oxidized pig-insulin. Both types of sera react with native as well as with photooxidized pig-insulin, with a second sample of crystalline pig-insulin (Novo) and with 2 different samples of repeatedly recrystallized ox-insulin (Farbwerke Hoechst and Novo). On the other hand, antisera either against native or photooxidized pig-insulin did not react with oxidized pig-insulin or with single or mixed A- and B-chains from ox-insulin prepared by the method of sulphitolysis[15,16]. Finally, the precipitating activity of antisera to native and photooxidized pig-insulin could be completely removed by addition of native or photooxidized pig-insulin. No precipitation line appeared after immunoelectrophoretic analysis of the supernatants of such absorbed antisera.

On the other hand, no precipitating insulin antibodies could be demonstrated in the sera of guinea-pigs immunized with the oxidized pig-insulin preparation. The loss of immunogenicity in this preparation may be related, at least partly, to the fact that the oxidation procedure results in a reduction of the sedimentation coefficient to 1,53 *S*, compared with 3,2 *S* and 3,3 *S*, respectively, for native and photooxidized pig-insulin, as found in sedimentation studies using analytical ultracentrifugation methods.

Should the observed precipitins be directed to antigenic impurities possibly present in repeatedly recrystallized insulin preparations, then one would expect these antibodies to react with similar impurities present in the oxidized insulin preparation. That the demonstrated antibodies are really directed to the insulin can be seen by the fact that in immunoelectrophoretic studies the precipitation lines formed with photooxidized pig-insulin were always localized much more towards the anode than those formed by the reaction of antisera to native or photooxidized pig-insulin with native pig- or ox-insulin. This means that photooxidized insulin has a higher electrophoretic mobility than the native preparation. This phenomenon can be explained by the observation[13] that photooxidized insulin in comparison with native insulin shows an isoelectric precipitation range which is shifted about 1.5 pH units towards the acid side. The results presented here may allow one to conclude that insulin has more than one antigenic determinant site.

Zusammenfassung. Die serologische Untersuchung von gegen natives und photooxidiertes Schweineinsulin gerichteten Meerschweinchenantiseren führte zu dem Ergebnis, dass die Antikörper gegen Insulin gerichtet sind, nicht aber gegen möglicherweise in den kristallinen Insulinpräparationen vorhandene antigene Verunreinigungen.

W. SCHAEG and H. FINGER

Institute of Biochemistry and Endocrinology, University of Giessen and Institute of Hygiene and Microbiology, University of Würzburg (Germany),
15th June 1967.

[15] J.-c. DU, R.-q. JIANG and C.-I. TSOU, Scientia sin. *14*, 229 (1965).
[16] The isolated A- and B-chains of ox-insulin as S-sulphonates were obtained through the courtesy of Dr. HÖRNLE, Institute of Biochemistry, University of Tübingen (Germany).

Production of Antibodies Against Synthetic Angiotensin II in Various Animal Species

Circulating antibodies to angiotensin II have been produced in the rabbit by injection of benzoylangiotensin-azo-bovine γ-globulin[1], by injection of conjugates containing albumin and the peptide bound covalently by means of carbodiimide reagents[2], and by immunization with synthetic branch-chain copolymers comprising backbones of poly-L-lysine and branches of angiotensin[3]. The in vitro immunochemical properties of anti-angiotensin have been determined by hapten inhibition tests using a quantitative micro-complement fixation[4] and, more recently, by means of a modified ammonium sulphate precipitation technique[5]. Some evidence has been found for immunization against angiotensin-amide in the rat after treatment with an angiotensin-II-carbodiimide-rat albumin complex[6]. In the present investigation an attempt was made to immunize animals from several species against angiotensin-amide.

Asp(NH_2)[1]-Val[5]-angiotensin-II (AII) was conjugated to bovine γ-globulin (BGG) or to human γ-globulin (HGG) by means of carbodiimide (CDI) condensation according to the procedure of GOODFRIEND et al.[2]. This antigen was given in complete Freund's adjuvant (Difco). Alum-precipitated AII ('coarse') was prepared as described earlier[7]. Rabbits (3–3.5 kg) were injected twice with 0.4 mg AII-CDI-BGG in multiple s.c. sites and once with the same amount of antigen in the foot pads. The injections were given at 1 month intervals. Rats (60–130 g initial weight) received 1–3 injections of 0.4–0.6 mg AII-protein conjugate s.c. or in the foot pads followed by an i.p. injection of 0.3 or 0.6 mg antigen 4–6 weeks later. Cats (3–5 kg) were injected at multiple s.c. sites with a total amount of 1.6 mg AII-CDI-BGG. Two mongrel dogs (20 and 21 kg) received 3 injections of AII-CDI-BGG. Over a period of 18 weeks a total amount of 22 mg was administered either i.m. or s.c. One month after the last injection of the emulsion 4 i.v. injections of 40 mg alum-precipitated AII were given every second week. Anti-angiotensin activity was determined 10–21 days after the final antigen injection in the rabbit and the rat and 6 weeks after the last antigen injection in the cat. The dogs were bled at intervals during the whole immunization period, as well as 4 weeks after the last injection in order to analyze small samples of serum for anti-AII activity. The pressor

[1] S. D. DEODHAR, J. exp. Med. *111*, 419 (1960).
[2] T. L. GOODFRIEND, L. LEVINE and G. D. FASMAN, Science *144*, 1344 (1964).
[3] E. HABER, L. B. PAGE and G. A. JACOBY, Biochemistry *4*, 693 (1965).
[4] T. GOODFRIEND, G. FASMAN, D. KEMP and L. LEVINE, Immunochemistry *3*, 223 (1966).
[5] F. M. DIETRICH, Immunochemistry *4*, 65 (1967).
[6] T. U. L. BIBER and D. E. OKEN, Clin. Res. *14*, 371 (1966).
[7] F. M. DIETRICH, Int. Arch. Allergy appl. Immun. *30*, 497 (1966).

response to AII in the dog was determined 5 weeks after the last injection of AII-protein complex and again 5 weeks after the last injection of alum-precipitated AII.

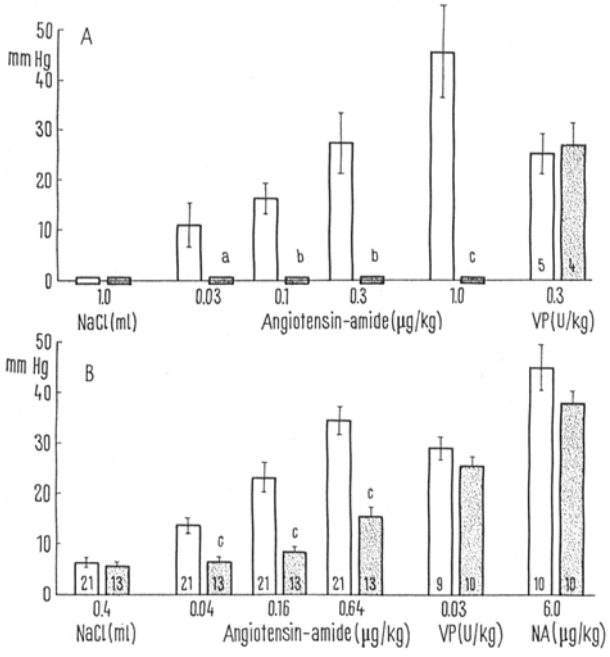
The determination of circulating anti-AII antibodies was carried out in vitro with a modified ammonium sulphate precipitation technic as described previously[5]. In all experiments I^{131}-AII was used at concentrations of 2 µg/ml. The results are expressed as % of trace-labelled AII specifically bound at the serum dilution of 1:10.

The pressor response to AII was determined in immunized and control animals. In the urethane-anaesthetized rabbit and rat (1.5 g/kg i.v. and 1.7 g/kg s.c. respectively) and in the allobarbitalurethane anaesthetized cat (Dial® 35 mg/kg i.p. + 35 mg/kg s.c.) blood pressure in the carotid artery was measured with a mercury manometer. In the pentobarbital-Na anaesthetized dog (ca. 30 mg/kg i.v.) blood pressure was measured in the femoral artery by means of a pressure transducer. Intravenous injections were given in the jugular vein in the rabbit, rat and cat and in a superficial vein of the hind limb in the dog. In order to test the specificity of the inhibition of the pressor response to AII, the responses to norepinephrine and to synthetic Lys^8-vasopressin were also determined.

The renin concentrations in the kidneys of rabbits and rats were determined by the indirect method of Gross et al.[8]. The concentrations are expressed as µg angiotensin produced from nephrectomized rat plasma/g of fresh kidney tissue.

All values are given as means ± S.E.

In rabbits immunized against AII, no pressor response to i.v. injected AII was seen (Figure). The highest AII dose tested, 1 µg/kg i.v., produced a marked increase in blood pressure in untreated control animals, while the response was completely suppressed in immunized animals. The pressor effect of vasopressin, on the other hand, was the same in both groups.



Pressor responses to angiotensin-amide, vasopressin (VP) and noradrenaline (NA) in immunized (dark columns) and control (open columns) rabbits (A) and rats (B). The columns represent the means, the cross bars S.E. The numbers on the columns give the number of animals in each group. [a] significantly different from control values at $p < 0.05$; [b] at $p < 0.01$; [c] at $p < 0.001$.

No significant difference in the renin concentration of kidney tissue was found: in the immunized group ($n = 4$) the renin concentration was 72 ± 17 µg angiotensin/g fresh tissue and 94 ± 23 µg angiotensin/g in the control group ($n = 5$).

A significant decrease in the pressor response to AII was seen in immunized rats (Figure). The effect of AII doses of 0.04 and 0.16 µg/kg i.v. did not greatly exceed that of the same volume of 0.9% NaCl solution, while the response to 0.64 µg/kg i.v. was less than half the response in control animals. The pressor effects of noradrenaline or vasopressin did not differ significantly in treated and control rats. Neutralization of AII in vitro in the rat was surprisingly effective in view of the low levels of circulating antibodies measured in vitro. The sera of 6 immunized rats exhibited a small, but distinct, specific binding of trace-labelled AII ranging from a few % to 14%, the average being 6.3 ± 1.2%. In the rabbit, on the other hand, far higher antibody levels have been measured in vitro[5]. The renin concentrations in kidney tissue of 10 immunized rats (38 ± 1 µg angiotensin/g fresh tissue) and 10 control rats (39 ± 4 µg angiotensin/g) were in the same range.

In the dog and cat no evidence for a reduction in the response to AII was found in treated animals. The AII test doses used ranged from 0.1–10.0 µg/kg i.v. in the dog and from 0.01–0.3 µg/kg i.v. in the cat. Likewise, no in vitro antibody activity, as judged by the absence of specific binding of I^{131}-AII, was seen. Immunization of the dog against AII after treatment with AII-protein conjugates has been reported[9], but the antibody levels were described as being very low and no details of the immunization procedure were given.

In the dog angiotensin has been reported to inhibit renin secretion by a 'feed-back' mechanism[10], but in the present investigation no change in the renin content of kidneys of actively immunized animals was found.

Active immunization to AII may offer the opportunity to gain further knowledge concerning the physiological and pathological role of angiotensin.

Zusammenfassung. Bei Ratten und Kaninchen, jedoch nicht bei Hunden und Katzen, konnten durch wiederholte Injektion von Angiotensin-II-Amid-Carbodiimid-Proteinkomplex in Freund'schem Adjuvans zirkulierende Antikörper gegen Angiotensin erzeugt werden. Durch diese Immunisierung wurde die pressorische Wirkung von exogenem Angiotensin aufgehoben bzw. abgeschwächt, während die von Noradrenalin oder Vasopressin unbeeinflusst blieb. Der Reningehalt der Nieren von Ratten und Kaninchen wurde durch die Immunisierung nicht verändert.

P. R. Hedwall and F. M. Dietrich

Research Laboratories of the Pharmaceutical Department of CIBA Limited, Basel (Switzerland), 3 August 1967.

[8] F. Gross, H. Brunner and M. Ziegler, Recent Prog. Horm. Res. *21*, 119 (1965).

[9] S. D. Deodhar, E. Haas and H. Goldblatt, J. exp. Med. *119*, 425 (1964).

[10] A. J. Vander and G. W. Geelhoed, Proc. Soc. exp. Biol. Med. *120*, 399 (1965).