RADIOIMMUNOASSAY: ITS PAST, PRESENT, AND POTENTIAL

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I am appreciative of the honor bestowed on me by receiving the Anachem Award for distinguished service to analytical chemistry. It is perhaps worth noting that this is the first time that the award has been presented for investigations in bioanalytic methodology and the first time that the recipient was trained as a nuclear physicist. In these days when there is a tendency for increasing support for contract research, for crash programs for instant solutions to problems such as cancer or the energy crisis, it is important to remember that while these programs are designed to exploit most efficiently previous scientific breakthroughs, revolutionary ideas continue to arise unpredictably from individual scientists or small groups and the benefits derived therefrom may well transcend the borders of any particular scientific discipline.

Today I would like to share with you the history of the development of radioimmunoassay and to discuss some aspects of the applicability of this bioanalytical tool in clinical medicine, physiology and biochemistry.

In the early 1950's the late Dr. Solomon A. Berson and I began our investigative careers in the application of radioisotopes in thyroid physiology and diagnosis and in blood volume determinations with radioisotope-labeled red cells and serum proteins. We soon turned our attention to the in-vivo distribution and metabolism of ^{131}I-labeled proteins (1-3) and, from this, extension to studies with ^{131}I-labeled pep-

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tidal hormones appeared promising.

At that time insulin was the hormone most readily available in a highly purified form. Dr. I. Arthur Mirsky had just proposed a hypothesis (4) that maturity-onset diabetes might not be due to a deficiency of insulin secretion but rather to abnormally rapid degradation of insulin by hepatic insulinase. We therefore began to investigate the distribution and metabolism of ^{131}I-labeled insulin administered intravenously to non-diabetic and diabetic subjects (5). Contrary to the predictions of the Mirsky hypothesis, we observed that radioactive insulin generally disappeared more slowly from the plasma of diabetic subjects than from the plasma of non-diabetic controls (Fig. 1).

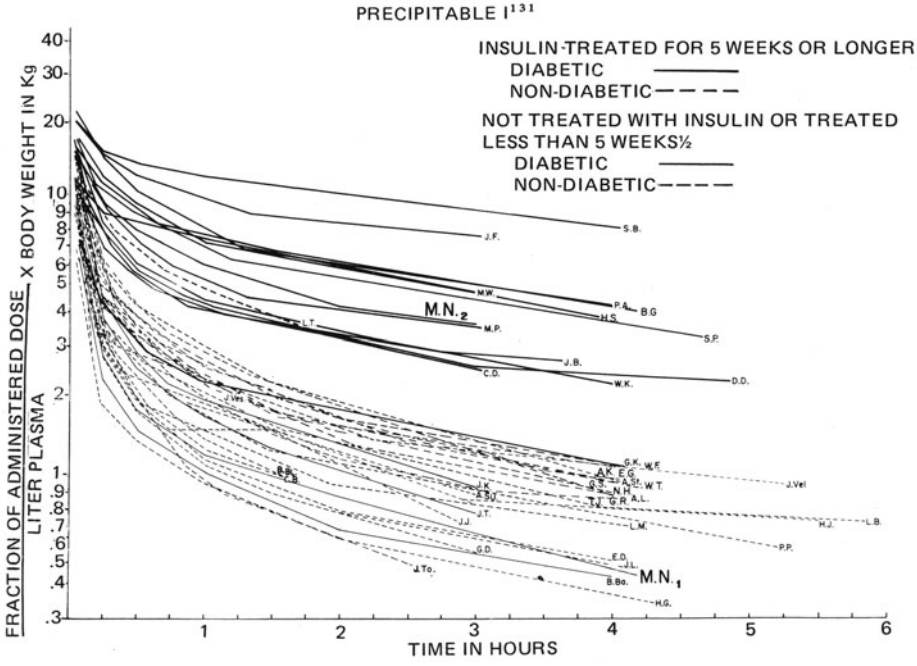

Fig. 1 - Trichloracetic acid precipitable radioactivity in plasma as a function of time following intravenous administration of ^{131}I-insulin in diabetic and non-diabetic subjects. M. N.1 - after treatment with insulin for 2 1/2 weeks; M. N.2 - after treatment with insulin for 4 1/2 months. (Reproduced from ref. 5)

However, one should remember that these studies were performed in the early 1950's, before the era of oral hypoglycemic agents, such as the sulfonylureas, and that most diabetic subjects had received insulin therapy. We soon appreciated that the difference between the two groups was not due to diabetes per se but rather that those patients from whose plasma the insulin disappeared slowly had a previous history of insulin therapy.

We suspected that the retarded rate of insulin disappearance was caused by binding of labeled insulin to insulin antibodies which had developed in response to administration of exogenous insulin. However classic immunologic techniques were not adequate for the detection of the low concentrations of antibody presumed to be present. We therefore developed new and highly sensitive techniques employing radioiodine-labeled insulin for the demonstration of binding to antibody in vitro. These included paper electrophoresis and chromato-electrophoresis (Fig. 2). In common with many other peptidal

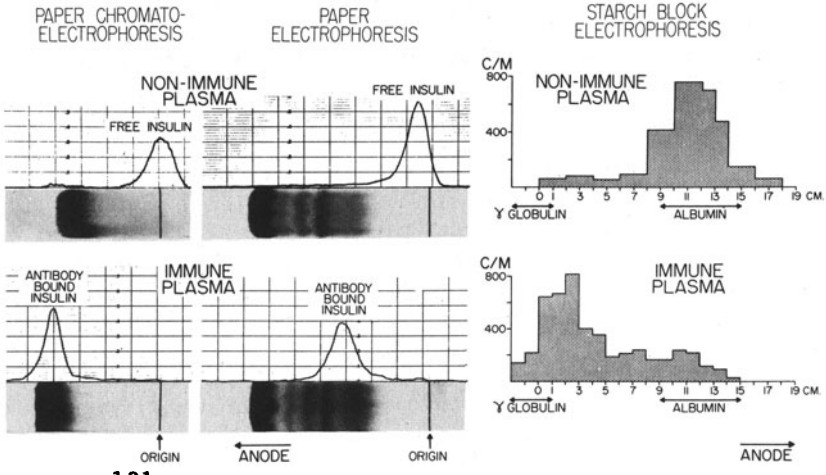

Fig. 2 - ^{131}I-insulin was added to the plasmas of insulin-treated (bottom) and untreated (top) human subjects and the mixtures were applied to a starch block (right) or to paper strips (middle) for electrophoresis or to paper strips for hydrodynamic flow chromatography combined with electrophoresis (left). After completion of electrophoresis, segments were cut out of the starch block for assay of radioactivity and the paper strips were assayed in an automatic strip counter. (Reproduced from Berson and Yalow: The Harvey Lectures, Series 62, Acad. Press, N.Y. 1968, p. 107.)

hormones, insulin in its free state tends to absorb firmly to paper. In contrast, it does not do so when bound to antibody. Therefore, if an incubated mixture of labeled insulin and plasma is applied to a strip of filter paper for electrophoresis or chromatoelectrophoresis, the labeled insulin in the plasmas of untreated subjects remains at the site of application, i.e., the origin, whereas, in the plasma of treated patients in whom antibody had developed, the insulin bound to the antibody moves between the gamma and the beta globulins. On chromatoelectrophoresis, the serum proteins do not separate significantly from each other, but, within a matter of only 20-30 minutes, all of the serum proteins carrying the insulin-antibody complexes have migrated away from the origin. On starch block electrophoresis, labeled insulin in the immune plasma remains with the inter β-γ globulins close to site of application and the free insulin which is not absorbed to the starch has an electrophoretic mobility almost that of albumin (Fig. 2). During ultracentrifugation, labeled insulin, having a molecular weight of about 6000, sedimented more slowly than serum albumin in the plasma of untreated subjects, but sedimented with the globulins in the plasma of insulin-treated subjects (5). Binding of insulin to γ-globulin was demonstrable on salt fractionation as well (5).

The demonstration of the ubiquitous presence of insulin-binding antibodies in insulin-treated subjects was not readily acceptable to the scientific community 20 years ago. It is perhaps worth noting that the original paper describing these finding was rejected by Science. Although the paper was eventually accepted by the Journal of Clinical Investigation we were not permitted to use the term 'insulin antibody' in the title of the paper (5). In the paper itself we were required to document our conclusion that the insulin-binding globulin was indeed an antibody by referring to the definition of antibody given in a textbook of bacteriology and immunity (6).

In this early work (5) we also observed that the binding of labeled insulin is a quantitative function of the amount of insulin present when the antibody concentration is kept fixed. It was this observation that provided the basis of the radioimmunoassay of plasma insulin. However several years were to pass from the presentation of the immunoassay principle (5, 7) to its practical application to the measurement of plasma insulin in man. During that

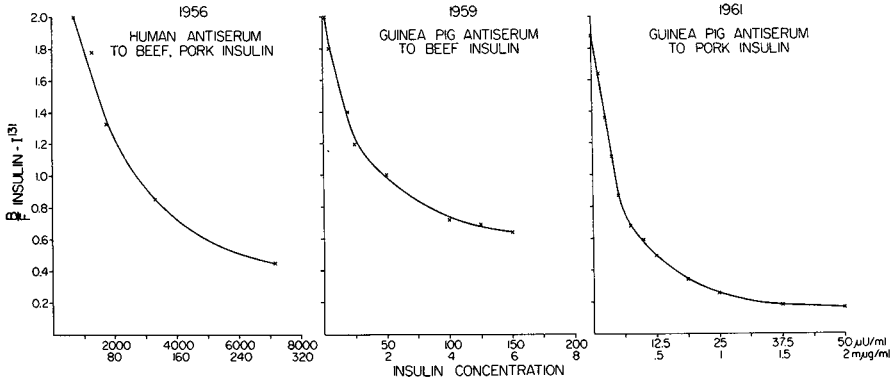

Fig. 3 - Standard curves for the measurement of insulin during the period 1956 to 1961. The human antiserum (left) could be used to detect 1000 µU beef insulin/ml. More than a 10 fold higher concentration of human insulin were required to effect the same reduction in B/F. The guinea pig antiserum to beef insulin (middle) could be used to detect 10 µU human insulin/ml. The guinea pig antiserum to pork insulin (right) had a 5-10 fold greater sensitivity for the detection of human insulin. This improvement in sensitivity was required to make radioimmunoassay of human plasma insulin possible since the average insulin concentration after an overnight fast is less than 20 µU/ml.

time we studied the quantitative aspects of the reaction between insulin and antibody (8), evaluated the species-specificity of the available antisera (9) and measured the disappearance of exogenous beef insulin administered to rabbits (10). These investigations provided the theoretical and experimental basis for the measurement of insulin in unextracted human plasma (11, 12). As shown in Fig. 3, the

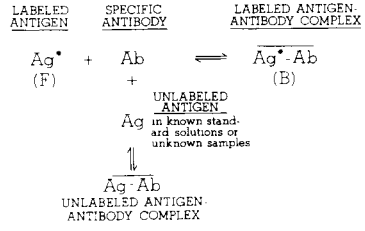

Fig. 4 - Competing reactions that form the basis of the radioimmunoassay.

PEPTIDE HORMONES

- PITUITARY HORMONES
  - Growth hormone
  - Adrenocorticotropic hormone (ACTH)
  - Melanocyte stimulating hormone (MSH)
    - α-MSH
    - β-MSH
  - Glycoproteins
    - Thyroid stimulating hormone (TSH)
    - Follicle stimulating hormone (FSH)
    - Luteinizing hormone (LH)
  - Prolactin
  - Lipotropin (LPH)
  - Vasopressin
  - Oxytocin
- CHORIONIC HORMONES
  - Human chorionic gonadotropin (HCG)
  - Human chorionic somatomammotropin (HCS)
- PANCREATIC HORMONES
  - Insulin
  - Proinsulin
  - C-peptide
  - Glucagon
- CALCITROPIC HORMONES
  - Parathyroid hormone (PTH)
  - Calcitonin (CT)
- GASTROINTESTINAL HORMONES
  - Gastrin
  - Secretin
  - Cholecystokinin-pancreozymin (CCK-PZ)
  - Enteroglucagon
  - Vasoactive intestinal polypeptide (VIP)
  - Gastric inhibitory polypeptide (GIP)
- VASOACTIVE TISSUE HORMONES
  - Angiotensins
  - Bradykinins
- HYPOTHALAMIC RELEASING FACTORS
  - Thyrotropin releasing factor (TRF)
  - Gonadotropin releasing factor (GnRF)

NON-PEPTIDAL HORMONES

- THYROIDAL HORMONES
  - Triiodothyronine
  - Thyroxine
- PROSTAGLANDINS
- STEROIDS
  - Aldosterone
  - Corticosteroids
  - Estrogens
  - Androgens
  - Progesterones

NON-HORMONAL SUBSTANCES

- DRUGS
  - Digoxin
    - Digitoxin
  - Morphine
  - LSD
  - Barbiturates
- CYCLIC NUCLEOTIDES
  - cAMP
  - cGMP
  - cIMP
  - cUMP
- ENZYMES
  - C_1 esterase
  - Fructose 1,6 diphosphatase
- VIRUS
  - Australia antigen (Hepatitis B antigen)
- TUMOR ANTIGENS
  - Carcinoembryonic antigen
  - α-Fetoprotein
- SERUM PROTEINS
  - Thyroxin binding globulin
  - IgG, IgE
  - Properdin
  - Anti-Rh antibodies
- OTHER
  - Intrinsic factor
  - Rheumatoid factor
  - Folic acid
  - Neurophysin
  - Staphylococcal
    - β-Enterotoxin

Fig. 5 - Partial listing of peptidal and non-peptidal hormones and other substances measured by radioimmunoassay.

principal improvement in sensitivity for the detection of human insulin was made possible by the use of guinea pig anti-bovine insulin sera instead of human anti-beef, pork insulin sera (13). Further improvement in sensitivity was effected by the use of guinea pig anti-porcine insulin sera (14).

Radioimmunoassay is simple in principle. It is summarized in the competing reactions shown in Fig. 4. The concentration of the 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. The validity of a radioimmunoassay procedure is dependent on identical immunologic behavior of antigen in unknown samples with the antigen in known standards. There is no requirement for identical immunologic or biologic behavior of labeled and unlabeled antigen. Furthermore, as will be considered in some detail later, there is no requirement for standards and unknowns to be identical chemically or to have identical biologic activity. An incomplete listing of substances measured by radioimmunoassay is given in Fig. 5. The rapid rate of growth in this field precludes continuous updating of the listing.

The radioimmunoassay principle can be generalized and extended to non-immune systems to which a more general set of equations (Fig 6) and a more general term, "competitive radioassay" can be applied. Even this term is not completely satisfactory since the principle can be applied in situations in which the marker is other than a radioisotopic tracer. Using competitive radioassay Rothenberg (15) and Barakat and Ekins (16) independently developed assays for serum vitamin B_{12} using intrinsic factor as the specific reactor. Ekins

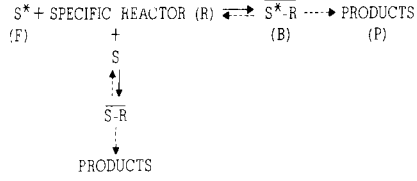

Fig. 6 - Application of competitive radioassay principle to physical or chemical reactions in non-immune systems. Degree of competitive reactions may be determined from changes in concentration of labeled free substrate (S*) or substrate-reactor complex (S*-R) or, as in the case of enzymes, inhibition of product formation.

first reported on the measurement of thyroxine in human plasma using thyroxine-binding globulin as the specific reactor (17). Further development of the thyroxine assay and the application of competitive radioassay to the measurement of plasma cortisol using cortisol binding globulin were carried out by Murphy (18). More recently there has been extensive application of the principle to the measurements of peptidal and non-peptidal hormones using tissue receptor sites as specific reactors (See 19 for review).

Dr. Rothenberg reviews his recent studies with competitive radioassay in a subsequent paper in this symposium.

Before considering more recent work from our laboratory involving radioimmunoassay, let us return to Dr. Mirsky's hypothesis. The first application of the radioimmunoassay of plasma insulin in man was for the determination of the plasma insulin response to a standard oral glucose tolerance test (100 g glucose) in non-diabetic and in adult mild maturity-onset diabetic subjects (Fig. 7) (12). An unexpected

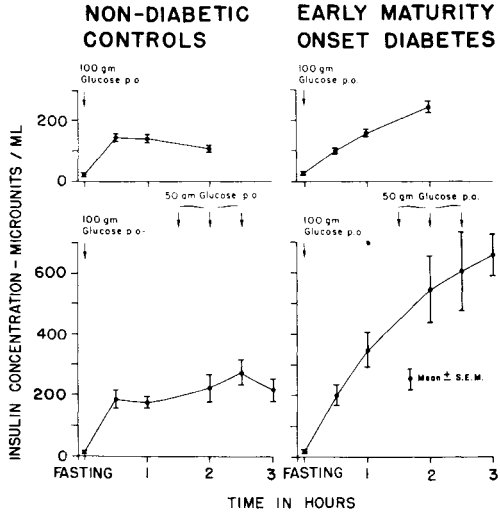

Fig. 7 - (Top) Mean plasma insulin concentration before and during a standard 100 gm oral glucose tolerance test in subjects never treated with insulin (30 non-diabetic subjects and 38 diabetic subjects). (Bottom) Same as above except that additional loads of 50 gm glucose were administered during the test at 1 1/2, 2 and 2 1/2 hours. (4 diabetic subjects and 5 non-diabetic subjects). (Reproduced from Yalow and Berson: Diabetes 10:339, 1961)

discovery was that although the non-diabetic subjects showed, on the average, a more brisk early insulin-secretory response, most of the diabetic patients revealed a capacity to secrete even greater amounts of insulin than would have been sufficient to restore the non-diabetic subjects rapidly to a euglycemic state. That the response of the diabetic in these instances does not represent maximal stimulation of islet tissue is indicated by the very much higher plasma insulin levels that can be achieved in these patients under the stimulus of further heavier glucose loading (Fig. 7) (12). From these investigations we concluded that in the mild maturity-onset diabetic subject absolute insulin deficiency per se is not the cause of hyperglycemia but rather that there is some inherent reason for the reduced responsiveness of the diabetic to apparently adequate amounts of plasma insulin. Now some 14 years later the problem has still not been resolved although, using radioimmunoassay, it is well appreciated that insulin-insensitivity can be associated not only with diabetes but can be induced by obesity, fasting, etc.

Radioimmunoassay has proven useful not only for studies of the physiologic regulation of hormonal secretion, one example of which has just been given, but also in the diagnosis of diseases associated with hormonal excess or hormonal deficiency. Proper interpretation of hormone measurements in these situations requires a clear understanding of the factors involved in the regulation of hormonal secretion. Generally hormonal secretion is stimulated by some departure from the state of biologic "homeostasis" that the hormone is designed to modulate. A model for one such system is shown in Fig. 8. Regulation is effected through the opera-

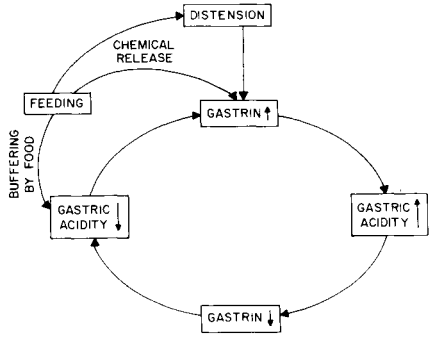

Fig. 8 - Feed-back control loop for gastrin regulation of gastric acidity : effect of feeding.

tion of a feed-back control loop which contains the hormone at one terminus and, at the other, the substance which it regulates and by which it is in turn regulated. Gastrin secretion increases gastric acidity, which in turn suppresses secretion of antral gastrin. Modulation of this system can be effected by a number of factors, perhaps the most important of which is feeding. Feeding promotes gastrin release directly through a chemical effect on antral cells and indirectly through gastric distension and through the buffering action of food (Fig. 8). In Fig. 9 are compared gastrin concentrations in normal subjects, in patients with pernicious anemia (PA) and in patients with Zollinger-Ellison (Z-E) syndrome, who have gastrin-producing tumors. Z-E patients have a very high acid secretion. In these patients the high level of plasma gastrin is inappropriate and demonstrates the failure of the feed-back mechanism either because the tumor is autonomous or because it is generally not located in the stomach and is therefore not bathed by the acid secretion (20). Patients with pernicious anemia have gastric

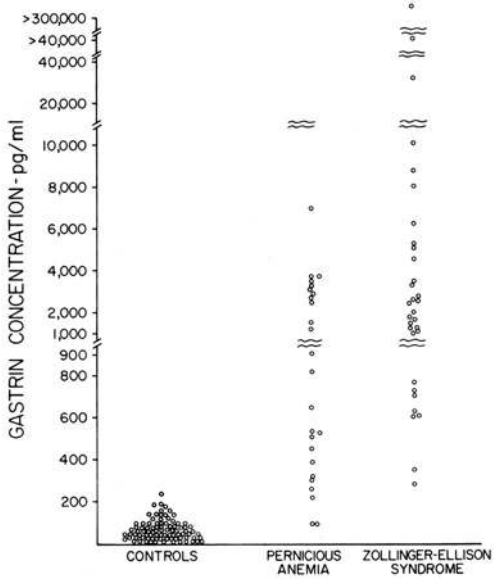

Fig. 9 - Basal plasma gastrin concentrations in normal subjects and in patients with pernicious anemia and Zollinger-Ellison syndrome. (Reproduced from Berson and Yalow: Rhode Island Med. J. 54:501, 1971)

hypoacidity. Since gastric hydrochloric acid normally suppresses gastrin secretion, the continued absence of acid and the repeated stimulation by feeding eventually produces secondary hyperplasia of gastrin-producing cells. The high level of plasma gastrin in pernicious anemia patients is quite appropriate in view of the absence of the inhibitory effect of HCl on the secretion of antral gastrin (20). In pernicious anemia patients the introduction of HCl into the stomach provokes a precipitous fall in plasma gastrin (20).

The radioimmunoassay of plasma gastrin in patients with marked hyperchlorhydria generally provides a valuable diagnostic test for Z-E syndrome since the values in this condition are usually much greater than in the normal or in the usual patient with duodenal ulcer. However over the past several years we have encountered a group of patients with a syndrome we have termed "hypergastrinemic hyperchlorhydria", which resembles Z-E syndrome but which can be distinguished from it. In this syndrome, the plasma gastrin levels are quite high and overlap with those of Z-E patients, the patients may or may not suffer from peptic ulcer and have rates of acid secretion which are generally only modestly elevated and within the range of that found in duodenal ulcer patients rather than in the much higher Z-E range. This syndrome appears to rise from overactivity of the gastrin-secreting cells of the gastrointestinal tract and, as a result, patients with this syndrome manifest an excellent gastrin-secretory response to feeding (Fig. 10) (21). In contrast, the Z-E patient shows no significant elevation of plasma gastrin on feeding (Fig. 10) probably because the long-standing hyperchlorhydria suppresses the gastrin-secreting cells of the antral and duodenal mucosa and because the tumor itself is not in the gastrointestinal tract and is not in contact with food.

Thus in the application of radioimmunoassay to problems of hormonal hypo- or hypersecretion we seldom should rely on a single determination of plasma hormone. Generally, to test for deficiency states, plasma hormonal concentrations should be measured not only in the basal state but also following administration of appropriate physiologic or pharmacologic stimuli. When hypersecretion is suspected and high hormonal levels are observed, one must determine whether the hormonal level is appropriate or inappropriate and whether the hormonal secretion is autonomous or can be modulated by appropriate physiologic or pharmacologic agents. Requirements

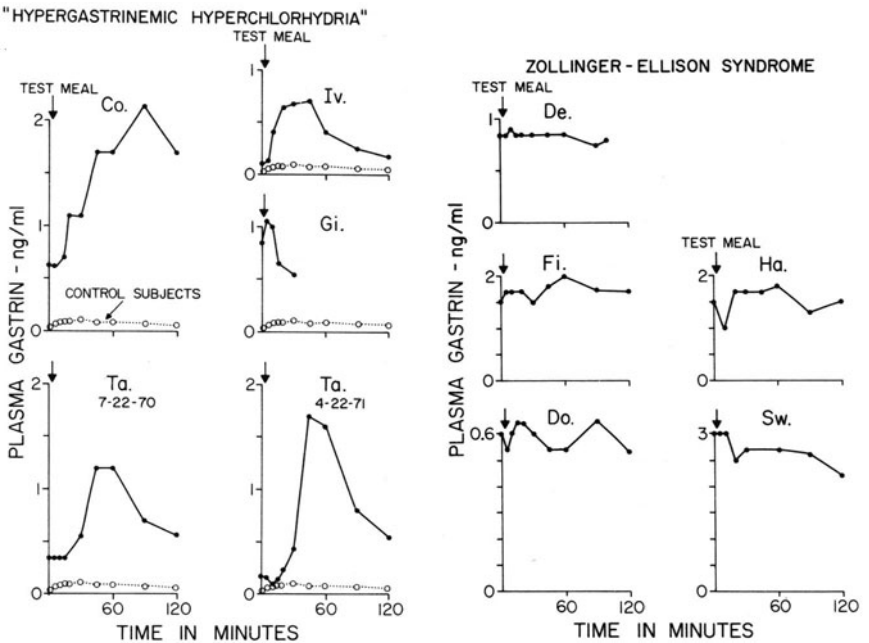

Fig. 10 - Plasma gastrin concentrations in basal state and following test meal (4 oz. orange juice, 2 eggs, 1 piece dry toast): (Left) In gastrin hypersecretors with hyperchlorhydria. Co, Gi and Ta have had duodenal ulcers. Iv has carried a diagnosis of peptic esophagitis but has had neither a typical ulcer history nor roentgen evidence of ulcer; (Right) In patients with proven Zollinger-Ellison syndrome. (Reproduced from ref. 21)

for absolute precision in the radioimmunoassay of peptidal hormones are much less stringent than in the usual clinical laboratory determinations of plasma concentrations of electrolytes, serum proteins or even of steroidal hormones. For instance the normal serum sodium concentrations have a range from 135 to 145 mEq/l; thus the entire normal range has a variation of less than 10% so that an error in the experimental determination of 10% would be intolerable. In the fasting state in normal subjects gastrin concentrations may range from undetectable (<5 pg/ml) to 100 pg/ml and values greater than a thousand-fold higher have been measured in Z-E patients. Diagnostic studies are frequently made not on the basis of a single hormonal concentration but rather on dynamic changes in response to provocative stimuli and suppressants. Therefore it is seldom necessary to have absolute precision of determination to better than 10% and, under some circumstances, errors of considerably greater magnitude are tolerable. When radioimmunoassay is applied to measurement of substances of biologic interest other than the peptidal hormones greater precision may be required and is usually readily achievable.

Over the past decade radioimmunoassay has had its principal impact on endocrinology. Not only has it made possible increased accuracy in the diagnosis of pathologic states characterized by hormonal excess or deficiency but it has been the major tool used in investigations concerned with the regulation of hormonal secretion, with the interrelationships between hormones and with our understanding of hormonal physiology in general. More recently radioimmunoassay has led to the discovery in plasma and tissue of new forms, presumably precursors or products of the commonly recognized peptidal hormones. It is to some aspects of this problem that the remaining portion of this presentation will be directed.

As discussed earlier, the validity of a radioimmunoassay procedure requires that the immunochemical behavior of standards and unknowns be identical. This condition can be tested by making multiple dilutions of an unknown sample and determining whether the curve of competitive inhibition of binding is superposable on the standard curve used for assay. Failure to meet this condition precludes a truly quantitative measurement. Immunochemical heterogeneity was first demonstrated in 1968 when studies from our laboratory established that there were striking immunochemical differences between human parathyroid hormone (hPTH) in plasma and in glandular

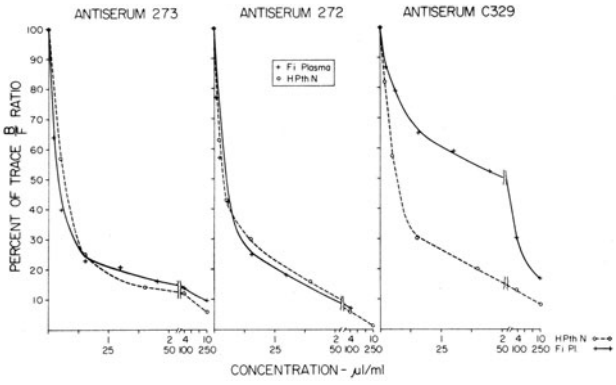

Fig. 11 - Inhibition of binding of ^{125}I-Bpth in three antisera by pooled plasma from a patient with 2° hyperparathyroidism (+) and by extract of a normal parathyroid gland (°). (Reproduced from ref. 22)

extracts (22). Thus while a single factor could be used to superimpose a plasma dilution curve on a curve of standards obtained from a normal parathyroid gland for two antisera, 272 and 273, the same factor resulted in discrepant results with another antiserum, C329 (Fig. 11) (22). Furthermore the rate

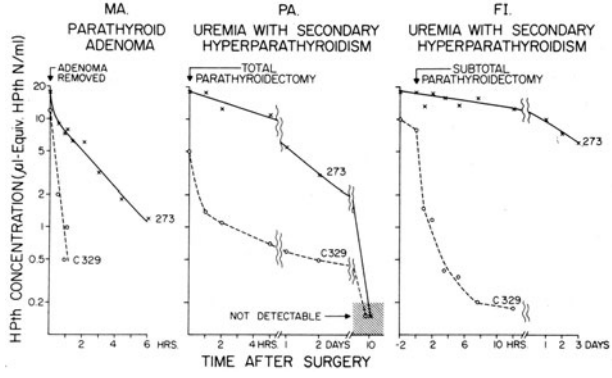

Fig. 12 - Disappearance of immunoreactive parathyroid hormone from plasma following parathyroidectomy in patients with 1° or 2° hyperparathyroidism. Plasma samples were assayed in antiserum C329 and antiserum 273 using extract of a normal human parathyroid gland (hPTH N) as standard and ^{125}I-bPTH as tracer. (Reproduced from ref. 22)

of disappearance of immunoreactivity following parathyroidectomy appeared to depend on the antiserum used for assay (Fig. 12) (22). The heterogeneity of plasma PTH has been widely confirmed by other workers (23-27) as well as in our own laboratory (28). From our studies (28) we have reached the following conclusions: One form of immunoreactive PTH secreted by the gland (Fraction A) (Fig. 13) has Sephadex gel filtration characteristics corresponding to those of intact PTH, bears a reciprocal relationship to serum calcium and disappears from the plasma, even of uremic subjects, with

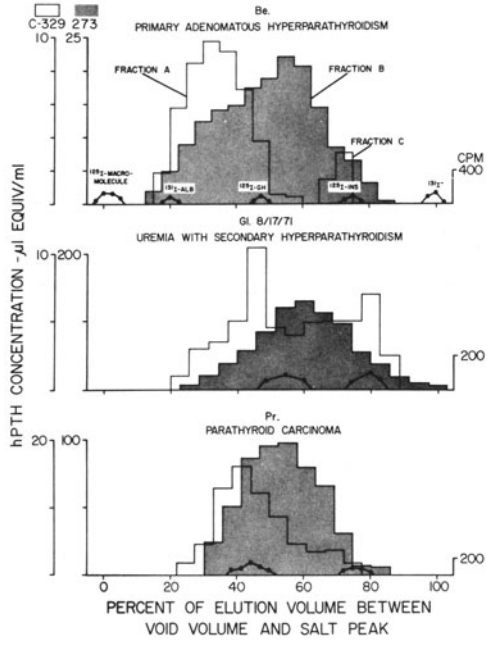

Fig. 13 - Sephadex G-100 gel fractionation of immunoreactive hPTH in plasma of patients with primary adenomatous, secondary uremic, and carcinomatous hyperparathyroidism. Volume of plasma applied to columns was 1 ml in primary and carcinomatous, and 3 ml in secondary hyperparathyroidism. Note variation in magnitude of ordinate scales of antisera 273 and C329 in these fractionations. The component of immunoreactivity that peaked with an elution volume less than that of ^{125}I-hGH was designated Fraction A; that peaked with an elution volume between ^{125}I-hGH and ^{125}I-insulin was designated Fraction B; that peaked with an elution volume equal to or greater than ^{125}I-insulin as Fraction C. (Reproduced from ref. 28)

a half-time of 20 minutes or less. This component reacts strongly with both antisera, 273 and C329, which we usually use and corresponds to the biologically active form of the hormone in the circulation. The predominant component in the plasma, Fraction B, (Fig. 13) is seen primarily by 273, has a molecular weight about 2/3 that of the intact hormone, is probably biologically inactive since it remains elevated even in the presence of clinical postparathyroidectomy hypoparathyroidism and has a disappearance rate from the plasma of a uremic subject more than 100 times longer than that of Fraction A. Fraction B appears to be a C-terminal fragment of the intact hormone. There is also present in plasma another generally minor immunoreactive form (Fraction C) (Fig. 13) which is presumably a N terminal fragment. This component, like B, is biologically inactive and disappears even more slowly than B. We have concluded (28) that the evidence favors a glandular origin for the fragments as well as for the intact hormone and that the relative prominence of the fragments in the plasma arises from their prolonged turnover times.

Dr. Arnaud reviews his recent studies on the heterogeneity of parathyroid hormone in a subsequent paper in this symposium.

After the brilliant discovery by Steiner and associates (29, 30) of pancreatic proinsulin, a biologically relatively inactive precursor of insulin in the pancreas, Roth et al (31) noted in plasma an immunoreactive insulin component ("big" insulin) with Sephadex gel filtration characteristics identical with that of proinsulin. Proinsulin is a single chain peptide, (MW $\sim$9000) half again as large as insulin, in which a connecting peptide runs from the amino terminal of the A chain to the carboxyl terminal of the B chain. It has an elution volume on Sephadex G-50 gel filtration about half that of insulin (Fig. 14). The findings of Roth et al (31) were soon confirmed by Steiner's group (32) and by ourselves (33, 34).

Studies in a number of laboratories have now shown that proinsulin usually comprises only a minor component of total immunoreactive plasma insulin in the stimulated state (Fig. 14). However in some though not in all cases of insulinoma, the major component of immunoreactivity has an elution volume on Sephadex gel corresponding to that of proinsulin (34, 35). Whether the component is intact proinsulin or an intermediate

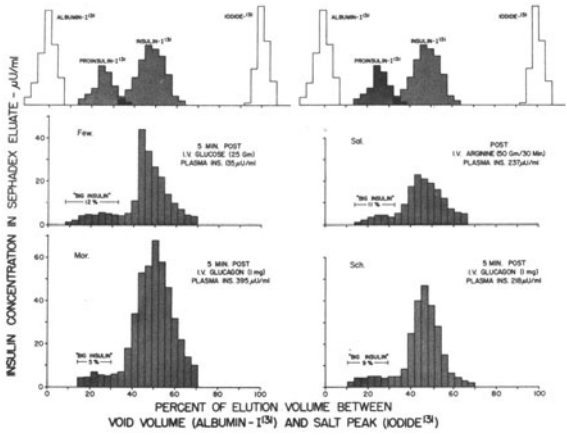

Fig. 14 - Insulin concentration in Sephadex eluates following intravenous glucose, glucagon or arginine stimulation of insulin secretion. The regions of elution of labeled standard insulin and proinsulin are depicted at the top of the figure. (Reproduced from ref. 34)

molecule with an opening at one of the ends of the connecting peptide that joins the A and B chains has not been determined with certainty. Since insulinoma patients usually present with hypoglycemia and the immunoreactive insulin levels are not always markedly elevated, the question arises as to whether the proinsulin-like component could in fact be biologically inactive proinsulin.

We have also demonstrated that a still larger form of insulin, "big, big insulin", predominated in the plasma of an insulinoma suspect (36). This patient was quite unusual in that she had only occasional hypoglycemia in spite of inordinately high plasma immunoreactive insulin (600 μU/ml fasting; 2000 μU/ml post feeding). Thus this new hormonal form of insulin must be devoid of biologic activity in vivo. On Sephadex G-200 gel filtration it has an elution volume smaller than that of labeled albumin and almost coincident with that of labeled γ-globulin (Fig. 15). This new hormonal form of insulin maintains its integrity on refractionation and cannot be distinguished immunochemically from 6000 MW insulin with the antiserum we use for radioimmunoassay. Big big insulin is stable in 8M urea but is rapidly converted by trypsin to an insulin-like component (36). It is a very minor component of extracts of normal pancreas and of insulinomas

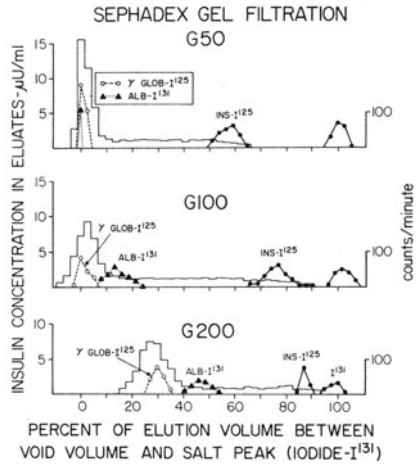

Fig. 15 - Immunoreactive insulin concentrations in Sephadex G-50 (top), G-100 (middle) and G-200 (bottom) eluates of plasma of patient Kc. (Reproduced from ref. 36)

(<1%). The evidence is suggestive that this new hormonal form is a precursor of the insulin family preceding even proinsulin but biosynthetic studies are required to confirm this suggestion.

The nature of the antral hormone, gastrin, was first elucidated only a decade ago by Gregory and Tracy (37) by the extraction and purification from hog antra of two heptadecapeptides, gastrin I and gastrin II. These differ, in the same species, only in the presence of an esterified SO_3H group on the tyrosine in position 12 in gastrin II. In a Conference on Gastrin held in September 1964, these authors had the foresight to state (38) "We have termed the peptides we isolated "Gastrins" I and II, but we do not mean to imply by this that either is considered to be in the same form as the hormone is when released from antral mucosa. Clearly, there may be present in antral mucosa other "gastrins" composed of part of the peptides we have isolated, or indeed incorporating them, or the active parts, within a larger molecule. This consideration must apply also to the substance produced by Zollinger-Ellison tumors." Our studies using the radioimmunoassay of gastrin have confirmed this prediction. When we fractionated the plasma of gastrin hyper-

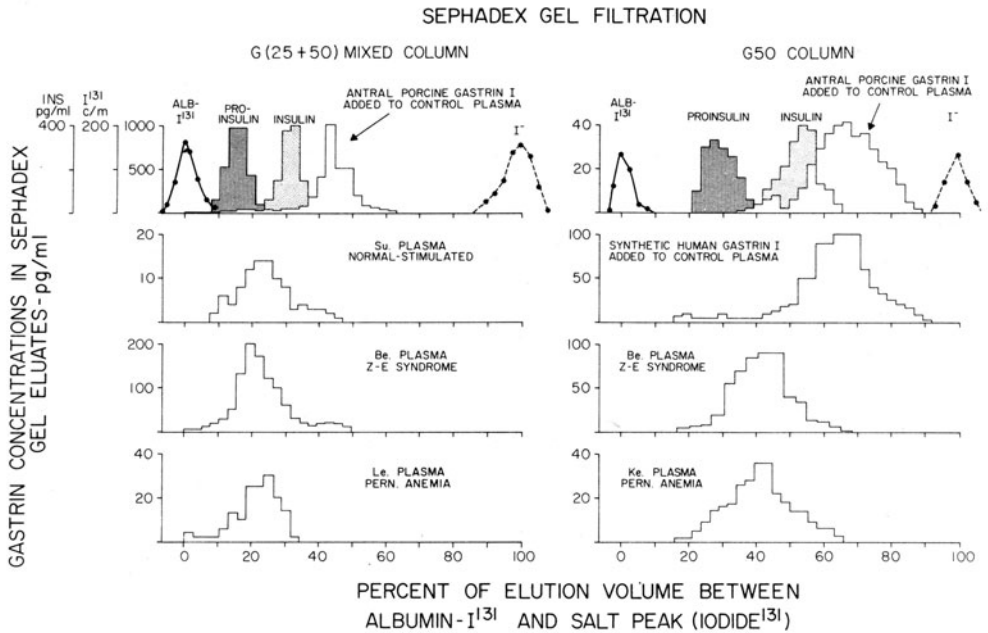

Fig. 16 - Distribution of immunoreactive gastrin in samples of endogenous plasma or plasma-gastrin mixtures added to columns of Sephadex G-50 (right), or mixtures of G-50 and G-25 (left) for gel filtration. The zones of elution of the marker molecules are shown in the top frames. (Reproduced from ref. 39)

secretors (primarily patients with Zollinger-Ellison (Z-E) syndrome or pernicious anemia (PA)), we found that the major component of immunoreactive gastrin in these samples had an elution volume on Sephadex gel filtration between insulin and proinsulin (Fig. 16) and on electrophoretic analysis (Fig. 17) appeared to be more basic than heptadecapeptide gastrin (HG) (39, 40). We termed this new hormonal form "big gastrin" (BG). We demonstrated that with the various antisera we use for radioimmunoassay it was immunochemically indistinguishable from HG; it cannot be transformed to HG by incubation in 8M urea, 2N HCl, or neuraminidase (500 U/ml) but can be converted to HG virtually instantaneously and quantitatively by tryptic digestion (40). Both BG and HG are stimulated by feeding PA patients, although BG disappears from plasma at a slower rate (40). Both BG and HG are present in extracts of antrum and proximal small bowel (41). BG becomes more and more prominent in relation to HG as one proceeds distally down the gastrointestinal tract (Fig. 17) (41). Gregory and Tracy (42) have since purified BG from Z-E tumors, have determined its amino acid composition, and have confirmed the properties which had initially been

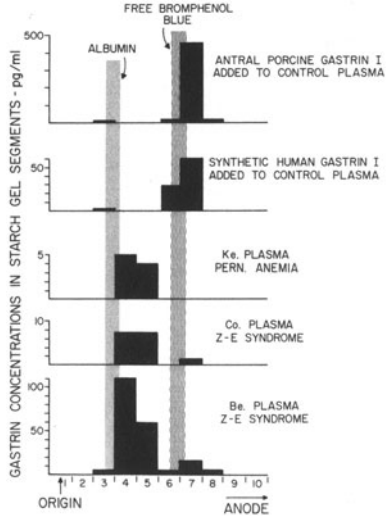

Fig. 17 - Distribution of immunoreactive gastrin components, on starch gel electrophoresis. The zones of migration of bromphenol blue-stained albumin and of free bromphenol blue were noted prior to sectioning. (Reproduced from ref. 39)

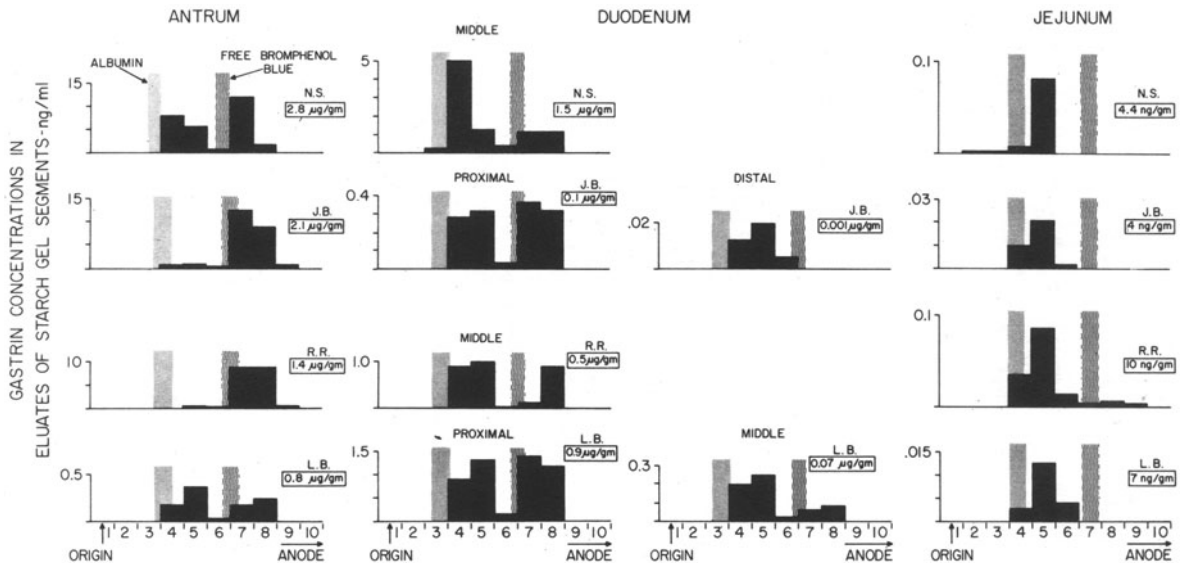

Fig. 18 - Distribution of immunoreactive gastrin. on starch gel electrophoresis, in extracts of antrum, duodenum, and proximal jejunum in postmortem material. The total concentration of gastrin (boxed values) in the crude extract for each sample is expressed as micrograms or nanograms gastrin per g of mucosa. Since gel eluates from different gels were assayed at different dilutions only the relative abundance of the components in each gel is significant. (Reproduced from ref. 41)

determined 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 biologic potency, the biologic activity in vivo of BG is about equivalent to that of HG for infusion doses of equal immunoreactivity (43).

Stimulated by our discovery of big big insulin (36) we looked for and found an even larger form of immunoreactive gastrin, "big big" gastrin (BBG) (44, 45). BBG eluted near ^{131}I-albumin in the void volume on Sephadex G-50 gel filtration. It is a minor component (<2% of immunoreactivity) in the plasma of Z-E and PA patients and in extracts of Z-E tumor (44, 45). It is virtually undetectable (<<1% of immunoreactivity) in antral and proximal duodenal extracts. However like BG, it becomes relatively more prominent distally down the gastrointestinal tract, amounting to as much as 24% of total immunoreactivity in some jejunal extracts (44). BBG is a major fraction of immunoreactive gastrin in normal human (Fig. 19), canine and porcine plasma in the non-stimulated states (45). Unlike BG and HG, BBG is not detectably stimulated by feeding (Fig. 19) (45). The half-times for disappearances of HG, BG and BBG from plasma are approximately 3, 9 and 90 minutes respectively (46). Since the plasma concentration of each component under steady state conditions is determined not only by its secretion rate but also by its distribution into extravascular spaces and its rate of degradation, it should be appreciated that even the relatively high concentration of BBG in the fasting state in normal subjects would be consistent with a low secretory rate for BBG compared to BG and HG. Whether BBG is the ultimate precursor of the gastrin family and whether or not it has significant biologic potency have not been evaluated.

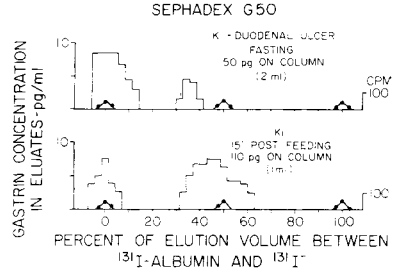

Fig. 19 - Distribution of immunoreactive gastrin on Sephadex G-50 gel filtration in plasma of a human subject before and after feeding. (Reproduced from ref. 45)

Immunoreactive ACTH is also heterogeneous in plasma and tissue (47-50). Highly purified human ACTH (1-39 peptide, "little" ACTH) added to plasma and fractionated on Sephadex G50 columns emerges as a single peak midway between the void volume and the salt peak. However fractionation of plasma or tissue extracts on these columns reveals an immunoreactive component which elutes in or immediately after the void volume. We have designated this component "big" ACTH. In plasma there is great variation in the relative distribution of big and little ACTH (Fig. 20). The only form detectable when plasma ACTH is elevated in response to stimulation of a normal pituitary is little ACTH. This form is found in patients with low plasma cortisol levels whether arising from hypoadrenal function (Addison's disease),

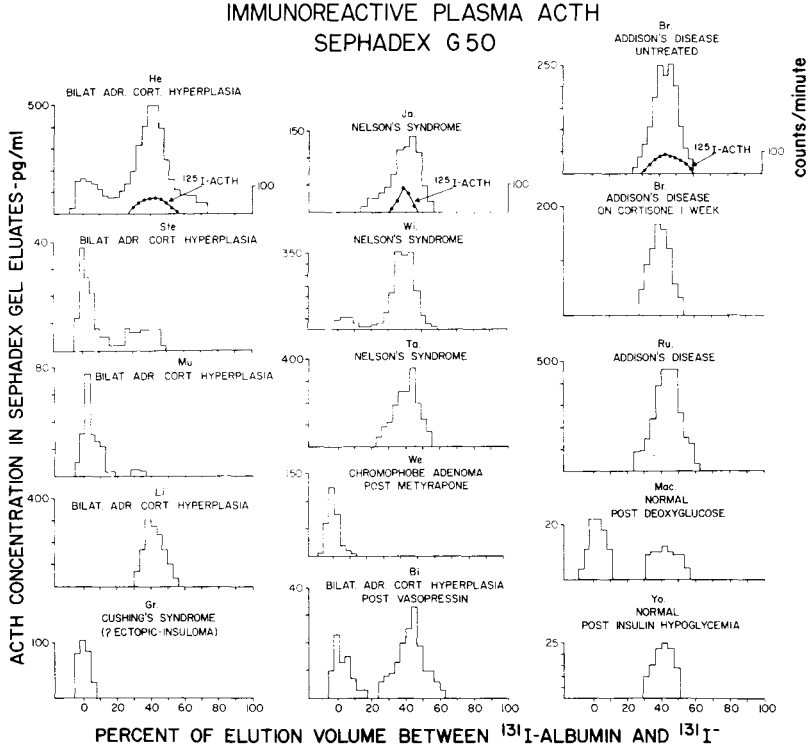

Fig. 20 - Distribution of ACTH immunoreactivity following Sephadex G-50 gel filtration of plasmas. Concentrations of immunoreactive ACTH are shown in open columns. The elution volumes of labeled marker molecules added to the plasma before application of the columns are shown in solid circles. (Reproduced from ref. 48)

(Fig. 20) post bilateral adrenalectomy, or subsequent to administration of metyrapone, an agent that blocks 11 β-hydroxylase activity in the adrenal cortex and leads to a fall in plasma cortisol levels (47, 48, 50). In the plasma of patients with ectopic ACTH production with (47, 48) or without (50) clinical Cushing's syndrome big ACTH predominates. In patients with bilateral adrenal hyperplasia or Nelson's syndrome who are presumed to have autonomous pituitary hypersecretion, the fraction in the big form may range from 0 to 100% (Fig. 20) (48). We have not as yet evaluated what factors are responsible for this very variable fraction in this group of patients.

Big ACTH maintains its integrity on refractionation, is immunochemically indistinguishable from little ACTH with the antiserum we use for radioimmunoassay and, from its behavior on starch gel electrophoresis, appears to be a more acidic peptide than little ACTH (48). Big ACTH is virtually devoid of biologic activity (49) as measured by the adrenal cell dispersion method of Sayers et al (51). Controlled tryptic digestion of big ACTH results almost instantaneously in virtually quantitative conversion to a peptide with many physical chemical characteristics resembling the authentic 1-39 peptide (48) and with biologic activity equivalent to its immunologic activity (49) (Fig. 21).

Although biosynthetic studies have not as yet been performed, these observations are consistent with big ACTH having a precursor relationship to the usual 1-39 ACTH peptide.

A recent observation of considerable interest was the finding of immunoreactive ACTH, predominantly in the big form, in all but one of 30 extracts of primary or metastatic carcinoma of the lung (50). The tissues were obtained as surgical or autopsy specimens from patients with no clinical evidence of Cushing's syndrome. In these same tissues we did not find evidence for ectopic production of human growth hormone, parathyroid hormone, insulin or gastrin. Control extracts of normal lung and lung tissue remote from the tumor did not contain ACTH so that accidental contamination or another non-specific artifact was not responsible (50). Subsequent extractions of another 20 tumor specimens confirm these studies (unpublished observations).

More than half of 83 patients with carcinoma of the

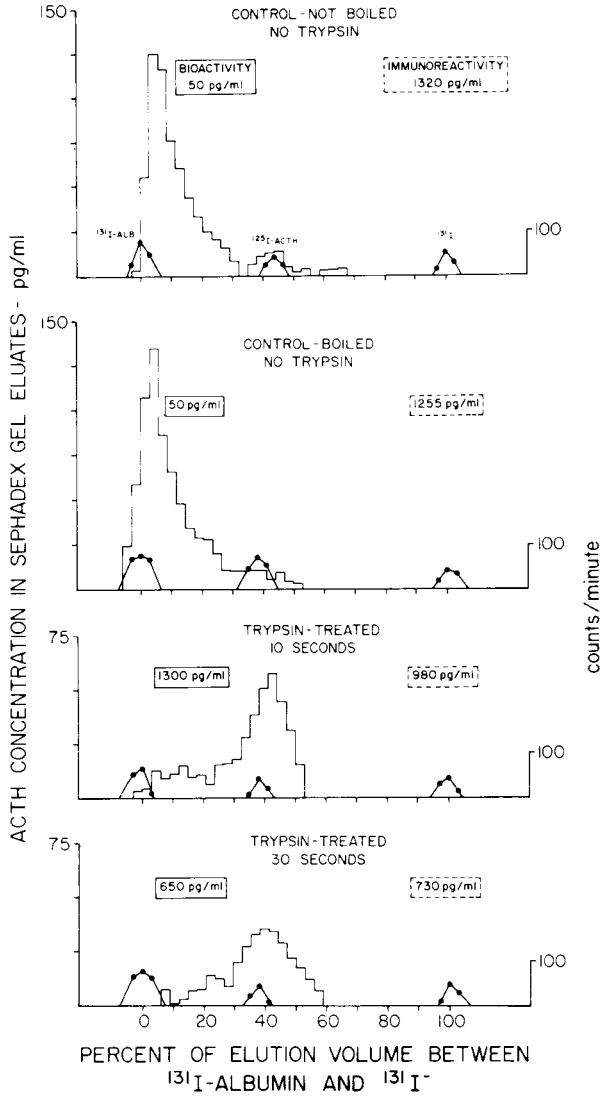

Fig. 21 - Sephadex G-50 gel filtrations of big ACTH (obtained from a boiled water extract of a bronchogenic carcinoma) before and after trypsin treatment. Bioactivity of each sample placed on the columns is indicated in the boxes on the left [ ] and immunoreactivity in the boxes on the right [- - -]. (Reproduced from ref. 49)

lung had afternoon plasma ACTH levels greater than 150 pg/ml; more than 90% of those patients with plasma concentrations less than 150 pg/ml had received radiation therapy or chemotherapy for treatment of the tumor (Fig. 22) (50). Only 7% of laboratory controls and other hospital patients without lung disease had plasma ACTH concentrations greater than 150 pg/ml.

Currently under investigation in our laboratory is the possible usefulness of measurement of plasma ACTH concentrations in screening procedures for detection of carcinoma of the lung or as a simple technique for determining the extent and activity of lung tumors and for evaluating the response of such tumors to therapeutic procedures.

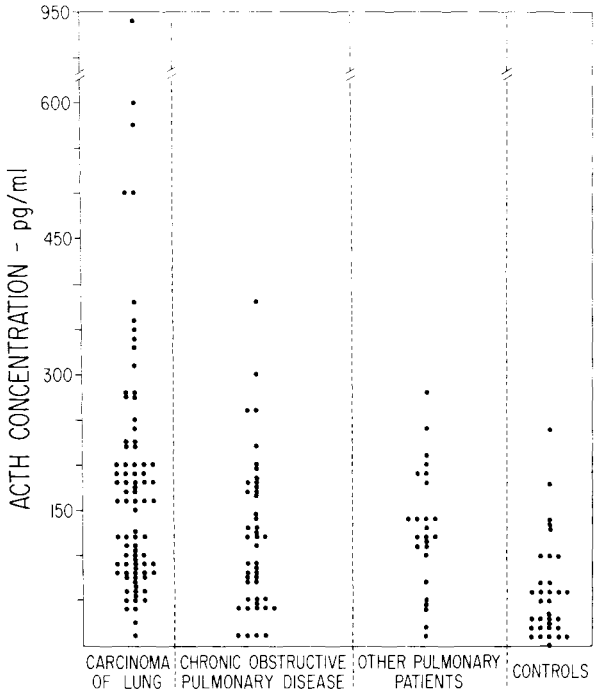

Fig. 22 - Scattergram of immunoreactive ACTH concentrations in afternoon plasma specimens from patients with carcinoma of the lung, chronic obstructive pulmonary disease, other pulmonary patients and controls. (Reproduced from ref. 50)

In this presentation I have selected from past and present endocrinologic investigations of our laboratory in which radioimmunoassay was a necessary tool. These represent but a minute fraction of the total contribution of radioimmunoassay to endocrinology. In other fields the impact of radioimmunoassay is just starting to be felt. It can be anticipated that in the decade to come its burgeoning applications in enzymology, oncology, pharmacology and toxicology and other areas will transcend in importance even its significance in endocrinology. From present indications it seems likely that if there is no other simple way to measure or to detect a substance of biologic interest some imaginative investigator will exploit radioimmunoassay to find a solution to the problem.

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