A Permeability Measurement of Small Unilamellar Vesicles by 6-Carboxyfluorescein*

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In order to characterize the permeability of small unilamellar vesicles (SUV), efflux of 6-carboxyfluorescein (6-CF) from the vesicles was monitored spectrophotofluorometrically. Since the entrapped highly quenched 6-CF (200 m*M*) became fluorescent upon release from the vesicles, the 6-CF could be used as an efflux probe. SUV containing entrapped 6-CF was prepared from egg phosphatidylcholine and separated by gel filtration on Sepharose 4B. Observed change of relative fluorescent intensity with time was sigmoidal. From this curve, the parameter of permeability was determined either by half-time or a relearsed amount per unit time from the initial slope. Half-time of efflux of prepared SUV having 302 ng phospholipid /m*l* in 10 m*M* Tris-HCl buffer pH 7.4 was 21.0 min at 37°C. Various factors which could affect the half-time were examined including temperature, pH, salt, and vesicle concentration. In particular the effect of vesicle concentration on the efflux revealed that the permeability can be a function of the concentration.

Introduction

Since Bangham[1] demonstrated that aqueous dispersions of phospholipids formed closed structures that were relatively impermeable to entrapped materials, there has been a great deal of physical and biochemical studies of lipid bilayer properties[2]. The phospholipid vesicles can be defined by various characters such as size, entrapment capability, permeability, phase transition, or surface charge[3]. These characters are different according to preparation methods, lipid composition, temperature, pH and ionic strength of the medium. It is therefore necessary to characterize some properties of prepared vesicles prior to use as a model for biological membrane. Among the properties, permeability and entrapment capability are the main feature of lipid vesicles that have made them a valuable investigative tool. Permeability property of vesicles demonstrates a great deal of similarity to that of the natural membranes. The permeabilities of various cations and anions from vesicles have been examined[4] and a large number of papers described changes in permeability by the lipid composition of vesicles and many other factors[5,6].

There have been many methods to measure the permeabliity of vesicles including dialysis[5,7], coupled enzyme method[8], nmr technique[9], and UV spectrophotometery[10]. Another method is a quenched fluorescence technique originally employed to monitor the fusion of vesicles with natural membranes by Weinstein et al.[11] Since then a couple of laboratories developed this method as a tool for the permeability test[12,13]. However there have been no detailed permeability studies such as time course of the permeablity and a possible effect of vesicle concentration.

In this study the permeability of small unilamellar vesicles (SUV) was examined by the fluorescence technique using 6-carboxyfluorescein (6-CF). SUV containing entrapped 6-CF was prepared from egg lecithin and the fluorescence intensity of 6-CF released from the vesicles was monitored. We found a couple of ambiguity from the intensity measurement and initiated a detailed study to overcome some of shortcomings. The detailed study revealed that the plot of fluorescence intensity vs. time showed sigmoidal curves and that the permeablity depended on vesicle concentration. These observations are important in view of the fact that in most cases experimenters tested the efflux in a fixed time and used arbitrary concentration of vesicles to measure the permeability[5,12,14]. Experimental conditions of the permeability measurement by 6-CF were optimized and some basic properties of the permeabiltiy were reexamined.

Experimental

Materials. Egg phosphatidylchloline from Sigma was purified by aluminum oxide (neutral) column and stored under nitrogen at −20 °C[15]. 6-Carboxyfluorescein was obtained from Eastman Kodak and treated with activated charcoal and recrystalized from enthanol/water (1:2)[16]. Cholesterol, insuline, and norepinephrine was purchased from Sigma. All other chemicals were reagent grade commercialy available.

Preparation of Lipid Vesicles. Small unilamellar vesicles were prepared by sonication of a phospholipid suspension according to Huang[17]. A portion of 25 mg egg lecithin was dried under nitrogen gas into a thin film on a glass tube and dried for 6 hrs in vacuum. The thin film was hydrated with 4 m*l* of aqueous solution of 200 m*M* 6-CF (PH 7.4). After agitation of thin layer film by a vortex mixer, the mixture was sonicated with one minute interval for one hour, approximately ten times of the period required for the optical clarity at 4 °C. Undispersed lipids and titanium particles were removed by centrifugation at 40,000g for 20 min. Free 6-CF was eliminated by passing the sonicated suspension through Sepharose 4B column (1.8 cm×40 cm) with 10 m*M* Tris-HCl buffer PH 7.4 as eluent. Elution pattern of the sonicated suspension was recorded by a UVicord at 206 nm.

Permeability Measurement. Since the entrapped highly quenched 6-CF became fluorescent upon release from the vesicle, 6-CF could be used as an efflux probe[11]. 6-CF has excitation

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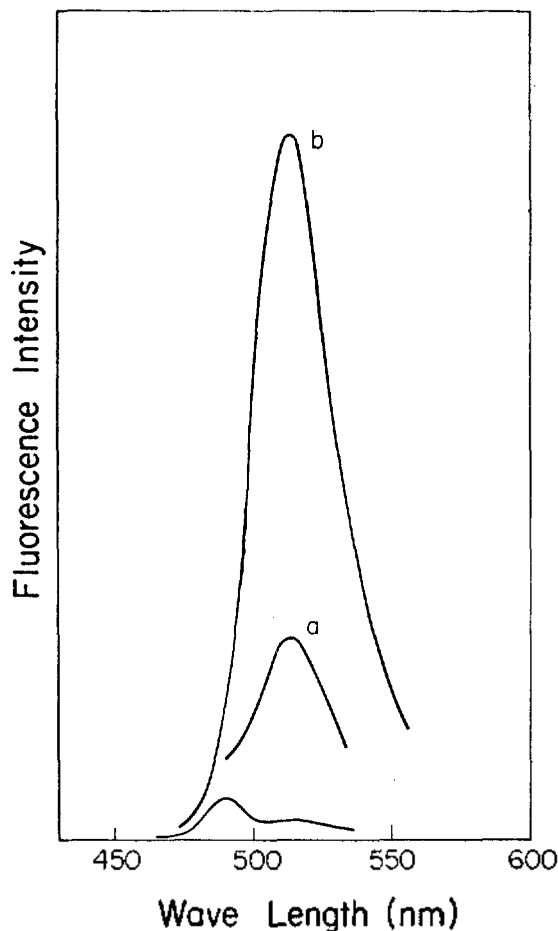

Figure 1. Fluorescence intensity of vesicles containing 200 mM 6–CF. Peak a represents an initial intensity (I_i) immediately after mixing with the buffer. Peak b represents the final intensity (I_f) when all 6–CF is released from the vesicles. Intensity of peak b is about 10–fold over the peak a .The unmarked line represents a base intensity without vesicles.

maximum at 492 nm and emission maximum at 516 nm. Efflux of 6–CF from SUV was monitored on a spectrofluorometer (Farrand Optical Co.) attached with a temperature controller. Immediately after the separation of vesicles containing 200 mM 6–CF, the fluorescence intensity was relatively small owing to the self–quenching, but the intensity increased more than 10–fold when the dye was released completely into entire solution as illustrated in Figure 1. Peak a is the initial intensity (I_i) of 6–CF entrapped in vesicles and peak b is the final intensity (I_f) when all 6–CF was released. When the intensity was monitored continuously, peak a increased with time until it reached to peak b. The final intensity of the prepared vesicles was also obtained by heating the vesicles for one min at 100°C. In actual experiments, a series of intensities at various time intervals (I_t) were recorded. The measured fluorescence intensity was always within the portion of linear relationship between the intensity and the concentration of 6–CF and and was converted into the relative fluorescence intensity, (I_t–I_i/I_f–I_i), for permeability calculation. The permeability was expressed by either half–time or amount released per unit time. Phospholipid contents of prepared vesicles were determined by phosphorous assay[18].

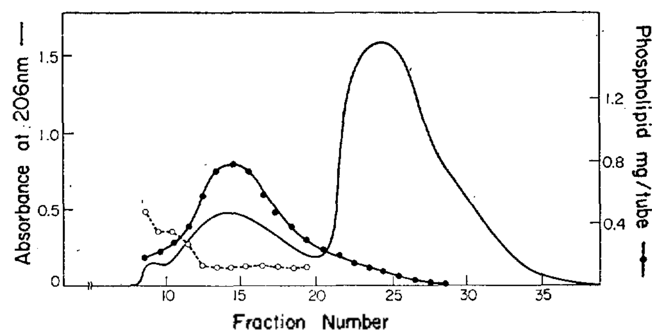

Figure 2. Elution profiles of phosphatidylcholine vesicles with 6–CF on a Sepharose 4B column, Open circles are the specific turbidity at 206 nm per μmole of phospholipid.

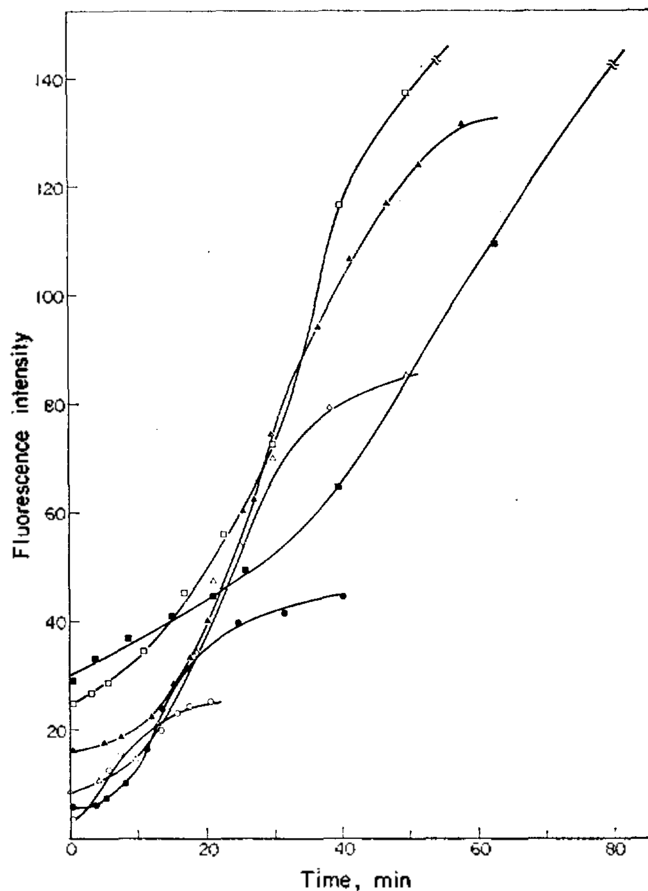

Figure 3. Time dependence of efflux of 6–CF from visicles. The fluorescence was monitored in 10 mM Tris–HCl buffer pH 7.4 at 37 °C. Each curve corresponds to different amount of phospholipid; —○—, 75ng phospholipid/ml; —●—, 151 ng/ml; —△—, 302ng/ml, —▲—, 453ng/ml; —□—, 604ng/ml, —■—, 755ng/ml.

Results

Seperation of Small Unilamellar Vesicles. A typical elution profile of sonicated mixture of lipid vescles with 6–CF on Sepharose 4B column was illustrated in Figure 2. A solid line with three peaks was obtained by use of UVicord at 206 nm. From the phospholipid assay of each fraction (closed circle), it was found that the first two peaks correspond to lipid vesicles and the last peak contained exclusively 6–CF. The specific turbidity (turbidity at 206 nm per μmole of

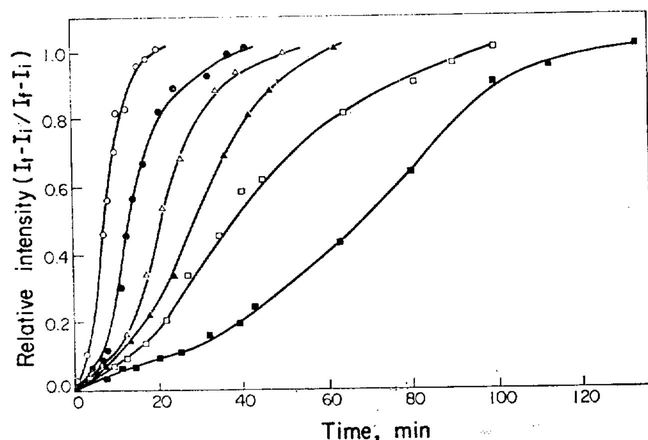

Figure 4. Time dependence of relative intensity of effluxed 6-CF. The relative intensities were obtained from the data of Figure 3.

phospholipids, open circle)[19] was calculated and found to be constant from fraction 12 to 19. Therefore the first peak near the void volume was large vesicles and the second peak corresponds to homogeneous small unilamellar vesicle. The fractions of 14, 15, and 16 were pooled and used for the following experiments.

Permeability Measurement by 6-Carboxyfluoresceine. The time dependence of the fluorescence intensity of 6-CF released from the vesicles showed sigmoidal curve (Figure 3). Shapes of the curve were changed depending on the concentration of vesicle. In order to estimate the efflux from the vesicles the observed fluorescence intensities were converted to relative intensities and replotted (Figure 4). From these curves, the half-times (I_t-I_i/I_f-I_i=0.5) and initial slopes were calculated and they were plotted against vesicle concentrations (Figure 5). Initial slope was obtained from the initial linear part of the relative intensity curve and expressed in the increase of relative intensity per min. When 100λ of vesicle sample (ca. 15.1 mg of phospholipid) was diluted into 100 m*l* of 10 m*M* Tris-HCl buffer, pH 7.4 that was 151 ng of phospholipid per m*l*, the half-time of efflux was 13.5 min at 37°C and the calculated initial slope was 0.123. When 200λ of the sample was used, the half-time was 21 min and the initial slope was 0.093. As the concentration of vesicle increased, the half-time increased (the initial slope decreased) and the shape of curve of relative intensity over time became less slant.

Basic Properties of the Vesicular Efflux. After the efflux procedure was established, the amount of 200λ of vesicle sample was selected as the standard procedure and the efflux was measured at 37°C. We reexamined several factors which could affect the permeability of prepared vesicles. As expected, the leakage of 6-CF was markedly dependent on temperature (Figure 6). As temperature increased, the half-time was reduced. When the ionic strength of medium was increased with 100 m*M* KCl, 6-CF was released rapidly (Figure 6). Without salt, the efflux rate was too slow to measure at lower temperature. When effect of PH on the efflux was examined, the efflux rate of 6-CF was reduced as

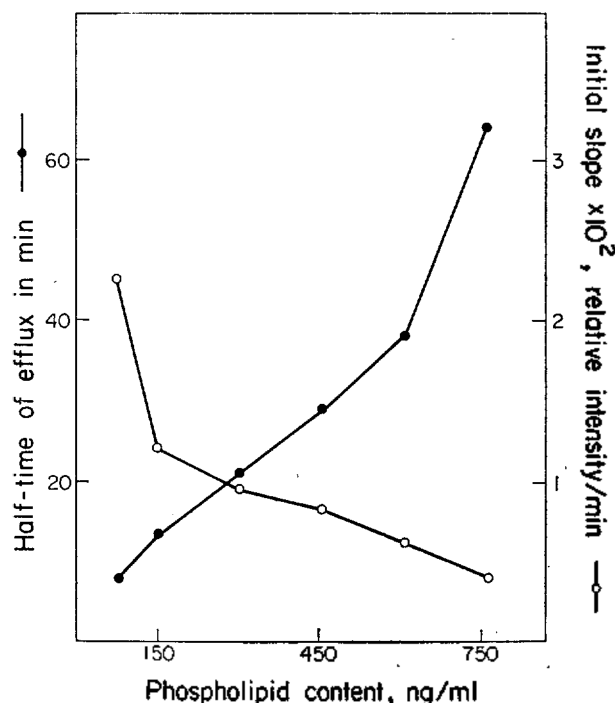

Figure 5. Effect of vesicle concentration on the half-time and inital slope of efflux. The data were calculated from Figure 4.

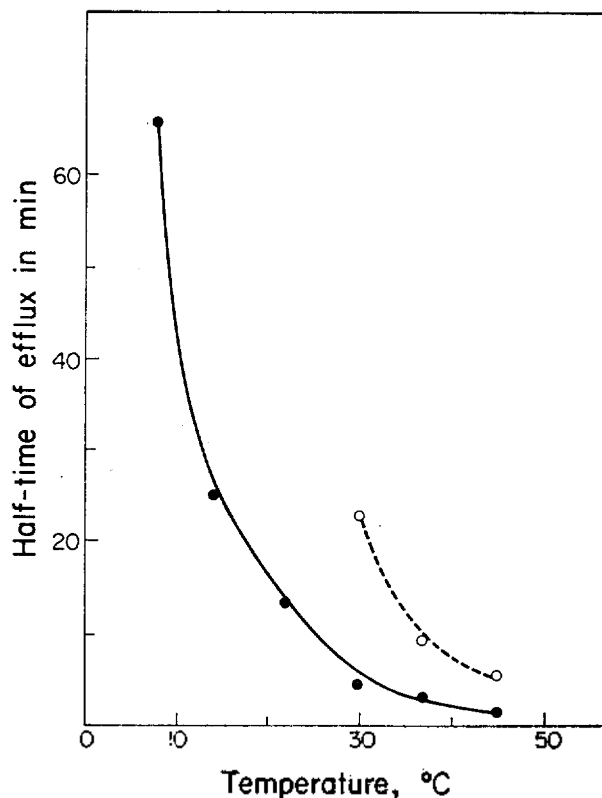

Figure 6. Effect of temperature on the half-time of efflux. The efflux measurement was carried out in 0.1*M* citrate-phosphate buffer pH 6.0 with the vesicle concentration of 302ng phospholipid/m*l*. Solid line has no KCl, while dashed line has extra 100mM KCl in the medium.

PH increased (Figure 7). In order to examine this method further, the effects of hormones on the permeability were tested. 1μM of insuline and norepinephrine decreased the half-time almost two fold respectively (Table 1). The effect

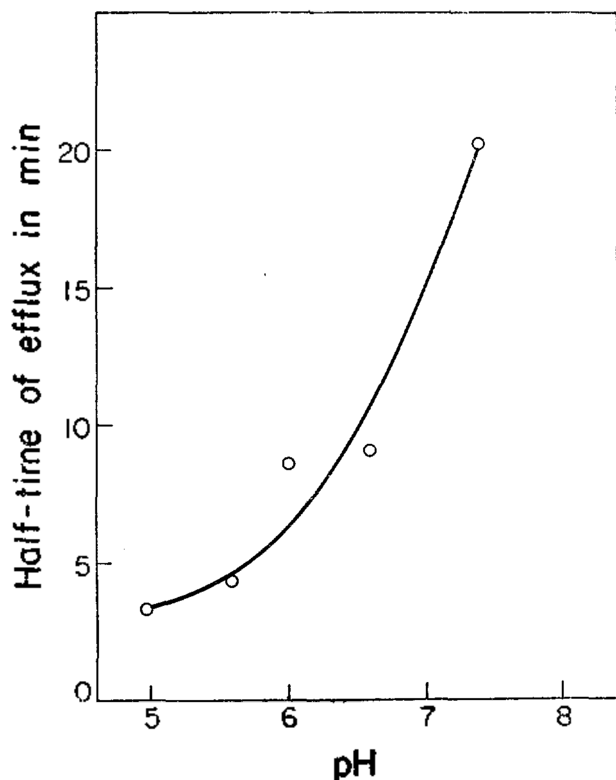

Figure 7. Effect of pH on the half-time of efflux. The efflux was measured under the standard conditions as described in text except the pH of media. The acidic buffer was 0.1*M* citrate–phosphate.

TABLE 1: Half-times of Efflux of 6–CF from Phosphatidylcholine Vesicles in 10m*M* Tris–HCl Buffer pH 7.4 at 37 °C

Lipid composition	Phospholipid content, ng/m*l*	Effector	Half–time min (n)[a]
Phosphatidyl	151[b]		13.5±2.21 (5)
Choline (egg)	302		21.0±1.77 (5)
	453		29.0±1.00 (5)
	302	Insuline 1 μM	11.0, 11.5
	302	Norepine-phrine, 1 μM	9.8, 10.0
	302	KCl–free	8.5[c]
	302	KCl–100m*M*	1.3[c]
phosphatidyl choline:	60		59.7±2.52 (3)
cholesterol	120		60.7±7.30 (3)
(4:6)	180		61.7±1.53 (3)

[a] *n* represents the number of measurements. Average half time ± standard deviation; [b] This amount of lipid corresponds to the vesicle number of 7.8×10^{8} per m*l* (ca. 1.3×10^{-12} *M*); [c] The efflux measurement was done in 0.1 *M* citrate–phosphate buffer pH 6.0.

of lipid composition on the efflux was also examined with egg lecithin: cholesterol vesicle (4:6 weight ratio). The half-time of the mixed vesicles was increased remarkably because of the stabilization effect of cholesterol on the bilayer structure. Furthermore the half-time of cholesterol containing vesicle appeared to be independent on the concentration of vesicles in contrast with the pure phosphatidylcholine vesicles. The permeability data obtained by the 6–CF efflux method were summarized in Table 1.

Discussion

The measurement of 6–CF intensity released from vesicles provided a simple and direct method to study the permeability of lipid bilayer vesicles. In conventional dialysis method entrapped molecules first leaked out from vesicle, and then penetrated dialysis membrance once more. Hence it gave only an average rate of efflux in a fixed period of time. Therefore it was not rapid enough to measure a dynamic characteristics of permeability. In this method, the fluorescence of 6–CF became fluorescent as soon as the molecules were released from vesicles, thus the permeability could be monitored directly.

The relative intensity data derived from the fluorescence intensity vs. time showed a singmoidal curve (Figure 4). This characteristic curve about permeability has not been revealed by other methods and it implies that the permeability mechanism is not simple as generally presumed. It indicates that the 6–CF efflux from vesicles does not follow a first order kinetics. And from the facts that the shapes of sigmoidal curves were less slant as the concentration of vesicles increased and the half-times were also dependent on the concentration (Figure 5), the efflux may be influenced by a intermolecular interation between vesicles. The possibility of the intermolecular interaction could be an important factor since the 6–CF molecule has a nature of significant hydrophobicity and this compound was originally used to monitor the fusion of vesicles with natural membrane[11]. Therefore it is desirable to study whether the sigmoidal behavior is a common feature of the efflux or it is stemmed solely from the 6–CF's own character. Our result that the permeability depends on the vesicle concentration suggests that some experiments of permeability taking arbitary portions of vesicle do not warrant their results. For example, since Papahadjopoulus et al.[5] used arbitary portion of vesicles to calculate the diffusion rate of various vesicles by use of dialysis method, their results could not be same when they chose different concentrations of vesicles.

In order to verify the efflux method further, some basic properties of the permeability of phosphatidylcholine vesicles were examined. When the experiments were performed at 8 °C with the buffer containing 100 m*M* KCl, the half-time was 23 times longer than that at 37 °C as expected (Figure 6). This temperature dependence of the permeability seems to indicate a rather high activation energy and agrees with other data obtained by different methods. The enhanced efflux by addition of salt appears to be very interesting with respect to the possibility that the electrostatic microenvironment of surface of the vesicle can be altered by changing ionic strength and this in turn alters the permeability. Lowering the PH of medium from 7.4 to 5.0, the half-time of 6–CF efflux from vesicles was decreased almost 7–fold (Figure 7). This dramatic effect of PH on the efflux may be originated from either the protonation of carboxyl group of 6–CF itself or the perturbation of the bilayer structure by protons. Although this phenomenon seems to be very important to

understanding of the efflux mechanism, it was not attempted to differentiate these two factors in the present study. If 6–CF is replaced with other probes which have no protonated groups such as calcein[12], it can be helpful to explain the cause of the PH effect on the permeability.

This method allows to calculate size and concentration of the prepared vesicles without knowing the molecular weight of vesicles. This is possible because the final concentration of released 6–CF can be measured directly and hence the number of entrapped 6–CF per vesicle can be calculated[11,20]. When 100λ of sample was diluted to 100 ml of buffer, radius of inner aqueous part was estimated to be 53Å and the number of vesicles was 7.8×10^{8}/ml (ca. 1.3×10^{-12} M). In this case, therefore, vesicle diameter is approximately 210Å and inner volume is 6.2×10^{-19} cm^3. This means that each vesicle contains 75 molecules of dye. The effect of biologically active materials such as insulin and norepinephrine on the permeability was examined and it was observed that these materials influenced significantly the efflux of 6–CF from the vesicles. The effect of lipid composition on the permeablity was also tested with phosphatidylcholine: cholesterol system and the preliminary data showed that the presence of cholesterol in the vesicle eliminated effectively the concentration dependency of the efflux (Table 1). However the pattern of the overall efflux did not change and remained as sigmoidal. In view of the present studies and the simplicity of the procedure, this method can help to produce a large number of permeability data from various vesicles and contribute to the study of permeability mechanisn.

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The Crystal and Molecular Structure of Chloramphenicol Base

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The crystal structure of chloramphenicol base, $C_9H_{12}N_2O_4$, the deacylated base of antibiotic chloramphenicol, has been determined by X–ray diffraction techniques using diffractometer data obtained by the ω–2θ scan technique with CuKα radiation from a crystal with space group symmetry $P2_12_12_1$ and unit cell parameters a = 22.322(6), b = 7.535(6), c = 5.781(5) Å. The structure was solved by direct methods and refined by full–matrix least–squares to a final R = 0.051 for the 573 observed reflections. The overall conformation of the base is quite different from those of the chloramphenicol congeners which are similar despite the presence of many rotatable single bonds. The propane chain in the base is bent with respect to the phenyl ring, while it is extended in the chloramphenicol congeners. There is no intramolecular hydrogen bond between the hydroxyl groups of the propanediol moiety. All of the molecules in the crystal lattice are connected by a three-dimensional hydrogen bonding network.