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STRUCTURAL CHANGES IN BILAYER MEMBRANES BY MULTIVALENT IONS

IVAN HALLER and MARVIN J. FREISER

IBM Thomas J. Watson Research Center, Yorktown Heights, N.Y. 10598 (U.S.A.)

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SUMMARY

Formation of microlenses, a reduction in thickness, and a change in mechnical properties have been observed when Ca^{2+} or other multivalent ions are added to bathing solutions of Mueller-Rudin membranes of acidic phospholipids. The observations are interpreted as an extrusion of residual solvent from the hydrocarbon core of the bilayer due to changes in packing imposed by the reduction of electrostatic repulsion of the head groups.

INTRODUCTION

Several recent papers have demostrated that addition of divalent cations raises the temperature of the order-disorder transition of pure negatively charged phospholipid bilayers [1–3]. Since the surface area requirement per molecule is lower in the ordered phase, the increase in the transition temperature can be interpreted as due to the reduction of the repulsive energy of the phospholipid head groups. This is expected as a result of the reduction in surface charge density either by specific absorption or electrostatic screening by divalent cations [1].

In lamellar structures of phospholipids that are inhomogeneous either in head-group or fatty acid composition, the same forces lead to a somewhat different effect. As expected, in binary mixtures in the vicinity of the phase transition temperature of at least one component, addition of divalent ions can lead to lateral phase separation [4–9]. Spin label studies have also indicated that the fluidity in lamellar phases of negatively charged phospholipids decrease in the presence of calcium ions [10, 11].

In this paper we report the gross structural changes that are observed in Mueller-Rudin type phosphatidylserine bilayers upon addition of multivalent ions to the bathing solution. We interpret the observed formation of microlenses, the reduction in thickness of the bilayer, and its altered mechanical properties, in terms of the change of solubility of hydrocarbons in the membrane. We demonstrate through thermodynamic arguments that a small concentration of multivalent ions can, merely by

Abbreviation: EDTA, ethylenediamine tetraacetic acid.

its influence on the electrostatic energies of the double layer, cause significant changes in the stess of the bilayer membrane. We ascribe the observed change of the hydrocarbon solubility to the stress-induced change in the packing in the hydrocarbon core of the bilayer.

EXPERIMENTAL

Materials. Phosphatidylserine (bovine) was purchased as 25 mg/ml solutions in decane, or in heptane, from Applied Science Laboratories, Inc., State College, Pa. Phosphatidylcholine (hen's egg, "chromatographically pure"), phosphatidylinositol (plant), glycerol monooleate (99 %), and cholesterol ("Ultrex") were obtained from General Biochemicals Co., Applied Science Laboratories, Sigma Chemical, and J. T. Baker Chemical Co., respectively. All lipids showed a single spot in thin-layer chromatograms, and were stored below -10 °C. All inorganic salts were reagent grade. The water was deionized and subsequently distilled in an all glass apparatus.

For optical observations the Mueller-Rudin films were formed over a hole 0.20 cm dia carefully machined in a thinned-down portion of a flat vertical Teflon support. The support was suspended diagonally in a cube shape all-glass cell (edges 2.4 cm) containing 10.0 ml bathing solution. The light source (tungsten halogen lamp, or the He-Ne laser) and the observing low power microscope were mounted on goniometer arms, and were usually set 90 ° with respect to each other and 45° to plane of the membrane. The absence of a partition in the cell insured that no pressure or concentration gradients acted across the membrane.

For capacitance measurements the flat support was replaced with a Teflon cup of 2.0 cm inner diameter, and a larger (edge 4.0 cm) glass cell was used. The General Radio Type 1656 Impedance Bridge was connected through Ag/AgCl electrodes. The equilibrium capacitance was independent of measuring frequency (10 Hz to 10 KHz range) and of measuring voltage (up to 60 mV), but the rate at which the capacitance changed with changes in the environment was not. The data reported were obtained at 1 KHz and 35 mV peak-to-peak.

For the observation of changes in the mechanical properties, after the formation of the bilayer, the teflon cup could be moved with a micrometer screw smoothly in the vertical direction. The hydrostatic head resulting from the raising of the cup caused an outward bulging of the membrane, the extent of which was measured with a filar eyepiece. The viewing direction was tangential to the cup at the point where the bilayer was located. The addition of salt solutions was immediately followed by the withdrawal of an equal volume of bathing solution to restore the pressure head. As a further precaution the minimum necessary solvent was used in making up to solutions.

In all measurements, the bathing solution was 0.1 M NaCl; the pH was continuously monitored and adjusted if necessary with dilute NaOH or HCl to 6.3 ± 0.8. Lipids, generally in 25 mg/ml solutions in decane, were painted over the support with a camel hair brush. Experiments were performed only if the film thinned to a "black" membrane free of multilayer spots in less than 1 h.

Solutions of multivalent ions were added as chlorides after the film thinned down. A magnetic stirrer was spun at a rate that avoided lasting changes in the properties of the membrane due to hydrodynamic effects, and for a length of time

that was shown in separate experiments to be ample to achieve complete mixing.

EDTA was added as the di-sodium salt in 0.1 M solution which also contained 0.4 M sodium acetate as buffer.

RESULTS

Phosphatidylserine bilayer membranes formed from decane solution in 0.1 M bathing solutions develop highly reflecting spots when Ca^{2+} are added to the bathing solution to a concentration of 1 mM. The time required for the appearance of the spots is typically 1 min, and several hours are required for their spontaneous decay. Upon addition of excess EDTA (buffered with 2 equivalents of sodium acetate) the spots disappear in a few minutes. Entirely analogous spot formation is observed

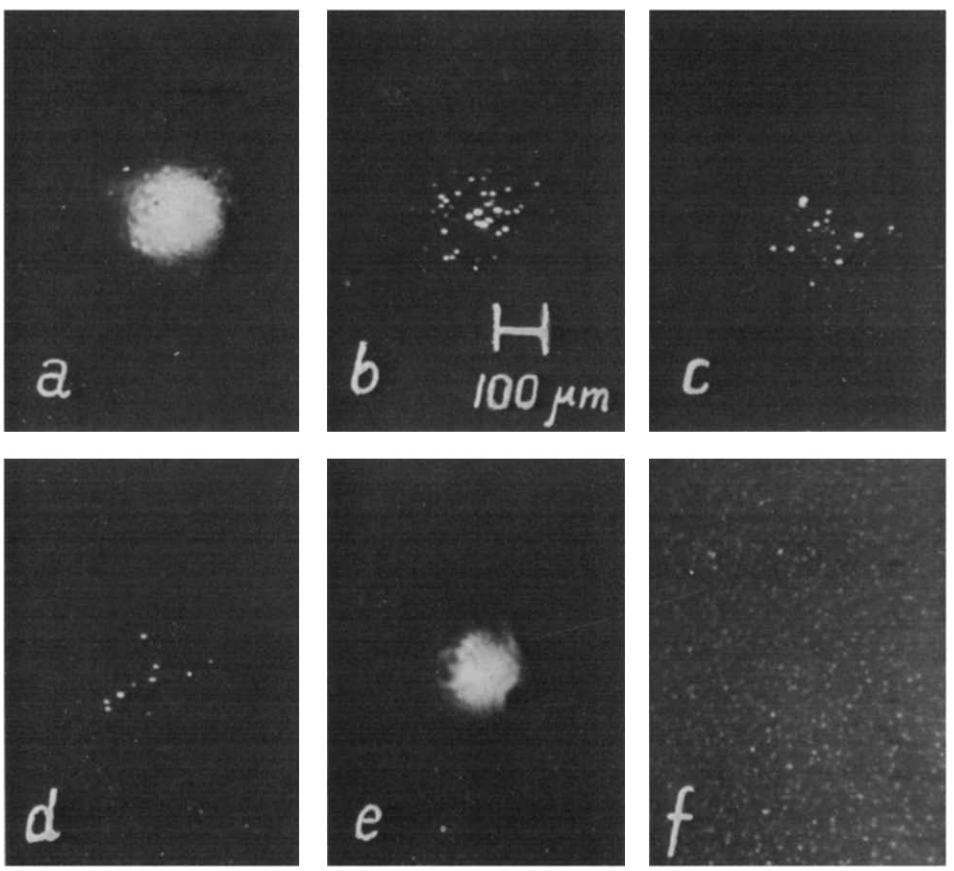

Fig. 1. Multivalent ion induced spots on phosphatidylserine/decane bilayers, as viewed in focused (convergence angle 2°) HeNe laser light (a–e) and in broad white light (f). In the first four photographs $[Ca^{2+}] = 10^{-3}$ M, and the angles of incidence and viewing, respectively, are (a) 45°, 45°, (b) 43°, 47°, (c) 40°, 50° and (d) 35° and 55°. In (e) the spots were removed by EDTA, $c = 2 \cdot 10^{-3}$ M; specular reflection at 45°. In (f) $[Ce^{3+}] = 10^{-5}$ M.

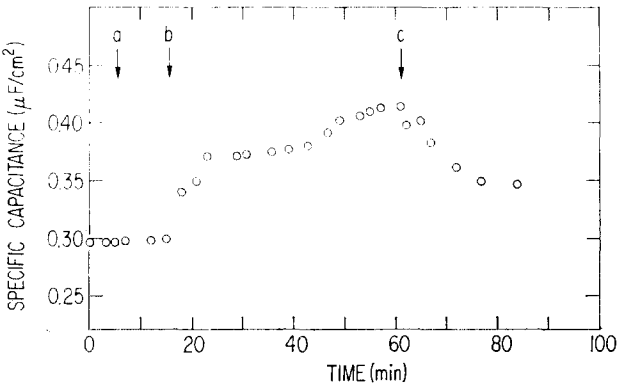

Fig. 2. Changes in the specific capacitance of a phosphatidylserine/decane bilayer. Marked arrows indicate: (a) stirring of the bathing solution; (b) addition of Ce^{3+} to $c = 3 \cdot 10^{-6}$ M, and (c) addition of EDTA to $c = 3 \cdot 10^{-5}$ M.

when instead of Ca^{2+} two orders of magnitude lower concentration of Ce^{3+} are added.

The spots show a distribution of sizes with the largest ones approximately 10 μm in diameter. The spots are not flat reflectors; as shown in the series of photographs in Fig. 1, considerable, light intensity is scattered even at angles 20° off from specular.

The visual appearance of the spots is very similar to the "microlenses" [12–14] that form spontaneously (that is without the addition of multivalent cations) in glycerol monooleate membranes, except for the absence of larger lenses showing multiple interference fringes. The similarity extends to the fact that no spots are formed upon addition of Ca^{2+} if the phosphatidylserine membranes are formed from a heptane solution. Following Haydon and coworkers [13, 14] we identify the spots as islands of decane that form when the lipid core of the membrane becomes supersaturated with hydrocarbon, heptane being soluble enough to escape through the aqueous phase. We conclude that multivalent cations reduce the solubility of hydrocarbons in the lipid region of phosphatidylserine membranes.

Supporting evidence for this picture was obtained from capacitance measurements. As Ca^{2+} or Ce^{3+} are added to the bathing solution, the specific capacitance of a phosphatidylserine bilayer membrane shows a stepwise increase, which is partially reversed by excess EDTA. A typical run is shown in Fig. 2. The multivalent cation induced increase in capacitance is interpreted as a decrease in thickness over the major part of the bilayer area which comes about as decane is extruded into the torus and into microlenses. We note that the specific capacitance of the phosphatidylserine bilayer is small even at its maximum compared with solvent-free bilayers of $C_{18}-C_{24}$ lecithins [16]. Since the fatty acid distribution of bovine phosphatidylserine is usually dominated by C_{18} acids [15], it appears likely that the amount of dissolved decane in the bilayer is substantial even in the presence of multivalent cations.

We have attempted to quantify the dependence of microlens formation on Ca^{2+} concentration. For this purpose we (1) counted the surface density of bright spots under fixed illumination conditions and (2) measured the flux of light scattered

in a direction not coinciding with the specular reflection. We were frustrated in achieving our goal in the first case by the broad distribution in size and brightness of the spots, and by the fact that the distribution, as in the studies of Requena et al. [14], appeared to change in time. In the second case the presence of imperfections in the bilayer, which were easily distinquishable from the microlenses by visual observations, made a large and variable contribution to the light scattered, and prevented the measurement of the flux due to scattering by microlenses at low ion concentrations. Thus while occasionally the microlens formation was elicited by concentrations as low as 10^{-4} M in Ca^{2+} or 10^{-6} M in Ce^{3+}, for reliable observations higher concentrations were used.

In order to further probe the nature of the cation-lipid interaction causing the extrusion of decane, we extended the observations to other multivalent ions and to other lipids. The results are summarized in Table I. The effectiveness of various ions to induce spot formation on phosphatidylserine/decane bilayer appears to depend to a good approximation, only on the number of positive charges carried. The presence of negative charge in the head-group of the phospholipid is also a requirement, as no spot formation was observed upon addition of Ca^{2+} to bilayers of phosphotidylcholine-cholesterol. On the other hand, the effect did occur when phosphatidylserine in the bilayer was diluted with phosphatidylcholine or cholesterol, or was replaced with another acidic lipid, phosphatidylinositol. It is clear that the change in decane solubility in the hydrocarbon portion of the bilayer is caused by electrostatic interactions involving the head groups.

The accumulation of multivalent cations in an interfacial region (be that

TABLE I

ION INDUCED MICROLENS FORMATION IN BILAYER MEMBRANES

Membrane forming solution				
Lipid (conc. mg/ml)	Solvent	Ion added (molar conc.)	Formation of spots	Removal by excess EDTA
PS(25)	decane	$Ca^{2+}(5 \cdot 10^{-4})$	+	+
PS(25)	decane	$Ba^{2+}(5 \cdot 10^{-4})$	+	[a]
PS(25)	decane	$Mg^{2+}(10^{-3})$	faint	+
PS(25)	decane	$Zn^{2+}(5 \cdot 10^{-4})$	+	[b]
PS(25)	decane	$Ce^{2+}(2 \cdot 10^{-6})$	+	+
PS(25)	decane	$La^{3+}(2 \cdot 10^{-6})$	+	[b]
PS(25)	heptane	$Ca^{2+}(5 \cdot 10^{-4}$ to $1.5 \cdot 10^{-3})$	—	—
PS(25)	heptane	$Ce^{3+}(2 \cdot 10^{-6}$ to $6 \cdot 10^{-6})$	—	—
PS(25)	1 : 1 decane heptane	$Ca^{2+}(1 \cdot 10^{-3})$	+	+
PS(25)	1 : 1 decane heptane	$Ce^{3+}(2 \cdot 10^{-6})$	+	[b]
PS(20)+Ch(10)	decane	$Ca^{2+}(2 \cdot 10^{-4})$	+	?
PS(20)+Ch(10)	decane	$Ce^{3+}(4 \cdot 10^{-6})$	+	+
PC(20)+Ch(10)	decane	$Ca^{2+}(5 \cdot 10^{-4}$ to $2 \cdot 10^{-3})$	—	—
PS(10)+PC(10)	decane	$Ca^{2+}(5 \cdot 10^{-4})$	+	[b]
PI(8)+Ch(4)	1 : 1 decane $CHCl_3$	$Ce^{3+}(4 \cdot 10^{-6})$	+	[b]

[a] Complexation constant too low to reduce free Ba^{2+} concentration significantly at pH 5.5.
[b] Membranes broke before disappearance of spots.
PS, phosphatidylserine, PC, phosphatidylcholine; PI, phosphatidylinositol; Ch, cholesterol.

diffuse or strongly adherent) between the bilayer and the bulk of the aqueous phase results in a screening of the negative charges of the phospholipid head groups. If the in-plane Coulombic repulsive forces constitute a significant contribution to the over-all interfacial tension, their diminution by screening by multivalent cation should alter the mechanical properties of the bilayer.

This is indeed observed. Bilayer membranes formed from phosphatidylserine/decane were bulged to less than hemispherical by applying a small hydrostatic pressure difference. Upon addition of Ce^{3+} to an approximate concentration of $3 \cdot 10^{-6}$ M we observed a flattening of the membrane, that is a contraction of its area. We estimate that the increase in the radius of curvature was at least three-fold. Ca^{2+} in a concentration of $8 \cdot 10^{-4}$ M acted on phosphatidylserine/decane bilayers in an entirely analogous way. The effect of both Ce^{3+} and Ca^{2+} was completely reversible by complexation with an excess of EDTA. In phosphatidylcholine-cholesterol/decane bilayers no change of bulging was observed upon addition of multivalent ions.

The effect of multivalent ion on phosphatidylserine decane/bilayers is symmetrical. That is bulged bilayers flatten whether the ions are introduced on the concave or the convex side, and no curvature is created when prior to addition the bilayer is flat.

It is known that the area requirement per phosphatidylserine headgroup is very different in the presence and absence of multivalent ions [17], and that the phosphatidylserine bilayer membrane is an effective barrier to ion transport*. The symmetry of the mechanical effect then implies that the bilayer expansion or contraction must be regarded as uptake or deposition of material from the torus. It also requires that the hydrocarbon core be fluid in the sense of being incapable of supporting shear stress.

DISCUSSION

The addition of multivalent cations to the solution bathing a negatively charged phosphaditylserine membrane decreases the magnitude of the surface potential of the membrane [19] as calculated from the Gouy-Chapman theory. In addition to the effect of these cations on the conductance [19] of charged membranes one also expects a change in the internal pressure of the membrane which may manifest itself as an upwards shift in the lipid transition temperature [1]. In any event the purely electrostatic effect of the multivalent cations, through the reduction of the repulsive forces in the membrane, should lead to a condensation of the membrane, that is, a reduction of the area per phosphatidylserine molecule. We interpret the occurrence of the microlenses as resulting from the reduced solubility of decane in the condensed film which initially, as formed in the purely monovalent solution, had been saturated with the decane.

In order to estimate the magnitude of the increased packing density of the phosphatidylserine molecules due to the addition of the multivalent cations one must consider the electrostatic contribution to the interfacial tension. If in the absence

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* Assuming that the increased conductivity of 1.25 $\Omega^{-1} \cdot cm^{-1}$ [18] of phosphatidylserine/decane in the presence of calcium ions at pH 5–6 is all due to transport by Ca^{2+}, and a membrane potential of 60 mV due to a concentration ratio of 10, to obtain a coverage of 140 A per Ca^{2+} on the back side of a phosphatidylserine bilayer would take $3 \cdot 10^4$ s.

of electrostatic repulsion the interfacial tension is γ, then if the stress due to the charges is denoted $-\pi_e$, the resulting interfacial tension is $\gamma' = \gamma - \pi_e$. π_e will depend on the composition of the solution. A change in π_e produces a change in packing density. If κ denotes the two dimensional compressibility of the membrane, then the fractional change in area per molecule due to the change $-\delta\pi_e$ in the electrostatic contribution to the stress is

$$\frac{\delta A}{A} = \kappa\delta\pi_e. \tag{1}$$

We obtain π_e from the Gouy-Chapman theory. It is convenient to consider the membrane as two monolayers back-to-back. Under conditions in which a surface has a fixed surface potential, the free energy per unit area of the associated double layer, which is the energy of charging, is a function of the surface potential Ψ_0 [20]. If we denote the free energy density for this condition as F_ψ, then the total energy of charging of a surface of area S is $F_\psi S$. The work done by the charged system when the area is changed by δS is $-F_\psi\delta S$ so one has the electrostatic contribution to the surface stess $-F_\psi$. This is the result used by Davies [21]. For a phosphatidylserine membrane, however, constant total charge appears to be the more relevant situation [19]. At constant total charge the area density of the energy of charging can be written as [22]

$$F_\sigma = \int_0^1 \frac{d\lambda}{\lambda} 2U(\lambda) \tag{2}$$

where $U(\lambda)$ is the electrostatic contribution to the internal energy of the system at the stage λ in the charging process. Now the electrostatic contribution of the free energy of an area S of interface is $F_\sigma S$. The work done by the charged system when the area is changed by δS is $-[\partial(F_\sigma S)/\partial S]\delta S$. Thus the electrostatic contribution to the surface stress is

$$\pi_e = -\frac{\partial(F_\sigma S}{\partial S} = -S\frac{\partial F_\sigma}{\partial S} - F_\sigma \tag{3}$$

At fixed total charge (that is, with no change in the dissociation of the phosphatidylserine head groups), $\delta(\sigma S) = \sigma\delta S + S\delta\sigma$, one can put $S\partial F_\sigma/\partial S = -\sigma\partial F_\sigma/\partial\sigma$ and so

$$\pi_e = \sigma\frac{\partial}{\partial\sigma}F_\sigma - F_\sigma. \tag{4}$$

It is shown in the appendix that it follows from the Gouy-Chapman treatment of the double layer that

$$\sigma\frac{\partial}{\partial\sigma}F_\sigma - F_\sigma = 2U \tag{5}$$

and hence

$$\pi_e = 2U \tag{6}$$

where U is the internal energy (not the free energy) per unit area of the double layer

and this is valid regardless of the composition of the salt solution. At a surface potential that is large compared with kT/e, the two results for π_e are the same in the crudest approximation but the distinction is not generally negligible.

At high surface potential in a 1:1 salt solution one obtains approximately*

$$\pi_e = (2kT/e)|\sigma|,$$

while in a z-1 solution one obtains

$$\pi_e = (2kT/ze)|\sigma|.$$

Thus in going from a NaCl solution to a $CaCl_2$ solution the stress on each surface of the membrane changes by $kT|\sigma|/e$. A more accurate numerical calculation of the effect of adding 1 mM of $CaCl_2$ to a 0.1 M solution of NaCl bathing a phosphatidylserine membrane yields about half of this. Thus, if one takes the phosphatidylserine membrane as having one electronic charge per 50Å [2], one obtains for the effect of the addition of the $CaCl_2$ a change $\delta\pi_e \approx 4.2$ dynes/cm. The two-dimensional compressibility of a condensed monolayer of dipalmitoyl phosphatidylcholine on water has been found to be $4 \cdot 10^{-3}$ cm/dyne [23]. If this value is assumed for the phosphatidylserine membrane one obtains as a result of the addition of the Ca^{2+} a fractional change in area of between 1 and 2 %. We then expect that approximately this fraction of the dissolved decane will be forced out of the membrane interior into microlenses.

With a multicomponent material such as natural phosphatidylserine it is possible that with the addition of Ca^{2+} or Ce^{3+} lateral phase separation occurs with some components undergoing the transition to their low temperature phase, with a subsequent reduction of solubility of decane, but this is not a necessary consequence of our observations.

It appears well established both experimentally and theoretically that the multivalent ion concentration in the aqueous phase strongly influences the packing and composition of the hydrocarbon core of the bilayer. One can speculate whether the reverse of this effect, that is the release of Ca^{2+} from an acidic phospholipid layer should be observable if the hydrocarbon core is expanded by some other means. Perhaps the easiest test would be to measure the extent of Ca^{2+} binding to a phosphatidylserine monolayer by following surface radioactivity [24] while the packing and composition of the hydrocarbon section is varied by changing the partial pressure of a volatile hydrocarbon in gas phase. This inverse process may be relevant to the mechanism of the translation of the photochemical conformation changes of rhodopsin embedded in the hydrocarbon core of the disc membrane into the release of calcium ions which has been postulated as an amplification stage in vision [25].

APPENDIX

Proof of Eqn. 4

The free energy of the double layer is the energy of charging. That is to say,

* If this result is employed in the two-dimensional Clausius-Clapeyron equation for the lipid phase transition one recovers the Träuble and Eibl analysis [1], of the effect of Ca^{2+} on transition temperature.

all charges, both in solution and on the surface, are set equal to λ times their final value and the work done in bringing the charges to their final values is calculated. For a surface of fixed charge density the result can be written

$$F_\sigma = \int_0^1 \frac{d\lambda}{\lambda} 2U(\lambda) \tag{A.1}$$

where $U(\lambda)$, electrostatic energy per unit area, is given by

$$2U(\lambda) = \frac{\varepsilon}{4\pi}\int_0^1 dx \left(\frac{d\psi}{dx}\right)^2 = \frac{\varepsilon}{4\pi}\int_{\psi_s}^0 d\psi \frac{d\psi}{dx} \tag{A.2}$$

Here ψ is the potential at the stage λ in the charging process and $\psi_s(\lambda)$ is the surface potential. In the Gouy-Chapman theory, for any mixture of salts regardless of the valences, $d\psi/dx$ is a function of $\lambda\psi$ and $\lambda\psi_s$ is a function of $\lambda\sigma$. It follows from Eqn. A.2 that $2U(\lambda)$ if of the form

$$2U(\lambda) = \frac{1}{\lambda}\phi(\lambda\sigma) \tag{A.3}$$

where ϕ is some function of $\lambda\sigma$. Therefore Eqn. A.1 can be written

$$F_\sigma = \int_0^1 \frac{d\lambda}{\lambda^2}\phi(\lambda\sigma) \tag{A.4}$$

The use of this form for F_σ in Eqn. 4 then yields

$$\pi_e = +\sigma\frac{d}{d\sigma}\int_0^1 \frac{d\lambda}{\lambda^2}\phi(\lambda\sigma) - F_\sigma. \tag{A.5}$$

The derivative with respect to σ can be taken inside the integral. The use of the relation

$$\frac{\partial}{\partial\sigma}\phi(\lambda\sigma) = \frac{\lambda}{\sigma}\frac{\partial}{\partial\lambda}\phi(\lambda\sigma) \tag{A.6}$$

then yields

$$\pi_e = +\int_0^1 \frac{d\lambda}{\lambda}\frac{\partial}{\partial\lambda}\phi(\lambda\sigma) - F_\sigma \tag{A.7}$$

and partial intergration leads to the result

$$\pi_e = +2U \tag{A.8}$$

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