Histol Histopath (1987) 2: 391-400

Histology and Histopathology

Ultrastructure of the tubular nephron of *Testudo graeca* (Chelonia). A comparison between hibernating and non-hibernating animals

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Summary. The tubular nephron of hibernating and non-hibernating specimens of *Testudo graeca* (Chelonia) was studied by means of conventional light and electron microscopy and histochemistry. The tubular nephron was composed of proximal, intermediate, distal and collecting tubules in both hibernating and non-hibernating animals. The cells of the proximal tubule showed long microvilli, cytoplasmic vacuoles, a developed endoplasmic reticulum and abundant mitochondria. Fat droplets were also observed. The intermediate segment was lined by ciliated and non-ciliated cells. The lining cells of the distal tubule presented few microvilli, abundant dense mitochondria and clear vesicles of mucous appearance in the terminal portion. Collecting ducts are composed of mucous and non-mucous cells. Mucous cells presented strong reaction to the histochemical techniques detecting sialo- and sulpho-mucins.

During hibernation, a progressive vacuolar degeneration of the endoplasmic reticulum was observed in all the segments of tubular nephron, which may be caused by a massive intake of extracellular water into the cell.

Key words: Ultrastructure - Tubular nephron - Hibernation - *Testudo graeca*

Introduction

In recent years the reptilian kidney has been increasingly studied due to the pivotal role in the phylogenetical scale of this group of vertebrates (Anderson, 1960; Roberts and Schmidt-Nielsen, 1966; Davis and Schmidt-Nielsen, 1967; Schmidt-Nielsen and Davis, 1968; Davis et al., 1976; Fernández et al., 1978, a, b; Peek and McMillan, 1979, a, b; Gabri, 1983, a, b,; Gabri and Butler, 1984; Soares and Fava de Moraes, 1984; Solomon, 1985) Previous reports described the reptilian nephron as composed of: renal corpuscle, proximal, intermediate and distal tubules and collecting duct (Bishop, 1959; Kahlil et al., 1974, a, b; Gabri, 1983 a); however, marked variability has been found in the ultrastructure of the tubular cells which has been attributed to the enormous variety of species and habitats of the animals included in this group (Gabri, 1983 a). Most studies, however, have been concerned with the ultrastructure of the Lacertidae. Little attention has been given to the Chelonia (Solomon, 1985) and no studies have been conducted to elucidate the alterations in the nephron produced by the hibernation period.

This study was undertaken in an attempt to establish the ultrastructural characteristic of the tubular component of the nephron of *Testudo graeca* and the morphological changes which take place during the hibernation periods. A similar study was previously carried out in the renal corpuscle of the same specie (Zuasti et al., 1986).

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Materials and methods

Adult specimens of *Testudo graeca* (n=10) of both sexes were caught in their own habitat in August (non hibernating period) and February (hibernating period). The animals were anaesthetized by intraperitoneal injection of 3 mg of pentobarbital for 100g of body weight. The specimens were sacrified and the kidneys were exposed.

Samples for light microscopic study were fixed in 10% buffered formalin or Bouin's solution for 24h and processed routinely. 5μm thick sections were stained by the following methods, H.&E., PAS (Martoja and Martoja, 1970), alcian Blue (AB) at pH 2.5 and pH 1 (Pearse, 1985), aldehyde fuchsin (AF) (Gabe, 1968), alcian Blue (ph 2.5)-PAS (AB-PAS) (Ganter and Jolles, 1969), alcian Blue at pH 2.5 after methylation and saponification (Met + Sap + AB 2.5) (Pearse, 1985), high iron diamine (HID) (Spicer, 1965), high iron

diamine-alcian blue at pH 2,5 (HID-AB) (Spicer, 1965) and alcian Blue at different concentrations of $C1_2Mg$ (Scott and Dorlin 1965).

Samples for electron microscopy were fixed for 2h. in 3.5% cold glutaraldehyde buffered with 0.1M sodium cacodylate with $CaC1_2$ at pH 7.4. The fragments were postfixed for 1 h. in buffered 1% osmium tetroxide, washed in buffer, dehydrated and embedded in Epon resin (Bruijin, 1973). Ultrathin sections, obtained using a LKB Ultratome III, were stained with uranyl acetate and lead citrate, and examined in a Zeiss EM10C electron microscope.

Results

Light microscopy

The nephron of *Testudo graeca* is composed of the following segments: renal corpuscle, proximal tubule, intermediate segment, distal tubule and collecting duct (Fig. 1).

The proximal tubule is lined by simple columnar epithelium. The cells of this epithelium have abundant clear vesicles and basophilic granules in the apical cytoplasm. The cell apex exposed to the lumen of the tubule exhibits a brush border. The intermediate segment is lined by columnar cells larger than those observed in the proximal tubule. The cells also present a brush border in their apical poles. The distal tubule is a short segment which shows a wide lumen, and is lined by a cuboidal epithelium. No brush border is observed. The collecting duct is composed of large columnar cells with basally located nuclei. The apical pole of the cells is very irregular showing small processes towards the lumen.

During hibernation, the nephron contains the same segments as described above; a progressive vacuolization of the lining cells of all segments was observed in hibernating specimens (Fig. 2).

Histochemically, the apical portion of the proximal tubule shows a PAS-positive reaction both in the microvilli and cytoplasmic granules; thus, indicating the presence of neutral mucosubstances. The apical third of the cells lining the distal tubule present a weak staining for the techniques detecting sialo- and sulpho-mucins. In the collecting ducts, the lining cells present a strong reaction to sialo and sulpho-mucins (Fig. 3). Table 1 summarizes the results obtained in the different areas studied. No differences are observed between hibernating and non-hibernating animals in the mucin content of the different areas studied.

Electron microscopy

Proximal tubule

The luminal surface of the proximal tubule is provided with long microvilli. The apical aspect of the cytoplasm contains numerous short apical tubules, small vesicles and several lysosomes and vacuoles. There are big cytoplasmic protrusions. (Fig. 4). Each cell contains a large, oval, centrally located nucleus. Scattered throughout the cytoplasm there are mitochondria with an electrodense matrix and few cristae and a large number of smooth and rough reticulum cisternae (Figs. 4, 5). In the basal third of the cells there are several fat droplets and a wide space occupied by cytoplasmic projections (Fig. 5).

During hibernation, the cells lining the proximal tubule show a lower amount of cytoplasmic organelles. Some cells were detected in a degenerative process presenting few organelles and a vacuolised cytoplasm (Fig. 6). Occasionally, the lipid droplets show myelin figures (Fig. 6).

Intermediate segment

The intermediate segment is lined by two cell types: ciliated and non-ciliated, the latter being more abundant. Non-ciliated cells are morphologically similar to the cells of the proximal tubule although the cell organules are fewer and the microvilli shorter and less numerous than in the proximal cells (Fig. 7).

Ciliated cells show an irregular, basally located nucleus, abundant mitochondria and RER. Basal bodies and cilia were similar to those found in mammals (Fig. 7).

During hibernation non-ciliated cells showed an increase in number of the cisternae of SER which appear irregularly dilated and there were mitochondria with long cristae and a dense matrix. The ultrastructural characteristics of ciliated cells were similar to those observed in non-hibernating animals. In some areas, a progressive vacuolisation and loss of organules was detected in the cell cytoplasm (Fig. 8).

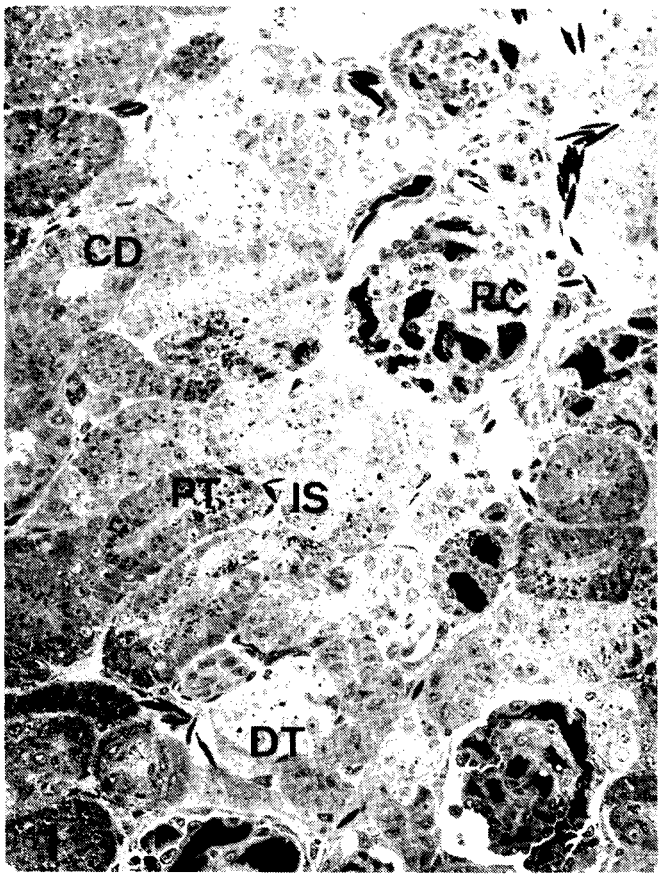
Distal tubule

The apical pole of the lining cells of the distal tubule present few microvilli (Fig. 9). Deep interdigitations with the neighbouring cells were observed (Fig. 10). The cytoplasm show abundant dense mitochondria, few cisternae of SER and RER and, occasionally, lysosomes (Figs. 9, 10). Abundant clear vesicles of mucous appearance are observed in the apical cytoplasm of the cells lining the terminal portion of the distal tubule (Fig. 11).

The morphology of the distal tubule in hibernating animals is similar to that observed in non-hibernating specimens; only the SER appear to increase in number showing a vacuolised aspect (Fig. 12).

Collecting duct

The collecting ducts are composed of two kinds of cells: mucosus and non-mucous cells. The mucous cells are the most abundant in the collecting ducts. These cells show a basal nuclei and interdigitations between the lateral margins of adjacent cells. The cytoplasm is filled by mucous granules and few mitochondria and cisternae of RER (Fig. 13). The non-mucous cells are characterised by the presence of abundant long mitochondria, with a dense matrix and numerous cristae;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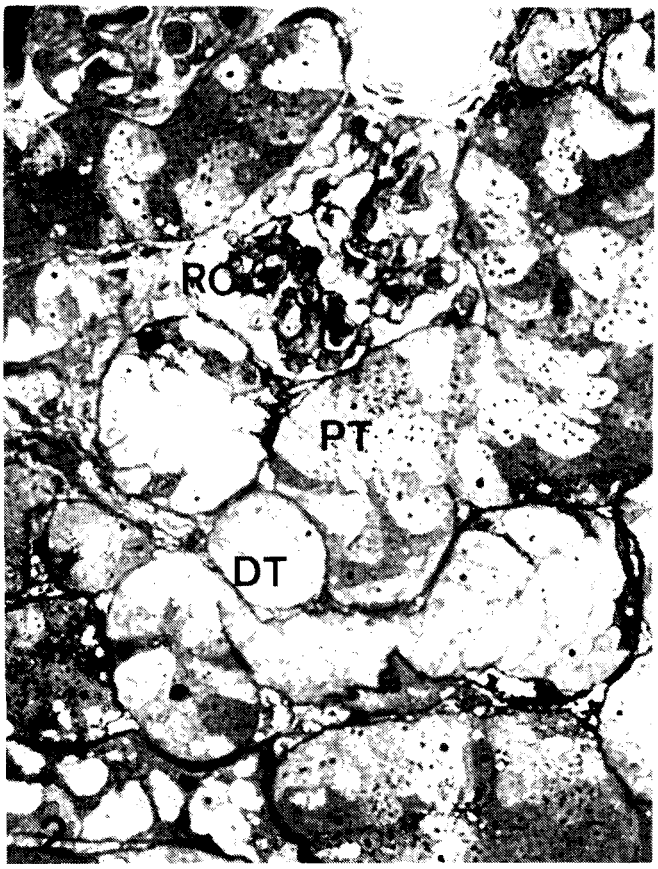
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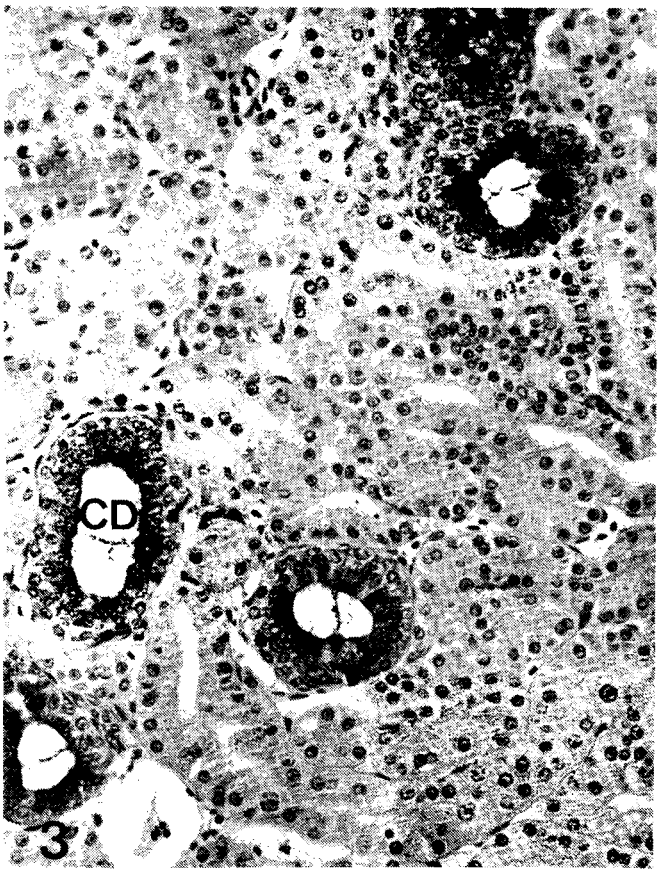
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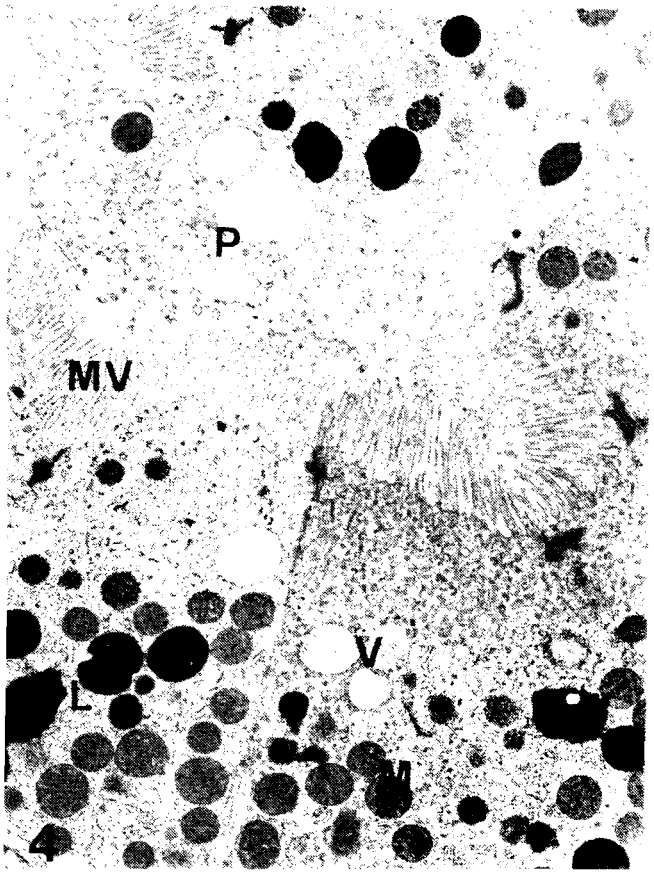
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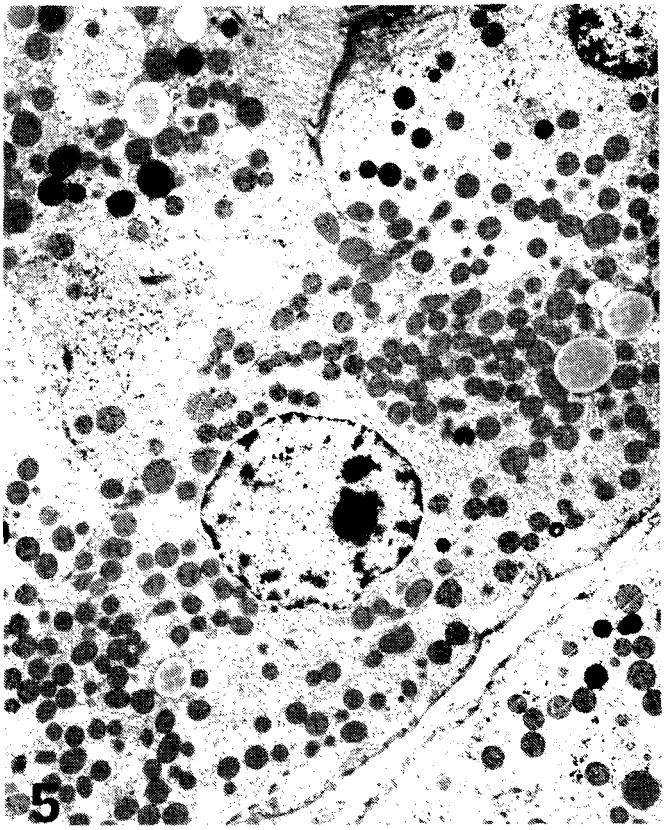
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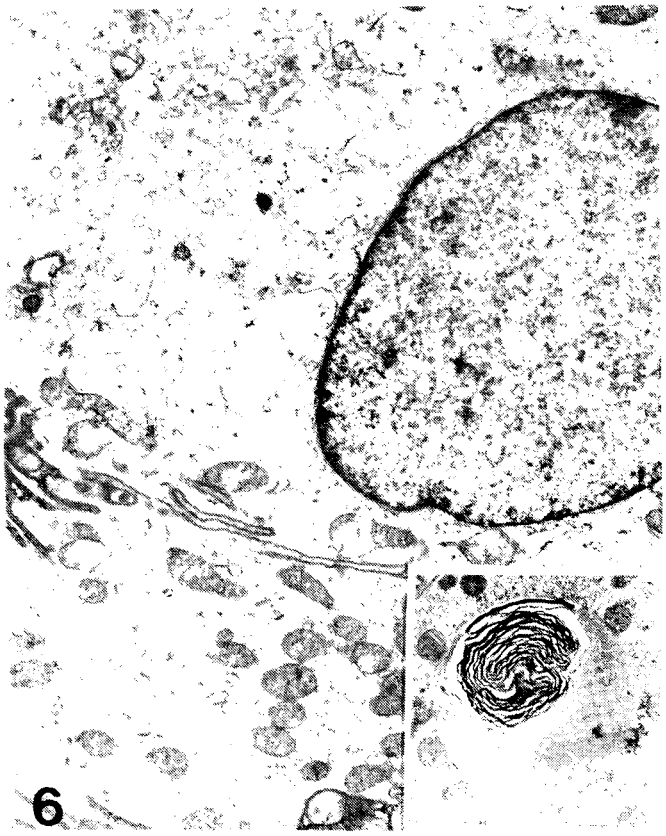
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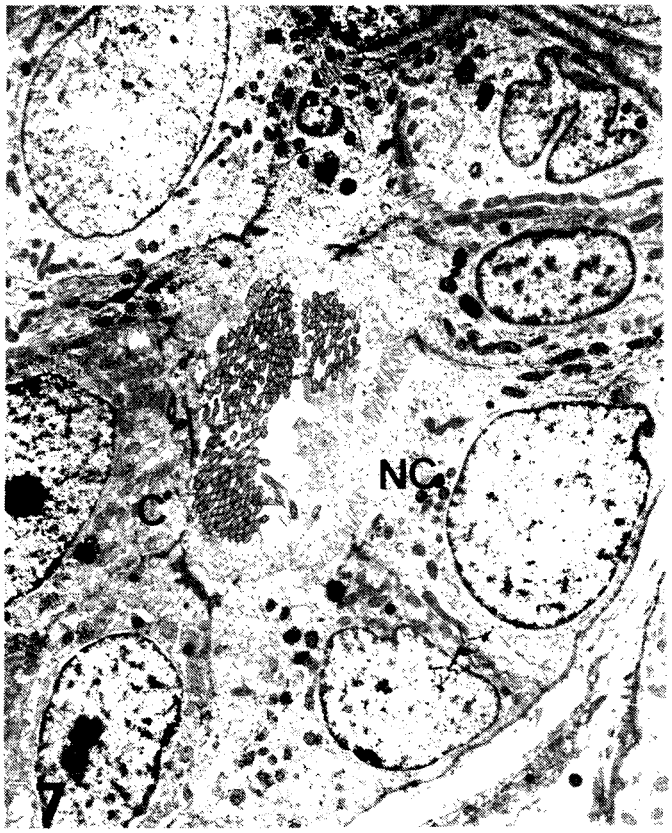

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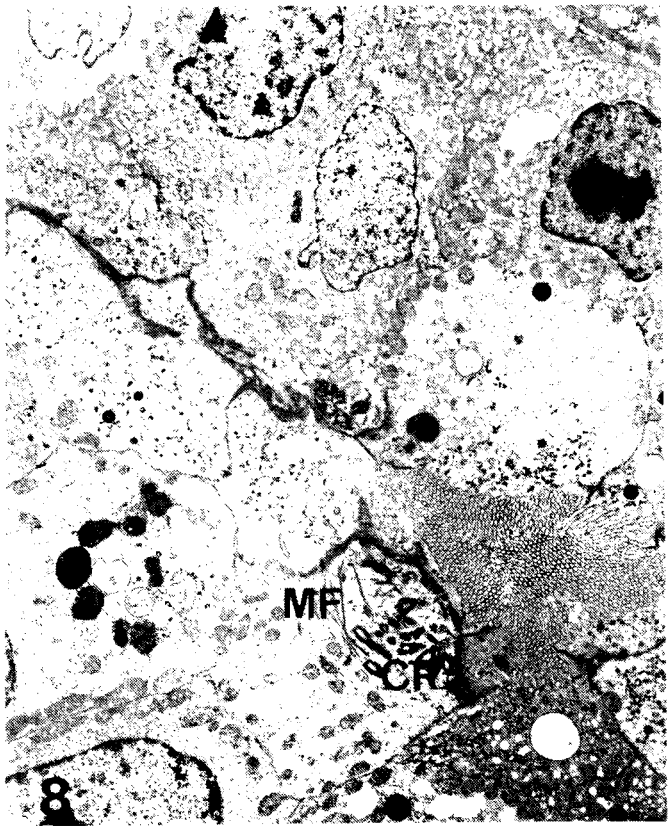

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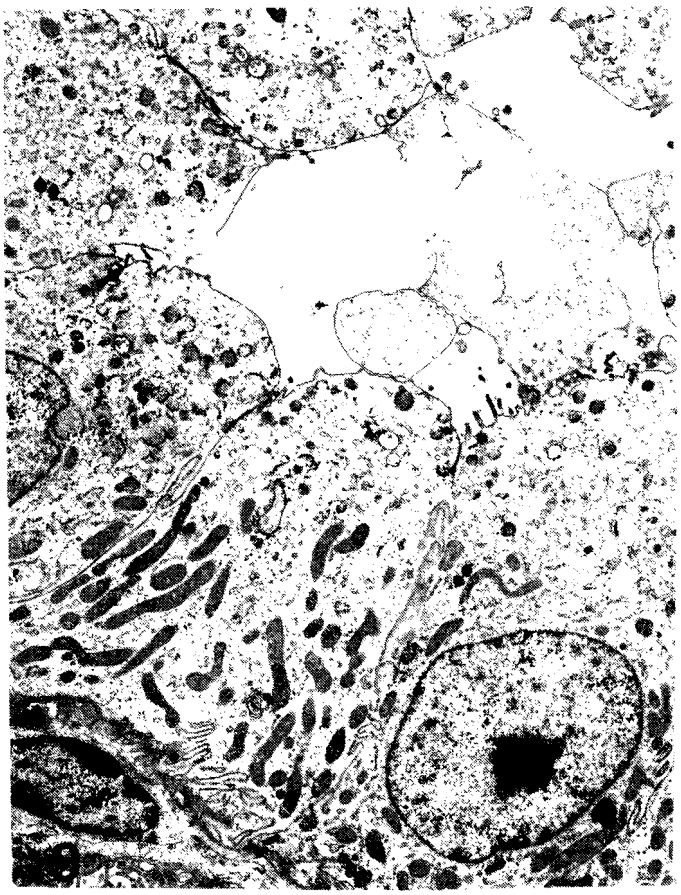

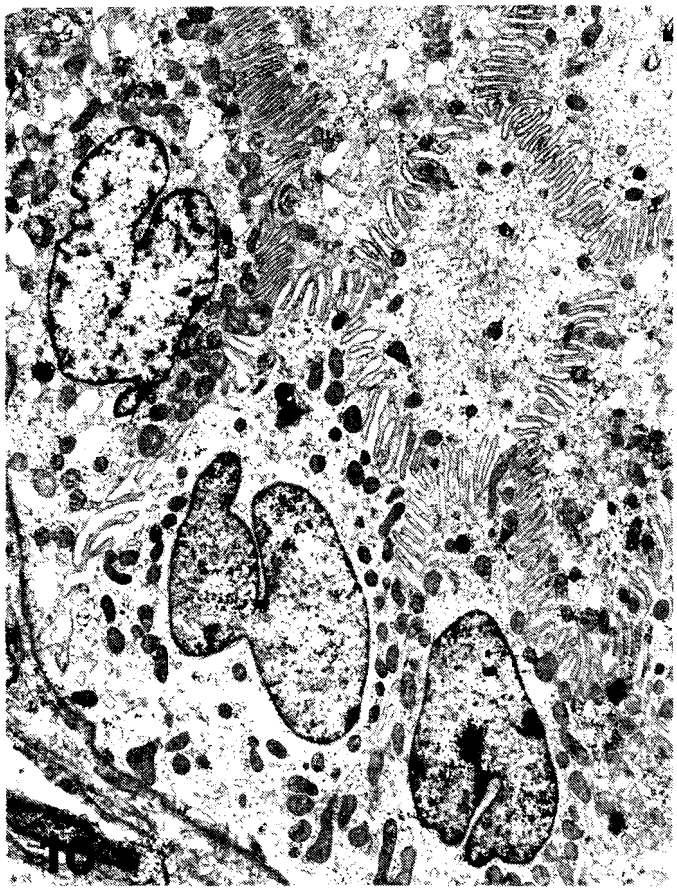

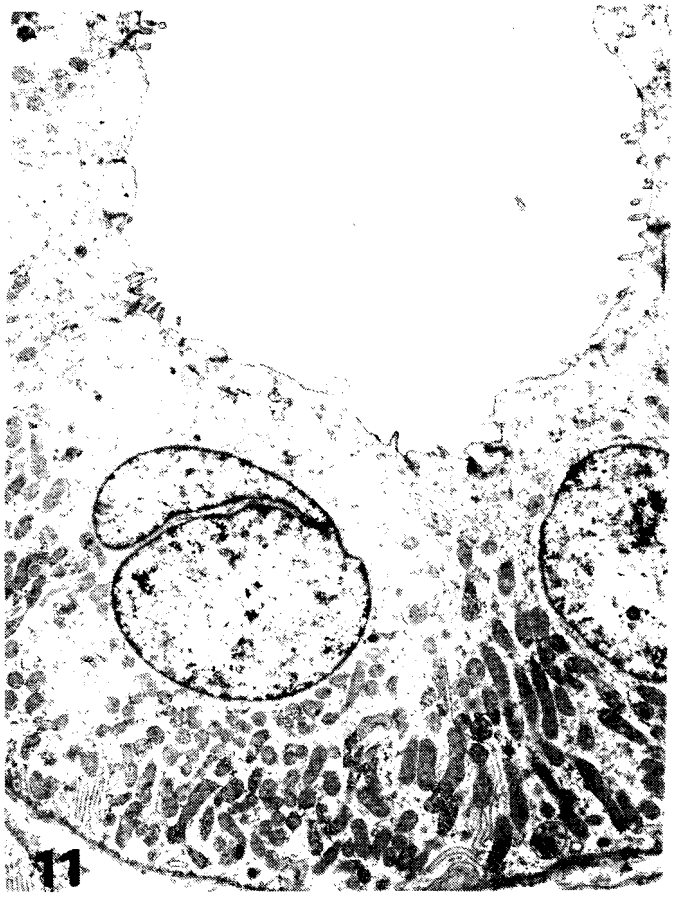
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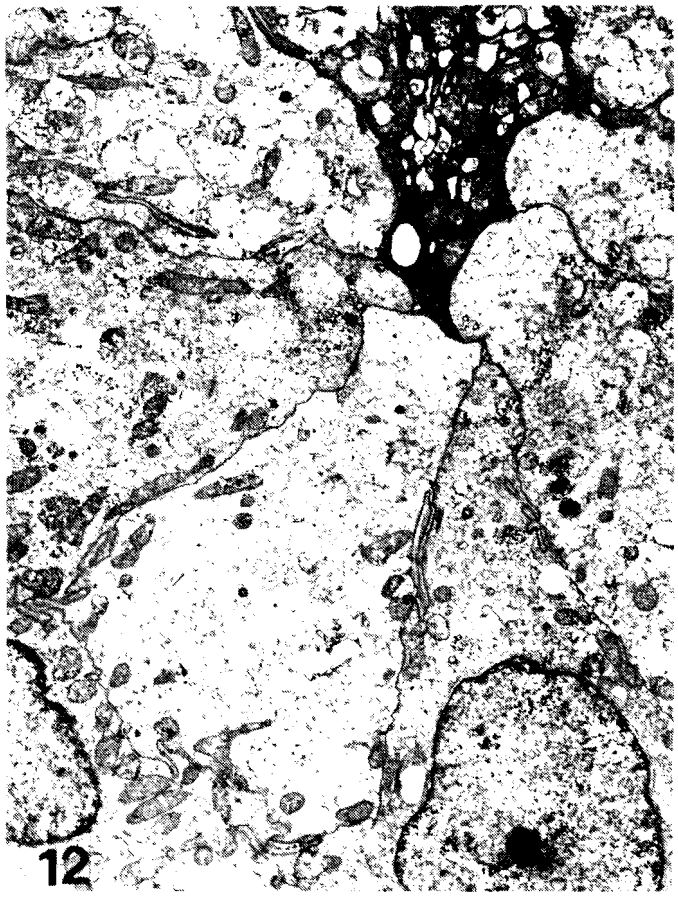
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Fig. 1. Kidney of non-hibernating *Testudo graeca* showing the different segments of the nephron. RC: renal corpuscle. PT: proximal tubule. IS: intermediate segment. DT: distal tubule. CD: collecting duct. ×400

Fig. 2. Kidney of hibernating *Testudo graeca*. Note the vacuolization of the lining cells of all segments. RC: Renal corpuscle. PT: proximal tubule. DT: distal tubule. ×1,000

Fig. 3. Kidney of non-hibernating *Testudo graeca*. The collecting ducts (CD) have mucous cells with a strong reaction to sialo- and sulpho-mucins. HID-AB. ×500

Fig. 4. Electron micrograph of the apical region of the proximal tubule of non-hibernating *Testudo graeca*. The cells have tightly packed microvilli (MV), cytoplasmic protrusions (P), large endocytic vacuoles (V), mitochondria (M) and lysosomes (L). ×10,000

Fig. 5. Proximal tubule of non-hibernating *Testudo graeca* showing cells with a prominent nucleus and numerous mitochondria. Abundant cytoplasmic processes are noted in the basal and lateral aspects of the cells. ×9,500

Fig. 6. Proximal tubule of hibernating *Testudo graeca*. The cells are largely devoid of organelles. ×8,000. Inset: Some cells show lipid droplets with myelin figures. ×8,000

Fig. 7. Intermediate segment of non-hibernating *Testudo graeca*. Cross section showing ciliated (C) and non-ciliated cells (NC). ×5,000

Fig. 8. Transition from the proximal tubule to the intermediate segment of hibernating *Testudo graeca*. Ciliated cells show ciliary roots (CR) and microfilaments (MF). Vacuolisation of some cytoplasmic areas can be observed. ×5,000

Fig. 9. Electron micrograph of the distal tubule of non-hibernating *Testudo graeca*. The cells display nuclei located in a basal position, apical microvilli and basal mitochondria. ×5,000

Fig. 10. Section through the cells of the distal tubule of non-hibernating *Testudo graeca* showing lateral folds between the adjacent cells. ×5,000

Fig. 11. Terminal region of the distal tubule of non-hibernating *Testudo graeca* showing clear mucous vesicles in the apical cytoplasm. ×5,000

Fig. 12. Distal tubule of hibernating *Testudo graeca* showing scarce organules and a characteristic electron-dense cell. ×6,500

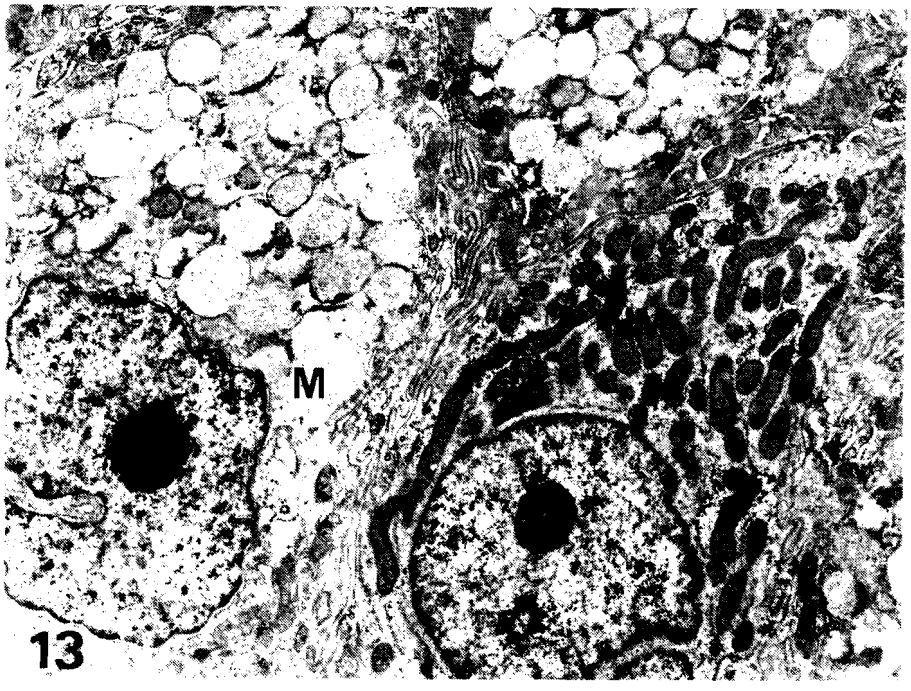

Fig. 13. Cross section of a collecting duct of non-hibernating *Testudo graeca* showing mucous (M) and non-mucous cells (NM). The cytoplasm of the mucus cells is filled by mucus granules. Non-mucous cells has abundant long mitochondria. ×9,500

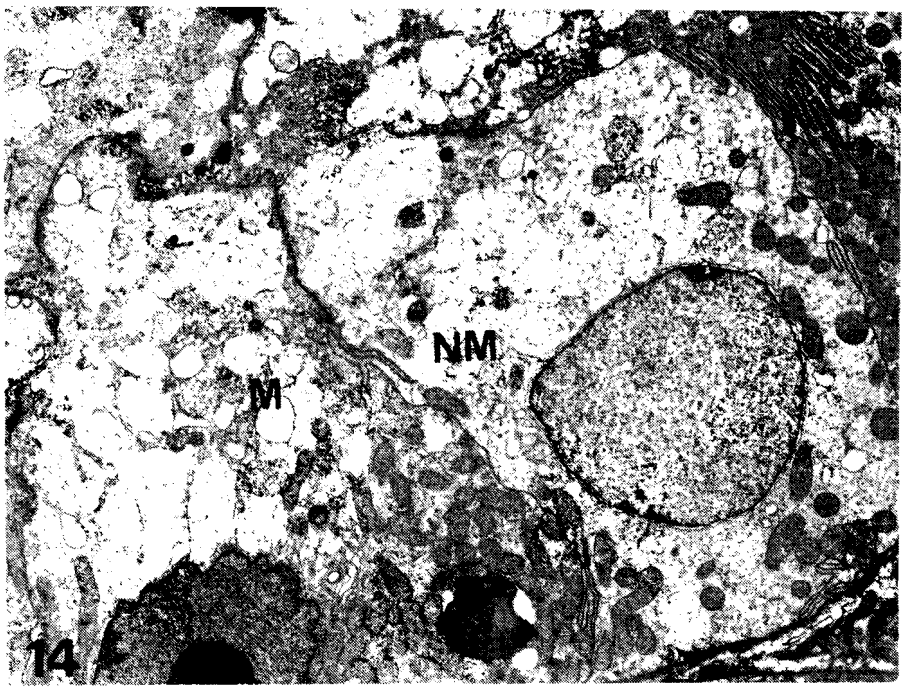

Fig. 14. Collecting duct of hibernating *Testudo graeca*. The granules of the mucous cell (M) appear disorganized. The non-mucous cell (NM) has scarce organules. ×8,500

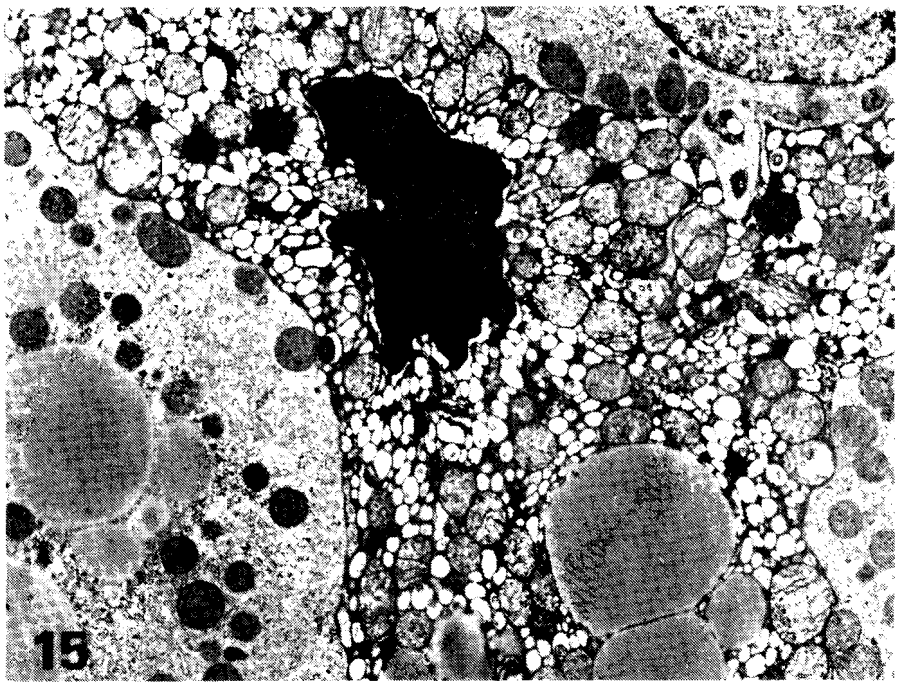

Fig. 15. Dark cell of the proximal tubule of hibernating *Testudo graeca*. The nucleus is deeply indented. Mitochondria, clear vesicles and lipid droplets can be seen in the cytoplasm. ×9,500

Table 1. Distribution of mucosubstances in the epithelium of proximal tubule (PT), distal tubule (DT) and collecting tubule (CT) of the kidney of *Testudo graeca*.

		PAS	Am + PAS	AB2.5	AB1	AB2.5 − PAS	AF	Met+ Sap+ AB2.5	HID	HID + AB2.5	AB Cl_2Mg 0.004mol/1	AB Cl_2Mg 0.1mol/1	AB Cl_2Mg 0.4mol/1	AB Cl_2Mg 0.7mol/1	AB Cl_2Mg 1mol/1
P.T.	Brush border	2P	2P	−	−	2P	−	−	−	−	−	−	−	−	−
	Apical granules	1P	1P	−	−	1P	−	−	−	−	−	−	−	−	−
D.T.	Apical cytoplasm	−/1P	−/1P	−/1B	−/1B	−/2BP	−/1R	−/1B	−/1N	−/2BN	−/1B	−/1B	−/1B	+/1B	−
	Cellular border	1P	1P	1B	1B	2BP	1R	1B	1N	2BN	1B	1B	1B	−/1B	−
C.T.		−/3P	−/3P	−/2B	−/2B	−/4BP	−/2R	−/2B	−/2N	−/4NB	−/2B	−/2B	−/2B	−/1B	−

Key to symbols in table:
P, PAS-positive
B, AB-positive
N, HID-positive
−, negative
R, AF-positive
BP, a mixture of AB− and PAS-positive mucins, with AB−positive mucins predominating
PB, a mixture of PAS AND AB-positive mucins, with PAS-positive mucins predominating
BN, a mixture of AB− and HID-positive mucins, with AB-positive mucins predominating
NB, a mixture of HID− and AB-positive mucins, with HID-positive mucins predominating

Symbols separated by/indicate a mixture of cells with different mucin content. Numerical values from 1 to 4 correspond to increasing intensity of staining.

some cisternae of RER are also observed (Fig. 13).

During hibernation the granules of the mucous cells appeared disorganized, the cytoplasm was almost devoid of organules and some cells underwent degenerative changes. The non-mucous cells presented a decrease in number of mitochondria and vacuolisation of cytoplasm (Fig. 14).

A characteristic cell type was found in all the segments of the tubular nephron of hibernating *Testudo graeca*. These cells had a large, deeply indented nucleus and an electron-dense cytoplasm containing mitochondria, lipid droplets and abundant clear vesicles (Fig. 15).

Discussion

The tubular portion of the nephron of *Testudo graeca* is composed of proximal, intermediate, distal and collecting tubules in both hibernating and non-hibernating specimens.

The epithelial cells of the proximal tubule show a well-developed brush border. Marked protrusions of the apical cytoplasm, suggesting a secretory activity, are observed in some cells (Anderson, 1960; Davis and Schmidt-Nielsen, 1967; Gabri, 1983 a). These protrusions have been related to the secretion of uric acid by the cells (Gabri, 1983 a). In the basal portion, there exist marked intercellular spaces similar to those described in other reptiles (Anderson, 1960; Roberts and Schmidt-Nielsen, 1966; Davis and Schmidt-Nielsen, 1967; Schmidt-Nielsen and Davis, 1968; Davis et al., 1976; Solomon, 1985). These spaces may be important in the exchange of fluids (Gabri, 1983 a). In *Testudo graeca*, the typical basal labyrinth of the mammalian species is absent as occurs in other reptilian species (Roberts and Schmidt-Nielsen, 1966; Peek and McMillan, 1979; Soares and Fava de Moraes, 1984; Solomon, 1985). The brush border and apical cytoplasmic granules of the proximal tubule are PAS-positive. This finding has been previously reported in other vertebrates (Soares and Fava de Moraes, 1983; Solomon, 1985).

The morphology of the intermediate segment of *Testudo graeca* is similar to that of both Henle's loop of mammals (Soares and Fava de Moraes, 1984) and intermediate segments of other reptiles (Davis et al., 1976; Fernández et al., 1978). Ciliated cells are frequently observed in this segment of the nephron of lower vertebrates. The role of ciliated cells may be related to the low blood pressure of these animals which produces a low filtration pressure in the kideny, cilia having then a propulsive action of the ultrafiltrate (Davis et al., 1976). In higher vertebrates, where the blood pressure increases, no cilia are usually found in the tubular nephron (Marshall, 1934; Giebrish, 1973; Soares and Fava de Moares, 1984).

The small amount of microvilli observed in the distal tubule of *Testudo graeca* indicates that the absorptive function of this segment is very low (Fernández et al., 1978). In the present study, mucus-secreting cells has been demonstrated by histochemistry and electron microscopy in the distal tubule. The presence of mucous cells in the distal tubule has been previously observed in the kidney of some lizards (Soares and Fava de Moares,

1983). It may be related to a gradual differentiation of distal tubular cells towards mucous cells of the collecting duct. The lateral interdigitations detected in this portion are similar to those found in other reptiles (Solomon, 1985).

The collecting duct of *Testudo graeca* is formed by mucous and non mucous cells. Mucous cells are very abundant, as occurs in lizards and snakes (Bishop, 1959; Gabe, 1959; Anderson, 1960; Fernández et al., 1978; Gabri, 1983b; Soares and Fava de Moares, 1984), and contain both sialo- and sulpho-mucins; however, in other Chelonia, Solomon (1985) reported that mucous cells were located in the distal portion of the intermediate segment and not in the collecting duct. The role of mucins in the tubular nephron of reptiles has been related to the excretion of non-soluble urates; thus, the urate crystals are surrounded by mucus which acts as a lubricating agent and avoids any injury of the epithelium (Minnich, 1972; Davis et al., 1976; Peek and McMillan, 1979; Gabri, 1983b; Solomon, 1985).

During hibernation, a progressive vacuolar degeneration of the endoplasmic reticulum is observed in all segments of the tubular nephron of *Testudo graeca* . A similar process has been reported in the renal corpuscle of this turtle (Zuasti et al., 1986). During hibernation of terrestrian reptiles, a reduction of extracellular fluid occurs without a considerable loss of weight by the animal, indicating that the water is redistributed inside the body (Gregory, 1982; Lawrence and Hawkey, 1986). Thus, the vacuolar degeneration observed in the tubular epithelium of the nephron of *Testudo graeca* may be caused by a massive intake of extracellular water into the cell (Ghadially, 1982).

During hibernation, characteristic, very electron-dense cells have been found diffusely distributed throughout the tubular nephron of *Testudo graeca* . These cells have been previously described in the renal corpuscle of *Testudo graeca* (Zuasti et al., 1986) and in the neprhon of a teleost fish (Zuasti et al., 1983) and birds (Nicholson and Kendall, 1983) where they seem to play an important role in the secretion-reabsorption of potassium.

Acknowledgements. We are grateful to Mrs. M. García, Mrs. M.C. González and Mrs. F. Calderón for technical assistance and to Ms. R. Vicente who typed the manuscript.

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Accepted July 14, 1987