METABOLIC INFLUENCES IN EXPERIMENTAL THROMBOSIS*

Harry N. Antoniades and Nelson Westmoreland

Center for Blood Research and Department of Nutrition
Harvard University School of Public Health
Boston, Massachusetts 02115

The role of thrombosis in atherosclerosis has been discussed and debated since the original von Rokitansky[1] thrombogenic theory of this disease. It has been the subject of recent reviews by Moore,[2] Haust,[3] and Mustard and Packham.[4] Conversely, atherogenesis appears to predispose one toward arterial thrombosis and toward venous thrombosis, as judged by the susceptibility to phlebothrombosis in individuals prone to atherogenesis. The role of platelets in thrombosis and atherosclerosis has attracted considerable attention from the recognition that, in addition to their function in thrombosis, platelets contain potent factors that stimulate cellular permeability and induce endothelial and smooth muscle cell proliferation. These properties of platelets are of particular importance in view of the increasing awareness that the vascular system represents not only a blood delivery system but also a metabolically active tissue, as evidenced by metabolic studies presented by other workers in this monograph. Thrombosis and atherogenesis should be viewed therefore in the context of a metabolically active environment, which includes the platelets and the vascular wall and which can be affected by several factors, including nutritional, metabolic, and endocrinologic influences.

This paper will review some of our studies [5–7] that provide direct evidence that intravascular coagulation and thrombosis in the whole animal can be greatly influenced by the nutritional, metabolic, and endocrinologic status of the animal. Thrombosis was induced by a single rapid injection of a partially purified procoagulant fraction obtained from human sera. The thrombotic effects of the procoagulant fraction were most pronounced in fasted, diabetic, and obese animals, whereas fed normal animals were shown to be resistant and remained unaffected by the injection of the procoagulant fraction. Injection of glucose in fasted rats or crystalline insulin in diabetic rats greatly reduced and reversed the thrombogenic effects of the serum procoagulant fraction.

Materials and Methods

Preparation of Human Serum Procoagulant Fraction

The procoagulant fraction used in these studies was obtained from human blood serum by ion-exchange chromatography, as described elsewhere.[6] The partially purified preparations obtained by this process exhibited about a 600-fold protein purification compared to original serum. Examination for coagulation factors indicated the presence of various concentrations of factors IXa–XIa. Thrombin was present in insignificant concentrations or was absent, as judged by its failure to clot fibrinogen. There were no significant amounts of factors V, VII, and VIII.

*Supported by a grant from the John A. Hartford Foundation.

The molecular weights of the proteins associated with the procoagulant activity were more than 100,000 daltons, as judged by Sephadex® G-100 filtration. Isoelectric focusing studies indicated the presence of two components with procoagulant activity, a major one with a pI of about 6.2 and a minor one with a pI of about 5.1. Heating at 60° C for 1 hr completely destroyed the procoagulant activity of the serum preparations.

Descriptions of Animals and Animal Studies

The following animals were used in these studies under the experimental conditions described.

Fed Rats. CD male Charles River Laboratory rats were used and weighed between 110 and 120 g. These animals were fed ad lib. on Purina chow.

Fasted Rats. Fed rats described above were fasted for about 48 hr before experimentation. The body weight of these animals ranged from 95 to 105 g.

In some studies, groups of fasted rats were injected intraperitoneally with 1 ml of glucose in 0.15 M NaCl (2 g glucose/kg of body weight) 60 min before intravenous administration of the serum procoagulant fraction.

Alloxan-Diabetic Rats. Groups of fed rats described above were injected into the jugular vein with 0.5 ml of alloxan in 0.15 M NaCl (65 mg/kg of body weight). For the injection, the animals were narcotized lightly with 50% O_2–50% CO_2. Blood glucose determinations were made in tail blood samples (25 μl) 48 hr after the alloxanization. Rats that exhibited blood glucose concentrations over 300 mg% were selected for these studies. In parallel studies, several alloxan-diabetic rats were injected intraperitoneally with 1.0 ml of a solution that contained 50 mU of crystalline bovine insulin and 5 mg of human albumin in 0.15 M NaCl 60 min before injection of the procoagulant fraction.

Fed Rats Infused with Free Fatty Acids. Fed rats (170–180 g body weight) anesthetized with pentobarbital (60 mg/kg) were infused with free fatty acid emulsion stabilized by albumin, as described by Benzman-Tarcher.[8] The final solution contained 300 μmol of fatty acid (oleic or linoleic) and 40 mg of albumin/ml. The fatty acid was infused at a rate of 0.014 ml/min with a Harvard pump through a polyethylene catheter inserted in the femoral vein and threaded into the inferior vena cava to approximately the level of the renal veins. In parallel control studies, some of the rats were infused only with albumin (40 mg/ml).

Genetically Obese Zucker Rats. These rats, along with littermate nonobese control rats, were obtained through the courtesy of Dr. Lois Zucker (Harriet G. Bird Memorial Laboratory). The body weight of the obese rats ranged from 500 to 700 g. These animals are hyperlipemic,[9] normoglycemic, have high concentrations of serum immunoreactive insulin, and exhibit strong resistance to insulin.[10]

Rats Fed Atherogenic High-Fat Diets. Rats with initial body weight of 110 or 120 g were placed on the following purified diets: coconut: 15% casein, 60% dextrose, and 15% coconut oil; corn oil: 15% casein, 60% dextrose, and 15% corn oil. Ten percent of both diets was composed of a mixture of Celluflour®, minerals, and vitamins. Control animals were fed Purina chow ad lib.

Injection of Serum Procoagulant Fraction

Lyophilized preparations of the serum procoagulant fraction, obtained by the technique described above, were dissolved in distilled water and dialyzed at 2° C for

24 hr with two changes against 2 liters of cold 0.15 M NaCl. After dialysis, the preparations were centrifuged, and aliquots of the clear supernatant fluid that contained about 10 mg of protein were pipetted into plastic tubes and kept frozen at −15° C. For the animal studies, 0.5 ml of either the serum procoagulant fraction or the control heat-inactivated (60° C for 1 hr) preparation was injected into the jugular veins of the animals. During injection, which lasted 8–10 sec, the animals were narcotized lightly with 50% O_2–50% CO_2. Blood samples were obtained from the animals before and after injection to assess the effects of the procoagulant fraction, as described below.

Criteria Used for the Assessment of Intravascular Coagulation and Thrombosis in Animals

Blood Clotting Time. Whole blood clotting time was measured in blood samples obtained in 25-μl glass capillaries from the tail of the animals before, and at various intervals after, injection of the serum procoagulant fraction. The capillaries were tilted gently every 10 sec until the blood ceased to flow inside the capillary.

Plasma Fibrinogen, Prothrombin Time, and Platelet Count. Plasma fibrinogen was determined by the method of Ratnoff and Menzie,[11] prothrombin time by the technique of Quick *et al.*,[12] and platelets were counted by phase-contrast microscopy.

Disappearance of Radioactive Human [^{125}I]Fibrinogen from Blood. The animals were injected into the jugular vein with 0.1 ml of human [^{125}I]fibrinogen (about 1 μCi/rat) 3 hr before injection of either the serum procoagulant fraction or control physiologic saline. During injections, which lasted 8–10 sec, the animals were narcotized lightly with 50% O_2–50 CO_2. Blood samples (25 μl) were collected from the tail veins of the animals just before injection of the serum fraction or control saline and at various intervals thereafter. The blood was placed in test tubes (13 × 100 mm) that contained 1 ml of 0.15 M NaCl. Bovine thrombin (50 μl, 20 U) was added, and the tubes were maintained at 2° C for 10 min and then centrifuged for 15 min at 2° C. The supernatant fluid was decanted into a clean test tube. The radioactivity in the supernatant fluid is referred to as *nonclottable* radioactivity and that in the precipitate as *clottable* radioactivity. The sum of the two values represents the *total* radioactivity. Control [^{125}I]fibrinogen was also added into test tubes that contained 25 μl of blood sample (25 μl) from uninjected animals, thrombin was added, and the tubes were centrifuged and counted as above.

The human [^{125}I]fibrinogen (Abbott Laboratories) was obtained through the courtesy of Dr. James L. Tullis. About 70–80% of the radioactivity of the preparations used in these studies was clottable with thrombin.

Light and Electron Microscopy Studies for Evaluation of Thrombohemorrhagic Effects in Animals

Light microscopy studies were performed in rats fasted for 24 hr after injection of the procoagulant fraction or a control preparation (heated-inactivited fraction or 0.15 M NaCl). The rats were anesthetized with pentobarbital (60 mg/kg), and a transparent area of membrane tissue that connected the vas deferens and the testes was selected for the microcirculation studies. Micromovies were recorded with a Bolex 16-mm camera mounted on a model-152 Sage cinephotomicrographic apparatus. For electron microscopy, fasted rats injected with the procoagulant fraction were anesthetized at various intervals. The areas to be examined were fixed by perfusion, excised, and processed for electron microscopy.

Distribution of Radioactivity in Various Organs

Groups of fasted rats (48 hr) were injected with [^{125}I]fibrinogen and 3 hr later were injected either with the serum procoagulant fraction or control saline as described above. Forty minutes later, the animals were anesthetized with pentobarbital (60 mg/kg) and then perfused for 5 min with physiologic saline to remove the blood. After perfusion, the major organs of the animals were excised and counted for radioactivity.

Estimation of Glucose, Cholesterol, Free Fatty Acid, and Triglyceride in the Blood of Animals

Blood glucose was estimated by an Auto Analyzer with the ferricyanide-reducing method of Hoffman,[13] plasma free fatty acid by the method of Dole,[14] serum triglycerides by a modification of the method of Moore,[15] and serum cholesterol by the method of Carpenter *et al.*[16]

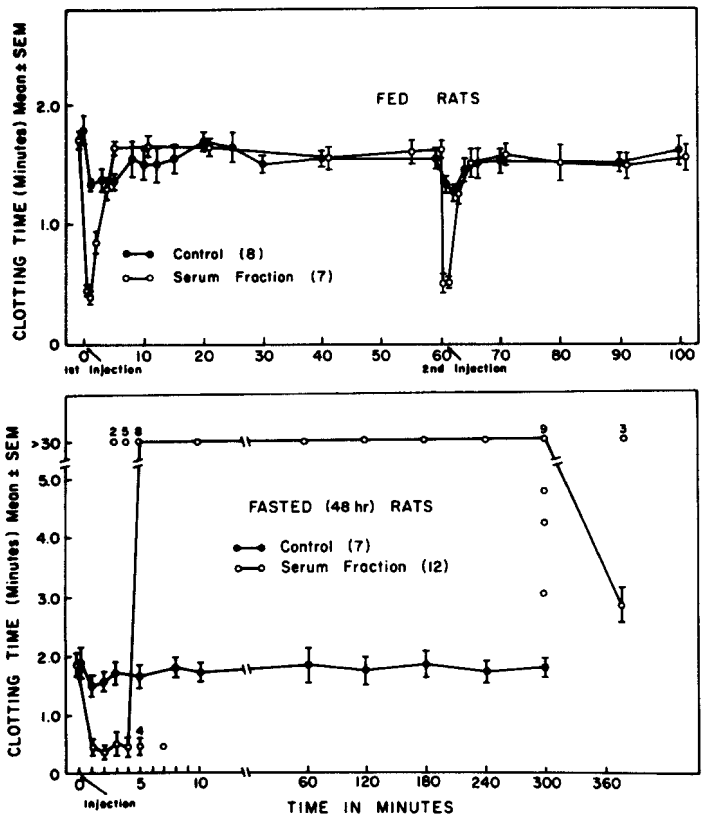

FIGURE 1. Effect of human serum fraction on the blood clotting time of fed and fasted (48 hr) rats. Each fed rat received a second injection 1 hr after the first one. Control animals were injected with identical amounts of heat-inactivated (60° C for 1 hr) preparations. At each injection, each rat received 10 mg of protein/kg of body weight. Adapted from Antoniades *et al.*[6]

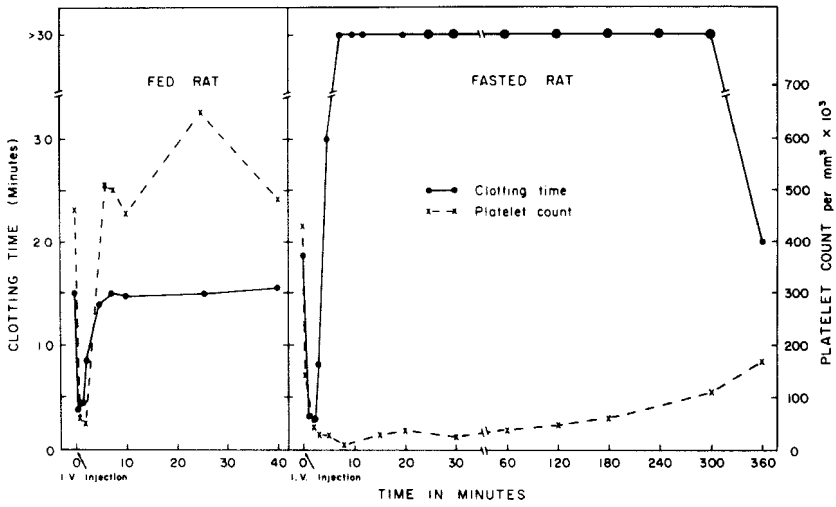

FIGURE 2. Effect of human serum fraction on the blood clotting time and platelet count of fed and fasted (48 hr) rats. Each animal received 10 mg of protein/kg of body weight. (From Antoniades *et al.*[6] By permission of *Thrombosis et Diathesis Haemorrhagica.*)

RESULTS

Thrombohemorrhagic Effects in Fed and Fasted Rats

FIGURES 1 and 2 show the effects of the serum procoagulant fraction on the blood clotting time and platelet count of fed and fasted rats. Injection in fed rats produced only a transient hypercoagulability of blood and a transient decline in blood platelet count. In contrast, a similar injection in fasted rats resulted in an initial

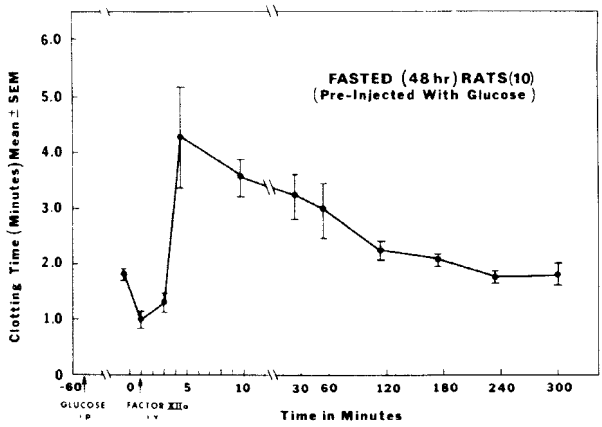

FIGURE 3. Effect of glucose on the blood clotting time of fasted rats injected with the procoagulant fraction. Compare this figure with data in fasted rats without glucose injection shown in FIGURE 1. (From Antoniades *et al.*[6] By permission of *Thrombosis et Diathesis Haemorrhagica.*)

hypercoagulability of blood, followed by a prolonged state of hypocoagulation, and a significant and prolonged decline in the platelet count that lasted for about 6 hr. Injection of control heat-inactivitated serum preparation did not produce any significant effect on the blood clotting time and platelet count of fed and fasted rats. The effect of the serum procoagulant fraction in fasted rats was reduced significantly by preinjection with glucose (FIGURE 3). Thus, glucose injection in fasted rats appears to reduce the susceptibility of these animals to intravascular coagulation. This observation was further confirmed by the data shown in FIGURE 4, which illustrates the effect of the serum procoagulant fraction on plasma fibrinogen and prothrombin time of fed and fasted rats and of fasted rats preinjected with glucose. In fed rats, the plasma fibrinogen concentration and prothrombin time remained unaffected by injection of the serum procoagulant fraction. However, when the same injection was given to fasted rats, a very different result was obtained. There was a significant decline in

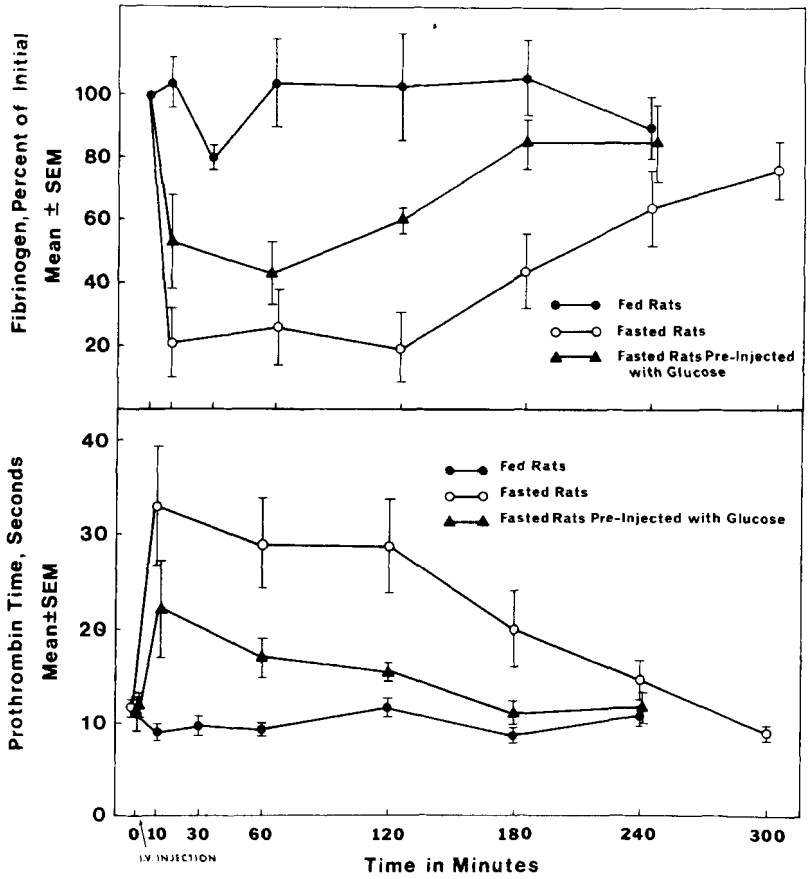

FIGURE 4. Effect of human serum fraction on prothrombin time and on the concentration of plasma fibrinogen of fed and fasted (48 hr) rats with or without glucose administration. Each rat received 10 mg of protein/kg of body weight. Each point represents the mean ± SEM of five values, and each value represents a single animal bled only once after injection. Adapted from Antoniades *et al.*[6]

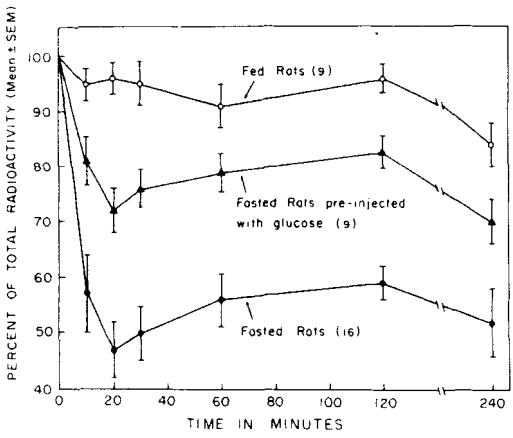

FIGURE 5. Turnover of total radioactivity in the blood of fed and fasted (48 hr) rats with or without glucose administration. Each rat was injected with 0.1 ml of human [^{125}I]fibrinogen (1 μCi) 3 hr before injection of the serum procoagulant fraction (10 mg/kg of body weight). The results are expressed as the percentage of total radioactivity of a blood sample obtained just before injection of the serum fraction. Values are expressed as means ± SE.

plasma fibrinogen concentrations and an increase in prothrombine time. These values returned to normal at about 6 hr after injection. Glucose injection in fasted rats, again, appeared to significantly minimize the effects of the serum procoagulant fraction when compared to those observed in fasted rats without preinjection of glucose.

These observations were further strengthened by the results obtained from studies with human [^{125}I]fibrinogen. FIGURE 5 shows the disposition of human [^{125}I]-fibrinogen in fed and fasted rats with or without preinjection with glucose. In all of these studies, [^{125}I]fibrinogen was injected 3 hr before injection of the serum procoagulant fraction, and the results were expressed as the percentage of radioactivity in the blood samples collected just before injection of the serum procoagulant preparation. As shown in FIGURE 5, in fed animals, despite injection of the procoagulant fraction, the turnover of [^{125}I]fibrinogen was essentially unaffected. However, when the same injection was given to fasted rats, there was a precipitous fall in circulating total radioactivity (about 50%), which increased again 1–2 hr after the injection. Injection of glucose into fasted rats 1 hr before injection of the procoagulant fraction greatly reduced the intravascular disappearance of radioactivity.

To explain the secondary rise of radioactivity in fasted rats (FIGURE 5), the total radioactivity was separated into two fractions by its clottability with thrombin. The results are shown in FIGURE 6; the data are expressed in terms of the percentage of initial clottable and nonclottable radioactivity. As shown in FIGURE 6, the clottable radioactivity declined sharply (about 60%) after injection of the procoagulant fraction in fasted rats. The nonclottable radioactivity, which decreased within the first 10 min, gradually increased in concentration above the preinjection levels. This nonclottable radioactivity apparently represents fibrinolytic products, which explains the secondary rise in total radioactivity seen in the fasted rats in FIGURE 5.

The question was raised of the fate of fibrinogen in fasted rats after injection of the serum procoagulant fraction. To answer this question, fasted rats were injected with control saline or the serum procoagulant fraction 3 hr after injection of [^{125}I]-fibrinogen. Forty minutes later, the animals were anesthetized, perfused for 5 min with physiologic saline, and then the major organs were excised and counted for ra-

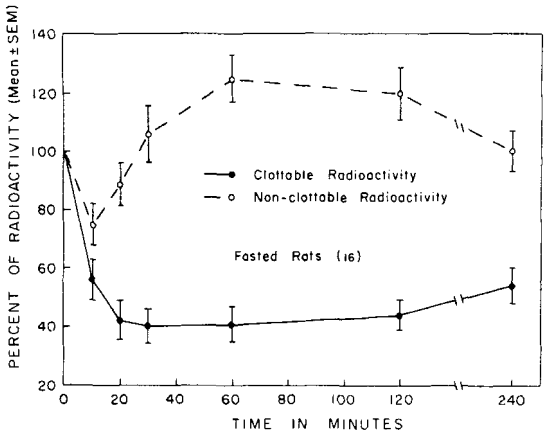

FIGURE 6. Turnover of clottable and nonclottable radioactivity in the blood of fasted (48 hr) rats. Each rat was injected with 0.1 ml of human [^{125}I]fibrinogen (1 μCi) 3 hr before injection of the serum procoagulant fraction (10 mg of protein/kg of body weight). The results are expressed as the percentage of total radioactivity of a blood sample obtained just before injection of the serum fraction. Values are expressed as means ± SE.

dioactivity. As shown in TABLE 1, a 400% increase in radioactivity was apparent in the kidneys of fasted rats injected with the serum procoagulant fraction as compared to rats injected with control saline. There was also an increase in the lungs, although this increase was not statistically significant. Thus, this study indicated that the major site of insoluble radioactivity, presumably fibrin, was in the kidney. Electron microscopy studies of kidneys of fasted rats injected with the serum procoagulant fraction confirmed the presence of thrombi in the glomerular capillary of the kidney after injection of the serum procoagulant fraction (FIGURE 7).

Light and Electron Microscopy Studies

The studies presented above described the effects of the serum procoagulant fraction in fed and fasted rats, as judged by changes in blood clotting time and

TABLE 1

DISTRIBUTION OF INSOLUBLE RADIOACTIVITY (cpm × 10^3) IN VARIOUS ORGANS OF FASTED RATS*

	Control Rats	Rats Injected with Serum Factor	Percentage of Control Rats Values
Heart	13.2 ± 2.1	10.7 ± 0.5	81.0
Liver	158.5 ± 16.5	161.6 ± 19	101.9
Lung	212 ± 25	310 ± 34	146.2
Kidney	28 ± 3.5	112.6 ± 22.6	400.0
Testicle	43 ± 3	40 ± 3.5	93.0

*Rats were injected either with control saline (control rats) or with the serum procoagulant factor 3 hr after injection of [^{125}I]fibrinogen. Forty minutes after injection of control saline or serum factor, the rats were perfused for 5 min with physiologic saline, and the organs were removed and counted. The values represent the means of seven animals ± SE.

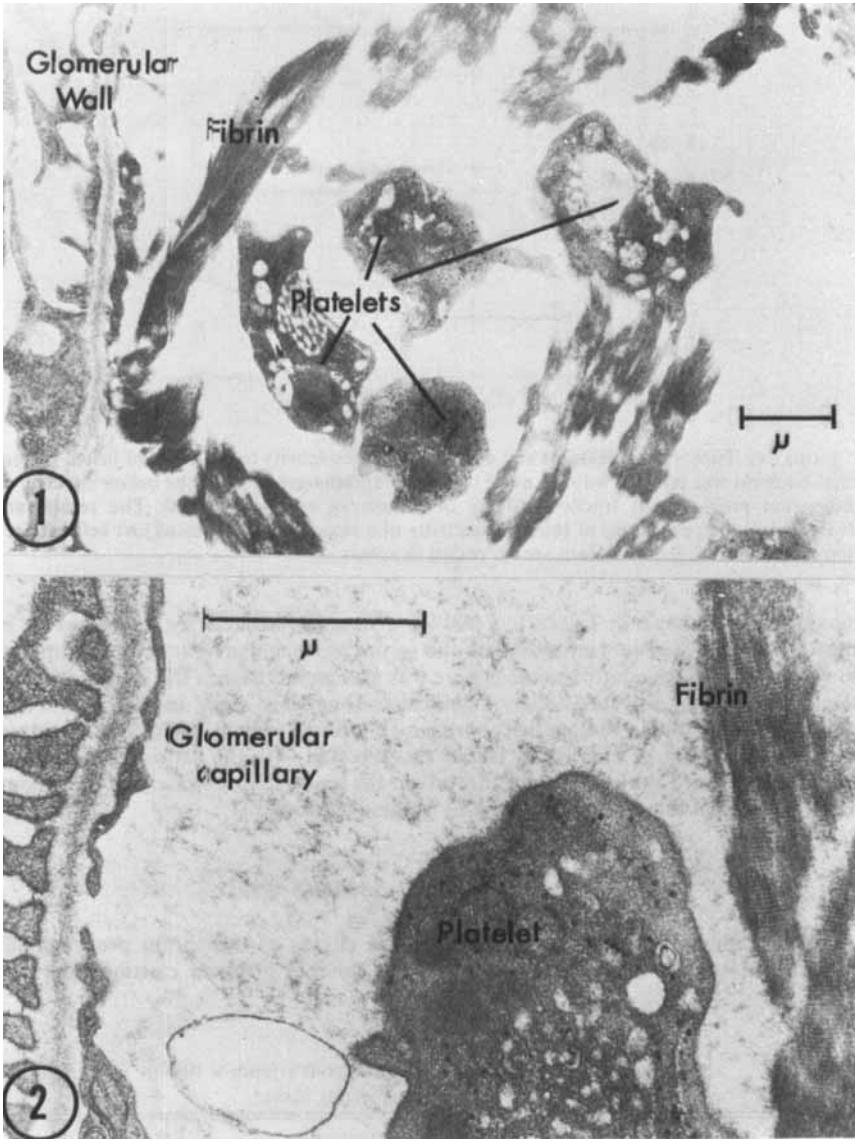

FIGURE 7. Electron microscopy of a glomerular capillary in the kidney of fasted rats 30 min after injection of the procoagulant fraction. 1, ×11,400; 2, ×2750.

platelet count, plasma fibrinogen concentration and prothrombin time, and by disposition of preinjected radioactive fibrinogen. These coagulation effects were most pronounced in fasted rats and were accompanied by the appearance of microthrombi in the vessels and hemorrhage in the tissues of these animals, as shown by electron and light microscopy studies. The rapid appearance of microthrombi in the microcirculation could be observed in fasted rats by light microscopy studies. An example is

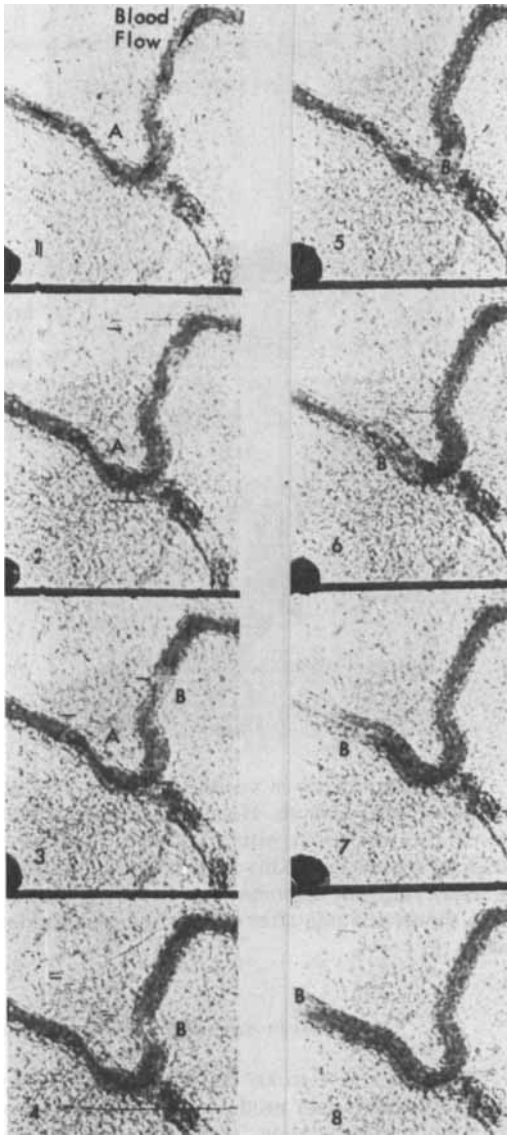

FIGURE 8. Light microscopy of the microcirculation of a transparent area of membranous connective tissue of a fasted rat (48 hr) 2 min after injection of the serum procoagulant fraction. ×200. (From Antoniades *et al.*[6] By permission of *Thrombosis et Diathesis Haemorrhagica.*)

given in FIGURE 8, which shows a small vein of a fasted rat 2 min after injection of the procoagulant fraction. The eight consecutive frames of FIGURES 8 represent slightly less than 0.5 sec. The dark color inside the small vein is the flowing column of red cells. In frames 1–5, there is a colorless constriction of the vessel at A, which most likely represents a clump of platelets and fibrin, as indicated by electron microscopy studies of fresh thrombi.[6] Beginning at frame 3, there is a light area at B, which probably represents a similar small clump. In the succeeding frames (4–8), clump B moves through the small vein, and as it passes spot A, it sweeps the thrombus away with it, leaving only red cells.

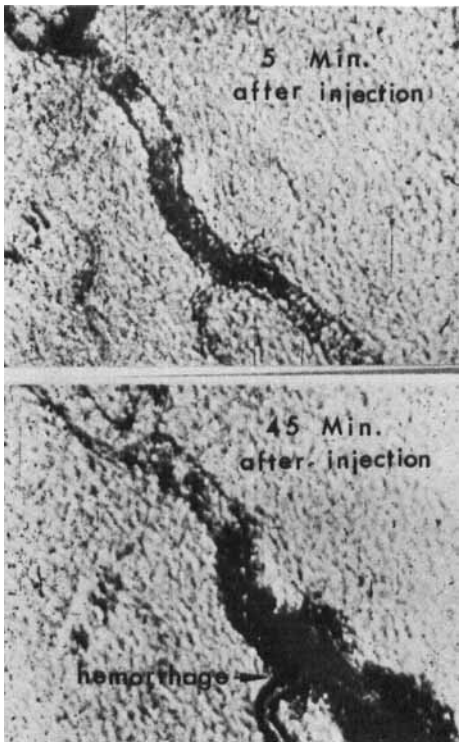

FIGURE 9. Hemorrhage in a fasted (48 hr) rat at 5 min (*top*) and 45 min after (*bottom*) injection of human serum fraction (10 mg of protein/kg of body weight). ×450. (From Antoniades *et al.*[6] By permission of *Thrombosis et Diathesis Haemorrhagica.*)

Microthrombosis in vessels of fasted rats was accompanied by hemorrhage in tissues of these animals. Hemorrhage in the testicles and epididymal adipose tissue of fasted rats was visible within 2 hr after injection of the procoagulant fraction. At this stage of hypocoagulability in fasted rats, bruising was easily induced. Hemorrhage in a fasted rat from thrombosis and stasis is shown in FIGURE 9. A small hemorrhagic area shown at 5 min after injection (*top*) expanded greatly over the next 45 min (*bottom*).

Effects in Alloxan-Diabetic and Genetically Obese Rats

As shown in FIGURE 10, injection of the serum procoagulant fraction in fed alloxan-diabetic rats produced an initial hypercoagulability of blood, followed by marked hypocoagulation. These effects in the fed diabetic rats were similar to those observed in fasted rats injected with the procoagulant fraction. The coagulation effects in diabetic rats were also accompanied by microthrombosis in the vessels and hemorrhage in the tissues of these animals, as shown by electron and light microscopy studies. The increased susceptibility of the alloxan-diabetic rats to thrombosis was reversed by preinjection with crystalline insulin (FIGURE 10). Thus, injection of insulin in diabetic rats produced effects similar to those observed in fasted rats preinjected with glucose: the animals became resistant to the thrombotic effects of the procoagulant fraction.

Genetically obese rats also exhibited a susceptibility to injection of the procoagulant fraction.[6] The animals developed an initial hypercoagulability, followed by

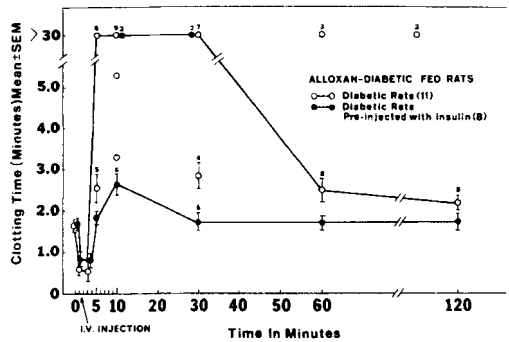

FIGURE 10. Effect of human serum preparations on the blood clotting time of alloxan-diabetic rats with or without preinjection of crystalline insulin (50 mU/rat, ip). Each rat received 10 mg of protein/kg of body weight. (From Antoniades *et al.*[6] By permission of *Thrombosis et Diathesis Haemorrhagica.*)

hypocoagulation; a second injection given 1 hr after the first one produced an even more pronounced effect. However, the obese rats were much less sensitive to the procoagulant fraction than were fasted and diabetic rats. Control nonobese littermate fed rats were resistant to injection of the procoagulant fraction, as were the fed rats shown in FIGURE 1.

Effect of Free Fatty Acid Infusion in Fed Rats

As shown above, intact fed rats exhibited a marked resistance to the thrombotic effects of the serum procoagulant fraction. In contrast, rats fasted for 48 hr, alloxan-diabetic fed rats, and, to a lesser degree, obese fed rats demonstrated an increased susceptibility to intravascular coagulation and thrombosis when challenged with the procoagulant preparation. This finding raised the question as to whether these animals shared a common characteristic that might explain their susceptibility to thrombosis. TABLE 2 lists the values of several blood metabolites of these animals,

TABLE 2

BLOOD GLUCOSE, TRIGLYCERIDE, FREE FATTY ACID, AND CHOLESTEROL CONCENTRATIONS IN FED, FASTED, DIABETIC, AND OBESE MALE RATS

	Blood Glucose (mg/100 ml)	Serum Triglycerides* (mg/100/ml)	Plasma Free Fatty Acids* (μequiv/ 100 ml)	Serum Cholesterol* (mg/100 ml)
Fed rats	102 ± 4 (10)	149	26.0	104
Fasted (48 hr) rats	82 ± (11)	36	92.0	84
Fasted rats 1 hr after glucose administration (ip)	135 ± 16 (10)	30	28.0	76
Alloxan-diabetic rats	408 ± 29 (11)	227	67.0	86
Alloxan-diabetic rats 1 hr after insulin administration (ip)	168 ± 8 (10)	141	36.0	85
Zucker obese fed rats (15–20 weeks old)	108 ± 3 (11)	2800	48.0	320

*Means of duplicate determinations in pooled samples.

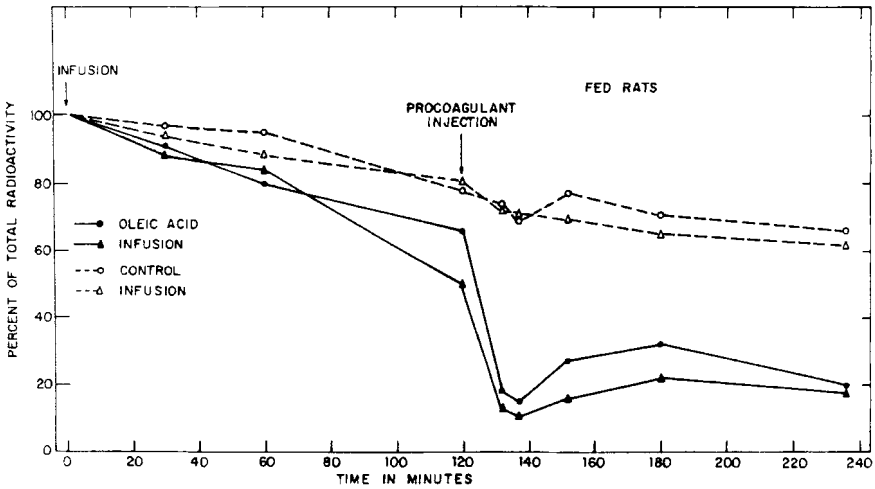

FIGURE 11. Effect of oleic acid infusion on the disposition of radioactive fibrinogen in fed rats before and after injection of the serum procoagulant fraction. Control fed rats were infused with albumin solution alone (control infusion) without oleic acid. At the end of the infusion period, the serum free fatty acid concentrations of rats infused with oleic acid were 1580 and 1440 μequiv/liter, and those that received the control infusion were 440 and 490 μequiv/liter. The oleic acid infusion medium consisted of 300 μmol of fatty acid and 40 mg of albumin/ml. Infusion rate was 0.014 ml/min.

such as glucose, cholesterol, triglyceride, and free fatty acid. It is apparent from these data that glucose, cholesterol, and triglyceride concentrations do not correlate with the susceptibility to thrombosis of the animals studied. For example, fasted rats with low blood glucose and triglyceride concentrations exhibited a marked susceptibility to thrombosis when challenged with the procoagulant fraction. On the other hand, the hyperlipemic obese rats, with significantly higher serum triglyceride and cholesterol concentrations, were much less susceptible to thrombosis than were fasted or diabetic rats. The free fatty acid concentrations, however, appear to be related to the susceptibility of the animals to thrombosis. The fasted rats, which were shown to be the most susceptible, exhibited the highest free fatty acid concentrations, followed by the diabetic and obese rats. Reversal of the thrombotic effects by injection with glucose in fasted rats and crystalline insulin in diabetic rats may also be explained by the fact that glucose and insulin injections caused a rapid decline in free fatty acid concentrations in fasted and diabetic rats, respectively.

To test the possible role of circulating free fatty acids, fed rats were infused with oleic or linoleic acid, or with albumin in controls, and then challenged with the serum procoagulant fraction. As shown in FIGURES 11 and 12, infusion of oleic or linoleic acid rendered the fed animals susceptible to thrombosis, as judged by the rapid disappearance of radioactive fibrinogen from the blood of the animals after injection of the serum procoagulant fraction. Microthrombosis in the vessels and hemorrhage in the tissues of these animals were demonstrated, similar to those observed in fasted rats after injection of the procoagulant preparation. Fed control rats infused with albumin alone did not respond to injection of the procoagulant fraction (FIGURES 11 & 12). As shown in FIGURES 12, the effect of the procoagulant fraction in fed rats infused with linoleic acid was similar to that observed in fasted rats without free fatty acid infu-

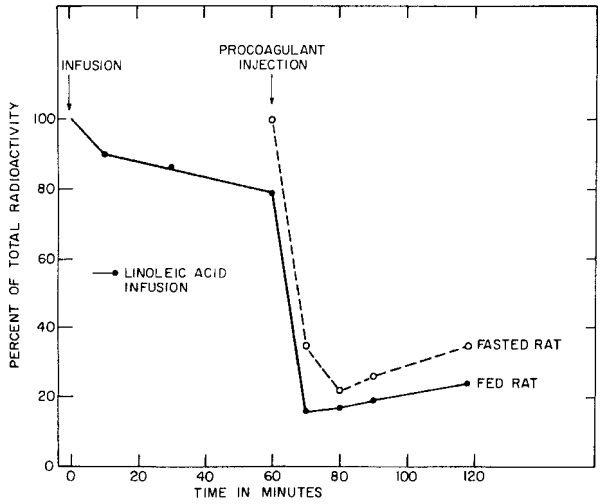

FIGURE 12. Effect of linoleic acid infusion on the disposition of radioactive fibrinogen in a fed rat before and after injection of the serum procoagulant fraction. The effects are compared to those observed in a fasted rat injected with the procoagulant fraction without free fatty acid infusion. At the end of the infusion period, the serum free fatty acid concentration was 1580 μequiv/liter. The linoleic acid infusion medium consisted of 300 μmol of fatty acid and 40 mg of albumin/ml. Infusion rate was 0.014 ml/min.

sion. Data not presented here indicate that free fatty acid infusion in fed rats, as described here, render the fed animals even more susceptible to thrombosis than fasted rats, as judged by the effects of low doses of the serum procoagulant fraction in these animals. Under these conditions, fasted rats either remained unaffected by the low dose of the procoagulant fraction or developed a mild hypocoagulation, whereas the same low dose in free fatty acid (oleic)-infused rats caused a dramatic thrombotic effect.

Free fatty acid in these studies was infused at a rate of 0.014 ml/min. At the end of the infusion period, the serum free fatty acid concentrations were about fourfold higher than those in animals infused with control albumin alone.

As shown in FIGURES 11 and 12, free fatty acid infusion alone, before injection of the procoagulant fraction, also appeared to cause a gradual decline in the radioactive fibrinogen concentrations of the recipient animals when compared to those infused with albumin alone. This finding may further indicate that a rise in free fatty acid concentration may, indeed, predispose the animals to thrombosis, as illustrated by the dramatic effects of procoagulant injection in these animals.

Effect of Atherogenic High-Fat Diet

Rats fed high coconut and corn oil diets for 10–20 weeks did not exhibit an increased sensitivity to injection of the serum procoagulant fraction. This lack of an effect, especially in the hyperlipemic rats fed the high coconut oil diet, was somehow surprising. However, because only high coconut and corn oil diets were used in these studies, this lack of an effect should relate only to the two diets used. It is possible

that other high fat diets, such as butter and cacao butter, may produce different results.

Discussion

The studies presented in this report demonstrate that thrombosis in the whole animal can be greatly influenced by noncoagulation factors, such as the metabolic, endocrinologic, and nutritional states. Normal intact fed rats were shown to be resistant to the thrombotic effects of a partially purified human serum procoagulant fraction. However, when these animals were exposed to prolonged fasting, or when they became diabetic by injection of alloxan, they exhibited a marked susceptibility to thrombosis when challenged with the procoagulant preparation. This susceptibility in fasted and diabetic rats was significantly reversed by injection of glucose or crystalline insulin, respectively. Hyperlipemic genetically obese rats were also shown to be susceptible to the procoagulant injection, although to a lesser degree than fasted and diabetic rats.

A common characteristic among fasted, diabetic, and obese rats is their relative insulin deficiency. The free insulin concentrations in fasted rats are extremely low and are undetectable in the allonxan-diabetic rats. The obese rats have large amounts of insulin in their blood; however, this insulin does not appear to be effective in these animals, which were shown to exhibit a marked tissue resistance to the hormone.[10] Thus, this relative or absolute lack of insulin results in the breakdown of triglycerides in the adipose tissue of these animals and in a rise in blood free fatty acid concentrations. It has therefore been postulated that the observed susceptibility to thrombosis in fasted, diabetic, and obese rats may be related to the rise in their free fatty acid concentrations.[6] This hypothesis is consistent with the reversal of susceptibility to thrombosis by injection of glucose in fasted rats and of crystalline insulin in diabetic rats. Glucose injection stimulates insulin release, which, in turn, promotes rapid oxidation of circulating free fatty acids by the tissues. A similar effect occurs when crystalline insulin is injected in diabetic rats. The studies with intravenous infusion of free fatty acids in intact fed rats described here appear to confirm this hypothesis. As shown above, fed rats infused with free fatty acids became sensitive to the thrombotic effects of the serum procoagulant fraction.

High blood cholesterol and triglyceride concentrations do not seem to be related to the increased susceptibility to thrombosis in the animals studied. As shown above, the obese rats that exhibited high blood concentrations of both cholesterol and triglyceride were much less sensitive to the procoagulant injection than fasted rats, which have low blood cholesterol and triglyceride concentrations (Table 2). Animal studies with free fatty acid infusion are not easy to perform, and the interpretation of poorly controlled studies can be misleading. In the present studies (Figures 11 & 12), the effect of oleic acid in the rat was monitored for 2 hr, and that of linoleic for 1 hr, before injection of the procoagulant fraction. The rate of free fatty acid infusion is critical. A high rate of infusion can cause hemolysis and thrombosis formation at the tip of the catheter inserted into the femoral vein of the rat. Saturation of albumin with large doses of free fatty acid may render the albumin insoluble, and precipitation may occur in the vessels. It is therefore important to carefully control the composition of the free fatty acid and albumin mixture. In the present studies, at the end of the infusion period, the mole ratio of free fatty acid to albumin in the blood of the rats was estimated at 4:1. Finally, one should keep in mind that the binding of infused free fatty acid to albumin can cause displacement of other biologically active molecules in blood transported by albumin, such as free amino acids, steroid hormones, and thyroxin.

Several investigators have implicated free fatty acids in thrombosis. Long-chain saturated free fatty acids have been shown to produce massive thrombosis in animals.[17–22] Short-chain fatty acids or long-chain unsaturated fatty acids did not produce these results. These thrombotic effects of the saturated free fatty acids were attributed to their ability to stimulate the clotting system[23] by activating the Hageman factor[24] and by inducing platelet aggregation.[25–27] Lipolytic hormones, such as ACTH, have been shown to cause hypercoagulability in rabbits and thrombosis in an isolated clamped jugular vein.[19] As early as 1914, Cannon and Gray[28] described a shortening of the blood clotting time in cats after injection of epinephrine, a powerful lipolytic hormone. More recently, McKay *et al.*[29] reported on the production of a generalized Shwartzman reaction in pregnant rats by infusion of factor XIIa and norepinephrine. Factor XIIa alone or norepinephrine alone did not produce these effects. A combination of phospholipid and factor Xa was shown to produce stasis thrombi in rabbits, and addition of phospholipid significantly prolonged the hypercoagulability induced by factor Xa.[30] Phospholipid or factor Xa alone did not produce stasis thrombi in rabbits, and addition of phospholipid did not make inactive factor X thrombogenic.

The effect of dietary fats on the development of atherosclerosis and arterial and venous thrombosis has been explored by many investigators. For an extensive review of the effect of dietary fats on arterial and venous thrombosis, the reader is referred to recent reviews by Renaud[31,32] and Honstra.[33] Renaud has shown that experimental animals fed high saturated fat diets, such as butter and cacao butter, become susceptible to thrombosis when challenged by endotoxin or epinephrine.[34–36] The locations of thrombi depended on the stimulus given. Endotoxin-induced thrombi were located primarily in the hepatic vein, whereas epinephrine-induced thrombi were located solely in the left atria.[35] Fats rich in polyunsaturated fatty acids, such as corn oil, antagonized the thrombotic effect of the saturated fats. It has been suggested that the thrombotic effects of the saturated free fatty acids may be mediated through platelets, by increasing their susceptibility to aggregation and their platelet factor 3 (PF_3) activity.[32,37] The latter may result from an increase in the phospholipid molecule concentration of oleic and stearic acid, at the expense of the polyunsaturated fatty acids.[32,37]

Thrombosis induced by a standardized damage of the jugular vein has been reported in rats fed a diet that contained 10% hydrogenated coconut fat and 1% cholesterol.[38] The incidence of thrombosis was reduced by addition of linseed oil but not by corn oil. In rats on a high fat diet that contained cholesterol, thrombosis was induced by intravenous injection of adenosine diphosphate, which indicates a possible relation between susceptibility to thrombosis and saturated fat and platelet levels.[39] An increased tendency to thrombosis induced by a high fat diet was also observed in pigs with an extracorporeal circulation.[40]

With a filter-loop technique in rats, Honstra and Vendelmans[41] have concluded that the thrombogenicity of a dietary fat depends primarily on its content in saturated fatty acid. An increase in the intake of unsaturated fatty acid at the expense of saturated fatty acids produced a decrease in the thrombotic tendency of dietary fat.[33]

These animal studies with dietary fats are of special interest, because studies in man indicate a relationship between coronary heart disease and intake of saturated fats.[42–44]

The metabolic studies reviewed here, and animal studies with atherogenic high fat diets reported by other investigators, provide strong evidence on the important role of dietary, endocrinologic, and metabolic factors in thrombosis. It is possible that the various factors and conditions that enhance or inhibit the susceptibility to thrombosis exert their action by affecting coagulation factors, platelets, and platelet metabolism and, possibly, the metabolic activity of the vascular wall.

Summary

Studies presented in this report demonstrate that intravascular coagulation and thrombosis in the whole animal can be greatly influenced by noncoagulation factors, such as metabolic, endocrinologic, and nutritional states. Injection of a partially purified human serum procoagulant fraction produced no significant clotting abnormalities in normal fed rats; however, injection of an identical preparation in fasted, diabetic, and obese rats produced hypercoagulability of blood, thrombosis, and hemorrhage. Glucose injection in fasted rats and insulin injection in diabetic rats reversed their susceptibility to thrombosis. The concentrations of serum free fatty acids were shown to be elevated in the susceptible animals; however, they returned to normal in fasted and diabetic rats after injections of glucose and insulin, respectively. Infusion of free fatty acid-albumin preparations in normal fed rats rendered the animals susceptible to thrombosis when challenged with the serum procoagulant fraction.

References

1. VON ROKITANSKY, C. 1842. Handbuch der Pathologicken Anatomie. Vol. 2: 534. Braunmuller and Seidel. Vienna, Austria (1852. Transl. by G. E. Day.: 261. Sydenham Society. London, England).
2. MOORE, S. 1974. Thrombosis and Atherosclerosis. *In* Platelets, Thrombosis and Inhibitors. P. Didisheim, Ed.: 205–212. F. K. Schattauer Verlag. Stuttgart, Federal Republic of Germany.
3. HAUST, M. D. 1975. Platelets, thrombosis and atherosclerosis. *In* Platelets, Drugs, and Thrombosis. J. Hirsh, J. F. Cade, A. S. Gallus & E. Schonbaum, Eds.: 94–110. S. Karger. Basel, Switzerland.
4. MUSTARD, J. F. & M. A. PACKHAM. 1975. The role of blood and platelets in atherosclerosis and the complications of atherosclerosis. Thromb. Diath. Haemorrhag. **33**(3): 444–456.
5. ANTONIADES, H. N., P. G. IATRIDIS, S. N. GERSHOFF & E. A. SWEENEY. 1969. Thrombohemorrhagic effects in fasted rats produced by the injection of partially purified human serum concentrates. Vox Sang. **17**: 66, 67.
6. ANTONIADES, H. N., P. G. IATRIDIS, N. WESTMORELAND, J. D. SIMON, K. C. HAYES & D. MACN. SURGENOR. 1973. Effects of nutritional, endocrine and metabolic state on the development of intravascular coagulation induced by human serum preparations with procoagulant activity. Thromb. Diath. Haemorrhag. **29**: 33–49.
7. ANTONIADES, H. N., N. WESTMORELAND & D. MACN. SURGENOR. 1973. Disposition of (^{125}I) fibrinogen in fed and fasted rats following injection of partially purified human serum preparations with potent procoagulant activity. *In* IVth International Congress on Thrombosis and Haemostasis, Vienna, Austria: 333.
8. BENZMAN-TARCHER, A. 1969. Method for continuous intravenous infusion of large amounts of oleic acid into rats. J. Lipid. Res. **10**: 197–206.
9. ZUCKER, L. M. 1965. Hereditary obesity in rats associated with hyperlipemia. Ann. N. Y. Acad. Sci. **131**: 447–458.
10. ZUCKER, L. M. & H. N. ANTONIADES. 1972. Insulin and obesity in the Zucker genetically obese rat "fatty." Endocrinology **90**: 1320–1330.
11. RATNOFF, O. D. & C. MENZIE. 1951. A new method for the determination of fibrinogen in small samples of plasma. J. Lab. Clin. Med. **37**: 316–320.
12. QUICK, A. J., M. STANLEY-BORWIN & F. W. BANCROFT. 1935. A study of the coagulation defect in hemophilia and in jaundice. Amer. J. Med. Sci. **190**: 501–511.
13. HOFFMAN, W. S. 1937. A rapid photoelectric method for the determination of glucose in blood and urine. J. Biol. Chem. **120**: 51–55.
14. DOLE, V. P. 1956. A relation between non-esterified fatty acids in plasma and the metabolites of glucose. J. Clin. Invest. **35**: 150–154.

15. MOORE, J. H. 1962. A modified method for the determination of glyceride glycerol. J. Dairy Res. **29:** 141–145.
16. CARPENTER, K. J., A. GOTSIS & D. M. HEGSTED. 1957. Estimation of total cholesterol in serum by a micromethod. Clin. Chem. **3:** 233–238.
17. CONNOR, W. E., J. C. HOAK & E. D. WARNER. 1963. Massive thrombosis produced by fatty acid infusion. J. Clin. Invest. **42:** 860–866.
18. CONNOR, W. E., J. C. HOAK & E. D. WARNER. 1966. The role of lipids in thrombosis. Thromb. Diath. Haemorrhag. **21**(Suppl. 21): 193.
19. CONNOR, W. E., J. C. HOAK & E. D. WARNER. 1969. Plasma free fatty acids, hypercoagulability, and thrombosis. *In* Thrombosis. S. Sherry, K. M. Brinkhous, E. Glenton, J. M. Stengle, Eds.: 355–373. National Academy of Science. Washington, D.C.
20. HOAK, J. C. & D. S. ROBINSON. 1963. Thrombosis associated with mobilization of fatty acids. Amer. J. Pathol. **43:** 987–998.
21. ZBINDEN, G. 1964. Transient thrombopenia after intravenous injection of certain fatty acids. J. Lipid Res. **5:** 378–384.
22. HOAK, J. C. 1964. Structure of thrombi produced by injection of fatty acids. Brit. J. Exp. Pathol. **45:** 44–47.
23. CONNOR, W. E., J. C. HOAK & E. D. WARNER. 1965. The effects of fatty acids on blood coagulation and thrombosis. Thromb. Diath. Haemorrhag. **13:** 89–102.
24. DIDISHEIM, P. & R. S. MIBASHAN. 1963. Activation of Hageman factor (factor XII) by long-chain saturated fatty acids. Thromb. Diath. Haemorrhag. **9:** 346–353.
25. WARNER, E. D., J. C. HOAK & W. E. CONNOR. 1967. The role of fatty acids in platelet aggregation and thrombosis. Thromb. Diath. Haemorrhag. **26:** 249–259.
26. KERR, J. W., R. PIRRIE, I. MACAULAY & B. BRONTE-STEWART. 1965. Platelet-aggregation by phospholipids and free fatty acids. Lancet **1:** 1296–1299.
27. PROST-DVOJAKOVIC, R. J. & M. SAMAMA. 1973–1974. Clot-promoting and platelet-aggregating effects of fatty acids. *In* Dietary Fats and Thrombosis. Haemostasis **2:** 73–84.
28. CANNON, W. B. & H. GRAY. 1914. Factors affecting the coagulation time of blood. II. The hastening or retarding of coagulation by adrenalin injections. Amer. J. Physiol. **34:** 232–242.
29. MCKAY, D. G., J. G. LATOUR & A. M. LOPEZ. 1971. Production of the generilized Shwartzman reaction by activated Hageman factor and α-adrenergic stimulation. Thromb. Diath. Haemorrhag. **26:** 71–76.
30. WESSLER, S. & E. T. YIN. 1968. Experimental hypercoagulable state induced by factor X: comparison of the nonactivated and activated forms. J. Lab. Clin. Med. **72:** 256–260.
31. RENAUD, S. & P. GAUTHERON. 1973–74. Dietary fats and experimental (cardiac and venous) thrombosis. *In* Dietary Fats and Thrombosis. Haemostasis **2:** 53–72.
32. RENAUD, S. 1974. Dietary fats and arterial thrombosis. Thromb. Res. **4**(Suppl. 1): 25–35.
33. HORNSTRA, G. 1973–74. Dietary fats and arterial thrombosis. *In* Dietary Fats and Thrombosis. Haemostasis **2:** 21–52.
34. RENAUD, S. 1965. Endotoxin-induced hepatic vein thrombosis in hyperlipemic rats. Sequence of events. Lab. Invest. **14:** 424–434.
35. RENAUD, S. & I. GODU. 1969. Induction of large thrombi in hyperlipemic rats by epinephrine and endotoxin. Lab. Invest. **21:** 512–518.
36. RENAUD, S. 1968. Thrombogenicity and atherogenicity of dietary fatty acids in rats. J. Atheroscler. Res. **8:** 625–636.
37. RENAUD, S., K. KUBA, C. GOULET, Y. LEMIRE & C. ALLARD. 1970. Relationship between fatty-acid composition of platelets and platelet aggregation in rat and man. Circulation Res. **26:** 553–564.
38. NORDØY, A. 1965. The influence of saturated fats, cholesterol, corn oil and linseed oil on experimental venous thrombosis in rats. Thromb. Diath. Haemorrhag. **13:** 244–256.
39. NORDØY, A. & A. B. CHANDLER. 1964. The influence of dietary fats on the adenosine diphosphate induced platelet thrombosis in the rat. Scand. J. Haematol. **1:** 202–211.
40. MUSTARD, J. F., H. C. ROWSELL, E. A. MURPHY & H. G. DOWNIE. 1963. Diet and thrombus formation: quantitative studies using an extracorporeal circulation in pigs. J. Clin. Invest. **42:** 1783–1789.
41. HORNSTRA, G. & A. VENDELMANS. 1973. Induction of experimental arterial occlusive thrombin in rats. Atherosclerosis **17:** 369–382.

42. MIETTINEN, M., O. TURPEINEN, M. J. KARNOVEN, E. ELOSUO & E. PAAVILAINEN. 1972. Effect of cholesterol-lowering diet on mortality from coronary heart disease and other causes. A twelve-year trial in men and women. Lancet **2:** 835–838.
43. STAMLER, J. 1973. Epidemiology of coronary heart disease. Med. Clin. N. Amer. **57:** 5–46.
44. STOMORKEN, H. 1973–1974. Epidemiological studies. Relation between dietary fat and arterial and venous thrombosis. *In* Dietary Fats and Thrombosis. Haemostasis **2:** 1–12.