Glycoconjugate Journal (1993) **10**:170–174

Effects of cyclodextrins on the hydrolysis of ganglioside G_{M1} by acid β-galactosidases

TAKAYUKI SHIRAISHI, MASAO HIRAIWA and YUTAKA UDA*

Laboratory of Health Chemistry, Niigata College of Pharmacy, 5-13-2 Kamishin'ei-cho, Niigata, Niigata 950-21, Japan

Received 2 November 1992

The hydrolysis of ganglioside G_{M1} by acid β-galactosidases was greatly enhanced by the inclusion of heptakis(2,6-di-*O*-methyl)-β-cyclodextrin or α-cyclodextrin in the assay mixture. The other cyclodextrins tested were not effective. The extent of stimulation by these cyclodextrins was relatively smaller than those by taurodeoxycholate and taurochenodeoxycholate. However, it is suggested that stimulation by bile salts may be partly a reflection of the detergent effects of bile salts on G_{M1} and partly a reflection of the interaction between bile salts and the enzyme itself. On the other hand, the stimulation by the cyclodextrins seems to correlate to the formation of an inclusion complex between G_{M1} and cyclodextrin without enzyme protein interaction.

Keywords: G_{M1} ganglioside β-galactosidase assay, dimethyl-β-cyclodextrin, α-cyclodextrin, ganglioside G_{M1}

Introduction

This laboratory has recently studied glycosidases [1, 2]. Synthetic substrates, such as *p*-nitrophenyl or 4-methylumbelliferyl glycosides, are usually used for the assay of glycosidase activities. While this approach offers the practical advantage of simplicity, it is often necessary to use natural substrates when detailed studies of specific glycosphingolipid glycosidases are intended [1]. In this instance bile salts are included in the assay systems as necessary components. They probably act by converting the glycosphingolipid substrates to mixed micellar forms more suitable for enzyme reaction. However, it has been pointed out that different brands and batches of bile salts lead to various activities for a particular enzyme [3]. In addition, bile salts may act on enzyme proteins themselves [4]; although this activity was shown to be an advantage in the study of two genetically distinct acid β-galactosidases [5].

Alpha-, β- and γ-cyclodextrins are cyclic oligosaccharides composed of six, seven and eight glucose moieties, respectively. These natural cyclodextrins can form water-soluble inclusion compounds with substances which are sparingly soluble in water [6]. α-Cyclodextrin was demonstrated to solubilize long chain fatty acids, ceramide and cerebroside [7], and was included in the reaction mixture of fatty acyl CoA ligase assay [8]. Recently, chemically modified cyclodextrins have become of interest because their physicochemical properties and inclusion behaviour are different from those of natural cyclodextrins [9]. Cyclodextrins and their methylated derivatives have been used in the study of *Mycobacterium phlei* fatty acid synthetase [10].

In the present investigation, natural and chemically modified cyclodextrins have been examined by introducing them into an assay system for acid β-galactosidase, which is known to be involved in a lysosomal storage disease, G_{M1} gangliosidosis [11].

Materials and methods

Materials

Tritiated sodium borohydride was purchased from American Radiolabeled Chemicals Inc. (USA), and galactose oxidase from Worthington Biochemical Corporation (USA), 4-methylumbelliferyl β-galactoside from Koch-Light Laboratories (UK), and diethylaminoethyl cellulose DE-52 from Whatman Ltd. (UK). Ganglioside G_{M1} was prepared from bovine brain as previously described [12], except that the isolated G_{M1} was purified by Iatrobeads chromatography. Sodium taurocholate, sodium taurodeoxycholate, sodium taurochenodeoxycholate, sodium cholate, and sodium deoxycholate were all from Calbiochem (USA). α-Cyclodextrin was purchased from Hayashiba Biochemical Laboratories, Inc. (Japan), and γ-cyclodextrin from Wako Pure Chemical Industries, Ltd. (Japan). Hexakis(6-*O*-hydroxypropyl)-α-cyclodextrin (hydroxypropyl-α-cyclodextrin), heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (dimethyl-β-cyclodextrin), heptakis(6-*O*-hydroxyethyl)-β-cyclodextrin

* To whom correspondence should be addressed.

0282-0080 © 1993 Chapman & Hall

(hydroxyethyl-β-cyclodextrin), heptakis(6-*O*-hydroxypropyl)-β-cyclodextrin (hydroxypropyl-β-cyclodextrin), β-cyclodextrin-polymer, and octakis(6-*O*-hydroxypropyl)-γ-cyclodextrin (hydroxypropyl-γ-cyclodextrin) were kindly donated by Nippon Shokuhin Kako Co. Ltd.

Preparation of tritium labelled ganglioside G_{M1}

Ganglioside G_{M1} was labelled in the terminal galactose moiety by the galactose oxidase/tritiated sodium borohydride method [13] with modification. The galactose oxidase-oxidation product and tritiated ganglioside G_{M1} were isolated from the respective reaction mixtures by using Bond Elut C_{18} columns (Analytichem, USA) [14]. The tritiated G_{M1} was finally purified by preparative thin layer chromatography with developing in chloroform:methanol:0.25% $CaCl_2 \cdot 2H_2O$ (55:45:10 by vol). The specific radioactivity of purified tritium labelled ganglioside G_{M1} was 2.46 mCi/μmol. Radioactivity was determined with an Aloka LSC-700 scintillation counter. Ganglioside G_{M1} was quantified by a fluorescamine assay for sphingosine [15].

G_{M1} ganglioside β-galactosidases

The enzymes were purified from human placenta and bovine brain as previously reported [16, 17] with modification as follows: the post *p*-aminophenyl β-D-thiogalactopyranoside affinity chromatography fraction from each tissue was further purified by using a Shim pack Diol 300 HPLC column [18].

Enzyme assay

1, Hydrolysis of 4-methylumbelliferyl β-galactoside was assayed by the procedure described previously [19]. 2, Hydrolytic liberation of tritiated galactose from the labelled G_{M1} was determined as previously described [19] with some modification as follows: tritiated G_{M1} (10 nmol, 40 000 disintegrations min^{-1}) was suspended in 50 μl of 0.2 M citrate-phosphate buffer (pH 4.7) containing 10 μmol NaCl, 10 μg bovine serum albumin (BSA) and various amounts of solubilizers (cyclodextrins or bile salts). The mixture was sonicated for 10 min prior to adding 50 μl (2–2.4 u) enzyme solution. After incubation for 1 h at 37 °C, the reaction was terminated with 400 μl cold 40% methanol containing 0.2% galactose and 0.4% sodium dodecylsulfate, and then cooled in an ice bath. After brief sonication, the mixture was applied to a dry column of diethylaminoethyl cellulose DE-52, which was prepared as follows: 500 μl of diethylaminoethyl cellulose DE-52 was packed into a blue tip (Iwaki Lab. Ware, Japan) plugged with fine glass wool, and washed successively with 1 mM galactose once, methanol twice, and diethyl ether twice by centrifugation, followed by drying on phosphorus pentoxide in a vacuum desiccator. The mixture on the column was allowed to stand for 10 min prior to elution by centrifugation. Radioactivity of 200 μl aliquots of the eluate was measured in 3 ml of ACS II scintillant (Amersham International). Activity of G_{M1} hydrolysis is expressed as nmol h^{-1} per unit; one unit is the amount of enzyme which releases 1 μmol galactose from 4-methylumbelliferyl β-galactoside per min at 37 °C.

Sucrose density gradient centrifugation

Tritiated G_{M1} (10 nmol, 40 000 disintegrations min^{-1}) was sonicated for 10 min in 100 μl of 0–10 mM cyclodextrin solution and applied to the top of a linear sucrose density gradient (2–30% sucrose in 1 M NaCl, 5 ml). Centrifugation was performed at 35 000 rev min^{-1} for 5 h at 20 °C with a Hitachi SCP 85H ultracentrifuge in an RPS-50 rotor. The gradient solution was taken out from the bottom of the tube and collected in 6 drop portions. Radioactivity of 100 μl aliquots of each fraction was counted in 3 ml of ACSII scintillant.

Results and discussion

In the study of lipid enzymology, there is a principal and continuing problem with the insolubility of lipid substrates. This problem is addressed by the inclusion of solubilizers such as detergents or cyclodextrins. This study investigated the effect of unsubstituted and chemically modified cyclodextrins on the hydrolysis of ganglioside G_{M1} by acid β-galactosidase. As listed in Table 1, unsubstituted and chemically modified cyclodextrins were included in the reaction mixtures for the hydrolysis of ganglioside G_{M1} catalysed by purifed human placental acid β-galactosidase. The reaction was carried out in the presence of 5 mM or 10 mM concentrations of various cyclodextrins under standard assay conditions. The [^{3}H]galactose liberated was separated by using a dry DEAE-cellulose column prior to counting, as described in the Materials and methods section. Although ganglioside G_{M1} should disperse in the reaction mixture as micelles, the hydrolysis of G_{M1} hardly proceeded without solubilizers. On adding cyclodextrin to the reaction mixture, liberation of [^{3}H]galactose from [^{3}H]G_{M1} was observed as shown in Table 1. The relative effects of the cyclodextrins were compared by expressing the results obtained for each reaction relative to the activity (designated arbitrarily as 100) obtained in the presence of 10 mM α-cyclodextrin. The rates of hydrolysis of G_{M1}, in the presence of α-cyclodextrin or dimethyl-β-cyclodextrin, were considerably greater than in their absence, whereas slower rates of hydrolysis were obtained with other cyclodextrins. As shown in Fig. 1, the activities of the two acid β-galactosidases purified from human placenta and bovine brain, respectively, were dependent on the concentration of α-cyclodextrin and dimethyl-β-cyclodextrin. In the presence of dimethyl-β-cyclodextrin at a concentration of 5 mM, maximum activities for human placental enzyme and bovine brain enzyme were 1.69 nmol h^{-1} per unit and 1.55 nmol h^{-1} per unit, respectively. It should be noted here that the activity is expressed as nmol per h per unit, not per mg protein, in order to compare the activations of the enzymes

Table 1. Effect of cyclodextrins on the hydrolysis of ganglioside G_{M1} by acid β-galactosidase. The cyclodextrins were included in the assay mixture at concentrations of 5 mM or 10 mM. For details see the text and the Materials and methods section.

	5 mM		*10 mM*	
	Hydrolysis rate (nmol h^{-1} per unit)	*Relative rate (%)*	*Hydrolysis rate (nmol h^{-1} per unit)*	*Relative rate (%)*
α-Cyclodextrin	0.54	50.5	1.07	100
Hydroxypropyl-α-cyclodextrin	0.05	4.7	0.08	7.5
Dimethyl-β-cyclodextrin	1.89	176	1.59	148
Hydroxyethyl-β-cyclodextrin	0.08	7.5	0.11	10.3
Hydroxypropyl-β-cyclodextrin	0.11	10.3	0.13	12.1
β-Cyclodextrin · polymer	0.20	18.7	0.33	30.8
γ-Cyclodextrin	0.11	10.3	0.26	24.3
Hydroxypropyl-γ-cyclodextrin	0.02	1.9	0.04	3.7

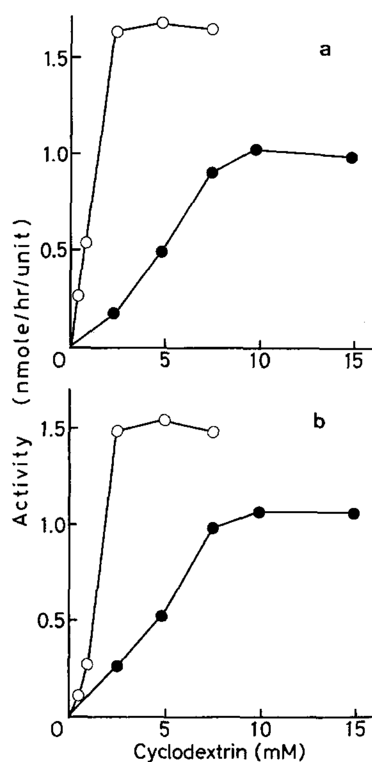

Figure 1. Effects of cyclodextrins on G_{M1} hydrolysis by the acid β-galactosidases. Assays were carried out with the enzymes purified (a) from human placenta and (b) bovine brain according to the standard procedure described in the Materials and methods section, except that the amounts of solubilizers (●, α-cyclodextrin; ○, dimethyl-β-cyclodextrin) were varied as indicated.

from different sources by solubilizers. In the case of α-cyclodextrin, activation of each enzyme reached a maximum (1.03 nmol h^{-1} per unit and 1.05 nmol h^{-1} per unit, respectively) at a concentration of 10 mM. Thus, both acid β-galactosidases were stimulated to a similar extent by dimethyl-β-cyclodextrin and α-cyclodextrin, respectively.

In order to compare the effects of the cyclodextrins with those of the bile salts, which are usually used as solubilizers in assaying the hydrolysis of glycosphingolipids, bile salts were included in the G_{M1} hydrolysis reaction mixtures instead of cyclodextrins. Human placental acid β-galactosidase was greatly activated, especially by taurodeoxycholate (17.3 nmol h^{-1} per unit at 2.3 mM) and taurochenodeoxycholate (16.2 nmol h^{-1} per unit at 2.3 mM) as shown in Fig. 2. Bovine brain acid β-galactosidase was also activated, but the extent of its activation was smaller than that of human placental enzyme (3.11 nmol h^{-1} per unit at 3.8 mM taurochenodeoxycholate and 3.66 nmol h^{-1} per unit at 3.8 mM taurodeoxycholate). Although G_{M1} hydrolysis proceeded very rapidly in the presence of bile salts, except for deoxycholate, compared with that in the presence of the cyclodextrins, the stimulation profiles of these enzymes were very different from each other (Fig. 2). These different profiles lead us to presume that the enzyme activity may be affected by not only detergent effect of bile salts but also direct interaction between bile salts and enzyme proteins. Figure 3 shows the influence of these stimulants on the hydrolysis of 4-methylumbelliferyl β-galactoside, a water soluble substrate, which does not require any solubilizers for its hydrolysis. The addition of bile salts slowed down the hydrolysis reaction, while the cyclodextrins did not affect the reaction significantly. Moreover, the addition of bile salts changed the K_M value of the hydrolysis of 4-methylumbelliferyl β-galactoside. The K_M value was 49.6 μM without solubilizers. On the other hand, K_M values were 23.1 μM with 7.7 mM taurodeoxycholate, 26.6 μM with 7.7 mM taurochenodeoxycholate, 25.3 μM with 8.1 mM cholate, and 20.9 μM with 7.5 mM taurocholate. These results suggest that stimulation by the addition of bile salt may be partly a reflection of the detergent effects of the bile salt on G_{M1}, and partly a reflection of the interaction between bile salts and the enzyme itself [4].

Effect of pH on the hydrolysis of G_{M1} was examined in

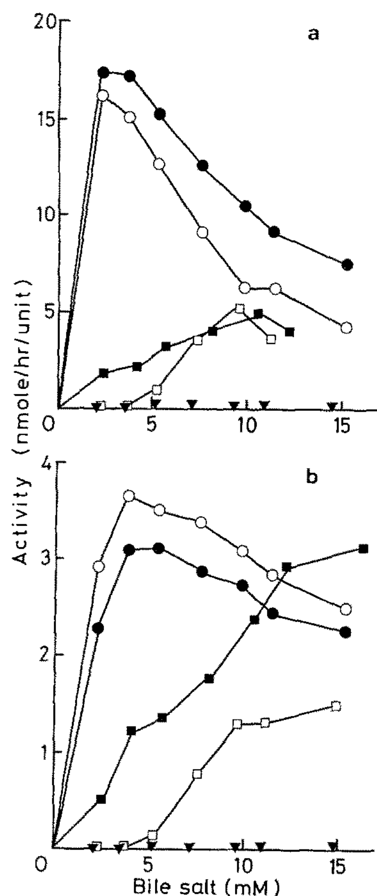

Figure 2. Effects of bile salts on G_{M1} hydrolysis by the acid β-galactosidases. Hydrolysis with (a) the human placental enzyme was carried out according to the standard procedure except for the amount of enzyme (1 unit) and incubation time (30 min), using various amounts of bile salts. The conditions of the reaction with (b) bovine brain acid β-galactosidase were the same as the standard procedure except that the amount of bile salts varied as indicated. ●, Taurodeoxycholate; ○, taurochenodeoxycholate; ■, cholate; □, taurocholate; ▼, deoxycholate.

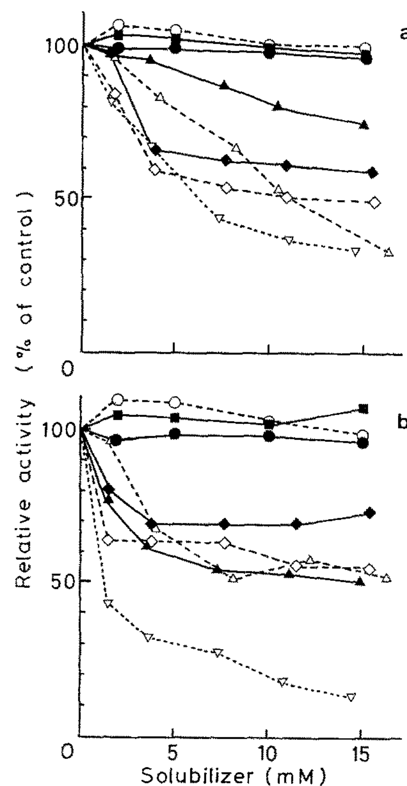

Figure 3. Effects of bile salts and cyclodextrins on the hydrolysis of 4-methylumbelliferyl β-galactoside by acid β-galactosidases from (a) human placenta and (b) bovine brain. The incubation mixtures contained 1 mM 4-methylumbelliferyl β-galactoside, 50 mM citrate-phosphate buffer, pH 4.7, 100 mM NaCl, 0.12 unit of the enzyme, and various amounts of bile salts or cyclodextrins as indicated in a total volume of 0.5 ml. ●, α-Cyclodextrin; ■, hydroxypropyl-α-cyclodextrin; ○, dimethyl-β-cyclodextrin; ◆, taurodeoxycholate; ◇, taurochenodeoxycholate; ▲, taurocholate; △, cholate; ▽, deoxycholate.

the presence of α-cyclodextrin as a solubilizer and compared with enzyme activity in the presence of taurodeoxycholate. The pH curve obtained in the presence of α-cyclodextrin is bell-shaped with a pH optimum at 4.7 (Fig. 4). In the case of taurodeoxycholate, although the same pH optimum was obtained, the activity fall on the acidic side of the optimum pH was greater than in the case of α-cyclodextrin. This was probably due to a change of a detergent property in the acidic region [20]. Thus the solubilizing properties of α-cyclodextrin do not seem to be affected by pH.

As already shown in Table 1, significant differences in the degree of activation by the respective cyclodextrins were observed. As the influence of cyclodextrins on the enzyme itself is thought to be negligible, as discussed above, the differences in the activation effects of the respective cyclodextrins may be involved in the formation of inclusion complexes of cyclodextrins and ganglioside G_{M1}. To

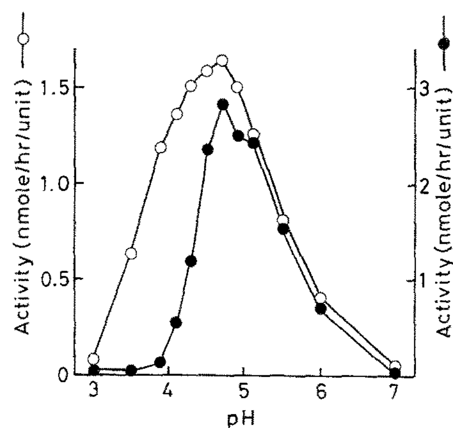

Figure 4. Effect of pH on G_{M1} hydrolysis by bovine brain acid β-galactosidase in the presence of α-cyclodextrin or taurodeoxycholate. Assays were carried out using ○, 10 mM α-cyclodextrin, or ●, 7.6 mM taurodeoxycholate, according to the standard procedure at various pHs as indicated.

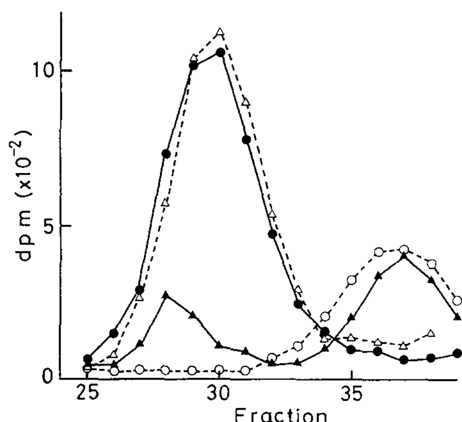

Figure 5. Complex formation between G_{M1} and cyclodextrins. The solution of tritiated G_{M1} was sonicated with or without cyclodextrins, then analysed by using the sucrose density gradient centrifugation method described in the Materials and methods section. ●, None; ○, 10 mM α-cyclodextrin; ▲, 10 mM γ-cyclodextrin; △, 10 mM hydroxypropyl-α-cyclodextrin.

examine this, the formation of inclusion complex was analysed by a sucrose density gradient centrifugation method. When a 0.1 mM solution of tritium labelled G_{M1} (a concentration in excess of CMC [21, 22]) without added cyclodextrin was fractionated after centrifugation, a radioactivity peak appeared in the relatively large molecular weight region (Fig. 5). This shows that G_{M1} was recovered as micellar forms. On addition of 10 mM α-cyclodextrin, which effectively stimulated the hydrolysis (Table 1, Fig. 1), the radioactivity of G_{M1} appeared in the smaller molecular weight region rather than in micellar forms. In the case of 10 mM dimethyl-β-cyclodextrin, the profile was almost identical to that obtained with α-cyclodextrin (data not shown). These findings indicate that all of the G_{M1} was present as inclusion complexes with these cyclodextrins. In the presence of 10 mM γ-cyclodextrin, which stimulated the reaction less effectively than α-cyclodextrin and dimethyl-β-cyclodextrin as shown in Table 1, radioactive peaks were observed in both the micellar forms and the inclusion complexes. In the presence of 10 mM hydroxypropyl-α-cyclodextrin, one of the poor stimulants shown in Table 1, a peak of radioactivity was obtained not in the inclusion complexes but in the micelles. These results suggest that the activating effects of cyclodextrins correlate to the ability of inclusion complex formation.

This paper investigated the effect of cyclodextrins on the hydrolysis of ganglioside G_{M1} by acid β-galactosidases and has revealed that α-cyclodextrin and dimethyl-β-cyclodextrin activate the hydrolytic reactions. The results obtained in this study suggest that the activation correlates with the inclusion complex formation without enzyme protein interaction, although the detailed mechanism of activation is still obscure. In addition, we propose here the utilization of these cyclodextrins in the assay systems of glycosphingolipid hydrolases.

References

1. Hiraiwa M, Uda Y (1988) *Japan J Exp Med* **58**:129–38.
2. Nashida (nee Itoh) T, Shiraishi T, Uda Y (1988) *Chem Pharm Bull* **36**:4000–7.
3. Tanaka H, Suzuki K (1975) *J Biol Chem* **250**:2324–32.
4. Poulos A, Beckman K (1980) *Clin Chim Acta* **107**:27–35.
5. Kobayashi T, Shinnoh N, Goto I, Kuroiwa Y (1985) *J Biol Chem* **26**:14982–87.
6. Saenger W (1980) *Angew Chem Int Ed Engl* **19**:344–62.
7. Singh I, Kishimoto Y (1983) *J Lipid Res* **24**:662–65.
8. Singh I, Singh R, Bhushan A, Singh AK (1985) *Arch Biochem Biophys* **236**:418–26.
9. Uekama K (1985) *Pharmacy Int* 61–65.
10. Bergeron R, Machida Y, Bloch K (1975) *J Biol Chem* **250**:1223–30.
11. Norden AGW, O'Brien JS (1975) *Proc Natl Acad Sci USA* **72**:240–44.
12. Svennerholm L (1972) *Methods Carbohydr Chem* **6**:464–74.
13. Radin NS, Hof L, Bradly RM, Brady RO (1965) *Brain Res* **14**:497–505.
14. Williams MA, McLeur RH (1980) *J Neurochem* **35**:266–69.
15. Higgins TJ (1984) *J Lipid Res* **25**:1007–9.
16. Lo J-T, Mukerji K, Awasthi YC, Hanada E, Suzuki K, Srivastava SK (1979) *J Biol Chem* **254**:6710–15.
17. Hiraiwa M, Uda Y (1986) *J Biochem (Tokyo)* **100**:707–15.
18. Hiraiwa M, Uda Y, Nishizawa M, Miyatake T (1987) *J Biochem (Tokyo)* **101**:1237–79.
19. Hiraiwa M, Shiraishi T, Uda Y (1986) *J Biochem (Tokyo)* **100**:255–58.
20. Helenius A, Simons K (1975) *Biochem Biophys Acta* **415**:29–79.
21. Formisano S, Johnson ML, Lee G, Aloj SM, Edelhoch H (1979) *Biochemistry* **18**:1119–24.
22. Rauvala H (1979) *Eur J Biochem* **97**:555–64.