OXIDATIVE AND HYDROLYTIC ENZYMES IN THE NEPHRON OF *NECTURUS MACULOSUS*

Histochemical, Biochemical, and Electron Microscopical Studies

S. RALPH HIMMELHOCH and MORRIS J. KARNOVSKY, M.B.B.Ch.

From the Department of Pathology, Harvard Medical School, Boston. Dr. Karnovsky's present address is Institut d'Histologie et d'Embryologie, École de Médecine, Geneva, Switzerland

ABSTRACT

The distribution of oxidative and hydrolytic enzyme activities along the nephron of *Necturus maculosus* Rafinesque was studied histochemically. The proximal tubule possessed all the demonstrable enzyme activities associated with the hexose-monophosphate shunt and glycolysis, but lacked detectable succinic dehydrogenase and cytochrome oxidase activities. Krebs cycle enzymes other than succinic dehydrogenase were easily detectable. The distal tubule, on the other hand, possessed no detectable hexose-monophosphate shunt enzyme activities, but all demonstrable glycolytic and Krebs cycle enzymes and cytochrome oxidase were present in high activity. These data indicate that the proximal tubule of *Necturus* probably cannot depend, as can the distal tubule, on the Krebs cycle and cytochrome system to provide energy for its transport processes, an inference supported, in general, by available physiological evidence. The question of the importance of the hexose shunt to proximal tubular function arises. Evidence is presented that the proximal tubular blood supply is primarily venous in nature, a hypothesis which would correlate well with its anaerobic metabolic pattern. In addition, the absence of cytochrome oxidase and succinic dehydrogenase from the proximal tubular cells implies either that they possess very few mitochondria, or that their mitochondria have a very unusual enzymatic pattern. Electron microscopical observations and data obtained from the measurement of the enzyme activities of homogenates of *Necturus* kidney are presented which indicate that the second hypothesis is more probably correct.

Physiological investigations of the metabolic basis of active transport in the kidney, summarized by Eggleton (12), indicate that proximal tubular "reabsorbing mechanisms probably derive their energy from active phosphate, but do not appear to be dependent on the coupled oxidative-phosphorylation system." Proximal tubular secretory mechanisms do appear to depend on oxidative phosphorylation (12).

The proximal tubular cells of *Necturus*, like those of other species, actively reabsorb many substances (50). Unlike other species, however, the majority of *Necturus* have never been shown to secrete any substance spontaneously into their proximal tubular urine. For example, the majority of *Necturus* actually reabsorb Diodrast and related substances, although these compounds are invariably secreted into the urine of all other species which have been studied. A minority of *Necturus* spontaneously exhibit a small net secretion of Diodrast (27).

In view of Eggleton's generalization, and the primarily reabsorptive function of the proximal tubular cells of the nephron of *Necturus*, it was of

interest to determine whether the proximal tubules had an anaerobic metabolic pattern. As a first step, the localization in the *Necturus* kidney of those enzymes concerned with carbohydrate metabolism for which histochemical methods are now available was studied. An attempt was then made to correlate these findings with spectrophotometric measurements of enzyme activities in homogenates, and with electron microscopical observations on certain aspects of the cytology of proximal and distal tubular cells. The distribution of renal portal and aortic blood to various segments of the nephron was also reinvestigated.

INTRODUCTION

MATERIALS AND METHODS

Fifty-five adult male and female *Necturus*, whose weights ranged from 80 to 120 grams, were studied.[1] Fifty were received from the dealer between the months of October and December ("winter animals"), and five were received after June 1 ("summer animals"). All were kept, in trays of tap water through which air was continually bubbled, in an ordinary refrigerator. They were fed earthworms.

Enzyme Histochemistry

The histochemical techniques employed were of three types: (1) methods to demonstrate activity of dehydrogenases, (2) methods to demonstrate activities of cytochrome oxidase and peroxidase, and (3) methods to demonstrate hydrolytic enzyme activities (esterase; acid, alkaline, and glucose-6-phosphatases).

All histochemical reactions were performed on tissue quenched as rapidly as possible, after removal from the living, pithed animal, in isopentane (2-methyl butane) cooled to −70°C. with acetone and solid carbon dioxide. For convenience tissues were stored in tightly sealed bottles at −70°C., but they were never used later than 6 hours after removal from the animal. Sections 16 microns thick were cut in a cryostat at −20°C., mounted on clean half-coverslips or slides, allowed to dry for 2 minutes at 22°C., and then placed immediately in the incubating medium.

For better cytological localization of acid phosphatase than could be achieved by the usual methods, the preparatory procedures of Holt (23) were used. Small blocks of kidney were fixed for 24 hours in formol-calcium at 4°C. The blocks were then placed in gum-sucrose at 4°C. for 5 days. Sections 4 microns in thickness were cut on a cryostat and the Gomori

---

[1] The *Necturus* used in this study were obtained from the Lemberger Company, Oshkosh, Wisconsin.

acid phosphatase reaction was performed in the usual manner (17).

1. *Dehydrogenases:* Routinely, incubations were performed at 37°C. and pH 7.2. In addition, all reactions were performed at least once at three temperatures (37°C., 22°C., and 4°C.) and three levels of hydrogen ion concentration (pH 6.0, pH 7.0, and pH 8.0).

Sections incubated in a medium lacking substrate and others boiled before incubation in the full medium served as controls in every experiment. In addition, each reaction was run at least once in the absence of coenzyme.

The details of the methods employed to demonstrate dehydrogenating enzyme activities are given in Table I.

A series of experiments was performed to test the effect of ouabain on every dehydrogenase activity studied. To do this, alternate sections were incubated in the usual incubating medium for the dehydrogenase being studied, and in the same medium altered only by the incorporation of ouabain in a final concentration ranging from 0.1 mg./ml. to 0.4 mg./ml. Two samples of ouabain were employed (ouabain Lilly and ouabain Pennick).[2] Since the Lilly product is provided in a phosphate-buffered solution, phosphate buffer in the same concentration had to be added to the control tubes whenever it was used.

2. *Cytochrome Oxidase and Peroxidase:* For cytochrome oxidase, both the modified Nadi (7) and the amine[3] (8) methods of Burstone were employed, modified (a) by the routine incorporation of catalase (0.02 mg./ml.) into the medium (7), (b) by the incorporation of cytochrome c (0.1 mg./ml.) into the medium (32), and (c) by coating the slides with coenzyme Q_{10} (56). Sections were incubated in a medium containing 10^{-3} M sodium azide as routine controls. The enzymatic nature of the catalase effect was tested by boiling the catalase before incorporating it into the medium. Incubation periods were routinely 1 hour both with the amine and with the modified Nadi reactions. This optimal period of incubation was determined by experiments in which sections were incubated for periods ranging from 10 minutes to 4 hours.

Peroxidase was demonstrated by incorporating 10^{-3} M hydrogen peroxide into the cytochrome oxidase media (7). The range of hydrogen peroxide concentration employed in determining this optimal concentration was 10^{-4} to 10^{-2} M. Controls consisted of boiling the sections prior to incubation and incubating sections in media from which hydrogen peroxide had been omitted.

Differentiation of peroxidase activity from cyto-

---

[2] Kindly provided by Dr. Otto Krayer.

[3] A sample of p-methoxy,p-amino diphenylamine was kindly provided by the Carbic Hoechst Corporation, New York 13, New York.

TABLE I

Histochemical Methods for Dehydrogenases

Enzyme	Reference	Substrate concentration	Incubation period	Modifications and special controls
		moles/liter	*min.*	
DPN diaphorase	(40)	*	*	—
TPN diaphorase	(40)	*	*	—
Glyceraldehyde-3-phosphate	(22)	0.015	7	1. Inhibition by 10^{-3} M iodoacetate. 2. Substitution of TPN for DPN.
Glucose-6-phosphate	(21, 35, 40)	0.010	60	1. Incorporation of 0.5 mg./ml. sodium versenate.
			(40–180)	2. Incorporation of 0.1 M glucose.
6-Phosphogluconic	(35, 40)	0.020	60	1. Incorporation of 0.5 mg./ml. sodium versenate.
		(sodium salt)	(40–180)	2. Incorporation of 10^{-3} M manganous chloride.
Succinic	(33, 40)	0.050	60	1. Incorporation of 10^{-4} M phenazine methosulfate.
		(0.05–0.10)	(40–180)	2. Incorporation of 10^{-2} M aluminum chloride.
TPN isocitric	(21, 35, 40)	0.020	60	1. Incorporation of 0.5 mg./ml. sodium versenate.
		(0.01–0.02)	(40–180)	
DPN isocitric	(34, 40)	0.020	30	—
		(0.01–0.02)	(30–180)	
DPN malic	(34, 40)	0.040	20	—
			(20–180)	
TPN malic	(35, 40)	0.040	30	1. Incorporation of 0.5 mg./ml. sodium versenate.
			(20–180)	
DPN lactic	(34, 40)	0.040	30	—
			(10–30)	
TPN lactic	‡	0.040	60	1. Incorporation of 0.5 mg./ml. sodium versenate.
			(60–180)	

* Recommendations of the reference followed exactly. Where changes in the quoted methods were made, these are given in the columns.
Numbers in parentheses indicate the range tested.
— No special tests performed.
‡ Authors' unpublished method.

chrome oxidase activity was further achieved, (*a*) because short fixation in 10 per cent formalin abolished cytochrome oxidase but not peroxidase activity, and (*b*) because the addition of cyanide (10^{-3} M) or azide (2×10^{-3} M) to the incubation medium abolished cytochrome oxidase activity but left peroxidase activity unaffected. This indicates that the peroxidase of *Necturus* kidney is unusual, since most animal peroxidases are sensitive to cyanide.
3. *Hydrolytic Enzymes: Esterase* was demonstrated by the alpha-naphthyl acetate method of Gomori (17). *Alkaline phosphatase* was demonstrated both by the calcium method of Gomori (17) and by the Pearse modification of the alpha-naphthyl phosphate method of Gomori (40). *Acid phosphatase* was demonstrated by the lead nitrate method of Gomori (17) and by the

technique of Seligman and Mannheimer as modified by Grogg and Pearse (19). *Glucose-6-phosphatase* was demonstrated by the Wachstein and Meisel modification of the method of Chiquoine (54).

Miscellaneous

Mitochondria were demonstrated by the method of Cain (9) using fixed tissues. Fixatives successfully employed included Elftman's (13), Baker's formol-calcium, Helly-Maximow's (43), Bouin's plus 1 per cent calcium chloride, and Romeis' (40). *Bound lipids* were shown by the methods of Berenbaum (4, 5). *Protein amino groups* were shown by the ninhydrin method as employed by Burstone (6), and *protein carboxyl groups* by the method of Barrnett and Seligman

(1).[4] The McManus modification of the *periodic acid–Schiff* reaction was employed (31).

Electron Microscopy

Small fragments of tissue were cut from those areas of the kidney in which proximal or distal tubules predominate and were fixed at 4°C. by immersion for 1½ hours in 1 per cent osmium tetroxide adjusted to pH 7.4 with *s*-collidine (3). The tissues were dehydrated in a graded series of acetone (70, 80, 95, 100 per cent), embedded in Vestopal W (44), and sectioned on a Porter-Blum (Servall) microtome. Mounted sections were stained for 30 minutes in a basic solution of lead tungstate at pH 10.5 (24) and were examined with an RCA model EMU-3F electron microscope at original magnifications of 3000 to 8000.

Homogenate Studies

Both kidneys were removed from a pithed *Necturus* and chilled immediately to 4°C. by immersion in cold 0.25 M sucrose. The wolffian and müllerian duct and large vessels were then dissected away and the kidneys separated. One kidney was weighed and placed in a glass homogenizer with about 9 times its own weight of 0.25 M sucrose at 4°C. The second kidney was divided longitudinally along a line about 1 mm. lateral to the demarcation between the proximal tubular zone and the remainder of the kidney (Fig. 2). This demarcation was clearly visible on the ventral surface of the kidney by virtue of the yellowish-brown pigmentation of the proximal tubules. That portion of the divided kidney which consisted primarily of proximal tubules was weighed and placed in another glass homogenizer with about 9 times its own weight of 0.25 M sucrose at 4°C. All remaining operations were carried out either on ice or in a refrigerator.

Both pieces of kidney were homogenized with a Teflon® pestle for 1 minute, and a drop of each homogenate was inspected microscopically to ascertain good cell breakage. Microscopic examinations were made both of unstained drops of homogenate under a phase microscope and of drops of homogenate stained with 1 per cent gentian violet under a light microscope. The homogenates were then centrifuged at 500 *g* for 10 minutes to remove nuclei, red cells, the majority of the large droplets characteristic of the cells of the proximal tubule, and debris; the pellet thus obtained was discarded. Experiments showed that less than 5 per cent of the total cytochrome oxidase and 3 per cent of the malic

[4] This method, which was originally believed to be specific for terminal carboxyl groups of proteins, has recently been shown to demonstrate side-chain carboxyl groups as well (25).

dehydrogenase activities were present in this "nuclear fraction." Microscopic examination revealed that this centrifugation removed all nuclei and red cells, though a small amount of debris remained unsedimented.

The remainder of the original homogenate was then centrifuged at 20,000 *g* for 20 minutes, the supernatant thus obtained decanted, and the pellet resuspended in one-fourth the initial volume of 0.25 M sucrose. No particulate component was evident in the supernatant fluid obtained by this centrifugation. After preliminary experiments which showed that it contained essentially no cytochrome oxidase activity and only about 10 per cent of the malic dehydrogenase activity of the whole homogenate, it was not assayed routinely. In the suspension obtained from the pellet a few of the large droplets characteristic of proximal tubular cells, numerous small granules (presumably mitochondria), and occasional debris were evident microscopically. Enough sodium deoxycholate to attain a final concentration of 0.7 per cent was added to this suspension and 15 minutes at 4°C. allowed for clearing. These preparations will be referred to as "mitochondrial fractions" of whole kidney or of proximal tubular zone.

Cytochrome *c* oxidase activity was measured at 25°C. on a Beckman DU spectrophotometer by following the disappearance of the extinction at 550 mμ of reduced cytochrome *c* according to the method of Smith (51). Malic dehydrogenase activity was also assayed spectrophotometrically at 25°C. by following the increase in optical density at 340 mμ according to the method of Beaufay et al. (2). Protein was determined by the method of Lowry et al. (30) using human serum albumin (Cohn fraction IV) as a standard. Activities of both enzymes were calculated as described in the references (2, 51) and expressed as specific activities on a unit protein basis.

Injection Studies

The *Necturus* were pithed and pinned out with their gills in a pool of water. The renal portal vein on one side was exposed by a mid-line longitudinal incision which extended from the cranial end of the kidneys through the pelvic girdle to the anterior lip of the cloaca, and a second transverse incision just anterior to the pelvic girdle. The vein was cannulated with a fine polyethylene cannula, and a cold gelatin injection mass (20) was injected by syringe under a pressure monitored by a manometer attached to the system through a T tube. Pressures higher than 40 cm. of water were employed. Outflow was established, once injection had begun, by opening the heart.

Aortic injections were made in a similar manner. The aorta was entered high in the thorax.

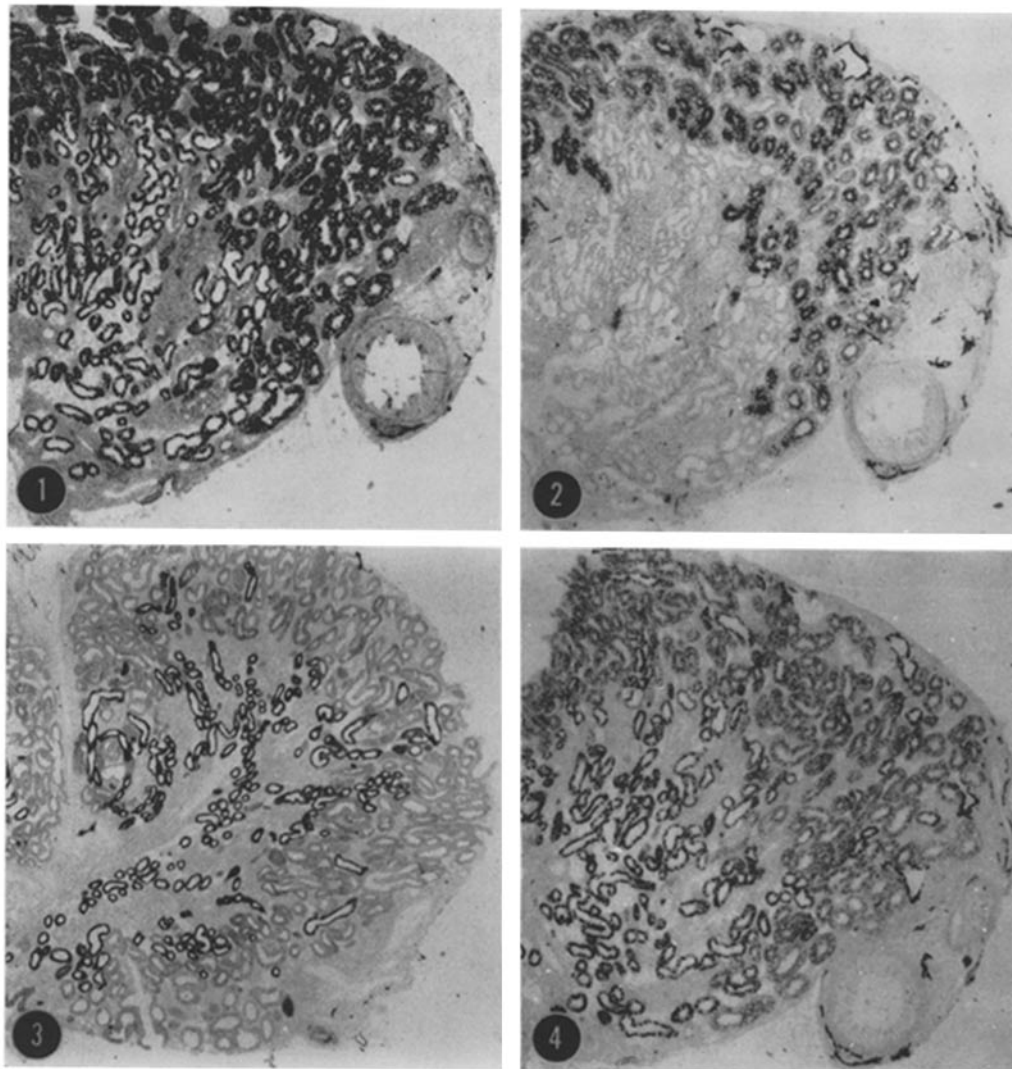At the completion of the injection the cannulated vessel was ligated as was the outflow opening, and

FIGURE 1

Glyceraldehyde-3-phosphate dehydrogenase activity in the kidney of *Necturus*: both proximal and distal tubules give a strong reaction. The lateral border of the kidney is on the right; thus the proximal tubules are above and to the right, and the distal tubules below and to the left. × 10.

FIGURE 2

Glucose-6-phosphate dehydrogenase activity in the kidney of *Necturus*: only the proximal tubules (black) are active. The distal tubules (lower left) are completely unstained. × 10.

FIGURE 3

Succinic dehydrogenase activity in the kidney of *Necturus*: only the distal and junctional tubules (black) are active. The proximal tubules are completely unstained. × 10.

FIGURE 4

Cytochrome oxidase activity in the kidney of *Necturus*: the concentration of catalase was just below that required for complete inhibition of endogenous peroxidase. Thus both tubular segments stain, but only the distal (lower left), which is far more active, is truly positive for cytochrome oxidase. × 10.

the whole animal with the abdomen open fixed for 24 hours in 10 per cent formalin to harden the injection mass.

Frozen sections, 100 microns in thickness, were cut, cleared, and mounted in glycerin. They were studied under a dissecting microscope.

RESULTS

The anatomical arrangement of the kidney of *Necturus* is described in detail by Chase (10). Viewed in transverse section, the proximal tubules lie restricted within a crescentic band comprising the dorsal and lateral portions of the organ (Fig. 2). The distal tubules, on the other hand, lie in a small zone comprising the ventral and medial portion of the kidney (Fig. 3). Terminal extensions of the distal tubules referred to as "junctional tubules" spray out into the proximal tubular zone (Fig. 3). In this paper the term "distal tubule" is taken to refer both to distal and to junctional tubules, since they are enzymatically identical. "Proximal tubule," however, refers only to proximal tubules proper. The ciliated portions of the nephron will not be considered.

Table II summarizes the localization in the nephron of *Necturus* of the enzymes studied. In all the fifty-five animals studied, the pattern was the same. No differences were noted between summer and winter animals.

The proximal tubule had high activities both of glyceraldehyde-3-phosphate dehydrogenase (Fig. 1) and lactic dehydrogenase (representatives of the glycolytic pathway) and of glucose-6-phosphate dehydrogenase (Fig. 2) and 6-phosphogluconic dehydrogenase (the two dehydrogenating enzymes of the hexose-monophosphate shunt). In addition, diphosphopyridine nucleotide–linked malic and diphosphopyridine and triphosphopyridine nucleotide–linked isocitric dehydrogenases (all Krebs cycle enzymes) were present, and their activities in the proximal tubules were as high as or higher than they were in the distal tubules.

Succinic dehydrogenase activity, however, was completely absent from the proximal tubule (Fig. 3) despite every effort to increase the sensitivity of the method with activating substances (phenazine methosulfate, aluminum chloride), prolonged incubation (up to 3 hours), and thick sections (up to 30 microns). In addition, no cytochrome oxidase activity could be elicited in the proximal tubule (Fig. 4), again in spite of the

use of activating substances (coenzyme Q_{10}, cytochrome c), prolonged incubation, and thick sections.

Although no reaction could be detected in the proximal tubular cells when catalase was included in the cytochrome oxidase media used, some reaction was observed in the proximal tubular cells when sections were incubated in the absence of added catalase (Fig. 4). This cannot be regarded as a true cytochrome oxidase reaction, since true cytochrome oxidase activity would have remained unaffected by the presence of catalase, and enzyme the only action of which was presumably to destroy hydrogen peroxide. The possibility that some non-enzymatic or non-specific inhibition of the cytochrome oxidase reaction was produced by catalase can be effectively excluded: first, because boiled catalase had no inhibitory effect; second, because distal tubular activity was little if at all affected by catalase; and, third, because activity in other tissues (heart of *Necturus;* heart, kidney, and liver of rat) was unaffected by catalase when sections of these tissues were incubated simultaneously and in the same medium with sections of *Necturus* kidney.

Thus this reaction simply indicates that in the absence of catalase, the tissue sections produced enough hydrogen peroxide to give a peroxidase reaction in the proximal tubule even when no hydrogen peroxide was added to the medium. This interpretation is consistent with the high peroxidase activity found in the proximal tubule (Table II) when hydrogen peroxide was added to the medium. The manner in which cytochrome oxidase activity was further distinguished clearly from peroxidase activity has been described in the section on Materials and Methods.

The distal tubule showed strong glyceraldehyde-3-phosphate dehydrogenase (Fig. 1) and lactic dehydrogenase activities, as well as high activities of all of the Krebs cycle enzymes studied including succinic dehydrogenase (Fig. 3). Strong cytochrome oxidase activity was easily elicited (Fig. 4). However, the distal tubule, in marked contrast to the proximal tubule, had no detectable activities of the dehydrogenases of the hexose-monophosphate shunt (Fig. 2).

In experiments with ouabain, glucose-6-phosphate dehydrogenase alone of all of the enzymes studied was inhibited by the glycoside. For total inhibition, concentrations of 0.4 mg./ml. were required, and below 0.2 mg./ml. no detectable

Localization of Enzymes in the Nephron of Necturus

Enzyme	Proximal tubule	Distal tubule
Diaphorases:		
DPN diaphorase	++++	++++
TPN diaphorase	+++	++
Enzymes related to glycolysis:		
Glyceraldehyde-3-phosphate dehydrogenase	++++	+++
DPN lactic dehydrogenase	+++	+++
TPN lactic dehydrogenase	+	+++
Enzymes of the hexose shunt:		
Glucose-6-phosphate dehydrogenase	++++	0
6-Phosphogluconic dehydrogenase	++++	0
Enzymes of the krebs cycle:		
Succinic dehydrogenase	0	++++
TPN isocitric dehydrogenase	++	++
DPN isocitric dehydrogenase	++	+++
DPN malic dehydrogenase	++++	+++
Enzymes related to electron transport to oxygen:		
Cytochrome oxidase	0	++++
Peroxidase	+++	++++
Miscellaneous dehydrogenase:		
TPN malic dehydrogenase	+++	++
Hydrolytic enzymes:		
Alkaline phosphatase	++++	0
Acid phosphatase	+	0
Glucose-6-phosphatase	++	0
Esterase	+++++	++++

TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide. Zero indicates no detectable activity above controls. The number of plus signs can only be compared for the same enzyme, and represents relative activities.

inhibition occurred. These concentrations, of course, are far beyond the concentrations necessary to achieve most of the pharmacological effects of ouabain *in vivo*, but are the same as those which have been used in the study of the effect of ouabain on the function of *Necturus* proximal tubule (46). The nature of this inhibition is problematic, however, inasmuch as ouabain had no effect on the glucose-6-phosphate dehydrogenase activity of homogenates of *Necturus* kidney when the spectrophotometric assay of Glock and McLean (16) was employed.

Since the proximal tubules lacked activities of two enzymes so characteristic of mitochondria (cytochrome oxidase and succinic dehydrogenase), it was decided to investigate the mitochondria of this tissue with conventional staining techniques. In the sections stained by Cain's method, the tubules contained large numbers of rod-shaped mitochondria lined up along the striations so characteristic of this tubular segment. The proximal tubular cells, on the other hand, were packed with large numbers of droplets, varying in size from 1 to 10 microns, globular in shape, and not localized to any particular part of the cells. Their abundance made it extremely difficult to determine whether mitochondria were present with the light or phase-contrast microscope. What information is available concerning their nature is described later.

Under the electron microscope, the distal tubular cells possessed considerable numbers of mitochondria which contained numerous transversely arranged cristae (Fig. 5). The proximal tubular cells, on the other hand, contained smaller mitochondria which characteristically possessed few, irregularly oriented cristae (Figs. 6 and 7). The distal tubular mitochondria tended to be concentrated toward the base of the cells and around the nucleus, whereas proximal tubular mitochondria were scattered throughout the cells. There seemed to be fewer mitochondria in the proximal tubular cell than in the distal tubular cell, but direct estimation of absolute numbers of mitochondria is very difficult.

Mitochondrial enzyme activities were present in "mitochondrial fractions" from pieces of *Necturus* kidney consisting primarily of proximal tubule. The proximal tubular mitochondrial fractions showed specific activities of cytochrome *c* oxidase which were 25 per cent of the specific activity observed in whole kidney mitochondrial

fractions. Malic dehydrogenase specific activity in the proximal tubular mitochondrial fractions, however, was fully 50 per cent of that observed in whole kidney mitochondrial fractions (Table III).

Since the proximal tubular cell, both metabolically and structurally, seemed oriented toward anaerobic existence, the distribution of venous (renal portal) and arterial (renal arterial) blood to the various parts of the *Necturus* kidney was reinvestigated.

When the renal portal system was injected, large vessels derived from the renal portal vein were seen to form an anastomotic network around the proximal tubules in such a manner that every proximal tubule was in intimate contact with a branch of the renal portal vein. Microscopically these vessels were lined by a single highly flattened layer of endothelial cells. All the portal vessels then joined to form a few fine vessels which ran directly medially to the vena cava, passing through only a small part of the distal tubular zone.

When the aorta was injected it was evident that no arterial blood reached the proximal tubules without first traversing the distal tubular zone and usually (but not always) a glomerulus. In hematoxylin and eosin sections all the vessels traversing the distal tubular zone were lined only by endothelial cells. Presumably, therefore, exchange could occur across their walls. The aortic blood, on finally reaching the proximal tubular zone, emptied into the same anastomotic channels utilized by the portal system, and presumably, therefore, always is mixed with venous blood. As far as can be determined anatomically, no arterial blood reaches the proximal tubules that has not already served to nourish the distal tubules, and further, the arterial blood reaching the proximal tubular zone mixes with renal portal venous blood; thus the proximal tubules appear to have an essentially venous blood supply.

The nature of the droplets appearing in proximal tubular cells in slides prepared according to the method of Cain was investigated further. They could be clearly distinguished both in fixed and in fresh-frozen preparations stained with hematoxylin and eosin. They were visible as refractile droplets of varying size (up to 10 microns) under the phase microscope in impression preparations of fresh cells. In homogenates they could be seen to sediment at or below 1000 g.

Histochemically the droplets gave an amylase-resistant periodic acid–Schiff reaction of varying intensity, and stained strongly for bound lipids (Fig. 8) and protein amino groups. In the acid phosphatase reaction performed on tissues prepared by the methods of Holt (23) granules of similar dimensions and distribution could be seen. At least one type of droplet or vacuole, however, usually located just beneath the brush border, was acid phosphatase–negative.

In electron microscope preparations irregularly shaped vacuoles of variable size, ranging up to about 3 microns in greatest diameter, were seen in proximal tubular cells, usually situated beneath the brush border (Fig. 7). These vacuoles were lined by a single membrane, and contained evenly dispersed finely granular and filamentous material, and occasional dense bodies. These structures are presumed to correspond to those seen in the same situation with the light microscope which did not give a positive reaction for acid phosphatase.

Scattered throughout the cytoplasm of the proximal tubular cells were large, somewhat oval droplets, ranging in greatest diameter from about 0.5 to 4 microns. These were enclosed by a single membrane, and had a homogeneous moderately electron-opaque matrix (Fig. 9). They are believed to be identical with the large refractile, acid phosphatase–positive droplets seen with the light microscope, although absolute proof of this contention is not yet available.

FIGURE 5

Electron micrograph of mitochondria (*m*) in distal tubular cell of *Necturus* kidney Note numerous, transversely oriented cristae. Nucleus at *n*. × 23,000.

FIGURE 6

Electron micrograph of mitochondria (*m*) in proximal tubular cell of *Necturus* kidney. Note relative dearth of cristae as compared with those in the mitochondria of the distal tubular cell shown in Fig. 5. Nucleus at *n*. × 23,000.
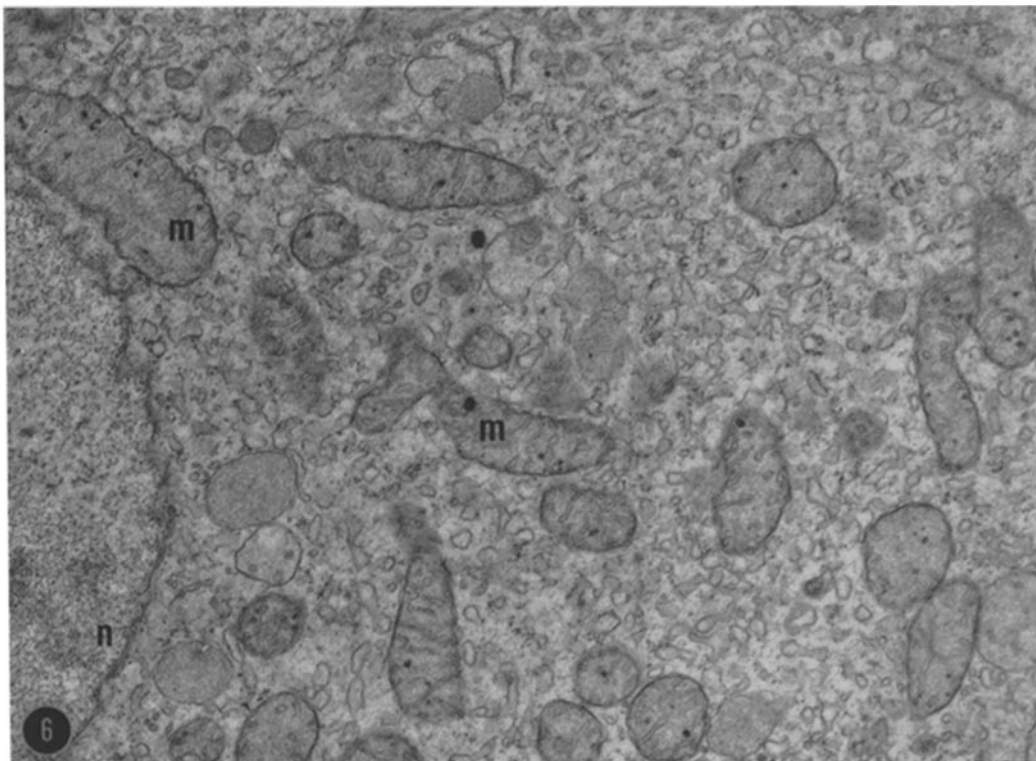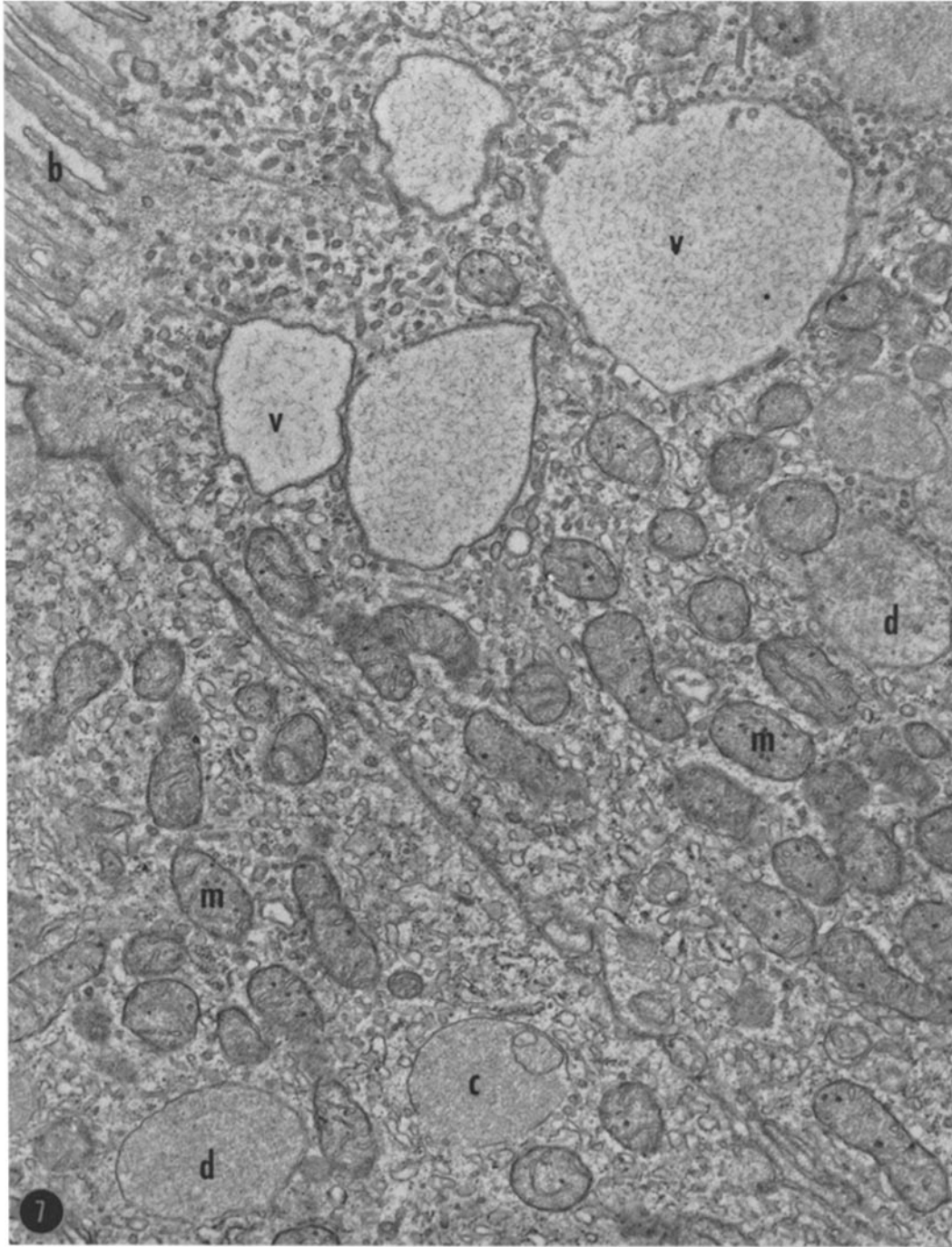
FIGURE 7

Electron micrograph of apical part of proximal tubular cell of *Necturus* kidney. Note mitochondria (*m*) containing relatively few cristae as compared with those in Fig. 5. Large vacuoles (*v*) are situated under the brush border (*b*). Cytoplasmic bodies (*d*) are seen, the matrices of which have varying degrees of granularity and density. Membranes are sometimes seen coiled in the matrix of these bodies (*c*). × 23,000.

TABLE III

Specific Activities of Cytochrome C Oxidase and Malic Dehydrogenase in Mitochondrial Preparations of Necturus Kidney

	Malic dehydrogenase specific activity		Cytochrome Oxidase Specific Activity	
	Units*	% of specific activity of whole kidney preparation	Units‡	% of specific activity of whole kidney preparation
Whole kidney	340 (290–420)	100	0.12 (0.10–0.15)	100
Proximal tubular preparation	180 (150–250)	50	0.03 (0.03 or less)	25

* Unit is calculated as the change in optical density/minute/mg. protein.
‡ Unit is calculated as in Smith (51).
Numbers in parentheses indicate the range of values obtained.

Other types of structures were seen (Figs. 7 and 9), somewhat intermediate in appearance between those described above. These were enclosed by a single membrane, and had a less dense and less homogeneous matrix than the large droplets. They frequently contained accumulations of membranes sometimes arranged in whorls, small membrane-bounded vesicles, granular material, and rhomboidal, crystal-like structures, which had a homogeneous electron-opaque matrix and an appearance similar to that of the matrix of the large type of droplet described above (Fig. 9). These crystal-like structures were about 0.4 × 0.8 microns in size. The exact identity of these cytoplasmic bodies in terms of the histochemical reactions at present remains undetermined. They may represent some of the smaller acid phosphatase–positive granules, and may also be a stage in the formation of the large, electron-opaque droplets.

DISCUSSION

Before discussing the implications of these results, certain technical problems inherent in enzyme histochemistry must be dealt with.

Nitro BT (2,2'-di-p-nitro-phenyl-5,5'-diphenyl 3,3'-(3,3'-dimethoxy-4,4'-biphenylene) dietrazolium chloride) cannot accept electrons from reduced pyridine nucleotides directly. This transfer requires a tissue enzyme ("diaphorase") specific for the coenzyme in question. Thus, before an enzyme is said to be absent from a histological

site, it must be demonstrated that adequate diaphorase is present. Both DPN and TPN diaphorases were present in high activity in both segments of the renal tubule of *Necturus* (Table II), and therefore did not constitute limiting factors.
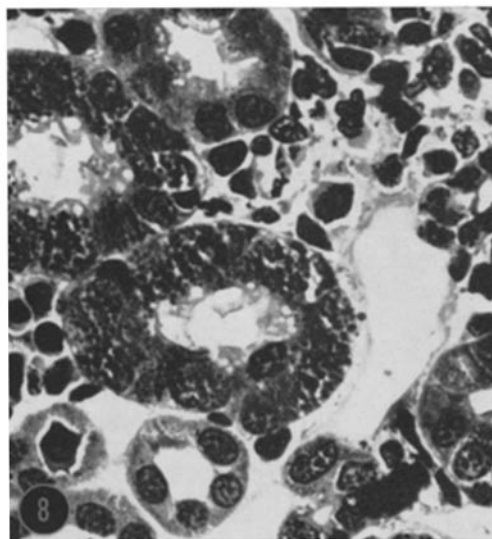

FIGURE 8

Proximal tubules of the *Necturus* kidney in a preparation stained by the method of Berenbaum for bound lipids. Large numbers of droplets of varying sizes (black) are seen throughout the proximal tubular cells. The two junctional tubules present in the field (lower left) contain no droplets. × 240.
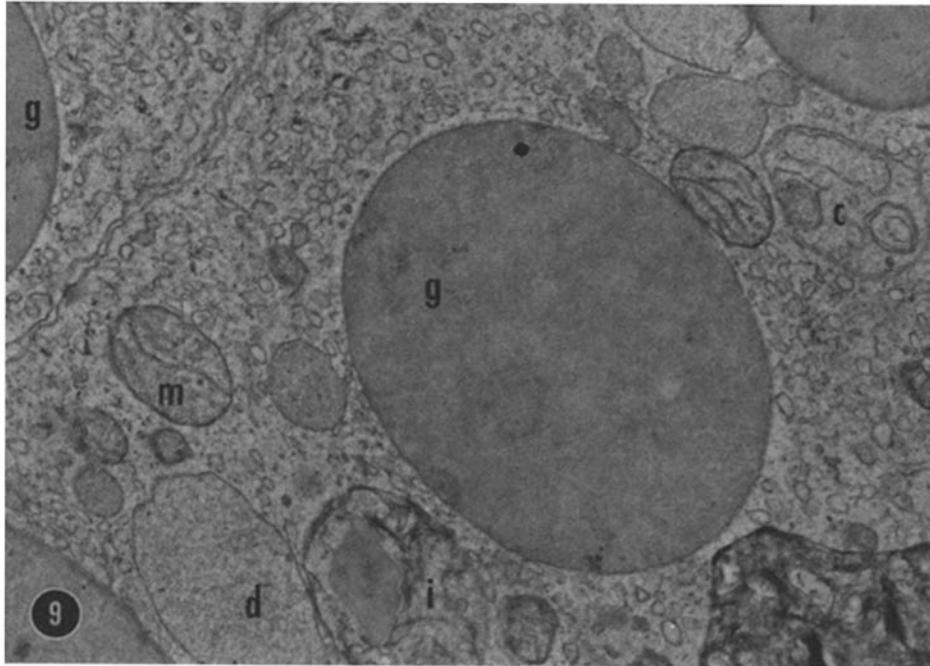
FIGURE 9

Electron micrograph of a portion of a proximal tubular cell of *Necturus* kidney. Large droplets are seen (*g*) which contain a dense, homogeneous matrix. These may correspond to some of the large bound lipid (Berenbaum) droplets seen in Fig. 8. Adjacent to this droplet is a cytoplasmic body (*i*) containing an accumulation of membranous material, and a rhomboidal, crystal-like mass, the matrix of which has an electron opacity and appearance similar to that of the large droplets (g); *m*, *d*, and *c* as in Fig. 7. (× 23,000)

Since substrate and coenzyme specificity, heat lability, and a pH optimum resembling that found in studies of homogenates and purified enzymes were demonstrated for each enzyme in this study, the specificity of the methods is in little doubt.

Assessment of the sensitivity of the methods is more difficult. This is of particular relevance in this study with regard to cytochrome oxidase and succinic dehydrogenase in the proximal tubule and the hexose-monophosphate shunt dehydrogenases in the distal tubule. Using tetrazolium methods far less sensitive than those used in the present study, Farber reported that the papilla of rat kidney, in which he had been able to demonstrate no succinic dehydrogenase histochemically, was almost negative for succinoxidase activity measured manometrically (14). The histochemical method used in this study easily elicits activity in this site (33). Direct comparison also shows that the histochemical methods for glucose-6-phosphate and 6-phosphogluconate dehydrogenases are of comparable sensitivity to the spectrophotometric methods, since homogenates of samples of *Necturus* kidney containing primarily distal tubules, essentially free from proximal tubular contamination, showed nearly unmeasurable activities of both these enzymes by the method of Glock and McLean (16).

Therefore, although the possibility remains that some activity lies below the sensitivity of the dehydrogenase methods, it may be regarded as established that cells reported histochemically negative for a given enzyme are indeed inactive or nearly so. On the other hand, sufficient data are not at present available to enable similar estimates of the sensitivity of the histochemical methods for cytochrome oxidase to be made.

Despite the fact that it is at present impossible to demonstrate histochemically many of the steps in the glycolytic pathway, Krebs cycle, and cytochrome system, our data and the available physiological evidence indicate that the distal tubule

depends for the major part of its energy for active transport on the process of oxidative phosphorylation (12).

The proximal tubular cell, on the other hand, has so little cytochrome oxidase, if any at all, that it is highly improbable that it could carry out adequate oxidative phosphorylation to support its energy requirements. Furthermore, the absence of sufficient proximal tubular succinic dehydrogenase to produce a histochemical reaction implies that the Krebs cycle activity of the proximal tubule must be very small. The only source of high-energy phosphate clearly present is that produced at the substrate level by glyceraldehyde-3-phosphate oxidation, and inferentially at the phospho-enol pyruvate stage.

The homogenate studies support these findings. It must be realized that the presence in the proximal tubular zone of the junctional tubules, high in succinic dehydrogenase and cytochrome oxidase activity (Figs. 3 and 4), makes it impossible to estimate accurately from studies of homogenates just how deficient the proximal tubular cells are in these enzymes. This is of particular importance when it is considered that the preparations of whole *Necturus* kidney studied had only 12 per cent of the cytochrome oxidase activity of similar preparations of rat kidney. The activity of the proximal tubular preparations, even with junctional tubular contamination, therefore, was only about 3 per cent of that of rat kidney. At such low activities, small degrees of contamination can produce large increases in the ratio of proximal tubular to whole kidney activity. It can be assumed that the actual ratio of cytochrome oxidase activity in the proximal tubules to that of whole kidney is lower than was observed experimentally.

More important than the absolute value of this ratio is the fact that malic dehydrogenase was found in the proximal tubular mitochondrial preparations in higher specific activity, relative to whole kidney mitochondrial preparations, than was cytochrome oxidase. Since both these enzymes are restricted to mitochondria (2), this implies that proximal tubular cells are much more deficient in cytochrome oxidase activity than in "mitochondrial activity" in general. Indeed, in conjunction with the histochemical evidence, it would appear that though there may indeed be fewer mitochondria in the proximal tubular cell than in the distal, those mitochondria which are present are almost completely or even wholly

deficient in cytochrome oxidase. Furthermore, it cannot easily be argued that there are simply too few mitochondria to give a histochemical reaction, since activities of other mitochondrial enzymes, such as DPN diaphorase and DPN-linked isocitric dehydrogenase, were easily detectable.

The view that there are quite different types of mitochondria in the proximal and distal tubular cells (the former low in succinic dehydrogenase and cytochrome oxidase and the latter high in both enzymes) is further strengthened by the fact that there are clear differences in the ultrastructure of the mitochondria in these two cell types. Green (18) and Siekevitz and Watson (48) have presented evidence that the cristae mitochondriales are the cytochrome oxidase– and succinic dehydrogenase–bearing parts of the mitochondrion. Others have presented evidence that malic dehydrogenase is contained within the mitochondrion in such a manner that it can be released in a soluble form by rupturing the mitochondrial membrane (2). The observation that proximal and distal tubular mitochondria differ precisely in that the former have only a few, irregularly arranged cristae, whereas the latter have numerous cristae, thus correlates suggestively with the enzymatic differences observed and supports this view of the role of the cristae. Of course, the differences in enzyme activities found in the studies of mitochondrial fractions could also be explained in part by a low specific activity of these enzymes in those cristae which are present in proximal tubular mitochondria.

In view of the anaerobic metabolic pattern observed in the proximal tubular cell, it is not surprising that the injection studies confirm the drawing of Chase (10) in indicating that the blood supply to the proximal tubule is predominantly venous in nature. The morphologically unbalanced distribution of portal and aortic blood supply between the proximal and distal tubular segments should be borne in mind when physiological experiments are interpreted.

Although it seems remarkable that the part of the nephron responsible for the reabsorption of all the bicarbonate, all the glucose, and 30 per cent of the sodium, chloride and water of the glomerular filtrate (42) should depend on anaerobic sources for its energy supply, there is much corroboratory physiological evidence.

In the dog, Nicholson (36) showed that cyanide has little or no effect on proximal tubular glucose,

salt, or water reabsorption in doses which completely abolish distal tubular transport processes. Shideman and Rene (47) showed also in the dog that proximal tubular glucose and phosphate transport were entirely unaffected by concentrations of dehydroacetic acid (an inhibitor of succinic dehydrogenase) which reduced para-aminohippurate secretion to one-fifth of its control value. In several species, dinitrophenol has been found to have no effect on proximal reabsorption processes (12). Thus, as early as 1951, Smith was led to remark that "the results are suggestive that cyanide may have a specific action on the distal tubule; . . . in the proximal tubule the only indicated action is the arrest of tubular excretion; the proximal reabsorption of glucose and sodium appeared to be unimpaired" (50).

However, Schatzmann et al. (46) found that dinitrophenol inhibited sodium and water reabsorption in the proximal tubule of Necturus. We have no certain explanation of this anomalous finding, but believe that the observed inhibition may have been the result of one of the effects of dinitrophenol on cellular processes other than oxidative phosphorylation (29).

Diodrast transport, of course, is well known to be inhibited in most species by all the poisons of aerobic metabolism (12). The problem has not been studied in Necturus, however, and since the usual direction of Diodrast transport in Necturus is opposite to that observed in any other species, it seems not unlikely that there are underlying differences in the mechanism of transport, possibly including the energy source. It is also possible, of course, that the secretion of Diodrast which can be induced in all Necturus and which is observed spontaneously in a minority of them (27) is supported by a small aerobic metabolism below the sensitivity of our methods.

The evidence at present available for an important role of the hexose-monophosphate shunt in renal tubular reabsorptive processes is largely circumstantial. Schatzmann et al. (46) found that ouabain inhibited proximal sodium and water transport. It is possible that the histochemically observed inhibition of glucose-6-phosphate dehydrogenase by the glycoside at the dose levels employed in the physiological studies thus implicates the hexose shunt in this transport process. In view of the apparent absence of cytochrome oxidase from the proximal tubule, however, it seems unlikely that the shunt provides an energy

source in the conventional sense. In the mammalian leucocyte, a strong burst of shunt activity has been found to accompany the phagocytic act, and the question was raised whether this activity is related to the utilization of reduced triphosphopyridine nucleotide in cellular membrane turnover (45). Speculatively, it is suggested that the high shunt activity of the Necturus proximal tubule may be related to turnover of cellular membranes in actively reabsorbing cells.

Histochemical studies of dehydrogenating enzymes in mammalian kidney fit well with the data gathered in Necturus. The distal tubular pattern of the rat kidney as reported by Nachlas et al. (33–35), Hess et al. (21), Burstone (8), and Himmelhoch and Karnovsky (22) is exactly similar to that found in Necturus. With some technical improvements in the hexose shunt enzyme methods, however, Karnovsky and Himmelhoch (26) did find some distal tubular hexose shunt enzyme activity in the rat.

There are two major differences between the histochemically determined metabolic pattern reported for the rat kidney and that described here for Necturus, however. The mammalian proximal tubule possesses plentiful cytochrome oxidase activity histochemically (8). This is not a puzzling finding since the proximal tubule of the mammal, unlike that of Necturus, does carry out secretory processes known to require an aerobic energy supply (12). The inhibitor data presented above suggest that those functions which the mammalian proximal tubule shares with that of Necturus can be performed by it in the absence of aerobic metabolism. The hypothesized importance of the hexose shunt to proximal tubular function would, however, be weakened considerably by the reported restriction of shunt enzyme activity to the macula densa of the rat kidney (21, 35). Karnovsky and Himmelhoch, however, found quite good shunt enzyme activities in the proximal tubular cells of the rat kidney using methods slightly different from those of earlier workers (26).

The nature of the droplets present in the proximal tubular cells remains an only partially solved problem, although much about their properties suggests that they are related to intracellular structures already at least partially defined. It has long been known that the proximal tubular fluid of Necturus may contain protein in a concentration approximating 10 per cent of that of Necturus

plasma (55). Since the work of Oliver and his coworkers clearly showed that the reabsorption of protein from the proximal tubule of the mammalian kidney involves droplets similar in appearance to those under discussion (28, 38, 39), a relationship of the proximal tubular droplets of *Necturus* to protein reabsorption is suggested. Further similarities between the droplets in the mammal and those in *Necturus* are evident from the studies of Straus and of Straus and Oliver (52, 53), who found that "protein reabsorption droplets" vary in size from 0.5 to 5 microns, sediment with the nuclei upon centrifugation, and contain acid phosphatase. If the droplets of *Necturus* are indeed analogous to those of the mammal, deDuve's hypothesis (11) that the three main types of droplets isolated by Straus are related to the "microbodies, big granules, and hyaline droplets observed by the electron microscopists" would be corroborated by the general similarities between the structures described by electron microscopists and those described here (15, 41, 49). Furthermore, the analogy between "protein reabsorption droplets" and the "lysosomes" of deDuve would be strengthened by the fact that the droplets of *Necturus* are periodic acid–Schiff–positive—a property ascribed to lysosomes by Novikoff (37).

In conclusion, it is believed that the clear differences observed between the metabolic patterns of the proximal and distal tubules of the kidney of *Necturus* render it uniquely suited to the systematic study of the effects of metabolic inhibitors on renal function. The existence of different types of mitochondria in the two tubular segments and the presence in the proximal tubular cells of large numbers of droplets, possibly of lysosomal nature, should also render this tissue of value in the study of these cellular organelles.

REFERENCES

1. BARRNETT, R. J., and SELIGMAN, A. M., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 169.
2. BEAUFAY, H., BENDALL, D. S., BAUDHUIN, P., and deDUVE, C., *Biochem. J.*, 1959, **73**, 623.
3. BENNETT, H. S., and LUFT, J. H., *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 113.
4. BERENBAUM, M. C., *Nature*, 1954, **174**, 190.
5. BERENBAUM, M. C., *Quart. J. Micr. Sc.*, 1958, **99**, 231.
6. BURSTONE, M. S., *J. Histochem. and Cytochem.*, 1955, **3**, 32.
7. BURSTONE, M. S., *J. Histochem. and Cytochem.*, 1959, **7**, 112.
8. BURSTONE, M. S., *J. Histochem. and Cytochem.*, 1960, **8**, 63.
9. CAIN, A. J., *Quart. J. Micr. Sc.*, 1948, **89**, 229.
10. CHASE, S. W., *J. Morphol.*, 1923, **37**, 457.
11. deDUVE, C., *in* Subcellular Particles, (T. Hayashi, editor), New York, Ronald Press Co., 1959.
12. EGGLETON, M. G., *in* Modern Views on the Secretion of Urine, (F. R. Winton, editor), London, Churchill, 1956, 34.
13. ELFTMAN, H., *Stain Technol.*, 1957, **32**, 29.
14. FARBER, E., and BUEDING, E., *J. Histochem. and Cytochem.*, 1956, **4**, 357.
15. GANSLER, H., and ROUILLER, C., *Schweiz. Z. Path. u. Bact.*, 1956, **19**, 217.
16. GLOCK, G. E., and McLEAN, P., *Biochem. J.*, 1953, **55**, 400.
17. GOMORI, G., Microscopic Histochemistry, Chicago, University of Chicago Press, 1952.
18. GREEN, D. E., *in* Subcellular Particles, (T. Hayashi, editor), New York, Ronald Press Co., 1959.
19. GROGG, E., and PEARSE, A. G. E., *J. Path. and Bact.*, 1952, **64**, 627.
20. GUYER, M. F., Animal Micrology, Chicago, University of Chicago Press, 1953, 98.
21. HESS, R., SCARPELLI, D. G., and PEARSE, A. G. E., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 753.

22. HIMMELHOCH, S. R., and KARNOVSKY, M. J., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 573.

23. HOLT, S. J., *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 383.

24. KARNOVSKY, M. J., unpublished method.

25. KARNOVSKY, M. J., and FASMAN, G. D., *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 319.

26. KARNOVSKY, M. J., and HIMMELHOCH, S. R., *J. Histochem. and Cytochem.*, 1961, **9**, 203.

27. KINTER, W. B., *Am. J. Physiol.*, 1959, **196**, 1141.

28. LEE, Y. C., *J. Exp. Med.*, 1954, **99**, 621.

29. LOTSPEICH, W. K., Metabolic Aspects of Renal Function, Springfield, Illinois, Charles C. Thomas, 1959, 35.

30. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.*, 1951, **193**, 265.

31. McMANUS, J. R. A., and HOCH-LIGETI, C., *Lab. Invest.*, 1952, **1**, 19.

32. NACHLAS, M. M., CRAWFORD, D. T., GOLDSTEIN, T. P., and SELIGMAN, A. M., *J. Histochem. and Cytochem.*, 1958, **6**, 445.

33. NACHLAS, M. M., TSOU, K. C., DESOUZA, E., CHENG, C. S., and SELIGMAN, A. M., *J. Histochem. and Cytochem.*, 1957, **5**, 420.

34. NACHLAS, M. M., WALKER, D. G., and SELIGMAN, A. M., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 29.

35. NACHLAS, M. M., WALKER, D. G., and SELIGMAN, A. M., *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 169.

36. NICHOLSON, T. F., *Biochem. J.*, 1949, **45**, 112.

37. NOVIKOFF, A. B., *J. Histochem. and Cytochem.*, 1960, **8**, 319.

38. OLIVER, J., MACDOWELL, M., and LEE, Y. C., *J. Exp. Med.*, 1954, **99**, 589.

39. OLIVER, J., MOSES, M. J., MACDOWELL, M. C., and LEE, Y. C., *J. Exp. Med.*, 1954, **99**, 605.

40. PEARSE, A. G. E., Histochemistry, Theoretical and Applied, Boston, Little Brown and Co., 1960.

41. RHODIN, J., Stockholm, Aktiebologet Godvil, 1954.

42. RICHARDS, A. N., *Harvey Lectures*, 1934–1935, 93.

43. ROMEIS, B., Mikroskopische Technik, München, Leibniz, 1948.

44. RYTER, A., and KELLENBERGER, E., *J. Ultrastruct. Research*, 1958, **2**, 200.

45. SBARRA, A. J., and KARNOVSKY, M. L., *J. Biol. Chem.*, 1960, **235**, 2224.

46. SCHATZMAN, H. J., WINDHAGER, E. E., and SOLOMON, A. K., *Am. J. Physiol.*, 1958, **57**, 195.

47. SHIDEMAN, F. E., and RENE, R. M., *Am. J. Physiol.*, 1951, **166**, 104.

48. SIEKEVITZ, P., and WATSON, M. L., *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 653.

49. SJÖSTRAND, F. S., and RHODIN, J., *Exp. Cell Research*, 1953, **4**, 426.

50. SMITH, H. W., The Kidney, Structure and Function in Health and Disease, New York, Oxford University Press, 1951, 344.

51. SMITH, L. C., *in* Methods of Biochemical Analysis, (David Glick, editor), New York, Interscience Publishers, Inc., **2**, 1955.

52. STRAUS, W., *J. Biol. Chem.*, 1954, **207**, 745.

53. STRAUS, W., and OLIVER, J., *J. Exp. Med.*, 1955, **102**, 1.

54. WACHSTEIN, M., and MEISEL, E., *J. Histochem. and Cytochem.*, 1956, **4**, 592.

55. WALKER, A. M., and HUDSON, C. L., *Am. J. Physiol.*, 1937, **118**, 130.

56. WATTENBERG, L. W., *J. Histochem. and Cytochem.*, 1960, **8**, 346.