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EXTRAVASCULAR AND INTRATUBULAR DIFFUSION OF LABELED SERUM PROTEINS IN THE RAT TESTIS*

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Rat whole serum, albumin, globulins, and fibrinogen were labeled with fluorescent dyes (sulforhodamine B, CI No. 45100 and fluorescein isothiocyanate); albumin was also tagged with radioactive iodine (I^{131}) and tritium (H^3). In addition heterologous albumin was also labeled with fluorescent dyes and radioiodine. The proteins were intravenously injected in prepubertal, pubertal and adult rats, and their decay in the circulation and histological distribution in the testis and epididymis was studied. As controls other animals were similarly injected with free labels alone and with labeled denatured and degraded albumin; also unlabeled homologous and heterologus albumins were administered followed by incubation of both organs with the corresponding labeled antisera applying the Coons' technique. It was observed: 1) with the exception of fibrinogen, labeled serum proteins rapidly appeared in the lumina of vessels, diffused extravascularly in the intertubular spaces and finally arrived inbetween the germinal cells and in the lumina of seminiferous tubules. Also labeled material was present in the lumen of epididymal canaliculi but not in the ductus deferens. 2) This extravascular and intratubular diffusion was parallel to the fast component of the time decline curve of labeled homologous serum proteins in the circulation. 3) There was no great difference between young and adult rats in the extravascular diffusion process, but intratubular passage was higher in pubertal and adult animals. 4) Control experiments revealed the presence of some fluorescent material only in the vessels and macrophages, whereas the immunofluorescent technique showed a very similar localization of unlabeled proteins to that provided by the injection of directly labeled proteins.

It has been demonstrated that radioactive serum albumin leaves the circulation immediately after intravenous injection and may be detected in skin and tendon and radioautographically in the extravascular spaces of many organs (Abdou and Tarver, 1951; Everett and Simmons, 1958; Humphrey *et al.*, 1957).

In previous works (Mancini *et al.*, 1961; Mancini *et al.*, 1962) using fluorescent dyes for labeling, the decay in the circulation of serum protein fractions and microscopically the dynamics of their extravascular diffusion in the stromal connective tissue of various organs were studied. It was also found that in the ovary, the serum proteins were transferred not only to the extravascular spaces but also to the ovocyte in the growing follicles (Mancini *et al.*, 1963).

Since an accumulation of serum proteins in the intertubular spaces of normal human testis was histoimmunologically presumed (Mancini *et al.*, 1964), a study on the dynamics of a similar extravascular diffusion and subsequent fate of serum proteins in the rat testis during sexual maturation seemed to be of interest.

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MATERIAL AND METHODS

Ninety-four albino male rats divided into three different lots corresponding to prepubertal, pubertal and adult ages, weighing around 15, 60, and 150 gm respectively, and fed *ad libitum* with a complete standard diet were used. Albumin and globulin fractions were separated from a pool of rat serum by a standard saline fractionation method. Fibrinogen was similarly obtained from a pool of plasma (Mancini *et al.*, 1962). In all cases the whole serum and the protein fractions were separately labeled with sulforhodamine B (Chadwick *et al.*, 1958) and with fluorescein isothiocyanate (Riggs *et al.*, 1958) and dialysis performed by passing through a column of Sephadex G. 25.

Concentration of the serum proteins was adjusted to 20 mg per 0.5 ml controlled by the biuret method, dialyzed against Carbowax, and the amounts of fluorescent dyes bound to proteins measured spectrophotometrically. Paper electrophoretic studies were made to control possible modifications after labeling, and the clotting time of fibrinogen was also checked. Labeled whole

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serum in doses of 1 to 2 ml and 20, 40, or 60 mg/100 gm body weight of labeled serum fractions were intravenously injected into different lots of rats belonging to the three different age groups.

Specificity of the results regarding the interpretation of the presence of fluorescence in a given area as evidence of localization of serum proteins and not due to degradation products or to free fluorescent dyes was checked by injecting other animals with: 1) 1 ml/100 gm body weight of 1 to 3% free fluorescent dyes in normal saline solution; 2) the same free fluorescent dyes simply mixed *in vitro* with serum fractions at a ratio of 0.5 ml of dye (3% in saline solution) per 0.5 ml saline solution containing 20 mg of serum proteins; 3) fluorescent heat-denatured albumin fraction (30 minutes at 60°C) injected in same concentration and amount as the "intact" fluorescent one; 4) degraded fluorescent albumin administered in the same amount as the nondegraded fluorescent one. This was intended to be accomplished with acid hydrolysis (concentrated hydrochloric acid for 3 to 6 hrs) and digestion with purified trypsin (0.5 mg of the enzyme per 30 mg of protein) (Dellacha, 1962); determination of breakdown products was attempted by ultraviolet absorption for proteins and paper electrophoresis; 5) unlabeled rat, rabbit, and human albumin fraction at doses of 40 mg/100 gm body weight. As controls other rats were injected with 1 ml of saline solution. In these cases the animals were killed at 1 and 5 hours and the Coons' technique was performed using unfixed or formaldehyde vapor-fixed 5μ sections of testis and epididymis incubated with horse globulin anti-human, anti-rat and anti-rabbit albumin fraction (Sylvana Corp. New Jersey). Specificity of the histoimmunological results was checked with normal horse globulin and the standard blocking reactions.

In all the experiments the animals injected with the fluorescent proteins were killed at 5, 10, 20, 30, 60 and 120 minutes and at 3, 6, 9, 12, 24, 48, 72 and 96 hr after injection. Blood samples were taken by aortic puncture before killing and spectrophotometric determinations of the albumin and globulin bound fluorescent dyes were made to assess the concentration of labeled proteins in the sera of injected animals. The kidney, liver, testis (equatorial zone), and epididymis were cut unfixed in the cryostat at −20°C, and sections 4 μ thick mounted in glycerine were examined directly, or after 30 minute fixation in formaldehyde vapor and mounting with Permount. Other blocks of tissues were immersed in cold buffered 10% formalin and then embedded in paraffin. Sections 4–6 μ thick were mounted with Permount and examined under the Leitz Ortholux fluorescence microscope using the appropriate source and ocular filters for both fluorochromes.

As radioactive iodine (I^{131}) is considered a nonspecific tag for proteins, rat and human albumin fractions, also prepared by the saline fractionation technique and adjusted to a concentration of 20 and 60 mg/ml, were labeled with this radioisotope (McFarlane, 1956) in a ratio of one to two atoms of iodine per albumin molecule, with a specific activity of 25 mC/gm of protein (National Atomic Commission, Buenos Aires). Tritiated rat albumin (20 and 40 mg/ml) was also prepared in a ratio of two atoms of H^3 per albumin molecule, with a specific activity of 40 mC/gm of protein (Commissariat l'Energie Atom., France). Rats of the three different age groups were intravenously injected with 1 to 1.5 ml/100 gm body weight of the two different concentrations of radioactive albumin, carrying an approximate activity of 2 to 3 μc of I^{131} and 4 μc of H^3 per gm/body weight. As controls other animals were injected with similar amounts of radioiodine alone (NaI^{131}). In all cases animals were sacrificed at 5, 10, 30, and 60 minutes and at 3, 6, 12, 24, 48 and 72 hr. The gonad, epididymis, liver, kidney, and thyroid were fixed in Carnoy or Bouin's fluid and 4 to 10 μ sections submitted to a radioautographic AR 10 stripping film procedure. Preparations were finally counterstained with hemotoxylin or toluidine blue.

RESULTS

Experimental: In vivo injections of fluorescent serum proteins. To avoid repetition the results and details concerning the spectrophotometric analysis of the free fluorescent dyes, the amount bound to serum fractions, the corresponding changes in the absorption peaks, nnd the controls demonstrating that there were some physicochemical changes of proteins after fluorescent label may be found in previous papers (Mancini *et al.*, 1961, Mancini *et al.*, 1962). These papers also illustrate that the time concentration curves of labeled serum fractions in the circulation were very much alike and revealed a fast and slow component with a 2.8 day half life for whole serum, 2.6 days for albumin, and 3.1 days for globulins.

Fluorescent microscopical observations of the testis and epididymis showed the presence of a moderate spontaneous green fluorescence in the heads of spermatozoa and some yellow fluorescence in scattered interstitial cells of the testis. This picture was clearly different from that obtained in animals injected with proteins labeled with both fluorescent dyes, as far as distribution and color of the fluorescent material were concerned.

Preservation of fluorescent proteins in the tis-

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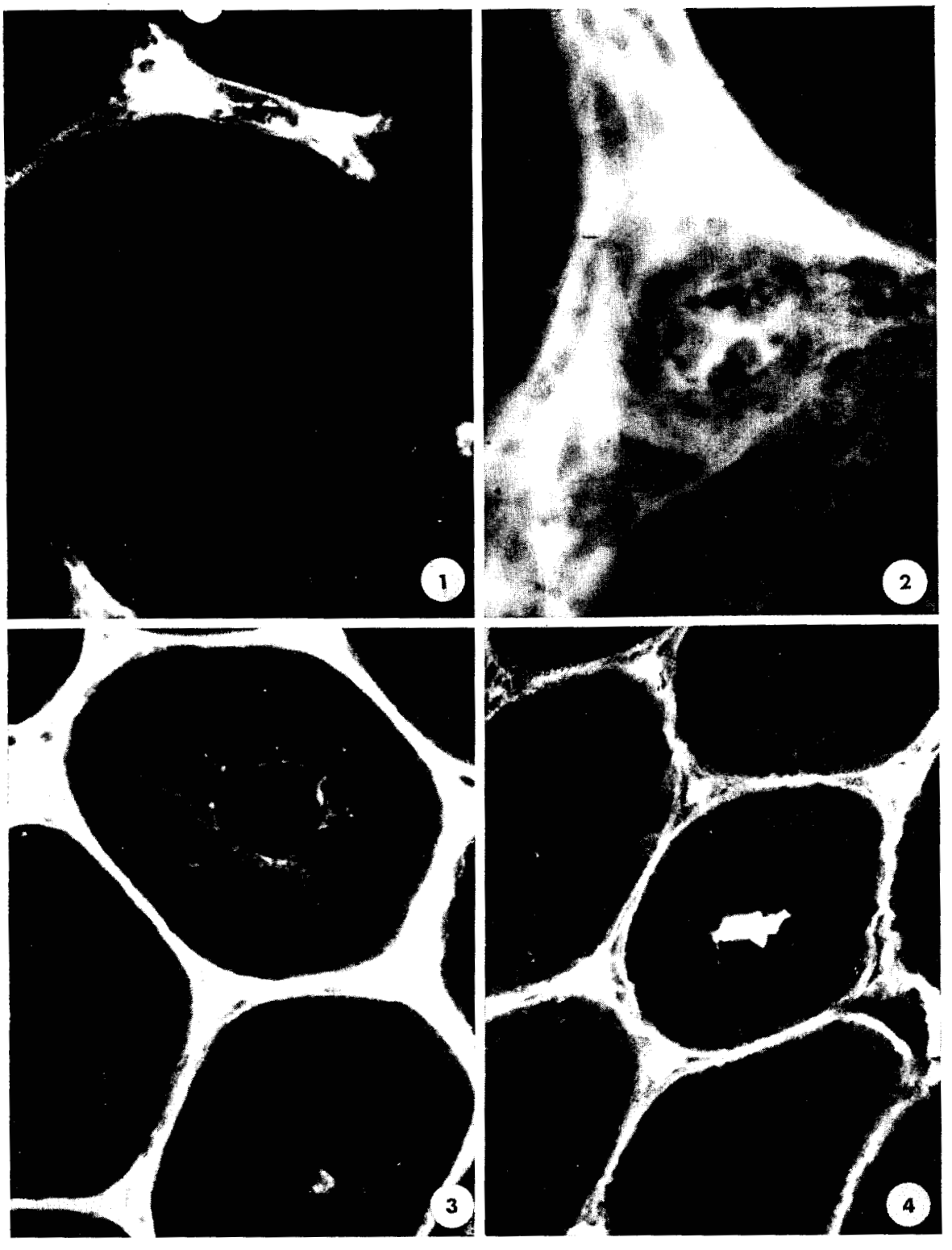

FIGS 1–4

FIG. 1. Section of testis of an adult rat killed 5 minutes after intravenous injection of homologous albumin labeled with sulforhodamine B. Fluorescent protein is seen in the lumen of vessels of intertubular spaces and spreading in the extravascular connective tissue. × 220.

FIG. 2. Same as preceding at higher magnification. An intertubular space showing the labeled protein in the lumen of a vessel, diffusely accumulated in the connective tissue and impregnating the basal membrane of seminiferous tubules. × 400.

FIGS. 3 and 4. Section of testis 30 minutes after injection of fluorescent albumin. The protein is now filling the intertubular spaces and is also seen as granules in between the germinal cells in one tubule and as small mass in the lumen of another. × 120.

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sue was better in cryostat sections either unfixed or post-fixed with formaldehyde vapor. On the other hand, the resolution attained was higher in homologous paraffin sections although an appreciable loss of fluorescent material was observed. Results were the same with both fluorochromes, but labeling with sulforhodamine B appeared for *in vivo* experiments to be a more efficient dye than fluorescein, due to its higher contrast with the dark green autofluorescence of the background.

The sequence in the vascular and extravascular distribution of the labeled whole serum and serum fractions in the gonad was essentially the same. Differences were restricted to the lack of extravascular accumulation of fibrinogen and to the intensity of the fluorescence which was higher for whole serum to decrease progressively from albumin to globulin and fibrinogen. Also the amount of labeled proteins injected was proportional to the brightness of the images, thus maximum was obtained with 60 mg of labeled serum fractions or 2 ml of whole serum per 100 gm body weight. In adult rats a few minutes after intravenous injection the fluorescence appeared localized in the lumen of all blood vessels of the albuginea and intertubular spaces of the testis and epididymis, reaching maximum in the areas corresponding to the deep layers of the albuginea. Up to one hour the labeled proteins were seen not only in the lumen but also increasingly impregnating the walls of small vessels and accumulating in the extravascular areas of the albuginea and intertubular spaces of testis (Fig. 1, 2). There also appeared to be high accumulation in some cells presumably identified as interstitial Leydig cells. This vascular and extravascular distribution could be simultaneously observed in the connective tissue of epididymis. At the same time the fluorescent material appeared to coat the thin walls of most of the seminiferous tubules and to penetrate a few of them. In this location the fluorescence was observed as small granules or masses in between the cells of the germinal epithelium and in the lumen of the tubules where clumps of spermatozoa were present (Fig. 3, 4). It was presumed that this intratubular diffusion took place preferentially in those tubules showing the final stages of the spermatogenic cycle. In the epididymis some fluorescent material appeared after 1 hour in the lumen of the canaliculi of the head and body portions but no indication of transepithelial passage from the vessels of surrounding connective tissue was detected. After 6–8 hours the fluorescence began to disappear from vessels, from intertubular spaces of testis and epididymis, and from basal membranes of seminiferous tubules but persisted until 12 hours inside the testicular and epididymal tubules and longer in the interstitial cells. In no case was fluorescence seen in the lumen of the ductus deferens although some appeared in the connective tissue wall during the first hours.

Testis and epididymis of prepubertal and pubertal rats appeared little different from adult animals as far as the sequence of extravascular distribution of labeled proteins was concerned. However, in the prepubertal rats intratubular diffusion was less intense and present in a lesser number of seminiferous tubules. It was difficult to detect any fluorescence inside the epididymal canaliculi or in the lumen of the ductus deferens with the exception of that found in the neighboring connective tissue structures.

Comparison between the decay of labeled serum proteins in the circulation (Mancini *et al.*, 1961; Mancini *et al.*, 1962) and its appearance in the intertubular spaces and within seminiferous tubules in adult animals showed that the fast component of the curve was correlated with the phase of extravascular diffusion. Conversely, disappearance of the proteins from these structures was not related with a new peak of this substance in the circulation but rather with a sustained decay expressed by the slow component of the curve.

In vivo injection of radioactive serum albumin. Although of less sharpness than the picture provided by the fluorescent dyes, the pattern of distribution of radioactive albumin was very similar in young and adult animals. As was expected, the intensity detected by radioautography was higher in the animals injected with the higher doses of labeled protein. Resolution was better than that obtained with fluorescent proteins, especially when H^3 albumin was compared with I^{131} albumin. Definition of the images was also higher with tritium; however, radioiodine, in spite of some diffusion artifacts, provided radioautographs of higher contrast. No clear differences were noted in the sequence of distribution between homologous rat albumin and that of human origin. From the first minutes and increasing until about 30 minutes to 1 hour, radioactive granules appeared first in the lumina and walls of all vessels of testis and epididymis, then in extravascular connective tissue, and finally concentrated in some cells presumably macrophages in the epididymis and interstitial Leydig cells in the testis (Fig. 5, 6). In contrast with the lack of images in the epithelium of canaliculi of the epididymis, the labeled material was seen impregnating the basal membranes of a great part of the seminiferous tubules, into the interior and spreading through the germinal epithelium to finally appear in the central lumen (Fig. 7). These images were better seen in thick and overexposed sections where the silver granules appeared preferentially distributed in narrow spaces lying among the germinal cells. In some tubules, most frequently those showing the final

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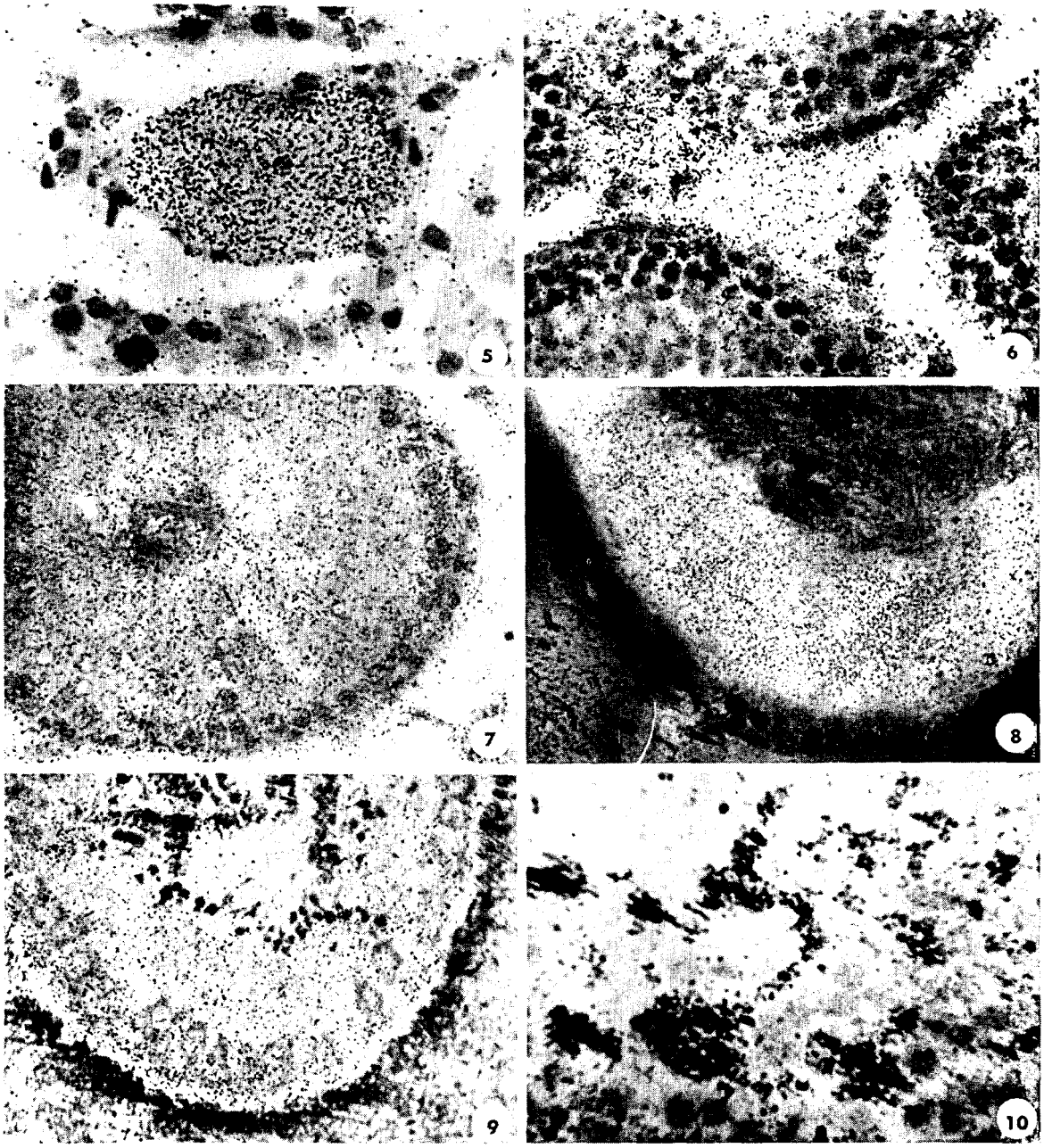

FIGS. 5–10

FIG. 5. Radioautograph of testis of adult rat injected intravenously with tritiated homologous albumin and killed after 10 minutes. Silver granules are seen concentrated in the lumen of a small vessel in the intertubular space. Counterstained with hematoxylin. × 800.

FIG. 6. Radioautograph of testis of another animal 15 minutes after injection. Granules are seen distributed in the intertubular spaces, and some grouped around nuclei of interstitial cells and diffused inside seminiferous tubules. Counterstained with hematoxylin. × 500.

FIG. 7. Same as preceding after 30 minutes. Silver granules appear inside the seminiferous tubule over the germinative epithelium and also in the lumen. Counterstained with hematoxylin. × 600.

FIG. 8. Radioautograph of the epididymis 40 minutes after the injection. Granules are observed in the lumen of the canaliculi and some intermingled with a clump of spermatozoa. Counterstained with hematoxylin. × 600.

FIG. 9. Overexposed radioautograph of testis of a rat injected with radioiodinated homologous albumin and killed after 10 minutes. Dense granules appear accumulated in the wall of seminiferous tubules and in less number in the germinae epithelium. Counterstained with toluidine blue. × 400.

FIG. 10. Same as preceding 30 minutes after injection. Conglomerated granules apparently accumulated in the intercellular spaces between germinal cells of a seminiferous tubule, which presumably correspond to the bodies and branches of Sertoli cells. Counterstained with hematoxylin. × 800.

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stages of spermatogenesis, the localization of granules of iodinated albumin apparently corresponded to the bodies of Sertoli cells (Fig. 9, 10). The radioautographs of the tubules appeared unchanged up to 8–12 hr, whereas it began to fade from the vessels at 10 hr and finally disappeared from both structures around 18–24 hours. Some granules were still observed in the interstitial cells at 48 and 72 hours. Radioactive material was detected in the lumen of canaliculi in the epididymis after 30 minutes, especially in the head portion (Fig. 8). This picture reached its maximal intensity at 3 hours and began to disappear at 16 to 24 hours. No indication of radioactivity was noted in the epithelium but traces were observed in the lumen of ductus deferens. Examination of the thyroid gland revealed the known classical images in the follicles around thirty minutes after injection.

Controls: Free labels alone. Free fluorescent dyes intravenously injected disappeared rapidly from the circulation in 20 to 50 minutes, irrespective of the doses used. Electrophoretic study of the serum showed no binding of the dyes to serum protein fractions. They apparently were eliminated by the liver via the bile ducts into the small intestine and through glomerular filtration into the urine. Histologically the dyes were mainly detected in fresh frozen sections, since fluorescence was greatly diminished when tissues were fixed. They appeared localized in macrophages of intertubular spaces of testis and epididymis as well as in Kupffer cells and proximal convoluted tubules of the kidney. The same results were obtained when free labes were injected together with unlabeled serum proteins, indicating that no binding had taken place between the two substances in the circulation or in the tissues. When free radiodine was injected, radioautographs of testis and epididymis showed only a few granules in the lumen of vessels and in the walls of small arterioles. No signs of any insoluble iodinated compound were observed in any other sites, with the exception of the thyroid gland where the typical radioautographic picture was obtained.

Denatured and degraded albumin. Heat denatured homologous albumin appeared in the lumen of vessels after injection as fast as the fluorescent "native" one. Accumulation in the extravascular connective tissue was neglible in contrast with the incorporation of this protein by interstitial cells of the testis and other connective tissue cells in the epididymis. The denatured proteins were not detected in the seminiferous tubules or canaliculi of epididymis. By 2 to 4 hours the fluorescent material disappeared from vessels and connective tissue but persisted until 72 and 96 hr in the interstitial cells. Also fluorescence was observed in a great number of Kupffer cells and in proximal convoluted tubules of the kidney.

Hydrolytically degraded albumin revealed by electrophoretic studies and ultraviolet absorption that, in the conditions used, no albumin molecules remained. The microscopical pattern of these still fluorescent degraded products showed soon after the injection the presence of a negligible fluorescence in the lumen of vessels of both organs under study and more constantly in the interstitial cells of the testis. Between 2 and 5 hours this fluorescent picture had disappeared and only macrophages in the liver and the convoluted tubules of kidney revealed a moderate accumulation of the labeled product. The enzymatically degraded albumin also suggested that no great amount of intact protein was present as judged by the methods used and results obtained which were much like those provided by the preceding experiment. This assumption was reinforced by the fluorescent histological pattern observed after injection, which showed only during 1 to 2 hours the presence of fluorescent material in the vessels and some in the neighboring interstitial cells, but none in the intertubular spaces, seminiferous tubules, or in the epididymal canaliculi. Again an appreciable amount of labeled product appeared in macrophages in the liver as well as in the epithelium of convoluted tubules of the kidney, where the material persisted for many days.

Histoimmunological detection of unlabeled serum proteins. The localization of injected unlabeled albumin detected as antigen with the corresponding fluorescent antibody by the Coons' technique was in general not so constant and sharp as that provided by the *in vivo* injection of labeled albumin. This was especially true in the experiments with rat albumin where detection in sections of the testis after varying periods of time was rather uncertain. On the other hand the presence of heterologous rabbit and human albumin in the testis was better detected, and preservation and resolution were enhanced when formaldehyde post-fixed frozen sections were used. Images were also more intense in sections of testis and epididymis of rats 5 hours after injection of the antigen. Beginning at 1 hour and increasing with time, fluorescent antialbumin reacted strongly with material inside the vessels, much less with that in the intertubular connective tissue of both testis and epididymis (Fig. 11, 12), slightly with granules in the wall of seminiferous tubules, moderately with small masses irregularly distributed in the germinal epithelium and in the lumen of testicular and epididymal tubules (Fig. 13, 14). No induced fluorescence was observed in the cells of the intertubular connective tissue of either organ. Sometimes clumps of spermatozoa showed a mod-

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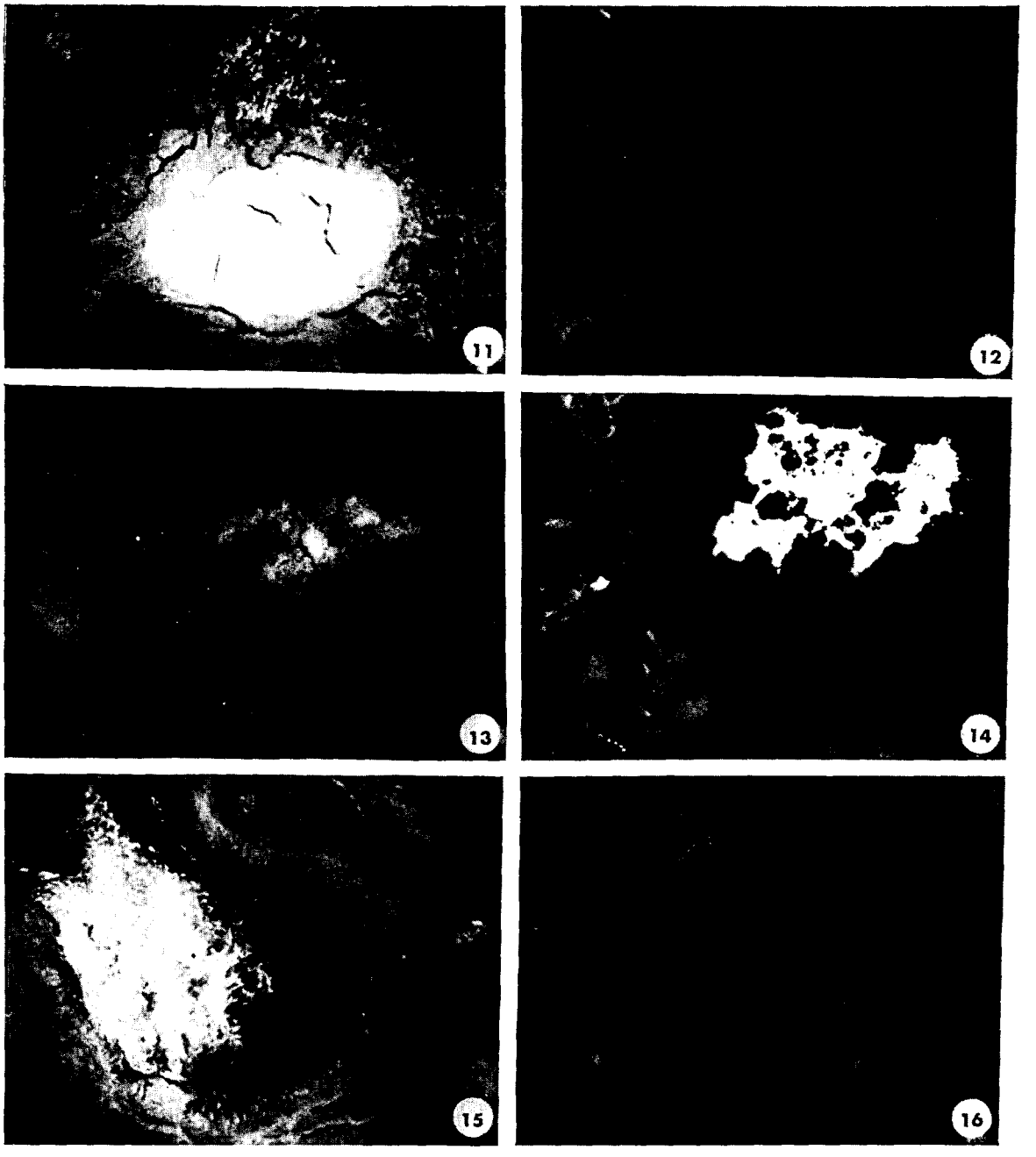

FIGS. 11–16

FIG. 11. Section of testis of a rat 30 minutes after intravenous injection of rabbit unlabeled albumin and incubated *in vitro* with the corresponding fluorescent antibody globulins. Coons's technique. Fluorescence is seen in the lumen of a vessel and some appears as small granules in the extravascular connective tissue. × 400.

FIG. 12. Same as preceding incubated with normal fluorescent globulins. No fluorescence is observed in the vessel, intertubular spaces, or seminiferous tubules. × 400.

FIG. 13. Same as Fig. 11, showing a small vessel with slight fluorescence and a mass of moderately fluorescent material in the intercellular spaces of germinae epithelium of an adjacent seminiferous tubule. × 400.

FIG. 14. Same as preceding, showing a bright fluorescent mass in the lumen of a seminiferous tubule. Some unspecific fluorescence appears in heads of spermatozoa. × 400.

FIG. 15. Section of the epididymis of rat, 40 minutes after the injection of unlabeled heterologous albumin. Coons's technique as in Fig. 11. Accumulation of fluorescent material in the lumen of the canaliculi. × 400.

FIG. 16. Same as preceding incubated with normal fluorescent globulins. No fluorescence is seen in the canaliculi or in other structures. × 400.

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erate light green autofluorescence which should not be confused with the specific fluorescence described above. Specificity of the immunofluorescent technique used was established after negative or dubious results with the following control procedures: a) no specific fluorescence when sections of organs of uninjected animals are subjected to the same immunofluorescent technique; b) no staining in sections exposed to labeled normal globulins; c) inhibition by prior treatment of the sections with unlabeled antibodies but not with normal globulin; d) no staining with labeled globulin anti-rat kidney saline extract.

DISCUSSION

Fluorescent and radioactive microscopical evidence here presented suggest that with the exception of fibrinogen, homologous or heterologous serum proteins may diffuse extravascularly in the connective tissue of the testis, traverse the walls of seminiferous tubules, and passing through the germinal epithelium, appear in the tubular lumen. This route is not apparently followed by the circulating proteins to reach the lumen of canaliculi in the epididymis, since no transitional stages were observed between the extravascularly diffused proteins and the epithelium. Therefore it is assumed that its presence in the canaliculi indicates that tagged proteins migrate from the seminiferous tubules to the epididymis via excretory ducts. That this material is probably reabsorbed or degraded there is supported by the absence of similar pictures in the lumen of the ductus deferens.

The rate and intensity of extravascular accumulation in the testis is high, increasing progressively from the first minutes up to 1 or even 3 hours and equally observable with fluorescent and radioautographic techniques. The intratubular diffusion and migration to the epididymis is a little slower, less intense, and better defined with radioautographs. As regards the rate of the extravascular accumulation, our findings confirm and amplify previous data demonstrating that testis is characterized by a radioactive albumin content increasing with time, (Everett and Simmons, 1958) and also resembles the results encountered in the growing follicles of the ovary (Mancini *et al.*, 1963). Comparison between the decay of labeled proteins in the circulation and their appearance in the stromal connective tissue and seminiferous tubules, show that the fast component of the curve is correlated with the phase of extravascular diffusion. The disappearance of labeled material from the same structures is not related with a new peak of these proteins in the circulation but rather with a sustained decline revealed by the slow component of the curve.

The validity of the microscopical basis of the direct transference of serum protein molecules from circulation to seminiferous tubules depends fundamentally on: a) proteins should not be modified by the label, and b) the tagged proteins should traverse the vessels in an unmodified form. It was demonstrated that the physicochemical properties of proteins show no gross modification after binding with fluorescent dyes (Chadwick *et al.*, 1958; Mancini, 1963; Schiller *et al.*, 1953) and practically none with radioisotopes. Our finding of slight changes in the electrophoretic pattern of serum proteins after labeling, their half-life in the circulation which agrees with that observed by others who used radioisotopes for labeling (Abdou and Tarver, 1951; Gitlin and Janeway, 1960; McFarlane, 1956), and the slight reduction of the clotting time of fibrinogen (Mancini *et al.*, 1962), also support the above mentioned concepts.

That labeled proteins have passed unchanged from vessels to connective tissue, seminiferous tubules, and epididymis and that they probably are not degraded products is backed by: a) stability of the binding between the fluorescent dyes (Chadwick *et al.*, 1958; Redetzki, 1958) and radioisotopes (Gitlin and Janeway, 1960; McFarlane, 1956) with proteins; b) injected free fluorescent dyes or radioiodine do not appear in the connective tissue, seminiferous tubules, or epdididymis, suggesting that binding of these free labels with testicular structures or substances resulting in the formation of new insoluble compounds do not occur; and c) hydrolytically or enzymatically degraded products, presumably polypeptides and amino acids (Dellacha, 1962), which still retain the fluorescent tag, are not present in extravascular sites, unless it is admitted that degradation products of albumin obtained *in vitro* are not identical with those produced *in vivo*. However, that these substances might diffuse extravascularly to accumulate as an insoluble compound, is proven by the appearance of fluorescent material in the macrophages of both organs. d) The half-life of fluorescent albumin, 2.6 days, and of globulins, 3.1 days, in the circulation indicates that the fractional rate of their disappearance is greater for several hours after intravenous injection, and later decreases slowly over a few days. Since the extravascular distribution is high a few minutes after injection

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and reaches a peak at 1 hour, it seems unlikely that sufficient protein was degraded to account for the accumulation in the extravascular structures on the basis of breakdown products alone. Moreover, if the half-life of the latter substances is too short to permit their intra- and extravascular localization, as was deduced from our experiments, it then seems highly unlikely that it could have been observed in the tissues after similar time intervals if it had arisen from the breakdown products of labeled proteins. That breakdown of these proteins may occur is proven by the appearance of radioactive granules in the thyroid gland 30 minutes after injection; even so fluorescent or radioactive proteins are present earlier in the vessels and extravascular sites, as was described. e) The fluorescent antibody technique demonstrates a very similar distribution of the injected unlabeled homologous or heterologous albumin to that obtained with the labeled proteins, suggesting that molecules with reactive groups identical to the serum fractions are present and retain their antigenicity after passing to connective tissue and to seminiferous and epididymal tubules. From all these findings it may be concluded that microscopical evidence strongly indicates but does not unquestionably prove that "intact" protein molecules pass unmodified to the extravascular structures. It should be added that at least compounds of high molecular weight are present, since those of lower molecular weight would be soluble and removed from tissues by the histological processing.

It is well known that capillary walls may differ in their submicroscopic structure and permeability to plasma components (Bennett *et al.*, 1959; Pappenheimer, 1953; Tschirgi, 1959). In this respect the ovary (Mancini *et al.*, 1963) and the testis show microscopically, as compared with other tissues, a rapid passage of proteins through vessel walls. Whether the subsequent accumulation of proteins outside the vessels is related with the extremely loose type of connective tissue present in the intertubular spaces of the testis is not yet known. Also in the human testis, histoimmunological techniques have demonstrated that serum proteins are a conspicuous component of this tissue, especially at pubertal stages (Mancini *et al.*, 1964).

The extravascular diffusion is apparently not greatly different in prepubertal and adult animals, but passage into the seminiferous tubules seems to increase with age, suggesting a gonadotrophic influence parallel to the maturation of the gonad. That this may be the case is supported by the fact that this process is frequently seen in those tubules showing the final stages of the spermatogenic cycle. This finding which resembles a similar phenomenon seen in growing ovarian follicles (Mancini *et al.*, 1963) suggests that special physicochemical properties should be attributed to the basal membrane of mature seminiferous tubules. This selective permeability might be related with the molecular weight of serum protein fractions, for the intratubular diffusion although of much lesser magnitude than the extravascular one is high for albumin and negligible for globulins.

Since the apparent route followed by the proteins through the germinal epithelium is not the germinal cells but rather the spaces lying among them, it is postulated that Sertoli cell bodies and cytoplasmic branches which occupy these intercellular areas (Bawa, 1963; Vilar *et al.*, 1962) may be acting as a transporting cell system. Current results of our group, using tagged serum proteins, with electron dense substances and electron microscope, are substantiating this assumption based on the old hypothesis concerning the "nutritional" function of this cell.

Much more work remains to be done to clarify the physiological significance of this extravascular and intratubular diffusion of serum proteins in the testis with subsequent passage to the epididymis. That a great part of this material constitutes a local extravascular pool of plasma proteins, with only a small portion of it being utilized by the seminiferous tubules and epididymis, is suggested by their progressive microscopical decrease in both structures without return into the circulation. It is not improbable that the transferred material may be involved in the metabolism of the germinal epithelium and also of spermatozoa during their travel to the epididymis.

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