taken from the electrophoresis runs were diluted to 1 ml. with phosphate buffer of 0.1 ionic strength and *p*H 7.0. Each sample was then used to inoculate 18 primary leaves of ten-day old Kentucky Wonder bean plants using a method described by Price.[17] Lesions were counted four to five days later. This method, although not as precise as the half-leaf method,[18] met adequately the requirements of this problem.

Experimental Results

Variation of Electrophoretic Mobility with *p*H.—A study of the variation of the electrophoretic mobility with *p*H was made for both the infectious entity and the characteristic particle using phosphate and acetate buffers of 0.02 ionic strength. The *p*H was limited to the range over which the infectious entity is stable,[17] *i.e.*, *p*H 4.0–8.0.

The resulting values of the mobility of the characteristic particle are given in Table II and in Fig. 1. These results are in excellent agreement with those reported by Miller, Eitelman and Golder.[19]

TABLE II

THE MOBILITY OF SBM VIRUS CHARACTERISTIC PARTICLE IN VARIOUS BUFFERS

*p*H	Mobility, cm.²/v. sec. × 10⁵	Buffer[a]	Ionic strength
7.77	−5.98	P	0.02
7.30	−5.01	P	.02
6.80	−3.34	P	.02
6.39	−1.84	P	.02
5.90	−0.02	P	.02
5.66	0.56	P	.02
6.28	−1.05	A	.02
5.68	0.90	A	.02
5.00	2.88	A	.02
4.50	3.54	A	.02
4.03	4.19	A	.02

[a] P = phosphate; A = acetate.

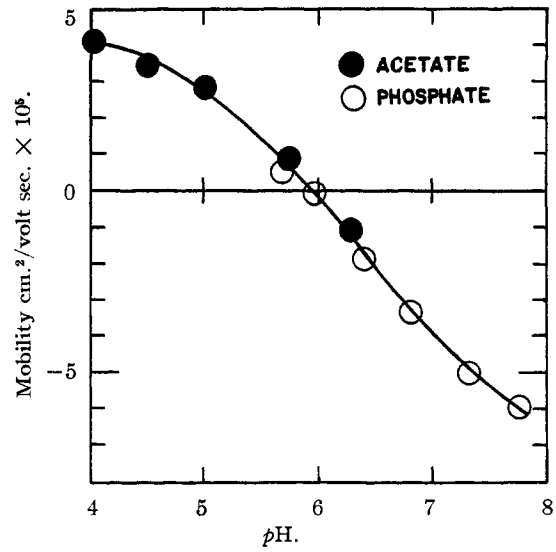

Fig. 1.—Electrophoretic mobility at 0.02 ionic strength of Southern bean mosaic virus as a function of *p*H.

In all cases the infectivity determinations showed coincidence of the mobilities of the infectious entity and the characteristic particle. Coincidence was assumed to have been demonstrated if the number of lesions obtained from the sample taken from below the characteristic particle boundary exceeded those produced by the sample taken from above the boundary by a factor of at least five. In the actual experiments, the median ratio was 26; in a third of the cases it exceeded 100, and in only a fifth was it less than 10.

A complication arose with all determinations made for *p*H values below the isoelectric point; in these cases the boundaries were resolved, after prolonged electrophoresis, into two components. These samples were extremely turbid and most of the protein precipitated after long standing. During the runs, the faster peak showed no turbidity and the slower peak was extremely turbid. At the conclusion of one such run, nine equally-spaced samples, taken from the region in each limb of the cell containing the boundaries, were analyzed for infectivity. In each case the first sample was removed just above the upper boundary and the ninth just below the lower boundary. For the ascending boundary the interval between samples was 0.25 cm.;

(13) W. C. Price, *Am. J. Bot.*, **33**, 45 (1946).
(14) M. A. Lauffer and W. C. Price, *Arch. Biochem.*, **15**, 115 (1947).
(15) L. G. Longsworth, *Chem. Revs.*, **30**, 323 (1942).
(16) P. Johnson and E. M. Shooter, *Science*, **109**, 39 (1949).
(17) W. C. Price, *Arch. Biochem.*, **8**, 13 (1945).
(18) W. C. Price, *Phytopathology*, **38**, 213 (1948).
(19) G. L. Miller, E. S. Eitelman and R. H. Golder, *Arch. Biochem. Biophys.*, **34**, 162 (1951).

for the descending, 0.36 cm. The results are given in Table III. From these data it is obvious that both components were infectious. The results also demonstrate the adequacy of the sampling procedure. The observations described above are in qualitative agreement with the hypothesis that the double peaks resulted from aggregation of a portion of the material. In all cases, the difference in mobility of the two components was about 6%. It then follows that, if only one peak represented the characteristic particle, the mobility determined from the unresolved peak was in error by about 3%. Since it is not possible to obtain an unequivocal answer to this question, it seemed most reasonable to report this average value.

TABLE III

INFECTIVITY OF SAMPLES WITHDRAWN FROM ELECTROPHORESIS CELL AT VARIOUS LEVELS IN THE REGION OF THE ASCENDING AND OF THE DESCENDING BOUNDARIES

Ascending boundary sample no.	Average no. of lesions per leaf	Descending boundary sample no.	Average no. of lesions per leaf
1	0.06	1	0.4
2	1.3	2	12
3	11	3	36
4	37	4	48
5	43	5	82
6	70	6	85
7	64	7	86
8	67	8	49
9	115	9	116

Variation of Mobility with Ionic Strength.—Two determinations were made in 0.10 ionic strength in phosphate buffers. The *p*H values were selected so that the two runs determined the isoelectric point in that buffer. The results are given in Table IV. The isoelectric point was then found, by extrapolation, to be 5.10. The infectivity tests showed coincidence of mobilities.

TABLE IV

DETERMINATION OF THE ISOELECTRIC POINT OF SBM VIRUS IN PHOSPHATE BUFFER AT 0.10 IONIC STRENGTH

*p*H	Mobility, cm.²/v. sec. × 10⁵	*p*I
6.23	−2.00	5.10
5.29	−0.28	

Discussion

The data from the *p*H *vs.* mobility study for the characteristic particle were fitted to a cubic regression line by the method of least squares.[20] The resulting equation is

$$\bar{v} = -36.085 + 23.732(p\mathrm{H}) - 4.3404(p\mathrm{H})^2 + 0.22942(p\mathrm{H})^3 \quad (2)$$

In this equation, v means the derived mobility in (cm.²)/volt sec. × 10⁵. This equation is strictly empirical in the sense that it is only an accurate description of the data within the experimental range. The equation cannot be extrapolated to predict mobility values below *p*H 4.03 or above *p*H 7.77, and it has no direct significance in relation to the possible origin of the charges responsible for electrophoretic mobility.

Since the mobility of the infectious entity agreed with the mobility of the characteristic particle at each of the eleven experimentally determined points, the regression equation for the infectious entity is obviously the same as that for the characteristic particle. Since equation 2 has four arbitrary constants, the eleven experimental coincidences between the mobility of the infectious entity and of the characteristic particle really describe only four definitive coincidences; *i.e.*, it would be sufficient to state that at four specific *p*H values in the range from *p*H 4.03 to 7.77 the mobilities of the characteristic particle and of the infectious entity have identical values. The precision of these coincidences can be estimated in the following manner.

In each experiment the distance from the center of the characteristic particle boundary to the point of sampling for the infectivity determination was measured. From these data a mobility increment can be calculated by use of equation 3.

$$\Delta v = d/tF \quad (3)$$

In this equation Δv is the mobility increment, d the distance from the center of the characteristic particle boundary to the point of sampling, t the duration of experiment in seconds, and F the field strength. The average of such values calculated for the data represented in Fig. 1 was found to be 0.3×10^{-5} cm.²/volt sec. This means that each of the eleven infectious entity mobilities agreed with the eleven characteristic particle mobilities within $\pm 0.3 \times 10^{-5}$ cm.²/volt sec. Thus, it is reasonable to regard this figure as twice the standard error of the sampling procedure. Since the 11 points represent only four definitive coincidences, each definitive coincidence is a sort of mean of 11/4 experimental values. Therefore, the standard error of each of the four definitive coincidences should be approximately $0.3/2 \div \sqrt{11/4}$ or approximately 0.1 mobility unit (× 10⁵). This would mean that the data have established that the electrophoretic mobility of the infectious entity does not differ from that of the characteristic particle at each of the four definitive points of coincidence by more than $\pm 0.2 \times 10^{-5}$ cm.²/volt sec. The total range of the mobility determinations in this experiment was 10×10^{-5}. Thus, if the total range of mobility values is divided into 25 equal units, it is virtually certain that at each of the four definitive coincidences the mobility of the characteristic particle and of the infectious entity are in the same category.

Since the isoelectric point is probably approximately a linear function of the ionic strength, isoelectric point values at two ionic strengths will serve to define this relationship. Since the isoelectric point at ionic strength 0.02 is already defined by the four definitive mobility values already discussed, the studies at other ionic strengths provided only one additional definitive coincidence between the mobilities of the infectious entity and of the characteristic particle. A satisfactory way of describing this coincidence would be to specify the isoelectric point at 0.1 ionic strength, namely, *p*H 5.10. The standard error of this isoelectric point should be the standard error for mobility divided by the slope of the mobility *vs.* *p*H relationship, or $0.15/\sqrt{2} \div 1.83$, or ± 0.06 *p*H unit.

Thus, five quantitative coincidences between properties of the operationally defined infectious entity of Southern bean mosaic virus and the operationally defined characteristic particle of Southern

(20) M. G. Kendall, "The Advanced Theory of Statistics," Vol. 2, Hafner, New York, N. Y., 1951, p. 145 ff.

bean mosaic virus have been established. Four of these coincidences can be taken as the mobility values at four different *p*H values at ionic strength 0.02. The standard error of the coincidences is approximately 0.1×10^{-5} cm.^{2}/volt sec., and the range of mobilities covered in the study is approximately 10×10^{-5} cm.^{2}/volt sec. The fifth coincidence can be expressed as the isoelectric point in phosphate buffer at ionic strength 0.1. This isoelectric point occurs at a *p*H of 5.10, and the standard error of the coincidence is ± 0.06 *p*H unit. The previously reported study of Epstein and Lauffer[3] showed that two such quantitative coincidences can be obtained in sedimentation studies carried out in media with different densities. Thus, seven quantitative parameters of the infectious entity of Southern bean mosaic virus are indistinguishable from seven similar quantitative parameters for the characteristic particle. Since these parameters of the infectious entity are identical with those of the characteristic particle, one must conclude that the probability that the infectious entity is something different from the characteristic particle is indeed very low.

There has never been much real doubt that the characteristic particle of Southern bean mosaic virus was in effect identical with the infectious entity, and in that sense the present study contributes very little new information. However, there are many viruses and many other materials possessing biological activity for which sound information concerning the relationship or non-relationship between the biologically active entity and the characteristic particle is lacking. The real contribution of the present report is that it provides a method for investigating identity in such cases.

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[CONTRIBUTION FROM THE LABORATORY OF ORGANIC CHEMISTRY OF THE TOKYO INSTITUTE OF TECHNOLOGY]

On the Thermal Dissociation of Organic Compounds. V.[5-8] The Effect of the Solvent (Fatty Acids) on the Thermal Dissociation of Urea

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On heating, a urea dissociates into an isocyanic acid and ammonia. In the absence of kinetic data on the thermal dissociation of urea in fatty acid, we have measured the rate constants of this reaction in various fatty acids by noting the rate of carbon dioxide evolution. The rate of dissociation, which is first order in urea, increases as the acidity of the solvent acid increases. In a large excess of *n*-caproic acid as solvent, the rate is accelerated by the presence of less than one mole of strong acid and is retarded by more than 1.5 moles of strong acid per mole of urea. The behavior of urea and monochloroacetic acid in cumene, tetralin or anisole was also studied.

Introduction

It is known that, on heating, urea dissociates thermally into isocyanic acid and ammonia. Earlier investigators[1] obtained ammonium carbonate on heating urea in aqueous solution. They explained the result by assuming isocyanic acid and ammonia, formed as a result of dissociation of urea, further react with water to form ammonium carbonate.

Although a similar experiment[2] was made to clarify the behavior of urea in dilute acids and alkalies, the results were not analyzed from the viewpoint of modern reaction kinetics.

Later, Mohon[3] studied the behavior of urea in fatty acids, and, identifying the reaction products by their melting points, assumed that in the case where acetic acid was the solvent, the first step was the dissociation of urea into ammonia and isocyanic acid and subsequent steps were the reactions of these with acetic acid to form (1) ammonium acetate and (2) acetamide and carbon dioxide, respectively.

Recently Cherbuliez and Landolt[4] have reported that acid amides are prepared by treating carboxylic acids with urea at elevated temperatures.

The kinetics of the thermal dissociation of substituted ureas in fatty acids has been studied[5-7] and, in the case of *sym*-diphenylurea in acetic acid, for example, the mechanism shown by equations 1 to 3 was suggested.

$$C_6H_5NHCONHC_6H_5 \rightleftarrows C_6H_5NCO + H_2NC_6H_5 \quad (1)$$

$$C_6H_5NCO + CH_3COOH \rightleftarrows C_6H_5NHCOOCOCH_3 \quad (2)$$

$$C_6H_5NHCOOCOCH_3 \longrightarrow C_6H_5NHCOCH_3 + CO_2 \quad (3)$$

By analogy, the steps in the dissociation of urea in a fatty acid solvent are believed to proceed *via* equations 4 to 7.

$$H_2NCONH_2 \rightleftarrows HNCO + NH_3 \quad (4)$$

$$HNCO + RCOOH \rightleftarrows RCOOCONH_2 \quad (5)$$

$$RCOOCONH_2 \longrightarrow RCONH_2 + CO_2 \quad (6)$$

$$NH_3 + RCOOH \rightleftarrows RCOONH_4 \quad (7)$$

The present investigation was undertaken to gain a better understanding of the dissociation.

The reaction can be followed by measuring the rate of evolution of carbon dioxide (eq. 6), provided it is evolved as soon as formed. Since the reaction temperatures were near 140° it is reasonable to believe that this is true, and also that the reverse reaction is negligible under the conditions of the

(1) C. E. Fawsitt, *Z. physik. Chem.*, **41**, 601 (1902).
(2) E. A. Werner, *J. Chem. Soc.*, **113**, 84 (1918).
(3) J. Mohon, *J. Indian Chem. Soc.*, **10**, 117 (1933).
(4) E. Cherbuliez and F. Landolt, *Helv. Chim. Acta*, **29**, 1438 (1946).
(5) T. Hoshino, T. Mukaiyama and H. Hoshino, THIS JOURNAL, **74**, 3097 (1952).
(6) T. Hoshino, T. Mukaiyama and H. Hoshino, *Bull. Chem. Soc. Japan*, **25**, 392 (1952).
(7) T. Hoshino, T. Mukaiyama and H. Hoshino, *ibid.*, **25**, 396 (1952).