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Modification of L-triiodothyronine binding sites from rat erythrocyte membrane by heating and by proteinase treatments

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The number of binding sites for L-triiodothyronine in rat erythrocyte membranes was increased 2-fold by incubation at 37°C for 60 min. An increase of approximately 3-fold was found when the incubation was carried out at 50°C. The proteinase inhibitor phenylmethylsulfonyl fluoride abolished the effect. Similar increments in the number of binding sites were obtained by treatment of the membranes with proteinases. The K_d values ($0.09 \cdot 10^{-10}$ M and $3.6 \cdot 10^{-10}$ M for the high-affinity and the low-affinity binding sites, respectively) remained unchanged after the treatment, as did the free-SH group requirements, storage stability and stereospecificity. Our results suggest that endogenous proteolytic activity could be involved in the increase of the number of membrane latent sites for L-triiodothyronine.

Introduction

Several reports have described binding sites for L-T_3(L-triiodothyronine) in the plasma membrane [1–8]. We have reported the presence of specific, high-affinity L-T_3 binding sites in rat [7] and in human [8] erythrocyte membrane. We have also shown that depending upon the species, the binding sites behave as integral [7] or as peripheral [8] membrane proteins. Extending these studies, we now present evidence that the number of binding sites can be increased by pre-incubation of the membranes at 37°C or 50°C. This increase is abolished by the addition of a non specific proteinase inhibitor before the preincubation. A similar increase in the number of binding sites is found when membranes are treated with exogenous proteinase. These facts strongly suggest a proteinase-mediated process as responsible for the increase of L-T_3 binding sites in erythrocyte membranes.

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Materials and Methods

Erythrocyte membrane preparations. Rat red cells ghosts were prepared by osmotic lysis using the method of Dodge et al. [9] with addition of 1 mM EDTA to the phosphate buffer. Peripheral protein-free erythrocyte membranes, obtained as described [8], were used in all the experiments of this paper.

Binding assays. All binding assays were carried out at 4°C for 45 min in phosphate-buffered saline (138 mM NaCl/10 mM sodium phosphate buffer/5 mM β-mercaptoethanol (pH 7.4), at a final volume of 0.5 ml. The amount of L-[^{125}I]T_3 is

indicated in each figure legend. Bound and free hormone were separated using Dowex exchange resin, as described by Botta et al. [8]. All assays were performed in duplicated.

Preincubation. 4 to 10 ml of membrane suspension (1 mg of protein/ml) were incubated in a water bath at different temperatures, and intermittently stirred. At different times aliquots were removed, chilled on ice and assayed. When the non-specific proteinase inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to the preincubation medium, the membranes were pelleted at $36000 \times g$ for 20 min at 4°C, washed twice in phosphate-buffered saline, resuspended, and adjusted to the original volume, prior to the binding assays. Control experiments were carried out without PMSF addition.

Proteinase treatment. 4 to 10 ml of membrane suspensions (150 μg of protein/ml) were preincubated in phosphate-buffered saline during 60 min at 4°C, 37°C or 50°C, in the presence of protease (40 μg/ml); chilled in ice, centrifuged, washed as explained above, resuspended and adjusted to the original volume. Control incubations were carried out in the absence of proteolytic enzyme. Proteins were determined by the method of Lowry et al. [10] or the Coomassie blue dye assay [11].

Reagents. L-[^{125}I]T_3 (spec. act. 2820 μCi/μg) was purchased from New England Nuclear; L-T_3 thyroid hormone analogues, proteinase from *Bacillus subtilis* type VIII (Subtilopeptidase A) and from *Bacillus thermoproteolyticus rokko* (Thermolysin) type X and PMSF were obtained from Sigma Chemical Co.

Results

Effect of heat treatment on L-T_3 binding activity

Fig. 1A shows that when the membrane preparations were heated for 60 min at different temperatures and then assayed at 4°C, a stimulation in the L-T_3 binding activity was observed at 37°C and at 50°C. A time curve performed at 50°C showed that the binding reached the maximal level within 20 min and maintained for up to 2 h (Fig. 1B). A summary of several experiments is shown in Table I. A two-fold increase in the specific binding was obtained with a preincubation at 37°C, while a 3.6-fold increase was observed when the preincubation was performed at 50°C (row 1). The presence of a proteinase inhibitor eliminated the stimulatory effect (row 2), suggesting that an endogenous proteinase activity could be involved.

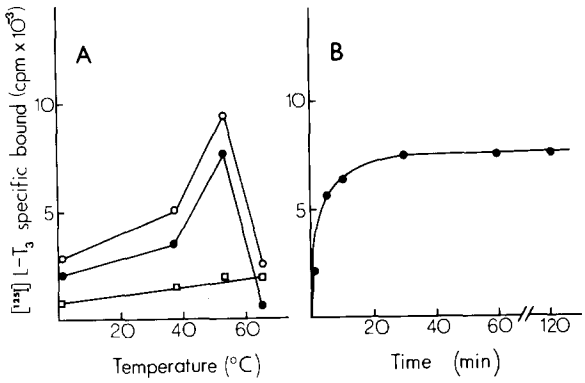

Fig. 1. (A) Effect of heating on L-T_3 binding. Total binding assays were performed at 4°C as described in Materials and Methods, using $5 \cdot 10^{-11}$ M L-[^{125}I]T_3 (40000 cpm per assay) and 60 μg of membrane protein per assay. Nonspecific binding was determined in the presence of 10^{-6} M unlabeled hormone. Heating was performed as indicated in Materials and Methods for 60 min. Total (○), specific (●) and nonspecific (□) binding. (B) Specific binding of heated (50°C) membranes.

Effect of proteinase on L-T_3 binding activity

In view of the foregoing results, preincubations with exogenous proteinases were performed. The dependence of L-T_3 binding site activation on proteinase type VIII treatment at 37°C for 1 h is shown in Fig. 2. With increasing enzyme concentration the binding was increased, reaching a plateau in the range concentration of 12.5 to 50 μg/ml. A total inactivation of binding was observed by increasing, both proteinase concentration (200 μg/ml for 1 h) or incubation time (40 μg/ml for 3 h). As shown in rows 3 and 4 of Table I, significative increases were obtained when the preincubations were performed in the presence of proteinases at 4°C (3-fold) or at 37°C (2-fold), but not when the incubations were performed at 50°C (compared values in row 1 to values in rows 3 and 4). The last finding could be explained as that top activation is reached with the heating at 50°C and that a subsequent activation is not possible by the addition of the proteinase type X

TABLE I

EFFECT OF HEATING AND PROTEINASE TREATMENT ON THE L-T_3 SPECIFIC BINDING

The stimulation was calculated as the specificaly bound L-T_3 divided by values obtained in parallel experiments with membranes preincubated at 4°C. The experimental conditions are indicated in the table. Incubations were carried out for 60 min. The values are means ± S.D. followed by the number of independent experiments, between parenthesis.

Preincubation	Fold stimulation		
	4°C	37°C	50°C
(1) No addition	–	1.9 ± 0.4(5)	3.6 ± 0.8(8)
(2) + PMSF 3 mM	1.0 ± 0.1(3)	0.9 ± 0.2(3)	0.6 ± 0.1(4)
(3) + proteinase type VIII	3.0 ± 0.4(3)	4.2 ± 0.5(5)	–
(4) + proteinase type X	–	–	4.5 ± 0.5(7)
(5) + proteinase type X + PMSF 3 mM	–	–	1.1 ± 0.5(3)

(active at 50°C). The recovery of membrane protiens after a proteinase treatment was of about 65% at the different temperatures, and polyacrylamide slab-gel electrophoresis showed a modified protein pattern in all cases after the treatment (not shown). The effect proteinase treatment, also, was abolished by the presence of a proteinase inhibitor (row 5).

Effect of storage

The L-T_3 binding activities remained constant after the heat at 50°C or the proteinase type VIII

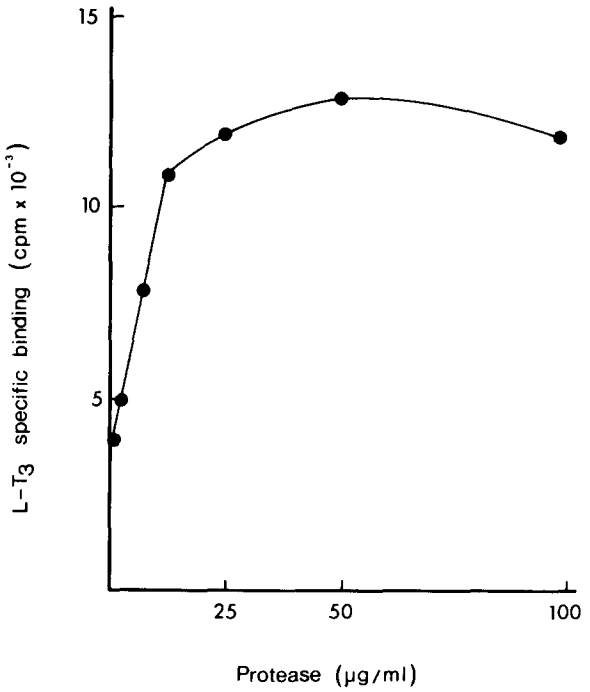

Fig. 2. Effect of proteinase concentration on L-T_3 binding sites. The treatment with proteinase type VIII was carried out as indicated in Materials and Methods at 37°C for 60 min. The binding assay was performed as in Fig. 1.

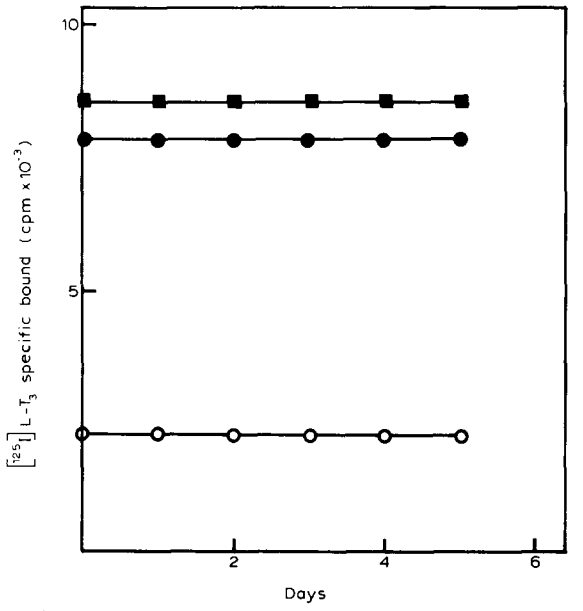

Fig. 3. Effect of storage at 4°C on the L-T_3 binding. Heating and proteinase treatment were carried out as indicated in Materials and Methods. The membranes (1 mg of protein per ml) were stored at 4°C in phosphate-buffered saline and aliquots were removed at the indicated times. Binding assays were performed as indicated in Fig. 1. Heated at 50°C (●); the proteinase type VIII treated at 37°C (■) and control (○) membranes.

TABLE II

EFFECT OF PCMB ON BINDING OF L-T_3 AND REVERSIBILITY

Membrane suspensions (400 μg of protein) were incubated with PCMB at 4°C for 30 min. Then, binding was assayed in the presence or absence of 5 mM of β-mercaptoethanol. Other assays conditions are in Fig. 1.

Preincubation	L-T_3 binding (cpm)	
	Specific	Nonspecific
(1) No addition	3000	1500
(2) +30 μM PCMB	667	1800
(3) +30 μM PCMB + 5 mM β-mercaptoethanol	3100	1200

treatments at 37°C, when the membranes were stored at 4°C during periods of up to 5 days (Fig. 3). Thus, the modifications produced during the preincubation of membranes at high temperature, or in the presence of exogenous proteinase led to a stable stimulation of the L-T_3 binding activity, suggesting that the activation process obtained by heating or protease treatment is irreversible, at least in this time period.

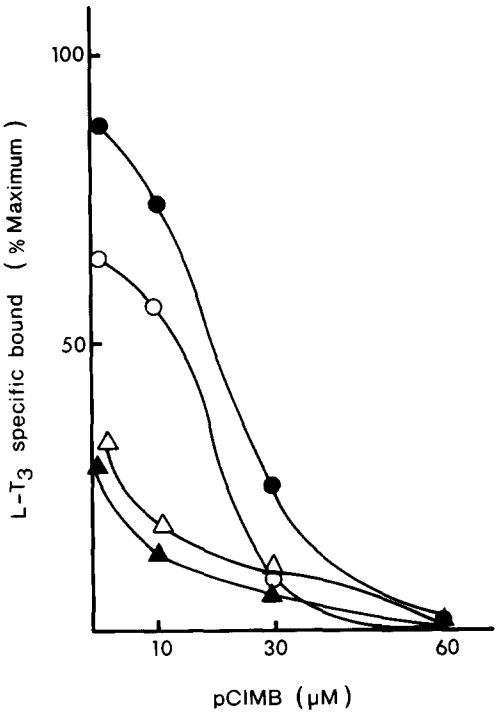

Fig. 4. Specific L-T_3 binding versus PCMB concentration from control (○, △) and proteinase-treated (37°C) (●, ▲) membranes. Specific binding was calculated by substraction of nonspecific binding (measured in the presence of 10^{-9} M (△, ▲) and 10^{-6} M (○, ●) of unlabeled L-T_3) from the total bound L-[^{125}I]T_3. The binding assay was performed in phosphate-buffered saline in the absence of β-mercaptoethanol. Specific binding activity is expressed as the percentage of total bound L-T_3 determined in the absence of PCMB.

Effect of thiol blocking agents

The membrane L-T_3 binding site is dependent upon the presence of free-SH groups. Table II shows that 30 μM of *p*-chloromercuribenzoate (PCMB) inhibited the specific binding of L-T_3 to the membrane, and that after the addition of 5 mM of β-mercaptoethanol, a total recovery of the initial value was obtained. The inhibition profiles of PCMB were similar in control and in proteinase-treated membranes (Fig. 4). This is also true for membrane heated at 50°C (not show). Another SH inhibitor drug, *N*-ethylmaleimide, was also effective in blocking the L-T_3 binding site. This avoids objections about the aromatic PCMB competing with L-T_3.

Competition of L-T_3 binding sites

The competition studies between L-[^{125}I]T_3 and L-T_3 showed that the hormone was a effective competitor in the heated, proteinase type VIII treated and control membranes (Fig. 5). We have previously shown [8] that 1000-fold higher concentrations of L-T_4 and D-T_3 were needed to obtain a 50% depression of L-[^{125}I]T_3 binding. The same competition results were found in the present study when the assays were performed with heated or with proteinase-treated membranes (data not shown).

Scatchard plots

Specific binding data were analyzed according to Scatchard [13]. Experiments performed with control (A), heated at 50°C (B) and proteinase-treated at 37°C (C) membranes are shown in Fig. 6. Data from control membranes were compatible with the existence of two sets of binding sites, confirming previous results of our laboratory [7]. The K_d values of about $0.09 \cdot 10^{-10}$ M and $3.5 \cdot 10^{-10}$ for the high-affinity and the low-affinity binding sites remained unchanged after heat or proteinase treatments. However, the binding capacities increased: from 20 fmol/mg protein in

control membranes to 40–50 fmol/mg protein in treated membranes; and from 350 fmol/mg protein to 700 fmol/mg protein (heated membranes) and to 1125 fmol/mg protein (proteinase-treated membranes). The membrane protein content was 35% less than the control value after proteinase treatment (see above). Then, when this correction by initial protein concentration, is applied to the capacity values, the above difference between proteinase-treated and heated membranes disappears.

From Fig. 5 a low-affinity site with a K_d of about 10^{-7} M would be expected. This additional site was not detected in our Scatchard determinations since the range of L-[^{125}I]T_3 used in the experiment, as shown in the legend of Fig. 6, did no cover those possible binding sites. In any case, the extention of the experiment to ranges higher than 10^{-8} M with L-[^{125}I]T_3 does not appear to be of physiological relevance [7].

Heat treatment and L-T_3 'latent' binding sites

After the preincubation of membrane with 30 μM PCMB the specific binding of L-T_3 to membrane was inhibited by approx. 60%. Using this membrane preparation, previously washed we carried out an additional heating at 50°C. As can be seen in Table III the fold stimulation of binding site above the existing (PCMB inhibited) basal values does not differ from the control fold stimulation. The 'new' L-T_3 binding sites are also dependent on the presence of the free-SH groups since they were blocked by PCMB (not shown). Additionally, when the heating at 50°C is carried out in the presence of PCMB (membrane prepara-

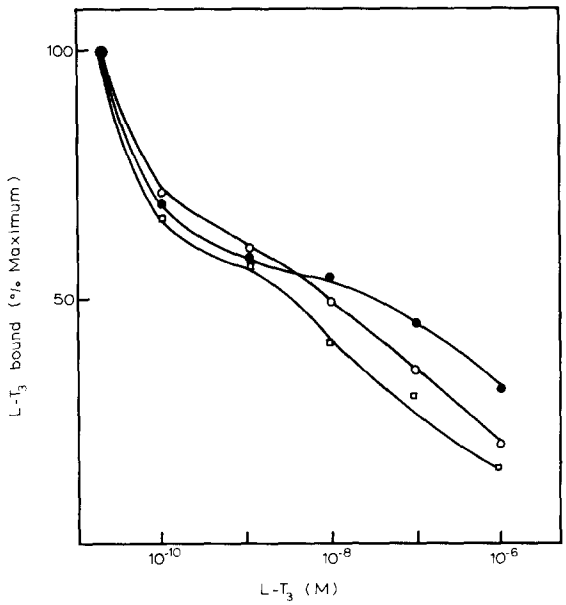

Fig. 5. L-[^{125}I]T_3 bound versus unlabeled L-T_3: Heated at 50°C (○) proteinase type VIII treated at 37°C (□) and control (●) membranes. The binding assays were performed as explained in Fig. 1.

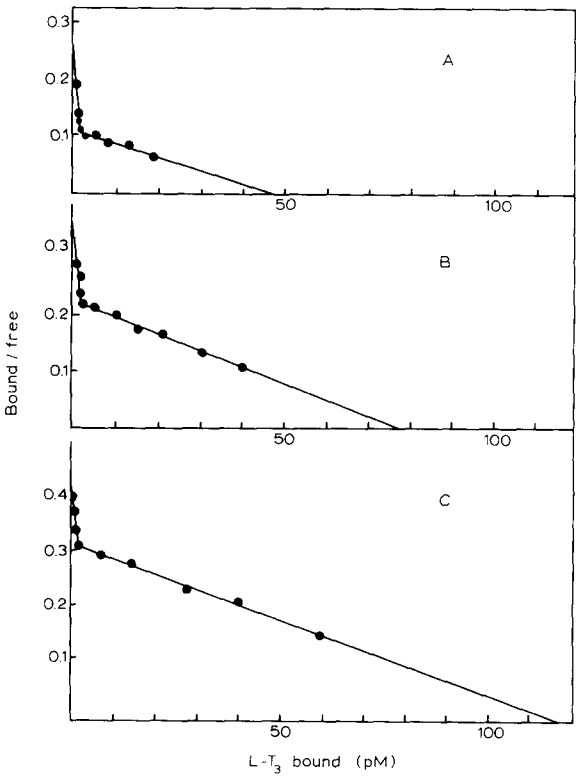

Fig. 6. Scatchard analysis of L-[^{125}I]T_3 binding to control (A), heated at 50°C (B) and proteinase type VIII treated at 37°C (C) membranes. Binding assays were performed with concentrations of L-[^{125}I]T_3 which varied from 4 to 135 pM for control membranes, and from 2 to 500 pM for heated and proteinase type VIII treated ones. To determine the specific binding, the nonspecific binding (determined in the presence of 10^{-6} M unlabeled hormone) was substracted. The K_d values for high- and low-affinity sites were in the range $(0.08–0.10)\cdot 10^{-10}$ M and $(34–39)\cdot 10^{-11}$ M for the three preparations. The binding capacities for the high-affinity site were 20 fmol/mg for control membranes, and 40 fmol/mg for heated and 50 fmol/mg proteinase-treated membranes. The binding capacities for low affinity sites were of 350, 700 and 1,120 fmol/mg for control, heated and proteinase type VIII treated membranes, respectively. Similar results and differences were obtained in two independent experiments.

TABLE III

EFFECT OF HEATING ON L-T_3 BINDING SITES INACCESSIBLE TO PCMB INHIBITION

The membranes were incubated in the absence (A) or in the presence of 30 μM PCMB (B) for 30 min at 4°C and washed twice in phosphate-buffered saline previous to preincubation, performed as indicated in Table I. Similar results were obtained in three independent experiments. The binding assay was performed, in the absence of β-mercaptoethanol, as indicated in Fig. 1.

Preincubation	Specific binding (cpm), membrane preparation	
	A	B
Not heated	3000	1147
Heated at 50°C	10401	3900
Stimulation	3.46	3.40

tion, unwashed) no effect of heating was found. From last experiments, it is not clear whether the PCMB effect is on the binding site or on the endogenous proteinase. However, since the heating activation event is detected after PCMB treatment and washing of the membrane (Table III), the action of the PCMB on endogenous proteinase can be ruled out. All these experiments suggest that the heating activates 'latent' L-T_3 binding sites that are inaccessible to previous PCMB treatment. Again, this activation was blocked by the presence of proteinase inhibitor (not shown).

Discussion

Alterations of thyroid hormone's binding specificity to nuclear receptors by heating the crude receptor preparations at 50°C were reported [14]. Heating produced a selective loss of L-T_3, but not of L-T_4 binding activity. Using more purified preparations, the treatment decreased both L-T_3 and L-T_4 binding sites activities [15]. A clearly different situation is described in the present paper when rat erythrocyte membranes are used; heating at 50°C increases the concentration of L-T_3 binding sites. These sites have properties similar to those exhibited by the binding sites of the intact membrane, i.e. same free-SH group requirement, stability to storage, stereospecificity and constant of dissociation. The activation by heat treatment of the L-T_3 binding sites of the membrane was independent of the previous inhibition of PCMB of the -SH exposed group, indicating that the heat activates 'latent' forms of the L-T_3 binding sites. The inhibition of the effect caused by an nonespecific proteinase inhibitor, and the stimulation obtained by the addition of exogenous proteinases to the preincubation medium suggests an endogenous proteolytic activity is involved in the heating effect. Several reports indicated the existence of proteinase activity in the erythrocyte membrane and its relationship with membrane protein degradation [16–21]. Moreover, a latent form of proteolytic activity from red cell membrane has been isolated [22]. Further work is needed to understand the relationship between L-T_3 binding, membrane-bound proteinase activities and thyroid hormone action on membrane systems. The L-T_3 binding sites have been postulated to be involve in mediating the entry of L-T_3 into cells [23]. A good correlation has been found between L-T_3 binding to rat erythrocyte membranes [24] and the biological effect of the hormone on changes of the kinetic properties of membrane-bound enzymes [25–27] and membrane fluidity [28].

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References

1 Pliam, N.B. and Goldfine, I.D. (1977) Biochem. Biophys Res. Commun. 79, 166–172
2 Gharbi, J. and Torresani, J. (1979) Biochem. Biophys. Res Commun. 88, 177–183
3 Gharbi-Chihi, J. and Torresani, J. (1981) J. Endocrinol Invest. 4, 177–183
4 Maxfield, F.R., Willingham, M.C., Pastan, I., Dragsten, P and Cheng, S.Y. (1981) Science 211, 63–65

5 Arnott, R.D. and Eastman, C.J. (1983) J. Receptor Res. 3, 393–407
6 Cheng, S.Y. (1985) J. Receptor Res. 5, 1–26
7 Botta, J.A., De Mendoza, D., Morero, R.D. and Farías, R.N. (1983) J. Biol. Chem. 258, 6690–6692
8 Botta, J.A. and Farías, R.N. (1985) Biochem. Biophys. Res. Commun. 133, 442–448
9 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
10 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
11 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
12 Laemmli, U.K. (1970) Nature 227, 680–685
13 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672
14 Eberhardt, N.L., Ring, J.C., Lathman, K.R. and Baxter, J.D. (1984) J. Biol. Chem. 254, 8534–8539
15 Apriletti, J.W., David-Inouye, Y., Eberhardt, N.L. and Baxter, J.D. (1984) J. Biol. Chem. 259, 10941–10948
16 Moore, J.L., Kocholaty, W.F., Cooper, D.A., Gray, J.L. and Robinson, S.L. (1970) Biochim. Biophys. Acta 212, 126–133
17 Tokes, Z.A. and Chambers, S.M. (1975) Biochim. Biophys. Acta 389, 325–338
18 Balias, S.K. and Burka, E.R. (1979) Blood 53, 875–881
19 Tarone, G., Hamasaki, N., Fukuda, M. and Marchesi, V.T. (1979) J. Membrane Biol. 48, 1–12
20 Pontremoli, S., Meldoni, E., Sparatore, B., Salamino, F., Michetti, M., Sacco, O. and Horecker, B.L. (1985) Biochem. Biophys. Res. Commun. 117, 859–866
21 Siegel, D.L., Goodman, S.R. and Branton, D. (1980) Biochim. Biophys. Acta 598, 517–527
22 Golovichenko-Matsumoto, A.M., Matsumoto, I. and Osawa, T. (1982) Eur. J. Biochem. 121, 463–467
23 Horiuchi, R., Johnson, M.L., Willingham, M.C., Pastan, I. and Cheng, S.Y. (1982) Proc. Natl. Acad. Sci. USA 79, 5527–5531
24 Farías, R.N. (1981) Adv. Lipid. Res. 17, 251–282
25 DeMendoza, D. and Farías, R.N. (1978) J. Biol. Chem. 253, 6249–6254
26 DeMendoza, D., Moreno, H. and Farías, R.N. (1978) J. Biol. Chem. 253, 6255–6259
27 Galo, M.G., Uñates, L.E. and Farías, R.N. (1981) J. Biol. Chem. 256, 7113–7114
28 DeMendoza, D., Moreno, H., Massa, E.M., Moreno, R.D. and Farías, R.N. (1977) FEBS Lett. 84, 199–203