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**Age-related increases in methylguanidine: Inhibitory effect of  
Bio-catalyzer in aged rat brain**

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**Running title: Inhibitory action of Bio-catalyzer on methylguanidine during ageing**

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## ABSTRACT

By HPLC-guanidino compound analyzer coupled with electrochemical detector using the phenanthrenequinone reaction, methylguanidine (MG) - a potent uremic toxin and a hydroxyl radical-mediated peroxidative of creatinine (CRN) - was found to increase with age in rat brain. Likewise, age-related increases were observed on creatinine (CRN) and - guanidinoacetic acid (GAA).

Oral administration of 0.1 g/kg body weight of Bio-catalyzer for 27 weeks on 89 week-old rats decreased significantly the elevated levels of MG and CRN in the brain - indicative of the antioxidant role of Bio-catalyzer in inhibiting hydroxyl radical-mediated synthesis of MG from CRN.

**Keywords:** methylguanidine, creatinine, <sup>件</sup>guanidine, lipid peroxidation, hydroxyl radical, free radical scavenger/antioxidant, Bio-catalyzer.

## INTRODUCTION

Methylguanidine (MG), a known potent uremic toxin<sup>1-2</sup> is distributed, aside from kidney, in the rat heart<sup>3</sup>, erythrocyte<sup>3</sup>, intestine<sup>3</sup>, liver<sup>4</sup>, plasma<sup>3,4</sup>, and brain<sup>5-8</sup>. It is formed from creatinine (CRN) via reactive oxygen species (ROS) in particular the hydroxyl radical-generated Fenton reagents<sup>10,11</sup>. Free hemoglobin acting as a biological Fenton reagent<sup>12</sup> and iron promoter forms hydroxyl radical. Since hydroxyl radical is implicated in various diseases and disorders, determination of MG as a marker of this radical could prove useful in studying the mechanism of these free-radical related diseases and disorders.

The hydroxyl radical has been used as a marker in uremic and hemodialyzed patients<sup>14</sup>, rat hepatocytes<sup>15</sup> and brain<sup>16-18</sup>. High levels of methylguanidine have been demonstrated to induce convulsion in cats and rabbits<sup>19</sup> and epileptic seizures in mice<sup>20</sup>. No data however is available on the formation of MG and its derivatives during the ageing process. By high performance liquid chromatographic and fluorometrical analyses using the phenanthrenequinone (PQ) reaction<sup>21,22</sup>, we determined MG and its precursor CRN<sup>9</sup> and - guanidinoacetic acid (GAA) in adult and aged rat brain.

Bio-catalyzer, a commercial natural fermented health food supplement in Japan with proven scavenging action on free radicals and lipid peroxide in epileptic foci in rats<sup>23</sup> - indicative of its anti-epileptic property, increased the mitochondrial and cytosolic superoxide dismutase and decreased thiobarbituric acid-reactive substances in aged rat brain (unpublished report) - suggesting its anti-ageing property. Since free radicals are normal metabolic products of oxidative stress, we studied their effects and the effect of oral administration of Bio-catalyzer on these vital guanidino compounds in the brain of aged rats.

## MATERIALS AND METHODS

**Bio-catalyzer** Bio-catalyzer  $\alpha$ -p No. 11 (Bio-normalizer) is a white, sweet health food preparation produced by fermentation of Carica papaya Linn. ( a Philippine medicinal plant) and other tropical herbal plants<sup>23</sup>; and is available commercially from Sun-O International Inc., Gifu, Japan.

**Animals** Male Sprague-Dawley rats from Clea Japan Inc., (Tokyo, Japan) aged 12 and 89 weeks were used. Thirty five aged rats (89 week-old) were housed in individual aluminum cages for 27 weeks (including 1-week acclimatization) at 20-26°C, at humidity of 40-70%, ventilation of 15-25 times/h, light of 150-160 lux for 12 h/day from 7:00 to 19:00, noise < 60 phon, ammonia of < 20 ppm, air stream of 25 cm/sec and were maintained under strict sanitary conditions. The animals were allowed free access to water and a standard diet (CE-2 pellet supplied by Clea Japan, Inc., Tokyo). The rats were divided randomly into two groups; the control group consisting of 20 rats were orally administered with tap water boiled for 30 min while the second group of 15 rats were orally administered with 0.1 g/kg body weight (BW) of Bio-catalyzer for 27 consecutive weeks (189 days) at a volume dosage of 0.1 ml/100g animal weight. This dose approximates the recommended dose for humans which is 3-9 g/day. The administration was done from 14:00 to 17:00 after 2-3 h period of restricted food and water. The rats were sacrificed on the 27th week of Bio-catalyzer treatment by cervical decapitation and were immersed in dry ice, with the whole brain rapidly removed. The cortex was dissected on an ice plate<sup>24</sup>, frozen in liquid nitrogen and kept at - 80°C until analysis.

**Chemicals** Guanidino standards such as guanidinoacetic acid (GAA), creatinine (CRN), guanidine (G) and methylguanidine (MG) were obtained from Wako Pure Chemical Ind. Ltd., Osaka; homoarginine (HArg), guanidinosuccinic acid (GSA) and guanidinopropionic acid (GPA) from Sigma Chemical Co., St. Louis, MO; arginine (Arg) from Katayama Chemical, Osaka; guanidinoethanesulfonate (GES) and guanidinobutyric acid (GBA) from Ono Pharmaceutical Co., Osaka; N-acetylarginine (NAA) from Eastman Kodak Co., NY; and guanidinoglutaric acid (GGA) was synthesized from glutamic acid and S-methylisothiurea<sup>25</sup>. Sodium hydroxide (NaOH), sodium citrate, sodium capric acid, boric acid, 9,10-phenanthrenequinone (PQ), and N,N'-dimethylformamide (DMF) were purchased from Wako Pure Chemical, Osaka while hydrochloric acid (HCl) was from Nacalai Tesque, Inc., Kyoto. All other chemicals and reagents used were of the highest grade available from commercial suppliers.

**Analysis of Guanidino compounds** The guanidino compounds in vacuo-concentrate prepared as shown in Fig. 1 was dissolved in 1.8 ml of HCl solution, pH 2.2 and analyzed by high pressure liquid chromatography system JASCO G-250 guanidino compound analyzer (Tokyo) with electrochemical detector JASCO FP-100B fluorometer (Tokyo) using the PQ reaction<sup>21,22</sup>. The analytical conditions employed



were as column, Guanidino pak III (6 x 35 mm, JASCO, Tokyo); temperature, 70°C; flowrate, 1.0 ml/min (eluent); flowrate, 0.5 ml/min (0.025% PQ in DMF); flowrate, 0.5 ml/min (0.1 M NaOH); reaction coil, 0.5 mm I.D. x 5 m L.; detector, fluorometer (JASCO FP-110, Ex 365 nm, Em 495 nm), and sample volume of 200 µl. Four eluents of 0.4 M sodium citrate buffer with different pH values and elution time corresponding to pH 3.0 at 10.2 min, pH 3.5 at 4.0 min, pH 5.25 at 4.2 min, and pH 10.0 at 9.4 min were used. The fifth eluent of 1 N NaOH was eluted within 5.2 min. The fluorescence reagent consists of 2 N NaOH and 0.025% PQ in DMF. Details are given in previous studies<sup>21,22</sup>.

**Statistical analysis** Statistical analysis was performed using Student's t-test.

## RESULTS

Important guanidino compounds related to the synthesis of MG such as CRN and GAA including MG were processed (as shown in Fig. 1), identified in the brain cortex of adult rats, aged rats and Bio-catalyzer-treated aged rats. Figure 2 shows the typical HPLC chromatogram of different guanidino compounds in the brain of these three groups of animals and the authentic samples of guanidino standards. The chromatograms reveal 5 peaks corresponding to GAA, CRN, Arg, HArg, and MG which were eluted at about 2.0, 11.5, 16.5, 20.0, and 26.5 min, respectively. For this study however, only GAA, CRN and MG were quantified. Compared to the adult brain, MG ( $p < 0.025$ ), CRN ( $p < 0.025$ ) and GAA ( $p < 0.001$ ) were increased in the aged rat brain (Fig. 3, 4 and 5). The elevated levels of CRN ( $p < 0.005$ ) and MG ( $p < 0.025$ ) but not GAA in the brain of aged rats however, were suppressed by the 27-week oral administration of 0.1 g/kg BW of Bio-catalyzer.

## DISCUSSION

Reactive oxygen species (ROS) such as hydroxyl radicals with their ability to react with MG<sup>17,18</sup> have been used as markers in the serum and plasma of hemodialyzed patients<sup>14</sup> and in rat hepatocytes<sup>15,16</sup>. The convulsive activity and epileptic seizures in cats and rabbits<sup>19</sup> and mice<sup>20</sup> and spike discharges in the rat encephalograms<sup>26</sup> were attributed to increased level in MG. The synthesis of MG has been suggested to be mediated by ROS. ROS, in particular hydroxyl radicals, may be generated from free hemoglobin which acts as biological Fenton reagent<sup>12</sup> or iron promoter of Fenton reaction<sup>13</sup>. The neurotoxicology of MG has been reported<sup>27</sup> and has been implicated in brain dysfunctions, such as epilepsy<sup>28,29</sup> but its effect on ageing has not been studied. Most recently, we found that while Bio-catalyzer

increased the elevated levels of superoxide dismutase, it inhibited the elevated levels of thiobarbituric acid-reactive substances (TBARS) in aged rat brain (unpublished data). Augmenting these observations, our findings reveal that MG and its precursors CRN and GAA were significantly increased in the brain of aged rats. Figure 6 shows the possible metabolic pathway for the synthesis of methylguanidine in the brain. MG, which is hydroxyl radical-mediated and is a peroxidative product of CRN<sup>12,13</sup> and creatol<sup>30</sup>, increases with a increase in CRN - indicative of the active participation of hydroxyl radical during the ageing process<sup>31</sup>. CRN was synthesized from GAA, hence an increased GAA led to an increase in CRN. These age-related increases in MG and CRN but not in GAA, were suppressed significantly by Bio-catalyzer at 0.1 g/kg body weight of the animals - a dose that approximates the recommended dose for humans. Bio-catalyzer, a proven scavenger of hydroxyl radicals and an inhibitor of TBARS in iron-induced lipid peroxidative damage in epileptic foci in rats<sup>23</sup>, is thought to have inhibited the hydroxyl radical-mediated peroxidative conversion of CRN to MG, thus decreasing the levels of MG and CRN in aged rat brain. The results also indicate that the synthesis of CRN from GAA does not involve hydroxyl radical intermediates.

The current findings add to the evidence that a precursor CRN to MG synthesis is mediated by hydroxyl radical. MG may therefore be used as a marker radical formation in the brain and could prove useful in studying the mechanism of ageing and other brain dysfunctions believed to be caused by free radicals. The proposed metabolic pathway of CRN and MG in the brain may be similarly linked to that in liver and kidney in cases of acute and chronic renal failures<sup>10,11,14-16,32</sup>. We speculate that Bio-catalyzer, a good inhibitor of hydroxyl radicals, may be an effective agent in arresting elevated levels of toxic convulsant MG in the brain of aged rats - indicative of its anti-convulsive and/or anti-ageing properties. As an inhibitor of methylguanidine, Bio-catalyzer may also be a useful clinical agent in the amelioration of epilepsy, acute and chronic renal failure, among others.



guanidino compounds  
入ると自動修正で guanidine になってほう

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## FIGURE LEGEND

Fig. 1. Isolation of guanidino compounds in rat brain tissue.

Fig. 2. HPLC chromatogram of guanidino compounds in the cortex of rats orally administered with 0.1 g/kg body weight of Bio-catalyzer for 27 consecutive weeks (A), and of the standard sample. (1) guanidinoethane sulfonate, GES; (2) guanidinosuccinic acid, GSA; (3) guanidinoglutaric acid, GGA; (4) guanidinoacetic acid, GAA; (5) N-acetylarginine, NAA; (6) guanidinopropionic acid, GPA; (7) creatinine, CRN; (8) guanidinobutyric acid GBA; (9) arginine, ARG; (10) homoarginine, HARG; (11) guanidine, G; and (12) methylguanidine, MG.

Fig. 3. Effect of oral administration of 0.1 g/kg body weight of Bio-catalyzer on elevated level of methylguanidine in aged rat brain. (A) adult rat, (B) aged rat, and (C) treated-aged rat. Values are means + SEM of 5-6 animals. <sup>a</sup>p < 0.025 versus (A); <sup>b</sup>p < 0.025 versus (B).

Fig. 4. Effect of oral administration of 0.1 g/kg body weight of Bio-catalyzer on elevated level of creatinine in aged rat brain. (A) adult rat, (B) aged rat, and (C) treated-aged rat. Values are means + SEM of 4 animals. <sup>a,b</sup>p < 0.025 versus (A); <sup>c</sup>p < 0.005 versus (B).

Fig. 5. Effect of oral administration of 0.1 g/kg body weight of Bio-catalyzer on elevated level of - guanidinoacetic acid in aged rat brain. (A) adult rat, (B) aged rat, and (C) treated-aged rat. Values are means + SEM of 6 animals. <sup>a</sup>p < 0.001 versus (A). EDIT

Fig. 6. Metabolic pathway of methylguanidine synthesis in the brain.



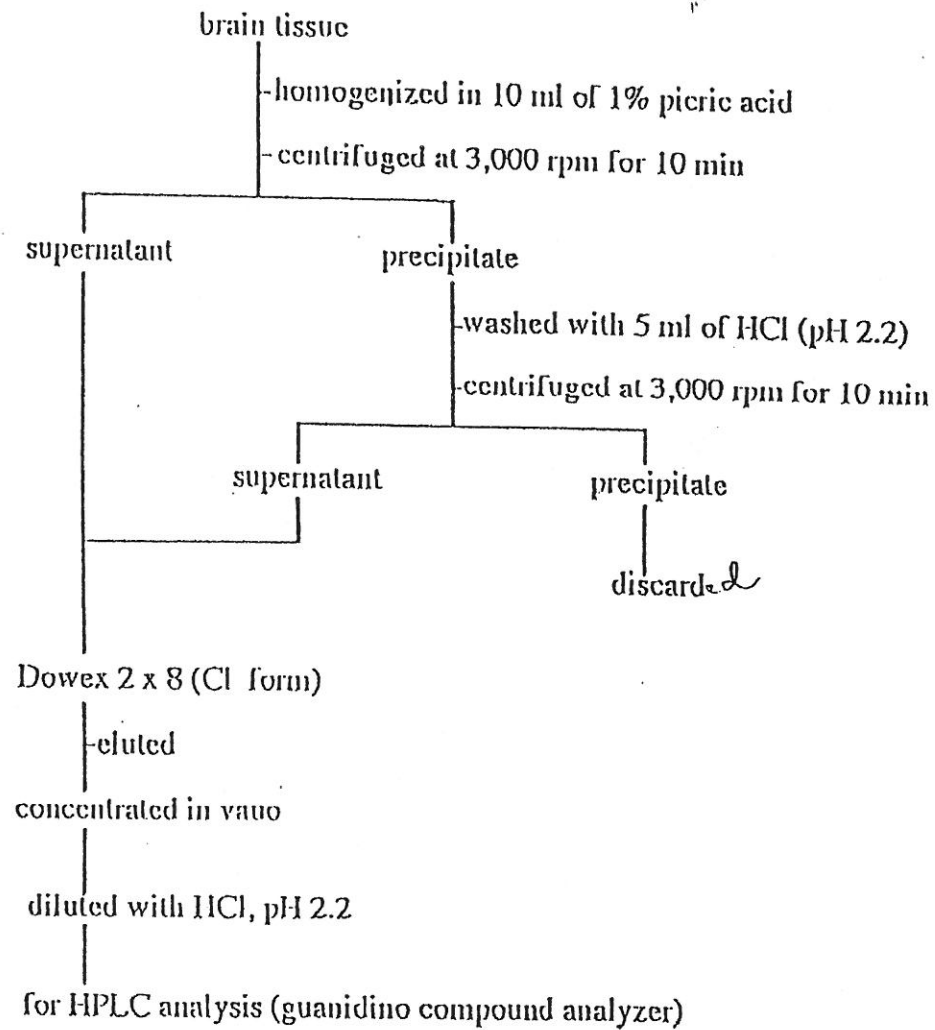


Figure 1

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Inhibitory action of Bio-catalyzer on methylguanidine

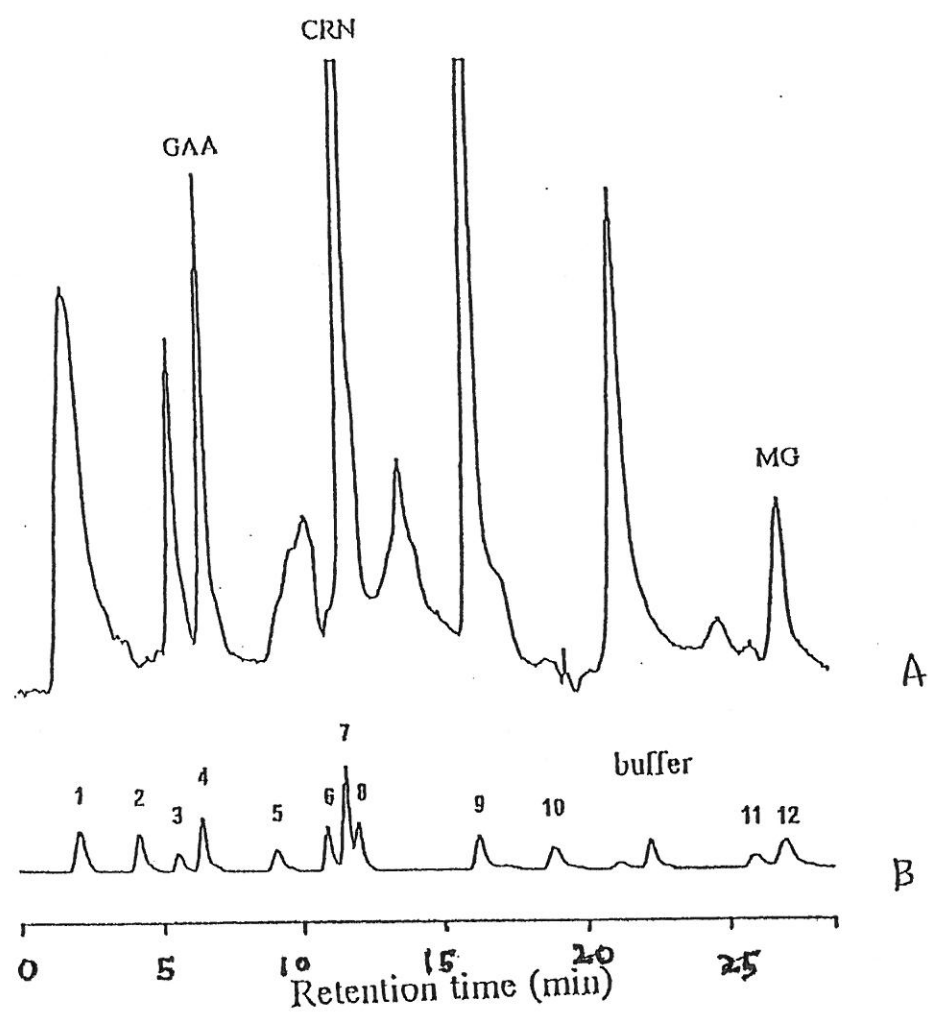


Figure 2

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Inhibitory action of Bio-catalyzer on methylguanidine



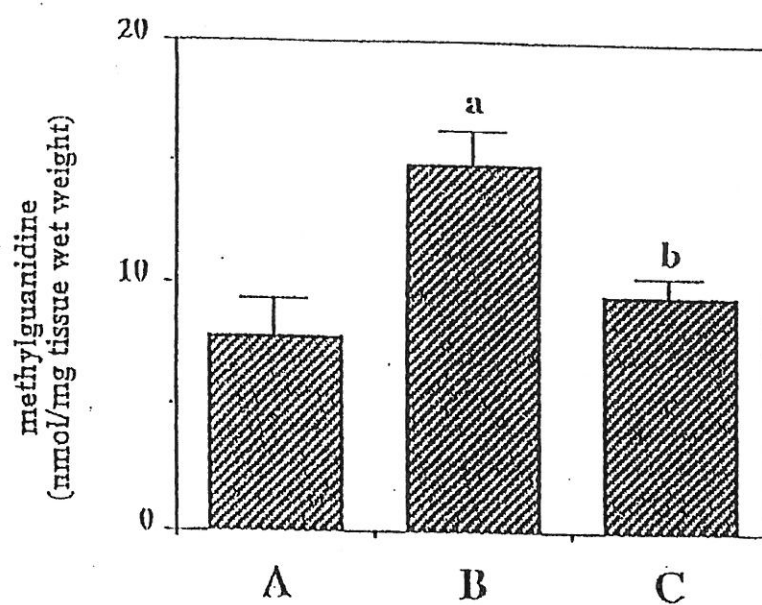


Figure 3

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Inhibitory action of Bio-catalyzer on methylguanidine

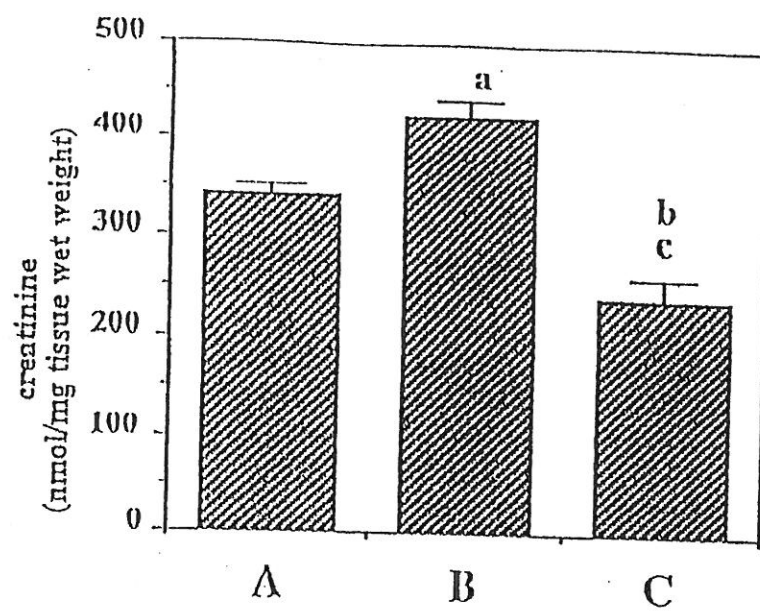


Figure 4

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Inhibitory action of Bio-catalyzer on methylguanidine



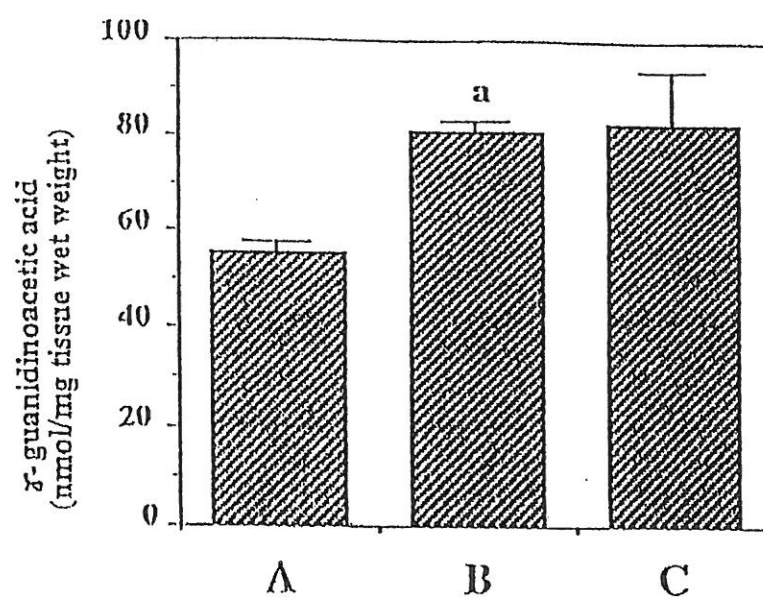


Figure 5

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Inhibitory action of Bio-catalyzer on methylguanidine

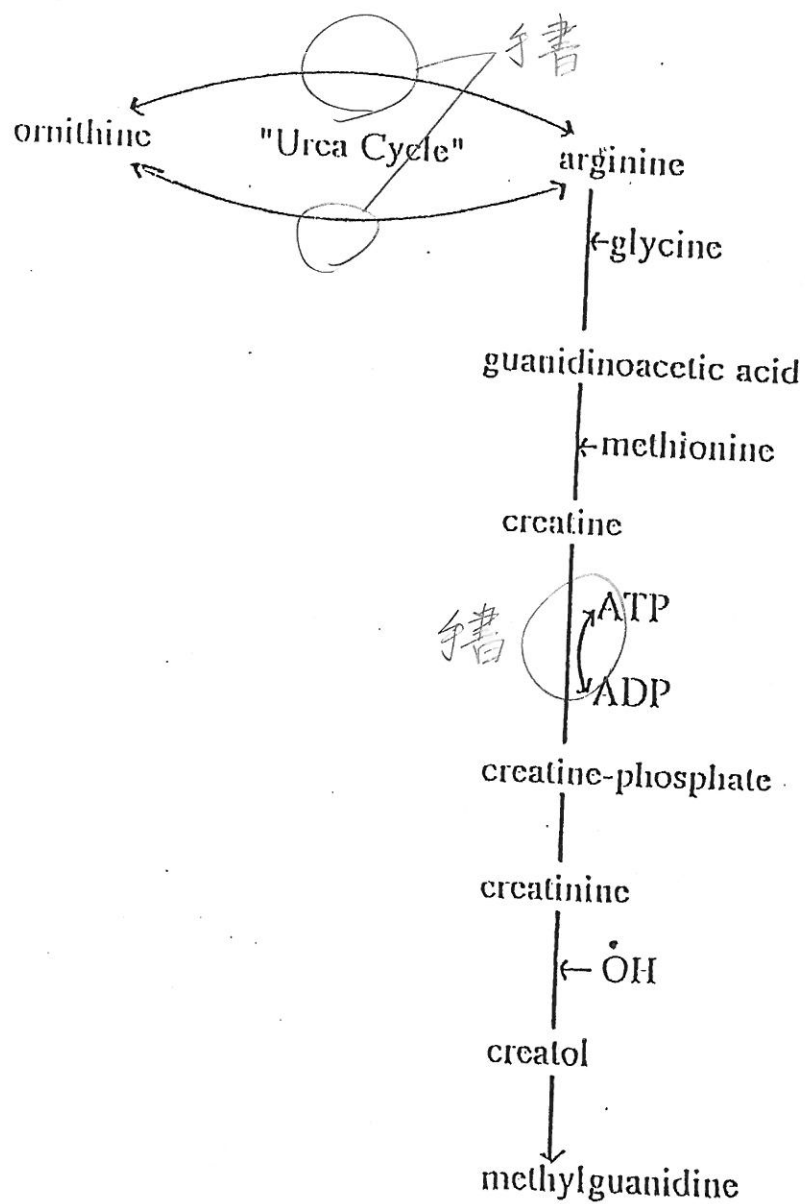


Figure 6

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