A selective uptake of LH-RH by the pituitary can therefore be postulated, while kidney and liver may be the primary sites of inactivation and/or removal of the injected material from the circulation.

It remains to be established whether the radioactivity found in the blood, liver and kidney represents all the decapeptide molecule, or if part of it (the active core) remains in the pituitary to exert a long term synthetic action and also a very rapid releasing or activating action.

The concentration of radioactivity in the other organs, including brain and gonads, is negligible. The clinical effects of synthetic LH-RH therefore seem to be mediated through the release of gonadotropins into the blood stream. The administration of the oestrogens seems to 'sensitize' the hypophysis, enhancing its uptake of labelled LH-RH, while kidney and liver are not affected.

Riassunto. Viene descritta la distribuzione della radioattività dopo iniezione intracarotidea di LH-RH marcato con I^{125} in ratti maschi e femmine. L'ipofisi ha mostrato la maggiore capacità di concentrazione dell'ormone marcato, 1 min dopo la somministrazione. L'ormone quindi viene rapidamente dismesso mostrando una riduzione del 50% già dopo 3 min. Altri organi captanti si sono rivelati il rene ed il fegato.

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Extraction from Hog Duodenum of a High Molecular Weight Protein with Gastric Antisecretory Activity

In 1930 Kosaka and Lim[1] showed that an inhibitor of the gastric secretory activity can be obtained from the intestinal mucosa. Later on (1942–1952), a low molecular weight compound, having peptidic nature according to some authors[2–4] and a non-peptidic one for others[5,6], was believed to be responsible for this inhibiting activity.

Recently, Lucien et al.[7] and Ichimura obtained[8] from hog duodenum an agent very active in blocking the gastric acid secretion. In both cases the inhibitory activity was found to be associated with a low mol wt. compound. In 1970 Brown et al.[9] extracted a polypeptide chain of 43 residues[10] which inhibits gastric secretion.

In this work we show that by using a new procedure it is possible to extract from hog duodenum a protein which behaves as a highly active inhibitor of the gastric secretion and that, in contrast to the above-mentioned agents, it is characterized by a high molecular weight.

Methods. The protein content was determined by the ninhydrin test according to Moore and Stein[11]. The carbohydrate content was determined as follows: hexoses by Winzler[12]; methylpentoses following Dische[13]; hexosamines by the method of Elson and Morgan[14]; sialic acid by Warren[15]; uronic acids according to Bitter and Muir[16]. Sulphate was determined by Terho[17].

Tryptic treatment was performed in 0.24 *M* phosphate buffer, pH 8.3; papain digestion in 0.1 *M* glycine-HCl buffer, pH 2; pronase digestion in 0.1 *M Tris*-HCl buffer, pH 8.7; in all cases at 37 °C for 24 h. The ratio E/S was 1/10.

[1] T. Kosaka and R. K. S. Lim, Proc. Soc. exp. Biol. Med. *27*, 890 (1930).
[2] S. C. Harris, J. S. Gray and A. C. Ivy, Fedn Proc. *1*, 37 (1942).
[3] A. N. Wick, F. Pauls, A. J. Irish and E. M. MacKay, Fedn Proc. *6*, 226 (1947).
[4] H. M. Millen, L. L. Gershbein and A. C. Ivy, Archs Biochem. Biophys. *35*, 360 (1952).
[5] K. J. Öbrink, Acta chem. scand. *2*, 49 (1948).
[6] K. J. Öbrink and H. Winberg, Acta chem. scand. *4*, 789 (1950).
[7] H. W. Lucien, Z. Itoh, D. C. H. Sun, J. Meyer, N. Carlton and A. V. Schally, Archs Biochem. Biophys. *134*, 180 (1969).
[8] M. Ichimura, J. pharm. Soc. Japan *91*, 245 (1971).
[9] J. C. Brown, V. Mutt and R. A. Pederson, J. Physiol., Lond. *209*, 57 (1970).
[10] J. C. Brown and J. R. Dryburgh, Can. J. Biochem. *49*, 867 (1971).
[11] S. Moore and W. H. Stein, J. biol. Chem. *211*, 907 (1954).
[12] R. J. Winzler, in *Methods of Biochemical Analysis* (Ed. D. Glick; Interscience, New York 1955), vol. 2, p. 279.
[13] Z. Dische and L. B. Shettles, J. biol. Chem. *175*, 595 (1948).
[14] L. A. Elson and W. T. J. Morgan, Biochem. J. *27*, 1824 (1933).
[15] L. Warren, J. biol. Chem. *234*, 1971 (1959).
[16] T. Bitter and H. M. Muir, Analyt. Biochem. *4*, 330 (1962).
[17] T. T. Terho, Analyt. Biochem. *41*, 471 (1971).

Table I. Gastric antisecretory activity of fraction E_3 and of fraction E_3 treated with proteolytic enzymes

Treatment	Dose administered as dry weight (μg/kg)	No. of rats	Gastric secretion per 2 h (ml ± S.D.)	Inhibition (%)	Hydrochloric acidity per 2 h (mEq ± S.D.)	Inhibition (%)
Controls		39	3.81 ± 0.43	—	0.201 ± 0.031	—
E_3	500 (385)	37	1.49 ± 0.21	61	0.032 ± 0.005	84
E_3	300 (231)	38	1.79 ± 0.24	53	0.049 ± 0.009	76
E_3	200 (154)	36	1.94 ± 0.30	49	0.057 ± 0.008	72
E_3	150 (115)	37	2.40 ± 0.32	37	0.088 ± 0.014	56
E_3	75 (57)	36	2.74 ± 0.31	28	0.131 ± 0.022	35
E_3, pronase treated	500 (385)	14	3.27 ± 0.40	14	0.165 ± 0.028	18
E_3, papain treated	500 (385)	15	2.97 ± 0.38	22	0.143 ± 0.023	29
E_3, trypsin treated	500 (385)	15	2.68 ± 0.35	27	0.133 ± 0.023	34

In parentheses the protein content.

Neuraminidase treatment was performed by incubating for 24 h at 37 °C 5 mg of sample with 500 U of enzyme (Behringwerke, from *Vibrio cholerae)*, in 3 ml of 0.05 *M* sodium acetate-acetic acid buffer, pH 5.5, containing 0.9% NaCl and 0.1% $CaCl_2$.

For the antisecretory activity determination[18], the sample was injected i.v. into 150–160 g Wistar male rats, fasted for 48 h. Two h after injection and pylorous ligation, the gastric juice volume, the hydrochloric, organic and total acidities were measured. The antisecretory unit is defined as the amount of inhibitor which reduces the gastric secretion volume to 50%. The gastric antiulcerous activity was determined according to the method of SHAY et al.[19], as modified by LUGARO et al.[18]. The sample was injected i.v. into 125-130 g Wistar male rats, fasted for 48 h. Eight h after injection and pylorus ligation (water ad libitum), the ulcus index after PAULS[20] was calculated. The antiulcerous unit is defined as the amount of inhibitor wich reduces the ulcus index to 0.25.

Results and discussion. The first 3 feet of hog duodenal mucosa were finely minced and extracted overnight using diluted HCl at pH 3. After centrifugation, the supernatant was left at pH 2.5 for 24–48 h, centrifuged, and saturated with NaCl. After 12–16 h the suspension was filtered and the residue washed and dried with acetone and diethyl ether (fraction E_1); 30 g of E_1, suspended in 1 l of 0.1 *M* acetic acid, were added with 250 ml of benzoic acid saturated acetone under stirring. After 12–16 h the precipitate was filtered and washed with water. The cake was resuspended in acetone and the solid washed and dried with acetone and diethyl ether (fraction E_2); 100 mg aliquots of fraction E_2 were gel filtered on a Bio-Gel A-1.5 column, as described in the Figure. The active fraction (fraction E_3) was eluted in the column void volume. Since the exclusion limit of the gel is claimed to be 1.5×10^6, the molecular weight of fraction E_3 should have this or a greater order of magnitude. All the extraction procedure was carried out in the cold (+2 °C). The fractions obtained during the purification were assayed using pylorous-ligated rats. All the active fractions (E_1, E_2 and E_3) showed a linear dose-response correlation.

A 50% inhibition is obtained with the fraction E_3 at a dosage of 250 μg/kg when testing the volume of gastric secretion and at 100 μg/kg measuring the hydrochloric acidity (Table I). This inhibitor is also active in preventing the development of induced ulcers in the Shay rat (approx. 14,000 U/g, Table II). A summary of the extraction of the gastric inhibitor is reported in Table III.

The benzoic acid treatment (fraction E_2) appears to be the most effective stage in the purification procedure; the total activity increases 10 times, probably owing to the separation from contaminants interfering with the bioassay in the crude extract. The chemical composition of fraction E_3 is approx. as follows: 77% protein, 9% hexoses, 1.9% fucose, 2.1% sialic acid, 5.1% hexosamines, 2.4% uronic acids, 2.5% sulphate. The protein nature of the gastric inhibitor is demonstrated by the remarkable reduction of antisecretory activity following treatment with papain, trypsin and pronase (Table I).

Neuraminidase treatment, which removes approx. 70% of its sialic acid, does not significantly alter the activity. Ultracentrifugal investigation confirms the gel filtration evidence about the high mol wt. of the inhibitor. However, it shows also that fraction E_3 is still heterogeneous.

In conclusion, a high mol wt. protein ($\geqslant 1.5 \times 10^6$), which is strongly active in inhibiting the gastric secretion, is present and extractable from hog duodenum.

Table II. Gastric antiulcerous activity of fraction E_3

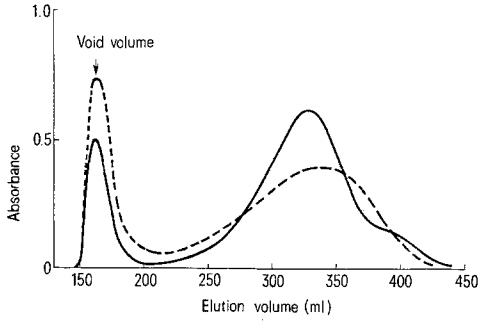
Dose administered as dry weight (μg/kg)	No. of rats	Ulcus index
Controls	33	2.52
1000 (770)	34	0.15
500 (385)	31	0.42
250 (192)	32	1.15

In parentheses the protein content.

Table III. Weight and degrees purification in various fractions of the hog duodenum

Stage of purification	Weight (g)	Specific activity (antisecretory U/g dry wt.)
Hog duodenal mucosa	10,000	—
Fraction E_1	400	22
Fraction E_2	6	13,000
Fraction E_3	2.1 (1.62)	41,000

In parentheses the protein content.



Gel filtration pattern of 100 mg of fraction E_2 on a Bio-Gel A-1.5 m column (2.5 × 80 cm), equilibrated with 0.05 *M* ammonium acetate buffer, pH 7. Flow rate 25 ml/h. ——, Absorbance of the eluent monitored at 254 nm with an L.K.B. Uvicord I; ------, Folin-Lowry colour at 750 nm. Activity eluted between 150 and 180 ml.

Riassunto. Un nuovo metodo di estrazione ha condotto alla parziale purificazione dal duodeno di maiale di una proteina ad alto peso molecolare, fortemente attiva nell'inibire la secrezione gastrica nel ratto a piloro legato. 250 μg/kg e 100 μg/kg inibiscono del 50% rispettivamente il volume di secrezione gastrica e l'acidità cloridrica.

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[18] G. LUGARO, I. LUPI and A. CORBELLINI, Ital. J. Biochem. *14*, 126 (1965).

[19] H. SHAY, S. A. KOMAROV, S. S. FELS, D. MERANZE, M. GRUENSTEIN and H. SIPLET, Gastroenterology *5*, 43 (1945).

[20] F. PAULS, A. N. WICK and E. M. MAC KAY, Gastroenterology *8*, 774 (1947).