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Review

# Carbonic anhydrases and their industrial applications

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## ABSTRACT

Carbonic anhydrases (CAs) include three structurally distinct families (α, β, and γ) of mostly zinc-metalloenzymes that catalyze the reversible hydration/dehydration of carbon dioxide/bicarbonate. Historically, CAs have been extensively studied because of their broad physiological importance in all kingdoms of life and clinical relevance as drug targets. Recently, there has been an increasing industrial interest in using CAs as biocatalyst for carbon sequestration out of flue gas from coal-fired power plants and in exploiting CAs in algae as a way to capture $CO_2$ and convert it into biofuels or other valuable products. In addition, there is the continuing development of CAs for medical devices such as artificial lungs and biosensors. This review highlights the current state of these industrial processes and discusses their biotechnological applications.

**KEYWORDS:** carbonic anhdyrase, carbon sequestration, artificial lungs, biosensor, biofuel

## INTRODUCTION

Carbonic anhydrases (CAs, EC 4.2.1.1) are mostly-zinc containing metalloenzymes that catalyze the reversible hydration of carbon dioxide ($CO_2$) to bicarbonate and a proton [1]. Carbonic anhydrases are ubiquitously expressed throughout nature and are classified according to at least three structurally distinct families (α, β, and γ) [2, 3]. There is no sequence homology between the classes, reflecting their independent evolution. The α class includes mammalian CAs, the β class is found in plants and some bacteria, while the γ class is an archaeal CA [4-7]. This review will focus primarily on the α-CAs and their role in industrial applications.

In the α CAs, human CAII (HCAII), the catalytic zinc ion is located at the bottom of a conical cavity 15 Å deep and is coordinated by the nitrogen atoms of three conserved histidine residues, His94, His96, His119, and a highly polarized water molecule ($pK_a$ 5 to 8 depending on the specific CA (Fig. 1A). The active site, is divided into two sides, a hydrophobic region (Val121, Val143, Leu198, Val207 and Trp209) and a hydrophilic region (Tyr7, Asn62, His64, Asn67, Thr199 and Thr200) (Fig. 1B) [8, 9].

CA catalyzes the reversible hydration of $CO_2$ to form bicarbonate and a proton via a two-step ping-pong mechanism [10]:

$$E{:}Zn\text{-}OH^- + CO_2 \underset{}{\overset{H_2O}{\rightleftarrows}} E{:}Zn\text{-}H_2O + HCO_3^- \quad \text{(Reaction 1)}$$

$$E{:}Zn\text{-}H_2O + B \rightleftarrows E{:}ZnOH^- + BH^+ \quad \text{(Reaction 2)}$$

In the first reaction, in the hydration direction, the zinc-bound hydroxide converts $CO_2$ to bicarbonate via a nucleophilic attack. The bicarbonate ion is then displaced from the zinc by binding of a water molecule to the zinc (Reaction 1). The zinc-bound water then transfers a proton to the bulk solvent via a series of intramolecular and intermolecular proton-transfer steps [11, 12] regenerating the zinc-bound hydroxide in the process (Reaction 2). Note that B represents a proton acceptor and has

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been proposed to be His64 in HCAII [8, 13]. In several X-ray crystallographic structures of HCAII, His64 has been observed in two orientations, one pointed into the active site toward the zinc ion and the other pointed into the bulk solvent (Fig. 1B) [14]. CA is an extremely efficient catalyst with the $CO_2$ catalytic turnovers for several human CA being among the highest known ($k_{cat} \approx 10^6\ s^{-1}$), and the second-order rate limit approaching the diffusional rate control ($k_{cat}/K_M \approx 10^8\ M^{-1}\ s^{-1}$) [10].

There are fifteen human α-CAs, all of which differ in characteristics such as kinetics, susceptibility to different inhibitors, cellular localization and tissue distribution [9, 15, 16]. Of the fifteen isozymes, twelve isoforms (CAI – VA, VB – VII, IX, XII-XIV) exhibit enzymatic activity whereas three are acatalytic due to one or more substitutions at the metal-coordinating histidine residues, known as CA-related proteins (CA-RPs VIII, X and XI) [16, 17]. Since the CAs are required in many physiological processes, the inhibition of enzymatic activity of the CAs has been linked with various disease treatments including; glaucoma, epilepsy, and osteoporosis [1, 15, 16].

This review will cover several industrial settings where CA is being utilized including carbon sequestration, biosensors, artificial lung systems and in biofuel production. There are several characteristics that make CA an ideal candidate for these industrial applications including the relative stability of the protein [18], the ability to be cloned and expressed in large quantities in *E. coli* [19], easy purification by either affinity or conventional chromatography [20, 21] and the abundance of high-resolution structures enables the rational design of CA variants for industrial applications based on structure-function relationships [22].

## Carbon Sequestration

The atmospheric concentrations of greenhouse gases such as $CO_2$, methane, chloroflurocarbons and nitrous oxides are increasing due to human induced (anthropogenic) activities [23]. $CO_2$ is the most abundant greenhouse gas, being produced primarily by the burning of fossil fuels such as coal, oil and natural gas. The atmospheric concentration of $CO_2$ has increased since preindustrial era from ~280ppm to ~390ppm in 2008 [24]. These levels are significantly higher than at any time during the past 800,000 years according to Antarctic ice core extraction measurements [25-28]. Less direct geological evidence based

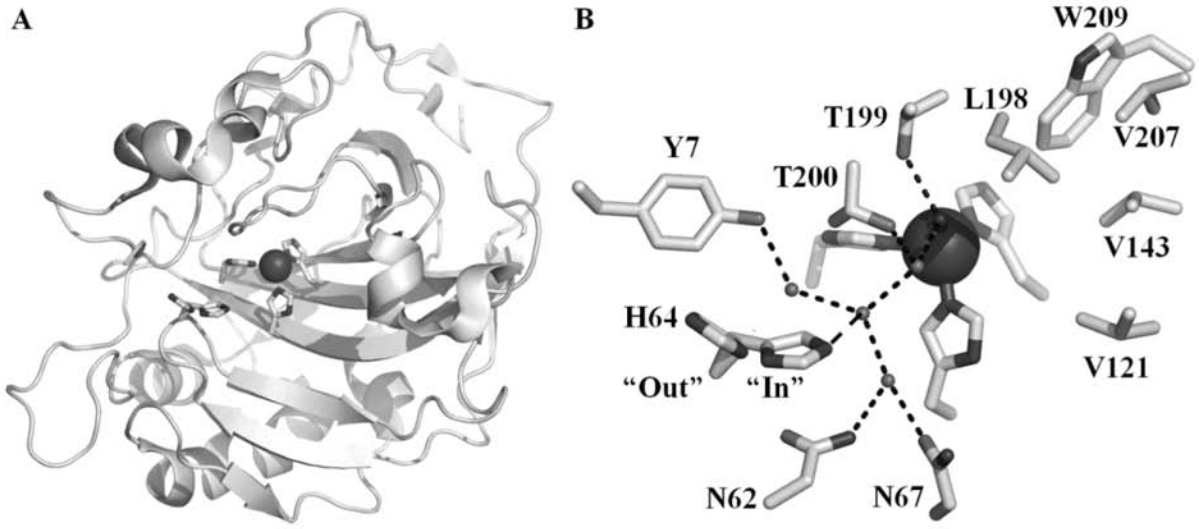


**Fig. 1.** Structure of HCAII. (A) Overall topology of HCAII shown as a cartoon model with the $Zn^{2+}$ ion shown as a sphere. The three coordinating histidines and H64 are shown as sticks. (B) Active site of HCAII. The water network where the proton transfer occurs is shown as small spheres with hydrogen bond networks shown as dashed line. The hydrophilic and hydrophobic residues are as labeled. Figure is of the high resolution structure of HCAII (PDB: 3KS3) [100].

on boron-isotope ratios of ancient planktonic foraminifer shells indicates that higher atmospheric concentrations of $CO_2$ than today were last seen about 20 million years ago [29]. The effect of rising $CO_2$ levels in the atmosphere has been associated with an increase in the average global temperature since 1896 [30]. From 1906-2005, the average surface temperature of the Earth rose by 0.7 ± 0.2°C, compared to the temperature remaining relatively stable over the past two thousand years prior to 1850 [31]. The rise in surface temperatures has been associated with a number of observations such as rise in sea levels, melting of glacier and polar ice caps and increase in ocean acidity [32-34]. Fossil fuel burning has produced about three-quarters of the increase in $CO_2$ from human activity over the past 20 years, with the rest of this increase being associated primarily with deforestation [35]. As such, the Kyoto Protocol signed in 1997 requires that industrialized nations to reduce their $CO_2$ emissions to 95% of their 1990 levels by 2012 [36]. Indirect carbon sequestration techniques include dissolution in an aqueous phase, hydration by water, ionization and carbonate formation which add up to about $41-72 per ton of $CO_2$ sequestered (44-46). To put this in perspective, about 300 million metric tons of $CO_2$ is emitted per year in the U.S., totaling ~$12.3-21.6 billion per year.

In carbon sequestration methods, the hydration of $CO_2$ is the slowest step leading to an interest in using CA as a carbon sequestration catalyst (Fig. 2) [37]. However, use of CA in solution has many drawbacks such as low stability that limits re-usability, recovery and cost in an industrial setting (temperatures in excess of 70°C and/or pH less than 6.0) [38]. Hence, several groups have immobilized HCAII on a variety of inorganic [39-44] and biopolymer surfaces [45, 46] including enriched microorganisms [47, 48] as a way to overcome the stability and recovery problems. CA has also been immobilized into a variety of matrices including acrylamide, alginate and chitosan-alginate [49, 50]. Alternatively, other groups have made HCAII more thermal stable via site-directed mutagenesis of surface hydrophobic to hydrophilic residues [14] and more chemical tolerant from introduction of a disulfide linkage [51]. Cyanobacteria has served as a model for evaluating the role of CA in $CO_2$ concentrating [52]. This environmental $CO_2$ can be used in energy production via CA hydration for a cost-efficient, large-scale carbon capture and allows for the use of energy-efficient chemicals such as amines, carbonates and amino acids [53].

**Biosensors**

Quantification of trace analytes in complex media containing chemically similar molecules is lacking in many traditional chemical systems. As such, the development of sensors based on biological molecules, termed biosensors, can achieve such specificity and sensitivity [54]. The high affinity of CA for zinc (4 pM) [55] has been used to quantify trace amounts of zinc in sea and waste water [56]. Traditional wet chemical techniques to detect zinc in sea water are slow, expensive, laborious and often results in contaminated samples [54]. Optimally, the detection of zinc would be performed in deep water with transmission of the signal being relayed back to the ocean surface via fluorescence resonance energy transfer upon binding of a strong inhibitor, dansylamide (DNSA), concurrent with zinc binding in the active site of CA [57]. However, the tight-binding of zinc to CA presents the technical problem of overcoming the slow dissociation constant of zinc from the enzyme ($t_{1/2} \approx 90$ days [55]). Mutation of the indirect zinc-binding residue E117, which coordinates H119 for optimal binding geometry, to a Gln decreased the binding affinity of zinc to CA to 4 nM and decreased the zinc-dissociation half-time to three seconds. This has been proposed to be a direct result of polarity reversal between the hydrogen bond of residues 117-119 that stabilizes the histidinate anion [58]. The first studies aimed at improving the signal detection of zinc-binding to CA involved randomly labeling surface lysines with a fluorescein derivative and the inhibitor azosulfamide as a fluorescence acceptor [59]. This random labeling resulted in suboptimal signal transduction as the fluorophores were at varying distances from the fluorescence acceptor. This problem was adverted via site-directed mutagenesis of cysteine residues, which can be selectively labeled with a thiol-reactive fluorphore [59, 60], at various positions in CA, with the best detection

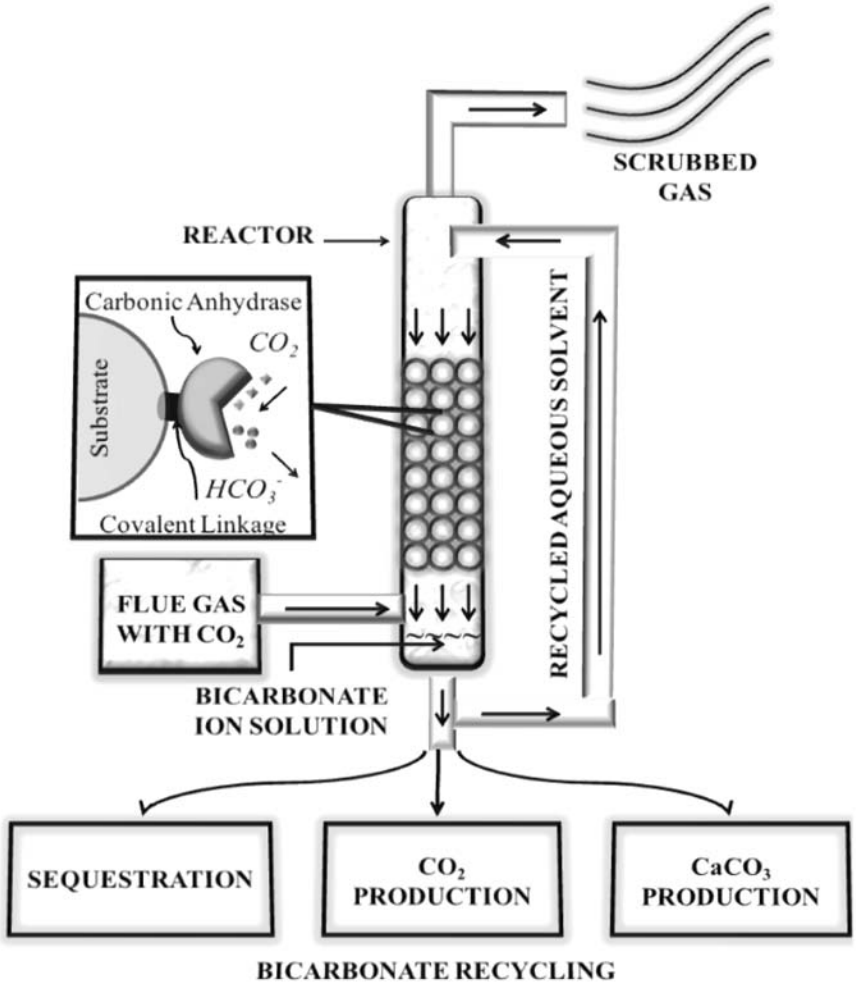


**Fig. 2.** Carbon sequestration techniques utilizing CA. Figure modified from Lee, S. W. *et al.* [101].

of zinc concentrations (0.1 - 1.0 μM) provided by the H36C variant [59].

Efforts to extend the CA-based biosensor to other trace metal ions other than zinc are currently in development. Metals that bind to CA consist primarily of transition metals in the +2 oxidation state including: $Cd^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Hg^{2+}$, $Fe^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Pb^{2+}$ and $In^{3+}$ [54, 55, 60, 61]. However, since $Cu^{2+}$ and $Hg^{2+}$ bind to CA more tightly than $Zn^{2+}$ [55], variants with a lowered binding affinity for these metals must be developed as discussed above. Alternately, other metals listed above bind with a lowered affinity to CA than $Zn^{2+}$, offering attractive candidates for the fluorescence resonance energy transferred as described above. Sulfonamide inhibitors, however, do not bind tightly to CA with metals other than $Zn^{2+}$ or $Co^{2+}$ bound in the active site [62], promoting the need of a new approach to measure binding of these other metals to CA. This limitation can been superseded with the fact that several of these divalent ions ($Cu^{2+}$, $Co^{2+}$ and $Ni^{2+}$ exhibit weak d-d absorbance bands in the visible regions which can be directly measured by the method of fluorescence energy transfer lifetimes [60]. This method can be extended to other metals that do not exhibit d-d absorbance bands, such as $Hg^{2+}$ and $Cd^{2+}$, since the binding of these metals to apo-CA causes a quenching of the fluorescence of an active site fluorophore, possibly through proximity effects such as spin-orbit coupling [63]. Since biologically prevalent divalent metals such as $Mg^{2+}$ and $Ca^{2+}$ do not bind to CA and interfere with the assay, biosensors employed in biomedical applications are especially useful [64-69].

**Artificial lungs**

Acute respiratory failure is a major health problem within the United States [70]. Treatment involves the use of mechanical ventilators [71], but these devices can have significant side effects, including damage to the lungs due to over-distending or over-pressurizing lung tissue [72]. Artificial lungs are respiratory assist devices that can function independently of the lungs, allowing this technology to potentially replace mechanical ventilators in treatment of respiratory failure. Progress, however, must still be made to improve the efficacy of artificial lungs before these devices can be implemented as an effective alternative treatment [73].

One of the major challenges currently facing the development of effective artificial lungs is the relatively inefficient transfer of $CO_2$ across the polymetric hollow fiber membranes (HFM) utilized by these devices as a blood-gas interface. Therefore, a very large surface area (1-2$m^2$) is presently required for sufficient gas exchange across the membrane [74-77]. This in itself causes various issues with hemocompatibility and biocompatibility [78, 79]. One potential way to solve these problems is by immobilizing CA onto the surface of the HFM to catalyze the removal rate of $CO_2$ (Fig. 3). The HFM is first activated via cyanogen bromide in acetonitrile. Covalent linkages with CA are formed within the HFM by immersing the HFM in a phosphate buffer that contains the enzyme. $CO_2$ exchange rates can then be measured in model artificial lungs. The "bioactive" HFM with covalently immobilized CA was shown to have as high as a 75% increased rate of $CO_2$ removal when compared to an untreated HFM. These results suggest that immobilizing $CO_2$ onto the HFM would allow for smaller yet fully functional HFMs-and thus smaller artificial lungs-to be created due to this increased efficiency. A smaller artificial lung could potentially eliminate the existing hemocompatibility and biocompatibility issues [80].

A separate technology that uses impeller devices to increase blood mixing has also been proven to be effective at increasing the rate of $CO_2$ transfer within the artificial lungs. When this technique is combined with immobilization of CA onto the HFM, however, the mixing shear forces lead to the denaturation of CA, preventing the enzyme from functioning efficiently and thus negating the purpose of immobilization. If a more stable variant of CA can be created to withstand these harsh conditions, the potential exists for these techniques to be combined, leading to an even smaller artificial lung [46].

**Biofuel production**

Many countries, including the U.S., have started to devise methods to replace fossil fuel energy sources with an environmental friendly alternative source [81]. An estimated 60 billion gallons of diesel and 120 billion gallons of gasoline are used in the U.S. per year [82]. Thus a total of ~141 billion gallons of biodiesel (gasoline is about 65% as energy efficient as diesel) is needed for total transportation fuel in the U.S. each year. However, if all of the arable land in the U.S. were used to grow soybean for oil production, it would produce only 21 billion gallons of biodiesel per year (based on 48 gallon/acre/yr), accumulating only ~15% of the total U.S. biodiesel need [81]. Current production of biofuels displaces croplands used for food and feed production, and has been blamed for elevated food prices [83, 84].

Algae are considered a promising alternate and renewable source for biofuels as they have higher oil production and carbon fixation rates compared to that of terrestrial plants [85, 86], can potentially produce 1,000-4,000 gallon/acre/yr and they do not compete with traditional agriculture because they can be cultivated in ponds or in closed photobioreactors located on non-arable land [81]. Additionally, biodiesel production by algae is preferential over conventional diesel as it does not contribute to the $CO_2$ or sulfur levels in the atmosphere, emits less gaseous pollutants and is non-toxic [87]. Thus algae are suitable systems for transportation among sensitive environments such as mining enclosures and marine ecosystems [88]. All of these properties make microalgae an attractive candidate for biofuel generation as well as $CO_2$ sequestration in an environmental friendly and sustainable manner. The continuous cultivation of algae would also yield additional beneficial bi-products such as proteins, fatty acids, vitamin A, minerals, pigments, dietary supplements and other bio-compounds [89].

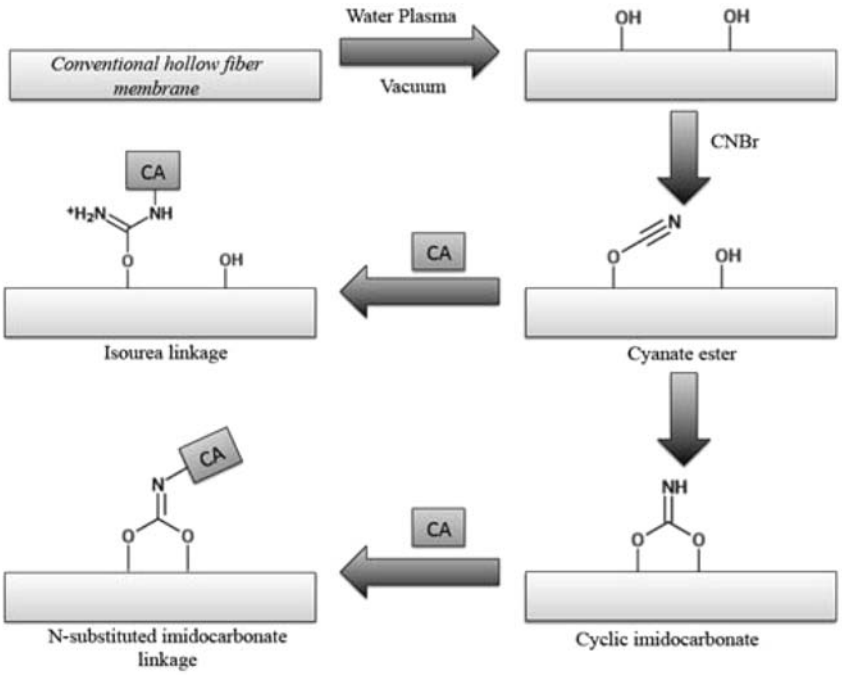


**Fig. 3.** Covalent immobilization of CA to the surface of HFMs. Initial deposition of hydroxyl groups occur via plasma deposition which are subsequently activated with cyanogens bromide to convert surface hydroxyls to cyanate esters and cyclic imidocarbonates. CA is covalently bound by forming isourea or N-substituted imidcarbonate linkages. Figure modified from Kaar, J. L. *et al.* [80].

The current challenge in algae biofuel production remains the selection of a suitable strain for optimal production of lipids as well as inefficient $CO_2$ utilization/sequestration ability [90-94]. In aquatic systems, algae use CA located on the surface of the cell to promote the conversion of dissolved $CO_2$ to bicarbonate, with the latter being transported into the cell and rehydrated to $CO_2$ by the cytosolic CA close to the Rubisco catalytic sites, where is subsequently converted into phosphoglyceric acid, enters the Calvin cycle and ultimately resulting in sugar [81].

Neutron and X-ray crystallography studies are currently being employed to elucidate the catalytic mechanism of CA that will allow rational design to improve catalytic performance and binding specificity of CA for increased carbon fixation during algal biofuels production [14, 95-97]. Structural knowledge of the proton shuttle mechanism in CA (the rate-limiting step; Reaction 2) has promoted the enhancement of this mechanism by 3-4 fold via the active site mutation Y7F [14, 98] whereas the X-ray crystallographic structure of $CO_2$ bound in the active site [8] has provided an eloquent understanding of the means by which $CO_2$ is held and orientated for optimal catalysis [99].

## CONCLUSIONS

The favorable properties of HCAII (high kinetic parameters, easy expression, high solubility, intermediate heat resistance) have made it an attractive candidate for numerous industrial applications [95]. There is an increasing industrial interest in using HCAII as a biocatalyst for carbon sequestration of flue-gas from coal-fired power plants [49]. Additionally, there are established protocols utilizing CA found in algae to capture carbon dioxide and convert it into biofuels and other valuable products [87, 92]. There is also interest in using apo-CAs as a biosensor for zinc and other transition metals in sea water or human serum [54]. For industrial applications, small improvements in stability without detriment to yield, activity or solubility, can accelerate the development of HCAII as a better biocatalyst. Use of the free enzyme in solution can also have disadvantages,

as the low stability can limit recycling and cost-efficiency in an industrial setting [38]. As such, there are numerous studies underway to enhance the stability of HCAII while also retaining its characteristic high catalytic efficiency. One such study deals engineering of a disulfide linkage in HCAII [51] whereas others include immobilization of HCAII on a variety of surfaces [38, 39] and directed-evolution of the enzyme involving mutagenesis of surface hydrophobic residues into hydrophilic moieties [95]. Further research is needed to maximize the stability of HCAII in a wide array of environments without the loss of catalytic efficiency for industrial use.

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