Biochimica et Biophysica Acta, 514 (1978) 198—205

BBA 78221

THE ARRHENIUS PLOT BEHAVIOUR OF RAT LIVER 5′-NUCLEOTIDASE IN DIFFERENT LIPID ENVIRONMENTS

KEITH K. STANLEY and J. PAUL LUZIO

Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR (U.K.)

(Received May 18th, 1978)

Summary

The plasma membrane enzyme 5′-nucleotidase (EC 3.1.3.5) was prepared from rat liver as a complex with sphingomyelin or in detergent-solubilized forms. Each preparation exhibited a K_m and Arrhenius break temperature indistinguishable from that of 5′-nucleotidase in plasma membranes. Measurement of fatty acid profiles, cholesterol and phospholipid content however showed a very wide variation between these preparations. We conclude that the biphasic nature of the Arrhenius plot of 5′-nucleotidase may be a property of the enzyme rather than its lipid environment.

Introduction

5′-Nucleotidase (EC 3.1.3.5) is a glycoprotein ectoenzyme which is tightly bound to the plasma membrane of most mammalian cells [1—4]. Reports of soluble 5′-nucleotidase activity refer to either a different enzyme activity [5] or to material solubilized by high bile detergent concentration in liver [6]. In common with other intrinsic membrane proteins 5′-nucleotidase may be purified as a phospholipid complex [7], although the phospholipid does not appear to be a strict requirement for activity [8] as has been reported for Ca^{2+}-activated ATPase [9]. 5′-Nucleotidase may be associated with sphingomyelin in vivo since it is found in the sphingomyelin-rich bilayer of the bile canaliculae of mouse liver membranes [10]. The ability to solubilize 5′-nucleotidase with a phosphatidyl inositol-specific phospholipase has suggested a role for this phospholipid in the anchoring of the enzyme in the membrane [11].

5′-Nucleotidase has been purified from plasma membranes solubilized in detergent in an apparently phospholipid-free form [12,13] but it has been noted that these preparations may be of low specific activity and less stable to heat denaturation than the sphingomyelin-enzyme complex [8].

We have used these preparations of 5′-nucleotidase to assess the relationship of biphasic Arrhenius plots to the lipid environment of the enzyme.

Materials and Methods

Detergents. Sarkosyl NL35 (sodium lauroyl sarcosinate) was a gift from CIBA-Geigy (Simonsway, Manchester, M22, 5LB, U.K.). Lubrol 12 A9 was a gift from ICI Ltd. (Organics Division, P.O. Box 42, Blackley, Manchester, M9 3DA, U.K.).

Plasma membranes. Plasma membranes were prepared from 250 g Wistar or Sprague-Dawley rats by the method of Fleischer and Kervina [14].

Partial purification of 5'-nucleotidase. Solubilization and partial purification of 5'-nucleotidase from plasma membranes was achieved using the method of Evans and Gurd [12] in which the membranes were solubilized in 2.5% Sarkosyl at 4°C for 5 min and purified by equilibrium centrifugation in a 5 to 15% sucrose gradient followed by gel chromatography. Alternatively, the plasma membranes were solubilized in 0.7% Lubrol at 37°C for 60 min followed by gel chromatography and DEAE-agarose ion-exchange chromatography according to the method of Slavik et al. [13].

High specific activity material was purified from Wistar or Sprague-Dawley rat liver as a phospholipid complex by the method of Widnell [7].

Assays

5'-Nucleotidase. 5'-Nucleotidase was assayed using the spectrophotometric assay of Ipata [15]. Cuvettes containing 50 mM Tris-HCl buffer, pH 7.5, and 2 mM $MgCl_2$ were pre-equilibrated to the temperature of the assay. Adenosine deaminase (2 units) and a sample (approx. 10^{-3} unit) were then added and the cuvette transferred to a Gilford model 250 spectrophotometer (Gilford Instruments Ltd., 44-48 Church Road, Teddington, Middlesex, U.K.). The temperature of the reaction mixture in the cuvette was measured with a 'thermolin' component (Sasco, P.O. Box 2000, Crawley, Sussex, U.K.) mounted on a probe built into the cuvette compartment lid, and displayed on a digital Voltmeter arranged in a bridge circuit with the sensor. The linear output of the device was calibrated against a mercury-in-glass thermometer. When the contents of the cuvette had reached a constant temperature, 100 μM AMP was added and the temperature averaged between the beginning and the end of each assay. This variation in temperature during an assay was less than 0.5°C. Arrhenius plots for CMP were performed on a thermal gradient block using the assay of Avruch and Wallach [16].

Phosphorus. Phospholipid was extracted from samples with 3 X 5 ml chloroform/methanol (2 : 1, v/v) and pyrolysed in H_2SO_4/perchloric acid (9 : 1, v/v) at 180°C for 3 h. Released phosphate was measured by the method of Fiske and Subbarow [17]. Alternatively, the sample was pyrolysed with magnesium nitrate [18] and phosphate measured by the method of Itaya and Ui [19].

Protein. Protein was estimated by the method of Lowry et al. [20].

Cholesterol. Total cholesterol plus cholesterol ester was measured using a Boehringer assay kit (Boehringer Corporation Ltd., Bell Lane, Lewes, Sussex, BN7 1LG, U.K.).

Fatty acids. Fatty acids were analysed by gas chromatography of chloroform/methanol extracts. After hydrolysis in 2 M NaOH for 30 min at 100°C the liberated fatty acids were extracted at low pH, dried down and methylated

with 14% BF_3 in methanol for 5 min at 60°C. Alternatively, samples were transmethylated with 14% BF_3 in methanol at 100°C for 90 min. Methyl esters were extracted in *n*-hexane, dried down in a stream of N_2 and injected in CS_2 solvent.

ESR

ESR spectra were recorded on a Varian E3 spectrometer and the temperature was monitored continuously during the experiment by a thermistor inserted immediately above the cavity. Samples were inserted in 1.0 mm internal diameter glass capillaries. 5-Doxyl stearic acid was used as spin label at a molar ratio to phospholipid of about 1 : 100.

Results

Fig. 1 shows the Arrhenius plots of 5′-nucleotidase in plasma membranes, and of three preparations extracted in different detergents and partially purified. Apart from the activation energy for plasma membrane 5′-nucleotidase below the break temperature there is no significant difference between these lines. The mean break temperature for all preparations was 30.4 ± 0.5°C.

Sphingomyelin 5′-nucleotidase. Differential detergent extraction of rat liver

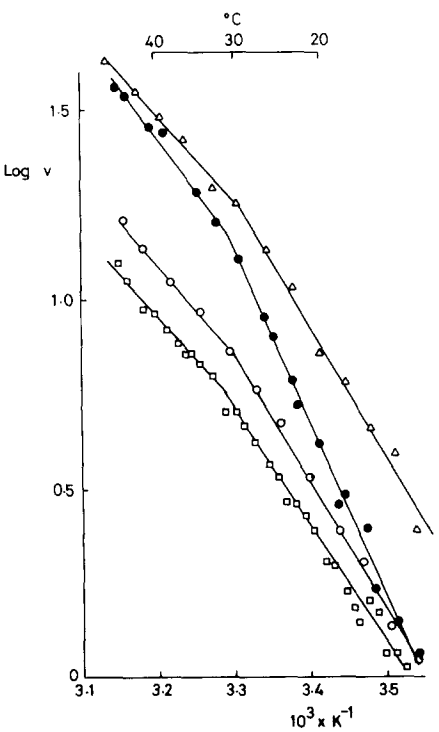

Fig. 1. Arrhenius plots of 5′-nucleotidase. 5′-Nucleotidase was assayed spectrophotometrically and initial rates were measured. Each point represents the mean of three assays. ●———●, plasma membranes; ○———○, sphingomyelin-enzyme complex; △———△, sarkosyl-solubilized plasma membranes; □———□, lubrol-solubilized plasma membranes.

TABLE I

THE COMPOSITION AND 5'-NUCLEOTIDASE ACTIVATION ENERGIES OF PARTIALLY PURIED PREPARATIONS FROM RAT LIVER

Data are expressed as a range of values for two experiments or as a mean ± S.E. with number of observations in parentheses.

	Specific activity (units/mg protein)	Phosphorus (μg/mg protein)	Cholesterol (μg/mg protein)	Activation energy (kJ/mol)		Break temperature (°C)
				Below break	Above break	
Plasma membranes	0.52—0.77	20.1—21.4	117—155	88—92		
Purified 5'-nucleotidase	54—72	133—169	116—140			
Sarkosyl-solubilized membranes	0.5—1.7	<0.09 *	<8 *	65.3 ± 3 (11)	42.3 ± 3 (11)	30.4 ± 0.5 (13)
Lubrol-solubilized membranes	1.7—1.8	<0.05 *	n.d.			

* No phosphorus or cholesterol could be detected in these samples, the figures shown are the limits of sensitivity of the assay taking into account the protein concentration of the sample.
N.D., not determined.

with Triton X-100 and deoxycholate followed by purification according to the procedure of Widnell [7], results in a material enriched in phospholipid relative to plasma membranes and with a similar cholesterol content (Table I). This phospholipid co-chromatographed with sphingomyelin on 2-dimensional thin layer chromatography plates [21]; no other phospholipids were present. Gas-chromatography showed it to have a radically different fatty acid composition compared to plasma membranes (Fig. 2). Very long chain fatty acids (not shown here) accounted for less than 10% of the total; at least 85% of the fatty acids were saturated.

Despite the 50% change in activation energy of this sphingomyelin-enzyme complex at 30°C, no discontinuity was observed over the temperature range 20—40°C in the ESR parameter $2T_{\parallel}$ of the spin label 5-doxyl stearic acid when it was incorporated into the same material (Fig. 3).

The Arrhenius plot of 5'-nucleotidase activity towards CMP (Fig. 4) showed a break temperature at 20.8 ± 0.7°C (S.E.M., 4 measurements) and activation energies of 86.5 and 41.8 kJ/mol below and above the break, respectively. In this case the enzyme shows a marked change in break temperature and activation energy despite a constant lipid environment. The K_m, however, was the same as that for AMP.

Detergent-solubilized plasma membranes. The cholesterol and phosphorus content of the detergent-solubilized plasma membranes was below the limits of detection of the assays used (Table I). Assuming a molecular weight of 140 000 for the protein in these preparations, this corresponds to 0.4 mol phospholipid/mol protein. Both the sarkosyl- and the Lubrol-solubilized preparations, however, display Arrhenius plots indistinguishable from the sphingomyelin enzyme (Fig. 1). The sarkosyl material was prepared in 0.25% deter-

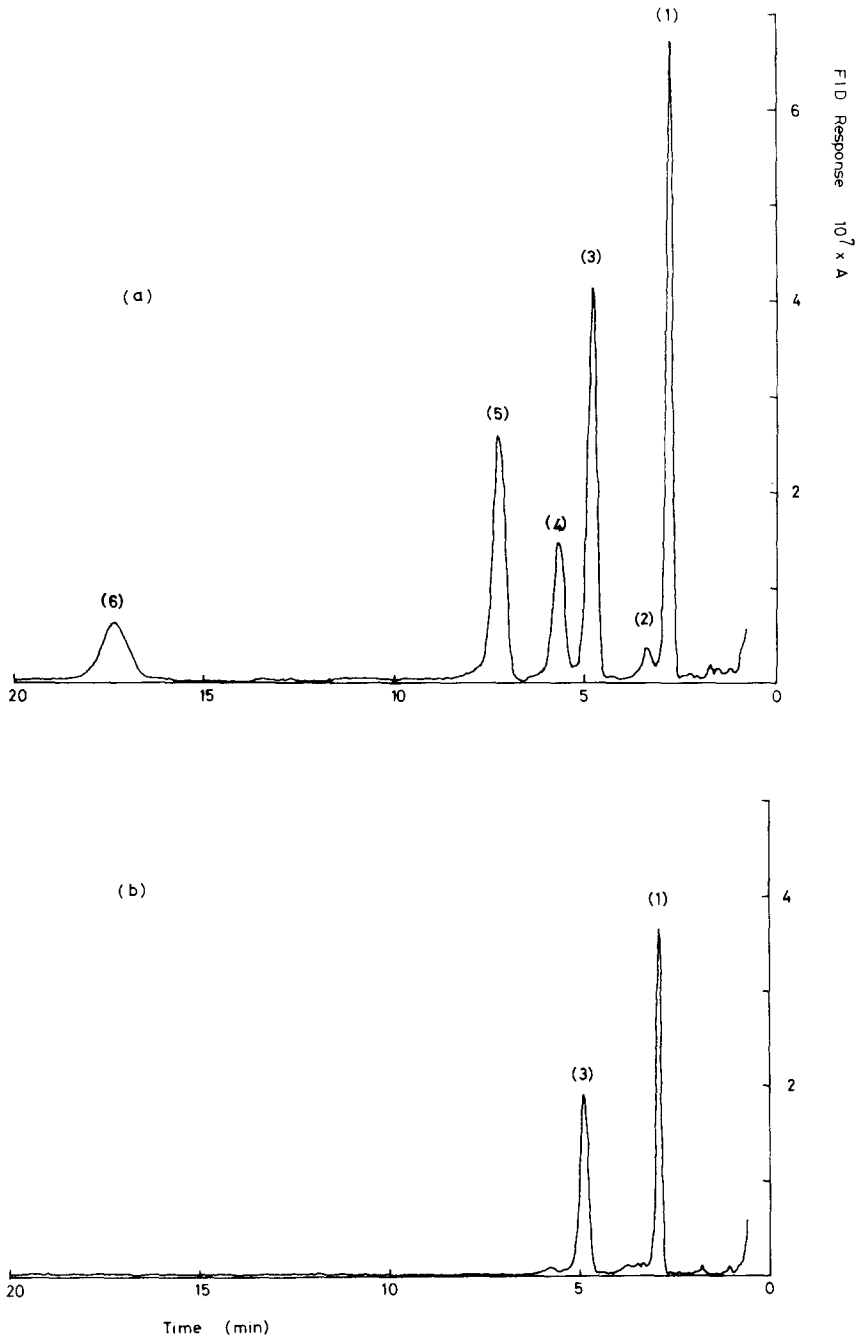

Fig. 2. The fatty acid composition of lipids extracted from 5'-nucleotidase preparation. Fatty acids from saponified lipids extracted from (a) plasma membranes and (b) purified 5'-nucleotidase-spingomyelin complex were methylated and injected in CS_2 solvent onto 10% ethylene glycol succinate columns at 180°C and 60 ml/min argon. Peaks were identified by comparison with standard methyl esters as follows: (1), hexadecanoic methyl ester (16 : 0); (2), hexadec-9-enoic methyl ester (16 : 1); (3), octadecanoic methyl ester (18 : 0); (4), octadec-9-enoic methyl ester (18 : 1); (5), octadec, 9-, 11-dienoic methyl ester (18 : 2); (6), Eicosa, 5-, 8-, 11-, 14-tetraenoic methyl ester (20 : 4). FID, flame ionization detector.

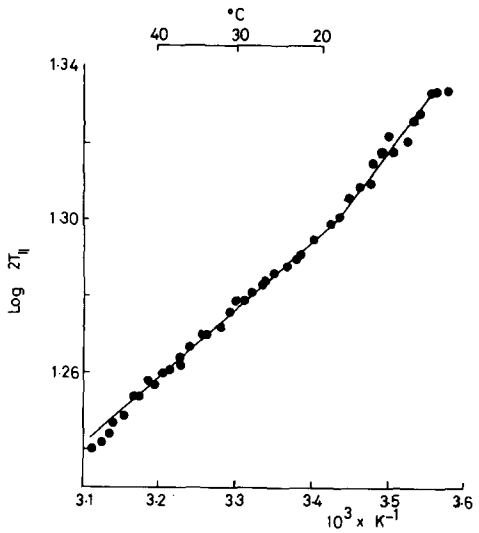

Fig. 3. ESR of sphingomyelin-5′-nucleotidase. Arrhenius plot of the parameter $2T_{\parallel}$ (arbitrary units) for the sphingomyelin-5′-nucleotidase complex spin labelled with 5-doxyl stearic acid (1 mol% of total lipid).

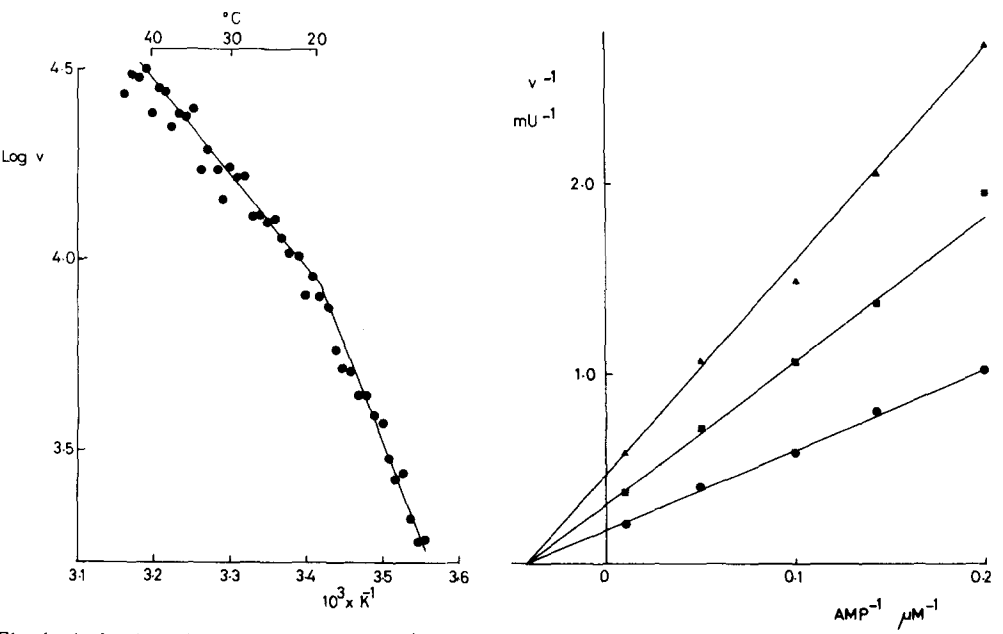

Fig. 4. Arrhenius plot of sphingomeylin-5′-nucleotidase using CMP as substrate. Enzyme activity was assayed at each temperature by measuring the [^{3}H]cytosine released from [^{3}H]CMP [16]. The mean break temperature from four measurements was 20.8 ± 0.7°C (S.E.M.) and the activation energies were 86.5 and 41.8 kJ/mol.

Fig. 5. Lineweaver-Burk plot of 5′-nucleotidase. Plasma membranes were prepared from rat liver [14] and incubated in 2.5% Sarkosyl or 2.5% Lubrol for 5 min at 4°C. The extract was centrifuged at 40 000 X g_{av} for 30 min and the supernatant assayed at 37°C in a final detergent concentration of 0.01%. ●——●, plasma membranes; ■——■, Sarkosyl extract; ▲——▲, Lubrol extract.

gent and assayed at less than 0.001% while the Lubrol material was prepared in 0.1% detergent and assayed at less than 0.0005%. Experiments in which plasma membranes were solubilized in 2.5% detergent and the activity measured in a supernatant after centrifugation showed no effect of the detergents on the K_m of the enzyme (24 μM) even at high concentration (Fig. 5). Sarkosyl, however, released more 5'-nucleotidase than Lubrol at the same concentration.

Discussion

Rat liver 5'-nucleotidase may be purified subsequent to detergent solubilization by a variety of different procedures. In contrast to the report by Nakamura [22] we have found no change in the K_m for AMP of the enzyme after solubilization in either Sarkosyl, Lubrol or deoxycholate. This suggests that the soluble enzyme functions, in most respects, like its counterpart in the plasma membrane. The four detergents used in preparing the enzyme bear little resemblance to each other or to sphingomyelin. They are also present at extremely low concentrations in the enzyme assays. It seems unlikely, therefore, that the detergents played a significant part in producing biphasic Arrhenius plots.

Cholesterol is a well known inhibitor of lipid phase transitions and Kreiner et al. [23] have suggested that enzymes exhibiting biphasic Arrhenius plots in mammalian membranes must either exist in cholesterol-depleted areas of the plasma membrane or, alternatively, show changes in activation energy that are dependent on the protein rather than the surrounding lipid. We have shown that the break temperature of 5'-nucleotidase Arrhenius plots in plasma membranes with a cholesterol : phospholipid ratio of 0.52 is indistinguishable from that of the sphingomyelin complex with a ratio of 0.07.

The sphingomyelin complex had a fatty acid profile consisting principally of saturated fatty acids, in agreement with published values [24,25]. No difference was observed however in the break temperature of this preparation compared with that of plasma membrane 5'-nucleotidase which contains a much greater proportion of medium chain-length unsaturated fatty acids. There was no evidence from ESR data of a phase transition in the sphingomyelin of purified 5'-nucleotidase corresponding to the break temperature of the Arrhenius plot of the enzyme. The discontinuity at 20°C, however, agrees with published values for bovine erythrocyte sphingomyelin [25] and the high degree of saturation of the fatty acid side chains. Preparations of 5'-nucleotidase containing no measureable phospholipid or cholesterol still exhibited a break in their Arrhenius plot at 30°C.

It is interesting to note that the higher activation energy of plasma membrane 5'-nucleotidase below the break temperature, which is the only significantly different feature of the Arrhenius plots from all the preparations, can be simulated in the sphingomyelin complex simply by changing the substrate. This change in the Arrhenius plot behaviour of 5'-nucleotidase using CMP rather than AMP as substrate is difficult to explain in terms of a modification of the lipid environment of the enzyme. A number of explanations for biphasic Arrhenius plots which do not involve changes in the environment have been suggested [26—28] and an example of such behaviour has been provided by the hydrolysis of ITP by the soluble protein myosin [29]. In this case a biphasic

Arrhenius plot was observed which was not present when the usual substrate, ATP, was used. This behaviour was interpreted as a conformational change of the protein induced by substrate binding, which allowed further folding of the proteins to occur at the break temperature of the Arrhenius plot. Presumably a similar mechanism operating in the enzyme 5′-nucleotidase could account for the changes in both break temperature and activation energies when the pyrimidine substrate CMP is used. The values of the activation energies above and below the break temperature for myosin are very similar to those reported here for solubilized 5′-nucleotidase.

5′-nucleotidase appears, therefore, to be an example of a membrane enzyme which displays a biphasic Arrhenius plot independent of its lipid environment.

Acknowledgements

We wish to thank Professor C.N. Hales for his advice and encouragement, the Medical Research Council for financial support, Malcolm Ryall for his help in building the 'Temperature probe', and Dr. J. Metcalfe for his help with ESR. Part of the work reported was carried out at the Department of Medical Biochemistry, Welsh National School of Medicine, Cardiff.

References

1 Hayman, M.J. and Crumpton, M.J. (1972) Biochem. Biophys. Res. Commun. 47, 923—930
2 DePierre, J.W. and Karnovsky, M.L. (1974) J. Biol. Chem. 249, 7111—7120
3 Gurd, J.W. and Evans, W.H. (1974) Arch. Biochem. Biophys. 164, 305—311
4 Newby, A.C., Luzio, J.P. and Hales, C.N. (1975) Biochem. J. 146, 625—633
5 Van Den Berghe, G., Pottelsberghe, C. and Hers, H. (1977) Biochem. J. 162, 611—616
6 Holdsworth, G. and Coleman, R. (1976) Biochem. J. 158, 493—495
7 Widnell, C.C. (1975) Methods Enzymol. 32, 368—374
8 Widnell, C.C. (1974) Fed. Proc. 33, 1254
9 Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Biochemistry 13, 5501—5507
10 Kremmer, T., Wisher, M.H. and Evans, W.H. (1976) Biochim. Biophys. Acta 455, 655—664
11 Low, M.G. and Finean, J.B. (1978) Biochim. Biophys. Acta 508, 565—570
12 Evans, W.H. and Gurd, J.W. (1973) Biochem. J. 133, 189—199
13 Slavik, M., Kartner, N. and Riordan, J.R. (1977) Biochem. Biophys. Res. Commun. 75, 342—349
14 Fleischer, S. and Kervina, M. (1974) Methods Enzymol. 31, 6—41
15 Ipata, I. (1968) Biochemistry 7, 507—515
16 Avruch, J. and Wallach, D.F.H. (1971) Biochim. Biophys. Acta 233, 334—347
17 Fiske, C.H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375—400
18 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769—775
19 Itaya, K. and Ui, M. (1966) Clin. Chim. Acta 14, 361—366
20 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
21 Rouser, G., Fleischer, S. and Hamamoto, A. (1970) Lipids 5, 494—496
22 Nakamura, S. (1976) Biochim. Biophys. Acta 426, 339—347
23 Kreiner, P.W., Keirus, J.J. and Bitensky, M.W. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1785—1789
24 Van Hoeven, R.P., Emmelot, P., Krol, J.H. and Oomen-Meulemans, E.P.M. (1975) Biochim. Biophys. Acta 380, 1—11
25 Demel, R.A., Jansen, J.W.C.M., van Dijck, P.W.M. and van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 465, 1—10
26 Dixon, M. and Webb, E.C. (1958) Enzymes, pp. 155—170, Longmans Green and Co., London
27 Silvius, J.R., Read, B.D. and McElhaney, R.N. (1978) Science 199, 902—904
28 Wynn-Williams, A.T. (1976) Biochem. J. 157, 279—281
29 Koshland, Jr., D.E. (1959) J. Cell. Comp. Physiol. Suppl. 1, 245—258