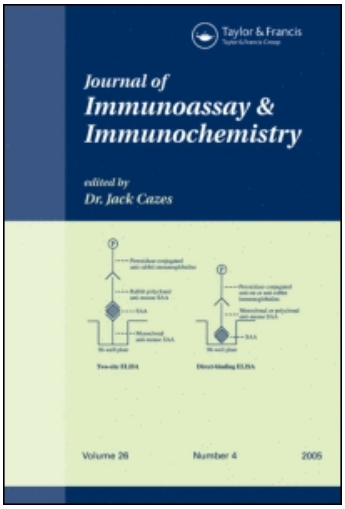
This article was downloaded by:
On: *16 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Iodination of [Tyr$_8$] - Bradykinin - Comparison of Chloramine-T and Lactoperoxidase Techniques

L. W. Redman[a]; E. Reno Tustanoff[b]
[a] Department of Clinical Pathology, Victoria Hospital, Ontario, Canada [b] Department of Biochemistry, University of Western Ontario London, Ontario, Canada

To cite this Article Redman, L. W. and Tustanoff, E. Reno(1984) 'Iodination of [Tyr$_8$] - Bradykinin - Comparison of Chloramine-T and Lactoperoxidase Techniques', Journal of Immunoassay and Immunochemistry, 5: 1, 29 — 57
To link to this Article: DOI: 10.1080/01971528408062997
URL: http://dx.doi.org/10.1080/01971528408062997

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF IMMUNOASSAY, 5(1&2), 29-57 (1984)

IODINATION OF [TYR^8] - BRADYKININ - COMPARISON OF CHLORAMINE-T AND LACTOPEROXIDASE TECHNIQUES

L.W. Redman and E. Reno Tustanoff*

Department of Clinical Pathology, Victoria Hospital

and

Department of Biochemistry, University of Western Ontario

London, Ontario, Canada. N6A 4G5

Keywords: bradykinin, R.I.A., iodination, chloramine-T, lactoperoxidase, isofocusing

Downloaded At: 12:23 16 January 2011

ABSTRACT

Antigen-antibody kinetics were studied using a hapten which was iodinated by two unique procedures. Using bradykinin, a vasopressor hormone as a model peptide, radioactive iodination (^{125}I) of its 8-tyrosyl analogue was carried out both enzymatically and chemically using

*This work was supported by the Medical Research Council of Canada through a grant in aid (MT-1460) to E.R.T. All correspondance dealing with this paper should be addressed to this author.

Copyright © 1984 by Marcel Dekker, Inc. 0197-1522/84/0501-0029$3.50/0

modified procedures. Two distinct chemical species were obtained which were characterized on a chromatographic, chemical as well as charge basis as a mono-iodinated form of [Tyr^8]-bradykinin using the lacto-peroxidase procedure and a di-iodinated entity using chloramine-T technique. The addition of a second iodine atom to the antigen lowers its immunoreactivity for its antibody and thus alters the kinetics of this reaction. Further experiments on the stability (temperature, time of storage, and chemical environment) of these iodinated peptides are described.

Downloaded At: 12:23 16 January 2011

INTRODUCTION

Historically, radioiodinated antigens and antibodies were first used by immunologists in the study of antigen-antibody reactions. Within a short time, the use of radiolabeled proteins was extended to *in vitro* studies of the metabolism of proteins (Berson *et al.*, 1953). Berson *et al.* (1956) were the first to point out the remarkable sensitivity that could be achieved when unlabeled antigens are measured by their ability to inhibit competitively the binding of highly radiolabeled iodide antigens by their antibodies. As a result of this initial description, radioimmunoassays (RIA) were rapidly developed for other proteins and polypeptide hormones (Grodsky and Forsham, 1960; Unger *et al.*, 1959; Utiger *et al.*, 1962; Pinto *et al.*, 1977).

The detection limits of the first bradykinin radioimmunoassay (Spragg *et al.*, 1966) were limited to a range of 10-100 ng/ml because of the unavailability of labelled antigens with high specific activities. Goodfriend and Ball (1965) using bradykinin analogues produced ^{125}I-labeled bradykinin with increased specific activities high enough to increase the sensitivity of the radioassay to measure the vaso-

pressor nonapeptide in physiological ranges. With the development of a procedure using chloramine T (Greenwood et al., 1963) to iodinate peptide hormones such as bradykinin, it soon became apparent that this strong oxidizing agent could cause structural changes to the protein being iodinated. To circumvent some of these shortcomings, several investigators (Theorell and Johansson, 1971; Pinto et al., 1977) introduced the use of the enzyme lactoperoxidase for the iodination of polypeptide hormones.

In this paper we have compared the iodination of [Tyr^8]-bradykinin using the two different labeling procedures and investigated the chemical and immuno-reactive properties of their products as they relate to radioimmunoassay procedures.

Downloaded At: 12:23 16 January 2011

METHODS

Antibodies were isolated from male while New Zealand rabbits that had been previously immunized with bradykinin (bradykinin triacetate, Schwartz-Mann Chemical Corp., Orangeburg, N.Y.) linked to ovalbumin via toluene diisocyanate by a procedure developed in our laboratory (Redman & Tustanoff, 1983).

Iodination of Bradykinin

a) Chloramine-T Method

[Tyr^8]-bradykinin triacetate was iodinated with carrier-free labeled sodium iodide ($Na^{125}I$) by a modification of the chloramine-T procedure described by Goodfriend and Ball (1969). The procedure which was maximized to ensure optimal labeling efficiency and to minimize over-oxidation, was carried out at 37°C. The reagents were added as fol-

lows: $Na^{125}I$ (1-5 mCi, 11-17 μgl/mCi) in 0.1N NaOH (pH 8-11) (50 μl); 0.25 M Tris-HCl buffer, pH 7.6 (20 μl); 0.5 μg Tyr^{8} -bradykinin (5 μl); chloramine-T, 1.25 mg/1.0 ml of 0.25 M Tris-HCl buffer, pH 7.6 (20 μl). After 2.0 minutes, the reaction was terminated by the addition of sodium metabisulphite, 25 mg/10 ml of 0.012 M sodium borate buffer, pH 7.6 (100 μl) and Kl 10 mg/ml (100 μl). After an initial protonation with 60% acetic acid (2 μl), the labelled peptide (specific activity 300-500 μCi/ g) was isolated from the reaction mixture by passage through an albumin-coated Amberlite IRA-401 (Cl^{-}) resin contained in a siliconized glass column. The radio-tagged bradykinin analog was eluted from the column with double-distilled water while the unreacted radioactive iodide was retained. 0.5 ml fractions were collected individually in tubes containing 20 μl of 0.5 M phosphate buffer (pH 7.4) supplemented with 0.2% albumin. Fractions 2 and 3, which contained the highest level of radioactivity were diluted 1 to 10 with saline (0.15 M) buffered with barbital buffer (Na barbitone (7 mM), Na acetate (12 mM)) (7 mM, pH 7.5) containing 1 mg/ml muramidase (EC 3.2.1.17) (lysozyme, Sigma Chemical Co. St. Louis, Mo.). The enzyme was added to prevent nonspecific adsorption of the peptide to glass as well as to protect its biological activity and then stored at -80°C. Aged preparations of iodinated bradykinin (>2 weeks) were rechromatographed prior to being used. These peptide solutions were passed through 3 cm siliconized glass columns containing bovine albumin-coated Amberlite IRC-50 (H+) resin. The columns were initially washed with 0.1 N acetic acid and the labeled ligand eluted with 16 ml of 50% acetic acid. The latter wash was taken to dryness under vacuum and the resulting residue was dissolved in 0.5 ml of the barbital buffer solution containing muramidase.

Downloaded At: 12:23 16 January 2011

Prior to use in an RIA assay all iodinated solutions were further diluted with barbital buffer so that 50 µl of the final solution contained approximately 30,000 cpm (ca. 60 pg of the nonapeptide).

b) Lactoperoxidase

This alternate procedure of iodinating [Tyr^8]-bradykinin was adapted from a method published by Thorell and Johansson (1971). The reaction was carried out at room temperature (ca. 25°C) in small polypropylene test tubes (12 x 75 mm). The reagents which were adjusted to ensure maximum labeling, were added in the following order: ammonium acetate buffer, 0.075 M, pH 5.0 (25 µl); 1-2 mCi Na ^{125}I in 0.1 N NaOH (11-17) µg I/mCi) pH 8-11 (20 µl); [Tyr^8]-bradykinin, 0.1 mg/ml 0.075 M ammonium acetate buffer, pH 5.0 (5 µl); lactoperoxidase 1 mg/ml of 0.075M ammonium acetate (pH 5.0 (a 1/20 dilution was prepared and used only once) (10 µl); 300 ng (5 µl) of H_2O_2 added, followed within 10 minutes by another 5 µl. The incubation time after the addition of the peroxide was 20 minutes. The reaction was terminated by the addition of 100 µl of Na metabisulphite (20 mg/20 ml 0.012 M sodium borate buffer, pH 7.6) followed by 100 µl potassium iodide (10 mg/ml). 2 µl of 60% acetic acid was added to ensure protonation. The reaction mixture was resolved by ion-exchange chromatography in the same manner as described for the product of the chloramine-T procedure.

c) Nonradioactive Iodination of [Tyr^8]-Bradykinin

The procedure used was essentially identical to the chloramine-T protocol except the concentrations of the reactants were changed. The reagents were added in the following sequence: 60 µl Na I (1.82 mg/ml water), 100 µl sodium phosphate buffer (0.2 M, pH 7.6),

Downloaded At: 12:23 16 January 2011

32 µl [Tyr^8]-bradykinin (100 µg in 0.1M acetic acid), 40 µl chloramine-T (1.25 mg/ml of 0.2 M Na phosphate buffer, pH 7.6). After two minutes the reaction was terminated as described in the radiolabelling procedure.

Chromatography

Iodinated preparations were resolved either by thin layer chromatography using cellulose coated plates (0.5 mm) (Merck) in a n-butanol: acetic acid:water (4:1:5 or 4:1:1.7) solvent system or by paper chromatography using Whatman #1 chromatography paper run in a two dimensional mode, initially in n-butanol:acetic:water (30:7.5:12.5) and then in a second dimension in isopropyl alcohol:formic acid: water (37.5:6.3:6.3). Compounds were located by either using specific spray reagents or by autoradiography (i.e. exposure of chromatograms to x-ray film for 13 hours).

Downloaded At: 12:23 16 January 2011

Iodide Detection

Qualitative detection of iodide containing compounds was carried out using a spray prepared by modifying a protein-bound procedure described by Barker et al. (1951). Five parts of solution A (2.7 gm $FeCl_3.6H_2O$ dissolved in 100 ml 2 N (HCl) were mixed with 5 parts solution B (3.5 gms $K_3Fe(CN)_6$ dissolved in 100 mls H_2O) and 1 part solution C (5.0 gm $NaAsO_2$ dissolved in 30 mls NaOH at 0° followed by the addition of 65 mls 2 N HCl). The quantitative determination of iodide was achieved using a procedure described by Henry (1965).

Peptide Detection

Fluorescamine, (4-phenyl spiro(furam-2(^{3}H)1-phthalan)-3-3'-dione) has been used by Weigle et al. (1972) to determine free amino acids,

peptides and proteins. This compound reacts with primary amines to form fluorphors which have excitation and fluorescence maxima of 390 and 480 nm respectively. Chromatograms were sprayed initially with 0.1 M sodium borate (pH 8.7) dried and resprayed with fluorescamine (LaRoche Chemical Corp. Nutley, N.J.) (15 mg/50 ml acetone). Peptides treated in this manner produced a billiant blue fluorescent color upon exposure to ultra-violet light. For quantitative determinations the solution extracts containing the bradykinin residues were added to 2.0 ml sodium borate buffer (0.1 M, pH 9). 0.5 mls of an acetone solution of fluorescamine (15 mg/50 ml acetone) were then added and the resulting fluorescence determined using a Perkin-Elmer spectrofluorometer (Norwalk, Conn.). A standard curve was prepared by arbitrarily setting the instrument to 100% fluorescence when a 3.0 μg peptide standard was used. Serial dilutions of this standard as well as unknown solutions were measured as relatives percentages of the 3 μg standard (detection limits were up to 0.15 μg peptide).

Downloaded At: 12:23 16 January 2011

Radioimmunoassay of Bradykinin

The protocol used to determine bradykinin levels employing radioimmunoassay techniques has been described in a previous publication from this laboratory (Redman, Regoli and Tustanoff, 1979). Incubations were carried out at 4°C for two hours using 1/800 dilution bradykinin antiserum which was isolated in our laboratory. The bound radioactive bradykinin was separated from unbound by using alkaline charcoal dextran mixture and counted for radioactivity. A dose-response curve was plotted as previously described (Redman, Rogoli and Tustanoff, 1979).

Isofocusing

35 mls of Sephadex G-200 superfine (Pharmacia, Uppsala Sweden) suspension (1.4 gms) containing 1% carrier ampholytes, pH 3-10 (LKB,

Stockholm, Sweden) supplemented with arginine and lysine hydrochloride (0.05% each) was spread over a 20 x 20 cm glass plate. After the plates had dried and the samples applied, they were placed on a precooled flat bed (4°C) of a Desaga Isofocusing apparatus (Desaga, Heidelberg, West Germany). The connection between the gel layer and electrode chamber was made with 0.4 M ethylenediamine (anode) and 0.2 M sulphuric acid (cathode). An initial potential gradient of 7-10 volts/cm was applied for 4 hours, followed by 15-30 volts/cm for an additional 3-4 hours. At the conclusion of the run the outer portion of the gel through which the full current travelled was divided into sequential segments (0.5 x 1.0 cms) and then scrapped off the plate and transferred to individual test tubes. After the addition of 1.0 ml of water, the pH of each test tube was measured. A double emulsion x-ray film was placed on the top of the plate and was exposed for 1½ hrs in the dark at -20° to prevent diffusion. Results of this separation were recorded graphically as a function of pH, fraction number and radioactivity.

Downloaded At: 12:23 16 January 2011

Enzymatic Digestion of Iodinated Bradykinin

Solutions of both radioactive and nonradioactive iodine labeled [Tyr^8] -bradykinin which was previously chromatographed by thin layer chromatography were digested enzymatically to liberate their iodinated amino acid residues. One unit each of carboxypeptidase A, carboxypeptidase B and aminopeptidase M (Sigma Chemical Corp. St. Louis, Mo.) were added to the iodinated bradykinin preparations (ca. 3 x 10^5 cpm or 5.3 µg) and to a sample of ^{125}I angiotensin I (New England Nuclear Corp. Boston, Mass) (1.57 µCi) (99% of its tyrosine residues were monoiodinated). The incubations for the digestion reactions $CaCl_2$ (5 mM), $ZnSO_4$ (0.1 mM) in a final volume of 600 µl of Tris-HCl buffer (0.25 M, pH 7.6) were carried on for 7½ days and then terminated by the addi-

tion of perchloric acid. The neutralized solutions were then resolved by paper chromatography using Whatman #1 paper in two separate solvent systems. After the papers were dried they were individually treated with either fluorescamine or the iodide spray. After the reactive areas were visualized they were cut into cm square sections and counted for their radioactivity content.

RESULTS

Extent of Iodination

In order to establish the degree of purity and integrity of the iodination products of the bradykinin analog produced by the chloramine-T and lactoperoxidase procedures, samples of [Tyr^8]-bradykinin were separately iodinated with radiolabeled iodine (^{125}I) and non-radiolabeled iodine (^{127}I). The products of these iodinations were applied individually to cellulose-coated thin layer plates along with marker compounds and resolved in a solvent system described in the Method Section. The chromatograms were shielded in such a manner that only specific vertical portions of the chromatograms were exposed. These areas were then either individually sprayed with fluorescamine (to detect peptide containing material) or ferric chloride-ferricyanide-arsenous acid reagent (to detect iodide compounds) or used to detect radioactivity. The results of these chromatographic separations are shown in Table 1. It can be seen that three areas were resolved by this separation. The non-radioactive iodination procedure gave rise to a slow moving area which travelled with the same Rf as Na ^{125}I, an intermediate area which reacted only with the protein spray and had Rf value similar to the [Tyr^8]-bradykinin and finally a faster moving area which reacted with both the iodide and protein reagents. With radioactively

Downloaded At: 12:23 16 January 2011

TABLE I

Chromatographic Separation of the Products of Iodination of [Tyr^8]-Bradykinin by the Chloramine-T Procedure

The products of chloramine-T iodination were resolved by thin layer chromatography on cellulose (0.5 mm thick) using n-butanol:acetic acid: water (4:1:1.7) as the solvent system. The resolved compounds were identified by either iodide, peptide or radioactivity content as described in the Method Section.

	Relative Rf Values
Standards	
[Tyr^8]-bradykinin	0.58
Na ^{125}I	0.39
Compounds Detected By	
Iodide Spray	0.39
	0.64
Peptide Spray	0.58
	0.63
Radioactivity	0.39
	0.63

Downloaded At: 12:23 16 January 2011

iodinated (^{125}I) bradykinin analog only two areas could be detected and these only by their radioactivity since the concentration of the labeled compounds was less than the limits of detection of the reagent sprays. These areas corresponded to the slow and faster moving components which travelled with an Rf of 0.39 and 0.64 respectively. Undeveloped areas from the chromatograms which corresponded to the fast moving protein and iodide reacting substance were extracted, eluted with formic acid and after drying *in vacuo* were separated in two different chromatographic systems (n-butanol:acetic acid:water (30:7.5:12.5) and 4:1:5)). Using

the identification criteria established above only one discrete area could be detected and that was ^{125}I-[Tyr^{8}]-bradykinin. This material was used as the standard in subsequent experiments. When the products of the lactoperoxidase iodination were subjected to the same chromatographic separations, qualitatively identical results were obtained.

Stability

It was observed that labeled preparations of bradykinin which had been labeled for at least one week began to break down with the liberation of free iodide. This resulted in reduced sensitivity when the labeled antigen was used in an RIA procedure. With the aid of thin layer chromatography, it was observed that the ratio of liberated iodide to iodinate peptide increased proportionately as the parent material aged at -80°C. When these aged bradykinin preparations were re-chromatographed, marked improvement in the slope, 50%, intercepts and Ka of the bradykinin dose response curve were achieved (cf. Table II). This was manifested by a shift in the dose response curve to the left along with an increase in sensitivity. Of the two procedures used to "clean up" aged labeled ligand, the albumin coated ion exchange procedure was superior to paper chromatography as can be seen in the kinetic data presented in Table 2.

Further experiments were undertaken to study the stability of the iodinated hapten during storage at different temperatures and in different chemical environments. Identical aliquots of chromatographically purified ^{125}I-[Tyr^{8}]-bradykinin which had been labeled using the chloramine-T procedure, were suspended in four different buffer solutions. Half of each of these peptide solutions was snap frozen in liquid nitrogen and stored at -80°C while the other portions were placed

Downloaded At: 12:23 16 January 2011

TABLE II

Purification of Aged Radiolabeled Bradykinin Preparations and Their Subsequent Effects on RIA Kinetics

Aged preparation of ^{125}I-[Tyr^{8}]-bradykinin (>2 weeks) were either chromatographed on Whatman #1 paper in a two dimensional solvent system or passed through an Amberlite IRC-50 ion exchange column as described in the Method Section. The isolated radioligands were then used as labeled haptens in a series of RIA assays using standard concentrations of bradykinin. The data from these experiments was plotted in dose-response curves: B/Bo against log of the peptide concentration. B = % of counts of labeled bradykinin bound by the antiserum in the presence of unlabeled peptide and Bo = % of counts bound in the absence of unlabeled peptide.

Radioligand	Bo	50% Intercept[1]	Slope of B/Bo	Ka x 10^{8} $^{-1}$ (2) litres/mole
Aged	52.0	1.63	-1.010	2.1
Purified by Paper Chroma-tography	48.0	1.29	-1.173	2.6
Purified by Colume Chroma-	47.0	0.99	-1.383	3.4

(1) 50% intercept = the dose (ng bradykinin required to reduce B/Bo from 1 to 0.5.
(2) Ka = everage affinity constant (Goodfriend et al. 1967).

in a -20° freezer. After two months of storage, the frozen samples were thawed and the intact radiolabelled [Tyr^{8}]-bradykinin was isolated, using cellulose thin layer chromatography. Each sample was then counted for radioactivity and the results of these experiments are tabulated in Table III. It can be seen that in the presence of muramidase the labeled ligand was fairly stable during the various temperature regimens. The nature of the buffer appears to be important in maintaining radioactive ligand integrity so can be seen in Table III. Repeated freezing and thawing of the labelled peptide has a profound

Downloaded At: 12:23 16 January 2011

TABLE III

Effect of Temperature and Chemical Environment on the Stability of Labeled Bradykinin

Newly chromatographed ^{125}I-[Tyr^{8}]-bradykinin was frozen either in liquid N_2 (stored at -80°) or at -20° (stored at -20°) in a series of different buffer systems and stored for two months. Half of each sample was thawed and refrozen to -80° five times over a period of 5 hours. Each sample was then subject to thin layer chromatography and the material travelling with the same Rf as the intact ^{125}I-bradykinin analog was isolated and counted for its radioactivity as described in the Method Section.

Buffer (1)	CPM x 10^6 Labeled Tyr^8-Bradykinin Thawed Once	Thawed and Refrozen 5x
	Slow Frozen and Stored at -20°	
a	2.04	1.31
b	2.06	0.72
c	0.72	0.61
d	2.51	1.54
	Snap Frozen and Stored at -80°	
a	1.34	0.61
b	0.90	0.54
c	0.73	0.00
d	1.36	0.90

(1) Buffers (a) - 0.075 M ammonium acetate (pH 5.0); (b) 0.1 M acetic acid with 10% ethanol (pH 5.0); (c) 7 mM barbital-acetate buffer (pH 7.4) and (d) 7 mM barbital acetate buffer with 1 mg/ml muramidase (pH 7.4).

effect on its stability as well. The method used to freeze the sample was important. Snap freezing in liquid N_2 resulted in up to 45% loss of the label when compared to the slow freeze method. As a result of these observations, all iodinated preparations of the bradykinin analog were diluted in standard barbital buffer supplemented with muramidase and the solution was slowly frozen at -20° and stored at -80°. Repeated thawing and freezing of the samples were avoided.

Downloaded At: 12:23 16 January 2011

Iso-electric Points of Iodinated Bradykinins

One must consider that the two different iodination procedures may give rise to two different iodinated compounds. On scrutinizing the number of potential ionizable groups that the bradykinin molecule has (the amino and guanidino groups that the N terminal arginine residue, the carboxyl and guanidino groups of the C terminal arginine residue as well as the hydroxyl groups of serine), it is possible to calculate its iso-electric point (pI) to be 9.0. Applying the same rationale to [Tyr^{8}]-bradykinin, mono-iodinated and di-iodinated bradykinin, pI values of 8.76, 8.66 and 8.36 were obtained. In order to substantiate the possibility that more than one iodinated compound may arise because of the two different labeling techniques, isofocusing electrophoresis was used. After ion-exchange chromatography, the products of both iodination procedures were separated by an isofocusing technique described in the Method Section and the results are graphically illustrated in Figure 1. It can be seen that ^{125}I-[Tyr^{8}]-bradykinin iodinated by the chloramine-T procedure had a pI of 7.9 whereas the enzymatically iodinated compound had a pI of 8.6.

To further document the degree of iodine insertion into the bradykinin molecule, iodinated nanopeptides were prepared using both labeling techniques. The compounds which were iodinated with both radioacrtive iodide and its stable isotope and then purified by two separate thin layer chromatographic procedures, were enzymatically digested with amino peptidase M and carboxy peptidase A and B to determine the nature of the liberated iodinated amino acid residues. The hydrolysates from these digestions were resolved by paper chromatography using two separate solvent systems. The resolved components of these chromatograms were identified either by their radioiodide content or their reactivity to

Downloaded At: 12:23 16 January 2011

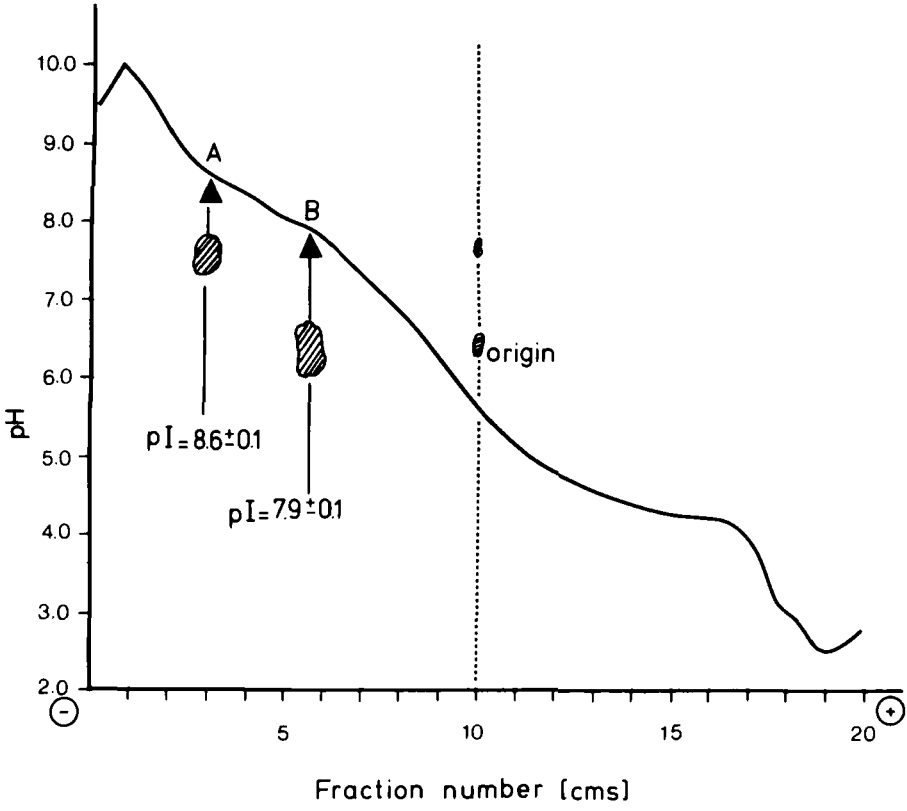


Figure 1. Isofocusing of Iodinated Bradykinin Prepared by Chloramine-T and Lactoperoxidase Labeling Procedures.

Preparations of ^{125}I-[Tyr^{8}]-bradykinin which had been iodinated by chemical and enzymatic methods and then passed through Amberlite ion exchange columns, were applied to glass plates coated with G-200 Sephadex containing 1% ampholines (pH 3-10) and lysine and arginine and resolved by an electrofocusing procedure described in the Method Section. A sample represent the lactoperoxidase prepared compound whereas B was iodinated by the chloramine-T procedure.

Downloaded At: 12:23 16 January 2011

TABLE IV

Rf Values of Chromatographically Separated Digestion Products of Enzyme Action on ^{125}I and ^{127}I-[Tyr^{8}]-Bradykinin

The bradykinin derivatives were iodinated by the two different procedures, purified by thin layer chromatography and then subjected to enzymatic digestion as described in the Method Section. The enzyme digests were chromatographed on Whatman #1 paper and run in two separate solvent systems n-butanol;acetic acid;water (30:7.5:12.5) and 2 isopropyl alcohol:formic acid:water (37.5:6.3:6.3) . Each chromatogram was run in duplicate, one being used to determine radioactivity and the other sprayed to detect iodide containing compounds.

Compounds	Relative Rf Values Solvent 1	Solvent 2
Na ^{125}I	0.24 [1]	0.18 [1]
3-iodo-L-tyrosine	0.60 [1]	0.52 [1]
3,5-diiodo-L-tyrosine	0.73 [1]	0.54 [1]
3-^{125}-iodo-L-tyrosine (hydrolysis product of ^{125}I angiotensin 1)	0.60 [2]	0.52 [2]
Enzymatic Digest of:		
1. ^{125}I-[Tyr^{8}]-bradykinin produced by:		
a) lactoperoxidase	0.60 [2]	0.50 [2]
b) chloramine-T	0.73 [2]	0.54 [2]
2. ^{127}I-[Tyr^{8}]-bradykinin produced by:		
a) chloramine-T	0.73 [1]	0.54 [1]

1. Detected by Radioactivity
2. Localized by Iodide Content

the iodide spray. The data from this experiment are recorded in Table IV and are given as relative Rf values for the major iodide containing components. These compounds were compared to mono- and di-iodotyrosine, the major potential products of the proteolytic enzyme activities. Both ^{127}I and ^{125}I-chloramine T iodinated [Tyr^{8}]-bradykinin produced iodinated hydrolysis products which migrated wih the same Rf values as diiodotyrosine in both chromatographic solvent systems. At the same time,

Downloaded At: 12:23 16 January 2011

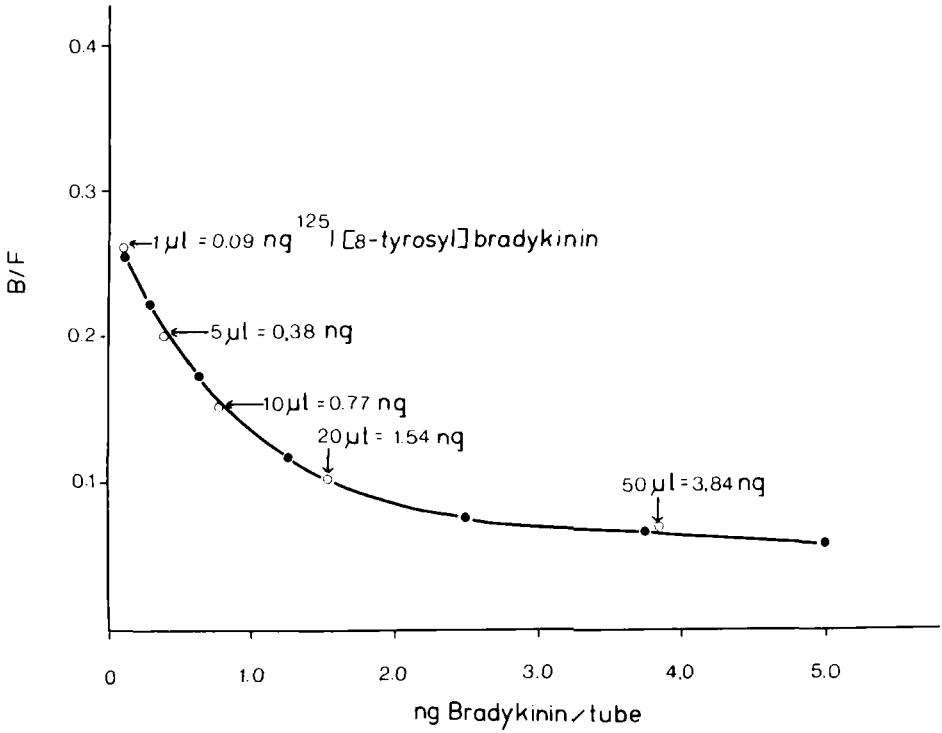


Figure 2. Comparison of Antiginic Reactivity of ^{125}I-[Tyr^8]-Bradykinin With That of Authentic Bradykinin.

Specific activities were determined by a procedure described by Cocke et al. (1965). Increasing volumes (1-50 µl) of a solution of ^{125}I-[Tyr^8]-bradykinin (4 x 10^4 to 6.5 x 10^6 cpm) iodinated by the chloramine-T procedure were incubated with a constant volume of buffered antiserum. These incubations were treated in the same manner as bradykinin standards in a dose response curve, i.e. separation of free (F) and bound (B) fractions with buffered dextran charcoal. The B/F response produced by these increasing doses were superimposed on the standard curve obtained using increasing amounts of unlabelled bradykinin. From these kinetics the amount of bradykinin in the labeled hapten was calculated. ^{125}I-[Tyr^8]-bradykinin (—○—○—), authentic bradykinin (—●—●—).

Downloaded At: 12:23 16 January 2011

the enzymatically iodination gave rise after proteolytic digestion to only the mono-iodinated tyrosine.

One further experiment was carried out to confirm these results. The iodide and protein content of each isolated iodinated peptide species was determined. Large scale iodinations were performed employing the two procedures and their products were isolated by means of preparative thin layer chromatography. The components which migrated with the same Rf as ^{125}I-[Tyr^{8}]-bradykinin were eluted from the chromatogram and analyzed for their iodide and peptide contents. The results of this experiment which are illustrated in Table IV, conclusively establish the fact that chloramine T produces a di-iodinated bradykinin species while lactoperoxidase forms a mono-iodinated peptide.

Downloaded At: 12:23 16 January 2011

When attempts were made to increase the sensitivity of the RIA method for bradykinin by using labeled haptens with higher specific activity it has been reported that certain problems arose which led to a loss in immunoactivity. The data in Figure 3 and Table VI summarize a typical experiment in which increasing amounts of labeled bradykinin analog were added to the standard concentration of antibody used in the RIA assay in the absence of unlabeled hapten. It can be seen that the observed B/F ratios are superimposable on a dose-response standard curve prepared using increasing concentrations of bradykinin. Each increment of the added radiolabeled bradykinin analog caused a proportional reduction in the B/F ratio, indicating that the labeled antigen reacts with the antibody in a manner indistinguishable from that of unlabeled bradykinin. It is possible to calculate the amount of ^{125}I-[Tyr^{8}]-bradykinin present from this type of experiment. It will be noted that the addition of each 5 µl increment of labeled hapten produced a reduction in the B/F ratio equivalent to an average of 0.38 ng of

Downloaded At: 12:23 16 January 2011

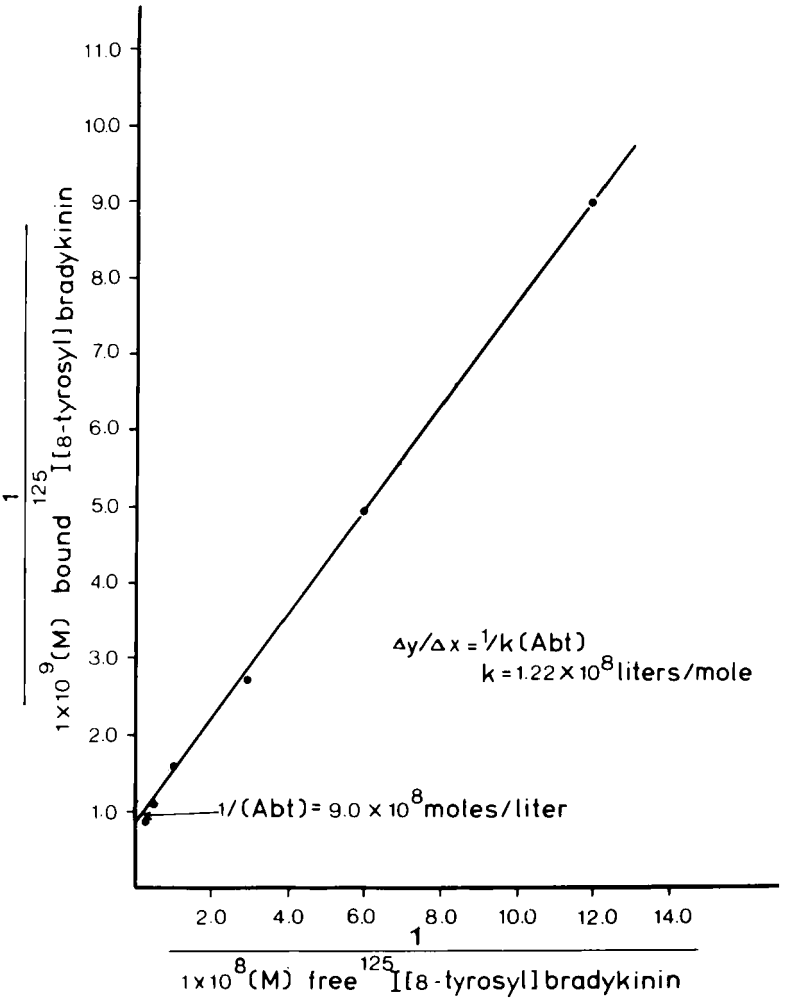


Figure 3. Reciprocal Plot Method of Determining the Average Affinity Constant of Antiserum (Ka).

The data in Table VI is graphically expressed as the reciprocal of the bound radiolabeled bradykinin analog (M) plotted against the reciprocal value of free radiolabeled bradykinin analog.

bradykinin. Since each 5 µl is equivalent to ca. 1.8×10^5 cpm, this preparation of iodide labeled [Tyr^8]-bradykinin has a specific activity of 288 mCi/ g, i.e. 1.8×10^5 cpm/0.38 ng bradykinin. If it can be assumed that the hapten is totally labeled and if compensation is made for the isotope's half-life, it can be calculated from the specific activity of bradykinin that there is approximately 2.0 mls of radioiodide per mole of peptide in the preparation used in the radioassay. Since chloramine T was used to prepare the labeled hapten this observation corroborates the evidence that we are dealing with a diiodinated species. Using the lactoperoxidase labeled ligand in a similar series of kinetic reactions evidence was obtained from specific activity measurement that there was only one mole of radioiodine per mole of the bradykinin analog.

Downloaded At: 12:23 16 January 2011

Using the data in Table VI it is possible to determine the average binding constant (Ka) for the antibody using a reciprocal plot procedure outlined by Weir (1973). From the kinetics of the plot (cf. Figure 3) the binding constant was found to be 1.22×10^8 liter/mole. Using the iodinated haptens prepared by the two labelling procedures in a series of standard dose-response curves, we have calculated the affinity constants Ka for each preparations and these are tabulated in Table VII. It can be seen by using chloramine T as an oxidizing agent a higher specific activity iodinated bradyinin species was prepared than that obtained with the enzyme preparation.

DISCUSSION

One of the important factors in a radioimmunoassay is the specific activity of the labeled hormone used in the assay itself. For

measurement in the picogram range, quantities of labeled hormone are required which have specific activities in excess of 100 μCi/ g of hormone. This is particularly true for a polypeptide of molecular weight of 1,000. Since radioiodine is ideally suited to this application it is used almost exclusively in such radioimmunoassays. It can be attached to polypeptides containing tyrosine as well as to those containing histidine residues. When the natural polypeptide or antigen which is to be labeled does not contain these residues, analogues of derivatives must be synthesized containing these amino acids. Originally, bradykinin which was labeled with either carbon or tritium isotopes had low specific activities. Spragg et at. (1966) used ^{3}H acetic anhydride to acetylate the amino terminal group of bradykinin and was able to achieve specific activities of approximately 1.2 μCi/μg while Talamo et al. (1968) using similar intrinsically labelled ^{14}C-2,3-proline-bradykinin could only produce a compound with a specific activity of 0.2 μCi/μg. Using these techniques these investigators were able to detect levels of bradykinin in the 10-100 ng range with their antisera.

Goodfriend and Ball (1969) adapted Greenwood and Hunter's chloramine T procedure (1963) to iodinate the analogue [Tyr^{8}]-bradykinin with a resultant specific activity of approximately 300 μCi/μg. When this high specific activity labeled ligand was used in a radioimmunoassay with antiserum developed by the carbodiimide technique, the detection sensitivity was increased to 0.1 - 10.0 ng of bradykinin. The experiments reported in this paper have optimized and modified Goodfriend and Ball's (1969) procedure for iodinating bradykinin using the oxidizing agent chloramine T. These modifications were necessary since the original conditions were severe, producing overoxidation of

Downloaded At: 12:23 16 January 2011

the iodide ion and oxidizing other amino acids which brought about structural changes in the protein being iodinated, as well as being difficult to control. The conditions for iodinating bradykinin reported here are less severe and readily more controllable, yielding labeled peptides of specific activities of approximately 600 μCi/μg.

Recently several authors have used lactoperoxidase for the iodination of polypeptide hormones (Thorell and Johanason (1971), Miyachi et al. (1972) and Wajchenberg et at. (1979)) since in addition to oxidizing iodide to "active iodine" it had the advantage of specifically incorporating the halogen into the tyrosyl radicals of the molecule. One of the aims of these experiments reported here was to compare and optimize the two methods that could produce radiolabeled haptens with specific activities high enough for radioimmunoassay but without loss of immunoreactivity.

Downloaded At: 12:23 16 January 2011

Bolton and Hunter (1973) compared ^{125}I-labeled human growth hormones prepared by direct iodination with the chloramine-T method and direct iodination using their coupling technique. They found that antisera differed with respect to binding to the ^{125}I-labeled growth hormones and that the sensitivity of an antiserum could be increased if the proper labeled antigen was employed. Recently, Odya et al. (1978) presented data that binding of various derivatives of bradykinin analogs followed the same pattern. Thus, the suggestion is in place that the subtle differences in the labeled hapten are important in ensuring maximum antisera binding. Since we have found evidence that it is possible to produce two distinct species of iodinated [Tyr^8] -bradykinins, the mono- and di-iodinated forms it follows that these compounds may respond differently in a radioimmunoassay system.

Most investigators when discussing the implications of radioiodining bradykinin with chloramine-T, infer that the iodinated product

is mono-iodo bradykinin. Goodfriend and Odya (1974) separated mono from di-iodo bradykinin using DEAE Sephadex but little mention was made of the distribution of these two species in their crude preparation. In a subsequent publication from that laboratory Odya et al. (1978) use the guarded phase "(presumed) mono-iodinated derivative" when describing the product of their chloramine-T oxidation of bradykinin. Recently this laboratory (Odya et al. (1980)) was able to simultaneously produce both the mono- and di-iodinated adjuncts of bradykinin by having a great excess of their peptide to iodine content during their oxidation step. This is in contrast to our method for this procedure where the iodine is in excess. Sipila and Larija (1976) subjected their chloramine-T preparation of ^{125}I-[Tyr^{8}]-bradykinin to isofocusing and found that the major iodinated component was located at pH 8.85. This observation is consistent with our pI for the mono-iodinated form of the nonapeptide. The data presented in this paper clearly shows that the product of the chloramine-T iodination is not a mono-iodinated form but a di-iodinated compound and furthermore, using the enzymatic procedure a mono-iodinated

Downloaded At: 12:23 16 January 2011

TABLE V

Determination of Iodide and Peptide Contents of Iodinated [Tyr^{8}] -Bradykinin

Chromatographically pure bradykinin preparations which were iodinated by the chloramine-T and lactoperoxidase procedures were subjected to quantitative analysis for their iodide and peptide contents by the procedures outlined in the Methods Section. Samples were run in triplicate and each determination was carried out three times (±S.E.M.).

^{127}I-Bradykinin Compound	Iodide Content (moles)	Peptide Content (moles)	Iodide / Peptide
Chloramine-T	8.1 ± 0.6	3.9 ± 0.4	2.1 ± 0.1
Lactoperoxidase	4.0 ± 0.9	3.9 ± 0.5	1.0 ± 0.1

Downloaded At: 12:23 16 January 2011

TABLE V I

Summary of an Experiment in Which Increments of ^{125}I-[Tyr^{8}]-Bradykinin Were Incubated With a Constant Amount of Antibody

µl	Total Amount of Added Bradykinin (ng)[2]	Total CPM Added	CPM Bound	Bound Antigen ng[3]	Bound Antigen M 10^{-11}	Bound Antigen 1/M 10^{9}	Free Antigen ng	Free Antigen M 10^{-11}	Free Antigen 1/M 10^{8}	B/F
1	0.09	$4.3x10^{4}$	$1.1x10^{4}$	0.02	5.73	17.50	0.07	22.04	45.37	.26
5	0.38	$2.0x10^{5}$	$1.8x10^{4}$	0.03	8.49	11.78	0.14	42.40	23.58	.20
10	0.77	$3.8x10^{5}$	$3.8x10^{4}$	0.07	19.10	5.24	0.47	127.40	7.85	.15
20	1.54	$7.7x10^{5}$	$7.1x10^{4}$	0.15	36.54	2.74	1.39	347.9	2.88	.10
50[1]	3.84	$1.9x10^{6}$	$1.2x10^{5}$	0.25	61.83	1.62	3.68	920.6	1.09	.07
100[1]	7.68	$3.5x10^{6}$	$1.7x10^{5}$	0.35	87.73	1.14	7.57	1893.8	0.53	.05
200[1]	15.36	$6.5x10^{6}$	$2.6x10^{5}$	0.53	132.40	0.76	14.83	3711.9	0.27	.04

[1] Amounts of ^{125}I [8-tyrosyl] bradykinin calculated by dilution
[2] Determined from B/F graph (Figure 2)
[3] Calculated by ratio of CPM bound/CPM added x ng total added bradykinin from B/F graph and expressed in moles/liter (M) or reciprocal $\frac{1}{(M)}$

TABLE VII

Affinity Constant (Ka) Determinations of Lactoperoxidase and Chloramine-T Iodinated [Tyr^8]-Bradykinin

Using the criteria of Weir (1973) the affinity constants were calculated from the reciprocal plot data obtained from a series of dose-response curves using labelled haptens prepared by the two different iodination procedures.

Method of Iodination	Amount $Na^{125}I$	Specific Activity uCi/ug	Ka x 10^8 L/mole
Lactoperoxidase	2 nCi	171.3	4.0
	3 mCi	275.7	5.4
	5 mCi	406.3	17.1
Chloramine-T	1.5 mCi	287.6	2.5
	3.0 mCi	662.5	8.9

Downloaded At: 12:23 16 January 2011

derivative of the vasopressor compound was produced. These observations were conclusively established by the isofocusing data (Figure 1), separation of iodinated amino acids (Table IV), quantitative determination of the iodide and peptide content (Table V) and finally the specific activity measurements using the two different labeled ligands (cf. Table VI and Figure 2).

Our affinity constant data (cf. Table VII) would indicate that the two labelled iodide compounds behave quite differently in their binding capacity for the antiserum used in these experiments. The inclusion of a second iodide atom onto the tyrosine residue which is attached to the parent bradykinin molecule lowers the immunoreactivity to the antiserum as can be seen by the high Ka value (cf. Table VII). These observations confirm those reported by Odya *et al.* (1980) who used a rat uterus bioassay in their experiments. The initial iodination of the [Tyr^8]-

bradykinin does not alter this activity. This can be seen clearly in Figure 3 where increasing concentrations of the radioligand were superimposed on the data obtained from response curves produced with trace amounts of radioligand and non-radioactive bradykinin. If the labeling process altered the immunoreactivity of the radio ligand the superimposable kinetic responses obtained with the parent molecule bradykinin would not be seen. Pinto et al. (1977) found similar results when they compared the radioiodination of h FSH by the two methods. The enzymatic labeled hormone revealed a greater binding affinity for their antibody than did the hapten prepared with chloramine-T.

Downloaded At: 12:23 16 January 2011

REFERENCES

1. Barker, S.B., Humphry, M.J. and Soley, M.H. (1951). The clinical determination of protein-bound iodine. J. Clin. Invest. 30, 55-62.

2. Berson, S.A., Yalow, R.C., Schruber, S.S. and Post, J. (1953). Tracer experiments with ^{131}I-labeled human serum albumin:distribution and regradation studies. J. Clin. Invest. 32, 746-768.

3. Berson, S.A., Yalow, R.S., Bauman, M.D., Rothschidd, M.A. and Newerly, K. (1956). Insulin ^{131}I metabolism in human subjects: demonstration of insulin binding globulin in the circulation of insulin treated subjects. J. Clin. Invest. 35, 170-190.

4. Bolton, A.E. and Hunter, W.M. (1973). The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J. 133, 529-539.

5. Goodfriend, T.L. and Ball, D.L. (1969). Radioimmunoassay of bradykinin: chemical modification to enable use of radioactive iodine. J. Lab. Clin. Med., 73, 501-511.

6. Goodfriend, T.L. and Odya, C.E. (1974). Bradykinin. In Methods of Hormone Radioimmunoassay (ed.) B.M. Jaffe and H.R. Behrnan. Academic Press Inc., New York, pg. 439-454.

7. Greenwood, F.C. and Hunter, W.M. (1963). The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. 89, 114-123.

8. Grodsky, G.M. and Forsham, P.H. (1960). An immunochemical assay of total extra stable insulin in man. J. Clin. Invest. 39, 1070-1079.

9. Henry, R.J. (1964). Clinical Chemistry, Principles and Technics. Harper and Row, Haeber Med. Div., New York, pg. 937-942.

10. Miyachi, K., Vaitukaistis, J.L., Nieschlag, B. and Lipsett, M.B. Enzymatic radioiodination of gonadotropins. J. Clin. Endoc. 34, 23-28.

11. Odya, C.E., Goodfriend, T.L., Stewart, J.M. and Pena, C. (1978). Aspects of bradykinin radioimmunoassay. J. Immunol. Methods, 19, 243-257.

12. Odya, C.E., Goodfriend, T.L. and Pena, C. (1980). Bradykinin receptor-like binding studied with iodinated analogues. Biochem. Pharmacol. 29, 175-185.

13. Pinto, H., Wajchenberg, B.L., Higa, O.Z., Souza, I.T.T., Werner, R.J. and Peironi, R.R. (1975). Preparation of high-quality iodine-^{125}-labeled pituitary luteinizing hormone for radioimmunoassay. Clin. Chim. Acta, 60, 125-135.

14. Pinto, H., Lerario, A.C., Souza, I.T.T., Wajchenberg, B.L., Matter, E. and Pieroni, R.R. (1977). Preparation of high-quality iodine-^{125}-labeled pituitary human follicle-stimulating hormone (hFSH) for radioimmunoassay:comparison of enzymatic and chloramine-T iodination. Clin. Chim. Acta. 76, 25-44.

Downloaded At: 12:23 16 January 2011

15. Redman, L.W., Regoli, D. and Tustanoff, E.R. (1979). Immunological and biological activities of fragments and analogs of bradykinin. Can. J. Biochem. 57, 529-539.

16. Redman, L.W. and Tustanoff, E.R. (1982). Determination of serum bradykinin levels using R.I.A. and bioassay procedures - a comparative study. Submitted for publication.

17. Sipila, R. and Larija, A. (1976). The application of bradykinin radioimmunoassay to plasma kininogen determination. Biochem. Pharmacol. 25, 543-545.

18. Spragg, J., Austen, K.F. and Haber, E. (1966). Production of antibody against bradykinin: demonstration of specificity by complement fixation and radioimmunoassay. J. Immunol. 96, 865-871.

19. Talamo, R.C., Haber, E. and Austen, K.F. (1968). Antibody to bradykinin. Effect of carrier and method of coupling in specificity and affinity. J. Immunol., 101, 333-341.

20. Thorell, J.I. and Johansson, B.G. (1971). Enzymatic iodination of polypeptides with ^{125}I to high specific activity. Biochim. Biophys. Acta. 251, 363-369.

21. Unger, R.H., Eisentraut, A.M., McCall, M.S., Keller, S., Lanz, H.C. and Madison, L.L. (1959). Glucagon antibodies and their use for immunoassay for glucagon. Proc. Soc. Exp. Biol. Med., 102, 621-623.

22. Utiger, R.D., Parker, M.L. and Daughaday, W.H. (1962). Studies on human growth hormone. I. A radioimmunoassay for human growth hormone. J. Clin. Invest. 41, 254-266.

23. Wajchenberg, B.L., Pinto, H., Souza, I.T.T., Lerario, A.C. and Pieroni, R.R. (1978). Preparation of iodine-^{125}-labeled insulin for radioimmunoassay:comparison of lactoperoxidase and chloramine-T iodination. J. Nucl. Med. 19, 900-905.

Downloaded At: 12:23 16 January 2011

24. Weigle, M., Blout, J.F., Tengi, J.P., Czaikowski, R.C. and Leimagruber, W. (1972). Flouorgenic ninhydrin reaction. Structure of the fluorescant principle. J. Amer. Chem. Soc. 94, 4052-4054.

25. Weir, D.M. (1973). In: Handbook of Experimental Immunology, 2nd Ed., Blackwell Scientific Publication, Oxford, Chapter 16.

Downloaded At: 12:23 16 January 2011