(N_v) of mitochondria in relation to the cytoplasmic volume. In order to determine N_v of mitochondria, the formula cited above was used. The factor K was again assumed to be close to unity and the coefficient β was estimated to be 2.25 in view of the size distribution of the mitochondrial profiles and their calculated average ratio[9]. In order to determine all the primary and secondary morphometric parameters, the methods described by Weibel et al.[10,11] were used. A multipurpose test grid of 50 short linear probes was used. No corrections were made in what concerns Holme's effect. Statistical analysis included calculations of the means, standard deviation and standard error, and examination of the significance by Student's 2-sided t-test. 2 means were considered to be significantly different if the probability of error (p) was smaller than 0.05.

Table 2. Values of secondary morphometric parameters

	Group 1	Group 2	Group 3
Cell volume (μm^3)	3521	3322	3425
Nuclear volume (μm^3)	292	252	288
Brush border volume (μm^3)	1028	1043	1021
Cytoplasmic volume (μm^3)	2201	2027	2116
Mitochondria volume (μm^3)	429	399	445
Mitochondria surface (μm^2)	3522	3728	3281
Volume of single mitochondrion (μm^3)	0.44	0.39	0.47
Surface of single mitochondrion (μm^2)	3.60	3.47	3.50

Values are expressed in means per average cell, with the exception of the 2 last ones that refer to single mitochondrion.

Results and discussion. The results obtained in the morphometric study of rat P3 cells are summarized in tables 1 and 2. No significant differences were found between the 3 groups, despite the artifactual distortion observed in the collapsed tubular segments of kidney fragments fixed by immersion.

The greater volumetric density of mitochondria found by Orsoni et al.[12] in the pars descendens of the female rat proximal tubule may depend upon the inclusion in the pars descendens of a portion of the P2 segment[8] which is richer in mitochondria than the P3 segment[13], although we cannot rule out the possible influence of the sex of the rats[8] and of the osmolality of the fixation media[4,7]. The similarity of results concerning mitochondria obtained in groups 2 and 3, on the other hand, confirms that the enlargement of mitochondrial profiles, when the tissue is fixed by glutaraldehyde and secondarily osmicated, only represents an artifact of bad fixation[5]. In view of these results it can be stated that the study of human kidney biopsies can be evaluated in comparison with those obtained under different conditions of fixation in animal experimental research, provided that the fixation media are made approximately isotonic.

11 E. R. Weibel and R. P. Bolender, in: Principles and Techniques of Electron Microscopy, p. 239. Ed. M. A. Hayat. Van Nostrand Reinhold Company, New York 1973.
12 J. Orsoni, H. P. Rohr and F. Gloor, Path. Eur. *4*, 345 (1969).
13 N. O. Jacobsen and F. Jørgensen, Z. Zellforsch. *136*, 479 (1973).

Specific immunostaining of CCK cells by use of synthetic fragment antisera[1]

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Summary. Antibodies to the central fragments 9–20 dodecapeptide sequence of CCK were used for specific immunostaining of the CCK cells of the mammalian gut. The use of high specific antibodies to synthetic fragment, essential when there is a possibility of immunochemical cross reactions between antisera and hormones of similar molecular structure provides the key to increased understanding of the nature and relationships of peptide hormones.

CCK has been localized in the human jejunum and ileum[2,3], and its cell of origin tentatively established using antibodies against pure natural CCK[4,5]. Gastrin is present in the intestine as well as in the antrum. The concentration of gastrin in human proximal duodenal mucosa is about a third of that in the antral mucosa and because of the greater bulk of duodenal mucosa, human duodenum contains as much gastrin as the antrum[2]. Highest concentrations of intestinal gastrin are found in the proximal duodenum, with progressively lower concentrations in the remainder of the duodenum and jejunum[6]. CCK and gastrin share a C-terminal amino acid sequence and antibodies to this sequence, raised against either hormone, will cross react. Specific immunocytochemistry of the CCK and gastrin producing cells thus requires that the antisera used should be completely free from any cross reacting components. Even minor subpopulations of antibodies, not normally detected by radioimmunoassay techniques, can produce significant cross reaction under cytochemical conditions.

This paper describes the solution of this problem by use of antisera raised to a synthetic CCK fragment chosen because its sequence avoids the areas of homology with other known hormones.

Material and methods. Synthesis of the CCK fragment.

9 10 11 12 13 14 15 16 17 18 18 20
H-Met-Ile-Lys-Asn-Leu-Gln-Ser-Leu-Asp-Pro-Ser-His-OH (I)

The central 9–20 dodecapeptide sequence (I) of CCK was synthetized on a Merrifield-type solid support using meth-

1 Grants from the Medical Research Council and the Volkswagenwerk-Stiftung made the work possible.
2 S. R. Bloom, Br. med. J. *30*, 62 (1974).
3 P. L. Rayford, T. A. Miller and J. C. Thompson, Gastrointestinal Hormones *294*, 1093 (1975).
4 J. M. Polak, A. G. E. Pearse, S. R. Bloom, Lancet *2*, 1016 (1975).
5 R. Buffa, E. Solcia and V. L. W. Go, Gastroenterology *70*, 528 (1976).
6 J. H. Walsh and M. I. Grossman, New Engl. J. Med. *292*, 1324 (1975).

ods which are known to minimize the formation of unwanted byproducts. After deprotection and removal from the solid support, the crude dodecapeptide was purified first by gel chromatography on Sephadex G-25 SF in 50% acetic acid and then by ion exchage chromatography on carboxymethyl cellulose in a buffer gradient of 0.01–0.2 M ammonium acetate (pH = 7).

The final product was shown to be homogeneous in the following TLC systems:

A. Ethyl acetate (5), Pyridine (5), Acetic acid (1), water (1); R_F^A (silica) 0.5

B. 1-Butanol (3), Acetic acid (1), water (1); R_F^B (silica) 0.01, (cellulose) 0.27

C. 1-Butanol (21), Pyridine (12), Acetic acid (2), water (15); R_F^C (silica) 0.20

The dodecapeptide was found to contain the correct ratio of amino acids both a) after acid hydrolysis (6N HCl, 110%/18 h) and b) after digestion with aminopeptidase M, indicating the absence of racemisation.

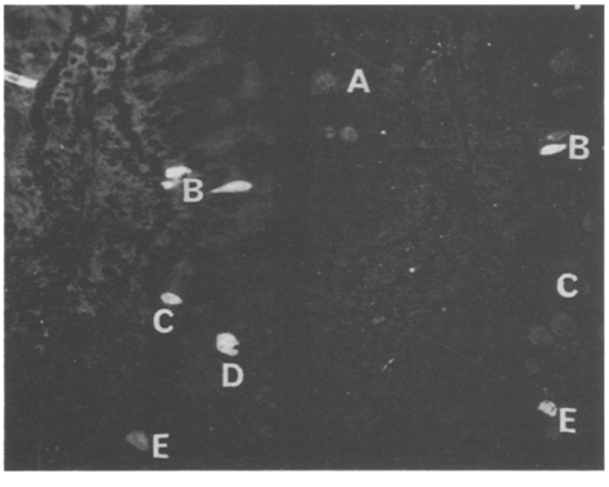
Found: a) Asp, 1.94; Glu, 1.00; His, 1.09; Ile, 0.97; Leu, 2.02; Lys, 1.05; Met, 0.96; Pro, 0.93; Ser, 1.90.

b) His, 1.09; Ile, 0.97; Leu, 1.94; Lys, 1.02; Met, 0.93; Ser, 2.05; Asn, Gln present but not determined; Asp, Glu, Pro and β-cyanoalanine absent.

Production of antibodies. Antibodies to pure natural CCK: Antibodies were raised in rabbits after multiple subcutaneous injections of both 99% and 16% pure CCK administered in complete Freund's adjuvant at 3 monthly intervals either directly or conjugated to bovine serum albumin. Antisera with titres of $^1/_{2000}$–$^1/_{8000}$ were harvested after a year. 2 antisera were selected which showed very low cross reactivity with synthetic human gastrin and no cross reactivity with other gut hormones including gastric inhibitory peptide and glucagon.

Antibodies to synthetic gastrin I: Human synthetic gastrin I (ICI Ltd) was coupled to bovine serum albumin (2:1 molar ratio) by carbodiimide condensation and administered to rabbits at above. After a year antisera with a titre from $^1/_{80\,000}$ to $^1/_{600\,000}$ were harvested.

Antibodies to synthetic CCK 9–20: The dodecapeptide was coupled to hen egg ovalbumin with glutaraldehyde. The conjugate contained 4.6 moles of peptide/mol ovalbumin as estimated via the tritium label (3 mCi/mmol) which was introduced at Ile[10] of the dodecapeptide during synthesis. Rabbits were immunized as above and antisera harvested at 6 months.



1 2

1 μm 'Araldite' serial sections stained 1. with antibodies to human Gastrin I and 2. with antibodies to synthetic CCK fragment (9–20). Note that some CCK cells (B and E) are also stained with antibodies to gastrin containing minor sub populations of IgG CCK cross reacting antibodies. ×628.

Histological techniques. Fresh samples of gastrointestinal mucosa (body and antrum of the stomach, duodenum, jejunum, ileum and pancreas) were taken from man (operative specimens) and also from dogs and pigs. Part of the material was immediately quenched in 'Arcton' (Freon 22) at –156 °C and then freeze-dried in a thermoelectric freeze-drier before being vapour fixed[7]. Serial 3 μm paraffin sections were then cut and sequential immunostaining was carried out using antibodies to synthetic human gastrin I and to CCK 9–20. The rest of the material was immediately fixed in p-benzoquinone and subsequently embedded in 'Araldite' mixture. From this, further serial sections were cut at 1 μm and used for immunohistochemical studies[4]. Control techniques included prior absorption of the antibodies with CCK synthetic fragment and synthetic human gastrin I. Gastrin and CCK-stained serial sections were then photographed and prints from corresponding areas compared.

Results. Antisera were raised in 3 of the 10 rabbits immunized with natural CCK but only the one with the highest titre gave adequate intensity for immunocytochemical staining. One antiserum was obtained from the 6 rabbits immunized with CCK 9–20. The CCK antiserum was used at a final titre of $^1/_{8000}$ and showed 0.01% cross reaction with gastrin as estimated by radioimmunoassay, and a similar binding with the CCK 9–20. The antiserum to CCK 9–20 showed no binding with gastrin I^{125}. Antibodies to CCK 9–20 stained many cells in the duodenum and jejunum of all species investigated and somewhat fewer cells in the ileum. G cells in the antral mucosa were not stained with antibodies to CCK 9–20, but antibodies to synthetic human Gastrin I and natural CCK demonstrated G cells in the antral mucosa and duodenal bulb, particularly in man, as well as cells in the small intestine. Comparison of the serially stained sections of intestine showed that some cells, presumed to contain CCK, stained with the antibodies to CCK 9–20 and also with gastrin antibodies which showed cross reaction with CCK, whereas others, presumably containing true intestinal gastrin did not react at all with the specific CCK antibodies. Quantitative studies revealed that CCK cells were about equal in number in the duodenum to cells containing gastrin.

Discussion. Antisera raised against synthetic gastrin 1–17 inevitably contain IgG subpopulations reacting strongly with the highly antigenic C-terminal portion of the molecule which is also present in CCK. A degree of cross reactivity is thus inevitable and such sera, when used for immunocytochemistry in the upper intestine, stain both gastrin cells and CCK cells. An antiserum raised against the 9–20 dodecapeptide sequence of CCK can in no way cross-react with gastrin, and when used for immunocytochemistry reveals only the population of CCK cells.

Our study thus emphasizes the value of using completely specific antisera. The synthesis of selected fragments of peptides, chosen for their lack of sequences common to other hormones, provides a unique antigenic stimulus to raise such antisera. The increasing use of such hormone fragment antibodies to achieve defined specificity is called for not only in distinguishing similar hormones as in the present study but also, more generally, in studies designed to elucidate the particular, and often unexpected, cross reactivities which whole hormone antisera may display in normal and, more frequently, neoplastic tissues.

7 A. G. E. Pearse and Julia M. Polak, Histochem. J. 7, 179 (1975).