# BIOCHEMICAL AND FUNCTIONAL CHANGES AFTER IMMUNIZATION OF RATS WITH ANGIOTENSIN II

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This paper describes an attempt to immunize rats with a conjugate of angiotensin II with bovine serum albumin (BSA) and to study the time course of activity of angiotensin converting enzyme (ACE), the angiotensin I level, and certain behavioral parameters.

## EXPERIMENTAL METHOD

Experiments were carried out on 40 male Wistar rats weighing initially 200-220 g, subdivided into four groups with 10 animals in each group. Rats of group 1 were immunized with a conjugate of angiotensin II with BSA, mixed with Freund's complete adjuvant, and rats of group 2 were immunized with a conjugate of BSA and carbodiimide. Animals of group 3 received injections of angiotensin II (in a dose of 150 μg per animal), and those of group 4 received injections of physiological saline. In accordance with the schedule of immunization with angiotensin II suggested in [3] the conjugate of angiotensin with BSA (in a dose of 150 μg angiotensin II, chemically bound with BSA, per animal) was injected four times with intervals of 7 days, after which maintenance doses were given every 30 days (seven injections altogether during the 6 months of the experiment). Injections of the substances used in the control experiments (groups 2, 3, and 4) wre given in accordance with the same schedule. All substances were injected subcutaneously into the upper third of the rats' limbs, in a volume of 0.1 ml per animal.

To prepare the conjugate of angiotensin II with BSA the carbodiimide method was used. The conjugate thus obtained contained 10-12 molecules of peptide per mole of protein [11].

Blood samples were taken from the subclavian vein of all the animals in the background period of the experiment and four times during immunization in order to determine titers of antibodies to angiotensin II and BSA, ACE activity, and the angiotensin I concentration. The antibody titer was determined by enzyme immunoassay [2], using polystyrene panels, sensitized with peptide or BSA, and subsequent binding of the test antiserum with conjugates of G immunoglobulins against rat IgG with peroxidase. The final determination was made with a substrate mixture containing o-phenylenediamine and hydrogen peroxide, with scanning on a "Titertek Multiscan" instrument (wavelength 492 nm). ACE activity in the blood serum and brain homogenates was investigated [5] in the modification [14], using carbobenzoxyphenylalanyl-histidyl-leucine as the substrate. Considering the small volumes of working material, we chose conditions for determination of ACE activity in 2 μl of serum or brain homogenate.

The angiotensin I level was determined in blood serum by radioimmunoassay [12].

The following reagents were used: angiotensin II (from the experimental factory, Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR), BSA (from "Sigma," USA), carbobenzoxyphenylalanyl-histidyl-leucine, histidyl-leucine, o-phthaleic dialdehyde, and o-phenylenediamine (from "Serva," West Germany), I-cyclohexyl-3(2)-morpholinoethyl)-carbodiimide (from "Sigma," USA); diagnostic antibodies against rat immunoglobulins, labeled with peroxidase (from the bacterial preparations factory of the N. F. Gamaleya Research Institute of Epidemiology and Microbiology), kits for radioimmunoassay of angiotensin I (from "CIS," France) and Freund's complete adjuvant (from "Difco," USA).

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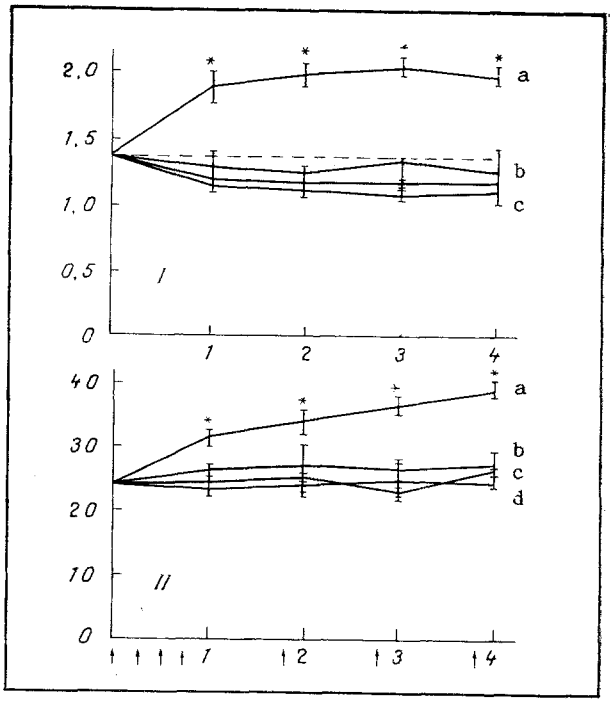


Fig. 1. Changes in biochemical and behavioral parameters of rats during immunization with conjugates of angiotensin II and BSA. Abscissa, time of immunization (months); ordinate: I) specific ACE activity (nmoles His-Leu/min/mg protein); II) daily consumption of water (ml); broken line — initial ACE activity before beginning of immunization; a) group 1; b) group 2; c) group 3; d) group 4. Arrows indicate periods of immunization, *$P < 0.05$ (compared with group 4).

During immunization of all the animals of groups 1-4 the quantity of water (in ml) and food (in g) consumed daily was measured. Body weight was recorded periodically.

The experimental results were subjected to statistical analysis by Student's t test and by the nonparametric Whitney—Wilcoxon test.

## EXPERIMENTAL RESULTS

Immunization of the rats with a conjugate of angiotensin II and BSA led to a gradual increase in the titer of antibodies to angiotensin II in the majority of animals. After the first 4 weeks of immunization antibodies were found in five of the 10 animals. Later the number of rats giving an immune response to the conjugate of angiotensin II and BSA increased to nine, and the titer of antibodies to angiotensin II rose from 1:8 to 1:128. Antibodies to BSA were detected in the same rats in titers of between 1:800 and 1:25,600. No direct correlation could be found between the values of the titers to BSA and angiotensin II in the same rats, but in rats with the maximal response to the angiotensin II conjugate (titer 1:128), a maximal titer of antibodies to BSA also was found.

In all the animals of group 2, which were immunized with a conjugate of BSA and carbodiimide, antibodies were found to BSA with a titer of between 1:400 and 1:25,600. Neither in this group nor in groups 3 and 4, in which angiotensin II and physiological saline were injected, were antibodies to angiotensin II found. Correspondingly, in rats of the last two groups, antibodies to BSA likewise were not found. Thus during immunization of rats with a conjugate of angiotensin II and BSA, a specific immune response was recorded in the animals with the formation of antibodies to this peptide.

Investigation of ACE activity in the blood serum showed a gradual increase in animals immunized with the conjugate of angiotensin II and BSA. After the second maintenance dose, activity of the enzyme reached its peak value, 50% higher than the initial level. This change also continued in the future. On immunization of the rats with the conjugate of BSA and carbodiimide (group 2) and after injections of unconjugated angiotensin II (group 3), on the other

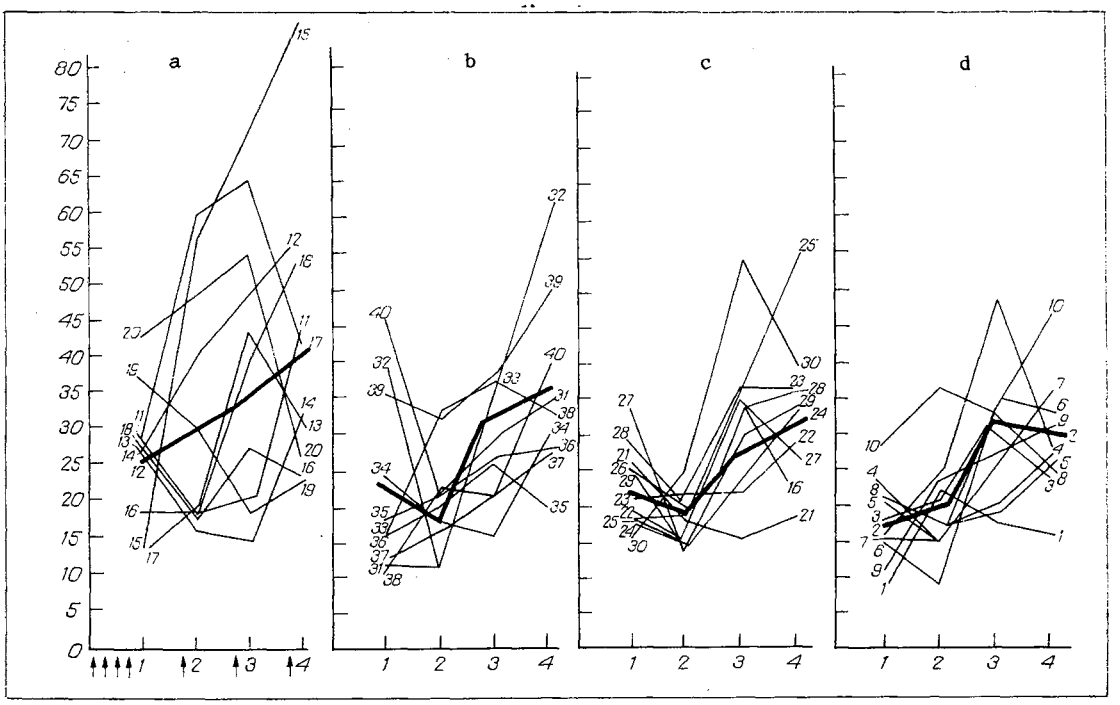


Fig. 2. Changes in serum angiotensin I concentration of rats during immunization by conjugate of angiotensin II with BSA. Ordinate, content of angiotensin I (ng-ml): thin lines (1-40) — in single animals; bold lines — mean values for each group. Remainder of legend as to Fig. 1.

hand, a tendency was noted for ACE activity to fall. Injection of physiological saline (group 4) had no effect on ACE activity throughout the period of investigation (Fig. 1).

Parallel determination of angiotensin I, which is the substrate for ACE and a precursor of physiologically active angiotensin II, in the blood serum revealed the clearest tendency for its concentration to rise in the rats of group 1, compared with animals of the control groups 2, 3, and 4. Incidentally, in animals immunized with the angiotensin II conjugate, individual differences were found to a greater degree than in the other groups in the time course of the change in angiotensin I concentration during immunization, evidently due to the different character of the increase in antibody titer in each of the animals composing this group (Fig. 2). Thus if it accepted that immunization of rats with the conjugate of angiotensin II and BSA leads to a fall in the body level of angiotensin II, the increase in ACE activity observed can be regarded as compensatory mobilization of its formation. It is in this context that the phasic changes in the angiotensin I level must be assessed.

The data described above on the effect of immunization with the conjugate of angiotensin II and BSA on ACE activity and on the serum angiotensin I level provided a basis for consideration of the problem of whether changes at the periphery could be reflected in activity of the brain renin-angiotensin system. It must be pointed out that the brain contains a complete set of components of that system, metabolism of which is not directly connected with the periphery [10]. We also assume that macromolecules of antibodies formed to angiotensin II and to BSA evidently do not penetrate into the brain. The unlikelihood that angiotensin can pass through the blood-brain barrier was discussed in [9]. The data in Fig. 3 are evidence of selectivity of the changes in ACE activity in the brain of the immunized animals (of both group 1 and group 2). In rats immunized with the conjugate of angiotensin II and BSA, ACE activity was depressed in the midbrain and also in the thalamo-hypothalamic region by 2.5-3 times. In control groups 3 and 4 ACE activity was unchanged. The results of these investigations demonstrated that immunization as such has a marked effect on ACE activity in individual brain zones.

Immunization with the conjugate of angiotensin II and BSA was found to cause significant changes in the realization of some forms of behavior of the animals, in the regulation of which this peptide plays an important role. For instance, it was shown that the water consumption was increased regularly (on average by 40-45%; Fig. 1) during immunization in the animals of group 1, unlike animals of all the control groups. No significant changes in feeding behavior were observed in animals of any of the groups studied.

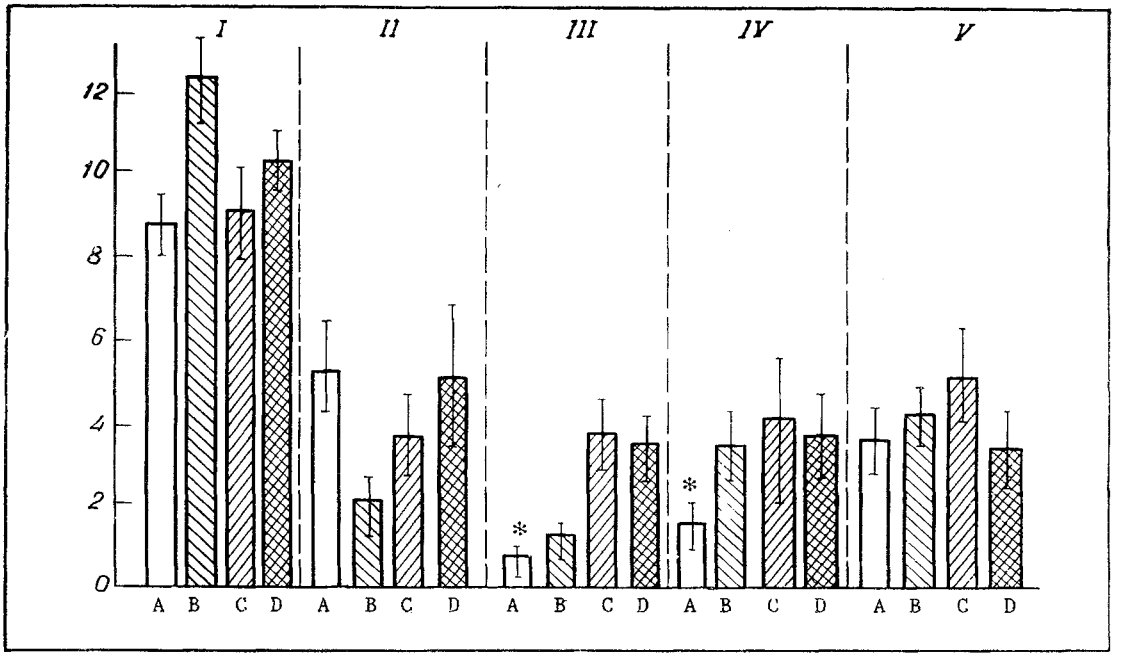


Fig. 3. Changes in ACE activity in individual zones of rat brain after immunization with conjugate of angiotensin II and BSA. Abscissa, groups of animals (Fig. 1); ordinate, specific ACE activity (nmoles His-Leu/min/mg protein). Brain zones: I) pituitary; II) medulla; III) midbrain; IV) hypothalamus and thalamus; V) striatum and basal ganglia. *P < 0.05 compared with group 4.

When the results are assessed, it must be emphasized that immunization with angiotensin II, which performs regulatory functions in the body, leads to changes in the metabolism of this peptide and also to behavioral changes (an increase in water consumption). Considering that angiotensin II plays an initiating role in the central mechanisms of thirst, it can be tentatively suggested that this change in drinking behavior is one of the results of compensatory adaptation of the animal to immunization with the peptide.

This conclusion indicates that immunization with regulatory peptides may be used as a method of demonstrating the mechanisms of their functional activity and correlation of physiological changes associated with involvement of the neuropeptide systems of the body.

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