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Dye Permeability and Alkaline Phosphatase Activity of Testicular Capillaries in the Postnatal Rat

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Summary. Staining of testicular and epididymal tissues after intravenous, intraperitoneal or subcutaneous administration of a number of dyes was investigated in rats at different stages of postnatal development. After light green injections heavy staining of both testis and epididymis was visible to the naked eye in neonatal animals up to the age of 10 days, while in rats over 15 days old no appreciable staining of the testis could be seen, although the caput epididymis was strongly coloured. From 3—8 hours after subcutaneous acriflavine administration, the nuclei in the blood vessel walls of the testis, as well as the nuclei in the rete testis, tubuli efferentes and caput epididymis, fluoresced in all age groups. The nuclei of the interstitial and tubular cells were stained intensely until the age of 5 days. Thereafter the intensity gradually diminished until the age of 20 days, when no nuclear fluorescence was visible in the seminiferous tubules and even the interstitial nuclei fluoresced weakly or not at all.

The histochemical alkaline phosphatase activity of the testicular capillaries was studied by GOMORI's method, using fresh and postfixed cryostat sections from postnatal rat testes. The testicular capillaries exhibited appreciable activity at the age of 10 days.

On the basis of the present and previous observations on the permeability of the testicular capillaries, the existence of a blood-testis barrier in the puberal and adult rat testis is suggested.

Development of the blood-testis barrier and the alkaline phosphatase activity of the testicular capillaries are suggested to reflect general vascular maturation at the beginning of puberty in the rat.

Introduction

The presence of a barrier mechanism in the testicular capillaries has been suspected since DE BRUYN et al. (1950) observed exceptionally weak staining of mouse testicular nuclei after subcutaneous administration of diaminoacridine dyes. In their studies on the blood supply of tumours, GOLDACRE and SYLVÉN (1959, 1962) noticed that lissamine green, when administered intravenously to mouse and rat, neither penetrated the blood-brain barrier nor stained the testis, ovary or adrenal gland. Other reports suggest that the testicular capillaries have a low permeability to a variety of substances. RO and BUSCH (1965) found the lowest concentration of C^{14}-actinomycin D in testis and brain 15 min after intravenous injection, and WAITES and SETCHELL (1966) observed a rubidium-rejecting compartment in the rat testis. On the other hand, COWIE et al. (1964) considered the high protein content of the testicular lymph in the ram to be a sign of the high permeability of these capillaries to plasma proteins. The high permeability of the testicular capillaries to plasma proteins has since been confirmed by MANCINI et al. (1965), using labelled serum proteins and rat testis.

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Reports on the permeability of the testicular capillaries thus suggest barrier phenomena similar to those of the CNS, with the exception that the testicular vessels, in contrast to those of the brain (KLATZO et al., 1962), are permeable to plasma proteins. The permeability characteristics of the testicular capillaries are undoubtedly of great importance in the physiology of the testis. A study was therefore undertaken to evaluate the staining properties of the rat testis with vital dyes in the course of postnatal development. A histochemical survey of alkaline phosphatase activity in the capillaries of the developing rat testis was also made, since this enzymatic activity may be related to transport phenomena across the capillary wall (LANDOW et al., 1942; WISLOCKI and DEMPSEY, 1948; LEDUC and WISLOCKI, 1952; BOURNE, 1958; NANDY and BOURNE, 1963).

Material and Methods

Altogether 151 white male Sprague-Dawley rats were used. They were 0, 5, 10, 15, 20, 25, 30, 35 and 40 days old; rats over 2 months of age were taken as adults. Each age group consisted of at least 12 experimental animals (at least 4 animals for each procedure). The rats were reared in constant environmental conditions and received rat cubes and water *ad libitum*. The young males were weaned at the age of one month.

For naked eye estimation of testicular staining with vital dyes, 40 animals were injected with various dyes, e.g. neutral red, trypan blue, light green yellowish. Light green yellowish (Merck, GURR: Soc. Dyers and Colourists Colour Index, 2nd ed., No. 42095) turned out to be most suitable, because of its better contrast and lesser toxicity. The anaesthetized animals received, intravenously or intraperitoneally, 15 ml/kg of 2% dye in saline. The rats were killed 5—10 min after intravenous and 3 hr after intraperitoneal injections. The staining of testicular tissue was compared with that of the epididymis, viscera and brain.

For microscopic examination, a series of 61 animals received a subcutaneous injection of 1% neutral acriflavine (B.D.H., C.I. No. 46000) 20 mg/kg. To a few older animals, intratesticular injections of 0.02 ml of 0.1% acriflavine were also given. The rats were killed 3—8 hours after the injection. Testes, epididymides and a piece of brain tissue were frozen with liquid air and sectioned at 10 μ in a cryostat. Fresh frozen sections were studied with a fluorescence microscope, using a high-pressure mercury lamp as a source of ultraviolet light. They were photographed at the same time.

For the demonstration of alkaline phosphatase activity fresh frozen 10 μ sections of testes were incubated unfixed (BITENSKY and COHEN, 1965) or fixed for 15 min in 3°C formol-calcium. The incubation solution was made up according to GOMORI (1952) with sodium α- and β-glycerophosphate as substrates. To test the relative activities of the enzyme at different ages, the sections were incubated at 37° C for different periods (5, 15 and 30 min).

Results

Staining of Testicular Tissue with Vital Dyes

Light Green Injection. — The visually estimated staining intensity of the testicular tissue was independent of the mode of injection or the post-injection time. The results are presented in Table 1. A green colour was constantly seen in the testes and epididymides of animals injected at 0 and 5 days but the brain tissue was unstained. At the age of 10 days, a clear decrease in the stainability of the testes was observed and at the age of 20 days and later, the testes were completely colourless or showed only traces of dye beneath the tunica albuginea.

The head of the epididymis was deeply and uniformly stained in all age groups. In other parts of the epididymis a peculiar distribution of the dye was observed in puberal and adult animals. While the caput and upper part of the corpus

Table 1. *Staining of the rat testicular tissue after injection of 2% light green yellowish (15 ml/kg)*

Age in days	Staining intensity		
	Testis	Caput epididymis	Brain
0	++	++	−
5	+±	++	−
10	+	++	−
15	− to +	++	−
20	− to ±	++	−
25	− to ±	++	−
30 to adult	− to ±	++	−

epididymis, as well as the vas deferens, were intensely green, the caudal portion had only a faint greenish hue. The brain did not stain in any age group, but most of the viscera in the abdominal cavity were distinctly stained.

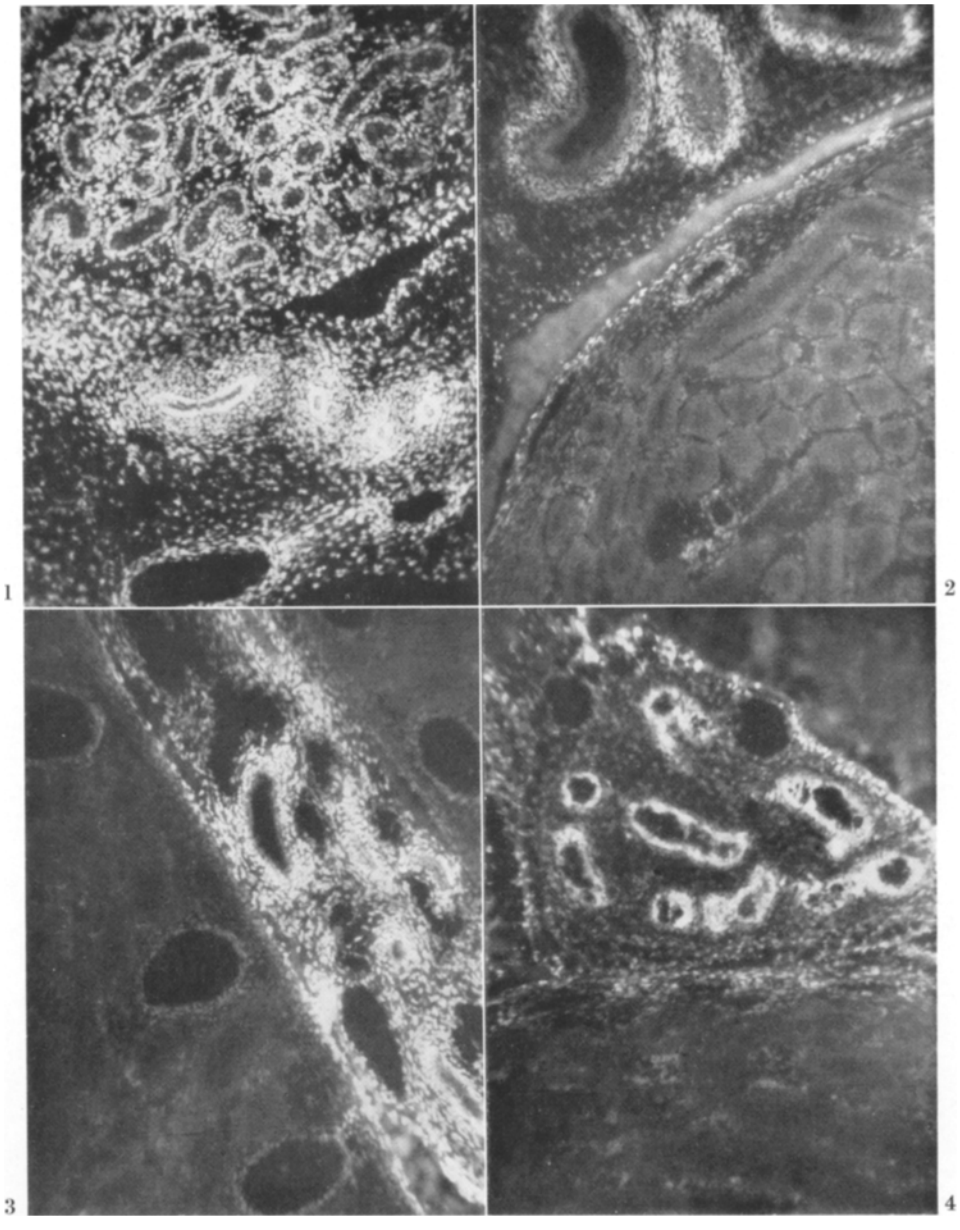
Acriflavine Fluorescence with Reference to Autofluorescence

The amount, location and colour of the lipid autofluorescence was studied in each age group. Before the age of 20 days, the testes did not fluoresce appreciably, but in older animals both the interstitial tissue and the seminiferous tubules exhibited fluorescence in the form of cytoplasmic granules. The epididymal duct epithelium showed autofluorescence in the luminal border. This cytoplasmic fluorescence was present even in the youngest animals and increased with age.

The results obtained with acriflavine injections are presented in Table 2. In the newborn animals, an intense nuclear fluorescence was seen throughout the testis and epididymis (Fig. 1). Some differences in the intensity of fluorescence could be observed, however; in the epithelium of the epididymis it was very intense but less so in the cells of the seminiferous tubules (Fig. 1). At the age of 5 and 10 days (Fig. 2) the seminiferous epithelium was definitely less fluorescent

Table 2. *Fluorescent staining intensity of testicular nuclei after subcutaneous administration of acriflavine (20 mg/kg) and alkaline phosphatase activity of testicular capillaries*

Age in days	Intensity of fluorescence in testicular nuclei				Intensity of fluorescence in other tissues		Relative alkaline phosphatase activity in testicular capillaries
	Tubules	Interstitial tissue	Blood vessel walls	Rete testis and tubuli efferentes	Caput epididymis	Brain	
0	++ to +++	+++	+++	+++	+++	± to +	− to ±
5	++	+++	+++	+++	+++	± to +	± to +
10	+	++	++ to +++	+++	+++	− to ±	+ to ++
15	±	+ to ++	++ to +++	+++	+++	−	++
20	−	+	++ to +++	+++	+++	−	+++
25	−	± to +	++ to +++	+++	+++	−	+++
30 to adult	−	− to +	++ to +++	+++	+++	−	+++



Figs. 1—4 (for legends see p. 331)

than the interstitial or epididymal nuclei. In the interstitial tissue, endothelial nuclei fluoresced as intensely as the nuclei of other cells. The nuclei of the rete testis and tubuli efferentes were all fluorescent as well.

In the 15-day group, the contrast between the staining of testis and epididymis was very clear (Fig. 3 and 4), only a trace of fluorescence being observable in the tubular nuclei. The interstitial tissue also fluoresced weakly, except the

nuclei of the vessel walls (Fig. 3). The rete testis, ductuli efferentes and caput epididymis showed undiminished fluorescence. In the 20- and 25-day-old rats no fluorescence was present in the tubular nuclei, and the interstitial nuclei fluoresced very weakly, except in the vicinity of blood vessels. From the age of 30 days onwards the appearance of autofluorescence disturbed the observations to some extent, but neither the seminiferous tubules nor the Leydig cells were fluorescent. An exception was the interstitial nuclei surrounding the blood vessels. The nuclei of the rete testis, ductuli efferentes and caput epididymis all fluoresced intensely. Both the stromal and epithelial nuclei were distinctly more fluorescent in the caput epididymis than in the caudal portion.

Even the nuclei of the brain tissue were weakly fluorescent in 0- and 5-day-old animals, but after the age of 10 days, acriflavine fluorescence was not seen there.

When small amounts of acriflavine were injected directly into the testis through the tunica albuginea, a bright fluorescence was observed in the interstitial nuclei, and some fluorescence appeared even in the tubular nuclei.

Alkaline Phosphatase Activity in Testicular Capillaries

After 5 min incubation, alkaline phosphatase activity was not present in the capillaries of 0-day-old rats and only a trace of activity was seen in 5-day-old animals. After this age, the capillaries were distinctly active, the intensity of the reaction reaching the level seen in adult animals at the age of 20 days. However, with incubation for 15 min or longer, some activity was seen in a few small blood vessels of the 0- and 5-day-old rat testis (Fig. 5), and the testicular capillaries of 10-day-old rats gave a distinct reaction (Fig. 6). In 15-day-old animals the number of vessels giving an intense reaction had increased still further and no difference could be detected between the stainability of these vessels and those of older animals of various ages.

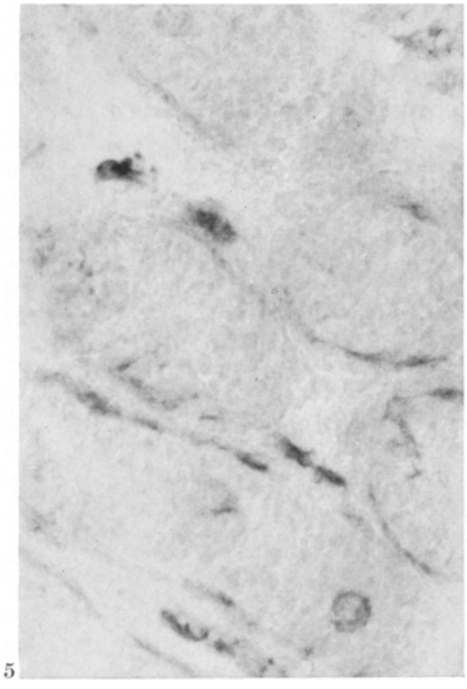
At the ages of 0 and 5 days, the endothelium of the testicular artery (being about 70 μ in internal diameter) gave a weak positive reaction. Smaller arterial branches also stained positively (Fig. 7), as well as the cytoplasm of the primordial germ cells. At the age of 5 days the basement membrane of the seminiferous tubules gave a weak positive reaction after incubation for 15 or 30 min, its activity being strong in older animals.

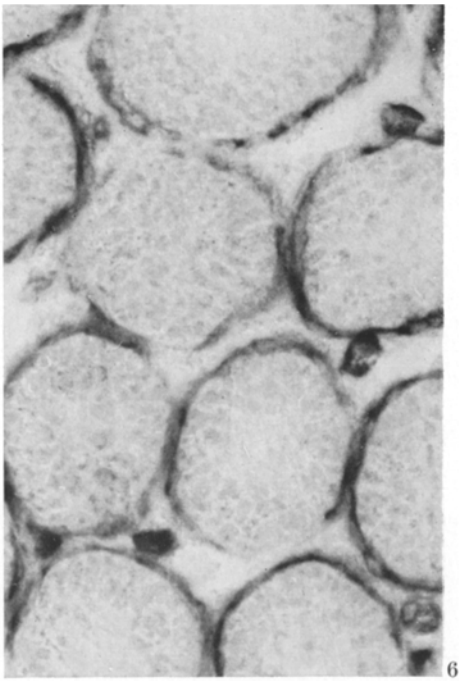
Fig. 1. A fresh frozen section through the testis and epididymis of a newborn rat killed 4 hours after subcutaneous injection of acriflavine (20 mg/kg). Note an intense nuclear fluorescence in both testicular and epididymal nuclei. × 260

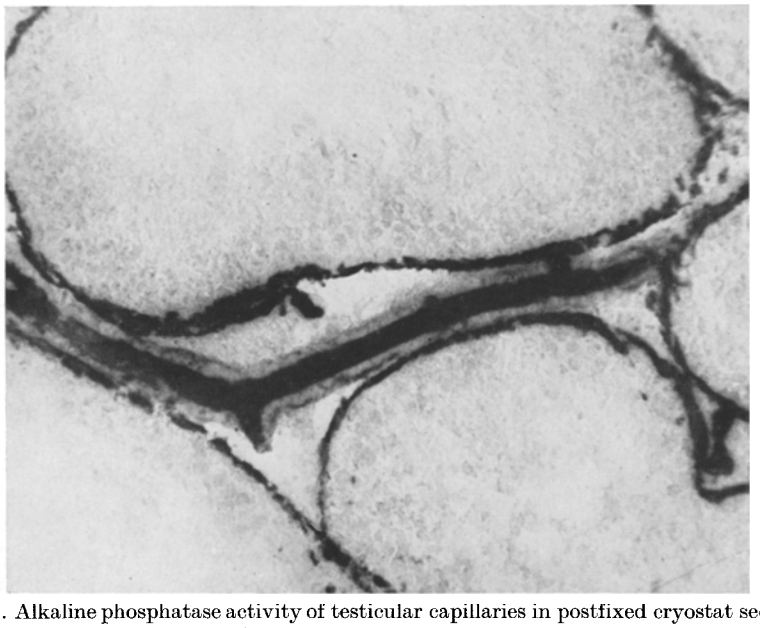
Fig. 2. Testis and epididymis of a 10-day-old rat killed 8 hours after acriflavine administration. The interstitial nuclei of the testis as well as those of the blood vessel walls and epididymis are more fluorescent than the nuclei of the seminiferous tubules. × 260

Fig. 3. A section of two testes with epididymal tissue between, from a 15-day-old rat 8 hours after subcutaneous acriflavine injection. Note the clear-cut contrast between strong epididymal fluorescence and weak testicular fluorescence. In the testes only the nuclei of the blood vessel walls and tunica albuginea and some of the interstitial nuclei are fluorescent. × 260

Fig. 4. A section of the testis with tubuli efferentes from a 15-day-old animal treated in the same way as in Fig. 3. Note the intense nuclear fluorescence in the ductuli efferentes in contrast to the weak testicular fluorescence. × 260







Figs. 5—7. Alkaline phosphatase activity of testicular capillaries in postfixed cryostat sections. Incubation time is 15 min

Fig. 5. A 5-day-old rat testis with weakly positive reactions in capillaries and in a few cells of the tubular wall. × 520

Fig. 6. A 10-day-old rat testis. Capillaries as well as tubular walls contain considerable alkaline phosphatase activity. × 520

Fig. 7. A 40-day-old rat. A small artery is seen to give off branches during its intertubular course. The arterial endothelium gives a positive reaction for alkaline phosphatase. × 520

Discussion

The Dye Test Method for Capillary Permeability

Light green was found to be the most suitable dye for demonstration of a vascular barrier because of its readily observable colour in animal tissues, its weak toxicity and its known inability to pass the blood-brain barrier (GOLDACRE and SYLVÉN, 1962). This dye does not penetrate the living cell membranes *in vitro* and therefore serves as an indicator of the movements of the interstitial fluid for a limited period (HOLMBERG, 1961; GOLDACRE and SYLVÉN, 1962). Trypan blue had certain disadvantages for use in the present study. Its colour was not so easily visually observable in the testis tissue as that of a green dye, and it gave variable results, the testes of adult animals sometimes being stained bluish, sometimes not.

Diaminoacridine dyes behave very differently from light green. These basic dyes penetrate readily into the living nuclei, where they can be detected even in low concentrations by virtue of their fluorescence near ultraviolet light (DE BRUYN et al., 1950). The original method of DE BRUYN et al. (1950) was successfully modified for use in fresh cryostat sections instead of freeze-dried tissue. The nuclear acriflavine fluorescence so induced was more intense and initially sharp, but became diffuse within one day in cryostat sections. When relatively thin (10 μ), fresh sections were used, little diffusion of the dye occurred in the present study.

The Blood-Testis Barrier

When failure of entry of a dye into a tissue is being investigated, it cannot be said that the restriction is due to a lower permeability of some structure separating it from the blood, unless it has been shown that there is no metabolic cause for the difference (DOBBING, 1961). In spite of criticism, there is convinving evidence in the recent literature that transport mechanisms are implicated, at least to some extent, in the inability of acid vital dyes to pass from the blood to the brain, and that these dyes are therefore useful for studying the barrier and alterations occurring in it (for references see BROMAN, 1955; STEINWALL, 1961, 1964; FLODMARK, 1965). The inability of acriflavine to pass the blood-brain barrier is also well documented (DE BRUYN et al., 1950; RODRIGUEZ-PERALTA, 1955, 1957, 1966; SAMORAJSKI and MCCLOUD, 1961).

On the basis of the well-known methods for blood-brain barrier demonstration, the existence of a barrier system in other organs besides the CNS has been suggested (e.g. adrenal gland, ELKELES, 1954; thymus, MARSHALL and WHITE, 1961a and b; lymph node, MENZIES, 1966). The testicular barrier mechanism, however, has not been subjected to systematic study. The presence of a dye-rejecting mechanism in the rat testis from the 20th postnatal day onwards has been clearly demonstrated in the present study. However, the possibility of other influences than a barrier mechanism must be considered when results of intravital staining are being evaluated. The testicular blood flow is known to be almost pulseless in contrast to other tissues in the ram (WAITES and MOULE, 1960), and its rate is known to be relatively low both in the ram (LINDNER, 1963; SETCHELL and WAITES, 1964), and in the rat (GOLDMAN, 1961; SETCHELL et al., 1964;

WAITES and SETCHELL, 1966). The peculiar blood flow, however, appears not to be the cause of the intravital staining properties of the testis in the present study, since acute cadmium damage to the testicular capillaries results in distinct staining of the testicular nuclei with acriflavine (KORMANO, unpublished observations), although cadmium is known to produce a sharp decrease in testicular blood flow in the rat (WAITES and SETCHELL, 1966). The postnatal development of the barrier is very similar to the maturation of the dye-rejecting capacity of the blood vessels of the rat brain (STERN and PEYROT, 1927). In the animals used in the present study the blood-brain barrier for acriflavine was functionally mature at the age of 15 days.

Protein conjugation is said to be at least one of the causes for the failure of many dyes to penetrate brain tissue (TSCHIRGI, 1950, 1958; DAVSON, 1956; DOBBING, 1961). The testicular capillaries however, are known to be highly permeable to plasma proteins (LINDNER, 1963 and COWIE et al., 1964, ram; MANCINI et al., 1965, rat).

It was thought that the basement membrane of the seminiferous tubules might be responsible for the weak staining of testicular tissue. The staining of the nuclei after intratesticular injection suggests, however, that the tubular basement membrane is not the only barrier.

Although there are some similarities between the blood-testis barrier and the blood-brain barrier, as reported in the present paper and by some earlier investigators (GOLDACRE and SYLVÉN, 1959, 1962; RO and BUSCH, 1965), the former cannot be of similar nature to or as effective as the blood-brain barrier. In the first place, a faint fluorescence was observed in some testicular cells, whereas the brain tissue remained totally negative in the acriflavine experiments. A second difference is the easy access of plasma proteins into the testicular tissue (MANCINI et al., 1965), whereas the blood-brain barrier is not penetrated by protein (KLATZO et al., 1962). It can be speculated that the peculiar permeability of adult testicular capillaries may be a reflection of an influence of the testicular Leydig tissue on the endothelium of its vessels. This hypothesis is supported by the observations on testicular grafts by WILLIAMS (1949). He observed that an intravenously injected dye did not appear in testicular grafts. There was a morphological difference in the graft capillaries also. The graft interstitial tissue thus probably altered the permeability of its capillaries.

The intense staining of the rete testis, ductuli efferentes and caput epididymis after systemic or intratesticular injection of dyes has been related to the well-known resorptive capacity of these structures in different species, including the rat (for the extensive literature see YOUNG, 1933; NICANDER, 1958; BURGOS, 1964; NIEMI and KORMANO, 1965a; MORITA, 1966). Dye transport from testis to epididymis cannot explain the very rapid and intense staining of the excurrent ducts after dye administration in the present study. It seems very likely that differences in staining properties between the testis and the other tubular systems a few minutes or hours after dye injection are related to differences in the permeability of the capillaries in these structures. In electron microscope studies LADMAN and YOUNG (1958) observed that in the guinea-pig the peritubular capillaries of the ductuli efferentes possess apertures in their endothelium, their

ultrastructure differing strikingly in this respect from that of the testicular capillaries (CRABO, 1963; ROSS, 1963; MURAKAMI, 1966), which are of the A-1-α type, like those of the brain (BENNETT et al., 1959). The capillary structure of the rat rete testis also seems to differ from that of the testis (LEESON, 1962).

Alkaline Phosphatase Activity in Testicular Capillaries

In the capillary endothelium and the tubular basement membrane of the adult rat testis, DEMPSEY et al. (1949) and WACHSTEIN and MEISEL (1954) demonstrated a high level of alkaline phosphatase activity. DEMPSEY et al. (1949) also observed the activity to be under hormonal influence. The present observations, as well as those of BAILLIE (1961), demonstrate that there is no alkaline phosphatase activity in the neonatal rat and mouse testis. On the other hand, the capillaries of the human testis have been shown to contain alkaline phosphatase activity in their walls already at birth and even earlier (IKONEN, 1965; NIEMI and IKONEN, 1965).

In the present study, appreciable alkaline phosphatase activity appeared in the blood vessels and in the tubular basement membranes at the age of ten days. This is considerably earlier than in the mouse, where BAILLIE (1961) did not observe activity until the end of the third postnatal weak. The appearance of alkaline phosphatase activity in the rat testis thus coincides with the beginning of endocrine (NIEMI and IKONEN, 1963) and tubular wall maturation (LEESON and LEESON, 1963; NIEMI and KORMANO, 1965b), when the dye barrier also becomes functional. The two observations in the present study may indeed reflect the same process of vascular maturation. This view is in agreement with numerous earlier suggestions on the relationship of alkaline phosphatase activity to transport phenomena across the capillary wall and the energy supply for this process (LANDOW et al., 1942; WISLOCKI and DEMPSEY, 1948; LEDUC and WISLOCKI, 1952; BOURNE, 1958; NANDY and BOURNE, 1963; TEWARI and BOURNE, 1963). However, an increase of transport through the capillary wall is usually followed by increased alkaline phosphatase activity (PEARSE, 1958; SAMORAJSKI and MCCLOUD, 1961; MAEKAWA et al., 1965). The possibility that alkaline phosphatase provides free energy for selective permeability in the normal mature testicular capillaries is compatible with an increase in its activity as active transport increases.

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