Comparative morphometric study of the cells of the third proximal segment of the rat kidney under different conditions of fixation[1]

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Summary. The morphometric study of the P3 segment of rat proximal tubule has shown that neither the method of fixation, nor the fixative itself, significantly change the relative and absolute volumes of the cell compartments, provided that the fixation media are made approximately isotonic.

It is well established that both the way in which the fixative is brought in contact with the tissue and the characteristics of the fixative itself influence the ultrastructure of cells and tissues[3–7]. The collapse of the proximal tubule in kidney fragments fixed by immersion considerably changes the size and shape of cells[5] and raises the question whether the results obtained in the morphometric study of human kidney biopsies, which are necessarily fixed by immersion, can be evaluated in comparison with those obtained under different conditions of fixation in animal experimental research. The purpose of this study was to determine whether any significant differences are found between renal tubular cells fixed by vascular perfusion and those fixed by immersion in the same fixatives, as well as between cells fixed by immersion in glutaraldehyde and secondarily osmicated and cells fixed by immersion in osmium tetroxide. A precise segment of the proximal tubule (P3) was chosen since the similarity of its ultrastructural appearance along the outer stripe of the outer zone of the medulla[8] makes it a good model to test the influence of different procedures of fixation.

Material and methods. Male Wistar rats weighing 220–240 g and having free access to food and water were used. The kidneys of 4 ether-anaesthetized rats (group 1) were fixed by vascular perfusion as described by Maunsbach (method 1)[5]; as a fixative 1% glutaraldehyde in Tyrode's solution containing only 75% of the regular amount of NaCl[5] was employed. After 6–8 min of perfusion, tissue fragments were obtained in the outer stripe of the outer zone of the medulla of the kidneys, immersed in the same fixative for 2 h and post-fixed in 1% osmium tetroxide in a veronal acetate buffer added with salts[5]. The kidneys of the second group of 4 rats (group 2) were also excised under ether anaesthesia and the tissue fragments were fixed by immersion in the same fixative as in group 1 and for the same period. Finally, tissue fragments of the kidneys of the third group of 4 rats (group 3) were proceeded just as in group 2, but as a fixative 2% osmium tetroxide in veronal acetate buffer, added with the same quantity of salts as in previous groups, was employed. The fragments were embedded in epoxy-resin following a graded series of ethanol and propylene oxide.

From each animal, 5 tissue blocks showing the required tubular segments were selected at random. From each block, a semi-thick section was cut and stained with toluidine blue and photographed at a primary magnification $\times 250$. From each semi-thick section, 1 light micrograph was recorded and used for determining the volumetric densities of the nucleus, the brush border and the cytoplasm in relation to cell volume (V_v) and the numerical density of nuclei in relation to total cellular volume (N_v). In order to determine N_v the formula of Weibel and Gomez[9] was used: $N_v = K(1/\beta) \cdot Na^{3/2}/V_v{}^{1/2}$. The coefficient β was estimated to be 1.38 assuming that the shape of the nuclei was similar to a sphere[9] and K was assumed to be close to unity for lack of adequate information[10]. From each block, a silver ultra-thin section was double-stained with uranyl acetate and lead citrate and micrographed at a primary magnification $\times 4{,}000$. From each ultra-thin section, 3 electron micrographs were recorded and used to calculate the volumetric density (V_v), the surface density (S_v) and the numerical density

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Table 1. Values of primary morphometric parameters

	Group 1	Group 2	Group 3
V_v of brush border (%)	29. 2 ± 1. 3 (20)	31. 4 ± 2. 0 (20)	29. 9 ± 1. 2 (20)
V_v of cytoplasm (%)	62. 5 ± 1. 2 (20)	61. 0 ± 2. 0 (20)	61. 7 ± 1. 4 (20)
V_v of nuclei (%)	8. 3 ± 0. 6 (20)	7. 6 ± 0. 9 (20)	8. 4 ± 0. 4 (20)
N_v of nuclei (no/10^6 μm^3)	284 ± 11 (20)	301 ± 34 (20)	292 ± 16 (20)
N_v of mitochondria (no/10^3 μm^3)	445 ± 32 (60)	505 ± 32 (60)	443 ± 54 (60)
V_v of mitochondria (%)	19. 5 ± 0. 4 (60)	19. 5 ± 0. 8 (60)	21. 0 ± 1. 8 (60)
S_v of mitochondria ($\mu m^2/\mu m^3$)	1.60 ± 0.10 (60)	1.75 ± 0.07 (60)	1.55 ± 0.14 (60)

No significant differences were found between the 3 groups. Each value is expressed in mean ± SEM. Number of micrographs examined are indicated in parenthesis.

(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.

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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-

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