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## AGMATINE IS TRANSPORTED IN LIVER MITOCHONDRIA BY A SPECIFIC ELECTROPHORETIC MECHANISM

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Running title: Agmatine transport in mitochondria

Abbreviations used: ADC, arginine decarboxylase; AGM, agmatine; AGMase, agmatinase; COX, cytochrome c oxidase; DMO, 5,5'-dimethyl-oxazolidine-2, 4-dione; E<sub>a</sub>, activation enthalpy; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; MAO, monoamine oxidase; MPT, mitochondrial permeability transition; mtNOS, mitochondrial nitric oxide synthase; NO, nitric oxide; P<sub>i</sub>, phosphate; RLM, rat liver mitochondria; TEA, tetraethylammonium; TPP<sup>+</sup>, tetraphenylphosphonium;  $\Delta\Psi$ , electrical membrane potential;  $\Delta\mu_{\text{H}^+}$ , transmembrane electrochemical gradient.

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## ABSTRACT

Agmatine, a divalent diamine having two positive charges at physiological pH, is transported into the matrix of liver mitochondria by an energy-dependent mechanism whose driving force is the electrical membrane potential ( $\Delta\Psi$ ). Although this process shows strict electrophoretic behavior, qualitatively similar to that of polyamines, agmatine is most probably transported by a specific uniporter. Common transport with polyamines, by means of their transporter, must be excluded, as the divalent putrescine and cadaverine are ineffective in inhibiting agmatine uptake. Indeed, use of the electroneutral transporter of basic amino acids is also to be discarded, as ornithine, arginine and lysine are completely ineffective in inducing inhibition of agmatine uptake. Involvement of the monoamine transporter or the existence of a leak pathway are also unlikely. Flux-voltage analysis and determination of activation enthalpy, depending on the valence of agmatine, are consistent with the hypothesis that the mitochondrial agmatine transporter is a channel or a single-binding center-gated pore.

The transport of agmatine is non-competitively inhibited by propargylamines, in particular clorgilyne, known to be inhibitors of monoamine oxidase (MAO). However, agmatine is normally transported in mitoplasts, thus excluding the involvement of MAO in this process. The  $I_2$  imidazoline receptor, which binds agmatine to the mitochondrial membrane, must also be excluded as a possible transporter since its inhibitor, idazoxan, is ineffective in inducing inhibition of agmatine uptake. Scatchard analysis of membrane binding revealed two types of binding sites,  $S_1$  and  $S_2$ , both with monocoordination and exhibiting high capacity and low affinity compared with polyamines.

Agmatine transport in liver mitochondria may be of physiological importance as an indirect regulatory system of cytochrome c oxidase activity and an inducer mechanism of mitochondrial-mediated apoptosis.

## INTRODUCTION

Agmatine [1-(4-aminobutyl)guanidine], the biogenic amine formed by decarboxylation of arginine catalyzed by arginine decarboxylase (ADC), is known to bind to  $\alpha_2$ -adrenergic and imidazoline receptors, and to have properties as a neurotransmitter or neuromodulator (for a review, see [1]). Clinical properties have been suggested, such as a neuroprotective effect, counteraction of tolerance to opiates [2] and tumor suppression [3]. Many other biochemical effects have also been shown: for instance, agmatine induces the ornithine decarboxylase antizyme [4,5] and spermidine/spermine acetyltransferase (SSAT) [6] and thus influences polyamine homeostasis; it inhibits nitric oxide synthase (NOS), most potently the inducible form [7]. It may also be a precursor of polyamines, when it is hydrolyzed to putrescine by agmatinase (AGMase) [8].

In mammals agmatine is not only formed “in situ” by ADC, but may also be taken up by exogenous sources, as it is known to be present in food and to be produced by the intestinal flora [9]. It follows that transport of agmatine across the plasma membrane may contribute to regulation of its cytosol concentration and therefore its biological action. Generally, after being absorbed from the stomach by means of an energy-dependent mechanism, it is taken up by several organs, but particularly by the liver [10]. The mechanism involved has been studied in various cell types such as hepatocytes in primary culture [11], endothelial [12] and kidney cells [13], and several other mammalian cell types [14] which take up agmatine using the same transporter as polyamines. In contrast, agmatine and putrescine appear to be transported by different carriers in gliomal cells [15] and in a cell line derived from human embryonic kidney, in which the organic cation transporter (OCT-2) and the extraneural monoamine transporter (EMT) are used [16].

Agmatine has recently been found to be present in neuronal mitochondria [17]. ADC and AGMase have also been located in mitochondria [18-20] as well as the  $I_2$  imidazoline receptor, which binds agmatine and, in particular, is located on monoamine oxidase (MAO) [21]. All these observations, revealing close relationships between this amine and mitochondria, led us to examine whether agmatine could be transported in these organelles and, if so, to characterize the mechanism and establish if uptake is mediated by some known transporter, e.g., that of polyamines.

## MATERIALS AND METHODS

Rat liver mitochondria (RLM) were isolated in 0.25 M sucrose and 5 mM HEPES (pH 7.4) by conventional differential centrifugation [22]. Mitochondrial protein concentration was assayed by a biuret method with bovine serum albumin as a standard [23].

Incubations were carried out at 20°C with 1 mg of mitochondrial protein/ml in the following standard medium: 250 mM sucrose, 10 mM HEPES-HCl (pH 7.4), 5 mM succinate, and 1.25  $\mu$ M

rotenone. Sodium salts were used. Other additions are indicated in the descriptions of specific experiments. A sucrose-based medium was chosen in order to compare obtained results with those of polyamine transport (for a review, see Toninello et al. [24]). However, the effects of higher ion strength media are also reported (Fig. 2, inset B). Uptake of [<sup>14</sup>C]agmatine and [<sup>14</sup>C]spermine was determined by a centrifugal filtration method, as previously described [25]. Uptake of [<sup>14</sup>C]agmatine was also measured by an HPLC method [11] (demonstrating a similar trend to that mentioned above) as well as the presence in RLM of [<sup>14</sup>C]guanidobutyric aldehyde and [<sup>14</sup>C]putrescine. Hydrogen peroxide was measured fluorometrically by the scopoletin method [26]. Membrane potential ( $\Delta\Psi$ ) was measured in an open, thermostatically controlled, stirred vessel by monitoring the distribution of the lipophilic cation tetraphenylphosphonium ( $\text{TPP}^+$ ) across the mitochondrial membrane with a selective electrode, prepared in our laboratory according to published procedures [27, 28] and an Ag/AgCl reference electrode.  $\Delta\Psi$  values were corrected as proposed by Jensen et al. [29]. Mitochondrial matrix volume was calculated from the distributions of [<sup>14</sup>C]sucrose and <sup>3</sup>H<sub>2</sub>O according to Palmieri and Klingenberg [30].  $\Delta\text{pH}$  was calculated from the distribution of [<sup>14</sup>C]5,5'-dimethyl-oxazolidine-2, 4-dione (DMO) across the mitochondrial membrane [31].

Kinetic parameters from initial rate measurements were estimated applying the analysis reported by Reich et al. [32].

Binding parameters were by applying thermodynamic treatment of ligand receptor interactions [33]. Scatchard analyses were performed using Eq. [1].

$$\frac{[B]}{[F]} = \sum_{i=1}^s \{ [B_{\max,i}] - [B_i] \} \cdot \left[ \frac{1}{K_{i,1}(t)} + \varepsilon_i(F) \right] \quad [1]$$

where:

$$\varepsilon_i(F) = \sum_{k=2}^{n_i} \frac{[F]^{k-1}}{\prod_{j=1}^k K_{i,j}(t)}$$

represents the appropriate measure of the extent of multiple coordination on the  $i$ -th sites.  $[B_{\max,i}]$  is the maximum concentration of  $i$ -th sites which may be bound by the ligand,  $[B_i]$  is the concentration of  $i$ -th sites bound by the ligand,  $[B_{\max}]$  is the maximum receptor-bound ligand concentration,  $[F]$  is the free ligand concentration,  $K_{i,j}(t)$  is the affinity constant of the ligand for the  $i$ -th site,  $j$  is the occupancy number, and  $t$  is time.

Fitting was performed as described earlier [33].

The distribution of total bound agmatine on its binding sites was calculated by parameter  $X_i(F)$ , obtained by means of Eq. 2:

$$X_i(F) = \frac{[B_{\max,i}] - [B_i]}{[B_{\max}] - [B]} = \frac{1}{1 + \beta_i[F]} \quad [2]$$

where  $\beta_i$ , is a parameter describing the influence of the parallel filling of the other  $k$ -th sites in comparison with filling of the  $i$ -th site [33].

[ $^{14}\text{C}$ ]agmatine was synthesized as reported in [11].

## RESULTS

At physiological pH, agmatine is a diamine with two net positive charges (Fig. 1A), since it has the lower pKa value of 9.07 [16]. It may thus be considered as a divalent cation. Fig. 1B shows the most stable of the monovalent forms, present at very high pH.

As shown in Fig. 2, RLM, incubated in standard medium, takes up about 50 nmol/mg prot of [ $^{14}\text{C}$ ]agmatine in 30 min of incubation. In the presence of FCCP or antimycin A, which completely collapse  $\Delta\Psi$  (Fig. 2, inset A), gradual transport of the diamine is completely inhibited. An almost identical effect is observed when the medium is deprived of succinate (results not reported). Indeed, in the presence of KCl, significant inhibition may also be observed. The inset B of Fig. 2 shows the dose-dependent effect of saline-sucrose media.

It should be emphasized that no oxidation products of agmatine such as  $\text{H}_2\text{O}_2$  or [ $^{14}\text{C}$ ]guanidobutyric aldehyde were detected; nor was [ $^{14}\text{C}$ ]putrescine, a catabolic product of agmatinase activity [8] (results not reported).

The inhibition of agmatine transport by de-energizing conditions indicates that it is energy-dependent and requires an electrochemical gradient ( $\Delta\mu_{\text{H}^+}$ ). This observation led us to identify which component of  $\Delta\mu_{\text{H}^+}$ ,  $\Delta\Psi$  or  $\Delta\text{pH}$  (or both) drives agmatine uptake, i.e., to verify if the mechanism is electrophoretic or electroneutral in nature.

Fig. 3 shows that, in the presence of phosphate (Pi), which raises the  $\Delta\Psi$  value from 150 to 180 mV (Fig. 3, inset), the amount of agmatine taken up by mitochondria is about 80 nmol/mg prot in 30 min of incubation. Furthermore, if RLM are incubated with the  $\text{K}^+/\text{H}^+$  exchanger, nigericin, instead of Pi - a condition which enhances  $\Delta\Psi$  up to 200 mV (Fig. 3, inset) and completely collapses  $\Delta\text{pH}$  - agmatine uptake is further increased in both initial rate and final extent. In contrast with the effect of Pi and nigericin, treatment of RLM with the ionophore valinomycin, in the presence of  $\text{K}^+$  - a condition which completely collapses  $\Delta\Psi$  (Fig. 3, inset) and raises  $\Delta\text{pH}$  - completely inhibits the uptake of the diamine (Fig. 3), as in the de-energizing conditions of Fig. 2. Fig. 3 clearly demonstrates that agmatine transport by RLM is electrophoretic and excludes the involvement of  $\Delta\text{pH}$ . This statement is further confirmed by the gradual drop of about 20 mV after 20 min of incubation in the  $\Delta\Psi$  value, paralleled by an identical increase in the 58  $\Delta\text{pH}$  value upon the

addition of agmatine to RLM (Fig. 4), also demonstrating that agmatine transport occurs in conditions of high  $\Delta\mu_{\text{H}^+}$  and that the driving force is  $\Delta\Psi$ .

The results of Fig 5A show the dependence of the initial rate of agmatine transport on  $\Delta\Psi$  which exhibits non-ohmic conductance, comparable to that of divalent putrescine [34]. A first consideration arising from this comparison is that agmatine exhibits significantly higher initial rates of transport than putrescine with increasing  $\Delta\Psi$  values.

The equation for influx rate  $J$  into the mitochondrial matrix of a cation is:

$$J = J_0 e^{z\beta F\Delta\Psi / RT} \quad [3]$$

where  $J_0$  is the exchange flux (transport rate at  $\Delta\Psi = 0$ ),  $z$  is the amine valence, and  $\beta$  is a parameter giving the shape and position of the energy barrier(s) for cation transport. It should be noted that, for sharp barriers,  $\beta$  equals the fractional distance from the external side of the membrane to the peak of the first barrier [35].

The observation of an apparently exponential relationship between agmatine transport and  $\Delta\Psi$  means that flux-voltage analysis can be applied to its transport, as also previously done for the polyamines [34] and here re-calculated also for putrescine for the best comparison. In this regard, Fig. 5B shows the semi-logarithmic plot of the data of Fig. 5A. This gives an estimate of rate constant  $k$  and intrinsic permeability coefficient  $P$ , since  $J_0 = kc$  ( $J_0$  is the intercept of the curves on the ordinate axis,  $c$  is the concentration of the cation in the medium) and  $P = k/400$  (400 is the inner membrane surface area measured in  $\text{cm}^2/\text{mg prot}$  [36]). Assuming that agmatine can cross the membrane with its net charge, at pH 7.4, 1.98 (the pK values of agmatine are 9.07 and >13 [16]), and also as a monovalent cation, the value of  $\beta$  can be estimated by the relative slope  $z\beta$  of the curves shown in Fig. 5B. All the above parameters for agmatine and putrescine are listed in Table I. The  $\beta$  value of agmatine, 0.25, is very close to those of putrescine and the other polyamines [34] and is the theoretical value for a channel [35]. Instead, the value of  $\beta$ , 0.5, is more appropriate for leaks or carriers (see below).

The initial rate of agmatine transport was also measured at varying temperatures from a linear Arrhenius plot in the range 5-35 °C (Fig. 6). The resulting slope gives the activation enthalpy,  $E_a$ , for agmatine transport. The obtained value of 22.5 kJ/mol is very close to that of putrescine (24 kJ/mol), previously calculated [34]. Also of note is the fact that the activation enthalpy/mol per charge of agmatine ( $E_a/z$ ), which is 11.25 kJ, is very close not only to that of putrescine (12 kJ) but also to those of spermidine (10.6 kJ) and spermine (14.5 kJ) [34].

In mitochondrial preparations with  $\Delta\Psi$  values of about 180 mV (presence of  $P_i$ ), agmatine transport exhibits saturation kinetics, as illustrated by the apparent hyperbolic curve obtained in a typical

experiment (Fig. 7A). Estimation of kinetic parameters from initial rate measurements gives  $K_m$  and  $V_{max}$  of 0.7 mM and 6.32 nmol/min/mg prot, respectively. For comparison, the  $K_m$  and  $V_{max}$  of putrescine transport are 1 mM and 1.14 nmol/min/mg prot, respectively [34]. All these observations indicate that the transporter of agmatine may be the same as that of polyamine [28, 34].

The experimental results shown in Fig. 8 aim at solving this question and at verifying if agmatine also uses other transporters, e.g., those of basic aminoacids, monoamines, or other monovalent cations. In this regard, polyamines, and the above-mentioned cationic molecules were used as possible inhibitors of agmatine transport. Fig. 8A shows that putrescine and cadaverine, also divalent, at 1 mM concentration, the same used for agmatine, completely fail to inhibit the transport of agmatine. It should be noted that both polyamines inhibit the initial binding of agmatine. Fig. 8A also shows that tetravalent spermine, at the same 1 mM concentration, exhibits marked inhibition of this binding and transport. However, this later effect is most probably due to the drop in  $\Delta\Psi$  caused by spermine transport (Fig. 8A, inset) rather than to direct interaction between the two amines. It should be noted that neither putrescine nor cadaverine affect the  $\Delta\Psi$  value (Fig. 8A, inset). Fig. 8A shows that agmatine transport is also not affected by 1 mM ornithine, lysine, arginine. An identical lack of efficacy is exhibited by the monoamines tyramine and benzylamine, and the monovalent cation tetraethylammonium (TEA) (Fig. 8B). None of these molecules affect  $\Delta\Psi$  (results not reported).

Fig. 9 shows that strong inhibition is observed with the propargylamines pargyline and clorgyline, inhibitors of MAO activity. It should be emphasized that they are completely ineffective in inhibiting spermine transport (Fig. 9, inset A). These inhibitors do not in fact affect  $\Delta\Psi$  (Fig. 9, inset B). It is noteworthy that propargylamines have a single protonated amino group [37], so that their inhibition sustain the hypothesis that agmatine is transported as a monovalent rather than a divalent cation.

As agmatine is able to bind to the  $I_2$  imidazoline receptor located on the mitochondrial membrane [21], the experiment shown in Fig. 9 was also performed with the aim of verifying whether this receptor is involved in agmatine transport. Results show that the  $I_2$  inhibitor idazoxan does not prevent its net transport but completely inhibits the initial binding of agmatine (see extrapolation of transport traces at zero time, Fig. 9).

The observation that propargylamines strongly inhibit the transport of agmatine led us to identify the type of inhibition induced. The results of Fig. 7A show that clorgyline, at 50  $\mu$ M concentration, causes marked inhibition of the initial rate of agmatine transport and that this inhibition is of non-competitive type as demonstrated by the double reciprocal plot of Fig. 7B. In this case  $K_m$  is 0.71 mM and  $V_{max}$  2.01 nmol/mg prot/min.

All experiments reporting agmatine transport (e.g., see Figs. 2, 3, 8, 9) show that the gradual phase of agmatine accumulation (in the absence of effectors) is preceded by a phase of very rapid uptake of approximately 15 nmol/mg prot, as calculated by rough extrapolation of the curves at zero time (see also description of results, Fig. 9). Indeed, as Fig. 2 shows, this uptake is sensitive to the presence of KCl. This almost instantaneous uptake is very probably due to the electrostatic binding of agmatine to the accessible surface of mitochondrial membranes, according to the following considerations: i) it also occurs in de-energizing conditions (see Fig. 2); ii) addition of 5 mM cold agmatine during the accumulation of the labeled diamine induces the very fast loss of an amount almost identical to that already bound at zero time (Fig. 2).

The observation that propargylamines inhibit agmatine transport with no significant inhibition of initial binding (see extrapolations at zero time, Fig. 9) and that idazoxan behaves in the opposite way, indicates more than one binding site for agmatine on the mitochondrial membrane. The experiments of Fig. 10 were performed in order to clarify this point. Fig. 10A shows the different amounts of agmatine that bind to the mitochondrial membranes at zero time, as a function of the total, external agmatine concentration. As shown, binding of the amine tends towards saturation. Fig. 10B, showing results of Scatchard analysis of agmatine binding, demonstrate that the theoretical curve for agmatine that satisfactorily fits the experimental data (Fig. 10B) is typical for two binding sites,  $S_1$  and  $S_2$ , both with monocoordination. Calculated total concentration of bound agmatine ( $B$ ),  $83.20 \pm 0.04$  nmol/mg prot, is distributed between  $S_1$  ( $B_{\max 1} = 3.20 \pm 0.03$  nmol/mg prot) and  $S_2$  ( $B_{\max 2} = 80.05 \pm 0.02$  nmol/mg prot) at 3.85% and 96.15%, respectively. Dissociation constants ( $K_1 = 25 \pm 3$  nmol/mg prot and  $K_2 = 4937 \pm 8$  nmol/mg prot) of  $S_1$  and  $S_2$ , respectively, demonstrate that  $S_1$  has an affinity about 200 times higher than that of  $S_2$ . Comparisons with the binding parameters of polyamines [38] show that both sites have high binding capacity and low affinity.

As the targets of propargylamines and idazoxan, that is MAO and  $I_2$  receptor respectively, are located on the outer membrane, in order to establish if their effects are related to these interactions, agmatine transport was also determined in mitoplasts compared with mitochondria. However, results (not reported) showed that agmatine is taken up by mitoplasts at the same rate and to the same extent as mitochondria, indicating that the effects of the inhibitors are not due to their interactions with the outer membrane and that the transport process involves neither MAO nor the  $I_2$  receptor.

## DISCUSSION

The results reported here provide evidence that agmatine is capable of binding to mitochondrial membranes and of being taken up into the matrix space of RLM. This binding, most probably electrostatic in nature, takes place in two sites  $S_1$  and  $S_2$  (Fig. 10), is affected by natural polyamines (Fig. 8A), idazoxan (Fig. 9) and saline media (Fig. 2), and is unaffected by de-energizing agents (Fig. 2), cationic aminoacids (Fig. 8A), monoamines or monovalent cations (Fig. 8B). Agmatine binding is followed by slow, long-lasting uptake which is highly dependent on mitochondrial energization (Figs. 2, 3) and electrophoretic in nature (Figs. 3, 4). It also exhibits a non-linear current voltage relationship (Fig. 5A), fitting the general behavior of monovalent and polyvalent cations (e.g. polyamines) in mitochondria [34, 35].

Considering flux-voltage analysis (Fig. 5), it should be noted that agmatine, depending on the valence of transported species, can exhibit two different  $\beta$  values (Table I). The calculation of  $\beta=0.25$  for the divalent form, very close to those of putrescine (Fig. 5B, Table I) and the other polyamines, suggests that agmatine is transported by a channel with two energy barriers, like that of polyamines [34].

This possibility is also supported by the calculation of the intrinsic permeability coefficient,  $P$  (Fig. 5, Table I). This value,  $26.8 \cdot 10^{-10}$  cm/sec, is higher than that of putrescine (Table I) and those of spermidine and spermine [34], but of their same order of magnitude. These permeabilities are very similar to that of the monovalent cation TEA -  $3.54 \cdot 10^{-10}$  cm/sec - which is transported by a leak pathway [34]. However, taking into account the divalency of agmatine, its permeability is very high, suggesting the presence of a uniport transporter which may be a channel.

This hypothesis is also strengthened by the  $E_a/z$  value of this uptake, which is 11.25 kJ/mol (Fig. 6), i.e., very similar to that of polyamines, which have an average value of 12.6 kJ/mol [34]. These values are much lower than that of TEA, which is 76 kJ/mol [34], and are comparable to that of the  $Ca^{2+}$  channel, which is 20 kJ/mol [39]. Indeed, the  $E_a$  for agmatine, 22.5 kJ/mol, is much lower than those of several mitochondrial carriers, which range from 64 to 92 kJ/mol [40, 41].

Force-flux analysis also demonstrates that monovalent agmatine is taken up by a transport system having a  $\beta$  value of 0.5 [Fig. 5B, Table I]. Bearing in mind that activation enthalpy values as low as 20-30 kJ/mol have also been identified for secondary transporters in mitochondria [42-44], this  $\beta$  value may be applied to a single-binding center-gated pore, of which a typical example is the ATP/ADP carrier [45]. The observation that the initial rate of agmatine transport is higher than that of putrescine (Fig. 5A) but also that of the other polyamines [34] is ascribed to the fact that agmatine in its divalent form (Fig. 1A) has a dipole moment,  $\mu=5.1$  D [46], whereas the polyamines have  $\mu=0$ . In this regard, it should be noted that the monovalent agmatine [Fig. 1B] has a very high dipole moment ( $\mu=15.8$  D) [46], suggesting that this form may be predominant in transport. As the

pH value of the channel or gated pore environment is not known, the question of whether the monovalent or divalent form of agmatine is actually transported is not solved. Because this information is critical for determining parameter  $\beta$ , the nature of the transport system still remains open.

As previously demonstrated, the polyamine transporter is common to all natural polyamines, so that they reciprocally inhibit their transport in a competitive manner [34]. Hence, putrescine, cadaverine, and spermine should exhibit the same type of inhibition on agmatine transport. Observations that the divalent putrescine and cadaverine are ineffective (Fig. 8A), indicate the existence of different transport systems for agmatine and polyamines, rather than a single common one. Indeed, the results of Fig. 8A clearly exclude the possibility that agmatine can use the electroneutral transporter of basic amino acids [47, 48]. Fig. 8B shows that TEA, tyramine and benzylamine, like other monoamines (results not shown), fail to exhibit any inhibition, suggesting that the leak pathway for monovalent cations [34] and the transporter for monoamines (A. Toninello, unpublished results), both electrophoretic, are not involved. Strong inhibition of agmatine transport is observable with clorgyline and pargyline, which act as non-competitive inhibitors of transport and independently of this action on MAO [Fig. 9]. Observations that some compounds, e.g., putrescine, cadaverine [Fig. 8A] and idazoxan [Fig. 9], reduce initial binding without affecting transport, whereas others, such as propargylamines, inhibit transport without inhibition of initial binding, indicate that there are at least two types of binding sites for agmatine on mitochondrial membranes. The results shown in Fig. 10 clearly confirm the existence of two binding sites,  $S_1$  and  $S_2$  which, as also observed for polyamine binding [33, 38], exhibit monocoordination, with high binding capacity and low binding affinity.

The non-competitive inhibition exhibited by clorgyline on agmatine transport (Fig. 7B) excludes the possibility that both molecules are taken up by the same transporter. The observation that clorgyline does not completely inhibit agmatine transport (Fig. 7A) is consistent with residual binding of agmatine to its transporter, putatively identified as binding site  $S_1$ . This site, in fact, has the same characteristics as the polyamine binding site involved in transport [33].

In conclusion, we report the existence of a specific selective transport system for agmatine in RLM, which may be a channel or, alternatively, a single-binding center-gated pore.

These results are considered of physiological importance, since the agmatine concentrations used (0.1-1 mM) and the  $K_m$  calculated for transport (0.7 mM) are compatible with the concentration of agmatine normally measured in liver cells, i.e., 0.5 mM [49]. It should also be taken into account that fluctuations in this concentration may be observed in various physiological and pathological situations [7]. For example, it may increase by about 20 times, as observed in rat aorta after

ischemic injury [50]. In particular, agmatine transport in RLM is able to induce apoptosis in hepatocytes by activating caspase 3 [51], due to release of cytochrome c from mitochondria promoted by agmatine, as a result of the mitochondrial permeability transition (MPT) induced in the presence of phosphate and high  $\text{Ca}^{2+}$  concentration. It is generally believed that MPT is closely connected with programmed cell death [for recent reviews, see Refs. 24, 52], the main role of which is probably to counteract tumor growth.

Another important point to be considered in evaluating the role of agmatine transport in RLM is the presence in these organelles of both biosynthetic and catabolic agmatine enzymes, such as ADC [18, 19] (the presence of ADC is strongly debated [53]), and AGMase [20]. In this regard, it is noteworthy that the presence of agmatine in cells is particularly high in liver [49]. Thus, identification of AGMase in the matrix of RLM indicates the great importance of finding a transport system for agmatine located on mitochondria. AGMase may represent a mechanism for regulating agmatine signaling properties in RLM via changes in agmatine levels in the organelles [54].

The observation that agmatine is an inhibitor of nitric oxide (NO) synthesis [7] raises the question of the important function of agmatine and AGMase at mitochondrial level. NO is produced by a group of NO synthases (NOSs) including three isoforms [55, 56]. Isoform mtNOS is constitutively present in the mitochondrial matrix, and is involved in altered mitochondrial regulation during hypoxia [57]. Indeed, mitochondrial NO has been identified as a physiological regulator of electron flux and ATP synthesis by inhibiting cytochrome c oxidase (COX) [58]. Increase in mitochondrial NO may follow the induction and activation of mtNOS, and is important in mitochondrial pathology, including the effects of aging, inflammation and cancer [58]. In this regard, it should be emphasized that mtNOS activity may be regulated by changes in agmatine matrix concentration, as the result of concerted activity by agmatine transporter and agmatinase [58]. In conclusion, the presence of agmatine in the mitochondrial matrix, if it inhibits mtNOS, favors ATP synthesis, whereas its fall in concentration by AGMase activity facilitates NO production, with consequent inhibition of ATP synthesis (see Fig. 11).

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## FIGURE LEGENDS

### Figure 1 Agmatine structure as divalent (A) or monovalent (B) cation

Structures determined by *ab initio* molecular orbital calculations, coupled to Raman spectroscopy [41].

### Figure 2 Agmatine uptake by rat liver mitochondria. Dependence on energized state

RLM were incubated in standard medium, as described in Materials and Methods, with 1 mM [<sup>14</sup>C]agmatine (50 µCi/mmol). When present in medium: 0.1 µg/mg prot FCCP, or 1 µg/mg prot antimycin A (Ant. A). KCl 50 mM, sucrose 150 mM. Where indicated, 5 mM cold agmatine was added. Dotted line and empty circle on ordinate axis: extrapolation of agmatine binding at zero time. Mean values ±SD of five experiments. *Inset A*: de-energizing effect of FCCP and Ant. A, obtained by incubating RLM in standard medium without agmatine. ΔE: electrode potential. *Inset B*: dose-dependent inhibition by KCl on agmatine transport. RLM were incubated in standard medium, final concentration together with sucrose was maintained at 250 mOsM. Results show amount of agmatine uptake after 30 min of incubation.

### Figure 3 Effect of phosphate, nigericin and valinomycin + K<sup>+</sup> on agmatine uptake and flux-voltage relationships

RLM were incubated in standard medium containing 1 mM [<sup>14</sup>C]agmatine (50 µCi/mmol). When present, 1 mM phosphate (P<sub>i</sub>), 0.33 µg/mg prot. nigericin (nig), 0.33 µg/mg prot. valinomycin (val)

and 10 mM KCl. Empty circle on ordinate axis: agmatine bound at zero time. Mean values  $\pm$ SD of six experiments. Inset: results obtained without agmatine, showing changes in  $\Delta\Psi$  due to different effectors.

**Figure 4 Changes in  $\Delta\Psi$  and 58 $\Delta$ pH values induced by agmatine uptake**

RLM were incubated in standard medium, with 1 mM  $P_i$ , 2  $\mu$ M TPP<sup>+</sup>, 400  $\mu$ M [<sup>14</sup>C]DMO (1  $\mu$ Ci/mmol), 5 mM [<sup>3</sup>H]glycerol (100  $\mu$ Ci/mmol). Where indicated, 1 mM agmatine was added. Both measurements of  $\Delta\Psi$  and  $\Delta$ pH were performed on same sample. A typical experiment is reported. Six other experiments gave almost identical results.

**Figure 5 Flux-voltage relationship (A) and flux-voltage analyses (B) for agmatine and putrescine**

*Panel A:* Agmatine and putrescine fluxes (nmol/min/mg prot) (J) plotted versus  $\Delta\Psi$ . RLM were incubated for 5 min in standard medium with 1 mM  $P_i$ . [<sup>14</sup>C]agmatine or [<sup>14</sup>C]putrescine (50  $\mu$ Ci/mmol) at 1 mM concentration.  $\Delta\Psi$  was manipulated by including limiting amounts (5-60 nM) of FCCP. Highest values of amine uptake were achieved by adding nigericin (0.33  $\mu$ g/mg prot.). Amine uptake values are corrected for instantaneous electrostatic binding [28]. Uptake of agmatine and putrescine was linear over incubation period. A typical experiment is reported. Six experiments were carried out with each compound, yielding almost identical results.

*Panel B:* Log-linear plots of data of panel A. Linear regression yielded values for slopes of curves representing product  $z\beta$  (see equation 3). Intercepts of curves on ordinate axis gave values of  $\ln J_0$ .

**Figure 6 Arrhenius plot of agmatine transport**

RLM with  $\Delta\Psi$  180 mV were incubated for 5 min over temperature range of 5-35 °C in standard medium with 1 mM  $P_i$ . [<sup>14</sup>C]agmatine (50  $\mu$ Ci/mmol) added at concentration of 1 mM. Binding correction as in Fig. 5A. Uptake was linear over incubation period. A typical experiment is reported. Five other experiments gave almost identical  $E_a$  values.

**Figure 7 Saturation kinetics (A) and double reciprocal plot (B) of agmatine uptake. Inhibitory effect by clorgyline**

*Panel A:* RLM were incubated for 5 min in standard medium with 1 mM  $P_i$  and [<sup>14</sup>C]agmatine (50  $\mu$ Ci/mmol) at indicated concentrations. When present, clorgyline was at 1 mM concentration. Uptake of agmatine was linear over incubation period. Binding correction as in Fig. 5. Mean values  $\pm$ SD of five experiments are reported.

*Panel B:* Double reciprocal plot of data of panel A. Inset: values of apparent  $K_m$  and  $V_{max}$  calculated by computer simulation.

**Figure 8 Effect of polyamines and basic amino acids (A) and monoamines and TEA (B) on agmatine uptake**

RLM were incubated in standard medium with 1 mM [ $^{14}\text{C}$ ]agmatine (50  $\mu\text{Ci}/\text{mmol}$ ) and 1 mM  $\text{P}_i$ . When present, putrescine, cadaverine, spermine, ornithine, lysine, arginine, tyramine, benzylamine and TEA were at 1 mM concentration. Inset A: effect of polyamine transport (at 1 mM ) on  $\Delta\Psi$ . Empty circles on ordinate axis: agmatine bound at zero time.

#### **Figure 9 Effect of propargylamines and idazoxan on agmatine uptake**

RLM were incubated in standard medium in presence of 1 mM [ $^{14}\text{C}$ ]agmatine (50  $\mu\text{Ci}/\text{mmol}$ ) and 1 mM  $\text{P}_i$ . When present, clorgyline and pargyline were at concentrations of 50 and 100  $\mu\text{M}$ , respectively, and idazoxan at 200  $\mu\text{M}$ . Empty circles on ordinate axis: agmatine bound at zero time. Insets A and B: effect of propargylamines (dashed lines), at above concentrations, on spermine transport and  $\Delta\Psi$ ; [ $^{14}\text{C}$ ]spermine (50  $\mu\text{Ci}/\text{mmol}$ ) at 1 mM concentration.

#### **Figure 10 Binding of agmatine to energized mitochondria**

*Panel A:* Concentration-dependent zero-time binding. Mitochondria incubated for 3 min in standard medium, as described in Materials and Methods, with varying [ $^{14}\text{X}$ ] agmatine concentrations, in range 100-3000  $\mu\text{M}$  (0.05  $\mu\text{Ci}/\text{ml}$ ), as indicated, and 1 mM  $\text{P}_i$ . B values obtained by measuring agmatine uptake (nmol/mg prot) at various times and extrapolating at zero time (on y axis) trend of curves, which is linear at all concentrations, in first 5 min of incubation (for a more complete description, see Fig. 1 in Ref. [32]). Results represent a typical experiment. Standard deviations of binding constants are reported in Table II.

*Panel B:* Binding analysis with thermodynamic treatment of Scatchard.

B values (agmatine bound at zero time) were calculated as mentioned above; free agmatine concentration (F) by subtracting bound agmatine (B) from total agmatine, shown in panel A. Continuous line is theoretical curve calculated using Eq. 1 for Scatchard-based analysis, as described in Materials and Methods.

#### **Figure 11 Proposed physiological role of agmatine in RLM**

For description, see text.

Figure 1

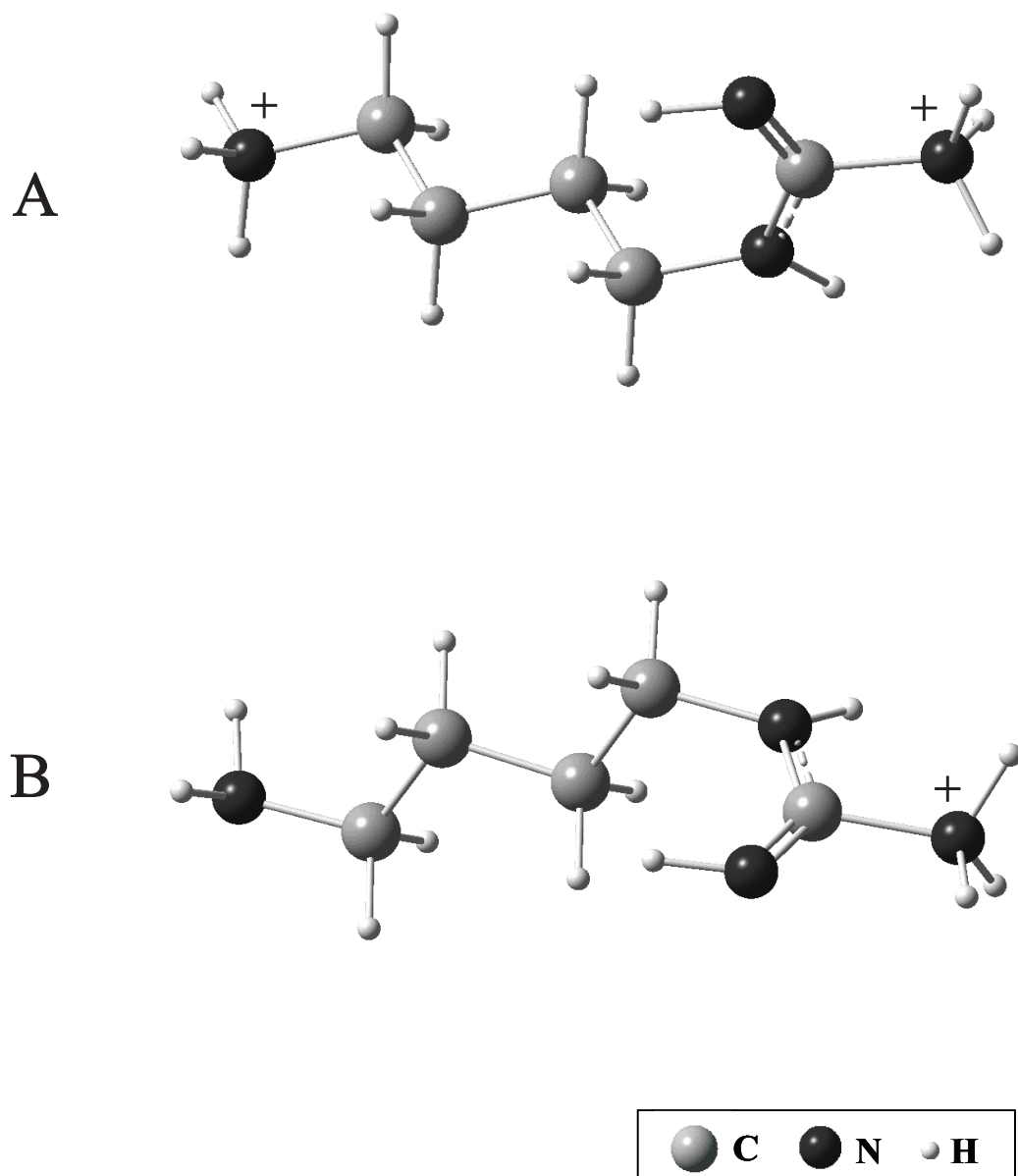


Figure 2

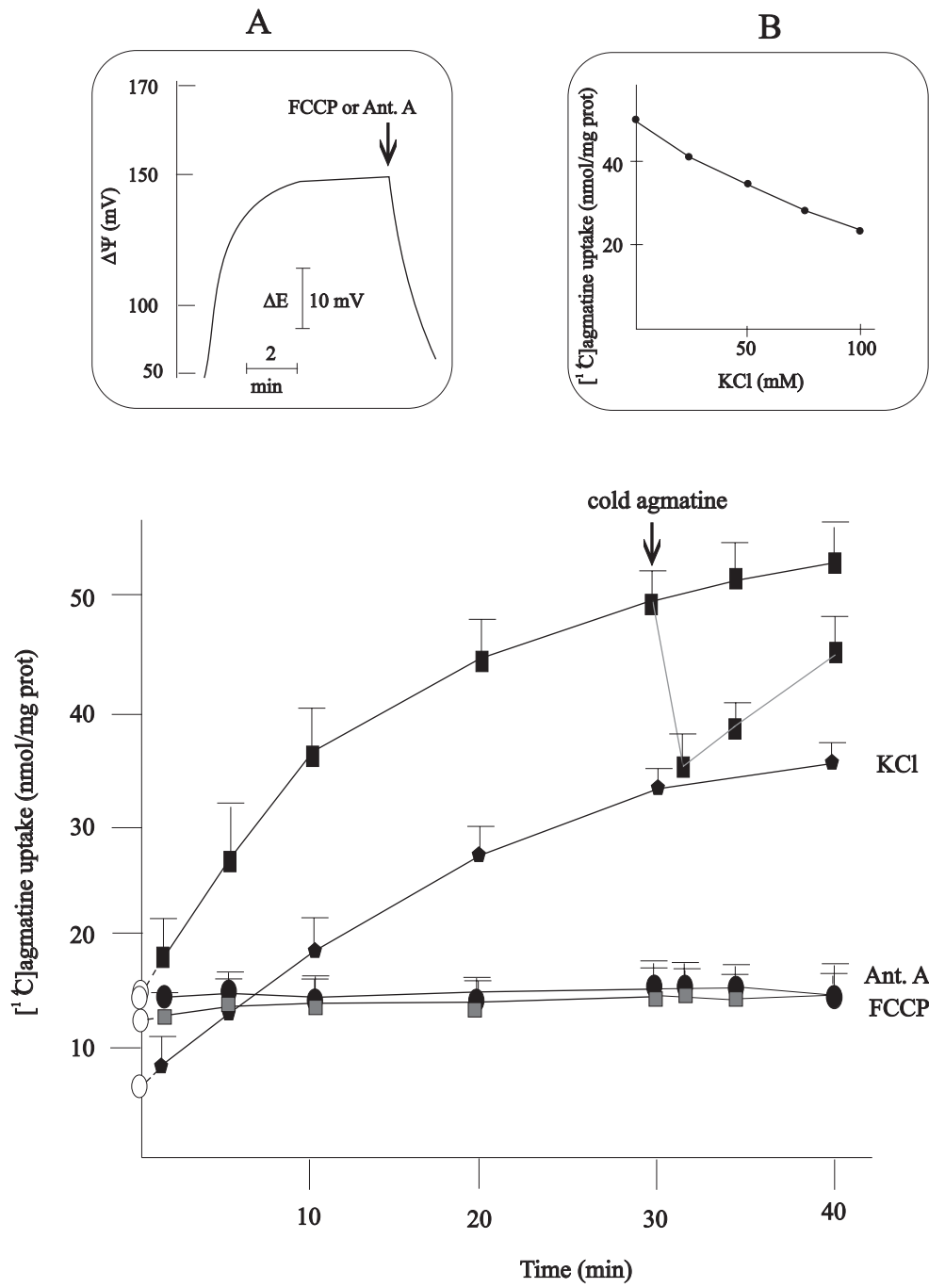


Figure 3

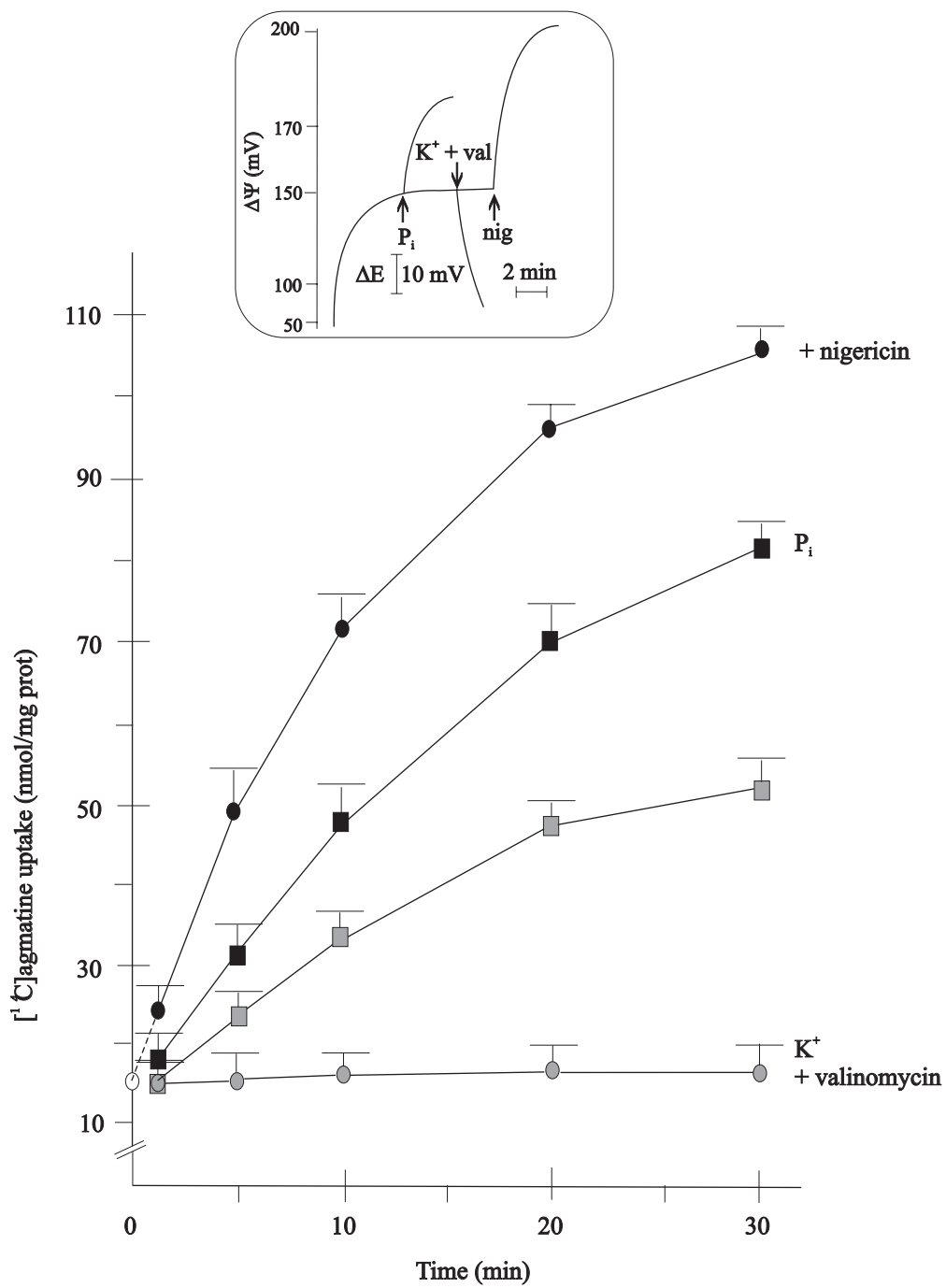


Figure 4

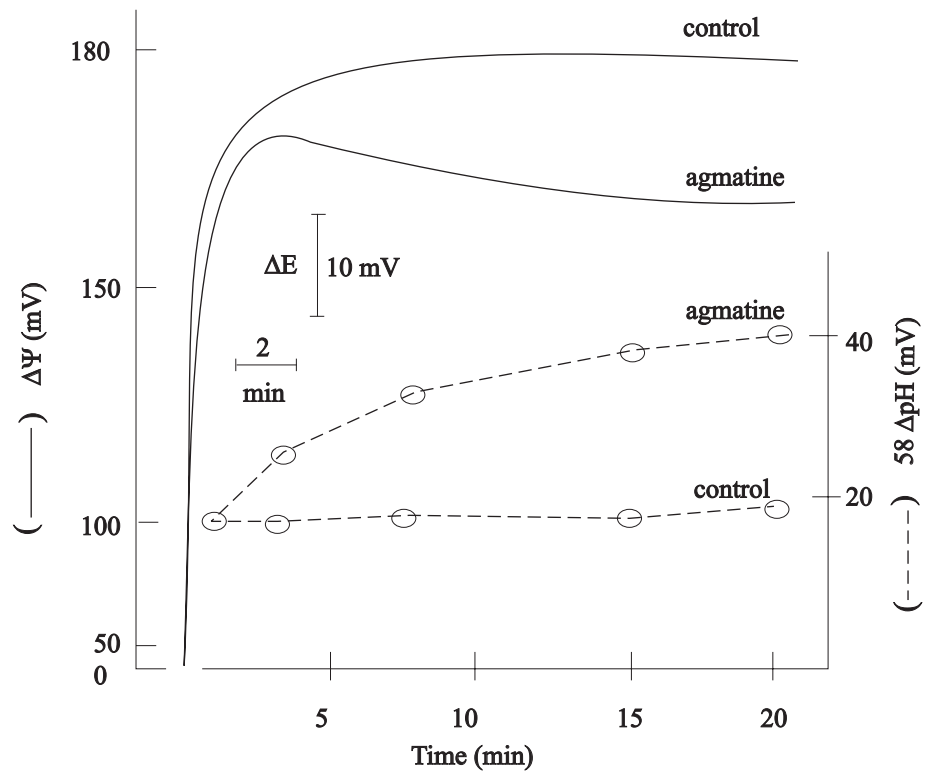
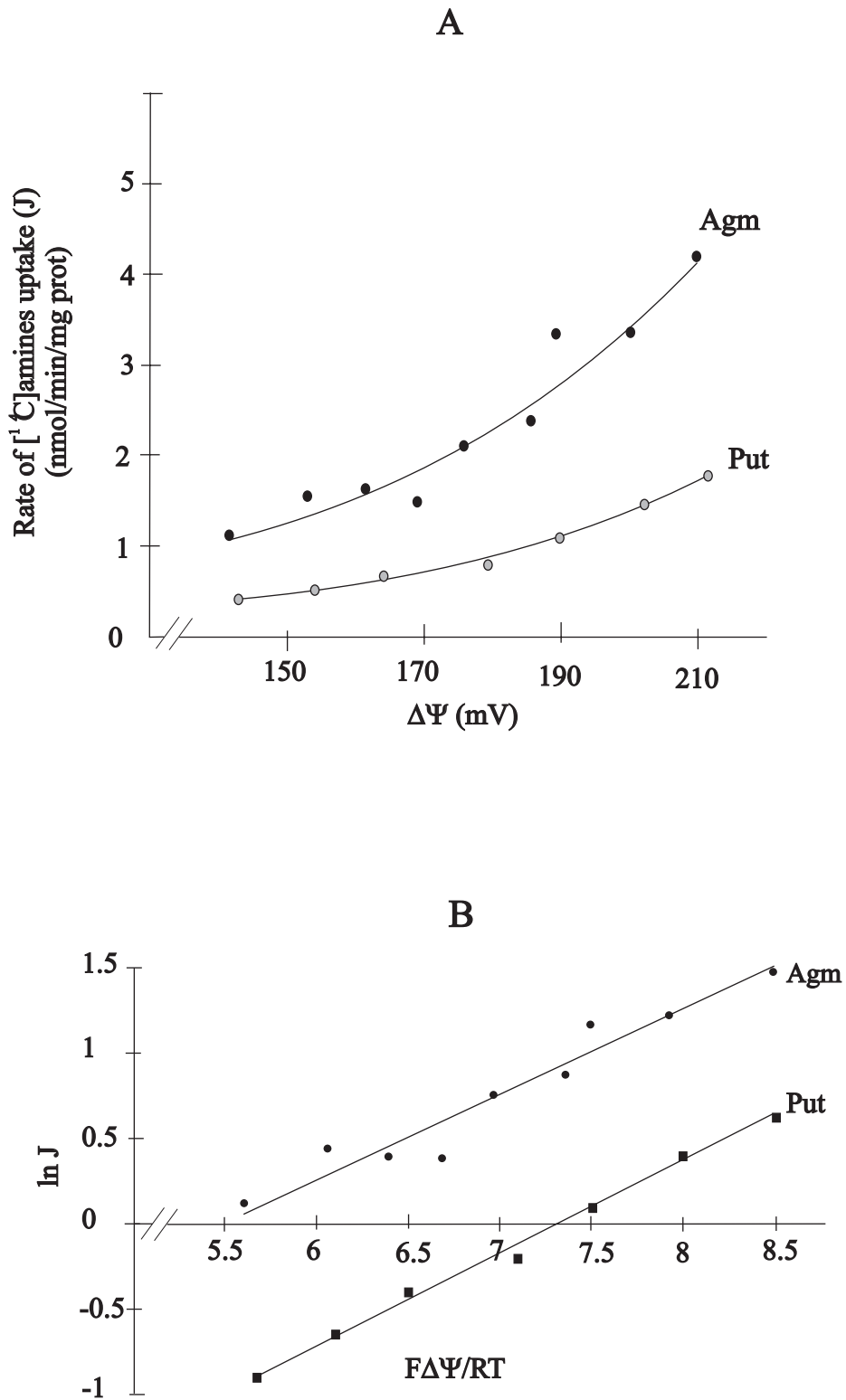


Figure 5



# Figure 6

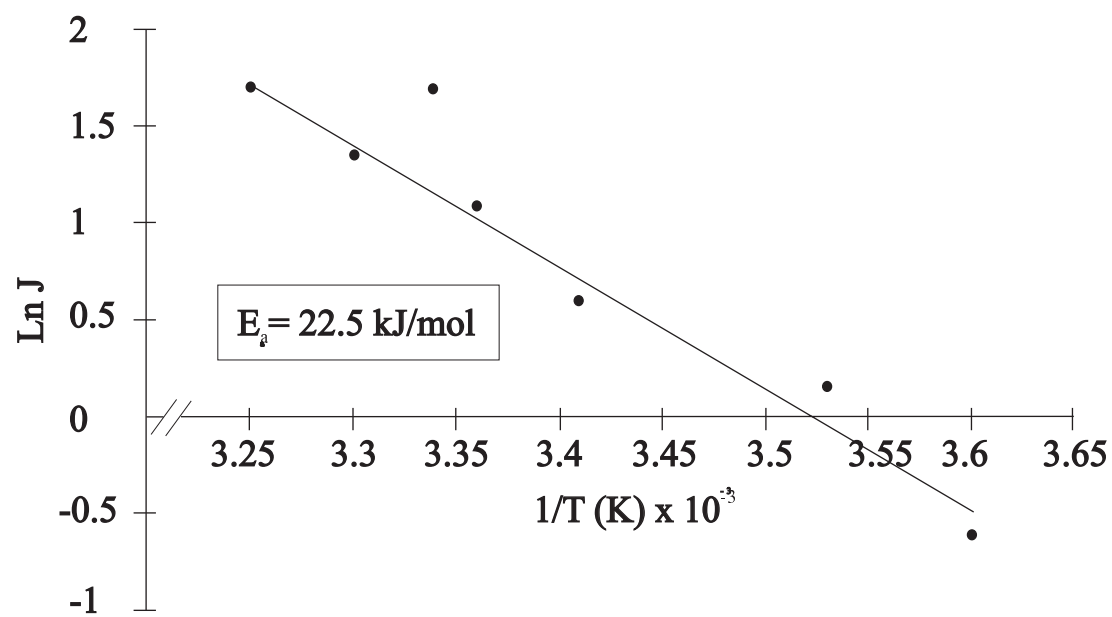


Figure 7

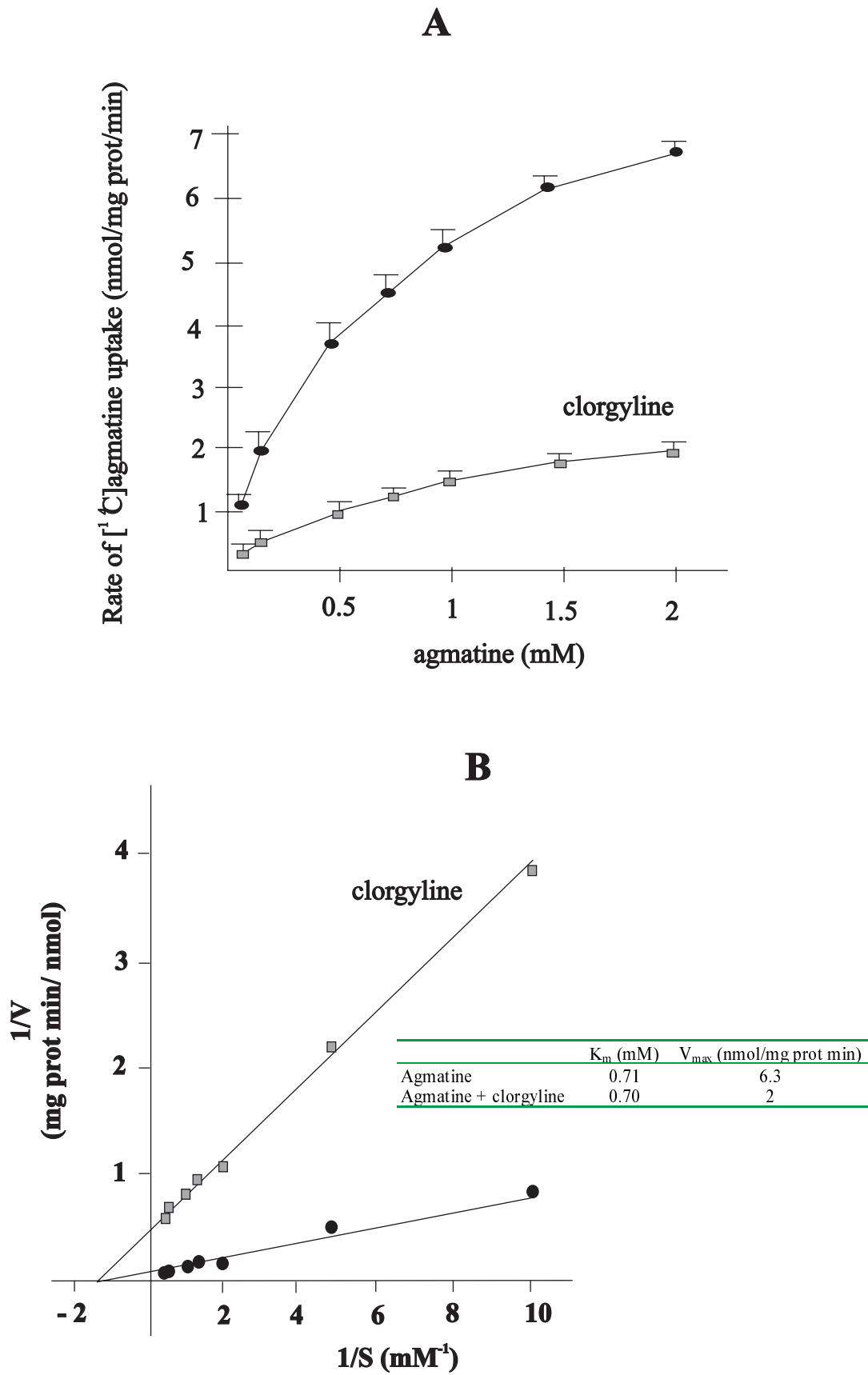
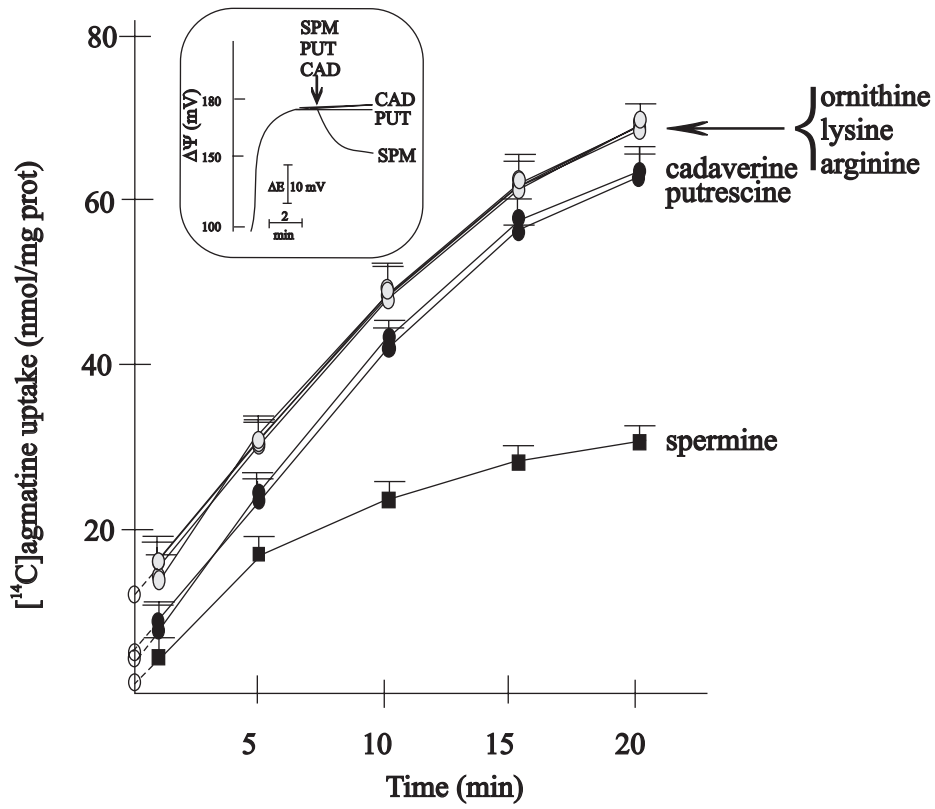


Figure 8

**A**



**B**

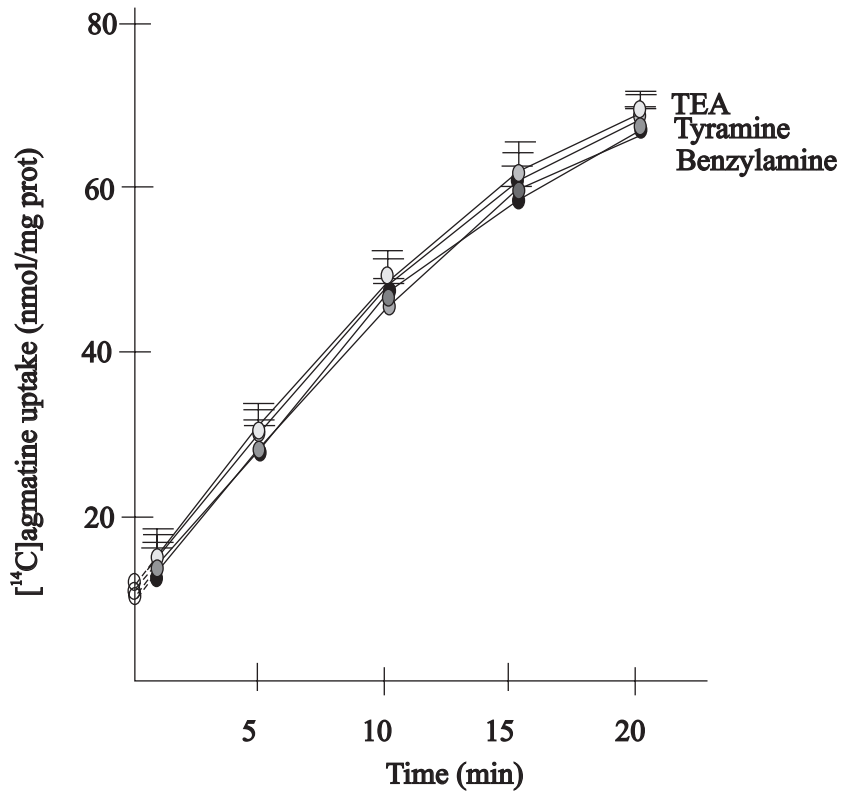
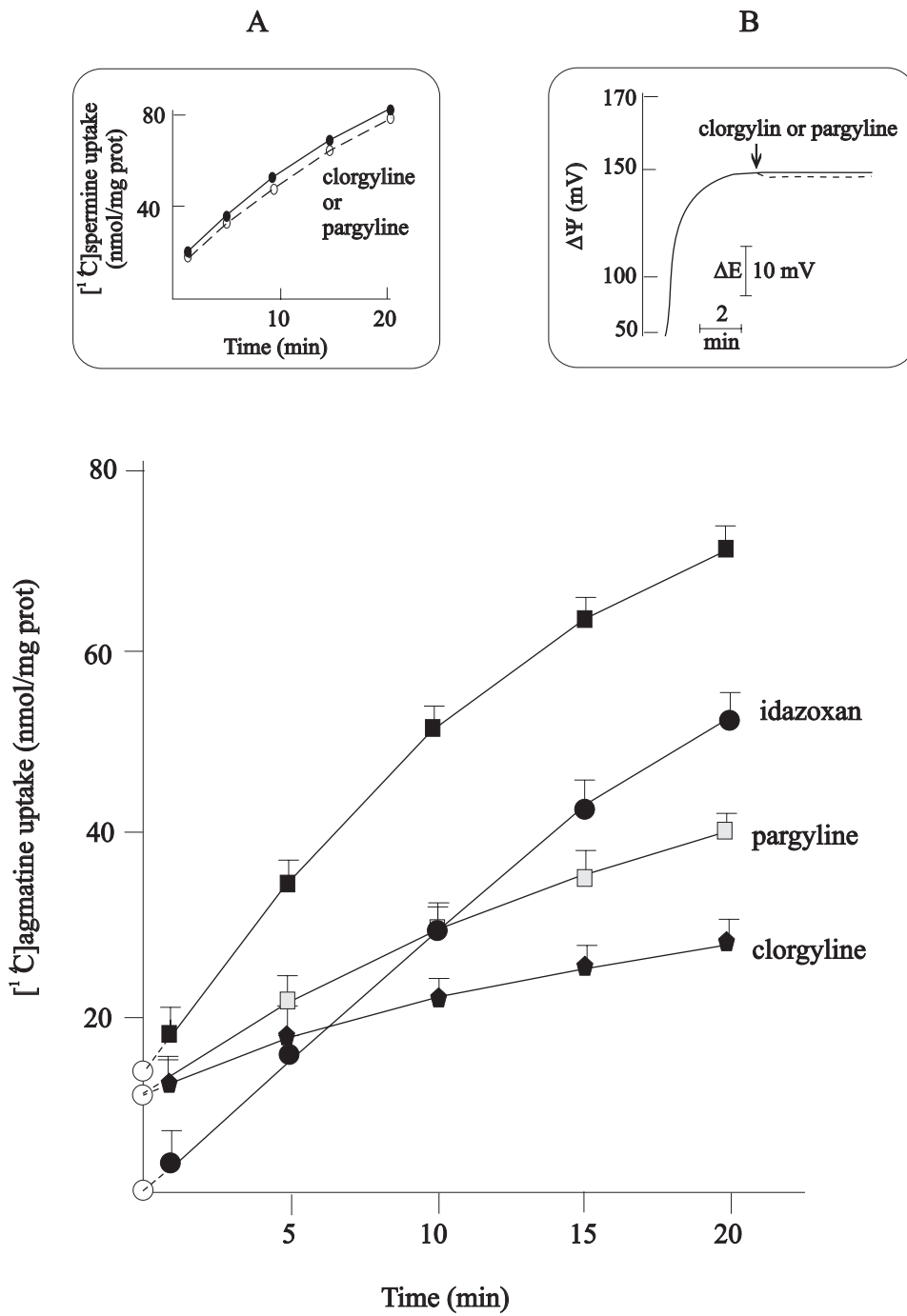
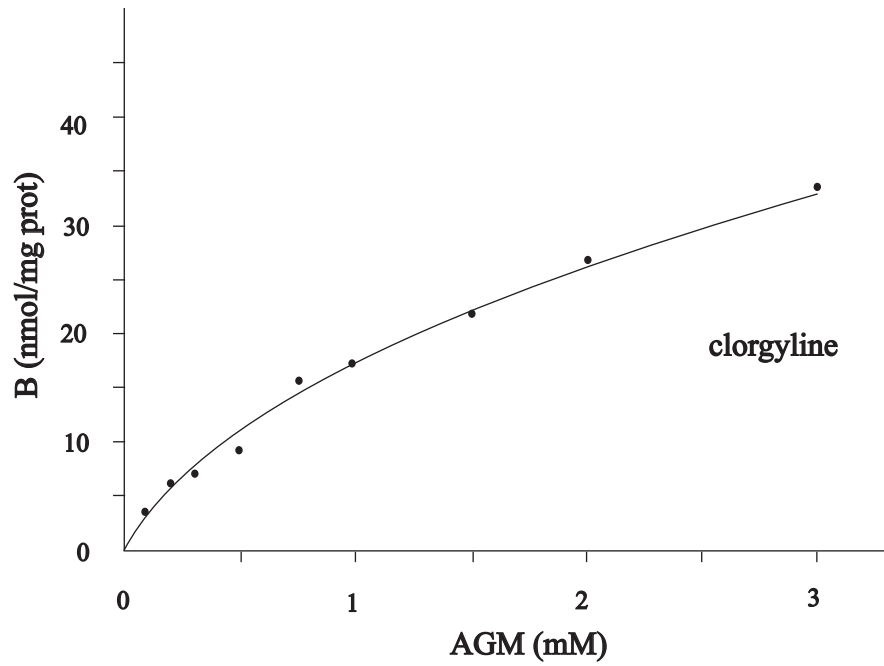


Figure 9



# Figure 10

## A



## B

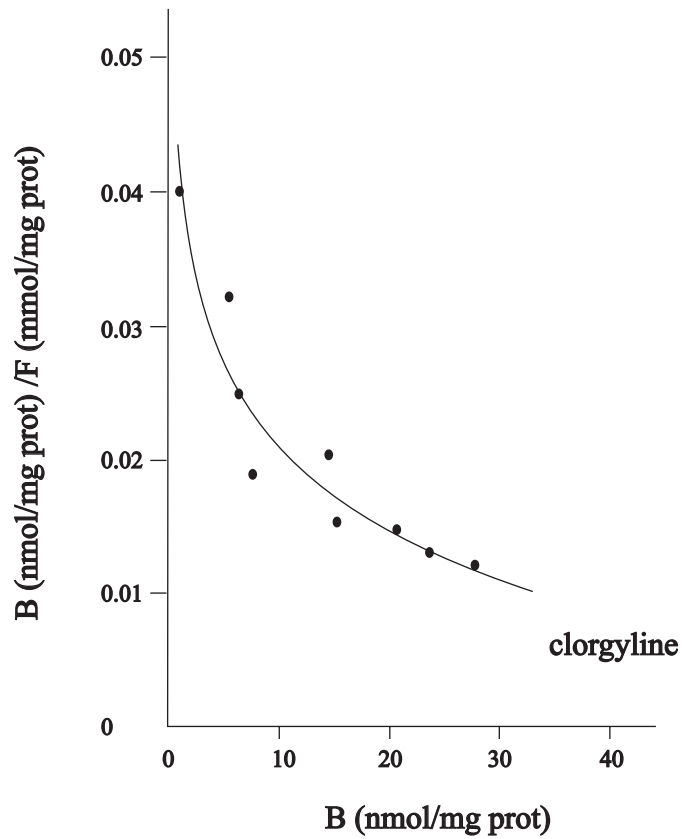


Figure 11

