THE METANEPHROS OF THE BIRD

by

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Abstract

Certain investigations were undertaken on the kidneys of various birds using that of the domestic fowl, as a type; and, where possible, comparing the results with those reported for other classes of animals.

The vascular system was considered, to establish the statement of Spanner (1924), that the bird kidney possesses a renal portal system. Embryological, histological, and anatomical evidence, were brought forward in favour of this venous arrangement.

The histology was examined with different techniques. In this connection, a comparative examination of fixation fluids was undertaken, as difficulty was experienced in the histological examination of bird kidney tissue.

The histological results indicated some degree of glomerular degeneration and an increase in proximal tubule development as compared to that of the mammal.

Cytological studies were carried out on mitochondria and the Golgi apparatus.

The mitochondria of the domestic fowl, and the pigeon, showed great concentration in the cells of the proximal tubule.

The Golgi apparatus was investigated in the fowl; and showed a development in the cells of the proximal segment of the nephron, in excess of that in the mammal.
The conclusion deduced from both these cytological studies, indicated an increase in activity of the proximal segment in the bird, over that of the mammal.

A histochemical test was performed to decide whether the reported glomerular degeneration in the bird is such that glucose elimination is reduced or absent. Alkaline phosphatase is an enzyme stated to assist in the reabsorption of glucose eliminated by glomerular filtration. The results were compared with those of the classes possessing good glomerular development. It was noted that the avian kidney shows considerable evidence of alkaline phosphatase activity.

Two conclusions are reached:

(1) That the bird kidney shows definite evidence of tubular activity.

(2) That in spite of apparent signs of degeneration, the glomerulus in the avian kidney functions comparably to that of the mammal.
Table of Contents

1. Introduction .................................................. 1
2. Acknowledgments .................................................. 4
3. Historical Review .................................................. 5
   I The microscopic units of the
      avian metanephros ........................................... 6
         Malpighian corpuscle
         Convoluted or uriniferous tubules
            Proximal segment
            Henle's loop
            Descending segment
            Ascending segment
            Distal segment
      Collecting tubules
   II Vascular Supply ............................................. 12
      Extrinsic
      Intrinsic
   III Cytology ................................................... 13
      Mitochondria
4. Materials and Methods ......................................... 13
      Vascular system .............................................. 13
      Histology .................................................... 14
      Cytology ..................................................... 20
      Mitochondria ................................................ 20
      Golgi Body .................................................. 23
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histochemistry</td>
<td>24</td>
</tr>
<tr>
<td>5. Observations</td>
<td>28</td>
</tr>
<tr>
<td>I Avian Metanephros: macroscopic features</td>
<td></td>
</tr>
<tr>
<td>II The Vascular System</td>
<td>29</td>
</tr>
<tr>
<td>Venous</td>
<td></td>
</tr>
<tr>
<td>Tympanic valve</td>
<td></td>
</tr>
<tr>
<td>Arterial</td>
<td></td>
</tr>
<tr>
<td>III The Histology</td>
<td>36</td>
</tr>
<tr>
<td>IV The Cytology</td>
<td>44</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>44</td>
</tr>
<tr>
<td>Golgi apparatus</td>
<td>52</td>
</tr>
<tr>
<td>V The Histochemistry</td>
<td>58</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td></td>
</tr>
<tr>
<td>6. Summary</td>
<td>65</td>
</tr>
<tr>
<td>7. Literature cited</td>
<td>66</td>
</tr>
</tbody>
</table>
THE AVIAN METANEPHROS

1. Introduction

It was Bowman (1842), the first investigator of the function of the Malpighian corpuscle, who remarked in his classical monograph on this structure that much knowledge might be gained by comparing the various parts of the kidney in different species of animals. The comparative approach stressed so long ago by Bowman, is often essential for a correct understanding of the physiological mechanisms of a particular group. This fact is again emphasized at the opening of this thesis, because it is felt that only by means of this method some light might be shed on the confused statements concerning the avian kidney in its various aspects, which meet the investigator at every turn.

Originally, this thesis was intended to be a study of the normal and pathological histology of the kidney of the domestic fowl, Gallus domesticus, in relation to the disease under such names as "Blue Comb" or "Pullets" disease, but owing to the confusion met with in the literature the writer
decided to collect the existing isolated facts concerning the
bird kidney in embryology, blood circulation, morphology,
histology and physiology, with these facts, and with fresh work in certain of the above fields, it was hoped to be able to sort out the contradictions, and to correlate the whole into a homogeneous presentation.

Moreover, it was felt that homogeneity could not be achieved unless comparison was made with those forms of animals which are closest in the phylogenetic scale. This fact is stated, because it is felt that certain theories, such as Cushny's (1917) and Richard's (1932), of filtration reabsorption theory, are inclined to dominate the field of kidney function to the exclusion of another theory which may be valid for other groups of animals.

Owing to the natural interest of man in his own body in health and disease the greater part of work on kidney function has been concerned with that of the mammal. Furthermore, because it is an animal easily obtained and experimented with, the kidney of the frog has been much worked on. Now, it is only fair to point out that these two classes of animals belong to the ureotelic type, that is to say animals in which the end-product of nitrogen metabolism is predominantly urea. The kidneys of these two classes will therefore to a great extent function somewhat similarly. Now it has been stated by Needham (1929) that the end product of nitrogen metabolism
depends on the embryological environment. Thus, an animal with an aquatic environment will, if it is small enough, excrete ammonia which, though highly toxic, diffuses out readily, or urea whose removal depends on a good water supply. With the assumption of a terrestrial life, as in the bird and reptile, it was necessary to conserve water and yet find some end-product which would be neither toxic, nor upset the osmotic gradient in a cleidoic egg, i.e., an "egg provided with water when laid and protected against its loss by being surrounded with a more or less impermeable shell or membranes". (Baldwin, 1940, p.38) Uric acid fulfils these requirements in most of the reptiles and in birds; it is non-toxic and sufficiently insoluble to be precipitated out and so exerts no harmful osmotic effects.

However, these animals already possessed a Malpighian body, a relic from a remote ancestor whose osmotic body gradient was higher than that of the external medium. Because of this condition water moved into the animal's body and was removed by filtration through the glomerular tuft. Thus the reptile and the bird, uricotelic terrestrial animals possessing an ancestral organ whose function is largely vestigial, except in some reptiles, must have made adaptations to conserve water with an apparatus designed to eliminate it.

Adaptations, therefore, have been directed towards modifying the glomerulus (Marshall and Smith, 1930) and
indications are shown of a return to a much more primitive mode of nitrogen excretion, namely tubular secretion. In addition the situation is further complicated in the bird by the fact that it is a warm-blooded animal and, therefore, will have a higher rate of metabolism with a correspondingly higher water intake and greater need for its conservation.

It will, therefore, be the purpose of this thesis, by various means to show to some small extent how the avian kidney is constructed; and to correlate the facts with the known physiology, and to produce some conclusions which may in the future be used in the study of bird kidney disease.

2. Acknowledgments

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3. Historical Review

In order to adequately present an historical review of the literature concerning the avian metanephros, it is necessary to give a brief summary of the subjects which will be dealt with in this section. Furthermore, to clarify the situation, a schematic representation (PLATE I.) of the avian renal unit adapted from Marshall (1934) is given, accompanied by explanatory notes.

If PLATE I is examined, it will be seen that the avian renal unit is made up of five morphologically distinct segments: 1, the renal or Malpighian corpuscle; 2, the proximal convoluted tubule; 3, the loop of Henle, which Huber (1917) by means of maceration and isolation, showed to vary from the reptilian type (PLATE I, fig. 1) with a short intermediate portion, to that of the mammal (PLATE I, fig. 2) with well developed descending and ascending regions. The reptilian
form is found at the periphery of the lobule, and the mammalian at the center. The distal tubule 4, extends from the region where the ascending loop of Henle approaches the glomerular root, to the initial collecting tubule, 5.

(1) Microscopic units of the avian metanephros.

Lobule, components:
- Malpighian body
- convoluted or uriniferous tubules
- proximal segment
- Henle's loop
- descending segment
- ascending segment
- distal tubule
- collecting tubule
- stroma (no references to aves)

(2) Lobular organization
(3) Vascular supply
(4) Cytology
- Mitochondria
- Golgi body (no references to aves)
(5) Histochemistry (no references to aves)

(1) Microscopic units of the avian metanephros

Lobular components
- Malpighian body

In comparison with the kidney of amphibia...
mammals that of the bird has been subjected to very little systematic work. No review of the subject has as yet been written and in many cases, particularly in text books, the data are contradictory.

Bowman (1842) proceeding by the comparative method, noted that in birds and reptiles the efferent glomerular artery "seldom divides, but dilates, instead, into a pouch-like cavity which after taking two or three coils contracts again and becomes the efferent vessel." Later Bowman described the glomerular vessel in the bird "as a coiled ampula." He also compared the size of the Malpighian bodies of various classes (and species) and gave the size of the parrot's as 1/430 of an inch (59 micra), incidentally the smallest he recorded. Bowman gives the diameter of the tubules as 1/600 - 1/700 (42 - 36) of an inch and also mentions that the "portal system of the kidney in the lower tribes, has a two-fold origin, one extraneous, the other in the organ itself. In both cases the extraneous source is the principal one, and the artery furnishing the internal source is very small."

Huber (1917) showed that the convoluted tubules of birds are of two types:

(a) Mammalian, i.e., with a fairly well-developed Henle's loop.

(b) reptilian, i.e., very short, with integrating forms between the two.

Huber also gives the lengths of the different segments.
Li Koue Tchang (1923, cited from Marshall and Smith, 1930) worked on the histology of the bird kidney. In this connection, Marshall and Smith (1930) believed that they were the first to describe the "central core of dense syncitial-like tissue" in the avian glomerulus, but it was first noticed by Tchang, to whom they gave the credit in a footnote. Tchang (1924) studied the brush border in the proximal tubule of the kidney of the species of exotic sparrow usually known as ignicolore (Euplectes franciscanus). He noted that the brush border is the same as that in the mammal with this difference, that it is much more highly developed in the bird i.e., one quarter of the height of the cell. He also stated that the striated appearance is in the bird a post-mortem artefact due to mal-fixation and that ordinarily it is a homogeneous structure.

Warner (1927) examined the avian kidney for vestigial and provisional uriniferous tubules which had been reported by Kampmeier to occur constantly in human fetuses, and which he alleged were a sign that not only the pronephros and mesonephros are retrogressive organs, but that the metanephros also is in some part vestigial. Warner showed that neither the first formed tubules nor the early glomeruli degenerate in the bird. He compared the relative diameters of Malpighian bodies of the central and peripheral areas of the metanephros, and found no marked disparity between the central and most peripheral bodies. The largest bodies were present in the
newly hatched chick, there being a gradual increase in the size of the Malpighian corpuscle from the thirteenth day on. von Mollendorf (1929) measured glomerular diameters in the pigeon and the ring sparrow and found that they were 55 x 55 micra in the pigeon, and 42 x 37 micra in the sparrow.

Marshall and Smith (1930) studied the glomerular development of the vertebrate kidney in relation to habitat. They gave the average diameters of the glomeruli of the pigeon as 48 micra, 38 micra for the ring-sparrow, 28 micra for the house sparrow and 24 micra for the finch. They also determined the average diameter of the Malpighian body of the chicken and pigeon, and found it to be 70 micra in the former and 50 micra in the latter. They noted syncytial-like tissue in the central part of the glomerular tuft mentioned previously. Marshall and Smith concluded their remarks on the bird kidney by saying that it is obvious that it shows glomerular degeneration, is indicated by the very small size and poor vascularization of the glomeruli, and by the replacement of the central part of the tuft by syncytial tissue. They also said that it is improbable that increased number of glomeruli can offset this reduction in filtering surface.

Marshall (1934) found that the total numbers of glomeruli in the kidneys of two chickens of about 2,500 grams body weight were 840,000 and 848,000.

Edwards and Schnitter (1933) studied the renal unit in the kidney of vertebrates. They stated that the proximal
region of the nephron is the most differentiated in the kidneys of all the vertebrates studied. This convolution in the kidney of the mammal, bird, reptile and frog is cytologically uniform throughout its length. They also noted that the distal convolution and duct portion is present and cytologically comparable in the tubule of the kidney in all of the vertebrates studied. They mentioned also that the glomerulus varies in size and degree of vascularity in various vertebrates. The least developed is present in the avian kidney and the most developed in that of the frog and mammal.

Vilter (1935) examined the morphology and development of the pigeon metanephros to confirm Bowman's original assertion that the glomerular vessel of the bird was a "coiled ampula". Using a reconstruction method he found that the afferent glomerular blood vessel enters the corpuscle and divides dichotomously, and that either or both of the resultant branches may show further subdivision, which can be quite extensive. Vilter also noted that the glomerulus of the pigeon shows a central avascular core.

Lobular organization

Spanner (1924) gave a clear account of the lobular organization in the bird kidney. He noted that each lobule, whose actual shape has not yet been worked out, consists of a ring of Malpighian corpuscles, at the periphery. The proximal tubules from Bowman's capsule take several turns, then descend towards the centre of the lobule, either still as proximal
tubule or as the descending limb of Henle (this depends on the position of the Malpighian corpuscle in the lobule). At the center of each lobule lies the efferent or intralobular vein. This vessel collects the blood brought to the proximal tubules by the afferent, or renal portal system. The efferent vein also receives the blood supplied to the glomeruli by the intralobular arteries which accompany this vein. The blood is finally discharged into the posterior vena cava by the efferent vein. The tubule now ascends back towards the glomerular pole to which it is always attached (as shown by Huber, 1917). It finally merges into the collecting tubule which is interlobular and runs with the afferent or interlobular (renal portal) vein. This renal portal vein runs, as stated above, between the lobules, giving off regular branches which supply the proximal tubules. The blood is collected up again by the central efferent vein and carried out of the kidney to the posterior vena cava. The small intralobular artery, that accompanies the efferent vein, gives off regular branches which supply the glomerular tufts, and these then run into the efferent blood capillaries which themselves run into the intralobular vein (see PLATE II).

Spanner remarks here on the very small diameter of the intralobular artery and says that its diameter and that of the afferent renal portal vein just approximate that of the efferent vein.
The Vascular Supply

Extrinsic and Intrinsic

The problem of the blood supply to the avian kidney is a controversial one. Jacobsen early in the last century, and after him Gratiolet and Jourdain, maintained that the bird metanephros is supplied not only by an arterial vascular system but also by a small renal portal system. This opinion was very soon opposed by many workers including Hyrtl. Spanner (1924) made a series of careful examinations of 178 birds of different species. He found that the bird possesses a renal portal system but that Jacobsen's original assertion was inaccurate. Spanner based his assumptions on the fact that a "tympanic" valve exists between the afferent or renal portal system and the efferent vein. He also showed that the diameters of the afferent vein and interlobular artery are together about equal to that of the efferent vein. Spanner's conclusions were accepted by von Mollendorf (1929) and later by Pitts (1933).

Das (1924, 1931) on the basis of perfusion experiments denied that the renal portal vein gives off an afferent supply to the kidney; he was unaware however of the existence of the valve described by Spanner, with whose work he was not acquainted, and he did not realize that the flow of blood in the renal-portal vein is increased in volume by blood coming in from the coccygo-mesenteric vein. Das, however, dissected a number of specimens of species of Indian birds, and showed
that (at least in the species examined) there are three main types of renal venous circulations, which he designated as "pigeon", "duck", and "parrot". In the kidney of the Grey Hornbill (*Ocyeros griseus*), of which species he examined eight specimens, he found that the kidney consisted of two lobes only, the anterior and the posterior, the middle lobe being absent. The two remaining lobes were completely separated from each other, being about 11 mm. apart and only joined by the afferent and efferent renal veins. Das also said, among other observations, that essentially all birds are endowed with a similar type of venous organization such as that demonstrated in the pigeon, in which animal the blood from the afferent renal has a direct communication with the posterior vena cava. He mentioned the fact that the afferent renal vein joins the post-caval vein "with undiminished calibre, and does not break up into sinusoids or capillaries in the substance of the kidney".

**Cytology**

Cowdry (1918) mentioned that mitochondria are found in the metanephric proximal tubules of birds.

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4. Materials and Methods

The Vascular Supply

Arterial

Venous

Tympanic valve
Material.

5 *Gallus domesticus*  
Domestic Fowl

and

1 *Gavia arctica pacifica* (Lawrence).  
Pacific Loon,

1 *Uria aalge californica* (Bryant)  
California Murre,

1 *Lunda cirrhata* (Pallas).  
Tufted Puffin

1 *Puffinus griseus* (Gmelin).  
Sooty Shearwater

1 *Columba livia livia* (Gmelin)  
Rock Dove,

Methods.

These specimens consisted of whole carcasses in some cases, in others the kidneys as removed from the specimen. All were preserved in:

- Commercial formalin ................. 10 cc.
- 0.9 per cent Sodium Chloride .......... 90 cc.

Dissections were carried out under a binocular microscope in all cases.

Histology

Material.

10 *Gallus domesticus*  
Domestic Fowl
2 *Columba livia livia* (Gmelin).

Rock Dove

1 *Diomedea nigripes* (Audubon).

Black-footed Albatross

1 *Melospiza melodia morphna* (Oberholser).

Rusty Song Sparrow

**Killing.**

The specimens used were killed by shooting, anaesthesia, by ether, and by having their necks wrung.

**Methods.**

**Fixation fluids.**

Such difficulty was encountered in fixation of bird kidney, that a comparative test was made on the merits of the following fluids, which are listed below, with remarks as to their effectiveness, in the hope that they may prove of use to workers on bird kidney.

(1) Allen's B-15 method (Guyer, 1947).

This fluid should be avoided as it gives most unsatisfactory results i.e., typical"Bouin" picture of kidney tissue, (see Bouin's fluid).

(2) Bouin's fluid (Guyer, 1947).

This fix should not be used for bird kidney as it causes cloudy swelling and disintegration of the cells, especially, those of the proximal convoluted tubule. Peacock (1945), states that Bouin's fluid is a bad fixative for mammalian kidney, and also for mitochondria. As the avian
proximal tubule cells are packed with these cellular constituents, their destruction may be a contributing cause to mal-fixation in this tissue.

(3) Carnoy's fluid (Lee, 1946). Although Lee quotes this as the best fixative for renal epithelium (presumably mammalian), it was found in bird kidney that it caused disintegration of the epithelium of the proximal tubule. However, staining with Heidenhain's azan after this fluid, gave quite good results in delineation of the connective tissue in the Malpighian corpuscle.

(4) Formol-saline. A 10 per cent solution of neutral formalin (4 per cent formaldehyde) in bird physiological salt solution (0.9 per cent sodium chloride). This fluid proved fairly satisfactory as a preservative of the cells of the proximal tubule; it has also the advantage that tissue may be left in it longer than any of the other fixes used for bird kidney. Stains are fairly brilliant after this fix.

(5) Helly's fluid (Baker, 1945). This fixative proved very disappointing; it hardened the tissue extremely; causing flattening on the side almost impossible; however it preserved the tissue fairly well, except mitochondria, which were not fixed at all.

(6) Regaud's fluid (Baker, 1945). This solution, while very variable in its results, gave the most brilliant staining of cell detail.
(7) Zenker's fluid (Guyer, 1947).

As a general fixative for kidney, this mixture proved the most useful. For this tissue, it is advised that mercury be removed with iodized alcohol on the slide, and not in bulk, or staining, which is usually brilliant after this fluid, will be greatly impaired.

(8) 80 per cent. alcohol.

The use of this fluid for 2 - 4 hours, followed by rapid changes of 95 per cent., 100 per cent. alcohol, toluene, 3 changes of paraffin for one half hour to one hour each, gave, with small kidneys, material which could be readily sectioned and easily flattened. Bird kidney is, if mammalian histological methods are followed, very easily hardened, therefore, flattened with difficulty on the slide. These facts prevent serial work, as some sections are usually lost, but, with 80 per cent. alcohol, satisfactory work can be done. Histological detail will not be very good, but the general organization of the tissue can be easily examined.

General remarks on fixatives.

No fixation fluid proved perfect. This conclusion is in accordance with the remarks on this phase of the histology, made by Regaud and Policard (1903-04), in their work on the snake kidney. Birds have a higher rate of metabolism than snakes, and it is therefore possible that autolysis is very rapid in their kidney tissue. In this connection, the author has noticed that the kidneys of fish and amphibians are much
more easily fixed, cut, flattened and stained. These facts would seem to support the remarks made above concerning autolysis.

Washing.

This process was carried out according to the method advised for the particular fixative employed.

Dehydration.

This process was effected by means of:

(1) dioxan for the usual periods of time;

(2) alcohol as usually employed; however it was found advantageous to shorten considerably the length of time in the solutions.

Before transference to wax, it was found preferable to place the tissue in toluene for 15-30 minutes, rather than in cedar, or wintergreen oil. These two oils were almost impossible to remove from kidney tissue, even after soaking in toluene, and caused crumbling of the tissue when it was sectioned.

Embedding.

Three changes of paraffin wax of melting point of 54-56 C. were used. It was found necessary to shorten considerably the time in the wax, using periods averaging one half to one hour for each.

Sectioning.

In order to see histological detail with clarity, sectioning was carried out at 5 - 8 micra.
Flattening and Affixing Sections.

Distilled water and Mayer's albumin were used for flattening and affixing sections on to the slides.

Great difficulty was encountered in successfully flattening the tissue which, probably, owing to its tubular nature, suffered badly from compression under the microtonic blade.

Descending Solutions Prior to Staining.

Owing to the reasons stated above, in connection with flattening and affixing, this tissue was very inclined to wash off the slide when descending through the fluids prior to staining, especially in alcohols lower than 95 per cent. In order to overcome this difficulty, all slides, after immersion in absolute alcohol, were soaked in a mixture of approximately one per cent. celloiden in ether-alcohol; they were then drained one minute, and hardened for 1-5 minutes in 80 per cent. alcohol.

After this treatment, even with very basic solutions, no loss of sections was encountered.

Staining.

1. The general stains.

Harris and Ehrlich's haematoxylin's counterstained with eosin, congo-red, or Van Giesen.

These stains proved the most satisfactory for general histological examination of kidney tissue. (Congo-red emphasized the hemopoietic-nodules satisfactorily.) Van Giesen
showed up the fibroblasts forming the core of the glomerulus.

2. Heidenhain's azan stain (Pantin, 1946)

A modification was made in this method, according to that suggested by McGregor (1929), who used this stain for the examination of the normal histology of the mammalian glomerulus.

Modification.

Instead of employing a 0.1 per cent. solution of azocarmine G, the concentration was increased to 1 per cent. Otherwise the stain was used as Pantin suggests.

3. Foot's short method for silver impregnation of reticulum (Bensley and Bensley, 1938).

This method was used according to the directions given by Bensley and Bensley. It was found very satisfactory with slides coated with celloidin. No counterstain was used, as the fine fibres showed up more clearly without it.

Ascending Solutions after Staining.

The usual procedures were followed as far as 70 per cent alcohol; after this step, celloidin was removed, and dehydration effected, by means of at least two changes of chemically pure acetone. The slides were then immersed in two changes of toluene, and finally mounted in thin balsam.

Cytology

Mitochondria.

Material

2 Gallus domesticus
Domestic Fowl

1. *Columba livia livia* (Gmelin)
   Rock Dove (Common Pigeon)

**Method.**

Small pieces were taken from the animal as soon after death as possible.

**Pigeon.**

**Fixation.**

Regaud's fluid.

Postchroming was carried out in three per cent. potassium dichromate for 6-7 days.

**Sectioning.**

Tissues were cut at 5 micra.

**Stain:** Altmann's acid-fuchsine, according to Lillie (1948) with the following modifications:

- Altmann's aniline acid fuchsin 2-3 minutes only from the commencement of heating.
- Toluidine blue: 1 minute.
- Dehydration: in two changes of acetone

**Counterstain:** Methyl green

  - Light green
  - Toluidine blue

Domestic Fowl

**Fixation:** Regaud's fluid, as above

Helley's fluid post-chromical 48 hrs. at 37 C.

Washed: in water, run through the alcohols, toluene,
3 changes of wax.
  Sectioned: at 5 micra.
  Stains: Altmann's acid-fuchsine
  Counterstains: Toluidine blue

Comments on Methods.

Helly's fluid:
  brittle, hard to cut,
  hard to flatten
  washed off slide
  mitochondria poorly preserved
  stained poorly

Regaud's fluid:
  variable i.e., some sections on slide excellent:
  beautiful histological detail, staining brilliant,
  mitochondria well preserved; other sections:
  tubular cells much swollen, staining dull.

Stains:
  Altmann's acid fuchsine, brilliant after good
Regaud's fixation.
  Counterstains:
  Toluidine blue - the best
  Methyl green)
  Light green) would not stain.

Omir
(Rana catesbiana: mesonephros

Method: adapted from Lee (1946)

Lillie, R.D. (1948)
Stain: Janus green B

1 per cent. aqueous salt Janus green B ... 1 cc.
0.9 per cent. Sodium Chloride ............ 99 cc.

Kidney was removed from freshly killed male bullfrog, cut into small pieces in 0.9 per cent sodium chloride in small petri-dish; saline poured off; 1:10,000 Janus green B added; incubated in 38 C oven 10-20 minutes. Small pieces were then crushed between two slides, cover-slip was added. Examination made by means of glycerine and oil-immersion lens, with x15 ocular.

Results.

The cytoplasm of the cell was clear, crowded with pale green spherical granules (mitochondria). Nucleus plainly seen with nucleolus; dark shadow in position of Golgi body.

Golgi Body

Material. Song Sparrow, No. 1.

Chicken No. X₁ X₂

Method. Small pieces of kidney tissue were taken and prepared according to Aoyama's method for the Golgi body, as given by Baker (1945). The periods of time allowed for silvering were 13, 15, and 17 hours respectively, as recommended.

Dehydration: 50, 70, 95 per cent alcohols
Clearing: Toluene
Embedding: 3 changes of wax
Sectioning: at 8 micra
Counterstainings: thionine
Histochemistry

Alkaline Phosphatase

Material. Small pieces were taken of

Pigeon - kidney and small intestine
Song Sparrow - kidney
Chickens (3) kidney
White rat kidney
Bullfrog Kidney

Method.

Killing.

Pigeon necks wrung
Chickens necks wrung
Song Sparrow shot
White rat anesthetized with sodium pentothal
Bullfrog pithed

Fixation, Dehydration, Clearing, Embedding

Chicken #1 Danielli (1946)

Fixation: 80 per cent alcohol for 2 hours in the refrigerator
Dehydration: absolute alcohol, 2-3 changes.
Clearing: cedar oil 2-3 changes
Embedding: wax 2-3 changes (1 hour each)

blocks were thereafter kept in the refrigerator until needed (in this case, one year, see Danielli (1946), who says they may be stored and still show unimpaired phosphatase activity).
Chicken #2,3 Lillie (1948)

Fixation: (1) acetone 24 hours in the refrigerator.

Dehydration: absolute alcohol
2 changes 24 hours

Clearing: cedar oil 6 hours

Embedding: wax, 3 changes 1 hour each
(2) 95 per cent. alcohol 24 hours thereafter as above.

White rat
Fixation: acetone 24 hours thereafter as above

Pigeon
Song Sparrow
Bullfrog

Fixation: 80 per cent alcohol for 4 hours in the refrigerator.

Dehydration: 4-5 changes of absolute alcohol for 1 hour in all.

Clearing: 3 changes of toluene of 15 minutes each.

Embedding: 3 changes of wax for one half hour each
Sectioned: all blocks were sectioned at 8 micra.

In all cases the blocks were sectioned at 8 micra mounted with distilled water and Meyer's albumin, dried at 37 C. overnight (or for 3-4 hours only), and tested for alkaline phosphatase activity according to the following
methods of Lillie (1948) and Danielli (1946). It should be explained that these two methods were combined owing to the fact that the substrate needed by Danielli's method was Sodium glycerophosphate. However, the only material in stock at the time in the Departmental stores was marked merely, "Sodium glycerophosphate". It was assumed that this was a mixture of alpha and beta, and, as Lillie gives a procedure using Eastman sodium glycerophosphate which is 52 per cent alpha, it was thought worth while to follow his method as follows:

(1) Serial sections were cut, mounted on consecutively numbered separate slides. Adjacent slides were used for phosphatase method and calcium control.

(2) Paraffin was removed with two changes of toluene, 2 changes of 95 per cent alcohol 100 per cent alcohol, slides were soaked in 0.5 - 1.0 per cent ether alcohol solution of collodion for one minute, drained for one minute, hardened for one minute in 80 per cent alcohol, and transferred to distilled water.

(3) Calcium control slides were incubated in 0.1 per cent Calcium nitrate at 37 C. for 12-14 hours, the phosphatase slides for 14 hours at 37 C. in:

3.2 per cent aqueous solution of sodium glycerophosphate (S.G. crystals, City Chemical Corp., N.Y.) 6 cc.
2 per cent aqueous calcium nitrate,
\[ \text{Ca(NO}_3\text{)}_2 - 4 \text{H}_2\text{O, crystalline} \] 9 cc.
10 per cent aqueous sodium barbital 6 cc.
2.465 per cent magnesium sulfate
\[ \text{MgSO}_4 \cdot 7\text{H}_2\text{O (0.1M)} \] 6 cc.
Distilled water 33 cc.
The final solution measured 60 cc., and had a pH of 9.0; this was checked with a pH meter.

Thereafter, Danielli's method was followed:

(4) After incubation both the control \( \text{Ca(NO}_3\text{)}_2 \) slides and the phosphatase slides were rinsed in 2 per cent \( \text{Ca(NO}_3\text{)}_2 \) solution, immersed in 2 per cent cobalt nitrate for 2 minutes. The \( \text{Co(NO}_3\text{)}_2 \) converts the calcium phosphate to cobalt phosphate.

(5) Washed in distilled water
(1 minute, 2 changes)

(6) Tested with dilute ammonium sulphide
(a few drops to a coplin jar of water)
for 1-2 minutes.

Here the site of enzyme activity is made visible by converting the calcium phosphate to black cobalt sulphide.

N.B. (use wax coated forceps were used.

(7) Washed for five minutes in tap water.

(8) At this point, some of both the control slides and the phosphatase slides were stained in:
0.1 per cent safranin
0. thionin
toluidine blue
Ehrlich’s haematoxylin and eosin

(9) The rest of the control and phosphatase slides were taken to 70 per cent (and with the stained slides), dehydrated with two changes of acetone, which also removed the celloidin, 2 changes of toluene and mounted in balsam.

Notes.

Danielli’s precautions were observed concerning the enzyme preservation by avoiding prolonged soaking in:

(1) distilled water
(2) alcohols
(3) toluene

All utensils used were chemically pure. All solutions of sodium glycerophosphate, sodium barbital etc., (should be) made up freshly as they are very quickly spoilt by growth of moulds.

5. Observations

I The Avian Metanephros: macroscopic features.

The kidneys of the bird are metanephroi. Each one is a somewhat flattened, usually tri-lobed, organ, lying on the ventral side of the dorsal body wall beneath the
synsacrum, and external to the peritoneum.

Amongst the birds examined in connection with macroscopic features, it was noted that there was considerable variation in the shape and extent of the lobes in different species.

In the pigeon, both anterior and posterior lobes, are about equal in size, whereas the middle lobe is somewhat reduced in extent. The situation in the domestic fowl closely approximates that in the pigeon. The Pacific loon shows no sharp division between the middle and posterior lobes, the latter being very much extended. The anterior lobe is the most developed in the California murre.

Discussion.

From the few observations recorded above, it can be seen that there is considerable lobular variation in the bird kidney. Das (1924) reports that the Indian pond heron shows two separated lobes, the anterior and posterior, which are joined only by the afferent and efferent venous systems. This situation, Das notes, is normal in this bird.

The Vascular System

Venous

Tympanic Valve

Arterial

Introduction.

As a foreword to this section on the vascular system of the avian kidney, it should be stated that the literature
generally, and in particular, that section which comprises the various text-books, is in a very confused state. This disorder is especially noticeable when the venous system is examined. There is apparently no agreement as to whether a renal portal system exists or not. Similarly, the nomenclature of the vessels themselves needs clarifying.

It is beyond the scope of this work, to give the various statements and their contradictions, which have appeared from time to time. Instead, with the aid of personal observations gained from anatomical and histological studies, combined with pertinent material from the literature of the avian kidney, an attempt will be made to give as unified a picture as possible of this very controversial subject.

Microdissection of the venous system, of the kidney coupled, with a histological examination of the vessel walls, brought the conclusion that there were two separate venous systems in this organ. One system, which can be said to be afferent, has a well-developed venous wall; the other system which can be termed efferent, has a thin vascular coat, consisting of an endothelial lining, one cell thick, resting on a small amount of connective tissue.

These observations were further confirmed by Spanner's (1924) study of the renal-portal system in the bird kidney, wherein he gives much evidence in support of this vascular plan. At this point, a brief description of the venous and arterial systems will be given, using Spanner's
diagramatic representation of the vascular supply as an outline (PLATE III). The nomenclature which, it has already been stated is not uniform, will be that used by Hyman (1947), with additions where necessary.

**Venous System**

Starting caudad to each kidney will be found the afferent, or renal portal vein. This vein is formed by the embryological union (Miller, 1903) of the two caudal parts of the posterior cardinals. Into this anastomosis, on each side, run the internal iliac veins (hypogastric) from the pelvic region, and a small single caudal vein. At about the centre of the union, the fairly large coccgeomesenteric (inferior mesenteric or Jacobsen's vein) enters from the gut. The latter vein is said to connect the two portal systems i.e., renal portal and hepatic portal (Hyman, 1947; Jungherr and Levine, 1941), and is probably homologous to the ventral abdominal veins of reptiles (Hyman). Each renal portal vein enters the posterior lobe of the kidney and gives off branches into the substance of the organ. These branches run perpendicularly between the lobules as far as the surface of the kidney, where they turn at right angles, and send out smaller venules in a rosette formation (Spanner, 1924). This rosette formation is readily discernible upon examining the surface of the kidney under a binocular microscope. The renal portal vein passes on through the posterior and median lobes as far as the external iliac, into which it flows. From the external
iliac, an afferent branch passes to the anterior lobe. The behaviour of this branch is similar to that supplying the other two lobes. It should be mentioned that the afferent system is interlobular, and runs with the collecting tubules which course in a similar manner. Branches of the afferent system, as capillaries, supply the proximal convoluted tubules. The behaviour of the efferent system, as Spanner has pointed out is radically different. The initial branches are intralobular. Under the microscope, cross-sections of these veins can be seen as large lacuna-like spaces, with thin walls, lying at the centre of the lobules. The capillaries from the afferent system, that supply the proximal tubules, finally merge with those running into the interlobular vein.

This blood vessel communicates with the large efferent renal vein (vena renales magna, great renal vein), which can be seen emerging as a thin walled vessel on the ventral surface of the posterior lobe. The efferent renal vein then runs forward and joins the so-called common iliac, at the point where the external iliac likewise enters. The two common iliacs together unite to form the posterior vena cava. The anterior lobe also has an afferent branch, called by Spanner the "superficial efferent vein". This vessel drains the lobe and joins the common iliac.

At the anastomosis of the afferent and efferent systems, Spanner (1924) made a very significant discovery, upon which he largely bases his theory for the existence of a
renal portal circulation in the bird. At this junction, he found a valve, shutting off the opening of the anastomosis into the efferent renal vein. He called it the "tympanic valve", and confirmed its presence in all the species he examined. This structure is described by Spanner as follows:

"This perforated tympanic membrane, pierced only by a fine communicating aperture in a direction towards the efferent renal vein, is comparable to an efficient valve. The opening usually lies eccentrically, and is frequently surrounded by a thick-lipped ring. Larger openings often show in addition a fine, floating membrane (segel)." (Spanner, p. 24, 1929)

In one species Spanner noted an almost completely closed-off valve. He suggests, that this tympanic valve serves as a highly efficient dam to the flow of blood, from the external iliac into the common iliac; and therefore, forces the blood to enter the afferent or renal portal system of the anterior lobe.

**Arterial System.**

Even a cursory examination of the arterial supply to the kidney, will show that, as compared to the venous system, it is small. Starting cephalad to the kidney, it will be apparent that the dorsal aorta gives off a very small renolumbar artery, which supplies the anterior lobe. The renofemoral (external iliac or crural) is the next artery arising from the dorsal aorta; it passes dorsal to the kidney, supplying some branches to the middle lobe; it then proceeds to
the lateral body wall. The third artery to enter the kidney by means of small branches, is the large sciatic (ischiadic), which courses ventrally over the kidney, and then runs out into the leg. Spanner has shown that the arterial branches which enter the kidney accompany the efferent system i.e., they are intralobular. In the lobule they circle the efferent vein sending off regular branches like the spokes of a wheel. These radiating arterioles ascend to the Malpighian corpuscles which they supply, entering and forming the glomerular tuft of capillaries. The blood from the glomeruli is finally transported to the capillary network between the afferent and efferent systems (see PLATE II), from thence it runs into the intralobular, or efferent vein, which carries it to the posterior vena cava.

Discussion.

Marshall (1934) has suggested that a tubular secreting kidney depends more on a venous, than an arterial blood supply. There is evidence in the bird kidney of a large, afferent venous system, which would indicate, if Marshall's statement is borne in mind, that the organ in this class, functions to a greater or less degree by tubular secretion. In this connection, it is significant to note, that the median lobe of the bird kidney is often the smallest. Das (1924) reported a kidney which consisted of two separate lobes, the median one being missing. This lobe is the most poorly furnished by an afferent system. It would appear that, in this
animal, there is a direct relationship, between the amount of venous blood entering the kidney, and its development. Uricotelic reptiles possess a renal portal system, however, this has often been denied in the bird. Miller (1903) states that this system is destroyed, during embryonic development in the bird, by the union of the efferent renal vein (great renal vein) with the posterior cardinals; these latter veins constituting part of the renal portal system in reptiles. However, Miller did not realize that a new renal portal system must surely arise when the anastomosis, previously mentioned, takes place. From a study of Miller’s illustrations, the conclusion can be drawn, that it is at this junction that Spanner’s tympanic valve is formed.

However, there is a possibility that there may be variations; from the schematic representation of the venous blood system described above. Das (1924), working on Indian birds, reported the existence of three main types of venous circulation. The author, during an investigation of Spanner’s valve in five species of birds, found four different patterns and its complete absence in one species (although it must be admitted that only one specimen was examined in this case). The author suggests, that more work should be undertaken on the chief end product of nitrogen metabolism in the different species of birds. Although it has been reported that uric acid accounts for the bulk of the total urinary nitrogen in the
chicken, (Davis, 1927; Coulson and Hughes, 1930), this animal, because of its economic importance, appears to be the only bird to have been investigated in any detail. Moreover, the chicken's venous pattern corresponds to that described above, in which there is a well-developed renal portal supply and an efficient tympanic valve. Perhaps, in other species, in which the venous scheme differs, and in which the tympanic valve is less developed, or lacking, there may be a difference in the chief end product of nitrogen metabolism.

Histology

The kidney of the birds enumerated under "Materials and Methods" were examined histologically. While minor differences were noted, that of the chicken will be given as a type of the histological components listed below:

Malpighian corpuscle

Bowman's capsule

epithelium

parietal

visceral

Glomerulus

Blood vessels

basement membrane

avascular core of fibroblasts

Proximal tubule

Henle's loop
limbs
descending
ascending
Distal tubule
Collecting tubule
Malpighian corpuscle

The Malpighian corpuscle or renal unit consists of Bowman's capsule, and the glomerulus or Malpighian tuft. Bowman's capsule is in the globular dilation which forms the beginning of an uriniferous tubule. It consists of an outer, or parietal layer, which is reflected onto the surface of the glomerular tuft as the visceral layer. The glomerulus is a coil of blood vessels projecting into Bowman's capsule.

Bowman's capsule
Parietal layer

This layer in the bird kidney is typical of that in other animals, i.e., it consists of a squamous epithelium. The cells contain scanty cytoplasm, the elongated, oval nucleus causes a bulging of the central part of the cell body into the subcapsular space. This epithelium rests on a basement membrane of fine reticular fibres which are continuous with the stroma of the rest of the kidney.

Visceral layer

This reflected layer is composed of cells which are unlike those seen in the mammal. They are cuboidal instead of squamous. The cell outlines can occasionally be seen, and there
is a considerable amount of cytoplasm. The nucleus is round or oval, and contains one to two well marked nucleoli. This visceral layer rests on a basement membrane, which is continuous with that upon which the endothelium of the glomerular capillaries is likewise based.

Glomerulus

At the centre of the glomerulus is the mass of fibroblasts recognized as such by Vilter (1935). These cells do not show any distinguishable outlines, possess a somewhat oval nucleus, containing chromatin, and one or more prominent nucleoli. An extensive fibrillar network separates the cells. These fibrils stain blue with Heidenhain's azan carmine stain (PLATE IV) and can also be demonstrated with silver impregnation methods used for reticular fibres (PLATE VII).

The glomerular capillary loops are peripherally placed and vary from two or three, in the renal units at the edge of the kidney to a number of branches, in those larger, centrally placed units. The endothelium lining the glomerular capillaries, appears to consist of squamous cells not very indistinguishable from fibroblasts, but with a rather darker nucleus, and very scanty cytoplasm. The afferent and efferent glomerular vessels are very small in the bird and are much less often seen than those in the mammal. They consist apparently of an endothelial layer, but whether smooth muscle cells are present such as exist in the mammalian kidney, the author is not prepared to state.
Proximal tubule

This part of the nephron which lies at the periphery of the lobule (PLATE ) has a length and diameter given by Marshall (1934) as 0.075 x 0.065 mm, and 0.110 x 0.100 mm. It extends from Bowman's capsule to the descending limb of Henle. The opening from Bowman's subcapsular space to the proximal tubule is seldom seen except in connection with the larger renal units in the vicinity of the small medullary area. In the more peripheral corpuscles it is only seen when the tissue is sectional at 5 micra or less.

In cross section the proximal tubule has a characteristic, somewhat irregular outline on the lumen side of the cell such as that seen in the mammal. The cells themselves, pass abruptly from the squamous type of epithelium of the parietal layer of Bowman's capsule, to a low columnar type somewhat irregular in height. In contradiction to the tubule in the mammal, in which the nuclei are reported as being relatively infrequent i.e., with only three to four nuclei in one transection of a tubule, they appear in every cell. In shape the nuclei are round, situated slightly basally. They contain a dust-like chromatin and from one to several nucleoli. The cytoplasm is abundant and often appears granular. It is not preserved well by routine histological fixes. Cell outlines are seen occasionally. Basal striations such as have been described for the kidney of higher vertebrates do not appear.

The brush border which characterizes this part of the nephron
presents a very variable appearance in accordance with the various fixation methods used. With Aoyama's cadmium chloride fluid (Baker, 1945), with which it is well preserved, the brush border has a height of about one third that of the cell. (Li Koue Tchang, year not given). With this fluid it has a homogeneous, rather than the striated appearance reported in the mammal.

Henle's loop

The extent of the development of this segment depends on the position of the nephron in the lobule. If the mammalian type is approached, it has a typically descending and ascending limbs.

Descending limb

This segment possesses a low cuboidal to squamous epithelium. The cells contain an elongated to round nucleus.

Ascending limb

That portion of the nephron opposed to the glomerular root, will be described here. This part is variously stated to be the ascending limb, or the distal tubule, in textbooks of mammalian anatomy and the situation likewise in the bird needs clarifying.

This segment shows in cross section, a low cuboidal epithelium, a round to oval nucleus, and slightly more cytoplasm than in the descending limb.

Collecting tubule

These tubules which run almost straight out from the
medullary region to the periphery of the kidney (PLATE II) are easily observed owing to this characteristic. Moreover, they take a basophilic stain as compared to the rest of the tubules. The cells are quite different to those in the other portions of the nephron. They are high cuboidal cells and the outlines are very distinct. The darkly staining nucleus is round. The cytoplasm is clear around the nucleus but granular at the supranuclear zone.

**Stroma**

With silver methods, the stroma of the avian kidney is seen to consist of a well defined network of reticular or argyrophil fibres, which form a framework around the tubules, renal units and blood vessels (PLATE VI). A fine network of these fibres also appears at the centre of the avascular core of the glomerulus (PLATE VI).

**Discussion:**

The chief points worthy of note, in connection with the histology of the bird kidney would appear to be the following:

**Malpighian corpuscle**

**Bowman's capsule:**

The small size of the subcapsular space as contrasted with that of the mammalian kidney; and the narrow diameter of the exit of the proximal tubule from the same region. The cuboidal epithelium of the visceral layer of the capsule. This epithelium is in contrast to that found in the mammal
in which it is squamous, except in the early postnatal life, when it consists of high columnar epithelium (Gruenwald and Popper, 1940). This epithelium, it is suggested, impairs filtration in the embryonic kidney of the mammal (Gruenwald and Popper).

Glomerulus

The glomerulus is characterized by a decrease in lobation and vascularization of the glomerulus; and the avascular core of fibroblasts. In connection with the latter structure, there is a counterpart in the central mass of dense connective tissue reported in the snake kidney (Regaud and Policard, 1903-1904; Marshall and Smith, 1930).

Proximal tubules

In contrast to the renal corpuscle, the proximal tubule shows greater development than that of the mammal. Marshall and Smith (1934) give the lengths and diameter of the proximal tubule of the rabbit and domestic fowl, as being 6.90 x 0.036 mm. for the former, and 7.00 x 0.063 mm. for the latter. An examination of the figures will show that the diameter of the tubule of the bird is almost double that of the mammal. To this fact should be added that histological evidence of an increase in height of the brush border of the cells of this area.

In summarizing one can say that, there is histological evidence of a decrease in development of the glomerulus in
the bird, as compared to the mammal; and a corresponding increase in the development of the proximal tubule.
The Cytology

Mitochondria

Results

Before commenting on the results obtained for mitochondria, the same general remarks can be made which are apparently to be expected in the fixation of avian kidney during the course of this work, i.e., the fixation shows disappointing results, especially in Helly's fluid.

On this point of fixation of kidney Baker (1945, p.45) says, "Some kinds of cells are very resistant to rough treatment, others are delicate. For instance, the intestinal of the newt is reasonably well fixed by almost any fixative, while the kidney and testis of mammals are wretchedly preserved by many well-known mixtures. The reason for this has not been explained." On examination of this statement, it will be seen that it is the tissue of a poikiloithermic organism which is easily preserved whereas that of the mammal, a homoithermic animal is poorly so. It could, therefore, seem as if those of the bird might be even less well fixed as in the case of these the body temperature is even higher than that of the mammal, and would on that account undergo autolytic changes even more rapidly. Again, the fact that kidney always fixes even more poorly than other tissues may be further ascribed to the reason that it is an intensely active organ.
Probably some method of fixation along the lines of Baker's (1944) for the Golgi body, using a formol-calcium fix with gelatine embedding, might be advantageous though time consuming.

**The Chicken**

**Helly's fluid.**

The results were uniformly poor: the mitochondria were not properly preserved and the general fixation itself inadequate. Staining by Altmann's method was, owing to the result of mitochondrial fixation, almost useless.

**Regaud's fluid.**

Here the results were better than with the method above, but not as good as could be desired.

**Proximal convoluted tubule.**

It can be seen under medium power that the contents of this segment stain almost entirely red with fuchsine, which on inspection by oil immersion, are found to consist of a solid mass of mostly spherical red granules which are too closely packed to indicate whether any polarity exists. The brush border is free of granules.

**Distal convoluted tubule.**

The cells of this area contain the same granules but to a lesser degree so that the appearance is not a solid mass of red stain at high power.

**Henle's loop.**

This part of the nephron found in close proximity
to the efferent intralobular vein, likewise includes the same granules in much the same density as the condition found in the distal tubule.

Collecting tubule.

This segment is ordinarily stained blue with metachromatic toluidine blue; however, it was not very apparent here except on the side bordering the lumen which is so colored. Most of the cells stain somewhat diffusely with no particular granulation; however, in each cross section one or two cells are filled with large red-staining granules. The side of these cells bordering on the lumen projects beyond the limits of the other cells. This condition would seem to indicate a difference in function of the cells in this area in which the cells stain uniformly with metachromatic stains in preparations not fixed for mitochondria; or rather perhaps a difference in secretory activity.

Malpighian Corpuscle.

This structure is not very well preserved in this specimen. As far as can be ascertained, the central avascular core of fibroblasts contain some small spherical mitochondria. The cells of the visceral layers do not show any visible evidence of granulation.

Pigeon Kidney.

This tissue gave better results than that of the chicken, however, even here the results were very variable. In places on the slide almost perfect fixation was shown with
very exact cellular detail, many mitochondria, and most brilliant staining of features. Other places showing much swelling of cells in the tubules, less easily distinguished mitochondria and duller staining.

Concerning this variability after Regaud's fluid, Baker (1945) says of this solution that it is, "less stable than Helly's, fixes some tissues badly (e.g., mammalian testis) it is poor for nuclei, and does not give such brilliant staining." Bensley and Bensley (1938) say in reference to the same subject, "for mitochondria good general fixation, especially for the kidney."

Proximal convoluted tubule. (PLATE VII, fig. 9(c))

As before the cells of this segment are under medium power of the microscope, stained a uniform red. Under oil immersion, it can be seen that a dense mass of spherical granules fills the cells so that no real polarity can be assigned. The brush border appears to be usually free of granules, except in some tubules, in which an eruption of granules takes place from the cell into the lumen.

Distal convoluted tubule.

This tubular area also contains granules, though to a much lesser degree than does the proximal segment; moreover, in general the granules appear to be larger.

Henle's loop.

Both descending and ascending portions contain red granules in lesser degree than in the proximal and distal
segments.

As this tissue was in a better state of preservation than that of the chicken, an examination was made for the macula densa (McManus, 1945) in connection with the ascending portion of Henle's loop. This structure consists of that part of the ascending loop which is adjacent to the glomerular pole, in particular to the vas afferens. Here the cells consist, according to Maximov and Bloom (1944) "of an elliptical disc of tall thin cells measuring 40 micra by 70 micra in man." Best and Taylor (1945) suggest that this structure probably should be considered functionally as an integral part of the juxta-glomerular apparatus, i.e., that cuff of myo-epithelial cells which lies on the walls of the glomerular arterioles. Maximov and Bloom (1944) say that this apparatus is reported as absent from the lower vertebrates. That side of the ascending loop of Henle against the glomerular pole in the pigeon does not possess significantly higher cells than does the other side, nor do they show any difference in mitochondrial arrangement. The afferent arterioles are very small in the bird and do not appear to possess myo-epithelial cells.

Collecting tubules.

These tubules stain a clear blue and are easy to pick out. The blue stain marks a few red granules.

Malpighian corpuscle.

No mitochondria appear in the avascular core or in
the visceral and parietal epithelia.

Discussion.

Mitochondria were first described by Flemming (1882), Altmann (1890) and by Benda (1897) at the end of the 19th century. Lazarow (1943) says that they are microscopic structures, varying in size and shape and found in practically all living cells where they can be stained intravitally with Janus green. They may be as large as several micra in size. They are morphological entities which can be seen dispersing into the surrounding medium upon crushing a cell under a cover slip. Then the mitochondria sometimes retain their exact size and shape and can be compared with those within the cell. Bensley and Hoerr first separated mitochondria in 1934, making accurate chemical analysis possible. These granules contain cholesterol, give a positive test for pentose and have a purine base, and therefore contain nucleoprotein. They also give a positive test for cytochrome oxidase with the Nadi reagent, they are also able to oxidize glutamic acid. The natural yellow color is due to riboflavin.

Lazarow notes that there is a confusion as to terminology, which fact can well be noted by referring to Cowdry's (1918) early exhaustive work in which he lists the nomenclature used up to that date. This confusion makes clarification of the subject somewhat difficult because it is not always clear as to what microscopic structures are under discussion.
Cowdry (1928) on this point in reference to renal secretion and mitochondria says that there are other granular constituents present in the cells of the proximal convoluted tubule. These constituents both resemble and differ from mitochondria in some respects and accordingly complicate the question. Maximov and Bloom (1944) state that although mitochondria may take part indirectly in secretory processes, they probably are not transformed into secretion granules. Cowdry says that the large granules which Claude separated from liver cells and which he (Claude) called secretion granules are without doubt mitochondria.

Cowdry (1928) says that mitochondria have been seen distributed in the protoplasm of the cells of the proximal convoluted segment in the metanephric tubule of reptiles, birds, and mammals, but, concerning birds he gives no specific reference, nor does he describe their form in this class of animal.

(Regaud and Policard (1903-04) did extensive studies on snake kidneys and report that the cells of the proximal convoluted tubule are riddled with many vacuoles of very diverse shape and form; they also note that intracellular inclusions (enclaves intracellulaires) of a lipoidal nature are found in the epithelium here. The lipoidal inclusions)
are sometimes found in Henle's loop. Cowdry (1918) gives an illustration of mitochondria in the cells of the kidney of the white mouse but does not state from which part of the nephron they were taken. From his illustration, these mitochondria appear to be disposed principally in an infranuclear position with a few in the supranuclear zone. The shape seems principally rod-like.

Covel (1927) studied mitochondria-cytoplasmic ratio of the renal tubules of albino rats. In his illustrations (drawings) the mitochondria in all tubules are rod-like. He found that the mitochondria-cytoplasmic ratio was highest in the cells of the proximal convoluted tubules.

Bailey (1936) gives an illustration (coloured drawing) of mitochondria in the proximal and distal tubules of the kidney of a mouse after Regaud's fluid fixation and Altmann's acid fuchsine. The granules are rod shaped in both tubules and prominently arranged in parallel lines, particularly in the proximal segment. The mitochondria are especially dense in the latter tubule, and there appear to be secretion granules leaving the apex of the cell and entering the lumen which also stain pink as do the mitochondria. The density of the mitochondria and the secretion granules compare favourably with those seen in the bird kidney.

That the condition concerning mitochondrial formation and concentration in the avian kidney should most approach that of the reptile might be expected when it is realized
that both phylogenetically and physiologically in renal function the bird is closer to this class than to the mammal. Although Regaud and Policard do not call either their vacuoles or their lipoidal inclusions mitochondria, it may be taken from their position and also from the fact of a similar position in other classes of animals, that these structures, at least the lipoidal "intracellular inclusions" do so represent them.

No mitochondria-cytoplasmic ratio was worked out on the avian kidney; moreover, it would be here, entirely unnecessary and even impossible to follow Cowdry and Covel's method. Even under medium power of the microscope it can be seen in the cells of the proximal convoluted tubule that their ratio is qualitatively very high. This condition far surpasses that in the mammalian kidney which is a predominantly glomerular filtration organ in urine formation.

Cowdry and Covel suggest on account of this high ratio that there is fair reason to believe that the part played by this segment in urinary secretion differs radically from that of the other segments. If this statement is made of the mammal, how much more should it be made of the bird in which animal the secretion of uric acid has shown to be independent of blood flow (Gibbs, 1929).

Golgi Body

Introduction.

Baker (1944) in his study of the structure and
chemical composition of the Golgi body says that in its fully developed condition, this apparatus of diverse cells consists of four parts:

(1) the "neutral-red vacuoles";

(2) the dense lipoid-containing substance, generally in close relation to the vacuoles in the form of strands, "lepidosomes", caps, crescents, rings or complete investments;

(3) the diffuse lipoid-containing substance, which fills all the space in the Golgi element not occupied by the other constituents;

(4) the Golgi-product, which arises in the vacuoles and is the result of the synthesis achieved by the apparatus.

Baker states that a review of the literature reveals that there had been no reliable evidence of the chemical composition of the osmophil substance of the Golgi element, apart from the fact that it contains lipoid (in the widest sense).

Therefore, in order to test the Golgi apparatus for its chemical composition, Baker used the lepidosomes (Golgi batonettes) of the spermatocytes and young spermatids of the common snail, Helix aspera. This material was chosen as it is visible without staining in living cells and there is therefore no possibility that the investigator is examining an artefact.

Baker, using the Smith-Dietrich test says that the
results strongly suggest that the lepidosomes contain lecithin, cephalin, or sphingomyelin. After further chemical tests he concluded that the lepidosomes of the material investigated consist of, or contain lecithin or cephalin or both.

As well as this method used by Baker, the Golgi element can be blackened either by osmium tetroxide or by one of the silver methods.

An important role in various cellular processes has been assigned to the Golgi body by many investigators, particularly in those dealing with secretion. Maximov and Bloom (1944) say that it is quite improbable that this structure is transformed directly into secretory vacuoles, therefore all that can be said at present of the Golgi element is that it is a center of cellular activity.

Results.

The only material in which the Golgi element showed in a characteristic fashion was in that of the chicken. Unfortunately that of the song-sparrow only showed this structure in the distal and collecting tubules. This was perhaps owing to a lapse of time between killing the animal and fixation, as it is known that the cells of the proximal tubule very rapidly undergo autolysis.

Chicken kidney.

The fixation of this material by Aoyama's cadmium chloride formaldehyde fluid was quite fair in reference to the histological picture and, in particular, to the brush border
which presents the homogeneous appearance spoken of by Li Koue Tchang (year not given).

**Malpighian Corpuscle.**

The central avascular core of fibroblasts shows only an occasional sign of the Golgi apparatus on deep impregnation.

In the larger corpuscles, in which the visceral layer is cuboidal, and not of the squamous type, Golgi bodies can be demonstrated. These structures consist of a very dark, cap-like shape closely applied to the nucleus.

**Proximal Tubule.**

The picture of the Golgi apparatus in the cells of this tubule (PLATE VIII fig. 11) appears to vary somewhat, perhaps, concomitant with the activity of the particular segment at the moment of death, or owing to the degree of impregnation which is variable (Baker, 1945). However, in general, it may be said that the supra-nuclear zone darkens somewhat, and that from one to several black filamentous projections (PLATE VIII fig. 12) extend outwards towards the brush border. The dark filaments are probably the "lepidosomes" or "batonnettes" described by Baker (1944).

**Henle's Loop.**

The shape of the Golgi element in this segment is somewhat difficult to estimate, as diffuse impregnation of the whole cell takes place. However, it apparently has the appearance of a dense, dark "cap" closely applied to the nucleus towards the lumen side of the cell.
**Distal Tubule.**

At the point of application at the distal tubule to the glomerular pole (Maximov, 1944), will be found a good area for observing the Golgi apparatus in this segment. The whole cell diffusely reduces the silver. Here the Golgi element has a very similar structure to that in Henle's loop. Against the glomerular pole, that side of the tubule which would be designated the macula densa in the mammal, there is usually such a dense impregnation that the Golgi body cannot usually be specifically located. However, reversal of polarity is not seen, such as that reported by McManus (1942-43).

**Collecting Tubules.**

In the collecting tubules some cells show no element; in others, the Golgi body appears as a black structure closely applied to the lumen side of the nucleus. Sometimes, lepidosomes are demonstrated which closely approximate those in the proximal tubule, although they are not as prominent. In most of the tubules, from one to several cells take up the silver so that the cytoplasm is completely blackened. This situation, it would appear, can be correlated with that shown during the study of mitochondria in the same tubule. In the latter case some cells were noticed to be filled with larger granules than in the other cells, and projected into the lumen beyond the border of the adjacent cells. These facts suggest a variation of the Golgi body with respect to secretory activity, which can be further confirmed by showing that the cells of this
area can be stained specifically for mucin with metachromatic dyes. These cells are known to vary in activity.

Discussion.

There are, as far as the writer is aware, no specific references to the Golgi element in the avian kidney. Therefore, comparison must be made with the somewhat scanty literature which is to be found concerning this structure in the amphibian and mammalian classes.

McManus (1944) says that Emmel (1938) gives a review of the Golgi element in the kidney. This statement is somewhat erroneous as most of Emmel's references are concerned with the Golgi body in tissue culture. Emmel does, however, give a good description of the situation in the frog kidney, and shows that the Golgi body closely approximates that seen in the bird, although in the proximal cells of this animal, the lepidosomes have a more distal position in reference to the nucleus. In the cells of the distal tubule, the Golgi apparatus lies in a more apical position. McManus (1944) gives some illustrations of the element in the mammalian nephron taken from Ramon-Cajal (1933), in which again the typical appearances of the Golgi body is repeated, varying from a small filamentous strand in the initial part of the proximal tubule, to a greatly extended projection in the active cells of this area. McManus (1943, 44) likewise describes the Golgi element in the rabbit, cat, and human proximal and distal cells, with special reference to those cells of the macula densa wherein the position
of the element is reversed, i.e., the polarity is infra-instead of supra-nuclear.

In retrospect it may be said that the Golgi element in the one species of bird examined, shows in the cells particularly of the proximal tubule, a great similarity in form and position to that seen in the amphibian and mammal. While it is still a highly controversial subject as to whether or not the Golgi body participates in secretion, the degree of its development demonstrated in the kidney proximal tubule cells in the chicken, taken in conjunction with the high concentration of mitochondria in the cells of this area, would indicate some special activity in this part of the nephron.

The Histochemistry

Alkaline Phosphatase

Introduction.

The existence of the enzyme "alkaline phosphatase" has been demonstrated in many organs and tissues of the mammalian body. This enzyme is capable of hydrolyzing various phosphoric esters, e.g., hexomonophosphate, glycerophosphate, etc. Among other tissues showing phosphatase activity the kidney has a fairly high place (Best and Taylor, 1945). Taking the arbitrary figure of 100 to indicate the enzyme activity in the jejunum of an adult man, the kidney is given as 35, adult cat jejunum 100, kidney 38. In the kidney this enzyme is
found in the "brush border" of the cells of the proximal tubule and the site of its activity can be shown histochemically by the method of Gomori (1939) and others. The glucose filtered out of the glomerular capillaries is reabsorbed in this region of the proximal tubule as can be shown by poisoning the tubular cells with phlorizin, in which case the sugar is passed out in the urine. In its transfer across the tubular membrane, phosphorylation of the glucose molecule appears to be a necessary step. This change is brought about by a scientific enzyme, kidney phosphorylase. The function of alkaline phosphatase appears to be at this point a dephosphorylation of the hexose phosphate previously formed.

The principles underlying the method for the histochemical detection of the site of the activity of alkaline phosphatase are as follows (Danielli, 1946):

the sections are incubated at 37 °C. in a solution containing glycerophosphate and calcium at a pH of approximately 9. Calcium phosphate is deposited in the sections as a result of the enzymatic splitting of glycerophosphate. The sections are treated with cobalt nitrate, washed with distilled water, treated with ammonium sulphide, washed in tap water, and mounted in balsam. Cobalt phosphate is less soluble than calcium phosphate, consequently the treatment of a section with cobalt solution changes any calcium phosphate present to cobalt phosphate. Also, cobalt sulphide is less soluble than cobalt sulphate, therefore the application to a section
of ammonium sulphide converts the cobalt phosphate into cobalt sulphide. This latter substance forms a black precipitate which appears in the tissue sections in the positions where initially calcium phosphate had been precipitated following enzymatic hydrolysis of glycerophosphate.

It has been suggested by Marshall and Smith (1930) that the glomerulus of the bird shows degeneration as indicated by the very small size and poor vascularization of the glomeruli, and by the replacement of the central portion of the tuft by a dense mass of fibroblasts. There are also other indications given by Spanner (1924), such as the small size of the arterial blood supply to the tuft, the double venous supply to tubules, and personal observations gleaned from an examination of the histology of the bird kidney. These latter include diminishing size of Malpighian corpuscles as they approach the periphery of the kidney, very poorly developed subcapsular space, cuboidal epithelium in the visceral layer overlying the glomerulus instead of the squamous layer found in the mammal, and the small diameter of the proximal tubule at its junction with Bowman's capsule. Grafflin (1933), in his study of glomerular degeneration in the kidney of the daddy sculpin (Myoxocephalus scorpius) lists the following indications (among others) of this condition:

(1) a loss of vascularity
(2) increase in cellular make up
(3) marked constriction of tubular outlet.
<table>
<thead>
<tr>
<th>CLASS</th>
<th>FIXATION FLUID</th>
<th>TIME IN FIXATION FLUID</th>
<th>EXTENT TO WHICH ALKALINE PHOSPHATASE ACTIVITY SHOWN IN TISSUE SECTION</th>
<th>LOCATION OF ENZYME ACTIVITY (ARBITRARY FIGURE ONLY)</th>
<th>DEGREE OF ACTIVITY (i.e. INCUBATED WITHOUT SUBSTRATE)</th>
<th>REMARKS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphibian</td>
<td>80% Alcohol</td>
<td>4 hrs.</td>
<td>Throughout tissue section except in central area (which contains collecting tubules only, which would therefore, not show activity)</td>
<td>Brush border of proximal tubule cells, also in supra-nuclear zone, Distal tubule, supra-nuclear zone.</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Bullfrog</td>
<td>80% Alcohol</td>
<td>12 hrs.</td>
<td>About half-way through the tissue section.</td>
<td>Proximal tubule: brush border of cells and in supra-nuclear zone. Henle's loop descending segment, supra-nuclear zone (of some segments only)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Aves</td>
<td>80% Alcohol</td>
<td>4 hrs.</td>
<td>At edge only of tissue section</td>
<td>Proximal tubule: brush border</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Pigeon</td>
<td>80% Alcohol</td>
<td>2 hrs.</td>
<td>No sign of activity</td>
<td>No sign of activity</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chicken</td>
<td>Acetone</td>
<td>24 hrs.</td>
<td>Throughout Tissue Section</td>
<td>Proximal tubule: confined to brush border i.e. not in supra-nuclear zone</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>No. 1</td>
<td>80% Alcohol</td>
<td>2 hrs.</td>
<td>Proximal tubule: brush border</td>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>No. 2</td>
<td>Acetone</td>
<td>24 hrs.</td>
<td>No sign of activity</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. 3</td>
<td>95% Alcohol</td>
<td>24 hrs.</td>
<td>No sign of activity</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Song Sparrow</td>
<td>80% Alcohol</td>
<td>4 hrs.</td>
<td>Through Tissue Section</td>
<td>Proximal tubule: confined to brush border i.e. not in supra-nuclear zone</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Song Sparrow</td>
<td>Acetone</td>
<td>24 hrs.</td>
<td>Edges of tissue section</td>
<td>Proximal tubule: brush border</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
Marshall (1934) says that one of the functional differences between the glomerular and the aglomerular tubules is the inability of the latter to eliminate glucose or other sugars which in the mammal pass by filtration from the glomerular capillaries through the squamous visceral layers. Glucose is reabsorbed by means of kidney phosphorylase and alkaline phosphatase in the proximal tubule as described previously. In view of these facts some tests were carried out to determine the degree of alkaline phosphatase activity in the avian kidney using, as stated under "Materials and Method" a combination of Danielli's (1946) and Lillie's (1948) methods.

Results.

The results of the histochemical detection of alkaline phosphatase are listed under Table 1.

An examination of Table 1 would seem to indicate that there is a fairly direct relationship between degree of enzyme activity and the length of time in fixation fluid. The most intense, and general black precipitation was obtained using as a fixation fluid, ice-cold 80 per cent alcohol for four hours.

The mesonephros of the frog and the kidney of the mammal were included as controls on the substrate, as the author was not certain as to the identity of the mixture.

The extent to which alkaline phosphatase activity was shown in the tissue of the classes examined is briefly
summarized below:

**Amphibia.**

Bullfrog (PLATE IX, fig. 13 and 14): the activity was noted throughout the tissue section except in the central region which contains collecting tubules only, which would therefore, not show any such function. In the dorsal region the proximal tubules are most abundant, while the ventral region of the mesonephros contains the distal tubules (Cole, 1941). The precipitation of cobalt sulphide was equally distributed between these areas, and was evident in the brush borders of the cells of the proximal tubule and also in the supra-nuclear zone. In the distal tubule, the precipitate likewise showed on this zone.

**Aves.**

Pigeon (PLATE X, fig. 15): location of enzymatic action was evident about half-way through the material. This result was probably due to the use of over-large blocks of tissue, and also leaving it too long a period in the fixation fluid, with subsequent destruction of the enzyme. The proximal tubules demonstrated alkaline phosphatase activity in both the brush border and the supra-nuclear zone. Henle's descending segment showed occasionally a fairly strong reaction.

Chicken: only one lot of tissue-sections manifested enzyme activity in the brush border at the very edge of the kidney. A glance at the length of times in the fixation fluids would indicate that they were, in the case of chicken nos. 2
and 3, unduly prolonged. The tissue from chicken no. 1 was one year old; a fact which may explain the lack of results; though Danielli (1946) states that tissue may be kept in refrigeration for a year and still show enzymic activity. Unfortunately, lack of time and material precluded further experimentation with this species.

Song-Sparrow (PLATE XI, fig. 16 and 17): the material obtained from this species was comparable to that obtained from the bullfrog in intensity. The precipitation occurred throughout the tissue and was very well shown in the brush border to which it was confined.

Mammalian.

White rat: the reaction was evident only at the edges of the kidney where it was located in the brush border.

Discussion.

There is, as far as the author is aware, only one reference to phosphatase activity in the bird and, in particular, to the metanephros. Moog (1943) worked on the localization of alkaline and acid phosphatases in the early chick embryo. She recorded the changes in phosphatase distribution in the principal soft organs, including the metanephros, up to the eighth day. Thus the relative enzymatic activity in the metanephric tubules at 4, 6, and 8 days of incubation shows that alkaline phosphatase activity is 10, 14, 14 at 4, 6, and 8 days respectively in the brush border of the mesonephric tubules. Incidentally, the mesonephros reaches its peak
functionally (in the production of urea) about the eighth day (Needham, 1929), and thereafter degenerates, giving place to the metanephros which starts to function at about eleven days with the production of uric acid. The alkaline phosphatase activity in the metanephric tubules at 6, and 8 days, is given as 4, 4. It would be inviting to attempt to correlate the decrease in alkaline phosphatase activity in the metanephros with an increase in glomerular degeneration in that organ, i.e., a decrease in filtration powers. However, as no data are given beyond eight days, it cannot be seen whether there is still further decrease in this species. Moreover, Moog suggests that the phosphatase activity shown here is, concerned with differentiation of tissues.

As far as the bird kidney is concerned, at least in the two species in which the results were positive, the findings of this histochemical experimentation, would seem to indicate that, in spite of apparent histological manifestations of glomerular degeneration, the organ in this class functions comparably to those of the amphibian and mammal. In these classes, well developed glomeruli exist, and they have been shown to reabsorb glucose by means of the proximal convoluted tubules. (Wearn and Richards, 1924; Best and Taylor, 1945).
Summary

The vascular supply has been considered; the histology, certain cytology, and a histochemical test of the avian metanephros have been carried out in as imperative a manner as possible.

Emphasis is placed on the following points:

(1) The vascular supply shows a definite renal portal system. There may be a variation in venous pattern here which should be further investigated.

(2) The histology shows signs of degeneration in the renal unit, with an increase in proximal tubule development.

(3) The cytological study, which consists of the examination of the mitochondria and the Golgi apparatus, indicates an increase in activity of the proximal tubule area as compared to that in the mammal.

(4) The histochemical work reveals signs of alkaline phosphatase activity in the bird, in spite of histological evidence concerning glomerular-degeneration.

Conclusions:

The avian proximal tubule shows signs of increased activity over that in the mammal.

The glomerulus is able to function comparably to that in the mammal in spite of histological evidence to the contrary.
Literature Cited


Lazarow, A. The chemical structure of cytoplasm as investigated in Professor Bensley's laboratory during the past ten years. Biol. Symposia, 10: 9, 1943.


PLATE I

Schematic representation of the two types of avian nephron (adapted from Spanner, 1924).

Fig. 1 Reptilian type
Fig. 2 Mammalian type
Schematic Representation of
The Two Types of Avian Nephron

Plate I

--- Malignant Corpses "
--- Proximal
--- Intermediate 
--- Henle's Loop, Thin Segment 
--- Distal Convoluted Segment 
--- Initial Collecting Tubule 

Fig. 1
Reptilian Type

(Adapted from Marshall, 1934) Fig. 2
Mammalian Type
PLATE II  Lobular Organization

Fig. 3  Schematic representation of lobular organization of the avian kidney
(adapted from Spanner, 1924).
PLATE II

Intralobular V. (Efferent)

Proximal Convoluted Tubule

Distal Convoluted Tubule

Intralobular Artery

Interlobular V.
Renal Portal (Afferent)

Gomerulus Efferent Vessel

Afferent Vessel

Fig. 3.

Schematic Representation of Lobular Organization in the Avian Kidney
(Adapted from Spanner, 1924)
PLATE III  Vascular system

Fig. 4  Diagramatic representation of the vascular system of the avian kidney (adapted from Spanner, 1924).
Diagrammatic Representation of Vascular System of Avian Kidney (Adapted from Spanner, 1924).
PLATE IV Histology

Fig. 5 Section of kidney of Domestic fowl
Heidenhain's azan stain

x 1214

Malpighian body, on renal unit,
showing glomerular avascular core
(fibroblasts) and basement membrane.
PLATE V  Histology

Fig. 6  Section of kidney of Song sparrow
Aoyama's method (no counter stain).

x 70

Proximal tubules are shown at
periphery of lobule.
PLATE VI  Histology

Fig. 7  Section of kidney of Domestic fowl
Foot's short method for silver impregnation of reticulum.  x 450

Malpighian body, showing reticular fibres at the centre of the glomerulus.

Fig. 8  Section of kidney of Domestic fowl
Foot's short method  x 450

Stroma of argyrophil or reticular fibres.
PLATE VII  Cytology
Mitochondria

Fig. 9  Section of kidney of Pigeon
Altmann's acid fuchsin stain,
counterstain toluidine blue.
  x 900
(a) Proximal tubule showing cells
     filled with mitochondria.
(b) Distal tubule showing cells
     with fewer mitochondria.

Fig. 10  Cell from proximal tubule showing
mitochondria (drawn to scale).
PLATE VIII  Cytology.

Golgi body

Fig. 11  Section of kidney of Song sparrow

Aoyama's Golgi method (no counter stain). x 1416

(a) Proximal convoluted tubules showing Golgi bodies in the supra-nuclear zone of the cells.

(b) Collecting tubules, showing deeply impregnated mucin producing cells.

Fig. 12  High power of a cell from the proximal tubule showing typical lepidosomes in the supra-nuclear region (not drawn to scale).
PLATE IX  Histochemistry

Alkaline phosphatase

Fig. 13  Section of kidney of Bull frog
Lillie's and Danielli's methods (combined)  x 900
Enzyme alkaline phosphatase activity showing in brush border and supra-nuclear zone.

Fig. 14  Section of kidney of Bull frog
prepared by Lillie's and Danielli's methods (combined) with the omission of substrate.  x 900
No enzyme activity shown.
Fig. 15 Section of kidney of Pigeon
Lillie's and Danielli's methods
(combined) x 900
Enzyme activity in brush border
and supra-nuclear zone.
PLATE XI  Histochemistry

Alkaline phosphatase

Fig. 16  Section of kidney of Song sparrow
  Lillie's and Danielli's methods  (combined)  x 900
  Enzyme activity showing in
  brush border.

Fig. 17  Section of kidney of Song sparrow
  control, no enzyme activity shown.