EFFECTS OF HYDROSTATIC PRESSURE ON LIPID BILAYER MEMBRANES

II. Activation and Reaction Volumes of Carrier Mediated Ion Transport

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SUMMARY Measurements of voltage relaxations following brief charge-pulses applied to lipid bilayers have been performed at different hydrostatic pressures in the presence of the neutral carriers cyclo (D-Val-L-Pro-L-Val-D-Pro)$_3$(PV) and valinomycin. From double-exponential relaxations observed in membranes containing PV-K^+ complexes estimates were obtained of the amount of membrane absorbed complexes, N_{MS}, and of the rate of complex translocation, k_{MS}. The pressure dependence of k_{MS} corresponded to an activation volume for translocation of ~12 cm^3/mol independent of ionic strength and K^+ concentration. The pressure dependence of N_{MS} strongly varied with K^+-concentration suggesting a major role of ion-complexation in solution which is estimated to involve a reaction volume of 25.5 cm^3/mol, while the volume of absorption of a PV-K^+ complex by the membrane was estimated −7.5 cm^3/mol. The relaxations observed in the presence of valinomycin contained three exponentials and could be used to estimate four rate constants and one absorption parameter which characterize the valinomycin-mediated transport. When the transport of Rb^+ was tested, the rate constant for the complex dissociation, k_D, and the total concentration of free and complexed carriers in the membrane, N_o, were found to be pressure insensitive. The translocation rates for the complex, k_{MS} and for the free carrier, k_S, were instead markedly pressure dependent according to estimated activation volumes in the range of 11 to 18 cm^3/mol. The recombination rate constant k_R was also pressure dependent according to an activation volume of 12–14 cm^3/mol. The study of the valinomycin-K^+ transport yielded similar results as far as N_o, k_S, and k_{MS} are concerned, but in this case k_R was pressure independent, while k_D was increased by pressure. The net volume change associated with the transfer of a free ion to the membrane in the form of a valinomycin-ion complex was nevertheless very similar for K^+ and Rb^+. It is concluded that pressure affects the transmembrane mobility of liposoluble molecules, whether charged or not, mostly by increasing the effective viscosity of the hydrocarbon core of the bilayer. The pressure dependence of the membrane uptake of amphipathic compounds seems also to obey the general rule: that of involving a negative volume change. However, when the compounds arise from a complexation reaction in solution or at the membrane solution interface possible positive volumes of complexation may make effective uptake to be reversed rather than increased by pressure.

INTRODUCTION

Studies of biological membranes under high hydrostatic pressure may provide information about membrane structure and thermodynamic properties of functional membrane proteins which are not obtainable by any other means (Heremans, 1982; MacDonald, 1984). Thus, the effects of pressure on excitable membranes (Conti et al., 1982*a*, *b*; 1984) and artificial bilayers (Bruner and Hall, 1983) can be interpreted in terms of activation volumes involved in the conformational changes of ionic channels. Although some direct studies of the pressure dependence of the lipid matrix structure of natural membranes were performed (Benz et al., 1984), model membranes provide a better controlled system for gaining information on such phenomena. We reported in the preceding paper (Benz and Conti, 1986) studies of the pressure dependence of membrane thickness and of the translocation and absorption of lipophilic ions. In this publication we proceed to the analysis of the activation and reaction volumes involed in the transport of ion carriers across membranes.

In an early work (Johnson and Miller, 1975) the overall activation volume of valinomycin induced potassium permeability of lipid vesicles was estimated to be ~40 cm^3/mol. Very recently, Moronne and Macey (1985) have reported similar results for the valinomycin and nonactin-

mediated potassium transport in planar lipid bilayers. Both studies, using steady state conductance, do not allow to decide if a single step is responsible for the observed activation volume or if several steps are influenced by the hydrostatic pressure. The experiments reported here allow to separte the activation and reaction volumes of all steps involved in the transport mediated by the neutral carriers cyclo-(D-Val-L-Pro-L-Val-D-Pro)$_3$(PV) and valinomycin. Our results suggest that the pressure dependence of these processes is governed by very general properties of the lipid bilayer system.

MATERIALS AND METHODS

The techniques used for the pressurizing system, for bilayer formation and for the charge pulse instrumentation were described in full detail in the preceding publication (Benz and Conti, 1986). Cyclo-(D-Val-L-Pro-L-Val-D-Pro)$_3$(PV) was a gift of the late Dr. B. F. Gisin (Gisin and Merrifield, 1972) and valinomycin was purchased from Calbiochem. (San Diego, CA). Concentrated stock solutions of the ion carriers were prepared in chloroform or in ethanol. Small amounts of PV stock solution were added to the aqueous phase bathing the membranes to get a final PV concentration of $5 \cdot 10^{-7}$ M. Valinomycin was added at a concentration of $1 \cdot 10^{-3}$ M to the membrane forming solution (Stark and Benz, 1971). The unbuffered aqueous solutions (pH ~6) contained various concentrations of KCl, RbCl, and LiCl (Merck, analytical grade). All measurements were performed at 20°C.

Description of the Transport Models

We used a simple model for valinomycin-mediated ion transport (Läuger and Stark, 1970) that has been already applied successfully to the analysis of charge pulse data as described in detail by Benz and Läuger (1976) and Benz (1978). The model assumes that the association between the ion, M^+ (aqueous concentration c_M) and the carrier, S, takes place at the membrane solution interface with rate constants for dissociation and recombination given by k_D and k_R, respectively. The translocation of S and of the ion-carrier complex, MS^+, occurs over a single energy barrier with rates k_S and (at zero membrane potential) k_{MS}. The voltage dependence of MS translocation is described according to an Eyring barrier or a modified Nernst-Planck model (trapezoid). However, the shape of the barrier is irrelevant for the description of our charge pulse relaxation measurements, where the initial voltage, V_m^o was of the order of 10 mV or smaller (Benz and Zimmermann, 1983). At $t = 0$ the membrane capacity is suddenly charged to V_m^o and the subsequent voltage decay, $V_m(t)$, is given by the sum of three exponentials, whose decay rates and relative amplitudes provide five experimental parameters from which it is possible to directly estimate the rate constants k_D, k_R, k_S, and k_{MS}, as well as the total carrier concentration in the membrane, N_o (Benz and Läuger, 1976).

PV is in principle an ion carrier. It has been shown, however, that the complexes of PV with the larger alkali ions, K^+, Rb^+, and Cs^+, have a high stability constant in the aqueous phase (Benz et al., 1976*a*). Therefore, the transport of PV-K^+ complexes has to be treated similarly to that of negatively charged lipophilic ions (Benz and Gisin, 1978), although some differences are expected from the fact that PV-K^+ complexes do indeed dissociate while the lipophilic ions do not. Consequently, for charge pulse experiments at small voltages, the membrane potential relaxation induced by the PV-K^+ complexes contains only two exponentials (Benz et al., 1976*b*; Benz and Conti, 1981). The translocation rate constant k_{MS} ($= k_i$) and the total concentration of complexes $2N_{MS}$ ($= N_t$) may be calculated from the experimental data using the same formalism given in the preceding paper for lipophilic ions.

RESULTS

Effect of Pressure on the PV-K^+-System

Fig. 1 shows the influence of hydrostatic pressure, P, on the voltage decay following a charge pulse applied to a DOPC/*n*-decane bilayer, separating identical solutions containing 0.1 M KCl and $5 \cdot 10^{-7}$ M PV. Traces 1 and 4 are control records, taken at atmospheric pressure (0.1 MPa) before and after pressurization. Traces 2 and 3 were taken at 50 MPa and at 150 MPa, respectively. The records of Fig. 1 were taken from a longer series of measurements listed in Table I. Several minutes intervals were left to elapse between any two measurements at different pressures to allow a good reequilibration of temperature and of the partitioning of the ion-carrier complexes between the membrane and the aqueous phases (Benz and Conti, 1986). It is apparent from the data of Fig. 1 that increasing P slows down the fast (first) relaxation associated with the PV-K transport system, while decreasing its amplitude. It is also clear that these effecs are fully reversible. The quantitative analysis of the experiment illustrated in Fig. 1 yielded the results that are summarized in Table I. Increasing P from 0.1 to 150 MPa gradually decreased the translocation rate constant of the PV-K^+ complex, k_{MS}, to -37% of its original value and produced a decrease of N_{MS} by ~50%.

Within the simple theortical framework adopted in this paper the translocation of PV-K^+ across the membrane is a first order reaction and the pressure dependence of k_{MS} can then be expressed according to Eyring's theory of absolute reaction rates (Johnson et al., 1974)

$$\ln[k_{MS}(P)/k_{MS}^o] = -\Delta V^{\ddagger}(P - P_o)RT, \qquad (1)$$

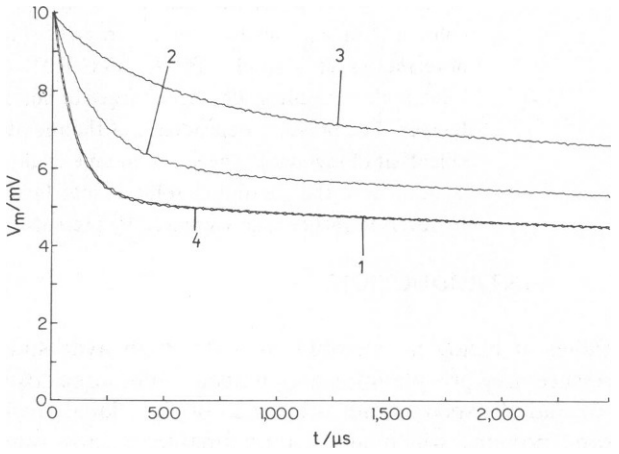

FIGURE 1 Voltage relaxation associated with intramembrane movements of PV-K^+ complexes, at different hydrostatic pressures. The membrane capacitance was charged to the initial voltage by a charge pulse of 20 ns duration. DOPC/*n*-decane membrane separating two identical solutions containing 0.1 M KCl and $5 \cdot 10^{-7}$ M PV. Temperature 20°C. Records 1 and 4 were obtained at atmospheric pressure (0.1 M Pa) before and after the series of pressurizations listed in Table I. Records 2 and 3 are measurements at 50 M Pa and 150 M Pa, respectively.

TABLE I
INFLUENCE OF HYDROSTATIC PRESSURE ON PV-K$^+$ TRANSPORT

P(MPa)	$\tau_1/\mu s$	τ_2/ms	a_1	$k_{MS}(s^{-1})$	$2N_{MS}$ (pmolcm^{-2})
0	134	21.4	0.43	2150	0.30
50	208	21.4	0.39	1480	0.26
100	332	21.8	0.30	1050	0.18
150	447	22.1	0.27	820	0.15
70	312	21.0	0.32	1100	0.20
0	121	19.9	0.44	2280	0.30

Analysis of the series of charge pulse relaxation measurements, from which were extracted the records of Fig. 1, according to Eqs. 1–3 of Benz and Conti (1986). The membrane capacitance of the DOPC/n-decane membranes was 3.7 · 10^{-7} F/cm^2 (Benz and Gisin, 1978). Bathing solutions: 0.1 M KCl; 5 · 10^{-7} M PV. Temperature: 20°C.

where P_o = 0.1 MPa, k^o_{MS} is the rate constant at P_o, and $\Delta V^{\ddagger}$ is the transient volume change (activation volume) associated with the movement of a complex from one of its interfacial equilibrium positions to the top of the free energy barrier inside the hydrocarbon core of the membrane.

Fig. 2 shows a semilogarithmic plot of $k_{MS}(P)$ data obtained from measurements on five different DOPC membranes. The data can be reasonably well fitted by a straight line according to Eq. 1, yielding a positive activation volume of 14 cm^3/mol. This value is practically identical to that found to describe the pressure dependence of the translocation rate constant of negative lipophilic ions (Benz and Conti, 1986). However, at variance with the latter work the present data were not corrected for effects of changes in membrane thickness, because the PV-K$^+$ transport system is insensitive to such changes (Benz and Gisin, 1978).

The pressure dependence of the concentration of membrane-absorbed PV-K$^+$ is illustrated in Fig. 3, which shows a semilogarithmic plot of $N_{MS}(P)$ data from the same

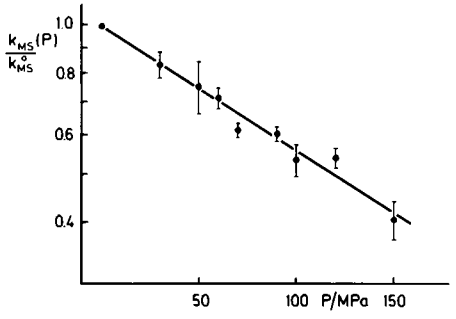

FIGURE 2 Pressure dependence of the rate, k_{MS}, of PV-K$^+$ translocation across the hydrocarbon core of DOPC/n-decane membranes immersed in 0.1 M KCl solutions containing 5·10^{-7} M PV. The data are mean (+ sem) of the ratio of k_{MS} estimates at hyperbaric pressures to the mean estimate, k^o_{MS}, at 0.1 M Pa before and after pressurization. Data from five different membranes. The straight line is the fit of the data according to Eq. 1, corresponding to an activation volume, $\Delta V^{\ddagger}$, of 14 cm^3/mol.

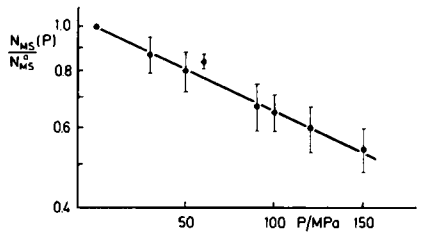

FIGURE 3 Pressure dependence of the concentration, N_{MS}, of PV-K$^+$ complexes absorbed on DOPC/n-decane membranes immersed in 0.1 M KCl solutions containing 5·10^{-7} M PV. The data are given as ratios of N_{MS} estimates at hyperbaric pressures to the mean estimate at 0.1 M Pa before and after pressurization. Same membranes as in Fig. 2. The straight line is a fit of the data according to Eq. 2, corresponding to a volume of 10 cm^3 for the formation of 1 mol of membrane-bound PV-K$^+$ complexes.

experiments of Fig. 2. The estimates of $N_{MS}(P)$ were corrected for the increase of specific membrane capacity with pressure discussed by Benz and Conti (1986). Also these data can be fitted by a straight line

$$\ln[N_{MS}(P)/N^o_{MS}] = -\Delta V^*(P - P_o)/RT, \qquad (2)$$

where ΔV^* = 10 cm^3/mol represents the volume of formation of membrane bound PV-K$^+$ complexes.

If ΔV^* was the volume of absorption in the partitioning of PV-K$^+$ between the aqueous phase and the membrane, the above data would reveal a striking difference between positive and negative lipophilic ions, because the latter partition with an absorption volume of about the same size but opposite sign (Benz and Conti, 1986). However, the following arguments will show that the data in Fig. 3 reflect mostly the pressure dependence of the formation of the PV-K$^+$ complex, a process which is likely to involve a positive volume change arising from the release of electrostriction in the hydration shell of potassium ions when the latter are incorporated into the PV ring (Low and Somero, 1975; Asano and Le Noble, 1978). The slow time course of the changes induced by pressure has been used as an argument supporting the interpretation of DPA$^-$ and TPhB$^-$ data in terms of changes in partition coefficients (Benz and Conti, 1986).

A similar test cannot be used here because the time constant for the diffusion controlled absorption of PV-K$^+$ is expected anyhow to be much shorter than the time required for pressurization and temperature reequilibration[1]. Indeed, apart from small oscillations due to tempera-

[1]The stability constant K for the PV-K$^+$ complex formation is ~10 M^{-1} (Benz et al., 1976*a*). The aqueous concentration c_{MS} of PV-K$^+$ complexes at 0.1 M KCl is ~2.5·10^{-7} M (Eq. A3). The absorption coefficient for PV-K$^+$, $K_a = N_{MS}/c_{MS}$ is ~6·10^{-4} cm at 0.1 M Pa. The characteristic time constant, τ_D, for the diffusion limited PV-K$^+$ absorption is given by K_a^2/D_{MS} (Conti et al., 1974), where D_{MS} is the diffusion coefficient of PV-K$^+$ in water. Even assuming $D_{MS} \approx 10^{-6}$ cm^2/s (~20 times smaller than for KCl) τ_D is expected to be of the order of 0.4 s, which is too small to be resolved.

ture variations, the measurements with PV did not show any systematic slow time course. Instead, a major role of PV-K^+ complexation involved in the change of N_{MS} was demonstrated by comparing experiments performed at different KCl concentrations. The stability constant of the PV-K^+ complex in the aqueous phase, K, has been calculated to be ~10 M^{-1} (Benz et al., 1976*a*). Thus, at 1 M KCl ~90% of the PV-molecules are complexed and the effect of pressure on the concentration of PV-K^+ in the aqueous solution should be rather low. Under these conditions N_{MS} was in fact not pressure dependent, whereas $V^{\ddagger}$ of k_{MS} was more or less the same. Intermediate results were obtained in 0.3 M KCl ($\Delta V^* = 3.5$ cm^3/mol). To rule out the possibility that the changes in ΔV^* arise from an ionic strength effect, we also studied 1 M solutions with 0.1 M KCl and 0.9 M LiCl instead of 0.1 M KCl alone, because Li^+ does not form complexes with PV (Benz et al., 1976*a*). This variation had only little effect on both $\Delta V^{\ddagger}$ and ΔV^*. Table II shows $\Delta V^{\ddagger}$ and ΔV^* as a function of different salt solutions. The results for ΔV^* suggest that the pressure dependence of PV-K^+ complexes in lipid bilayers is caused by the reaction volume of the complex formation, which will be discussed in more detail in Discussion.

Pressure Dependence of Valinomycin-mediated Ion Transport

The carrier transport mediated by valinomycin is characterized by as many as five parameters, but it has been shown that the charge pulse method allows fairly accurate measurements of all of them provided that three relaxation processes can be resolved (Benz and Läuger, 1976; Benz et al., 1977). Fig. 4 shows records of voltage relaxations of a GMO/*n*-decane membrane in a solution of 1 M RbCl, taken at 0.1 MPa, 80 MPa, 150 MPa, and again after return to 0.1 MPa (traces 1–4). It is obvious that the whole relaxation process is markedly and reversibly slowed by the hydrostatic pressure, the effect being most evident for its fastest component. The analysis of the voltage relaxations of Fig. 4 in terms of the four rate constants, k_R, k_D, k_{MS}, k_S, and of the total surface concentration of carrier molecules, N_o, is given in the figure legend. Increasing P from 0.1 MPa to 150 MPa decreased k_R, k_{MS}, and k_S by 40–60%, whereas k_D and N_o, appeared to be practically constant.

TABLE II
ACTIVATION AND REACTION VOLUMES OF PV-K^+ TRANSPORT

Salt solutions	$\Delta V^{\ddagger}$/cm^{3}mol^{-1}	ΔV^*/cm^{3}mol^{-1}
0.1 MKCL	14	10
0.1 M KCl + 0.9 M LiCl	12	8.5
0.3 M KCl	12	3.5
1.0 M KCl	11	<2

Dependence of the activation volumes of PV-K^+ translocation, $\Delta V^{\ddagger}$, and of the apparent volumes of PV-K^+ absorption, ΔV^*, upon KCl concentration and ionic strength of the bathing solutions. DOPC/*n*-decane membranes. Temperature: 20°C. The standard deviations of $\Delta V^{\ddagger}$ and ΔV^* are about ±2 cm^3/mol.

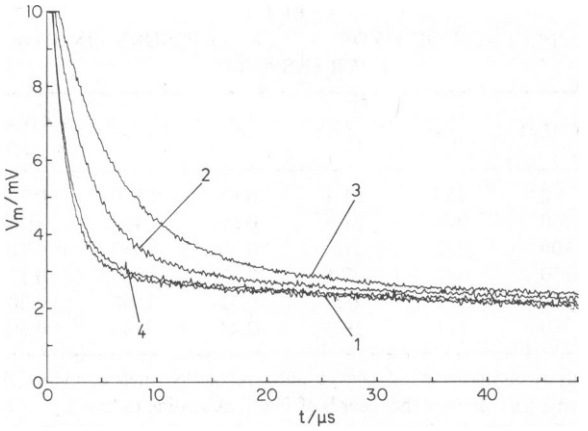

FIGURE 4 The effect of pressure upon the charge pulse voltage relaxations induced by the valinomycin-Rb^+ system. The membrane was formed from a solution of 1% GMO in *n*-decane, containing 10^{-3} M valinomycin. The bathing solution contained 1 M RbCl; T = 20°C. Traces 1 and 4 are control records taken at the beginning and at the end of the experiment at atmospheric pressure. Traces 2 and 3 are records obtained at 80 MPa and 150 MPa, respectively. The following rate constants were calculated from the three relaxation processes:

P/MPa	$k_R/10^4$ $M^{-1}s^{-1}$	$k_D/10^4$ s^{-1}	$k_{MS}/10^4$ s^{-1}	$k_S/10^3$ s^{-1}	N_o/ pmol/cm^2
0.1	19	10	10	11	1.6
80	16	9.5	6.0	7.8	1.5
150	11	10	3.9	5.8	1.6
0.1	18	11	10	9.8	1.8

Fig. 5 shows overall data on the pressure dependence of the rate constants k_R and k_D obtained from similar experiments on four different GMO/*n*-decane membranes. The semilogarithmic plot of $k_R(P)$ data is fitted by a straight

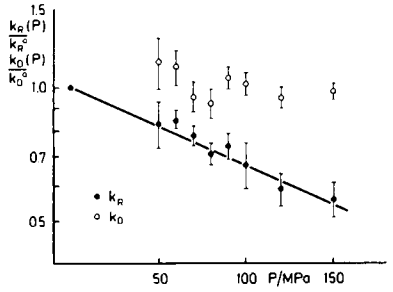

FIGURE 5 Pressure dependence of the rate constants, k_R and k_D, characterizing the formation and the dissociation of the valinomycin-Rb^+ complex at the membrane-water interface. The data are given as ratios of estimates at hyperbaric pressures to the mean estimate obtained at 0.1 MPa before and after pressurization. Data from four different GMO *n*-decane membranes formed from solutions containing 10^{-3} M valinomycin and separating aqueous solutions of 1 M RbCl. Temperature: 20°C. The straight line is a fit of the $k_R(P)$ data according to an equation similar to Eq. 1, yielding an estimated activation volume for complex formation of 10 cm^3/mol. Since k_D appears to be pressure independent, 10 cm^3/mol is also the volume of formation of a membrane absorbed valinomycin-Rb^+ complex.

line according to an apparent activation volume of the formation of valinomycin-Rb^+ complexes at the membrane-solution interface of 10 cm^3/mol. The k_D data indicate that the dissociation of the complex occurs with an activation volume close to zero. This implies that the heterogeneous stability constant $K_h = k_R/k_D$, decreases with increasing hydrostatic pressure, the number of complexes in the membrane at 150 MPa being reduced to ~55% of its value at normal pressure.

Fig. 6 shows the pressure dependence of the translocation rate constants, k_S and k_{MS}, of the complexed and of the free carrier, respectively. The semilogarithmic plots of the data can be fitted by straight lines that correspond to an activation volume of 18 cm^3/mol for the translocation of the MS^+ complex and of 12 cm^3/mol for that of the free carrier. It is known that k_S and k_{MS} are rather insensitive to changes in membrane thickness produced by changing the membrane solvent (Benz et al., 1977). Therefore, at variance with what discussed for the translocation of lipophilic ions (Benz and Conti, 1986), the data were not corrected for thickness changes induced by pressure.

Some experiments on the valinomycin-mediated Rb^+ transport were also performed using *n*-hexadecane as a solvent for membrane formation, although with this system we could not raise the hydrostatic pressure above 60 MPa without breakage of the membranes. The reason of this membrane instability is not clear, and could arise from phase separation between lipid and solvent. The observations made in the pressure range of 0.1 to 60 MPa were in qualitative agreement with those obtained with *n*-decane.

The influence of the salt concentration on the pressure effects was studied by comparing the results described above with those of similar measurements of 0.1 M RbCl solution. The pressure dependence of the various parameters of the valinomycin-Rb^+ system was found to be very similar in both conditions. This is more directly illustrated in Table III listing the estimates of the activation volumes

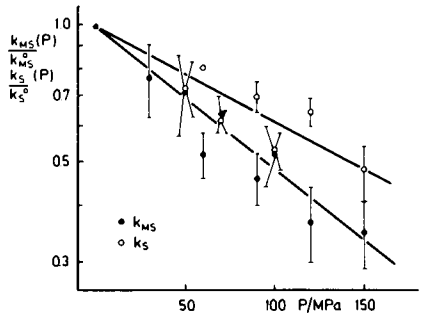

FIGURE 6 Pressure dependence of the translocation rates, k_S and k_{MS}, of valinomycin and valinomycin-Rb^+ complexes, respectively, k_S^0 and k_{MS}^0 are the mean estimates of the rate constants at normal pressure before and after a series of measurements at various P values. The data were obtained from the same experiments of Fig. 5. The straight lines were drawn according to the theoretical pressure dependence of the type of Eq. 1. They correspond to activation volumes of 12 cm^3/mol and 18 cm^3/mol for translocation of the free carrier and of the complex, respectively.

TABLE III
ACTIVATION VOLUMES OF VALINOMYCIN MEDIATED ION TRANSPORT

Solution	$\Delta V^{\ddagger}$/$cm^3 \cdot mol^{-1}$			
	(k_R)	(k_D)	(k_{MS})	(k_S)
1 M RbCl	10	<2	18	12
0.1 M RbCl	14	<2	11	16
1 M KCl	<2	−6	12	14

Activation volumes for the valinomycin mediated ion transport for different cationic concentrations in solution. The standard deviations of $\Delta V^{\ddagger}$ are about ±2 cm^3/mol.

for complex formation and dissociation and for carrier and complex translocation, obtained with different ionic solutions. The table does not include the pressure dependence of N_o, which was found in all cases to be insignificant.

Besides the valinomycin-Rb^+ system we studied also the influence of hydrostatic pressure on the valinomycin-K system using 1 M KCl solutions under conditions otherwise identical to those described above. Fig. 7 shows voltage relaxation records obtained with such system at different hydrostatic pressures. As in Figs. 1 and 4, only two traces at high pressure (80 MPa and 150 MPa) are shown for simplicity together with the control records taken at 0.1 MPa at the beginning and the end of the experiment. The analysis of the relaxation data in terms of transport and

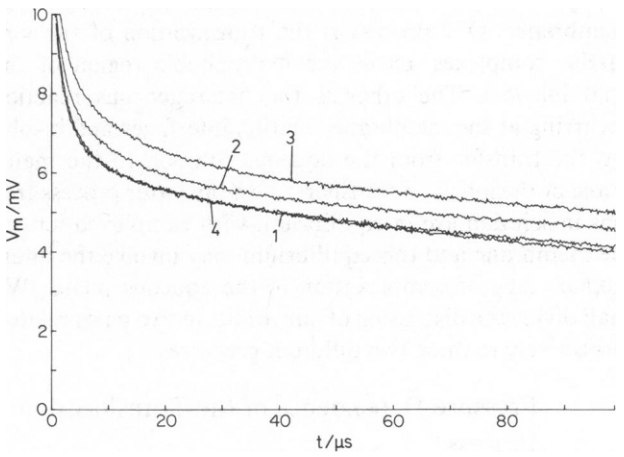

FIGURE 7 Pressure dependence of the charge plus voltage relaxation associated with the valinomycin-K^+ transport system in a GMO/*n*-decane membrane. The experimental conditions were the same as Fig. 4 except that the membrane was formed in 1 M KCl instead of 1 M RbCl. Traces 1 and 4 are control records taken at the beginning and at the end of the experiment at atmospheric pressure. Traces 2 and 3 are records taken at 90 MPa and 150 MPa, respectively. The following rate constants were calculated from the three relaxation processes:

P/MPa	$k_R/10^4$ $M^{-1}s^{-1}$	$k_D/10^4$ s^{-1}	$k_{MS}/10^4$ s^{-1}	$k_S/10^3$ s^{-1}	N_o/ pmol/cm^2
0.1	4.3	3.6	14	9.8	0.55
90	4.5	4.9	8.1	6.0	0.50
150	5.0	6.1	5.8	4.1	0.56
0.1	4.7	4.0	13	9.5	0.58

complexation is given in the figure legend. The comparison of these data with those obtained for the Rb^+ transport system (Table III) shows substantial similarities as far as the pressure dependence of the translocation rate constants, k_S and k_{MS}, and the insensitiveness of N_o to pressure, are concerned. On the other hand, the effect of pressure on the complexation reaction, characterized by the rates k_R and k_D, seems qualitatively different for the Rb^+ and the K^+ system. In the latter case k_R appears to be little sensitive to pressure, while k_D is increased at high pressures. For the Rb^+-valinomycin complexation we found a strong decrease of k_R with pressure, while k_D was little affected. Interestingly, despite these differences, the pressure dependence of the heterogeneous stability constant, $K_h = k_R/k_D$, is similar in both cases so that the net volume change associated with the formation of membrane bound complex is always positive. A summary of the results obtained from six different experiments on the K^+ transport system similar to those just described is given in Table III in terms of estimated activation volumes for complex formation and dissociation, and for carrier and complex translocation. The table gives also a similar summary of the data obtained for the Rb^+ transport system.

DISCUSSION

The experiments reported in this paper provide information about two distinct processes that are involved in the transport of ions mediated by neutral carrier through lipid membranes. One process is the translocation of the ion-carrier complexes across the hydrophobic region of the lipid bilayers. The other is the heterogeneous reaction occurring at the membrane-solution interfaces and involving the transfer from the aqueous solution to the membrane of the ion to be transported. In the latter process free ions in solution are in equilibrium with complexed ions in the membrane and this equilibrium may involve the intermediate step of complexation in the aqueous phase. We shall divide the discussion of our results in two parts related respectively to these two different processes.

Pressure Dependence of the Translocation Process

Our measurements provide estimates of the activation volume, $\Delta V_T^\ddagger$, involved in the translocation across the aliphatic chains region of a lipid bilayer of four different molecular aggregates: the three positively charged ion-carrier complexes. PV-K^+, valinomycin-K^+, and valinomycin-Rb^+, and the neutral carrier valinomycin. The values of $\Delta V_T^\ddagger$ that we have obtained for all these species lie in the narrow range of 11–18 cm^3/mol, their scatter having most likely to be attributed to experimental errors and to the simplification of the theoretical analysis. Indeed, the largest variation (from 11 to 18 cm^3/mol) was not found between the $\Delta V_T^\ddagger$ of different species but between the $\Delta V_T^\ddagger$ estimates for the valinomycin-Rb^+ complex obtained with 0.1 M and with 1 M RbCl solutions, respectively. It is interesting to compare these results with those obtained from the study of the pressure dependence of the translocation of the negative lipophilic ions dipicrylamine and tetraphenylborate (Benz and Conti, 1986). For both ions $\Delta V_T^\ddagger$ was estimated ~14 cm^3/mol. Altogether these data suggest that the activation volumes involved in the transport of liposoluble substance across the hydrophobic core of a lipid bilayer reflect mostly average properties of this phase which might be described in first approximation by an effective viscosity. This view is in sharp contrast with that of Moronne and Macey (1985), who conclude from their studies with carriers and lipophilic ions that "pressure produces little or no viscosity change in the membrane interior."

Such conclusion was based upon the comparison of a large pressure coefficient of the carrier transport as opposed to a very small effect on the lipophilic ion translocation. We have argued in our preceding paper (Benz and Conti, 1986) that the low $\Delta V^\ddagger$ estimated by Moronne and Macey (1985) for DPA^- and $TPhB^-$ translocation may result from not having properly accounted for membrane thickness changes and temperature effects. On the other hand, our present dissection of the different steps involved in the carrier meditated ion transport shows indeed, that also free carrier and ion-carrier complexes have strongly pressure dependent transloction rate constants.

We notice that the values of $\Delta V_T^\ddagger$ in lipid bilayer membranes containing solvent are significantly larger for lipophilic ions than those measured in the nerve membrane (Benz et al., 1984). As already pointed out in the preceding paper (Benz and Conti, 1986), it is possible that this difference mainly arises in the case of the lipophilic ions because of thickness effects. However, ion carriers are insensitive to membrane thickness (Benz et al., 1977; Benz and Gisin, 1978) and the pressure effect on the translocation rate constants reflects thus at least in this case a change of the apparent microviscosity within the hydrocarbon core. Nevertheless we think that experiments with solvent-free bilayers, which we have so far been unable to perform at high pressure, are needed to clarify this point in more detail.

Pressure Dependence of Ion-carrier Complexation

The pressure dependence of the incorporation into the membrane of ion-carrier complexes appears to vary a lot in different experimental situations. Variability includes dependence on the aqueous concentration of the transported ion (in the case of PV mediated transport). This variability is consistent with the idea that the complexation reaction between carrier molecule and ion is pressure dependent.

In the case of the PV-K^+ system a simple analysis (see Appendix) shows that the apparent volume change, ΔV^*,

revealed from our results should consist of two contributions

$$V^* = \Delta V_a^* + \alpha \Delta V_c^*, \quad (3)$$

where ΔV_a^* is the volume of absorption of an already formed PV-K^+ complex to the membrane, ΔV_c^* is the volume of formation of a PV-K^+ complex in solution, and α is a factor varying between 0 and 1 depending on the aqueous concentration of ions (compare Eq. A8). Using the data for ΔV^*, given in Table II, for $c_M = 0.1$ M and 0.3 M and assuming $K^o = 10\ M^{-1}$ (Benz et al., 1976*a*), ΔV_a^* and V_c^* are estimated from Eqs. 3 and A8 to be about -7.5 cm^3/mol, and 25.5 cm^3/mol, respectively. These estimates are consistent with the results at 1 M KCl because they predict for this KCl-concentration a ΔV^* of -2.5 cm^3/mol, i.e., practically no dependence of N_{MS} on pressure as was actually observed.

The above analysis leads to the interesting conclusion that the actual absorption of an already formed PV-K^+ complex occurs with a negative volume change, as in the case of the negative lipophilic ions dipicrylamine and tetraphenylborate (Benz and Conti, 1986). This reinforces the argument of the latter work that such negative absorption volumes are due to the amphipathic character of lipophilic ions and are, therefore, independent of the sign of the ions. On the other hand, the large positive volume of complexation, ΔV_c^*, contains most likely a large contribution due to the release of electrostriction in the solvation shell of potassium ions when they are incorporated in the PV ring (Asano and Le Noble, 1978; Low and Somero, 1975).

The largest variation in the pressure dependence of the valinomycin-ion complexation was found by varying the species of the transported ion (Rb^+ or K^+), while changing the aqueous concentration from 1 M RbCl to 0.1 RbCl affected only little the estimated activation volumes for formation and dissociation of membrane bound Rb^+-valinomycin complexes. The latter result provides further support for the assumption that the complexation reaction governing the valinomycin-mediated transport occurs directly in heterogeneous phase at the membrane solution interface (Läuger and Stark, 1970; Stark and Benz, 1971). The differences of the activation volumes measured for Rb^+ or K^+ indicate that the complexation of these two ions with valinomycin occurs via transition states that have different structural properties. However, it is remarkable that the net volume of these reaction, ΔV^*, is not very different. If we indicate the activation volumes of recombination and dissociation of the complexes by $\Delta V_R^\ddagger$ and $\Delta V_D^\ddagger$ respectively, we have $\Delta V^* = \Delta V_R^\ddagger - \Delta V_D^\ddagger$. From the data of Table III we estimate ΔV^* to be ~ 8 cm^3/mol for Rb^+ and ~ 12 cm^3/mol for K^+. This suggests that ΔV^* is mostly determined by common properties of the reactants (a free ion from the aqueous phase and a neutral carrier from the membrane-solution interface) and the product (an amphipathic ion-carrier complex at the membrane solution interface).

Altogether, our present results and the preceding study (Benz and Conti, 1986) suggest a simple general interpretation of the effects of hydrostatic pressure on the uptake and intramembrane mobility of both charged and neutral lipophilic compounds in lipid bilayer membranes. Our data are consistent with the idea that the estimated volume changes arise mainly from general properties of the lipid bilayer system. The negative volumes of absorption for amphipathic ionic compounds, like DPA^-, $TPhB^-$, and PV-K^+ are simply explained by assuming that these compounds find a best packing efficiency by embedding their hydrophobic moiety into the hydrocarbon core of the bilayer, while leaving their charged region exposed to the interface. The positive ΔV^* estimated for the valinomycin-ion complexes is not inconsistent with this view, because it refers to a heterogeneous complexation reaction rather than to the absorption of a preformed complex. The quantitative agreement between the positive activation volumes for the translocation across the hydrocarbon core of different ionic compounds (PV-K^+, Val-K^+, Val-Rb^+) and of the neutral valinomycin, suggests that such volumes are mostly related to properties of the effective viscosity of the hydrocarbon phase and they are fairly independent of the actual shape or ionic character of the mobile species. The opposite conclusion reached by Moronne and Macey (1985) is due to the fact that they estimated much smaller activation volumes for lipophilic ions and did not measure the single steps involved in carrier mediated ion transport.

The conclusions made above provide clues for deciding the role played by the lipid matrix and by membrane proteins in the pressure dependence of the properties of natural membranes. For example, the properties of channel gating mechanism in nerves (Conti et al., 1982*a*, *b*; 1984) appears to be too large to be attributed to the changes in intramembrane viscosity estimated here. This reinforces the hypothesis of the latter authors that their estimated activation volumes reflect mostly structural properties of the channel protein transformation. Our present results may also be useful for better understanding the mechanism of ion complexation and of the anthagonistic effect of pressure on anesthesia (Spyropoulos, 1957; Kendig and Cohen, 1977). The simple extrusion of the anesthetics from the membrane lipids appears to be an unlikely mechanism for charged and possibly also for polar drugs, since their absorption might be favored rather than antagonized by pressure. At least for such compounds, a pressure reversal effect should more reasonably be taken as evidenced that their action involves specific sites of membrane proteins (Franks and Lieb, 1982).

APPENDIX

The surface concentration of PV-K^+ complexes in the membrane, N_{MS}, is determined by the concentration of PV-K^+ in solution, c_{MS}, according to

an absorption coefficient, K_a which can be defined as

$$K_a = N_{MS}/c_{MS}. \quad (A1)$$

In turn, c_{MS} is determined by the total PV concentration in solution, c_o, and by the concentration of free K^+ in solution, through the equilibrium constant for the PV-K^+ complex formation, K

$$K = c_{MS}/c_M(c_o - c_{MS}) \quad (A2)$$

According to Benz et al. (1976*a*), $K \approx 10\ M^{-1}$. Thus, the pressure dependence of N_{MS} will result from the combination of the influence of pressure on the two equilibrium constants K_a and K, according to

$$N_{MS} = c_o c_M K K_a/(1 + Kc_M) \quad (A3)$$

or, since c_o and c_M are independent of pressure

$$d \ln N_{MS}/dP = d \ln K_a/dP + (d \ln K/dP)/(1 + Kc_M). \quad (A4)$$

If we indicate with ΔV_a^* the volume of absorption of a PV-K^+ complex by the membrane, with ΔV_c^* the volume of formation of a PV-K^+ complex in solution and with ΔV^* the apparent net volume associated with the formation of membrane bound PV-K^+ complex starting from PV and K^+ in solution, as defined by Eq. 2, Eq. A4 can also be written as

$$V^* = -RT(d \ln N_{MS}/dP) = \Delta V_a^* + \Delta V_c^*/(1 + Kc_M) \quad (A5)$$

with

$$K = K^\circ \exp[-\Delta V_c^*(P - P_o)RT], \quad (A6)$$

K° being the value of K at $P = P_o = 0.1$ MPa. Eqs. A5 and A6 show that ΔV^* is expected to vary with pressure even if we assume that the individual reaction volumes, ΔV_a^* and ΔV_c^* are pressure independent. An accurate analysis would require, therefore, the fitting of data such as those of Fig. 3 according to Eqs. A5 and A6, rather than with Eq. 2 which implicitly assumes that ΔV^* is constant. However, as a first approximation and considering also the large scatter of the data, Eq. 2 can be used, as we did, to estimate a mean apparent ΔV^*, and the dependence of ΔV^* on c_M can be interpreted according to Eq. A5 to estimate the order of magnitude of the individual reaction volumes, ΔV_a^* and ΔV_c^*. Taking the mean of Eq. A5 over the interval from P_o to P_1, and taking into account Eq. A6, we obtain

$$\Delta V^* = \Delta V_a^* + \alpha \cdot \Delta V_c^*, \quad (A7)$$

where

$$\alpha = 1 - [RT/\Delta V_c^*(P_1 - P_0)] \cdot \ln((1 + K^\circ c_M)/\{1 + K^\circ c_M \exp[-\Delta V_c^*(P_1 - P_o)/RT]\}) \quad (A8)$$

The authors wish to thank Mr. Thomas Apel for the analysis of the data.

This work was financially supported by the Deutsche Forschungsgemeinschaft grants Be 865/3-1 and 3-3.

Received for publication 25 July 1985 and in final form 31 December 1985.

REFERENCES

Asano, T., and W. J. LeNoble. 1978. Activation and reaction volumes in solution. *Chem. Rev.* 78:407–489.

Benz, R. 1978. Alkali ion transport through lipid bilayer membranes mediated by enniatin A and B and beauvericin. *J. Membr. Biol.* 43:367–394.

Benz, R., and F. Conti. 1981. Structure of the squid axon membrane as derived from charge-pulse relaxation studies in the presence of absorbed lipophilic ions. *J. Membr. Biol.* 59:81–104.

Benz, R., F. Conti, and R. Fioravanti. 1984. Extrinsic charge movement in the squid giant axon membrane. Effect of pressure and temperature. *Eur. Biophys. J.* 11:51–59.

Benz, R., and F. Conti. 1986. Effects of hydrostatic pressure on lipid bilayer membranes. I. Influence on membrane thickness and activation volumes of lipophilic ion transport. *Biophys. J.* 50:91–98.

Benz, R., O. Fröhlich, and P. Läuger. 1977. Influence of membrane structure on the kinetics of carrier-mediated ion transport through lipid bilayers. *Biochim. Biophys. Acta.* 464:465–481.

Benz, R., and F. Gisin. 1978. Influence of membrane structure on ion transport through lipid bilayer membrane. *J. Membr. Biol.* 40:293–314.

Benz, R., B. F. Gisin, H. P. Ting-Beall, D. C. Tosteson, and P. Läuger. 1976*a*. Mechanism of ion transport through lipid bilayer membranes mediated by peptide cyclo-(D-Val-L-Pro-L-Val-D-Pro)$_3$. *Biochim. Biophys. Acta.* 455:665–684.

Benz, R., and P. Läuger. 1976. Kinetic analysis of carrier-mediated ion transport by the charge-pulse technique. *J. Membr. Biol.* 27:171–191.

Benz, R., P. Läuger, and K. Janko. 1976*b*. Transport kinetics of hydrophobic ions in lipid bilayer membranes. Charge-pulse relaxation studies. *Biochim. Biophys. Acta.* 455:701–720.

Benz, R., G. Stark, K. Janko, and P. Läuger. 1973. Valinomycin-mediated ion transport through neutral lipid membranes: influence of hydrocarbon chain length and temperature. *J. Membr. Biol.* 14:339–364.

Benz, R., and U. Zimmermann. 1982. Evidence for the presence of mobile charges in the cell membrane of *Valonia utricularis*. *Biophys. J.* 43:13–26.

Bruner, L. I., and J. E. Hall. 1983. Pressure effects on alamethicin conductance in bilayer membranes. *Biophys. J.* 44:39–47.

Conti, F., R. Fioravanti, F. Malerba, and E. Wanke. 1974. A comparative analysis of extrinsic fluorescence in nerve membranes and lipid bilayers. *Biophys. Struct. Mechanism.* 1:27–45.

Conti, F., R. Fioravanti, J. R. Segal, and W. Stühmer. 1982*a*. Pressure dependence of the sodium currents of squid giant axon. *J. Membr. Biol.* 69:23–34.

Conti, F., R. Fioravanti, J. R. Segal, and W. Stühmer. 1982*b*. Pressure dependence of the potassium currents of squid giant axon. *J. Membr. Biol.* 69:35–40.

Conti, F., I. Inoue, F. Kukita, and W. Stühmer. 1984. Pressure dependence of sodium gating currents in the squid giant axon. *Eur. Biophys. J.* 11:137–147.

Davis, D. G., B. F. Gisin, and D. C. Tosteson. 1976. Conformational studies of peptide cyclo-(D-Val-L-Pro-L-Val-D-Pro)$_3$, a cation-binding analogue of valinomycin. *Biochemistry.* 15:768–774.

Franks, N. P., and W. R. Lieb. 1982. Molecular mechanisms of general anesthesia. *Nature (Lond.).* 300:487–493.

Gisin, B. F., and R. B. Merrifield. 1972. Synthesis of a hydrophobic potassium binding peptide. *J. Am. Chem. Soc.* 94:6165–6170.

Heremans, K. 1982. High pressure effects on proteins and other biomolecules. *Annu. Rev. Biophys. Bioeng.* 11:1–21.

Johnson, F. J., H. Eyring, and B. Stover. 1974. The theory of rate processes in biology and medicine. John Wiley & Sons, Inc., New York.

Johnson, S. M., and K. W. Miller. 1975. The effect of pressure and the volume of activation of the monovalent cation and glucose permeabilities of liposomes of varying composition. *Biochim. Biophys. Acta.* 375:286–291.

Kendig, J. J., and E. N. Cohen. 1977. Pressure antagonism to nerve conduction block by anesthetic agents. *Anesthesiology.* 47:6–10.

Läuger, P., and G. Stark. 1970. Kinetics of carrier-mediated ion transport across lipid bilayer membranes. *Biochim. Biophys. Acta.* 211:458–466.

Low, P. S., and G. N. Somero. 1975. Activation volumes in enzymatic catalysis: their sources and modification by low-molecular-weight solutes. *Proc. Natl. Acad. Sci. USA.* 72:3014–3018.

MacDonald, A. G. 1984. The effects of pressure on the molecular structure and physiological functions of cell membranes. *Phil. Trans. R. Soc. Lond. B.* 304:47–68.

Moronne, M., and R. I. Macey. 1985. Apparent activation volumes of hydrophobic ions and carriers in planar lipid bilayers. *J. Membr. Biol.* 84:221–227.

Spyropoulos, C. S. 1957. The effects of hydrostatic pressure upon the normal and narcotized nerve fiber. *J. Gen. Physiol.* 40:849–957.

Stark, G., and R. Benz. 1971. The tranpsort of potassium through lipid bilayer membranes by the neutral carriers valinomycin and monactin. *J. Membr. Biol.* 5:133–153.