Update Radioimmunoassay In 1984

Rosalyn S. Yalow, Ph. D

Solomon A. Berson Research Laboratory Veterans Administration Medical Center, Bronx, NY
Department of Clinical Sciences Montefiore Medical Center, Bronx, NY

Radioimmunoassay (RIA) is now employed in thousands of nuclear medicine and clinical laboratories throughout the world to measure hundreds of substances of clinical or biologic interest. In most routine laboratories the assays are performed with reagents supplied by commercial sources and the users are often unfamiliar with potential pitfalls in the use of these procedures. Probably the two major problems associated with the application of RIA for the measurement of peptide hormones are the limited sensitivity of the assay which requires the use of virtually undiluted plasma resulting in the introduction of non-specificity in the assay procedure and the fact that some peptides may be found in the circulation in more than one molecular form having different biologic activities. In this presentation I shall first review briefly the assay for parathyroid hormone which was first described in 1966 [1] but which is the assay whose interpretation remains the most difficult because of the problems of heterogeneity. Then I shall discuss some recently completed work from my laboratory on the ontogeny and post-translational processing of some peptides common to the brain and gut and will conclude with some preliminary clinical studies that describe a different approach to determination of integrated hormonal output.

The first evidence for immunochemical heterogeneity of peptide hormones in plasma was the demonstration that different antisera recognized that human parathyroid hormone (hPTH) in plasma was not identical with that in glandular extracts[2]. It was observed that a constant factor which could be used to superpose a plasma dilution curve on a curve of standards obtained from an extract of a normal parathyroid gland for two antisera (272 and 273) did not permit superposability of dilution curves employing the same standard and plasma with another antiserum (C329) (Fig. 1). Furthermore, the apparent disappearance of immunoreactivity following surgical removal of the secreting tissue depended on the antiserum used for the assay, a more rapid rate being observed with C329 than with 273 (Fig. 2). The rapid rate of disappearance of immunoreactivity as measured with C329 paralleled the decrease in serum calcium. The discrepancies between assay results with these two antisera were more marked in uremic patients with secondary hyperparathyroidism. It was hypothesized that the presence of metabolic fragments of hPTH produced in the gland or in the periphery could account for the observed heterogeneity.

The nature of immunoreactive hPTH in plasma was revealed by Sephadex gel filtration method-

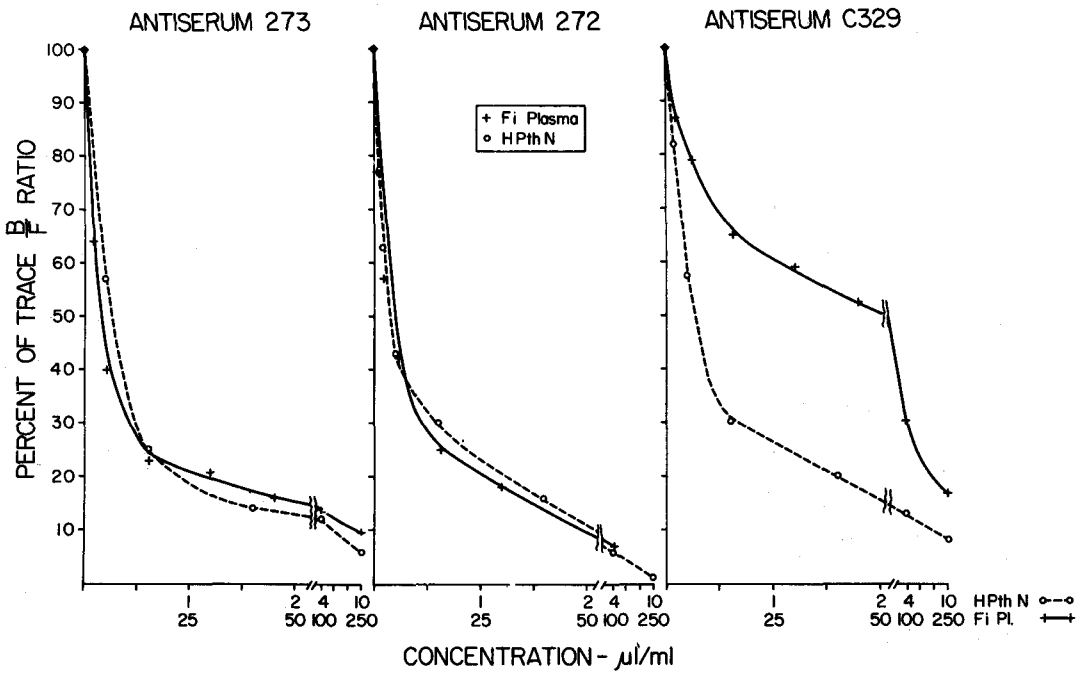

Fig. 1. Inhibition of binding of ^{125}I-Bpth in 3 antisera by pooled plasma from a patient with 2° hyperparathyroidism (+) and by an extract of a normal parathyroid gland (°). (Reproduced from reference 2).

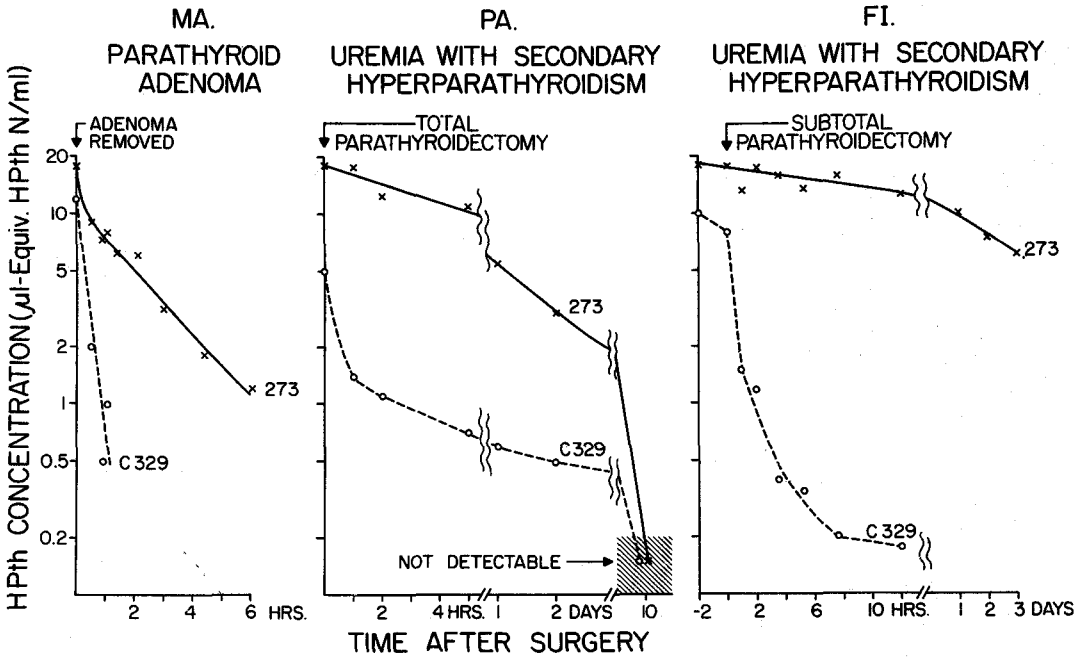

Fig. 2. Disappearance of immunoreactive parathyroid hormone from plasma following parathyroidectomy in patients with 1° or 2° hyperparathyroidism. Plasma samples were assayed in antiserum C329 and antiserum 273 using extract of a normal human parathyroid gland as standard and ^{125}I-Bpth as tracer.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

ology and assay with antiserum C329, an N-terminal antiserum, and antiserum 273, a C-terminal antiserum[2]. It was demonstrated that a biologically inactive C-terminal fragment is the major immunoreactive form of hPTH in plasma. During a calcium infusion that elevates serum calcium in a uremic patient with secondary hyperparathyroidism the biologically active intact hormone as determined with antiserum C329 disappears rapidly and the C-terminal fragment, as measured with antiserum 273, persists (Fig. 3). One can conclude from this study either that the C-terminal

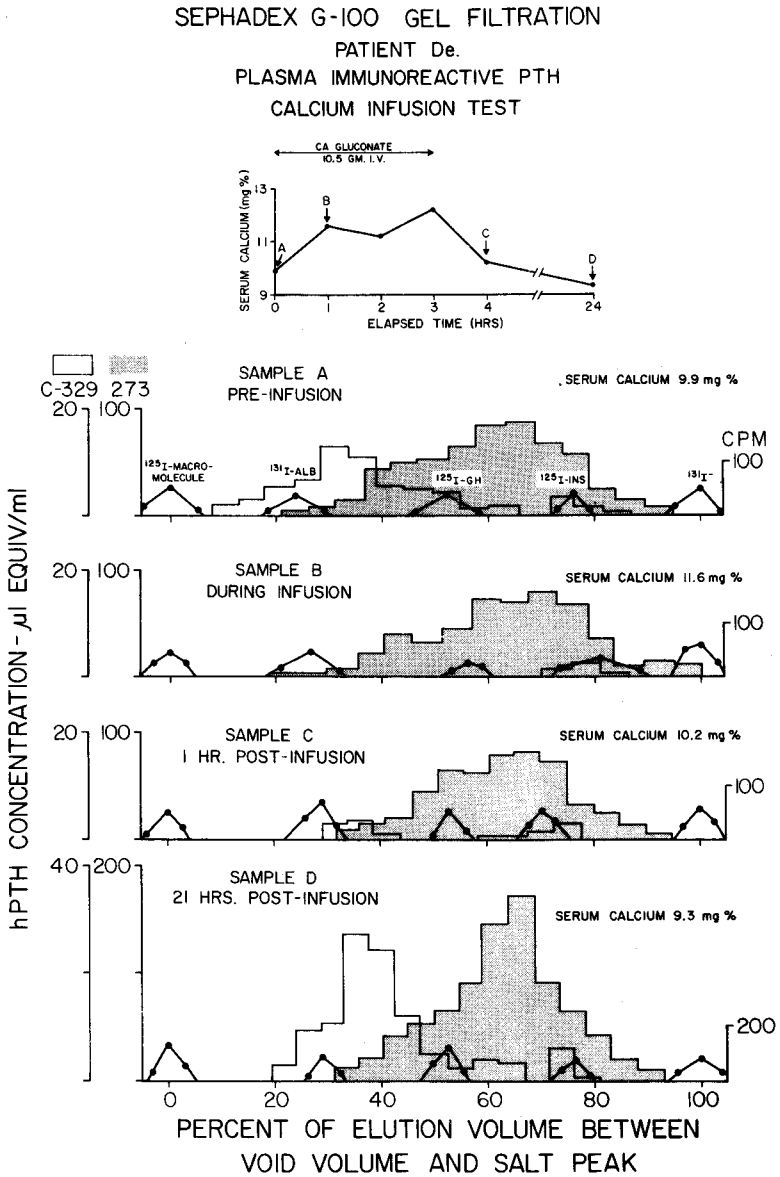

Fig. 3. Sephadex G-100 gel fractionation of immunoreactive hPTH in plasma of a patient with secondary uremic hyperparathyroidism before, during, and after a calcium infusion test. Serum calcium concentrations are shown at the top of the figure. (Reproduced from reference 3).

fragment is not suppressed by calcium administration or that its turnover time is long compared to the time of the infusion. That the latter is the case is evidenced by the observation that following total parathyroidectomy in patients with end-stage renal disease the half-time for disappearance of the C-terminal fragment averages 1 to 2 days, about 20-fold longer than its disappearance half-time in patients with normal kidney function[3,4] (Fig.4). The half-time for disappearance of intact hPTH is much more rapid; in patients with normal kidney function it averages about 15 minutes and in patients with end-stage renal disease about 30-40 minutes (Fig. 5). Thus it would appear that about half of intact hPTH is removed by the kidney and half by other sites in the body. However, the kidney appears to be almost solely responsible for removal of the C-terminal fragment.

The clinical relevance of these observations relates to the choice of antiserum suitable for the diagnosis of primary or secondary hyperparathyroidism. Assuming equal secretory rates and spaces of distribution for the different hormonal forms, the steady state plasma concentrations are proportional to their respective turnover times. Therefore the concentration of the C-terminal fragment is amplified by a factor which depends on the relative turnover times for the two hormonal forms, a factor of about five in patients with normal kidney function. This amplification is useful in that it permits distinction between euparathyroidism and primary hyperparathyroidism which otherwise might not have been possible if only the biologically active component were assayed. However, at least two assumptions must be made if the assay of a biologically inactive form is to be used to reflect the biologically active hormone even in patients

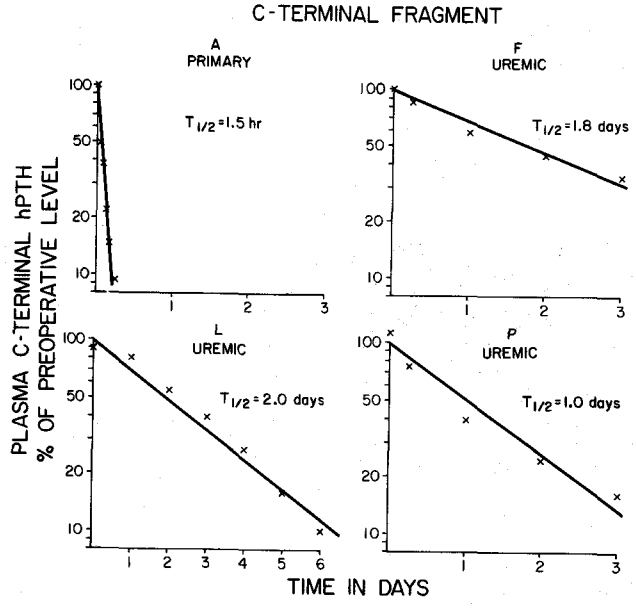

Fig. 4. Disappearance of immunoreactive hPTH as measured by antiserum 273, a C-terminal antiserum, from the plasma of patients with primary or uremic secondary hyperparathyroidism after surgery. The concentrations are plotted in terms of percent of preoperative level. (Reproduced from reference 4).

without renal disease. The assumptions are that there is a constant ratio of turnover times of the active and inactive hormonal forms and that there is a constant ratio in the production rates of intact hormone and fragment. To my knowledge no one has attempted to investigate how "constant" the assumed ratios are. In uremic patients the turnover time of the C-terminal fragment is greatly prolonged and the extent of prolongation is related to the degree of renal dysfunction. Therefore an increase in its immunoreactivity in plasma may or may not reflect increased secretion of biologically active hormone from a hyperplastic parathyroid gland. It may simply represent build-up of that fragment because of a decrease in its rate of removal by the damaged kidney. A systematic quantitative investigation into the relation between the disappearance half-time of the C-terminal fragment and any measure of kidney dysfunction has not been reported. Furthermore it is also not known whether the kidney is involved in peripheral conversion of intact hormone to the specific C-terminal fragment which is removed from the body primarily by the kidney. The clinical interpretation of elevation of immunoreactive plasma PTH using a C-terminal antiserum therefore requires a number of assumptions and considerable additional information. A more direct approach would be the development of simple methodology to extract and concentrate biologically active hPTH from plasma and assay it directly with an N-terminal antiserum or to prepare N-terminal antisera with greatly increased sensitivity. Although there have been some claims concerning the availability of specific N-terminal assays, the direct demonstration of appropriately short turnover times following extirpation of the secreting organ has not been decribed.

Our laboratory continues to be interested in the heterogeneity of molecular forms of peptides. I use the more general term "peptides" rather than "peptide hormones" since it is now appreciated that there are many peptides common to the brain and gut, which are not hormones in the classical sense. Together with Dr. Kiyoshi Ichihara, when he was in our laboratory as a Fellow of the

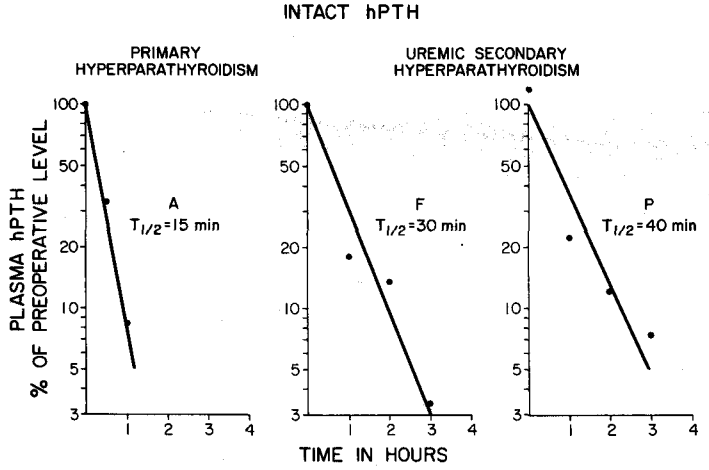

Fig. 5. Disappearance of immunoreactive intact hPTH as measured by antiserum C329 from the plasma of patients with primary or uremic secondary hyperparathyroidism after surgery. The concentrations are plotted in terms of percent of preoperative level. (Reproduced from reference 4).

Solomon A. Berson Fund for Medical Research, we have investigated the ontogeny of cholecystokinin (CCK) and vasoactive intestinal peptide (VIP) in rat and pig[5,6] and secretin only in the rat[5]. Since secretin appears to be less stable post-mortem[7] than many of the other peptides with which we have had experience, we were reluctant to report the secretin concentrations in pig tissues which were obtained by sacrifice of the animals other than in our laboratory[6]. We chose these three peptides because of similarities and differences. In the gastrointestinal tract, CCK is present in neuronal as well as in mucosal tissues[8] whereas VIP is found predominantly in neurons in the muscular layer[9]. Secretin is structurally related to VIP and although secretin-like activity has been reported in brain[10], we have failed to find it there in concentrations in excess of 1 ng/g.

The concentrations and total amounts of iCCK in methanol and HCl extracts of rat tissues are shown in Figure 6[5]. The concentration in the brain was very low at day 3, showed a sigmoidal increase with a maximal change observed between day 7 and day 21 and reached the adult level by day 28. At each age brain iCCK concentrations in HCl extracts were about one-tenth those in methanol extracts. In contrast, the concentrations in duodenum and jejunum were higher

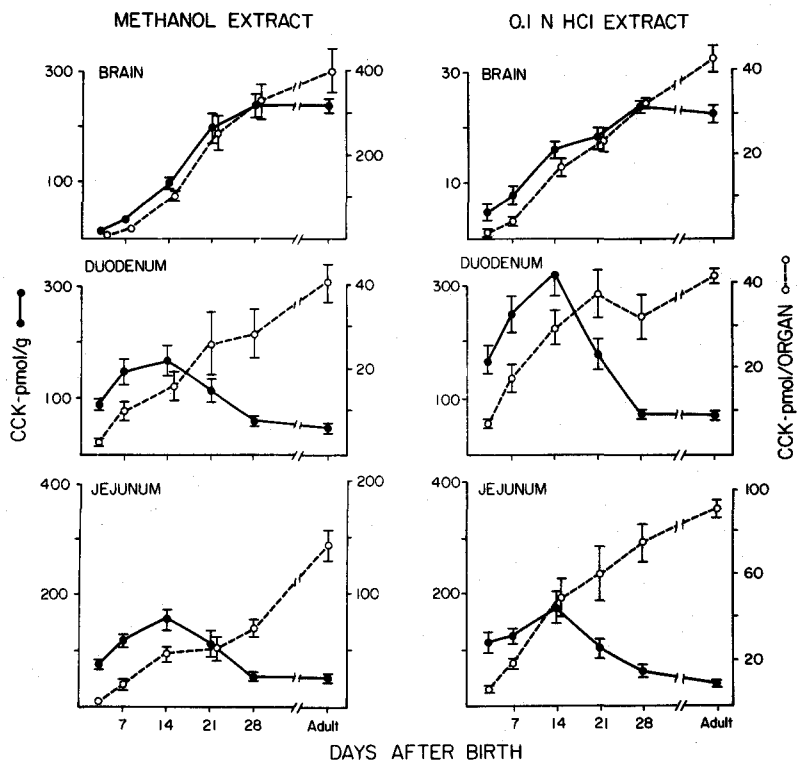

Fig. 6. Immunoreactive cholecystokinin (iCCK) concentration in methanol (left) and 0.1N HCl (right) extracts of rat brain (top), duodenum (middle) and jejunum (bottom) as a function of time after birth. Shown also are the total organ contents for the various tissues. In this and in Figures 7-10 the bars (I) represent the standard error of the mean. (Reproduced from reference 5).

in the day 3 rat than those in the adult. They continued to rise until day 14 and decreased rapidly thereafter reaching adult levels by day 28. It should be noted that iCCK concentrations in the HCl extracts of duodenum and jejunum were comparable to the concentrations found in the methanol extracts. Note that the total iCCK in brain of the adult rat is much greater than that in the duodenum. The nature of the molecular forms of CCK in the acid and methanol extracts were studied by Sephadex gel fractionation and will be described below.

In contrast to the findings with iCCK, iVIP concentrations continued to increase in the duodenum, jejunum and ileum as well as in brain, until day 28, at which time they approximately equalled adult levels (Fig. 7). Peak concentrations in stomach, colon and cecum occurred between day 14 and 21, then fell thereafter to adult levels. Also in contrast with iCCK, the brain content

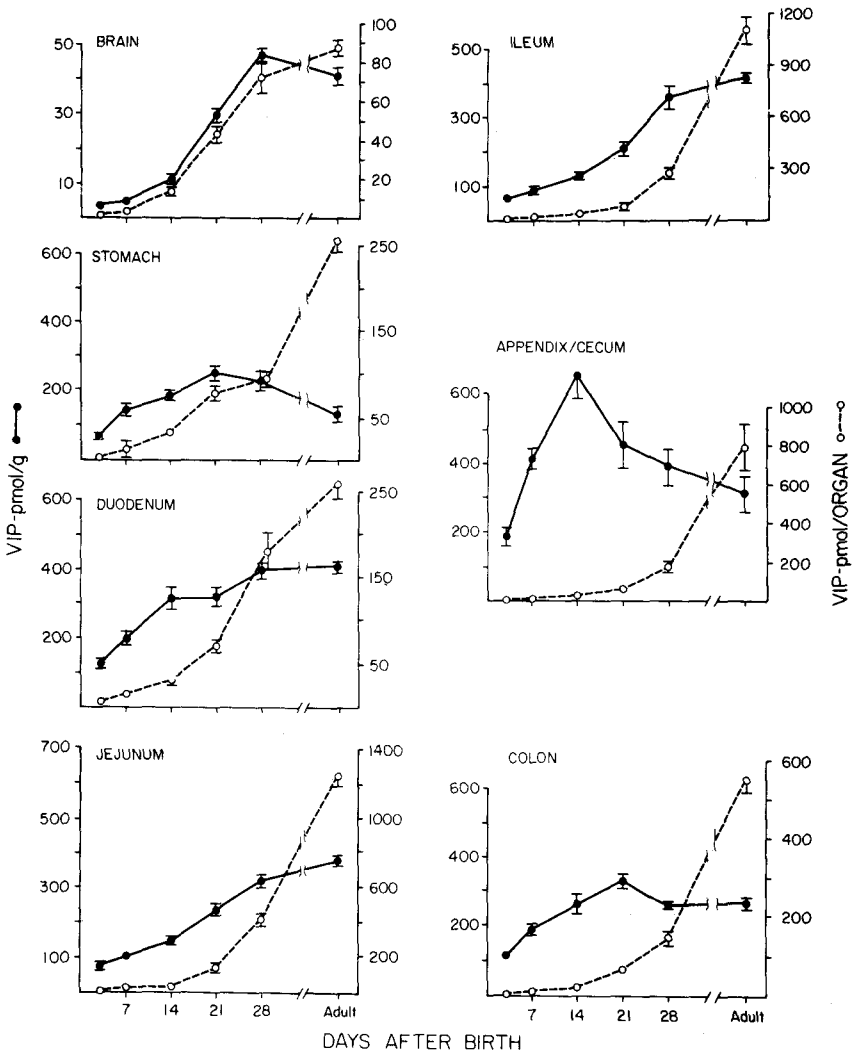

Fig. 7. Immunoreactive vasoactive intestinal peptide (iVIP) in 0.1N HCl extracts of rat brain and in various regions of the gastrointestinal tract. Shown also are the total organ contents. (Reproduced from reference 5).

of VIP in the adult rat is only 40% of that in the duodenum and less than 10% of that in the jejunum or ileum. The ontogeny of VIP in the jejuno-ileal region of the rat has previously been studied by radioreceptor assay[11] and in the brain[12,13] and in duodenum[12] by RIA and although the time periods studied were not identical to those shown here, there is general agreement that VIP concentrations in brain and gut increase strikingly from birth.

Acid extracts of brain, stomach, appendix/cecum and colon contained no measurable immunoreactive secretin (iSEC). Thus, although SEC and VIP have considerable homologies in structure, they are not found in the same organs. Furthermore, unlike VIP, iSEC concentration in the 3 day rat was either not significantly different from (in duodenum and ileum) or much greater than (in jejunum, $p<0.01$) the adult concentrations (Fig. 8).

The differences observed among the patterns of development of these three hormones in the different tissues are that the concentrations appear to increase from birth in neuronal tissues (CCK in brain or VIP) whereas the concentrations in endocrine tissues (SEC) seem to reach or

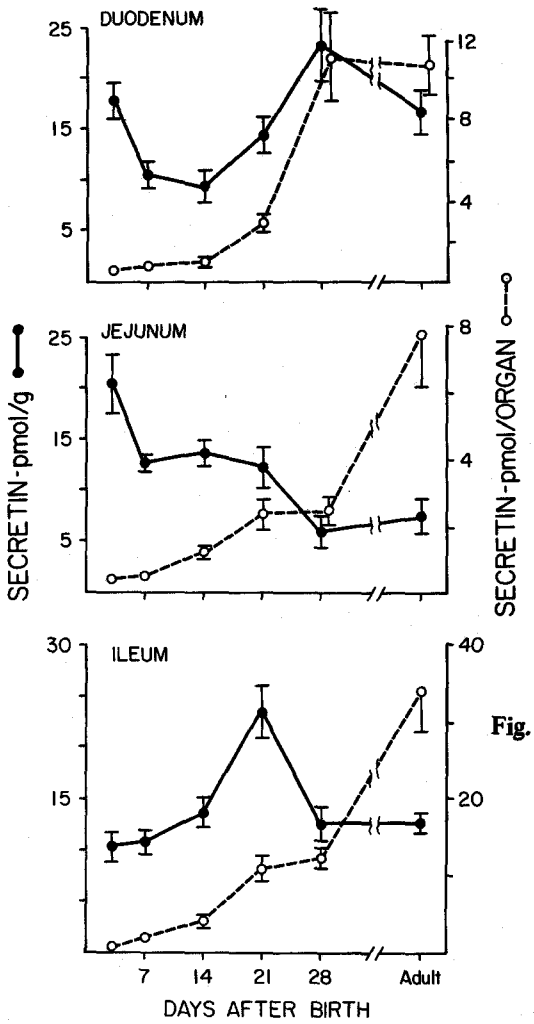

Fig. 8. Immunoreactive secretin (iSEC) concentrations in 0.1N HCl extracts of rat duodenum (top), jejunum (middle) and ileum (bottom). Secretin was not found in brain, stomach, appendix/cecum or colon. Shown also are the total organ contents. (Reproduced from reference 5).

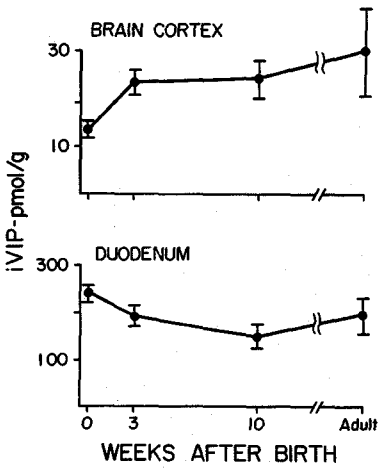

Fig. 9. Immunoreactive VIP as a function of age in acid extracts of pig brain cortex (top) and duodenum (bottom). (Reproduced from reference 6).

exceed adult levels at day 3, the first point in time studied. However, since CCK is found in the gut both in the mucosal and muscle layers, it is possible, therefore, that the peaking at two weeks is due to a rising concentration in the neuronal tissues in the muscle layer and a falling concentration in mucosal tissues.

The concentrations of VIP as a function of age in acid extracts of pig brain, cortex and duodenum are shown in Figure 9[6]. In contrast with the studies in the rat, the concentration of VIP in the pig duodenum is essentially constant from birth to adult life and in the brain is more than one-third that in the adult, reaching adult levels within the first three weeks. The ontogeny of CCK in the pig resembles that of VIP (Fig. 10). Thus CCK concentrations in methanol extracts and acid extracts of the duodenum as measured either with the C- or N-terminal antiserum are essentially constant from birth. In the brain, the concentrations at birth are about one-half of adult levels.

The conclusion to be reached from these studies in that with respect to these two hormones, the pig duodenum is fully mature at birth and that the pig brain is closer to maturity at birth than is the rat brain. Currently under study is the ontogeny of these peptides in other species in order to relate maturity of behavior of the neonate with the concentrations of brain-gut peptides.

Our studies on Sephadex gel filtration of VIP in brain or gut or SEC in gut have shown only one molecular form for each of these peptides. However, in rat brain we find a molecular form of CCK corresponding to the C-terminal octapeptide (CCK8) in methanol extracts of brain and a

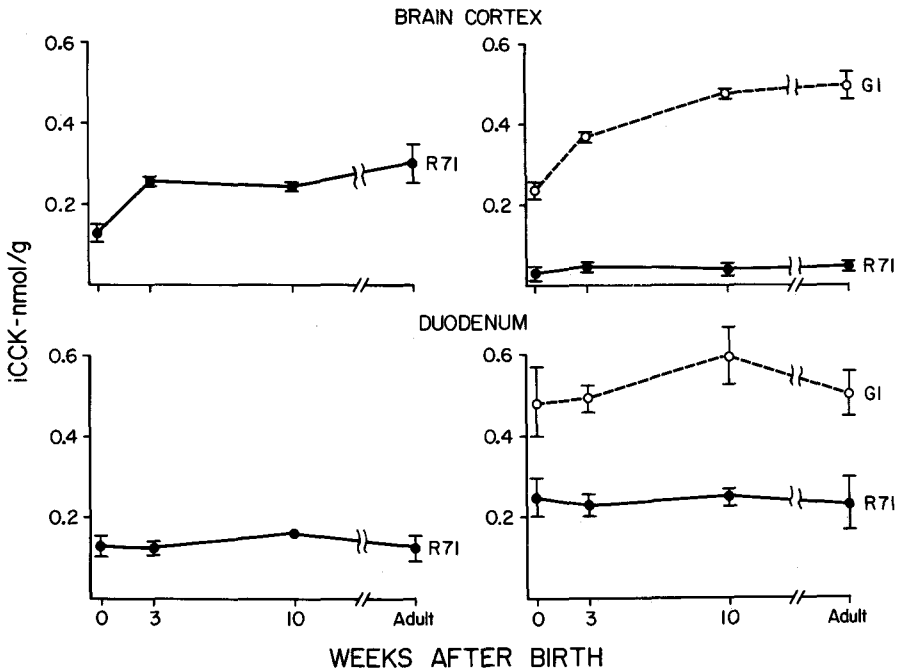

Fig. 10. Immunoreactive CCK as a function of age in methanol (left) and acid (right) extracts of pig brain cortex (top) and pig duodenum (bottom) as determined with N-terminal (G1) and C-terminal (R71) radioimmunoassays. (Reproduced from reference 6).

precursor peptide, probably CCK58, in acid extracts[14] (Fig. 11). Thus in rat brain as we had previously shown in pig brain[15], there is very rapid and complete post-translational processing resulting in cleavage of CCK8 from larger precursor forms.

The post-translational processing in the rat duodenum is quite different from that in brain and the relative concentrations of the different molecular forms in the acid extracts appear to be age-related. The distribution pattern observed on fractionation on Sephadex G50 of the methanol extracts of duodenum appears to be independent of age although the concentration is greatest at 2 weeks (Fig. 12, left). There are two peaks of approximately equal size: one eluting in the salt region that corresponds to CCK8; the other peak elutes earlier than CCK12 and corresponds to a larger peptide. The distribution of molecular forms in the acid extracts of the duodenum appears to be age-related. At 1 week or earlier a peak corresponding to CCK33 or 39 predominates but this peak becomes less prominent with age. At 3 weeks or later a precursor form, probably CCK58, predominates. However, at 2 weeks, there is a very prominent peak corresponding to a molecular weight form smaller than CCK33. The presence of this peak largely accounts for the higher concentration of iCCK at two weeks; this peak is no longer prominent in acid extracts of the adult duodenum. We now believe that the molecular forms observed are CCK peptides in various states of post-translational processing and we hope to be able soon to report on their purification and sequencing.

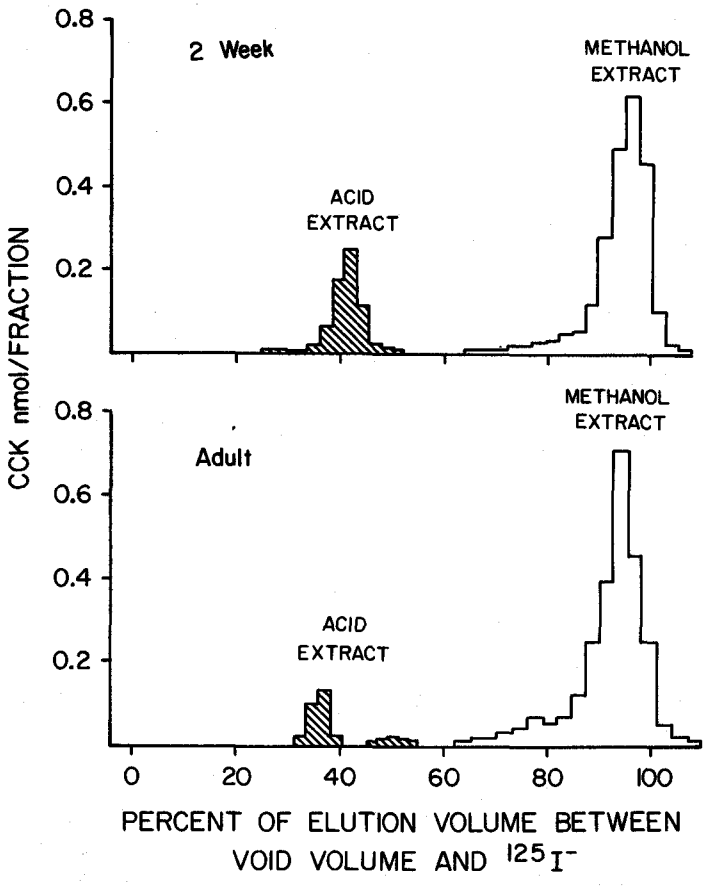

Fig. 11.
Sephadex G50 superfine gel filtration of extracts of rat brain. The methanol extracts were dried in a vacuum oven, reconstituted in 0.02M barbital buffer and applied to the Sephadex columns which were equilibrated and eluted with the same buffer. The single peak has the same elution volume as CCK8. The acid extracts were applied to columns equilibrated and eluted with 0.1M HCl. The major peak corresponds to a form with a molecular weight corresponding to CCK58. Elution volumes for authentic CCK8, 12 and 33 in columns eluted with 0.02M barbital are 96, 80, and 50 percent, respectively. The elution volume for authentic CCK33 in columns eluted with 0.1M HCl is 58%. (Reproduced from reference 14).

The observations that the processing of CCK in gut and brain appears to be quite different raises the question as to whether the brain and gut peptides have a common mRNA coding for CCK in these two tissues. This question is of some importance since it has been shown that the biosynthetic pathway of the enkephalins in adrenal tissue[16] is different from that observed in pituitary and brain[17,18]. However, a recent study based on molecular cloning of CCK mRNA from porcine cerebral cortex and duodenal mucosa demonstrated that CCK is synthesized via identical precursor proteins in both tissues[19]. The finding of several molecular forms of CCK with different relative concentrations in intestinal tissues of different species raises a still unanswered question - namely, in what form is CCK found in the circulation in the various species?

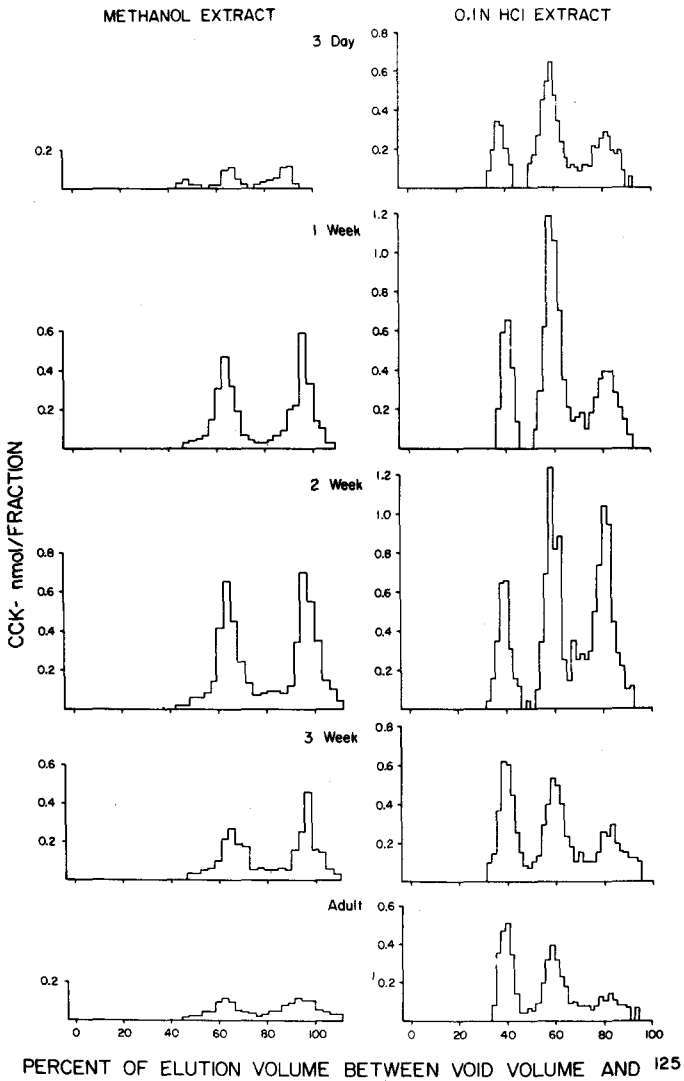

Fig. 12. Sephadex G50 gel filtration of rat duodenal extracts. The conditions for equilibration and elution of columns are given in the legend for Figure 12. (Reproduced from reference 14).

This has been a difficult question to answer since the concentration of CCK in the unstimulated state in humans is less than 0.5 fmol/ml[20,21]. The hormonal effects of CCK are well-established, i.e., its effect on gall bladder contraction and release of pancreatic enzymes. However, its role as a neuronal peptide has yet to be fully defined.

The last question to be addressed is to what extent can studies of urinary excretion of peptide hormones replace multiple blood sampling in evaluation of integrated hormonal output. Bioassay of urinary gonadotropins had been the preferred methodology for the diagnosis of pregnancy and of hypogonadal states in the 1950's and 1960's. However, early attempts to measure peptide hormones such as growth hormone (GH) in urine by RIA appeared to have been plagued by non-specificity[22]. Since then, there have been a series of studies on determination of urinary GH. The latest report demonstrated that after concentration of a 24 hour urine collection by polyionic membrane ultrafiltration the major component of the immunoreactive GH appeared to be the 22000 dalton peptide and the integrated 24 hour collection did reflect the clinical state[23].

Recently there has been increased interest in assay of urinary peptide hormones as a reflection of integrated hormonal output. For instance, over the past few years, it has been demonstrated that urinary calcitonin (CT) concentration (24-300 pg/mg creatinine in normals) is usually higher

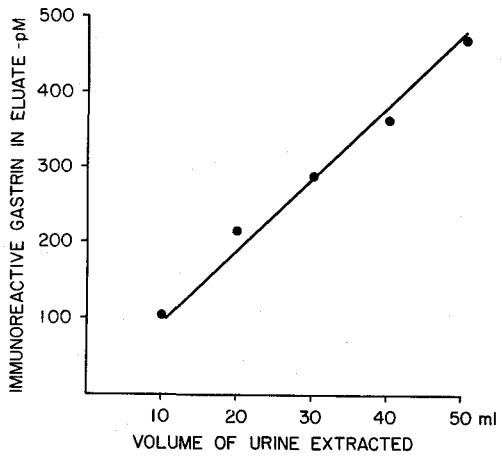

Fig. 13.

Immunoreactive gastrin in eluates from C_{18} cartridge as a function of volume of urine concentrated. (Reproduced from reference 29).

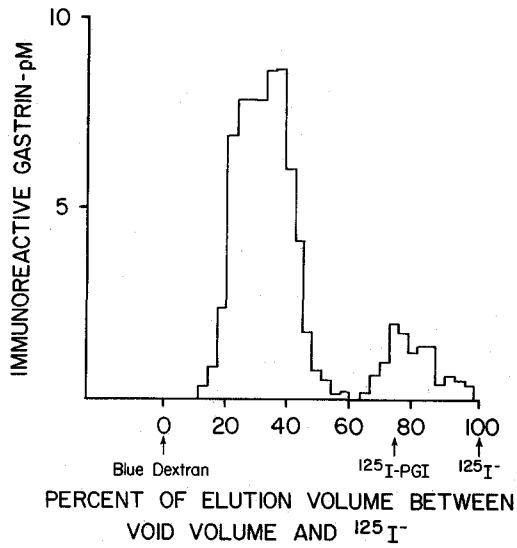

Fig. 14.

Sephadex G50 gel filtration of gastrin in 50 ml of urine from a normal fasted subject which was concentrated using a C_{18} cartridge. Recovery of immunore activity applied to this column was 74%. (Reproduced from reference 29).

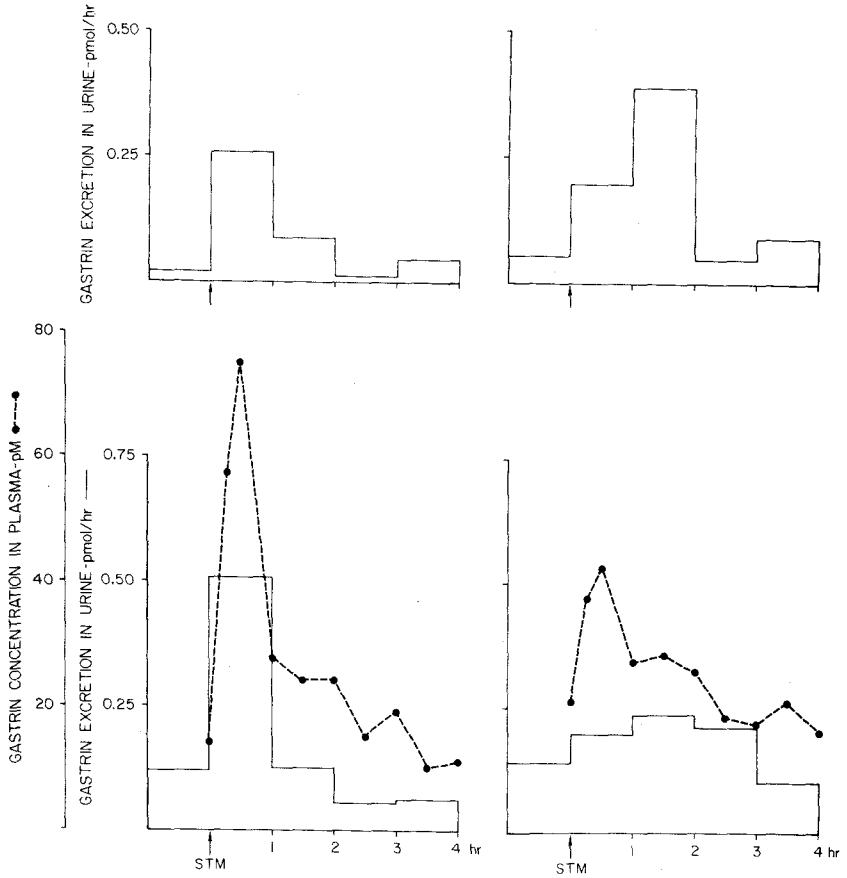

Fig. 15. Hourly urinary gastrin output following a standard test meal (STM). In two (bottom) of the four subjects shown, plasma gastrin was determined in samples obtained half hourly. (Reproduced from reference 29).

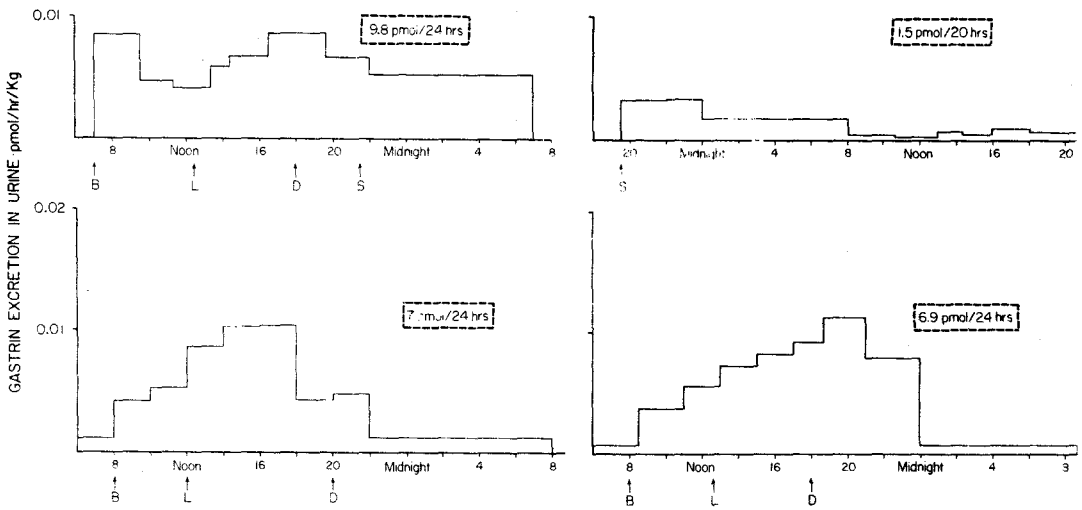

Fig. 16. Average hourly rate of excretion of immunoreactive gastrin in 3 normal subjects on their usual diet and in 1 normal subject during a 20-hour fast. The integrated output over the entire period is shown in the box in each frame. B, breakfast; L, lunch; D, dinner; S, snack. (Reproduced from reference 29).

than plasma CT and that this observation has been useful in the diagnosis of medullary thyroid carcinoma (24-27) and pseudohypoparathyroidism[27].

Schwartz and associates[28] had considered that the 24 hour urinary excretion of gastrin might serve as a useful measure of diurnal gastrin secretion[28]. However, because of the limited sensitivity of their assay for gastrin they concluded that there is a renal threshold for gastrin secretion corresponding to plasma gastrin levels of 100-200 fmol/ml. Recently we have employed octadecylsilyl (ODS) silica columns (C_{18} Sep-Pak cartridges - Waters Associates) for the concentration of several peptides from tissue extracts and body fluids. The methodology is quite simple[29]. For concentration of gastrin from urine, the cartridges are prepared by washing with 10 ml 75% ethanol in 0.01M HCl followed by 10 ml distilled water. Volumes of urine up to 50 ml are passed through the cartridge under pressure at a rate of 10 ml/min. The cartridge is then washed with 10 ml distilled water and eluted with 75% ethanol in 0.01M HCl. The optimal elution volume was determined and those fractions saved and evaporated to dryness. The dried residue was reconstituted in assay buffer. The cartridges can be rewashed and used many times. Recovery of both the 17 amino acid gastrin (G17) and the 34 amino acid gastrin (G34) added to urine is in excess of 90%. Gastrin is quite stable in urine at an acid pH. More than 95% is recovered when stored at a pH4 at 4°C for 24 hours. The recovery of endogenous gastrin from urine is essentially independent of the volume of urine extracted up to a volume of 50 ml (Fig. 13). Sephadex gel filtration of immunoreactive gastrin in 50 ml of urine from a normal fasted subject reveals the presence of both G34 and G17 - in the case shown G34 predominates as it frequently does in plasma in the unstimulated state (Fig. 14). Measurement of hourly urinary gastrin following a standard test meal reveals that the output reflects plasma levels (Fig. 15). During a 20 hour fast (Fig. 16, upper right) urinary gastrin output is considerably reduced compared to the output when subjects are on their usual diet. The urinary gastrin output is relatively constant in normal subjects - total urinary gastrin output ranged from 6.8 to 10.2 pmol/24 hours with an average of 8.5+1.5 (SD) pmol/24 hours. Renal clearance of gastrin appears to be independent of the fasted or fed state and ranged from 0.09 ml/min to 0.26 ml/min with an average of 0.16+0.05 (SD) ml/min in the 5 subjects studied. We have not as yet studied the effect of renal disease on the clearance of gastrin. This study would suggest that the determination of renal clearance from a single plasma specimen and a timed urinary output during a steady state would permit subsequent determination of integrated plasma levels by measurement of timed urinary outputs.

Methodology similar to this should be applicable to other peptide hormones as well. As yet unanswered are such questions as the range of urinary clearances for various peptides, the dependence of urinary clearance on molecular size and configuration and the effect of renal and non-renal pathology on these clearances. We are currently exploring the answers to these questions. Even now 25 years after the introduction of RIA there remain new fields that can be explored with its help.

REFERENCES

1) Berson, S.A. and Yalow, R.S.: Parathyroid hormone in plasma in adenomatous hyperparathyroidism, uremia, and bronchogenic carcinoma. Science 154: 907-909, 1966.
2) Berson, S.A. and Yalow, R.S.: Immunochemical heterogeneity of parathyroid hormone. J. Clin. Endocrinol. Metab. 28: 1037-1047, 1968.
3) Silverman, R. and Yalow, R.S.: Heterogeneity of parathyroid hormone: Clinical and Physiologic implications. J. Clin. Invest. 52: 1958-1971, 1973.
4) Yalow, R.S.: Significance of the heterogeneity of parathyroid hormone. In: Endocrinology of Calcium Metabolism, Excerpta Medica Internat'l Congress Series No.421, 1977, pp. 308-312.
5) Ichihara, K., Eng, J. and Yalow, R.S.: Ontogeny of immunoreactive CCK, VIP and secretin in rat brain and gut. Biochem. Biophys. Res. Commun. 112: 891-898, 1983.
6) Ichihara, K., Eng, J., Pond. W.G., Yen, J.T., Straus, E. and Yalow, R.S.: Ontogeny of immunoreactive CCK and VIP in pig brain and gut. Peptides 5: 623-626, 1984.
7) Straus, E. and Navarro, N.: Mucosal secretin content in post-mortem tissues. Lab. Invest. 44: 97-98, 1981.
8) Ryder, S., Eng, J., Straus, E. and Yalow, R.S.: Alkaline extraction and characterization of cholecystokinin-immunoreactivity from rat gut. Gastroenterology 81: 267-275, 1981.
9) Reinecke, M., Schlüter, P., Yanaihara, N. and Forssman, G.W.: VIP immunoreactivity in interic nerves and endocrine cells of the vertebrate gut. Peptides 2, (Suppl.2) 149-156, 1981.
10) Mutt, V., Carlquist, M. and Tatemoto, K.: Secretin-like bioactivity in extracts of porcine brain. Life Sciences 25: 1703-1708, 1979.
11) Laburthe, M., Bataille, D. and Rosselin, G.: Vasoactive intestinal peptide (VIP): Variation of the jejuno-ileal content in the developing rat as measured by radioreceptorassay. Acta Endocrinol. 84: 588-599, 1977.
12) Emson, P.C., Gilbert, R.F.T., Loren, I., Fahrenkrug, J., Sundler, F. and Schaffalitzky de Muckadell, O.B.: Development of vasoactive intestinal polypeptide (VIP) containing neurones in the rat brain. Brain Res. 177: 437-444, 1979.
13) Maletti, M., Besson, J., Bataille, D., Laburthe, M. and Rosselin, G.: Ontogeny and immunoreactive forms of vasoactive intestinal peptide (VIP) in rat brain. Acta Endocrinol. 93: 479-487, 1980.
14) Ichihara, K., Eng, J. and Yalow, R.S.: Ontogeny of hormonal forms of CCK-peptides in rat brain and gut. Life Sciences 34: 93-98, 1984.
15) Eng, J., Shiina, Y., Pan, Y.-C. E., Blacher, R., Chang, M., Stein, S. and Yalow, R.S.: Pig brain contains CCK-octapeptide and several CCK-desoctapeptides. Proc. Natl. Acad. Sci.

USA 80: 6381-6385, 1983.

16) Stern, A.S., Jones, B.N., Shively, J.E., Stein, S. and Udenfriend, S.: Two adrenal opioid polypeptides: Proposed intermediates in the processing of proenkephalin. Proc. Natl. Acad. Sci. USA 78: 1962-1966, 1981.

17) Lewis, R.V., Stern, A.S., Rossier, J., Stein, S. and Udenfriend, S.: Putative enkephalin precursors in bovine adrenal medulla. Biochem. Biophys. Res. Commun. 89: 822-829, 1979.

18) Brownstein, M.J.: Opioid peptides search for the precursors. Nature (London) 287: 678-679, 1980.

19) Gubler, U., Chua, A.O., Hoffman, B.J., Collier, K.J. and Eng, J.: Cloned cDNA to cholecystokinin mRNA predicts an identical preprocholecystokinin in pig brain and gut. Proc. Natl. Acad. Sci. USA 81: 4307-4310, 1984.

20) Calam, J., Ellis, A. and Dockray, G.J.: Identification and measurement of molecular variants of cholecystokinin in duodenal mucosa and plasma. J. Clin. Invest. 69: 218-225, 1982.

21) Walsh, J.H., Lamers, C.B. and Valenzuela, J.E.: Cholecystokinin-octapeptidelike immunoreactivity in human plasma. Gastroenterology 82: 438-444, 1982.

22) Girard, J. and Greenwood, F.C.: Radioimmunoassay for human growth hormone in urine. Aspecific factors imitating the presence of growth hormone. In: Protein and Polypeptide Hormones (M. Margoulies, ed.) Part 2, Excerpta Medica Foundation, Amsterdam, pp.332-334, 1968.

23) Baumann, G. and Abramson, E.C.: Urinary growth hormone in man: Evidence for multiple forms. J. Clin. Endocrinol. Metab. 56: 305-311, 1983.

24) Snider, R.H., Moore, C.F., Silva, O.L. and Becker, K.L.: Radioimmunoassay of calcitonin in normal human urine. Anal. Chem. 50: 449, 1978.

25) Silva, O.L., Snider, R.H., Moore, C.F. and Becker, K.L.: Urine calcitonin as a test for medullary thyroid carcinoma. Ann. Surg. 189: 269, 1979.

26) Morimoto, S., Onishi, T., Okada, Y., Lee, S., Inanaka, S., Fukuo, K. and Kumahara, Y.: Radioimmunoassay of urinary calcitonin in normal subjects and in patients with medullary carcinoma of the thyroid. Endocrinol. Jpn. 29: 1, 1982.

27) Morimoto, S., Onishi, T., Kumahara, Y. and Avioli, L.: Differentiation of pseudo- and idiopathic hypoparathyroidism by measuring urinary calcitonin. J. Clin. Endocrinol. Metab. 57: 1216-1220, 1983.

28) Schwartz, T.W., Sask, P. and Rehfeld, J.F.: Immunochemical studies on gastrins in the urine. Clin. Chim. Acta 89: 381-386, 1978.

29) Du, B.-H., Zhang, J.-Y., Eng, J. and Yalow, R.S.: Urinary immunoreactive gastrin in normal subjects. Horm. Metab. Res. 16: 132-135, 1984.