yielded as the main product *n*-propylbenzene and toluene. Aromatic hydrocarbons were also formed when hexahydroindan was destructively hydrogenated in the presence of copper–alumina catalyst.

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The Fractionation of γ-Globulin by Electrophoresis-convection

BY JOHN R. CANN, RAYMOND A. BROWN AND JOHN G. KIRKWOOD

Introduction

A method of fractionation of proteins in solution utilizing an electrophoretic adaptation of the principles of the Clusius column was suggested by Kirkwood[1] in 1941 and tested experimentally by Nielsen and Kirkwood[2] several years later. Recently an electrophoresis-convection apparatus of improved design has been described and successfully used to fractionate horse diptheria antitoxin pseudoglobulin.[3] Fractionation occurs in a narrow vertical convection channel between two semi-permeable membranes, connecting an upper and lower reservoir. Separation depends upon the superposition of differential horizontal electrophoretic transport of the components on vertical convective transport of the solution as a whole. The vertical convective transport is controlled by the horizontal density gradient produced by the electrophoretic migration of the proteins across the channel.

The utility of the method of electrophoresis-convection and the effectiveness of the new fractionation unit have been well illustrated by the partial separation of the bovine serum proteins reported recently by the authors.[4] These separations were accomplished by the isoelectric procedure, *i.e.*, by successively immobilizing the components at their respective isoelectric points and transporting the mobile components from the top reservoir of the apparatus. It was suggested that electrophoresis-convection should supplement the ethanol fractionation of biological tissues and fluids as carried out by Cohn, *et al.*[5] It was apparent that subfractionation of the plasma protein fractions obtained by alcohol precipitation could be accomplished by electrophoresis-convection.

The sub-fractionation of bovine γ-globulin prepared by ethanol precipitation, Fraction II of bovine plasma, is the subject of this paper. γ-Globulin was chosen because of its known heterogeneity and its immunological importance. In the case of a protein which migrates as a single boundary in an electric field but has a specified mobility distribution as revealed by reversible electrophoretic boundary spreading, fractionation is accomplished by means of a modified isoelectric procedure. In this procedure the *p*H of fractionation is so chosen as to be close to the average isoelectric point of the protein. Transport in the apparatus leads to a redistribution of the protein ions such that the fractions withdrawn from the top and bottom reservoir possess mobility distribution differing from that of the original protein. Using this procedure γ-globulin has been separated into four fractions of different mean mobilities and isoelectric points.

Experimental

Material.—The bovine γ-globulin[6] used in this investigation had a mobility of -1.73×10^{-5} cm.^{2} volt^{-1}sec.$^{-1}$ at a concentration of 1% in barbital buffer, *p*H 8.7 and ionic strength 0.1. In this buffer the γ-globulin migrated as a single peak during electrophoresis.

Electrophoretic Analysis.—The moving boundary technique of Tiselius[7] as modified by Longsworth[8] was used in the electrophoretic analysis. Mobilities were determined by electrophoresis of a 1% protein solution in barbital buffer *p*H 8.7 and ionic strength 0.1, at a field strength of 4 volts/cm. for four hours. Mobilities were calculated in accordance with the suggestions of Longsworth and MacInnes.[9]

Boundary spreading experiments were carried out on 0.5% solution of globulin and its fraction equilibrated against cacodylate buffer (0.08 *N* sodium chloride–0.02 *N* sodium cacodylate). These experiments were performed at the average isoelectric points of the proteins. The power consumption in boundary spreading experiments did not exceed 0.015 watt/cc. The refractive-index gradient curves were recorded photographically on Eastman Kodak Co. CTC plates using both the schlieren scanning technique of Longsworth and the cylindrical lens schlieren technique. In the cylindrical lens method a diagonal knife edge brought in from below the optical axis was used in the optical system. The standard deviations of the mobility distributions determined by the two methods agreed to within 5%. Since the γ-globulin fractions possessed Gaussian mobility distributions, the heterogeneity constant, *h*, is tabulated for these proteins. However, the original γ-globulin had a non-gaussian distribu-

(1) J. G. Kirkwood, *J. Chem. Phys.*, **9**, 878 (1941).

(2) L. E. Nielsen and J. G. Kirkwood, THIS JOURNAL, **68**, 181 (1946).

(3) J. R. Cann, J. G. Kirkwood, R. A. Brown and O. Plescia, *ibid.*, **71**, 1603 (1949).

(4) J. R. Cann, R. A. Brown and J. G. Kirkwood, *ibid.*, **71**, 1609 (1949).

(5) (a) E. J. Cohn, J. A. Leutscher, Jr., J. L. Oncley, S. H. Armstrong, Jr., and B. D. Davis, THIS JOURNAL, **62**, 3396 (1940); (b) E. J. Cohn, L. E. Strong, W. L. Hughes, D. J. Mulford, J. M. Ashworth, M. Melin and H. L. Taylor, *ibid.*, **68**, 459 (1946).

(6) Armour Laboratories, Armour and Company, Chicago, Illinois, kindly supplied the bovine γ-globulin, Fraction II of Bovine Plasma.

(7) A. Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).

(8) (a) L. G. Longsworth, *Chem. Rev.*, **30**, 323 (1942); (b) L. G. Longsworth, *Ind. Eng. Chem., Anal. Ed.*, **18**, 219 (1946).

(9) L. G. Longsworth and D. A. MacInnes, THIS JOURNAL, **62**, 705 (1940).

tion of mobilities; and the standard deviation of the mobility distribution, β, is reported (see appendix).

Fractionation.—The details of construction and operation of the electrophoresis-convection apparatus employed in this investigation have previously been described.[3] The fractionation cell consists of a narrow vertical channel connecting upper and lower reservoir. The channel is formed by the space between two sheets of semi-permeable membranes. The cell containing the protein solution to be fractionated was immersed in buffer solution and a homogeneous electric field applied across the channel by means of external platinum electrodes. The nominal field strength was calculated from measurements of the conductivity of the solutions and the average current density. Electrolysis products were prevented from reaching the membranes by circulation of the external buffer solution. Fractionations were carried out in a constant temperature cold room operating at 4°.[10]

Fractions withdrawn from the top and bottom reservoirs at the conclusion of the runs were centrifuged lightly or filtered, dialyzed against distilled water at 4° to remove buffer salts, and then lyophilized. The dried proteins dissolved readily to form clear and stable solutions at their isoelectric points. In a few cases it was necessary to filter off a small amount of suspended material. Solutions of the various fractions were equilibrated against barbital or cacodylate buffer and analyzed electrophoretically.

Results

The fractionation of γ-globulin has been carried through two stages. The first stage of fractionation was carried out on 100–120 ml. of a 2–3% γ-globulin solution equilibrated against phosphate buffer, *p*H 6.7 and ionic strength 0.1. This *p*H was several tenths of a *p*H unit removed from the average isoelectric point of γ-globulin. Eight runs were made in order to study the influence of field strength and duration of electrolysis upon the efficiency of fractionation.[11] The results of a representative series of runs are presented in Table I, where E is nominal field strength in volt/cm.; t is duration of run in hours; c_i is initial concentration in g. protein/100 ml.; u is mobility (sq. cm. volt^{-1} sec.$^{-1}$) at *p*H 8.7 barbital buffer; I.P. is isoelectric point in cacodylate buffer (0.08 *N* NaCl–0.02*N* Na cacodylate); h is heterogeneity constant (sq. cm. volt^{-1} sec.$^{-1}$).

With the exception of Run 1 these initial separations all yielded the same top fraction, designated as Fraction A. Fraction A had a mobility of -1.35×10^{-5} and an isoelectric point of 7.03, about 0.5 *p*H unit greater than the mean isoelectric point of γ-globulin. The heterogeneity constant determined for this fraction is 0.65×10^{-5}. This fraction corresponds roughly to the bovine γ_2-globulin of Hess and Deutsch,[12] reported to have a mobility of -1.25×10^{-5}.

The top fraction resulting from Run 1 had a mobility of -1.52×10^{-5}, which is significantly greater than that of Fraction A, and the same heterogeneity constant as Fraction A. The isoelectric point of this fraction was approximately the same as that of Fraction A. It appears that the optimum conditions for fractionation are 2.6–1.0 volts/cm. at operating times of 42–118 hours, respectively. A number of routine fractionations of γ-globulin, employing a field strength of 1.6 volts/cm. and an operating time of fourty-eight hours, have been carried out in this Laboratory. The results obtained in these runs have shown that fractionations accomplished by electrophoresis-convection are very reproducible.

The bottom fractions, Fractions B, resulting from the first stage of fractionation had mobilities ranging from -1.98 to -2.12×10^{-5}. This fraction appears to correspond to the bovine γ_1-globulin of Hess and Deutsch,[12] which has a mobility of -2.1×10^{-5}.

The second stage of fractionation was carried out on 120 ml. of about a 2% solution of a composite of Fractions B resulting from Runs 1,

TABLE I

FIRST STAGE OF FRACTIONATION OF BOVINE γ-GLOBULIN

(a) Experimental Conditions

Run	E, v./cm.	t, hr.	c_i, g./100 ml.
1	3.1	24	3.0
2	2.6	47	3.2
3	1.6	42	2.4
4	1.6	77	2.4
5	1.0	118	2.8

(b) Properties of γ-Globulin Fractions

Run	Fraction	Yield, g. protein	$-10^5 \times u$	$10^5 \times h$
1	Top		1.52	0.64
	Bottom		2.10	
2	Top	1.4	1.36	.62
	Bottom	1.6	2.07	
3	Top	1.1	1.33	.67
	Bottom	1.4	2.12	
4	Top	1.4	1.33	.66
	Bottom	1.4	2.02	
5	Top	1.3	1.38	
	Bottom	1.6	1.98	

TABLE II

SECOND STAGE OF FRACTIONATION OF BOVINE γ-GLOBULIN

(a) Experimental Conditions

E, v./cm.	t, hr.	c_i, g./100 ml.
1.6	48	2

(b) Properties of γ-Globulin Fractions

Fraction	Yield, g. protein	$-10^5 \times u$	I. P.	$10^5 \times h$
Top	1.0	1.63	6.47	0.67
Bottom	1.7	2.20	6.01	.65

(10) During two runs the temperature rose well above 4° due to refrigeration breakdown. Because of Joule heating some denaturation occurred in the channel resulting in recovery of only 75% of the protein. In all other runs denaturation was negligible. It is recommended that fractionations by electrophoresis-convection be carried out in a cold room operating at 0-4°. Depending upon the field strength and the conductance of the buffer, the operating temperature of the protein solution in the convection channel will be several degrees higher than the cold room temperature due to Joule heating.

(11) Osmotic transport of solvent into the channel from the exterior buffer solution increased the volume of protein solution during these runs. The rate of influx of solvent was found to be an increasing function of the field strength. Thus, at field strengths of 3.1-2.6 volts/cm. the rate of influx was 0.9 ml./hr., at 1.6 volts/cm., 0.4 ml./hr., and at 1.0 volt/cm. negligible.

(12) E. L. Hess and H. F. Deutsch, THIS JOURNAL, **70**, 84 (1948).

2 and 4. The protein solution was equilibrated against phosphate buffer, *p*H 6.7 and ionic strength 0.1. The data[13] are presented in Table II. The resulting top fraction, Fraction C, had a mobility of -1.63×10^{-5} and an isoelectric point of 6.47, which is very close to the mean isoelectric point of γ-globulin itself. The bottom fraction, Fraction D, had a mobility of -2.20×10^{-5} and an isoelectric point of 6.01, about 0.5 *p*H unit lower than the mean isoelectric point of γ-globulin. Both fractions were found to have the same heterogeneity constant as Fraction A.

Discussion

Fraction A represented 45% of the original γ-globulin, Fraction C 19% and Fraction D 36%. These fractions possessed Gaussian mobility distributions. The mobility distribution of the γ-globulin was not Gaussian, of course. However, as a first approximation the mobility distribution can be adequately represented as a Gaussian probability function, whose standard deviation is taken as that of the actual mobility distribution.[14] The standard deviation, β, of the actual mobility distribution was found to be 0.67×10^{-5}. The mobility distribution of each fraction at *p*H 6.5 has been normalized to an area corresponding to its weight fraction of the γ-globulin and plotted in Fig. 1. The solid curve in Fig. 1 represents the sum of the three distributions. In Fig. 2 this composite curve is compared with the Gaussian probability function which represents to a first approximation the mobility distribution of γ-globulin.[15] The mo-

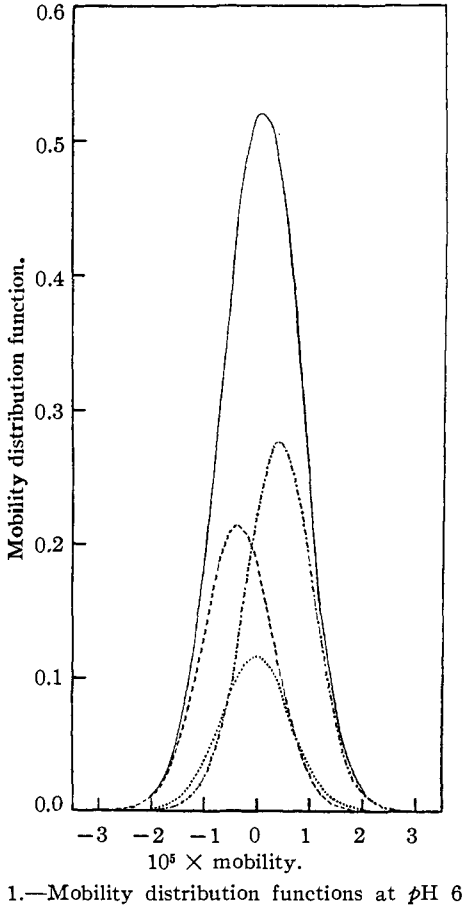

Fig. 1.—Mobility distribution functions at *p*H 6.5 in cacodylate buffer: —.—, Fraction A; . . . , Fraction C; - - - , Fraction D; ——, sum of the distributions of Fractions A, C and D.

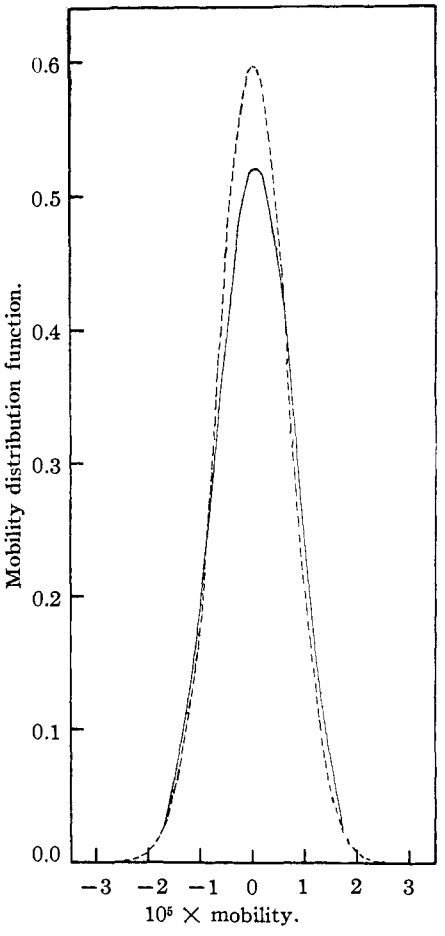

Fig. 2.—Mobility distributions at *p*H 6.5 in cacodylate buffer: - - -, bovine γ-globulin; ——, sum of the distributions of Fractions A, C and D.

(13) In this experiment the rate of osmotic transfer of solvent into the channel from the external buffer solution was only 0.1 ml./hr.

(14) A complete representation of the mobility distribution entails, of course, the determination of the third and higher moments of the refractive-index gradient curves. An attempt was made to compute the third and fourth moments of the gradient curve. However, the precision and accuracy involved in the computation did not warrant inclusion of those terms defining the departures from the Gaussian in the expression for the mobility distribution, equation 1 of the appendix.

(15) In making this comparison it is assumed that within the ranges of concentration and *p*H considered, the standard deviations of the mobility distributions of γ-globulin and its fractions are independent of protein concentration and *p*H. It is also assumed that the slopes of the mobility-*p*H curves of all the constituent proteins of a given fraction are the same and equal to the mean $\Delta u/\Delta p\mathrm{H}$ of the fraction.

bility distribution of γ-globulin is normalized to unit area. It will be noted that the agreement between the two curves is good.

On the basis of electrophoretic characterization the differences between Fraction C and γ-globulin are relatively small. At *p*H 8.7 the mobility of Fraction C was found to be -1.63×10^{-5} and that of γ-globulin -1.73×10^{-5}. The two proteins have approximately the same isoelectric points in cacodylate buffer. The chief difference between the two proteins is that Fraction C has a gaussian and γ-globulin a non-gaussian distribution of mobilities. Fraction C appears to be a center cut of γ-globulin resulting from the separation of two fractions, A and D, possessing mobility distributions the centroidal axes of which are situated on either side of the centroidal axis of the mobility distribution of γ-globulin.

The theory of transport in an electrophoresis-convection channel, to be published elsewhere, predicts that the fractionation of a protein possessing a gaussian mobility distribution, with specified first and second moments, will result in top fractions which also possess gaussian mobility distributions. Furthermore, the second moments of the mobility distributions of the top fractions will be the same as that of the original protein. The first moments will, of course, be different than that of the original protein. The theory also predicts that transport in the apparatus will be stopped by attainment of a stationary state when the first moment of the mobility distribution of the material in the top reservoir vanishes. Both of these predictions have been approximately realized in the experiments reported in this paper.

The results of the investigation presented in this paper illustrate the ease with which a heterogeneous protein can be fractionated by electrophoresis-convection. The large quantities of material that can be fractionated in a relatively short time and the reproducibility of the fractionations promise to make electrophoresis-convection a valuable tool in the sub-fractionation of the plasma fractions obtained by ethanol precipitation.

Employing a procedure similar to the one used in this investigation it should be possible to further separate Fractions A, C and D into subfractions by electrophoresis-convection. Such a program is now in progress in this Laboratory. Preliminary results of the sub-fractionation of Fraction A are very encouraging.

Appendix

The electrophoretic inhomogeneity of γ-globulin and its fractions is evidenced by reversible electrophoretic boundary spreading. The rate of spreading of an electrically homogeneous protein boundary under conditions such that convection and anomalous electrical effects are avoided should be no greater than that due to diffusion alone. However, in the case of an inhomogeneous protein the refractive-index gradient curve is spread simultaneously by diffusion and by the differences in the mobilities of the constituent protein ions. For the case in which diffusion is not negligible, Alberty, *et al.*,[16] have developed a method for the determination of the electrophoretic mobility distribution of a heterogeneous protein having a gaussian mobility distribution. In this case the apparent diffusion constant calculated from the gradient curves is a linear function of the time of electrophoresis; and a heterogeneity constant, h, may be calculated from the slope. h is the standard deviation of the mobility distribution.

A general theory of reversible boundary spreading applicable to proteins possessing either gaussian or non-gaussian mobility distributions has been developed recently in this Laboratory. It is a result of this theory, which will be published elsewhere, that the mobility distribution, $q(u)$, can be expressed in terms of the moments of the gradient curve taken about the centroidal axis by means of an infinite series, equation 1. D is the diffusion constant

$$q(u) = (1/\beta\sqrt{2\pi})e^{-u^2/2\beta^2}\left\{1 + \sum_{j=3}^{\infty} \frac{C_j}{j!}(-i)^j\alpha^j H_j(iu\sigma/Et_E\beta^2\alpha)\right\}$$
$$\beta^2 = (\sigma^2 - \sigma_0^2 - 2Dt_E)/E^2t_E^2 \qquad (1)$$
$$\alpha = \sqrt{1 - 2(\sigma/\beta Et_E)^2}$$

and E the electric field strength. σ_0^2 and σ^2 are the second moments of the gradient curves at the moment of application and at time t_E after application of the electric field. β is the standard deviation of the mobility distribution. H_j is the j-th Hermite polynomial. The coefficients C_j are related to the higher moments of the gradient curve, *e.g.*

$$C_3 = \overline{x^3}/\sigma^3, \; C_4 = \overline{x^4}/\sigma^4 - 3$$

If the gradient curves are gaussian in form, *i.e.*, the mobility distribution is gaussian, β is identical with the heterogeneity constant h. Deviations from a gaussian distribution of mobilities are given by the third and higher moments of the gradient curves. For proteins possessing either gaussian or non-gaussian mobility distributions, β may be calculated from the equation

$$D^* = (\sigma^2 - \sigma_0^2)/2t_E = D + (E^2\beta^2/2)t_E \qquad (2)$$

D^* is the "apparent diffusion constant" calculated from the second moments of the gradient curves during electrophoresis. A plot of D^* *vs.* t_E is a straight line which extrapolates back to the normal diffusion constant at zero time. β may be calculated from the slope $E^2\beta^2/2$.

Summary

The separation of bovine γ-globulin into four fractions of different mobilities and isoelectric points has been accomplished by electrophoresis-

(16) (a) R. A. Alberty, E. A. Anderson and J. W. Williams, *J. Phys. Colloid Chem.*, **52**, 217 (1948); (b) R. A. Alberty, THIS JOURNAL, **70**, 1675 (1948); (c) E. A. Anderson and R. A. Alberty, *J. Phys. Colloid Chem.*, **52**, 1345 (1948).

convection. These fractions were found to have gaussian mobility distributions, which, when properly normalized and added together, yield a mobility distribution in agreement with that possessed by γ-globulin itself.

This investigation demonstrates the applicability of electrophoresis-convection to the subfractionation of the plasma fractions obtained by ethanol precipitation.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF WISCONSIN]

The Kinetics of the Decomposition of Malonic Acid in Aqueous Solution

BY GEORGE A. HALL, JR.

Recent studies of the thermal decomposition of trichloroacetic and trinitrobenzoic acids, as well as of salts of these acids, in water and in mixed solvents,[1] have led to a reasonable understanding of the factors which affect the rate of decarboxylation for those acids studied. In order to see how the theory proposed as a result of these investigations might apply to other acids, particularly dicarboxylic acids, it seemed desirable to study the similar reaction of malonic acid. Kinetic studies on the decarboxylation of dilute aqueous solutions of malonic acid were first carried out by Bernoulli and Wege[2] who reported that the reaction was of first order for temperatures around 100° and for concentrations in the neighborhood of 0.2 *N*. Fairclough[3] reported studies on the two sodium salts and found that, while disodium malonate was thermally stable up to 125° sodium acid malonate decomposed by a first order reaction. He suggested that, since the rate constants for the acid malonate ion were less than those reported for the acid by Bernoulli and Wege, the mechanism of the decomposition of malonic acid differed from that of the monocarboxylic acids where it had been shown[1] that the rate determining step is the decomposition of the unassociated acid anion.

The rate of decomposition of dibromomalonic acid as a function of *p*H was studied by Muus[4] who found that the rate was proportional to the concentration of the acid dibromomalonic ion.

This paper reports a study of the effect of changing concentration and of changing *p*H on the rate of decomposition of malonic acid and the results obtained confirm Fairclough's suggestion that the mechanism is not the same as in the case of the monocarboxylic acids previously studied.

Experimental

Materials.—Malonic acid obtained from the Eastman Kodak Company was dissolved in a minimum amount of methyl alcohol and recrystallized in a Dry Ice–acetone-bath. The product gave a neutral equivalent of 52.1 (52.02 calcd.). The water used as a solvent was doubly distilled and was of conductivity grade. Solutions of *p*H outside the range obtainable with pure malonic acid were prepared by using as solvents either standard hydrochloric acid solutions or standard sodium hydroxide solutions of appropriate strengths.

Procedure.—The reaction was followed by the previously described procedure[1c] of preparing a series of identical samples of the desired concentration and removing them from the constant-temperature bath at appropriate times. Each sample was analyzed by bubbling air through it for several minutes to remove the carbon dioxide and then titrating with standard sodium hydroxide solution. Since during the course of the reaction the dibasic malonic acid is changed to the monobasic acetic acid the amount of malonic acid present at any time is proportional to the quantity $(v_t - {}^1/_2 v_0)$ where v_t is the volume of standard base used at time t and v_0 is the volume used at zero time. For those solutions containing hydrochloric acid a correction for the amount of additional acid was applied and the solutions containing sodium hydroxide were corrected for the amount of sodium acid malonate present. The *p*H measurements were made on samples of the solutions used for the reaction velocity measurements using a Beckman glass electrode *p*H meter at 25°.

Results

The rate of decomposition of malonic acid was investigated at temperatures of 80 and 90° for a series of concentrations of malonic acid ranging from 0.005 to 0.05 *M*. The results obtained are given in Table I. In addition to the range of *p*H available from the dissociation of the acid a series of measurements were carried out in which hydrochloric acid or sodium hydroxide was added to the solution giving a total *p*H range of 0.4 to 4.89. Table II gives the data for the decomposition of malonic acid in the presence of hydrochloric acid and sodium hydroxide.

It was found that while each experiment gave a good first order rate constant, the values of these constants increase regularly with an increase in malonic acid concentration. With a decrease in *p*H beyond the range available in pure malonic

(1) (a) Verhoek, THIS JOURNAL, **56**, 571 (1934); (b) Verhoek, *ibid.*, **61**, 186 (1939); (c) Trivich and Verhoek, *ibid.*, **65**, 1919 (1943); (d) Verhoek, *ibid.*, **67**, 1062 (1945); (e) Hall and Verhoek, *ibid.*, **69**, 613 (1947); (f) Cochran and Verhoek, *ibid.*, **69**, 2897 (1947).

(2) Bernoulli and Wege, *Helv. Chim. Acta*, **2**, 511 (1919).

(3) Fairclough, *J. Chem. Soc.*, 1186 (1938).

(4) Muus, *J. Phys. Chem.*, **39**, 343 (1935).