EXCESS LIPID LEAKAGE: A PROPERTY OF VERY YOUNG VASCULAR ENDOTHELIUM*

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Received for publication December 23, 1961

IN our earlier studies (Friedman, Byers and Pearl, 1960 ; Friedman and Byers, 1961 ; Byers and Friedman, 1962, and unpublished) we observed that the introduction of a thrombus into the aorta or inferior vena cava of either the normal rabbit or rat elicited an intimal response in these vessels that resulted in a thrombosclerotic process. Such a process, whether in the aorta (Friedman and Byers, 1961*a*) or in the inferior vena cava of the rabbit (Byers and Friedman, 1962), quickly becomes thromboatherosclerotic (*i.e.* accumulates large excesses of both triglyceride and cholesterol) if the animal is allowed to become hypercholesteraemic by ingestion of a cholesterol-rich diet. This excess lipid and cholesterol accumulation, however, was not found to be due to any increased synthesis *in situ* by the hyperplastic intima, nor to any significant penetration of the lipid and cholesterol contained in the luminal blood passing over the thrombotic process. Rather, it appeared (Friedman *et al.*, unpublished) that these substances were being supplied to the thrombosclerotic process in excess amounts, primarily from the capillaries newly sprung from the adventitia and accompanying the hyperplastic ingrowth of intimal cells. In short, the transmedial capillaries were found excessively permeable (*i.e.* " leaking ") to lipid and cholesterol as well as to Evans Blue, Trypan Blue and colloidal iron particles. A similar phenomenon also was observed in the new transmedial capillaries of the rat's aorta responding to an induced thrombus (Friedman, Byers and St. George, 1962).

Although these studies indicated that the newly formed capillaries of the aortic adventitia of the rabbit and rat exhibited such excessive permeability, it was not ascertained whether this permeability was characteristic of all newly formed capillaries, or whether it resulted from some type of inflammatory agent acting upon such capillaries. In other words, do *all* very young capillaries " leak " excess amounts of lipid and cholesterol ? Is an inflammatory stimulus needed for this phenomenon ?

The following studies were designed to answer these two questions. The results of the studies indicate that all of the various types of very young vascular tissues examined do exhibit this excessive permeability to lipid and cholesterol, with or without the presence of inflammation.

* Aided by grants from the National Institutes of Health, National Heart Institute, The Life Insurance Medical Research Fund, the American Heart Association, Sacramento County Heart Association, Contra Costa County Heart Association, and Humboldt County Heart Association.

METHODS

A. *Permeability of New Capillaries Arising in Tissues other than Aorta and Inferior Vena Cava, and Subject to Inflammation*

1. *Thromboatherosclerotic plaque of right common carotid artery*

Thrombi were induced in the right common carotid artery of ten male rabbits (age : 12 weeks) by incising the artery and insertion of a 1-cm. gutter segment of polyethylene tubing (Clay Adams PE–160, Intramedic (Medical Formulation PHF) polyethylene tubing, i.d. = 0·023 and o.d. = 0·038) employing the same technique as was described (Friedman and Byers, 1961*a*) for similar thrombus formation in the aorta. Five of the rabbits after operation were fed Wayne rabbit chow enriched with cholesterol (2 per cent) and cottonseed oil (2 per cent) and the remaining five, Wayne chow alone. Two of the latter rabbits were killed 72 hr. later ; the other 8 rabbits were killed at the end of 4 weeks. A terminal blood sample was obtained and analyzed for total cholesterol content (Friedman and Byers, 1961*a*). The thrombotic processes resulting from these tubing insertions were inspected grossly and then a cross section of each lesion was obtained, fixed and stained with Sudan IV.

2. *Peritoneal granulomatous tissue*

Cylinders were made of Millipore (WSWP Filters, Nylon netting, White-Millipore Filter Corp., Bedford, Mass., filter sheet having a pore size of 3·0 μ) by approximating the two ends of a flat section of the material (2·5 × 2·0 cm.) and then sealing this juncture and both open ends with an " Epoxy " glue (Devcon "2-ton" Epoxy-Devcon Corporation, Danvers, Massachusetts). Following such closure, each capsule was pierced in 10–12 separate areas with a 25-gauge hypodermic needle. Each capsule possessed a volume of approximately 0·5 c.c.

Four male rabbits (age : 12 weeks) were anaesthetized with pentobarbital and ether, each abdomen opened and four of the Millipore capsules were secured by silk suture to the retroperitoneal wall of each rabbit. Following operation, two of the four rabbits were fed a cholesterol (2 per cent) cottonseed oil (2 per cent) enriched Wayne chow diet. The other two rabbits were fed ordinary Wayne chow.

At the end of 3 weeks, blood samples were obtained and each rabbit was injected intravenously with 20 mg. of Evans Blue. All animals were killed 24 hr. after injection of the dye. A sample of peritoneal fluid was collected, the capsules then were obtained, opened, and, after a section of the contained tissue was removed for staining with Sudan IV, the remainder was reserved for analysis. Blood, peritoneal and capsular tissue samples were analyzed for total cholesterol (Friedman and Byers, 1961*a*).

3. *Subcutaneous granulomatous tissue*

Ten male rabbits (age : 10 weeks) were injected with 20 ml. of a 1 per cent suspension of carrageenan (Sea Kem type 21 carrageenan, Marine Colloids, Inc., New Bedford, Massachusetts) in the subcutaneous portion of their dorsum as described by McCandless, Bailey and Zilversmit (1960) in order to induce the growth of new connective tissue. Five of the ten rabbits were fed the cholesterol-oil enhanced Wayne chow diet. At the end of three weeks, a blood sample for cholesterol analysis was obtained and then each rabbit was injected intravenously with 20 mg. of Evans Blue. They were killed 24 hr. later and a section of each granulomatous area was stained with Sudan IV.

4. *Pulmonary granulomatous tissue*

This section of the study was done in collaboration with Doctors Maurice L. Cohn and Gardner Middlebrook of the National Jewish Hospital at Denver, Colorado. Ten mice of a total group of twenty were fed laboratory chow diet enriched with cholesterol (2 per cent) and cottonseed oil (2 per cent) for approximately 28 days. All mice were then infected by the airborne route with a virulent strain of tubercle bacilli (H37Rv). Twenty-eight days after infection, the mice were bled and killed. The lungs were stained with Sudan IV and the blood samples were analyzed for cholesterol.

B. *Permeability of New Capillaries in Non-inflammatory Tissues*

1. *Intimal hyperplasia of occluded aortic segment*

Six male rabbits (age : 12 weeks) were anaesthetized with pentobarbital, each abdomen incised and a ligature placed about the abdominal aorta just below the orifices of the renal arteries. Then a second ligature was applied approximately 2 cm. distal to the first, thus, totally occluding a segment of aorta. Continuity of aortic flow was secured by the immediately subsequent insertion of a segment of the animal's own right jugular vein in such a manner as to conduct blood past the obstruction. (Dr. Laurence M. Rivkin, Director of Surgical Research, Mount Zion Hospital and Medical Center devised and performed for us this new operative procedure which he will describe in a forthcoming communication).

Three of the six animals were fed the cholesterol-oil enriched diet. At the end of 4 weeks, blood samples were obtained and the animals were killed. A section of the intimal tissue partially filling the occluded aortic segment was stained with Sudan IV and a sample was submitted for cholesterol analysis.

2. *Placental tissue*

Twelve female rabbits were bred and then eight of them were fed the cholesterol-oil enriched Wayne chow diet. Approximately 25–27 days after conception, blood samples were obtained for cholesterol determination and then two of the cholesterol fed rabbits and two of the rabbits fed the ordinary diet were injected intravenously with 20 mg. of Evans Blue dye. All rabbits were killed the following day. The placentas were inspected grossly and then sections were obtained from two placentas of each rabbit for Sudan IV staining. In addition, sections obtained from the portions of the uterus not involved in the placental attachment were also stained with Sudan IV.

C. *Permeability of Aortic Endothelium of Very Young Rabbits*

A series of nine very young rabbits (age : 14 days, aver. wt. : 182 g.) were injected intravenously with 2 mg. of Evans Blue dye daily for three days. Twenty-four hr. after the last injection, blood samples for cholesterol determination were obtained, the animals killed and the aortas removed and examined for possible dye extravasation. In addition, sections from four of these aortas were obtained and stained with Sudan IV. For comparative purposes, an older series of nine rabbits (age : 12 weeks, aver. wt. : 2340 g.) were studied similarly except that they received 20 mg. of Evans Blue dye in each daily injection.

A second series of five very young rabbits (age : 12–14 days) received a single intravenous injection of Trypan Blue (15 mg.). Twenty-four hr. later they were killed and their aortas were studied for dye extravasation.

RESULTS

A. *Permeability of New Capillaries Arising in Tissues other than Aorta and Inferior Vena Cava, and Subject to Inflammation*

1. *Thromboatherosclerotic plaque of carotid artery*

As was observed in our aortic studies (Friedman *et al.*, 1960), polyethylene segments introduced into the common carotid artery of the rabbit quickly led to the formation of a greyish-white, semitranslucent thrombus occupying the space between the trough of the tubing and the artery wall. At 72 hr. this thrombus was well developed (Fig. 1) in the two rabbits that were killed at this time.

At time of killing (4 weeks) a moderate hypercholesteraemia (aver. serum cholesterol : 571 mg./100 ml.) was present in each of the 5 cholesterol fed rabbits. The average cholesterol of the other 3 rabbits fed ordinary lab. chow was 42 mg./100 ml. Similar to our earlier observations of aortic plaques (Friedman and Byers, 1961*a*) each of the five hypercholesteraemic rabbits exhibited a relatively dense, fibrous tissue filling the trough of the tubing and also covering its convex surface. Similar plaques also were observed in the three normocholesteraemic rabbits, but they were approximately half the size of those in the hypercholesteraemic rabbits.

On microscopic examination, again similar to the earlier findings in the aorta studies (Friedman and Byers, 1961*a*), the plaques of the hypercholesteraemic rabbits exhibited intense Sudanophilia in the basal areas of the plaque (see Fig. 2) with the more peripheral tissue encircling the tubing almost totally free of fat staining. The plaques of the normocholesteraemic rabbits were completely devoid of Sudanophilic staining. These results suggest that, similar to the capillaries present in the basal areas of aortic plaques of hypercholesteraemic rabbits, those present in the basal area of carotid plaques likewise " leak " excessive amounts of lipid.

2. *Peritoneal granulomatous tissue*

When the abdomen of each rabbit bearing Millipore capsules was opened and the site of capsule fixation inspected, it was found that a luxuriant growth of new connective tissue had partially covered and penetrated each of the capsules. It also was observed that considerable extravasation of dye had occurred both in the new connective tissue surrounding the capsule (see Fig. 3), and also in the tissue contained in the capsule (see Fig. 4) in each of the rabbits previously injected with Evans Blue. This finding indicated excessive permeability of the blood vessels accompanying the newly formed connective tissue.

On microscopic examination, each of the eight capsular tissues contained in the two hypercholesteraemic rabbits exhibited a rich vascularity and intense Sudanophilia primarily intracellular (see Fig. 5). However, the eight capsular tissues contained in the two normocholesteraemic rabbits, while exhibiting comparable vascularity, did not show any Sudanophilia (Fig. 6).

TABLE I.—*Cholesterol Content of Peritoneal Granulomatous Tissue of Hypercholesteraemic Rabbits*

Number of rabbits	Aver. serum cholesterol (mg./100 ml.)	Number of capsules	Capsular tissue: Aver. Dry Wt. (mg.)	Capsular tissue: Aver. cholesterol (g./100 g.)	Aver. peritoneal fluid cholesterol (mg./100 ml.)
A.		*Control Normocholesteraemic Rabbits*			
2	37	8	2·1	2·59	84
Range	(32–42)		(0·7–3·4)	(1·55–3·54)	(70–98)
S.E. mean			±0·28	±0·21	
B.		*Hypercholesteraemic Rabbits*			
2	1927	8	3·0	10·44	705
Range	(1245–2610)		(1·9–4·1)	(6·2–18·7)	(394–1015)
S.E. mean			±0·4	±1·87	

As Table I illustrates, the cholesterol content of the capsular tissues of the hypercholesteraemic rabbits (aver. : 10·44 g./100 g. dry weight) was approximately four times greater than that found in comparable tissues of normocholesteraemic rabbits (aver. : 2·59 g./100 g. dry weight).

Apparently then, the capillaries accompanying new peritoneal connective tissue exhibited the same excess permeability to the Evans Blue dye, lipid and cholesterol that newly developed aortic intimal capillaries were found (Friedman *et al.*, unpublished) to show.

3. *Subcutaneous granulomatous tissue*

The results obtained in the ten rabbits injected 21 days previously with carrageenan were almost identical with those observed in the rabbits bearing Millipore capsules. Thus, on gross observation, each of the granulomatous masses of the 5 hypercholesteraemic rabbits (aver. serum cholesterol: 540 mg./100 ml.) exhibited large areas of dye extravasation once more suggesting excessive permeability of the new blood vessels to the previously injected dye. On microscopic examination, the masses were observed to consist of relatively undifferentiated fibroblastic tissue well supplied with capillaries and almost identical in appearance (Fig. 7 and compare with Fig. 5) with that observed in the capsular tissues. Like the latter, too, each of the 5 tissue sections abounded in Sudanophilia confined intracellularly almost exclusively.

On the other hand, although the granulomas of the 5 normocholesteraemic rabbits (aver. serum cholesterol: 42 mg./100 ml.) exhibited similar areas of gross extravasation of injected dye and a similar fibroblastic tissue replete with numerous capillaries, none exhibited Sudanophilic infiltration. In this they resembled the capsular tissues of the normocholesteraemic rabbits.

4. *Pulmonary granulomatous tissue*

Although 10 of the 20 mice inoculated with the strain of acid-fast bacilli had been on the cholesterol-oil enriched diet for approximately 56 days, their terminal average serum cholesterol content (128 mg./100 ml.) was identical with that of the remaining 10 mice fed ordinary laboratory chow. Nevertheless, 9 of the 10 mice fed the cholesterol enriched diet bore pulmonary tuberculomas that were rich in Sudanophilia (Fig. 8), whereas the tuberculomas of 8 of the 10 stock fed mice exhibited no Sudanophilia (Fig. 9) and those of the remaining two mice, only very slight traces of such staining.

B. *Permeability of New Capillaries in Non-inflammatory Tissues*

1. *Intimal hyperplasia in occluded aortic segment*

Extensive proliferation of the intima occurred in each of the 6 occluded aortic segments (Fig. 10). On microscopic examination fibroblastic tissue very richly supplied with arteries, arterioles and capillaries was observed and in all essential respects it resembled the capsular tissues and the subcutaneous granulomas described above. Similar to the findings obtained in these latter tissues, only the aortic segments of the three hypercholesteraemic rabbits (aver. serum cholesterol:

EXPLANATION OF PLATES

FIG. 1.—*Rabbit Z-3, No.* 412.—Thrombus resulting from insertion of polyethylene gutter 72 hours previously into right common carotid artery of the rabbit. Note the grey-white appearance of the thrombus. It has not yet become firmly adherent to the underlying intima. Later, hyperplastic intimal tissue will totally encompass the gutter and its contained thrombus. In this figure the polyethylene (nearly transparent) has been moved in order to exhibit the thrombus.

FIG. 2.—*Rabbit L-1, No.* 4.—*Thromboatherosclerotic Plaque in Common Carotid Artery.* (Sudan IV Stain ×60).—The plaque shown is that usually observed four weeks after insertion of the polyethylene tubing. The central lacuna indicates the space occupied by the tubing. The red staining (i.e. Sudanophilia) is confined to the basal portions of the plaque, whereas none is present in the peripheral hyperplastic intima that had encircled the tubing, again demonstrating the adventitial capillary source of the excess triglyceride and cholesterol that are present in these plaques.

FIG. 3.—*Rabbit MP*1, *No.* 12.—Gross appearance of two Millipore capsules *in situ* 24 hr. after the parenteral administration of Evans Blue dye to the rabbit host. The new connective tissue growing from the peritoneal wall and partially covering the capsules exhibits profound extravasation and concentration of the previously injected dye thus giving it the dark aspect shown in the photograph.

FIG. 4.—*Rabbit W*–14, *No.* 734.—New connective tissue in an opened Millipore capsule 24 hr. after the parenteral administration of Evans Blue dye to the rabbit host. The dark areas represent the extravasation and concentration of the injected dye that had entered the capsule via the latter's capillary supply.

FIG. 5.—*Rabbit Be*–3, *No.* 55.—*Peritoneal Granulomatous Tissue in an "Open" Millipore Capsule of an Hypercholesteraemic Rabbit.* (Sudan IV ×400).—Tissue obtained from an "open" millipore capsule placed three weeks previously in the peritoneal cavity of a cholesterol-oil fed hypercholesteraemic rabbit. The extensive Sudanophilia is obvious and contrasts strikingly with the Sudan-free tissue shown in Fig. 6. Note the vascularity of this tissue.

FIG. 6.—*Rabbit Be*–3, *No.* 61.—*Peritoneal Granulomatous Tissue in an "Open" Millipore Capsule of a Normocholesteraemic Rabbit* (Sudan IV ×400). Tissue obtained from an "open" millipore capsule placed three weeks previously in the peritoneal cavity of a stock fed, normocholesteraemic rabbit. Although this tissue, similar to that shown in Fig. 5, exhibits vascularity, no Sudanophilia has occurred.

FIG. 7.—*Rabbit C*–3, *No.* 9.—*Subcutaneous Granulomatous Tissue of an Hypercholesteraemic Rabbit* (Sudan IV ×400).—Tissue obtained from the area injected three weeks previously with carrageenan. Its extensive vascularity and Sudanophilia appear almost identical with that of the peritoneal granulomatous tissue obtained from the hypercholesteraemic rabbit (Fig. 5).

FIG. 8.—*Mouse C*–4.—*Pulmonary Tuberculoma of the Cholesterol-Oil Fed Mouse* (Sudan IV × 400. Tissue obtained four weeks after mouse was inoculated with the tubercle bacillus. The intracellular Sudanophilia is clearly shown and contrasts with its absence in comparable tissue depicted in Fig. 9.

FIG. 9.—*Mouse D*–4.—*Pulmonary Tuberculoma of the Stock Fed Mouse* (Sudan IV ×400). Tissue also obtained four weeks after mouse was inoculated with the tubercle bacillus. No Sudanophilia can be discerned.

FIG. 10.—*Rabbit V*–1, *No.* 4.—Microscopic section of an aortic segment four weeks after occlusion in a hypercholesteraemic rabbit (H. and E. ×25). The luxuriant growth of the new intimal tissue is striking. Note also the rich vascularity of this tissue. See Fig. 11 for demonstration of Sudanophilia also in this tissue. A capillary may be seen penetrating the media, at the central base of the new growth.

FIG. 11.—*Rabbit V*–1, 4—*Hyperplastic Intimal Tissue of Occluded Aortic Segment of an Hypercholesteraemic Rabbit.* (Sudan IV × 400).—Tissue obtained from the interior of an aortic segment four weeks after its obstruction in a cholesterol-oil fed rabbit. The similarity of the intensive Sudanophilia of this tissue to that exhibited by the peritoneal (Fig. 5) and subcutaneous (Fig. 7) granulomas of the hypercholesteraemic rabbit is striking.

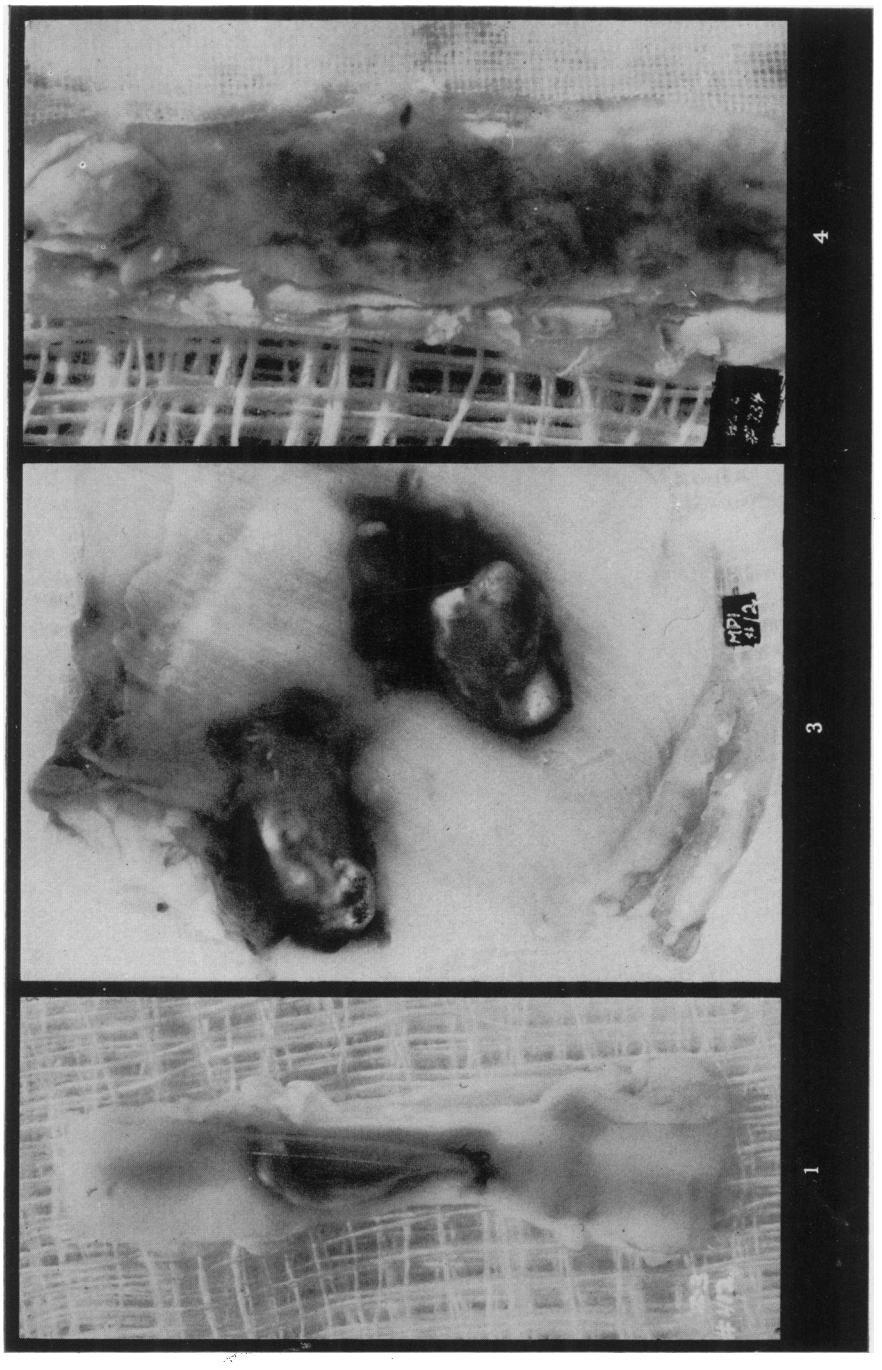
FIG. 12.—*Rabbit P*–9.—Cross section of the placenta of a pregnant rabbit (27 days) 24 hr. after parenteral administration of Evans Blue dye. The dark areas represent areas of dye extravasation and concentration.

FIG. 13.—*Rabbit P*–3.—*Placental Decidual Basalis of a Pregnant Hypercholesteraemic Rabbit* (Sudan IV ×60).—The marked concentration of Sudanophilia in maternal derived tissue of the placenta is well shown in this photograph and contrasts strongly with the total absence of such Sudanophilia in both the uterine mucosa of the same rabbit (Fig. 14) and in the placental decidua basalis of the pregnant normocholesteraemic rabbit (Fig. 15).

FIG. 14.—*Rabbit P*–3.—*Uterine Mucosa of a Pregnant Hypercholesteraemic Rabbit* (Sudan IV ×60).—This section of uterine mucosa approximately 2 cm. distant from the placental attachment was obtained from the same hypercholesteraemic rabbit from whom a section of the decidua basalis is shown in Fig. 13. Note here, however, that no Sudanophilia can be discerned.

FIG. 15.—*Rabbit P*–9.—*Placental Decidua Basalis of a Pregnant Normocholesteraemic Rabbit* (Sudan IV ×60).—In striking contrast with similar tissue of similar age obtained from the cholesterol-oil fed hypercholesteraemic rabbit (Fig. 13), this tissue exhibits no Sudanophilia.

FIG. 16.—*Rabbit W*–12, *No.* 326.—Aorta of young rabbit (2 weeks) after multiple intravenous injections of Evans Blue dye. The dark areas just distal to the semilunar valves (towards the top of the photograph) are areas of dye extravasation and concentration. The remainder of the aorta is clear.



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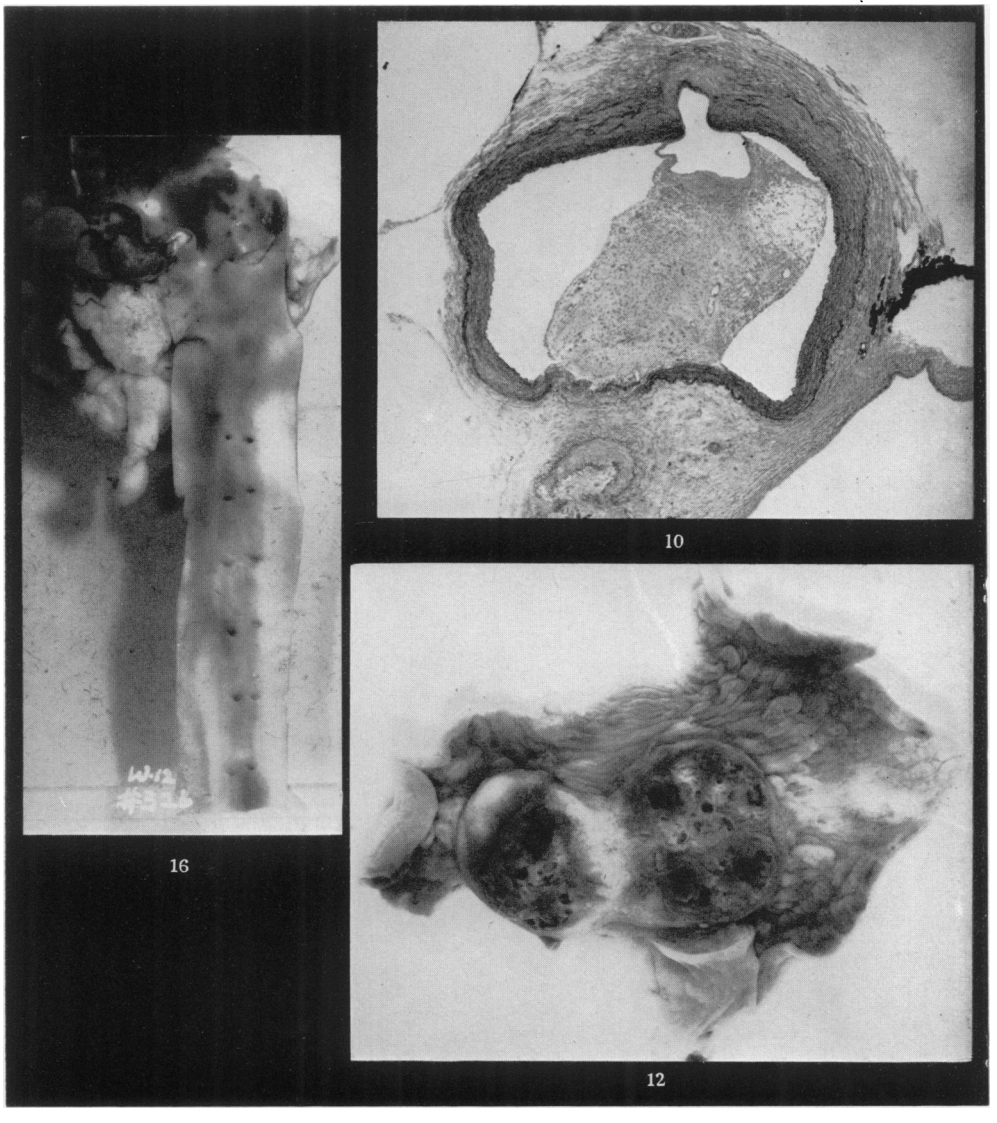

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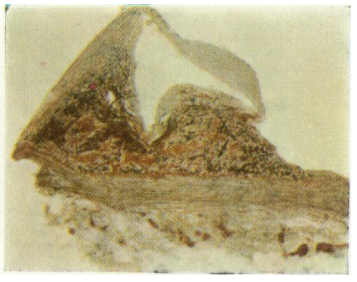

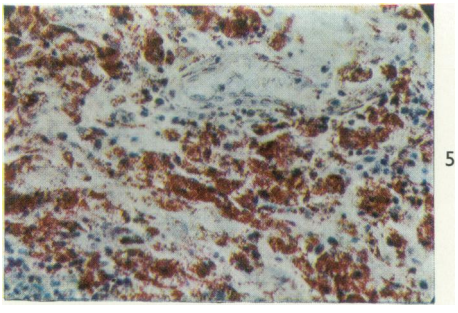

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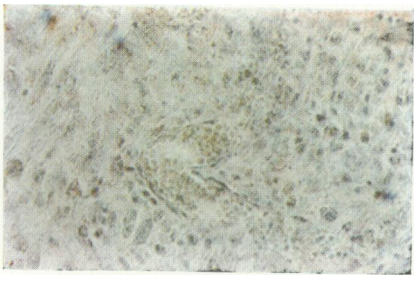

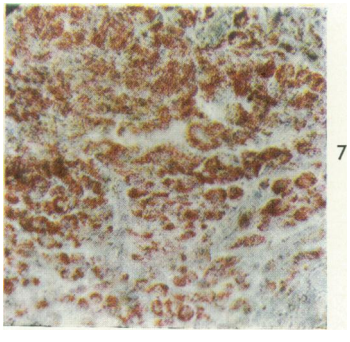

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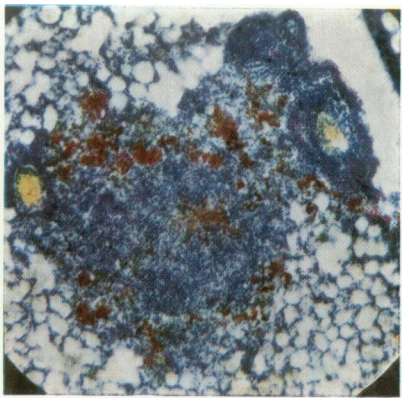

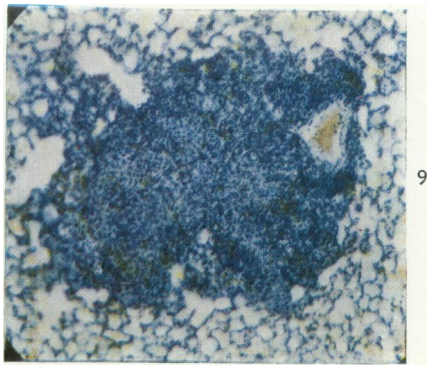

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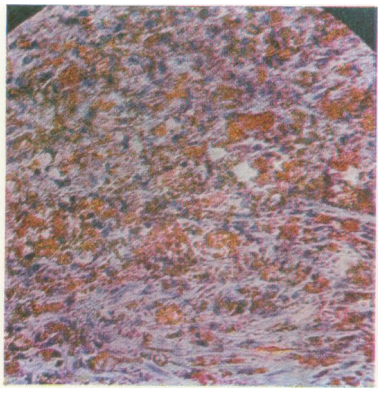

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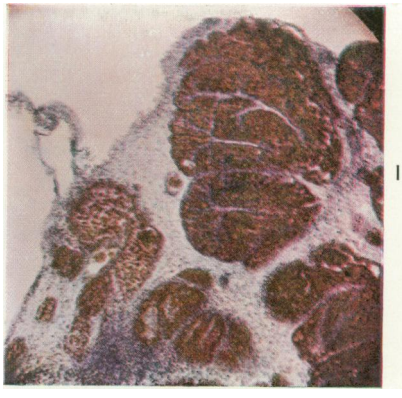

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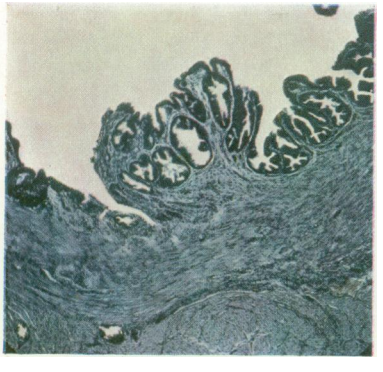

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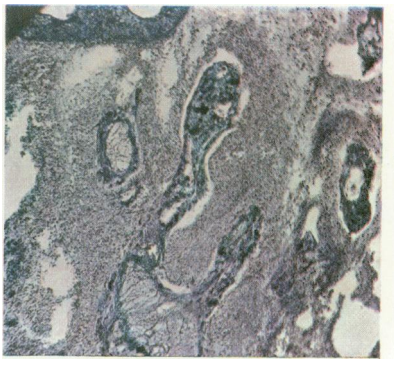

879 mg./100 ml.) exhibited an hyperplastic intima containing Sudanophilic infiltration (Fig. 11). As found in the other tissues studied, the lipid stain was exclusively intracellular. Cholesterol analysis of this aortic hyperplastic intimal tissue also indicated that a marked concentration of cholesterol had taken place in the intimal tissue of the hypercholesteraemic rabbit; thus, the average cholesterol content of the latter three intimal samples was 9·4 g./100 g. of dry tissue compared to 1·8 g./100 g. of dry tissue found in the three intimal samples of the normocholesteraemic rabbits.

2. *Placental tissue*

The average serum cholesterol of the eight hypercholesteraemic pregnant rabbits when killed was 587 mg./100 ml. The placentas of the two hypercholesteraemic rabbits injected with Evans Blue dye 24 hr. before killing exhibited areas of marked extravasation of dye (Fig. 12), suggesting again the excess permeability of the new vessels contained in this tissue. Moreover, sections of the 16 placentas obtained from these hypercholesteraemic rabbits exhibited without exception marked Sudanophilia in the decidua basalis areas (Fig. 13). On the other hand, sections of the pregnant uteri of the same rabbits not in the immediate vicinity of the placental attachment (hence not possessing newly formed capillaries) uniformly failed to exhibit Sudanophilic infiltration (Fig. 14).

The four normocholesteraemic pregnant rabbits (aver. serum cholesterol: 46 mg./100 ml.), on the other hand, bore placentas which on microscopic examination failed to exhibit any significant Sudanophilia (Fig. 15). However, the placentas of the two rabbits injected with Evans Blue 24 hr. prior to killing showed areas of dye extravasation similar to those observed in the hypercholesteraemic animals.

C. *Permeability of Aortic Endothelium of very Young Rabbits*

Eight aortas of the 9 young nursing rabbits injected with Evans Blue were found to exhibit several or more areas of dye extravasation—almost always limited to the first 3–5 cm. of aorta distal to the semilunar valves—following injection of Evans Blue dye (Fig. 16). Similarly, 4 of the 5 aortas of young rabbits that had received an injection of Trypan Blue showed similar areas of dye concentration. These aortic areas of dye concentration were inspected under the dissecting microscope and in each instance, the dye was found to be concentrated in the intimal area and fading in intensity as the adventitia was approached. This latter inspection was performed in order to make certain that the dye had penetrated from the intimal surface according to the criteria Duff (1932) had established.

On the other hand, only 1 of the 9 aortas of the older rabbits exhibited similar areas of dye extravasation.

It was of interest to us that despite the high average serum cholesterol (aver.: 289 mg./100 ml.) of the very young rabbits, none of the aortic sections obtained from these animals showed any discernible Sudanophilia.

DISCUSSION

The preceding studies showed that whenever and wherever growth of new connective tissue was induced together with the feeding of excess cholesterol and cottonseed oil, regardless of the species used, such new tissue invariably exhibited

an excess of triglyceride as indicated by the positive Sudan staining. Such tissue also probably accumulated excess cholesterol since, when analyzed for this substance, two of our varieties of connective tissue (*i.e.* the peritoneal granulomatous and the hyperplastic intimal tissue of the occluded aortic segment) exhibited such excess cholesterol and similar types of connective tissue were also found both by Boucek and Noble (1957) and McCandless *et al.* (1960) to contain huge excesses of cholesterol. The placental tissue of the hypercholesteraemic rabbit also has been found by Popjak (1946) to accumulate excess cholesterol.

Is this excess of triglyceride and cholesterol, which accumulates in new connective tissue, due to an enhanced synthesis of these substances *in situ*, or is it due to a greater provision of these substances from the capillary blood supplying this tissue ? For a variety of reasons, we believe that the latter cause is primary. First, in an earlier study of hyperplastic aortic intimal tissue (which is a form of connective tissue) we found (Friedman *et al.*, unpublished) that such tissue did not synthesize cholesterol more rapidly than intact adjacent resting aortic intima but, on the other hand, was far more capable of accumulating or concentrating the already formed cholesterol present in the capillary blood supplying it. This suggests, of course, that the excess cholesterol observed in the hyperplastic intimal tissue came primarily from the blood and not from an enhanced rate of local synthesis. Second, in the same study, when the hyperplastic intimal tissue was isolated from its usual capillary blood supply, the excess of triglyceride and cholesterol usually observed did not occur. Third, the capillary vessels of this new aortic intimal tissue were found to " leak " not only Evans Blue, Trypan Blue and Thorotrast, but also carbonyl iron particles having a diameter many times that of the low density lipoproteins bearing triglyceride and cholesterol.

Fourth, the present studies indicated that none of the various types of connective tissue studied ever exhibited excess amounts of triglyceride and cholesterol unless the animal bearing them also had ingested excess cholesterol and triglyceride. If, however, enhanced local synthesis of triglyceride and cholesterol had been chiefly responsible for the observed excess accumulations of these substances in the connective tissues, such accumulations might reasonably be expected in animals not fed excess amounts of the substances under consideration. In this connection it should be mentioned that the tubercular lung of the mouse has been found (Bhattathiry, Venkitasubramanian and Viswanathan, 1961) to exhibit no enhancement in rate of cholesterol synthesis and an actual decrease in fatty acid synthesis but, nevertheless, in the present study the granulomas of such lungs were found to exhibit a significant amount of excess triglyceride if, and only if, the mice were fed excess cholesterol and oil. It was of interest too that only the granulomas of the cholesterol-oil fed mice exhibited a significant Sudanophilia, although the serum cholesterol of these mice was the same as that of the control animals. Finally, whenever studied, the capillaries of these connective tissues, similar to those studied earlier in aortic hyperplastic intimal tissue (Friedman *et al.*, unpublished) were found to be excessively permeable to Evans Blue dye—an indication that a molecule at least as large as albumin was " leaking " rapidly from the capillary system. All of these observations, considered *in toto*, strongly suggest that capillary " leakage " plays a predominant role in causing the excess triglyceride and cholesterol invariably found in new connective tissue of cholesterol fed animals.

This concept of excess capillary permeability amply explains the, at first

glance paradoxical, findings of Boucek and Noble (1957) and Noble and Boucek (1955) who observed that whereas the cholesterol content of the new connective tissue forming in their Ivalon sponges was far greater than that of the recipient animal's liver, the latter was found to synthesize cholesterol at a rate hundreds of times faster than this same cholesterol-rich connective tissue. The concept also explains the findings of McCandless *et al.* (1960) who, in analyzing new connective tissue following the injection of carrageenan, found it excessive in cholesterol content after feeding cholesterol to the animals so injected.

The present study also strongly suggests, we believe, that the " leaking " of vessel endothelium need not be a function solely promoted by inflammatory processes (Boyd, 1928 ; Waters, 1954). Although the induction of a thrombus, the insertion of a Millipore capsule and injection of carrageenan ostensibly could bring about endothelial " leaking " of lipid and cholesterol by the process of inflammation, the latter phenomenon does not seem involved in the hyperplasia of the intima of an occluded arterial segment. It certainly cannot be assumed to exist in the decidua basalis of the pregnant rabbit where abnormal extravasation of triglyceride, dye, or both were observed to occur ; nor does inflammation exist in the aortic lining of the very young rabbit where extravasation of dye is also seen.

The penetration of vascular endothelium by large molecules and particles has been observed often enough previously (Menkin, 1940) and the possible mode of such penetration has been partially elucidated by the newer anatomical studies (Buck, 1957 ; Bennett, Luft and Hampton, 1959 ; Parker, 1960 ; Palade, 1961) of this tissue. What has not been widely realized, however, is that various lipoproteins and macromolecules such as chylomicrons also may be capable of such penetrations. The present study emphasizes the probability of this type of penetration. It suggests, too, that significant penetration probably occurs in any newly developing vascular system regardless of site. It finally implies that the intensity of such penetration is a function in great part of the concentration and possibly quality of the lipoproteins of blood.

SUMMARY

Various types of tissues served by newly developing capillaries were observed to accumulate excessive amounts of Sudan staining material and cholesterol when triglyceride and cholesterol were supplied in the diet, and to accumulate Evans Blue when this dye was injected intravenously. Such tissues include rabbit thromboatherosclerotic plaques, peritoneal, subcutaneous and intra-aortic granulomas and mouse pulmonary granulomas. Rabbit placenta and some areas of the aortic endothelium of the very young rabbit were also found to accumulate the above mentioned substances.

The current and earlier observations, taken as a whole, suggest that excessive permeability to blood lipids is a property of probably all types of very young vascular tissue regardless of the presence or absence of inflammation.

The authors wish to express their thanks to Burroughs Wellcome and Company for underwriting the cost of the colour reproductions published with this study.

REFERENCES

ABELL, R. G.—(1946) *Amer. J. Physiol.*, **147**, 237.
BENNETT, H. S., LUFT, J. H. AND HAMPTON, J. C.—(1959) *Ibid.*, **196**, 381.
BHATTATHIRY, E. P. M., VENKITASUBRAMANIAN, T. A. AND VISWANATHAN, R.—(1961) *Dis. Chest*, **39**, 609.
BOYD, A. N.—(1928) *Amer. J. Path.*, **4**, 159.
BUCK, R. C.—(1957) *Circulation*, **16**, 484.
BOUCEK, R. J. AND NOBLE, N. L.—(1957) *Circulation Res.*, **5**, 27.
BYERS, S. O. AND FRIEDMAN, M.—(1962) *Brit. J. exp. Path.*, **43**, 198.
DUFF, G. L.—(1932) *Amer. J. Path.*, **8**, 219.
FRIEDMAN, M. AND BYERS, S. O.—(1961*a*) *J. clin. Invest.*, **40**, 1139.—(1961*b*) *Proc. Soc. exp. Biol., N.Y.*, **106**, 796.
Iidem AND PEARL, F.—(1960) *Amer. J. Physiol.*, **199**, 770.
Iidem and ST. GEORGE, S.—(1962) *J. clin. Invest.* **41**, 828.
MCCANDLESS, E. L., BAILEY, R. E. AND ZILVERSMIT, D. B.—(1960) *Circulation Res.*, **8**, 724.
MENKIN, V.—(1940) ' Dynamics of Inflammation '. New York City (Macmillan).
NOBLE, N. L. AND BOUCEK, R. J.—(1955) *Circulation Res.*, **3**, 344.
PALADE, G. E.—(1961) *Circulation*, **24**, 368.
PARKER, F.—(1960) *Amer. J. Path.*, **36**, 19.
POPJAK, G.—(1946) *J. Physiol.*, **105**, 236.
WATERS, L. L.—(1954) *Proc. nat. Acad. Sci., Wash.*, publ. 338, p. 91.