IMMUNOLOGIC PRODUCTION OF ANTIANGIOTENSIN

II. Production and Detection of Antiangiotensin

By SHARAD D. DEODHAR, Ph.D.

(From the L. D. Beaumont Memorial Laboratory, The Mount Sinai Hospital, Cleveland)

(Received for publication, November 16, 1959)

Landsteiner's original studies (1) on hapten-antibody interaction were extended by other workers to the production of antibodies against certain biologically active, low molecular weight, organic compounds. Thus, Clutton *et al.* (2) were able to produce an antibody to thyroxine, by the injection of a thyroxine-protein complex into rabbits, and this antibody neutralized the ability of thyroglobulin to raise the basal metabolic rate of rats. Similar studies with histamine (3) and aspirin (4) have demonstrated that antibodies capable of neutralizing the biological action of each of these compounds could be produced by the employment of suitable hapten-protein complex antigen. However, a hapten-antibody reaction does not always result in neutralization of the biological activity of the hapten; for example, serologically demonstrable antibody to adrenaline, fails to inhibit its pressor action (5). The present report describes the procedures used in the production and detection of antiangotensin by the employment of benzoylangiotensin II-azo-BGG as the hapten-protein antigen. The preparation of this antigen has been described previously.

Materials and Methods

Valine^5-angiotensin II (aspartyl-β-acid)-synthetic product obtained through the courtesy of Dr. Schwyzer, Ciba Ltd., Switzerland.

Isoleucine^5-Angiotensin I and II.—These were natural products prepared by the action of hog renin on horse serum angiotensinogen and were obtained through the courtesy of Dr. Skeggs and Dr. Kahn, Crile Veteran's Administration Hospital, Cleveland. Purity over 98 per cent for type II and 50 per cent for type I on the basis of dog units per milligram N.

Immunization Procedure.—Female rabbits weighing between 3 to 3.5 kg. were divided into four groups, with three of the groups consisting of two rabbits each and the fourth group of three rabbits. Group 1 was a control group not subjected to the immunization procedure, while groups 2, 3, and 4 were immunized with bovine gamma globulin (BGG) alone, with benzoylangiotensin II-azo-BGG or with angiotensin II alone, respectively. Immunizations were carried out according to the following procedure. The three antigen solutions were made up in buffered saline (9 parts of isotonic saline + 1 part of 0.1 M sodium phosphate buffer, pH 7.4) to which was added either 10 mg. BGG/ml., or 10 mg. benzoylangiotensin II-azo-BGG/ml. or 2.5 mg. angiotensin II/ml. All injections were given intraperitoneally. For the first two injections of each series 1 ml. of each antigen solution was thoroughly mixed with 1 ml. of Freund's complete adjuvant to form a homogeneous colloidal suspension. The same

procedure was repeated a week later and from then on the injections consisted of 0.5 ml. of antigen solutions without Freund's adjuvant given three times a week for a period of 5 weeks.

Isolation of Rabbit γ-Globulin.—At the end of the immunization period all rabbits were bled from the femoral artery and the plasma collected from each rabbit was fractionated for γ-globulin according to the procedure described by Goldstein and Anderson (6). The purity of these γ-globulin fractions was checked by paper electrophoresis on a Spinco model R apparatus. The outstanding advantage of this procedure was that the antibody containing γ-globulin fraction prepared in this manner was free of serum angiotensinase activity which would have interfered with the assay procedure for anti-angiotensin. All γ-globulin fractions were lyophilized and stored under vacuum, as dry powders.

Assay for Antiangiotensin.—The γ-globulin fraction obtained from the serum of each rabbit was incubated with a known amount of angiotensin II, and, at the end of the incubation period, the reaction mixture was assayed for angiotensin activity by the dog assay procedure previously described. In addition, the guinea pig ileum assay procedure, as described by Picarelli *et al.* and Collins and Hamilton (7, 8), was used, especially when amounts of angiotensin were too small to be assayed in the dog. For the calculation of unknown amounts of angiotensin II from ileum responses, the method described by Burn (9) was used.

Serological Procedures.—Antigen-antibody precipitin reactions were carried out by the incubation of immune γ-globulin fraction with the appropriate antigen, for 1 hour, at 26–27°C., followed by 16 hours at 2–3°C. For incubation, all solutions were made up in buffered saline. The extent of precipitation was determined by measuring optical density (at 750 mμ) of the reaction mixture in a Coleman Junior spectrophotometer (8 x 8 x 100 mm. cuvettes). Because of certain obvious drawbacks in this approach for the study of precipitin reactions, these tests must be considered only qualitative. In general, the serological demonstration of antiangiotensin involved (*a*) a study of its precipitin reaction with benzoylangiotensin II-azo-BGG and with BGG alone, (*b*) partial inhibition of this precipitin reaction by angiotensin II, and (*c*) precipitin reaction with an angiotensin II-protein complex containing a protein other than BGG.

EXPERIMENTAL

Table I summarizes the results of experiments in which a known amount of angiotensin II was incubated with the γ-globulin fraction from each group of immunized rabbits. These experiments were carried out under conditions described below Table I.

Adequacy of the Goldstein-Anderson procedure for the isolation of an angiotensinase-free γ-globulin from rabbit plasma is evident from the results shown in Table I (Experiments 2, 3, and 5). We have further observed that the incubation of angiotensin II with normal rabbit γ-globulin for as long as 2 to 2½ hours at 37°C. does not result in any loss of angiotensin activity. Similarly, after incubation with γ-globulin from rabbits immunized with BGG alone (group 2), the total initial amount of angiotensin II was recovered. On the other hand, γ-globulin from group 3 rabbits (Table I, Experiment 4) inactivated a small but significant portion of added angiotensin II. The fact that in Experiment 5 no disappearance of angiotensin II activity was observed indicates that angiotensin II is probably too small a molecule to be antigenic by itself, since each rabbit in this group was immunized with an amount of angiotensin II equivalent to that used in the form of azo-BGG complex for the immunization of group 3 rabbits.

TABLE I

Inactivation of Angiotensin II by Antiangiotensin

No.	Experiment	Units of angiotensin II recovered	Inactivation of angiotensin
		*	*per cent*
1.	No γ-globulin added	2.88 ± 0.2	0
2.	γ-globulin from untreated rabbits, group 1	2.76 ± 0.2	2
3.	γ-globulin from rabbits given BGG, group 2	2.94 ± 0.21	0
4.	γ-globulin from rabbits given angiotensin II-BGG-complex, group 3	1.90 ± 0.18	33‡
5.	γ-globulin from rabbits given angiotensin II alone, group 4	2.80 ± 0.21	0
6.	Group 3 γ-globulin alone (no angiotensin II added)	0	

Reaction mixture, 20 mg. γ-globulin in 0.5 ml. buffered saline, 0.1 ml. (1.0 millimicromole or 2.8 dog units) angiotensin II. Incubated at 37°C. for 75 minutes, at the end of the incubation period 2.2 ml. of buffered saline was added and the solution was then assayed in the dog. Each experiment in Table I was carried out in duplicate and each reaction mixture was assayed in two different dogs so that each figure indicated in the above table represents an average of four determinations.

* Standard deviation.

‡ This figure is significantly different from the "control" values ($P < 0.01$).

TABLE II

Specificity of Antiangiotensin

Experiment No.	Angiotensin analog	Units of angiotensin recovered	Inactivation of angiotensin
		*	*per cent*
1.	Valine^5-angiotensin II (aspartyl-β amide)	1.94 ± 0.18	30.7
2.	Valine^5-angiotensin II (aspartyl-β acid)	1.22 ± 0.14	56.7
3.	*p*-Aminobenzoylangiotensin II (aspartyl-β amide)	0.8 ± 0.1	71.5
4.	Isoleucine^5-angiotensin II	1.36 ± 0.15	51.5
5.	Isoleucine^5-angiotensin I	3.0 ± 0.22	0

Reaction mixture, 20 mg. of group 3 γ-globulin in 0.5 ml. of buffered saline, 0.1 ml. (2.8 dog units) of the respective angiotensin analog. Remainder of the procedure same as that described below Table I.

There is no significant difference between the values of Experiments 2 and 4. All other results are significantly different from each other (P value <0.01).

* Standard deviation.

Specificity of Antiangiotensin.—Table II shows a comparison of the effects of antiangiotensin on different analogs of angiotensin. Experimental conditions used in this case are described below Table II.

As indicated in Table II, antiangiotensin possesses greater activity in neutralizing the free acid form of angiotensin II than the amide form. Since, in

the preparation of the azo-protein antigen, the amide and not the free acid form was used as the starting product, one would expect the opposite result. It is possible, however, that during one of the reactions employed in the coupling process the β-amide group of aspartic acid was converted to the free acid form. The most likely reaction in this respect appears to be that involving diazotization of *p*-aminobenzoylangiotensin II with nitrous acid (HNO_2), since this reagent is known to convert organic amides into the corresponding free acids under comparable conditions (10). During this investigation, no direct attempts were made to prove the occurrence of such a transformation.

Table II also shows that, of all the different analogs, *p*-aminobenzoylangiotensin II is inactivated to the largest extent. This may be attributed to the presence of the *p*-aminobenzoyl group, which brings this compound structurally closer to the hapten moiety of the benzoylangiotensin II-azo-BGG complex antigen.

A comparison of Experiments 2 and 4, in Table II, shows that the two forms of angiotensin II, (synthetic, valine[5] and the natural, isoleucine[5]) are inactivated to about the same extent by antiangiotensin. The substitution of valine for isoleucine, the only difference between these two octapeptides, does not affect the hapten-antibody interaction. On the other hand, antiangiotensin has no effect on angiotensin I (Experiment 5, Table II) which differs from angiotensin II in possessing the additional dipeptide, histidyl-leucine, at the phenyl alanine carboxyl end. This observation is in agreement with the studies of Landsteiner (11) on peptide haptens. He demonstrated that the amino acid with the free carboxyl group at the carboxyl end of the peptide determines, to a large extent, the specificity of the respective antibody. Since the structure of angiotensin I and angiotensin II differs markedly at the free carboxyl end, it follows from Landsteiner's conclusion that antibody to angiotensin II would show little or no affinity for angiotensin I.

The observation that antiangiotensin inactivates angiotensin II (Table II, Experiment 4) but not angiotensin I (Table II, Experiment 5), further rules out the possibility that the antiangiotensin activity of group 3 γ-globulin, may be due to a contamination of this fraction with serum angiotensinase, since the latter inactivates both forms of angiotensin. Figure 1 represents an experiment in which normal rabbit serum was incubated with known, equal amounts of angiotensin II (aspartyl-β-amide), angiotensin II (aspartyl-β-acid) and angiotensin I and the amount of angiotensin left in each incubate at different time intervals was determined. The conditions used for this experiment are described below Fig. 1.

Serological Studies.—The group 3 rabbit γ-globulin solution, containing antiangiotensin, readily showed a precipitin reaction upon addition of benzoylangiotensin II-azo-BGG. Precipitation was also observed with BGG alone, but larger amounts of protein were needed in this case to bring about

maximum precipitation. This is illustrated in Fig. 2, in which the amount of precipitate formed, as indicated by the optical density of the solution, is plotted against the corresponding amount of antigen protein added in each case.

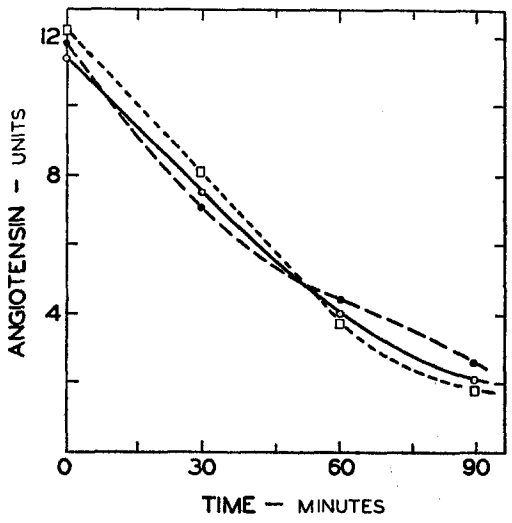

FIG. 1. Inactivation of angiotensin derivatives by serum angiotensinase

□-----□, angiotensin I; O——O, angiotensin II (acid); ●-----●, angiotensin II (amide).

(Reaction mixture—2 ml. normal rabbit serum; 0.4 ml. (12 dog units) of the respective angiotensin solution; incubated at 37°C.

At 0, 30, 60, and 90 minutes, 0.6 ml. of the reaction mixture was withdrawn, and to this was added 0.04 ml. of 1 N HCl. The acidified solution was heated in boiling water for 5 minutes to terminate angiotensinase activity and to coagulate and precipitate serum proteins. 0.04 ml. of 1 N NaOH and 2.7 ml. of buffered saline were then added and the suspension was centrifuged. Supernatant obtained from this centrifugation was assayed for angiotensin in the dog).

Fig. 2 indicates that the introduction of *p*-aminobenzoylangiotensin II residues into BGG changes the antigenic nature of the latter, so that the antibody produced in response to benzoylangiotensin-azo-BGG shows a distinctly higher affinity for the BGG-azo complex than for BGG alone. A similar example of this phenomenon may be found in the studies of Heidelberger *et al.* (12) on the quantitative precipitin reactions of anti-azo-egg albumin with azo-egg albumin and with native egg albumin. The type of curve illustrated in Fig. 2 is often seen for many of the so called cross-reactions, in which the antigen and antibody do not fit very well and large amounts of antigen are needed for maximal precipitation.

The precipitate formed after the addition of BGG to group 3 γ-globulin did

not remove antiangiotensin, and, after centrifugation, the supernatant was found to contain the full antiangiotensin activity of the original solution.

It was not possible to carry out a similar experiment with the supernatant obtained from the precipitin reaction of group 3 γ-globulin with benzoylangiotensin II-azo-BGG, since this latter preparation itself was found to have considerable angiotensin II (pressor) activity (13).

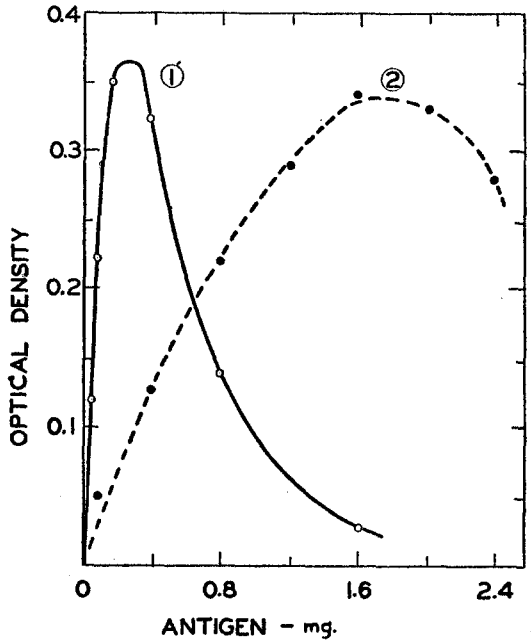

FIG. 2. Precipitin reactions of antiangiotensin.

①, angiotensin II-azo-BGG; ②, BGG alone.

(To 0.25 ml. of group 3 γ-globulin (20 mg./ml.) containing antiangiotensin were added increasing amounts of each antigen, dissolved in buffered saline. Final volume 1 ml. in each tube; incubated at 26°C. for 1 hour and at 2–3°C. for 16 hours. The resulting suspension was diluted to 2 ml. with buffered saline and its optical density determined. Controls, γ-globulin solution, in buffered saline, and each antigen alone, in buffered saline.)

Inhibition of Precipitin Reaction.—Ability of a hapten to inhibit the precipitin reaction between the hapten-protein complex and its antiserum is usually considered as evidence for the existence of antibodies to the hapten. In the case of antiangiotensin, the phenomenon of hapten inhibition was demonstrated in the following manner. A given amount of group 3 γ-globulin (antiangiotensin) was incubated with increasing amounts of angiotensin II and, following this incubation, the precipitin reaction was carried out in the usual manner by the addition of the same amount of benzoylangiotensin II-azo-BGG

to all the reaction mixtures. Fig. 3 represents the results obtained from such an experiment.

It is apparent from Fig. 3 that angiotensin II shows a significant inhibition of the precipitin reaction between antiangiotensin and its corresponding antigen, whereas it has practically no effect on the reaction between anti-BGG and BGG. The phenomenon of hapten inhibition is usually explained by the

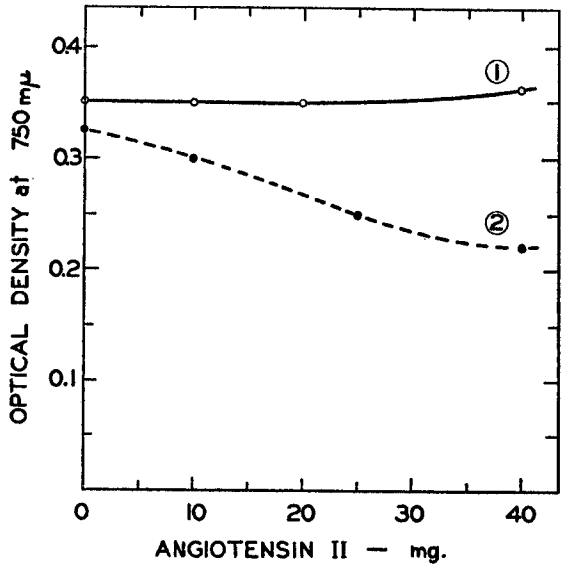

FIG. 3. Inhibition of the precipitin reaction by angiotensin II.

①, BGG; ②, angiotensin II-azo-BGG.

To 0.25 ml. of group 3 γ-globulin (20 mg./ml.) were added 0, 10, 20, and 40 mg. of angiotensin II; total volume 0.5 ml.

Incubated at 37°C. for 1 hour. To each incubate was added 0.4 mg. of benzoylangiotensin II-azo-BGG in 0.5 ml. solution and the precipitin reaction was studied in the manner previously described. An identical experiment was set up to study the effect of angiotensin II on the precipitin reaction between group 2 γ-globulin (from rabbits immunized with BGG alone) and BGG.

hypothesis that the hapten molecules block the active sites on the corresponding antibody, thereby preventing later combination with the homologous antigen. The possibility that larger amounts of angiotensin II might produce a greater degree of inhibition could not be tested, because the amount of angiotensin II available was limited. For the same reason, inhibition by angiotensin II (free acid form) was not tested at all. In the case of several synthetic peptides that were studied by Landsteiner (11), the precipitin reaction between benzoylpeptide-azo-protein antigen and the corresponding antiserum was inhibited by the *p*-aminobenzoyl derivative, but not by the free peptide itself.

On the other hand, both histamine and *p*-aminobenzoylhistamine could effectively inhibit the precipitin reaction between histamine-azo protein and its antiserum (3). It was pointed out previously that *p*-aminobenzoylangiotensin II was the angiotensin derivative most effectively antagonized by antiangiotensin (Table II, Experiment 3); one might expect this derivative to be highly effective in inhibiting the precipitin reaction as well. Unfortunately, the solubil-

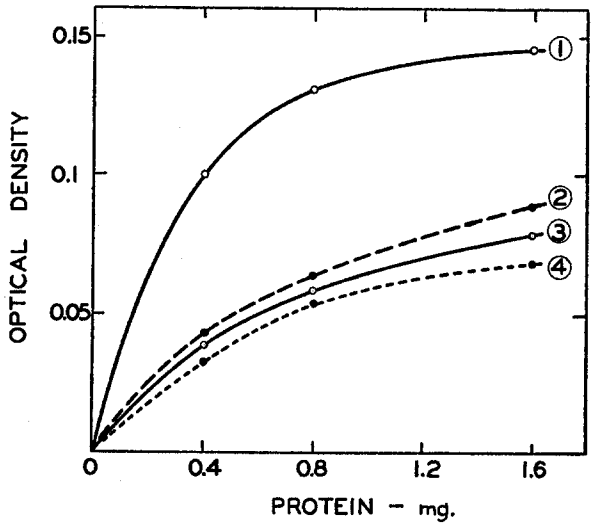

FIG. 4. Precipitin reaction of antiangiotensin with a different angiotensin II-protein complex.

①, angiotensin II-azo–cat serum + γ-globulin 3; ②, cat serum + γ-globulin 3; ③, cat serum + γ-globulin 2; ④, angiotensin II-azo–cat serum + γ-globulin 2.

10 mg. of dialyzed, lyophilized cat serum was coupled with 2.5 mg. of *p*-aminobenzoyl-angiotensin II according to the procedure described previously. To 0.25 ml. of group 3 or group 2 γ-globulin (20 mg./ml.) were added increasing amounts of cat serum protein and benzoylangiotensin II-azo–cat serum protein. Final volume 1 ml. Precipitin reactions were studied, as described previously, with appropriate controls.

ity of *p*-aminobenzoylangiotensin II at or near the pH of neutrality is too low to permit such a study.

Precipitin Reaction of Antiangiotensin with a Different Angiotensin II-Protein Complex.—Further evidence for the presence of antiangiotensin in group 3 γ-globulin was derived from the observation that a precipitin reaction could be demonstrated not only with its corresponding antigen, but also with *p*-aminobenzoylangiotensin II coupled to cat serum protein. Group 3 γ-globulin cross-reacts to a certain extent with cat serum alone; however, the degree of precipitation is increased significantly upon the addition of benzoylangiotensin-azo–cat serum protein. This experiment is illustrated in Fig. 4.

This type of evidence has been used to demonstrate the presence of antibody to a hapten. During their studies on the production and detection of antibody to histamine, Fell and collaborators (3) observed that the antiserum obtained in response to administration of histamine-azo-globulin (horse) to rabbits, could enter into a precipitin reaction with histamine coupled to a variety of proteins, including rabbit serum, casein, egg albumin, and others. Many examples of this phenomenon may be found in the classical studies of Landsteiner.

DISCUSSION

By the use of many of the criteria estabished for the detection of antibody to a hapten, it was possible to show that the parenteral administration of the angiotensin-BGG complex, (benzoylangiotensin II-azo-BGG) to rabbits, results in the production of an antiserum containing antiangiotensin activity. The ability of this antiserum to neutralize (*in vitro*) the biological action (pressor activity, and smooth muscle contraction) of angiotensin II, and the specificity of this neutralization, the partial inhibition of the precipitin reaction between the antiserum and the angiotensin complex by angiotensin II, and the precipitin reaction of the antiserum with another angiotensin protein complex (benzoylangiotensin II-azo–cat serum) are among the most pertinent findings that establish conclusively the formation of antiangiotensin. Since our interests at present are directed mainly towards the neutralization of the pressor activity of angiotensin by antiangiotensin, and also because of the limited amount of angiotensin II available to us, the serological tests described in this report were used only for a qualitative demonstration of antiangiotensin.

In comparing the two antigen-antibody systems investigated so far in relation to the humoral mechanism of renal hypertension, namely the renin-antirenin (14) and angiotensin II-antiangiotensin systems, the rapidity of interaction and the high titers of antibody as seen in the former case become worthy of consideration. Thus, in the rabbit, antirenin titers of up to 10 units per ml. serum, (and even higher in other animals) were produced after the injection of hog renin, whereas the highest titer of antiangiotensin was 0.8 units per ml. serum (*i.e.* 10 mg. of γ-globulin). Also the inhibition of renin by antirenin, *in vitro*, occurs instantaneously, while an incubation period of about 75 minutes is necessary to produce the maximal effect of antiangiotensin. It remains to be seen how these factors influence the activity of antiangiotensin *in vivo*. Studies on the effect of this immunization procedure with benzoylangiotensin II-azo-BGG on the hypertensive course of renal hypertensive dogs and rabbits are currently in progress in our laboratories.

SUMMARY

The parenteral administration of benzoylangiotensin II-azo-BGG to rabbits produced an antiserum with antiangiotensin activity.

Antiangiotensin inhibited the biological action of *p*-aminobenzoylangiotensin II, valine^5-angiotensin II (free acid form), isoleucine^5-angiotensin II and valine^5-angiotensin II (amide form), but it was totally inert towards angiotensin I.

Antiangiotensin activity was distinguished from that of serum angiotensinase by the following observations: (*a*) an angiotensinase-free γ-globulin fraction contained antiangiotensin, (*b*) angiotensinase inactivated angiotensin II (both the amide and free acid forms) and angiotensin I in contrast to the remarkable specificity for angiotensin II exhibited by antiangiotensin.

Serological demonstration of antiangiotensin included: (*a*) a comparison of its precipitin reaction with the angiotensin BGG complex with the reaction with BGG alone, (*b*) the partial inhibition of the precipitin reaction with the angiotensin BGG complex by angiotensin II, (*c*) a precipitin reaction with a different angiotensin II protein complex (cat serum).

Angiotensin II administered parenterally as the free polypeptide was not antigenic.

The author wishes to express his sincere appreciation to Dr. Harry Goldblatt and Dr. Erwin Haas for their suggestion of this problem and their guidance throughout the course of this work and in preparation of this manuscript. The competent technical assistance of Mrs. Mary Picken and Mr. James Malone is gratefully acknowledged.

BIBLIOGRAPHY

1. Landsteiner, K., The Specificity of Serological Reactions, Cambridge, Harvard University Press, 1946.
2. Clutton, R. F., Harrington, C. R., and Yuill, M. E., Studies in synthetic immunochemistry, *Biochem. J.*, 1938, **32,** 1119.
3. Fell, N., Rodney, G., and Marshall, D. E., Histamine-protein complexes: synthesis and immunologic investigation, *J. Immunol.*, 1943, **47,** 237.
4. Butler, G. C., Harrington, C. R., and Yuill, M. E., Studies in synthetic immunochemistry, *Biochem. J.*, 1940, **34,** 838.
5. Went, S., Kesztyiis, L., and Szilagyi, T., Weitere untersuchungen über die physiologische wirkung der adrenalylazoprotein-antikörper, *Arch. exp. Path. u. Pharmakol.*, 1943, **202,** 143.
6. Goldstein, J., and Anderson, J. W., The isolation of γ-globulins from rat, guinea pig, and rabbit plasma, *J. Biol. Chem.*, 1957, **224,** 775.
7. Picarelli, Z. P., Kupper, R., Prado, E. S., Prado, J. L., and Valle, J. R., Assay of renin and hypertensin with the isolated guinea pig ileum, *Circulation Research*, 1954, **2,** 354.
8. Collins, D. A., and Hamilton, A. A., Changes in the renin-angiotonin system in hemorrhagic shock, *Am. J. Physiol.*, 1944, **140,** 499.
9. Burn, J. H., Biological Standardization, Oxford University Press, 2nd edition, 1950, 183–186.
10. Fieser, L. F., and Fieser, M., Organic Chemistry, Boston, T. C. Heath and Co., 1956, 3rd edition, 242.

11. Landsteiner, K., and Van der Scheer, J., On the serological specificity of peptides, *J. Exp. Med.*, 1932, **55,** 781.
12. Heidelberger, M., and Kendall, F. E., Quantitative studies on the precipitin reaction, *J. Exp. Med.*, 1934, **59,** 519.
13. Deodhar, S. D., Immunologic production of antiangiotensin. I. Preparation of angiotensin-protein complex antigen, *J. Exp. Med.*, 1960, **111,** 419.
14. Lamfrom, H., Haas, E., and Goldblatt, H., Studies on antirenin, *Am. J. Physiol.*, 1954, **177,** 55.