Arterial lesions resembling arteriosclerosis produced by reticuloendothelial system blockade have been demonstrated by Patek and Bernick who injected carbon or thorium dioxide into rats and rabbits; by Shimamoto who injected tissue and bacterial polysaccharides, bacterial exotoxins, killed bacteria, dextran and glycogen, and kaolin into rabbits, rats and guinea pigs; by Lautsch et al. who injected thorium dioxide into rabbits and by Hueper who injected polyvinyl alcohol into dogs. The appearance of particles of the suspensions in endothelial cells or macrophages of the arterial intima has been observed following massive or repeated injections of these substances and has indicated the possibility of a direct effect of these substances on endothelium. Single, relatively small doses of colloidal suspensions, however, can produce vascular lesions which do not contain any of this particulate material. Therefore, it can be assumed that the resultant vascular pathology was not directly caused by the particulate materials themselves, but was probably related to the effects of particulate material upon the reticuloendothelial system of macrophages.

Experiments described below were designed to test the hypothesis that a humoral factor may be involved in production of the arterial lesions which follow blockade of the reticuloendothelial system by particulate matter.

Methods

A series of twenty Holtzman strain male rats, approximately 150 g in weight were each given an intravenous injection of carbon (Higgins India ink) in the amount of five mg carbon per 100 g animal body weight. Three days later these injected animals were united by parabiosis to non-injected male animals of the same weight and inbred genetic strain. Eight pairs of non-injected male rats of the same weight and genetic strain were united by parabiosis to serve as controls. The animals were maintained on natural food stock diet. (Wayne Lab-Blox, Allied Mills Inc., Chicago).

One group of four experimental and two control pairs was killed two months after parabiosis, another similar group after the fourth month, and a third group at the end of the sixth month. Specimens of heart, aorta, liver, thyroid gland and adrenal glands were removed and prepared for paraffin imbedding. Alternate cut sections were stained with hematoxylin and trisron, periodic acid Schiff and aldehyde fuchsin.

After the fourth month of the experiment, four pairs of parabiosed rats (carbon injected with non-injected) and control pairs were placed on a diet containing 1% cholesterol and 5% cotton seed oil. One-half of this group was killed on the 10th day after the change in diet and the other half on the 20th day. Tissues removed from these animals were fixed in 10% formalin and frozen sections were prepared and stained with oil red 0 to demonstrate lipids.

Four pairs of animals died within the first two weeks after surgery. The remaining animals showed no signs of toxicity; there were no significant differences in size or weight between individuals of a union.

Results

LIVER

The livers of the parabiont which received the carbon injection showed uniform pick-up of carbon by the Kupffer cells of the liver (fig. 1a). The livers of the parabiont which did not receive the carbon injection demonstrated only occasional carbon granules in single widely separated Kupffer cells (fig. 1b). Following addition of 1% cholesterol-5% fat to the diet, lipid not previously stainable was seen in large quantities in both Kupffer and
hepatic parenchymal cells. The gross distribution of lipid was similar in both experimental and control animals in that its deposition was heavier in the peripheral than in the central portions of the lobule. There was, however, a difference in the detailed appearance of the lipid droplets and in their intracellular organization. In the control animal the large lipid droplets in parenchymal cells tended to be located parallel to the sinusoidal walls while the smaller lipid droplets were fairly uniformly dispersed within these cells (fig. 2). In both experimental parabions the lipid droplets, both large and small, appeared to have a more haphazard distribution of irregularly sized and shaped droplets. In addition, lipid droplets appeared to be free within the sinusoids (fig. 3a and b).

THYROID GLAND
The thyroid glands of control parabiosed rats appeared normal. The follicular cells were cuboidal in shape and contained fine PAS positive colloid droplets. Each follicle was filled with homogeneous, strongly positive PAS colloid (fig. 4). In contrast, the carbon-receiving parabions and also the non-injected partners exhibited thyroid gland hyperplasia. The follicles, as compared to controls, varied in size and shape. The follicular cells became columnar and there was an increase in number of large PAS positive thyroglobulin droplets in their cytoplasm. There was a decrease in the stored colloid in the lumina of the follicles (fig. 5a and b).

CORONARY ARTERIES
Two months following the beginning of the experiment, a few of the medium-sized ventricular coronary arteries in all hearts of injected and non-injected parabions showed slight arteriosclerotic changes. In control animals the internal elastic membrane was continuous, narrow and sharply defined (fig.

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6a). In experimental animals the internal elastic membrane was thickened, discontinuous and its margins were fuzzy (fig. 6b). At four months there was an additional change in the media adjacent to the internal elastic membrane. The smooth muscle cells stained poorly and contained numerous small and large vacuoles. In some small regions the intima was somewhat thickened and more cellular (fig. 7a and b). At the sixth month the same changes persisted; however, there was an increased thickening of the intima and an increase in fragmentation of the internal elastic membrane (figs. 8a and b, 9a and b). Also, the number of arteries involved had increased. No lipid was demonstrable in these arteries. Those animals receiving 1% cholesterol-5% fat as an additive to their diet, four months after the parabiosis, demonstrated an abundance of fine lipid droplets in the endothelial cells and in macrophages of the intima of damaged arteries (fig. 10a and b). Control parabiosed animals demonstrated no coronary arterial changes, either before or after the addition of cholesterol to their diet.

Discussion

The technique of parabiosis was used to investigate the hypothesis that reticuloendothelial system blockade by an inert colloidal suspension causes production of a humoral substance which is injurious to arteries. In this study, rats receiving colloidal carbon intravenously were united to non-treated rats following the time necessary for removal of free circulating carbon particles from the blood stream.14 Thus, neither the hepatic cells nor the Kupffer cells of the non-injected parabionts could be influenced directly by the carbon particles. In spite of the absence of carbon in the non-injected animals, they developed coronary arteriosclerosis in like frequency and degree as did their carbon-injected partners.

The effects of stress or the presence of a toxic agent produced by the parabiosis itself cannot be eliminated in the evaluation of this finding. Non-parabiosed rats, given a single similar carbon injection, showed coronary arterial lesions in four to five months. In the parabiosis experiment, lesions appeared somewhat earlier, and progressed a little more rapidly indicating some effect by the parabiosis itself on the lesions. However, it must be remembered that no lesions were observed when neither parabiont received a carbon injection.

It has been reported that commercial India ink contains dead and live bacteria including gram negative rods and that the medium and particles themselves have been shown to have endotoxic activity.15 Also, it has been contended that the shellac in the suspension medium of India ink may be another factor contra-indicating its use in biological experimentation.14 On the other hand, similar vascular lesions have been produced by other particulate suspensions such as thorium diox-
FIGURE 3

Sections of liver from (a) carbon-injected and (b) non-injected parabionts. The lipid, both small and large droplets, demonstrates no uniform or organized distribution within the hepatic cells as seen in control (fig. 2). Note phagocytized carbon (–) in Kupffer cells of parabiont (a). Oil red O; × 300.

ide\textsuperscript{1, 2, 4} or kaolin\textsuperscript{3} and by macromolecular suspensions such as bacterial polysaccharides, dextran and glycogen and polyvinyl alcohol\textsuperscript{3, 5}. Cholesterol itself is cleared from the blood by the reticuloendothelial system and may, perhaps, be considered as a reticuloendothelial blocking agent\textsuperscript{16, 17}.

Previous investigations from this laboratory have shown that 1% cholesterol-5% fat added to the diet of non-injected rats does not result in atherosclerosis. However, if lesions were first produced by reticuloendothelial blockade, and then 1% cholesterol-5% fat was added to the diet, the lesions regularly contained lipid; prior alteration of the intima may therefore be a factor in the development of atherosclerosis. Similarly, in the parabiosed rats, no lipid was demonstrated in the coronary arterial lesions unless 1% cholesterol-5% fat was subsequently added to the diet of the experimental pairs. Since the control, non-injected pairs of parabiosed rats on the cholesterol-fat containing diet presented no atheromatous coronary lesions, it can be assumed that the parabiosis itself did not cause even minimal lesions.

Previous work has shown that the feeding of 1% cholesterol-5% fat\textsuperscript{18} or the injection of carbon\textsuperscript{19} in the rat caused a hyperplasia of the thyroid gland. In this study, both the carbon-injected parabiont and its non-injected partner demonstrated thyroid gland hyperplasia. However, there is no evidence, in these experiments, to indicate that the thyroid gland alone is a causative factor in the resultant arteriosclerosis or atherosclerosis.

It has also been shown that reticuloendothelial cell blockade caused a change in distribution of lipid in the parenchymal cells of the liver of both the carbon-injected parabiont and its non-injected partner when compared with control parabionts.
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Section of thyroid gland of control animal. The follicular cells are cuboidal and contain fine PAS positive colloid droplets. Periodic Schiff (PAS) stain; X 175.

These experiments suggest that reticuloendothelial cell blockade resulted in production of a humoral substance which was injurious to arteries, stimulated thyroid gland hyperplasia and altered lipid deposition in hepatic cells. The origin of the humoral substance is unknown, but it is reasonable to suggest that it was a product of reticuloendothelial cells and/or related hepatic parenchymal cells. That reticuloendothelial (Kupffer) cells play a role in lipid metabolism has been demonstrated by Friedman, Byers and Rosenman, Brown, Philips and Kagan, and Di Luzio. Neveu et al. have shown that reticuloendothelial blockade interferes with uptake of cholesterol. That the reticuloendothelial cells of the liver also have a role in removal of protein and probably in its metabolism, has been demonstrated by Freeman, Gordon, and Humphrey and by Hyman and Paldino. The latter have also demonstrated that blockade of these cells interfered with uptake of protein. Since the reticuloendothelial cells and related hepatic parenchymal cells are involved in lipid and protein transport and metabolism, it is conceivable that carbon blockade may inhibit or alter an enzymic activity necessary for the complete metabolism of one of these substances and that an intermediate metabo-

FIGURE 4

Thyroid glands of (a, top) carbon-injected and (b, bottom) non-injected parabionts. The follicles from both animals show large columnar cells containing numerous large PAS positive colloid droplets. Periodic acid Schiff (PAS) stain; X 175.

FIGURE 5

(a, left) Coronary artery from control animal. The internal elastic membrane is thin, discrete and not interrupted. (b, right) Coronary artery from a non-injected rat, two months following its union to a carbon-injected rat. The internal elastic membrane is markedly thickened and shows interruptions and irregularities. Aldehyde fuchsin stain; X 350.
Coronary arteries from (a, left) carbon-injected and (b, right) non-injected parabionts four months following their union. The internal elastic membranes are interrupted, thickened and irregular. Areas of smooth muscle cells just external to internal elastic membrane stain poorly and contain small and large vacuoles. Aldehyde fuchsin stain; × 350.

**FIGURE 7**

Coronary arteries from (a, left) carbon-injected and (b, right) non-injected parabionts six months following their union. These arteries show a progression of the changes seen in figure 7 (a and b) as well as small areas of intimal thickening. Aldehyde fuchsin stain; × 350.

**FIGURE 8**

Coronary arteries from (a, left) carbon-injected and (b, right) non-injected parabionts. These animals were united for four months. They were then removed from the stock diet and placed on a diet containing 1% cholesterol-5% fat. Ten days following the change in diet they were killed. Note the lipid infiltration into endothelial cells and intimal macrophages. (10b is an oblique cut.) Oil red O; × 350.

100 g animal body wt). Three days later the injected animals were united by parabiosis to non-injected animals of the same sex, weight and genetic strain.

Carbon granules were found in the RES of injected rats, but few or no granules were found in the RES of non-injected rats. When accumulated in adequate quantity, carbon is injurious to arteries.

**Summary**

The experimental production of arteriosclerosis and atherosclerosis by RES blocking agents such as colloidal carbon and thorium dioxide has implicated the RES as a factor in the etiology of these diseases. Holtzman strain male rats each received a single intravenous injection of colloidal carbon (5 mg/

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found in Kupffer cells of their non-injected parabiotic partners. Coronary arteries of both injected and non-injected animals showed thickening and fragmentation of the internal elastic membrane after two months. After four to six months the lesions included degenerative changes of muscle cells just external to the internal elastic membrane and thickening of the intima. Hyperplasia of the thyroid was found in both parabionts.

Addition of 1% cholesterol-5% fat to the diet caused deposition of lipid in endothelial cells and intimal macrophages of the damaged arteries of both parabionts. Distribution of lipid within the hepatic parenchymal cells was also altered in the experimental animals but not in controls.

It is suggested that uptake of particulate matter by cells of the RES causes them to release a substance capable of producing arterial lesions.

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Production of Arterial Lesions by a Humoral Factor in Parabiotic Rats
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