Biochemical Pharmacology, Vol. 19, pp. 651–662. Pergamon Press. 1970. Printed in Great Britain

PREPARATION AND PROPERTIES OF IODINATED ANGIOTENSINS*

SHOEI-YN LIN,† HENRY ELLIS, BERNARD WEISBLUM and THEODORE L. GOODFRIEND‡

Departments of Pharmacology and Internal Medicine, University of Wisconsin School of Medicine, Madison, Wisc. 53706, U.S.A.

(*Received* 8 *March* 1969; *accepted* 16 *May* 1969)

Abstract—Monoiodoangiotensin II amide and diiodoangiotensin II amide were synthesized and purified. The monoiodo derivative was less active than native angiotensin II in both biologic and immunologic assays. Its potency was from 25 to 80 per cent of the parent compound depending on the assay system. Diiodoangiotensin II amide was less active than monoiodoangiotensin II amide. Its potency was 2 to 24 per cent of angiotensin. Conflicting results in the literature regarding the activity of iodinated angiotensins may have resulted from the use of different mixtures of the two compounds and different assay methods.

INVESTIGATIONS into the role of angiotensin in health and disease have been aided by radioactive derivatives of the hormone. A tritiated polypeptide was synthesized by Khairallah *et al.*,[1] and a sulfur-35 derivative by Osborn *et al.*[2] Derivatives of higher specific activity have been obtained by iodination of the tyrosine residue in position 4 (Table 1).[3] However, alteration of angiotensin by introduction of 1 or 2 iodine atoms or by the reagents used for iodination have raised questions about the usefulness of

TABLE 1. AMINO ACID SEQUENCES OF ANGIOTENSINS AND DERIVATIVES

Position:	1	2	3	4	5	6	7	8
	COOH							
	Asp	Arg	Val	Tyr	Val	His	Pro	Phe
	Angiotensin II acid							
	$CONH_2$							
	Asp	Arg	Val	Tyr	Val	His	Pro	Phe
	Angiotensin II amide							
	$CONH_2$			I				
	Asp	Arg	Val	Tyr	Val	His	Pro	Phe
	Monoiodoangiotensin II amide							
	$CONH_2$			I_2				
	Asp	Arg	Val	Tyr	Val	His	Pro	Phe
	Diiodoangiotensin II amide							

*Supported by research grants from the National Heart Institute (HE-09922), from the Wisconsin Heart Association, and the National Science Foundation (GB-7145).

†Submitted in partial fulfillment of the requirements for the Ph.D. degree in Pharmacology.

‡Recipient of a U.S. Public Health Service Research Career Development award.

iodinated products for physiologic studies.[4-6] We report here the synthesis and purification of monoiodoangiotensin II and diiodoangiotensin II and their biologic and immunologic properties.* Our results indicate that the purified monoiodo derivative is suitable for physiologic studies, but the diiodo derivative is not. Conflicting physiological data from various laboratories may be explained by the use of different mixtures of these two forms.

MATERIALS

Angiotensin II was the gift of Dr. J. J. Chart, Ciba Pharmaceuticals, Summit, N.J. The material was labeled 83 per cent pure. Impurities were not described. Angiotensin II acid was the gift of Dr. W. Rittel, Ciba Pharmaceuticals, Basle, Switzerland; samples of commercial radioactive iodinated angiotensin were supplied by Mr. E. Lieberman, Cambridge Nuclear Corp., Cambridge, Mass.; ^{125}I was purchased from Cambridge Nuclear Corp., Cambridge, Mass.; 3-monoiodotyrosine and 3,5-diiodotyrosine were purchased from Calbiochem, Los Angeles, Calif.; anion exchange resin AG1-X8 was purchased from Bio-Rad, Richmond, Calif.; anion-exchange resin-impregnated paper WB-2 was purchased from Reeve–Angel, Clifton, N.J.; cation-exchange resin, type 15A, for the model 120 amino acid analyzer was purchased from Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.; and rats of the Rolfsmeyer strain were obtained locally.

METHODS

Iodination. Angiotensin was iodinated with ^{127}I by the method of Simpson and Vallee.[7] One ml of a fresh solution of iodine (0·1 M) in KI (0·5 M) was added dropwise to a solution containing 10 mg angiotensin in 15 ml of sodium barbital (0·02 M)-NaCl (2 M), pH 7·5. The reaction mixture was stirred at 4° for 2 hr, and the reaction terminated by the addition of sufficient sodium thiosulfate (0·1 M) to decolorize the solution.

Radioactive iodine was added to angiotensin by the Chloramine-T method of Hunter and Greenwood,[8] as modified by Kwa and Verhofstad.[9] To a vial containing 3–4 mc of carrier-free ^{125}I in 50 μl of 0·1 N NaOH was added 20 μl of sodium borate buffer, 0·12 M, pH 7·6. An initial aliquot of sodium metabisulfite, 10 μl of a solution containing 2·5 mg/ml in borate buffer, 0·012 M, pH 7·6, was then added. Angiotensin was introduced, 1 μg in 2 μl water, followed by Chloramine-T, 20 μl of a fresh solution containing 5·0 mg/ml in 0·012 M buffer. The contents of the vessel were mixed quickly, and within 20 sec the reaction was stopped by the addition of a second aliquot of sodium metabisulfite, 0·1 ml of the above solution. Finally, 1 mg of potassium iodide in 0·1 ml, and 2 μl of acetic acid, 4 N, were added to the vial. The acetic acid assured the conversion of angiotensin to the cationic form, so that it would not adhere to anion exchange resins described below.

Purification. Radioactive iodinated derivatives were purified by two preliminary anion exchange chromatographic procedures before the definitive cation exchange step. (Nonradioactive iodination reaction mixtures were brought to pH 3·1 and applied directly to the cation exchange column.) Immediately after iodination, the

*Abbreviations. Unless indicated otherwise, the terms angiotensin and angiotensin II will refer to asparagine-arginine-valine-tyrosine-valine-histidine-proline-phenylalanine, (val^5-angiotensin II amide).

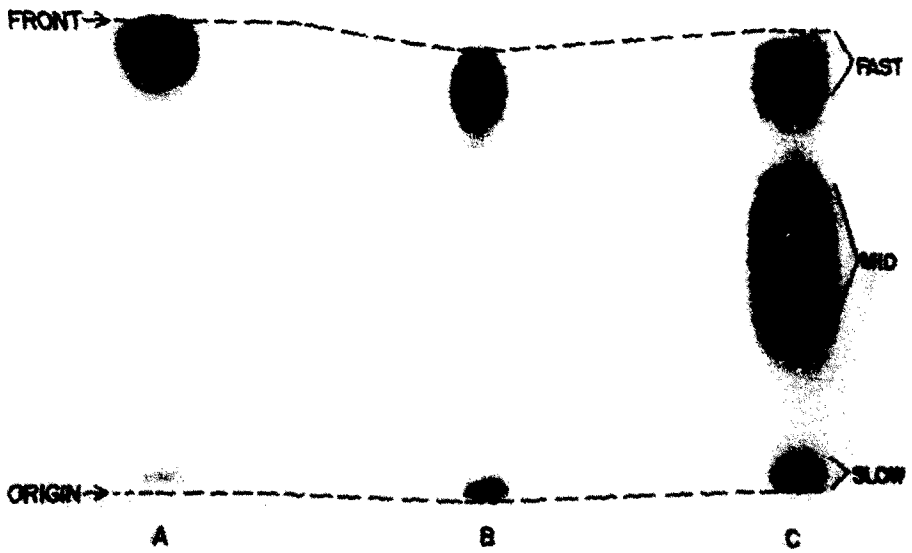

FIG. 1. Radioautogram of paper chromatographic purification of iodinated angiotensins. The products of Chloramine-T iodination, using ^{125}I and angiotensin II amide were passed through anion exchange resin, then applied to ion-exchange resin-impregnated paper and developed as described in the text. The specimens labeled A, B and C are the products of three different iodinations. The iodinated angiotensins and some residual impurities migrated in the fast fraction. Iodide remained near the origin.

P.—facing page 652

reaction mixture was applied to a short column (7 × 120 mm) of anion exchange resin AG1-X8 in the chloride form and eluted with water. Iodinated polypeptide appeared within the first 3 ml. The second anion exchange purification was performed on Reeve–Angel paper type WB-2 by descending chromatography in a solvent system composed of pyridine (65): acetic acid (57): ethanol (400): water (278). Iodinated angiotensin migrated with the front, and impurities remained at the origin or middle of the paper. These could be visualized by radioautography as shown in Fig. 1.

Cation exchange chromatography was performed according to the method of Jones,[10] using sulfonated styrene divinylbenzene copolymer resin, 8% cross-linked (Dowex 50-X8). The iodinated angiotensins were applied in aliquots of 1–3 ml to the column (1 × 20 cm), which had been equilibrated with starting buffer (pyridine, 64·5 ml; acetic acid, 1115 ml; water, 2820 ml; pH 3·1). Elution was achieved with a linear gradient to the limit buffer (pyridine, 653 ml; acetic acid, 529 ml; water, 2798 ml; pH 4·8). The gradient was delivered from two bottles, the first of which contained 500 ml of starting buffer. The column was maintained at 50° with a water jacket. Flow was maintained at 30 ml/hr with a pump.

Chemical analyses. Because acid hydrolysis causes conversion of iodotyrosines to tyrosine, separate aliquots of iodoangiotensins were hydrolyzed with HCl, 6 N at 105° for 18 hr, and with barium hydroxide, 5 M, at 105° for 18 hr. Barium was removed with ammonium carbonate as described by Ray and Koshland,[11] and the mixture titrated to the pH of the eluting buffer with HCl.

Amino acid analyses were performed with the Spackman–Stein–Moore apparatus,[12] operated at 50° and eluted at 70 ml per hr. Neutral amino acids were measured with standard techniques. Diiodotyrosine was measured using the short column and a buffer containing sodium citrate, 0·117 M; BRIJ-35, 0·9 g per l.; and *n*-propanol, 17·5 ml per l., brought to pH 4·43 with concentrated HCl. Monoiodotyrosine was measured using the long column equilibrated and eluted with the aforementioned buffer at pH 5·27. The long column was made necessary by the presence of an alkaline degradation product of arginine, probably ornithine, which eluted close to monoiodotyrosine when the short column was used.

Iodine and iodinated compounds were detected in chromatographic fractions and measured by a method which takes advantage of the catalytic effect of iodine on the reduction of ceric sulfate by arsenious acid.[13, 14] Two ml of 2% ceric sulfate was added to a cuvette containing a suitable amount of iodinated compound (e.g. 7–70 μg of iodoangiotensin, or 1–10 μg of monoiodotyrosine). The reaction was initiated by adding 0·4 ml of 1% As_2O_3 at room temperature. Optical density at 450 mμ was read after 30 sec. The decrease in optical density, corrected for a blank containing no iodine, correlated linearly with the quantity of iodinated compound. The effect of iodine differed depending on its presence as iodide, or organic iodine, and the organic compounds differed among themselves. For this reason, quantitative measurement of the iodine content of unknown organic compounds was accurate only after acid treatment to dissociate the iodine. The reaction was used without standardization for qualitative detection of iodinated compounds in column fractions.

Immunologic and biologic analyses. Antibody to angiotensin was induced in rabbits as previously described.[15] The antiserum used in these studies was from rabbit No. 4 in reference 15. Immunologic reactivity of the iodinated derivatives was determined

by two methods. Radioactive derivatives were tested for their binding to antibody by incubating them in serial dilutions of antiserum at 4° for 18 hr, then separating antibody-bound radioactivity from free radioactivity by ammonium sulfate precipitation.[15] Nonradioactive compounds were tested for their capacity to inhibit the antibody binding of radioactive antigens in a similar assay.

Smooth muscle preparations were suspended in de Jalon's solution at 30°, and aerated with 95% oxygen, 5% CO_2. Isometric contraction was recorded with a Grass strain gauge (model FT 03) and polygraph (model 5B).

Pressor responses were determined in rats which had undergone vagotomy and had received pentolinium, pentobarbital, and atropine.[16]

RESULTS

Purification and characterization of iodoangiotensins. When ^{125}I was added to angiotensin II amide by the Chloramine-T procedure, a mixture of products was formed which was resolved best by column chromatography. Figure 2A shows the results of fractionating the products of one iodination. Figure 2C is the pattern obtained from a sample supplied by Cambridge Nuclear Corp. Different iodinations in our own laboratory yielded different proportions of the major peaks. The cause of this variation was not clear. Two separate samples of partially purified angiotensin-^{125}I from Cambridge Nuclear Corp. contained 60% and 85% of peak IV.

The chemical composition of radioactive peaks IV and V was determined by

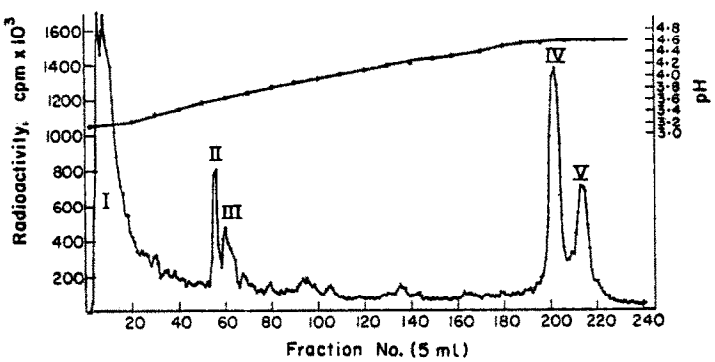

FIG. 2A

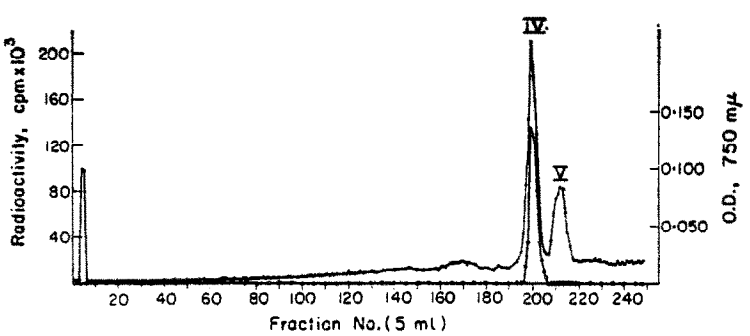

FIG. 2B

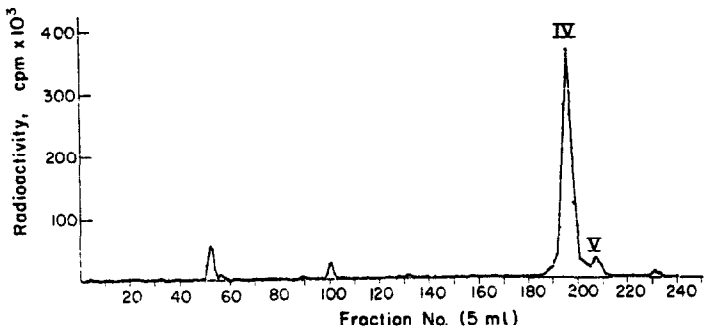

FIG. 2C

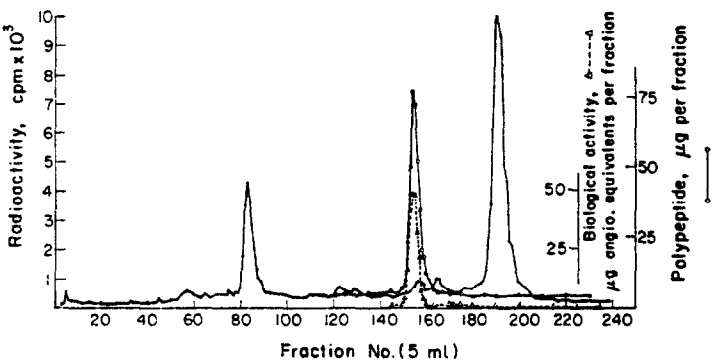

FIG. 2D

FIG. 2. Column chromatographic purification of iodinated angiotensins. The column contained Dowex 50-X8 cation exchange resin, and was 1 cm in diameter and 20 cm long. The pyridine acetate buffer was applied under pressure, and elutions performed at 30 ml per hr, 50°. Fractions were tested as follows: Panel A, radioactivity; Panel B, radioactivity (closed circles) and polypeptide by the Lowry *et al.* method,[17] (open circles); Panel C, radioactivity; and Panel D, radioactivity (closed circles); polypeptide by the Lowry *et al.* method,[17] (open circles); and bioassay by rat uterine contractile response compared to synthetic angiotensin II amide (triangles and dotted line). Panel A is a purification of the products of iodination by the Chloramine-T method, using ^{125}I and angiotensin II amide. Panel B is the pattern of a mixture of columns IV and V from Panel A, plus 1 peak from a purification of ^{127}I-angiotensin (non-radioactive) prepared in a separate procedure. Panel C is the purification of a sample of ^{125}I-angiotensin supplied by Cambridge Nuclear Corp., Isoserve Division. Calculations based on this pattern show that peak IV represented 85 per cent of the total radioactivity. Panel D is the pattern of a mixture of iodinated angiotensins plus 3 mg of fresh angiotensin II amide. All of these purifications were performed with the same column, and the same gradient, which is indicated in Panel A only.

synthesizing and purifying larger quantities of iodinated angiotensins using non-radioactive iodine (^{127}I). The identity of these derivatives with the radioactive ones was established by refractionating them, one at a time, with the combined radioactive products of a Chloramine-T reaction. The stable isotopic polypeptides were detected by the Lowry *et al.* method[17] and by the iodine reaction described above. Figure 2B shows the results of one of the two repeat chromatographic separations. These repurifications showed that the derivatives of angiotensin resulting from iodination with radioactive iodine were probably the same as those resulting from

iodination with stable iodine. The non-radioactive compounds were then analyzed for amino acid and iodine content.

Separation of basic amino acids and iodotyrosines by the amino acid analyzer is illustrated in Fig. 3. The results of amino acid analyses of acid and alkaline hydrolysates of aliquots from peaks corresponding to IV and V showed that peak IV contained monoiodotyrosine in place of tyrosine, and peak V contained diiodotyrosine in place of tyrosine. The other amino acids were present in the predicted ratios. Representative analyses are summarized in Table 2.

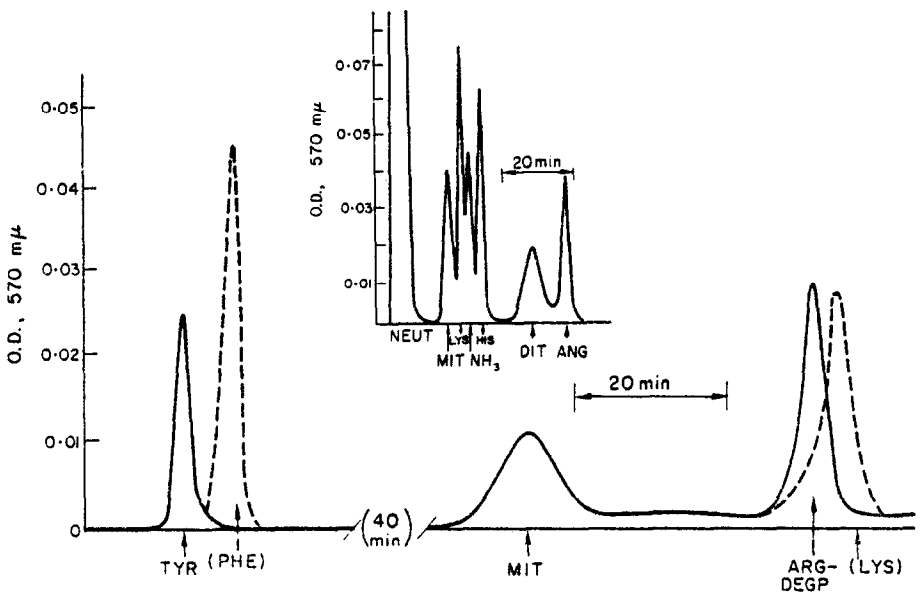

FIG. 3. Amino acid analyzer separation of iodotyrosines and amino acids from hydrolyzed angiotensin. The long tracing (lower figure) has been abbreviated by beginning at the 30th min, and omitting 40 min where indicated. The solid line is the pattern from an alkaline hydrolysis of monioidoangiotensin, and contains tyrosine (TYR), monoiodotyrosine (MIT), and a product derived from arginine (ARG-DEGR). Superimposed is the probable location of phenylalanine (PHE) and lysine (LYS) as seen in a separate elution of a standard amino acid mixture. Not shown in this figure is the fact that the pen indicating optical density at 440 mμ rose above the pen for optical density at 570 mμ (short path) in the case of lysine and the arginine degradation product; all other amino acids showed the opposite relationship. The short tracing (insert) is derived from a standard amino acid mixture plus monoiodotyrosine (MIT) and diiodotyrosine (DIT) separated on the short column at pH 4·43. The locations of neutral amino acids (NEUT), lysine (LYS), ammonia (NH_3), and arginine (ARG) are also shown.

In acid hydrolysates, the iodinated residues were converted to tyrosine, and it was possible to measure the amount of iodide released. These analyses showed the presence of twice as much iodine per mole in peak V as in peak IV. These data show that peak IV was probably monoiodoangiotensin, and peak V was probably diiodoangiotensin. Peak I is presumed to be free iodide, because it was brown in color when the non-radioactive reaction mixture was fractionated, and was negative for biological activity and polypeptide content. Peaks II and III are discussed below.

TABLE 2. AMINO ACID ANALYSES OF IODINATED ANGIOTENSINS*

Polypeptides	Monoiodoangiotensin			Diiodoangiotensin		
Hydrolysis Conditions:	Alkaline		Acid	Alkaline		Acid
Column:	Long	Long	Long	Long	Short	Long
Eluting buffer pH:	3·26–4·28	5·27	3·26–4·28	3·26–4·28	4·43	3·26–4·28
Propanol in eluting buffer:	No	Yes	No	No	Yes	No
Phenylalanine	1·00	1·00	1·00	1·00		1·00
Aspartic acid	0·42		0·96	0·35		0·94
Proline	0·99		0·93	1·08		1·07
Valine	2·33		1·98	2·05		1·96
Tyrosine	0	0·068	0·79	0		0·96
Histidine					1·00	
Monoiodotyrosine		1·13				
Diiodotyrosine					1·01	

*The polypeptides were purified on a Dowex 50-X8 column, and hydrolyzed in barium hydroxide or hydrochloric acid as described in the text. The terms "monoiodoangiotensin" and "diiodoangiotensin" are used here to denote the polypeptides whose alkaline hydrolysates contained monoiodotyrosine and diiodotyrosine when fractionated under the special conditions described. The values have been compared to phenylalanine or histidine as standards. Arginine was not measured,

TABLE 3. IMMUNOLOGIC PROPERTIES OF IODINATED ANGIOTENSINS

Radioactive column peak	Antibody dilution for 33% binding of radioactive peaks	Polypeptide required for 50 per cent inhibition of the binding of each peak by antiserum in the dilutions listed (ng)*			
		Angio II amide†	Angio II acid†	Monoiodo-angiotensin	Diiodo-angiotensin
II	1 : 67,000	0·45	10·0		
III	1 : 6500	0·90	10·0		
IV	1 : 6600	0·15	0·12	0·20	5·10
V	1 : 650	0·14	0·14		

*Inhibitory potencies of nonradioactive polypeptides were determined as follows: Aliquots of 1 fraction from each radioactive peak were incubated at 4° with antiserum in appropriate dilution and serial dilutions of nonradioactive polypeptide in a final volume of 0·5 ml. The appropriate dilution of antiserum (column II) was determined from data presented in Fig. 4. After 18 hr, the radioactivity bound to antibody was precipitated with ammonium sulfate, washed once and counted in a gamma ray counter. The 50 per cent point was read from a curve.

†The angiotensin II amide used in this experiment was the stock powder, supplied as "Hypertensin, 83 per cent pure," by Ciba Pharmaceuticals, whereas the angiotensin II acid was a purified polypeptide provided by the research laboratories of Ciba Pharmaceuticals.

Separation of iodoangiotensins from precursor (native) angiotensin is shown in Fig. 2D. To 1 aliquot of peak IV (67,000 cpm) was added 3 mg of angiotensin II amide. The mixture was rechromatographed on the Dowex 50X-8 column, and the fractions were tested for radioactivity, biological activity (rat uterus), and polypeptide concentration (Lowry *et al.* method).[17] The results showed that the maximum contamination of radioactive monoiodoangiotensin by unlabeled angiotensin was about 0·0001 times the initial quantity of angiotensin purified. The specific activity of monoiodoangiotensin-^{125}I purified on the Dowex column would therefore be

approximately 2 mc/μg. Unpurified samples could be as dilute as 0·3 mc/μg if the iodination efficiency were approximately 10%.

Immunologic properties of iodoangiotensins. The capacity of anti-angiotensin to combine with the principal purified fractions of Chloramine-T iodination is summarized in Table 3. The data for peaks IV and V are presented graphically in Fig. 4. Inhibition of this antigen-antibody reaction by nonradioactive polypeptides is also summarized in Table 3. The results indicate that the four major iodinated polypeptide

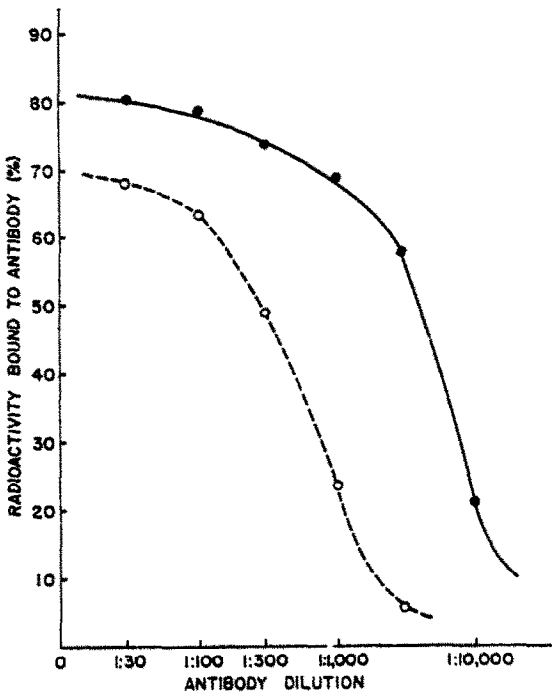

FIG. 4. Binding of radioactive iodinated angiotensins by antiserum to angiotensin. The antiserum was induced in a rabbit, as described in the test and reference 15. Iodinated angiotensins were purified as described in the text and depicted in Fig. 2. Binding to antibody was determined by incubating polypeptide and antiserum (at the dilutions indicated) for 18 hr, then precipitating antibody and bound polypeptide with ammonium sulfate. The ordinate is the percentage of total radioactivity added which precipitated in the absence of antibody. The solid line shows the results with monoiodoangiotensin (peak IV, Fig. 2A), and the dotted line with diiodoangiotensin (peak V, Fig. 2A).

peaks combine with antiserum. This is to be expected, since the immunizing antigen probably contained the precursors of these peaks, and the antiserum is probably heterogeneous. However, the relative resistance of antibody binding of peaks II and III to inhibition by purified angiotensin II acid indicates that these peaks are not angiotensin derivatives, but are iodinated derivatives of impurities in the stock angiotensin amide. The chemical composition of these peaks, or of minor peaks, was not elucidated further.

Comparison of binding of radioactive mono- and diiodoangiotensins by antibody shows that monoiodoangiotensin is bound roughly ten times more avidly than the

diiodo derivative by the antiserum tested. Comparison of nonradioactive compounds as inhibitors of the binding of labeled compounds to antibody shows that introduction of one iodine atom causes a reduction of only 33 per cent in the haptenic potency of angiotensin, whereas two iodine atoms reduce this by 97 per cent. It should be noted that this potency refers to the ability of the free polypeptides to inhibit the reaction between a specific antigen and a specific antibody. Different relative haptenic potencies might be revealed if a different radioactive test antigen or a different antiserum were studied.

TABLE 4. BIOLOGICAL ACTIVITY OF IODINATED ANGIOTENSINS*

Polypeptide	Relative biological activity		
	Rat uterus	Rat colon	Rat blood pressure
Angiotensin	1·00	1·00	1·00
Monoiodoangiotensin	0·25 (9)	0·80 (2)	0·33 (2)
Diiodoangiotensin	0·02 (3)	0·24 (2)	0·13 (2)

*The responses of three different rat assay systems were measured after administration of val^5-angiotensin II amide and its iodinated derivatives (see text). Short dose–response curves were constructed, and the quantities of polypeptides required for a given response were compared. The dose–response curves were parallel for the three polypeptides. The response chosen for comparison were on the straight-line segments of the curves. The quantities of polypeptides were measured by the Lowry *et al.* method,[17] which was shown in a separate experiment to give equivalent color with equal quantities (by amino acid analysis) of the three analogues. The numbers in parentheses refer to the number of separate experiments. The values shown are the means of closely matching experiments.

TABLE 5. STABILITY OF MONOIODOANGIOTENSIN DURING STORAGE

Buffer	Additions	Temperature (°C)	Time (days)	Free iodide after storage (per cent of total radioactivity)
Phosphate, 0·01 M, pH 7·6		4	48	40
Phosphate, 0·01 M, pH 7·6		−20	48	36
Phosphate, 0·01 M, pH 7·6		−40	48	25
Phosphate, 0·01 M, pH 7·6	Bovine albumin, 0·5%	−20	48	12
Phosphate, 0·01 M, pH 7·6	Casein, 0·12%	−20	48	8
Glycine, 1·0 M, pH 9·3 NaCl, 0·2 M	Bovine albumin, 0·25%	−40	34	4

Biological properties of iodoangiotensins. Table 4 summarizes the results of bioassays on rat uterus, rat colon and rat blood pressure by purified angiotensin II amide and its two iodinated derivatives. The results show that monoiodoangiotensin was fairly active compared to angiotensin, but diiodoangiotensin was only 2–24 per cent as active as native angiotensin.

Stability. The stability of the iodine–tyrosine bond during storage was estimated by using radioactive monoiodoangiotensin and testing the stored sample with the paper chromatography system depicted in Fig. 1. Table 5 summarizes the results.

This experiment shows that storage at −40° in a 1 M glycine–0·2 M NaCl buffer at pH 9·3 with albumin (0·25%) is superior to other methods.

Stability of the polypeptides during Dowex-50 chromatography was tested by heating purified radioactive fractions to 50° for 24 hr in starting buffer and limit buffer and testing the treated and untreated samples for their capacity to bind to anti-angiotensin. No loss of binding of radioactivity to antibody was observed. In view of the specificity of this antiserum,[15] the results indicated that mild acid conditions do not affect the primary structure, optical activity, or degree of iodination of iodoangiotensin.

DISCUSSION

The radioactive isotopes of iodine have made it possible to detect picogram quantities of substances which contain them. The specific activities of the isotopes give them considerable advantages over tritium and carbon-14. However, addition of the bulky iodine atom to compounds which do not contain it in nature may introduce considerable variation from normal in the metabolism and effects of biologically active substances. The smaller the substance to be studied, and the more critical the site iodinated, the more likely that addition of iodine will introduce artifacts. Variations from normal have been discussed with respect to iodinated insulin[18, 19] and other hormones,[20] and have been suspected in studies with iodinated angiotensin.[3–6] For these reasons we have synthesized and purified mono- and diiodoangiotensin and studied some of their properties.

The results reported here suggest that monoiodoangiotensin shares all the properties of native angiotensin, but is only 25–80 per cent as active, whereas diiodoangiotensin is less potent by an additional factor of 3 to 10

All of our data on biological properties of iodinated angiotensins assume that the derivatives do not undergo de-iodination to the native hormone. Although we have evidence for the stability of iodoangiotensins in various buffer solutions for as long as 12 months, we have not tested their stability in serum or in target tissues. Therefore, all estimates of biologic activity are maximum estimates. If de-iodination takes place, the true potency of the iodoangiotensins is lower than stated. Deiodination *in vivo* may account for the relatively high potency of iodoangiotensins in the intact rat compared to that in the isolated uterus or antibody.

A second assumption which underlies the reported relative biological activities of the iodinated derivatives is the purity of the samples tested. Evidence for the separation of native angiotensin from monoiodoangiotensin is presented in the text and in Fig. 2D. Evidence for the purity of unlabeled monoiodoangiotensin and diiodoangiotensin with respect to cross-contamination is based on four observations: (1) the separation of the peaks by column chromatography (Fig. 2); (2) the smooth curve of hapten–inhibition, by the unlabeled derivatives, of antigen–antibody reactions between the purified radioactive analogue and anti-angiotensin; (3) the molar ratios of iodine in the acid hydrolysates of the two polypeptides; and (4) amino acid analysis. The data pertaining to each of these points are subject to error. (1) Although the samples tested were from separate chromatographic peaks, tailing of the other iodinated derivative was not excluded. To minimize cross-contamination, samples were drawn from the far sides of the two peaks. (2) Although smooth hapten–inhibition curves were obtained, the possibility exists that diiodoangiotensin was

totally inactive and all activity resided in a contaminating quantity of monoiodoangiotensin. This is unlikely because of the close correspondence of antibody affinities deduced from direct antibody binding of labeled analogues (clearly separable on the column), and inhibition of antibody binding by unlabeled analogues. (3) Although the iodine determination is inexact, in two separate experiments, the ratio of iodine in presumed diiodotyrosine was exactly twice that in monoiodotyrosine. The range of error in this method would permit 10 per cent contamination. (4) Amino acid analysis showed the absence of detectable diiodotyrosine from monoiodoangiotensin. However, we were unable to prove the absence of tyrosine from monoiodoangiotensin and the absence of monoiodotyrosine from diiodoangiotensin. Purified samples of diiodotyrosine yielded some monoiodotyrosine and tyrosine on alkaline hydrolysis. Monoiodotyrosine yielded some tyrosine. Thus, the presence of tyrosine in hydrolyzed monoiodoangiotensin, and the presence of monoiodotyrosine and tyrosine in hydrolyzed diiodoangiotensin were probably the result of chemical degradation during hydrolysis. This degradation limits the use of amino acid analysis as a proof of purity.

Iodination reactions are difficult to control, and the discrepant results from different laboratories using iodinated angiotensin may be explained by the use of different mixtures of the two analogues, or the presence of other impurities such as our peaks I, II, and III. Our values for the relative biological potencies of angiotensin and its mono- and diiodinated derivatives agree with those reported by Dietrich.[21] The range of relative immunologic potency is wider without antiserum than with Dietrich's, illustrating the intrinsic variability among antisera from different rabbits and different immunization techniques.

In radioimmunoassays, we have not found that purification of monoiodoangiotensin improves sensitivity or reproducibility. In this assay there is a relatively high specificity and availability of antibody. On the other hand, our studies of angiotensin receptors, in which nonspecific binding and low ligand concentration are problems, were facilitated by purification of the monoiodoangiotensin derivative.*

Acknowledgements—Pressor responses were determined by Dr. Harold Itskovitz, Marquette University School of Medicine. Synthetic angiotensin II amide and angiotensin II acid were gifts of Ciba Pharmaceuticals. The glycine buffer was suggested by Dr Arthur Freedlender, Boston University School of Medicine.

*Shoei-Yn Lin and Theodore L. Goodfriend, unpublished observations.

REFERENCES

1. P. A. Khairallah, I. H. Page, F. M. Bumpus and R. R. Smeby, *Science, N.Y.* **138**, 523 (1962).
2. E. C. Osborn, W. J. Louis and A. E. Doyle, *Aust. J. exp. Biol. Med. Sci.* **44**, 475 (1966).
3. R. L. Wolf, M. Mendlowitz, S. E. Gitlow and N. Naftchi, *Circulation Res.* **11**, 195 (1961).
4. E. Cruz-coke, in *Experimental Hypertension*, vol. III, p. 32. New York Academy of Sciences, New York (1946).
5. B. Barbour and F. C. Bartter, *J. clin. Endocr. Metab.* **23**, 313 (1963).
6. E. C. Osborn, *J. Pharm. Pharmac.* **20**, 399 (1968).
7. R. T. Simpson and B. L. Vallee, *Biochemistry, N.Y.* **5**, 1760 (1966).
8. W. M. Hunter and F. C. Greenwood, *Nature, Lond.* **194**, 495 (1962).
9. H. G. Kwa and F. Verhosftad, *Biochim. biophys. Acta* **133**, 186 (1967).
10. R. T. Jones, *Cold Spring Harb. Symp. quant. Biol.* **29**, 297 (1964).
11. W. J. Ray and D. E. Koshland, Jr., *J. biol. Chem.* **237**, 2493 (1962).
12. D. H. Spackman, W. H. Stein and S. Moore, *Analyt. Chem.* **30**, 1109 (1958).

13. E. B. SANDELL and I. M. KOLTHOFF, *J. Am. chem. Soc.* **56**, 1426 (1934).
14. C. H. BOWDEN, N. F. MACLAGAN and J. H. WILKINSON, *Biochem. J.* **59**, 93 (1955).
15. T. L. GOODFRIEND, D. L. BALL and D. B. FARLEY, *J. Lab. clin. Med.* **72**, 648 (1968).
16. P. T. PICKENS, F. M. BUMPUS, A. M. LLOYD, R. R. SMEBY and I. H. PAGE, *Circulation Res.* **17**, 438 (1965).
17. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
18. J. L. IZZO, A. RONCONE, M. J. IZZO and W. F. BALE, *J. biol. Chem.* **239**, 3749 (1964).
19. K. BRUNFELDT, B. A. HANSEN and K. R. JORGENSEN, *Acta endocr. Copen.* **57**, 307 (1968).
20. J. LANDON, T. LIVANOU and F. C. GREENWOOD, *Biochem. J.* **105**, 1075 (1957).
21. F. M. DIETRICH, *Immunochemistry* **4**, 65 (1967).