Functional reconstitution of the hepatic System N amino acid transport activity

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Hepatic System N is responsible for the active plasma-membrane transport of glutamine, histidine and asparagine. This report describes the solubilization and reconstitution of System N activity. Differential solubility resulted in an approximate enrichment of almost 600-fold compared with total cell homogenate. The results indicate that reconstitution can be utilized as a functional assay during purification of the hepatic System N carrier protein.

INTRODUCTION

Glutamine is the most abundant free amino acid in the plasma and serves as a unique carrier of carbon and nitrogen between different tissues in the body [1,2]. Glutamine carbon may be utilized to generate energy in rapidly dividing cells such as the enterocyte [3], or it may support gluconeogenesis in tissues such as the liver [4]. NH_4^+ generated by catabolism in extrahepatic tissues is converted into glutamine, a non-toxic carrier of nitrogen, and then taken up by the liver, where the nitrogen may be utilized for ureagenesis and subsequently removed from the body. Within the liver acinus there is a division of labour with regard to glutamine metabolism. The periportal cells contain high levels of glutaminase and the majority of the liver's total activity for the urea cycle [5]. In contrast, glutamine synthetase activity is localized in a small population of hepatocytes, termed the perivenous cells, which are localized around the central vein [6]. Thus NH_3 by-passing the ureagenic periportal cells is scavenged by the perivenous cells, where the NH_3 is incorporated into glutamine, which is released into the circulation. This process has been termed the 'Intercellular Glutamine Cycle' by Haussinger and colleagues [5,7]. The key role played by glutamine translocation across the plasma membrane of the periportal hepatocytes is underscored by the observation that glutamine transport may represent one rate-limiting step for its hepatic metabolism [8].

The concentrative Na^+ -dependent transport of glutamine across the hepatocyte plasma membrane is mediated by a carrier termed System N, which also transports histidine, and to a lesser extent asparagine [9,10]. Although the general characteristics have been described at the cellular level, little is known about the membrane protein responsible for this activity. In order to isolate and characterize the carrier protein fully, a functional assay is required. Reconstitution of the solubilized carrier into proteoliposomes would allow one to monitor System N activity, and therefore serve as a reliable functional assay during purification. Quesada & McGivan [11] have reported the reconstitution of Na^+ -dependent alanine and glutamine transport from rat liver plasma membranes. The latter presumably is the result of System N mediation. Lectin affinity chromatography of the solubilized proteins resulted in an enrichment of alanine transport activity, but glutamine transport activity was not retained by the column. In the present study, we have described an alternative procedure for the solubilization and subsequent reconstitution

into proteoliposomes of the System N carrier. The optimal conditions for the solubilization and storage of the carrier are presented, as well as a description of general properties of the carrier in both plasma-membrane vesicles and proteoliposomes.

EXPERIMENTAL

Preparation of rat liver plasma-membrane vesicles

Plasma-membrane vesicles were isolated from intact rat liver by a modification of the protocol described by Prpic *et al.* [12]. The liver of an anaesthetized rat was perfused with ice-cold phosphate-buffered saline (150 mM- NaCl /10 mM-sodium phosphate, pH 7.5), removed, weighed and placed in a small volume of SEB buffer (250 mM-sucrose/1 mM-EGTA/10 mM-Hepes, pH 7.5). The liver was minced and homogenized with a Dounce homogenizer, with 10 strokes of a loose-fitting pestle followed by 4 strokes of a tight-fitting pestle. The homogenate was diluted with SEB buffer to 6% (w/v) liver, and then centrifuged at 132 g for 2 min. The supernatant was kept, and then centrifuged at 1500 g for 10 min at 4 °C. The resulting pellet was resuspended in SEB buffer and homogenized with one stroke of a loose-fitting pestle in a Dounce homogenizer. The mixture was filtered through cheese-cloth and adjusted to a final volume of 62.4 ml in SEB buffer. To this suspension 8.4 ml of Percoll was added, and the mixture was centrifuged in six Corex tubes at 35000 g for 30 min at 4 °C. Plasma membranes were collected by using a 18-gauge needle from a band in the upper one-third of the gradient, but below a fluffy lipid layer near the top of the gradient. The vesicles were washed free of Percoll by low-speed centrifugation in 5 vol. of SMB buffer (250 mM-sucrose/1 mM- MgCl_2 /10 mM-Hepes, pH 7.5). The plasma-membrane suspension in SMB buffer was centrifuged at 35000 g for 30 min, and the pellet was resuspended in a minimal volume of SMB. Batches were stored frozen at -70 °C and thawed only once, just before use.

Solubilization of plasma-membrane proteins

System N activity was solubilized from the plasma membranes by using a detergent extraction protocol described previously [13,14]. Membrane vesicles were diluted with an equal volume of solubilization buffer containing 2.5% cholate, 4 M-urea, 0.1 mM-EDTA, 100 mM-KCl, 1 μM -phenylmethanesulphonyl fluoride and 10 mM-Hepes, pH 7.5. The mixture was incubated for 30 min at 4 °C and then centrifuged at 100000 g for 30 min. Insoluble

Abbreviations used: PEG, poly(ethylene glycol) (8000); MeAIB, 2-(methylamino)isobutyric acid.

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membrane remnants were discarded, and the supernatant, which contained most of the System N transport activity, was used for further study.

Detergent removal with poly(ethylene glycol)

Solubilized membrane protein (1 vol.) was mixed with 2 vol. of 30% poly(ethylene glycol) 8000 (PEG) (Sigma P-2139) unless indicated otherwise. The mixture was gently swirled and placed on ice. After incubation for 15 min at 4 °C, the mixture was then centrifuged at 100 000 *g* for 30 min. The resulting supernatant was discarded and, without resuspension, the pellet was gently rinsed three times in KMB buffer (200 mM-KCl/1 mM-MgCl₂/10 mM-Hepes, pH 7.5) to remove residual PEG. The pellet was then resuspended in a minimal volume of KMB buffer in preparation for reconstitution.

Reconstitution of PEG-precipitated protein

After precipitation with PEG, the solubilized membrane protein was reconstituted into liposomes as described by Fafouroux *et al.* [14]. Briefly, asolectin stock (soybean phospholipid) was prepared by dissolving 40 mg of asolectin in 1 ml of KMB buffer under N₂. The tube was capped and sonicated in a bath-type sonicator (Laboratory Supplies, Hicksville, NY, U.S.A.) for 15 min or until the cloudy solution became translucent. The asolectin stock was added to the solubilized protein at a lipid:protein ratio of 20:1 (w/w) unless indicated otherwise. A typical experiment utilized 0.5 mg of protein and 10 mg of asolectin in a 1 ml volume. The mixture was frozen in liquid N₂, thawed at room temperature, and then diluted with 10 vol. of KMB buffer and sonicated for 5 s. The resulting proteoliposomes were pelleted by centrifugation at 100 000 *g* for 30 min at 4 °C and resuspended in a minimal volume of KMB.

System N transport assay

The assay for System N-mediated transport was initiated by adding 20 μ l of uptake buffer (either 200 mM-KSCN or -NaSCN, 2 mM-MgCl₂ and 20 mM-Hepes, pH 7.5) containing 100 μ M-[³H]histidine (0.02 mCi/ml) and unlabelled 20 mM-2-(methyl-amino)isobutyric acid (MeAIB) to 20 μ l (10–20 μ g of protein) of proteoliposomes or plasma-membrane vesicles in KMB. The function of the unlabelled MeAIB is to serve as a competitive inhibitor of the System A carrier [15], thus assuring that all of the Na⁺-dependent histidine uptake observed is mediated by System N [9]. Both vesicles and uptake buffer had been pre-warmed to 37 °C. The uptake mixture was vortex-mixed and incubated at 37 °C for the time indicated for each experiment, diluted with 1 ml of ice-cold phosphate-buffered saline, and then filtered over a 0.45 μ m-pore-size nitrocellulose filter under vacuum. The filter was washed with 3 $\times$ 4 ml of ice-cold phosphate-buffered saline and placed in a scintillation vial containing 5 ml of scintillation fluid. In general, each assay utilized 10–20 μ g of reconstituted membrane protein. Transport rates were proportional to the amount of protein added over the range 5–100 μ g. Control incubations were performed in the absence of membrane vesicles to determine the non-specific binding of radioactivity by the filter. This 'blank' value was typically less than 5% of the total radioactivity associated with the filters containing vesicles, and was subtracted from all samples.

Protein determination

Proteoliposomes or plasma-membrane vesicles were incubated in 0.2 M-NaOH containing 0.2% SDS, and then the solubilized proteins were precipitated by adding ice-cold 24% (w/v) trichloroacetic acid. Protein was pelleted by centrifugation, resuspended in 100 μ l of 0.2 M-NaOH/0.2% SDS, and then 650 μ l of copper reagent (0.58 mM-Cu-EDTA, 189 mM-Na₂CO₃,

100 mM-NaOH and 1% SDS) was added. After incubation for 10 min, 60 μ l of Folin-Ciocalteu reagent (diluted 1:1 with water) was added, and the A₅₀₀ was determined after a 30 min incubation [16,17].

Materials

Cholic acid was obtained from Sigma and was recrystallized three times as described by Kagawa & Racker [18]. Ultra-pure urea was purchased from Pierce, and asolectin was obtained from Associated Concentrates Inc. (Woodside, NY, U.S.A.). [³H]Histidine was purchased from ICN Pharmaceuticals. Nitrocellulose filters were Gelman type GN-6 (0.45 μ m). Male Sprague-Dawley rats (150–200 g) were obtained from a colony maintained by the University of Florida Animal Resources Facility.

RESULTS AND DISCUSSION

The transport of amino acids by the hepatic System N carrier has been characterized in whole cells [9,19,20] and plasma-membrane vesicles [21,22], but the membrane protein responsible for this activity has not been identified, and a complete description of the transporter necessitates the development of a functional assay. Consequently, we have developed a protocol for the solubilization of System N activity from rat liver plasma membranes and its subsequent reconstitution into proteoliposomes. Detergent extraction of plasma membranes with 2.5% cholate/4 M-urea had been employed to solubilize System A activity in Ehrlich ascites cells [13] and rat liver [14]. A similar protocol effectively extracted approx. 90% of the total System N activity from rat liver plasma-membrane vesicles. Quesada and McGivan have shown that 5% decanoyl-*N*-methylglucamide will also solubilize System N activity [11].

Time course of System N-mediated uptake by proteoliposomes

After solubilization, precipitation with PEG and reconstitution into proteoliposomes as described in the Experimental section, the hepatic System N activity was measured by assaying Na⁺-dependent histidine uptake (Fig. 1). The time course for accumulation by the proteoliposomes showed that uptake was linear for the first 2–3 min and began to slow considerably over the next 15 min. Over 90% of the uptake was Na⁺-dependent, in agreement with results from intact cells [9]. The data demonstrate that transport assays of 1 min or less provide an estimate of initial rate.

Fig. 1. Time course of histidine uptake in proteoliposomes

For this, 20 μ l of proteoliposomes (10–20 μ g of protein) were incubated at 37 °C with 20 μ l of uptake buffer containing 100 μ M-[³H]histidine, 20 mM-MeAIB and either 200 mM-NaSCN (●) or 400 mM-KSCN (○). Histidine uptake was terminated at 0.5, 1, 3, 5, 10 or 20 min by addition of 1 ml of ice-cold phosphate-buffered saline as described in the text, and is expressed in pmol/mg of protein (△, Na⁺-dependent uptake). The results are means $\pm$ S.D. of at least four determinations. Where not shown, the S.D. bars are contained within the symbol.

Fig. 2. Optimization of PEG precipitation and the lipid:protein ratio for reconstitution of System N activity

Plasma-membrane proteins were solubilized as described in the text. Solubilized proteins were precipitated with PEG at final concentrations of 3% (○), 10% (●), or 20% (△). Precipitated proteins were resuspended in KMB buffer and mixed with asolectin phospholipid from a 40 mg/ml stock. The amount of asolectin added to the precipitated protein was varied to give lipid:protein ratios of 5, 10, 20, or 30. The mixture was subjected to the standard reconstitution protocol described in the Experimental section, and the proteoliposomes were collected by centrifugation at 100 000 *g* for 20 min at 4 °C. System N activity in proteoliposomes was measured for 1 min at 37 °C as the Na⁺-dependent uptake of 50 μM-[³H]histidine in the presence of 10 mM-MeAIB. The results are means ± S.D. of at least four determinations. Where not shown, the S.D. bars are contained within the symbol.

Optimization of PEG precipitation and lipid:protein ratio

After detergent extraction of transport activity, detergent removal was essential before reconstitution. Several other techniques have been employed for the removal of detergents, including dialysis, (NH₄)₂SO₄ precipitation and gel filtration [23,24]. We have demonstrated previously that the solubilized System A carrier was precipitated in an active form after extended dialysis, and went on to show that the carrier could be selectively precipitated with 3% PEG [14,25]. Therefore, in the present study, final PEG concentrations of 20%, 10% or 3% were used to precipitate proteins from the STAB (20% glycerol, 2 mM-EDTA, 2 mM-dithiothreitol, 0.05% Triton X-100, 0.25 mg of asolectin/ml and 10 mM-Tris, pH 7.4)-treated protein fraction (Fig. 2). The System N activity was not subject to selective precipitation over this range of PEG. However, to ensure the recovery of as much of the carrier activity as possible, a final concentration of 20% PEG was used routinely in all subsequent experiments.

The reconstitution of membrane proteins in a functionally active state is dependent on the composition of the artificial phospholipid bilayer as well as the relative lipid:protein ratio [25–27]. Therefore, the lipid:protein ratio was varied from 5 to 30 during the reconstitution of solubilized System N activity (Fig. 2). The functional reconstitution of the carrier was clearly sensitive to the amount of lipid added during the procedure. A significant decrease in System N activity was observed when the lipid:protein ratio was either greater than or less than 20:1. When a final PEG concentration of 10% was used, the decrease in recovered activity at low lipid:protein ratios was not as large as that observed for PEG values of 3% or 20%. Nevertheless, a ratio of 20:1 was consistently the most effective. Therefore, all further experiments were performed with the 20:1 ratio to achieve optimal System N activity.

Stability of the carrier in STAB buffer

To test the stability of the System N carrier during storage, the activity was solubilized, precipitated with 20% PEG, and then

Fig. 3. Stability of System N carrier activity stored in STAB buffer

Solubilized plasma-membrane proteins were precipitated with 20% PEG, and subsequently resuspended in STAB buffer at a concentration of 1 mg/ml. The suspension was sonicated for 15 s in a bath-type sonicator, and insoluble material was removed by centrifugation at 15 000 *g* for 2 min. The clear supernatant was divided into batches, which were incubated at 4 °C (○), -20 °C (●), or -70 °C (△) for 24 or 48 h. One sample (*t* = 0) was immediately mixed with 2 vol. of 30% PEG and the precipitated protein was reconstituted into proteoliposomes. At 24 or 48 h after storage at the appropriate temperature, the STAB-solubilized protein was precipitated with 20% PEG (final concn.) and reconstituted in proteoliposomes. System N activity was determined as the Na⁺-dependent uptake of 50 μM-[³H]histidine in the presence of unlabelled MeAIB. The results are expressed as percentage of System N activity at 0 h. The control (*t* = 0) values were 2683 ± 253 and 1455 ± 139 pmol/min per mg of protein. Independent experiments with different membrane preparations yielded quantitatively similar results.

resuspended in STAB buffer. Portions were stored at 4 °C, -20 °C or -70 °C for 2 days. The transporter could be stored for 24 h at 4 °C with minimal loss of activity (Fig. 3). Freezing at either -20 °C or -70 °C even for 24 h was detrimental to transporter recovery. System N activity was decreased by only 25% after 48 h of storage at 4 °C, whereas storage of the protein for the same length of time at -20 °C or -70 °C led to the loss of 70% or more of the activity.

Substrate specificity of the reconstituted carrier

To establish that the apparent System N activity in proteoliposomes was the result of solubilization and partial purification of the same activity present in intact rat hepatocytes, some properties of the carrier in plasma-membrane vesicles and proteoliposomes were compared. The substrate specificity of the carrier was monitored by inhibition analysis of Na⁺-dependent [³H]histidine uptake in the presence of 10 mM unlabelled MeAIB to block System A-mediated transport [15]. Glutamine, a preferred substrate of System N [9], caused 86% inhibition of histidine uptake by plasma-membrane vesicles and 74% inhibition of the activity in proteoliposomes (Table 1). Amino acids which are predominately System ASC substrates, such as serine and threonine [28], did not significantly decrease System N-mediated histidine uptake in either plasma-membrane vesicles or proteoliposomes. These results parallel earlier observations on the substrate selectivity of System N in whole cells [9] and plasma-membrane vesicles [21,22], and demonstrate the specificity of MeAIB-insensitive Na⁺-dependent histidine uptake toward the System N carrier.

Sensitivity of the reconstituted System N carrier to pH

System N-mediated uptake of amino acids by whole cells is decreased in response to extracellular pH values below 7.4 [9]. To determine the sensitivity of the reconstituted System N carrier to pH, histidine transport by plasma-membrane vesicles or proteoliposomes was measured for 1 min in uptake buffer adjusted to

Table 1. Substrate specificity of the System N carrier in rat liver plasma-membrane vesicles and proteoliposomes

System N activity was monitored as the Na⁺-dependent uptake of 50 μ M-[³H]histidine in the presence of 10 mM-MeAIB alone (None) or of 10 mM-MeAIB and 5 mM-glutamine, -serine or -threonine. Transport was measured for 1 min at 37 °C. Results are expressed as the percentage of System N-mediated histidine uptake in the absence of inhibitors. The control values for plasma-membrane vesicles and proteoliposomes were 330 $\pm$ 9 and 1479 $\pm$ 87 pmol/min per mg of protein respectively. Duplicate experiments utilizing different membrane preparations resulted in quantitatively similar data.

Inhibitor	Histidine uptake (%)	
	Proteoliposomes	Plasma-membrane vesicles
None	100	100
Glutamine	26	14
Serine	87	92
Threonine	89	100

Table 2. Reconstitution and partial purification of the hepatic System N activity

Rat liver plasma-membrane proteins were solubilized, mixed with 2 vol. of 30 % PEG, and then pelleted by centrifugation at 100000 *g* for 30 min. Precipitated proteins were resuspended in KMB buffer (4–5 mg/ml) and then reconstituted into liposomes as described in the Experimental section. In a second series of experiments, the PEG-precipitated proteins were resuspended in STAB buffer, the solution was clarified by centrifugation as described in the text, and then the proteins were PEG-precipitated again before resuspension in KMB buffer and reconstitution. Proteoliposomes were collected by centrifugation at 100000 *g* for 30 min. System N activity was monitored in either the plasma-membrane vesicles or the proteoliposomes as the Na⁺-dependent uptake of 50 μ M-[³H]histidine in the presence of 10 mM unlabelled MeAIB. The reported specific activities (pmol/min per mg of protein) are means $\pm$ s.d. of four determinations. The protein values are expressed in mg, and the total units of transport are given as pmol/min. The values in parentheses are calculated enrichments compared with the total cell homogenate and are based on the actual enrichment of plasma-membrane marker activity of approx. 30-fold (Na/K-ATPase; 0.77 and 24.0 μ mol/h per mg of protein for homogenate and plasma-membrane vesicles respectively).

Sample	Total protein	Specific activity	System N enrichment	Total units of transport
Plasma-membrane vesicles	3	90	1 (30)	270
Proteoliposomes	0.23	1070	12 (360)	246
STAB-treated proteoliposomes	0.15	1705	19 (570)	256

pH 6.0, 7.0, 7.5 or 8.0. With the exception of changing the assay pH, System N activity was measured as described in the Experimental section. Maximal histidine uptake was observed at pH 7.5, and the alkaline pH of 8.0 had little or no effect. In contrast, a decrease in extravesicular pH below 7.0 resulted in a significant decline in System N activity. At pH 6.0 the activity present in either proteoliposomes or membrane vesicles was approx. 25 % of that at pH 7.5.

Effect of substitution of Li⁺ for Na⁺ on System N activity

System N-mediated glutamine uptake functions equally well

when energized by a gradient of Li⁺ or Na⁺ [9,21]. System N-mediated uptake of histidine by proteoliposomes or plasma-membrane vesicles was measured in buffers containing either 100 mM-NaCl or 100 mM-LiCl. Histidine uptake in proteoliposomes was approx. 70 % of that with Na⁺ when Li⁺ was substituted (427 and 285 pmol/20 s per mg of protein for Na⁺ and Li⁺ respectively), and the carrier in native membrane vesicles behaved similarly (242 and 174 pmol/20 s per mg of protein for Na⁺ and Li⁺ respectively). The data indicate that the activities present in plasma-membrane vesicles or proteoliposomes are not different with respect to the substitution of Li⁺ for Na⁺. The similarity of the response in vesicles and proteoliposomes indicates that the reconstituted carrier is functioning in a manner similar to the native membrane.

Selective solubilization of System N activity

System N activity, extracted from plasma membranes in 2.5 % cholate/4 M-urea and precipitated with 20 % PEG, was reconstituted into proteoliposomes. Detergent extraction and PEG precipitation led to recovery of 8 % of the total membrane protein, and consequently the specific activity of the reconstituted System N carrier was increased 12-fold compared with plasma-membrane vesicles (Table 2). Despite the recovery of only 8 % of the protein, 91 % of the total carrier activity present in the plasma-membrane vesicles was recovered in the proteoliposomes. To test for further purification of the transporter by differences in solubility [14], the PEG-precipitated proteins were resuspended in STAB buffer, followed by centrifugation to clarify the turbid suspension. This procedure results in the selective extraction of certain plasma-membrane proteins, and the System A carrier was enriched significantly by this protocol [14,29]. For System N, the additional step increased the carrier specific activity 19-fold compared with plasma-membrane vesicles (Table 2). Once again, over 90 % of the total carrier activity was recovered in the proteoliposomes after solubilization and reconstitution. The relative enrichment of specific activity is consistent with the degree of protein recovery, and suggests partial purification of the carrier protein.

Summary

This report has described conditions for the effective solubilization and reconstitution of the hepatic System N carrier. Conditions for short-term stabilization and storage were investigated, and the data suggest that storage in STAB buffer at 4 °C is considerably less harmful than freezing. Despite the overall recovery of only 5 % of the total protein, the achievement of an estimated enrichment of almost 600-fold over the cell homogenate, while recovering over 90 % of the total transporter activity, is an important initial step in purification. Although the proteoliposomes prepared after enrichment for System N activity also contain System A-mediated transport, the two activities are not enriched by the same procedures, suggesting significant differences in molecular structure. In future investigations, reconstitution will be used as an assay to monitor System N activity and to facilitate the purification and characterization of the carrier.

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REFERENCES

1. Welbourne, T. C. (1987) *Am. J. Physiol.* **253**, F1069–F1076
2. Bulus, N., Cersosimo, E., Ghishan, F. & Abumrad, N. N. (1989) *Metab. Clin. Exp.* **38** (Suppl. 1), 1–5

3. Windmueller, H. G. (1982) *Adv. Enzymol.* **53**, 202–237
4. Joseph, S. K. & McGivan, J. D. (1978) *Biochim. Biophys. Acta* **543**, 16–28
5. Haussinger, D. (1983) *Eur. J. Biochem.* **133**, 269–275
6. Gebhardt, R. & Mecke, D. (1983) *EMBO J.* **2**, 567–570
7. Haussinger, D., Sies, H. & Gerok, W. (1984) *J. Hepatol.* **1**, 3–14
8. Haussinger, D., Soboll, S., Meijer, A. J., Gerok, W., Tager, J. M. & Sies, H. (1985) *Eur. J. Biochem.* **152**, 597–603
9. Kilberg, M. S., Handlogten, M. E. & Christensen, H. N. (1980) *J. Biol. Chem.* **255**, 4011–4019
10. Bode, B., Tamarappoo, B. K., Mailliard, M. & Kilberg, M. S. (1990) *JPEN, J. Parenter. Enteral Nutr.* **14**, 51S–55S
11. Quesada, A. R. & McGivan, J. D. (1988) *Biochem. J.* **255**, 963–969
12. Prpic, V., Green, K. C., Blackmore, P. F. & Exton, J. H. (1984) *J. Biol. Chem.* **259**, 1382–1385
13. McCormick, J. I., Tsang, D. & Johnstone, R. M. (1984) *Arch. Biochem. Biophys.* **231**, 355–365
14. Fafournoux, P., Dudenhausen, E. E. & Kilberg, M. S. (1989) *J. Biol. Chem.* **264**, 4805–4811
15. Christensen, H. N., Oxender, D. L., Liang, M. & Vatz, K. A. (1965) *J. Biol. Chem.* **240**, 3609–3616
16. Zak, B. & Cohen, J. (1961) *Clin. Chim. Acta* **6**, 665–670
17. Markwell, M. A. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206–213
18. Kagawa, Y. & Racker, E. (1971) *J. Biol. Chem.* **246**, 5477–5487
19. Weissbach, L. & Kilberg, M. S. (1984) *J. Cell. Physiol.* **121**, 133–138
20. Gebhardt, R. & Kleemann, E. (1987) *Eur. J. Biochem.* **166**, 339–344
21. Jacob, R., Rosenthal, N. & Barrett, E. J. (1986) *Am. J. Physiol.* **251**, E509–E514
22. Leonardi, M. G., Comolli, R. & Giordana, B. (1988) *Pflugers Arch.* **411**, 328–332
23. Allen, T. M., Romans, A. Y., Kercret, H. & Segrest, J. P. (1980) *Biochim. Biophys. Acta* **601**, 328–342
24. Ingham, K. C. (1984) *Methods Enzymol.* **104**, 351–356
25. Bracy, D. S., Schenerman, M. A. & Kilberg, M. S. (1987) *Biochim. Biophys. Acta* **899**, 51–58
26. Hirata, H. (1986) in *Techniques for the Analysis of Membrane Proteins* (Ragan, C. I. & Cherry, R. J., eds.), pp. 77–96, Chapman and Hall, London
27. Pick, U. (1981) *Arch. Biochem. Biophys.* **212**, 186–194
28. Kilberg, M. S., Handlogten, M. E. & Christensen, H. N. (1981) *J. Biol. Chem.* **256**, 3304–3312
29. Kilberg, M. S. (1989) in *Hepatic Transport of Organic Solutes* (Petzinger, E., Kinne, R. K. H. & Sies, H., eds.), pp. 167–176, Springer-Verlag, Berlin

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