their cages in LD 12:12 (06.00 h on) and then were exposed to LL from 50 days of age.

Experiment 2. Animals were exposed to LL for 70 days from 50 days of age. On the 120th day, they were given 3 h darkness (D_1, LD 21:3, dark time; 18.00-21.00 h) for 20 days. Afterwards, the lighting schedule was changed to D_2 (LD 21:3, dark time; 03.00-06.00 h) and continued for 20 successive days (140 to 160 days of age) and then changed again to D_1 (160 to 180 days of age). In order to determine whether light information is conveyed only via the retina, bilateral ophthalectomy was performed under ether anesthesia in the morning (09.00-10.00 h) at 160 days of age.

Result. The WR rhythm of a representative animal in experiment 1 was shown in figure 1. In the early prolonged estrous state from 50 to 67 days of age, the WR thythm with a free running rhythm of approximately 25.5-h period was observed. In the late prolonged estrous state, from 68 to 83 days of age, there was hardly any WR rhythm. After this WR arrhythmia, PE followed. A time lag between the onset of both arrhythmias existed (9.6 ± 1.7 day); the WR rhythm disappeared earlier than did the estrous cycle in LL.

As shown in figure 2, there was hardly any WR rhythm in the complete PE state. This was coincident with experiment 1 (fig. 1). When an animal showing both arrhythmias was given 3-h darkness (D_1), the WR rhythm reappeared in this short dark period within at least 3 days. The reappearance of this WR rhythm was found at off-time (18.00 h) and entrained with an approximately 24.0-h period to D_1. The WR activity period was about 10 h. In order to check whether the reappearance of the rhythm is driven by internal controlling elements, the dark time was shifted 9 h later (D_2). The WR rhythm shifted with an approximately 25.5-h period and then entrained with a 24.0 h period to D_2; the reappearance of the rhythm seems to be driven by internal controlling elements. Although the WR rhythm was clearly produced by an atypical light cycle, the PE state continued up to the time of the ophthalectomy. After the ophthalectomy, a free-running period of the WR rhythm was about 24.0 h. These blinded animals could not entrain again to the new lighting schedule, indicating that light informtion could be conveyed only via the retina.

Discussion. During the early prolonged estrous state in LL, the WR rhythm clearly showed a free-running pattern and its period was about 25.5 h. During the late prolonged estrous state, however, there was scarcely any WR rhythm. After this arrhythmicity of WR, the PE state was invariably observed. There was an apparent time lag (average 10 days) between the onset of arrhythmicity of WR and that of PE. This time lag was also found in atypical light cycles[9]. Conversely, when animals showing both arrhythmias in LL were exposed to a LD 21:3 photoperiod, the activity rhythm apparently returned to normal but the estrous cycle did not. These results suggested that 2 circadian rhythms might be separable in LL and in LD 21:3.

Recent reviews[10-12] have shown that the suprachiasmatic nucleus (SCN) might act as a central circadian controlling element. When it is destroyed or isolated, both the behavioral and the estrous rhythms become arrhythmical in nocturnal mammals[13-15]. An intact circadian system, as revealed by a regular activity rhythm, does not, however, guarantee a regular estrous rhythm because this is apparently disrupted in LD 21:3. The dissociation of the 2 rhythms in LL and in LD 21:3 suggests that a central circadian controlling element, probably in the SCN, might couple more strongly with the elements controlling the activity rhythm than with those controlling the estrous cycle.

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Synthesis of [1-Aib]-angiotensin II, an analogue with higher potency than [1-Asn,5-Val]-angiotensin II[1]

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Summary. [1-Aib]-angiotensin II was synthesized by Merrifield's solid-phase procedure. The analogue, tested on rabbit aorta strips, showed intrinsic activity $\alpha_E = 1$, and when tested on rat blood pressure it gave a pD_2 of 8.06; a 3.2 ± 1.3-fold higher potency than the Ciba-Hypertensin standard.

It is recognized that the residue in position 1 of angiotensin II contributes primarily to the duration of action through 2 possible mechanisms: a) it makes the molecules more-or-less resistant to aminopeptidases; b) it increases the binding affinity of the peptide for the receptor[3-5]. Moreover, the basicity of an α-nitrogen at the N-terminus is important for maximum agonistic properties of angiotensin II. Thus, [1-Sar]- and [1-diMeGly]- angiotensin II were found to be respectively 1.5 and 1.7 times as active as angiotensin II as pressor agents[6,7]. On the other hand, model compounds containing α-methyl-amino acids indicate that these compounds are resistant to chemical hydrolysis and to enzymatic attack by both endopeptidases and exopeptidases[8,9]; moreover, α,α-dialkyl-amino acids showed, on the basis of theoretical analysis, considerable restriction of conformational freedom of peptides[10]. Recent studies indicate that

the presence of Aib (α-aminoisubutyric acid or α-methyl-alanine) introduces considerable stereochemical constraints on the conformations of acyclic peptides, initiates β-turns and generates helical structure in solution and in the solid state[11].

Since it is an accepted concept that linear peptides of the size of angiotensin II are characterized by a certain flexibility which corresponds to an equilibrium of conformational families, while the peptide backbone is C-shaped with a C-terminal bend around proline and a β-turn involving the N-terminal tetrapeptide[5] we thought it of interest to investigate whether the replacement of the 1-Asp with Aib would yield an analogue of more stable and active conformation.

Therefore, [1-Aib]-angiotensin II was prepared by the solid phase method of Merrifield[12] on a 1% crosslinked chloromethyl polystyrene containing 0.75 meq Cl/g (Bio-Beads S-X1, 200–400 mesh). A substitution of 0.56 mmole of Boc-Phe per g of resin was obtained according to Loffet[13]. The remainder of the synthesis was performed on a Schwarz-Bioresearch Model 105 Peptide Synthesizer starting with 0.71 g of substituted resin. Boc group was used for the protection of the α-amino functions of the residues with the side-chain protecting groups indicated in parentheses: Pro, His(Tos), Ile, Tyr(O-2-Br-Z), Val, Arg(NO_2), Aib[8]. Couplings were performed with 2.5 equiv. of t-Boc-protected amino acids and DCC (0.25 M in CH_2Cl_2) for 120 min. The completion of the coupling reaction was confirmed by the Kaiser ninhydrin test[14]. For the incorporation of Boc-Aib the coupling reaction was repeated twice for 4 h each time. Boc protecting groups were removed at each stage by treatment with 25% CF_3COOH in CH_2Cl_2 containing 2% anisole (20 ml, twice for 15 min). Neutralization was effected with 10% triethylamine in CH_2Cl_2 (20 ml, 5 min). Between individual operations, extensive washings (4×20 ml for 2 min) was performed with CH_2Cl_2 or DMF, followed by absolute methanol (4×20 ml).

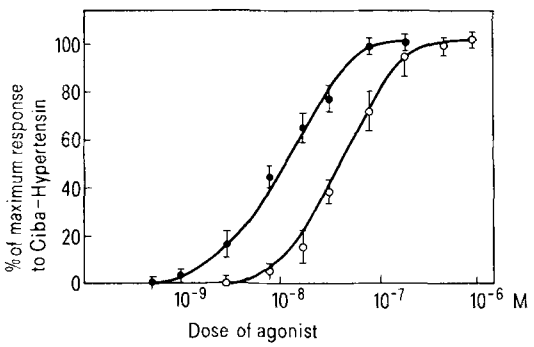
Cleavage of the peptide from the resin and removal of the protecting groups was accomplished with HF containing 10% by volume of anisole for 1 h at 0 °C[15]. After removal of HF and drying under vacuum the resin was washed several times with ether and then was extracted with acetic acid (2 M). Lyophilization of the latter extract yielded the crude peptide (333 mg, 82%) in a solid form. A portion of this product (250 mg) was partially purified by gel filtration on Sephadex G-15 column, using 2 M acetic acid as the eluent, and finally by partition chromatography on Sephadex G-15 and Sephadex G-25M columns using the solvent system n-butanol-acetic acid-water (4:1:5 v/v). Peptide material was detected in the collected fractions by monitoring the absorbancy at 254 and 280 nm; yield 60 mg (19% overall yield based on initial phenylalanine anchored to the polymer).

The final product gave single spots on TLC (precoated plates of silica gel G) when loads of 15–20 μg were used, with R_f 0.15 in the solvent system (upper phase) n-BuOH-AcOH-H_2O (4:1:5) and R_f 0.35 in n-BuOH-AcOH:pyridine:H_2O (30:6:20:24). Compound was detected on the chromatogram with ninhydrin, chlorine peptide spray and with diazotized sulfanilic acid. The HPLC profile of [1-Aib]-angiotensin II (4 mm×25 cm column Licrosorb RP 18 Merck, load 100 ng, flow rate 3 ml/min, solvent system: gradient 15% to 30% CH_3CN/1% formic acid-triethylamine pH 3.5, UV-absorbance 275 nm, back pressure 234 at, D.O (F.S. 0.1) revealed that the compound was 97% pure. Amino acid analysis gave the following molar ratios: Arg, 0.96; Val, 0.96; Tyr, 0.95; Ile 0.92; His, 0.98; Pro, 0.86; Phe, 1.08; Aib, 0.89. Anal. calcd for $C_{54}H_{81}N_{13}O_{10} \cdot C_2H_4O_2 \cdot 2\,H_2O$ (1152.36) C, H, N.

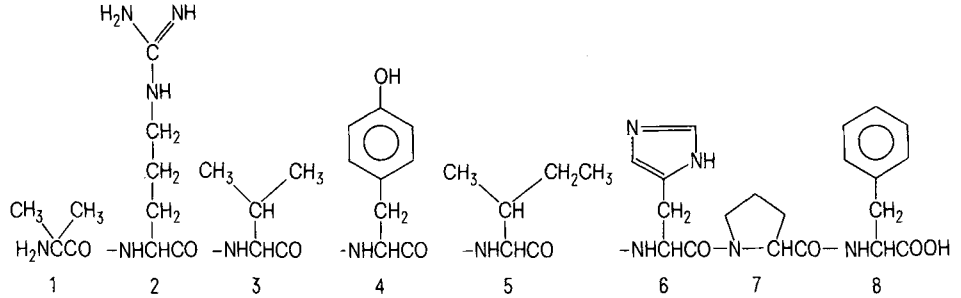
The compound was tested on thoracic rabbit aorta strips[16] (in vitro) and in the rat blood pressure test (in vivo), according to Regoli[17] and its biological activity was compared with that of Ciba-Hypertensin. The results indicate that the replacement of aspartic acid with α-aminoisobutyric acid in position 1 of angiotensin II significantly increases the potency of the analogue which is 3.2± 1.3-fold more active in vivo than Ciba-Hypertensin but 80% relative to [1-Sar]-angiotensin II.

Moreover, [1-Aib]-angiotensin II shows identical intrinsic activity with Ciba-Hypertensin and a PD_2 8.06 (fig.). Interestingly enough, the [1-Ala]-angiotensin II (1 methyl group onto the α-carbon atom) shows 37% pressor activity relative to Ciba-Hypertensin[4].

The high relative affinity and potency of the tested analogue with 2 methyl groups attached onto the α-carbon atom, could be explained by the assumption that the β-turn of the N-terminal part of angiotensin II is favorably stabilized due to stereochemical constraints imposed by the Aib. This may lead to a proportionally increased amount of the active conformation as reflected by its higher affinity.



Dose-responce relationship of [1-Aib]AT II (●—●) and Ciba-Hypertensin (○—○) on rabbit aorta strips.



Aminoacid sequence of [1-Aib, 5-Ile]-angiotensin II; numbers indicate sequence positions of individual residues.

Certainly this assumption deserves further investigation and it cannot be entirely ruled out at present that increased potency may be due to a reduced enzymatic rate of degradation[9]. An interesting correlation between [1-Aib]angiotensin II and [1-N^4, N^4-diMe-Asn]-angiotensin II reveals that dimethylation of the N^4-asparaginyl carboxamide reduces the affinity of the analogue, probably because of repulsion of the side chains of essential residues like 3-Val and 4-Tyr[18,19].

1 All optically active amino acids are of the L configuration. Abbreviations used follow the recommendations of IUPAC-IUB as found in Biochemistry *14* (1975) 449 and Biochem. J. *126* (1972) 773. Other abbreviations: Aib, α-aminoisobutyric acid or α-methyl-alanine.

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An early effect of testosterone propionate upon hypothalamic function in the neonatal rat

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Summary. Rats treated neonatally with testosterone propionate exhibit a reduced uterine growth response to estradiol administration prepubertally. This androgen-induced impairment is the consequence of developmental effects on both the ovary and the hypothalamic-pituitary complex, although the latter is the more sensitive.

Neonatal treatment with estrogens or androgens is known to result in impaired reproductive function in the adult rat at hypothalamic[1], ovarian[2], and uterine[3] sites. Futhermore, the etiology of this impaired or reduced reproductive tissue function is clearly evident in the immature rat[4-6]. The impaired uterine responsivity evident in the androgenized or estrogenized prepubertal rat appears to be the consequence of decreased function of the hypothalamic-pituitary-ovarian axis[6].

The purpose of this work was to gain some insight into the relative influence of altered hypothalamic or ovarian function upon the development of the uterine response syndrome observed in the prepubertal rat injected with sex steroids during infancy. Only neonatal testosterone propionate (TP) exposure was utilized in this study since, unlike estradiol, it results in an impaired uterine response to exogenous estrogen without a concomitant reduction in cytoplasmic estrogen receptor in the immature rat uterus[5,6].

Materials and methods. Sprague-Dawley derived rats were obtained from Charles River Breeding Labs and bred in the UAH animal facility. The morning on which pups were found was designated day 1 of life. Testosterone propionate (Sigma Chemicals) was injected s.c. on day 3 in 0.1 ml peanut oil at a dose of 1250 µg. Controls received an equal volume of the oil vehicle. On day 8 ovaries were reciprocally transplanted between animals which had been previously treated with TP or peanut oil. Control transplants to the dorsal neck region were also performed on animals having received the peanut oil injection on day 3. All transplant procedures were carried out under hypothermia-induced anesthesia and involved short-term culture in ice-cold Eagles Medium (Difco).

At 21 days of age the animals in the various treatment groups were injected s.c. with either 0.1 µg or 1.0 µg estradiol-17β in physiological saline. Uteri were excised, trimmed of connective tissue, and weighed on an analytical balance 24 h after the estradiol injection. The weight of the uteri from the experimental treatment groups was expressed relative to the weight of the uteri from the control treatment groups. The presence of a functional ovarian graft in the experimental animal was also confirmed at autopsy.

Results. Neonatal exposure to TP produces an apparent impairment of hypothalamic and ovarian function as indicated by the parameter of prepubertal uterine growth response to exogenous estradiol (table). These results are in general agreement with the reduced uterine responsivity noted in intact prepubertal rats injected neonatally with TP[4-6]. However, normal rats with ovarian transplants from TP-treated individuals exhibited a less severe impairment of uterine responsiveness than those animals treated with TP and possessing normal ovarian transplants. 21-day-old