Studies on Phosphatidylcholine Vesicles. Formation and Physical Characteristics*

Ching-hsien Huang†

ABSTRACT: A method is described for the separation of phosphatidylcholine vesicles, formed by prolonged ultrasonic irradiation under nitrogen in 0.1 M buffered NaCl solution, by molecular sieve chromatography on large-pore agarose gels. One fraction of the separated vesicles was found to be homogeneous by the criteria of gel filtration, sedimentation velocity ultracentrifugation, and electron microscopy. Experimental data which support the homogeneous vesicle as a shell-like structure, about 250 Å in diameter, with a continuous phosphatidylcholine bilayer surrounding a volume of solvent are presented. The following physical parameters were established for the homogeneous vesicles: $s_{20,w}$, 2.1 S; $D_{20,w}$, 1.87×10^{-7} cm² sec^{-1}; $\bar{v}$, 0.9885 ml g^{-1}; [η], 0.041 dl g^{-1}; and vesicle weight, 2.1×10^6.

The study of experimental model membranes has proven to be of considerable value in understanding some of the relations between structure and function exhibited by biological membranes. The examination of simple models has not only provided insight into the relations between the basic parameters of the biological system, but has also, in some cases, influenced the direction of experimental work on biological membranes. In recent years two types of model systems have been developed both of which are comprised of the lipid component of natural membranes, and in addition, have transverse dimensions similar to that of biological systems (Mueller *et al.*, 1962; Huang *et al.*, 1964; Hanai *et al.*, 1964; Bangham *et al.*, 1965a,b; van den Berg, 1965; Liberman, 1965; Läuger *et al.*, 1967). The orientation of the lipid component in both of the models is the bimolecular lamellar arrangement suggested by Davson and Danielli (1952) to be the structural form of the lipid component of biological membranes. The physical properties of one model system, the phospholipid bilayer membrane separating two aqueous phases, have proven to be strikingly similar to the corresponding properties of natural membranes (Huang and Thompson, 1965, 1966). The other model system, consisting of an aqueous dispersion of liquid crystals of phospholipid, has been shown to have permeability properties similar to those of biological membranes (Bangham *et al.*, 1965a,b) and to be particularly well adapted for study of interactions between phospholipid lamellae and proteins in aqueous system (Litman and Thompson, 1967).

The basic question to which studies of the physical properties of the model membrane systems are addressed is the relation between the structure of the lipid component in natural membranes and certain of the properties of these biological systems. In order to answer this question it is necessary that the structures and compositions as well as the properties of the models be determined. In the case of the liquid crystal dispersion, although the compositions of the lamellar phase are readily determined and its structure known, it has been difficult if not impossible to relate the biologically relevant properties of this model to the surface area of the lipid lamellae. This is due to the fact that in all systems of this type the liquid crystal dispersions are heterogeneous collections of multiconcentric, lamellar structures which vary greatly in size and shape.

The purpose of the present communication is to describe the preparation, properties, and structure of an aqueous liquid crystal dispersion in which the liquid crystals are spherical vesicles, remarkably homogeneous in size and surrounded by a single lamella of bilayer dimension. These structures are formed from purified egg phosphatidylcholine by prolonged sonication under nitrogen in 0.1 M buffered NaCl solution followed by molecular sieve chromatography on large-pore agarose gels. Aqueous phospholipid dispersions, which in certain respects are similar to the system described in this paper, have been described by Green and Fleisher (1964) and Saunders *et al.* (1962).

Experimental Section

Formation of Phosphatidylcholine Vesicles. The phosphatidylcholine was isolated from chicken egg yolks by the alumina column method (Singleton *et al.*, 1965). To remove small amounts of the lysophosphatide, sphingomyelin, and free fatty acid, the preparation was chromatographed on silicic acid (Rouser *et al.*, 1963). The purified phosphatidylcholine was dissolved in twice recrystallized benzene and was then lyophilized

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† Research Fellow of the Helen Hay Whitney Foundation (1966–1969).

for 6 hr. The product obtained from these procedures showed a single component on thin-layer chromatography (Skipski *et al.*, 1962).

To prepare the lipid dispersion, lyophilized phosphatidylcholine was suspended in buffer at a concentration of approximately 3%. The suspension was then ultrasonically irradiated with a 20-kc Branson Sonifier (Model S-125) at power level 6, under a nitrogen atmosphere, for 160 min in a jacketed vessel maintained at 2° by a constant-temperature circulating bath. The buffer solution used throughout this work was NaCl (0.1 M) in Tris-HCl (0.01 M), adjusted to pH 8.5.

After removal of an undispersed phospholipid by centrifugation at 105,000*g* for 60 min at 4°, the clear dispersion was collected and filtered through an extensively washed 0.1-μ Sartorius filter (type MF 4). The amount of lipid lost in passage through the membrane filter, as determined in P_i content (Gomori, 1942) between the solution before and after the filtration, was less than 10%. The filtrate was then subjected to molecular sieve chromatography on a Sepharose 4B column (2.5 × 50 cm) at 4°. Since the Sepharose 4B column was found to contract slightly after application of the dispersion, and in addition not all the phospholipid was recovered in the effluent, it was apparent that some adsorption of the phosphatidylcholine to the Sepharose gel particles occurred. To eliminate absorptive effects, the column was first saturated with the lipid dispersion and then washed and equilibrated with the buffered NaCl solution. The recovery of phosphatidylcholine in the effluent of this lipid-treated column proved to be satisfactory (~93%), and similarly prepared columns were used for the separation and subfractionation of all phosphatidylcholine dispersions. The absorption of column effluent at 300 mμ was continuously recorded by a spectrophotometer (Bausch & Lomb Spectronic 600) equipped with flow cell and chart recorder. The effluent solution, collected in a refrigerated fraction collector, had a concentration of phospholipid directly proportional to the recorded absorbance. The isolated fractions were concentrated by placing the solution in a Sartorius collodion bag under vacuum. The concentrated solution was then equilibrated with fresh buffer by dialysis overnight at 4°. Prior to studies of the physical properties, the dialysate and the dispersion were filtered once again through a 0.1-μ Sartorius filter. All filters and collodion bags were exhaustively washed with buffer solution before use.

Phosphatidylcholine concentrations were calculated from the inorganic phosphate content (Gomori, 1942) or from dry weight determinations. With the latter method, aliquots of the solution and dialysate were placed in separate weighing bottles. Both were dried at 110° over silica gel for 6 hr, left under vacuum for 1 hr, and then weighed again. The difference between these weights was used to calculate the phosphatidylcholine concentration.

Sedimentation Velocity Measurements. Sedimentation velocity studies were carried out at 20 ± 0.05° in a Spinco Model E ultracentrifuge, equipped with a RTIC temperature-control unit and a schlieren optical system fitted with a phase plate. Kodak metallographic plates were used to photograph the schlieren patterns which were then read on a two-dimensional microcomparator (Leitz). A double-sector, capillary-type synthetic boundary cell with a 12-mm optical path was used in all experiments.

In early studies, two rotational speeds were used. The velocity was first maintained at 42,040 rpm for about 1 hr and was then increased to 59,780 rpm. Sedimentation coefficients obtained at these two speeds were compared and no appreciable differences were found. Subsequently, 42,040 rpm was used in all experiments. Since the schlieren patterns were highly symmetrical, sedimentation coefficients were calculated from plots of time *vs.* log *r* determined from measurements of the position of the maximum ordinate (Schachman, 1957). Sedimentation coefficients were reduced to values in water at 20°, $s_{20,w}$, using densities and viscosities determined for the dialysates and the partial specific volume of the vesicles.

The heterogeneity parameter, p, was calculated for the dispersions with the following equation (Fugita, 1962)

$$\frac{\sigma^2(r_0/r)}{2t} = D + \left(\frac{p^2\omega^4 r_0}{2}\right)(rt) \qquad (1)$$

where r is the distance from the center of rotation to the point of the maximum ordinate on the moving-boundary gradient curve, r_0 is the distance at time zero, ω is the angular velocity of the rotor, and σ^2 is the square of the second moment of the gradient curve.

Diffusion Measurements. Diffusion studies were carried out in the analytical ultracentrifuge under conditions identical with those employed for the sedimentation velocity studies, except that the experiments were performed at a lower speed of 10,580 rpm for 3–4 hr. At this speed the boundary did not move appreciably. The apparent diffusion coefficient, D_{app}, was calculated by the maximum ordinate–area method from the following relationship (Ehrenberg, 1957).

$$D_{app} = \frac{1}{4\pi t}\left(\frac{A}{kH_{max}}\right)^2 \qquad (2)$$

where A is the area under the gradient curve as measured on the photographic plate at time t, H_{max} is the maximum height of that curve, and k is the magnification factor along the radial coordinate.

Area measurements were made with a planimeter on enlarged tracings of the schlieren patterns. The maximum height measurements were also made from the same tracings. Apparent diffusion coefficients obtained by this method were reduced to values in water at 20°, $D_{20,w}$ (Svensson and Thompson, 1961).

Determination of Partial Specific Volume. Partial specific volumes were calculated from density measurements made in a 10-ml calibrated pycnometer. All measurements were performed in duplicate at 20 ± 0.05°. The partial specific volume, $\bar{v}$, was calculated from

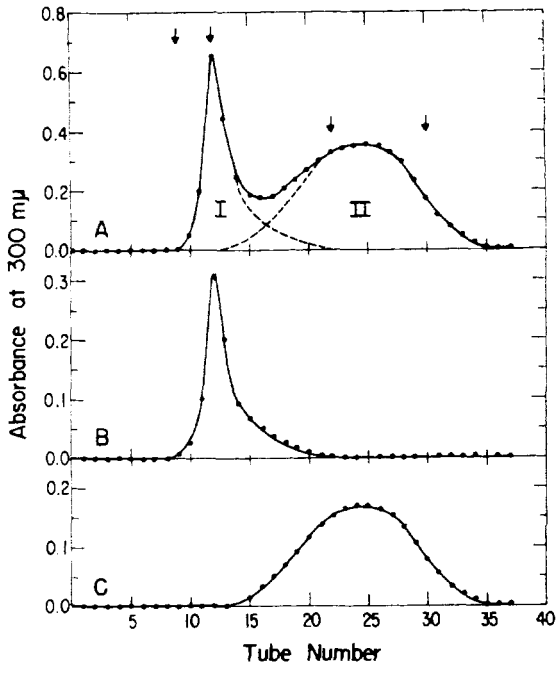

FIGURE 1: Elution patterns of phosphatidylcholine. (A) Dispersion obtained from ultrasonic irradiation. Phosphatidylcholine dispersion (8 ml of 3%) was applied to a 2.5 × 50 cm lipid-treated Sepharose 4B column. (B) Vesicles collected and concentrated from fraction I of part A as indicated by the first two arrows. (C) Vesicles collected and concentrated from fraction II of part A as indicated by the last twoar rows.

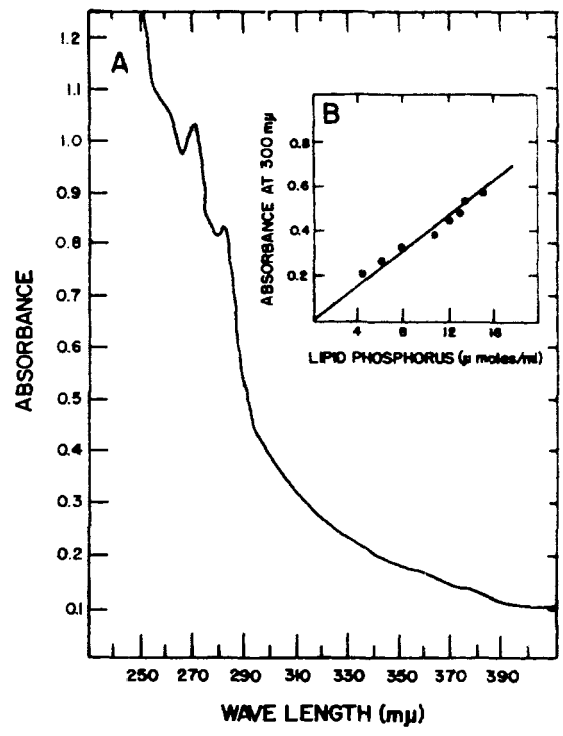

FIGURE 2: Studies of fraction II. (A) Absorption spectrum of fraction II phosphatidylcholine vesicles. (B) The scattering (absorption at 300 mμ) of fraction II phosphatidylcholine vesicles *vs.* concentration.

density data using the following relationship (Cassassa and Eisenberg, 1961)

$$\bar{v} = \frac{1}{\rho_0}\left[1 - \frac{100(\rho - \rho_0)}{c}\right] \quad (3)$$

where ρ_0 is the density of the dialysate and ρ is the density of the phosphatidylcholine vesicle solution at concentration c in grams per deciliter. The partial specific volume of one phosphatidylcholine dispersion was determined by Dr. D. W. Kupke using a magnetic densitometer (Ulrich *et al.*, 1964).

Viscosity Measurements. Cannon-Ubbelhode semimicro dilution viscometers with a shear rate of 1500 sec^{-1} and a water flow time of 300 sec were used in most experiments. Reduced viscosities obtained at concentrations of 0.4, 1.0, and 1.4 g/dl using viscometers with shear rates of 500, 1000, and 1500 sec^{-1} were identical, indicating that deviations from Newtonian flow were negligible. Flow times determined with an electric timer were reproducible to 0.05% in all cases. Measurements were carried out at 20 ± 0.01°.

The intrinsic viscosity, $[\eta]$, was obtained by extrapolating the reduced viscosity, $\eta_{sp/c}$, to infinite dilution according to the following equation (Huggins, 1942)

$$\frac{\eta_{sp}}{c} = [\eta] + K[\eta]^2 c \quad (4)$$

where η_{sp} is the specific viscosity, c the concentration in grams per deciliter, and K the Huggins' constant.

Analytical Molecular Sieve Chromatography. The partition coefficient of the phosphatidylcholine dispersions was determined on a Sepharose 4B column with a scanning optical system (Brumbaugh and Ackers, 1968). The Stokes' radius, a, of the vesicles was calculated from the partition coefficient according to the following equation (Ackers, 1967)

$$a = a_0 + b_0 erfc^{-1}\epsilon \quad (5)$$

where $erfc^{-1}\epsilon$ is the inverse error function complement of partition coefficient ϵ of the system. $erfc^{-1}\epsilon$ can be determined from ϵ following the procedures of Ackers (1967). In these experiments, tobacco mosaic virus and glycylglycine were used to determine the void and total column volumes, respectively. Turnip yellow mosaic (Stokes' radius = 140 Å) and γ-globulin (Stokes' radius = 52 Å) were used to determine the two column constants a_0 and b_0.

Electron Microscopy. Liquid crystal dispersions were diluted with a 2% solution of potassium phosphotungstate adjusted to pH 6.5. A drop of the mixture was placed on a Formvar–carbon-coated grid and excess solution was drained off. After drying, the grid was examined immediately in a Siemens Elmiskop IA electron microscope, operated at 80 kV.

"Freeze-etching" experiments were carried out following the procedures of Steere (1957).

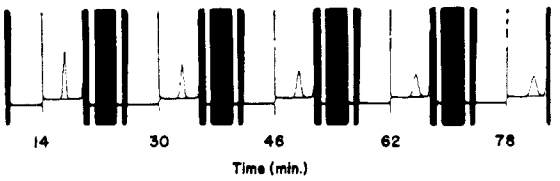

FIGURE 3: Ultracentrifugal schlieren pattern of a sedimentation velocity experiment on the phosphatidylcholine vesicle solution. Time in minutes after rotor attained a speed of 42,040 rpm. The phase plate angle was 70° throughout. Initial concentration 0.98 g/dl.

Results

Sonication and Gel Filtration. Figure 1A shows a typical elution pattern of a phosphatidylcholine dispersion on a Sepharose 4B column. Fraction I was eluted with the void volume of the column and consisted of particles totally excluded from the internal volume of the gel. Fraction II, which distributed with the internal volume of the column, eluted as a broad, symmetrical peak. Rechromatography of either fraction resulted in a single peak eluting at a volume corresponding quantitatively to its position in the original chromatogram (Figure 1B,C). Thus there appeared to be no equilibration between these two fractions on the column. The relative amounts of the two fractions depended upon the duration of ultrasonic irradiation. In general, longer periods of ultrasonic irradiation resulted in a relatively larger proportion of fraction II. However, under no conditions was fraction I completely absent after sonication. It is the physiochemical properties of fraction II that are of primary interest in this communication.

The development of absorption maxima in the 265–285-mμ range after sonication (Figure 2A) indicates the formation of conjugated "triene" chromophores as a result of partial oxidation of the unsaturated fatty acid moieties of the phosphatidylcholine (Holman and Burr, 1946). The amount of "triene" chromophore relative to total phospholipid in fraction II vesicles can be estimated to be less than 0.3%, using the absorbancy value of C_{16}-triene reported by Stoffel and Ahrens (1958).

Sedimentation velocity experiments were performed at concentrations of fraction II vesicles ranging from 0.3 to 1.1 g/dl. The schlieren patterns illustrated in Figure 3 were the results of an experiment in which the initial concentration was 0.98 g/dl. The sedimentation coefficient decreased with increasing vesicle concentration (Figure 4). The concentration dependence calculated by a least-squares procedure is given by the expression $s_{20,w} = s^0_{20,w}(1 - 0.164c)$, where c is the phosphatidylcholine vesicle concentration in grams per deciliter and $s^0_{20,w}$ is 2.10×10^{-13} sec, the value of the sedimentation coefficient extrapolated to infinite dilution. The slope of $s_{20,w}$ plotted against c was negative over the concentration range studied, thus excluding the existence of association–dissociation equilibria (Schachman, 1959). The sedimentation coefficient of fraction II vesicles was unchanged after storage for 1 week at 4°.

Homogeneity. Application of eq 1 to the centrifuge data obtained from fraction II lead to a value for p, the heterogeniety parameter, less than 0.5×10^{-14} sec. A plot of $\sigma^2 r_0/2rt$ *vs.* rt is presented in Figure 5. It is apparent from the linear, horizontal plot that this fraction is essentially homogeneous. In contrast to this result, the unfractionated dispersion gave a value of $p = \pm 5 \times 10^{-14}$ sec.

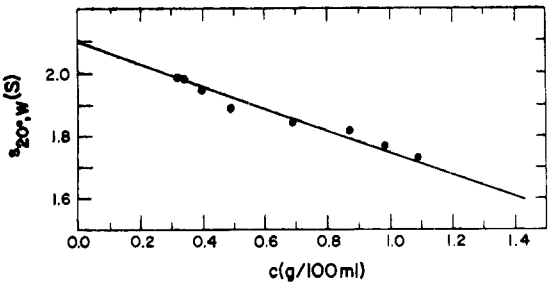

FIGURE 4: Plot of sedimentation coefficient, $s_{20,w}$, of the phosphatidylcholine vesicle *vs.* concentration.

The essential homogeneity of fraction II was further demonstrated by electron microscopic studies. As shown in Figure 6A,C, the size distribution of the spherical phosphatidylcholine vesicles is very narrow with an average diameter of either 300 ± 30 Å determined from negatively stained material or 250 ± 20 Å by the freeze-etching method. Difference in dimension between negatively stained and freeze-etched vesicles is to be expected because of the different procedures involved. Each method, however, demonstrates the homogeneity of phosphatidylcholine vesicles. For comparison, an electron micrograph of fraction I is presented in Figure 6B. It is quite apparent that the vesicles in fraction I are very heterogeneous with respect to both size and shape. The general appearance of fraction I is similar to the dispersion studied by Bangham *et al.* (1965a,b).

Diffusion. All the schlieren patterns obtained in diffusion experiments on fraction II were symmetrical. The experimental results are summarized in Figure 7. Diffusion coefficients are seen to be slightly concentration dependent with the data best fitted by least squares to the expression: $D_{20,w} = D^0_{20,w}(1 - 0.024c)$, where $D^0_{20,w}$, the apparent diffusion coefficient extrapolated to infinite dilution, was found to be $1.87 \pm 0.04 \times 10^{-7}$ cm² sec⁻¹. $D^0_{20,w} = 3.20 \times 10^{-7}$ cm² sec⁻¹ was obtained for the unfractionated dispersion. This high value of $D^0_{20,w}$ is due to a heterogeneity in s and is consistent with the

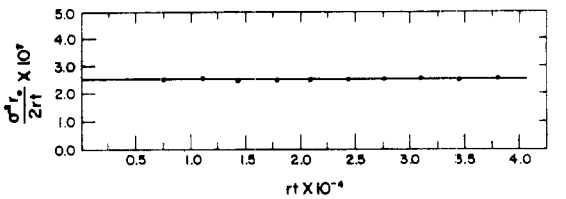

FIGURE 5: Analysis of boundary spreading during sedimentation velocity centrifugation of phosphatidylcholine vesicles. For practical reason, the zero time in this experiment was taken as the time at which full speed (42,040 rpm) had been reached.

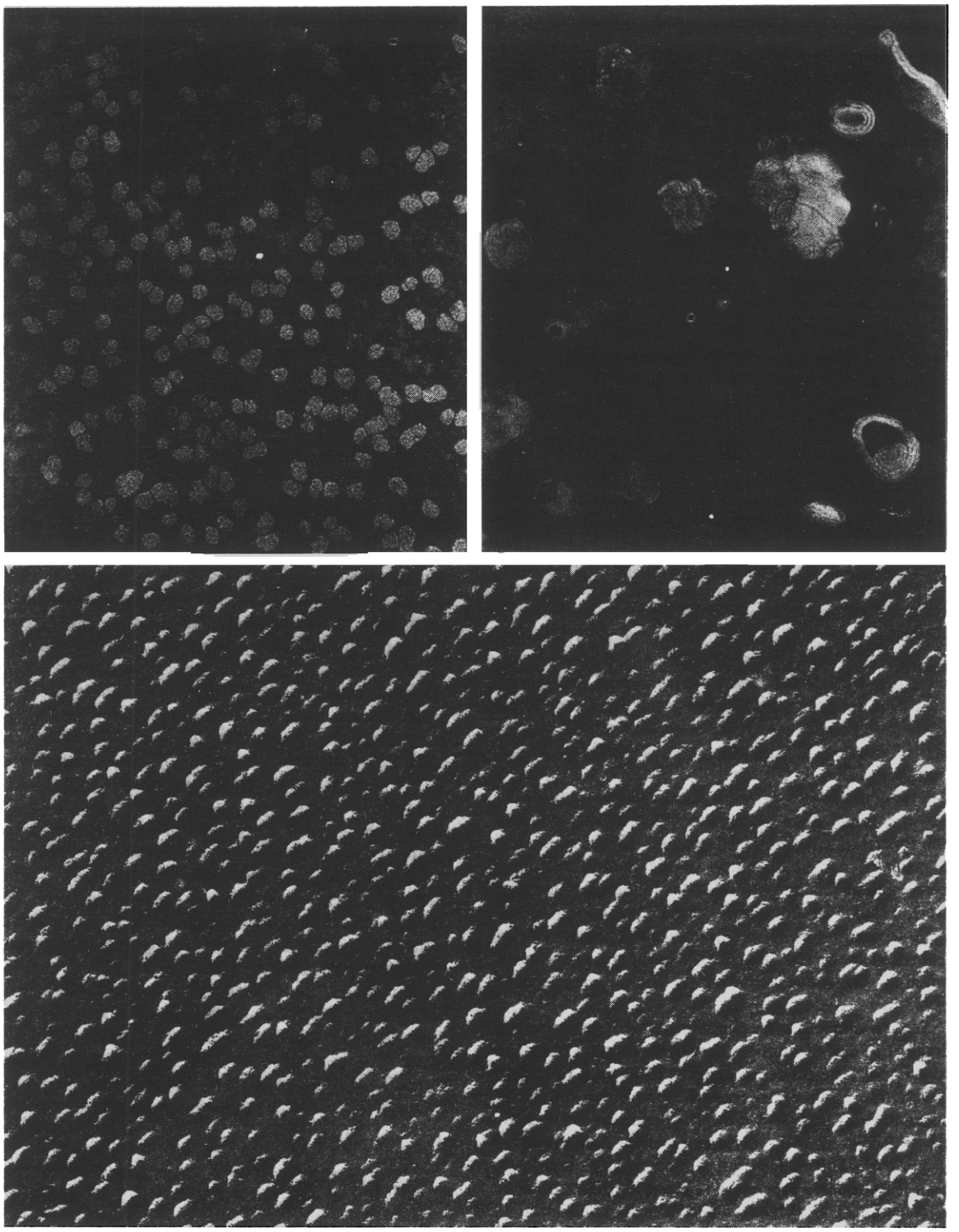

FIGURE 6: Electron micrograph studies. (A) Of fraction II phosphatidylcholine vesicles negatively stained with potassium phosphotungstate. × 100,000. (B) Of fraction I phosphatidylcholine vesicles negatively stained with potassium phosphotungstate. × 100,000. (C) Of a platinum-shadowed carbon replica of fraction II phosphatidylcholine vesicles prepared by the freeze-etching technique. × 100,000.

value of p (eq 1) calculated from data obtained from this unfractionated material. If it is assumed that the Stokes' law ($f = 6\pi\eta a$) is applicable to these vesicles, then the Stokes' radius, a, can be calculated from the experimentally determined diffusion coefficient by the Einstein relation ($a = RT/N6\pi\eta D$). The diameter of the sphere calculated by this method is 228 ± 5 Å, in excellent agreement with the value of 250 ± 20 Å es-

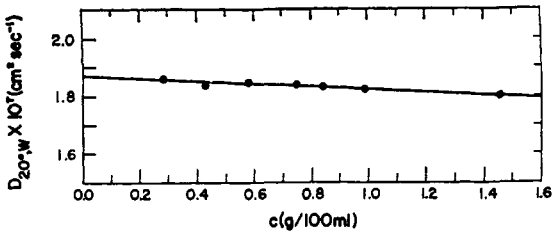

FIGURE 7: Plot of diffusion coefficient, $D_{20,w}$, of the phosphatidylcholine vesicle *vs.* concentration.

timated from the electron micrograph of freeze-etched material.

Partial Specific Volume. The difference in density between dispersion and solvent was very small ($<2 \times 10^{-4}$). As a result, the over-all precision in determining $\bar{v}$ was considerably lower than that obtained in the other physical measurements. The partial specific volume of fraction II vesicles was found to be 0.986 ± 0.003 and 0.9885 ± 0.0005 ml/g as measured by the pycnometer and magnetic densitometer method, respectively. In subsequent calculation, a value of 0.9885 was taken as the partial specific volume of the phosphatidylcholine vesicles.

Viscosity. Figure 8 shows the reduced viscosity and specific viscosity of the vesicles as a function of concentration. The intrinsic viscosity, $[\eta]$, obtained by extrapolating the reduced viscosity to infinite dilution, was found to be 0.041 dl/g. Some information can be obtained about the hydration and shape of the phosphatidylcholine vesicles in solution from the following relation (Tanford and Buzzell, 1956)

$$100[\eta] = \nu(\bar{v} + \delta_1 v_1^0) \qquad (6)$$

where $\bar{v}$ is the partial specific volume, v_1^0 is the specific volume of pure solvent, δ_1 is the number of grams of solvent incorporated in the hydrodynamic particle per gram of dry phosphatidylcholine, and ν is the Einstein–Simha constant, equal to 2.5 for spheres, and increasing for more asymmetric shapes. If the homogeneous vesicles are taken to be spherical on the basis of electron microscopy (Figure 6A,C), then the maximum amount of hydration for the vesicle can thus be calculated from eq 6 to be 0.64 g/g of dry phosphatidylcholine.

Analytical Molecular Sieve Chromatography. The partition coefficients of phosphatidylcholine vesicles, turnip yellow mosaic virus (TYMV), and γ-globulin, calculated from the experimentally determined slopes of plots of elution volume *vs.* peak position, are shown in Table I. The column calibration constants a_0 and b_0 in eq 5 were determined to be −10 and 270, respectively, from the calculated ϵ and the known radii of γ-globulin and TYMV. An average value of 140 ± 5 Å can thus be obtained by eq 5 as the Stokes' radius of the phosphatidylcholine vesicle.

In order to test the sensitivity of the molecular sieve chromatography method in determining particle sizes of the dimensions of the phosphatidylcholine vesicles, southern bean mosaic virus (286 Å in diameter, Klug and Casper, 1960) and tomato bushy stunt virus (309 Å

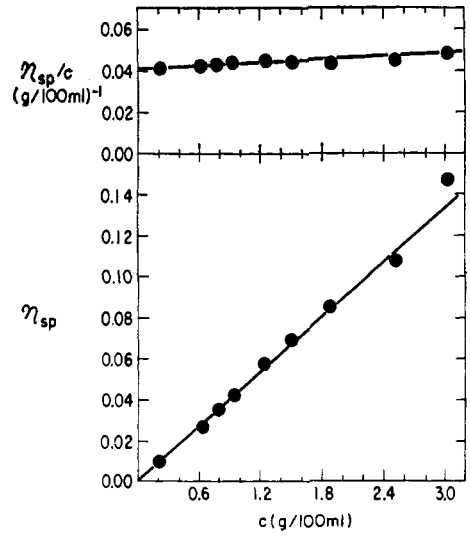

FIGURE 8: Dependence of the viscosity of the phosphatidylcholine vesicle upon concentration at 20°. Top: Reduced viscosity, η_{sp}/c, plotted against the phosphatidylcholine vesicle concentration. Bottom: Specific viscosity, η_{sp}, plotted against the phosphatidylcholine vesicle concentration.

in diameter, Klug and Casper, 1960) were run through the column. It was found that the partition coefficients in Sepharose 4B gels for phosphatidylcholine vesicles and these small viruses could be directly compared from the slope of a plot of elution volume *vs.* peak position as shown in Figure 9. It was thus demonstrated that the Sepharose 4B column was indeed sensitive enough to differentiate between two particles in this size range differing in diameter by 23 Å.

The value of 280 ± 10 Å obtained as the diameter of the phosphatidylcholine vesicle from molecular sieve chromatography must be regarded as an upper limit value for two reasons. First, the partition coefficient of the vesicle in Sepharose 4B gel was determined at a single concentration (1 g/dl). Since the partition coefficient, in analogy to the sedimentation coefficient, is concentration dependent, results obtained at higher concentration give a value of Stokes' radius larger than the value corresponding to infinite dilution. Secondly, the partition coefficient for the phosphatidylcholine vesicle could be underestimated due to the fact that the column had been pretreated with phosphatidylcholine

TABLE I: Partition Coefficients for the Phosphatidylcholine Vesicle and Calibrated Molecules in Sepharose 4B.[a]

Molecules	ϵ	$erfc^{-1}\epsilon$	a (Å)
γ-Globulin	0.805	0.22	52
TYMV	0.440	0.55	140
Phosphatidylcholine vesicle	0.429	0.56	

[a] See Experimental Section for experimental details.

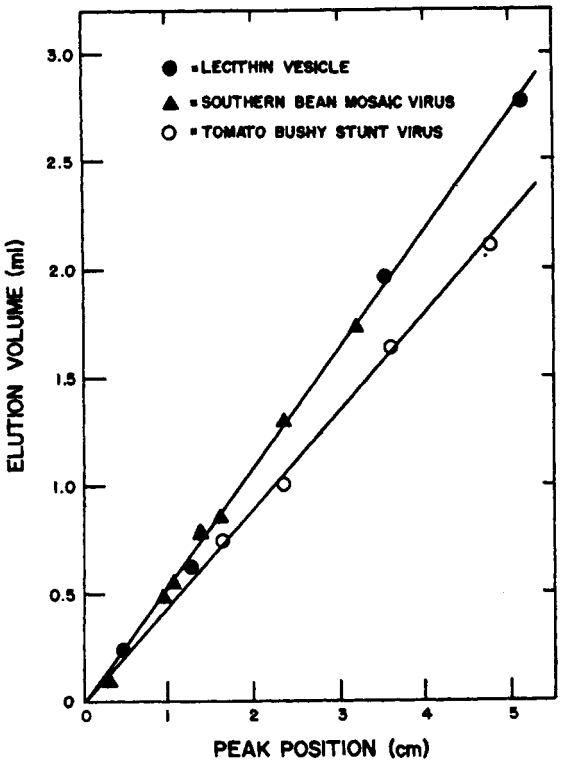

FIGURE 9: Comparison of partition coefficients for phosphatidylcholine vesicles and small viruses in Sepharose 4B gel. The peak position (centimeters) of the sample within the column, corresponding to its elution volume (milliliters), was measured by a scanning optical system along the column (0.935 × 6 cm).

vesicles so that the newly introduced vesicles were distributed to a lesser extent throughout the interior of the gel.

Vesicle Weight. The apparent weight-average vesicle weight can be calculated from the Svedberg equation (Svedberg and Pederson, 1940) by combining the experimentally measured sedimentation and diffusion coefficients of the phosphatidylcholine vesicles. This method gives an apparent weight-average vesicle weight of $2.06 \pm 0.05 \times 10^6$.

The vesicle weight, M, of phosphatidylcholine in dispersion can also be estimated from the β function of Scheraga and Mandelkern (1953) by combining the intrinsic viscosity with the sedimentation coefficient together with the partial specific volume, $\beta = Ns[\eta]^{1/3}\eta_0/M^{1/3}(1 - \bar{v}\rho)$, where N is Avogadro's number (6.02×10^{23}). If the reasonable assumption is made that vesicles are effectively spherical in solution, then 2.12×10^6 can be taken as the value of β. The vesicle weight estimated from the β function using the sedimentation coefficient, intrinsic viscosity, and partial specific volume is 1.94×10^6.

Discussion

From X-ray studies, it is known that only one liquid crystalline phase, the lamellar phase, occurs in the water–egg phosphatidylcholine system within the temperature range of 5 to 35° (Luzzati *et al.*, 1966; Reiss-Husson, 1967). From electron microscopy studies, a continuous bimolecular lamella of about 50–60 Å in thickness can be seen if the phosphatidylcholine vesicles are formed in the presence of ammonium molybdate (Thompson and Henn, 1969). Recent work of Staehelin (1968) further indicates that in the phosphatidylcholine–water system the lipids are arranged either in stacks of concentric lamellae separated by layers of water or in the form of various sized vesicles bound by a single or at most a few bilayer lamellae each 50–60 Å in thickness. A shell-like structure, about 250 Å in diameter, with a continuous phosphatidylcholine bilayer surrounding a volume of solvent can thus be postulated for the homogeneous phosphatidylcholine vesicle under study. Such a structure is morphologically very similar to the well-characterized synaptic vesicles of the presynaptic nerve terminals (Whittaker, 1968).

The correctness of this structure postulated for the phosphatidylcholine vesicle is further corroborated by combining the X-ray data for the phosphatidylcholine molecule and the average molecular weight of the phosphatidylcholine vesicle. The number of phosphatidylcholine molecules per vesicle can be calculated, on the average molecular weight for the egg phosphatidylcholine (770) and that of the phosphatidylcholine vesicle (2.06×10^6), to be 2678. Taking the X-ray data of 60 Å as the average surface area per hydrophilic group of phosphatidylcholine in the liquid crystalline phase and 40 Å as the bilayer thickness as determined by X-ray diffraction studies (Small, 1967), the internal radius of the spherical lamella can be calculated as follows: total surface area $= 2678 \times 60$ Å$^2 = 4\pi r_i^2 + 4\pi(r_i + 40)^2$. The internal radius, r_i, would thus be 60 Å. This value of 60 Å for the internal radius of the vesicle should be taken as the minimum since the X-ray data of 60 Å^2 is a minimum surface area per phosphatidylcholine hydrophilic group obtained by extrapolating the degree of hydration to a minimum. On the basis of this calculation, the minimum diameter of structure consisting of a single bilayer of phosphatidylcholine is 200 Å.

Considering the hydrophobicity of the hydrocarbon core of the phosphatidylcholine bilayer membrane, it is reasonable to assume that most of the incorporated water in the vesicle is inside the spherical shell. The amount of incorporated water can be calculated, for the system under study, by utilizing eq 6 which gives a value of 0.64 as the maximum number of grams of solvent incorporated in the hydrodynamic particle per gram of dry phosphatidylcholine. Employing this value, 0.64, and assuming that all the incorporated water of the phosphatidylcholine vesicle is inside the shell, the maximum radius of the spherical aqueous space inside the vesicle can be calculated to be 80 Å. Adding the maximum thickness of the phospholipid bilayer, 73 Å (Levine *et al.*, 1968), to this maximum internal aqueous radius, 306 Å can be considered as the upper limit of the diameter of the sphere. Comparing all the values on hand with their theoretical as well as experimental limitations, a shell-like structure, about 250 Å in diameter, with a continuous phosphatidylcholine bilayer sur-

rounding a volume of solvent can be taken as the most probable structure of the phosphatidylcholine vesicle under investigation.

The experiments described in the present report show that a homogeneous fraction of the phosphatidylcholine vesicles, formed by prolonged ultrasonic irradiation in 0.11 M NaCl-Tris buffer at pH 8.5, can be isolated by molecular sieve chromatography on large-pore agarose gels. The physical parameters of the preparation have been determined and all the data are summarized in Table II. This well-characterized system can thus be

TABLE II: Physical Characteristics of Phosphatidylcholine Vesicles.

Physical Parameter	Value
Intrinsic viscosity (dl g^{-1})	0.041
Partial specific volume, $\bar{v}$ (ml g^{-1})	0.9885
Diffusion coefficient, D^0_{20} (cm^2 sec^{-1} $\times 10^7$)	1.87
Sedimentation coefficient, $s^0_{20,w}$ (S)	2.10
Molecular weight (g)	2.06×10^6

quantitatively used as a model biological membrane for studies on the permeability of the ions through this defined structure. Furthermore, this homogeneous phosphatidylcholine vesicle system provides a new basis for study of the interactions between the phospholipid and membrane- or lipid-bound enzymes and proteins in a definite stoichiometric manner.

Acknowledgments

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References

Ackers, G. K. (1967), *J. Biol. Chem. 242*, 3237.
Bangham, A. D., Standish, M. M., and Watkins, J. C. (1965a), *J. Mol. Biol. 13*, 238.
Bangham, A. D., Standish, M. M., and Weissmann, G. (1965b), *J. Mol. Biol. 13*, 253.
Brumbaugh, E. E., and Ackers, G. K. (1968), *J. Biol. Chem. 243*,6317.
Cassassa, E. F., and Eisenberg, H. (1961), *J. Phys. Chem. 65*, 427.
Davson, H., and Danielli, J. F. (1952), The Permeability of Natural Membranes, 2nd ed, Cambridge, University Press, p 57.
Ehrenberg, A. (1957), *Acta Chem. Scand. 11*, 1257.
Fujita, H. (1962), Mathematical Theory of Sedimentation Analysis, New York, N. Y., Academic.
Gomori, G. (1942), *J. Lab. Clin. Med. 27*, 955.
Green, D. E., and Fleischer, S. (1964), *in* Metabolism and Physiological Significance of Lipid, Dawson, R. M. C., and Rhodes, D. N., Ed., New York, N. Y., Wiley, p 584.
Hanai, T., Haydon, D. A., and Taylor, J. (1964), *Proc. Roy. Soc. (London) A281*, 377.
Holman, R. R., and Burr, G. O. (1964), *J. Am. Chem. Soc. 68*, 562.
Huang, C., and Thompson, T. E. (1965), *J. Mol. Biol. 13*, 183.
Huang, C., and Thompson, T. E. (1966), *J. Mol. Biol. 15*, 539.
Huang, C., Wheeldon, L., and Thompson, T. E. (1964), *J. Mol. Biol. 8*, 148.
Huggins, M. L. (1942), *J. Am. Chem. Soc. 64*, 2716.
Klug, A., and Caspar, D. L. D. (1960), *Advan. Virus Res. 7*, 225.
Läuger, P., Lesslauer, W., Marti, E. and Richter, J. (1967), *Biochim. Biophys. Acta 135*, 20.
Levine, Y. K., Bailey, A. I., and Wilkins, H. M. F. (1968), *Nature 220*, 577.
Liberman, E. A. (1965), *Biophys. USSR 10*, 354.
Litman, B. J., and Thompson, T. E. (1967), *Fed. Proc. 26*, 834.
Luzzati, V., Reiss-Husson, F., Rivas, E., and Gulik-Krzywicki, T. (1966), *Ann. N. Y. Acad. Sci. 137*, 409.
Mueller, P., Rudin, D. O., Tien, H. T., and Wescott, W. C. (1962), *Nature 194*, 979.
Reiss-Husson, F. (1967), *J. Mol. Biol. 25*, 363.
Rouser, G., Kritchevsky, C., Heller, D., and Lieber, E. (1963), *J. Am. Oil Chemists' Soc. 40*, 425.
Saunders, L., Perrin, J., and Gammack, D. (1962), *J. Pharm. Pharmacol. 14*, 567.
Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, N. Y., Academic.
Schachman, R. K. (1957), *Methods Enzymol. 4*, 32.
Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.
Singleton, W. S., Gray, M. S., Brown, M. L., and White, J. L. (1965), *J. Am. Oil Chemists' Soc. 42*, 53.
Skipski, V. P., Peterson, R. F., and Barclay, M. (1962), *J. Lipid Res. 3*, 467.
Small, D. M. (1967), *J. Lipid Res. 8*, 551.
Staehelin, L. A. (1968), *J. Ultrastruct. Res. 22*, 326.
Steere, R. L. (1957), *J. Biohphys. Biochem. Cytol. 3*, 45.
Stoffel, W., and Ahrens, E. H., Jr. (1958), *J. Am. Chem. Soc. 80*, 6604.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University.
Svensson, H., and Thompson, T. E. (1961), *in* Analytical Methods of Protein Chemistry, Alexander, P., and Block, R. J., Ed., Vol. 3, New York, N. Y., Pergamon, p 58.
Tanford, C., and Buzzell, J. C. (1956), *J. Phys. Chem. 60*, 225.
Thompson, T. E., and Henn, F. A. (1969), *in* Structure and Functions of Membranes of Mitochondria and Chloroplasts, Racker, E., Ed., New York, N. Y.,

Reinhold (in press).
Ulrich, D. V., Kupke, D. W., and Beams, J. W. (1964), *Proc. Natl. Acad. Sci. U. S. 52*, 349.
van der Berg, H. J. (1965), *J. Mol. Biol. 12*, 280.
Whittaker, V. P. (1968), *Proc. Natl. Acad. Sci. U. S. 60*, 1081.

Rate of Isotope Exchange in Enzyme-Catalyzed Reactions*

Gad Yagil and Henry D. Hoberman

ABSTRACT: The methodology of treating the kinetics of isotope exchange catalyzed by enzymes is discussed. A relatively simple way of deriving the equations relating velocity at equilibrium of an enzymatic reaction with the kinetic parameters of the reaction is described. The following general relations are helpful. The equilibrium rate of n consecutive reactions is given by

$$1/V_e = \sum_1^n 1/V_i$$

The equilibrium rate of n parallel reactions is

$$V_e = \sum_1^n V_i$$

These relations also make possible the calculation of exchange rates through coupled enzymatic reactions which occur in metabolizing systems. In the experimental part the rate of tritium equilibration between position 4A of reduced nicotinamide–adenine dinucleotide and position 2 of L-lactate is measured. Series of measurements in which lactate is varied between 0.37 and 37 mM at constant pyruvate and reduced nicotinamide–adenine dinucleotide concentrations, as well as series in which pyruvate is varied between 4.3 and 86 μM at constant lactate and pyruvate concentrations, are reported. The results, in conjunction with the equations derived in the first part, make possible the evaluation of three rate constants involved in the lactate dehydrogenase reaction. These constants are summarized in Table II, and are shown to be close to the ones obtained from initial rate studies.

When a labeled substrate is added to an actively metabolizing system the label may appear in a particular product either as a result of net synthesis or because the product is in a state of dynamic equilibrium with the initially labeled metabolite. The latter process falls into the class of isotope-exchange reactions; for example, when L-[2-^{3}H]lactate is infused into an isolated perfused rat liver, tritium appears in the liver malate (Hoberman, 1965). It is important to know whether the labeling of malate results from an exchange between lactate and endogenous malate or by net formation of malate. The labeling by exchange may be attributed to coupling of the lactate and malate dehydrogenase systems (eq 1–3). In the normal rat liver these reactions appear to be in a state of equilibrium (Bücher and Klingenberg, 1958). When an isotopic compound is used to measure the

L-[2-^{3}H]lactate + NAD $\rightleftharpoons$ pyruvate + [4A-^{3}H]NADH + H$^+$ (1)

oxalacetate + [4A-^{3}H]NADH + H$^+$ $\rightleftharpoons$ L-[2-^{3}H]malate + NAD (2)

L-[2-^{3}H]lactate + oxalacetate $\rightleftharpoons$ L-[2-^{3}H]malate + pyruvate (3)

metabolic throughput of a particular process, it is essential to evaluate the contribution of the isotopic-exchange reaction to the observed rate of appearance of the labeled product. In order to evaluate this contribution it is necessary to derive and evaluate an expression for the rate of the exchange at equilibrium of each of the coupled enzymatic reactions in terms of the rate constants of the individual steps.

The kinetics of isotope-exchange reactions catalyzed by enzymes have been discussed by a number of investigators (Koshland, 1955; Boyer, 1959; Alberty *et al.*, 1962). The subject has recently been reviewed by Rose (1966) and Cleland (1967). The present communication describes a simplified way to derive the relations between the measured exchange rates at thermodynamic equilibrium and the kinetic parameters on which the rates depend. Using the derived equations three of the rate constants characterizing the reaction catalyzed by

* From the Department of Biochemistry of the Albert Einstein College of Medicine, Bronx, New York 10461 (H. D. H.), and the Department of Cell Biology of the Weizmann Institute of Science, Rehovoth, Israel (G. Y.). *Received May 28, 1968.* This work was supported by Grant GB-4559 from the National Science Foundation and by U. S. Public Health Service Grant No. CA-03641-03 from the National Cancer Institute, both to H. D. H. A preliminary account of this work was presented at the 142nd National Meeting of the American Chemical Society, Atlantic City, N. J., Sept 1962, p 23C.