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INTERACTION OF N-BROMOALKYL DERIVATIVES OF IODOTHYRONINES WITH THE NUCLEAR TRIIODOTHYRONINE RECEPTOR

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SUMMARY. Several N-bromoalkylderivatives of triiodothyronine (T_3) and thyronine (T_0) were synthesized and analyzed for their ability to bind to the nuclear receptor of T_3 in competition experiments with ^{125}I-L-T_3 using either the crude nuclear extract or a partially purified preparation of the T_3 receptor. N-bromoacetyl-L-T_3 was the most potent with K_d varying between 10 and 100 nM within nuclear material preparations, higher values being found in crude nuclear extract. N-bromoacetyl-DL-T_0 was not bound. Incubating the partially purified nuclear T_3 receptor with N-bromoacetyl-L-T_3 led to a significant loss of T_3 binding site concentration (up to 32 %) without significantly altering the affinity for T_3, while N-bromoacetyl-T_0 was ineffective. This suggests that a specific covalent interaction could occur between N-bromoacetyl T_3 and the T_3 binding site region of the nuclear receptor.

The existence of high affinity sites for 3,5,3'-triiodo-L-thyronine (T_3) in nuclei from various target tissues is now clearly demonstrated (1-5); T_3 binds to acidic non-histone proteins of the chromatin which have been solubilized and partly characterized (6-11). The nuclear T_3 binding protein(s) (NTBP) complexed with ^{125}I-labeled T_3 (T_3^*) binds to DNA (12, 13) and chromatin (14, 15). Following several indirect arguments reviewed in 16, and as suggested by the perichromatin localization of T_3^*-NTBP complexes after radioautography (17), NTBP could mediate the regulatory effects of thyroid hormones at a transcriptional level; they have been considered as a nuclear receptor for T_3. Nevertheless the mechanism of thyroid hormone action and the exact role of NTBP remain unknown. A better characterization of NTBP and of their interaction with chromatin constituents could help to investigate this role. Few attempts at NTBP purification have appeared (18, 19); we reported a 500-fold enrichment in NTBP using conventional purification methods and DNA-Sepharose chromatography but the partially purified NTBP became unstable and rapidly undetectable in the absence of added nuclear constituents (histones and DNA) (18). The importance of histones in T_3 binding site stabilization has been recently confirmed by Eberhardt et al (20). A covalent affinity labeling of the T_3 binding site would be useful in further characterization and purification of NTBP.

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It was shown in several studies using structural analogues of T_3 that the amino group of T_3 is probably not essential for binding to the nuclear receptor (triiodothyroacetic acid is more strongly bound that T_3) (5, 7, 21). In the present report, we demonstrate that several N-bromoalkyl derivatives of T_3 are able to compete with T_3 for the T_3 binding site of NTBP either in the whole nuclear extract or after partial purification. The affinity of amino-blocked derivatives of T_3 for the T_3 binding site of NTBP is lower than that of T_3 (1 to 3 orders of magnitude lower), the most active being N-bromoacetyl T_3. The bromoalkyl group is able to react with several nucleophilic groups in proteins, and a covalent affinity labeling of serum prealbumin using N-bromoacetyl thyroxine has been reported (22). Our results strongly suggest a specific covalent interaction of N-bromoacetyl T_3 with the T_3 binding site region of NTBP.

METHODS AND MATERIAL

Nuclear material. Liver nuclei were prepared as reported (5) from male Sprague-Dawley rats (250-350 g) and washed once with 0.25 % Triton X-100. NTBP and about 50 % of nuclear proteins (nuclear extract NE) were solubilized by 0.4 M KCl. Partially purified NTBP ($NTBP_{pp}$) were obtained after Sephadex G-100 gel filtration and DEAE-Sephadex chromatography as previously described (18), with a yield of about 30 % and an approximate 80 purification factor as compared to nuclei. They were then concentrated 3 to 4 fold in Visking dialysis bags using Sephadex G-200 or sucrose and dialyzed overnight at 0° C against 0.1 TKEM (0.1 M KCl, 20 mM Tris-Cl, 1 mM $MgCl_2$, 2 mM Na_2 EDTA pH 7.9). No or negligible loss in T_3 binding activity was noticed provided T_3^* binding was measured in the presence of dithiothreitol (DTT) and histones + DNA (18). Proteins were estimated according to Hartree (23) using bovine serum albumin as the standard and applying the Ross and Schatz modification (24) when reducing agents were present.

Bromoalkyl derivatives of iodothyronines and thyronine. N-bromoalkyl derivatives of iodothyronines were essentially synthesized as described by Cheng et al. for N-bromoacetyl T_4 (22). For the synthesis of N-bromoacetyl-L-T_3 ($BrAcT_3$), a solution of N,N'-dicyclohexylcarbodiimide (100 µmol) in 100 µl of anhydrous dimethylformamide (filtered and stored over 4 Å molecular sieves) was added to bromoacetic acid (100 µmol). After a 45 min mixing at 20° C, the precipitated dicyclohexylurea was removed by centrifugation. To the supernatant were added 90 µmol T_3 (Na salt), generally supplemented with trace amounts of [^{125}I] T_3 (T_3^*) (0.5 µCi, a quantity which could not significantly affect subsequent NTBP binding assays utilizing T_3^*). After 2 hr at 20° C, $BrAcT_3$ was purified by thin layer chromatography on preparative silicagel plates (PLK 5 80 Å, 1000 µ thickness, 20 x 20 cm, Whatman) with the solvent system ethylacetate-acetic acid (9 : 1). The major band detected in UV light and after radioautography had a $R_F \approx 0.32$. It was identified as $BrAcT_3$ on the following basis : 1) it gave no reaction with ninhydrin indicating a blocked α-amino group, 2) it gave a colored reaction with α-nitroso-β-naphtol which showed that the 4'-OH group was free (25). $BrAcT_3$ was fairly distinct from T_3 (R_F nearly 0) and N-acetyl T_3 ($N\text{-}AcT_3$)[x] which migrates with a $R_F \approx 0.53$. The bromoacetyl band was scapped off and eluted with ethanol; the solvent was evaporated (< 30° C) and the residue immediately used in binding studies with NTBP. $BrAcT_3$ concentrations were estimated using the radioactivity recovered and assuming a similar specific radioactivity as that of initial T_3^*.

[x] $N\text{-}AcT_3$ was synthesized from T_3 using acetic anhydride and following suggestions of S.Y. Cheng to whom we are indebted.

In spectrophotometric titrations, the wavelenght of maximum light absorption was shifted from 320 to 302 nm between alkaline (0.01 N NaOH in ethanol) and acidic (0.01 N HCl in ethanol) medium, indicating once more the persistence of a free 4'-OH group (26); the measured extinction coefficient was 4528 at 320 nm (alkaline medium). The same procedure was used for the synthesis of BrAcD-T_3. Similar procedures were also applied for the synthesis of N-3-bromo-propionyl T_3 (BrPropT_3) and N-α-bromo-p-toluyl T_3 (BrTolT_3) using 3-bromopropionic acid and α-bromo-p-toluic acid respectively in place of bromoacetic acid. After thin layer chromatography on preparative plates, BrPropT_3 and BrTolT_3 had $R_F \simeq 0.34$ and 0.54 respectively. N-bromoacetyl thyronine (BrAcT_0) was synthesized from DL-thyronine (T_0) as described for T_3. T_0 is less soluble than T_3 in dimethyl formamide; the reaction time was prolonged for about 15 hr until a solution was obtained. BrAcT_0 with a $R_F \simeq 0.33$ was eluted with ethanol; since it was not radiolabeled, its concentration was approximated from spectrophotometric titration in alkaline medium assuming an extinction coefficient of 4065 at 320 nm; this value was extrapolated from that of T_0 assuming a correction factor similar to that observed between T_3 and BrAcT_3.

Incubations. T_3^*-NTBP complexes used in dissociation experiments were formed by incubating freshly prepared NE or $NTBP_{pp}$ with T_3^* (0.5 nM, a nearly saturating concentration), up to equilibrium at 0° C in 0.1 TKEM. Displacement of bound T_3^* with T_3 or the N-bromoalkyl derivatives was studied at 20° C in the same medium. In competition experiments, NE or $NTBP_{pp}$ (20-40 fmol NTBP) were incubated with T_3^* (0.5 nM) and various concentrations of T_3 or N-bromoalkyl derivatives (0.2 ml of 0.1 TKEM, for 90 min at 20° C). Bound T_3^* was estimated after adsorption of free T_3 on a Dowex 1 x 8 anion exchange resin as described (7); radioactivity was counted in a Biogamma Beckman spectrometer. Estimation of residual T_3 binding sites after incubation of NTBP with bromoalkyl derivatives was performed on aliquots of NE or $NTBP_{pp}$ preincubated with these derivatives (≃ 2 μM) for 90 min at 20° C and withdrawn from unreacted products by filtration through 1 ml Sephadex G-25 fine microcolumns. T_3 binding site concentration was determined by Scatchard analysis of saturation experiments with T_3^* in the presence of 5 mM DTT (10). For $NTBP_{pp}$ Scatchard analyses, calf thymus DNA (15 μg) and histones (10 μg) were added to each incubate with T_3^* as previously described (18).

[^{125}I] T_3 (T_3^*) specific activity ≃ 1200 μCi/μg was from the Radiochemical Centre (Amersham, England); T_3,D-T_3 and T_0, calf thymus DNA and histones H_{IIA} were from Sigma (St Louis, Miss., USA). Other products were from Merck (GFR) or Fluka (Switzerland); organic solvents were repurified just prior delivery by S.D.S. (Peypin, France).

RESULTS

Bromoalkyl derivatives react with nucleophilic groups in a protein, essentially with-SH groups and to a lesser extent with $-NH_2$ and -OH groups (27). A covalent bond formation between a bromoalkyl ligand and its specific binding site region in a protein would be more probable at short times of contact while longer exposure times might provoke the formation of other non-specific randomly distributed covalent bonds. Fig. 1 illustrates the dissociation time-course at 20° C of T_3^*-NTBP complexes, in NE, by unlabelled T_3 and several N-bromoalkyl derivatives of T_3 and T_0; similar results were obtained with $NTBP_{pp}$. Half-dissociation times were of the same order of magnitude with T_3, BrAcT_3 and BrPropT_3 (about 35 min for T_3, slightly less for both latter). At equivalent concentrations of bromoalkyl T_3, BrAcT_3 was always the most effective after T_3; BrTolT_3 had a weaker effect. A complete dissociation of bound T_3^*, even with T_3, could not be

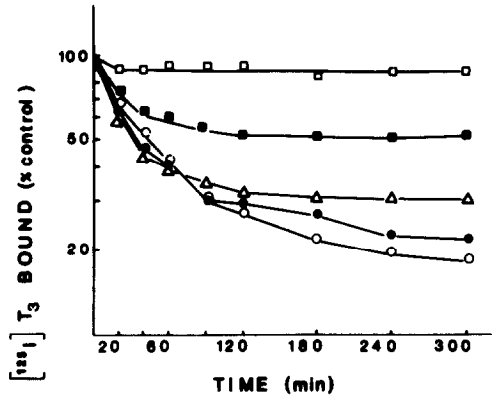

Fig. 1. Dissociation time-course of T_3^*-NTBP complexes in NE at 20° C. Six batches of NE preincubated with T_3^* (0.5 nM; for 48 hr at 0° C) were incubated at 20° C in the presence of : L-T_3, 0.5 µM (○—○); $BrAcT_3$, 2.1 µM (●—●); $BrPropT_3$, 2.1 µM (△—△); $BrTolT_3$, 1.9 µM (■—■); $BrAcT_0$, 2.1 µM (□—□) or buffer alone. At the indicated time points aliquots were pipetted and analyzed for bound T_3^*. 100 % bound T_3^* was that found in buffer alone at each time point. Assays were in duplicate, with variations not exceeding 3 % of mean value.

attained in the absence of reducing agents while it is regularly observed with the same concentration of T_3 in the presence of 5 mM DTT (in agreement with previous studies under slightly different medium conditions (10)). $BrAcT_0$ had no or a negligible effect, as was also demonstrated for T_0 which has no thyromimetic activity (21). Nevertheless, since a slow decrease of bound T_3^* generally occured in the presence of $BrAcT_0$ after 90 min exposure, an incubation time of 90 min at 20° C was chosen.

Dissociation and competition experiments were performed with several concentrations of the bromoalkyl derivatives of T_3 and T_0. Fig. 2 illustrates typical competitions obtained in NE and $NTBP_{pp}$. Concentrations which provoke a half-maximum inhibition of T_3^* binding (approximate Kd) were : in NE, 1.2 nM T_3, 55 nM $BrAcT_3$; 360 nM $BrPropT_3$ and 600 nM $BrTolT_3$; in $NTBP_{pp}$ from the above NE, 1 nM T_3, 12 nM $BrAcT_3$, 21 nM $BrTolT_3$ and 35 nM $BrPropT_3$. $BrAcT_0$ was always ineffective in the concentration range studied. Similar results were obtained in dissociation experiments where $BrAcT_3$ was also the most active derivative in displacing bound T_3^* with Kd ranging between 10 and 100 nM, closer to that of T_3 in $NTBP_{pp}$ than in NE. $BrPropT_3$ and $BrTolT_3$ were less effective with similar Kd in $NTBP_{pp}$ (≃ 70 nM) and low Kd in NE (500 nM and > 1 µM respectively). In one NE, BrAcD-T_3 was found about 3.5 fold less effective than BrAc L-T_3 in inhibiting T_3 binding (Kd 90 and 24 nM respectively); this result, which reproduces the differences found between D-T_3 and L-T_3 Kd for NTBP (7) and the inability of $BrAcT_0$ to interact with the T_3 binding site suggest that a biospecific interaction

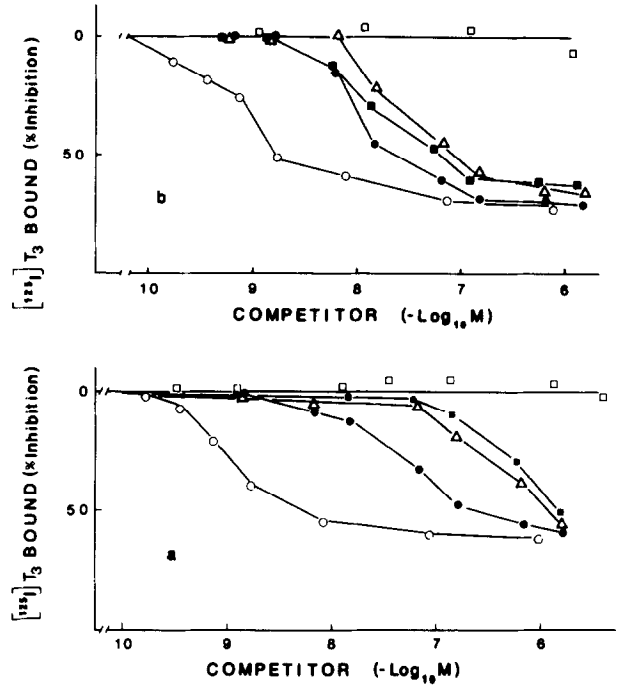

Fig. 2. Inhibition of $[^{125}I]$ T_3 binding to NTBP in NE (2a) or $NTBP_{pp}$ (2b) by increasing concentrations of T_3 (O—O), $BrAcT_3$ (●—●), $BrPropT_3$ (△—△) $BrTolT_3$ (■—■) and $BrAcT_o$ (□—□). Incubations in duplicate were performed for 90 min at 20° C in 0.2 ml of 0.1 TKEM.

occurs between NTBP and the bromoalkyl derivatives of T_3. The observed variations in Kd values as well for T_3 as for the bromoalkyl derivatives, and the relatively high Kd obtained for T_3 (≃ 1 nM) are probably due to the absence of reducing agents and some consecutive modifications of the NTBP molecule conformation (10). When studied in the presence of 5 mM DTT, T_3^* was generally bound with a lower Kd (0.5 - 0.1 nM) while $BrAcT_3$ displaced bound T_3^* with an approximate Kd of 600 - 800 nM close to that found for $N\text{-}AcT_3$; this suggests that a reaction of $BrAcT_3$ with -SH groups of the medium could have inactivated the bromoalkyl derivative.

In order to check the existence of a covalent interaction between $BrAcT_3$ and the T_3 binding site region, NTBP, in NE or after partial purification, were preincubated with the bromoalkyl derivatives; after elimination of excess unbound reactant, they were incubated under standard conditions with T_3^* for binding site concentration determination. As shown in Fig. 3, pretreatment with $BrAcT_3$ decreased the T_3 binding site concentration; this was not observed with $BrAcT_o$; a single class of binding sites was always found with no significant

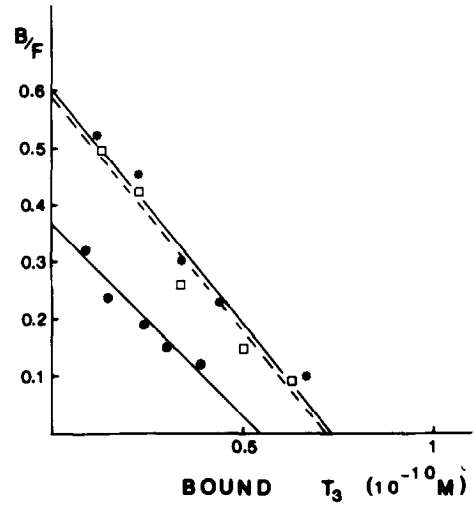

Fig. 3. Scatchard analysis of $[^{125}I]$ T_3 binding in $NTBP_{pp}$ preincubated for 90 min at 20° C in buffer alone (0.1 TKEM) (✱——✱) or in the presence of 2.1 x 10^{-6}M $BrACT_3$ (●——●) or $BrAcT_\theta$ (□——□) and filtered on Sephadex G-25 microcolumns to eliminate the unbound reactant. Saturation with T_3* was studied with T_3 concentrations ranging from 0.04 nM to 0.090 nM on duplicate aliquots in 0.2 ml of 0.1 TKEM supplemented with 5 mM DTT, calf thymus DNA (15 µg) and histones H_{IIA} (10 µg).

variation of K_a for T_3. Mean values obtained with $NTBP_{pp}$ are summarized in Table I. In 6 experiments a 19 % loss of T_3 binding sites was obtained with minimal and maximal losses of 10.4 and 26.7 % respectively. The right part of the table shows the results obtained after preincubation with BrAcT3 in the presence of DNA +

TABLE I. Effect of preincubation with $BrAcT_3$ and $BrAcT_\theta$ on binding site characteristics in $NTBP_{pp}$.

Preincubation with	0.1 TKEM			0.1 TKEM + DNA + Histones		
	Ka x 10^{10}L/M	n	Capacity pmol T3/ mg protein	Ka x 10^{10}L/M	n	Capacity pmol T3/ mg protein
0	1.27 ± 0.19	6	9.9 ± 0.8	1.07 ± 0.11	4	12.8 ± 2.6
$BrAcT_3$	1.05 ± 0.29	6	8.0 ± 0.8	0.98 ± 0.10	4	8.6 ± 1.6
$BrAcT_\theta$	1.17 ± 0.29	4	12.9 ± 1.9	0.75 ± 0.05	2	15.4 ± 3.8

Preincubation and subsequent incubation with T3 were as described in Fig. 3 except that in experiments reported in the right part, preincubation was performed in 0.1 TKEM + calf thymus DNA (75 µg/ml) and histones H_{IIA} (50 µg/ml).
n : number of experiments

histones. As expected (18), a higher number of T_3 binding sites were recovered in control samples; furthermore the effect of $BrAcT_3$ was more pronounced with a mean 32.8 ± 0.6 % (n = 4) loss of T_3 binding sites. A similar loss of about 29 % was obtained in NE preincubated with $BrAcT_3$. $BrPropT_3$ and $BrTolT_3$ also decreased the T_3 binding site concentration but to a lesser extent (9.0 and 7.5 % respectively) without altering Ka for T_3. $BrAcT_o$ was always ineffective as also was T_3 when used itself at 0.2 µM for the preincubation step. This suggests that a specific covalent interaction could have occured between the N-bromoalkyl derivatives of T_3, particularly $BrAcT_3$, and the T_3 binding site region. In 2 experiments without DNA + histones, $BrAcT_o$ slightly enhanced the T_3 binding site concentration as if had exerted a protective role the origin of which is at present unknown

DISCUSSION

The reported results indicate that a biospecific interaction is likely to occur between the T_3 binding site of the nuclear T_3 receptor and some N-bromoalkyl derivatives of T_3; $BrAcT_3$ was the most potent, while $BrAcT_o$ was unable to interfere with T_3 binding. The apparent affinity of these bromoalkyl derivatives of T_3 for the T_3 binding site is lower than that of T_3 with Kd ranging between 10 and 100 nM for $BrAcT_3$, variations between NE and $NTBP_{pp}$ or between different batches of $NTBP_{pp}$ being probably enhanced by the absence of DTT in incubation medium. These values are in the range of apparent Kd values reported by Cheng et al (28) for rhodamine-T_3 derivatives in which rhodamine was attached to the α-amino group of T_3; they are lower than those found for N-AcT_3 which were constantly in the range of 1 µM. Preincubation of NTBP preparations with the N-bromoalkylderivatives of T_3 provoked a significant loss in T_3 binding site concentration; the ineffectiveness of $BrAcT_o$ and the inability of T_3 itself to alter NTBP capacity strongly suggest that a specific interaction is involved and that a covalent bond could have occured with the T_3 binding site region. This interaction is more important when a shorter chain length is attached to the α-NH_2 group of T_3. $BrAcT_3$ could then be a candidate for affinity labeling of the T_3 nuclear receptor. Experiments using $[^{125}I]$ $BrAcT_3$ of high specific radioactivity are in progress.

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REFERENCES

1. Oppenheimer, J.H., Koerner, D., Schwartz, H.L. and Surks, M.I. (1972) J. Clin. Endocrinol. Metab. 35 : 330-333.
2. DeGroot, L.J. and Strausser, J.L. (1974) Endocrinology 95 : 74-83.
3. Samuels, H.H. and Tsai, J.S. (1973) Proc. Nat. Acad. Sci. USA, 70 : 3488-3492.
4. Yoshizato, K., Kistler, A. and Frieden, E. (1975) Endocrinology 97 : 1030-1035.

5. DeGroot, L.J. and Torresani, J. (1975) Endocrinology 96 : 357-369.
6. Thomopoulos, P., Dastugue, B. and Defer, N. (1974) Biochem. Biophys. Res. Comm. 58 : 499-506.
7. Torresani, J. and DeGroot, L.J. (1975) Endocrinology 96 : 1201-1209.
8. Samuels, H.H., Tsai, J.S., Casanova, J. and Stanley, F. (1974) J. Clin. Invest, 54 : 853-865.
9. Surks, M.I., Koerner, D., Dillman, W. and Oppenheimer, J.H. (1973) J. Biol. Chem. 248 : 7066-7072.
10. Torresani, J., Anselmet, A. and Wahl, R. (1978) Mol. Cell. Endocrinol. 9 : 321-333.
11. Latham, K.R., Ring, J.C. and Baxter, J.D. (1976) J. Biol. Chem. 251 : 7388-7397.
12. MacLeod, K.M. and Baxter, J.D. (1976) J. Biol. Chem. 251 : 7380-7387.
13. Anselmet, A. and Torresani, J. (1977) Ann. Endocrinol. (Paris) 38 : 18 A.
14. DeGroot, L.J. Hill, L. and Rue, P. (1976) Endocrinology 99 : 1605-1611.
15. Anselmet, A., Bismuth, J. and Torresani, J. (1980) Ann. Endocrinol. (Paris) 41 : 97 A.
16. Oppenheimer, J.H. (1979) Science 203 : 971-979.
17. Torresani, J., Charbi, J., Lissitzky, S., Mirre, C., Delgrossi, M.H. and Stahl, A. (1979) Biochem. Biophys. Res. Comm. 86 : 371-378.
18. Torresani, J. and Anselmet, A. (1978) Biochem. Biophys. Res. Comm. 81 : 147-153.
19. Silva, E.S., Astier, H., Thakare, U., Schwartz, H.L. and Oppenheimer, J.H. (1977) J. Biol. Chem. 252 : 6799-6805.
20. Eberhardt, N.L., Ring, J.C., Johnson, L.K., Latham, K.R., Apriletti, J.W., Kitsis, R.N. and Baxter, J.D. (1979) Proc. Nat. Acad. Sci. USA 76 : 5005-5009.
21. Koerner, D., Schwartz, J.L., Surks, M.I., Oppenheimer, J.H. and Jorgensen, E.C. (1975) J. Biol. Chem. 250 : 6417-6423.
22. Cheng, S.Y., Wilchek, M., Cahnmann, H.J. and Robbins, J. (1977) J. Biol. Chem. 252 : 6076-6081.
23. Hartree, E.F. (1972) Anal. Biochem. 48 : 422-427.
24. Ross, E. and Schatz, G. (1973) Anal. Biochem. 54 : 304-306.
25. Acher, R. and Crocker, C. (1952) Biochim. Biophys. Acta 9 : 704-705.
26. Gemmill, C.L. (1955) Arch. Biochem. Biophys. 54 : 359-367.
27. Wilchek, M. and Givol, D. (1977) in Methods in Enzymology vol. 46 pp 153-157 ed. Jakoby, W.B. and Wilchek, M., Academic Press.
28. Cheng, S-Y, Eberhardt, N.L., Robbins, J., Baxter, J.D. and Pastan, I. (1979) FEBS Lett. 100 : 113-116.