Enzyme immunoassay for arginine vasopressin

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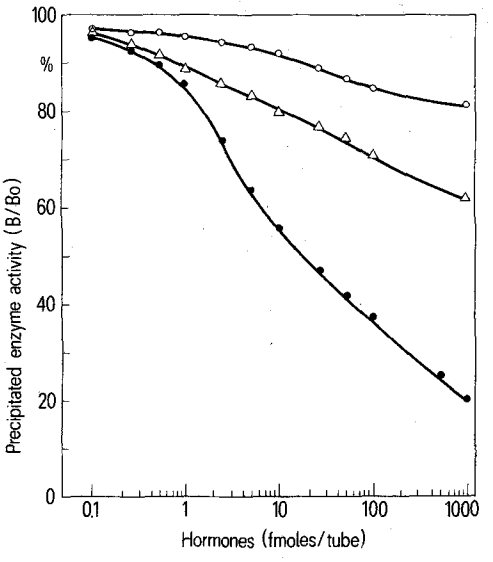
Summary. A highly sensitive enzyme immunoassay for arginine vasopressin (AVP) was described. The enzyme used was β-D-galactosidase from *Escherichia coli*, and it was coupled to AVP by using the N-hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl)-maleimide. The complex was then utilized for the enzyme immunoassay, which could detect 0.5 fmoles (or 0.5 pg) of AVP in the assay tube.

Arginine vasopressin (AVP) can be measured by radioimmunoassay[1-3], which is sufficiently sensitive to determine physiological levels (1-10 pg/ml) of AVP in plasma. The radioimmunoassay method is, however, associated with the hazards of radioactivity and the short life of the radionuclides used. Therefore, we tried to standardize an enzyme immunoassay system for the measurement of AVP. In this study, we synthesized the AVP-β-D-galactosidase complex and examined the competitive binding of the complex with AVP, lysine vasopressin (LVP) and oxytocin to the anti-AVP antibody (Calbiochem-Behring Corp., La Jolla).

The AVP-β-D-galactosidase complex was synthesized by using the N-hydroxysuccinimide ester of N-(4-carboxycyclohexylmethyl)-maleimide as described by Ishikawa et al.[4]. Synthetic AVP (0.5 mg, Calbiochem-Behring Corp., La Jolla) in 1.5 ml of 0.1 M sodium phosphate buffer (pH = 7.0) was mixed with the above maleimide ester (0.5 mg) which had been dissolved in 50 µl of 1-4-dioxan and the mixture was allowed to stand at 30 °C for 30 min. The reaction mixture was then lyophilized, and the lyophilized powder was washed with 5 ml of acetone to remove the unreacted maleimide ester. The residues were dissolved in 2 ml of 0.1 M sodium phosphate buffer (pH = 7.0) and were used as maleimide-treated AVP. The maleimide-treated AVP (0.3 ml) was incubated with β-D-galactosidase from *E. coli* (0.2 mg, Boehringer, Mannheim) and then the reaction mixture was applied to a Sepharose 6B column (1.5 × 40 cm) which had been washed with buffer A (0.01 M sodium phosphate buffer, pH = 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin and 0.1% NaN_3). The fractions containing the major peak of the enzyme activity were used as the AVP-β-D-galactosidase complex. When an aliquot of the complex (190 µunits) was incubated with an excess amount of anti-AVP antibody, and the bound form of the complex was precipitated by the anti-rabbit IgG serum, about 80% of the enzyme activity in the complex was found to bind with the anti-AVP antibody. 1 unit of β-D-galactosidase is defined as that which hydrolyses 1 µmole of 4-methylumbelliferyl-β-D-galactoside per min under the conditions described below, and 190 µunits of the complex were used for each assay. The amount of anti-AVP antibody used for each assay was that which could bind the complex (190 µunits) to give about 40% of the maximum binding. Enzyme immunoassay was performed by the double antibody technique. Standards of AVP, LVP and oxytocin in serial dilutions from 1000 to 0.1 fmoles in 0.4 ml of buffer A were incubated in a borosilicate tube with or without the anti-AVP antibody at 4 °C for 24 h. Then, the AVP-β-D-galactosidase complex (40 µl, 190 µunits) was added to each mixture and incubated at 4 °C for an additional 24 h. Subsequently, 10 µl each of 2.5% normal rabbit serum and anti-rabbit-IgG (Goat) serum were added successively in each assay tube to separate the complex bound to the anti-AVP antibody. The mixture was incubated at 4 °C for 24 h. Then, 1 ml of buffer A was added to each tube and the mixture was centrifuged at 3000 rpm at 4 °C for 30 min. The supernatants were carefully aspirated, drained and wiped off. The precipitates were resuspended in 0.1 ml of buffer A and incubated with 50 µl of 0.3 mM 4-methylumbelliferyl-β-D-galactoside at 30 °C for 40 min with shaking. The reaction was stopped by adding 2.5 ml of 0.1 M glycine-NaOH buffer (pH = 10.3), and the 4-methylumbelliferone formed was measured by a fluorophotometer (360 nm for excitation and 450 nm for emission analysis) against a freshly prepared standard of 1 µM 4-methylumbelliferone[4].

A blood sample (10 ml) was collected into a heparinized and chilled syringe and immediately centrifuged at 4 °C to separate plasma. AVP in plasma was extracted by acetone[1], and the dried residue from 1 ml of plasma was dissolved in 0.5 ml of buffer A. Each reconstituted plasma extract was serially diluted with buffer A or supplemented with a known amount of AVP and then 0.4 ml of each sample was subjected to the assey. Furthermore, the reproducibility of this method was examined for a subject who had fasted overnight and was then hydrated with 1000 ml of 5% dextrose. Two blood samples were drawn before and after the infusion of 5% dextrose. After the extraction, the AVP values of these 2 samples were measured in triplicate, and the measurements were performed at 3 separate periods.

A standard curve for AVP, and the cross-reactivities of the assay with LVP and oxytocin are shown in the figure. The assay cross-reacted slightly with LVP, but little with oxyto-



Competitive binding curves of the AVP-β-D-galactosidase complex with the standards of AVP, LVP and oxytocin. Various amounts of AVP (-●-), LVP (-△-) or oxytocin (-○-) were mixed with 190 µunits of the AVP-enzyme complex and the anti-AVP antibody in a final volume of 0.46 ml with buffer A. Bo: Enzyme activity precipitated without added hormone. B: Enzyme activity precipitated with added hormones.

cin. The minimal detectable amount of AVP was 0.5 fmoles/tube at which the enzyme activity was lower than the value of $\bar{x}-2$ s calculated from that of the blank. This indicates that the enzyme immunoassay for AVP is sufficiently sensitive for the determination of physiological levels of AVP in plasma. Furthermore, the inhibition-curve with the samples which were prepared with a plasma extract by serial dilution or addition of known amount of AVP, was parallel to the standard curve of AVP. The mean values of AVP determined at 3 separate periods were 3.2, 3.6 and 3.5 fmoles/tube in the dehydrated state and were 1.6, 1.4 and 1.3 fmoles/tube after the hydration. This indicates that the enzyme immunoassay for AVP is highly reproducible. The AVP-β-D-galactosidase complex was stable for at least 1 year with respect to the enzyme activity and to the binding activity to the anti-AVP-antibody. Therefore, the enzyme immunoassay for AVP described here could be applicable in clinical chemistry.

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Effect of tea consumption on the levels of α-ketoglutarate and pyruvate dehydrogenase in rat brain[1]

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Summary. Administration of tea to rats fed on a normal diet results in a marked drop in brain levels of total thiamine as well as of α-ketoglutarate and pyruvate dehydrogenase activities. The patterns of decrease in both enzyme activities are similar to that of total thiamine content; they drop to about 65% of the control at 14–20 weeks after continuous consumption of tea.

Tannin, the main antithiamine component in tea has been reported to interact with thiamine, resulting in a decrease in thiamine absorption[2,3]. A thiamine deficiency status has been shown among people who have the habit of chewing fermented tea leaves[4]. Since thiamine deficiency can lead to a dysfunction of the nervous system, it is of considerable interest to investigate the possible effects of prolonged tea consumption on the thiamine status of the brain. Moreover, the thiamine pyrophosphate dependent enzymes, α-ketoglutarate and pyruvate dehydrogenase are involved in the synthesis of γ-aminobutyric acid and acetylcholine respectively. Impairment of these enzyme activities could affect neurotransmitter synthesis and might contribute to disturbances in neurological function[5,6]. Therefore, in addition to brain thiamine content, the brain levels of α-ketoglutarate and pyruvate dehydrogenase were also determined in the rat at various times after tea administration.

Materials and methods. Male albino weanling rats (aged 3 weeks, weight 30–40 g), fed ad libitum on a normal basal diet were divided into 2 groups of 60 animals, namely tea-treated and control. The experimental group was given black tea (The Raming Tea, Thailand), 1:50 w/v while drinking water was given to the control group. At different times after tea administration, 8 animals from the tea-treated group and 8 animals of the same ages from the control group were decapitated, and their brains were removed and separately processed immediately. 4 brains from each group were used for the determination of thiamine content in brain while another 4 brains were used for the assay of α-ketoglutarate and pyruvate dehydrogenase activity. For determination of total thiamine content, the whole brain was homogenized in 8 vol. of cold 0.3 M $HClO_4$ and then centrifuged at $6000\times g$ for 30 min. The supernatant obtained was digested with Takadiastase[7] and the total thiamine content was assayed by the thiochrome method[7].

The activities of brain α-ketoglutarate and pyruvate dehydrogenase were assayed in the mitochondrial fraction which was prepared from freshly removed brain. Each brain was homogenized in 2 vol. of Krebs-Ringer phosphate buffer, pH 7.4 and centrifuged at $1000\times g$ for 10 min. The pellet was discarded and the resulting supernatant was further centrifuged at $12{,}000\times g$ for 20 min to isolate the mitochondrial fraction. The mitochondrial pellet obtained after washing was suspended in Krebs-Ringer phosphate buffer, pH 7.4 and used while fresh for assaying the enzyme activity. The α-ketoglutarate dehydrogenase activity was measured by using $Fe(CN)_6^{3-}$ as an electron acceptor[8,9]. The rate of reduction to $Fe(CN)_6^{4-}$ was followed spectrophotometrically at 420 nm and the enzyme activity was expressed as changes in OD_{420} per min per mg mitochondrial protein. For pyruvate dehydrogenase activity, the assay system as described by Wieland et al.[10] involved the oxidative decarboxylation of pyruvate, resulting in the formation of acetyl phosphate. Pyruvate dehydrogenase exists as an active nonphosphorylated and an inactive phosphorylated form[11]. To determine the total pyruvate dehydrogenase activity, the mitochondrial fraction was preincubated at 25 °C for 1 h with 10 mM $MgCl_2$, prior to the assay[10].

Results and discussion. The fluid intake and body weights of rats in the 2 groups, consuming either drinking water or tea (1:50 w/v) were not significantly different. However, in the tea-treated group, as early as 2 weeks after tea consumption, the brain total thiamine content had already decreased. When compared to the nontreated control, a progressive decline was observed in the tea-treated group from 2 to 14 weeks, where about 60% of the control value remained (fig.a). After 14 weeks, there was no further decrease in brain thiamine content even though the rats were still maintained on tea drinking for 24 weeks. The results suggest that prolonged consumption of tea can lead to a status of thiamine deprivation in the brain although the rats still do not yet show neurological symptoms. It has been reported that when the brain thiamine declines to less than 25% of the normal value, neurological signs are observed[12,13]. The decrease in brain thiamine content following tea consumption could possibly result from interfer-