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[^{3}H]ACETAZOLAMIDE BINDING TO CARBONIC ANHYDRASE IN NORMAL AND TRANSFORMED CELLS

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Abstract—The binding of [^{3}H]acetazolamide (AZ), a carbonic anhydrase (CA) inhibitor, to soluble and particulate forms of CA was investigated. Sources for the assays were purified CA II, adult rat cortical, oligodendrocyte and neuronal enriched preparations; cultured murine glial cells, rat C-6 glioma, rat hepatoma and human glioblastoma cells. CA enzyme activity in the same preparations was also assayed by following change in pH during incubation. A gel permeation chromatographic method was developed to assess [^{3}H]AZ binding to soluble CA, while glass fiber filter vacuum filtration was used for particulate CA binding. Saturable specific binding of [^{3}H]AZ to rat cortical soluble and particulate CA preparations was demonstrated. Computer-assisted data analysis estimated the binding parameters of [^{3}H]AZ to soluble rat cortical CA to be B_{max} = 0.38 ± 0.13 pmol/mg protein and K_d = 34.7 ± 17.5 nM. The rat cortical particulate fraction B_{max} was 2.05 ± 0.28 pmol/mg protein with a K_d of 107.1 ± 24.2 nM. Purified bovine CA-II bound 1.15 ± 0.19 pmol [^{3}H]AZ/mg protein with a K_d of 54.0 ± 3.4 nM. The pH optima for [^{3}H]AZ binding to soluble and particulate CA was between 6.5 and 7.5. Binding was linear with respect to protein up to 1.0 mg/mL. The particulate fraction bound 3–4 times more [^{3}H]ligand per unit protein than the soluble fraction. Interestingly, no detectable CA enzyme activity or [^{3}H]AZ binding was observed in the soluble or particulate fractions of human glioblastoma, rat C-6 glioma or rat hepatoma cells. Binding of [^{3}H]AZ to other soluble enzymes or proteins was negligible. In competition binding experiments, a rank order of inhibition of [^{3}H]AZ binding to rat cortical CA by established CA inhibitors was: dichlorphenamide > acetazolamide ≥ benzolamide > methazolamide > hydrochlorothiazide ≥ sulfanilamide. [^{3}H]AZ binding was not affected by other classes of pharmacologic characterizing agents. The binding of [^{3}H]AZ to the CA enzyme molecule is highly specific and sensitive and may prove useful *in vitro* or *in situ* as a probe for this enzyme.

Carbonic anhydrase (CA) (EC 4.2.1.1.) is a zinc-containing metalloenzyme ubiquitously distributed in many vertebrate and invertebrate tissues, plants and bacteria [see Refs. 1 and 2 for reviews]. The enzyme catalyzes both the hydration of CO_2 to H_2CO_3 and the reverse dehydration [1]. Functionally, this enzyme participates in ionic fluxes and acid-base regulation [1].

Mammals have three soluble isozyme forms of CA which are distinct genetically and immunologically. One isozyme form, designated CA II also known as CA_C, is the predominate soluble form of the enzyme and is present in blood. A deficiency of CA II may be related to metabolic disorders such as osteopetrosis, cerebral calcification and renal tubular acidosis [3, 4]. A second form of the enzyme also present in blood is CA I and is alternatively known as CA_B. Skeletal muscle is a source of a third type of CA [5].

Brain CA activity is present in both white and gray matter and in the rodent shows a subcellular distribution of 60% in membrane-bound myelin and 40% in the soluble fraction [6]. CA is enriched 10-fold in glia compared to neurons [7] and has been used as a marker for oligodendrocytes and astrocytes [7].

The use of immunological or radiochemical probes for the identification and characterization of recognition sites is well documented. Studies on radioligand receptor binding are numerous; however, only a limited number of studies have used equilibrium radioligand binding to characterize enzyme recognition domains. For example, immunocytochemical probes for the CA II isozyme have been utilized as an enzyme marker for identification of choroid plexus papillomas [8]. Additionally, CA activity in brain increases with age [9] and exhibits an unequal regional distribution in homogenates, using either immunocytochemistry or direct enzyme activity measurements [10].

Sulfonamides are potent inhibitors of CA [11]. The well-known sulfonamide, acetazolamide, is a non-competitive reversible enzyme inhibitor with respect to CO_2 [12] and is used clinically for the effective management of glaucoma, edema and epilepsies [13]. [^{3}H]AZ has been used to label human CA I employing equilibrium dialysis techniques [12].

The present study characterizes the binding parameters of [^{3}H]AZ to soluble and particulate CA enzyme sources from normal rat brain tissue homogenates, bulk isolated cells, transformed rodent and human cells, and purified bovine CA-II.

MATERIALS AND METHODS

Reagents and pharmaceuticals. All chemicals were

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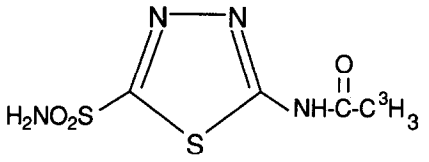

Acetazolamide [Acetamide-^{3}H]

Fig. 1. Chemical structure of [^{3}H]acetazolamide.

of the highest purity commercially available. Various chemical and pharmacological agents were obtained as follows: acetazolamide (*N*-[5-(aminosulfonyl)-1,3,4-thiadiazol-2-yl] acetamide (Diamox®) (Lederle); cytochrome *c* (bovine heart), α_1-acid glycoprotein (human), aldolase (rabbit muscle), elastase, metallothionein (rabbit liver), dichlorphenamide, hydrochlorothiazide, methazolamide, sulfanilamide, pepsin, pyrilamine, and propranolol (Sigma); albumin (human) (Calbiochem); acetylcholine, norepinephrine, dopamine, and serotonin (Regis); apomorphine and quipazine (Research Biochemicals, Inc.); haloperidol (McNeil); mianserin (Organon); and scopolamine and atropine (Aldrich). Acetazolamide was custom tritiated (New England Nuclear) to a specific activity of 4.375 Ci/mmol (Fig. 1). Purified bovine carbonic anhydrase II, ribonuclease A, soybean trypsin inhibitor and trypsin TPCK were obtained from Worthington Biochemicals. Crude carbonic anhydrase was derived from animal cortical preparations or from cultured cell types from the American Type Culture Collection.

Rat cortical CA preparation. For cortical carbonic anhydrase preparations, mature male Sprague–Dawley rats (Charles River, Wilmington, DE) weighing 250 g were decapitated and brains quickly removed and rinsed in 0.32 M sucrose at 4°. The olfactory bulbs were removed and the anterior portion of the frontal cortex (approximately 350 mg wet weight) was excised. The tissue was placed in 10 vol. of either 50 mM sodium phosphate buffer, pH 7.5, or 20 mM Tris buffer, pH 8.0, and homogenized using an Ultra-Turrax Tissumizer (Tekmar) at 50% power for 30 sec at 4°. The homogenate was then centrifuged at 100,000 *g* for 1 hr at 4° using an SW28 swinging bucket rotor in a Beckman model L8-80M ultracentrifuge. The soluble and particulate fractions were either used fresh or aliquoted and stored at −20° for later use. The stored aliquots exhibited no measurable loss of binding or enzyme activity from one freeze–thaw cycle.

Bulk isolation of rat brain cell types. Adult rat brain neurons, glia and oligodendrocytes were bulk isolated by established procedures based upon gradient centrifugation techniques [14, 15].

Cell culture. Fetal murine glial cell cultures were initiated from Swiss–Webster 15-day fetuses (Charles River, Wilmington, DE) using established procedures [16]. Transformed cell cultures were A172 human glioblastoma, rat hepatoma and C-6 glioma cells from the American Type Culture Collection. All cell cultures were grown in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 mg/mL streptomycin and 0.25 μg/mL fungizone, and incubated at 37° in an atmosphere of 5% CO_2/95% air. When flask populations reached confluence, cells were harvested by scraping with a Teflon spatula into 0.32 M sucrose at 4°. Cells were centrifuged at 1500 *g* for 30 min and the pellets were washed in 50 mM sodium phosphate buffer, pH 7.5, centrifuged at 1500 *g* and resuspended in the same buffer. The cell suspensions were then homogenized using an Ultra-Turrax Tissumizer at 50% power for 20 sec and centrifuged at 100,000 *g* for 1 hr. The resultant soluble and particulate fractions were either used immediately or aliquoted and stored at −20°.

Enzyme activity measurements. CA activity was assayed according to electrometric procedures [17]. Briefly, the time (sec) was measured in which added enzyme lowers the pH of CO_2-saturated water (4 mL) and 20 mM Tris buffer (6 mL), by 2.0 units, from pH 8.0 to 6.0. All solutions and reagents were maintained at 0–4° during the assay. "T_0" is the time recorded for the reaction containing no enzyme ."T" is the time recorded for the reaction containing 1 μg pure CA II enzyme, or an unknown amount in a sample, in Tris buffer, pH 8.0. The final volume of the assay mixture was kept constant at 10 mL. Units of enzyme activity were calculated using the formula:

$$\text{Units}/\mu\text{g} = \frac{2 \cdot (T_0 - T)}{T \cdot \mu\text{g protein used in assay}}.$$

Binding of [^{3}H]*AZ to soluble CA.* Binding of [^{3}H]AZ to soluble CA enzyme preparations was assessed using the following paradigm. Supernatant protein (200 μg) was incubated at 22° for 30 min in 50 mM Tris buffer, pH 7.0, containing [^{3}H]AZ in a final volume of 500 μL. Saturation isotherms were conducted using varied radioligand concentrations between 1 nM and 1 μM. Non-specific binding was determined in the presence of 100 μM methazolamide. Following incubation, the contents of the assay tubes were applied to Pharmacia PD-10 columns (bed vol., 9.1 mL) which were pre-equilibrated by passage of 25 mL of 50 mM sodium phosphate buffer, pH 7.5. The columns were eluted with 4 mL of the equilibration buffer. The fractions containing [^{3}H]AZ bound to carbonic anhydrase (mol wt = 30,000 daltons) were collected in scintillation vials, to which 5 mL of Beckman Ready-Solv CP scintillation fluid was added. Radioactivity was determined using a Packard Tri-Carb 4640 liquid scintillation spectrometer at 55% efficiency. Specific [^{3}H]AZ bound to soluble CA was calculated by subtracting values obtained for non-specific binding from total binding. For competition studies, CA inhibitors (at various concentrations) or general pharmacologic agents (at 100 μM) were included in the incubation mixture, at a [^{3}H]AZ concentration of 50 nM.

Binding of [^{3}H]*AZ to membrane CA.* Binding of [^{3}H]AZ to particulate fraction preparations was

assessed using vacuum filtration techniques. Briefly, incubations (500 μL total volume) were conducted as described above except that 125–500 μg of protein from particulate preparation was added. Following a 30-min incubation at 22°, binding was terminated by rapid filtration through Whatman GF/B filters (pre-soaked in 50 mM Tris buffer, pH 7.0, containing 0.1% bovine serum albumin) using a Brandel M-24R Cell Harvester, followed by four 5-mL buffer washes. The filter disks were then placed into scintillation vials to which 10 mL of Beckman-Ready-Solv HP scintillation fluid was added. The vials were mixed by vortexing and radioactivity was determined using liquid scintillation spectrometry. For [^{3}H]AZ displacement studies, the methodology as outlined above was followed using the particulate CA preparations and [^{3}H]AZ at 100 nM. Displacing agents were tested at the concentrations stated above.

Binding specificity of [^{3}H]*AZ with other proteins.* The binding of [^{3}H]AZ to other soluble proteins was investigated. Selected proteins (100 μg) were incubated at 22° for 20 min with 100 nM [^{3}H]AZ in 50 mM Tris buffer, pH 7.5. Methazolamide (100 μM) was added to parallel incubations to assess non-specific binding. Binding analysis was performed as described above.

Effect of protein concentration on [^{3}H]*AZ binding.* [^{3}H]AZ binding to soluble and particulate rat cortical preparations was performed across a varied protein (50–500 μg) concentration range. Incubations were conducted at 22° for 20 min with 50 nM [^{3}H]AZ for soluble binding and 100 nM [^{3}H]AZ for binding to particulate preparations. Methazolamide (100 μM) was added to parallel incubations to assess non-specific binding. Analysis of binding to soluble and particulate preparations was performed as described above.

pH Optima for [^{3}H]*AZ binding.* The effect of pH on [^{3}H]AZ binding to rat cortical soluble and particulate preparations was examined. Sodium pyrophosphate (50 mM) was used as the buffer for pH ranges of 6.0 to 7.0 and 8.0 to 9.0. Sodium phosphate (50 mM) was used as the buffer salt from pH 7.0 to 8.0. [^{3}H]AZ was used at 50 nM for soluble preparations while 100 nM [^{3}H]AZ was employed for particulate preparations. Incubations and binding analysis were performed as described above.

Protein determination. Protein concentrations were determined by the protein-dye binding method of Bradford [18] in kit form from BioRad Laboratories (Richmond, CA) with bovine γ-globulin as the standard.

Curve fitting. [^{3}H]AZ saturation isotherms and displacement assays were analyzed by computer-assisted programs (Lundon I and Lundon II VAX/VMS versions; Lundon Software, Inc., Cleveland, OH).

RESULTS

Separation of CA and [^{3}H]*AZ by chromatography.* [^{3}H]AZ bound to bovine CA II as well as free [^{3}H]AZ were baseline separated using PD-10 gel filtration mini-columns (Fig. 2). The elution profiles for CA and [^{3}H]AZ were highly reproducible and

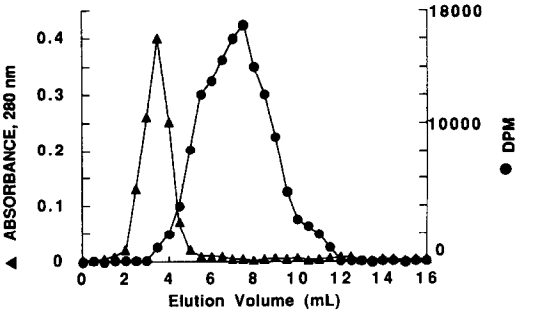

Fig. 2. Representative PD-10 column chromatographic elution profile of free [^{3}H]AZ (●) and [^{3}H]AZ bound to carbonic anhydrase (▲). Absorbance was monitored at 280 nm and radioactivity was detected in eluted fractions by liquid scintillation spectrometry.

enabled accurate estimation of the amount of [^{3}H]AZ bound to the soluble enzyme. The enzyme eluted between 3.5 and 4.5 mL while the free [^{3}H]AZ eluted in a wider band from 4.0 to 12.0 mL peaking at 7.5 mL. CA activity in eluted column fractions was also assessed by electrometric methods to determine percent recovery of the chromatographed enzyme. Recovery of CA activity from PD-10 gel permeation columns was between 95 and 100% (data not shown). This method was employed to separate bound from free [^{3}H]AZ in all soluble enzyme or protein preparations.

[^{3}H]*AZ binding to cell/tissue CA.* [^{3}H]AZ bound to purified bovine CA-II in a specific and saturable manner with a B_{max} of 350 pmol/mg protein. Saturable specific binding of [^{3}H]AZ to soluble and particulate CA preparations from rat brain homogenates was also observed. The maximal number of binding sites (B_{max}) was 0.38 ± 0.13 pmol/mg protein for the soluble preparation, with an affinity (K_d) of 34.7 ± 17.5 nM. The particulate CA preparations showed a B_{max} of 2.05 ± 0.28 pmol/mg protein and a K_d of 107.1 ± 24.2 nM. Specific binding accounted for 58 and 45% of total binding for the soluble and particulate CA preparations respectively. A representative Scatchard plot of [^{3}H]AZ binding to soluble and particulate rat cortical preparations is shown in Fig. 3.

The binding of [^{3}H]AZ to particulate and soluble forms of bulk cell isolates and cultured cells is shown in Table 1. When bulk isolates of neurons or oligodendrocytes were prepared, a striking difference was observed. In rat neurons, 95% of specific [^{3}H]AZ binding resided in the soluble fraction while the oligodendrocytes had 60% of the [^{3}H]AZ binding in the soluble fraction. [^{3}H]AZ binding to the cultured murine glial preparations was also performed. Soluble and particulate preparations of these cells specifically bound [^{3}H]AZ. At an AZ ligand concentration of 50 nM the soluble preparation bound 0.5 ± 0.03 pmol [^{3}H]AZ/mg protein while the particulate preparation bound 0.2 ± 0.04 pmol [^{3}H]AZ/mg protein at a ligand concentration of 100 nM.

[^{3}H]AZ did not specifically bind to human

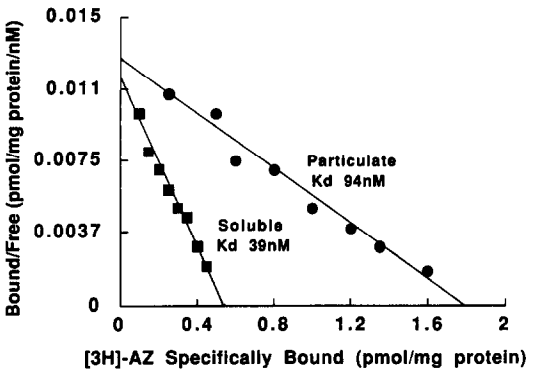

Fig. 3. Representative Scatchard plot of [^{3}H]AZ binding to soluble and particulate rat cortical preparations. Saturation data were evaluated with computer-assisted curve fitting (Lundon I, Lundon Software, Inc.). Each data point is the mean of triplicate determinations.

Table 1. Specific binding of [^{3}H]AZ to cell or tissue carbonic anhydrase

Cell/Tissue	Specific bound [^{3}H]AZ (pmol/mg protein) Particulate	Soluble
Bulk isolates		
Rat neurons	0.09 ± 0.02	1.82 ± 0.11
Rat oligodendrocytes	2.53 ± 0.08	3.61 ± 0.27
Cultured cells		
Fetal murine neurons	0.20 ± 0.04	0.50 ± 0.03
Rat C-6 glioma	ND	ND
Rat hepatoma	ND	ND
Human glioblastoma	ND	ND

Binding of [^{3}H]AZ to soluble and membrane preparations from bulk isolated cells or harvested cultured cells was measured. [^{3}H]AZ was used at concentrations of 50 nM for soluble fractions and 100 nM for particulate fractions. Methazolamide (100 μM) was used to assess non-specific binding. Data are means ± SEM from three separate determinations. ND = no specific binding detectable.

Table 2. Binding of [^{3}H]acetazolamide to selected proteins

Protein	[^{3}H]AZ bound (pmol/mg protein)
Carbonic anhydrase II (bovine)	303.8 ± 22
Lysozyme	0.8 ± 0.06
Aldolase	0.3 ± 0.02
Ribonuclease A	0.2 ± 0.03
Cytochrome *c*	ND
Pepsin	ND
Metallothionein	ND
Soybean trypsin inhibitor	ND
Albumin (human)	ND
Elastase	ND
Trypsin TPCK	ND
α_1-Acid glycoprotein (human)	ND

Specific binding of [^{3}H]AZ was conducted in the presence of 100 μg specified purified protein and 100 nM [^{3}H]AZ. Methazolamide (100 μM) was used to assess non-specific binding. G-25 columns were used to separate bound from free [^{3}H]AZ. Values are means ± SEM for three separate experiments. ND = binding of [^{3}H]AZ to specific protein was not detectable.

Table 3. Inhibition of [^{3}H]acetazolamide binding to rat brain particulate and soluble preparations by established CA inhibitors

Compound	IC_{50} (μM) Particulate	Soluble
Dichlorphenamide	0.080 ± 0.027	0.039 ± 0.008
Acetazolamide	0.199 ± 0.007	0.049 ± 0.012
Benzolamide	0.220 ± 0.013	0.049 ± 0.007
Methazolamide	0.666 ± 0.056	0.122 ± 0.025
Hydrochlorothiazide	35.14 ± 7.0	30.1 ± 20.3
Sulfanilamide	22.59 ± 7.9	174.9 ± 11.9

The IC_{50} values were generated by a computer-assisted Lundon-II radioligand competition analysis program. Data are means ± SEM from three separate experiments.

glioblastoma, rat hepatoma or rat C-6 glioma cell preparations in the conditions shown in Table 1 or across a wide range of protein or ligand concentrations (data not shown).

Binding specificity with other proteins. The binding of [^{3}H]AZ to eleven selected soluble proteins and enzymes was examined. Low level binding to lysozyme, aldolase and ribonuclease A was observed, while no detectable binding was seen with the remaining proteins tested (Table 2).

Competitive binding studies. The binding of [^{3}H]AZ to rat cortical soluble and particulate preparations was examined in the presence of known CA inhibitors (Table 3). The IC_{50} values were derived for the inhibitors used and a close rank order of [^{3}H]AZ binding inhibition to soluble and particulate preparations was found to be: dichlorphenamide > acetazolamide ≥ benzolamide > methazolamide > hydrochlorothiazide ≥ sulfanilamide.

Binding of [^{3}H]AZ to rat brain 100,000 *g* supernatant and pellet CA preparations was examined in the presence of histaminergic, dopaminergic, serotonergic, adrenergic or cholinergic characterizing agents. Apomorphine, atropine, acetylcholine, dopamine, haloperidol, mianserin, norepinephrine, quipazine, pyrilamine, propranolol, scopolamine or serotonin when tested at 100 μM produced less than 5% inhibition of [^{3}H]AZ binding (data not shown).

Effect of protein concentration on [^{3}H]*AZ binding.* The effect of increasing protein concentration on [^{3}H]AZ specific binding is shown in Fig. 4. Both rat cortical soluble and particulate preparations exhibited linearity of binding with respect to increasing assay protein concentration. The particulate preparation specifically bound 3–4 times more [^{3}H]AZ per milligram of protein compared to the soluble preparation.

[^{3}H]*AZ Binding pH optima.* The pH dependency

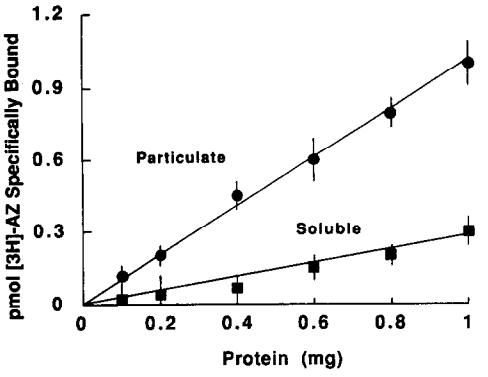

Fig. 4. Effect of protein concentration on the binding of [3H]AZ to particulate or soluble rat cortical preparations. Data points are means ± SEM of three separate determinations.

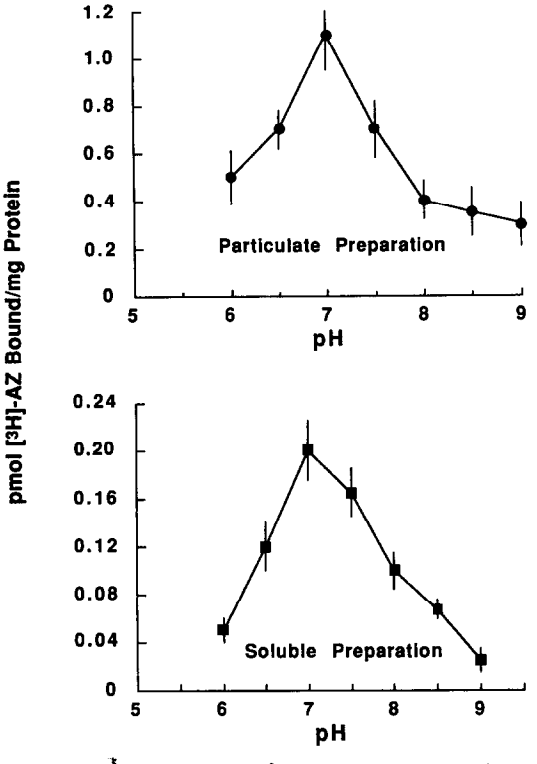

Fig. 5. The pH optima of [3H]AZ binding to particulate or soluble rat cortical preparations. Data points are means ± SEM of three separate determinations.

for the specific binding of [3H]AZ to rat cortical soluble and particulate preparations is shown in Fig. 5. The maximum for specific binding of [3H]AZ was between pH 6.5 and 7.5 for both preparations.

DISCUSSION

Carbonic anhydrase is an important enzyme for the maintenance of cellular microenvironmental pH by participating in fluid and ion movement. The present study characterizes the binding of [3H]AZ to both soluble and membrane bound forms of carbonic anhydrase. Binding of [3H]AZ to the particulate form of CA was accomplished through conventional vacuum filtration methods, while a gel permeation chromatography method was developed to separate free ligand from enzyme-bound ligand for soluble CA. Radioligand binding to CA is a sensitive assay for the detection of the enzyme and is technically simpler than immunological or electrometric techniques.

[3H]AZ binding to CA was specific, reversible and saturable, demonstrating nanomolar affinity for the enzyme. The labeling of CA by [3H]AZ appeared specific for the enzyme since its binding was not displaced by other classes of pharmacologic agents. The pharmacological properties of CA derived from different sources appeared homogeneous. The rank order of potency for CA inhibitors was similar for rat cortical particulate and soluble enzyme forms.

In rat cortex, binding of [3H]AZ to the soluble preparation was of higher affinity and lower density compared to the particulate preparation. Trachtenberg and Sapirstein [6] found that membrane bound CA represents 63% of the total CA population in cortical areas of rat brain measured by pH change assay. In the present study, the crude rat cortical particulate preparation represented 79% of the total CA as measured by the binding of [3H]AZ. Therefore, a reasonable correlation exists in identifying CA activity in rat brain fractions either by pH change assay or binding of [3H]AZ. When bulk isolates of rat neurons or oligodendrocytes were prepared, a striking difference was observed. In neurons, 95% of the total CA was soluble whereas in oligodendrocytes, 60% of the total was located in the soluble fraction. This supports the hypothesis that the different subcellular forms of CA may be responsible for different cellular functions. Coleman [12] performed [3H]AZ binding to purified human carbonic anhydrase B which resulted in a total binding of 33 nmol/mg protein with an optimal pH for binding of 7.0. The present study agrees with the pH optimum finding; however, [3H]AZ binding was 16,000 times less in a crude rat cortical particulate preparation and 100 times less in purified bovine CA-II. The binding discrepancy could be due to the purity and source of the enzyme as well as corrections made for specific binding which was not performed in the former study [12].

CA activity assays were performed on various preparations to assess the activity of enzyme present in each sample. Rat cortical and cultured murine glial cell preparations consistently exhibited measurable CA activity. The lack of binding of [3H]AZ to human glioblastoma, rat C-6 glioma and rat hepatoma cells is rather unusual. However, a similar observation was made using rat C-6 glioma and mouse neuroblastoma cell lines employing a pH change assay [9]. The possibility exists that the CA enzyme molecule expressed in transformed cells is altered from that in normal cells.

[3H]AZ is not selective or preferential for a specific CA isozyme. Thus, the binding of [3H]AZ is useful as a probe for assessing all forms of CA in various

normal and pathological tissues. AZ is a very polar sulfonamide, unlike the moderately polar methazolamide or the very lipophilic ethoxzolamide. The more hydrophobic sulfonamides due to their increased lipophilicity have a greater potential to cross the blood–brain barrier. One could exploit the finding that in transformed cells [^{3}H]AZ does not bind to CA. Thus, a gamma emitting form of lipophilic ethoxzolamide may prove useful in reverse positron emission tomography imaging of CA in brain tumors.

REFERENCES

1. Maren TH, Carbonic anhydrase: Chemistry, physiology and inhibition. *Physiol Rev* **47**: 595–781, 1967.
2. Lindskog S, Carbonic anhydrase. In: *Zinc Enzymes* (Ed. Spiro TG), pp. 77–121. John Wiley, New York, 1983.
3. Sly WS, Hewett-Emmett D, Whyte MP, Yu YSL and Tashian RE, Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. *Proc Natl Acad Sci USA* **80**: 2752–2756, 1983.
4. Tashian RE, Hewett-Emmett D, Dodgson SJ, Forster RE and Sly WS, The value of inherited deficiencies of human carbonic anhydrase isozymes in understanding their cellular roles. In: *Biology and Chemistry of the Carbonic Anhydrases* (Eds. Tashian RE and Hewett-Emmett D), pp. 262–275. New York Academy of Sciences, New York, 1984.
5. Holmes RS, Purification, molecular properties and ontogeny of carbonic anhydrase isozymes: Evidence for A, B and C isozymes in avian and mammalian tissues. *Eur J Biochem* **78**: 511–520, 1977.
6. Trachtenberg MC and Sapirstein VS, Carbonic anhydrase distributions in central and peripheral nervous system of the rat. *Neurochem Res* **5**: 573–581, 1980.
7. Hillman H, *Cellular Structure of the Mammalian Nervous System*, p. 45. MTP Press, Lancaster, 1985.
8. Weller RO, The immunopathology of brain tumours. In: *Tumours of the Brain* (Ed. Bleehen NM), pp. 19–29. Springer, New York, 1986.
9. Kimelberg HK, Narumi S and Bourke RS, Enzymatic and morphological properties of primary rat brain astrocyte cultures, and enzyme development *in vivo*. *Brain Res* **153**: 55–57, 1978.
10. Snyder DS, Zimmerman TR Jr, Farooq M, Norton WT and Cammer W, Carbonic anhydrase, 5′-nucleotidase, and 2′,3′-cyclic nucleotide-3′-phosphodiesterase activities in oligodendrocytes, astrocytes, and neurons isolated from the brains of developing rats. *J Neurochem* **40**: 120–127, 1983.
11. Mann T and Keilin D, Sulphanilamide as a specific inhibitor of carbonic anhydrase. *Nature* **146**: 164–165, 1940.
12. Coleman JE, Mechanism of action of carbonic anhydrase. *J Biol Chem* **242**: 5212–5219, 1967.
13. Maren TH, Carbonic anhydrase: General perspectives and advances in glaucoma research. *Drug Dev Res* **10**: 255–276, 1987.
14. Norton WT and Poduslo SE, Neuronal soma and whole neuroglia of rat brain: A new isolation technique. *Science* **167**: 1144–1145, 1970.
15. Snyder DS, Raine CS, Farooq M and Norton WT, The bulk isolation of oligodendroglia from whole rat forebrain: A new procedure using physiologic media. *J Neurochem* **34**: 1614–1621, 1980.
16. Hertz L, Juurlink BHJ, Fosmark H and Schousboe A, Methodological appendix: Astrocytes in primary cultures. In: *Neuroscience Approached Through Cell Culture* (Ed. Pfeiffer SE), Vol. 1, pp. 175–186. CRC Press, Boca Raton, FL, 1982.
17. Wilbur KM and Anderson NG, Electrometric and colorimetric determination of carbonic anhydrase. *J Biol Chem* **176**: 147–154, 1948.
18. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.