

## Development of [<sup>125</sup>I]RB104, a potent inhibitor of neutral endopeptidase 24.11, and its use in detecting nanogram quantities of the enzyme by "inhibitor gel electrophoresis"

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**ABSTRACT** Neutral endopeptidase 24.11, also known as the common acute lymphoblastic leukemia antigen, is a zinc metallopeptidase involved in the inactivation of biologically active peptides, such as the enkephalins and atrial natriuretic peptide. The highly potent radiolabeled inhibitor 2-[(3-[<sup>125</sup>I]iodo-4-hydroxy)phenylmethyl]-4-*N*-[3-(hydroxyamino-3-oxo-1-phenylmethyl)propyl]amino-4-oxobutanoic acid ([<sup>125</sup>I]RB104;  $K_i = 30$  pM) has been developed for the enzyme. [<sup>125</sup>I]RB104 is highly specific, its  $K_i$  for another widely distributed zinc peptidase, angiotensin-converting enzyme, being 15  $\mu$ M. In binding studies using rat brain slices, [<sup>125</sup>I]RB104 was shown to have a high affinity ( $K_d = 300 \pm 20$  pM) and high specific binding at the  $K_d$  concentration (90%). With rat brain homogenates the  $K_d$  of [<sup>125</sup>I]RB104 was  $26.8 \pm 0.9$  pM, close to the kinetically derived  $K_d$ ,  $7.0 \pm 0.8$  pM. Using the inhibitor, we have developed a simple, rapid, and quantitative technique to detect low nanogram quantities of the endopeptidase directly from tissue extracts after SDS/PAGE. The method has been used to show the presence of low quantities of the enzyme in rabbit bone marrow. Apart from its sensitivity, "inhibitor gel electrophoresis" using [<sup>125</sup>I]RB104 has the advantage over immunohistochemical methods of being able to label the enzyme in all tissues and species. It will therefore be of great value in determining the exact role of this important regulatory peptidase in a number of biological systems. Moreover, this one-step characterization of neutral endopeptidase 24.11 could be extended to other zinc metallopeptidases such as angiotensin-converting enzyme or collagenases, and inhibitors with affinities as high as RB104 could open the way to visualization of zinc metallopeptidases in different tissues by electron microscopy.

The zinc endopeptidase neutral endopeptidase-24.11 (NEP; EC 3.4.24.11) is a 94-kDa ectoenzyme involved in the extracellular metabolism of biologically active peptides (1). The enzyme has a relatively broad specificity and generally cleaves peptides on the amino side of hydrophobic residues. The most widely studied *in vivo* substrates of NEP are the enkephalins and atrial natriuretic peptide, as it has been shown that, due to the inactivation of these peptides by the enzyme, NEP inhibitors have antinociceptive and antihypertensive properties (reviews in refs. 2 and 3). In addition NEP has been found to be identical to the common acute lymphoblastic leukemia antigen (CALLA) (4), a differentiation marker of B lymphocytes and identified as a lymphocyte surface marker in most acute non-B-lymphoblastic leukemias and in some T-cell acute lymphoblastic leukemias and lymphoblastic lymphomas as well being present in myelomas,

gliomas (review in ref. 5), and fibroblastic cells in meningiomas (6).

The distribution of NEP has been reasonably well documented in the rat and pig, both in the central nervous system and in the periphery, by using either tritiated inhibitors such as *N*-[(2*RS*)-4-(hydroxyamino)-1,4-dioxo-2-(phenyl[<sup>3</sup>H]methyl)[2-<sup>3</sup>H]butyl]glycine (<sup>3</sup>H]HACBO-Gly) (7, 8) and [<sup>3</sup>H]thiorphan (9) or antibodies (10-12). There are, however, several questions that remain to be answered. For instance, although it is clear that NEP inhibitors prolong both the half-life and the actions of atrial natriuretic peptide *in vivo*, their sites of action have yet to be established (3, 13). In addition, a number of hematopoietic cells and cell lines, classed as being "CALLA-negative" by fluorescence-activated cell sorting using monoclonal antibodies, have since been shown to have NEP activity by using a radioactive substrate (14). Although this apparent anomaly is probably due to the difference in sensitivity of the two techniques, it is necessary to demonstrate formally the presence of the enzyme on the surface of these cells.

For distribution studies, radioactive inhibitors have the advantage over antibodies of being able to label the active site, quantitatively, in all tissues and species. This is important for an enzyme like NEP as, although the primary sequences of the molecule from different species are highly conserved (15), the enzyme appears to be differentially glycosylated not only in different species but also in different tissues of the same species (16), which might affect its recognition by antibodies. A recent study has shown, for example, that out of 20 monoclonal antibodies raised against the human lymphocyte antigen only 4 cross-reacted with NEP from a rat cell line (17). There has also been a report that 1 out of 6 anti-CALLA antibodies tested cross-reacted with amyloid plaques present in patients with Alzheimer disease (18). It still remains to be established whether the antibody is marking NEP in the plaques or a protein with which NEP shares an epitope. Another advantage of radioactive inhibitors, such as [<sup>3</sup>H]HACBO-Gly, is that their *in vivo* distribution can easily be followed after different routes of administration (19), an important factor, considering the potential clinical applications of such molecules.

It is of course essential to ensure that the labeled inhibitor is specific for the enzyme, as zinc metallopeptidases have many active-site features in common (20). Thiorphan, for instance, has a relatively good affinity for the widely distrib-

Abbreviations: NEP, neutral endopeptidase 24.11 (identical to CALLA, common acute lymphoblastic leukemia antigen); HACBO-Gly, *N*-[(2*RS*)-4-(hydroxyamino)-1,4-dioxo-2-(phenylmethyl)butyl]glycine; RB104, 2-[(3-iodo-4-hydroxy)phenylmethyl]-4-*N*-[3-(hydroxyamino-3-oxo-1-(phenylmethyl)propyl]amino-4-oxobutanoic acid; TFA, trifluoroacetic acid.

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uted angiotensin-converting enzyme (21). This problem was overcome with [ $^3\text{H}$ ]HACBO-Gly (22), but the main disadvantage of this molecule, as with all tritiated inhibitors, lies in its low specific activity, necessitating long exposure times for autoradiograms and giving relatively low sensitivity.

We report here the synthesis and radiolabeling with  $^{125}\text{I}$  of the highly selective potent NEP inhibitor 2-[(3-iodo-4-hydroxy)phenylmethyl]-4-*N*-[3-hydroxyamino-3-oxo-1-(phenylmethyl)propyl]amino-4-oxobutanoic acid (RB104). This molecule, which has a  $K_i$  of 30 pM for NEP, not only is an excellent ligand for classical autoradiography and binding studies but also can be used to detect nanogram quantities of the enzyme directly from tissue extracts after SDS/PAGE.

## MATERIALS AND METHODS

**Materials.** The NEP inhibitor (*R*)-retrothiorphan was synthesized as previously described (23). Aminopeptidase N from hog kidney was purchased from Boehringer Mannheim and angiotensin-converting enzyme was a generous gift from P. Corvol (College de France, Paris).  $\text{Na}^{125}\text{I}$  (2100 Ci/mmol; 1 Ci = 37 GBq) and [ $^3\text{H}$ ][Leu $^5$ ]enkephalin (50 Ci/mmol) were from Amersham and [ $^3\text{H}$ ][DAla $^2$ ,Leu $^5$ ]enkephalin (51 Ci/mmol) was from Dositek, France. [ $^3\text{H}$ ]HACBO-Gly (45 Ci/mmol) was from the Commissariat à l'Énergie Atomique (Saclay, France). Unless otherwise stated other products were from Sigma.

**Synthesis and Radioiodination of RB104.** The synthesis of RB104 (6) is summarized in Fig. 1. *t*-Butyl-2-(*p*-hydroxy)benzylidene succinate was synthesized as previously described for *t*-butyl-2-benzylidene succinate (24). Starting from diethyl succinate and *p*-hydroxybenzaldehyde, compound 2 was obtained as an oily product.  $R_f$  in  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (9:1, vol/vol) = 0.45. *Step a* (Fig. 1). To a solution of 2.00 g (7.2 mmol) of 2 in dry tetrahydrofuran (THF) were added at 0°C a solution of 2.86 g (7.2 mmol) of the trifluoroacetate of

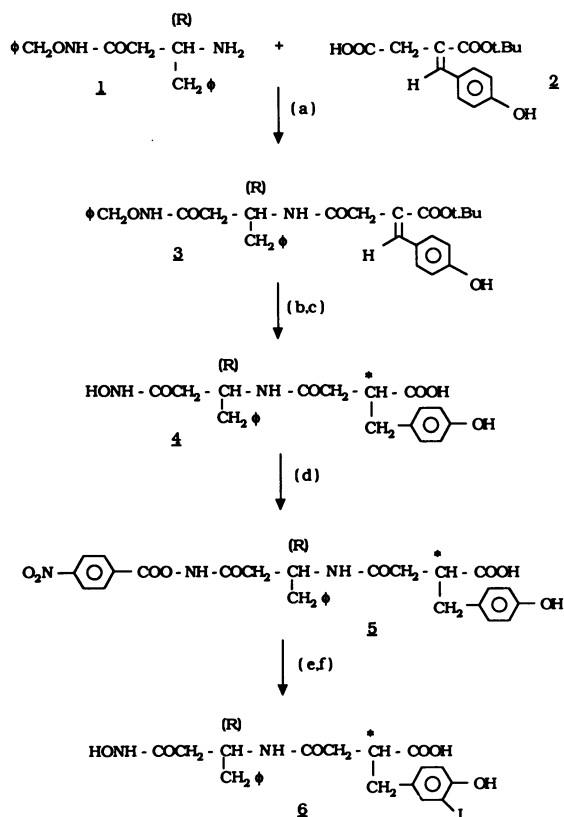


FIG. 1. Synthesis of RB104. See text for details.

3-amino-4-phenylbutane-(*O*-benzyl)hydroxamate (1) (24), a solution of 1.1 g (7.2 mmol) of 1-hydroxybenzotriazole in dry THF, and 1.63 g (7.9 mmol) of *N,N'*-dicyclohexylcarbodiimide in  $\text{CHCl}_3$ . After 1 hr at 0°C, the mixture was stirred overnight at room temperature, the precipitate was concentrated, the solvent was evaporated under reduced pressure, and the residue was dissolved in EtOAc. The organic layer was washed, dried over  $\text{Na}_2\text{SO}_4$ , and filtered, and the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel with  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (10:0.5, vol/vol) as eluent. Compound 3 was obtained as an oily product.  $R_f$  in  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  (9:1, vol/vol) = 0.47; yield = 60%.

*Steps b and c in Fig. 1.* Five milliliters of trifluoroacetic acid (TFA) was added to 1.9 g (3.5 mmol) of 3 in 10 ml of  $\text{CH}_2\text{Cl}_2$  at 0°C. The mixture was stirred for 2 hr at 0°C and 1 hr at room temperature, and solvent was evaporated under reduced pressure. The residue was taken off with  $\text{Et}_2\text{O}$  and the white precipitate was intensively washed with  $\text{Et}_2\text{O}$ . The white solid obtained [yield = 70%;  $R_f$  in  $\text{CHCl}_3/\text{MeOH}$  (9/1, vol/vol) = 0.18] was dissolved in MeOH and hydrogenated over Pd/carbon. A white solid (4) was obtained [yield = 92%; mp = 110°C; 15- $\mu\text{m}$  TLC  $R_f$  in  $\text{CHCl}_3/\text{MeOH}/\text{AcOH}/\text{H}_2\text{O}$  (7/3/0.6/0.3, vol/vol) = 0.46]. *Step d in Fig. 1.* To a solution of 4 (0.3 g, 0.75 mmol) in EtOH were added, at 0°C, 0.205 g (0.79 mmol) of the *p*-nitrophenyl ester of *p*-nitrobenzoic acid and 0.126 ml (1.2 eq) of triethylamine. The mixture was stirred for 2.5 hr at room temperature and solvent was evaporated under reduced pressure. The residue was dissolved in  $\text{H}_2\text{O}$  and the aqueous layer was washed with  $\text{Et}_2\text{O}$ , taken to pH 1.0 with 1 M HCl, and extracted three times with ethyl acetate. The organic layer was washed with saturated NaCl, dried over  $\text{Na}_2\text{SO}_4$ , and filtered, and the solvent was evaporated under reduced pressure. A pale yellow solid (5) was obtained [yield = 58%; mp 117°C; HPLC retention time on Ultrabase C $_8$  5- $\mu\text{m}$  in  $\text{CH}_3\text{CN}/0.05\%$  TFA, 45/55 (vol/vol) = 9.0 and 9.7 min; 15- $\mu\text{m}$  TLC  $R_f$  in  $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{acetic acid}$  (9/1/0.5, vol/vol) = 0.43]. Analysis. Calculated for  $\text{C}_{28}\text{H}_{27}\text{N}_3\text{O}_9$ : C, 61.20; H, 4.95; N, 7.65. Found: C, 60.82; H, 4.86; N, 7.57.

*Steps e and f in Fig. 1.* The iodination of 5 was carried out on ice and in 5 mM phosphate buffer, pH 7.0. To 35 mg of the protected hydroxamate 5 were added 1 eq of NaI followed by 1.2 eq of chloramine T, and the reaction was stopped after 5 min by adding 25 eq of  $\text{Na}_2\text{S}_2\text{O}_5$ . The pH was then increased to 9 with 6 M NaOH, the solution was left for 10 min at room temperature and the pH was adjusted to 1.5 with 6 M HCl, and the reaction mixture was immediately injected onto a C $_8$  Ultrabase 5- $\mu\text{m}$  HPLC column (Société Française de Chromato Colonne Shandon, Eragny, France). The products were separated at a flow rate of 1 ml/min under isocratic conditions for the first 30 min (23%  $\text{CH}_3\text{CN}$  in 0.05% TFA) followed by a gradient of 23–43%  $\text{CH}_3\text{CN}$  in 20 min. The two stereoisomers of the monoiodinated inhibitor RB104 6 were found to have retention times of 32.5 and 40.6 min. Analysis. Calculated for  $\text{C}_{21}\text{H}_{23}\text{N}_2\text{O}_6\text{I}$ : C, 47.92; H, 4.40; N, 5.32. Found: C, 47.82; H, 4.46; N, 5.36.

[ $^{125}\text{I}$ ]RB104 was synthesized by using 0.7 mCi of  $\text{Na}^{125}\text{I}$ , 10 eq of 5, and 40 eq of chloramine T in a total reaction volume of 40  $\mu\text{l}$  of 50 mM phosphate buffer, pH 7.5. The reaction was stopped after 1 min at room temperature by adding 100 eq of  $\text{Na}_2\text{S}_2\text{O}_5$ . The pH was then increased to 10 with 1 M NaOH, the solution was left for 5 min at room temperature, and the pH was adjusted to 1.5 with 6 M HCl prior to HPLC as above. The two stereoisomers, which have similar inhibitory potencies towards NEP, were not separated during the radioiodination.

**Membrane Preparations and Purification of Rabbit Kidney NEP.** All procedures were carried out at 4°C. Tissue or cell preparations were homogenized in 10 vol of 50 mM Tris-HCl,

pH 7.4, and centrifuged at  $1000 \times g$  for 10 min. The supernatant was then centrifuged at  $100,000 \times g$  for 40 min, the pellet was resuspended in Tris buffer containing 125 mM NaCl, and proteins were solubilized by adding *n*-octyl glucoside to a final concentration of 1%. A 1-hr incubation at 4°C, with gentle agitation, was followed by centrifugation at  $100,000 \times g$  for 40 min. The supernatant was stored at  $-80^\circ\text{C}$  until required. No change in NEP activity was noted after up to 3 months of storage under these conditions. NEP was purified from solubilized rabbit kidney cortex membranes by affinity chromatography, using a monoclonal antibody as previously described (25).

**Enzyme Assays.** NEP activity and  $\text{IC}_{50}$  were assayed by using 20 nM [ $^3\text{H}$ ][DAla<sup>2</sup>,Leu<sup>5</sup>]enkephalin as substrate. Incubations were carried out in 100  $\mu\text{l}$  of 50 mM Tris-HCl, pH 7.4, at 25°C. When a membrane preparation was used, 1  $\mu\text{M}$  captopril, an inhibitor of angiotensin-converting enzyme, and 10  $\mu\text{M}$  bestatin, an inhibitor of aminopeptidase, were added. The reaction was stopped by adding 10  $\mu\text{l}$  of 0.5 M HCl and the product [ $^3\text{H}$ ]Tyr-DAla-Gly was separated from intact substrate by using Porapak Q beads (Waters) as previously described (26). Aminopeptidase N activity was assayed as described above, using 10 nM [ $^3\text{H}$ ][Leu<sup>5</sup>]enkephalin as substrate. Angiotensin-converting enzyme was assayed as previously described (14), using 50 mM Z-Phe-His-Leu as substrate (Z, benzyloxycarbonyl).

**Binding Assays.** Rat brain striatal sections were cut on a cryostat at  $-17^\circ\text{C}$ , thaw-mounted onto gelatin-coated slides, and stored at  $-80^\circ\text{C}$  until used. The sections were warmed to room temperature immediately prior to incubation and were placed in chambers maintained at constant (20°C) temperature and 60–80% relative humidity. The sections were incubated as above in 300  $\mu\text{l}$  of buffer containing the iodinated ligand, with or without unlabeled ligand. After 60 min, or at various times for association studies, the slices were drained and washed twice in a large excess of cold buffer. The sections were wiped from the slides with a Whatman GF/B filter disk and the radioactivity was determined.

Rat brain membranes from whole brain minus cerebellum were prepared as described above and the resulting pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4. Binding assays were performed in the same buffer in a final volume of 1 ml and contained 0.6–0.7 mg of protein and the iodinated ligand. Incubations were carried out at 35°C and were terminated by filtration through Whatman GF/B filters. The filters were rinsed twice with 5 ml of ice-cold buffer and the radioactivity was measured. For kinetic studies [ $^{125}\text{I}$ ]RB104 was used at 30 pM and dissociation was initiated by addition of 1  $\mu\text{M}$  retrothiorphan, a highly potent and specific NEP inhibitor (23), after a 60-min incubation. For saturation studies incubations were for 1 hr and the radioactive ligand was used at 3 pM with various concentrations of unlabeled RB104 added. In all studies the nonspecific binding was determined by using 1  $\mu\text{M}$  retrothiorphan.

**Characterization of NEP in Membrane Preparations After Inhibitor Gel Electrophoresis.** Nonreducing SDS/PAGE was carried out as previously described (27) on 9% polyacrylamide slab gels (70  $\times$  50  $\times$  0.75 mm). After electrophoresis at a constant 180 V, the gels were washed twice for 15 min in 50 ml of 10 mM Tris-HCl, pH 7.4, containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and finally in Tris containing 0.5% CHAPS, 0.15 M NaCl, and 0.3 M urea. The gels were then incubated for 1 hr at room temperature with 100 pM [ $^{125}\text{I}$ ]RB104 in the presence or absence of 0.1 mM retrothiorphan. After washing to reduce radioactive background (four times, 15 min each, in 50 ml of 10 mM Tris-HCl, pH 7.4, at room temperature), the gels were placed in sealed plastic bags and opposed to Amersham Hyper  $\beta$  max film for 1–4 days at 4°C. The films were developed as described above and analyzed by densitometry

with a Biocom 200 image analyzer. Alternatively, the gel lanes were cut into 2-mm horizontal slices and the radioactivity was determined directly. In some experiments NEP was incubated with 150 pM [ $^{125}\text{I}$ ]RB104 before SDS/PAGE and the gels were sliced or subjected to autoradiography directly after electrophoresis.

**Protein Determination.** Protein was determined by the method of Bradford (28).

## RESULTS

**Synthesis and Radioiodination of RB104.** This compound was synthesized by using previously described methods (24). As outlined in Fig. 1, the precursor 4 was obtained by a coupling step between the *O*-benzyl hydroxamate 1 and the succinyl derivative 2 by using the dicyclohexylcarbodiimide/hydroxybenzotriazole method, followed by successive deprotection of the *t*-butyl ester in acidic medium and of the hydroxamate group by catalytic hydrogenation. Iodination of 4 was carried out by the chloramine-T method and, as preliminary experiments indicated that the hydroxamate group had to be protected in this step, the *p*-nitrobenzoyl group was chosen, both for its easy introduction as an active ester and easy removal under mild alkali conditions.

**Inhibitory and Binding Properties of RB104 and [ $^{125}\text{I}$ ]RB104.** RB104 inhibited pure rabbit kidney NEP with a  $K_i$  of 30 pM. Its  $K_i$  for angiotensin-converting enzyme, a zinc peptidase for which NEP inhibitors often show some degree of cross-reactivity (29, 30), was 15  $\mu\text{M}$  and that for aminopeptidase N, another widely distributed zinc peptidase, was 2.5  $\mu\text{M}$ . Association kinetics of 20 pM [ $^{125}\text{I}$ ]RB104 to rat brain striatal slices showed that specific binding reached a steady state at around 1 hr (Fig. 2). The specific binding was saturable and the  $K_d$  and  $B_{\text{max}}$  values, derived from Scatchard analysis, were  $300 \pm 20$  pM and  $3.5 \pm 0.12$  fmol per slice, respectively (Fig. 2). Specific binding was 90% at the  $K_d$  value, and computer analysis of the saturation isotherm showed that the inhibitor interacted with a single class of binding sites. With rat brain homogenates and 30 pM [ $^{125}\text{I}$ ]RB104, specific binding reached a steady state at around 40 min. The calculated association rate constant,  $k_{+1}$ , was  $1.43 \pm 0.22$  min $^{-1}\cdot\text{M}^{-1}$ , and, assuming a monophasic dissociation, the off-rate constant,  $k_{-1}$ , was calculated to be  $1.01 \pm 0.3$  10 $^{-2}$  min $^{-1}$ . The kinetically derived dissociation constant ( $k_d = k_{-1}/k_{+1}$ ) was  $7.0 \pm 0.8$  pM. Binding was saturable

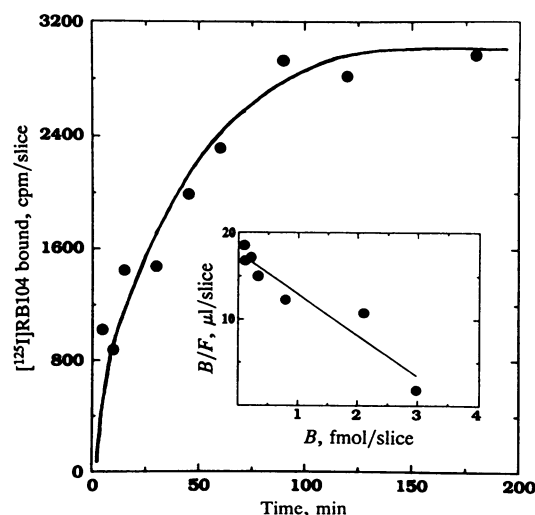


FIG. 2. Association kinetics for the specific binding of 20 pM [ $^{125}\text{I}$ ]RB104 to rat brain sections. (Inset) Scatchard analysis of binding over a concentration range of 0.9 pM to 1.5 nM;  $B$ , bound;  $F$ , free.

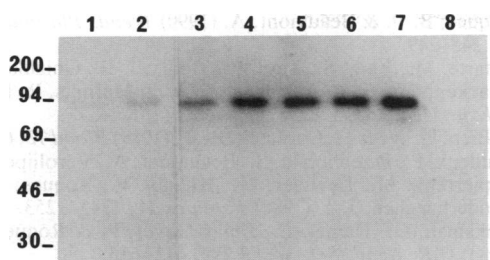


FIG. 3. Labeling of different quantities of rabbit kidney NEP after SDS/PAGE and incubation of the gel with 100 pM [<sup>125</sup>I]RB104 with (lane 8) or without 0.1 mM retrothiorphan. The quantity of enzyme loaded in each lane was as follows: 1, 2 ng; 2, 5 ng; 3, 10 ng; 4, 20 ng; 5, 30 ng; 6, 40 ng; and 7, 50 ng. Lane 8 was 50 ng. The running positions of molecular mass standards (kDa) are shown on the left.

when concentrations between  $3 \times 10^{-12}$  and  $1 \times 10^{-8}$  M with a 1-hr incubation were used, and Scatchard analysis of the binding isotherms showed a single class of binding sites with a  $K_d$  of  $26.8 \pm 0.9$  pM and a  $B_{max}$  of  $8.1 \pm 0.2$  fmol/mg of protein. Specific binding was about 90% at the  $K_d$  concentration and greater than 70% at saturation.

**Detection of NEP by Inhibitor Gel Electrophoresis with RB104 After SDS/PAGE.** Fig. 3 shows the autoradiogram obtained when different amounts of pure rabbit kidney NEP were subjected to SDS/PAGE and the gel was subsequently incubated with [<sup>125</sup>I]RB104 and treated as described in *Materials and Methods*. A single band of radioactivity was observed, corresponding to NEP, the intensity of which increased with the quantity of enzyme, allowing a calibration curve to be constructed (Fig. 4). The minimum quantity of enzyme detectable under the conditions used (exposure time, 3 days) was 2 ng. The band was completely displaced by including retrothiorphan in the incubation mixtures with the iodinated ligand, though higher concentrations (0.1 mM) were required than for binding or autoradiography studies. When the gels were sliced and the radioactivity was determined directly it was estimated that 0.25% of the enzyme was labeled. When incubated with the enzyme before electrophoresis, [<sup>125</sup>I]RB104 was also found to specifically comigrate with NEP (not shown). However, although this method removed the necessity of washing the gels after electrophoresis, the amount of inhibitor bound to the enzyme was reduced by approximately 95%, thus lowering the sensitivity of the technique.

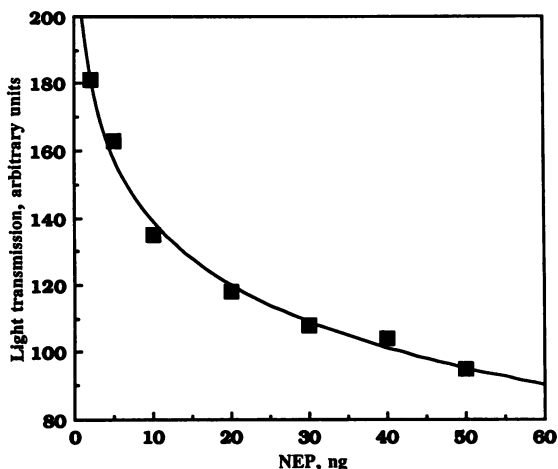


FIG. 4. Calibration curve constructed from the data obtained from the autoradiogram shown in Fig. 3 and relating the quantity of rabbit kidney NEP subjected to SDS/PAGE with the intensity of labeling by [<sup>125</sup>I]RB104.

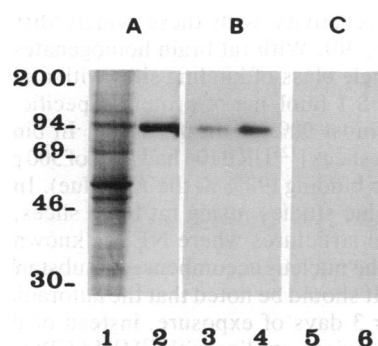


FIG. 5. Autoradiogram of the labeling of solubilized rabbit kidney protein with [<sup>125</sup>I]RB104 after SDS/PAGE. After electrophoresis the gel was divided into three pieces and lanes 1 and 2, which contained 15 μg of rabbit kidney membrane protein or 2.5 μg of pure rabbit kidney NEP, respectively, were stained with Coomassie blue. Lanes 3 and 5, containing 4 μg of kidney protein, and 4 and 6, containing 50 ng of pure enzyme, were incubated with 100 pM [<sup>125</sup>I]RB104, in the presence (lanes 5 and 6) or absence (lanes 3 and 4) of 0.1 mM retrothiorphan. The running positions of molecular mass standards are shown on the left.

The procedure was then tried on different tissue extracts. As shown in Fig. 5, [<sup>125</sup>I]RB104 labeled a protein from rabbit kidney, which had a molecular mass of 94 kDa and migrated in the gel at the same position as the pure enzyme. This labeling was abolished by 0.1 mM retrothiorphan. From a calibration curve constructed from standards run on a parallel gel it was estimated that there was 9.6 μg of enzyme per mg of protein in the extract. Similar results were found with extracts of cells from rabbit hind leg bone marrow (Fig. 6), although NEP, 0.38 μg/mg of protein, was 1/25th the concentration in the kidney.

**DISCUSSION**

RB104 was found to be a potent inhibitor of NEP, with a  $K_i$  of 30 pM for pure rabbit kidney NEP. As expected from a molecule with such a strong affinity, it also proved to be highly specific. A comparison of the  $K_i$  of RB104 for NEP with its  $K_i$  values for angiotensin-converting enzyme and aminopeptidase N shows that the inhibitor has selectivity factors for NEP of  $5 \times 10^5$  and  $8.34 \times 10^4$ , respectively. Angiotensin-converting enzyme and aminopeptidase N were chosen for comparison because NEP inhibitors often show a

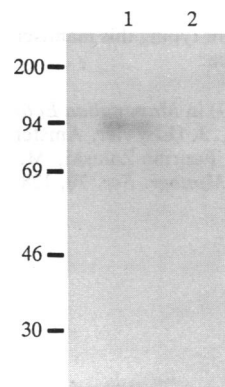


FIG. 6. Autoradiogram of the labeling of solubilized rabbit bone marrow membrane protein by [<sup>125</sup>I]RB104 after SDS/PAGE. Lanes 1 and 2 contained 40 μg of rabbit bone extract. After electrophoresis the gel was divided into two pieces and incubated with 100 pM [<sup>125</sup>I]RB104 in the presence (lane 2) or absence (lane 1) of 0.1 mM retrothiorphan. The running positions of molecular mass standards are shown on the left.

certain cross-reactivity with these widely distributed zinc peptidases (29, 30). With rat brain homogenates [<sup>125</sup>I]RB104 bound to a single class of binding sites with a  $K_d$  of 26.8 pM and a  $B_{max}$  of 8.1 fmol/mg of protein. Specific binding was high, being almost 90% at the  $K_d$  value. In binding studies using rat brain slices [<sup>125</sup>I]RB104 had a  $K_d$  of 300 pM and again a high specific binding (90% at the  $K_d$  value). In preliminary autoradiographic studies, using rat brain slices, [<sup>125</sup>I]RB104 labeled typical structures where NEP is known to be localized, such as the nucleus accumbens and substantia nigra (not shown here). It should be noted that the autoradiograms were obtained after 3 days of exposure, instead of the 3 months required for previous studies with [<sup>3</sup>H]HACBO-Gly, with an even better precision.

[<sup>125</sup>I]RB104 was also found to bind to pure rabbit kidney NEP after SDS/PAGE. This is in line with a previous report showing that the enzyme retains some activity under these conditions (31). However no additional band was found at 200 kDa, as occurred with the substrate gel electrophoresis. Binding was quantitative and, with an exposure time of 3–4 days, as little as 2 ng of enzyme could be detected. This simple method can be made even more sensitive if required, using longer exposure times and intensifying screens. The inhibitor was also found to bind to a single protein with a molecular mass of 94 kDa from a rabbit kidney extract subjected to SDS/PAGE, and this binding was inhibited by the NEP inhibitor retrothiorphan, confirming that the binding was specific to NEP. In addition the inhibitor was found to label NEP in extracts of rabbit bone marrow. Autoradiography with [<sup>3</sup>H]HACBO-Gly has previously shown the presence of NEP in rat bone tissue (19), and this has now been confirmed in the rabbit.

[<sup>125</sup>I]RB104 is thus an ideal molecule for characterizing NEP, either by classical or autoradiography studies or by inhibitor gel electrophoresis. One of the reasons for its favorable properties is that it is endowed with a balance between hydrophobic aromatic side chains and hydrophilic groups. "Inhibitor gel electrophoresis" is an accurate, simple, and relatively quick method of characterizing and measuring the NEP in tissues and cell lines. It is also reliable because, as previously discussed, inhibitors have the advantage of marking the active site of the enzyme in all tissues and species. Furthermore, in preliminary experiments the same binding profile in unfixed and paraformaldehyde-fixed brain tissues was observed with [<sup>125</sup>I]RB104, opening the way towards electron microscopic visualization of NEP in different tissues.

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