THE EFFECT OF ENDOTOXIN ON FACTOR VII IN RATS: *IN VIVO* AND *IN VITRO* OBSERVATIONS*

By S. A. Evensen, M. Jeremic and P. F. Hjort

From the Hematology Unit, Medical Department A, Rikshospitalet, (University Clinic), Oslo, Norway

Severe Gram-negative septicemia may provoke acute intravascular coagulation, resulting in small vessel thromboses and in low levels of platelets, fibrinogen, and Factors V and VIII (see Hjort & Rapaport 1965). Endotoxin probably acts as the trigger: it can be demonstrated in the blood from such patients (Douglas, Beller & Debrovner 1963), and it can produce similar reactions in experimental animals.

Factor VII is not consumed during *in vitro* coagulation. Nevertheless, low levels of Factor VII are found in patients with Gram-negative septicemia and acute intravascular coagulation (Pfau, Lasch & Günther 1960, Lasch, Krecke, Rodriguez-Erdmann, Sessner & Schütterle 1961 a, McGehee, Hjort & Rapaport 1965, Rapaport, Tatter, Coeur-Barron & Hjort 1964) and in animals injected with endotoxin (Kleinmaier, Goergen, Lasch, Krecke & Bohle 1959, Lasch, Rodriguez-Erdmann & Schimpf 1961 b, Hardaway & Johnson 1963). This finding may have at least two explanations. First,

Factor VII is activated during intrinsic clotting (Johnston & Hjort 1961), probably because part of its molecule is split off (Prydz 1965). This activated form of Factor VII is not inactivated during *in vitro* incubation, but it disappears more quickly from the blood stream than does native Factor VII (Rapaport, Hjort, Patch & Jeremic 1966). Low levels of Factor VII, therefore, could be caused by *in vivo* coagulation followed by rapid removal of activated Factor VII. Secondly, endotoxin might suppress the synthesis of Factor VII in the liver.

In this work, we have investigated the second of these hypotheses. We have studied the effect of a single sublethal dose of endotoxin on the Factor VII level in rats and also on the synthesis of Factor VII by rat liver slices. Under our experimental conditions, endotoxin *increased* Factor VII levels, probably because of an increased rate of synthesis in the liver.

MATERIALS

Animals. Male albino rats of the Wistar-Møllegaard strain weighing 150—250 g were used. The animals were fed commercial rat food (Felleskjøpet, Oslo) and housed for 5—7 days before the experiment.

Buffer. An imidazol buffer, pH 7.35 and ionic strength 0.154, was prepared as described by Stormorken & Newcomb (1956).

Citrated dilution fluid. One part 3.13 % sodium citrate dihydrate was mixed with five parts of 0.9 % sodium chloride.

EDTA. A neutral 2 % solution of disodium-ethylenediaminetetraacetate was used.

Endotoxins. Two preparations (type W) were obtained from Difco Laboratories (Detroit, Mich.) : lipopolysaccharide from *S. marcescens* (control No. 456047) and lipopolysaccharide from *E. coli* 0111 : B4 (control No. 476141). They were dissolved in 5 % glucose as suggested by Braude (1964).

Tissue slicer was obtained from Harvard Apparatus Co., Inc. Dover, Mass.

Warfarin sodium from Nyegaard & Co., Oslo, was used.

METHODS

Administration of drugs. Intravenous injections were given in the lateral tail vein under light ether anesthesia. For surgical procedures Nembutal Sodium (Abbott Laboratories, Chicago, Ill.) 6 mg per 100 g body weight, was administered intraperitoneally.

Blood sampling. The rat was wrapped in a towel and the tail warmed for 2—3 minutes in a waterbath at 42°C. The tail was thoroughly dried, and a thin layer of vaseline was applied to provide a non-wettable surface. The lateral tail vein was then punctured with a 23-gauge needle. From the first or second rapidly increasing drop, 0.05 ml of blood was collected with silicone technique and mixed with 0.95 ml of citrated dilution fluid in a plastic vial. The samples were then frozen at —28°C for 1—5 hours before testing. This procedure gave an evenly hemolysed sample that was used without further dilution in assays of Factor VII.

Hematocrit was measured in a micro-capillary centrifuge.

Incubation of liver slices. Liver slices about 0.5 mm thick were prepared as described by Pool & Robinson (1959), except that we preferred the Harvard tissue slicer (Merskey & Wohl 1965. It took about 30 minutes from the perfused liver was taken out of the animal until incubation was star-

ted. Two slices (total weight 150—200 mg) were placed in a flat-bottomed Erlenmeyer flask which contained 4.5 ml of the bicarbonate buffered medium described by Peters & Anfinsen (1950) and 0.5 ml of 5 % glucose *or* endotoxin in glucose *or* warfarin in glucose. Slices for 4—8 flasks were prepared from the same liver. The flasks were placed in a 37°C waterbath shaker and equilibrated with a moistened gas mixture of 95 % oxygen and 5 % carbon dioxide for 5 minutes. Control flasks were stored at 4°C for 1—10 minutes before sampling.

Sampling from the incubation flasks. To a flasks, at zero time for the controls or at 4 hours for the experimental flasks, 1 ml of EDTA and 5 ml of buffer were added. The contents of the flasks were homogenized in a glass homogenizer for 3 minutes. From each homogenate one uncentrifuged and undiluted sample was assayed for Factor VII. The rest of the homogenate was centrifuged at 1250 g for 15 minutes. The supernatant was stored at —28°C for later assays of soluble protein (method of Folin-Ciocalteu).

Factor VII assay. Factor VII activity was assayed in the following test system: 0.05 ml of citrated plasma from a patient with congenital deficiency of Factor VII, 0.05 ml of barium sulphate absorbed oxalated ox plasma, 0.1 ml of human thromboplastin, and 0.1 ml of test material were incubated for 4 minutes at 37°C, and then recalcified with 0.1 ml of 35 mM calcium chloride. A "blind" technique was used for the assays.

Calculation of Factor VII activity. Factor VII activity in the lysed blood samples was translat into percentage values from a calibration curve prepared from dilutions of a pool of five lysed normal rat blood samples. Factor VII activity in the homogenates was read from a calibration curve prepared by diluting pooled rat plasma, said to contain 100 units of Factor VII per ml, in a mixture containing one part EDTA, five parts incubation medium, and five parts buffer. Units of Factor VII per ml of homogenate were read from the curve and expressed as number of units per 100 mg of soluble protein. Net Factor VII synthesis was then calculated by subtracting the mean content of two blank flasks from the mean content of two flasks incubated for 4 hours.

Statistical calculations. The results were plotted on a logarithmic scale because the calibration curves are double logarithmic plots. Consequently, logarithmic values were used for the statistical calculations, except for the hematocrit. To evaluate the significance of differences between groups, Student's t-test was used. A p-value of less than 0.05 was considered "significant". In the *in vivo* part of this study, the calculations were based on net increase in Factor VII activity. The coefficient of correlation and its significance was determined as described by Snedecor (1964).

RESULTS

Effect of endotoxin on the blood level of Factor VII.

In these experiments we measured the effect of a single intravenous injection of endotoxin on the blood level of Factor VII. The lethality of the two endotoxins used was determined in a preliminary experiment (see Table I). The results were clearcut for the endotoxin from *E. coli,* and for this endo-

T a b l e I. *Lethality of the two endotoxins in rats.*

Dose (per 150 g body weight)	Lethality of endotoxin from:	
	E. coli	S. marcescens
8 mg		1 (2)
4 »	2 (2)	1 (4)
3 »		2 (7)
2 »	3 (4)	0 (5)
1 »	12 (23)	
0.5 »	0 (3)	

A single intravenous injection of endotoxin freshly dissolved in 5 per cent glucose was given early in the morning. The table shows the number of animals dying within 24 hours; the total numbers of animals injected are given in parentheses.

toxin we chose a dose of 1 mg per 150 g body weight for the main experiment, *i.e.,* approximately an LD-50 dose. The lethality of the *S. marcescens* endotoxin was more difficult to evaluate; the concentrated solutions were also quite viscous and difficult to inject. Arbitrarily, we used 3 mg per 150 g body weight of this endotoxin for the main experiment. In these doses both endotoxins produced marked clinical symptoms. Within half an hour the rats were quiet, cyanotic, ruffled, and appeared to be severely ill.

When the tip of the tail was snipped off from these shocked animals, there was hardly any bleeding from the wound. However, by the venepuncture technique described under Methods, we always obtained 1—2 rapidly increasing drops of blood. In five rats, we measured Factor VII both in the first and in the second drop. There was no difference, and adequate samples could always be obtained from one of these drops.

In rabbits, Factor VII can be reliably measured in hemolysed capillary blood (Rapaport *et al.* 1966). We developed a similar technique for rat blood and found Factor VII to be stable for at least 24 hours at —28°C in such samples.

Fig. 1 shows the effect of endotoxin on the Factor VII levels. After a lag phase of about 10 hours, endotoxin markedly increased Factor VII activity. A maximum value of about 250 per cent was measured after 48 hours. At this time, there was no overlapping between the individual values in the control and in the experimental groups, and the mean difference from the control group was significant below the 2.5 per cent level for the *S. marcescens* group and below the 0.1 per cent level for the *E. coli* group.

Fig. 1. *Effect of endotoxin on the Factor VII level in rats.* Groups of rats were injected intravenously with glucose (10 animals), *S. marcescens* endotoxin (7 animals, 3 mg per 150 g body weight), or *E. coli* endotoxin (15 animals, 1 mg per 150 g body weight). At intervals, Factor VII was measured as described in Methods. Each point on the graph represents group means: for the control group of 10 animals (except for the last point, 5 animals), for the *S. marcescens* endotoxin of the 5 surviving animals, and for the *E. coli* endotoxin for the 8 surviving animals (except for the last point, 5 animals).

● —————— ● Controls (5 per cent glucose)
○ —————— ○ *E. coli* endotoxin
× —————— × *S. marcescens* endotoxin

Factor VII was determined in hemolysed whole blood, and changes in the hematocrit would therefore influence the results. The hematocrit was followed in all animals in order to correct the Factor VII results, if necessary. The initial mean hematocrit value for 23 animals was 44.1 ± 2.5 per cent (range 40—49 per cent). Following the injection, the endotoxin treated rats became mildly anemic. The differences from control animals, which never exceeded 3 per cent, were not significant, and the Factor VII levels were therefore not corrected for changes in the hematocrit.

Effect of endotoxin on the in vitro synthesis of Factor VII by liver slices.

When liver slices were incubated *in vitro*, Factor VII activity steadily increased in the homogenate, as has been previously demon-strated (Pool & Robinson 1959, Merskey & Wohl 1965). Theoretically, this activity could depend on activation of the clotting mechanism during incubation or on synthesis of Factor VII. Three observations strongly suggest that synthesis is the correct explanation.

First, warfarin markedly reduced this rise of Factor VII during incubation. Fig. 2 shows that Factor VII activity increased 10-fold during incubation for 4 hours at 37°C. In the presence of warfarin this activity was halved. The difference between these two groups was significantly below the 0.1 per cent level. Fig. 3 shows a necessary control experiment: warfarin did not block the *in vitro* reactions between tissue thromboplastin, calcium, and serum. These two observations fit the synthesis theory but not the activation theory. A dose of only 1 mg per kg body weight blocks the synthesis of Factor VII in
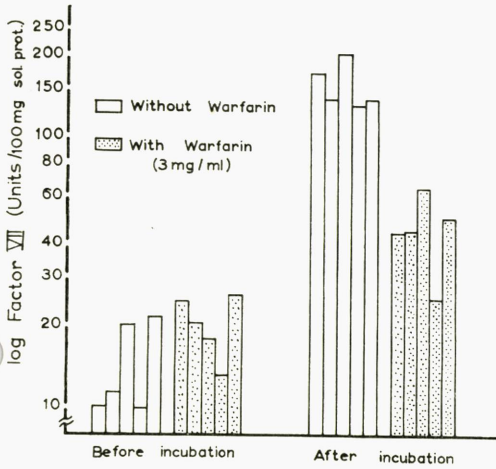
Fig. 2. *Synthesis of Factor VII in vitro, effect of warfarin.* Liver slices were incubated with and without warfarin, as described in Methods. In the diagram each column represents the mean of two flasks prepared from the same liver. Columns representing flasks with slices prepared from the same liver occupy corresponding places in each of the four groups.

Fig. 3. *Effect of warfarin on the formation of "convertin" in the extrinsic system.* The following mixture was incubated at $37°C$: 0.5 ml human thromboplastin, 0.05 ml rat serum diluted 1:20 in buffer, 3.95 ml 6.3 mM calcium chloride, and 0.5 ml warfarin (30 mg per ml saline) or saline. At intervals, subsamples were tested for Factor VII activity; see Methods.

the living rat (Pyörälä 1965), but much larger doses are required to block the *in vitro* synthesis. The reasons for this discrepancy are not known.

Secondly, decalcifying agents reverse activation in the extrinsic system (Hjort 1957), but EDTA did not reduce the Factor VII levels after incubation. In fact, EDTA was added to all flasks upon completion of the incubation.

Thirdly, the increase in activity was greater than could be expected from activation only, as also pointed out by Prydz (1964).

At last, it may be mentioned that one should expect a close correlation between the activity before and after incubation of slices prepared from the same liver, if the in-
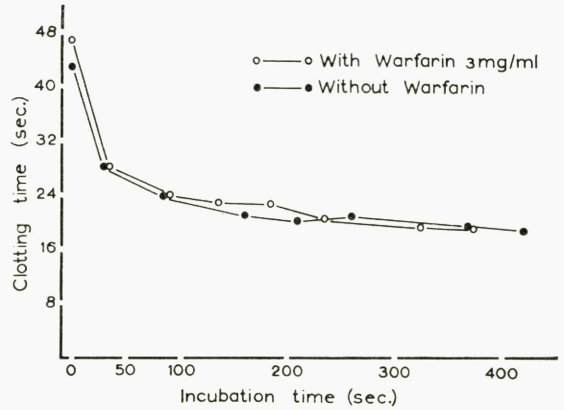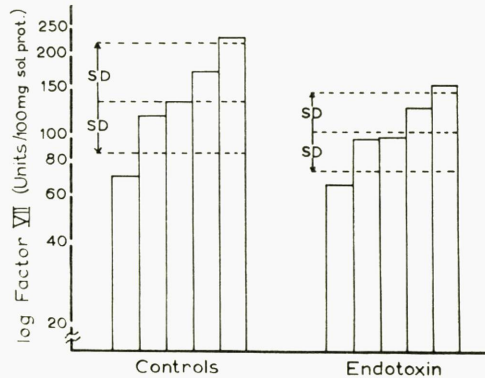

Fig. 4. *Effect of added endotoxin on the in vitro synthesis of Factor VII.* Liver slices were incubated with or without *E. coli* endotoxin (final concentration 0.2 mg per ml medium). Each column represents net Factor VII synthesis during 4 hours incubation as described in Methods. Mean ± 1 SD is shown for each group.
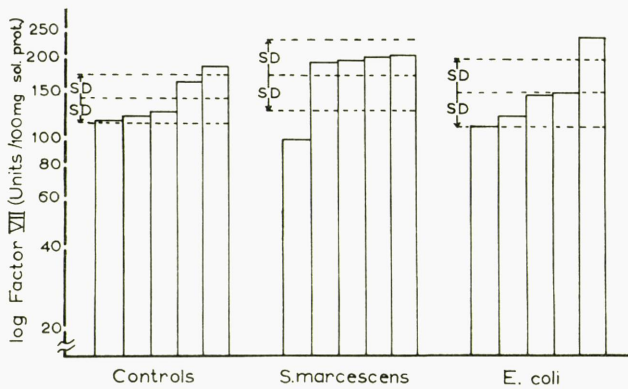
Fig. 5. *Effect of injected endotoxin on the in vitro synthesis of Factor VII.* Liver slices were prepared from rats 48 hours after an intravenous injection of glucose, *S. marcescens* endotoxin (3 mg per 150 g body weight), or *E. coli* endotoxin (1 mg per 150 g body weight). Each column represents net Factor VII synthesis during 4 hours incubation as described in Methods. Mean ± 1 SD is shown for each group.

creased activity depended upon activation. For 15 pairs of such observations we calculated a correlation coefficient of 0.501, which is nearly significant at the 5 per cent level. Thus, only a moderate correlation was found.

Endotoxin added *in vitro* had no significant effect on the synthesis of Factor VII by liver slices (see Fig. 4). In the *in vivo* experiment we found a maximum effect of endotoxin 48 hours after the intravenous injection of a sublethal dose, as shown in Fig. 1. Therefore, we also incubated liver slices from animals that had received endotoxin 48 hours earlier. Fig. 5 and Table II show that such slices did not differ significantly from control slices in their Factor VII synthesis.

Table II. *Effect of endotoxin on the in vitro synthesis of Factor VII.*

Experiment	Endotoxin	No. of animals	Net synthesis of Factor VII (Mean ± 1SD)	Test for significance	
				t	p
Effect of endotoxin added *in vitro*	*E. coli* 200 µg/ml	5	103.4 $+41.3$ -29.6	1.052	0.4 > p > 0.2
	Controls (glucose)	5	134.3 $+74.6$ -48.0		
Effect of endotoxin injected 48 hours previously	*S. marcescens* 3 mg/150 g	5	172.2 $+60.6$ -44.8	1.258	0.4 > p > 0.2
	Controls (glucose)	5	140.4 $+31.4$ -25.7		
	E. coli 1 mg/150 g	5	147.9 $+50.1$ -37.5	0.333	p > 0.5

The table summarizes the results of the experiments illustrated in Figs. 4 and 5. The net synthesis of Factor VII is given in units per 100 mg of soluble protein; incubation for 4 hours at 37°C. The last two columns show significance of differences between means.

DISCUSSION

Patients with severe Gram-negative septicemia (Pfau *et al*. 1960, Lasch *et al*. 1961 a, McGehee *et al*. 1965, Rapaport *et al*. 1964), rabbits receiving two injections of endotoxin (Kleinmaier *et al*. 1959, Lasch *et al*. 1961 b), and dogs receiving a lethal dose of endotoxin (Hardaway & Johnson 1963) all develop low Factor VII levels within hours. In contrast, we found that a sublethal dose of endotoxin, after an initial lag phase of about 10 hours, increased Factor VII in rats (see Fig. 1). At no time after the injection could we demonstrate low values of Factor VII. Our findings indicate that endotoxin, at least in the rat, does not directly depress the synthesis of Factor VII. Thus, the low levels of Factor VII found in other species may be due to accelerated removal of activated Factor VII following intravascular coagulation (see Rapaport *et al*. 1966). Another possible explanation is, of course, that more severe and prolonged shock may sufficiently interfere with liver function to depress the synthesis of Factor VII.

The increased Factor VII levels in our rats could be due to activation of Factor VII, either directly by endotoxin or indirectly through intravascular coagulation. However, both the lag period of about 10 hours and the fact that the increase lasted for 3—4 days argue against this explanation, since the half-life of native Factor VII is only 2.1 hours in the rat (Pyörälä 1965), and the half-life of activated Factor VII is probably even shorter (see Rapaport *et al*. 1966). The non-pregnant rat also appears to be resistant to the triggering of intravascular coagulation by endotoxin (Wong 1962). Increased rate of synthesis, therefore, better explains our *in vivo* findings, even if we failed to confirm this in the *in vitro* experiments with liver slices. This technique does not provide the synthesizing cells with the optimal conditions present *in vivo,* and these results, therefore, carry less weight than the *in vivo* observations.

The liver is the main source of Factor VII in the body, but the cellular source — parenchymatous cells or reticuloendothelial cells — has not been determined. Clinicians favor the parenchymatous cells, since Factor VII is depressed by liver disease. Our finding, that endotoxin probably increases the synthesis of Factor VII, may be taken to support the other possibility, and for two reasons. First, endotoxin is rapidly cleared from the blood, mainly by the liver (Braude 1964, Herring, Herion, Walker & Palmer 1963). Immunohistochemical studies show that most of it is found in the Kupffer cells (Cremer & Watson 1957), a finding which fits with its high molecular weight and colloidal characteristics. Secondly, endotoxin has a biphasic effect on the granulopectic capacity of the reticuloendothelial system as evaluated by the clearance of colloidal carbon. The maximum activity is found after about 48 hours (Biozzi, Benacerraf & Halpern 1955, Benacerraf & Sebestyen 1957); *i.e.,* at a time when Factor VII activity is also reaching its maximum (see Fig. 1).

These observations are both indirect. Strictly speaking, they apply only to the increased synthesis following endotoxin and not to the normal synthesis. However, they do suggest that the reticuloendothelial cell should still be considered a possible source of Factor VII.

SUMMARY

1. In rats Factor VII was measured at intervals after a sublethal dose of endotoxin. After a lag phase of about 10 hours, Factor VII rose to a maximum level of about 250 per cent 48 hours after the injection. At no time was Factor VII depressed.

2. Incubation experiments with liver slices *in vitro* confirmed previous findings of Factor VII synthesis. This synthesis was not influenced by endotoxin.

3. In spite of these negative *in vitro* findings, we conclude that endotoxin probably increased the synthesis of Factor VII *in vivo*. The results are discussed with regard to two problems: 1) What is the explanation of the low Factor VII levels in patients with Gram-negative septicemia? 2) Is Factor VII synthesized by the parenchymatous cells or by the reticuloendothelial cells in the liver?

REFERENCES

Benacerraf, B. & Sebestyen, M. M.: Effect of bacterial endotoxins on the reticuloendothelial system. Fed. Proc. *16*, 860, 1957.

Biozzi, G., Benacerraf, B. & Halpern, B. N.: The effect of *Salm. typhi* and its endotoxin on the phagocytic activity of the reticulo-endothelial system in mice. Brit. J. exp. Path. *36, 226*, 1955.

Braude, A. J.: Absorption, distribution and elimination of endotoxins and their derivatives, in Landy, M. & Braun, W., ed.: Bacterial endotoxins, Rutgers Univ. Press, New Brunswick, N. J. 1964, 691 pp.

Cremer, N. & Watson, D. W.: Influence of stress on distribution of endotoxin in RES determined by fluorescein antibody technic. Proc. Soc. exp. Biol. (N.Y.) *95,* 510, 1957.

Douglas, G. W., Beller, F. K. & Debrovner, C. H.: The demonstration of endotoxin in the circulating blood of patients with septic abortion. Amer. J. Obstet. Gynec. *87,* 780, 1963.

Hardaway, R. M. & Johnson, D.: Clotting mechanism in endotoxin shock. Arch. intern. Med. *112, 775*, 1963.

Herring, W. B., Herion, J. C., Walker, R. J. & Palmer, J. G.: Distribution and clearance of circulating endotoxin. J. clin. Invest. *42, 79*, 1963.

Hjort, P. F.: Intermediate reactions in the coagulation of blood with tissue thromboplastin. Convertin, accelerin, prothrombinase. Scand. J. clin. Lab. Invest. *9* Suppl. 27, 1957.

— Rapaport, S. I.: The Shwartzman reaction: pathogenetic mechanisms and clinical manifestations. Ann. Rev. Med. *16*, 135, 1965.

Johnston, C. L., Jr. & Hjort, P. F.: Development of increased Factor VII activity during the spontaneous coagulation of blood. J. clin. Invest. *40, 743*, 1961.

Kleinmaier, H., Goergen, K., Lasch, H. G., Krecke, H.-J. & Bohle, A.: Untersuchungen zur Frage der Gerinnungsstörung beim Sanarelli-Shwartzman-Phänomen (sog. generalisierten Shwartzman-Phänomen) des Kaninchens. Z. ges. exp. Med. *132, 275*, 1959.

Lasch, H. G., Krecke, H.-J., Rodriguez-Erdmann, F., Sessner, H. H. & Schütterle, G.: Verbrauchskoagulopathien (Pathogenese und Therapie). Folia haemat. *6, 325*, 1961 a.

— Rodriguez-Erdmann, F. & Schimpf, K.: Antithrombin III und Anti-Blutthrombokinase bei experimenteller Verbrauchskoagulopathie. Klin. Wschr. *39, 645*, 1961 b.

McGehee, W. G., Hjort, P. F. & Rapaport, S. I.: Intra-vascular coagulation in overwhelming meningococcemia. Clin. Res. *13, 125*, 1965.

Merskey, C. & Wohl, H.: *In vitro* production of coagulation factors VII-X by liver slices from rats fed atherogenic diets. Proc. Soc. exp. Biol. (N. Y.) *118, 703*, 1965.

Peters, T., Jr. & Anfinsen, C. B.: Net production of serum albumin by liver slices. J. biol. Chem. *186,* 805, 1950.

Pfau, P., Lasch, H. G. & Günther, O.: Sanarelli-Shwartzmann-Phänomen bei febrilen Fehlgeburten und schweren Schock- und Blutungszuständen in der Geburtshilfe. Gynaecologia (Basel) *150,* 17, 1960.

Pool, J. G. & Robinson, J.: *In vitro* synthesis of coagulation factors by rat liver slices. Amer. J. Physiol. *196,* 423, 1959.

Prydz, H.: Studies on proconvertin (Factor VII). V. Biosynthesis in suspension cultures of rat liver cells. Scand. J. clin. Lab. Invest. *16,* 540, 1964.

— Studies on proconvertin (Factor VII). IX. Some characteristics of purified Factor VII preparations. Ibid. *17,* Suppl. 84, 78, 1965.

Pyörälä, K.: Determinants of the clotting factor response to warfarin in the rat. Ann. Med. exp. Fenn. *43,* Suppl. 3, 1965.

Rapaport, S. I., Tatter, D., Coeur-Barron, N. & Hjort, P. F.: Pseudomonas septicemia with intravascular clotting leading to the genera-lized Shwartzman reaction. New Engl. J. Med. *271,* 80, 1964.

— Hjort, P. F., Patch, M. J. & Jeremic, M.: Consumption of serum factors and prothrombin during intravascular clotting in rabbits. Scand. J. Haemat. *3,* 59, 1966.

Snedecor, G. W.: Statistical methods. The Iowa State University Press, Ames, 1956, 534 pp.

Stormorken, H. & Newcomb, T. F.: The use of buffers in blood coagulation studies. Scand. J. clin. Lab. Invest, *8,* 237, 1956.

Wong, T.-C.: A study on the generalized Shwartzman reaction in pregnant rats induced by bacterial endotoxin. Amer. J. Obstet. Gynec. *84,* 786, 1962.