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Membrane-structuring properties of bacterial long-chain alkylresorcinols

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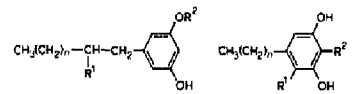
To investigate the mechanism by which 5-n-alkyl(C_{19}-C_{25})-resorcinols synthesized by certain bacteria of the *Azotobacter* genus affect the lipid bilayers of cellular membranes, planar bimolecular membranes were formed from these alkyl-resorcinols and from mixtures of those and typical bacterial phospholipids such as phosphatidylethanolamine, phosphatidylglycerol, and diphosphatidylglycerol. The electrical properties and, in some instances, the stability of the prepared membranes have been studied. The alkylresorcinols have been found to associate with phospholipids to form oligomeric and polymeric complexes, thereby giving rise to modifications in the bilayer structure and properties. It has been shown that the same compounds suppress the mitochondrial respiration in the presence of NAD-dependent substrates, but they activate it if succinate is used as substrate. This fact is explained in terms of the interaction between the alkylresorcinols and membrane phospholipids.

Introduction

Despite an extremely simple chemical structure the bacterial 5-n-alkylresorcinols (5-ARs) of type I (Fig. 1) have received widespread attention among researchers due to their unusual biological activity and biological functions. 5-ARs and their derivatives (II–V) have been shown to be the main membrane lipids of the metabolically dormant cysts of a Gram-negative, nitrogen-fixing bacterium, *Azotobacter vinelandii*, whereas the vegetative cells fail to synthesize them, the lipid fraction of their membranes consisting of phospholipids typical for Gram-negative bacteria, i.e., largely of PE, PG, and DPG [1–4]. 5-ARs become the predominant cell lipids of another representative of the *Azotobacter* genus, *Azotobacter chroococcum*, when the majority of cultivated cells turn into cysts [5,6]. Recently the same resorcinol lipids have been isolated from the culture medium of a Gram-negative, hydrogen-oxidizing bacterium, *Pseudomonas carboxydoflava* [7], and there is a good reason to suggest that microorganisms of various taxa would produce 5-ARs as extracellular metabolites [8]. Similar compounds have been found in higher plants (see, for example, Refs. 9–13). However, the plant alkylresorcinol fractions differ from the bacterial ones in containing considerable amounts (sometimes more than 70%) of unsaturated, mainly monoenic, species. In addition, the hydrocarbon chains of



I–III, VI IV, V, VII

Fig. 1. The structures of resorcinolic lipids isolated from bacteria of the genus *Azotobacter*, and the structure of M-5-ARs.

Lipids	R^1	R^2	*n*	Ref.
I (5-ARs)	H	H	16, 18, 20, 22	1, 2, 4–6
II	H	galactosyl	18, 20	1, 2, 4
III	OH	H	16, 18,	2, 4
IV	$COOCH_3$	H	20, 22	2, 4
V	$COCH_3$	H	20, 22	2, 4
VI (M-5-ARs)	H	CH_3	16, 18, 20, 22	this study
VII	H	α-D-talo-pyranosyl	18, 20	6, 33

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Abbreviations: 5-AR, 5-n-alkylresorcinol (the abbreviation refers exclusively to the long-chain species of type I, Fig. 1); BLM, bimolecular lipid membrane; DNP, 2,4-dinitrophenol; DPG, diphosphatidylglycerol; EGTA, 1,2-bis-[2-di(carboxymethyl)aminoethoxy-1]ethane; GC-MS, gas chromatography-mass spectrometry; M-5-AR, mono-*O*-methylated 5-AR (3-methoxy-5-alkylphenol); PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

plant 5-ARs vary in length within a much wider range, from 11 to 27 carbon atoms. Information about the biological activity and membrane-modulating properties of the plant resorcinol lipids is available in Refs. 14–19 and the publications cited therein.

The bacterial 5-ARs exhibit antioxidant properties and, when incorporated into biomembranes, make the latter more resistant to peroxidation [20]. It has been found [7,21–24] that 5-ARs (referred to as 'factor d_1' in the communications cited) can suppress the respiration of vegetative bacterial cells and thus induce their transition into a hypometabolic or anabiotic state. According to the available information [25,26], this effect may be attributed to the association of 5-ARs with the membrane phospholipids through hydrogen bonds, which results in disturbing the lipid bilayer and deactivating the membrane-bound respiratory enzymes. Probability of this kind of association was confirmed by Kaprelyants et al. [26], who investigated certain physical properties of monolayers and liposomes formed from a mixture of synthetic 5-*n*-decyl-resorcinol and DPG as well as from a mixture of the former and the total cell lipid of *Micrococcus lysodeikticus*.

In this paper, the data on the interactions occurring in planar BLMs between the bacterial 5-ARs and the phospholipids (PE, PG, DPG) common to the membranes of Gram-negative bacteria, including the *Azotobacter* genus, are presented. The BLMs were formed either from 5-ARs alone, or from mixtures of 5-ARs and one or two of the above mentioned phospholipids which varied in ratios of their constituents. The electrical conductivity measurements carried out with these BLMs definitely indicated the existence of lipid association caused by the presence of 5-ARs. This fact was confirmed in measuring the lifetime of the BLMs formed from mixtures of PE and 5-ARs. These findings as well as the aforecited data made it interesting for us to find out whether 5-ARs will affect the mitochondrial respiration in the same way as they affect the respiration of bacterial cells. Relevant experiments revealed a pronounced inhibitory effect, when NAD-dependent substrates were used, as well as a certain respiration-stimulating effect when succinate was used as substrate.

Materials and Methods

Lipids. A fraction of 5-ARs (I) was isolated from *A. chroococcum* 92 as described previously [5]. The fraction consisted of the homologues (see Fig. 1) with $n = 16$ (30 mol%), $n = 18$ (64 mol%), $n = 20$ (4 mol%), and $n = 22$ (2 mol%); foreign substances (largely neutral glycerides) accounted for about 0.5% of the total fraction.

For the preparation of M-5-ARs (Fig. 1, VI), a solution of 5% 5-ARs in ether was treated with excess ethereal diazomethane for 2 h at 20°C. The resultant mixture of mono- and di-*O*-methyl derivatives (the molar ratio is approximately 2:1) was separated by column chromatography on silica gel L-100/160 (Lachema, Czechoslovakia). For elution, a mixture of *n*-hexane and ether was employed, with the content of the latter gradually increasing from 10 to 50 vol%. The structure of M-5-ARs (VI) was confirmed by ^{1}H-NMR and mass spectra. Homologous composition of the product obtained was essentially identical with that of the parent 5-AR fraction as found by GC-MS.

TABLE I

Fatty acid composition (mol%) of the phospholipids used for the BLM formation (as found by GC-MS)

Fatty acids	Phospholipids			
	PE (*A. chroococcum*)	PE (egg yolk)	PC	DPG
14:0	5			
16:0	46	14	32	2
16:1	24	4	2	2
18:0	19	23	18	4
18:1	4	32	38	6
18:2		16	7	74
18:3		1	1	6
20:2		1		2
20:4		8	1	
Others	2	1	1	4

The aforementioned *A. chroococcum* strain was used also for obtaining PE. The method previously proposed for isolating PE from actinomycetes [27] was employed for this purpose. Egg yolk PE and bovine heart DPG were isolated by routine techniques [27]. PG was prepared by means of phospholipase D-catalyzed trans-esterification of egg yolk phosphatidylcholine [27]. The fatty acid composition of phospholipids used in the present study are summarized in Table I.

Other chemicals. Glutamate, α-oxoglutarate, pyruvate, and EGTA were purchased from Serva; malate and Tris were from Fluka and Sigma, respectively. ADP was obtained from Reanal (Hungary). All other chemicals were of highest pure grade.

BLMs. BLMs were formed from *n*-heptane solutions of either individual lipids or lipid mixtures (10–20 mg/ml) in an aqueous medium containing 10 mM Tris-HCl and 150 mM KCl by applying a lipid solution on a 1.5 mm diameter hole in a Teflon beaker. The membranes were observed with a microscope under reflected light, the diameter of the black area being measured with an eyepiece micrometer. The physical parameters of the BLM prepared from 5-ARs were determined at pH 8.0 ± 0.1; the parameters of the BLMs formed from mixtures of lipids were determined at pH 7.2 ± 0.1. All the measurements were made at

25°C. Ag/AgCl electrodes were used to stud, the electrical properties of BLMs.

For determining the electrical conductivity of a BLM, transmembrane current was measured under a constant transmembrane potential with the use of a Model U 1-6 electrometer amplifier (USSR) and a Model KSP-4 potentiometer (USSR). Breakdown voltage was measured with a Model pH-673 M pH-meter millivoltmeter (USSR) 5 s after the black membrane formation; the voltage applied to the membrane was gradually increased until it ruptured. The BLM capacitance was measured with a MIE-0.2 alternating current bridge (USSR).

The average values of the conductance, specific capacitance, breakdown voltage, and lifetime of BLMs are given below. Each of these average values was derived from at least twenty measurements. The standard error did not exceed 10%.

Mitochondria. Mitochondria were isolated from the livers of adult male Wistar rats following the previously described procedure [28], with a buffer containing 10 mM Tris-HCl, 0.5 mM EGTA, and 0.3 M sucrose (pH 7.5). The mitochondria were washed in a buffer containing 10 mM Tris-HCl and 0.3 M sucrose (pH 7.5), then resuspended in the same buffer (80–100 mg protein/ml), and the suspension was stored in a sealed glass test tube; the storage time did not exceed 4 h. The protein content of the mitochondria preparation was determined according to Lowry et al. [29]. Mitochondrial respiration was followed polarographically at 25°C with the use of a closed thermostated 1 ml cell equipped with a magnetic stirrer. A medium of 50 mM KCl, 3 mM KH_2PO_4, 10 mM Tris-HCl, and 150 mM sucrose (pH 7.5) was used for incubation.

ADP up to 1 mM, 40–100 μl of the mitochondria suspension and a substrate were added to the incubation medium placed in the polarographic cell. The concentrations of substrates are given in the legend to Fig. 8. DNP up to 0.2 mM was added to the incubation medium in experiments with uncoupled respiration. 5-ARs and their derivatives were dissolved in ethanol at a concentration of 6–8 mg/ml, and the solutions were brought into the cell in 10–20 μl portions.

Results and Discussion

After a solution of 5-ARs in *n*-heptane had been applied to the hole in a Teflon beaker, a coloured film was observed. Within 2–3 min a black area appeared on the film, indicating that a BLM was formed. The lifetime of this BLM depended on pH of the aqueous medium (Fig. 2). The BLM was unstable at pH $\leq$ 7 and, at most, existed for 5 min. However, at pH > 7 its stability increased sharply with an increase of the medium pH. At pH $\geq$ 9, only a thick stable coloured film was observed in the hole and this film did not

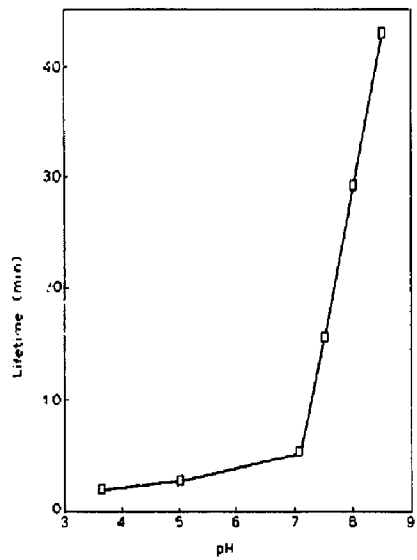

Fig. 2. pH dependence of the lifetime of BLMs formed from 5-ARs.

convert into a BLM. Under the conditions employed in the present study for determining the physical parameters of alkylresorcinol BLM, i.e. at pH 8.0 $\pm$ 0.1, the average lifetime was 30 min, which was sufficient for accomplishing all the necessary measurements. In some cases the BLM remained intact for more than 1 h. The found value of specific capacitance of the membrane (0.50 $\pm$ 0.03 $\mu F/cm^2$) confirmed its bimolecular structure since for the BLMs made from lipids of various classes with the use of *n*-heptane or *n*-decane as solvent, the same parameter ranges from 0.35 to 0.60 $\mu F/cm^2$ [30]. Thus, it is probable that, in principle, 5-ARs are able to form a bilayer in the azotobacter cysts where the phospholipids peculiar for vegetative cell membranes are present, if any, only as traces (cf. Ref. 4).

In the experiments carried out with the alkylresorcinol BLMs, the value of their specific resistance varied from $2 \cdot 10^6$ to $4 \cdot 10^7$ Ohm/cm^2 and was within the limits characteristic for the BLMs formed from widespread phospholipids and glycolipids [31]. Hence, there is no reason to assume that the abovementioned transformation of the lipid composition of the azotobacter cell membranes occurring upon encysting would have any appreciable effect on the ion permeability of these membranes. The same conclusion has been drawn from the data obtained on measuring the breakdown voltage for the BLMs formed from both the individual 5-ARs fraction and mixtures of the latter and PE, the predominant phospholipid of the vegetative azotobac-

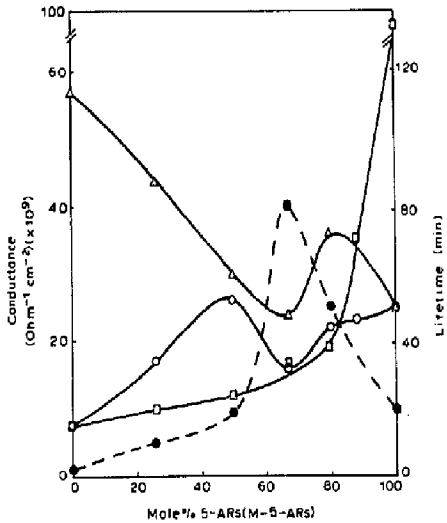

Fig. 3. The conductance and lifetime of BLMs formed from mixtures of 5-ARs (M-5-ARs) and PE as a function of the molar percentage of 5-ARs (M-5-ARs) in the parent lipid mixture A (○), the conductance of BLMs formed from mixtures of 5-ARs and bacterial PE; B (●, interrupted curve; the ordinate on the right-hand side), the lifetime of BLMs formed from mixtures of 5-ARs and bacterial PE; C (□), the conductance of BLMs formed from mixtures of M-5-ARs and bacterial PE; D (△), the conductance of BLMs formed from mixtures of 5-ARs and egg yolk PE.

ter cell. With the 5-ARs content increasing from 0 to 100%, the parameter value varied negligibly (from 240 to 230 mV) and was in conformity with the values (150–300 mV) reported earlier for various BLMs [31].

The conductance and lifetime of the BLMs formed from mixtures of 5-ARs and PE are of particular interest because of their pronounced dependence on the molar ratio of these components (Fig. 3) *. As the molar content of 5-ARs increased from 0 to 50 mol%, the conductance of the BLMs was rising. Further enrichment of the mixture in these components, at first, caused a decrease and then an increase of the parameter value. Accordingly, Curve A (Fig. 3), demonstrating these variations, has a maximum and a minimum corresponding to the molar ratio of 5-ARs to PE of 1:1 and 2:1, respectively. The incorporation of 5-ARs into a PE bilayer contributed to its stability (Fig. 3, Curve B), increasing the lifetime to a maximum at the molar ratio of 5-ARs to PE of about 2:1 and then decreased. It should be noted that on the ascending part of Curve B a bend point is seen which corresponds to the equimolar mixture of the lipids, that is, this point and the maximum of Curve A take the same position relative to the composition of the parent lipid mixture.

The findings described evidently show that 5-ARs and PE, when present together in a BLM, are not inert to each other and enter into interaction. This conclusion is consistent with the observations made by other authors [19,26], regarding the behaviour of related lipids in monolayers and liposomes. The interaction between 5-ARs and phospholipids (in this case PE) is likely to involve the formation of hydrogen bonds, which correlates with the suggestion of the authors of the communications cited. Since a 5-AR molecule has two free hydroxyl groups capable of forming intermolecular hydrogen bonds, this molecule is able to associate with one or two PE molecules (or a similar phospholipid). Therefore, it is reasonable to suppose that di- and tri-molecular complexes (Fig. 4(a)) occur in the BLM prepared from mixtures of these lipids with low concentrations of 5-ARs. A gradual increase in the 5-ARs molar proportion should result in the appearance of oligomeric complexes which will extend in size.

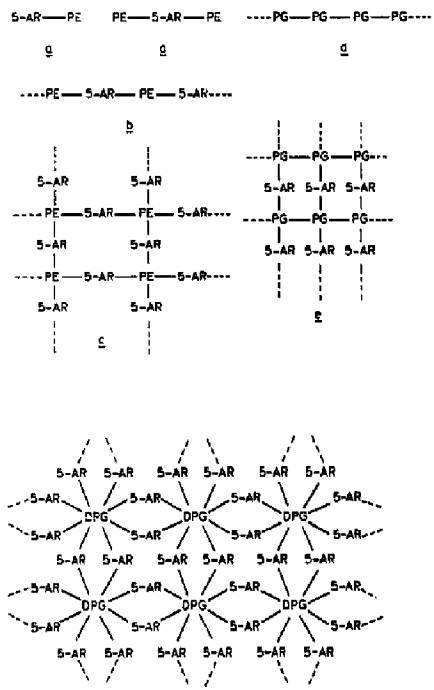

Fig. 4. Schematized hypothetical structures of the 5-AR-phospholipid associates occurring in BLMs (see the explanation in the text).

* In this paper, the 'average molecular mass' of a lipid fraction calculated on the basis of the data on the homologous composition of this fraction implies the molecular mass of a lipid.

When the equimolar ratio of the components is reached, polymeric chains resembling those presumed by Kaprelyants et al. [26] are likely to be formed (Fig. 4(b)). The associates (a) and (b) appear to be too labile to affect significantly the stability of the membrane. Meanwhile, the presence of 5-ARs in the BLMs loosens their hydrophobic regions (cf. Ref. 4), resulting in a certain increase of the BLM conductance. With a further increase of the 5-ARs proportion in the lipid mixture, their molecules may link to the PE molecules involved in the polymeric chains (b) and 'bridge' the latter. As a result, these chains and the membrane as a whole become more stable. At the molar ratio of 5-ARs to PE of about 2:1, a polymeric network presented schematically in Fig. 4(c) is likely to arise. It is obvious that the lifetime of this structure should be the longest; on the other hand, its conductance must be the lowest as compared with the other patterns considered (Figs. 4(a), (b)).

As seen from Curves A and B in Fig. 3, further addition of 5-ARs to the lipid mixture decreases the BLM lifetime and increases the conductance. An explanation of this fact may be the following: a PE molecule is able to directly associate with four 5-AR molecules at most (see below) and the presence of 'excessive' 5-AR molecules in the bilayer prevents from the formation of a regular polymeric network illustrated in Fig. 4(c). It should be noted that the formation of the above associates (a), (b) and (c) due to the hydrogen bonds of phenolic hydroxyl groups is favoured by the chemical structure of 5-ARs: 1,3,5-substitution in the benzene nucleus minimizes the steric hindrance from the association of a 5-AR molecule with two phospholipid molecules.

To ascertain indirectly whether the proposed type of polymeric association may take place, the conductance of the BLMs made from mixtures of PE isolated from *A. chroococcum* and *M*-5-ARs (Fig. 1, VI) was measured. The *M*-5-AR molecule has only one hydroxyl group and, consequently, it fails to play the role of a linking unit in a polymeric structure. The results of the measurements are given in Fig. 3 (Curve C). It can be seen that the conductance increases gradually with increasing the *M*-5-ARs content of the parent lipid mixture and the corresponding curve has neither extremes nor bends. Thus, it follows that the appearance of the associates (a)–(c), depicted in Fig. 4, in the bilayer seems to be quite possible.

It is of interest to consider in detail how the conductance described by Curve C (Fig. 3) tends to change. With increasing the molar concentration of M-5-ARs in the lipid mixture from 0 to 80%, that is, until the molar ratio of M-5-ARs to PE is approximately 4:1, the conductance changes slightly, but a further increase in the M-5-ARs content sharply raises the conductance. This fact may be explained as follows: a M-5-ARs molecule having a free hydroxyl group retains the ability to associate with PE to form two-, three-, four-, and five-molecular complexes, i.e., PE·(M-5-AR), PE·$(M\text{-}5\text{-}AR)_2$, PE·$(M\text{-}5\text{-}AR)_3$ and PE·$(M\text{-}5\text{-}AR)_4$. Associated in such a manner the molecules of M-5-ARs appear to loosen the hydrophobic region of the membrane to a relatively small degree. If the lipid mixture contains more than four molecules of M-5-ARs per PE molecule, the 'excess' molecules of the former either associate into separate oligomeric structures, or link to the molecules of M-5-ARs involved in the mentioned PE·$(M\text{-}5\text{-}AR)_4$ complexes. In any case the hydrophobic region of the BLMs is loosened to a great extent, which is followed by an abrupt rise of the conductance. At this point, a certain resemblance to the trends in the conductance changes of the above discussed BLMs formed from PE/5-ARs mixtures is observed, namely, in both cases the conductance starts to increase just when a PE molecule becomes surrounded by more than four molecules of the resorcinolic lipids.

To assess the influence of fatty acyl structures on the interaction between PE and 5-ARs, the conductance of the BLMs formed from mixtures of 5-ARs and the egg yolk PE was measured. Unlike the bacterial PE, the PE of egg yolk is much more unsaturated and contains, in particular, di- and tetraenoic fatty acyl residues (see Table I). As would be expected [32], the conductance of the BLM made from this PE proved to be much higher than that of the BLM formed from the bacterial PE. Curve D in Fig. 3 shows the dependence of the conductance of the BLMs prepared from the mentioned mixtures on the 5-ARs content. In this case the effect of 5-ARs manifests itself in decreasing the BLM conductance as their concentration increases. Nevertheless, the lowest conductance was again recorded at a 5-ARs/PE ratio close to 2:1. Hence, the structuring of a bilayer resulting in its decreasing conductance is due solely to the interaction of polar groups of lipid molecules.

PG is the second major membrane lipid in the vegetative cells of *Azotobacter*. It accounts for 14% of the total membrane lipids in *A. vinelandii* [4] (which is approximately the same as in *A. chroococcum*). Therefore, the interrelation between PG and 5-ARs in a bilayer is of certain interest. Contrary to the molecule of PE, that of PG has two free hydroxyl groups, due to which the PG molecules are able to associate with each other through hydrogen bonds and are likely to form linear polymeric chains schematically presented in Fig. 4(d). The measurements of the conductance of the BLMs made from PG/5-ARs mixtures revealed that the value of this parameter diminished with an increase of the 5-ARs molar content, became minimal at equimolar amounts of the components and finally rose with a further increase of the 5-ARs concentration in

the lipid mixture (Fig. 5, Curve A). By analogy with the above discussed features of the BLMs formed from 5-ARs/PE-mixtures, the conclusion suggests itself that the minimal conductance corresponds to the formation of a polymeric network in the bilayer (Fig. 4(e)). This type of structure may result from 'bridging' the linear chains (d) by the molecules of 5-ARs. Apparently, this structure hinders the ion current to the greatest extent. Incorporated into the lipid mixture, the additional amount of 5-ARs would impede the formation of the regular polymeric associate and thus would give rise to increasing conductance.

In order to assess the adequacy of the above described molecular interactions the conductance of the BLMs formed from PG/M-5-ARs mixtures was measured. As shown by Curve B (Fig. 5), the conductance gradually increases as the molar proportion of M-5-ARs increases, the conductance changing only to a small extent until the molar ratio of M-5-ARs to PG reached about 4:1 (80 mol% M-5-ARs), and then abruptly increasing. In other words, a complete analogy with the BLMs formed from M-5-ARs/PE mixtures was observed in this case. Thus, the occurrence of the regular polymeric network (e) (Fig. 4) in the bilayer is quite possible.

The results of the present investigation lead to the assumption that 5-ARs occurring in the membrane during encysting in the *Azotobacter* cells interact with the two major lipids (PE and PG) and, as a result,

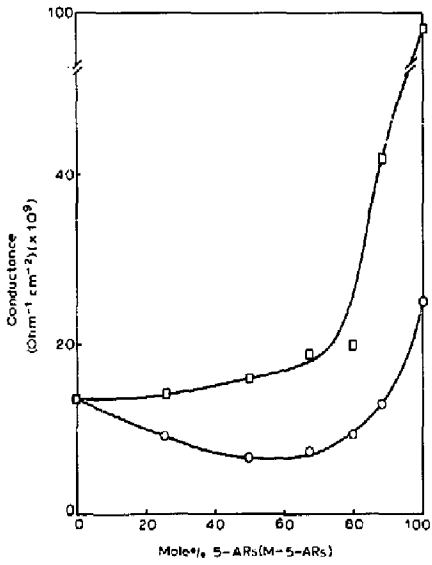

Fig. 5. The conductance of BLMs formed from mixtures of 5-ARs and PG (A: ○), M-5-ARs and PG (B: □) as a function of the molar percentage of 5-ARs and M-5-ARs in the parent lipid mixture.

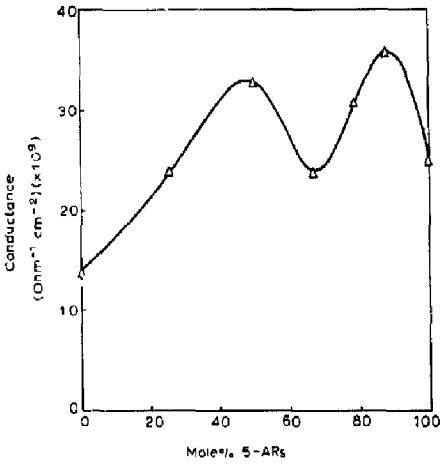

Fig. 6. The conductance of BLMs formed from mixtures of 5-ARs, PG and bacterial PE as a function of the molar percentage of 5-ARs in the parent lipid mixture. The molar ratio of PE/PG is constant as 80:14.

modify the structure and properties of the membrane. To elucidate the general features of these interactions, BLMs were used to model the process of variation in the lipid composition of the cell membrane. The initial phospholipid bilayer was formed from the mixture of PG and the bacterial PE with the molar ratio of 14:80, since the latter coincides with the ratio of these phospholipids in the cell membrane of *A. vinelandii* [4]. The conductance of the BLMs formed from this mixture with incorporation of various amounts of 5-ARs was measured. The dependence of the found conductance on the 5-ARs concentration is shown in Fig. 6. The comparison of this curve with Curve A in Fig. 3 shows that the conductance variation pattern in the experiment under discussion and in the case with BLMs made from PE and 5-ARs is similar. Consequently, the observed variations in the conductance of the three-component BLMs are set by the interaction between 5-ARs and PE. However, this may be determined by the substantial predominance of PE over PG in the bilayers.

Both PG and 5-ARs have two free hydroxyl groups and, therefore, they could exhibit, in principle, a similar structure-forming ability. The measurements of the dependence of the conductance of the BLMs formed from PG/PE mixtures on the ratio of the components demonstrated certain interaction between these phospholipids. However, the effect of the interaction was exhibited to a rather weaker degree as compared with the cases where BLMs contain 5-ARs. The minimal conductance was recorded at the molar ratio of PE to PG in the parent lipid mixture as about 3:2, but a

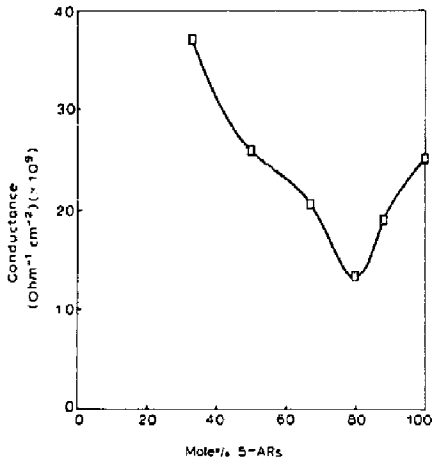

Fig. 7. The conductance of BLMs formed from mixtures of 5-ARs and DPG as a function of the molar percentage of 5-ARs in the parent lipid mixture.

distinct extreme was not observed. This fact emphasizes that the unique chemical structure of 5-ARs as a membrane lipid is related to their peculiar function in bacterial cells under unfavourable conditions.

DPG is a minor lipid of the vegetative cells of *A. vinelandii* [4] and *A. chroococcum* (2–4% of the total cell lipids). Nevertheless, the interaction between DPG and 5-ARs in a lipid bilayer is of special interest since DPG ranks among dominant lipids in the microorganisms which have been shown to be sensitive to 5-ARs [8]. The BLMs formed from an individual DPG fraction were unstable. However, it proved to be possible to form relatively stable BLMs from mixtures of DPG and 5-ARs. Investigation of the dependence of conductance of the BLMs made from these mixtures on the content of 5-ARs in the parent lipid mixture showed a picture different from those considered above. With an increase of the molar proportion of 5-ARs, the conductance of the BLMs reached a minimum at the molar ratio of 5-ARs to DPG of 4:1, and then rose as the 5-ARs content of the lipid mixture increased further (Fig. 7).

By analogy with the BLMs described, the minimal conductance can be attributed supposingly to the formation of a regular polymeric network in the bilayer. Since a DPG molecule contains two residues of phosphatidic acid, it can be represented formally as a double phospholipid one. In order for the polymeric network associate to be formed from 5-ARs and DPG, the molar ratio of the constituents should be 4:1 rather than 2:1 as it is in the case of the 5-ARs/PE bilayer. In such a network, each DPG molecule is apparently surrounded by eight molecules of 5-ARs as shown schematically in Fig. 4(f). The formation of homogeneous oligomeric or polymeric chains consisting of DPG molecules and similar to the PG associates (Fig. 4(d)) seems to be quite unlikely for the following reasons. The DPG molecule has only one free hydroxyl group which is sterically hindered by two phosphatidyl residues and, moreover, located in between two anionic orthophosphate groups which create electrostatic hindrance from association.

All the above discussed results concern the interactions of 5-ARs and the main phospholipids of bacterial cell membranes. However, similar interactions are certain to occur in bilayers formed from 5-ARs mixed with phosphatidylcholine, phosphatidylserine or other phospholipids characteristic of other biomembranes, because all these phospholipids differ only in the structure of the polar group linked to the orthophosphate residue. The aforesaid is in agreement with the data reported by Kozubek et al. [19]. The authors revealed the ordering effect of 5-nonadecyl- and 5-nonadecenyl resorcinols isolated from rye grain, when these compounds were incorporated into the membranes of erythrocytes and dimyristoylphosphatidylcholine liposomes. This effect is also attributed to the association of the resorcinolic lipids with the membrane lipids. According to the authors, this association is realized through hydrogen bonds.

The results of the present study show that the variations caused by 5-ARs in the conductance of BLMs are relatively not large. The appearance of these compounds in a bacterial membrane seems to affect its ion permeability only scarcely. On the other hand, the structural modifications produced by 5-ARs in the lipid bilayer of the membrane are most likely to affect critically the activity of the membrane-bound enzymes, including the respiratory ones. In the Introduction, the communications concerning the inhibitory effect of alkylresorcinols on the respiration of bacteria have been cited. In this connection and following the results described herein we raised the question whether the respiration of mitochondria is sensitive to the presence of 5-ARs.

Relevant experiments were carried out with rat liver mitochondria. Glutamate, α-oxoglutarate, malate, pyruvate (NAD-dependent substrates), and succinate were used as substrates. DNP served as uncoupler. The mitochondrial preparations showed the respiratory control index (V_3/V_4) in the range from 3.3 to 4.5 and the uncoupled respiration index (V_{DNP}/V_4) in the range from 3.4 to 6.1. In the experiments with uncoupled respiration, the oxygen consumption was 85 ± 15 nmol O per mg protein per min with succinate as a substrate, and it was 49 ± 9 nmol O per mg protein per min when the NAD-dependent substrates were used.

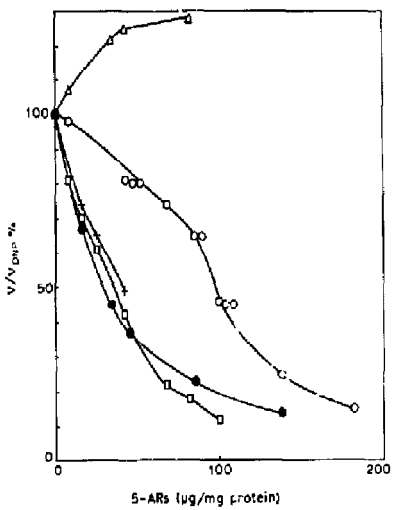

Fig. 8. The effect of 5-ARs on the uncoupled respiration of rat liver mitochondria. The abscissa is the amount (μg) of 5-ARs present in the incubatory medium per 1 mg of mitochondrial protein. The ordinate is the relative rate of uncoupled respiration. Substrates: ○, 4 mM pyruvate + 4 mM malate; □, 4 mM pyruvate; +, 4 mM glutamate; ●, 8 mM α-oxoglutarate; △, 4 mM succinate.

The measurements of the rate of uncoupled mitochondrial respiration in the presence of NAD-dependent substrates at various concentrations of 5-ARs in the incubation medium exhibited unambiguously the inhibitory effect of these lipids, the evident correlation being observed between the degree of inhibition and the quantitative ratio of 5-ARs and mitochondrial protein (Fig. 8). When 0.20–0.22 mg of 5-ARs per mg of protein were added to mitochondria, the respiration rate decreased by 90–95%. Since 5-ARs were applied to a polarographic cell as a solution in ethanol, the effect of this solvent on uncoupled respiration was estimated. It was found that ethanol, even being present in the incubation medium at 6% concentration (this exceeded more than twice the concentration of ethanol which was present upon addition of a maximal amount of 5-ARs), retarded the respiration by not more than 40%.

With uncoupled respiration, the presence of 5-ARs accelerated the oxidation of succinate to some extent (Fig. 8). The addition of ethanol to the mitochondrial suspension in the same amount which was added with 5-ARs, markedly inhibited the oxidation of succinate, i.e., in this case the stimulatory effect of 5-ARs was masked in part by the action of alcohol. The examination of the effect of 5-ARs on mitochondrial respiration in the absence of uncoupler (DNP) brought out a pattern similar to that described above, namely, a certain acceleration of the oxidation of succinate and retardation of that of the NAD-dependent substrates were observed.

Taken at the same concentrations, M-5-ARs did not affect the oxidation of all the aforementioned substrates both in the presence and the absence of DNP. This suggests that 5-ARs affect the mitochondrial respiration (as well as the respiration of bacterial cells) and the properties of phospholipid bilayers by identical mechanisms which consists in associating with the molecules of membrane lipids and, consequently, modifying the membrane structure. It is worth adding that 2-*C*-α-D-talopyranosyl-5-ARs (Fig. 1, VII), a minor lipid component of *A. chroococcum* 92 [33], also had no effect on the mitochondrial respiration. The inertness of this natural derivative of 5-ARs is thought to be caused by the polyhydroxylated bulky *C*-glycosidic residue located in close proximity to the phenolic hydroxyls. Therefore, these hydroxyles are sterically hindered and, furthermore, may be involved in intramolecular hydrogen bonds to a great extent.

The molecules of 5-ARs are quite simple as to their chemical structure. However, they possess all the structural features needed for functioning as a regulator of the membrane structure. Their long hydrocarbon chains enable them to be involved in lipid bilayers or cell membranes (see [19,20]) as well as to form a bilayer; the phenolic hydroxyls of their polar heads allow the association with two phospholipid molecules in the membrane; the location of the long-chain alkyl residue at C-5 and the absence of substituents at C-2, C-4 and C-6 of the benzene ring provide the most favorable conditions for this association.

In conclusion, it is worthwhile to touch upon the possible role of 5-ARs in the *Azotobacter* cells during encystment. Reusch and Sadoff [4] were the first to establish that the membrane lipid bilayer in the cysts of these bacteria consists mainly of 5-ARs, their derivatives (Fig. 1, II–V) and 6-alkyl(C_{21}–C_{23})-4-hydroxypyran-2-ones. These authors have also shown that this bilayer, being highly hydrophobic, is able to protect the cyst more efficiently than the phospholipid bilayers do. The results of the present study and the data reported by other investigators [7,25,26] lead to the assumption that the role of 5-ARs is not limited by their function as a barrier. Being the first among the specific lipids which are synthesized in the process of cell encysting, 5-ARs are able to inhibit the membrane-bound enzymes, including the respiratory enzymes, and to suppress the metabolic activity of the bacterial cell. Moreover, the antioxidant properties of 5-ARs [20] seem to be of considerable importance for protecting the cysts.

References

1 Reusch, R.N. and Sadoff, H.L. (1979) J. Bacteriol. 139, 448–453.
2 Su, C.-J., Reusch, R.N. and Sadoff, H.L. (1981) J. Bacteriol. 147, 80–90.
3 Su, C.-J. and Sadoff, H.L. (1981) J. Bacteriol. 147, 91–96.
4 Reusch, R.N. and Sadoff, H.L. (1983) Nature 302, 268–270.
5 Batrakov, S.G., Pridachina, N.N., Kruglyak, E.B. and Novogrudskaya, E.D. (1977) Khim. Prirod. Soedin. (USSR), 494–499.
6 Batrakov, S.G., Pridachina, N.N., Kruglyak, E.B., Novogrudskaya, E.D. and Chekasina, E.V. (1984) Izv. Akad. Nauk SSSR. Ser. Biol., 177–184.
7 Osipov, G.A., El-Registan, G.I., Svetlichny, V.A., Kozlova, A.N., Duda, V.I., Kaprelyants, A.S. and Pomazanov, V.V. (1985) Mikrobiologiya (USSR), 54, 186–190.
8 El-Registan, G.I., Khokhlova, Yu.M., Duzha, M.V., Kozlova, A.N., Poplaukhina, O.G. and Duda, V.I. (1979) Izv. Akad. Nauk SSSR. Ser. Biol., 869–877.
9 Madrigal, R.V., Spencer, G.F., Plattner, R.D. and Smith, C.R., Jr. (1977) Lipids 12, 402–406.
10 Tyman, J.H.P., Tychopoulos, V. and Chan, P. (1984) J. Chromatogr. 303, 137–150.
11 Kozubek, A. (1985) Acta Aliment. Polon. 25, 185–198.
12 Bullock, L.M. and Drewes, S.E. (1989) S. Afr. J. Chem. 42, 76–80.
13 Scannell, R.T., Barr, J.R., Murty, V.S., Reddy, K.S. and Hecht, S.M. (1988) J. Am. Chem. Soc. 110, 3650–3651.
14 Hecht, S.M. (1989) Pure Appl. Chem. 61, 577–580.
15 Itokoawa, H., Totsuka, N., Nakahara, K., Takeya, K., Lepoittevin, J.P. and Asakawa, Y. (1987) Chem. Pharm. Bull. 35, 3015–3020.
16 Kozubek, A. (1984) Z. Naturforsch. 39c, 1132–1136.
17 Kozubek, A. (1987) Acta Biochim. Polon. 34, 357–367.
18 Sikorski, A.F., Michalak, K., Bobrowska, M. and Kozubek, A. (1987) Stud. Biophys. 121, 183–191.
19 Kozubek, A., Jezierski, A. and Sikorski, A.F. (1988) Biochim. Biophys. Acta 944, 465–472.
20 Erin, A.N., Davitashvili, N.G., Prilipko, L.L., Boldyrev, A.A., Lutshak, V.I., Batrakov, S.G., Pridachina, N.N., Serbinova, A.P. and Kagan, V.E. (1987) Biokhimiya (USSR) 52, 1180–1185.
21 El-Registan, G.I., Duda, V.I., Kozlova, A.N., Duzha, M.V., Mityushina, L.L. and Poplaukhina, O.G. (1979) Mikrobiologiya (USSR) 48, 240–244.
22 Duda, V.I., El-Registan, G.I., Kaprelyants, A.S. and Mityushina, L.L. (1982) Mikrobiologiya (USSR) 51, 77–81.
23 Pronin, S.V., El-Registan, G.I., Shvetsov, V.V. and Duda, V.I. (1982) Mikrobiologiya (USSR) 51, 314–317.
24 Vostroknutova, G.N., Kaprelyants, A.S., Svetlichny, V.A., El-Registan, G.I., Shvetsov, V.V. and Ostrovsky, D.N. (1983) Prikl. Biokhim. Mikrobiol. (USSR) 19, 547–551.
25 Kaprelyants, A.S., Skrypin, V.I., El-Registan, G.I., Kozlova, A.N., Ostrovsky, D.N. and Duda, V.I. (1985) Prikl. Biokhim. Mikrobiol. (USSR) 21, 376–381.
26 Kaprelyants, A.S., Suleimenov, M.K., Sorokina, A.D., Deborin, G.A., El-Registan, G.I., Stoyanovich, F.M., Lille, Yu.E., Ostrovsky, D.N. (1987) Biol. Membr. (USSR) 4, 254–261.
27 Lipid Biochemical Preparations (1980) (Bergelson, L.D., ed.), Elsevier/North-Holland, Amsterdam.
28 Schneider, W.C. (1948) J. Biol. Chem. 176, 259–264.
29 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
30 Fellipface, R., Andrews, D.M. and Haydon, D.A. (1971) J. Membr. Biol. 5, 277–296.
31 Tien, H.T. (1971) in The Chemistry of Biosurfaces, Vol. 1 (Hair, M.L., ed.), pp. 23–248, M. Dekker, New York.
32 Macdonald, R.C. (1976) Biochim. Biophys. Acta 448, 193–198.
33 Batrakov, S.G., Pridachina, N.N., Kruglyak, E.B., Novogrudskaya, E.D. and Chekasina, E.V. (1982) Bioorg. Khim. (USSR) 8, 980–995.