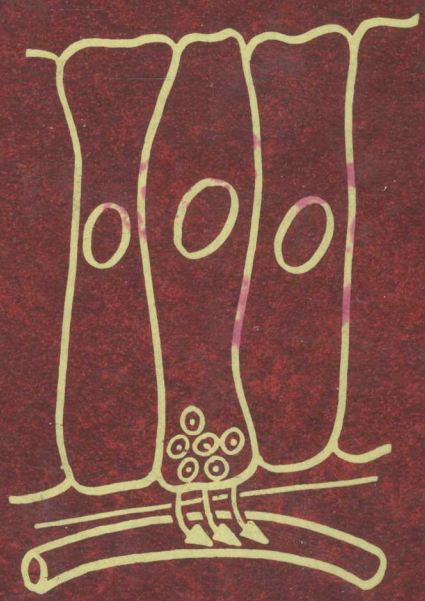
Gastrointestinal Endocrinology



James C. Thompson
George H. Greeley, Jr.
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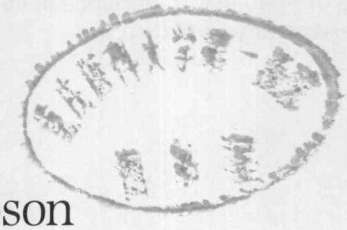

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Writing and editing this book has been great instructive fun. We believed that we could provide a service to students of gut hormones by bringing together in one place as much information as we could practically assemble. The evolution of our plan is detailed in the Introduction, Chap. 1.

Development of gastrointestinal endocrinology was slow. After demonstration of secretin and gastrin, a long period of relative inactivity followed. Since 1964, there has been explosive development (Figure). Not all of the agents are true hormones, some have not yet been isolated. We are all greatly indebted to Viktor Mutt who has provided the intellectual stimulus and the substrate for isolation and purification of most recently discovered peptides.

We are grateful to our colleagues in research, visiting scientists and surgical residents, in the Department of Surgery at UTMB, Galveston, and to our colleagues at the University of Arkansas for Medical Sciences and in the Department of Pharmacology and Toxicology, UTMB. We are especially grateful to our superb manuscript secretary, Julie Gips.

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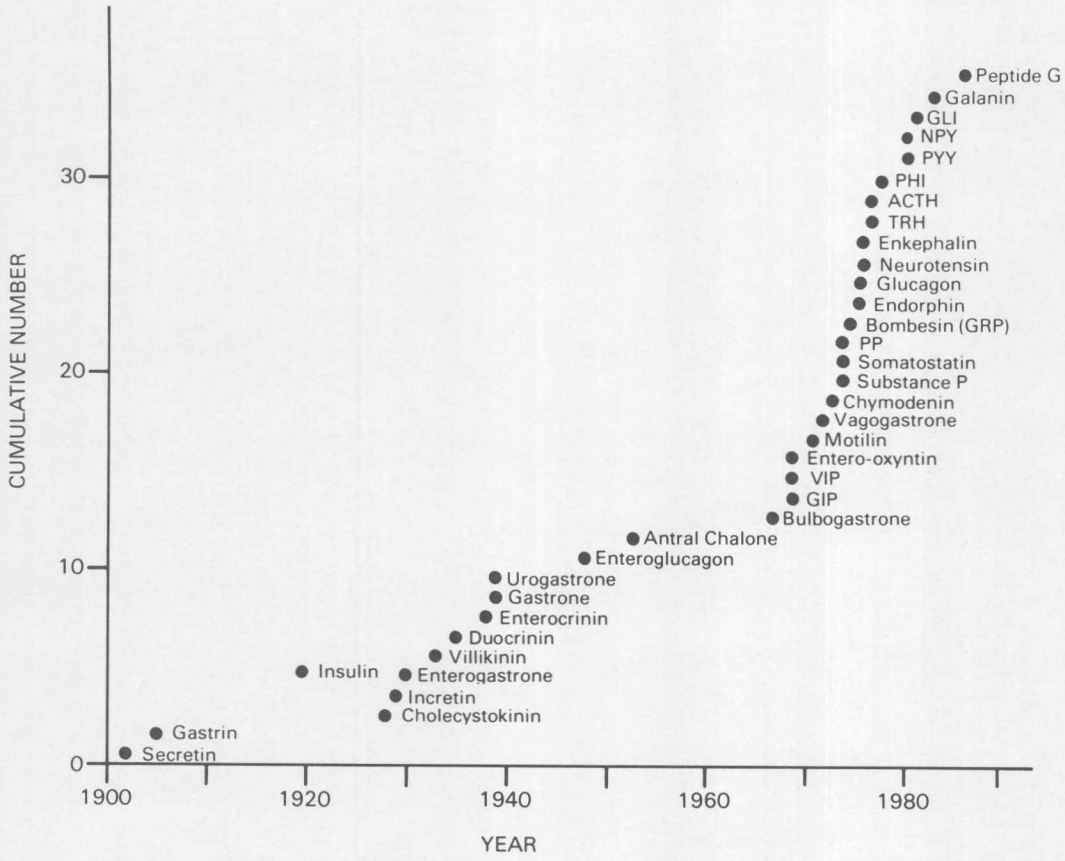

Adapted from Rehfeld JF, in SR Bloom and JM Polak (eds): *Gut Hormones*. New York, Churchill Livingstone, 1981, p. 11. Reproduced with permission.

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Section One

Overview

Chapter 1

Introduction

James C. Thompson, M.D.

The entire field of endocrinology began with gut hormones. The beginning can be pinpointed with precision: the time was the afternoon of 16 January 1902, the place, the University College Hospital, London, and the event was the discovery of secretin. W. M. Bayliss and E. H. Starling [1] elicited the brisk secretion of pancreatic juice following the irrigation of a denervated loop of small bowel with dilute HCl. They understood the significance of their finding immediately; they suggested that a chemical messenger was released from the acidified intestine to mediate the response. According to Babkin [2], the word "hormone" was introduced by Starling on the suggestion of W. B. Hardy [3]. The word is from the Greek $\delta\rho\mu\alpha\omega$, meaning "I arouse to activity," or, "I excite."

The discovery of secretin activity was followed shortly by Babkin's discovery of gastrin [4,5], but more than 50 years elapsed before the physiologic and pathologic implications of gastrointestinal hormones were realized. The Zollinger-Ellison syndrome [6] was described in 1955, but the actual breakthrough occurred in the early 1960s, when gastrin [7] and secretin [8] were isolated almost simultaneously. Gregory and Tracy [9] announced the constitution and properties of gastrin in 1964, initiating the biochemical era of gut endocrinology. Secretin [10] and cholecystokinin [8] were next isolated and chemically characterized and, by now, a wide variety of new hormones and related substances are recognized [11,12]. New developments in the field have been celebrated and analyzed in conferences at Los Angeles [13], Edmonton [14], Aalborg [15], Stockholm [16], Erlangen [17], Rochester, NY [18], Galveston [19], Asilomar [20], Rome [21], Lausanne [22], Bieto [23], Cambridge [24], Stockholm [25], and Rochester, MN [26], among others. Much of the rapid development of gastrointestinal endocrinology is due to the brilliant contributions of Gregory, Jorpes, Mutt, and Grossman. Important advances have been made by surgeons, especially Dragstedt, Woodward, Zollinger, and Ellison.

Gastrointestinal hormones are chemical messengers that regulate gut function. Most of them—perhaps all, depending on definitions—are peptides. They are secreted by endocrine cells that are

widely distributed throughout the gastrointestinal mucosa in such profusion that the gut has been called the largest endocrine organ in the body. Although we call these agents hormones, they do not always function in a truly endocrine fashion, that is, the active peptides are not always discharged into blood vessels to act on a distant site. Sometimes they are discharged and act locally in a paracrine fashion. They also may serve as transmitting agents for nervous impulses, or they may be discharged into blood vessels following nervous stimulation in a true neuroendocrine fashion (Fig 1-1). Gastrointestinal endocrinology includes all of these functions, and we do not always know when an agent acts locally as well as at a distant site.

What about the present volume? More than 2 years ago, my colleagues and I began the periodic ritual of preparation for renewal of the grant that has supported our work for several years (AM-15241), but this time, things were different. Several coinvestigators (my coeditors, as well as Cary Cooper, Ph.D., and Pomila Singh, Ph.D.) and I had been encouraged at this same time to prepare and submit a Program Project Grant, which would provide support for our coordinated efforts in the field of gastrointestinal endocrinology. As part of this combined effort in grant writing, we decided to embark on a review of the literature concerning gastrointestinal hormones; we assigned these reviews to one another and to various visiting scientists and research fellows in our laboratory. The early drafts were concise, and a brief précis of these reviews was published in 1984 [27].

In short order, however, a brisk competition arose regarding the scope of these reviews; they became increasingly definitive. The senior investi-

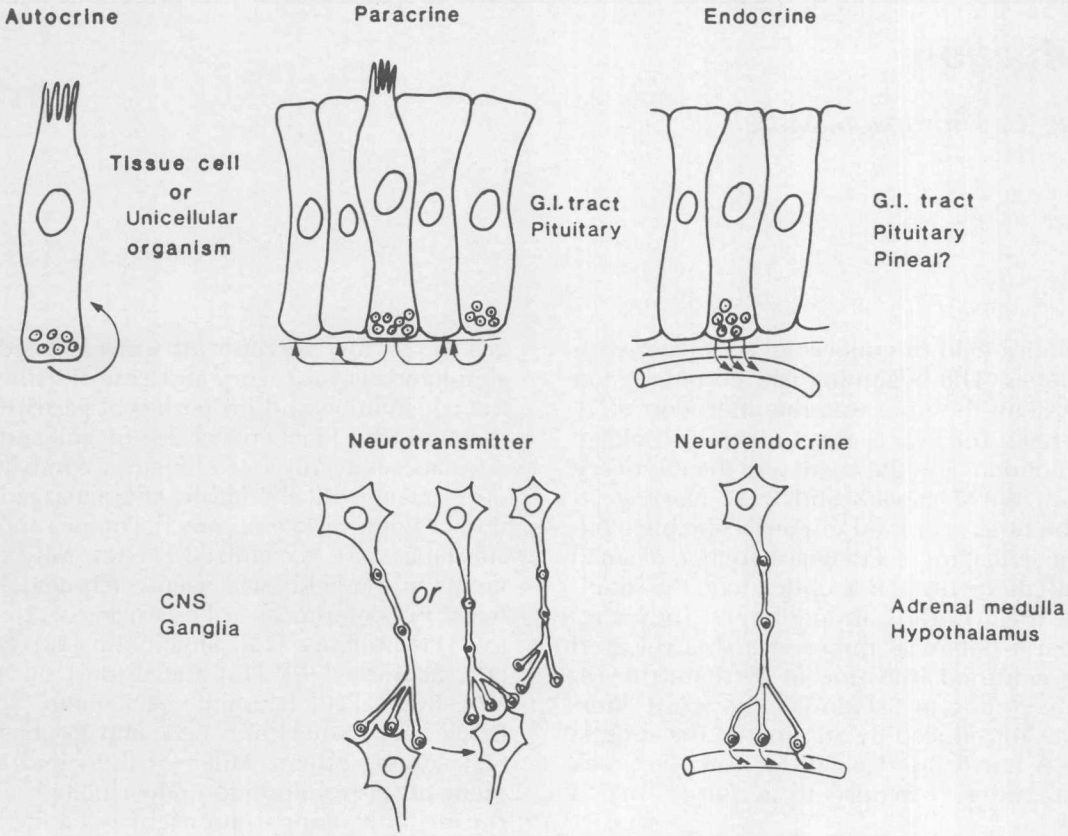

Figure 1-1. Modes of action of regulatory peptides. The secreted autocrine product acts locally on the cell of origin. Paracrine secretion involves local action of a peptide on neighboring cells, either by systems of extracellular fluid transport or by means of gap junctions between cells. Endocrine secretion involves secretion of a regulatory peptide into the bloodstream to affect distant targets. Nerve cells, shown in the lower portion of the figure, may transmit impulses from cell to cell by means of axodendritic or presynaptic axoaxonic synapses (neurotransmitter). Neurohumoral secretion (neuroendocrine) refers to release of a peptide product of the neuron into the bloodstream to act on other tissues. (From Krieger DL, Clin Res 31:342, 1983.)

gators in our group were frankly unprepared for the enthusiastic manner in which our colleagues responded to the challenge of providing a summary of all that is known about our field. We were overwhelmed by searching, thoughtful, critical reviews that appeared to deserve more exposure than to appear in truncated form as a synopsis in a grant request (however vital). We decided to write a book. And here it is.

We have tried to provide fellow students with information of use in the study of gastrointestinal endocrinology. We have provided a summary of the investigative techniques that we use in studying the synthesis, storage, release, transport, actions and interactions, and catabolism of regulatory peptides of the gut. There is a relatively detailed series of descriptions of various surgical procedures that we have found useful. We have discussed in detail some models for study: receptors for gastrin and cholecystokinin, the blood-brain barrier, and the various prototypic actions of gut hormones. Additionally, we have provided information on the ontogeny of gut peptides and on the effects of aging, on the possible role of gut hormones in cancer, and on the effects of circadian rhythms on gut peptides.

We have discussed the classic gut peptides, other agents that have been closely studied, nonpeptide agents, and other peptides that we have arbitrarily called candidates (clearly recognizing that the veil between anointed and nonanointed is constantly in motion).

We have discussed important regulatory interrelationships, the calcium-gut hormone interactions, the brain-gut axis, the entero-insulinar axis, and the gonad-gut axis. We finish with a short section on the clinical significance of gastrointestinal hormones.

At the end is an appendix that provides the amino acid composition and molecular weights of gut hormones and related substances. We provide this information for about 60 peptides, plus histamine, serotonin, and some prostaglandins. These range alphabetically from bombesin to urogastrone, and in familiarity from gastrin to sauvagine.

The editors are grateful to colleagues in our laboratory who have put most of this information together. My coeditors and I have gone over every page in our very best effort to weed out all errors and to include all pertinent information. Long familiarity with such projects forces us to recognize that errors will be present. We apologize for them and invite the reader to notify us so that they can be corrected. We have provided an extensive bibliography that we hope will help any student embark upon further closer study of any area of interest. Interested readers may wish to consult reviews that have been helpful and informative to us [11,12,27–49].

We have found the study of gastrointestinal hormones to be a wonderful, exciting, and highly rewarding field in which to spend a lifetime. We commend it to all potential and fellow students. We hope that this book will provide worthwhile information to gastroenterologists and to surgeons with interest in the gut, as well as to endocrinologists, physiologists, pediatricians, internists, and all clinicians and scientists who are interested in gut function.

Last, I wish to acknowledge the immense contributions of research fellows (listed below), who have come from all over the world to work in our laboratory. Their efforts are responsible—in large part—for whatever has been achieved. 1) B. Guy Clendinnen, F.R.C.S., 1970, UK. 2) B. Michael Jackson, M.D., 1970, USA. 3) Herbert H. Bunchman, M.D., 1970, USA. 4) Jack L. Conlee, M.D., 1971, USA. 5) Larry C. Watson, M.D., 1971, 1976, 1979, USA. 6) J. Robert Searcy, M.D., 1971, USA. 7) Ulf B. E. Hjelmquist, M.D., 1972, Sweden. 8) John C. W. Evans, F.R.C.S., 1972, UK. 9) Melvyn Lerman, M.D., 1972, USA. 10) H. Dieter Becker, M.D., 1972, 1975, 1976, West Germany. 11) Charles S. Clark, Jr., M.D., 1972, USA. 12) Robert A. D. Booth, F.R.C.S., 1973, UK. 13) Takaho Watayou, M.D., 1973, Japan. 14) Hugo V. Villar, M.D., 1973–1975, USA. 15) N. Ian Ramus, F.R.C.S., 1975, UK. 16) H. Roberts Fender, M.D., 1975, USA. 17) Peter J. Curtis, F.R.C.S., 1976, UK. 18) Osvaldo L. Llanos, M.D., 1975–1977, Chile. 19) Janusz S. Swierczek, M.D., 1976, 1977, 1979, 1983, Poland. 20) Reinhard K. Teichmann, M.D., 1977–1978, West Germany. 21) Thomas A. Miller, M.D., 1977, USA. 22) Talaat Khalil, M.D., 1977, 1984, Egypt. 23) J. Tom Peurifoy, M.D., 1977, USA. 24) Anton Schafmayer, M.D., 1977–1978, West Germany. 25) Webster S. Lowder, M.D., 1978, USA. 26) Masahiko Miyata, M.D., 1978, 1980, Japan. 27) Jean-Alain Chayvialle, M.D., 1978, France. 28) Sergio Guzman, M.D., 1977–1979, Chile. 29) Janos Lonovics, M.D., 1979, 1984, Hungary. 30) William A. Banks, 1978, USA. 31) Peter G. Devitt, F.R.C.S., 1980, UK. 32) Amram Ayalon, M.D., 1980, Israel. 33) Raul Yazigi, M.D., 1980, Chile. 34) Patricia M. Simon, Ph.D., 1980, France. 35) Kazumoto Inoue, M.D., 1980, 1981, 1982, Japan. 36) Isodoro Wiener, M.D., 1981, Mexico. 37) Per Lilja, M.D., 1981, Sweden. 38) Gerald M. Fried, M.D., 1982, Canada. 39) Xue-Guang Zhu, M.D., 1982, China. 40) Owen Winsett, M.D., 1982, USA. 41) Beverly Lewis, M.D., 1982, USA. 42) W. David Ogden, M.D., 1982, USA. 43)

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Section Two

Methods

Chapter 2

Radioimmunoassay of Gut Peptides

George H. Greeley, Jr., Ph.D.

A radioimmunoassay (RIA) is a biochemical technique in which the concentration of almost any naturally occurring or fabricated substance can be measured quantitatively. Radioimmunoassays, in comparison to most biologic assays, are exquisitely sensitive and highly specific.

The RIA is founded on a simple concept that utilizes the inherent specificity of the immune reaction between an antigen and its antibody [1–3]. In a majority of cases, a fixed amount of antibody is incubated with a standard amount of radioactive antigen (i.e., ^{125}I-peptide) and graded amounts of nonradioactive antigen (reference standard or test sample). During this incubation period, the radioactive antigen and the nonradioactive antigen compete for binding sites on the antibody (Fig 2-1). A dose-response curve can be generated from the reference standard, and this is used to determine the amount of antigen in test samples.

There are three basic requirements for the development of a radioimmunoassay. These are 1) a specific and sensitive antibody; 2) a radiolabeled antigen; and 3) a quick and reproducible method for separating antibody-bound antigen (bound) from unbound antigen (free), since the immune complex does not normally precipitate in a test tube. Parenthetically, the radioactive and nonradioactive ligands need not exhibit identical immunologic behavior. The standard and test samples, however, should compete similarly for antibody sites, but they need not be chemically or biologically identical.

The generation of an antibody in a laboratory animal is usually the first step to establishing an RIA. However, other substances that possess high affinity binding sites for specific biologic molecules, such as a membrane receptor, enzyme, or blood protein (e.g., cortisol-binding globulin, thyroid hormone-binding globulin), can be used in place of a specific antibody. In order to generate an antibody, a semipure antigen is adequate, although a pure antigen is preferred. If the antigen alone is inadequately antigenic, it can be linked chemically to a larger molecule, such as bovine serum albumin, using the carbodiimide or glutaraldehyde conjugation method [4]. If the peptide antigen consists of less than 30 amino acid residues, it is usually advisable to link it to a larger

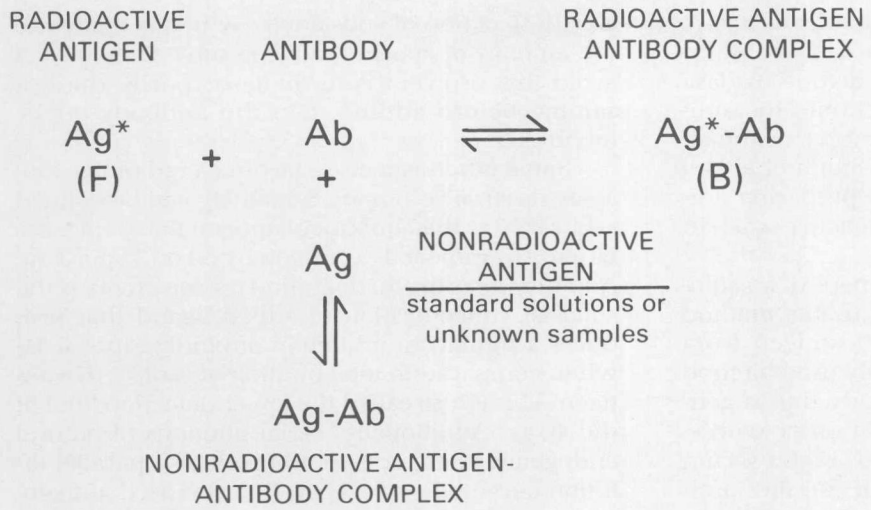

Figure 2-1. The competing reaction that is the basis of a radioimmunoassay. (F) = free; (B) = bound.

molecule. The antigen may also need to be modified chemically, which allows attachment to a larger molecule. Alternatively, a small fragment of the antigen (determinant) can be used in place of the entire antigen.

Antibodies are usually generated in rabbits, guinea pigs, goats, or sheep. There is, however, no set protocol that can be relied upon to ensure success. In our laboratory, the antigen is always emulsified with complete Freund's adjuvant, and pertussis vaccine (0.5 mL) is given along with the antigen. Antigen (100 μg to 1 mg) is injected into 50–100 intradermal sites on the shaved back of a rabbit [4]. It is advisable to collect some preimmune serum, which may be necessary in control experiments. The formula of 1:1 (vol:vol) of antigen to Freund's adjuvant is used. The frequency of booster injections varies from every 2 weeks to every 2–3 months, depending upon the response of the immunized rabbit. The amount of the antigen given in the booster injection also varies, depending upon the response of the rabbit and the purity and availability of the antigen. Blood is collected for titering every week after the second or third booster and checked for antibodies. When blood is collected, an antibody titer curve, using dilutions of 1:100–1:100,000, is constructed. An antibody with a titer of at least 1:500 is desirable at this time. Sometimes the immune response of the immunized animals will drop precipitously after repeated inoculations. If so, it may be better to start over with a different animal. At times, however, better results may be achieved with booster injections with smaller doses of antigen spaced farther apart. Patience is required in this stage of RIA development, as it may take months before a satisfactory antibody is developed. Generation of antibodies is not an exact science.

The second step in establishing an RIA is to obtain a radioactive antigen. This may, at times, be purchased from a commercial source; many radioactive pharmaceuticals, steroids, polypeptides, peptides, neurotransmitters, and vitamins with suitable specific activities are currently available. The radioactive ligand must be pure and stable, and most importantly, one must be confident of its identity. If a radioactive ligand cannot be purchased, it must be prepared. Most frequently, proteins, peptides, or other substances can be iodinated with ^{125}I or ^{131}I.

The Hunter and Greenwood method [5] for iodination is usually used with minor modifications that suit the particular antigen. In brief, a small amount of ligand (2–10 μg) is mixed with chloramine-T and ^{125}I, resulting in a radiolabeled ligand. The presence of a tyrosyl or histyl residue in proteins or peptides is necessary for this method of iodination; otherwise, a modified form of the peptide containing a tyrosine can be synthesized. The iodination reaction is halted with the addition of sodium metabisulfite or a small amount of bovine serum albumin. The next step is to separate the radiolabeled peptide from other contaminants. This can usually be done easily and quickly on a small gel or affinity column. High pressure liquid chromatography (HPLC) may also provide an excellent means to purify the radiolabeled peptide, that is, to separate it from free iodine and fragments of peptides. In fact, the HPLC method may be superior to the gel and affinity chromatography methods, since HPLC can separate monoiodo forms from polyiodo forms of the peptide.

Since chloramine-T is a strong oxidizing agent, use of this method may damage the antigen. Alternate methods include the milder lactoperoxidase method [6], the introduction of ^{125}I onto the antigen using the Bolton-Hunter reagent [7], or the iodogen method [8,9]. For example, Singh et al [10] have used the iodogen method in preparing a ligand for use in a method to measure gastrin receptors.

The next stage in the development of a radioimmunoassay is to decide upon a usable method for separating the antibody-bound antigen from the free antigen. The most frequently used method takes advantage of a second antibody that is generated against the first antibody. In other words, sheep or goats are immunized with rabbit serum (or the gamma globulin fraction) if the first antibody is generated in rabbits. The first antibody might be, for example, a rabbit-antigastrin antibody, and the second, a sheep antirabbit antibody. Other useful methods include the use of polyethylene glycol, dextran-coated charcoal [11], alcohol, and protein A. Protein A (Pansorbin) is a product derived from *Staphylococcus aureus* cells that selectively binds the IgG fraction of blood with a high avidity [12]. Protein A is commercially available from Calbiochem-Behring (San Diego, CA). Using a suitable separation method, the free and bound ligands are separated, and one or both are counted in a gamma or scintillation counter. A standard line is then constructed, and the amount of antigen in the test specimens can be calculated by a direct comparison with the standard line.

In the course of establishing a radioimmunoassay, the method must be validated. Despite the specificity of the immune reaction, a variety of nonspecific factors can interfere and disrupt the immune reaction taking place in the test tube. The specificity of the particular assay must be evaluated by testing all substances with structural similarities. If, for example, a radioimmunoassay for cholecystokinin-33 (CCK-33) is under development, substances related to the antigen-ligand must be examined, since any agent that is chemically related may cause interference immunologically. Whether CCK-8, CCK-4, CCK-12, or gastrin, for example, can compete with CCK-33 for antibody sites must be evaluated. Similar evaluations must be performed in the development of an assay for steroid hormones, certain drugs, polypeptide hormones, and their metabolites. A variety of nonspecific factors that are not related to the antigen can also cause interference, including the ionic environment, pH, or the presence of heparin in the specimen. Temperature also brings about a variable destruction of substances, which is related to the amount of specimen in the tube. One way to avoid this problem is to dilute or purify the test sample before adding it to the antibody-buffer mixture.

Some other aspects regarding a radioimmunoassay deserve comment. Sensitivity can be defined arbitrarily as that amount of antigen that depresses binding of radioactive antigen by 50 or 33 percent. Another more useful definition for sensitivity is the smallest concentration of added ligand that produces a significant inhibition of binding ($p < 0.05$) when compared to total binding of radioactive antigen. This is also called the lower detection limit of the assay. Additionally, serial dilutions of natural endogenous antigen should produce a parallel dilution curve when compared to standard antigen. In other words, dilution curves of blood- and tissue-derived antigen must be parallel to the dose-response line of the standard. Parallelism is not a conclusive test, since there can be substances that behave similarly, if not identically, to the antigen in the radioimmunoassay.

We will not review the numerous published RIAs for multiple gut peptides, since references will be provided in the discussion of each agent. We will, instead, provide some general remarks relative to the development of a radioimmunoassay for CCK-33/39 in order to demonstrate the specific steps in development.

In the process of achieving a specific assay for CCK-33/39 [13–15], rabbits were first immunized with pure or semipure preparations of CCK-33/39. A combination procedure of 16 percent pure CCK-33/39 and 99 percent pure CCK-33 without conjugation to a larger protein was used successfully. The antibody has been shown to be specific for the large variants of CCK with negligible to no cross-reactivity with small forms of CCK (CCK-8, CCK-4) or with any of the multiple forms of gastrin. The antibody that was initially developed by Rayford et al [13] is unique in that it requires the entire CCK molecule for complete cross-reactivity. Further development by Rayford and Greeley has improved this assay [14,15].

In this particular radioimmunoassay procedure, CCK-39 is radioiodinated using chloramine-T. The radiolabeled hormone is usable for 4–6 weeks, with negligible loss of binding to the antibody. Although unextracted plasma samples are used in some laboratories, other laboratories extract and concentrate CCK before measurement. The use of the C_{18} Sep-Pak (Waters Associates, Milford, MA) has proved useful and seems to result in lower basal levels of CCK, although these differ-

ences are not fully resolved [16]. On the other hand, other laboratories have used plasma extracts rather than plasma in their assays, and they have reported CCK-33 levels similar to those reported by a laboratory that uses ordinary plasma [17,18]. The use of Sep-Paks in extracting neurotensin from plasma specimens has also proved useful [19]. Extraction of neurotensin and CCK immunoreactivity from plasma specimens results in lower values when compared to unextracted specimens [20]. Another technique that appears promising is the use of HPLC for the isolation and separation of the molecular forms of CCK with subsequent radioimmunoassay [21,22].

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