IMMUNOLOGIC PRODUCTION OF ANTIANGIOTENSIN*

I. Preparation of Angiotensin-Protein Complex Antigen

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It is generally accepted that acute renal hypertension, produced experimentally by constriction of the main renal arteries, is caused by the renin-angiotensin pressor mechanism. The observation that chronic experimental renal, hypertension in the dog can be effectively prevented and treated by antirenin, actively produced in or passively administered to the animal (1, 2), lends considerable support to the view that this pressor mechanism may be operating actively even in the chronic stage of this disease. Whether the pressor mechanism participates in any way in the etiology of benign or malignant human essential hypertension remains to be clarified, but the fact that in man, too, all the constituents of the renal humoral hypertensive mechanism have been identified suggests that this mechanism may also play a part in human hypertension. One approach toward the elucidation of this problem was the study of the effect of the development of antirenin to heterologous renin in hypertensive patients (3). Unfortunately, although antirenin produced in an animal is nonspecific for most other animals, so that antirenin to heterologous renin can partially inactivate the renin of other animals, it has no effect on human renin. This is probably the explanation for the failure of the blood pressure to fall in hypertensive human beings with high titers of antirenin to hog renin (4).

Since angiotensin is a polypeptide, active in man and all animals no matter what the source of the renin and angiotensinogen used to produce the angiotensin, there was reason to believe that an angiotensin inactivator, or inhibitor, might be effective in lowering the blood pressure of hypertensive animals and perhaps of man.

In an elucidation of the structure and the synthesis of angiotensin II, the active octapeptide of the humoral mechanism (5–7), it became possible to approach the problem of producing an angiotensin inhibitor in a systematic fashion. A study of the structural analogs of angiotensin would appear to be one of the promising approaches; but another, which was adopted for this

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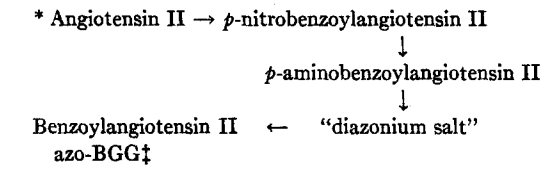
study, is the immunological production of anti-angiotensin. Angiotensin II, like most polypeptides, by itself, appears to be too small a molecule to be antigenic. It was considered, however, that coupling with a suitable protein carrier, might result in a hapten-protein complex antigen which would induce the development of an antibody capable of neutralizing the biological action of angiotensin II.

This report describes the chemical procedures used for the preparation of the angiotensin-protein complex. Briefly the reactions involved were as follows:



* *Angiotensin II*—unless otherwise specified, this refers to the synthetic product valine^5-angiotensin II (aspartyl-β-amide).

‡ BGG-bovine-γ-globulin.

Materials and Methods

Valine^5-Angiotensin II.—This was prepared synthetically as the aspartyl-β-amide derivative. We are very grateful to Dr. Wettstein, Dr. Schwyzer, and their colleagues of Ciba Research Laboratories, Basle, Switzerland, for making this compound available to us. Without their generous gift, this investigation would not have been possible. The synthetic product has the identical amino acid sequence as that of naturally occurring, beef angiotensin II. The aspartyl-β-amide form has the same specific activity as the aspartyl-β-acid form (8).

Bovine-γ-globulin.—This was obtained commercially from the Armor Laboratories, Kanakee, Illinois.

Assay Procedures.—Bioassay of angiotensin II was carried out according to the procedure described by Goldblatt, Lamfrom, and Haas (9). A sample of angiotensin was injected intravenously into a normal, trained, unanesthetized dog and the rise in the direct, mean, femoral blood pressure was determined. Pressor activity was then expressed in terms of dog units, one unit being the quantity required to raise the pressure by 30 mm. Hg.

Chemical Assay.—Color reaction of the tyrosine residue in angiotensin II with Folin's phenol reagent (10) was used for assaying samples of angiotensin II in cases in which contamination with any other phenolic derivatives was not possible. Free tyrosine and tyrosine bound in the peptide chain of angiotensin II were found to behave identically in their color reaction with Folin's phenol reagent. Optical density determinations were carried out at 550 mμ with a Coleman Junior spectrophotometer.

Aromatic Amine Determination.—The number of aromatic amine residues introduced per molecule of angiotensin II was determined by the α-naphthol procedure of Fantl (11) with slight modifications. Excess, solid sodium nitrite was added to a chilled (2 to 3°C.) solution of the aromatic amine in 0.2 N HCl and the excess HNO_2 was later destroyed by the addition of solid ammonium sulfamate. After adjustment of the diazonium salt solution to a concentration of approximately 0.002 M, 0.4 ml. of this solution was added to a mixture of 0.4 ml. (20 micromoles) of an alcoholic solution (95 per cent ethyl alcohol) of α-naphthol and 0.2 ml. of 1 N NaOH. The deep, red colored, azo dye solution was diluted 1:40 with water and optical densities were read at 520 mμ on the Coleman Junior spectrophotometer (8 x 8 x 100 mm. cuvette). The amount of aromatic amine was determined by comparison with stand-

ard solutions of *p*-aminobenzoylglycine; *i.e.*, *p*-aminohippuric acid. The choice of the standard in this assay was made on the basis of the observation that the absorption spectrum at 480 to 560 mμ of the azo dye of *p*-aminobenzoylglycine is identical with the spectra of the corresponding azo products from *p*-aminobenzoyl derivatives of different peptides. The *p*-aminobenzoyl derivatives of glutathione, leucylglycine, diglycylglycine, glycylglycine, and triglycylglycine were studied. These were prepared according to the procedures described by Landsteiner *et al.* (12, 13). Table I lists optical densities of the different azo products prepared by the procedure outlined above. It appears from the results in Table I that the nature of the peptide chain attached to the *p*-aminobenzoyl radical has little or no influence on the light absorption at 520 mμ of the final azo dye produced in this procedure.

UV Absorption Measurements.—The absorption spectra of benzoylangiotensin II-azo-BGG and those of other aromatic diazo-BGG complexes were studied at room temperature with a Beckman (DU) spectrophotometer using quartz cuvettes with a light path $d = 1.00$ cm.

TABLE I

Determination of Aromatic Amine Content of Different p-Aminobenzoylpeptides

Compound (*p*-aminobenzoyl derivative of)	Optical Density at 520 mμ
1. Glutathione	0.64
2. Leucylglycine	0.68
3. Diglycylglycine	0.69
4. Glycylglycine	0.65
5. Triglycylglycine	0.68
6. Glycine	0.67

0.4 ml. (0.8 μM) of a 0.002 M solution of the diazonium salt of each compound used for the determination.

EXPERIMENTAL

Preparation of p-Nitrobenzoylangiotensin II.—To a solution of 0.1 mM (105.3 mg., 280,000 dog units) of angiotensin II in 10 ml. of water was added slowly and with stirring 0.5 mM of *p*-nitrobenzoyl chloride in 0.5 ml. of ether. The addition was carried out at room temperature (25 to 27°C.) over a period of 90 minutes. The reaction mixture was kept alkaline at all times by the frequent additions of small amounts of 1 N NaOH of which a total of 2 ml. was added by the end of the reaction period. The resulting deep yellow colored solution was acidified (to Congo red) by the addition of 5 N HCl when a flocculent, white precipitate appeared. The *p*-nitrobenzoic acid present in this precipitate was removed by repeated extractions with ether (7 to 8 extractions with 25 ml. ether each time). The ether layer in each case was discarded. Excess *p*-nitrobenzoic acid was found to be completely removed by this procedure leaving behind the precipitate of *p*-nitrobenzoylangiotensin II which was then filtered.

Because of the small amount of angiotensin II used in this preparation no attempt was made to purify or crystallize the product obtained after ether extraction. Total tyrosine content of the product at this stage was found to be 80 per cent of the theoretical value.

Preparation of p-Aminobenzoylangiotensin II.—The precipitate of *p*-nitrobenzoylangiotensin II obtained above was dissolved by heating it in a mixture of 20 ml. of 50 per cent ethyl

alcohol and 1 ml. of concentrated NH_4OH (36 per cent NH_3). The clear yellow solution obtained in this manner was heated to 65°C. and 3 mM of powdered $FeSO_4$, $7H_2O$ was added slowly (15 to 20 minutes) and with vigorous stirring. The resulting dark brown suspension was kept at 60°–65°C. for 45 minutes. The Fe $(OH)_3$ precipitate was then centrifuged and the supernatant containing the *p*-aminobenzoylangiotensin II was evaporated to dryness under vacuum. The residue was dissolved in 5 ml. of 0.2 N HCl and the solution was analyzed for tyrosine and aromatic amine content by the respective assay procedures.

Tyrosine content—0.065 mM *i.e.* 65 per cent of the starting material

Aromatic amine content—0.0949 mM

$$\text{Ratio, } \frac{\text{Aromatic amine}}{\text{Tyrosine (angiotensin II)}} = 1.46$$

The ratio of aromatic amine to angiotensin II of 1.46 indicates that in the reaction between *p*-nitrobenzoyl chloride and angiotensin II some group(s) of the polypeptide chain, in addition to the terminal, free amino group of the aspartic acid must be reacting. During this investigation no attempt was made to determine the location of this additional *p*-nitro (amino) benzoyl group. The guanidine group of arginine and the phenolic hydroxyl group of tyrosine would appear to be two possibilities in this respect, although the findings of Miller and Stanley (14) on benzoylation of tobacco mosaic virus suggest that under the conditions of *p*-nitrobenzoylation used in our experiments (pH > 10), the phenolic group probably, would not be esterified.

Coupling of p-Aminobenzoylangiotensin II to BGG.—

To 4.5 ml. (0.0585 mM) of *p*-aminobenzoylangiotensin II solution in 0.2 N HCl kept at 2–3°C. was added an excess (1 mM) of solid, sodium nitrite slowly and the mixture was stirred. After 15 to 20 minutes, the excess HNO_2 was destroyed by the addition of solid ammonium sulfamate and the solution was tested with starch-iodide paper. The diazonium salt solution was then added slowly to a solution of BGG (200 mg.) in 20 ml. of 0.25 M sodium carbonate buffer, pH 9.6. The solution rapidly turned an intense, orange red, indicating the formation of an azo dye complex. After standing for 12 hours at 2°C., the solution was dialyzed against cold (2–3°C.), distilled water for 48 hours. Part of the protein precipitated during dialysis, but this could be redissolved by making the solution 0.1 M with respect to sodium phosphate buffer, pH 7.4. The solution was sterilized, by passing it through a Seitz filter, and stored under sterile conditions.

Absorption Spectrum of Benzoylangiotensin II-azo-BGG.—In the coupling reaction described above, the diazonium salt of *p*-aminobenzoylangiotensin II would be expected to couple primarily with the tyrosine and histidine residues of BGG. The formation of an azo dye complex is indicated by the characteristic intense, orange-red color of the resulting solution. Another indication of the formation of this azo dye complex was derived from a comparison of its absorption spectrum with that of the azo complexes between several para substituted, aromatic amines, and BGG. These azo complexes were prepared in the following manner: 0.5 ml. (0.5 micromole) of a diazonium salt solution prepared from each of different aromatic amines was added to a solution of BGG (10 mg. in

1.2 ml. of 0.25 M sodium carbonate buffer, pH 9.6) and the azo-protein complex was treated as before.

Figs. 1 and 2 illustrate absorption spectra (250 to 500 mμ) of these different R-phenyl-azo-BGG (R = para substituent) preparations studied in 0.1 M sodium phosphate buffer, pH 7.4. The ordinate in this figure represents optical density of a solution of the azo complex which is 10^{-3} M with respect to concentration of the aromatic amine.

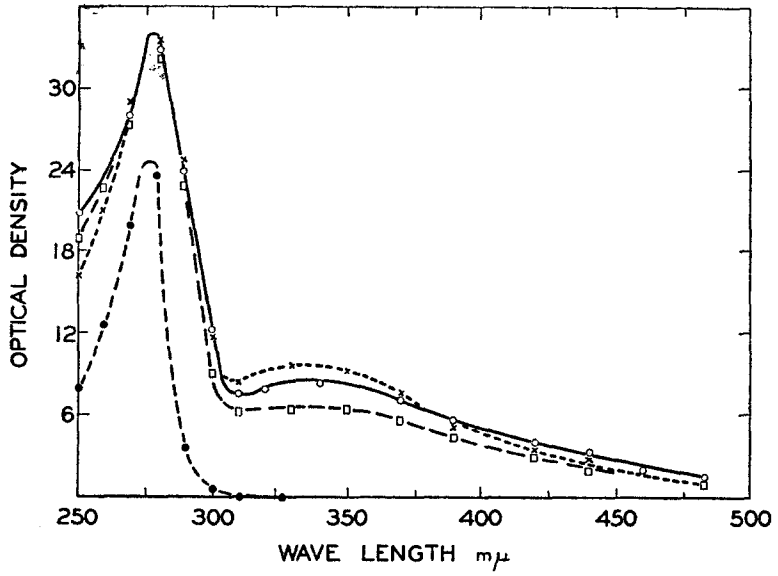


FIG. 1. Absorption spectra of R-phenyl-azo-BGG complexes. X------X, sulfanilic acid; O——O, *p*-aminobenzoylangiotension II; □---□, *p*-aminobenzoic acid; ●------●, BGG alone.

For comparison, Fig. 1 also shows the absorption spectrum of a solution of BGG carried in equivalent amounts through the entire procedure except for the addition of aromatic amine. It is apparent from these figures that introduction of the diazo linkage between an aromatic amine and BGG imparts a characteristic absorption spectrum to the complex. The nature of the substituent in the para position of the aromatic amine has more of a quantitative than qualitative effect on the absorption spectrum. Since *p*-aminobenzoylangiotensin II and BGG react to give a product with a similar absorption spectrum, the formation of a diazo linkage between the two is indicated.

Biological Activity of Angiotensin II Derivatives.—Table II shows the biological activity (dog units per millimicromole of angiotensin II) of different angiotensin II derivatives.

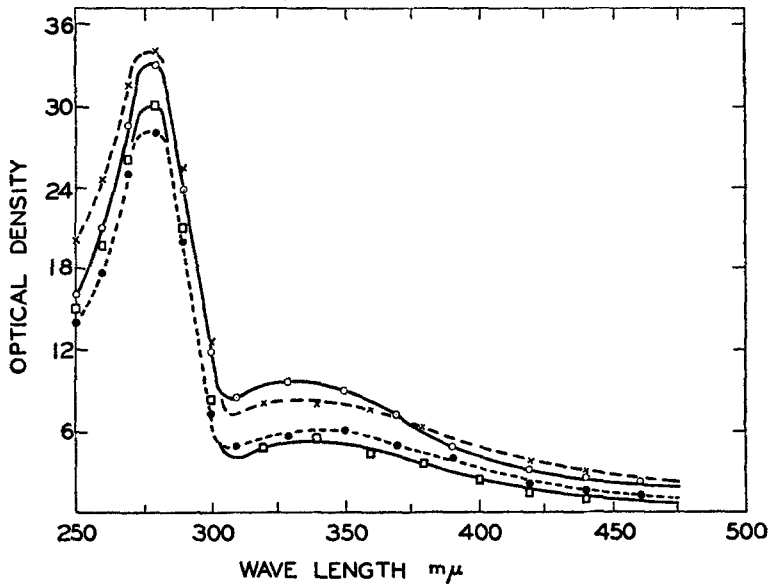


FIG. 2. Absorption spectra of R-phenyl-azo-BGG complexes. O——O, p-aminoacetophenone; X------X, *p*-aminohippuric acid; ●------●, aniline; □——□, *p*-anisidine.

TABLE II

Biological Activity of Angiotensin II Derivatives

Compound	No. of determinations	Biological activity, dog units/millimicromole Tyrosine
		*
1. Angiotensin II (aspartyl-β-amide)	14	2.80 ± 0.2
2. Angiotensin II (aspartyl-β-acid)	8	2.84 ± 0.21
3. *p*-Nitrobenzoylangiotensin II	4	1.2 ± 0.14
4. *p*-Aminobenzoylangiotensin II	6	1.2 ± 0.13
5. After coupling with BGG	5	0.1 ± 0.023

* Standard deviation.

It was shown in control experiments that under the conditions of the coupling procedure non-specific inactivation of angiotensin II can be ruled out. A sample of angiotensin II was carried through the entire procedure, except for the addition of *p*-nitrobenzoyl chloride, and was found to retain its original specific activity and 70 to 80 per cent of its total, initial activity. Therefore, the lower activity of *p*-nitro and *p*-aminobenzoylangiotensin II as compared to that of angiotensin II must be attributed to the introduction of the *p*-nitro (amino) benzoyl group. It was indicated previously that in the *p*-nitrobenzoylation of

angiotensin II some group(s) in addition to the free, terminal amino group of aspartic acid appeared to be reacting. The observation that *p*-nitro (amino) benzoylangiotensin II prepared under these conditions retains about 40 to 50 per cent of the biological, specific activity of the parent compound indicates that the presence of a free, terminal amino group is not an absolute requirement for the pressor action of angiotensin II.[1] This property of angiotensin II distinguishes it from angiotensin I since in the latter case, presence of a free, terminal amino group appears to be essential. Thus Braun-Menendez *et al.* (15) have reported that benzoylation of crude preparations of angiotensin by the Schotten-Bauman procedure resulted in a complete loss of activity. Although the distinction between angiotensin I and angiotensin II was not known at the time of this report, it would appear from their procedures that they were very likely dealing with angiotensin I. Using a partially purified preparation of horse angiotensin I (obtained through the courtesy of Drs. Skeggs and Kahn, Crile Veteran's Administration Hospital, Cleveland—purity 50 per cent, on the basis of dog units/mg. N) we have also observed that the introduction of *p*-aminobenzoyl groups under conditions identical with those used for angiotensin II, results in a complete loss of activity. Aside from the obvious possibility that this may be caused by certain impurities in the starting preparation, one may explain this difference in the behaviors of angiotensins I and II on the basis of the known enzymatic mechanisms involved in the formation of angiotensin II, the active pressor substance. It was shown by Skeggs *et al.* (16) that angiotensin I, the initial product of renin-angiotensinogen interaction, is not the active pressor substance but that it assumes this property only after conversion to angiotensin II, as catalyzed by the "converting enzyme." It is therefore possible that, for the action of the converting enzyme, the presence of a free amino group(s) in angiotensin I is essential; on the other hand, the specificity of the receptor site for angiotensin II may be such that the presence of the free, terminal amino group is not an absolute requirement.

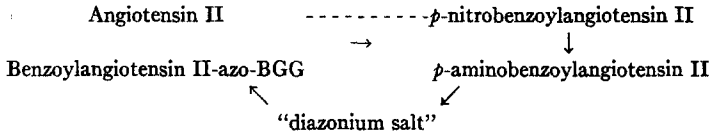
Coupling of the diazonium salt of *p*-aminobenzoylangiotensin II with BGG results in a tenfold decrease of its pressor activity. It has not yet been determined whether this small, residual activity truly represents the pressor action of benzoylangiotensin II-azo-BGG or whether this merely represents contamination with (*a*) uncoupled *p*-aminobenzoylangiotensin II diazonium salt that was not removed by dialysis or (*b*) intermolecular benzoylangiotensin II-azo-benzoylangiotensin II complex which may be formed by the coupling of the diazonium salt with the tyrosine or histidine residue in the polypeptide chain of angiotensin II. During this investigation, no attempt was made to fractionate

[1] Dr. Schwyzer and his coworkers at Ciba Ltd., Switzerland, have recently sent us a sample of *p*-aminobenzoylangiotensin II prepared by total synthesis starting from *p*-aminobenzoyl-aspartic acid (β-amide). From our assay procedures this compound was found to have aromatic amine to tyrosine ratio of 1.0 and a specific activity of 1.4 dog units per millimicromole.

the benzoylangiotensin II-azo-BGG preparation and it was used for immunological studies without further purification.

SUMMARY

Angiotensin II was coupled with bovine γ-globulin (BGG) through the following series of reactions.



By determinations of the aromatic amine and tyrosine contents of *p*-aminobenzoylangiotensin II, the number of *p*-aminobenzoyl residues introduced per molecule of angiotensin II was calculated.

Absorption spectra (between 250 and 500 mμ) of BGG complexes of *p*-aminobenzoylangiotensin II and six different para substituted aromatic amines were compared.

Specific activities (dog units/millimicromole) of the different intermediate products were determined. Presence of a terminal, free amino group does not appear to be an absolute requirement for the biological activity of angiotensin II, since substitution of a *p*-aminobenzoyl radical in this group yields a product with 40 to 50 per cent of the activity of the parent compound. Angiotensin I, on the other hand, is completely inactivated under identical circumstances. Possible implication of this finding has been discussed.

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BIBLIOGRAPHY

1. Wakerlin, G. E., Antibodies to renin as proof of the pathogenesis of sustained renal hypertension, *Circulation*, 1958, **17**, 653.
2. Helmer, O. M., Studies on renin antibodies, *Circulation*, 1958, **17**, 648.
3. Lamfrom, H., Haas, E., and Goldblatt, H., Studies on antirenin, *Am. J. Physiol.*, 1954, **177**, 55.
4. Goldblatt, H., Haas, E., and Lamfrom, H., Antirenin in man and animals, *Tr. Assn. Am. Physn.*, 1951, **64**, 122.
5. Lentz, K. E., Skeggs, L. T., Woods, K. K., Kahn, J. R., and Shumway, N. P., Amino acid sequence of hypertensin II, *J. Exp. Med.*, 1956, **104**, 193.
6. Rittel, W., Iselin, B., Kappeler, H., Riniker, B., and Schwyzer, R., Synthese eines hochwirksamen hypertensin II-amids, *Helv. Chim. Acta*, 1957, **40**, 614.
7. Schwarz, H., Bumpus, F. M., and Page, I. H., Synthesis of a biologically active octapeptide similar to natural isoleucine angiotonin octapeptide, *J. Am. Chem. Soc.*, 1957, **79**, 5697.

8. Schwyzer, R., Iselin, B., Kappeler, H., Riniker, B., Rittel, W., and Zuber, H., Synthese hochwirksamer oktapeptide mit der vermutlichen aminosäuresequenz des noch unbekannten hypertensins II aus rinderserum, *Helv. Chim. Acta.*, 1958, **41,** 1287.
9. Goldblatt, H., Lamfrom, H., and Haas, E., Physiological properties of renin and hypertensin, *Am. J. Physiol.*, 1953, **175,** 75.
10. Hawk, P. B., Oser, B. L. and Summerson, W. G., Practical Physiological Chemistry, Philadelphia, Blakiston Co. Inc., 1954, 13th edition, 939.
11. Fantl, P., The estimation of sulphanilamide and other primary aromatic amines in body fluids, *Australian J. Exp. Biol. and Med. Sc.*, 1940, **18,** 175.
12. Landsteiner, K., and Van der Scheer, J., On the serological specificity of peptides, *J. Exp. Med.*, 1932, **55,** 781.
13. Landsteiner, K., and Van der Scheer, J., On the serological specificity of peptides, *J. Exp. Med.*, 1934, **59,** 769.
14. Miller, G. L., and Stanley, W. M., Derivatives of tobacco mosaic virus, *J. Biol. Chem.*, 1942, **146,** 331.
15. Braun-Menendez, E., Fasciolo, J. C., LeLoir, L. F., and Munoz, J. M., The substance causing renal hypertension, *J. Physiol.*, 1940, **98,** 283.
16. Skeggs, L. T., Kahn, J. R. and Shumway, N. P., The preparation and function of the hypertensin converting enzyme, *J. Exp. Med.*, 1956, **103,** 295.