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INCORPORATION BY THE KIDNEY OF FLUORESCENT PITUITARY HORMONES

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SUMMARY

Several Armour and NIH pituitary hormone preparations as well as other homologous serum protein fractions were labeled with fluorescent dyes (sulphorhodamine B, C.I. 45100, and fluorescein isothiocyanate). Rats were injected intravenously with the different labeled proteins and the animals killed at various intervals from 3 minutes to 10 days. Sections of the kidneys were studied under ultraviolet light (3,900 Å). It was observed that all the labeled proteins passed the glomerular filter and were accumulated by the cells of the proximal convoluted tubules. Labeled pituitary hormones were, in addition, reabsorbed by the thin limb and collecting tubules and appeared in the lumen of bladder. Filtration and reabsorption of proteins by the kidney can proceed independently of their nature and origin. All the proteins were reabsorbed with the same sequence, but pituitary hormones appeared faster and in larger amounts in the same sites of the nephron. They were also detected in the lumen of the ureter and in the bladder. It is assumed that the molecular weight of the proteins may play an important role, since the hormones' molecules were significantly smaller. It is remarkable that although the different hormones showed a preferential accumulation in the vessels of the corresponding target glands, they behave similarly in the kidney.

INTRODUCTION

The passage of injected homologous or heterologous proteins through the kidney glomeruli and their subsequent reabsorption by the proximal convoluted tubule cells has been histologically demonstrated using unlabeled diazonium salt (16, 17) or labeled (23) proteins. Findings with the electron microscope suggest that ferritin molecules are picked up by the visceral epithelium of glomerular capillaries in pinocytotic vesicles and transported via the small vesicles to larger vacuoles which are subsequently transformed in dense bodies. The content of these bodies may then undergo partial digestion and be extruded into the urinary spaces (5). After intraperitoneal administration of ox hemoglobin, it was also found that this protein passes from the lumen of the nephron through the brush border of the renal epithelial cells and is transported into dense absorption droplets (12). Most of the previous studies (6, 14) were carried out using serum fractions or other high molecular weight proteins. From pathologic evidence it has been demonstrated that those proteins are reabsorbed in the proximal convoluted tubules (15). It appeared appropriate to study at the microscopical level, the participation of the kidney in the incorporation of proteins of lower molecular weight, like protein hormones, as little is known regarding their behavior in this organ.

Several pituitary hormonal preparations, labeled with fluorescent dyes, and similarly labeled homologous serum fractions were used for this experiment.

MATERIAL AND METHODS

Several preparations of follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactine, growth hormone (GH), adrenocorticotropic hormone (ACTH) and thyrotrophin (TSH) were labeled with different amounts of sulphorhodamine B (C.I. 45100) according to Chadwick's technique (1) for serum proteins; slight modifications were introduced to adapt this technique to the physicochemical properties of the hormones. Thyrotrophin, growth hormone and gonadotrophins were also labeled with fluorescein isothiocyanate following Riggs' technique (20) similarly modified. After labeling, the hormones were lyophilized before use. Homologous rat albumin, globulins and fibrinogen were prepared

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according to techniques previously described (9, 10) and also separately labeled with one or both fluorescent dyes. After labeling the amount of protein-bound dye was photometrically determined and the biological activity of the hormones controlled by the corresponding methods (Table I). Different lots of Wistar rats weighing between 70 and 100 gm were intravenously injected with different doses of the labeled hormones, 2, 5, 10 or 20 mg in 0.5 cc saline solutions. Other lots of animals were similarly injected with labeled homologous serum proteins at the same concentrations in which they are found in the rat blood. The rats weighed from 70 to 150 gm, and the doses used ranged from 0.5 to 2 cc per 100 gm of body weight; this represents 10 to 40 mg per 100 gm of body weight for albumin and 22 to 88 mg per 100 gm of body weight for globulins. Labeled fibrinogen was administered at doses from 5 to 10 mg per 100 gm of body weight. As a control, another lots were similarly injected with denatured thyrotrophin and growth hormones (heated in acid medium for one hour) in doses of 2 and 10 mg or with 0.8 cc of 1 to 3% solution of free fluorescent dyes.

The animals were killed at different intervals ranging from 3 minutes to 10 days after injection. Just before death, blood and urine samples were taken to determine spectrophotometrically the decay of the labeled proteins in the circulating blood and to check their elimination by the urine, as previously described (9, 10). The kidneys and other tissues were fixed in 4% formaldehyde, embedded in paraffin and adequately prepared for fluorescent microscopy. Sections were observed under light at a maximum wave length of 3900 Å.

TABLE I

Biological Activity of Pituitary Hormones after Labeling

Hormone	Bound Sulphorhodamine	Method	Activity Retained
	μg/mg hormone		*per cent*
TSH	23 41 70	Incorporation of radioiodine by newborn chicken	88 78 56
ACTH	62 26	Depletion of adrenal ascorbic acid	20 52
GH	78 28	Body weight of growing rats	30 50
FSH	56 27	Ovarian weight	37 56
LH	43 21	Ovarian ascorbic acid depletion	39 57
Prolactin	52	Not checked	

RESULTS

In microscopical observations of the kidney, sites of induced fluorescence due to the presence of labeled proteins, were better seen with sulphorhodamine B than with fluorescein isothiocyanate. The higher contrast provided by the former against the pale green autofluorescence of the background, rendered fluorescent images of better resolution, improving the sensitivity of the method used. Distribution of labeled proteins in the structures of the kidney was the same with both fluorescent dyes. Bright green native fluorescence was restricted to the elastic membranes of vessels and sometimes to granules of unknown nature present in the cells of proximal convoluted tubules. For comparative purposes, the results obtained with serum proteins will be described before those obtained with pituitary hormones.

1) **Labeled serum proteins:** Particulars may be found elsewhere (7) of spectrophotometric analysis of free fluorescent dyes, and the amount bound to serum fractions, the demonstration of some physicochemical changes of proteins after labeling and the time concentration curves of labeled serum proteins in the circulation.

Fluorescence induced by the bound labeling dyes revealed histologically that the labeled serum fractions localized in the lumen of all blood vessels of the kidney a few minutes after intravenous injections. The decay in the circulation of the injected labeled proteins, measured spectrophotometrically, paralleled the progressive decrease of the fluorescent material in the renal glomerulus and their subsequent reabsorption by the proximal tubules. The fluorescence localized in the lumen of the blood vessels of the kidney, diffused extravascularly and was accumulated at the basement membranes of the proximal tubules like that observed in the vascular bed of other tissue. However, in the vasa recta of the renal papillae, fluorescence was higher than in the cortex.

In the first few minutes, albumin, and in lesser amounts globulins and fibrinogen were observed in the lumen and were also attached to the basement membrane of vascular tufts of glomeruli in some areas of the cortex.

Subsequently, albumin and globulins, in very negligible traces, were seen in the lumen of the Bowman capsule and passed to the lumen of con-

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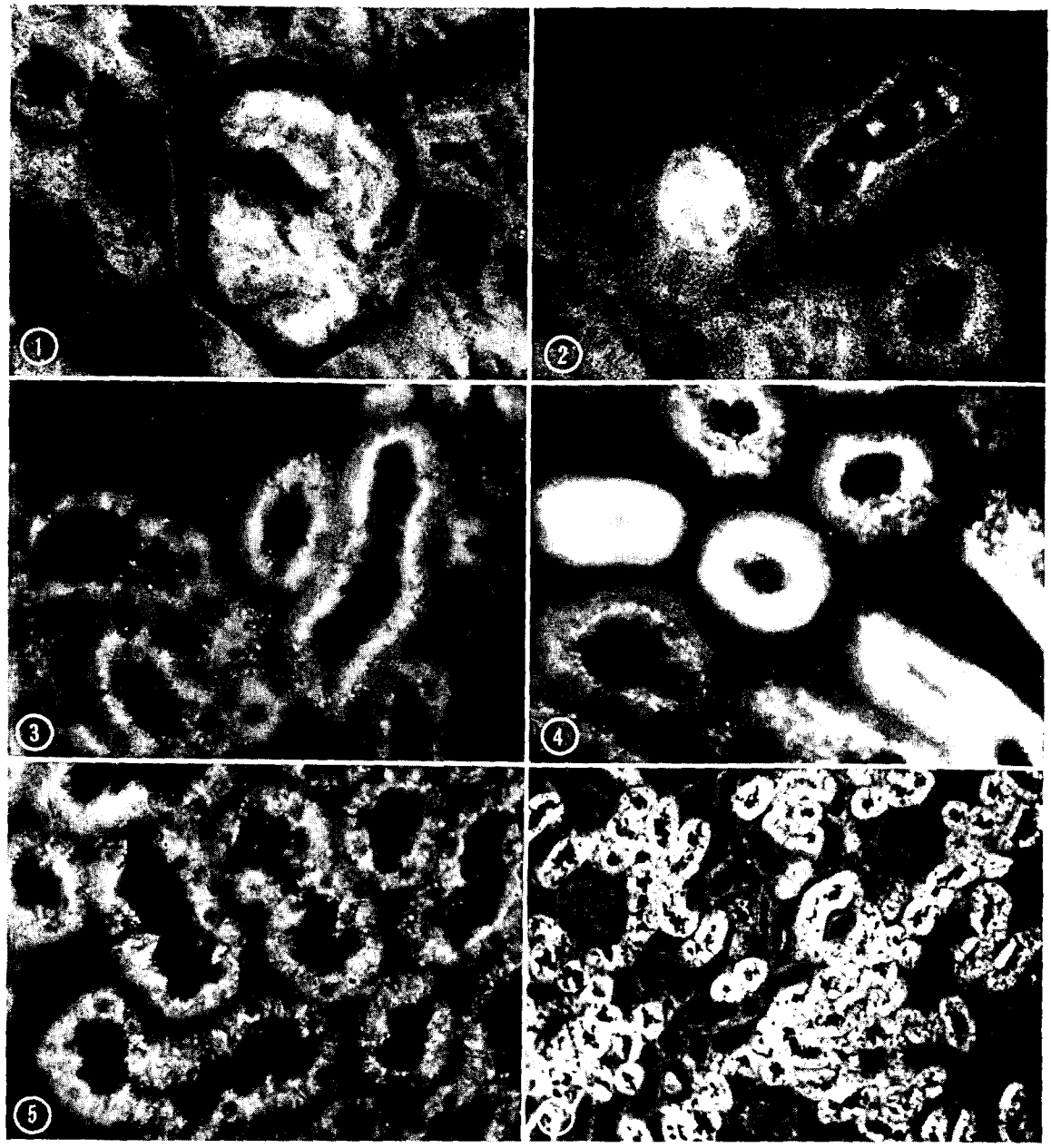

Images of bright fluorescence (white granules or diffuse masses) correspond to the presence of either FSH, LH. TSH preparations labeled with sulphorhodamine B. The hormones were detected in the kidney between 3 to 30 minutes after intravenous injection.

FIG. 1. Hormones appeared in the lumen and walls of capillaries of glomerular tuft. ×400.

FIG. 2. They are seen in the lumen and apical portion of convoluted proximal tubules. ×400.

FIG. 3. Diffusing from the apical portion to the interior of epithelial cells of same tubules. ×400.

FIG. 4. Varied degree of incorporation in the same cells. ×400.

FIG. 5. Occupying the cytoplasm of same cells; sometimes concentrated in the perinuclear zone. ×400.

FIG. 6. Massive incorporation by the proximal convoluted tubules. Glomeruli and distal convolutions show no fluorescence. ×400.

voluted proximal tubules. After six hours, fluorescence had almost dissappeared from the glomerulus, the capillaries and tubular basal membranes of the cortex, but persisted twelve or more hours in the vasa recta of the papilla. At about eight to twelve hours, it began to appear as small granules in the apical portion of the cells belonging to some groups of proximal convoluted tubules, surrounding glomeruli now empty of fluorescent material. After a slow increase lasting up to 72 hours and

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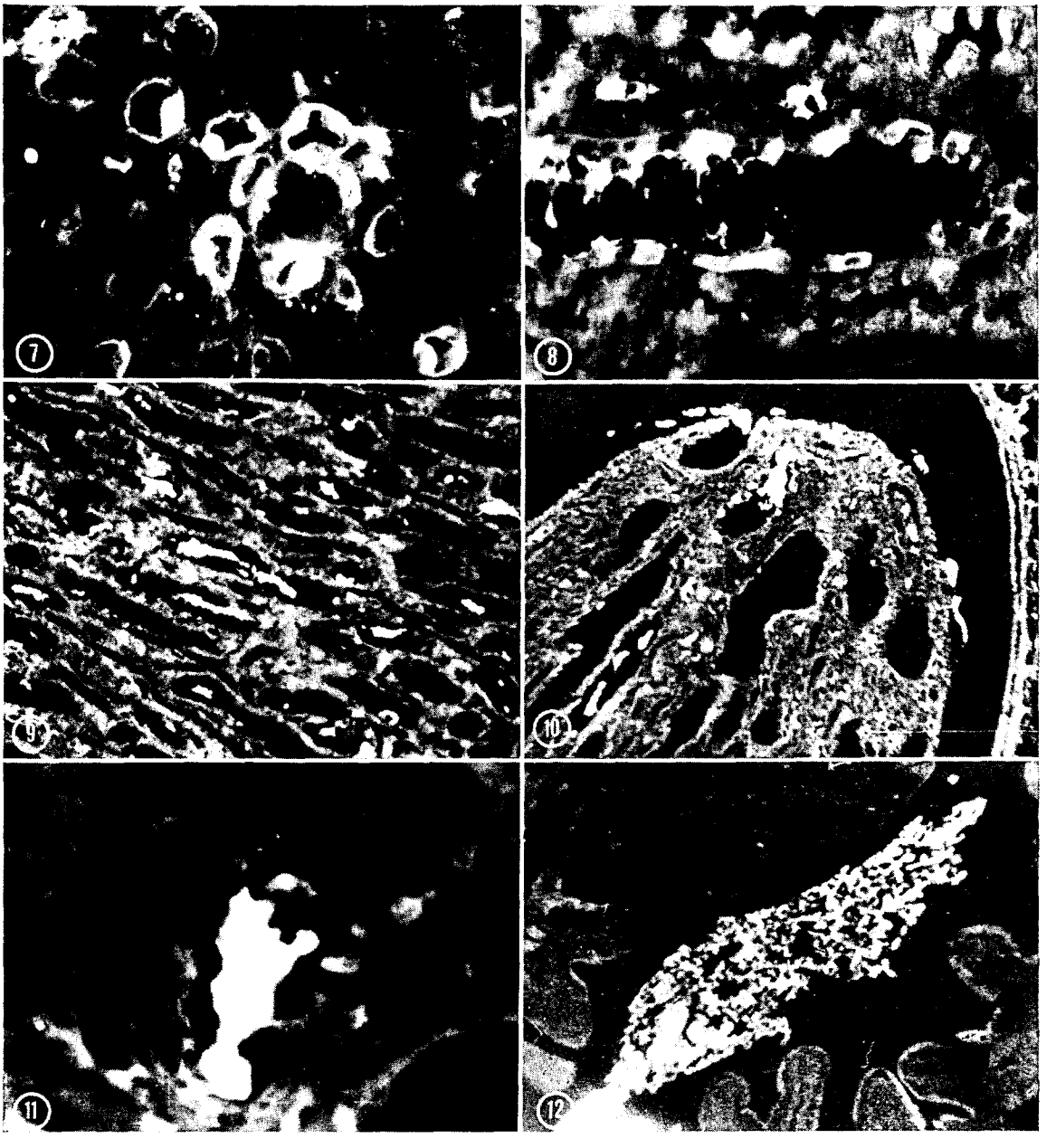

FIG. 7. Henle's loop, thin portion, with evident incorporation in the cytoplasmic band surrounding the nuclei. ×400.
FIG. 8. Hormones are seen in the perinuclear cytoplasm of cells of distal convoluted tubules. ×400.
FIG. 9. Lumen of collecting and distal tubules with masses of labeled hormones. ×100.
FIG. 10. Big collecting tubules opening in the renal papillae and containing fluorescent material. ×100.
FIG. 11. One of those collecting tubules showing the fluorescent hormones in the lumen and also in the cytoplasm of epithelial cells. ×100.
FIG. 12. Bladder with a large mass of fluorescent material in the lumen. ×100.

involving the whole cytoplasm of the cells, merely granules remained 6 to 8 days, disappearing gradually thereafter. Nuclei of the cells appeared free of fluorescent material. The images already described increased in intensity as doses were raised, but the proteins were not detected in the rest of the nephron even if a maximal amount was injected (2 cc per 100 gm of body weight). Neither the labeled albumin nor globulins could be detected in the collected urine as it was shown by spectrophotometry.

2) **Labeled pituitary hormones:** The biological activity of the hormones after labeling, as measured with standardized methods (Table I),

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showed a variable loss in relation to the amount of bound dye when compared with similar unlabeled batches. Only the hormones labeled with sulphorhodamine B were checked and the biological activity is expressed as a percentage of the average values obtained with the corresponding method in a group of animals for each hormone.

After intravenous injection, the labeled pituitary hormones appeared distributed in the circulation as serum proteins do, but they disappeared faster from it, between 15 and 190 minutes. Details concerning the differences in the decay among the different hormones may be found elsewhere (8).

Under the fluorescence microscope, the distribution of the labeled hormones in the blood vessels and in the kidney were similar. The intensity of the images was higher for TSH, FSH and LH and minimal for ACTH, GH and Prolactin. A few minutes after intravenous injection, the hormones were present in the lumen of the vascular system and in the wall of capillaries.

There was a preferential accumulation of labeled material in the vessels of the corresponding target gland (8, 9). The intensity of fluorescence in the vascular bed decreased after 10 minutes. The fluorescent material was also recognized in the vessels of the kidney where, as in the case of serum proteins, it disappeared more rapidly from the cortex than from the medulla.

Pituitary hormones appeared faster and in greater amounts than serum proteins in the same sites of the nephron, reaching their maximum at 6 hours and disappearing after 96 hours. The sequence of events was the same as far as the passage through the glomeruli via the lumen of convoluted proximal tubules were concerned. Images of absorption were first seen clearly in the brush border and in the cytoplasm of the cells, in the form of granules sometimes surrounded by vacuoles. In addition, small amounts of these labeled hormones were observed as homogeneous masses in the lumina of most of the distal convoluted tubules as well as in the collecting ones. Images of reabsorption by the epithelial cells were frequent. In some areas, fluorescent material was also present in the thin band of cytoplasm of cells belonging to the thin portion of Henle's loop. As in the case of serum proteins, no labeled hormones could be detected in the nuclei of epithelial cells. Neither could evidence of passage from these cells to the intertubular connective tissue be detected.

Finally, the labeled hormones were detected as gross spots of dense material in the lumen of the ureter and in the bladder, and at some sites became attached to the surface of the epithelium. In the collected urine, a labeled protein could be precipitated and was demonstrated as a diffuse band by paper electrophoresis. In no case could free dye be detected by dialysis in the urine.

The sensitivity of the method used increased with the amount of the labeled hormone preparation injected. The threshold of resolution was about 1 to 2 mg per injected animal. Hormones labeled with larger amounts of dye, also showed higher fluorescence, although in these cases the biological activity had considerably diminished.

When animals were similarly injected with the same amounts of labeled but denaturated hormones, they disappeared from the circulation faster than the labeled native hormones. TSH was almost gone after 60 minutes and ACTH was no longer detectable after 8 minutes. Histologically, they also decreased rapidly, as fluorescent material in the lumen of the vascular bed of different tissues, and of the cortex of the kidney. Although the sequence was the same, fluorescence along the different parts of the nephron from the glomeruli to the bladder was stronger when compared with undenaturated hormones.

3) **Solutions of free labels:** When these free solutions were intravenously injected, they disappeared from the circulation in about 20 to 40 minutes. Although such free labels could not be demonstrated in the lumen of blood vessels or in the glomeruli, they appeared as small and scarce granules in the cytoplasm of the proximal convoluted tubule cells a half hour after injection, and disappeared after 24 hours. Most of the dye passed rapidly into the intestine through the biliary tract, and was easily recovered by dialysis in the urine.

DISCUSSION

Present results using pituitary hormones mainly add direct histological evidence which reinforces previous views that injected proteins pass the glomerular filter and are incorporated by the cells of the renal convoluted tubules and accumulated there (16, 17, 23).

Though it seems that the filtering membrane of the glomerulus does not differentiate among homologous and heterologous proteins (6, 14, 17), it is evident that different proteins are not dealt with in the same way by the kidney. For example, fibrinogen was never seen inside the cells of the renal tubuli as was the case with other fractions of the serum proteins and hormones which were used in our experiments. Even with proteins which showed in all cases, the same sequence of events, differences were evident in the time for them to reach the epithelium of the tubules. The hormones were eliminated faster and reabsorbed in larger amounts. As the amount

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of labeled protein injected was similar or larger for serum fractions than in the case of hormones, it is reasonable to assume that the molecular weight of the proteins may play an important role, since the molecular weight of the hormones used was significantly smaller. Differences in the rate of filtration should also be considered. If it occurs slowly, as happens with serum proteins, this slow absorption enables the cells to metabolize absorbed proteins and so they are not accumulated. This could explain the differences in the histological picture representing the morphological expression of what is called the accessory mechanism of the droplet (16, 17), which is present when the capacity of the tubule cells is suppressed by the amount of protein reabsorbed. There are other differences among serum fractions and hormones which are observable in the thin limb and in the collectiong tubules. These cells showed fluorescence in their cytoplasm which probably could be interpreted as due to reabsorption, provided, as seems to be the case, that these proteins are not coming from another source, such as the intertubular vessels. In such a case, the loops of Henle, the distal convoluted and collecting tubules would act as an accessory mechanism of absorption when the capacity of the proximal convoluted tubules is saturated. This occurs in the case of pituitary hormones which could even be found in the urine of the injected animals. It appears then, that filtration and reabsorption of proteins by the kidney can proceed independently of their nature and origin, as proved by our results with slightly modified homologous serum proteins and heterologous hormones, partially inactivated by labeling with fluorescent dyes or completely denatured by heating. This is also backed by the finding of electrophoretically characteristic components of plasma proteins in the urine of rats and human beings (19, 22). Unlabeled hemoglobin and egg albumin have also been demonstrated after intravenous injection in the urine of rats (21) and tubular reabsorption revealed by light microscopy (16) or by labeling with fluorescent dyes or using immunological methods (11). By bio-assay, it was reported that nonlabeled ACTH (18) and radioactive GH and TSH are concentrated in the kidneys (24) while radioiodinated insulin and glucagon have been radioautographically localized in the convoluted proximal tubules after intravenous administration (13). The disappearance of fluorescent serum proteins and hormones from the renal tubular cells after several days strongly suggests a process of progressive degradation, as no passage of labeled material to the intertubular vessels could be detected and no rise in the time decline curve of labeled serum proteins appeared at this time (10). Thus, some of the fluorescent substance observed within the renal tubular cells may not be attached to the intact protein molecules, but to disrupted portions of these, or might even represent the split-off free label. This last assumption is supported by the fact that a few fluorescent granules are present in the proximal convoluted tubular cells when the free dye is intravenously injected.

Finally, the role of the kidney as an active site of protein catabolism was substantiated by many facts, such as the observation that the renal vein has a higher content of amino acids and polypeptides than does arterial blood (4) and that nephrectomy retards the rate at which radioactive serum albumin, insulin and glucagon are degraded (2, 3).

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