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DIFFUSION STUDIES OF BILE ACIDS, FATTY ACIDS AND SUCROSE–NaCl–WATER SYSTEMS AT 37 °C BY A MODIFIED CAPILLARY CELL APPARATUS AND THEIR APPLICATION TO MEMBRANE TRANSPORT STUDIES

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SUMMARY

A capillary cell apparatus is described that allows accurate measurement of solute tracer diffusion coefficients in biological solutions at 37 °C. The apparatus has a unique stirring mechanism to provide a uniform flow pattern over the capillaries with only 18 ml of the bulk solution. Four capillaries of 2 cm length are used. With this apparatus measurement can be made at relatively short time periods so that bacterial overgrowth in the solutions is minimized. Using this apparatus tracer diffusion coefficients of three bile acids, cholic, taurocholic and taurodeoxycholic acids, and four fatty acids, acetic, pentanoic, octanoic and decanoic acids, were measured in an isotonic phosphate buffer, pH 7.1, at 37 °C. Viscosity, density and diffusion coefficients of sucrose in physiological saline solutions were also measured.

INTRODUCTION

Recent work in the field of solute transport across biological membranes has emphasized the importance of unstirred water layers as a potentially major source of resistance to absorption of molecules into cells and across epithelial surfaces. In particular, studies using intestine have shown that such unstirred layers lead to serious artifacts in determination of permeability coefficients for passively absorbed substances and Michaelis constants for actively transported molecules[1–3]. In addition, in certain circumstances this diffusion barrier, rather than the cell membrane, may become rate limiting to cell uptake so that the rate of absorption of a particular substance is related directly to its rate of diffusion across the unstirred water layer[4,5].

In view of these recent findings it is apparent that far greater cognizance must be taken of unstirred layer effects in the study of solute transport across biological membranes than has been done heretofore. Correction for unstirred layer resistance, however, requires accurate values for free diffusion coefficients for molecules of biological importance under conditions of temperature and ionic strength encountered in physiological situations. While there are many published values for free diffusion coefficients in the physical–chemical literature, these seldom involve specific sub-

stances, solvent systems or temperatures that are directly relevant to biological systems.

Thus, the purpose of the present study is 2-fold. First, a new capillary diffusion cell is described that allows determination of tracer diffusion coefficients of various molecules in physiological solutions and at physiological temperatures accurately and over relatively short periods of time. Second, using this cell, tracer diffusion coefficients are reported for three types of molecules of biological importance—fatty acids, bile acids and sucrose—in physiological buffer and saline solutions.

METHODS AND MATERIALS

The diffusiometer which contains 4 capillary diffusion cells is shown in Fig. 1. It is 11 cm long, 5 cm in diameter and sealed with an O-ring and teflon arrangement at the top. The diffusiometer is essentially based on that of Anderson and Saddington[6] but has been modified to allow a well-controlled flow of liquid over the ends of the capillaries using only 18 ml of bulk solution. Also the initial and final conditions of the experiment are defined by opening and closing the capillaries against teflon seals.

The glass capillary cells were of 0.5 mm precision bore and 7.5 mm outside diameter and were approximately 2 cm long. The actual lengths were accurately measured with a McPherson microcomparator. Both ends of each capillary were ground flat and one end was closed off with a mini-torch to form a seal normal to the inside cylinder of the capillary.

To prepare for a diffusion experiment, the inner portion of the apparatus (A and B in Figs 1 and 2) is removed and set in a ring stand. Plates A and B are

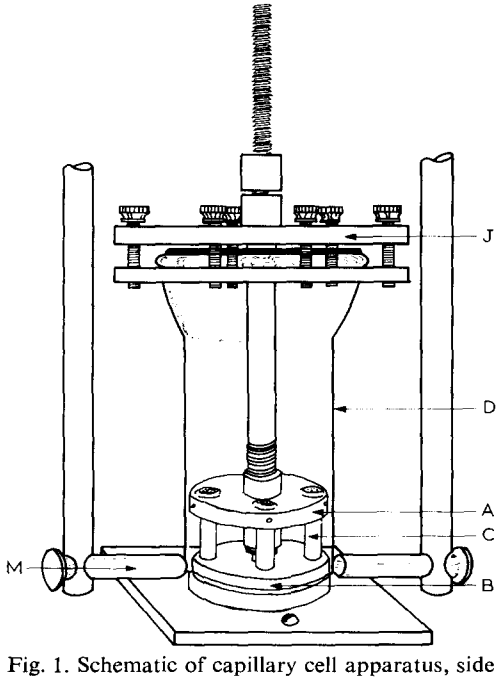

Fig. 1. Schematic of capillary cell apparatus, side view, see text.

made of teflon and are attached to two stainless steel shafts, one inside the other, such that the position of A is totally adjustable relative to the cover plate J while B is adjustable relative to A. Each capillary, C, is filled with radioactive tracer solution with a 10-μl syringe where care is taken to insure that there are no bubbles. The capillaries are clamped in holder A with the open ends set firmly on the 3.8 mm diameter flat ends of four teflon cylinders, T, embedded in plate B. 18 ml of non-radioactive bulk solution with the same chemical composition as the tracer solutions* is put into the bottom of D and the apparatus is assembled as in Fig. 1. Initially the capillaries remain sealed and suspended above the bulk solution. The top J is sealed onto D and the whole apparatus is lowered into a constant temperature bath maintained at 37 ± 0.005 °C and allowed to equilibrate for at least 20 min. After equilibration the capillaries, still sealed, are lowered until the ends are immersed 2–3 mm in the bulk solution. Diffusion begins as plate B is then separately lowered from the capillary tips and turned 45° so that each capillary is positioned between two slots F and G as in Fig. 2. During the experiment a uniform flow of bulk solution over the ends of the capillaries results when a three-pronged glass-encased stirring bar, K, in the bottom of the vessel is turned at 4.8 rev./min by a rotating external magnet, M, in Fig. 1. Solution is slowly circulated up through slots F in plate B which is now setting on ring H, across the ends of the capillaries and down through slots G. The flow rate is approx. 1 mm/s. This was measured independently by timing

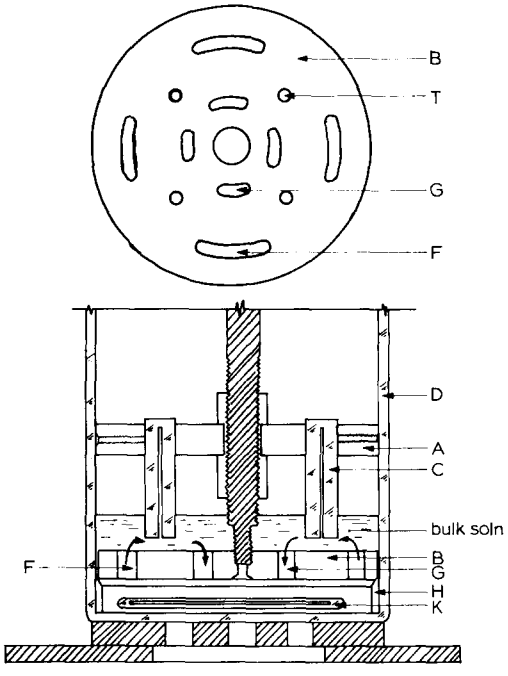

Fig. 2. Bottom teflon plate, B, vertical view, and detailed side view of apparatus emphasizing the bulk solution flow pattern, see text.

* Density stabilization is always maintained, thus the bulk solution is the same concentration as the radioactive solution or slightly denser.

the movement, across the capillary ends, of illuminated carbon tetrachloride–benzene mixture droplets adjusted to the same density as the bulk solution[7].

After sufficient time has elapsed for 40–60% of the tracer component to diffuse out of the capillaries, the experiment is terminated by again sealing the ends of the capillaries and withdrawing the inner portion of the apparatus. After carefully drying the outside of the capillaries, they are removed and their contents are then centrifuged into vials containing 12 ml of a scintillation solution containing 2,5-phenyloxazole (2.5 g/l) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (0.098 g/l) in an ethanol–toluene mixture for liquid scintillation counting.

The ratio for the average final concentration to initial concentration in the capillaries, c_{av}/c_0, is determined by scintillation counting of the vials containing the final solutions and vials to which known volumes of the initial radioactive solutions have been added with a calibrated μl syringe. The volume of the actual capillaries have been measured in separate experiments so that c_{av}/c_0 may be directly calculated. This procedure has been found to be more reproducible than directly filling the capillaries with initial solution and then centrifuging the contents into vials. All solutions were counted with a Packard Tri-Carb Scintillation Counter Model 3003. Care was taken to insure that all counting solutions had the same chemical composition. As a check a channels ratio measurement was always made and this remained the same within statistical errors for a set of measurements for a given experiment. Also for a given experiment c_{av} and c_0 vials were counted alternately to obviate any difficulties with instrument drift.

The tracer diffusion coefficient of the solute is determined by the equation[8].

$$c_{av}/c_0 = \sum_{n=0}^{\infty} \frac{8}{\pi^2(2n+1)^2} \exp\left[-\pi^2(2n+1)^2 \frac{Dt}{4l^2}\right] \tag{1}$$

where D is the diffusion coefficient, t is time of diffusion experiment in s and l is the effective length of capillary in cm. Data reduction was performed on a Wang 700 Series Programmable Calculator.

Viscosities of the solutions were measured in an Ubbelohde viscometer tube with a Rehovoth viscometer reader. The viscometer was calibrated with doubly distilled water, ethanol, 1,2-dichloroethane, benzene, and toluene at 37 ± 0.005 °C[9].

Density measurements were made in two calibrated Ostwald-type pycnometers.

All solutions were prepared gravimetrically with doubly distilled deionized water. Baker, reagent grade, urea was purified by two recrystallizations with water with centrifugal drainage. The urea was not heated above 65 °C. The biuret test was performed and no significant amount was found present. [^{14}C]Urea was obtained from Mallinckrodt Chemical Works, St. Louis, Mo., and recrystallized on a small scale with a small quantity of added purified non-radioactive urea in the same procedure as above. Sucrose that was 99.99% pure was obtained from the National Bureau of Standards. [U-^{14}C]Sucrose obtained from New England Nuclear Corp., Boston, Mass., Matheson, Coleman and Bell, analytical reagent grade, NaCl and Mallinckrodt, analytical reagent grade, $Na_2HPO_4 \cdot 7\ H_2O$ were used without further purification. [^{3}H]Taurocholic acid and [*carboxy*-^{14}C]taurodeoxycholic acid obtained from Mallinckrodt were purified by thin-layer chromatography (carried out by J. M. Dietschy). Cholic acid and taurocholic acid were obtained from Calbiochem,

Los Angeles, Calif. Purity of the radioactive and non-radioactive bile acids were further checked (B. C. Sherrill and J. G. Albright) by thin-layer chromatography and scintillation counting using Hofmann's solvent systems[10]. The fatty acids, [2-^{14}C]acetic and [1-^{14}C]octanoic acids were obtained from New England Nuclear. All unlabeled fatty acids and [1-^{14}C]pentanoic acid and [1-^{14}C]decanoic acid were obtained from Applied Science Laboratories, Inc., State College, Pa., and were used without further purification. All bile acid and fatty acid solutions were prepared in phosphate buffer, pH 7.1, containing 0.01 M sodium phosphate and 0.14 M NaCl.

RESULTS

Effective lengths of the capillaries, l, were determined in a series of calibration experiments[11]. These experiments were performed with the system urea–water over the concentration range 0.3 to 3.0 M at 25±0.005 °C. The calibrations were based on values of the tracer diffusion coefficients for urea that had been previously determined by the diaphragm cell method[12]. The difference, Δl, between the values of length determined by calibration and values obtained by direct measurement with a McPherson microcomparator are listed in Table I along with the lengths themselves. The values of Δl are relatively small and attest to ideal flow conditions within the apparatus.

TABLE I

CALIBRATION OF CAPILLARY CELL APPARATUS

Capillary	*l, measured (cm)*	*Δl (cm)*	*l, corrected (cm)*
8c	2.00290	0.000	2.00290
10c	2.00435	+0.022	2.02637
6c	2.00140	−0.015	1.98660
9c	1.99515	+0.015	2.01050

TABLE II

TRACER DIFFUSION COEFFICIENTS OF BILE ACIDS

Bile acids in 0.01 M sodium phosphate (pH 7.1), 0.14 M NaCl. Diffusion times are approx. 44 h. Values are the means ±S.E. (n determinations).

Bile acid	*Bile acid concn (mM)*	*η (37 °C) (cP)*	*D × 10⁶ (37 °C) (cm²·s⁻¹)*
Cholic acid	0.49	—	5.604±0.028 (6)
	0.25	0.7072	5.713±0.114 (4)
Taurodeoxycholic acid	0.50	0.7064	5.356±0.070 (8)
	0.25	0.7054	5.415±0.098 (3)
Taurocholic acid	0.56	0.7045	5.150±0.072 (7)
	0.25	0.7036	5.351±0.048 (4)

Analysis of the data from the calibration experiments indicates that the overall accuracy of the apparatus is better than $\pm 1.5\%$. It is of interest to note that the internal consistency of data from the four capillaries from a single experiment was generally better than $\pm 0.8\%$. The reason for the greater scatter of data between experiments may be associated with the difficulty of accurately determining the value of counts for c_0.

Tracer diffusion coefficients and viscosities for the bile acids at low concentrations are shown in Table II and Fig. 3. The densities of each of the solutions were 1.000 $g \cdot ml^{-1}$.

Tracer diffusion coefficients of the fatty acids, sodium acetate, pentanoic acid, octanoic acid and decanoic acid, made into solutions from the same phosphate buffer as for the bile acids are shown in Table III. Densities of all the solutions were 1.000

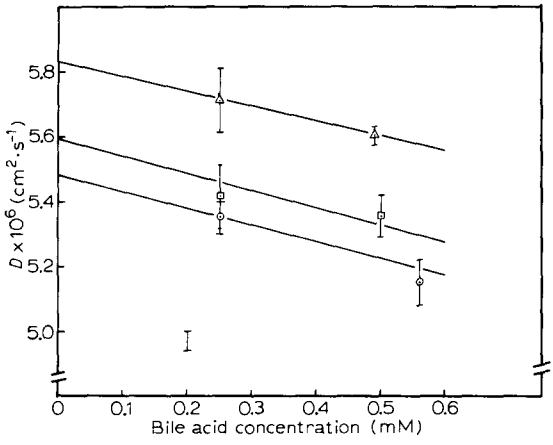

Fig. 3. Tracer diffusion coefficient as a function of the bile acid concentration; △, cholic acid; ⊡, taurodeoxycholic acid; and ⊙, taurocholic acid.

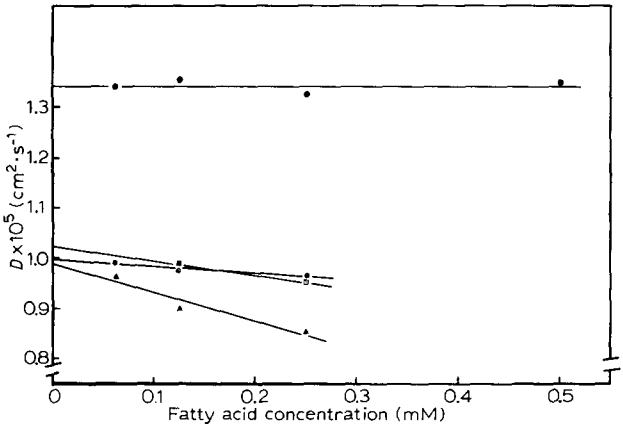

Fig. 4. Tracer diffusion coefficient as a function of the fatty acid concentration; ●, sodium acetate; ⊙, pentanoic acid; ⊡, octanoic acid; and △, decanoic acid.

$g \cdot ml^{-1}$. Fig. 4 shows that the slope of diffusion coefficients *versus* concentration seems to become more negative as the carbon chain length increases. This may be due to the formation of aggregates of the fatty acids even though the solutions were prepared at concentrations well below the critical micelle concentration. The fact that the extrapolated diffusion coefficient at infinite dilution of octanoic acid is greater than that of pentanoic acid is anomalous and is suggestive of experimental difficulty. The possibility of difficulty due to adsorption of the fatty acids on the walls of the capillaries has been suggested[13]. However, because of the consistency of results of duplicate experiments this problem would seem to be discounted. Nevertheless, to be cautious, the accuracy of the data of decanoic acid and octanoic acid should be considered to be only 10–15%.

TABLE III

TRACER DIFFUSION COEFFICIENTS OF FATTY ACIDS

Fatty acids in 0.01 M sodium phosphate (pH 7.1), 0.14 M NaCl. Diffusion times are approx. 24 h. Values are the means ±S.E. (*n* determinations).

Fatty acid	*Concn* (*mM*)	η (37 °C) (*cP*)	$D \times 10^5$ (37 °C) ($cm^2 \cdot s^{-1}$)
Sodium acetate	1.00	0.7059	1.347 ± 0.015 (4)
	0.50	0.7059	1.323 ± 0.036 (4)
	0.25	0.7056	1.352 ± 0.012 (4)
	0.125	0.7054	1.339 ± 0.033 (4)
Pentanoic acid	0.50	0.7066	0.962 ± 0.012 (19)
	0.25	0.7055	0.971 ± 0.011 (8)
	0.125	—	0.989 ± 0.009 (10)
Octanoic acid	0.50	0.7055	0.951 ± 0.019 (16)
	0.25	0.7053	0.988 ± 0.033 (3)
Decanoic acid	0.50	0.7053	0.854 ± 0.008 (12)
	0.25	0.7050	0.898 ± 0.008 (4)
	0.125	—	0.961 ± 0.006 (3)

TABLE IV

SUCROSE–0.9% NaCl SOLUTIONS AT 37 °C

NaCl concentration was approx. 0.150 M in each mixture. Fluidity, *f*, is the reciprocal of viscosity. Density is ρ.

Sucrose concn (*M*)	ρ ($g \cdot ml^{-1}$)	*f* (cP^{-1})	$D \times 10^6$ ($cm^2 \cdot s^{-1}$)
0.534	1.068	0.859	4.315 ± 0.043 (4)
0.400	1.051	0.987	4.942 ± 0.049 (4)
0.300	1.038	1.091	5.327 ± 0.043 (3)
0.199	1.025	1.202	5.954 ± 0.054 (4)
0.150	1.018	1.255	6.173 ± 0.025 (4)
0.099	1.012	1.309	6.343 ± 0.158 (4)
0.049	1.006	1.367	6.699 ± 0.040 (4)

The tracer diffusion coefficient of sucrose has been measured in a series of aqueous solutions containing sucrose and NaCl. Also mutual diffusion experiments at two dilute solutions of the system were performed to determine mutual diffusion coefficients in relation to tracer diffusion values[18]. In all cases the concentration of NaCl was 0.9%. The results of the investigation are listed in Table IV and Fig. 5.

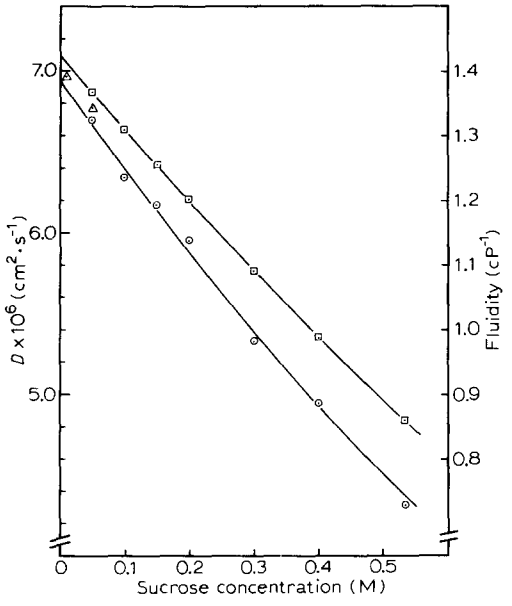

Fig. 5. Diffusion coefficient and fluidity as functions of the concentration of sucrose in physiological saline solutions; ⊡, f; ⊙, tracer diffusion coefficient; and △, mutual diffusion coefficient.

By using the method of least squares, the tracer diffusion coefficients of sucrose and the fluidity and density of the solutions were related to the concentrations by the following equations

$$D \times 10^6 = 6.945 - 5.502\, c + 1.085\, c^2 \tag{2}$$

$$f = 1.424 - 1.170\, c + 0.205\, c^2 \tag{3}$$

$$\rho = 0.99930 + 0.12877\, c \tag{4}$$

where c is molar concentration, f is fluidity and ρ is density.

DISCUSSION

While the presence of unstirred layer effects in biological systems has been recognized for many years, only recently have their major importance in altering the kinetics of both active and passive transport processes, particularly in the intestine, been fully appreciated[1]. As a molecule moves from the bulk solution to the cell interior it must, in effect, penetrate two barriers in series: a layer of unstirred water

and the lipid cell membrane. Movement across the unstirred layer presumably is by simple diffusion; the rate of such movement, J, is given by the expression

$$J=(C_1-C_2)\left(\frac{D}{d}\right) \tag{5}$$

where C_1 and C_2 are the concentrations of the solute molecule in the bulk solution and at the cell membrane surface, respectively, and d is the effective thickness of the unstirred water layer. In *in vitro* experiments with epithelial surfaces such as intestine and gallbladder d commonly varies from 70 to 500 μm in thickness depending upon the rate of stirring of the bulk solution[14,15]. *In vivo*, it is likely that the aqueous diffusion barrier is even thicker since such physiological processes as intestinal peristalsis probably are relatively ineffective in disrupting the unstirred layer.

Once the solute molecule has reached the aqueous lipid interface it is absorbed into the cell either by passive diffusion or by carrier-mediated, energy-linked transport. These membrane transport processes have been the subject of intense research interest in past years and the kinetic characteristics of the transmembrane movement of compounds such as steroids, fatty acids, amino acids and sugars across the various biological membranes have been described in detail. However, in most of these studies the presence of an unstirred water layer interposed between the bulk solution and the cell membrane has been ignored. Yet, recent studies from this laboratory on intestinal transport have demonstrated that the unstirred layer may have very significant physiological effects which, if ignored, lead to serious errors in interpretation of transport data. These effects fall into three categories.

First, with regard to passive absorption processes it is often desirable to determine permeability coefficients, P, for a series of molecules. If the unstirred layer resistance is not taken into account and P is calculated by dividing the rate of absorption, J, by the bulk phase concentration, C_1, of the probe molecule then the true permeability coefficient may be significantly under-estimated. Furthermore, the magnitude of the error is not constant for any given biological system but will vary with the absolute value of J; the greater the rate of absorption, the greater the error. True permeability coefficients can be calculated only if the concentration of the probe molecule at the aqueous–lipid interface, C_2, is known. This value can be determined from the relationship

$$C_2=C_1-J\left(\frac{d}{D}\right) \tag{6}$$

where the term $J\,(d/D)$ corrects the bulk phase concentration of the solute molecule for the unstirred layer resistance.

Second, for some highly permeant molecules such as medium and long chain fatty acids which penetrate the cell membrane very much more rapidly than they diffuse through the unstirred water layer, *i.e.* molecules where $P \gg D/d$, the aqueous diffusion barrier becomes absolutely rate limiting to the rate of passive absorption. In this circumstance, J for members of a series of compounds will vary directly with their respective free diffusion coefficients[4,5].

Third, the presence of unstirred layers significantly distorts the kinetic parameters of active transport processes such that apparent K_m values may be many-fold

too high. This artifact again derives from the fact that the solute concentration that determines the rate of active transport by a membrane carrier system is the concentration at the aqueous–lipid interface, C_2, and not the bulk phase concentration. Recently, the equations that properly describe the parameters of active transport have been reported from this laboratory (J. M. Dietschy) and they again require knowledge of both unstirred layer thickness and free diffusion coefficients[16].

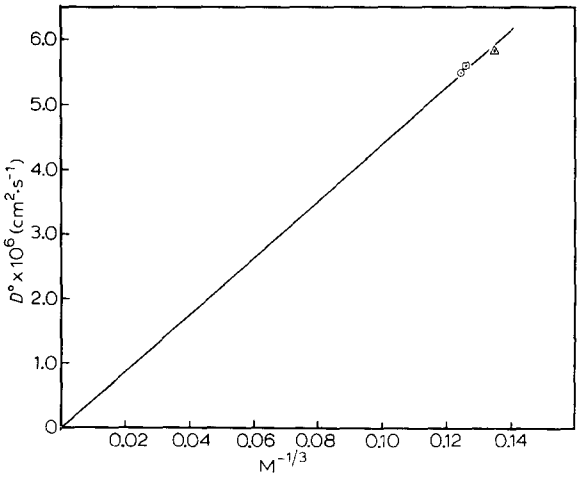

Fig. 6. Extrapolated diffusion coefficients at infinite dilution of bile acids in an isotonic phosphate buffer, pH 7.1, at 37 °C as a function of the reciprocal of the cube root of the molecular weight of the bile acid. The linear regression equation is: $D^\circ \times 10^6 = 43.787\ M^{-1/3}$ where D° is the extrapolated diffusion coefficient at infinite dilution from Fig. 3 and M is the molecular weight of the bile acid. Reasonable diffusion coefficients of other bile acids with similar molecular weights can probably be extrapolated from this linear regression equation.

From these considerations it is apparent that it is of critical importance for the further study of transport processes that accurate diffusion coefficients be available for molecules of biologic importance such as steroids, bile acids, amino acids and sugars in solutions of physiological ionic strengths and pH. While a few such data are available, these are extremely limited.

The apparatus described here has several major advantages in determining diffusion coefficients under these conditions. The unique stirring mechanism provides a uniform well-controlled flow pattern of bulk solution across the capillary openings. Small Δl corrections are observed and the accuracy is good, particularly in consideration of the relatively short capillaries. The short duration of the diffusion experiments minimizes bacterial growth contamination in physiological solutions. The small volume of bulk solution makes the device particularly suitable for the investigation of solutions containing solutes of biological interest that may be difficult or expensive to obtain in large quantities.

Basic diffusion data at temperatures and compositions relevant to studies of transport phenomena across membranes have been measured. It is probably not possible to meaningfully correlate the diffusion coefficients measured here with molar volumes or molecular weights. However, the diffusion coefficients of the bile acids seem to be linear with $M^{-1/3}$, Fig. 6, in accordance with analysis of Stein[17]. This

correlation allows for the possibility of extrapolating diffusion coefficients of other bile acids with analogous molecular weights. No useful correlation of this type can be derived for the fatty acids. Of course, the sucrose in physiological saline solution provides several useful correlations as shown in Eqns 2, 3 and 4.

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