Biochimica et Biophysica Acta, 613 (1980) 420—428

BBA 68983

DIFFERENTIAL BEHAVIOUR OF EEL AND ERYTHROCYTE ACETYLCHOLINESTERASE ON *N*-METHYLACRIDINE AFFINITY COLUMNS

IMPORTANCE OF LIGAND AFFINITY AND CONCENTRATION

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(Received September 4th, 1979)

Key words: Acetylcholinesterase: N-Methylacridine; Ligand affinity; (Eel, erythrocyte)

Summary

The retention and elution of acetylcholinesterase from bovine erythrocytes and electric eel on *N*-methylacridinium affinity columns have been compared at various ligand concentrations. A soluble 7.7 S dimeric form of bovine erythrocyte acetylcholinesterase required a ligand concentration of 2.0—2.8 μmol/ml in 0.1 M NaCl for retention, compared to 0.44 μmol/ml for various forms of the electric eel acetylcholinesterase. The difference in the retention of acetylcholinesterase from these two sources could not be explained by differences in their oligomeric structure. The affinity of bovine erythrocyte acetylcholinesterase for *N*-methylacridinium was 13-fold or more lower than the electric eel acetylcholinesterase at similar ionic strengths. *N*-Methylacridinium appeared to react selectively with the catalytic anionic site of both enzymes. It was concluded that the affinity of the side arm ligand was the major determinant of the differences in the retention properties of the eel and erythrocyte acetylcholinesterase. The difference in affinity for *N*-methylacridinium probably reflects differences in the organic cation binding region of the two enzymes.

Introduction

Affinity chromatography is one of the most widely used methods for the purification of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) from a variety of sources [1—3]. Berman [1] purified bovine erythrocyte and electric eel acetylcholinesterase to specific activities of 2300 and 10 000 units/

mg protein, respectively, with *meta*-(erythrocyte) and *para*-(eel) phenyltrimethylammonium affinity ligands. Subsequently, the phenyltrimethylammonium ligand was used for the purification of human erythrocyte acetylcholinesterase [3—5]. More recently, 10-methyl-9-[*N*-β-(6-amino-hexanoyl)-β-aminopropylamino]acridine has been used extensively for the purification of eel [6—9] and brain [10] acetylcholinesterase, as it offered a number of advantages over the phenyltrimethylammonium ligand. Specifically, *N*-methylacridinium exhibits a higher affinity for acetylcholinesterase than the phenyltrimethylammonium ligand at high ionic strength and allows preferential retention of the 18 S plus 14 S forms from a mixture of these and the 11 S proteolytic derivative [9]. Reavill and Plummer [10] found *N*-methylacridinium to be more efficient than the phenyltrimethylammonium ligand for the purification of acetylcholinesterase from pig cerebral cortex. In the present study we have investigated the suitability of *N*-methylacridinium for the purification of bovine erythrocyte acetylcholinesterase from a commercial source and have compared the affinity chromatographic behaviour of this enzyme with the behaviour of a dimeric species of eel acetylcholinesterase under similar condition.

Methods

The 6-aminohexanoyl derivative of *N*-methylacridinium was synthesized and coupled to Sepharose 2B as described by Webb and Clark [9], at nominal ligand concentrations of 0.5, 1.0, 2.0 and 2.8 μmol per ml Sepharose 2B.

Affinity chromatography of erythrocyte acetylcholinesterase. 250 units of bovine erythrocyte acetylcholinesterase (Sigma Chemical Company), dissolved in 200 ml of 0.1 M NaCl and 20 mM sodium phosphate, pH 7.4 (Buffer L), was loaded onto affinity columns (3 ml) equilibrated with Buffer L, as described previously [9]. Following washing of the column with 10 vol. Buffer L, enzyme retained by the column was eluted with 20 mM decamethonium bromide in Buffer L. Fractions eluted from the column were dialyzed for 16 h against Buffer L before assay. Acetylcholinesterase activity was assayed by a radiometric method in the presence of 0.1 M sodium phosphate buffer, pH 7.4, as previously described [11]. Protein was determined both by the method of Lowry et al. [12] and Murphy and Kies [13].

Preparation and affinity chromatography of guanidine-dissociated electric eel acetylcholinesterase. Acetylcholinesterase purified from high salt extracts of electric organ tissue as described previously [9], and comprised of approximately equal amounts of 18 S and 14 S acetylcholinesterase and negligible 11 S acetylcholinesterase, was dissociation with guanidine-HCl to obtain acetylcholinesterase dimers and dimers plus 'tail'. Acetylcholinesterase (1 mg/ml) was sonicated in 2.7 M guanidine-HCl and 1 M NaCl, 20 mM sodium phosphate (pH 7.0) in a final volume of 1.0 ml, using a Braun sonicator at setting 40. The sample was sonicated in three 10-s bursts with 30-s cooling periods in between in an ice bath. The sample was then dialyzed for 5 h against 500 ml of the above buffer and diluted with 100 ml of Buffer L containing 10 mg of bovine serum albumin. This solution was loaded onto a 5 ml *N*-methylacridine affinity column at a ligand concentration of 0.46 μmol/ml gel.

Following loading and washing [9] the column was eluted with 20 mM decamethonium in 0.1 M NaCl, then equilibrated in 1.0 M NaCl, 20 mM sodium phosphate, pH 7.0 (Buffer H) and finally eluted with 20 mM decamethonium in Buffer H. The guanidine-dissociated enzyme and the column eluates were characterized by gel permeation chromatography and sucrose gradient sedimentation as described previously [9]. In some characterizations of the guanidine-dissociated enzyme active-site-radiolabelled acetylcholinesterase, prepared as previously described [14], was used to facilitate the detection of small quantities of enzyme.

Kinetic studies. Inhibition of acetylcholinesterase by *N*-methylacridinium was determined using acetylthiocholine substrate, by the method of Ellman et al. [15]. The assay conditions were similar to those described by Wermuth and Brodbeck [16]. The reaction was started by the addition of various concentrations of acetylthiocholine to a mixture containing (in a final concentration) 5,5′-dithiobis(2-nitrobenzoic) acid (0.125 mM), bovine serum albumin (0.01%) and acetylcholinesterase (0.62 units/3 ml) in 25 mM sodium phosphate, pH 7.4. The initial velocity was determined during the first 1—2 min from a recording of the change in absorption at 412 nm with time.

The effect of calcium on *N*-methylacridinium inhibition was determined by the pH-stat method at 25°C (Radiometer). *N*-Methylacridinium-purified bovine erythrocyte acetylcholinesterase (0.5 units) was incubated with *N*-methylacridinium at the required concentration for 5 min in the absence or in the presence of 0.1 mM $CaCl_2$. The reaction was started by the addition of 0.4 mM acetylcholine. Experiments were done in the absence of added ion or in the presence of 40 mM NaCl.

Materials. *N*-Methylacridinium was synthesized and purified as described by Mooser et al. [17]. [1-^{14}C]Acetylcholine iodide (1—5 Ci/mol) was from New England Nuclear. Acetylcholine perchlorate was from British Drug Houses. Acetylthiocholine iodide, 5,5′-dithiobis(2-nitrobenzoic acid), decamethonium bromide, acridine and iodomethane were purchased from Sigma. All inorganic salts were of analytical reagent grade.

Results

Affinity chromatography of bovine erythrocyte acetylcholinesterase

The relative amounts of bovine erythrocyte acetylcholinesterase retained and eluted on *N*-methylacridinium-affinity columns are shown in Fig. 1. As the ligand concentration was increased from 0.5 to 2.8 μmol/ml gel, more of the enzyme activity was retained during the loading and buffer-wash steps. Whereas very little enzyme activity was retained at ligand concentrations of 0.5 and 1.0 μmol/ml, the enzyme was retained at ligand concentrations of 2 and 2.8 μmol/ml, and was eluted with 20 mM decamethonium. A single affinity purification of the bovine erythrocyte acetylcholinesterase at a ligand concentration of 2.8 μmol/ml of gel yielded preparations with a specific activity of 60 μmol/mg per min; this was increased to 420 μmol/mg per min after a second passage through the column.

Composition of guanidine-dissociated electric eel acetylcholinesterase

Sonication of 18 S plus 14 S eel acetylcholinesterase in the presence of

2.7 M guanidine hydrochloride, followed by brief dialysis, completely altered the sucrose gradient profile, as shown in Fig. 2. The 18 S form of the enzyme was greatly reduced while a considerable proportion of the total enzyme sedimented more slowly than the tetrameric 11 S form. Gel permeation chromatography carried out on the fractions designated I and II in Fig. 2 eluted in the same volume as previously reported for the tail-containing forms of eel acetylcholinesterase [14]. The fractions labelled III showed approximately equal amounts of 11 S and tail-containing acetylcholinesterase forms, while the fractions designated IV eluted after the tetrameric 11 S form on the gel column (data not shown). These data are consistent with the conclusion that the species arising from sonication of 18 S plus 14 S acetylcholinesterase in the presence of guanidine hydrochloride is the 8 S dimeric form of the eel enzyme previously described in a catalytically inactive or degraded state [18].

Affinity chromatography of guanidine-dissociation electric eel acetylcholinesterase

At low ionic strength (Buffer L), all forms of acetylcholinesterase generated by sonication of 18 S and 14 S eel acetylcholinesterase were retained on the affinity column. The column elution profile is shown in Fig. 3; three peaks of acetylcholinesterase activity were generated by the three solutions used (A—C). The composition of the three peaks was analyzed on sucrose gradients (results not shown). Tetrameric (11 S) and dimeric (8 S) forms of acetylcholinesterase co-eluted from the affinity resin in the presence of decamethonium at low ionic strength, while larger forms were released only at high ionic strength.

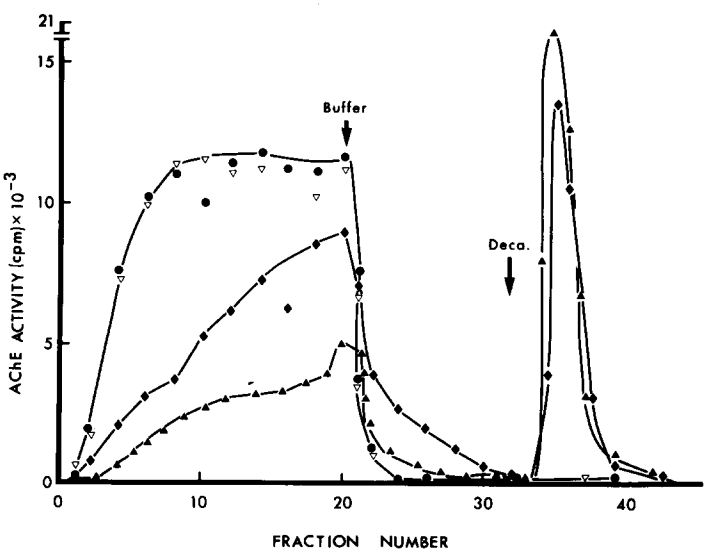

Fig. 1. Affinity chromatography of bovine erythrocyte acetylcholinesterase on *N*-methylacridinium-Sepharose 2B columns at various ligand concentrations. The columns were loaded as described in Methods. At the first arrow marked 'Buffer', Buffer L was applied to the columns to remove unbound acetylcholinesterase and nonspecifically adsorbed proteins. At the arrow marked 'Deca', 20 mM decamethonium was applied. Fractions (1.4 ml) were addayed for acetylcholinesterase activity by the radiometric assay. The ligand concentrations were 0.49 μmol/ml (●); 0.99 μmol/ml (▽); 1.97 μmol/ml (◆); and 2.8 μmol/ml (▲). AChE, acetylcholinesterase.

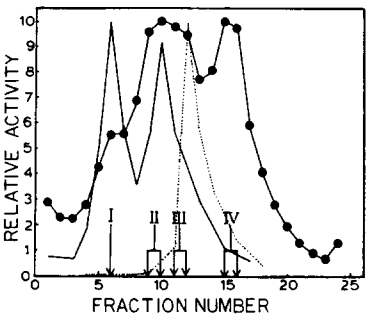

Fig. 2. Sucrose gradient sedimentation profile of guanidine hydrochloride sonicated eel acetylcholinesterase. Fresh affinity chromatography-purified and active-site-radiolabelled [14] 18 S plus 14 S eel acetylcholinesterase was sonicated in the presence of guanidine hydrochloride as described under Methods (●———●). Also shown are profiles obtained for intact 18 S plus 14 S acetylcholinesterase (———), and for trypsin-digested 11 S acetylcholinesterase (· · · · · ·), centrifuged simultaneously in separate sucrose gradients. The fractions designated I, II, III and IV were subjected to gel permeation chromatography as discussed in the text (figures not shown). Sedimentation was from right to left and acetylcholinesterase concentration is normalized to the same maximum value for all profiles.

Kinetics of N-methylacridinium inhibition of bovine erythrocyte acetylcholinesterase

To investigate the nature of the interaction between *N*-methylacridinium and bovine erythrocyte acetylcholinesterase, the kinetics of inhibition was studied. Lineweaver-Burk plots show that both $K_{m(app)}$ and V were altered by

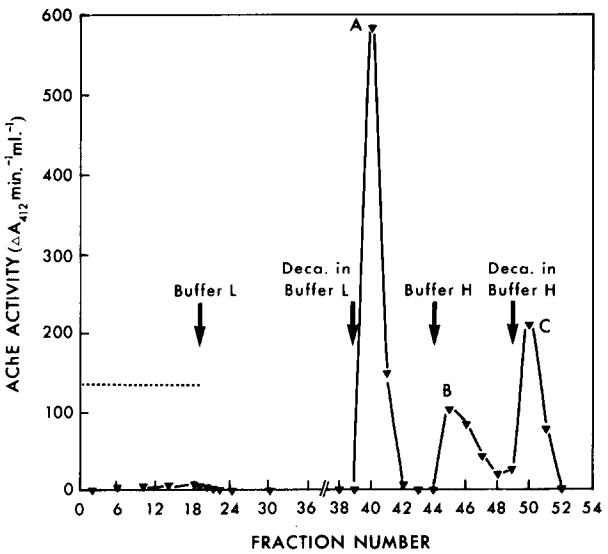

Fig. 3. Affinity chromatography elution profile of sonicated guanidine-dissociated acetylcholinesterase from electric eel. A 5 ml bed volume affinity column containing *N*-methylacridinium ligand at 0.46 μmol/ml was loaded with guanidine HCl-dissociated eel acetylcholinesterase in 0.1 M NaCl. The dotted line indicates the acetylcholinesterase activity of the solution applied to the column. After loading was complete, the column was washed with 20 vol. Buffer L and elution was carried out with 2 vol. 20 mM decamethonium (Deca) in the same buffer, followed by 2 vol. Buffer L (peak A). The column was then equilibrated with 4 vol. Buffer H (peak B) and then eluted with 20 mM decamethonium in this buffer (peak C). All operations were done at 4°C. The fraction size was about 2 ml and the flow rate throughout was 2 ml/h. AChE, acetylcholinesterase.

N-methylacridinium (Fig. 4), indicating mixed (competitive/uncompetitive) inhibition. Replots of the slopes and $1/v$-intercepts against *N*-methylacridinium concentration [16] yielded a K_i value of 0.1 μM for the competitive component and a K_i' value of 2.0 μM for the uncompetitive component (results not shown).

The effect of Ca^{2+} on the inhibition of bovine erythrocyte acetylcholinesterase by *N*-methylacridinium in the absence of other added ions was also examined. $CaCl_2$ (0.1 mM) shifted the inhibition curve (v/v_0) to higher *N*-methylacridinium concentrations, indicating that $CaCl_2$ antagonized the inhibition of the enzyme by *N*-methylacridinium (results not shown). $CaCl_2$ (0.1 mM) shifted the apparent K_i for *N*-methylacridinium inhibition from 0.1 μM (compared to 0.016 μM in similar ionic conditions for eel acetylcholinesterase [17]) to 0.22 μM (results not shown). The antagonism by $CaCl_2$ was abolished in the presence of 40 mM NaCl (results not shown).

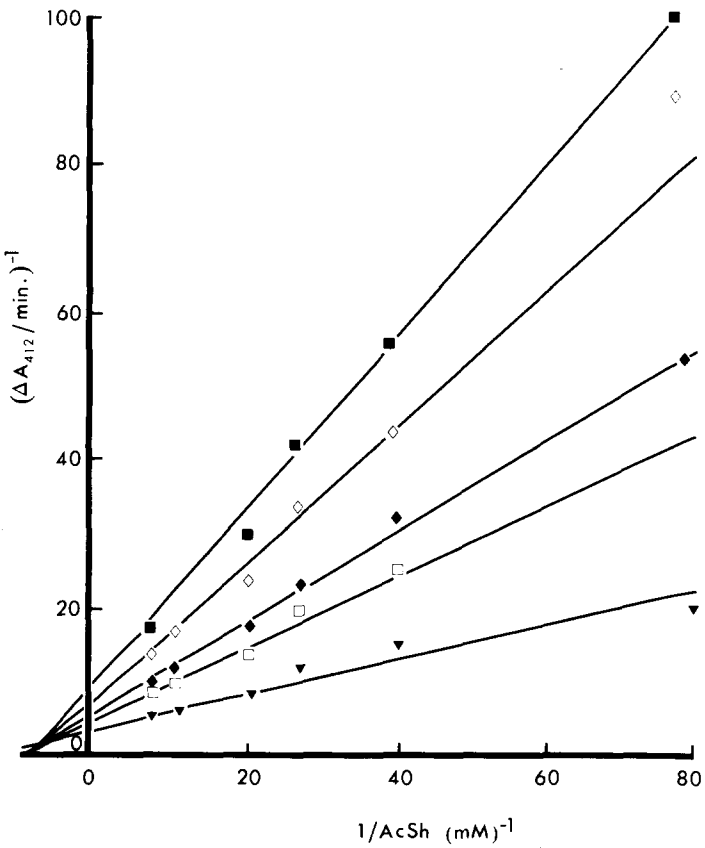

Fig. 4. *N*-Methylacridinium inhibition of bovine erythrocyte acetylcholinesterase. Acetylthiocholine (AcSh) was assayed by the colorimetric method [14]. The *N*-Methylacridinium concentration was zero (▼), 1.15 μM (□), 1.84 μM (◆), 2.76 μM (◇) and 3.58 μM (■).

Discussion

The present study shows that acetylcholinesterase derived from bovine erythrocytes and electric eel exhibit different retention and elution behaviour on *N*-methylacridinium-Sepharose 2B affinity columns. A high salt extract of eel acetylcholinesterase is optimally retained by the affinity column at a ligand concentration of 0.44—0.98 μmol/ml [9]. By contrast, the erythrocyte enzyme was not retained at these ligand concentrations, even at a salt concentration 1/10 that used for purification of the eel enzyme. Retention of the bovine erythrocyte enzyme occurred at a ligand concentration of 2.0—2.8 μmol/ml. Under these conditions the eel enzyme is very tightly retained, and is eluted in only poor or zero yield by decamethonium [9].

A number of possible reasons for the differential behaviour of the enzyme from these two sources were considiered. In particular, the differential behaviour could be due to differences in their structure, in their affinity for the *N*-methylacridinium ligand or in their mode of interaction with the ligand.

(a) Effect of structure

Since it was shown previously that differences in affinity chromatographic retention of various molecular forms of eel acetylcholinesterase was due to simultaneous interaction of more than one ligand with the larger oligomeric molecular forms, we have now compared the retention of the dimers of bovine erythrocyte and eel acetylcholinesterase. Sonication of 18 S plus 14 S forms of eel acetylcholinesterase in the presence of guanidine hydrochloride generates a catalytically active dimeric form of acetylcholinesterase with a sedimentation coefficient of approx. 8 S, which elutes after the 11 S form on a Sepharose 4B column. In the present study it has been shown that both the 8 S and 11 S forms from the guanidine-treated eel acetylcholinesterase were retained at a ligand concentration of 0.46 μmol/ml at a low salt concentration (0.1 M NaCl). Under similar ionic strength conditions, the bovine erythrocyte acetylcholinesterase, which is a dimer of sedimentation coefficient 7.7 S [19], was not retained, suggesting that the difference in behaviour of the two enzymes cannot be readily explained on a structural basis.

(b) Affinity for ligand

The inhibition constant, K_i, for the competitive component of *N*-methylacridinium inhibition of bovine erythrocyte acetylcholinesterase (1.0 μM) is 13-fold [20] or more [21] higher than for eel acetylcholinesterase at a similar ionic strength. A 10-fold decrease in binding affinity of the aminohexanoyl derivative of phenyltrimethylammonium with increasing ionic strength correlates with the marked dependence of acetylcholinesterase retention on ionic strength in affinity columns containing this ligand [22—24,9]. The difference in *N*-methylacridinium binding affinity between the eel and erythrocyte enzymes is entirely adequate, therefore, to account for the observed differences in affinity chromatographic behaviour between the two enzymes on *N*-methylacridinium affinity columns.

(c) Site of inhibition

The mixed competitive/uncompetitive inhibition of bovine erythrocyte

acetylcholinesterase by *N*-methylacridihium was also found in the inhibition of the electric eel enzyme [20], suggesting that the ligand reacts with both enzymes at similar sites. The uncompetitive component has been attributed to binding of *N*-methylacridinium to the acetylated-enzyme intermediate of acetylcholinesterase [20]. Alternatively, mixed inhibition could occur as a result of binding of *N*-methylacridinium to the peripheral anionic site on acetylcholinesterase (Ref. 25 and Roufogalis, B.D., unpublished observations). In a previous paper [25] it was shown that the influence of Ca^{2+} on the inhibition is diagnostic of the relative affinity of a ligand for the catalytic and peripheral anionic sites. Low Ca^{2+} concentrations enhance the inhibition of the enzyme when the ligand-affinity for the peripheral site is high, while Ca^{2+} antagonizes the inhibition when the peripheral site-affinity is low [25]. The antagonism by Ca^{2+} at low ionic strength, and the loss of antagonism in 40 mM NaCl in the present results, suggest that *N*-methylacridinium inhibits bovine erythrocyte acetylcholinesterase by binding predominantly at the catalytic anionic site. Linear slope replot of the Lineweaver-Burk plots and linear Dixon plots [26] obtained in this study support this conclusion. Although a similar kinetic study was not made with eel acetylcholinesterase, fluorescent measurements show that *N*-methylacridinium also binds selectively to the catalytic anionic site on this enzyme [20,21]. Thus the different behaviour of the erythrocyte and eel acetylcholinesterase on *N*-methylacridinium affinity columns canot be accounted for by a different site of interaction of the ligand on the side-arm with the two enzymes.

In summary, it appears that the lower retention of bovine erythrocyte acetylcholinesterase on *N*-methylacridinium-affinity columns is adequately explained by a 13-fold or more lower affinity of this enzyme, compared to the eel enzyme, for the side-arm ligand in the chromatographic conditions. The lower affinity of *N*-methylacridinium for bovine erythrocyte acetylcholinesterase also limits the usefulness of *N*-methylacridinium as a fluorescent probe for this enzyme (Sekar and Roufogalis, unpublished observation), while it has been used successfully with the eel enzyme [20,21]. Kinetic differences between the acetylcholinesterases of other tissues have also been reported [27]. The results obtained could be explained if the hydrophobic region surrounding the anionic site of eel acetylcholinesterase, thought to be a tryptophan moiety which avidly binds organic cations [28], is either lacking or is in a different spatial arrangement with respect to the anionic site in the bovine erythrocyte enzyme. This possibility is consistent with the different amino acid composition of acetylcholinesterase from these two sources [1]. Paradoxically, while bovine erythrocyte acetylcholinesterase appears to be an integral membrane protein [30,19], eel acetylcholinesterase is peripherally associated with the postsynaptic membrane [30], and does not have extensive hydrophobic areas on its surface [32]. The effect of environment and the contribution of cardiolipin tightly associated with the erythrocyte enzyme [19,32] on the kinetic properties remain to be determined.

Acknowledgement

Supported by the Medical Research Council of Canada (MA-5595).

References

1 Berman, J.D. (1973) Biochemistry 12, 1710—1715
2 Berman, J.D. and Young, M. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 395—398
3 Niday, E., Wang, C.S. and Alaupovic, P. (1977) Biochim. Biophys. Acta 469, 180—193
4 Ott, P., Jenny, B. and Brodbeck, U. (1975) Eur. J. Biochem. 57, 469—480
5 Grossman, H. and Lieflander, M. (1975) Hoppe-Seyler's Z. Physiol. Chem. 56, 663—669
6 Dudai, Y., Silman, I., Shinitzky, M. and Blumberg, S. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 2400—2403
7 Duadai, Y. and Silman, I. (1974) in Methods in Enzymology (Jacoby, W.B. and Wilchek, M., eds.), Vol. 34, Part B, pp. 571—580, Academic Press, New York
8 Rosenberry, T.L. and Richardson, J.M. (1977) Biochemistry 16, 3550—3558
9 Webb, G. and Clark, D.G. (1978) Arch. Biochem. Biophys. 191, 278—288
10 Reavill, C.A. and Plummer, D.T. (1978) J. Chromatog. 157, 141—151
11 Roufogalis, B.D., Quist, E.E. and Wickson, V.M. (1973) Biochim. Biophys. Acta 321, 536—545
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 251, 2113—2118
13 Murphy, I.B. and Kies, M.W. (1960) Biochim. Biophys. Acta 45, 382—384
14 Webb, G. (1978) Can. J. Biochem. 56, 1124—1132
15 Ellman, G.L., Courtney, K.D., Andreas, V., Jr. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
16 Wermuth, B. and Brodbeck, U. (1973) Eur. J. Biochem. 37, 377—388
17 Mooser, G. and Sigman, D.S. (1972) Biochem. Biophys. Res. Commun. 48, 559—564
18 Bon, S., Huet, M., Lemonnier, Rieger, F. and Massoulie, J. (1976) Eur. J. Biochem. 6, 523—530
19 Beauregard, G. and Roufogalis, B.D. (1979) Biochem. J. 179, 109—117
20 Mooser, G., Schulman, H. and Sigman, D.S. (1972) Biochemistry 11, 1595—1602
21 Rosenberry, T.L. and Neumann, E. (1977) Biochemistry 16, 3870—3878
22 Dudai, Y., Silman, I., Kalderon, N. and Blumberg, S. (1972) Biochim. Biophys. Acta 268, 138—157
23 Rosenberry, T.L. (1976) Enzymol. Biol. Memb. 4, 331—363
24 Rosenberry, T.L. (1975) Adv. Enzymol. 43, 103—218
25 Roufogalis, B.D. and Wickson, W.M. (1975) Mol. Pharmacol. 11, 352—360
26 Webb, J.L. (1963) Enzyme and Metabolic Inhibitors, Vol. 1, p. 151, Academic Press, New York
27 Moss, D.E. and Fahrney, D. (1978) Biochem. Pharmacol. 27, 2693—2698
28 Shinitzky, M., Dudai, Y. and Silman, I. (1973) FEBS Lett. 30, 125—128
29 Mitchell, C.D. and Hanahan, D.J. (1966) Biochemistry 5, 51—57
30 Anglister, L. and Silman, I. (1978) J. Mol. Biol. 125, 293—311
31 Millar, D.B., Christopher, J.P. and Burrough, D.O. (1978) Biophys. Chem. 9, 9—14
32 Beauregard, G. and Roufogalis, B.D. (1977) Biochem. Biophys. Res. Commun. 7, 211—219