Biochimica et Biophysica Acta, 774 (1984) 254–260
Elsevier

BBA 72134

CHROMATOFOCUSSING AND CENTRIFUGAL RECONSTITUTION AS TOOLS FOR THE SEPARATION AND CHARACTERIZATION OF THE Na^+-COTRANSPORT SYSTEMS OF THE BRUSH-BORDER MEMBRANE *

JIANN-TRZUO LIN **, KORNELIA SCHWARC and ANNEMARIE STROH

Department of Physiology and Biophysics, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY (U.S.A.)

(Received December 7th, 1983)

Key words: Chromatofocussing; Na^+ transport; Cotransport; Brush-border membrane; Membrane reconstitution; Carrier protein; (Calf kidney)

Chromatofocussing was used for the separation of brush-border membrane proteins from calf kidney into 4 or 5 fractions over the pH range 4.0 to 7.4. These fractions were reconstituted into proteoliposomes by gradient centrifugation. Determination of the sodium-dependent solute uptake by proteoliposomes reconstituted from different chromatofocussed fractions showed that the sodium-D-glucose cotransport system was present in the fraction eluted between pH 5.3 and 5.8, and that the sodium-phosphate cotransport was present in fractions eluted between pH 4.6 and 5.3 and between pH 5.8 and 6.6, sodium-alanine cotransport could be detected in almost all fractions. Marker enzymes of the brush-border membrane, such as alkaline phosphatase, γ-glutamyltransferase and aminopeptidase M etc. were also found to be eluted at pH 7.0–7.4, 4.0–4.1 and 5.6–5.8, respectively. These results suggest that chromatofocussing is a promising tool for the separation of membrane proteins and for pre-purification of the sodium-D-glucose cotransport system. It can be further concluded that the sodium-dependent phosphate transport across the brush-border membrane is not dependent upon alkaline phosphatase activity.

Introduction

Studies of isolated brush-border membranes from kidney and intestine [1–3] have shown that a variety of sodium-solute cotransport systems and enzymes are present in these membranes. On SDS gels, complex polypeptide patterns are visible when stained with Commassie blue R or Amido black [4–6]. In order to isolate the sodium-D-glucose cotransport system, an affinity chromatographic method utilizing phlorizin has been introduced [4] based on the high affinity of phlorizin for the sodium-D-glucose cotransport system [7]. However, only a partial purification of the sodium-dependent D-glucose transport activity has been achieved [4]. Since the D-glucose transport proteins represent only about 0.1% of the total membrane protein a K_D of 1.2 μM for phlorizin for the sodium-D-glucose cotransport system [8] appears to be too low to allow a one-step purification of the system by affinity chromatography on the phlorizin polymer.

In the present study we have used chromatofocussing to pre-purify the sodium-D-glucose cotransport protein of brush-border membranes in the presence of a non-ionic detergent, β-octyl-

* Part of the results have been published in abstract form for the 67th Annual Meeting of the Federation of American Societies for Experimental Biology, No. 6227, April, 1983, Chicago, U.S.A.

** Present address: Department of Physiology, Cornell University Medical College, New York, NY 10021, U.S.A.

Abbreviation: Hepes, *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

glucoside. Fractions thus obtained during the chromatofocussing were then reconstituted into proteoliposomes using a gradient centrifugation method described by Brethés and his co-workers [9]. This reconstitution method is faster and more reproducible than the classic dialysis method [10].

As described here, reconstitution of chromatofocussed fractions into artificial liposomes by using the centrifugation method served as a useful tool to study the sodium-dependent transport activities.

Materials and Methods

Chemicals. D-[^{3}H]Glucose, β-[^{3}H]octylglucoside, L-[^{14}C]alanine and [^{33}P]phosphate were purchased from New England Nuclear. β-Octylglucoside was synthesized in our laboratory [11].

Membrane preparation. Brush-border membranes were prepared from calf kidney cortex using the calcium-precipitation method described previously [12]. The D-glucose uptake of the membranes in a sodium gradient (100 mM NaSCN) showed an overshoot of 450% versus equilibrium.

Enrichments of marker enzymes, such as alkaline phosphatase, aminopeptidase M and γ-glutamyltransferase were found to be 11-, 14- and 11-fold, respectively.

Chromatofocussing. Brush-border membranes were solubilized in 20 mM Hepes-Tris buffer (pH 7.4), containing 1% β-octylglucoside at 4°C for one hour. The ratio of detergent to membrane protein was 4:1 (w/w) as described previously [11]. Residual non-solubilized protein was removed by centrifugation at 100000 × *g* for 1 h. In previous studies, no D-glucose transport activity was found in the pellet fraction; therefore it was discarded. The supernatant was then subjected to chromatofocussing. An ion-exchange column (15 cm × 1 cm) of type PBE-94 (Pharmacia Co.) was pre-equilibrated with a starting buffer (20 mM imidazole-HCl, 0.1% azide, 0.5% β-octylglucoside, pH 7.4 at 4°C). Before applying the solubilized protein to the column, approx. 6 ml of limit buffer (Poly-buffer 74, Pharmacia Co., 1:8 dilution with water, 0.5% β-octylglucoside, adjusted pH 4.0 at 4°C with HCl) was pumped to the column, thereafter protein (60 mg) was applied to the column, and the column was eluted with the limit buffer. The pH in the effluent was assessed at intervals during the chromatography process by a pH-sensitive electrode. The appearance of protein was followed by an UV-monitor (280 nm wavelength). The chromatofocussing was considered complete when the effluent pH was 4 and no further protein absorption was noted.

Reconstitution. For the reconstitution a slight modification of the centrifugation method described by Brethés and co-workers [9] was used. Fractions obtained from chromatofocussing were reconstituted separately into artificial liposomes. A discontinuous sucrose gradient (dissolving various amounts of solid sucrose in a 20 mM Hepes-Tris buffer (pH 7.4), containing 100 mM NaCl, 0.1 mM Ca^{2+} and Mg^{2+}) was formed as follows: starting from the bottom of a 15 cm centrifuge tube, 2 ml of 60% sucrose; 3 ml of 40% sucrose involving liposomes (5 mg lipid/ml), prepared from asolectin/cholesterol (see below); 1 ml of 20% sucrose containing 0.1% β-octylglucoside; 9 ml of 10% sucrose containing 1% β-octylglucoside and 10 mM sodium borate. Protein from fractions obtained during chromatofocussing (fraction 1 to 4) or membrane crude extract (fraction 0) was mixed into the 10% sucrose zone at a level of 0.5 mg/ml. In order to detect the distribution of detergent (β-octylglucoside) after reconstitution, 5 μCi of β-[^{3}H]octylglucoside (0.1 mCi/ml) was also added to the 10% sucrose zone before centrifugation.

Samples in the sucrose gradient were then centrifuged at 200000 × *g* for 20 h at 10°C. After centrifugation, sequential 1 ml fractions were taken starting from the top of the tube. Fractions 11 to 13 with maximal turbidity and the highest protein content as shown in Fig. 3 were collected, combined, and diluted with a large excess amount of vesicle-buffer (200 mM mannitol/20 mM Hepes-Tris (pH 7.4)). The proteoliposome suspensions were centrifugated at 160000 × *g* for 1 h, and resulting pellets were again resuspended in a small amount of vesicle buffer and stored at −80°C before peorforming the transport assay.

Preparation of liposomes. Purified asolectin [4] and cholesterol (20% in wt.) were dissolved in chloroform (10 mg/ml), the chloroform was then evaporated under high vacuum (0.01 Torr) at 40°C. The solvent-free lipid mixture was sus-

pended in a Hepes-Tris buffer (20 mM, pH 7.4) to give a final lipid concentration of 45 mg/ml. Two sonications of 3 min each with a 1 min interval, were carried out with a cell disrupter, model W-220 F (Heat System, Ultrasonic Inc.) at 8°C. The suspension was centrifuged at $30000 \times g$ for 15 min. The liposomes were then centrifuged at $100000 \times g$ for 1 h and the pellet resuspended in the vesicle buffer.

Protein and enzyme assays. Protein concentrations in chromatofocussed fractions and in proteoliposomes were determined according to Lowry et al. [13]. Protein content of the sucrose gradient fractions were determined by the biuret method (Bio-Rad Laboratories, CA, U.S.A.). Alkaline phosphatase (EC 3.1.3.1), aminopeptidase M (EC 3.4.11.2.) and γ-glutamyltransferase (EC3.2.2.2.) were measured as described previously [4].

Transport studies. Sodium-dependent D-glucose, phosphate, and L-alanine uptake by membrane vesicles and proteoliposomes were determined under gradient conditions using the millipore filtration technique [14]. Millipore filters of pore size 0.22 μm and 0.45 μm, type GSWP were used in the transport studies with membrane vesicles and proteoliposomes, respectively.

Results

The chromatofocussing profile of the solubilized calf brush border membrane (in 1% β-octylglucoside) is shown in Fig. 1. A linear pH-gradient of 7.4–4.3 was formed during the chromatofocussing. Routinely, four or five protein fractions, monitored at 280 nm wavelength, could be collected. In order to maintain the biological activity, the pH of the collected fractions were adjusted to 7.4, and the fractions were kept at −80°C for further use. The total protein recovery was between 45 and 70%, depending on the column conditions. Also resolution of the ion-exchange column could be altered in a small extent if a larger column (30 cm × 1.2 cm) was used instead of the regular one (15 cm × 1.0 cm) and the similar amount of solubilized membrane proteins was subjected. Under this condition, five fractions were obtained (Table I). Most of the protein (30 to 40%) was recovered in the first fraction, which was eluted between pH 6.8 and 7.4. The distribution of

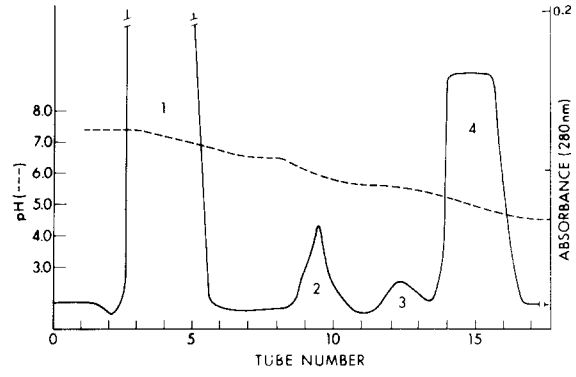

Fig. 1. Chromatofocussing of renal brush-border membrane proteins between pH 4 and 7.4. Membrane crude extracts (60 mg protein) of β-octylglucoside in 20 mM Hepes-Tris buffer (pH 7.4) were applied to a PBE -94 column (15×1 cm) pre-equilibrated with 25 mM imidazole buffer (pH 7.4 at 4°C) containing 0.5% β-octylglucoside. Protein fractions were eluted with the limit buffer (Polybuffer 74, pH 4.0, Pharmacia) containing 0.5% β-octylglucoside with an elution rate of 6 ml/h; 5 ml in each tube was collected.

protein in the various chromatofocussed fractions during chromatofocussing is shown in Table I and II. Electrophoresis of the fractions on SDS-gradient gels (4/30% gel, Pharmacia), at pH 5.6 showed significant differences in the polypeptide pattern between the fractions (Fig. 2). An actin-like band (43 kDa) was mainly found, for example, in fraction 2. Bands between 50 kDa and 60 kDa were visible in fraction 3, which was found to contain D-glucose transport activity (see below).

In order to examine specific transport proteins, fractions obtained during the chromatofocussing were incorporated into artificial liposomes. The sodium-dependent transport activity of D-glucose, phoshate and L-alanine of each fraction was determined. As shown in Fig. 3, after centrifugation, a continuous sucrose gradient was obtained. The turbidity maximum which indicates the presence of proteoliposomes and the protein absorption maximum were identicial, indicating an incorporation of protein into the liposomes fractions. Approximately 70% of the initial protein in the 10%-gradient fraction was found under the peak area (in the region between 20 to 30% sucrose). In contrast, most of the detergent, indicated by β-[^{3}H]octylglucoside, remained in the upper part of the gradient. In other words, only a small amount of β-octylglucoside was incorporated into the pro-

TABLE I

DISTRIBUTION OF BRUSH-BORDER (CALF KIDNEY) ENZYME ACTIVITIES (mU/mg PROTEIN) DURING CHROMATOFOCUSSING

Solubilized brush border membrane protein (71 mg) was subjected to a PBE-column (30 cm × 1.2 cm) that was pre-equilibrated with the starting buffer containing 0.5% β-octylglucoside. Aliquots of membrane crude extract (fraction 0) and fractions obtained at different pH ranges during chromatofocussing (fractions 1 to 5) were taken for enzyme assay. Data in parentheses show the protein recovery and the enrichment of enzyme activity, respectively. After chromatofocussing the pH of the protein fractions was adjusted to 7.4.

Fraction No.	pH	Protein (mg)	Enzyme activity		
			Alkaline phosphatase	Aminopeptidase M	γ-Glutamyl-transferase
0	7.4	71	667	471	2580
		(100%)	(1.00)	(1.00)	(1.00)
1	7.0–7.4	27	584	253	1672
		(38%)	(0.87)	(0.54)	(0.65)
2	6.5–7.0	1.6	0	242	332
		(5.9%)	–	(0.51)	(0.12)
3	5.6–5.8	0.4	0	1657	727
		(1.4%)	–	(3.51)	(0.28)
4	4.9–5.3	0.75	0	856	500
		(2.7%)	–	(1.81)	(0.19)
5	4.0–4.1	1.15	0	413	10937
		(4.4%)	–	(0.87)	(4.23)
Recovery		44%	33.4%	27%	32%

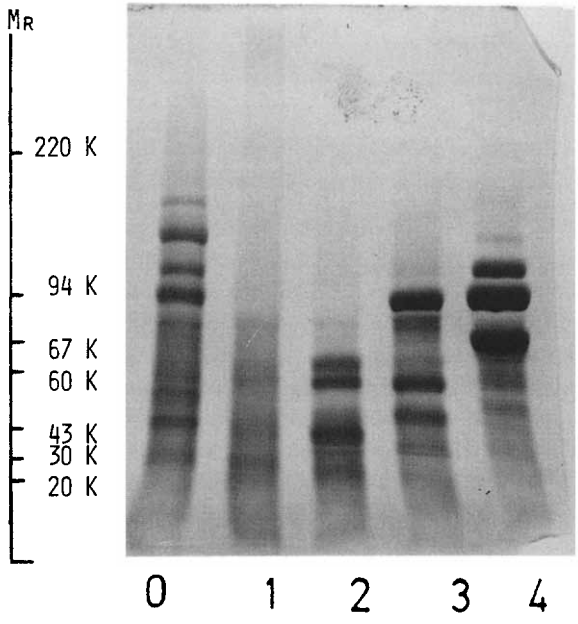

Fig. 2. Gradient SDS-gel (4/30% polyacrylamide gel) pattern. Electrophoresis was carried out in 10 mM sodium acetate/acetic acid buffer (pH 5.0), containing 2 mM Na-EDTA and 0.2% SDS. Samples were solubilized in 5% mercaptoethanol, 8% SDS and 20 mM Tris-HCl (pH 8.0) at 60 °C for 30 min. Approximately 80–100 μg protein per sample were applied to the gel: 0, membrane crude extract; 1, fraction between pH 6.8 and 7.4; 2, fraction between pH 6.3 and 6.8; 3, fraction between pH 5.5 and 6.3; 4, fraction between 4.0 and 5.5.

teoliposomes. The turbid fractions (proteoliposomes fractions) were collected, diluted with a large excess of vesicle-buffer (10- to 30-fold dilution) and sedimented at 150000 × *g* for 1 h. After resuspension, the proteoliposomes did not contain measurable ^3H (i.e., were free of detergent). Freeze-fracture of proteoliposomes showed a monolaminar structure and particles indicating an effective incorporation of protein (fig. not shown). The D-glucose uptake by proteoliposomes decreased linearly with increased osmolarity, varied from the external medium, indicating the presence of an intravesicular space and a translocation of D-glucose across the lipid bilayer of proteoliposomes [15]. However, the size of proteoliposomes was found to be heterogeneous and ranged between 0.2 to 1.0 μm. This heterogeneity may be due to a different lipid/protein ratio in the proteoliposomes.

The initial sodium dependent uptake of D-glucose, L-alanine and phosphate by proteoliposomes

TABLE II

SODIUM-DEPENDENT SOLUTE-UPTAKE BY PROTEOLIPOSOMES RECONSTITUTED FROM FRACTIONS DURING CHROMATOFOCUSSING

1. Proteins of one representative run of chromatofocussing on the PBE-column (15 cm × 1.0 cm) were used for the reconstitution. 2. Solute-uptake (glucose, L-alanine and phosphate) after 30 s incubation in the transport medium was performed under gradient conditions in the presence of 100 mM KNO_3 or $NaNO_3$ and either with 0.1 mM D-glucose, or 0.1 mM L-alanine or 0.5 mM phosphate. 3. Data represent the means ± S.D. of triplicate measurements. 4. Sodium-dependent uptake represents the difference between uptake in the presence of $NaNO_3$ and of KNO_3. 5. Data in parenthesis indicate the recovery of activity calculated on the base of protein recovery of fractions.

Fraction	Protein (recovery %)	pH	Protein (mg/ml) in proteoliposomes	D-Glucose (pmol/mg/30 s)	L-Alanine (nmol/mg/30 s)	Phosphate (nmol/mg/30 s)
0	100	7.4	0.42	71 ± 2.65 (100)	4.6 ± 0.24 (100)	13.6 ± 0.78 (100)
1	40	6.6–7.4	0.98	7 ± 1.4 (3.9)	1.3 ± 0.21 (11.1)	6.1 ± 0.46 (17.9)
2	5	5.8–6.6	0.2	84 ± 3.5 (5.9)	4.6 ± 0.32 (5.0)	26.2 ± 1.04 (9.6)
3	5.5	5.3–5.8	0.24	638 ± 8.62 (49.4)	4.7 ± 0.35 (5.6)	16.2 ± 1.23 (6.5)
4	28.3	4.6–5.3	0.29	126 ± 6.58 (35.3)	4.4 ± 0.28 (26.8)	27.9 ± 2.50 (58.0)

reconstituted from different protein fractions was evaluated as the difference in uptake in the presence of sodium and potassium under concentration gradient conditions as described in Methods and are summarized in Table II. Sodium-dependent D-glucose uptake was found to be highest in the chromatofocussing fraction obtained between pH 5.3 and 5.8, while proteins obtained at a pH above 5.8 showed only minimal D-glucose transport activity. The former fraction contained only 2–5% of the total protein applied to the column.

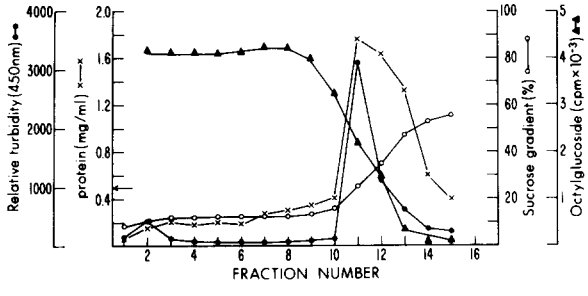

Fig. 3. Profile of the sucrose gradient after centrifugation. After centrifugation 1 ml fractions were taken, starting from the top of the centrifugation tube. Turbidity of the fractions were measured using a fluorescence spectrophotometer (Perkin Elmer 650-40). The wave length of excitation/emission was 450/450 nm. The sucrose gradient was determined in terms of the refraction indices at 22 °C, using the ABBE-3L refractometer (Baush and Lomb). 100 μl of each sample were mixed with 2 ml scintillation liquid and the radioactivity in the samples was determined as counts per minute (cpm). The initial protein level (approx. 0.5 mg/ml) is indicated by the arrow.

The sodium-dependent phosphate uptake was found to be present in the fraction eluted between pH 5.8 and 6.6, and in the fraction obtained at pH 4. This result suggests the possible existence of two phosphate transport systems or of two different forms (monomer-dimer) of one transport system, with different charge characteristics, in the brush-border membrane.

The sodium-dependent L-alanine uptake was present in all the other three protein fractions shown in Table II, except the fraction obtained above pH 6.6. This behaviour might reflect the multiplicity of transport systems for neutral amino acids in the brush-border membrane [3].

The results shown above indicate that most of the sodium cotransport proteins may have a negative charge under physiological conditions (pH 7.4), confirming the suggestion of Semenza et al. [16], Crane and Dorando [17] and Hilden and Sacktor [18] that the sodium-D-glucose cotransport system carries a net negative charge that may facilitate the suger transport process.

Analysis of the enzymatic activities of each fraction, as shown in Table I, reveals that alkaline phosphatase is localized in the protein eluted between pH 7.0 and 7.4, whereas phosphate transport was found in other fractions as mentioned above. This result indicates that different molecular entities catalyze phosphatase activity and phosphate transport. In accordance with this finding, Dousa and his associates [19] found that no effect

on the sodium-dependent phosphate transport activity was observed when alkaline phosphatase was removed from the brush-border membranes isolated from the kidneys of rabbits kept on normal P_i (inorganic phosphate) diet.

Aminopeptidase M was found in the fractions eluted between pH 5.0 and 6.0, and the activity of γ-glutamyltransferase was enriched in the fraction eluted at pH 4.0.

Discussion

We have demonstrated that an effective separation of functional proteins of brush-border membranes can be achieved between pH 7.4 and 4.0 by chromatofocussing in the presence of β-octylglucoside. β-Octylglucoside has a relatively high critical micell concentration (about 27 mM) [20] and the solubilization of membranes by β-octylglucoside is not affected by pH [21]. These two facts lead to a low interference of β-octylglucoside on the interaction between proteins and ion-exchanger over a wide pH-range during chromatofocussing. A control experiment showed that, for example, albumin was eluted at the same pH range (pH 4.5 to 5.5) from the chromatofocussing column in the presence and absence of β-octylglucoside (data not shown).

In a previous study [11], we demonstrated that most of membrane lipids were found in the supernatant after solubilization of brush border membranes with octylglucoside. Because of the high lipid content of this supernatant membrane proteins may not be separated strictly in order of their isoelectric points. Even so, useful separation of membrane proteins was achieved. Results of chromatofocussing indeed were reproducible in five independent runs.

The total recovery of protein during chromatofocussing can be affected by the size of ion-exchange column. While running chromatofocussing on a 15 cm × 1.0 cm column gave a 80% of protein recovery (Table II), only 44% recovery was obtained with a double length column (Table I). Taking loss of protein in the column into account, the recovery of alkaline phosphatase, aminopeptidase M and γ-glutamyltransferase was found to be 75%, 61% and 72%, respectively. An inactivation of enzyme activity due to the low pH or the possible removal of metal ions which are required for some enzymes in activated forms [22] could be the causes for the slight loss of enzyme activities. The total recovery of D-glucose and phosphate transport (see (Table II) are 95% and 92%, calculated on the basis of the total protein recovery, providing another information about the enrichment of transport activities in some fractions obtained during chromatofocussing. The recovery of L-alanine was low and enrichment in the transport activity was observed in all fractions. Amino acid transport systems are more complicated by the fact that at least three different amino acid transport systems are present in the brush-border membrane. The L-alanine uptake might not be representative for the entire amino acid transport systems. Another reason might be the sensitivity of the amino acid transporter to the pH-shift or to the possible removal of metal ions.

Our primary purpose was to isolate the sodium-D-glucose cotransport system of the renal brush-border membrane [4]. A protein fraction enriched in the sodium-dependent (D-glucose transport activity was obtained (see Table II). This fraction represents only 2–5% of the total membrane proteins subjected to chromatofocussing, that is, more than 95% of membrane proteins that are not related to the sodium-D-glucose cotransport were removed by chromatofocussing. Thus, it seems that chromatofocussing in fact can be regarded as a powerful tool to enrich the sodium-D-glucose cotransport system of the brush-border membrane.

Another interesting finding is that the sodium-D-glucose cotransport protein that was eluted between pH 5.3 and 5.8 should be negatively charged under physiological conditions, pH 7.4. The sodium coupled D-glucose translocation across the brush border membrane might be facilitated by this negative charge. We believe that this is a valuable insight for the understanding of the transport process across biomembranes.

Acknowledgments

The authors thank Dr. R. Kinne for his interest and constructive suggestions. This work was supported by USPHS Grant BRSG 613-4564 awarded to the Albert Einstein College of Medicine, and by Arthur Andersen & Company, a Research Grant

awarded by the New York Heart Association to J.-T.L. and by NIH Grant GM 27859.

References

1 Evers, C., Hasse, W., Murer, H. and Kinne, R. (1978) Membrane Biochem. 1, 203–219
2 Kenny, A.J. and Maroux, S. (1982) Physiol. Rev. 62, 91–128
3 Murer, H. and Kinne, R. (1980) J. Membrane Biol. 55, 81–95
4 Lin, J.T., Da Cruz, M.E.M., Riedel, S. and Kinne, R. (1981) Biochim. Biophys. Acta 640, 43–54
5 Malathi, P., Preiser, H. and Crane, R.K. (1980) Ann. N.Y. Acad. Sci. 358, 253–266
6 Klip, A., Grinstein, S. and Semenza, G. (1979) J. Membrane Biol. 51, 47–73
7 Bode, F., Bauman, K. and Diedrich, D.F. (1972) Biochim. Biophys. Acta 290, 134–149
8 Lin, J.T. and Hahn, K.-D. (1983) Anal. Biochem. 129, 337–344
9 Brethés, D., Averet, N., Gulik-Krzywicki, T. and Chevallier, J. (1981) Arch. Biochem. Biophys. 210, 149–159
10 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487
11 Lin, J.T., Riedel, S. and Kinne, R. (1979) Biochim. Biophys. Acta 557, 179–187
12 Riedel, S. (1980) Dissertation, Rhein. Westf. Tech. Hochschule, Aachen, F.R.G.
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
14 Murer, H., Hopfer, U., Kinne-Saffran, E. and Kinne, R. (1974) Biochim. Biophys. Acta 345, 170–179
15 Kinne, R., Murer, H., Kinne-Saffran, E., Thees, M. and Sachs, G. (1975) J. Membrane Biol. 21, 375–395
16 Todenburger, G., Kessler, M. and Semenza, G. (1982) Biochim. Biophys. Acta 688, 557–575
17 Crane, R.K. and Dorando, F.C. (1979) Function and Molecular Aspects of Biomembrane Transport (Quagliariello, E., Palmieri, F., Papa, S. and Klingenberg, M., eds.), pp. 271–278, Elsevier/North-Holland Biomedical Press, Amsterdam
18 Hilden, S. and Sacktor, B. (1982) Am. J. Physiol. F340–F345
19 Yusufi, N.K.A., Low, M.C., Turner, S.T. and Dousa, T.P. (1983) J. Biol. Chem. 258, 5695–5701
20 Baron, C. and Thompson, T.E. (1975) Biochim. Biphys. Acta 352, 276–285
21 Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta 415, 29–79
22 Komoda, T., Sakagishi, Y. and Sekine, T. (1981) Clin. Chim. Acta 117, 167–187