THE SITE OF ABSORPTION AND MODE OF STORAGE OF TRYPAN BLUE IN THE MESONEPHROS OF NECTURUS[1]

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ONE PLATE (THREE FIGURES)

This study of the behavior of trypan blue as an index of mesonephric activity in Necturus was undertaken to supplement a similar study made previously with iron salts (Dawson, '25). The dye was introduced into the blood stream and into the peritoneal cavity in order to compare the behavior of a freely diffusing chemical with that of a colloidal substance. In a recent paper, based on the frog, Hayman and Richards ('26) have anticipated some of my conclusions regarding the elimination and absorption of trypan blue. However, besides confirming their observations on the activity of the proximal convoluted portion of the tubule, I have been able also to demonstrate considerable activity in the distal convoluted portion.

The elimination and storage of trypan blue by the mesonephros have been noted incidentally by many authors who were not primarily interested in renal function (Hoskins and Hoskins, '18, dogfish; McClure, '18, Bufo and Rana, larva and adult; Wislocki, '21, chick embryo; Hanan, '27, chick embryo). Trypan blue is readily taken up and stored by cells such as those of the reticulo-endothelial system which actively ingest particulate matter. The passage of the dye into the mesonephric epithelium, however, cannot be regarded

[1]Read and material demonstrated at the twenty-fourth meeting of the American Society of Zoölogists, Philadelphia, 1927.

as the result of a process of phagocytosis, but must be a case of true vital staining or penetration of the cell membrane. This is noteworthy, since the blue component of the dye does not pass through an ordinary collodion membrane. The violet or red component, on the other hand, diffuses readily.

Other epithelia than renal, also incapable of phagocytic activity, are permeable to trypan blue. According to McClure ('18) this colloidal acid dye may, in both larval and adult frogs and toads, gain entrance to the body through the integument. Moreover, he finds in feeding experiments with adults that the solution of dye may be absorbed by the intestinal mucosa and reach the general circulation by way of the portal vein. More recent work by Huppert ('26) has extended the number of amphibian tissues found to be permeable to trypan blue. After feeding dye to adult frogs, she observed blue granules in the following locations: in the ciliated cells of the cardiac end of the stomach, in the basal protoplasm of active mucous cells of the fundus, in the cells of the fundic glands, and in the epithelium of both the small and large intestines. Following injections of the dye, no color was observed in the gastric epithelium and the amount of dye in the intestinal epithelium was negligible. The dye appearing in the intestinal cells was probably not obtained from the blood, but was absorbed from the lumen of the intestine following its elimination from the liver with the bile, since the intestinal content was stained light blue. In the case of the gastric epithelium, it is obvious that the dye entered the cells only from the lumen side.

The value of experiments utilizing dyes or other chemicals normally foreign to the kidney, in attempts to determine the mode of urine formation, has been questioned by many. This objection has been met, in part at least, by Cushny ('26) in his reply: "It may be asked why cells absorb a useless pigment, but it is to be remarked that they do not distinguish between the useful and the useless, but only between those substances having certain physical properties and those devoid of them" (p. 77). This more or less theoretical answer

is substantiated to some extent by experimental evidence in studies on the mesonephros of the chick. Boyden ('24) has shown that obstruction of the mesonephric ducts below the level of the twenty-fourth somite causes extensive hydronephrosis of the mesonephros. He concludes that this phenomenon provides an empirical basis for determining the beginning of the excretory activity in the mesonephros, thereby establishing it as early as the beginning of the fourth day of incubation. A recent study (Hanan, '27) has shown that trypan blue injected into the air chamber of the egg appears in the mesonephros and in the allantoic fluid of a chick of the fourth day, agreeing closely with the time established by Boyden. This definite correlation between the beginning of excretory activity and the ability to eliminate trypan blue indicates that the attainment of some distinctive stage of differentiation in the renal epithelium is essential for normal excretory activity and also for elimination of trypan blue. A significant observation has been made by v. Möllendorff ('20) that, in the tadpole, trypan blue is taken up by the cells of the tubules only when the corresponding glomerular capsule has developed its typical flattened epithelium. Similar observations were made by de Haan ('22) on the renal tubules of young kittens.

In most discussions of renal function it is customary to make little distinction between experimental results obtained while working with an amphibian kidney, a mesonephros, and results obtained while working with a mammalian kidney, a metanephros. It has been argued that, since these organs are structually similar and performing similar functions, results obtained in one are applicable without qualification to the other. The most striking difference between the two organs is their blood supply. In Amphibia, owing to the relation between the renal portal system and the renal veins, the circulation in the capillaries about the tubules is more or less independent of that of the renal arteries supplying the glomeruli. In the mammal, on the other hand, as recently demonstrated by MacCallum ('26), the entire circulation of the kidney is

essentially a glomerular one, so that glomerular and tubular activity in the mammalian kidney may be coordinated to some degree through the related common blood supply. Such a coordination of the activity of the glomerulus and tubules on the basis of blood supply cannot be postulated for the mesonephros, since the chemical composition of the blood brought in by renal portal veins must differ greatly from that received by way of the renal arteries. Unfortunately, MacCallum was not able to demonstrate a specific relation of the vas efferens and its capillary subdivisions to the tubule associated with the glomerulus from which the vas efferens takes its origin. He was only able to demonstrate that the efferent arteriole breaks up in the neighborhood of the glomerulus from which it arises, but whether its blood goes chiefly to its own uriniferous tubule or indifferently to parts of several was left undecided. If each uriniferous tubule in the mammalian kidney has a single and definite blood supply by way of its own glomerulus, the activities of the capsular and tubular portions may be definitely correlated through the blood stream. On account of these differences in blood supply, it would seem that observations based on a mesonephros may not be utilized unqualifiedly to elucidate problems of renal function in mammals.

It seems unnecessary to review in detail the extensive literature dealing with dye elimination by the kidney. Adequate summaries are given by Cushny ('26) and Hayman and Richards ('26).

In their work on the frog's kidney, Hayman and Richards introduced the dye either directly into the lumen of the uriniferous tubule by means of a capillary pipette or into the circulating blood by way of the abdominal vein or lymph sacs. In the present work on Necturus, instead of using the pipette method to introduce dye into the lumen of the tubules, the dye was injected intraperitoneally and then reached the lumina of the primary and secondary tubules by way of the nephrostomes and peritoneal canals connecting the necks of the mesonephric tubules with the peritoneal cavity. In this

method the animal is maintained in a normal physiological condition and the dye is enabled to pass continuously into the tubules over long periods of time. For comparison, dye was also injected intravenously.

INTRAPERITONEAL INJECTIONS

In this series of experiments 2 cc. of 0.4 per cent solution of trypan blue in 0.6 per cent NaCl was injected into the abdominal cavity and the animals killed from eight to forty-eight hours later. The dye leaves the body cavity by way of the ciliated nephrostomes and peritoneal canals entering the ciliated neck of the tubule a short distance from the capsule. Animals killed two to eight hours after injection show the dye in the lumina of the tubules, and blue granules are present within the cells of the proximal convoluted portion. The dye occupies a definite location within these cells, just beneath the brush border (fig. 2). Animals killed twelve hours later show the dye in the lumina throughout the whole extent of the tubules, and also in the wolffian ducts. Sections of such a mesonephros show an increased amount of trypan blue within the cells of the proximal convolute. Blue granules are evident much deeper in the cells some distance from the brush border. In the distal convoluted portion of the tubule, which is lined with peculiar 'striated' cells, dye is also present in many cells (fig. 3) but instead of appearing as blue granules it is in definite streaks which often extend from the luminal borders to the bases of the cells. Animals killed at longer intervals, twenty-two to forty-eight hours after intraperitoneal injection, show an accentuation of the conditions just described. In the proximal convolute many of the cells are completely tinted with blue, and in the distal convolute the dye has accumulated in large quantities between the bases of the 'striated' cells and the endothelium of the large sinusoidal capillaries which envelop this portion of the tubule (fig. 1). Some dye is also seen within certain cells of the interstitial lymphoid tissue. A very similar picture is obtained in animals which have received repeated injections at intervals of twenty-four hours over a period of two days.

INTRAVENOUS INJECTIONS

Injections into the blood stream were usually by way of the abdominal vein, using 0.2 per cent solution of the dye in 0.6 per cent NaCl. Dye can be detected in the cells of the proximal convolute two hours after the injection of 1 cc. of the dye solution. It is limited, however, to the proximal convolute and is found immediately beneath the brush border. After eight to twelve hours, more dye is found in the proximal portion of the tubule, but there is none in the distal convolute, and none was found in the latter location after any single injection. However, with three serial injections ten to fourteen hours apart, first by way of the abdominal vein, then by the vein of the fore leg, and then by the vein of the hind leg, it is possible to introduce sufficient dye that it can be detected in traces in the distal convolute, but the striking picture (fig. 3) which is obtained following intraperitoneal injections is never observed.

Following intravenous injection, the amount of dye observed in the lumina and walls of the proximal convoluted tubules varied notably. This is probably correlated with the degree of activity of the renal corpuscle, which is conditioned in turn by the intermittency of the circulation through the glomerulus (Richards and Schmidt, '22). Similar variations in the amount of dye eliminated into the various tubules following intravenous injections were noted by Hayman and Richards in the frog.

These experiments, as stated previously, confirm the observations of Hayman and Richards on the absorption of trypan blue by the proximal convoluted portion of the frog's mesonephros. In both studies the dye was introduced directly into the lumen, thereby avoiding the difficulties of interpretation which are encountered when the vascular route is employed. Moreover, the similarity of the picture obtained after intravenous injection is strong presumptive evidence that trypan blue passes through the glomerular capsule and is reabsorbed, although we have not actually observed the passage of the dye. The progressive accumulation of the dye within the

epithelial cells indicates that it does not enter the blood stream as rapidly as it passes into the cells from the lumen of the tubule. Consequently, there is a definite storage within the renal cells, and dye may be observed within these cells for some time after it has disappeared from the lumina of the tubules, and is also absent from the urine.

In Necturus the distal convoluted portion is separated from the proximal convolute by a straight tubule composed of ciliated cells, the so-called 'narrow' segment. The histological features of the distal convolute are totally unlike those of the proximal portion. They are devoid of granules and do not possess a 'brush border.' The most characteristic feature of the cells of the distal convolute is the presence of the definite rods in the cytoplasm giving them their striated appearance. Trypan blue is apparently absorbed by these cells only when it reaches a certain concentration, since dye is often present in small amounts in the lumen when none can be discerned within the cells. Unlike the cells of the proximal portion, these cells are apparently unable to store any dye, but allow it to pass toward the blood stream where it accumulates in a peritubular location. The accumulated dye does not seem to pass through the endothelium very rapidly, since the dye may persist in these locations after it has disappeared from the lumen of the tubule.

RELATION BETWEEN THE BEHAVIOR OF THE DYE AND THE HISTOLOGICAL DIFFERENTIATION OF THE EPITHELIUM

In the cells of the proximal convoluted tubules the following cytoplasmic differentiations are encountered: 'brush border,' granules, mitochondria, a hyalin body surrounded by a vacuole (fig. 2), and the reticular apparatus of Golgi. In the 'striated' cells only the rods or 'Stäbchen' and the reticular apparatus have been demonstrated. All of these, with the exception of the reticular apparatus, were discussed by Chase ('23), and the lack of any adequate knowledge of the significance and relationships of these structures was emphasized. The hyalin body of the proximal cells appears

to be associated with the reticular apparatus and with the formation of cytoplasmic granules. The nature of the relationship between these three structures, however, is difficult to determine. In several cells dye is found in the vacuole surrounding the hyalin body and the body itself may be tinted.

The origin and nature of the 'Stäbchen' or rods of the distal cells has not been satisfactorily explained. The most commonly accepted interpretation is that they are modified mitochondrial elements, since they react chemically like mitochondria, and no other elements giving a similar reaction can be definitely identified within these cells. Some have regarded them as pericellular structures, but, according to Chase ('23), they are intracellular differentiations and are grouped in a hollow cylinder investing the nucleus.

Following the observations of Cowdry ('22) on the position of the Golgi reticulum as an indicator of physiological polarity in the thyroid gland, many workers have attempted to use the location of this structure as an aid in determining the region of greatest secretory or excretory activity within the cell. Bowen ('26), in a recent paper, however, has obtained evidence which suggests that the reversal of polarity as expressed by the Golgi apparatus actually has little bearing on the secretory or excretory processes. Avel ('24) has studied the location of the Golgi network in the cells of both the proximal and distal convolutes of the mesonephros in Rana fusca and Triton alpestris. In the proximal cells the reticular apparatus varies from an equatorial to a basal position in respect to the nucleus, while in the striated portion of the tubule it is situated between the nucleus and the luminal border of the cell. In Necturus the reticular apparatus has a more or less basal position in the proximal tubule, but the writer has been unable to obtain a good differentiation of the apparatus in the distal portion. Until the function of the Golgi apparatus is more fully understood, a study of this structure in relation to the activity of the renal cells will not be a great aid in determining whether secretion into, or absorption from, the lumen is taking place.

From our observations on the location of trypan blue following both intraperitoneal and intravenous injection, it appears that in the proximal convolute the dye passes readily and quickly through the brush border and is adsorbed to the cytoplasmic granules, and not to mitochondria. Further, the dye does not leave the cell as rapidly as it enters, and a definite storage occurs. In the distal convolute, on the other hand, the dye does not appear to enter the cells so readily as in the proximal portion, and the degree of concentration has been suggested as a factor. The dye appears to pass readily through the striated cells and accumulates in masses between the renal epithelium and the endothelium of the sinusoidal capillaries investing the tubule, indicating that the renal cells are more permeable to the dye than is the endothelium. It should also be pointed out that the main blood supply of the striated portion of the tubules is composed of a complicated network of large sinusoidal capillaries which form efferent renal veins leading directly into the posterior vena cava.

DISCUSSION

Owing to their position in the mesonephros, the proximal convoluted portions of many tubules are easily seen on the ventral surface of the organ. The distal convoluted portions usually lie deeper and cannot be observed directly without disturbing the organ. Accordingly, the activity of the proximal portion has been followed more closely. Using direct methods, White and Schmitt ('26) have demonstrated the reabsorption of sugar and chlorides by the epithelium of the proximal convolute, and Hayman and Richards have observed absorption of several dyes and other chemicals following intracapsular injections, by the same portion of the tubule. Observations, however, on the distal portion are scanty and indefinite. Hayman and Richards noted that, following repeated intracapsular injections of trypan blue, the intensity of color in the proximal loop increased, and other loops apparently distal to this became clearly visible, though less intensely stained. When no injection was made for some

minutes, these latter slowly faded, while the stain in the first loop persisted unchanged. Working with methylene blue, they also noted that in places the dye seemed to extend from the ring of stain in the lumen as streaks between the cells. Their observations on trypan blue are in agreement with the results of the present study and indicate clearly, 1) that there is a definite tendency toward storage of the dye in the proximal convoluted portion and, 2) that the cells of the distal convolute do not retain the absorbed dye, but pass it on toward the blood stream. When, however, large amounts of dye are introduced by the intraperitoneal route over considerable periods of time, definite peritubular accumulations form, presumably because the dye penetrates the striated cells more rapidly than it does the endothelium. The picture they describe is found almost typically in Necturus following repeated intravenous injections where the dye is seen within the epithelium, but does not become accumulated between the renal cells and the capillary endothelium. This is probably due to the smaller amount of dye which enters the lumen of the tubule from the blood stream.

The intercellular streaks of methylene blue described by Hayman and Richards are probably similar to those of trypan blue described earlier in this paper as intracellular in the distal convolute and which I have attempted to explain as due to the peculiar histological differentiation of this portion of the tubule. The 'Stäbchen' or rods have a peripheral location within the cell, and it is difficult to be certain that the streaks of dye are intracellular, since the exact relation of the dye to the rods is not clear; that is, whether the dye is within the rods or in the cytoplasm between them. In earlier experiments with iron salts (Dawson, '25), the distribution in streaks was observed and reported for the distal convolute.

SUMMARY

In the mesonephros of Necturus the primary and secondary tubules are connected with the body cavity by peritoneal canals. Advantage is taken of this fact in the present study.

Trypan blue in normal saline was injected intraperitoneally and intravenously. Following intraperitoneal injections, trypan blue is found adsorbed to the cytoplasmic granules of the cells of the proximal convoluted tubules and a definite intracellular storage of dye occurs. In the striated cells of the distal convolute the dye is in streaks and an extracellular accumulation of dye between the bases of the cells and the endothelium of the capillary plexus is observed. The same distribution of dye is noted following intravenous injections, but much less dye is stored. There is, therefore, strong presumptive evidence that dye is reabsorbed from the tubular lumen following intravenous injection, since in intraperitoneal injections no dye finds its way into the circulation in the time period of the experiment, and evidence of absorption in the latter case is conclusive. The manner of absorption and storage appears to be correlated with the presence of a brush border and cytoplasmic granules in the cells of the proximal and of the rods in the striated cells of the distal portion of the tubule.

ADDENDUM

Since this paper was submitted to the editorial board, a report of a similar study by Edwards ('27) has appeared. He summarizes as follows: "Results have been obtained by injecting certain dyes intraperitoneally and intravascularly in Necturus, which show that when these dyes are allowed to get into the lumen of the renal tubule through the nephrostome, they are *not* absorbed by the cells of any portion of the tubule." Trypan blue was one of the several dyes utilized by Edwards. It is difficult to account for the divergent results obtained in these two studies. The physiological state of the animals may possibly account for it. Edwards does not specify the time at which the trypan-blue experiments were carried out. The animals used in my study were taken in the spring and early summer, and the experiments conducted during the ten days immediately following the receipt of the animals, while they were actively feeding on crayfish. (Edwards, J. G. 1927 The behavior of dyes in the kidney tubule of Necturus. Amer. Jour. Physiol., vol. 80, pp. 179–184.)

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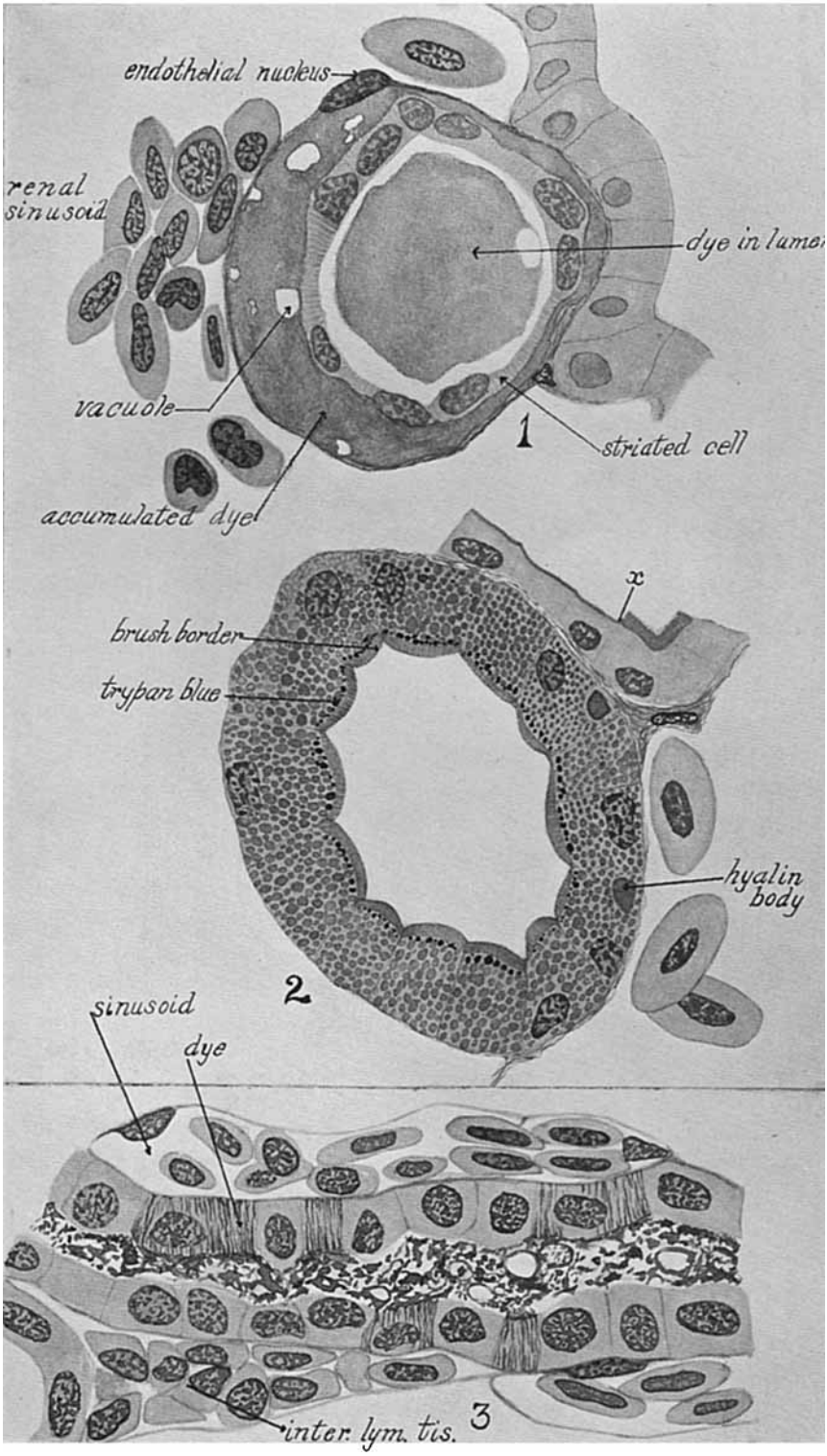
PLATE 1

EXPLANATION OF FIGURES

1 Transverse section through the distal convoluted portion of a tubule, showing the peritubular accumulation of trypan blue between the bases of the epithelial cells and the endothelium of the renal sinusoid. This portion of the tubule is composed of 'striated' cells, through which the trypan blue appears to pass readily. Note the dilated condition of the tubule and the dye in the lumen. Twenty-four hours after a single intraperitoneal injection.

2 Transverse section through the proximal portion of a tubule, eight hours after intraperitoneal injection with trypan blue. Note the definite location of the dye, just within the brush border. Dye adsorbed to cytoplasmic granules usually appears dense. An adjoining tubule shows the brush border well developed on some cells and very poorly developed in adjoining cells (*X*).

3 Longitudinal section through the distal convoluted portion, showing the dye passing through the striated epithelium. The distribution of the dye within the cells conforms to their histological differentiation. Twelve hours after intraperitoneal injection. *inter.lym.tis.*, interstitial lymphoid tissue.


endothelial nucleus
renal sinusoid
dye in lume
vacuole
striated cell
1
accumulated dye
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trypan blue
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