On the Measurement of Cholecystokinin

A hormone is a chemical transmitter that is secreted from one part of the body and circulates in the bloodstream to reach a distant target in another part of the body where it exerts its biologic effect. At its inception, this was a revolutionary concept that gave birth to an entire physiological and, later, medical discipline. Early on, hormones were discovered by their biologic actions. However, the physiology of hormones could not be assessed without quantification. Fundamental to the study of hormones was the ability to measure concentrations in the blood. Although biological assays have been the cornerstone of endocrinologic measurements, the development of the radioimmunoassay (RIA) completely changed the field of endocrinology *(1)*. The attractive features of RIA include (*a*) applicability to most hormones, (*b*) ease of performance, and (*c*) relatively low cost, as well as high degrees of (*d*) accuracy, (*e*) sensitivity, and (*f*) specificity.

Cholecystokinin (CCK) was discovered in 1928 on the basis of the ability of intestinal extracts to stimulate gallbladder contraction in dogs *(2)*. Later it was recognized that CCK was a potent stimulant of pancreatic enzyme secretion *(3)*. However, it was not until 1966, when CCK was purified, that the primary sequence was determined *(4, 5)*. Early estimates of CCK-like activity in blood were based on biological assays such as pancreatic secretion or gallbladder contraction. However, these estimates were fraught with confounding problems that existed in whole animals, such as the effects of other hormones or neural influences. To circumvent these problems, a sensitive and specific in vitro bioassay was developed *(6)*, but this method is labor-intensive and cumbersome and is not readily available to clinical laboratories. Attempts to develop an RIA for CCK had to overcome a number of unique challenges. Among these were (*a*) the multiple molecular forms of CCK, (*b*) amino acid sequence similarity between CCK and gastrin, (*c*) low blood concentrations of CCK, (*d*) limited peptide availability, and difficulties with (*e*) isotope labeling and (*f*) peptide synthesis. It should not be surprising then, that solutions to these many problems were slow in coming.

CCK was originally identified as a 33-amino acid peptide (CCK-33); however, since its discovery, multiple molecular forms have been described. In several species, biologically active forms ranging in size from CCK-83 to CCK-8 have been found to exist in intestine, brain, and blood *(7, 8)*. All forms are derived from a single *CCK* gene by posttranslational or extracellular processing *(9)*. The biologically active portion of the molecule is its amidated carboxyl terminus. All forms of CCK larger than CCK-8 are biologically active. Therefore, to measure physiologically relevant CCKs, assays must detect the carboxyl terminus of all molecular forms. A major difficulty in developing CCK assays has been its structural similarity to gastrin. CCK and gastrin comprise a family of gastrointestinal peptides that share an identical carboxyl-terminal pentapeptide sequence. To develop a specific CCK RIA that does not cross-react with gastrin, the antisera should recognize the tripeptide sequence at the amino terminus of CCK-8, which is common to all forms of CCK but is dissimilar to gastrin.

Over the last 20 years, a number of RIAs and a bioassay for CCK have been developed *(6, 10–24)*. Although most have not undergone the extensive validation described in the current study by Rehfeld *(25)* in this issue, there is general agreement that CCK concentrations in the circulation are relatively low (in the picomolar range). In contrast, plasma concentrations of gastrin are 20–100 times higher. Thus, even slight antibody cross-reactivity with gastrin poses a substantial problem for accurately measuring blood concentrations of CCK. Accordingly, the sensitivity and specificity of an accurate CCK assay must be extremely high.

Even after the discovery of CCK-33, only limited amounts of material were available for raising antibodies. The largest bioactive form of CCK that has been described is CCK-83 *(26)*. Abundant forms of CCK in tissue and blood include CCK-58, CCK-33, CCK-22, and CCK-8. Unfortunately, large forms of CCK are difficult to purify and still are not readily available. As such, CCK-83 and CCK-58 have not been used as standards for most assays, and the cross-reactivity with many antibodies is unknown. It has even been suggested that larger forms of CCK are less immunoreactive than smaller forms, perhaps because of tertiary structure *(27)*.

Sulfation of the tyrosine residue at position seven from the carboxyl terminus of CCK is critical for biological activity. Because of this, synthesis of moderately large forms of CCK had not been possible until recently, and these peptides are still not commercially available.

The final problem in the development of a CCK RIA has been difficulty with isotopic labeling of the peptide. Oxidative methods to label CCK tended to destroy biological activity of the molecule through oxidation of the methionine residue in position three from the carboxyl terminus. Oxidation of CCK reduces its biological activity 100- to 1000-fold.

Identifying the problems in developing a CCK assay is one thing, successfully overcoming those problems is another. In the current issue of *Clinical Chemistry*, Rehfeld has undertaken the ambitious task of developing an accurate RIA for measuring blood concentrations of CCK. He has tackled each of the problems listed above. Selection of a proper antigen for raising specific CCK antisera was a critical initial step. A variety of antigens, including natural porcine CCK-33 and synthetic nonsulfated CCK-33 and -29, as well as synthetic sulfated CCK-4, -12, and -13, were all used as immunogens. Two types of tracers were used, including CCK-33 labeled by nonoxidative conjugation and Bolton-Hunter-labeled CCK-8. Characteristics of the antisera that were critically evaluated included the titer, affinity, specificity, and homogeneity of binding kinetics. Seventy-eight rabbits, 29 guinea

pigs, and 8 mice were immunized. The most successful immunization strategy used an immunogen consisting of a CCK-12 analog corresponding to O-sulfated CCK-10 extended at the N-terminus with a diglycine bridge for carrier coupling. The resulting rabbit antiserum (Ab. 92128) was of high titer (>500 000) and bound sulfated CCK-8, CCK-22, CCK-33, and CCK-58 with nearly equimolar potency and with essentially no cross-reactivity with gastrin. This latter point was demonstrated in three ways. First, Ab. 92128 did not detect gastrin in chromatographically purified plasma extracts. Second, there was no correlation between CCK and gastrin plasma concentrations in humans. And third, infusion of gastrin-17 into human subjects did not increase plasma CCK immunoreactivity. With this RIA, plasma CCK concentrations averaged ~1 pmol/L under basal conditions and increased to ~5 pmol/L after ingestion of a meal. These estimates are in the range of other accepted assays.

The discovery of CCK-58 in tissue and the circulation required special treatment to preserve forms larger than CCK-33 *(28)*. It was suggested that immediate acidification of plasma was necessary to prevent the in vitro degradation of CCK. The current study confirmed that larger forms of CCK were detectable only after plasma was acidified and that only CCK-22 and smaller forms were present in neutral extracts, thus confirming that CCK-58 is a major component of CCK-like immunoreactivity in plasma. Unfortunately, large forms of CCK are not available as standards for RIAs, and it still remains to be determined whether the immunoreactivity and biological activity of CCK-58 are equivalent to other molecular forms. In future studies, these determinations and the ability to compare CCK values by various RIAs will depend on the manner in which plasma is collected and extracted, the antibody that is used, and the epitope to which it is directed. These challenges and the lack of CCK RIAs for general use are hurdles that remain to be overcome. Nevertheless, this report by Rehfeld represents an exhaustive and careful attempt to develop the best characterized CCK RIA to date.

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Rodger A. Liddle
Department of Medicine
Box 3913
Duke University Medical Center
Durham, NC 27710
Fax 919-684-8857