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THE EFFECTS OF COTYLEDON SENESCENCE ON THE COMPOSITION AND PHYSICAL PROPERTIES OF MEMBRANE LIPID

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Summary

The phospholipid content of rough and smooth microsomal fractions from cotyledons of germinating bean declines as the tissue becomes senescent. Both types of membrane contain comparable proportions of three major phospholipids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol, which collectively comprise about 90% of the total. This proportionality does not change appreciably during senescence. Only small quantities of lysophosphatides were noted at all stages of senescence. The unsaturated : saturated fatty acid ratio for total extracted lipid declined only slightly in both membrane systems, but pronounced differences in this ratio were observed among the major phospholipids of the membranes. The most striking alteration in lipid composition with advancing senescence was an increase in the sterol : phospholipid ratio; this rose by about 50% for rough microsomes and 400% for smooth microsomes. For both types of membrane the patterns of change in this ratio correlated with previously reported changes in bulk lipid transition temperature, suggesting that the increase in sterol level may contribute to changes in phase behaviour of the membranes during senescence.

Arrhenius plots of rotational correlation times for the electron spin label 2,2-dimethyl-5-dodecyl-5-methyloxazolidine-*N*-oxide (2N14) partitioned into the membrane lipid showed an increase in viscosity with advancing senescence and a corresponding increase in activation energy for both types of membrane. These changes in activation energy and viscosity correlated closely with the increase in sterol : phospholipid ratio. However, no phase transitions were detectable between temperatures of 2 and 55°C despite the fact that transitions from a liquid-crystalline to gel state are detectable within this temperature range by wide angle X-ray diffraction.

Abbreviation: 2N14, 2,2-dimethyl-5-dodecyl-5-methyloxazolidine-*N*-oxide.

Introduction

Cotyledons of *Phaseolus vulgaris* become senescent during the later stages of germination, and this is accompanied by major structural and metabolic modifications within the cells of the tissue. Initially, protein and starch reserves, which are abundant in cotyledon tissue of the ungerminated seed, become hydrolyzed and the hydrolysis products are translocated to the developing axis [1]. Metabolic activity is high in the young cotyledons, but as germination progresses and senescence of the tissue sets in, there is a general deterioration of cytoplasmic structure which culminates in complete autolysis of the cell cytoplasm and eventual death and abscission of the organ [1,2].

Microsomal membranes from this tissue are highly sensitive to the pressures of senescence. The activities of several microsomal enzymes decline dramatically during the later stages of germination and this appears to be accompanied by a progressive structural disassembly of the membranes [3]. We have also reported that with advancing senescence an increasing proportion of microsomal membrane lipid becomes crystalline at physiological temperature [4,5]. The enzyme activity and permeability properties of membranes are known to be modulated by the physical properties of the lipid matrix [6–8], and it is conceivable that such changes in phase behaviour of the lipid could contribute to the loss of membrane function and alterations in membrane permeability accompanying senescence. Many factors, both extrinsic and intrinsic, are known to influence the physical properties of lipid bilayers [9,10]. These include changes in lipid composition such as lysophosphatide formation [11, 12], alterations in fatty acid chain length and saturation [9,10] and in sterol content [9]. In the present study we have examined the extent to which the lipid composition of cotyledon microsomal membranes is altered by senescence and whether such alterations can be correlated with changes in physical properties of the membrane lipid.

Materials and Methods

Growth conditions and membrane isolation. Seeds of *Ph. vulgaris* (variety kinghorn) were germinated under etiolating conditions at 29°C in moist vermiculite. Cotyledons were harvested after 2-, 4-, 7- and 9-days of germination, and rough and smooth microsomes were isolated as previously described [3]. The tissue was homogenized in 0.3 M sucrose/0.05 M $NaHCO_3$ (pH 7.0) to yield a 33% (w/v) homogenate. The supernatant obtained after centrifugation at 10 000 × *g* for 20 min was made 15 mM with CsCl and layered over 4 ml of 1.3 M sucrose/15 mM CsCl in cellulose nitrate tubes. These tubes were centrifuged in a Beckman Type 50 Ti rotor at 165 000 × *g* for 3 h. Smooth microsomes collected at the interface and the rough microsomes formed a pellet at the bottom of the tube. The smooth microsomal fraction was removed from the gradient, diluted with 0.05 M $NaHCO_3$, pH 7.0, and pelleted by centrifugation at 165 000 × *g* for 1 h.

Lipid extraction. Lipids were extracted from the membrane pellets using the procedure of Nichols [13]. The membrane pellets from 20 g of tissue were resuspended in 10 ml of isopropanol using a Potter-Elvehjem homogenizer. The

suspension was centrifuged at 10 000 × *g* for 10 min and the pellet resuspended in 10 ml of chloroform/isopropanol (2 : 1, v/v). The pellet was then extracted three times with 10 ml of chloroform/methanol (2 : 1, v/v). The combined extracts were evaporated to dryness in vacuo. The residue was resuspended in 10 ml of chloroform/methanol and partitioned with 2 ml of 0.7% NaCl as described by Folch et al. [14]. The upper wash phase was discarded and the bottom phase was again evaporated to dryness in vacuo. The residue was resuspended in a small volume of chloroform/methanol and stored at −20°C under N_2 until analyzed.

Phospholipid analysis. The individual phospholipids were separated by thin-layer chromatography using prewashed, heat-activated Bakerflex 1B2-F plates coated with a 200 μm thick layer of silica gel, and developed in chloroform/methanol/acetic acid/water (13 : 3 : 3 : 2, v/v). The lipid spots were visualized by iodine vapour or by H_2SO_4 charring. The phospholipids were identified by their reactions with specific spray reagents, molybdenum blue, ninhydrin and dragendorf reagents [15], and by co-chromatography with authentic lipid standards (Sigma). For quantitative analysis the phospholipids were separated by thin-layer chromatography, detected by I_2 vapour, scraped off the plate and eluted from the silica gel in a Whatman Extraction Thimble with 4 ml of developing solvent, 2 ml of methanol and 2 ml of methanol/acetic acid/water (94 : 1 : 5, v/v) sequentially. The eluants were evaporated to dryness and digested with $HClO_4$. Inorganic phosphate was determined by the method of Fiske and Subba Row as outlined by Dittmer and Wells [16].

Fatty acid analysis. Fatty acid methyl esters were prepared from the total lipid extract as described by Morrison and Smith [17]. A volume of lipid extract containing at least 1 μmol of phospholipid phosphorus was evaporated to dryness under N_2. The sample was dissolved in 1 ml of 14% BF_3 in methanol (J.T. Baker), sealed under N_2 and heated at 90°C for 90 min. The methyl esters were extracted with 2 ml of pentane/water (2 : 1, v/v). The presence and purity of the fatty acid methyl esters were confirmed using thin-layer chromatography. The analysis was carried out on a Perkin-Elmer model 900 gas chromatography equipped with a flame ionization detector and using a 6 ft × 4 mm stainless steel column of 10% EGSS-X (ethylenesuccinatemethylsilicone copolymer) on 100—120 mesh Supelcoport at 170°C. Integration was performed using a mechanical disc integrator.

The fatty acid composition of the individual phospholipids was determined in the same manner after the phospholipid classes had been separated by thin-layer chromatography and detected by Rhodamine 6G.

Sterol analysis. Free sterols were prepared for analysis by gas-liquid chromatography using a method based on that of Grunwald [18]. A neutral lipid fraction, collected as the chloroform eluant from a silica gel column, was dissolved in 4 ml of acetone/ethanol (1 : 1, v/v). The free sterols were precipitated overnight with 2 ml of 0.5% digitonin. The sterol · digitonin complex was washed once with 3 ml of diethyl ether/acetone (1 : 1, v/v), twice with 3 ml of diethyl ether and was then dried under N_2. The internal standard cholestane was added in 2 ml of pyridine and the mixture was heated to 60°C for 1 h and left overnight at room temperature to break the sterol · digitonin complex. The digitonin was removed by precipitation with 25 ml of diethyl ether. The super-

natant, after centrifugation at 10 000 × *g* for 30 min, was evaporated to dryness. The resulting sterol residue was derivatized in equal volumes of acetonitrile and *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) for at least 1 h at room temperature. The trimethylsilyl derivatives were analyzed on a Perkin-Elmer model 900 gas chromatograph equipped with a flame ionization detector and a 6 ft × 2 mm glass column of 3% OV-17 on 100—120 chromosorb W at 255°C. Peak areas were determined with a mechanical disk integrator.

Total phospholipid content of the sample was determined on the lipid extract prior to preparation of the neutral lipid fraction by the method of Fiske and Subba Row, as outlined by Dittmer and Wells [16].

Electron spin resonance. The spin 2N14 (2,2-dimethyl-5-dodecyl-5-methyl-oxazolidine-*N*-oxide) used in this study has been described previously [19]. It consists of an alkane chain 14 carbons long with a nitroxide radical containing an oxazolidine ring attached to the second carbon. In membranes it undergoes isotropic rotation and a motional parameter, the rotational correlation time (τ_c), can be calculated from the electron spin resonance spectrum [19]. The τ_c was calculated from the relation [20]

$$\tau_c = 6.5 \cdot 10^{-10} W_1 \left[\left(\frac{h_1}{h_{-1}} \right)^{1/2} - 1 \right]$$

where W_1 is the low field line width measured from the first derivative spectrum and h_1 and h_{-1} are the low and high field line heights, respectively. The value of the constant was calculated from the spectral parameters of Libertini and Griffith [21]. The rotational correlation time of the spin label can be related to the viscosity, η, of its environment by the Stokes' relation $\tau_c = 4\pi r^3 \eta / {}_3KT$ as previously described [22,23].

The 2N14 was dissolved in ethanol and 0.04 μmol of the label were dried onto the inside of a small glass tube with a stream of nitrogen. A portion of membranes, which had been packed into a pellet by centrifugation, equivalent to about 25 mg of membrane protein was then added to the tube and the mixture was vortexed for 3 min. It has been previously demonstrated that under these conditions all of the label partitions into the membranes [19].

Results

Phospholipid analysis

To determine the extent to which cytoplasmic membranes actually break down in the face of intensified senescence, total levels of phospholipid in the microsomal fractions isolated from 20 g of tissue were determined at various ages. The data illustrated in Fig. 1 indicate clearly that microsomal phospholipid does decrease as germination advances. Both rough and smooth subfractions showed the greatest diminution between days 2 and 4, although the smooth membranes deteriorated more rapidly than the rough during this period.

Rough and smooth microsomal membranes from 2-day old cotyledons contained three major phospholipids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (Table I). Several other phospholipids, collectively comprising less than 10% of the total, were also detectable. These

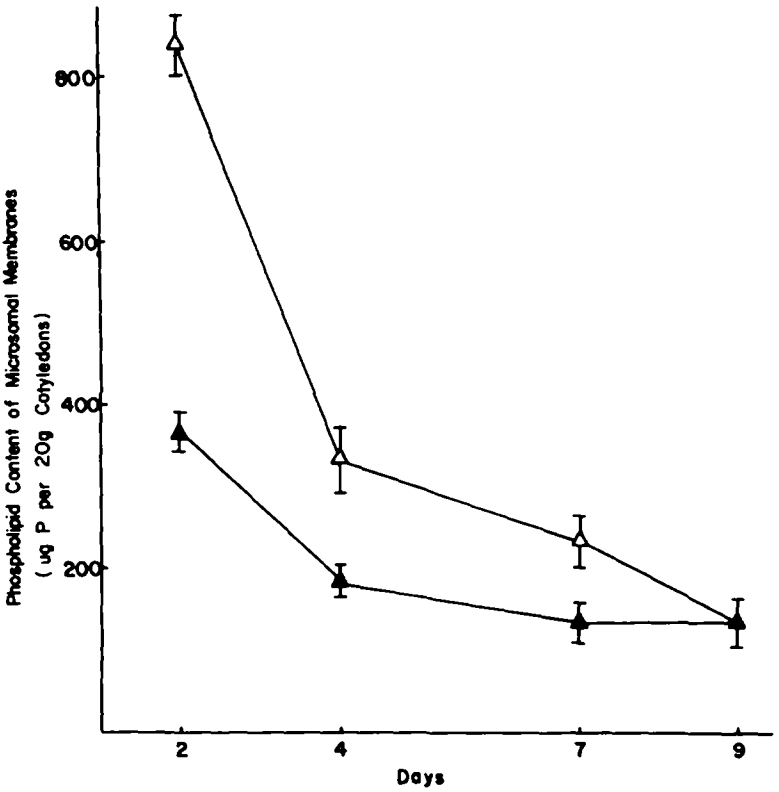

Fig. 1. Changes during senescence in total phospholipid recovered in the microsomal membranes isolated from 20 g cotyledon tissue. △, Smooth microsomes; ▲, rough microsomes. Standard errors of the means are indicated; $n = 3$—5.

TABLE I

PHOSPHOLIPID COMPOSITION OF MICROSOMAL MEMBRANES FROM SENESCING COTYLEDON TISSUE

Values shown are the means of three separate determinations. Least significant differences determined at the 1% level of significance after a one-way analysis of variance are indicated. PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol. Results are expressed in μmol/0.1 mmol total phospholipid.

Membrane	Age (days)	PC	LPC	PE	LPE	PI	PG	DPG
Smooth	2	47.3	1.6	24.1	3.0	21.2	1.8	1.0
	4	51.6	1.3	23.1	2.8	18.6	1.5	1.1
	7	49.0	1.2	23.9	3.2	17.1	3.0	1.8
	9	50.4	0.8	21.1	6.8	14.9	2.4	3.6
Rough	2	39.8	1.3	27.9	3.0	21.7	2.8	3.4
	4	43.7	1.3	27.9	2.6	16.2	3.4	5.0
	7	44.2	0.2	29.2	2.5	15.3	4.4	4.3
	9	41.2	1.6	26.9	5.6	15.2	4.6	4.8
Least significant difference		5.0	1.6	3.9	1.9	2.8	2.7	0.8

included lysophosphatidylcholine, lysophosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (Table I). For young 2-day old tissue the relative proportions of these phospholipids proved to be similar for the two membrane systems except for phosphatidylcholine, which was significantly higher in the smooth membranes. This proportionality did not change appreciably during senescence, and the changes that did occur were closely parallel for the rough and smooth membranes. For example, the level of phosphatidylinositol declined with senescence for both membrane systems (Table I). Lysophosphatide levels, which might be expected to rise in an autolysing system, did not change appreciably except for an increase in lysophosphatidylethanolamine from 3 to 6% between days 7 and 9 (Table I). Significant levels of phosphatidic acid were not detected.

Fatty acid composition

Both rough and smooth microsomal membranes were found to contain five major fatty acids, palmitic, linoleic, linolenic, oleic and stearic acid (Fig. 2). Others including arachidic and palmitoleic were also detectable, but accounted for less than 1% of the total fatty acid complement. The proportionality among the major fatty acids for the smooth microsomal fraction was comparable to that for the rough membranes, and changes in this proportionality during senescence were also quite similar for the two types of membrane (Fig. 2). The major changes occurred in the unsaturated 18-carbon fatty acids, and in effect comprised a reduction of linolenic and oleic acids and an increase in linoleic. These changes in proportionality did not, however, have any appreciable impact on the degree of lipid saturation. The unsaturated : saturated fatty acid ratio for the total lipid extract decreased slightly between days 2 and 4 as a result of the slight rise in palmitic acid and thereafter remained essentially constant (Table II).

There were, however, quite pronounced differences in the degree of saturation among the major phospholipids of both rough and smooth microsomes. For example, throughout the period of senescence the unsaturated : saturated fatty acid ratio for phosphatidylcholine remained 2—3-fold greater than that for phosphatidylinositol in smooth microsomes and about 2-fold greater in rough microsomes (Table II). This occurred despite changes in proportionality among the fatty acids of the phospholipids (Figs. 3 and 4). For example, in the case of phosphatidylcholine there was a notable increase in both palmitic and linoleic acids with advancing senescence, but this was compensated for by a decline in linolenic and oleic (Figs. 3A and 4A). Similarly, for phosphatidylethanolamine linoleic acid increased and linolenic and oleic acids both decreased, but palmitic remained constant (Figs. 3B and 4B). Palmitic acid comprised close to 50% of the total fatty acid complement in phosphatidylinositol for both membrane systems, thus accounting for the low unsaturated to saturated fatty acid ratio by comparison with phosphatidylcholine and phosphatidylethanolamine (Table II and Figs. 3C and 4C). The decrement in this ratio between days 2 and 4 for the phosphatidylinositol of smooth microsomes can be largely attributed to an increase in palmitic acid and a decrease in linolenic acid during this period.

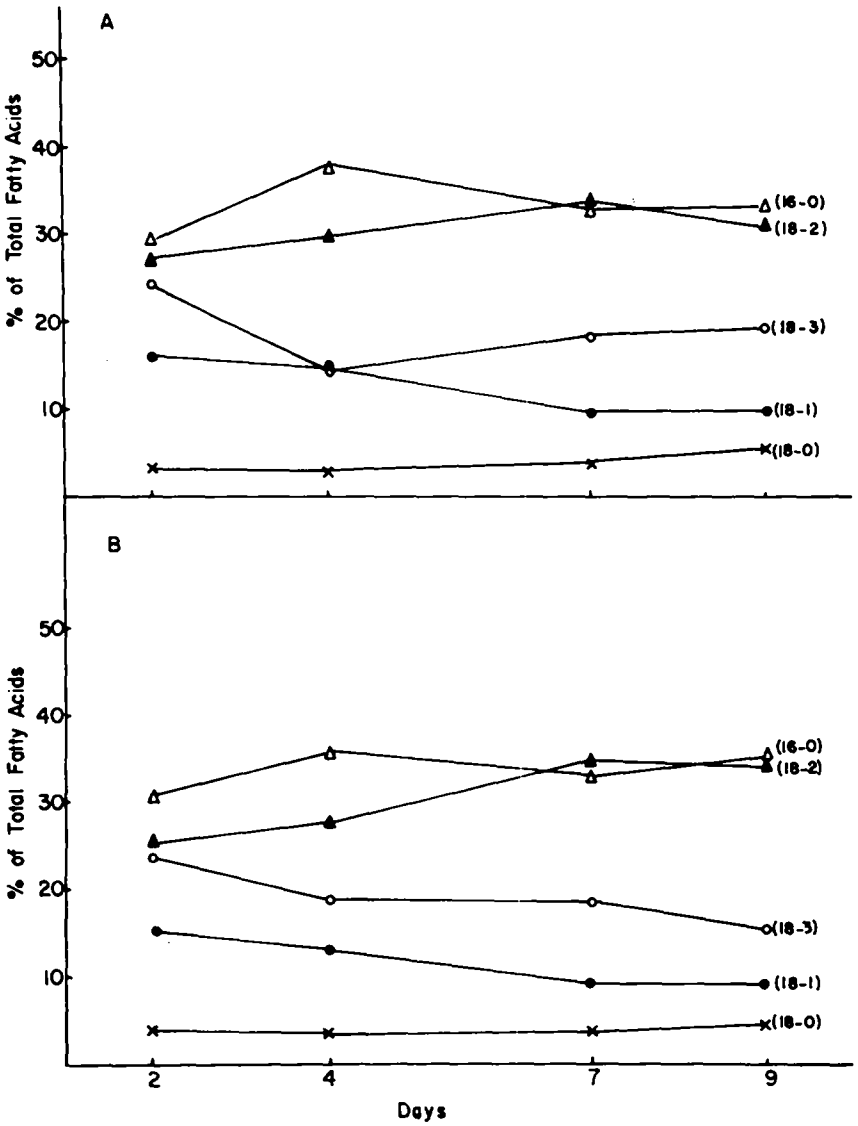

Fig. 2. Fatty acid composition of smooth (A) and rough (B) microsomal membranes from senescing cotyledon tissue. Values shown are the means of 3—6 separate determinations. Least significant differences determined at the 1% level of significance after a one-way analysis of variance are 3.7% for palmitic, 1.0% for stearic, 2.8% for oleic, 2.0% for linoleic and 4.1% for linolenic acid.

Sterol composition

Both rough and smooth microsomal membranes contained substantial amounts of free sterols. For 2-day old tissue stigmasterol, β-sitosterol and isofucosterol collectively comprised more than 90% of the total free sterol, campesterol about 5% and cholesterol less than 1% (Table III). Moreover, their relative proportions were virtually identical in the rough and smooth membranes, and although there were changes in this proportionality during senescence the changes were closely parallel for the two types of membrane (Table III). Stig-

TABLE II

UNSATURATED : SATURATED FATTY ACID RATIOS FOR TOTAL LIPID EXTRACTS AND THE MAJOR PHOSPHOLIPIDS OF ROUGH AND SMOOTH MICROSOMAL MEMBRANES FROM SENESCING COTYLEDON TISSUE

The values for total lipid extract are means of 3—6 separate determinations and those for the separated phospholipids are means of two separate determinations. Values for the separate determinations had a range of less than 0.20.

Membrane	Extract	Ratio			
		2 day	4 day	7 day	9 day
Smooth microsomes	Total	2.08	1.43	1.64	1.51
	Phosphatidylcholine	2.94	2.15	2.11	1.91
	Phosphatidylethanolamine	1.83	1.70	1.85	1.72
	Phosphatidylinositol	1.09	0.85	0.88	0.82
Rough microsomes	Total	1.90	1.51	1.67	1.44
	Phosphatidylcholine	2.19	2.02	1.71	1.80
	Phosphatidylethanolamine	1.57	1.52	1.61	1.44
	Phosphatidylinositol	1.03	0.93	0.94	0.92

masterol increased almost linearly from 31% of the total at day 2 to about 46% of the total at day 9. Isofucosterol levels declined from 18% at day 2 to about 8% at day 9. β-Sitosterol levels also dropped, but less dramatically; campesterol remained essentially unchanged and cholesterol levels rose slightly as senescence intensified (Table III).

By far the most striking change in lipid composition with age was a dramatic increase in the total level of free sterols in the membranes (Fig. 5). For smooth microsomes the sterol : phospholipid ratio rose from a value of 63 μmol sterol/mmol phospholipid at day 2 to 241 by day 9. The ratio also increased for rough microsomes from 149 at day 2 to 222 at day 9. However, the temporal patterns of change were different for the two types of membranes. For rough microsomes the increase in sterol : phospholipid ratio occurred between days 2 and 4, with virtually no change thereafter (Fig. 5). By contrast the smooth microsomes exhibited a progressive increase in this ratio throughout senescence, although the rate of increase was greatest in more senescent tissue (Fig. 5).

Electron spin resonance

The spin label 2N14 was partitioned into rough and smooth microsomes isolated from 2-, 4-, 7- and 9-day old tissue and spectra were obtained at temperatures ranging from 2 to 55°C. Arrhenius plots of τ_c for membranes from 4-day old tissue are illustrated in Fig. 6. These are representative of those obtained for both rough and smooth microsomes from all ages of tissue in that they show no discontinuities or changes in slope. Consequently, the lipid regions of the membranes being probed by 2N14 do not appear to undergo a phase transition between 2 and 55°C.

The slope of the Arrhenius plot can be expressed as an Arrhenius activation energy (E_a), and a comparison of these values indicates a general increase in E_a during senescence for both rough and smooth microsomes (Fig. 7). However, the patterns of change were different for the two types of membranes. For

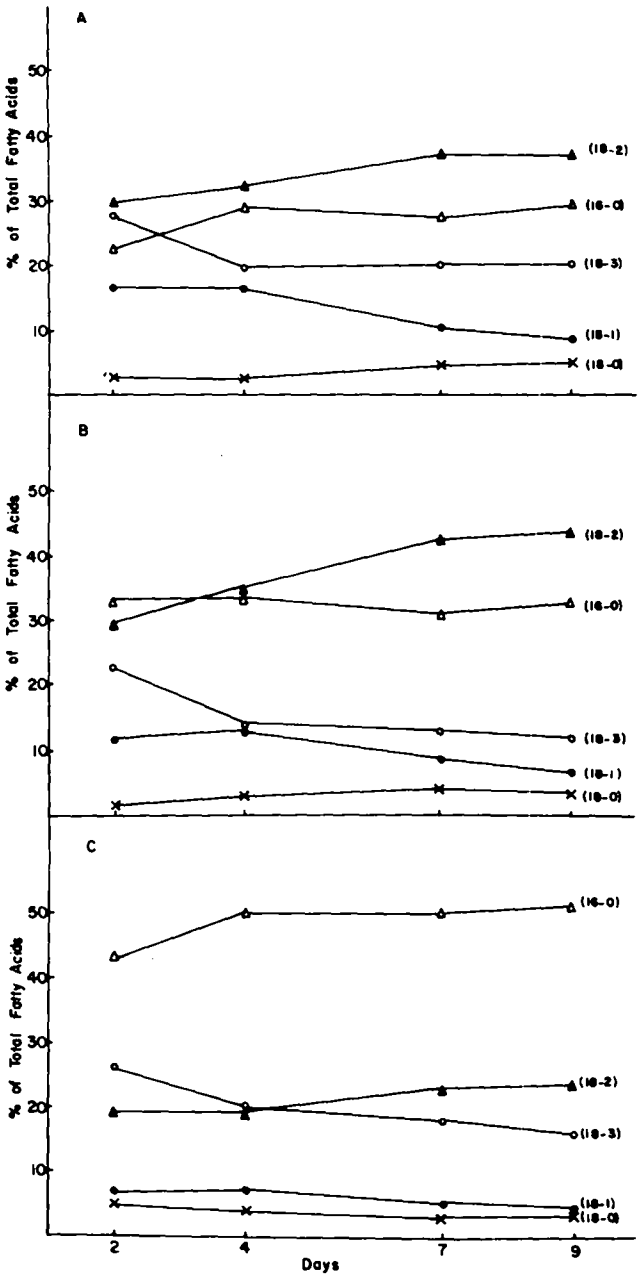

Fig. 3. Fatty acid composition of phosphatidylcholine (A), phosphatidylethanolamine (B) and phosphatidylinositol (C) from smooth microsomal membranes of senescing cotyledon tissue. Values shown are means of two separate determinations and are expressed as a percentage of the total fatty acid content of each phospholipid. Values for the separate determinations had a range of less than 4%.

rough microsomes E_a increased between days 2 and 4, and thereafter remained essentially constant. By contrast, the smooth microsomes showed no change during the early stages of germination, but between days 4 and 9 the value of E_a increased by about 75% (Fig. 7).

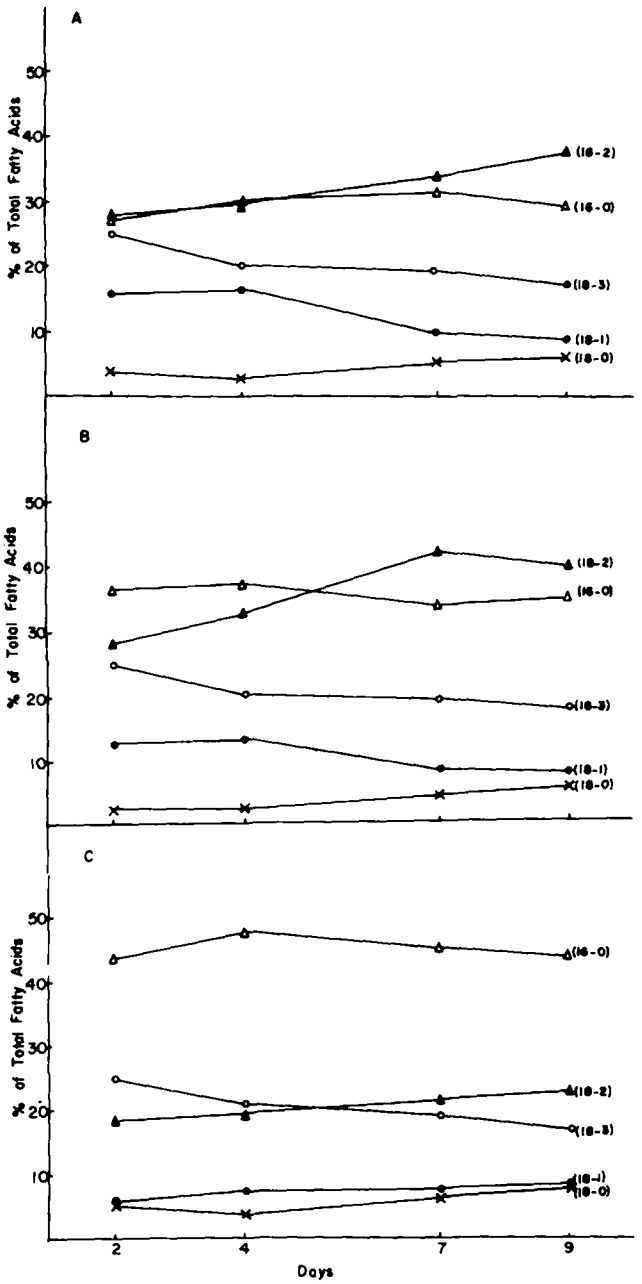

Fig. 4. Fatty acid composition of phosphatidylcholine (A), phosphatidylethanolamine (B) and phosphatidylinositol (C) from rough microsomal membranes of senescing cotyledon tissue. Values shown are for two separate determinations and are expressed as a percentage of the total fatty acid content of each phospholipid. Values of the separate determinations had a range of less than 4%.

The values for τ_c at 29°C give a relative indication of the viscosity at the growth temperature of the lipid region being probed, and the two types of membranes showed different patterns of change in this parameter during senes-

TABLE III

STEROL COMPOSITION OF MICROSOMAL MEMBRANES FROM SENESCING COTYLEDON TISSUE

Values shown are the means of three separate determinations. Least significant differences determined at the 1% level after a one-way analysis of variance were less than 1. Results are expressed in mol% of total sterol.

Membrane	Age	Cholesterol	Campesterol	Stigmasterol	β-Sitosterol	Isofucosterol
Smooth	2	0.5	4.6	31.7	44.7	18.6
	4	0.4	4.5	35.1	43.5	16.5
	7	1.1	4.9	44.6	39.0	10.3
	9	1.3	4.7	45.8	39.5	8.8
Rough	2	0.3	4.9	31.1	45.3	18.4
	4	0.3	5.3	36.0	42.6	15.9
	7	1.1	5.4	45.4	38.8	9.4
	9	2.4	5.0	47.7	37.5	7.4

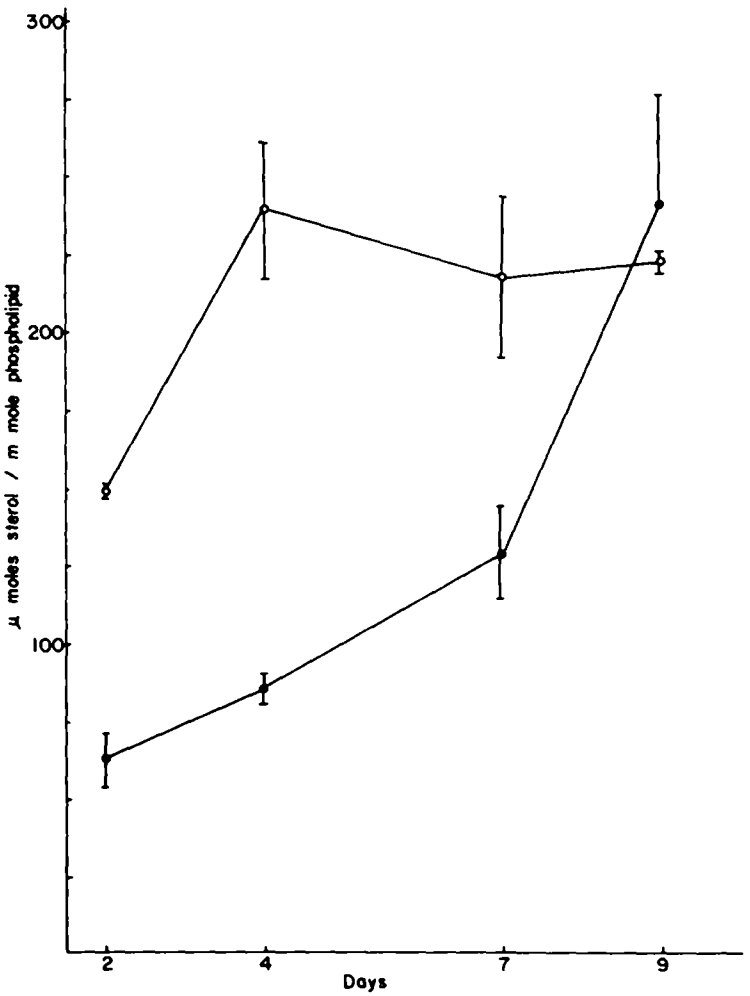

Fig. 5. Changes during senescence in the sterol : phospholipid ratio of microsomal membranes from cotyledon tissue. Ratios are expressed as μmol total free sterol per nmol phospholipid. ●, Smooth microsomes; ○ rough microsomes. Standard errors of the means are indicated; n = 3—5.

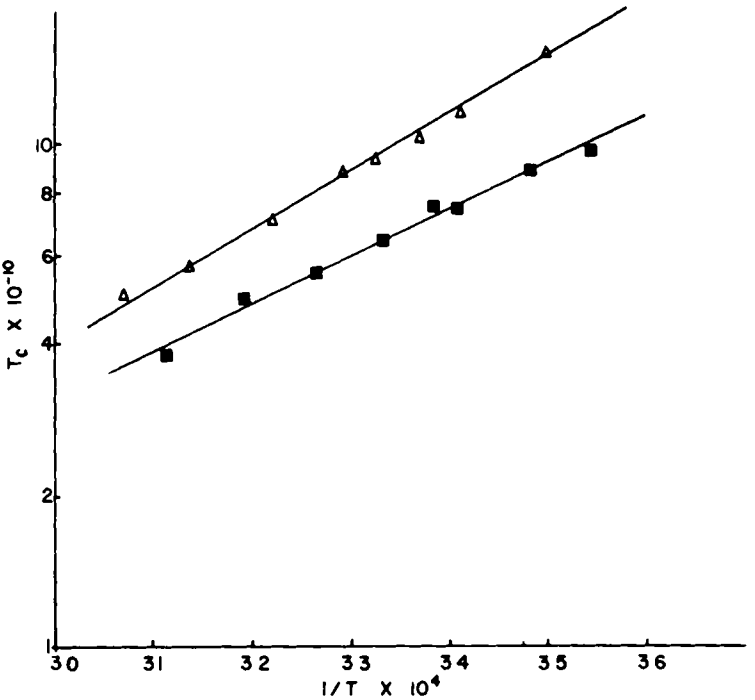

Fig. 6. Arrhenius plots of the rotational correlation time (τ_c) for microsomal membranes from 4-day old cotyledons. The spin label 2N14 was partitioned into the membranes. τ_c is expressed in s. ■, Smooth microsomes; △, rough microsomes.

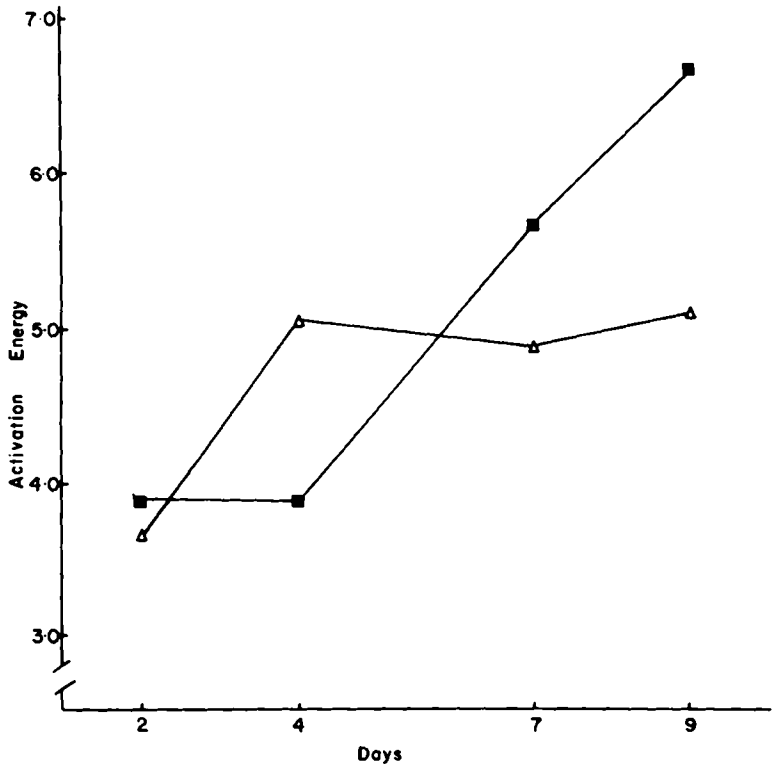

Fig. 7. Changes in activation energy (E_a) for microsomal membranes from cotyledons harvested at various stages of senescence. The spin label 2N14 was partitioned into the membranes. E_a is expressed as kilocalories/degree per mol. The data were analyzed by a one-way analysis of variance and determined significant at the 5% level. Least significant difference is 1.15. n = 3. ■, Smooth microsomes; △, rough microsomes.

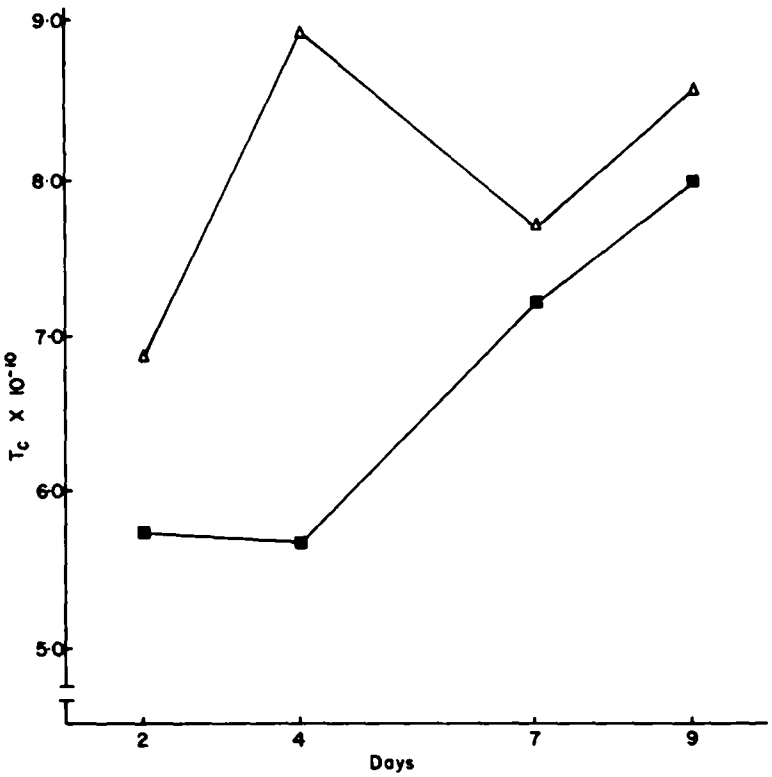

Fig. 8. Changes in the viscosity of microsomal membranes from cotyledons harvested at various stages of senescence. The spin label 2N14 was partitioned into the membranes. Rotational correlation time (τ_c) is expressed in s at 29°C. The data were analyzed by a one-way analysis of variance and determined significant at the 5% level. Least significant difference is 1.39. n = 3. ■, Smooth microsomes; △, rough microsomes.

cence. For rough microsomes the viscosity increased significantly between 2 and 4 days but then fluctuated, decreasing between days 4 and 7 and increasing again by day 9 (Fig. 8). The lipid viscosity of smooth microsomes remained essentially constant between days 2 and 4, but then increased progressively through to day 9. However, throughout the period of senescence the smooth microsomes remained more fluid than corresponding rough microsomes (Fig. 8).

Discussion

It has been previously reported for this tissue that the activities of four microsomal enzymes, glucose-6-phosphatase, 5′-nucleotidase, NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase, decline as germination progresses and the tissue becomes senescent [3]. In addition, wide-angle X-ray diffraction of these membranes has provided clear evidence that with advancing senescence an increasing proportion of the membrane lipid is converted from a liquid-crystalline (fluid) phase to a gel (crystalline) phase at physiological temperature [4,5]. For rough and smooth microsomes from 2-day old tissue the transitions occur at 0 and 3°C, respectively. By the fourth

day of germination the transition temperatures have increased to 32°C for smooth microsomes and 35°C for rough microsomes. During the later stages of germination the temperature at which the transition occurs continues to rise for smooth microsomes through 44°C at day 7 to 56°C at day 9. By contrast the rough microsomes show little change in transition temperature during this later period [5].

It is now clear that these structural and functional alterations are accompanied by a marked reduction in the phospholipid content of both rough and smooth microsomal fractions, indicating that the lipid bilayers of the membranes comprising these fractions break down in the face of intensified senescence. However, despite this disassembly, the proportionality among the various phospholipids remains relatively unchanged for both types of membrane. Moreover, with the exception of some increase between days 7 and 9, lysophosphatide levels do not change appreciably and do not correlate with either loss of phospholipid (compare Fig. 1 and Table I) or the changes in lipid transition temperature reported previously [5].

There is, however, a substantial concentration of free sterols within the microsomal membranes during senescence. The sterol : phospholipid ratio increased by about 50% between days 2 and 9 in the rough microsomes and by close to 400% in the smooth microsomes. These altered ratios may partly reflect a more rapid loss of phospholipids than of sterols from the membranes, but this is unlikely to be the sole cause because the temporal changes in sterol to phospholipid ratio and in total phospholipid levels are not in synchrony. For example, the decrease in phospholipid content is greatest between days 2 and 4 in the germination sequence, yet for smooth membranes, at least, the greatest increase in sterol : phospholipid ratio occurs between days 7 and 9 (compare Figs. 1 and 5). We were unable to detect sterol esters at concentrations in excess of 10% of the level of free sterols, and the amounts of glycosterol in this tissue are known to be low by comparison with free sterols [25]. Thus, while there may be some conversion of esterified and glycosterols to the free sterol form during senescence, it clearly contributes only minimally to the large change in free sterol concentration. Moreover, it has been previously reported that total sterols decline with age in this tissue [26]. It would appear, therefore, that sterols are retained, or perhaps accumulated, in the microsomal membranes against a translocation gradient.

The Arrhenius plots of τ_c for 2N14 show clearly that the physical state of the membrane lipids is modified by senescence. Of particular significance, however, is the absence of any breaks or discontinuities in slopes of the Arrhenius plots at all stages of senescence, which would suggest that there are no phase transitions in the lipid over the temperature range tested. Yet X-ray diffraction data indicate that a liquid-crystalline to gel phase transition does occur in these membranes within the temperature range tested by electron spin resonance [5]. The fact that this phase transition is not detected by electron spin resonance is given perspective by the knowledge that the spin label in effect behaves as an impurity dissolved in the membrane. As such, its solubility would be expected to be highest in regions of greatest fluidity. For example, Shimshick and McConnel [27] have shown that the partitioning of spin label into membranes is greater above the phase transition when the lipids are in a more

fluid state. It therefore seems reasonable to interpret the combined X-ray diffraction and electron spin resonance observations as indicating that the spin label 2N14 partitions into liquid-crystalline domains and does not probe lipids in the gel phase. Thus, X-ray diffraction and electron spin resonance appear to be complementary techniques for monitoring the phase behaviour of membranes.

It becomes clear, therefore, that changes during senescence in viscosity and activation energy as determined by electron spin resonance pertain only to localized domains of lipid that are probably liquid-crystalline. The parameter E_a in effect scores the energy barrier acting upon the rotation of the spin label. An increase in this parameter reflects an increase in the degree of interaction between the probe and its macromolecular environment and, more generally, an increase in the degree of macromolecular interaction within the lipid matrix. The increase in τ_c at 29°C reflects an increase in viscosity. Thus the changes in these parameters during senescence are compatible in that more extensive interaction between fatty acid side chains could be expected to result in a decrease in their mobility and an increase in viscosity.

These changes in physical properties of the membrane lipid correlate temporally with changes in sterol content. Sterols are known to modulate the physical properties of phospholipid bilayers and to exercise some control over membrane function. Increasing concentrations of cholesterol, for example, have been shown to increase the viscosity of phospholipid bilayers in a fluid state [28]. It has also been reported that cholesterol intercalates more effectively with liquid-crystalline phase lipid than with gel phase lipid [29]. Considerably less is known about the influence of plant sterols on phospholipid bilayers. However, the changes in E_a and viscosity in microsomal membranes of senescing cotyledon suggest that plant sterols behave in an analogous manner. Their sterol accumulation may well occur predominantly in the liquid-crystalline phase, where it could alter the molecular interaction and viscosity of the fatty acid side chains.

Cholesterol is also known to induce lateral phase separation of membrane phospholipids [30], and it is therefore conceivable that the higher sterol levels in the older microsomal membranes from cotyledon contribute to the change in phase behaviour detectable by X-ray diffraction. Changes in total fatty acid saturation are of insufficient magnitude to account for this increase in transition temperature [5]. However, the major phospholipids of these membranes have quite different fluidities, judging from their relative degrees of fatty acid saturation, and any lateral rearrangement of these within the plane of the membrane accruing from the higher concentration of free sterols should logically be reflected in the phase behaviour of the bulk lipid. Other factors such as protein breakdown and the presence of divalent cations may also be contributing to a separation of phospholipids.

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References

1 Opik, H. (1966) J. Exp. Bot. 17, 427—439
2 Opik, H. (1965) J. Exp. Bot. 16, 667—672
3 McKersie, B.D. and Thompson, J.E. (1975) Phytochemistry 14, 1485—1491
4 McKersie, B.D., Thompson, J.E. and Brandon, J.K. (1976) Can. J. Bot. 54, 1074—1078
5 McKersie, B.D. and Thompson, J.E. (1977) Plant. Physiol. 59, 803—807
6 Eletr, S., Williams, M.A., Watkins, T. and Keith, A.D. (1974) Biochim. Biophys. Acta 415, 231—251
7 Raison, J.K., Lyons, J.M., Mehlhorn, R.J. and Keith, A.D. (1971) J. Biol. Chem. 246, 4036—4040
8 Papahadjopoulos, D., Jacobsen, K., Nir, S. and Isac, T. (1973) Biochim. Biophys. Acta 311, 330—348
9 Chapman, D. (1975) in Biomembranes (Eidenberg, H., Katchalski-Katzir, E. and Manson, L.A., eds.), Vol. 7, pp. 1—9, Plenum Press, New York
10 Luzzati, V. (1968) in Biological Membranes (Chapman, D., ed.), Vol. 1, pp. 71—123, Academic Press, New York
11 Poole, A.R., Howell, J.I. and Lucy, J.A. (1970) Nature 227, 810—814
12 Lucy, J.A. (1970) Nature 227, 815—817
13 Nichols, B.W. (1964) in New Biochemical Separations (James, A.T. and Morris, L.S., eds.), pp. 321—327, D. Van Nostrand, Toronto
14 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497—509
15 Skipski, V.P. and Barclay, M. (1969) in Methods in Enzymology (Lowenstein, J.M., ed.), Vol. 14, pp. 530—598, Academic Press, New York
16 Dittmer, J.C. and Wells, M.A. (1969) in Methods in Enzymology (Lowenstein, J.M., eds), Vol. 14, pp. 482—530, Academic Press, New York
17 Morrison, W.R. and Smith, L.M. (1964) J. Lipid Res. 5, 600—608
18 Grunwald, C. (1970) Plant Physiol. 45, 663—666
19 Williams, J.C., Mehlhorn, R. and Keith, A.D. (1971) Chem. Phys. Lipids 7, 207—230
20 Keith, A.D., Bulfield, G. and Snipes, W. (1970) Biophys. J. 10, 618—629
21 Libertini, L.J. and Griffith, O.H. (1970) J. Chem. Phys. 53, 1359—1367
22 Keith, A.D. and Snipes, W. (1973) Science 183, 666—668
23 Morse, II, P.D., Ruhlig, M., Snipes, W. and Keith, A.D. (1975) Arch. Biochem. Biophys. 168, 40—56
24 Esfahani, M., Limbrick, A.R., Knutton, S., Oka, T. and Wakil, S.J. (1971) Proc. Natl. Acad. Sci. U.S. 68, 3180—3184
25 Duperon, R., Meance, J., Duperon, P. and Thiersault, M. (1975) C.R. Acad. Sci. Paris 280 D, 605—608
26 Duperon, P. and Duperon, R. (1965) C.R. Acad. Sci. Paris 261, 3459—3462
27 Shimshick, E.J. and McConnel, H.K. (1973) Biochemistry 12, 2351—2360
28 Oldfield, E. and Chapman, D. (1971) Biochem. Biophys. Res. Commun. 43, 610—616
29 De Kruyff, B., van Dijck, P.W.M., Demel, R.A., Schuijff, A., Brants, F. and van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 356, 1—7
30 Shimshick, J.T. and McConnell, H.M. (1973) Biochem. Biophys. Res. Commun. 53, 446—451