PHARMACOLOGICAL REVIEWS
Vol. 25, No. 2

Background—Theoretical and Practical

CHAIRMAN: ROSALYN S. YALOW, PH.D.

Radioimmunoassay Methodology: Application to Problems of Heterogeneity of Peptide Hormones

ROSALYN S. YALOW

Solomon A. Berson Research Laboratory, Veterans Administration Hospital, Bronx, New York and Department of Medicine, Mount Sinai School of Medicine, The City University of New York

Introduction

WITHIN the past two decades, a host of new diagnostic aids have been developed in virtually every specialty of medicine. Except for the common major trend towards automation and computerization of many laboratory procedures, these new techniques have been highly diverse both in principle and practice. There is, however, a broad group of quantitative assays which share a common principle, a principle which was conceived and adapted only about 15 years ago but with applications which seem to be penetrating into a myriad of fields of medical investigation and diagnosis. This principle is the basis of radioimmunoassay or, in more general terms, of competitive assay (fig. 1). This principle is simply that of competition between labeled and unlabeled substances for a limited number of combining sites on a specific reactor, which results in a progressive inhibition of the binding of labeled substance as the concentration of unlabeled substance is increased. The concentration of substance in an unknown sample is determined by comparing the degree of inhibition observed in the sample with that produced by known standards. The specific reactor might be, for instance, a specific binding protein in plasma (32), an enzyme (38), or a tissue receptor site (31). Although non-radioactive labels, *e.g.*, modified phages (20), have been used, by far the most common application of this principle is the one for which it was first employed (5–8, 46, 47), radioimmunoassay (RIA) (fig. 2) in which the specific reactor is an antibody and the marker molecule is radioisotopically labeled. Some appreciation of the general applicability of RIA can be gleaned from a listing of substances which have been measured by this technique (table 1). This list is certainly incomplete since the rapid rate of growth in this field precludes a truly up-to-date table.

What have been the accomplishments of RIA? The several thousand papers which have been published even in the single field of peptide hormones are not readily summarized. However, the ability to measure small amounts of peptide hormones in plasma which RIA has made possible has provided greatly increased accuracy of diagnosis of pathological states which are characterized by hormonal excess or deficiency, has resulted in an information explosion concerning the regulation of secretion and the interrelationships of hormones and has contributed greatly to our understanding of the mechanisms of hormonal release and of hormonal physiology in general. As will be discussed shortly, RIA has recently led to the discovery of new hormonal forms in

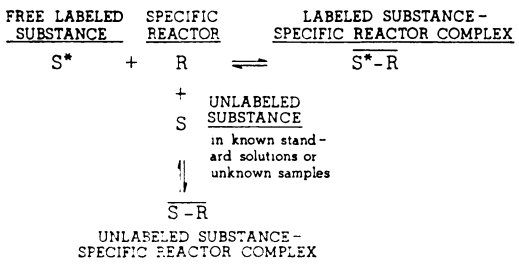

FIG. 1. Application of radioassay principle to physical or chemical reactions in non-immune systems.

blood and tissue. The impact of the methodology on other fields is quite new, but one can anticipate that, in the years to come, its contribution to fields as varied as toxicology, virology, and cancer detection will equal, if not exceed, its effect on endocrinology. From present indications, it seems likely that if there is no other simple way to measure or detect a substance of biological interest, some imaginative investigator will exploit RIA to find a solution to the problem.

Methodology

RIA, although simple in principle, is not always simple in practice. Let us now consider briefly the basic methodology with due consideration to some of the problems and pitfalls. The essential requirements for RIA (fig. 2) include suitable reactants, *i.e.*, labeled antigen and specific antibody and some technique for separating antibody-bound from free-labeled antigen since under the usual conditions of assay the antigen-antibody complexes do not precipitate spontaneously. A typical RIA is performed by the simultaneous preparation of standard and unknown mixtures in test tubes. To these tubes are added fixed amounts of labeled antigen and of antiserum. With currently available automatic pipetting devices, it is quite practical to set up several thousand samples in a typical working day. After an appropriate reaction time, which may range from hours to days depending on the association-dissociation rates for the particular reaction, the antibody-bound (B) and free (F) fractions are separated by one of many different techniques (table 2). The B/F ratios in the standards are then plotted as a function of the concentration of unlabeled antigen ("standard curve") and the concentration in an unknown sample is determined by comparing its observed B/F ratio with the standard curve (fig. 3). Alternately another of the measures of competitive binding such as percent bound, percent free, or their reciprocals could also be plotted as a linear or a logarithmic function of the standard concentration. Different investigators have used these alternate formulations. However, it is important not to use formulations which obscure the true experimental determinations. For instance, plots in terms of the percent of the trace binding, *i.e.*, the binding in the absence of unlabeled hormone, may fail to reveal that the maximal binding of the labeled antigen under trace conditions is only a few percent.

RIA has found wide application because of its potential for high sensitivity and specificity and because the methodology lends itself to automation and computerization. However, each of the diverse applications does not demand all of these attributes. The requirements for sensitivity are dependent on the minimal quantity that must be measured. For instance, the concentration of the peptide hormones in plasma in the unstimulated state are of the order of 10^{-12} M to 10^{-10} M; the concentrations of thyroidal and steroidal hormones are higher by a factor of about 10^5 and, therefore, the requirements

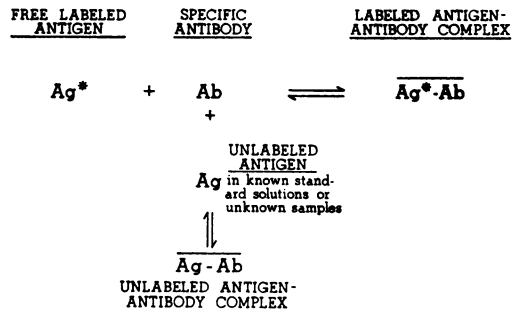

FIG. 2. Radioimmunoassay principle: specific reactor is specific antibody.

TABLE 1

Substances measured by radioimmunoassay

Peptide Hormones	Non-peptidal Hormones	Non-hormonal Substances
Pituitary hormones	Steroids	Drugs
Growth hormone	Aldosterone	Digoxin
Adrenocorticotropic hormone (ACTH)	Testosterone	Digitoxin
Melanocyte stimulating hormone (MSH)	Dihydrotestosterone	Morphine
α-MSH	Estradiol	LSD
β-MSH	Estrone	Barbiturates
Glycoproteins	Estriol	Cyclic nucleotides
Thyroid stimulating hormone (TSH)	2-Hydroxyestrone	cAMP
Follicle stimulating hormone (FSH)	Medroxyprogesterone	cGMP
Luteinizing hormone (LH)	17-Hydroxyprogesterone	cIMP
Prolactin	Prostaglandins	cUMP
Lipotropin (LPH)	Thyroidal hormones	Enzymes
Vasopressin	Triiodothyronine	C_1 esterase
Oxytocin	Thyroxine	Fructose 1,6-diphosphatase
Chorionic hormones		Virus
Human chorionic gonadotropin (HCG)		Australia antigen (HAA)
Human chorionic somatomammotropin (HCS)		Tumor antigens
Pancreatic hormones		Carcinoembryonic antigen
Insulin		α-Fetoprotein
Proinsulin		Serum proteins
C-peptide		Thyroxin binding globulin
Glucagon		IgG, IgE
Calcitropic hormones		Properdin
Parathyroid hormone (PTH)		Anti-Rh antibodies
Calcitonin (CT)		Other
Gastrointestinal hormones		Intrinsic factor
Gastrin		Rheumatoid factor
Secretin		Folic acid
Cholecystokinin-pancreozymin (CCK-PZ)		Neurophysin
Enteroglucagon		Staphylococcal β-Enterotoxin
Vasoactive tissue hormones		
Angiotensins		
Bradykinins		
Hypothalamic releasing factors		
Thyrotropin releasing factor (TRF)		

for sensitivity in the latter assays are correspondingly less stringent.

The maximal sensitivity of a radioimmunoassay is limited by the characteristics of the individual antiserum employed. Animals immunized on the same regimen vary considerably in the concentration and potential sensitivity (fig. 4) as well as the specificity of the antibodies they produce. Detailed formulation of the quantitative aspects of the antigen-antibody reactions and of the mathematical considerations governing optimization of the sensitivity and precision of radioimmunoassays have been considered previously (9, 51). One can gain some insight into the problem from the following highly simplified approach: Consider the biomolecular reaction between an antigen containing a single reactive site (Ag) and a single order of homogeneous combining-sites on antibody (Ab) and assume that labeled and unlabeled antigen behave identically. It is obvious that it is inadvisable in a RIA to employ an amount of labeled tracer antigen whose immunochemical concentration is large compared

TABLE 2

Techniques used for separation of antibody-bound and free-labeled antigen in radioimmunoassay

- I. Adsorption of free antigen to solid phase material
  - A. Cellulose
    1. Paper chromatoelectrophoresis
    2. Paper strip adsorption in test tubes
    3. Cellulose column adsorption
    4. Cellulose powder sludge adsorption
  - B. Charcoal
    1. Uncoated charcoal
    2. Charcoal coated with dextran, albumin, ficoll, *etc.*
  - C. Silicates
    1. Talcum powder
    2. QUSO*
    3. Silicic acid and other silicates
  - D. Ion exchange resins
    1. Anion resins
    2. Cation resins
- II. Precipitation of antigen-antibody complexes
  - A. Double antibody techniques
  - B. Salting out techniques
  - C. Organic solvents
    1. Alcohol
    2. Dioxane
- III. Adsorption or complexing of antibody to solid phase material
  - A. Adsorption to plastic tubes
  - B. Complexing to dextran
  - C. Complexing to glass
- IV. Miscellaneous
  - A. Sieve separation
  - B. Electrophoretic separation on paper strips

* Microfine precipitated silica.

to the concentration of unlabeled antigen in the unknown; *e.g.*, if the tracer concentration is 10 times the antigen concentration then a random 5% error in the tracer produces a 50% error in the hormone concentration. Let us assume, therefore, that tracer concentration and minimal concentration of antigen to be detected are about equal. From the mass-action law,

$$\underset{F}{[Ag]} + [Ab] \underset{k'}{\overset{k}{\rightleftharpoons}} \underset{B}{[\overline{AgAb}]} \qquad (1)$$

where

$F \equiv$ [Ag], the molar concentration of uncomplexed antigen

$B \equiv [\overline{AgAb}]$, the molar concentration of complexed antigen or antibody-combining sites

and

[Ab], the molar concentration of uncomplexed antibody

then

$$B/F = K([Ab^\circ] - B) \qquad (2)$$

where the equilibrium constant for the reaction $K = k/k'$ and $[Ab^\circ]$ is the total molar concentration of antibody binding sites, *i.e.*, $[Ab^\circ] = [Ab] + [\overline{AgAb}]$.

It is evident from reaction 2 that when $B \ll [Ab^\circ]$, B/F decreases only slightly for large changes in B; thus if B increases 10-fold from 0.001 $[Ab^\circ]$ to 0.01 $[Ab^\circ]$ the change in B/F is less than 1%. For a sensitive assay therefore $[Ab^\circ]$ must be reduced by dilution so that $[Ab^\circ]$ is not much larger than B and

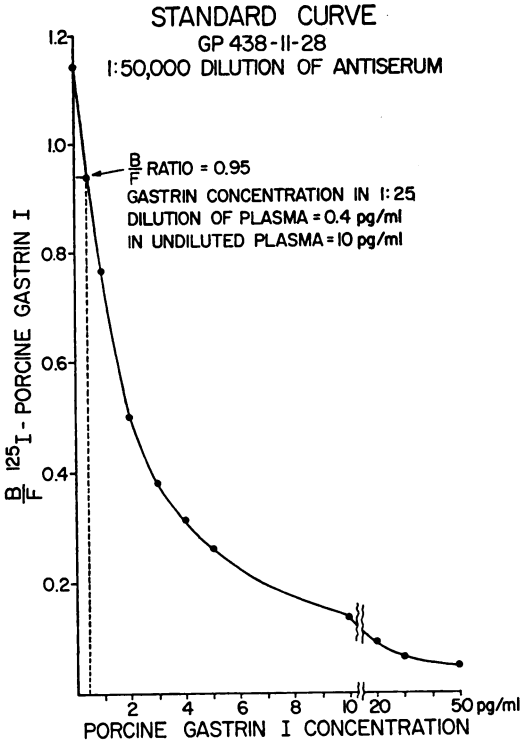

FIG. 3. Standard curve for gastrin immunoassay. Hormone concentration in unknown sample is determined as shown.

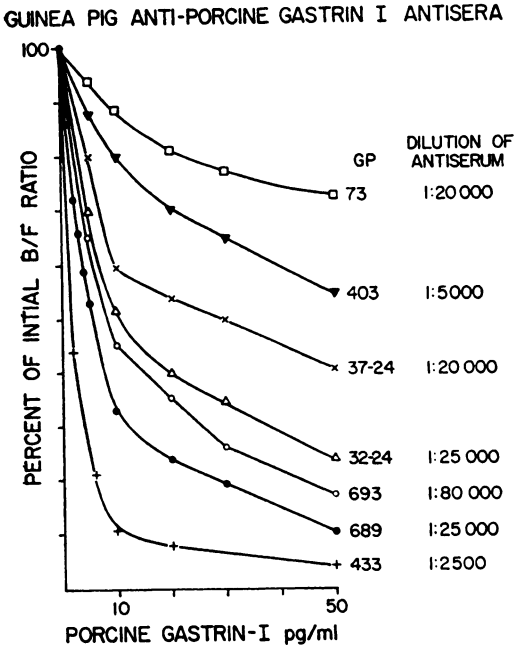

FIG. 4. Comparison of the sensitivity of various antisera for measurement of gastrin. Initial B/F ratios between 0.5 and 1.0 in all cases. (From S. A. Berson and R. S. Yalow: Radioimmunoassay in gastroenterology. Gastroenterology **62**: 1061–1084, 1972.)

since B, the bound antigen, must be less than the total antigen, it follows that $[Ab^\circ] \cong H$, the minimal antigen concentration to be detected.

If we wish to start with B/F = 1 in the absence of added unlabeled antigen ("trace" conditions), then from reaction 2

$$1 \leqq K\,[Ab^\circ] \lessapprox KH$$

and, therefore,

$$K \gtrapprox 1/H$$

Thus, there is an inherent sensitivity that can be achieved with any given antiserum which is dependent on the equilibrium constant, K, that characterizes the reaction of the predominating antibodies.

Extensions can be introduced into this mathematical treatment by considering non-identity of labeled and unlabeled antigen, more than one antibody-binding site, interactions between binding sites, experimental errors, *etc.* However, the more complicated formulations all arrive at the same conclusion, that is, in order to achieve high sensitivity assays, the primary reaction of antigen with antibody must be one of large equilibrium constant, hence of very high energy.

The availability of a suitable antibody is the *sine qua non* of RIA. As a rule, we have found guinea pigs to be the best species for immunization. Generally polypeptide hormones of molecular weight greater than 4000 are sufficiently immunogenic when the hormone is administered as an emulsion in Freund's adjuvant. Usually we have employed commercial or low purity native hormonal preparations to take advantage of the perhaps slight denaturation of such preparations which might render them more "foreign" and hence more antigenic. Immunization with highly purified native hormonal preparations seems to confer no advantage since non-specific contaminants are not likely to lead to immunological reactions which interfere with the assay. The antigen used for *labeling*, however, must be highly purified in order to avoid interaction of labeled contaminants with non-specific antibody.

Substances such as drugs, steroids, *etc.* may be rendered antigenic by coupling to a large protein or polypeptide by a variety of methods (19, 22, 34, 44). The antigenicity of small peptides such as angiotensin, oxytocin, and vasopressin may be enhanced by coupling. However, it is worthy of note that in spite of their low molecular weights, the vasopressins are antigenic in many species (rabbit, guinea pig, hog, chicken, man) irrespective of which vasopressin is the endogenous hormone in that species (4, 35, 37).

Since animals, even of the same species, vary considerably in the concentration, specificity, and potential sensitivity of the antibodies they produce, the probability of obtaining a satisfactory antiserum increases with the number of animals immunized. However, the presence of other immuno-

logical reactions does not interfere with the reaction between labeled antigen and its specific antibody. Therefore, immunization with several unrelated antigens can be performed simultaneously, affording an advantage in reducing the number of animals to be immunized and bled by a factor equal to the number of antigens employed.

The limiting size of the chemical amount of the tracer generally determines the choice of radioisotope used for labeling. When very high specific activity preparations are required, radioiodine (^{131}I or ^{125}I) is the radiochemical of choice. The radioiodine usually substitutes on a tyrosyl residue, although substitution on a histidyl residue or carbon double bond may occur. Because the isotopic abundance of ^{131}I ($T_{1/2}$, 8 days) is generally not more than 15 to 30% at the time of receipt into the laboratory and that of ^{125}I ($T_{1/2}$, 60 days) is closer to 100% (50, 51), the longer half-life of the latter is not disadvantageous and it has become the radioiodine isotope of choice. The specific activity of an ^{125}I labeled antigen may be increased by increasing the number of radioiodine substitutions but the more highly iodinated molecules manifest decreased immunoreactivity and decreased stability due to radiation self-damage (12, 15). This damage, which we have called "decay catastrophe" is manifest when decay of the first radioactive atom results in dissociation of the residual molecule into labeled molecular fragments or free radioiodide. Thus the radioactivity is no longer associated only with unaltered molecules and "damage" is said to have occurred. For maximal stability and immunoreactivity, antigen labeled with 1 radioiodine atom per molecule, therefore, is generally preferable.

The requirement for high specific activity preparations is much less stringent for assays of non-peptidal hormones, drugs, and other substances present in plasma in much higher concentrations than the peptide hormones. For these assays ^{3}H-labeled tracers, prepared and purified in commercial laboratories, may be preferable to radioiodinated tracers. Long-lived isotopes such as ^{14}C are of limited value except when the concentrations of antigen to be measured are quite high, *i.e.*, concentrations $\sim 10^{-6}$ M or higher.

It is important to realize that radioimmunoassay does *not* require that labeled and unlabeled antigens be identical, nor is it necessary that standards and unknown be biologically or chemically identical. A necessary but not sufficient condition for the validation of a RIA procedure is, however, that the antigen concentration measured in an unknown sample be independent of the dilution assayed. This requires immunochemical identity of standards and unknown. This condition is tested by making multiple dilutions of an unknown sample and determining whether the competitive inhibition curve is superimposable on the standard curve used for the assay (fig. 5A) or alternately whether the calculated concentration in the unknown sample falls linearly with dilution (fig. 5B). Failure to meet this condition may be due to a number of factors which can be classified as in table 3. Nonhormonal factors which interfere in the assay can and should be avoided since they simply interfere with the chemical reaction in a non-specific fashion. This is usually effected by having the incubation mixtures for standards and unknown prepared, stored, and separated under as near identical conditions as possible.

Hormonal Cross-Reactivity

The problems relating to hormonal cross-reactivity are not dealt with so simply. Let us consider first the problem of species differences. Crystalline insulins from most species have identical biological potencies despite differences in amino acid sequences. Where insulins are chemically very different, as in the case of mammalian and fish insulins, the immunological discrimination may be very marked (49) (fig. 6), *i.e.*, nearly equal biological potency is consistent with greater than a 1000-fold difference in immunoreactivity. However, some antisera

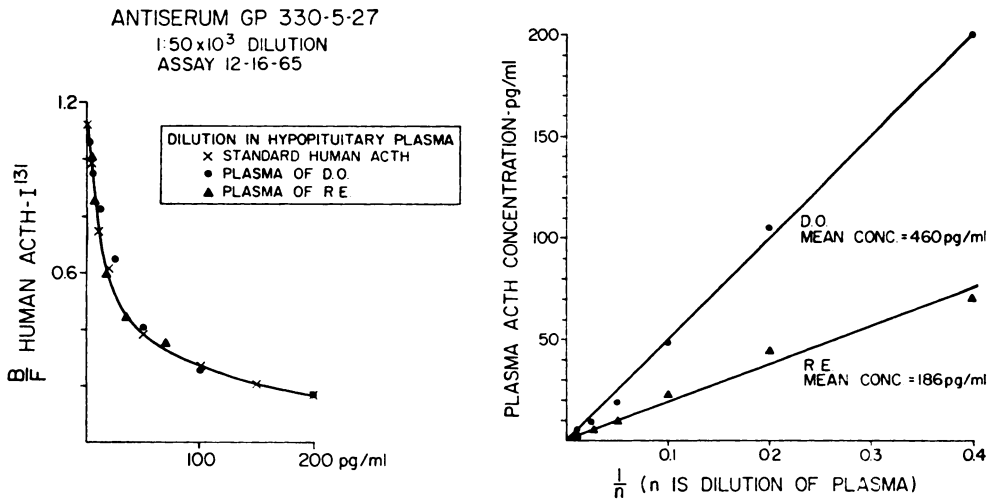

FIG. 5. Plasmas of 2 patients (D.O. and R.E.) with high concentrations of endogenous plasma ACTH were assayed at 6 different dilutions by mixing with appropriate volumes of hypopituitary plasma. All tubes including standards contained plasma at 1:2.5 dilution in 0.02 M barbital buffer and appropriate protective agents.

A. The concentration in whole plasma was determined from assay at 1:2.5; the concentrations at other dilutions were then calculated and plotted against the observed B/F ratios. (Based on data from S. A. Berson and R. S. Yalow: Radioimmunoassay of ACTH in plasma. J. Clin. Invest. 47: 2725–2751, 1968.)

B. Plot of concentration in sample as function of 1/n (where n is dilution factor).

can distinguish between insulins which differ in as few as 1 to 4 amino acid residues. With such antisera, if hormone from one species is used as standard for assay of immunologically distinct endogenous hormone in another species, inconsistencies will be found in apparent concentrations obtained at different plasma dilutions (fig. 7) (13). Certain antisera seem able to detect immunological differences even between insulins purported to have the same amino acid sequences. Thus we showed early that a few human antisera recognized pork and whale insulins as immunologically distinct (10) although their amino acid sequences were reported to be identical (28, 30). We concluded that the distinguishable aspects of the two insulins must reside in certain conformational features related to unknown differences in their secondary or tertiary structures. However, since changes undergone during extraction and purification may have been responsible for the differences, similar studies with native unextracted hormones appeared indicated. When Smith

TABLE 3

Factors relevant to validation of radioimmunoassay (RIA) procedures

A. Non-hormonal effects
1. pH; ionic environment
2. Anticoagulant; protective agents
3. Variable damage in unknowns as compared to standards
4. Temperature effects
B. Hormonal cross-reactivity
1. Heterologous hormonal standard
2. Immunologically related hormones in the same species
3. Heterogeneity of hormonal forms in standards and unknowns

(42) showed that dog insulin had the same amino acid sequence as pork and sperm whale insulins, it seemed practical to intercompare the relative immunoreactivity of dog and pig *plasma* insulins. The concentrations of insulin in the animal plasmas were determined with a guinea pig antiserum that does not distinguish between crystalline dog and pork insulins. Our studies revealed that dog plasma and dog crystalline insulins are

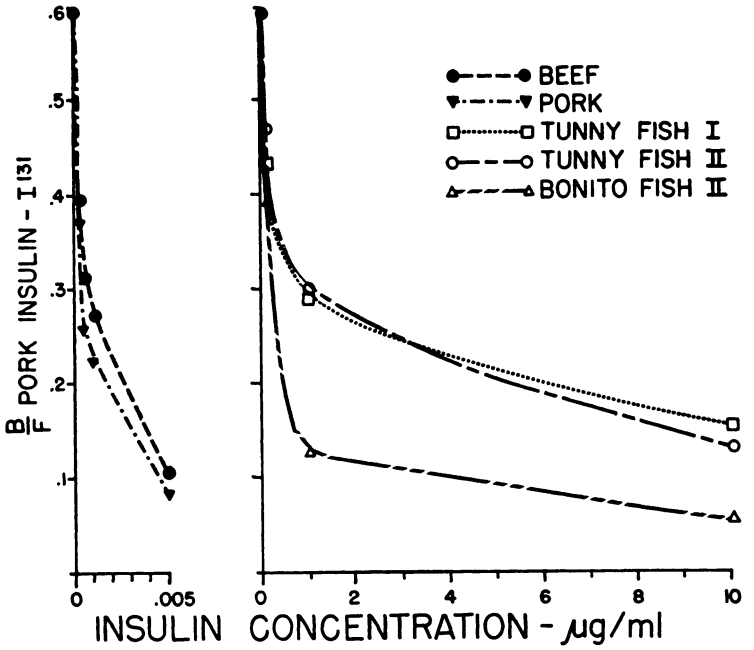

FIG. 6. Ratio of bound to free ^{131}I-labeled pork insulin in a guinea pig antiserum against pork insulin as a function of the concentration of unlabeled beef, pork and fish insulin. (From R. S. Yalow and S. A. Berson: Reaction of fish insulins with human insulin antiserums: potential value in the treatment of insulin resistance. N. Engl. J. Med. 270: 1171-1178, 1964.)

immunologically identical with each other but are distinguishable in certain human antisera from pig plasma and crystalline pork insulins (fig. 8) (11). We concluded, therefore, that if the data on the amino acid sequences were unimpeachable these insulins have conformational differences despite their identical amino acid sequences.

The mystery has perhaps been cleared by the discovery of proinsulin, if one can assume that the configuration of the insulin molecule is determined at the time of synthesis *via* its proinsulin precursor. The amino acid sequences of the connecting peptides in dog and pig proinsulins are strikingly different (33) and hence conformational differences between the two prohormones are not surprising. If the subsequent removal of the connecting peptide leaves the secondary and tertiary structures unaltered, then pig and dog insulins, in spite of the identity of primary structure, would remain distinguishable. Obviously studies on synthetic pork and dog insulin are required to determine the preferred configuration in the absence of the directing effect of the C-peptide.

The presence of plasma of immunologically related hormones of the same species has introduced problems into a number of RIA procedures. In the assay for growth hormone it was noted that apparent hormonal concentration in the plasma of pregnant women did not decrease linearly with dilution although it did in plasma from cord blood, from acromegalics, and from stimulated control subjects (21, 23). The interference was due to human chorionic somatotropin (HCS) which is of placental origin and resembles growth hormone, but is neither biologically nor immunologically identical with it. The synthesis by the placenta of other hormones, *e.g.*, chorionic gonadotropin, chorionic thyroid stimulating hormone (TSH), *etc.* that have biological and immunological properties similar to the pituitary hormones may also render nonspecific the assays for the glycoproteins in the plasmas of pregnant women. Even in the

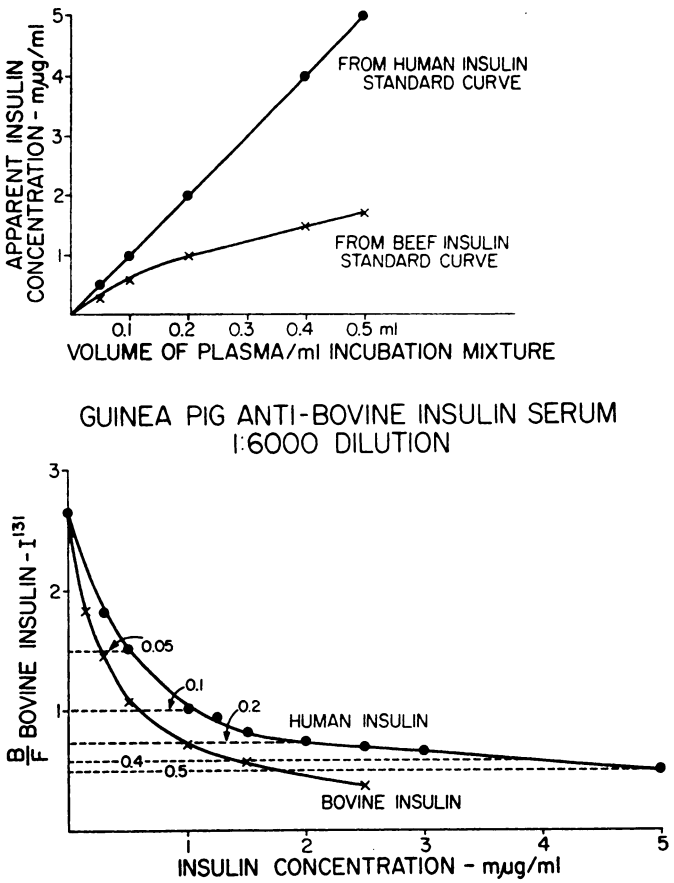

FIG. 7. Effect of dilution of plasma on apparent endogenous insulin concentration. Standard curves for human insulin and bovine insulin reacting against bovine ^{131}I-insulin in a guinea pig antibovine insulin serum are shown in the bottom graph. The B/F ratios in a sample of human plasma diluted from 1:20 (0.05) to 1:2 (0.5) are shown as horizontal dashed lines. The apparent concentrations of hormone in the various diluted samples of plasma as determined from bovine insulin and human insulin standard curves are plotted against the plasma dilutions in the graph at the top of the figure. (From S. A. Berson and R. S. Yalow: Principles of immunoassay of peptide hormones in plasma. *In* Clinical Endocrinology, ed. by E. B. Astwood and C. E. Cassidy, vol. II, pp. 699–720, Grune & Stratton, New York, 1967.)

absence of placental hormones, the assays of the pituitary glycopeptide hormones have been plagued by problems relating to lack of specificity. It is now appreciated that these problems arise, among other reasons, because these hormones share similar species specific *alpha* subunits and hormonally specific *beta* subunits. The common *alpha* subunits produce cross reactions which must be abolished by absorption of the antisera with the cross reacting hormones or subunits. When the antibodies are directed primarily against the *alpha* subunits, the antisera generally cannot be rendered specific. When the antibodies are directed against the *beta* subunits of the intact molecule or when the *beta* subunits are used for immunization, more specific antisera may be obtained. Problems of non-specificity must also be considered and evaluated whenever pairs of hormones share common amino acid sequences which might result in immunological or biological cross reactivity or both. Such systems include among others gastrin and cholecystokinin which share the same C terminal pentapeptide, adrenocorticotrophic hormone (ACTH) and the melanocyte stimulating hormone (MSH) which have

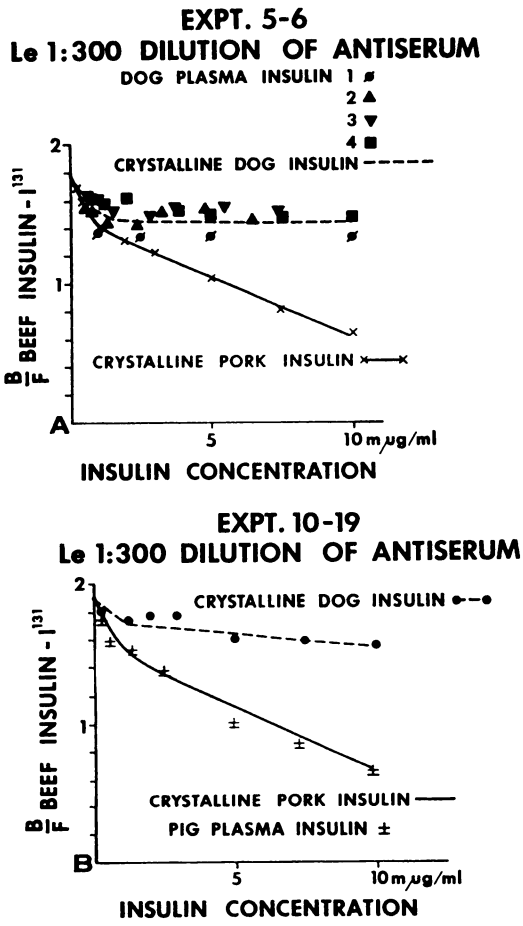

FIG. 8A. Cross reactivities of crystalline pork insulin and dog insulin and dog plasma insulin *versus* ^{131}I beef insulin in a human antiserum. Endogenous insulin concentrations in the dog plasma were determined by radioimmunoassay in guinea pig antipork insulin serum with crystalline dog insulin as standard.

B. Experimental conditions are same as in figure 8A. Endogenous insulin concentrations in pig plasma were determined in guinea pig antiserum with crystalline pork insulin as standard. (From S. A. Berson and R. S. Yalow: Insulin in blood and insulin antibodies. Amer. J. Med. 40: 676–690, 1966.)

similar N terminal sequences, and lipotropin which contains within it the complete structure of β-MSH (18).

Hormonal fragments may or may not competitively cross react with intact hormone in antisera raised against the intact hormone. Such fragments may or may not retain biological activity. Dissociation of biological and immunological activity can, therefore, occur and no general conclusions can be reached since the specificities must be evaluated for each hormone, each bioassay system, and each antiserum. Thus desoctapeptide insulin, insulin lacking the eight C-terminal amino acids of the B-chain, appears devoid of biological activity but is fully reactive with some antisera and only weakly reactive with others (48). With the antiserum used by us in the ACTH assay, the hormonally active N terminal 1–23 and 1–24 peptides are more than 20-fold less immunoreactive than intact ACTH and the biologically inactive 11–25 and 25–39 fragments are more than 100-fold less immunoreactive. However, others have reported ACTH antisera that have immunological reactivity of C-terminal hormonally inactive fragments equal to or greater than that of intact hormone (29). Thus consideration must always be given to the possibility that hormonal fragments, biologically active or inactive, may be either secreted by the gland or returned to the circulation after partial peripheral degradation and that these fragments may or may not be immunologically active and distinguishable from the hormone as usually extracted from the gland.

Immunochemical heterogeneity was first shown for parathyroid hormone (PTH) when it was observed that a constant factor could be used to superimpose a plasma dilution curve on a curve of standards obtained from a normal parathyroid gland for two antisera but this same factor resulted in discrepant results when a third antiserum was employed (fig. 9) (14). The interpretation that there was more than one form of immunoreactive PTH in plasma was further supported by the difference in disappearance rates after parathyroidectomy as measured by two different antisera (fig. 10) (14). The heterogeneity of plasma PTH has been widely confirmed by other workers (2, 3, 17, 27, 41) as well as in our own laboratory (41a). The conclusions we have reached (41a) are that one immunoreactive form of PTH se-

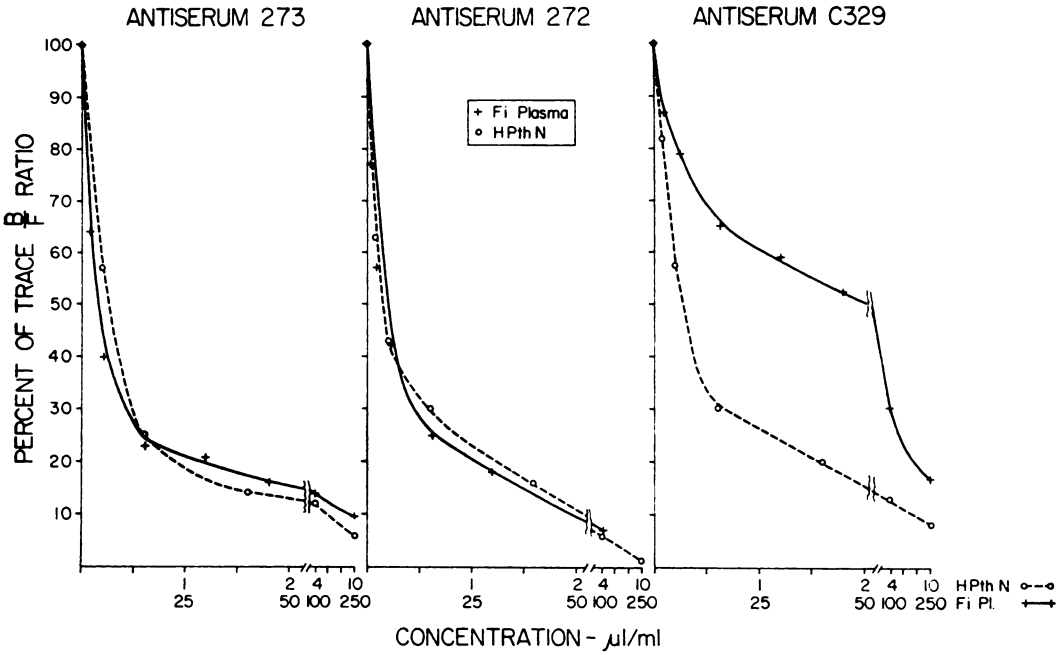

FIG. 9. Inhibition of binding of ^{125}I-Bpth in 3 antisera by pooled plasma from a patient with 2° hyperparathyroidism (+) and by extract of a normal parathyroid gland (°). (From S. A. Berson and R. S. Yalow: Immunochemical heterogeneity of parathyroid hormone in plasma. J. Clin. Endocrinol. Metab. 28: 1037–1047, 1968.)

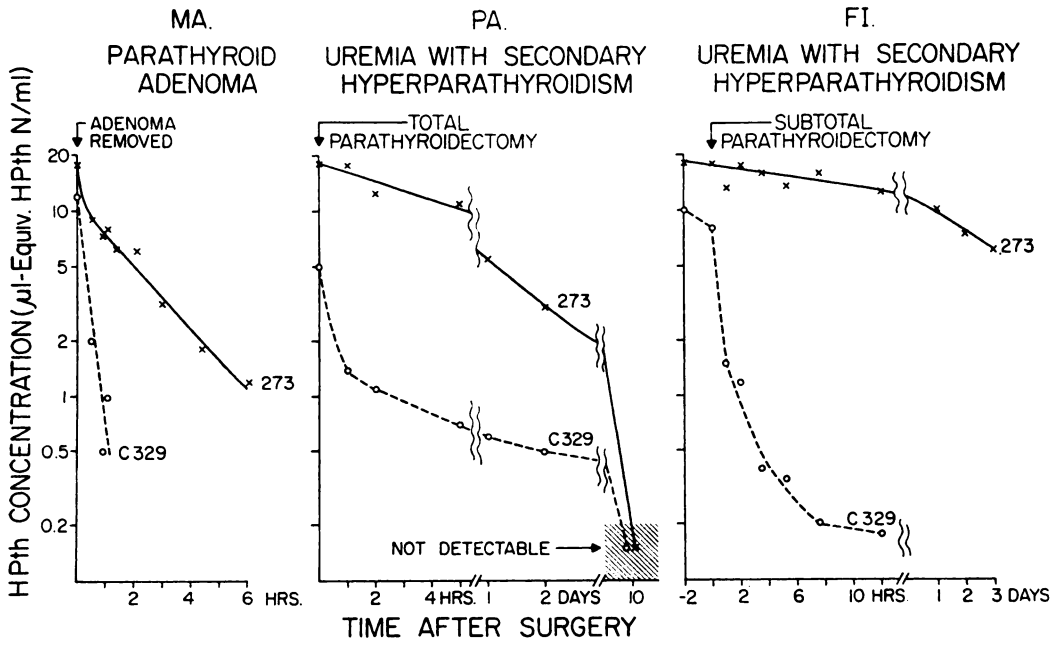

FIG. 10. Disappearance of immunoreactive parathyroid hormone from plasma after parathyroidectomy in patients with 1° or 2° hyperparathyroidism. Plasma samples were assayed in antiserum C329 and antiserum 273 with extract of a normal human parathyroid gland as standard and ^{125}I-Bpth as tracer. Plasma concentrations of hormone are given as µl-equivalents of standard/ml. Thus a plasma sample containing 1 µl-equiv Hpth N/ml produces the same inhibition of binding of ^{125}I-Bpth as 1 µl Hpth N/ml. (From S. A. Berson and R. S. Yalow: Immunochemical heterogeneity of parathyroid hormone in plasma. J. Clin. Endocrinol. Metab. 28: 1037–1047, 1968.)

creted by the gland has Sephadex gel filtration characteristics corresponding to those of intact PTH, that it bears a reciprocal relationship to serum calcium, and that its disappearance from the plasma of a uremic patient during calcium infusion or after parathyroidectomy corresponds to a half-time of no more than 20 min. This component reacts strongly with both antisera (C329 and 273) which we usually use and appears to be the biologically active form in the circulation. However, the predominant component of immunoreactive PTH is secreted by the gland, is seen primarily by antiserum 273, has a molecular weight about ⅔ that of the intact hormone, is probably biologically inactive since it remains elevated even in the presence of postparathyroidectomy hypoparathyroidism, and has a disappearance rate from the plasma of a uremic subject more than 100 times longer than the active form. Evidence presented elsewhere[1] suggests that this fragment is a C-terminal fragment. However, there is present in plasma another immunoreactive form, presumably an N terminal fragment, which is also biologically inactive and which disappears even more slowly in the same uremic subject. The presence of this complexity of immunoreactive forms with markedly different removal rates and biological potencies precludes the possibility of a truly quantitatively valid assay for PTH. It is interesting to note, however, that the use of antiserum 273 with increased sensitivity because it measures both biologically active and inactive forms of the hormone makes it possible to demonstrate greatly increased accuracy in the diagnosis of primary hyperparathyroidism when compared to antiserum C329 which measures primarily the presumably biologically active form of the hormone (41a).

Insulin seems to be characterized by a number of "firsts" among protein hormones. It was among the first to be crystallized, the first whose primary structure was elucidated, the first for which a radioimmunoassay was described, and the first to be totally synthesized chemically—and it is the only major hormone with a 2-chain structure. Two theories of its biosynthesis were current during the 1950's and 1960's; either the two polypeptide chains were synthesized as separate ribosomal units and then combined through the formation of disulfide bonds; or both chains were synthesized as part of a larger single chain with cleavage of a portion of the chain producing the common form of insulin. Through the brilliant work of Steiner *et al.* (43), the latter hypothesis was unequivocally confirmed and proinsulin, a precursor of insulin of low biological activity, was discovered in a culture of an islet cell adenoma *in vitro*. Proinsulin is a single chain peptide, half again as large as insulin (mw 6000) in which a connecting piece runs from the amino terminal of the A chain to the carboxyl terminal of the B chain. Shortly after the discovery by Steiner, Roth *et al.* (36) demonstrated, with radioimmunoassay of Sephadex fractions of plasma, that an immunoreactive component corresponding to proinsulin is present in plasma and this finding was soon confirmed by Steiner and coworkers (39) and our own group (52). Studies in a number of laboratories have now shown that proinsulin usually comprises a minor fraction of total immunoreactive insulin in plasma. However, in some, though not all, cases of insulinoma, proinsulin is the predominant form comprising as much as 75% of total immunoreactive plasma insulin (53). Generally, even in such cases, only a small fraction of the insulin extracted from the islet cell tumor is proinsulin (53). Proinsulin and its products are not the only immunoreactive insulin components to appear in plasma. In an unusual case of a patient suspected of having an insulinoma and who had a very high immunoreactive plasma insulin concentration (600 μU/ml, fasting; 2000 μU/ml, postglucose feeding) but not significant hypoglycemia, most of the immunoreactive insulin in several fasting specimens emerged in the zone of elution of serum albumin on Sephadex G-50 gel filtration (58). We have designated this new form "big, big insulin." It maintains its integrity on refractionation, even in 8 M urea, and is

immunochemically indistinguishable from, but is a more basic protein than, 6000 mw insulin. This new immunoreactive form of insulin is intermediate in molecular weight between albumin and γ-globulin, and is completely convertible on tryptic digestion to a molecule with characteristics similar to regular insulin (fig. 11). It is found as a minor component (generally <1%) of immunoreactive insulin in extracts of insulinomas or normal pancreas. These observations suggest that the newly described "big, big insulin" may well be the ultimate precursor of the insulin family. It seems likely that this component has diminished biological activity since the patient had only occasional hypoglycemia in spite of inordinately high plasma immunoreactive insulins.

We turn now to another peptide hormone, gastrin. In 1959 Gregory and Tracy (25) began a careful and elaborate series of investigations which led to the purification of gastrin, the elucidation of its structure (24), and its synthesis (1). This hormone is a 17-amino acid peptide [*i.e.*, heptadecapeptide gastrin (HG)], is a highly acidic molecule, and contains no lysine or arginine residues. Alerted by our previous experience with parathyroid hormone and cognizant of the size heterogeneity of insulin in plasma, we fractionated the immunoreactive gastrin in the plasma of gastrin hypersecretors with a number of physical chemical systems [primarily patients with Zollinger-Ellison (Z-E) syndrome, whose hypergastrinemia is secondary to a gastrin-secreting tumor, or pernicious anemia (PA), whose hypergastrinemia is secondary to gastrointestinal hyperplasia induced by prolonged hypoacidity]. It was surprising to find that the *major* component of immunoreactive gastrin in these plasma samples had an elution volume on Sephadex gel filtration between insulin and proinsulin and on electrophoretic analysis appeared to be more basic than HG (54, 55). We termed this new immunoreactive form of gastrin "big gastrin" (BG) and demonstrated that it was immunochemically indistinguishable from HG, maintained its integrity on refractionation, and

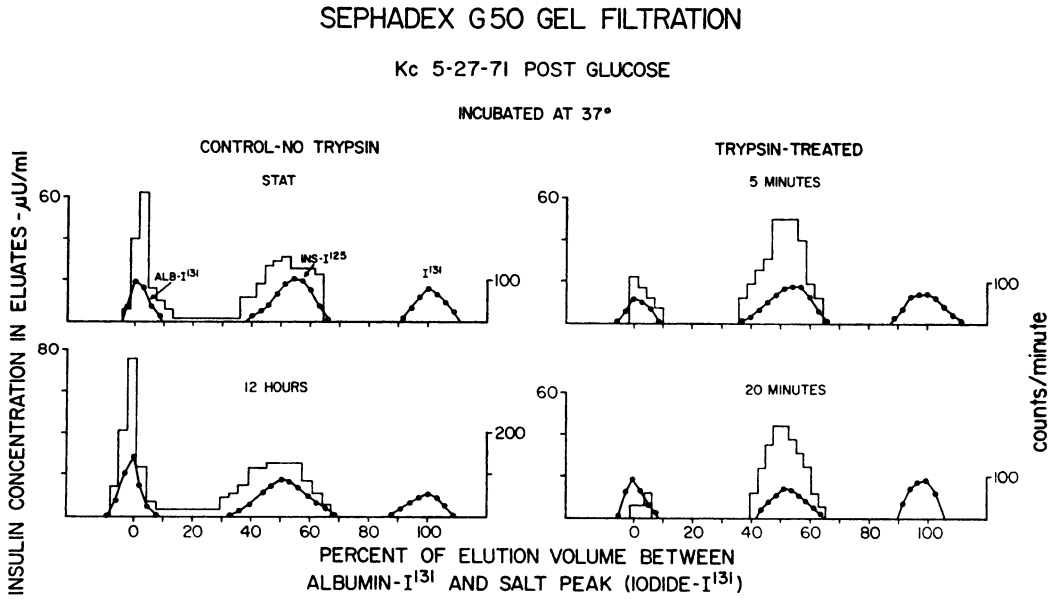

FIG. 11. Immunoreactive plasma insulin concentrations in Sephadex gel eluates of plasma of Kc incubated at 37°C in a 1:10 dilution in 0.05 M phosphate buffer (pH 7.4) without trypsin (left) and with trypsin (1 mg/ml). The "big, big insulin" peak comprised 38% and 41% of total immunoreactive insulin in the stat and 12-hr incubated controls, respectively. (From R. S. Yalow and S. A. Berson: Big, big insulin. Metabolism, 22: 703–713, 1973.)

was convertible to HG only on tryptic digestion (55). Both BG and HG are stimulated by feeding of PA patients, although BG disappears from plasma at a slower rate (55) (fig. 12). Similar components are present in extracts of antrum and proximal small bowel (16). Some antral extracts contain little or no BG but substantial amounts of this component are to be found in other antral extracts. BG becomes more and more prominent in relation to HG as one proceeds distally down the gastrointestinal tract (fig. 13). Gregory and Tracy (26) just recently have purified BG from Z-E tumors, have determined its amino acid composition, and have confirmed the properties which had been determined initially by radioimmunoassay of picogram to nanogram amounts of immunoreactive gastrin in plasma or tissue extracts containing a millionfold excess of other proteins. Unlike proinsulin which has low biological potency, the biological activity *in vivo* of BG is about equivalent to that of HG for infusion doses of equal immunoreactivity (45).

More recently we have described a still

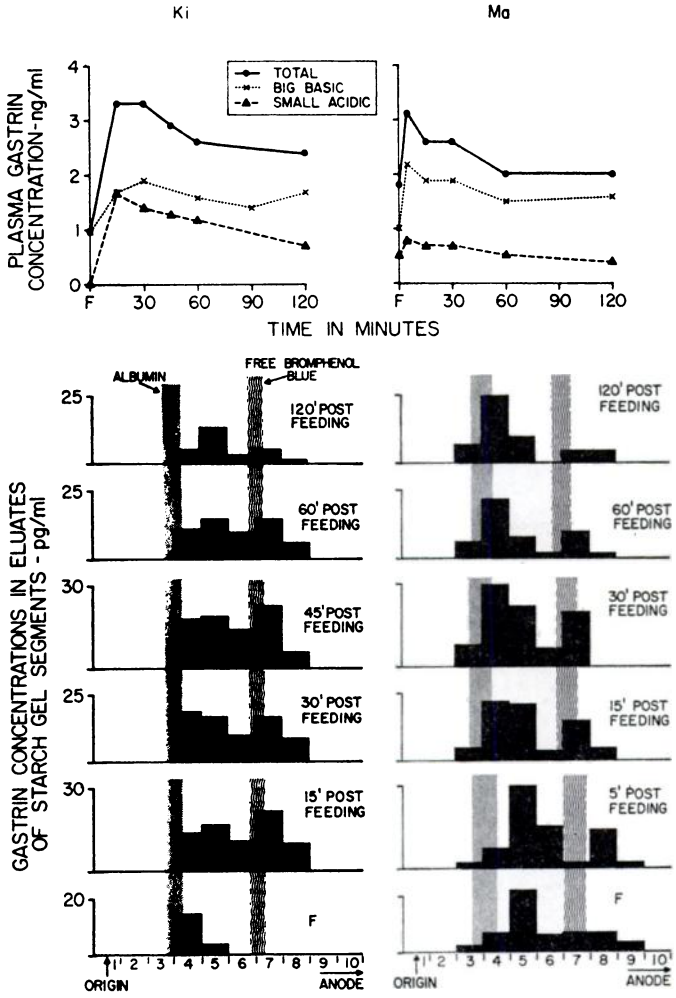

FIG. 12. Effects of feeding on concentrations of the immunoreactive plasma gastrin components fractionated on starch gel electrophoresis in 2 patients with pernicious anemia. (From R. S. Yalow and S. A. Berson: Further studies on the nature of immunoreactive gastrin in human plasma. Gastroenterology 60: 203-214, 1971.)

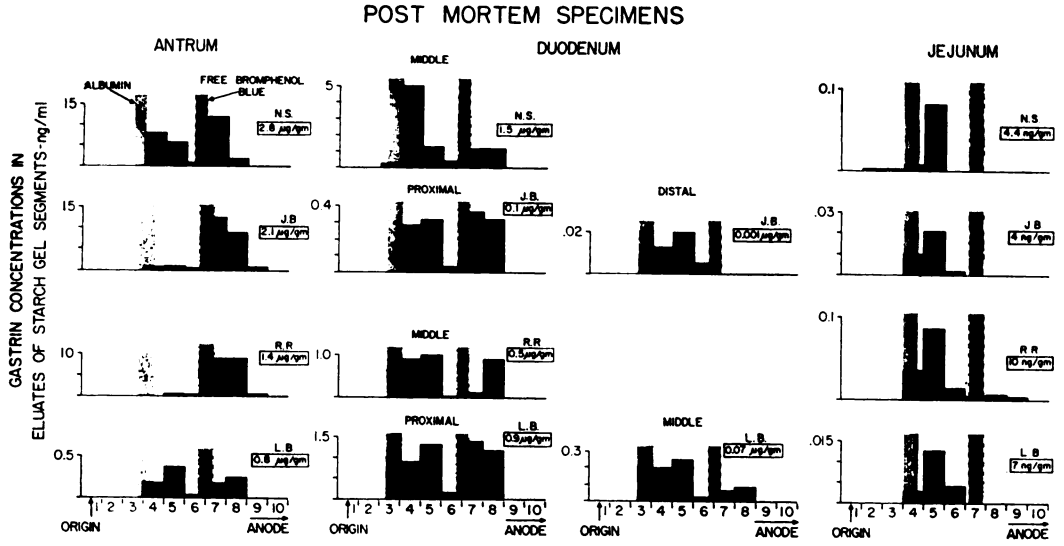

FIG. 13. Distribution of immunoreactive gastrin, on starch gel electrophoresis, in extracts of antrum, duodenum, and proximal jejunum in postmortem material. The total concentration of gastrin (boxed values) in the crude extract for each sample is expressed as micrograms or nanograms gastrin per g of mucosa. Since gel eluates from different gels were assayed at different dilutions only the relative abundance of the components in each gel is significant. (From S. A. Berson and R. S. Yalow: Nature of immuneractive gastrin extracted from tissues of gastrointestinal tract. Gastroenterology 60: 215–222, 1971).

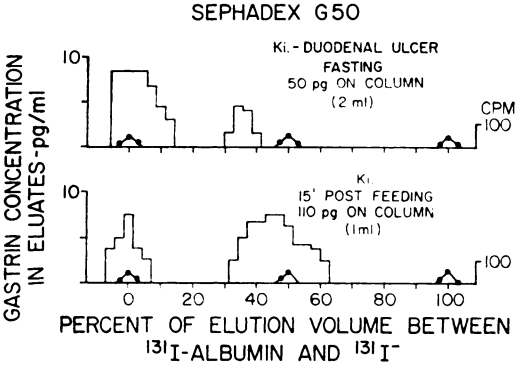

FIG. 14. Distribution of immunoreactive gastrin on Sephadex G-50 gel filtration in plasma of a human subject before and after feeding.

newer immunoreactive form of gastrin, "big, big" gastrin (BBG) (57, 60). This component elutes in the void volume on Sephadex G-50 gel filtration, has the same ultracentrifugal velocity as human growth hormone and an electrophoretic mobility on starch gel just in advance of serum albumin (60). It is immunochemically indistinguishable from HG and BG and is converted by tryptic digestion to smaller hormonal forms. Although BBG is only less than 2% of the total immunoreactivity in a Z-E tumor or in the plasma of patients with Z-E syndrome, PA, or who were gastrin hypersecretors for other reasons, it is a major fraction in normal human (fig. 14), canine, and porcine plasma in the nonstimulated state (60). Unlike BG and HG, BBG is not significantly stimulated by feeding (fig. 14). Whether BBG is the ultimate precursor of the gastrin family or whether or not it has significant biological potency has not been evaluated.

Stimulated by the evidence for the heterogeneity of several peptide hormones, we looked for and found in plasma and in extracts of pituitary and tumor a new form of ACTH, big ACTH (56, 59). This form is larger and more acidic than the well characterized 39 amino acid polypeptide (little ACTH) and is immunochemically indistinguishable from it with the antiserum employed. Big ACTH is without biological activity *in vitro* as measured by an adrenal cell dispersion method (40). However, after controlled tryptic digestion big ACTH is

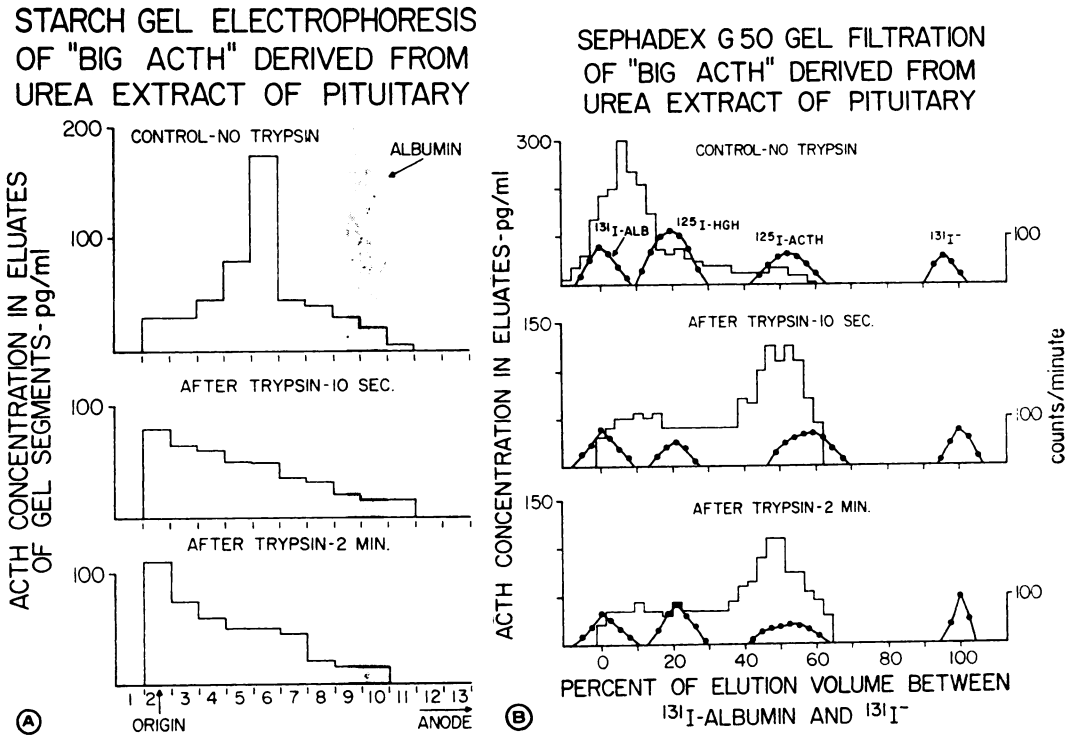

FIG. 15. A. Concentrations of immunoreactive ACTH in eluates from starch gel after electrophoresis of "big ACTH" component derived from Sephadex gel fractionation of the urea extract of a pituitary added to a 1:10 dilution of hormone-free plasma in 0.05 M phosphate buffer, pH 7.4 containing 0.5% mercaptoethanol and incubated without trypsin (top) and with trypsin (50 μg/ml) for 10 sec (middle) and 2 min (bottom).

B. Concentrations of immunoreactive ACTH in eluates from a Sephadex G-50 gel filtration of aliquots of same samples as in A. (From R. S. Yalow and S. A. Berson: Characteristics of "big ACTH" in human plasma and pituitary extracts. J. Clin. Endocrinol. Metab., 36: 415–423, 1973.

quantitatively converted to a product indistinguishable from authentic ACTH with respect to size and charge (fig. 15) (59) and with full biological potency (40). That controlled tryptic digestion can release a little ACTH-like moiety with full biological potency is consistent with a precursor role for big ACTH similar to the precursor relationship of proinsulin to insulin.

Summary and Conclusions

Competitive radioimmunoassay (RIA) is a general method for the determination of the concentration of virtually any substance which is or can be rendered immunogenic. The principle is simple. The concentration of an unknown unlabeled antigen is obtained by comparing its inhibitory effect on the binding of radioactively-labeled antigen to specific antibody with the inhibitory effect of known standards. Although up to now, the principal impact of RIA has been in the field of endocrinology, recent developments have demonstrated its applicability to fields as diverse as toxicology, virology, and cancer detection.

Problems encountered in RIA relate to the presence of non-hormonal factors which interfere in a non-specific fashion with the reaction of antigen with antibody and to the presence of cross reacting prohormones, molecular fragments, and related hormonal antigens which may alter the specificity of the immune reaction. However, the latter problem has been turned to an advantage in that the method has been instrumental

in broadening our understanding of the mechanisms involved in the synthesis and degradation of peptide hormones. It would appear that many, if not all, peptide hormones are synthesized in a "big" precursor form of low or no biological activity, followed by the enzymatic conversion to the smaller, better known biologically active form and then rapid degradation to peptide fragments or constituent amino acids or both. For none of these systems has there been developed a complete picture of the complex interrelationships among precursor, active form(s), and degradation products. Especially desirable is a model which correlates immunoreactivity and biological activity as measured in the intact animal and in receptor site assay. One can only be certain that radioimmunoassay will continue to be involved in both the questions and the answers which will be necessary for the solution of this problem, as it has been for so many others in medicine.

REFERENCES

1. Anderson, J. C., Barton, M. A., Gregory, R. A., Hardy, P. M., Kenner, G. W., MacLeod, J. K., Preston, J., Sheppard, R. C. and Morley, J. S.: Synthesis of gastrin. Nature (London) **204**: 933–934, 1964.
2. Arnaud, C. D., Sizemore, G. W., Oldham, S. B., Fischer, J. A., Tsao, H. S. and Littledike, E. T.: Human parathyroid hormone: glandular and secreted molecular species. Amer. J. Med. **50**: 630–638, 1971.
3. Arnaud, C. D., Tsao, H. S. and Oldham, S. B.: Native human parathyroid hormone: an immunochemical investigation. Proc. Nat. Acad. Sci. U.S.A. **67**: 415–422, 1970.
4. Beardwell, C. G.: Radioimmunoassay of arginine vasopressin in human plasma. J. Clin. Endocrinol. Metab. **33**: 254–260, 1971.
5. Berson, S. A.: *In* Resumé of Conference on Insulin Activity in Blood and Tissue Fluids, ed. by R. Levine, and E. Anderson, p. 7, National Institutes of Health, Bethesda, Md., May 9–10, 1957.
6. Berson, S. A. and Yalow, R. S.: Kinetics of reaction between insulin and insulin-binding antibody. J. Clin. Invest. **36**: 873, 1957.
7. Berson, S. A. and Yalow, R. S.: Isotopic tracers in the study of diabetes. Advan. Biol. Med. Phys. **6**: 349–430, 1958.
8. Berson, S. A. and Yalow, R. S.: Recent studies on insulin-binding antibodies. Ann. N. Y. Acad. Sci. **82**: 338–344, 1959.
9. Berson, S. A. and Yalow, R. S.: Quantitative aspects of reaction between insulin and insulin-binding antibody. J. Clin. Invest. **38**: 1996–2016, 1959.
10. Berson, S. A. and Yalow, R. S.: Immunochemical distinction between insulins with identical amino acid sequences from different mammalian species (pork and sperm whale insulins). Nature (London) **191**: 1392–1393, 1961.
11. Berson, S. A. and Yalow, R. S.: Insulin in blood and insulin antibodies. Amer. J. Med. **40**: 676–690, 1966.
12. Berson, S. A. and Yalow, R. S.: Iodoinsulin used to determine specific activity of iodine-131. Science **152**: 205–207, 1966.
13. Berson, S. A. and Yalow, R. S.: Principles of immunoassay of peptide hormones in plasma. *In* Clinical Endocrinology, ed. by E. B. Astwood and C. E. Cassidy, vol. II, pp. 699–720, Grune & Stratton, New York, 1967.
14. Berson, S. A. and Yalow, R. S.: Immunochemical heterogeneity of parathyroid hormone in plasma. J. Clin. Endocrinol. Metab. **28**: 1037–1047, 1968.
15. Berson, S. A. and Yalow, R. S.: Recent advances in immunoassay of peptide hormones in plasma. *In* Proceedings of International Diabetes Federation 6th Congress, Stockholm, Sweden, 1967, pp. 50–67, Excerpta Medica Foundation, 1969.
16. Berson, S. A. and Yalow, R. S.: Nature of immunoreactive gastrin extracted from tissues of gastrointestinal tract. Gastroenterology **60**: 215–222, 1971.
17. Canterbury, J. M. and Reiss, E.: Multiple immunoreactive molecular forms of parathyroid hormone in human serum. Proc. Soc. Exp. Biol. Med. **140**: 1393–1398, 1972.
18. Cretien, M.: Lipotropins. *In* Methods in Investigative and Diagnostic Endocrinology, Part II: Pituitary Hormone and Hypothalamic Releasing Factors, ed. by S. A. Berson and R. S. Yalow, ch. 9, pp. 617–632, North-Holland Publishing Co., 1973.
19. Erlanger, B. F. and Beiser, S.: Antibodies specific for ribonucleosides and ribonucleotides and their reaction with DNA. Proc. Nat. Acad. Sci. U.S.A. **52**: 68–74, 1964.
20. Givol, D.: Procedures for sensitive immunoassay. *In* In Vitro Procedures with Radioisotopes in Medicine, pp. 515–524, STI/Pub/237, International Atomic Energy Agency, Vienna, 1970.
21. Glick, S. M., Roth, J., Yalow, R. S. and Berson, S. A.: The regulation of growth hormone secretion. Recent Progr. Hormone Res. **21**: 241–283, 1965.
22. Goodfriend, T. L., Levine, L. and Fasman, G. D.: Antibodies to bradykinin and angiotensin: a use of carbodiimides in immunology. Science **144**: 1344–1346, 1964.
23. Greenwood, F. C., Hunter, W. M. and Klopper, A.: Assay of human growth hormone in pregnancy at parturition and in lactation. Detection of a growth-hormone-like substance from the placenta. Brit. Med. J. **1**: 22–24, 1964.
24. Gregory, H., Hardy, P. M., Jones, D. S., Kenner, G. W. and Sheppard, R. C.: The antral hormone gastrin. Structure of gastrin. Nature (London) **204**: 931–933, 1964.
25. Gregory, R. A. and Tracy, H. J.: The constitution and properties of two gastrins extracted from hog antral mucosa. I. The isolation of two gastrins from hog antral mucosa. Gut **5**: 103–117, 1964.
26. Gregory, R. A. and Tracy, H. J.: Isolation of two "big gastrins" from Zollinger-Ellison tumour tissue. Lancet **2**: 797–799, 1972.
27. Habener, J. F., Powell, D., Murray, T. M., Mayer, G. P. and Potts, J. T., Jr.: Parathyroid hormone: secretion and metabolism in vivo. Proc. Nat. Acad. Sci. U.S.A. **68**: 2986–2991, 1971.
28. Harris, J. I., Sanger, F. and Naughton, M. A.: Species differences in insulin. Arch. Biochem. **65**: 427–437, 1956.
29. Imura, H., Sparks, L. L., Grodsky, G. M. and Forsham, P. H.: Immunologic studies of adrenocorticotropic hormone (ACTH): dissociation of biologic and immunologic activities. J. Clin. Endocrinol. Metab. **25**: 1361–1369, 1965.
30. Ishihara, Y., Saito, T., Ito, Y. and Fujino, M.: Structure of sperm- and sei-whale insulins and their breakdown by whale pepsin. Nature (London) **181**: 1468–1469, 1958.
31. Lefkowitz, R. J., Roth, J., Pricer, W. and Pastan, I.: ACTH receptors in the adrenal: specific binding of ACTH ^{125}I and its relation to adenylcyclase. Proc. Nat. Acad. Sci. U.S.A. **75**: 745–752, 1970.
32. Murphy, B. E. P., Engelberg, W. and Pattee, C. J.: Simple method for the determination of plasma corticoids. J. Clin. Endocrinol Metab. **23**: 293–300, 1963.
33. Peterson, J. D., Nehrlich, S., Oyer, P. E. and Steiner, D. F.: Determination of the amino acid sequence of the monkey, sheep, and dog proinsulin C-peptides by a semimicro Edman degradation procedure. J. Biol. Chem. **247**: 4866–4871, 1972.
34. Richards, F. M. and Knowles, J. R.: Glutaraldehyde as a

protein cross-linking reagent (a letter to the editor) J. Mol. Biol. 37: 231–233, 1968.

35. Roth, J., Glick, S. M., Klein, L. A. and Petersen, M. J.: Specific antibody to vasopressin in man; preliminary communication. J. Clin. Endocrinol. Metab. 26: 671–675, 1966.
36. Roth, J., Gorden, P. and Pastan, I.: "Big insulin": a new component of plasma insulin detected by immunoassay. Proc. Nat. Acad. Sci. U.S.A. 61: 138–145, 1968.
37. Roth, J., Klein, L. A. and Petersen, M. J.: Vasopressin antibodies and a sensitive radioimmunoassay. J. Clin. Invest. 45: 1064, 1966.
38. Rothenberg, S. P.: A radio-enzymatic assay for folic acid. Nature (London) 206: 1154–1156, 1965.
39. Rubenstein, A. H., Cho, S. and Steiner, D. F.: Evidence for proinsulin in human urine and serum. Lancet 1: 1353–1355, 1968.
40. Schneider, B., Gewirtz, G., Krieger, D. and Yalow, R. S.: Big ACTH: conversion to biologically active ACTH by trypsin. Fifty-fifth Meeting of the Endocrine Society, Chicago, 1973.
41. Sherwood, L. M., Lundberg, W. B., Jr., Targovnik, J. H., Rodman, J. S. and Seyfer, A.: Synthesis and secretion of parathyroid hormone in vitro. Amer. J. Med. 50: 658–669, 1971.
41a. Silverman, R. and Yalow, R. S.: Heterogeneity of parathyroid hormone: clinical and physiologic implications. J. Clin. Invest., 1973, in press.
42. Smith, L. F.: Cited by F. G. Young, *in* Experimental Research on Diabetes Mellitus. Brit. Med. J. 2: 1449–1454, 1961.
43. Steiner, D. F., Clark, J. L., Noland, C., Rubenstein, A. H., Margoliash, E., Aten, B. and Oyer, P. E.: Proinsulin and the biosynthesis of insulin. Recent Progr. Hormone Res. 25: 207–282, 1969.
44. Talamo, R. C., Haber, E. and Austen, K. F.: Antibody to bradykinin: effect of carrier and method of coupling on specificity and affinity. J. Immunol. 101: 333–341, 1968.
45. Walsh, J. H., Debas, H. T. and Grossman, M. I.: Pure natural human big gastrin: biological activity and half life in dog (abstract). Gastroenterology, 1973, in press.
46. Yalow, R. S. and Berson, S. A.: Assay of plasma insulin in human subjects by immunological methods. Nature (London) 184: 1648–1649, 1959.
47. Yalow, R. S. and Berson, S. A.: Immunoassay of endogenous plasma insulin in man. J. Clin. Invest. 39: 1157–1175, 1960.
48. Yalow, R. S. and Berson, S. A.: Immunologic aspects of insulin. Amer. J. Med. 31: 882–891, 1961.
49. Yalow, R. S. and Berson, S. A.: Reaction of fish insulins with human insulin antiserums: potential value in the treatment of insulin resistance. N. Engl. J. Med. 270: 1171–1178, 1964.
50. Yalow, R. S. and Berson, S. A.: Labelling of proteins—problems and practices. Trans. N. Y. Acad. Sci. 28: 1033–1044 1966.
51. Yalow, R. S. and Berson, S. A.: General aspects of radioimmunoassay procedures. *In* In Vitro Procedures with Radioisotopes in Medicine, pp. 455–481, STI/Pub/237, International Atomic Energy Agency, Vienna, 1970.
52. Yalow, R. S. and Berson, S. A.: Dynamics of insulin secretion in early diabetes in humans. *In* Early Diabetes, Suppl. 1 to Advances in Metabolic Disorders, Proceedings of the First International Symposium, Oct. 23–26, 1968, ed. by R. A. Camerine-Davalos and H. S. Cole, pp. 95–103, Academic Press, New York, 1970.
53. Yalow, R. S. and Berson, S. A.: Fundamental principles of radioimmunoassay techniques in measurement of hormones. Proceedings of VII Pan-American Congress of Endocrinology, Sao Paulo, Brazil, August 16–21, 1970, Excerpta Medica International Congress Series No. 238, pp. 16–34.
54. Yalow, R. S. and Berson, S. A.: Size and charge distinctions between endogenous human plasma gastrin in peripheral blood and heptadecapeptide gastrins. Gastroenterology 58: 609–615, 1970.
55. Yalow, R. S. and Berson, S. A.: Further studies on the nature of immunoreactive gastrin in human plasma. Gastroenterology 60: 203–214, 1971.
56. Yalow, R. S. and Berson, S. A.: Size heterogeneity of immunoreactive human ACTH in plasma and in extracts of pituitary glands and ACTH-producing thymoma. Biochem. Biophys. Res. Commun. 44: 439–445, 1971.
57. Yalow, R. S. and Berson, S. A.: And now, "big, big" gastrin. Biochem. Biophys. Res. Commun. 48: 391–395, 1972.
58. Yalow, R. S. and Berson, S. A.: Big, big insulin. Metabolism 22: 703–713, 1973.
59. Yalow, R. S. and Berson, S. A.: Characteristics of "big ACTH" in human plasma and pituitary extracts. J. Clin. Endocrinol. Metab. 36: 415–423, 1973.
60. Yalow, R. S. and Wu, M.: Further studies on the nature of big, big gastrin. Gastroenterology, 1973, in press.