The Effects of Administration of Semicarbazide and Aminooxyacetic Acid on $\text{B}_6$ Vitamer Levels in Subcellular Fractions of Mouse Brain

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Summary The in vivo effects of semicarbazide (SC) and aminooxyacetic acid (AOAA) on the $\text{B}_6$ vitamer concentrations in brain subcellular fractions, especially in extrasynaptosomal, synaptosomal plasma, extrasynaptosomal and synaptosomal mitochondria, were investigated. Treatment of mice with SC decreased levels of pyridoxal phosphate (PLP) in extrasynaptosomal and synaptosomal plasma, while it had no effect on those in extrasynaptosomal and synaptosomal mitochondria. On the contrary, treatment with AOAA more significantly decreased pyridoxamine phosphate (PMP) levels in extrasynaptosomal and synaptosomal mitochondria than those in extrasynaptosomal and synaptosomal plasma. According to these results, the difference between in vivo effects of SC and AOAA on vitamin $\text{B}_6$-enzymes was discussed.

Key Words semicarbazide, aminooxyacetic acid, $\text{B}_6$ deficiency convulsions, $\text{B}_6$ vitamers in subcellular fractions of brain

Both semicarbazide (SC) and aminooxyacetic acid (AOAA) are known to be carbonyl-trapping agents and thereby to be vitamin $\text{B}_6$ depleters which form respective complexes with pyridoxal (PL) or pyridoxal phosphate (PLP). Administration of both agents to animals actually produces running fits as well as clonic and tonic convulsions which are observed in vitamin $\text{B}_6$-deficient animals. However, AOAA at a subconvulsant dose is known to have a powerful anticonvulsant action, and it actually prevents the convulsions induced by SC (1,2). Thus, the effect of AOAA at a subconvulsant dose on $\text{B}_6$ vitamer contents seems to differ from that of SC.

The present investigation was undertaken to determine the effect of SC and AOAA on the contents of $\text{B}_6$ vitamers in subcellular fractions of mouse brain.
MATERIALS AND METHODS

B₆ vitamers were obtained from Nakarai Chemicals. Acid phosphatase was purchased from Boehringer Mannheim.

Administration of drugs. Adult albino mice (DDY strain) were used throughout the present experiments.

Solutions of the drugs (SC and AOAA) were prepared daily in 0.9% NaCl, the pH being adjusted to 7.0 immediately before use. The final concentration of the drugs was adjusted in such a way as to give the required dose in a volume equivalent to 1 per cent of the body weight of the animal. The drugs were injected intraperitoneally and the injected animals were kept in a laboratory with minimal background noise. Control animals were similarly treated with a 0.9% NaCl alone.

Subcellular fractionation of brain. The animals were decapitated and the brains were quickly removed. A 10% brain homogenate in 0.32M sucrose was prepared in a Teflon-glass homogenizer under gentle conditions. The primary fractions (nuclei, crude mitochondria, microsomes and extrasynaptosomal plasma) were obtained following the procedure of Gray and Whittaker (3). Pellets containing each fraction were resuspended in cold water and used for assay.

Subfractionation of crude mitochondria. The pellet containing crude mitochondria was subfractionated by following the procedure of Sellström et al. (4). The pellet was resuspended in 3 ml of 0.32M sucrose solution and placed over layers of 5 ml of 4 and 15% Ficoll made up in the sucrose solution. This gradient was then centrifuged for 45 min at 63,000 g in an Hitachi RPS 40T swinging-bucket rotor. All the subfractions obtained (myelin, synaptosomes and extrasynaptosomal mitochondria) were manually separated with a Pasteur pipette and diluted with 0.32M sucrose and again centrifuged at 100,000 g for 30 min. The pellets were resuspended in cold water and used for assay.

Preparation of synaptosomal organelles. A modification of the procedure of Whittaker et al. (5) was employed. The synaptosome fraction was osmotically shocked in water (2 ml/g original brain), and the aqueous suspension was layered on a discontinuous density gradient consisting of three layers of 0.4, 0.6 and 1.2 M sucrose. This gradient was then centrifuged for 110 min at 75,000 g in a Hitachi RPS 40T swinging-bucket rotor. All the subfractions obtained (synaptosomal plasma, synaptic vesicles and synaptosomal mitochondria) were manually separated with a Pasteur pipette and used for assay.

Extraction, separation and assays of B₆ vitamers. Portions of the subcellular fractions were acidified with perchloric acid to a final concentration of 1 N and centrifuged. The deproteinized clear supernatant fluids were neutralized with 5 N KOH to pH about 6.0 and the formed K perchlorate was removed by centrifugation. The supernatants were adjusted to pH 3.5 with acetic acid and subjected to ion exchange chromatography.

The extract (0.7 ml) was applied to an Amberlite CG-120 column (4 × 35 mm) and the elution was performed according to the procedure of Loo and Badger (6).
The column was washed with 1.0 ml of water, and the first 1.7 ml of effluent was collected in one fraction and held for the assay of PLP and pyridoxine phosphate (PNP). The resin was washed successively with various buffers, as follows: for pyridoxamine phosphate (PMP), with 2.5 ml of 0.1 M acetate buffer at pH 4.0 after 1.5 ml of 0.01 M acetate buffer at pH 4.0; for PL, with 2.0 ml of 0.1 M Na phosphate buffer at pH 6.0 after 1.0 ml of 0.1 M acetate buffer at pH 5.0; for PN, with 2.0 ml of 0.1 M Na phosphate buffer at pH 6.5; for pyridoxamine (PM), with 2.5 ml of 0.1 M Na phosphate buffer at pH 8.5. Flow rate through the column was about 0.02 ml/min. Since PLP and PNP were eluted in the common fraction as described above, both the phosphate esters were hydrolyzed to PL and PN by the action of acid phosphatase (0.5 mg/ml, incubation for 60 min at 37°C) and then separated by rechromatography with the same resin. PMP was also determined after being hydrolyzed to PM by the phosphatase.

The B₆ vitamers in the eluates were individually assayed microbiologically with *Saccharomyces carlsbergensis* (ATCC 9080) against its own vitamer as a reference standard which was dissolved in saturated K-perchlorate and treated on the column in the same way. The assay method was modified from that of Chiao and Peterson (7): the concentration of each component except hydrolyzed casein and sugar was half that of their basal medium. Thiamine was added at 500 μg/liter. Appropriate aliquots (0.1–2.5 ml) of the eluates were inoculated with 0.05 ml of a diluted 20-hr culture of the yeast (optical density at 610 nm = 0.3), grown on limited B₆ media. The tubes were incubated for 20 hr at 30°C in an incubator. Growth was estimated by reading at 610 nm in a colorimeter.

**Determination of marker enzyme activities.** Succinate dehydrogenase [EC 1.3.99.1] (SDH) was assayed by the method of Slater and Bonner (8), as modified by De Robertis et al. (9). Lactate dehydrogenase [EC 1.1.1.27] (LDH) was assayed using the method of Kubowitz and Ott (10) with minor modifications; the reaction mixture contained 0.6 mM pyruvate, 0.18 mM NADH and 50 mM phosphate buffer pH 7.5.

Protein was measured according to Lowry et al. (11).

**Statistical analysis.** When required, statistical analysis was carried out using Student’s *t*-test. The term ‘significant’ is used in this paper to denote significance at *p* < 0.001.

**RESULTS**

The subcellular localizations of marker enzymes were similar to those previously reported (12), which were obtained from the subcellular fractions prepared by a different method from that described above. As shown in Table 1, LDH (known to be a soluble-cytoplasmic marker) was localized mainly in the extrasynaptosomal and the synaptosomal plasma; SDH (known to be a mitochondrial marker) was mainly in the extrasynaptosomal and the synaptosomal mitochondria. The observed distribution of protein in this experiment was also similar to that...
Table 1. Distribution of SDH, LDH and protein in the subcellular fractions from mouse brain.

| Fraction                  | Protein (mg/g of brain) | SDH ($\Delta E_{400}$/g/min) | LDH ($\Delta E_{340}$/g/min) |
|---------------------------|-------------------------|-------------------------------|-----------------------------|
| Extrasynaptosomal plasma  | 22.6                    | 0.3                           | 131.7                       |
| Extrasynaptosomal mitochondria | 10.4                | 5.0                           | 6.5                         |
| Synaptosomal plasma       | 3.3                     | 0.06                          | 26.6                        |
| Synaptosomal mitochondria | 4.1                     | 2.35                          | 1.6                         |

previously reported by Whittaker et al. (5).

Distribution of $B_6$ vitamers in subcellular fractions

Normal $B_6$ distribution in extrasynaptosomal plasma, synaptosomal plasma, extrasynaptosomal mitochondria and synaptosomal mitochondria is tabulated in Table 2 (only data of these four fractions are shown, since most of each $B_6$ vitamer was localized in plasmic and mitochondrial fractions). It will be observed that there are large amounts of PLP and PMP in all fractions, and that PLP is mostly localized in the extrasynaptosomal plasma. Among the free forms of the vitamers, PL was found in significantly high concentrations in the extrasynaptosomal and the synaptosomal plasma. PNP, PN and PM are found in only trace amounts in all the fractions.

Effect of SC administration on subcellular levels of $B_6$ vitamers

The effect of SC on subcellular levels of $B_6$ vitamers was studied using a convulsant dose (1.8 nmol/kg). The mice injected with SC were decapitated at the first onset of convulsions (85 ± 4 min after the injection), and $B_6$ vitamers in the subcellular fractions of brain were assayed. Table 3 shows only the contents of PLP, PMP and PL which are found in high concentration in every fraction. The SC treatment decreased PLP contents in the extrasynaptosomal and the synaptosomal plasma to about 42% and 66% of the control, respectively, whereas the PLP contents in the extrasynaptosomal and the synaptosomal mitochondria were not affected. PMP and PL levels were not significantly affected in any of the subcellular fractions.

Effect of AOAA administration on subcellular levels of $B_6$ vitamers

The effect of AOAA on $B_6$ vitamer levels was studied using an anticonvulsant dose (0.2 mmol/kg). The mice injected with AOAA were sacrificed 120 min after the injection, and the $B_6$ vitamers in the plasma and the mitochondria were assayed
Table 2. Distribution of B₆ vitamers in the subcellular fractions from mouse brain.

| Fractions               | No. of experiments | PLP  | PNP  | PMP (nmol/g of brain) | PL   | PN   | PM   |
|-------------------------|--------------------|------|------|-----------------------|------|------|------|
| Extrasynaptosomal plasma| 19                 | 3.90 | 0.192| 1.44                  | 0.694| 0.0056| 0.0696|
|                         | ± 0.21             | ± 0.006| ± 0.10| ± 0.034               | ± 0.0029| ± 0.0478|
| Extrasynaptosomal mitochondria | 18              | 1.54 | 0.09 | 1.56                  | 0.091| 0.0022| 0.0414|
|                         | ± 0.11             | ± 0.004| ± 0.19| ± 0.01               | ± 0.0009| ± 0.0300|
| Synaptosomal plasma     | 14                 | 0.458| 0.003| 0.414                 | 0.150| 0.0027| 0.0190|
|                         | ± 0.043             | ± 0.001| ± 0.050| ± 0.015             | ± 0.0002| ± 0.0108|
| Synaptosomal mitochondria | 14             | 0.489| 0.002| 0.336                 | 0.015| 0.0013| 0.0144|
|                         | ± 0.050             | ± 0.001| ± 0.045| ± 0.003             | ± 0.0004| ± 0.0021|

Values are the means ± SD.
Table 3. Effects of SC and AOAA administrations on the PLP and PMP levels in the subcellular fractions of mouse brain.

| Fraction treatment                     | No. of experiments | PLP (nmol) | PMP (nmol/g of brain) | PL (nmol/g of brain) |
|----------------------------------------|--------------------|------------|-----------------------|-----------------------|
|                                        |                    |            |                       |                       |
| Extrasynaptosomal plasma               |                    |            |                       |                       |
| Control                                | 19                 | 3.90 ± 0.21| 1.44 ± 0.10           | 0.694 ± 0.034         |
| SC (1.8 mmol/kg)                       | 12                 | 1.64 ± 0.46*| 1.32 ± 0.26           | 0.673 ± 0.059         |
| AOAA (0.2 mmol/kg)                     | 8                  | 3.59 ± 0.53| 1.30 ± 0.39           | 0.673 ± 0.069         |
| SC + AOAA (1.8 + 0.2 mmol/kg)          | 5                  | 1.68 ± 0.27*| 1.25 ± 0.38           | 0.724 ± 0.159         |
| Extrasynaptosomal mitochondria         |                    |            |                       |                       |
| Control                                | 18                 | 1.54 ± 0.11| 1.56 ± 0.19           | 0.091 ± 0.010         |
| SC (1.8 mmol/kg)                       | 11                 | 1.50 ± 0.23| 1.31 ± 0.19           | 0.079 ± 0.015         |
| AOAA (0.2 mmol/kg)                     | 8                  | 1.39 ± 0.56| 1.13 ± 0.10*          | —                     |
| SC + AOAA (1.8 + 0.2 mmol/kg)          | 6                  | 0.88 ± 0.11*| 0.68 ± 0.26*          | —                     |
| Synaptosomal plasma                    |                    |            |                       |                       |
| Control                                | 14                 | 0.458 ± 0.043| 0.414 ± 0.050    | 0.150 ± 0.015         |
| SC (1.8 mmol/kg)                       | 10                 | 0.306 ± 0.071*| 0.335 ± 0.082    | 0.129 ± 0.025         |
| AOAA (0.2 mmol/kg)                     | 9                  | 0.433 ± 0.071| 0.326 ± 0.124    | 0.122 ± 0.018         |
| SC + AOAA (1.8 + 0.2 mmol/kg)          | 6                  | 0.291 ± 0.081*| 0.244 ± 0.076*  | —                     |
| Synaptosomal mitochondria              |                    |            |                       |                       |
| Control                                | 14                 | 0.489 ± 0.050| 0.336 ± 0.046    | 0.015 ± 0.003         |
| SC (1.8 mmol/kg)                       | 9                  | 0.502 ± 0.088| 0.329 ± 0.053    | 0.015 ± 0.004         |
| AOAA (0.2 mmol/kg)                     | 8                  | 0.499 ± 0.078| 0.248 ± 0.022*   | 0.018 ± 0.003         |
| SC + AOAA (1.8 + 0.2 mmol/kg)          | 6                  | 0.576 ± 0.108| 0.250 ± 0.032*   | —                     |

Values are the means ± SD. *Significantly different from control values p < 0.001. — = not tested.
(Table 3). In contrast with SC treatment, the AOAA treatment resulted in significant decreases of PMP contents in the extrasynaptosomal (to 72% of the control) and the synaptosomal mitochondria (to 74% of the control) without altering PLP contents in either plasma. PMP levels in the synaptosomal plasma also appeared to be slightly decreased by this treatment, but the decrease was not statistically significant because of the relatively large standard errors. PLP and PL levels in all the subcellular fractions were hardly affected by this treatment.

Effect of administration of SC together with AOAA on subcellular levels of B₆ vitamers

The effect of SC together with AOAA on the vitamer levels was studied using mice which were pretreated with an anticonvulsant dose of AOAA (0.2 mmol/kg) 35 min prior to treatment with a convulsant dose of SC (1.8 mmol/kg). The animals treated with SC plus AOAA were decapitated 85 min after the SC treatment (corresponding to 120 min after the AOAA pretreatment). The administration of SC plus AOAA significantly decreased PLP levels in both the plasmas and PMP levels in both the mitochondria (Table 3). Moreover, a significant decrease of PLP and of PMP was observed in the extrasynaptosomal mitochondria and in the synaptosomal plasma, respectively. The decrease of PLP in both the plasmas was similar to that observed with SC alone, while the decreases of PLP and PMP in the extrasynaptosomal mitochondria as well as the decrease of PMP in the synaptosomal plasma appeared to be strengthened by cooperation of the two drugs.

DISCUSSION

The effects of SC and AOAA administrations on B₆ vitamer levels in four subcellular fractions (extrasynaptosomal plasma and mitochondria and synaptosomal plasma and mitochondria) were studied.

The treatment of mice with a convulsant dose of SC produced a decrease of PLP in the extrasynaptosomal and the synaptosomal plasma without altering its levels in the extrasynaptosomal and the synaptosomal mitochondria. In contrast, the same treatment did not have a significant effect on PMP levels in any of the fractions. The decrease of PLP in both plasmas can be explained by the formation of PLP- (or PL-) semicarbazone and by the known inhibitory action of the formed semicarbazones upon the activity of pyridoxal kinase (13, 14), which is a soluble-cytoplasmic enzyme (15). The lack of effect of SC on the PLP levels in both the mitochondria could reflect that SC can scarcely penetrate the organella.

The decrease of PLP in both plasmas would well explain the inhibition of glutamate decarboxylase (GAD) induced by the SC treatment, since the enzyme is located mainly in the plasmic fractions and, besides, the enzyme activity is closely dependent on PLP concentration (2, 16–21). The inhibition of the enzyme activity, with a consequent reduction of GABA levels, could be an important factor in the production of convulsions by SC (2, 19–21).

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On the other hand, the treatment of animals with an anticonvulsant dose of AOAA produced a decrease of PMP in the extrasynaptosomal and the synaptosomal mitochondria, as that previously reported for the whole brain (22). The decrease of PMP levels in both the mitochondria seems to be an unusual phenomenon, since it is usually expected that AOAA would attack and decrease PLP in cytoplasm rather than PMP in mitochondria. However, it might be possible that AOAA would be actively taken in the mitochondria and then in someway decrease the PMP content in the organella. The decrease of PMP in mitochondria may be explained by a postulation that AOAA decreases PLP content in mitochondria but the decreased PLP is immediately recovered at the expense of PMP through the deamination reaction from PMP forms to PLP forms of transaminase; the deamination is probably performed by a supply of $\alpha$-ketoglutaric acid which is synthesized in mitochondria. The decrease of PMP content or the lack of balance in PLP and PMP contents shown here might be related to the fact reported previously that activity of GABA-$\alpha$-ketoglutaric acid transaminase (GABA-T) was inhibited (to 20–30% of control) by AOAA treatment (2).

On the contrary, the AOAA treatment did not affect PLP contents in any of the subcellular fractions (Table 3). The lack of effect of AOAA on the PLP content in both plasmas may correspond to the fact that the AOAA treatment does not show inhibition on activity of GAD (2), which is found mainly in both plasmas.

When the convulsions by SC were protected by pretreatment of AOAA, the decreases of PLP in both plasmas as well as those of PMP in both mitochondria were found. The decreases by SC of PLP in both plasmas were not in the least strengthened by AOAA pretreatment, but the decreases of PLP and PMP in the extrasynaptosomal mitochondria and of PMP in the synaptosomal plasma were strengthened by a cooperation of the two drugs, though the explanation for these results is not easy. These results, that the treatment of AOAA prior to SC injection does not affect the PLP content decreased by SC in both plasmas but it decreases the PLP and PMP contents in both the mitochondria, may correspond to the fact that the AOAA pretreatment does not affect GAD activity inhibited by SC in both plasmas but it inhibits GABA-T activity in both mitochondria (2), since GAD is a PLP-dependent enzyme and it localized exclusively in both plasmas while GABA-T is both a PLP- and PMP-dependent enzyme and is localized exclusively in both mitochondria (2).

Recent studies in this laboratory suggested that the onset of convulsions by SC was accompanied by a fall in synaptosomal GABA levels while the cessation by AOAA pretreatment of the convulsions was accompanied by recovery in the GABA levels, and that the fall in GABA levels was probably due to an inhibition by SC of GAD activity in the synaptosomal plasma while the recovery by AOAA was probably due to an inhibition by AOAA of GABA-T in the synaptosomal mitochondria (1, 2). This inhibition of GAD by SC and of GABA-T by AOAA may be associated with the decrease by SC of PLP in the synaptosomal plasma and by AOAA of PMP in the synaptosomal mitochondria, respectively.
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