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Sodium Bicarbonate Enhances Membrane-bound and Soluble Human Semicarbazide-sensitive Amine Oxidase Activity *In Vitro*

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Semicarbazide-sensitive amine oxidase (SSAO) is a multifunctional enzyme with different biological roles that depend on the tissue where it is expressed. Because SSAO activity is altered in several pathological conditions, we were interested in studying the possible regulation of the human enzyme activity. It has been previously reported that SSAO activity is increased in the presence of Dulbecco's modified Eagle medium (DMEM) *in vitro*. The aim of the present work was to investigate the effects of the different constituents of DMEM on human SSAO activity. We found that sodium bicarbonate was the only component able to mimic the enhancement of both human aorta and plasma SSAO activity *in vitro*, suggesting a possible physiological role of bicarbonate as an intrinsic modulator of the human enzyme. Failure to take this activating effect into account could also result in inaccuracies in the reported tissue activities of this enzyme.

Key words: activity enhancement, dulbecco's modified eagle medium, semicarbazide-sensitive amine oxidase, sodium bicarbonate, vascular adhesion protein-1.

Abbreviations: DMEM, Dulbecco's modified Eagle medium; MAO, monoamine oxidase; SSAO, semicarbazide-sensitive amine oxidase.

The term 'semicarbazide-sensitive amine oxidase' (SSAO) is generally used to describe those enzymes classified as E.C.1.4.3.6 [amine: oxygen oxidoreductase (deaminating) (copper-containing)]. Semicarbazide inhibition allows SSAOs to be distinguished from monoamine oxidases (MAOs) [amine: oxygen oxidoreductase (deaminating) (flavin-containing); E.C.1.4.3.4 (MAO), which are sensitive to acetylenic inhibitors, such as clorgyline and L-deprenyl, but are less affected by semicarbazide. The substrate specificities of MAO and SSAO overlap to some extent but, whereas MAO catalyzes the oxidative deamination of primary, secondary and some tertiary amines, SSAO activity appears to be restricted to primary amines. Methylamine, which arises from the metabolism of adrenaline, lecithin, sarcosine and creatinine, is metabolized by SSAO from many sources. It has been proposed that methylamine and aminoacetone, which are not MAO substrates, are important physiological SSAO substrates (*1*, *2*).

SSAO is associated with cell membranes in mammalian tissues and is also present in blood plasma (*3*). Membrane-bound SSAO shows high activity in endothelial and smooth muscle cells of blood vessels (*4*, *5*). The soluble SSAO in blood plasma is believed to be derived from the membrane-bound enzyme, and it has recently been reported that soluble SSAO is shed from the adipocyte membrane by a metalloprotease activity (*6*). The physiological roles of SSAO are still far from clear, and it has been described as an enzyme with multifunctional behaviour that depends on the tissue where it is expressed (*7*). SSAO is also known as vascular adhesion protein-1 (VAP-1), which is involved in lymphocytes trafficking, and its expression in endothelial cells is induced during an inflammatory response (*8*).

SSAO activity has been shown to be altered in several pathological conditions. Plasma SSAO is increased in patients suffering from diabetes types I and II (*9*), in patients afflicted by congestive heart failure (*10*), in non-diabetic morbid obesity (*11*), in inflammatory liver diseases (*12*) and in severe Alzheimer's disease (*13*). It has also been implicated in atherosclerosis (*14*) and in the development of diabetic complications (*15*). Furthermore, it has been shown that plasma SSAO can induce apoptosis in smooth muscle cells through its catalytic action on methylamine as substrate, which might contribute to vascular cell damage (*16*) and in the development of diabetic retinopathy (*15*).

Although some factors in human plasma have been reported to modulate platelet MAO activity (*17*, *18*), little is known about the possible modulation of SSAO under physiological conditions.

We have previously described the activation of membrane-bound SSAO from human lung by a low molecular weight component present in human plasma (*19*). In addition, it has also been reported that the standard cell culture medium, Dulbecco's modified Eagle medium

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(DMEM), enhances the SSAO activity present in foetal calf serum *in vitro* (*20*).

The aim of the present work was to investigate the effects of the DMEM and its constituents on human SSAO activity *in vitro*. Here we report, for the first time, that sodium bicarbonate ($NaHCO_3$) is the only component of DMEM able to enhance SSAO activity. The kinetic behaviour of this modulator is reported and its possible physiological role suggested.

MATERIAL AND METHODS

Chemicals—[^{14}C]-Benzylamine was from Amersham (Amersham, UK). MDL72974A ((E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride) was a kind gift from Dr P. H. Yu (University of Saskatchewan, Saskatoon, Saskatchewan, Canada). DMEM, with or without $NaHCO_3$ (3.7 mg/ml), methylamine, semicarbazide, L-deprenyl and other chemicals were from Sigma-Aldrich (St Louis, MO, USA).

Human Samples—The Ethical Committee of Universitat Autònoma de Barcelona approved the experimental protocol used for human samples in this study.

Dialyzed human plasma

Human plasma samples were obtained from Hospital Universitari de la Vall d'Hebron, Servei d'Hematologia, Barcelona, Spain, and stored at −20°C until use. Plasma was thawed at 37°C and dialyzed against fresh saline solution (1 : 500) overnight at 4°C. Dialyzed samples were stored in aliquots at −20°C.

Human aorta homogenates

Human aorta was obtained from Hospital Universitari de la Vall d'Hebron, Servei de Transplantaments, Barcelona, Spain, and stored in PBS at –80°C until use. For the homogenization process, the tissue was thawed and the tunica media was detached and saved from the rest of the tissue. The endothelial cell layer was removed by rubbing the luminal side of the vessel with a cell scraper. The final homogenate was prepared in phosphate buffer (10 ml:1 g tissue) with a polytron homogenizer. The homogenate was then stored, in aliquots, at −20°C until use.

SSAO activity determination—SSAO activity towards benzylamine as substrate was determined radiometrically at 37°C as previously described (*21*), using 100 μM [^{14}C]-benzylamine (2 mCi/mmol). Samples were pre-incubated for 30 min at 37°C with 1 μM L-deprenyl to inhibit any possible platelet MAO B contamination. The reaction was carried out at 37°C in a final volume of 225 μl in 50 mM phosphate buffer (pH 7.2) and stopped by the addition of 100 μl 2 M citric acid. Radiolabelled products were extracted into toluene/ethyl acetate (1 : 1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole before liquid scintillation counting.

SSAO activity towards methylamine 500 μM as substrate was determined by following H_2O_2 formation, using a peroxidase-coupled continuous spectrophotometric method (*22*). In this system, 4-aminoantipyrine is oxidized by the hydrogen peroxide (H_2O_2) formed during amine oxidation and then condenses with vanillic acid to give a red quinone imine dye. The absorbance at 498 nm, which was monitored using a Cary spectrophotometer, is proportional to the amount of H_2O_2 generated. SSAO activity is expressed as pmol/min mg protein. All assays were performed in the presence of L-deprenyl 1 μM to ensure the inhibition of any MAO activity. Protein was measured by the method of Bradford, using bovine-serum albumin as standard.

Kinetic studies—The effects of $NaHCO_3$ concentration (0–1 g/l) on SSAO activity towards benzylamine (25–400 μM) were determined without pre-incubation with the enzyme. The pH of $NaHCO_3$ solution was adjusted to 7.0–7.2 with HCl at the beginning of each experiment.

Reversibility studies—The reversibility of the SSAO activation by $NaHCO_3$ was determined by dialysis. Enzyme samples were pre-incubated for 30 min at 37°C with 2 g/l $NaHCO_3$. Samples were then dialyzed using a Centricon Centrifugal Filter (2 ml capacity, 3.0 Molecular Weight-Limit Membrane; Millipore, USA), following the manufacturer's instructions. Briefly, three consecutive washings were performed and samples were centrifuged at 4°C for 30 min between washings. Total protein was measured and SSAO activity was determined as described previously.

Analysis and Statistics—Results were given as means ± SEM. Statistical analysis was done by one-way ANOVA and further Newman–Keuls Multiple Comparison Test using the program Graph-Pad Prism 3.0. A P value of < 0.05 was considered to be statistically significant. K_m and V_{max} values were determined by non-linear regression, using the same program. The double-reciprocal plot is used only for illustrative purposes.

RESULTS

The ability of complete DMEM to enhance SSAO activity towards benzylamine was tested, using two different enzyme sources; circulating SSAO from human plasma and membrane-bound SSAO from human aorta. The basal SSAO activities of the aorta homogenates and plasma were 915.2 ± 95.5 and 0.73 ± 0.02 pmol/min mg protein, respectively. Pre-incubation in the presence of DMEM for 30 min increased the activity of the plasma SSAO 2.48 ± 0.10 times and that of the membrane-bound SSAO 3.43 ± 0.38 times (Fig. 1). In order to elucidate which specific component(s) of DMEM was responsible for the activation effect, each constituent, shown in Table 1, was tested alone. A concentration range of each component, including the corresponding dose present in DMEM, was incubated for 30 min with human plasma or human aorta homogenate before the SSAO activity was assayed towards benzylamine as substrate. $NaHCO_3$ was the only constituent that caused activation, and this was the same as that obtained with complete DMEM. Other inorganic salts, including those containing the sodium cation, did not show any effect on SSAO activity (data not shown). Since the pH of DMEM is 7, all compounds tested were prepared at this pH to avoid possible alterations in the activity determination caused by pH differences.

To confirm that $NaHCO_3$ was the only component responsible for the activation, the same experiments were performed using a mixture equivalent to DMEM

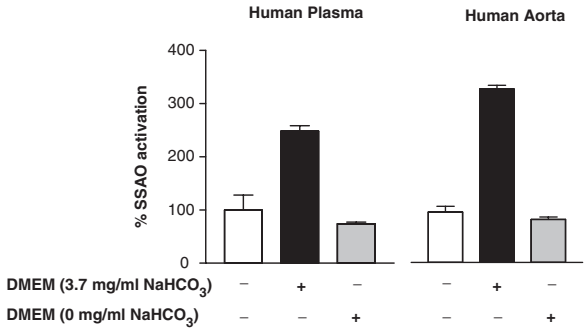


Fig. 1. **$NaHCO_3$ is the only component contained in DMEM responsible of SSAO activity enhancement.** Human aorta and human plasma were pre-incubated for 30 min with 50 µl of DMEM, with or without $NaHCO_3$. SSAO activity was determined towards 100 µM benzylamine as substrate in 50 mM phosphate buffer (pH 7.2). Data are mean ± SEM of three different experiments.

Table 1. **DMEM composition.**

Amino acid (g/l)		Vitamins (g/l)	
L-Arginine·HCl	0.084	Choline chloride	0.4
L-Cysteine·2HCl	0.0626	Folic acid	0.004
Glycine	0.03	Myo-inositol	0.0072
L-Histidine·HCl·H_2O	0.042	Niacinamide	0.004
L-Isoleucine	0.105	D-Pantothenic acid	0.004
L-Leucine	0.105	Pyridoxine·HCl	0.004
L-Lysine·HCl	0.146	Riboflavin	0.0004
L-Methionine	0.03	Thiamine·HCl	0.004
L-Phenylalanine	0.066		
L-Serine	0.042		
L-Threonine	0.095		
L-Tryptophan	0.016		
L-Tyrosine·2Na·2H_2O	0.10379		
L-Valine	0.094		
Inorganic Salts (g/l)		**Others (g/l)**	
$CaCl_2$ ·$2H_2O$	0.265	D-glucose	1.0
$Fe(NO_3)_3$ ·$9H_2O$	0.0001	Phenol Red·Na	0.015
$MgSO_4$	0.09767	Pyruvic Acid·Na	0.11
KCl	0.4		
$NaHCO_3$	3.7		
NaCl	6.4		
NaH_2PO_4	0.109		

Note: All components of DMEM were tested separately as possible SSAO modulators and $NAHCO_3$ was the only compoundable to enhance its activity.

with or without $NaHCO_3$. Figure 1 shows that the presence of $NaHCO_3$ in the medium is necessary to enhance both membrane-bound and plasma SSAO activity, suggesting this inorganic compound to be a, previously unrecognized, modulator of the SSAO activity.

Because there are significant concentrations of $NaHCO_3$ in human plasma (about 23 m Eq/l, which corresponds to 1.4 g/l), a prior dialysis process was required to study the net effect of this compound. The SSAO activity in dialyzed human plasma (0.31 ± 0.01 pmol/min mg protein) was lower than that determined without dialysis

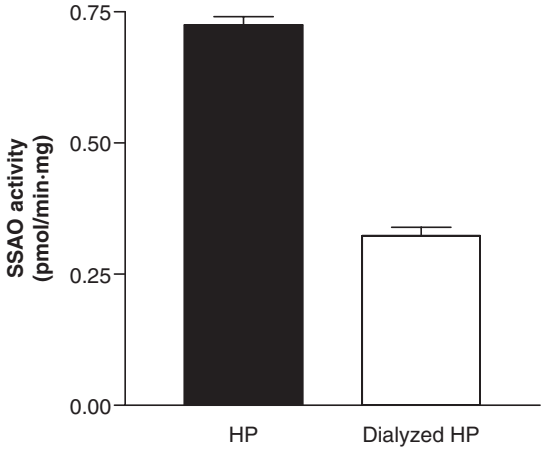


Fig. 2. **Dialysis of human plasma decreases SSAO specific activity.** Human plasma (HP) was dialyzed towards saline solution. SSAO activity present in dialyzed and non-dialyzed human plasma was assayed towards 100 µM benzylamine as substrate in 50 mM phosphate buffer (pH 7.2). Data are mean ± SEM of three different experiments.

(0.73 ± 0.02 pmol/min mg protein) (Fig. 2). These results suggest that the $NaHCO_3$ itself, as one of the components of human plasma, could be able to interact physiologically with the circulating enzyme.

Figure 3 shows the effects of varying concentrations of DMEM and $NaHCO_3$ on SSAO activity from dialyzed human plasma. The $NaHCO_3$ concentrations tested were equivalent to those contained in the different DMEM volumes used. The presence of the SSAO inhibitor, MDL 72974A, in the reaction mixtures completely destroyed the activity, confirming it to be due to SSAO. The activation of SSAO was sigmoidally dependent on the $NaHCO_3$ concentration, reaching a maximum at about 2 g/l NaHCO3 (Fig. 4). Fitting the data to the Hill equation (not shown) gave a Hill constant of 3.2 ± 0.7.

The possible time dependence of the activation was studied using high concentrations of $NaHCO_3$ (Fig. 4). The assay was initiated by the addition of the substrate to the mixture, containing the enzyme and $NaHCO_3$ that had been pre-incubated for 30 or 0 min. The enhancement of SSAO activity from dialyzed human plasma by $NaHCO_3$ was not time dependent, and, as shown in Fig. 5, this activation was completely reversible by dialysis.

The kinetic behaviour of $NaHCO_3$ towards plasma SSAO activity was determined from the initial rates in the presence of different amounts of the modulator (0–1 g/l) and increasing concentrations of the substrate, benzylamine (25–400 µM). $NaHCO_3$ behaved as a competitive activator of SSAO, as shown in the Lineweaver–Burk plot (Fig. 6A). The K_m values decreased as the amount of $NaHCO_3$ increased, whereas the V_{max} values remained constant (Fig. 6B). The decline in K_m was not a simple hyperbolic function of the $NaHCO_3$ concentration, as might be expected from the dependence shown in Figs. 3 and 4, and therefore a Ka value was not determined.

NaHCO3 also enhanced membrane-bound SSAO activity towards the physiological substrate methylamine (Fig. 7) and the presence of the classical SSAO inhibitor, semicarbazide, inhibited the enzyme activity completely.

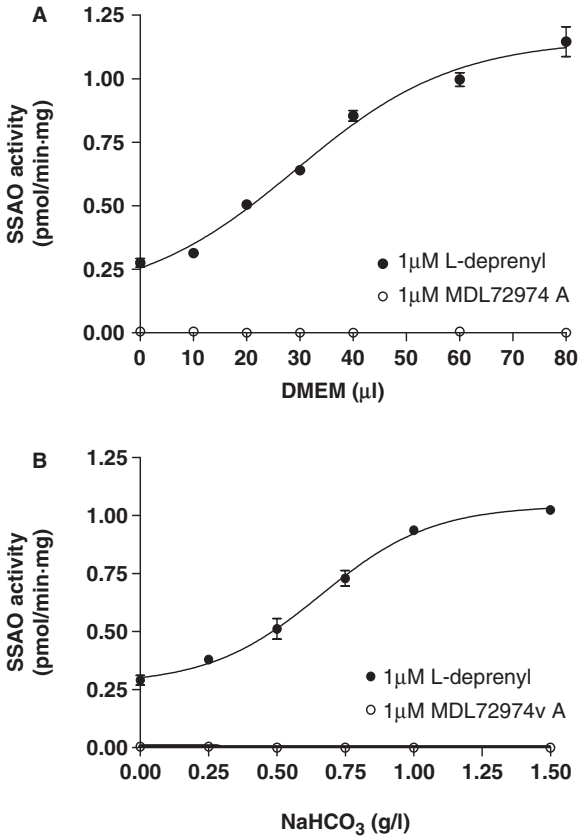


Fig. 3. **DMEM and $NaHCO_3$ enhance SSAO activity.** Dialyzed human plasma was pre-incubated with **(A)** DMEM (pH 7.0) or **(B)** $NaHCO_3$ (pH 7.2) in 50 mM phosphate buffer (pH 7.2) until a final volume of 200 µl for 30 min before adding 100 µM benzylamine as substrate. $NaHCO_3$ final concentration contained in DMEM was 3.7 g/l. Samples were previously inhibited with 1 µM L-deprenyl (black figures) or 1 µM MDL71974A (empty figures). Data are mean ± SEM of three different experiments.

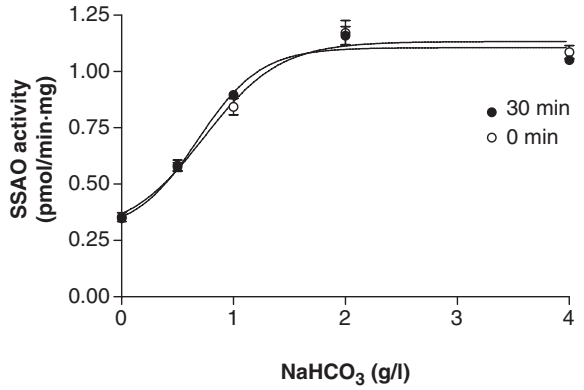


Fig. 4. **$NaHCO_3$ enhances SSAO activity in a non-time-dependent manner**. Dialyzed human plasma was pre-incubated with different $NaHCO_3$ solutions (pH 7.2) for 0 min (empty figures) or 30 min (black figures) before adding 100 µM benzylamine as substrate in 50 mM phosphate buffer (pH 7.2). Data are mean ± SEM of three different experiments.

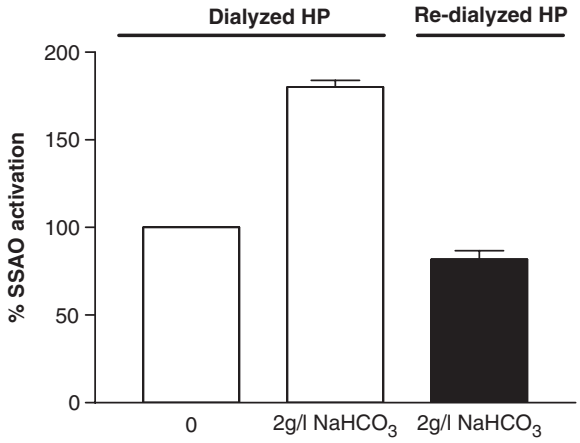


Fig. 5. **$NaHCO_3$ enhances SSAO activity in a reversible manner**. Dialyzed human plasma (Dialyzed HP) was pre-incubated for 30 min at 37°C with 2 g/l $NaHCO_3$ and then dialyzed again (see 'Materials and Methods'). Three consecutive washings were performed and samples were centrifuged at 4°C for 30 min between washings. Activity was measured by adding 100 µM benzylamine as substrate in 50 mM phosphate buffer (pH 7.2). Empty figures: dialyzed human plasma samples as control. Black figures: Samples of dialyzed human plasma that were dialyzed again after the 30 min SSAO activation process by $NaHCO_3$. Data are mean ± SEM of three different experiments.

However, the percentage of activation with methylamine was smaller than the enhancement observed using benzylamine as substrate.

DISCUSSION

SSAO activity has been reported to be altered in several pathological conditions (*7*), but little is known about the factors that may modulate its activity under physiological conditions. We have previously described the activation of human lung SSAO by a low molecular weight molecule present in human plasma, which had no effect on either MAO A or MAO B (*19*). Raimondi *et al*. (*23*) have reported bicarbonate to activate the histaminase activity of rat adipocytes at elevated pH values. Trent *et al*. (*20*) reported that culture medium was able to enhance the SSAO activity present in foetal calf serum, but they did not identify the component(s) responsible for the activation.

The present results show that DMEM enhances the activities of both the plasma and the tissue-bound forms of human SSAO and that $NaHCO_3$ is the sole component of DMEM that is responsible for this activation.

The activating effect on SSAO from dialyzed human plasma by $NaHCO_3$ was reversible and not time dependent. Kinetic studies showed the activation to be apparently competitive. Because amines can react with CO_2 to form carbamates (*24*), it is possible that these derivatives are better substrates than the free amines. Such a system is illustrated in Scheme 1A. This might account for the greater degree of activation seen with benzylamine than with methylamine because the ease of carbamate formation depends on the physico-chemical properties of the amine (*25*). However, it would be expected to give rise to complicated dependence of activity on both amine and bicarbonate concentrations (*26*). Furthermore, SSAO is

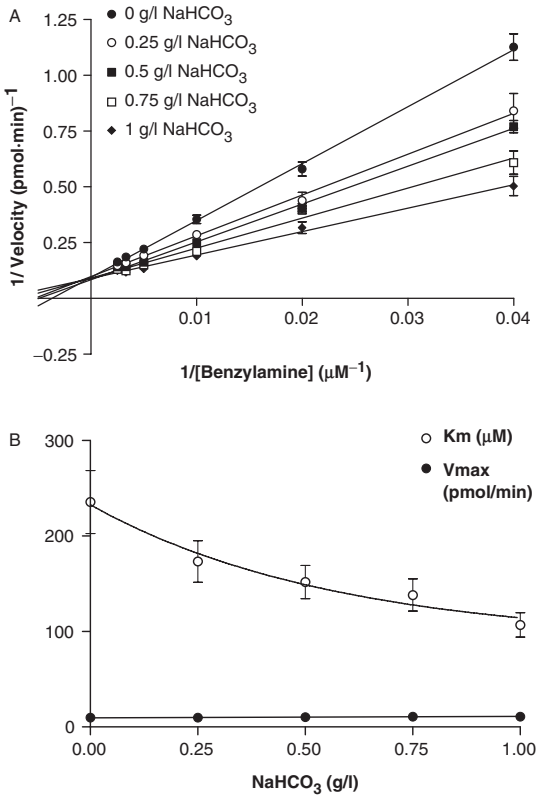


Fig. 6. **Kinetic behaviour of SSAO activation by $NaHCO_3$.** (A) Double reciprocal plots (Lineweaver–Burk transformation) of SSAO activation by $NaHCO_3$ towards benzylamine as substrate and (B) their corresponding kinetic constants towards $NaHCO_3$ concentration. Enzyme samples from dialyzed human plasma were incubated in the absence or presence of $NaHCO_3$ (0–1 g/l, pH 7.2) and, immediately, different benzylamine concentration (25–400 μM) were added to the reaction mixture in 50 mM phosphate buffer (pH 7.2). Data are mean ± SEM of six different experiments.

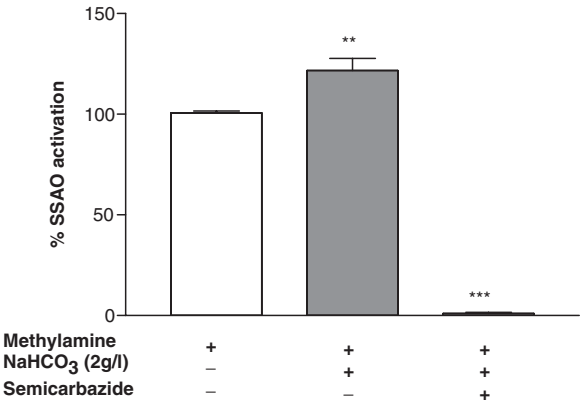
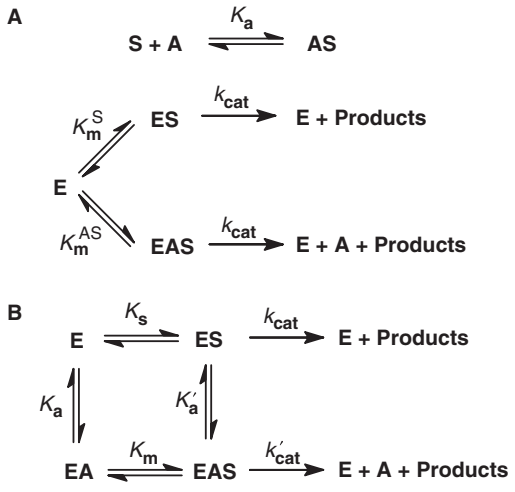


Fig. 7. **$NaHCO_3$ enhances SSAO activity towards methylamine as substrate.** Human aorta homogenate was preincubated with $NaHCO_3$ (2 g/l) in 50 mM phosphate buffer for 30 min and SSAO activity was assayed spectrophotometrically towards methylamine 500 μM as substrate. Semicarbazide (SC) 1 μM was used as SSAO inhibitor. Data are mean ± SEM of three different experiments; $^{***}P < 0.001$, $^{**}P < 0.01$ by a One-way ANOVA test and the addition of Newman–Keuls Multiple Comparison test versus control.

reported not to be active towards N-substituted amines. An alternative explanation, shown in Scheme 1B, would involve the binding of bicarbonate to the free enzyme resulting in a species (EA) with a higher affinity for substrate (S) without affecting the rate of product formation.



Under rapid-equilibrium conditions, this mechanism would result in competitive activation if $K_s > K_m$ and $k_{cat} = k'_{cat}$. Steady-state treatment of the above mechanism would, however, yield a complex equation containing squared reactant concentration terms. This might account for the apparently sigmoid dependence of activation on the concentration (Figs. 3 and 4). Interaction of more than one bicarbonate molecule with the enzyme might also contribute.

It has been reported that aminopeptidase A (PepA) from *Escherichia coli* is activated 10-fold by bicarbonate when L-leucine *p*-nitroanilide is used as substrate (*27*). In this case, the authors proposed that an exogenous bicarbonate anion as a catalytic group in an enzyme mechanism. Although our results would also seem to be consistent with such a process, more detailed protein structure studies would be necessary to investigate this hypothesis. It is also possible that activation might result from carbamoylation of lysine side chains in the enzyme itself, as a carbamoylated lysine has been shown to be essential for the activity of some class-D β-lactamases (*28*).

The report that bicarbonate increases the rate of oxidative deamination of histamine by SSAO from rat adipocyte [*24*] suggests that this activation may be a general phenomenon. It would be interesting to test such effect using different SSAO substrates.

Under physiological conditions, $NaHCO_3$ is an important buffering molecule in human plasma. The extent to which variations in the blood concentrations of bicarbonate might modulate the activity of the enzyme, enhancing the metabolism of circulating amines in respiring peripheral tissues, merits further investigation. This phenomenon could also result in inaccuracies in the SSAO activities previously reported because of the effects of dissolution of varying amounts of atmospheric CO_2 in the assay medium.

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