RADIOIMMUNOASSAY:

A Probe for Fine Structure of Biologic Systems

Nobel Lecture, 8 December, 1977

by
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To primitive man the sky was wonderful, mysterious and awesome but he could not even dream of what was within the golden disk or silver points of light so far beyond his reach. The telescope, the spectroscope, the radio-telescope - all the tools and paraphernalia of modern science have acted as detailed probes to enable man to discover, to analyze and hence better to understand the inner contents and fine structure of these celestial objects.

Man himself is a mysterious object and the tools to probe his physiologic nature and function have developed only slowly through the millenia. Becquerel, the Curies and the Joliot-Curies with their discovery of natural and artificial radioactivity and Hevesy, who pioneered in the application of radioisotopes to the study of chemical processes, were the scientific progenitors of my career. For the past 30 years I have been committed to the development and application of radioisotopic methodology to analyze the fine structure of biologic systems.

From 1950 until his untimely death in 1972, Dr. Solomon Berson was joined with me in this scientific adventure and together we gave birth to and nurtured through its infancy radioimmunoassay, a powerful tool for determination of virtually any substance of biologic interest. Would that he were here to share this moment.

Radioimmunoassay came into being not by directed design but more as a fall-out from our investigations into what might be considered an unrelated study. Dr. I. Arthur Mirsky had hypothesized 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 (1). To test this hypothesis we studied the metabolism of ^{131}I-labeled insulin following intravenous administration to non-diabetic and diabetic subjects (2). We observed that radioactive insulin disappeared more slowly from the plasma of patients who had received insulin, either for the treatment of diabetes or as shock therapy for schizophrenia, than from the plasma of subjects never treated with insulin (Fig. 1). We suspected that the retarded rate of insulin disappearance was due to binding of labeled insulin to antibodies which had developed in response to administration of exogenous insulin. However classic immunologic techniques were not adequate for the detection of antibodies which we presumed were likely to be of such low concentration as to be non-precipitating. We therefore introduced radioisotopic methods of high sensitivity

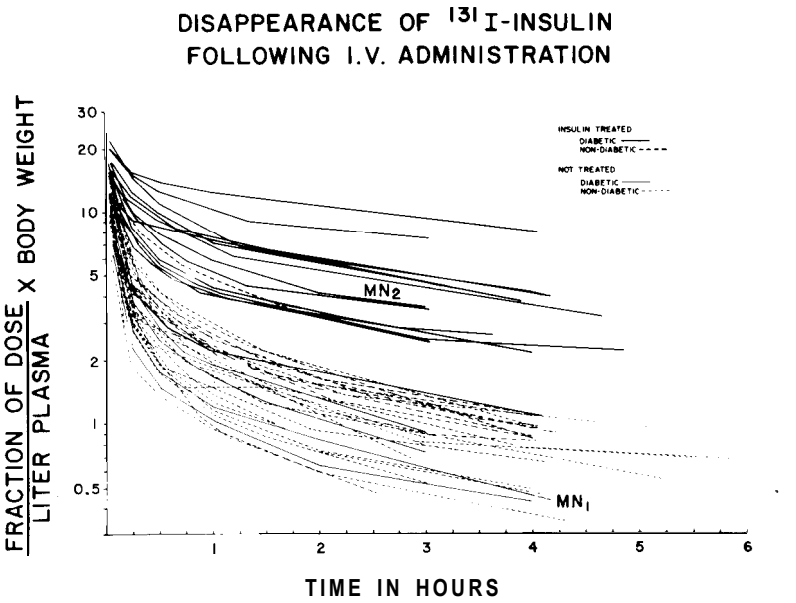

Fig. 1. Trichloractic acid precipitable radioactivity in plasma as a function of time following intravenous administration of ^{131}I-insulin to insulin-treated and non-insulin-treated subjects. The disappearance was retarded in the insulin-treated subjects irrespective of whether they had received the hormone for treatment of diabetes or for shock therapy for schizophrenia. The retarded rate is a consequence of binding to insulin antibodies generated in response to administration of animal insulins. Note the slower disappearance from the plasma of MN after 4 months of insulin therapy (curve MN,) than prior to such therapy (curve MN,). (Data reproduced from Ref. 2.)

for detection of soluble antigen-antibody complexes. Shown in Fig. 2 are the electrophoresis patterns of labeled insulin in the plasma of controls and insulin-treated subjects. In the insulin-treated patients the labeled insulin is bound to and migrates with an inter beta-gamma globulin. Using a variety of such systems we were able to demonstrate the ubiquitious presence of insulin-binding antibodies in insulin-treated subjects (2). This concept was not acceptable to the immunologists of the mid 1950's. The original paper describing these findings was rejected by Science and initially rejected by the Journal of Clinical Investigation (Fig. 3). A compromise with the editors eventually resulted in acceptance of the paper, but only after we omitted "insulin antibody" from the title and documented our conclusion that the binding globulin was indeed an antibody by showing how it met the definition of antibody given in a standard textbook of bacteriology and immunity (3). Our use of radioisotopic techniques for studying the primary reaction of antigen with antibody and analyzing soluble complexes initiated a revolution in theoretical immunology in that it is now generally appreciated that peptides as small as vasopressin and oxytocin are antigenic in some species and that the equilibrium constants for the antigen-antibody reaction can be as great as 10^{14} liters per mole, a value up to 10" greater than the highest value predicted by Pauling's theory of 1940 (quoted in 4).

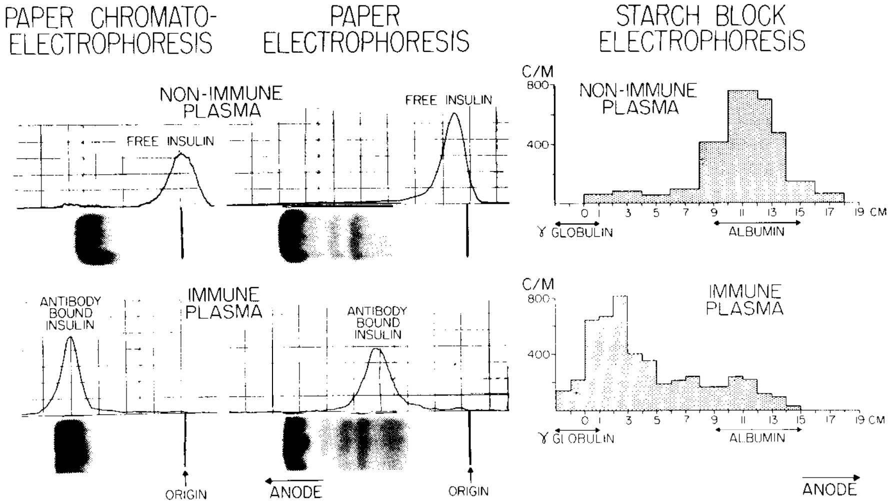

Fig. 2. **^{131}I-insulin was added to the plasmas of insulin-treated (bottom) and untreated (top) human subjects and the mixtures were applied to a starch block (right) or to paper strips (middle) for electrophoresis or to paper strips for hydrodynamic flow chromatography combined with electrophoresis (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. The zones of migration of albumin and y-globulin were identified on the starch block by running samples containing ^{131}I-albumin and ^{131}I-y-globulin on the same block. (Starch block reproduced from ref. 2; paper strips reproduced from Berson and Yalow, 1962, Ciba Found. Colloq. Endocrinol. 14, 182-201.)**

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September 29, 1955

Dr. Solomon A. Berson
Radioisotope Service
Veterans Administration Hospital
130 West Kingsbridge Road
Bronx 68, New York

Dear Dr. Berson:

I regret that the revision of your paper entitled "Insulin-I^{131} Metabolism in Human Subjects: Demonstration of Insulin Transporting Antibody in the Circulation of Insulin Treated Subjects" is not acceptable for publication in THE JOURNAL OF CLINICAL INVESTIGATION. - - - - - - - - - - - - -

- - - - - - - - - - - - - - - The second major criticism relates to the dogmatic conclusions set forth which are not warranted by the data. The experts in this field have been particularly emphatic in rejecting your positive statement that the "conclusion that the globulin responsible for insulin binding is an acquired antibody appears to be inescapable". They believe that you have not demonstrated an antigen-antibody reaction on the basis of adequate criteria, nor that you have definitely proved that a globulin is responsible for insulin binding, nor that insulin is an antigen. The data you present are indeed suggestive but any more positive claim seems unjustifiable at present.

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Sincerely,

Stanley E. Bradley

Stanley E. Bradley, M.D.
Editor-in-Chief

SEB/mcs
Encl.

Fig. 3. Letter of rejection received from Journal of Clinical Investigation.

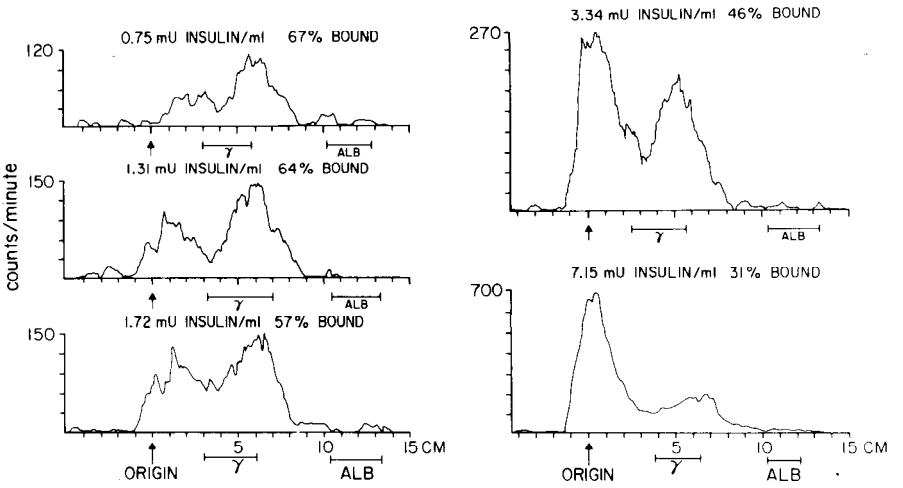

Fig. 4. Paper electrophoretograms showing the distribution of ^{131}I-insulin between that bound to antibody (migrating with serum protein) and that free (remaining at site of application) in the presence of increasing concentrations of labeled insulin. The antibodies were from an insulin-treated human subject. (Data reproduced from Ref. 2.)

In this paper we also reported that the binding of labeled insulin to a fixed concentration of antibody is a quantitative function of the amount of insulin present (Fig. 4). This observation provided the basis (5) for the radioimmunoassay of plasma insulin. However investigations and analysis which lasted for several years and which included studies on the quantitative aspects of the reaction between insulin and antibody (6) and the species specificity of the available antisera (7) were required to translate the theoretical concepts of radioimmunoassay into the experiments which led first to the measurement of plasma insulin in rabbits following exogenous insulin administration (8) and finally in 1959 to the measurement of insulin in unextracted human plasma (9).

Radioimmunoassay (RIA) is simple in principle. It is summarized in the competing reactions shown in Fig. 5. 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

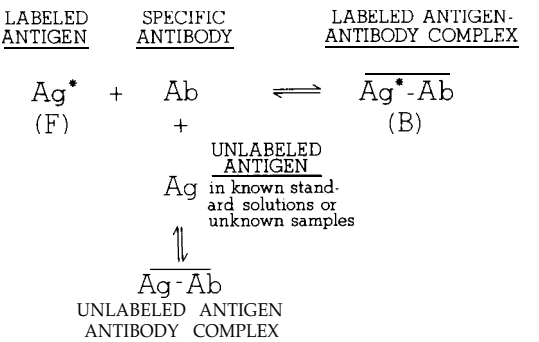

Fig. 5. Competing reactions that form the basis of radioimmunoassay (RIA).

of known standards (Fig. 6). The sensitivity of RIA is remarkable. As little as 0.1 pg gastrin/ml of incubation mixture, i.e., 0.05 picomolar gastrin, is readily measurable. RIA is not an isotope dilution technique, with which it has been confused, since there is no requirement for identical immunologic or biologic behavior of labeled and unlabeled antigen. The validity of RIA is dependent on identical immunologic behavior of antigen in unknown samples with the antigen in known standards. The specificity of immunologic reactions can permit ready distinction, for instance, between corticosterone and cortisol, steroids which differ only in the absence of or presence of respectively a single hydroxyl residue. There is no requirement for standards and unknowns to be identical chemically or to have identical biologic behavior. Furthermore it has been demonstrated that at least some assays can be clinically useful, even though they cannot be properly validated due to lack of immunologic identity between standards and the sample whose concentration is to be determined.

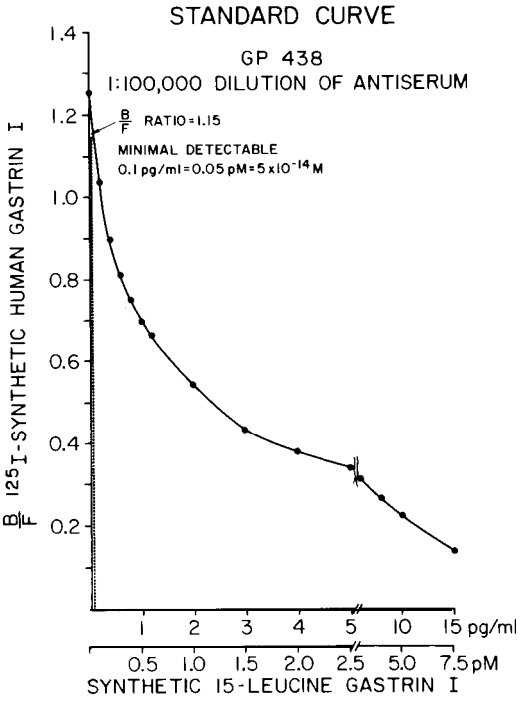

Fig. 6. Standard curve for the detection of gastrin by RIA. Note that as little as 0.2 pg gastrin/ml incubation mixture (0.1 picomolar) is readily detectable.

The RIA principle is not limited to immune systems but can be extended to other systems in which in place of the specific antibody there is a specific reactor or binding substance. This might be a specific binding protein in plasma, a specific enzyme or a tissue receptor site. Herbert and associates (10, 11) first demonstrated the applicability of competitive radioassay to the measurement of vitamin B_{12} in a liver receptor assay using "Co-vitamin B_{12} and intrinsic factor as the binding substance. However it remained for Rothen-

berg in our laboratory (12) and Ekins (13) to develop assays for serum vitamin B_{12} using this principle. Ekins (14) and later Murphy (15) employed thyroxine binding globulin as the specific reactor for the measurement of serum thyroxine.

It is not necessary that a radioactive atom be the "marker" used to label the antigen or other substance which binds to the specific reactor. Recently there has been considerable interest in employing as "markers" enzymes which are covalently bound to the antigen. Although many variations of competitive assay have been described, RIA has remained the method of choice and is likely to remain so at least in those assays which require high sensitivity. The receptor site assays for the peptide hormones have the presumed advantage of measuring biologic activity but are generally at least 10-to 100-fold less sensitive than RIA. Enzyme marker assays have several disadvantages; the most important is that the steric hindrance introduced into the antigen-antibody reaction because of the presence of the enzyme molecule almost inevitably decreases the sensitivity of the assay.

Two decades ago, when bioassay procedures were in the forefront, the first presentation on the potential of hormonal measurements by radioimmunoassay (16) went virtually unnoticed. Somewhat more interest was generated by the demonstration in 1959 of the practical application of radioimmunoassay to the measurement of plasma insulin in man (9). It became evident that the sensitivity and simplicity of radioimmunoassay permitted ready assay of hundreds of plasma samples, each as small as a fraction of a milliliter, and made possible measurement not only of single blood samples, as had been performed on occasion with in vivo bioassay, but also of multiple samples, thus permitting study of dynamic alterations in circulating insulin levels in response to physiologic stimuli (9, 17). Nonetheless in the early 60's the rate of growth of radioimmunoassay was quite slow. Only an occasional paper other than those from our laboratory appeared in prominent American journals of endocrinology and diabetes before 1965 (Fig. 7). But by the late 60's RIA had become a major tool in endocrine laboratories and more recently it has expanded beyond the research laboratory into the nuclear medicine and clinical

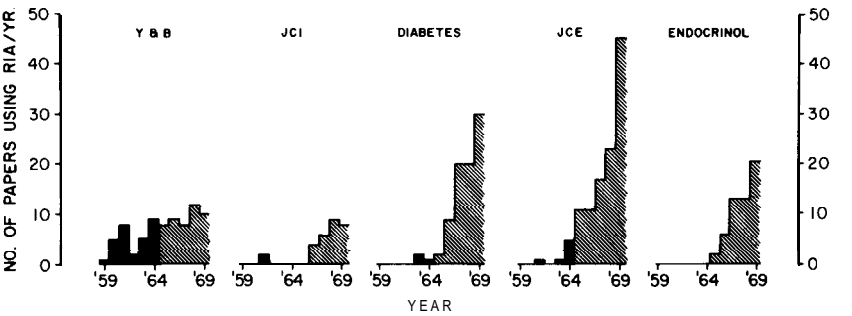

Fig. 7. Number of papers using radioimmunoassay published by Yalow and Berson (Y and B, left) and by all others in American journals of endocrinology and diabetes through 1969. Papers before 1965 are shown in black; 1965 and later are cross-hatched. (JCI, Journal of Clinical Investigation; JCE, Journal of Clinical Endocrinology; Endocrinol, Endocrinology.)

laboratories. It has been estimated (18) that in 1975, in the United States alone, over 4000 hospital and non-hospital clinical laboratories performed radioimmunoassays of all kinds, almost double the number of a year or two earlier and the rate of increase appears not to have diminished in the past two years. The technical simplicity of RIA and the ease with which the reagents may be obtained have enabled its extensive use even in scientifically underdeveloped nations.

The explosive growth of RIA has derived from its general applicability to many diverse areas in biomedical investigation and clinical diagnosis. A representative but incomplete listing of substances measured by RIA is given in Figure 8.

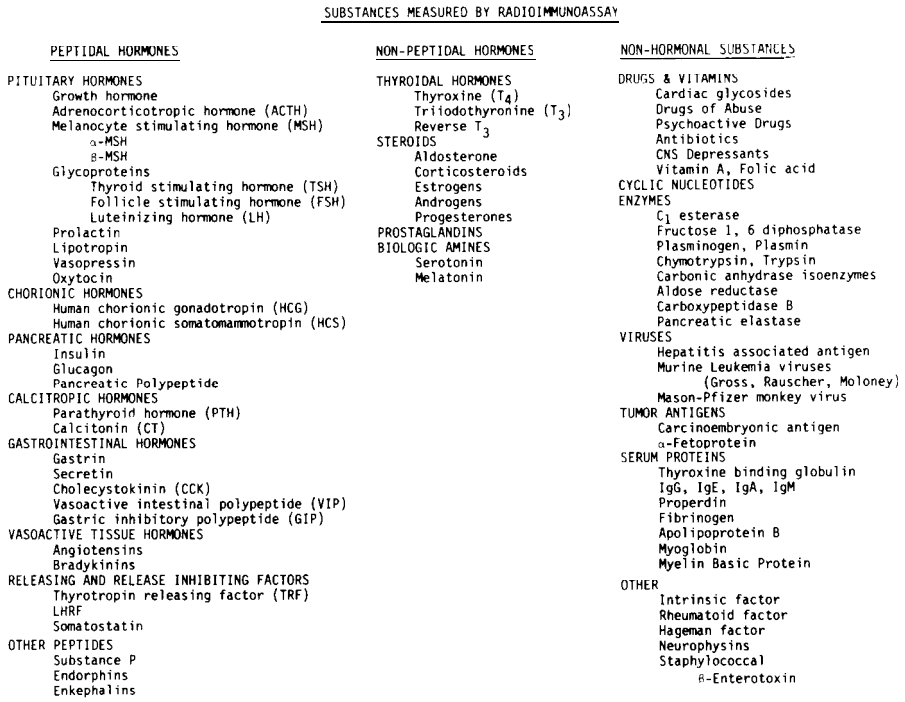

Fig. 8. Partial listing of peptidal and non-peptidal hormones and other substances measured by radioimmunoassay.

The exquisite sensitivity, specificity and comparative ease of RIA especially now that instrumentation and reagents are so readily and universally available, have permitted assay of biologically significant materials where measurements were otherwise difficult or impossible. Only if we can detect and measure can we begin really to understand, and herein lies the major contribution of RIA as a probe for insight into the function and perturbations of the fine structure of biologic systems.

For the first decade following the development of RIA and its first application to the measurement of plasma insulin in man, primary emphasis was given to its importance in endocrinology. The ability to measure in the presence

of billion-fold higher concentrations of plasma proteins the minute concentrations (10^{-10} to 10^{-12} M) of peptide hormones in plasma with the high specificity characteristic of immunologic reactions has provided greatly increased accuracy of diagnosis of pathologic states which are characterized by hormonal excess or deficiency. It has provided virtually all the information now known about the regulation of hormonal secretion and the interrelationships among hormones and has contributed greatly to our understanding of the mechanisms of hormonal release and of hormonal physiology in general. More recently, as perhaps will be discussed by Drs. Guillemin and Schally, it has been applied to investigations of the potential role of the hypothalamic releasing and release inhibiting factors; studies which have been made easier by RIA of the hormones they control as well as of the factors themselves. Over the past few years, RIA has had an important role in the discovery of new forms of hormones in blood and in tissue. These include the larger hormonal forms -- proinsulin (19), big gastrin (20--22), proparathyroid hormone (23, 24), big ACTH (25, 26), etc., and the hormonal fragments -- the biologically inactive COOH-terminal parathyroid hormone fragment (27, 28) among others. These studies have generated new insights concerning the biosynthesis of the peptide hormones.

Let us now consider some examples from our laboratory of older and of newer diverse applications of RIA. Proper interpretation of plasma hormone levels in clinical diagnosis requires a clear understanding of the factors involved in the regulation of hormonal secretion. Generally, such secretion is stimulated by some departure from the state of biologic "homeostasis" that the hormone is designed to modulate. A representative model for one such system is shown in Fig. 9. Regulation is effected through the operation 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 then suppresses secretion of antral gastrin. Modulation of this system can be effected by a number of

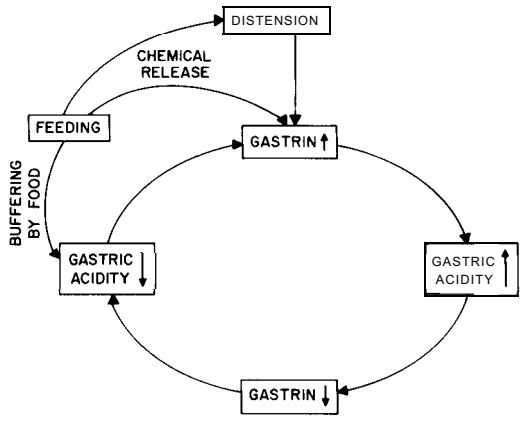

Fig. 9. Feed-back control loop for gastrin regulation of gastric acidity: effect of feeding.

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. 9).

In Figure 10 are compared basal gastrin concentrations in patients with pernicious anemia (PA), in patients with Zollinger-Ellison syndrome (ZE) and in a group of patients we have diagnosed as having non-tumorous hypergastrinemic hyperchlorhydria (NT-HH) (29--31). Gastrin levels are generally considerably higher in each of the three groups than the 0.1 ng/ml considered to be the upper limit for normal subjects. However the reasons are different. Patients with PA have gastric 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 PA is quite appropriate in view of the absence of the inhibitory effect of HCl on the secretion of antral gastrin.

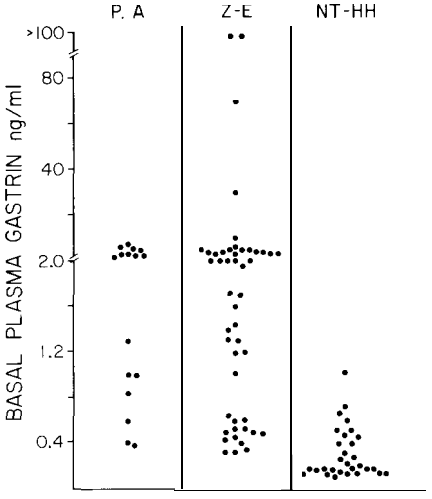

Fig. 10. Basal plasma gastric concentrations in gastrin hypersccretors, i.e., patients with pernicious anemia (PA). Zollinger-Ellison syndrome (ZE) and non-tumorous hypergastrinemit hyperchlorhydria (NT-HH). Most control subjects without known gastrointestinal disease have basal levels less than 0.1 ng/ml. (Data reproduced in part from Ref. 29-31.)

The elevated values in ZE and NT-HH are inappropriate since these patients have marked hyperacidity and the feed-back mechanisms which should suppress gastrin secretion have failed. How does one distinguish between patients with a gastrin-secreting tumor (ZE) and those whose inappropriate gastrin secretion appears to be due to overactivity of the gastrin-secreting cells of the gastrointestinal tract (NT-HH) ? Accurate diagnostic differentiation between these diseases is essential because procedures appropriate for their treatment are so markedly different that diagnostic error might be fatal. Some ZE patients have levels higher than those ever achieved by the non-tumorous group. However in the region of overlap the distinction between them is

readily made on the basis of responsiveness to various provocative agents. Patients with ZE respond to a calcium challenge (2 mg Ca^{++}/kg intravenously) or to a secretin challenge (4 U/kg intravenously) with a dramatic increase in plasma gastrin but fail to respond to a test meal; for patients with NT-HH the reverse is true (Fig. 11).

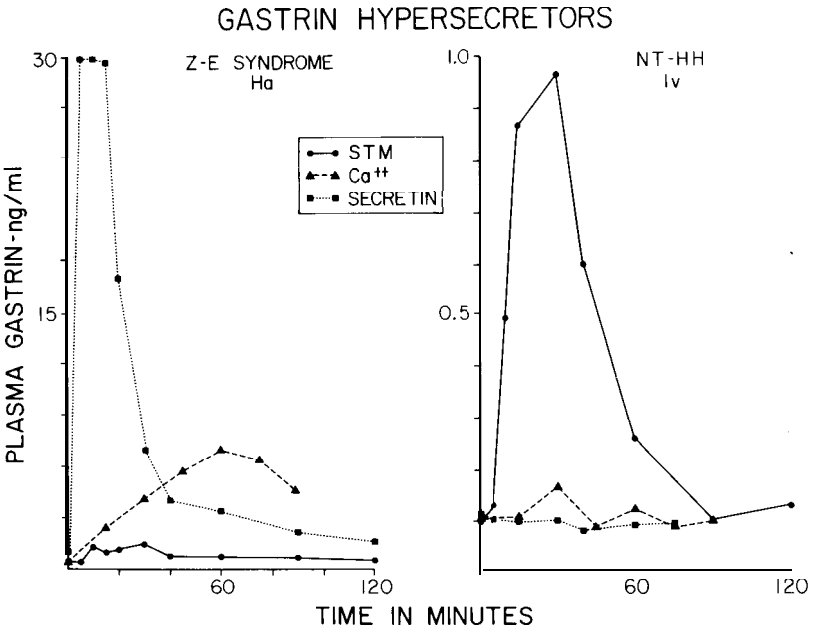

Fig. 11. Plasma gastrin concentrations in the fasting state and in response to three provocative stimuli in gastrin hypersecretors; patient Ha (left) has ZE; subject Iv (right) is in the non-tumorous (NT-HH) group. (Reproduced from Ref. 31.)

Thus, in the application of radioimmunoassay to problems of hypo- or hypersecretion we should seldom 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 concentrations are observed, one must determine whether the level is appropriate or inappropriate and whether the hormonal secretion is autonomous or can be modulated by appropriate physiologic or pharmacologic agents.

Studies such as these are now common in endocrinology and would not have been possible without RIA.

During the past decade our concepts of the chemical nature of peptide hormones and their modes of synthesis have changed dramatically. This change is due in large part to observations based on RIA which have demonstrated that many, if not all, peptide hormones are found in more than one form in plasma and in glandular and other tissue extracts. These forms may or may not have biologic activity and may represent either precursor(s) or metabolic

products(s) of the well-known, well-characterized, biologically active hormone. Their existence has certainly introduced complications into the interpretation of hormonal concentration as measured by RIA, and as measured by bioassay as well. A typical example of the work in this area is the current interest in the heterogeneity of gastrin.

Investigations concerning the possible heterogeneity of gastrin were stimulated by considerations in comparative endocrinology, in that the immunochemical heterogeneity of parathyroid hormone (27) and the demonstration of a precursor form for insulin, proinsulin, (19) spurred the search for heterogeneous forms of gastrin as soon as a radioimmunoassay for gastrin (29) had been developed with sufficient sensitivity to permit fractionation of plasma in a variety of physicochemical systems and assay of the immunoreactivity in the various fractions.

Several analytical methods were used to elucidate the nature of plasma gastrin. Quite unexpectedly it appeared that the major component of immunoreactive gastrin in the fasting state of patients with hypergastrinemia was a peptide clearly different from heptadecapeptide gastrin (HG), a 17 amino acid peptide that had earlier been purified and sequenced by Gregory and Tracy (32, 33). The newly discovered peptide eluted between insulin and proinsulin on Sephadex G50 gel filtration, in contrast with HG which eluted after insulin (Fig. 12). This peptide had an electrophoretic mobility on starch gel just greater than serum albumin, which is about half that of HG (20, 21). Characterization in other physical chemical systems helped verify that this

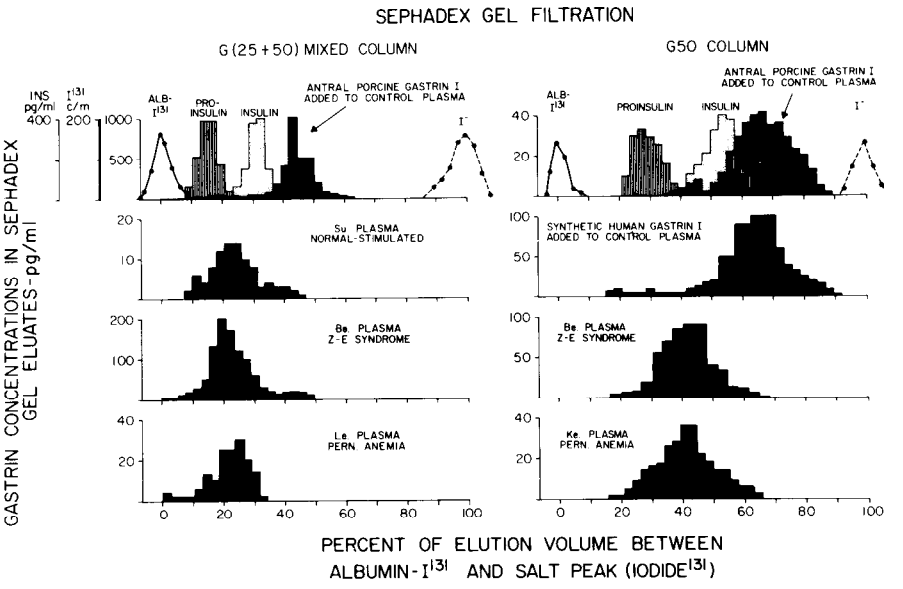

Fig. 12. Distribution of immunoreactive gastrin in samples of endogenous plasma or plasma-gastrin mixtures added to columns of Sephadex G-50 (right), or mixtures of G-50 and G-25 (left) for gel filtration. The zones of elation of the marker molecules are shown in the top frames. (Reproduced from Ref. 20.)

peptide was indeed larger and more basic than HG. In advance of its further characterization we called this new form big gastrin (BG). Both gastrins were found in extracts of a ZE tumor as well as in extracts of the antrum and proximal small bowel (22). We further demonstrated that HG could be generated by tryptic digestion of BG, with no significant change of total immunoreactivity. We predicted that the larger form was composed of the smaller form linked at its amino terminal end to a lysine or arginine residue of another peptide (2 1) .

Our predictions based on measurement of picogram to nanogram amounts of immunoreactive gastrin in the presence of billion-fold higher concentrations of other proteins stimulated Gregory and Tracy to purify and chemically characterize this material. Soon thereafter they succeeded in isolating, both from a ZE tumor and from hog antral extracts, pairs of gastrin peptides with physico-chemical behavior similar to that we had described for BG (34, 35). They then demonstrated that BG is a 34 amino acid peptide with two iysine residues adjacent to the N-terminal residue of HG (35).

Unlike proinsulin which is virtually devoid of biologic activity (36), the in vivo administration of immunochemically identical amounts of BG and HG resulted in the same integrated acid output in a dog (reported in 21). However, the turnover time for BG is prolonged 3 to 5 fold longer than for HG (37, 38). Therefore, following administration of' equivalent doses the plasma levels of BG are approximately 3 to 5 fold greater than that of HG. It is evident that the observed heterogeneity introduces complications into bioassay, as well as into immunoassay, since biologic activity as defined by the traditional dose-response method is certainly different from that defined by plasma concentration-response data in the case of the gastrins or any other groups of biologically active related peptides with different turnover times.

Our discovery of the immunochemical heterogeneity of parathyroid hormone (27) and Steiner's discovery of proinsulin (19) just over a decade ago initiated a revolution in concepts of biosynthesis of the peptide hormones. The original suggestion that a major function of proinsulin in biosynthesis was to facilitate disulfide bond formation (39) could not prognosticate that virtually all peptidal hormones, including those which consist simply of a linear peptide chain, appear also to have larger precursor forms. In many, but not all, peptide hormones the smaller peptide is joined into the larger form by two basic residues (gastrin, insulin, etc.). A notable exception to this rule is cholecystokinin (CCK). In the case of CCK and its COOH-terminal octapeptide (CCK-8), both of which are biologically active, cleavage to the smaller form occurs at the COOH-terminal side of a single arginine residue (40). As is discussed below, both forms are found in the tissues of origin.

At present, a decade after the concept of heterogeneity was developed and in spite of an enormous body of descriptive data in this field, we still do not know very much about the rules or reasons for this precursor-product synthetic scheme. Is the synthesis of the peptide hormones in a form in which they are linked to another peptide essential only for their proper storage or release or is some other mechanism involved? What are the enzymes involved in the con-

version process? Are the converting enzymes hormone specific or species specific? Is conversion effected only in the secreting tissue, or is there peripheral conversion from inactive to active form? What is the role of the part of the precursor molecule which is discarded after biosynthesis? Finding the answers to these and related questions will keep many of us busy for quite a while.

Since investigations concerned with the brain peptides as well as RIA have enjoyed prominence this year it is relevant to combine the two and discuss some applications of RIA to the understanding of peptides in the brain. Much interest has been generated recently in the finding that several peptides are common to the brain and the gastrointestinal tract. A determination of the location and concentration of these peptides has usually depended on immunologic techniques. The finding by Vanderhaeghen et al (41) of a new peptide in the vertebrate central nervous system that reacts with antibodies against gastrin has been confirmed by Dockray (42), who suggested that the brain peptide resembled cholecystokinin (CCK)-like peptides more closely than it did gastrin-like peptides. We extended these studies and demonstrated that the peptides in the brain are not from the gastrin family or simply CCK-like, but are in fact intact cholecystokinin (CCK) and its COOH-terminal octapeptide (CCK-8) (43-45). These observations depended on the use of two antisera with different immunochemical specificities. One was prepared in a goat by immunization with porcine CCK (pCCK). For all practical purposes this antiserum does not crossreact with CCK-8 or the gastrins, big or little, in spite of their sharing a common COOH-terminal pentapeptide. The other antiserum was prepared by immunization of a rabbit (Rabbit B) with the COOH-terminal gastrin tetrapeptide amide. With this antiserum the cross-reactivities of pCCK and of CCK-8 are virtually identical on a molar basis. Using the Rabbit B antiserum, we have observed that in all animal species studied the immunoreactive content as measured in the CCK-8 assay was about five-fold greater in gut extracts than in brain extracts (Table 1). However, the concentrations in the gut and brain extracts were comparable among the different species and did not change significantly on tryptic digestion (Table 1).

Sephadex gel filtration and assay in the CCK-8 system of the brain and gut extracts of the pig, dog and monkey generally revealed two peaks of comparable size, one with an elution volume resembling that of CCK and the other with an elution volume like CCK-8 (Fig. 13). A minor void volume peak whose significance has not yet been determined was also generally observed. Although there was no change in immunoreactivity following prolonged tryptic digestion (Table 1) there was complete conversion of all immunoreactivity to a peptide resembling CCK-8 (Fig. 13).

In the same monkey and dog extracts in which CCK-like material was present in about the same concentration as in the pig extracts we failed to detect immunoreactivity with the anti-pCCK serum (Table 1). The extracts of the gut and brain of the pig contained comparable molar amounts of CCK when measured with either antiserum (Table 1). The failure to detect intact CCK in dog and monkey brain and gut extracts, which were proven to have

Table 1. Immunoreactive content of brain and gut extracts

SPECIES	ORGAN	Goat 1 ASSAY μg pCCK equivalent/ml		Rabbit B ASSAY μg CCK-8 equivalent/ml	
		BEFORE TRYPSIN	AFTER TRYPSIN	BEFORE TRYPSIN	AFTER TRYPSIN
Pig (2)*	Brain	0.80 + 0.05+	ND	0.20 ± 0.01	0.15 ± 0.01
	Gut	1.80 ± 0.1	ND	0.60 ± 0.05	0.50 ± 0.03
Monkey (1)	Brain	ND	ND	0.05 ± 0.01	0.05 ± 0.02
	Gut	ND	ND	0.40 ± 0.05	0.35 ± 0.05
Dog (2)	Brain	ND	ND	0.10 ± 0.01	0.10 ± 0.01
	Gut	ND	ND	0.70 ± 0.02	0.70 ± 0.05

( )* no of animals
ND = not detected
+ mean ± standard error of the mean of multiple assays

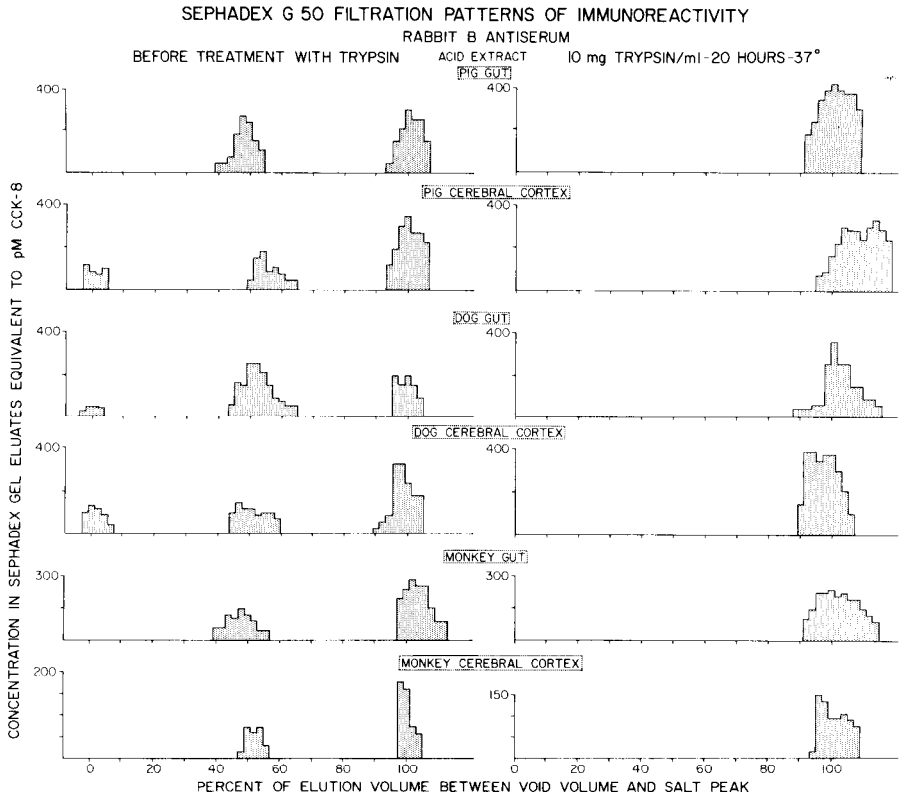

Fig. 13. Immunoreactivity in eluates following Sephadex G50 gel filtration was determined using an antiserum which reacts identically on a molar basis with intact porcine cholecystokinin (pCCK) and its COOH-terminal octapeptide (CCK-8). Purified pCCK has an elution volume midway between the void volume and the salt peak and CCK-8 coelutes with the salt peak in this system. Shown are the patterns for pig. dog and monkey brain and gut extracts before (left) and after (right) prolonged tryptic digestion. Note the complete conversion to a CCK-8-like peptide with no loss in immunoreactivity (Data reproduced from Ref. 4.5.)

this hormone when measured in the CCK-8 assay, forms the basis for our prediction based on RIA that there are major differences between pig and the other animal cholecystokinins in the amino terminal portion of the molecule. Since this portion of the molecule is not directly involved in its biologic action, it is not surprising that the amino acid sequences in this region of the molecule have diverged during the course of evolution. As yet the amino acid sequences of CCK from animals other than a pig have not been reported. Just as our predictions based on RIA stimulated Gregory and Tracy to purify and chemically characterize big gastrin, we hope our predictions of the nature of the amino terminal portion of CCK will encourage chemical verification by others.

Where in the brain is CCK found? Its concentration is highest in the cerebral cortex (43). Our immunohistochemical studies (Fig. 14) suggest that CCK-8, at least, appears to be concentrated in the cortical neurons (44).

The finding of peptides resembling CCK and CCK-8 in the central nervous system raises intriguing questions about their physiologic function particularly with respect to their potential roles as satiety factors. The observation of Gibbs et al (46, 47) that injection of purified CCK or CCK-8 evoked satiety, although pentagastrin and secretin did not, has suggested negative feedback from the gastrointestinal tract as the causative mechanism. These studies of

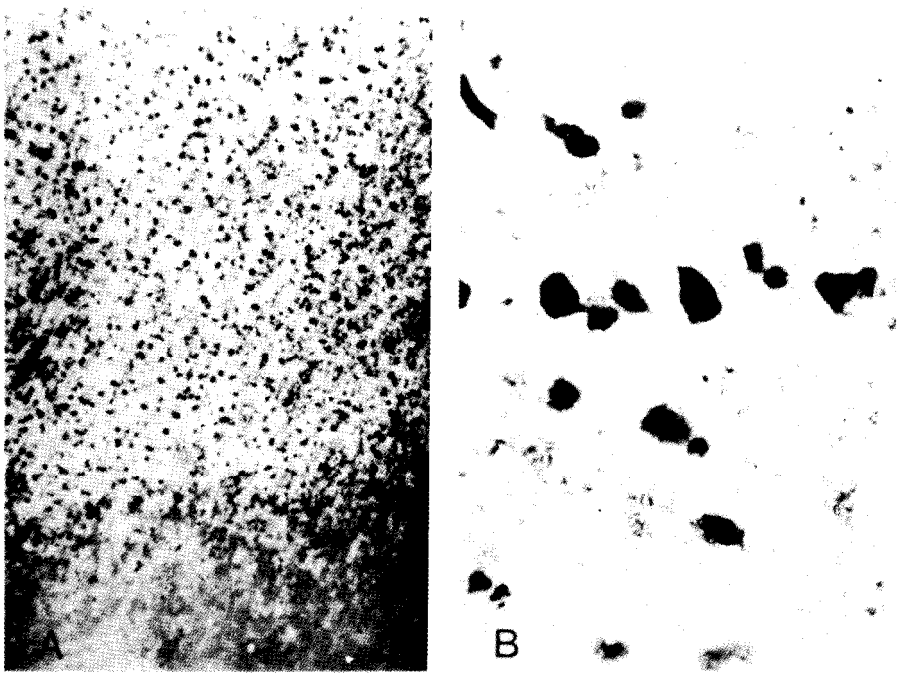

Fig. 14. Left: Low-power photomicrograph of rabbit cerebral cortex (frontal lobe). The tissue was stained by the immunoperoxidase technique using rabbit B antiserum in a 1: 10 dilution. Staining of individual cell bodies can be seen in all layers of cortical grey matter and diffuse staining can be seen at the bottom in subcortical white matter (X33). Right: Higher-power photomicrograph showing staining of cell bodies in cortical grey matter. (X208) (Data reproduced from Ref. 44.)

Gibbs et al (46, 47) confirm the earlier work of Schally et al (48) who had shown that enterogastrone, a gut extract undoubtedly rich in CCK, inhibited eating by fasted mice. The finding that CCK peptides appear to be endogenous in the brain suggests a more direct role for them as neuroregulators.

It is now commonly accepted that there are a group of peptides such as somatostatin (49, 50), substance P (51), vasoactive intestinal peptide (52) and cholecystokinin or its C-terminal octapeptide (43--45) which are found both in the gastrointestinal tract and in the central nervous system. Some evidence has also been presented that peptide hormones such as β lipotropin, ACTH and peptides structurally related to them, initially thought to be of pituitary origin, are found widely distributed in the brain in extrahypothalamic regions (53-56). We had considered the possibility that the finding of a pituitary hormone, such as ACTH in the brain of the rat might be a consequence of the small dimensions of its brain. Therefore, we recently undertook to study the distribution of ACTH in the brains not only of rodents such as the rat and rabbit but also of animals with large brains such as the dog, monkey and man (57). We observed that the dimensions within which ACTH is found is about the same for all five of these species but that the particular anatomical regions which contain ACTH depend on the brain size (57). Thus, ACTH is widely distributed in the brain of the rat, but is found in the brain only in the hypothalamic regions of primates (Table 2) (Fig. 15). Since there is no reason to assume that the synthetic mechanism is different in small brained animals than in the primates, we believe that these studies suggest that the pituitary is likely to be the sole site of synthesis of ACTH and that the hormone is found in other cranial sites due to mechanisms other than synthesis.

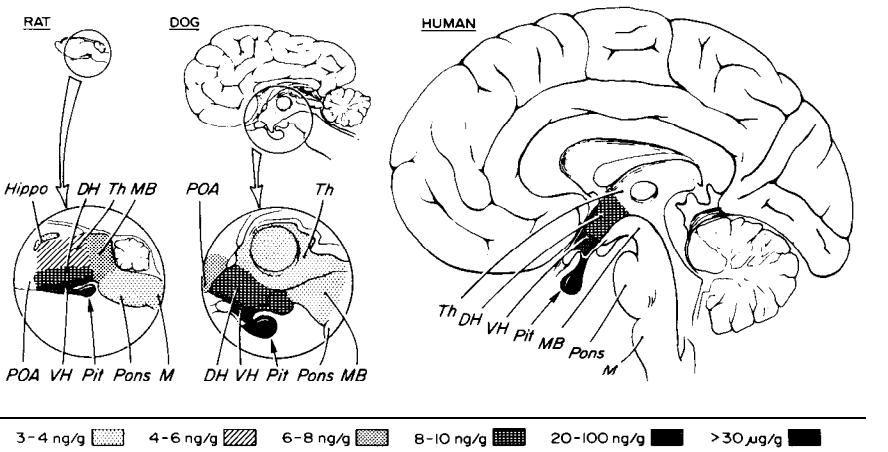

Fig. 15. Distribution of immunoreactive ACTH in the pituitary and brain of several animal species. The rat, dog and human brains are drawn to scale. The regions shown in circles have been enlarged to show in better detail the concentrations of ACTH in the brain of the rat and dog. ACTH was not detectable in regions shown in white. Abbreviations: Pit, pituitary gland; VH, ventral hypothalamus; DH, dorsal hypothalamus; POA, preoptic area; MB, midbrain; M, medulla; TH, thalamus; Hippo, hippocampus. (Reproduced from Ref. 57.)

Table 2. Regional distribution of ACTH in brains of several mammalian species ACTH Concentration (ng/g wet weight)

Brain Region	Human	Monkey			Dog			Rabbit			Rat Pool
		Craniot.	Sacrifice	Autopsy	1	2	3*	P	G	J*	n = 6
Hypothalamus								100	100	100	14
Ventral	33	—	76	10	28	26	17	—	—	—	40
Dorsal	10	4	4	3	—	5	11	—	—	—	10
Thalamus	ND†	ND	ND	ND	2	4	3	20	20	12	4
Preoptic Area	ND	ND	ND	ND	—	8	7	—	—	7	5
Amygdala	ND	ND	ND	ND	3	4	4	6	5	6	3
Hippocampus	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3
Striatum	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Midbrain	ND	ND	ND	ND	4	3	2	20	21	15	7
Pons	ND	ND	ND	ND	ND	ND	ND	10	8	10	4
Medulla	ND	ND	ND	ND	ND	ND	ND	3	6	10	3
Cerebral Cortex	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cerebellum	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

* Dissected frozen

† ND — not detectable, <1 ng/g

The presence of pituitary hormones in the brains of commercially prepared hypophysectomized rats has been taken as evidence for de novo synthesis of pituitary hormones in the brain (54-56, 58). We also have observed that in these animals residual pituitary tissue is rarely detected upon visual inspection of the sella (57). Nonetheless although there is an immediate decrease in stress-stimulated ACTH release in hypophysectomized rats, after two months the plasma ACTH concentrations can be stress-stimulated to about 80% of the level found in intact rats (Fig. 16). It would appear therefore that visual

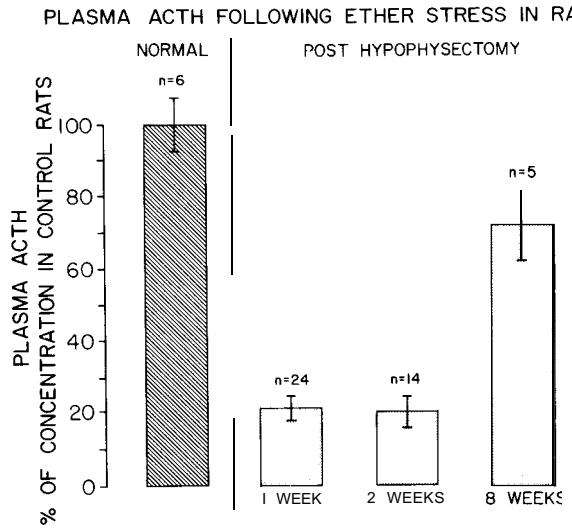

Fig. 16. Plasma ACTH following ether stress in control and hypophysectomized rats. (Reproduced from Ref. 57.)

inspection of the sella is not sufficient to insure that the hypophysectomy has been total. Scrapings from the sella have been shown to contain ACTH amounting to almost 5% that of the normal pituitary (57). This represents a considerable residual source of ACTH since the hypothalamic ACTH content is only about a fraction of a percent of that of the pituitary. Thus, even in these "hypophysectomized" rats we believe that residual pituitary fragments are the source of the brain ACTH (57).

At the present state of our knowledge, we consider it most likely that hormones known to be synthesized in the pituitary are synthesized only there and are transported to the brain by one or more mechanisms; perhaps by retrograde flow along the portal vessels or by leakage into the basal cistern. In addition, there is another group of peptides common to, and likely to have been synthesized in, the gastrointestinal tract and the central nervous system. We leave to others to determine where in this schema is the source of the enkephalins.

The examples chosen come from a sampling of studies in endocrinology since my Nobel citation specifically deals with the application of RIA in this subspecialty. Nonetheless RIA is rapidly growing beyond the borders of endocrinology, its first home.

RIA has already added a completely new dimension to the identification and measurement of pharmacologically active substances in plasma and tissue- and the list of compounds for which such assays are available is growing rapidly (Fig. 8). In general since the molar concentrations of drugs at pharmacologic levels are high compared, for instance, to the concentration of the peptide hormones in body fluids, achieving adequate sensitivity is not likely to be difficult. However, the requirements for the specificity of RIA of drugs merit some consideration. Structurally related compounds or metabolites may have significant immunoreactivity with some antisera but not with others and may or may not constitute a problem, depending on the purpose of the assay. For instance, if the clinical problem relates to the toxicity of a particular drug, then the question as to whether or not the assay measures only the biologically active form is relevant. If the question relates simply to whether or not a drug had been taken surreptitiously, then the reactivity of metabolites or variation of the immunoreactivity with the exact form of the drug may be irrelevant.

The application of RIA to the measurement of enzymes is a field of increasing interest. The very great sensitivity of RIA permits measurement of enzyme levels much lower than that possible by the usual catalytic methods. It permits direct assay of the enzyme rather than only its effects and is not influenced by inhibitors or activators of enzyme systems or variations in substrates. That in the same system one can with RIA measure both an enzyme and its proenzyme and other inactive forms has both advantages and disadvantages, depending on the problem under investigation. It must be appreciated that many enzymes may be species specific and biologic activity need not parallel immunologic activity. At present, RIA seems likely to complement rather than to replace catalytic methods for enzymatic analysis.

There is another field in which the potential application of RIA is in its

infancy. My crystal ball--or intuition--tells me that in the '80's the impact of RIA on the study of infectious diseases may prove as revolutionary as its impact on endocrinology in the '60's. A start has already been made in virology. RIA of hepatitis-associated-antigen (59, 60) has become the method of choice for testing for infected blood in Red Cross and other blood banks in the United States where transfusion-transmitted hepatitis has been a significant public health problem. The recent description of a RIA for intact murine leukemia virus (61) with sufficient sensitivity to detect virus in 0.5 μl of blood or of tissue extracts from animals with viral induced or spontaneous leukemia gives us a tool with which we may be able to determine where and in what concentration a virus resides during the period from infection to the time when the fully developed pathologic manifestations of the disease are present. Recently we have developed a sensitive and specific RIA for some constituent of tuberculin purified protein derivative (PPD) (62) which is shed into culture medium in vitro or in vivo by growing Mycobacterium tuberculosis. We have already reported (62) earlier detection of growth of tubercle bacilli in culture medium than is possible by other means and we envision its applicability to rapid and early detection of bacterial growth in biologic fluids. We anticipate that this preliminary work will lead the way to the more extensive use of RIA in bacteriology.

Infectious diseases have become less prominent as causes of death and disability in regions of improved sanitation and adequate supplies of antibiotics. Nonetheless they remain a major public health problem throughout the world and simple inexpensive methods of identifying carriers of disease would facilitate eradication of these diseases. RIA is likely to provide those methods and one can anticipate its fuller exploitation in this virtually untapped field.

The first telescope opened the heavens; the first microscope opened the world of the microbes; radioisotopic methodology, as exemplified by RIA, has shown the potential for opening new vistas in science and medicine.

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