abnormal specimens is indicated in the following tabulation:

Tibia	7	Hand	6
Mandible	8	Sternum	2
Ribs	6	Ulna	4
Fibula	5	Hindfoot	4
Radius	5	Cleft palate	2

One of the abnormal young showed in addition to the abnormalities of the "pattern of Diet I" a defect of the spine.

Summary. Congenital malformations were observed in the offspring of female rats fed a purified diet lacking riboflavin. The malformations were anatomically identical with those described previously as the "pattern of Diet I."

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Cephalin, Protamine and the Antithromboplastic Activity of Normal and Hemophilic Plasmas.

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Normal human plasma, collected and separated with especial precautions, has the property of reducing the clot accelerating activity of homologous brain tissue extracts. The clot decelerating activity of hemophilic plasma is several times greater than normal.[1] The thromboplastin of aqueous brain-tissue extracts, on which this activity of the plasma has been tested, is a lipoprotein; its clot accelerating properties reside chiefly in the lipid moiety, the active constituent of which is a cephalin.[2] From the evidence here presented it appears that cephalin is the constituent of the lipoprotein vulnerable to the antithromboplastic action of the plasma. When the lipoprotein is stripped of most of its lipid content by extraction with fat solvents, it loses most of its susceptibility to the antithromboplastin.

Methods for separating the plasma and testing antithromboplastin activity have been given in detail elsewhere.[1] Only clear, "platelet-free" plasmas may be used. The thromboplastic lipoprotein solution (Sol. A) was prepared by extracting 300 mg of acetone dried human brain powder with 5 cc of 0.85% NaCl.[3] Cephalin was obtained from acetone dried human brain by extraction at 5°C overnight, with ether of low peroxide content (1 g brain powder plus 20 cc ethyl ether). In view of the rapid loss of potency of cephalin solutions[4] the time of extraction was made short and only a small amount was prepared each time. The clear ether extract was rapidly concentrated *in vacuo* and poured into ice cold absolute ethanol. The white flaky precipitate was then washed with acetone, dried to a waxy consistency and suspended at once in 10 cc of saline. In a few instances the cephalin was purified further by repeating the passage of the lipid through absolute alcohol 2 or more times before suspending it in saline. A pale bluish grey suspension was obtained (Sol. B), 0.1 cc of which gave a clotting time between 60 and 80 seconds on 0.3 cc recalcified citrated normal human plasma. As is well known, the cephalin obtained by ether extraction of the thromboplastic lipoprotein does not have the potency of the latter (0.1 cc of a concentrated aqueous extract of brain tissue clots 0.3 cc of recalcified human plasma in 14-15 seconds). To the dried residue that remained after the twice-repeated ether extraction of 1 g of brain powder, 10 cc of 0.85% NaCl was added (Sol. C). All extracts were allowed to stand until the coarse particles had settled; the supernatant fluid was used for the

[1] Tocantins, L. M., *Am. J. Physiol.*, 1943, **139**, 265.

[2] Howell, W. H., *Am. J. Physiol.*, 1912, **31**, 1.

[3] Quick, A. J., *Science*, 1940, **92**, 113.

[4] McLean, J., *Am. J. Physiol.*, 1917, **43**, 586.

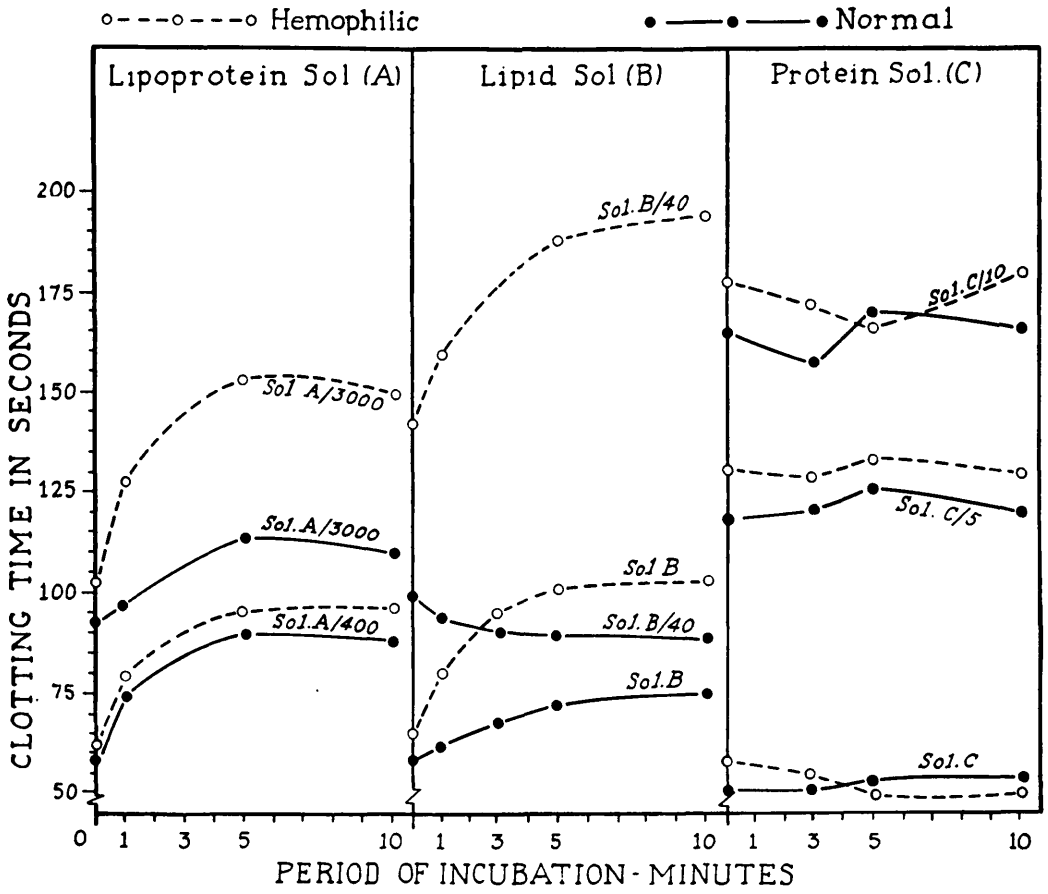

FIG. 1.

Effect of incubation of normal or hemophilic plasma with three types of clot accelerating solutions (Sols. A, B, or C). The numbers after each solution indicate its dilution.

0.3 cc plasma, 0.1 cc thrombopl. sol. (incubation), 0.1 cc $CaCl_2$. In the non-incubated mixtures, the calcium was placed in the tube first, the plasma and the thromboplastin solution immediately after. Mixing of the cephalin suspensions with the $CaCl_2$ before adding the plasma is to be avoided; flocculation of the cephalin that follows addition of the $CaCl_2$ interferes with the action of the lipid and so alters the structure of the clot as to make it difficult to determine the end point of coagulation.

tests, with appropriate dilution in order to obtain, on normal plasma, approximately similar times with the 3 clotting agents.

In Fig. 1 are compared the results of incubation of normal or hemophilic plasma with the thromboplastin lipoprotein or with either one of its 2 components, separately. A clear decelerating effect is observed with the thromboplastic lipoprotein or with the cephalin alone, but not with the protein alone. There is no further prolongation of the clotting time of the mixture even when the incubation is carried out for as long as 90 minutes. There is little difference between the effect of the protein alone on the 2 plasmas; this may be an indication that, since the thromboplastic protein does not seem to be vulnerable to the action of antithromboplastin, the latter is bypassed by the protein which acts directly on prothrombin. In this respect, the action of the thromboplastic protein resembles that of dilute Russel viper venom. This venom is likewise invulnerable to antithromboplastin[5] and acts equally well on normal and hemophilic blood.

It has been shown[1] that over-recalcifica-

[5] Tocantins, L. M., *Fed. Proc.*, 1943, **2**, 48.

TABLE I.

The prothrombin time, plasma clotting time and anti-thromboplastic activity of plasmas (normal and hemophilic) to which protamine has been added. Non-incubated plasma mixtures handled as stated under Fig. 1. All plasmas tested within 10 minutes after adding the protamine to avoid the fibrinogenolysis[13] and rapid loss of antithromboplastic activity that follows standing at room temperature.

	Normal						Hemophilic					
	Control .85% NaCl	Mg of protamine per cc plasma					Control .85% NaCl	Mg of protamine per cc plasma				
		.025	.05	.1	.2	.4		.025	.05	.1	.2	.4
Prothrombin time (sec.)*	14	16	17	18	21	47	14	16	18	21	26	35
Plas. clot. time (min.)†	3.5	4.2	11	17	25	44	45	50	76	90	125	147
Plas. + thrombopl. + $CaCl_2$‡												
0′ incub. (sec.)	80	112	149	227	400	560	89	125	140	165	180	233
5′ incub. (sec.)	115	145	215	375	630	710	167	225	275	300	315	345
Plas. + cephalin + $CaCl_2$§												
0′ incub. (sec.)	67	82	112	158	184	297	146	290	400	425	525	650
5′ incub. (sec.)	89	106	136	172	228	430	233	325	447	520	568	720

* Quick[3] method.
† 0.3 cc plasma, 0.1 cc $CaCl_2$.
‡ 0.3 cc plasma, 0.1 cc dilute thromboplastin (incubation), 0.1 cc 0.074 M $CaCl_2$.
§ 0.3 cc plasma, 0.1 cc cephalin sol. (incubation), 0.1 cc 0.074 M $CaCl_2$.

tion, standing, heating (65°C for 5 min), dilution or previous incubation with a small amount of tissue extract reduce or eliminate the antithromboplastin activity of plasma. Similar results were obtained when cephalin instead of the thromboplastic lipoprotein was used. The clot decelerating effect of incubation of the plasma with the cephalin was irregular and often missing when heterologous cephalins (sheep, dog) were used on human plasma. This suggests that a certain degree of species specificity applies to the reaction between cephalin and antithromboplastin as it does between the latter and the thromboplastic lipoprotein.[1]

It appears, therefore, that the antithromboplastin activity of plasma is exerted on the cephalin moiety of the thromboplastic lipoprotein. Mills[6] and Eagle[7] have pointed out that hemophilic plasma is unusually resistant to activation by cephalin. A cephalin-like substance is responsible for the clot accelerating properties of platelets and platelet extracts.[8,9] These facts would seem to indicate that the excessive amount of antithromboplastin in hemophilic blood[1] is probably responsible for the resistance of this blood to activation by cephalin (or platelets) as well as for its delayed coagulability.

Chargaff[10] has demonstrated that certain basic proteins (protamines, histones) possess the property of forming true salts with cephalin (which is acidic). These lipoprotein complexes once formed are unusually resistant to disruption by various chemical means. The anticoagulating effect of protamine on the blood has been known for some time.[11] Part of this effect, at least, must be due to the direct neutralization of cephalin by the protamine which may therefore, as Chargaff[10] pointed out, be considered a true antithromboplastin. Plasma to which an appropriate amount of protamine is added should therefore behave towards thromboplastic tissue extract and cephalin essentially like hemophilic plasma. Addition of protamine (salmine sulfate) to normal plasma in the amounts designated in Table I does, indeed, lead to a prolongation of the plasma

[6] Mills, C. A., *Am. J. Physiol.*, 1926, **76**, 632.

[7] Eagle, H., *J. Gen. Physiol.*, 1935, **18**, 813.

[8] Chargaff, E., Bancroft, F. C., and Stanley-Brown, M., *J. Biol. Chem.*, 1936, **116**, 237.

[9] Howell, W. H., *Physiol. Rev.*, 1935, **15**, 435.

[10] Chargaff, E., and Ziff, M., *J. Biol. Chem.*, 1939, **131**, 25.

[11] Waldschmidt-Leitz, E., Stadler, P., and Steigerwaldt, F., *Z. f. phys. chem.*, 1929, **183**, 39.

clotting time and the appearance of a degree of antithromboplastin activity closely resembling (in certain concentrations of the protamine) that observed in intact hemophilic plasma. Such protamine plasmas behave in other respects like plasmas with excessive amounts of antithromboplastin. Aging (24-48 hours at 5°C), dilution, over-recalcification, heating to 65°C (5′) or incubation with tissue extract reduce or abolish the antithromboplastin activity of protamine plasmas as they do that of normal and hemophilic plasmas. Protamine also exaggerates the antithromboplastic and anticephalin activity of hemophilic plasmas but, as shown in Table I and observed repeatedly, not to as great a degree, comparatively speaking, as when the protamine is added to normal plasma. While the plasma clotting time of protaminized (0.04 g %) normal plasma is about 12 times that of intact plasma, that of hemophilic plasma with an equivalent protamine concentration is prolonged only a little over 3 times. The antithromboplastic activity developed in normal plasma by addition of protamine is apparently greater than that developed in hemophilic plasma by equivalent amounts of the same substance.

It is plain, however, that the anticoagulating action of protamine is not solely due to its antithromboplastic properties. As Ferguson[12] has pointed out protamine has also antiprothrombic properties. The prolongation of the prothrombin time of the protaminized plasma (Table I) is probably due in part to this effect. Protamine is also known to precipitate fibrinogen to the extent of even "defibrinogenating" the blood and rendering it incoagulable.[13] 1.5 mg of protamine per cc of plasma precipitates approximately 80% of the total fibrinogen of that plasma.[13] By keeping the concentration of the protamine below 0.5 mg per cc of plasma the antithromboplastic effect is permitted to assert itself with a minimum precipitation of fibrinogen. Obviously, unlike the natural plasma antithromboplastin, protamine exerts its effects at several levels of the coagulation mechanism. Antithromboplastin is probably much simpler in action and composition than the protamines but may, like them, be strongly basic and thereby able to combine with cephalin, forming undissociable inert compounds. That this hypothesis may have some merit is indicated by the results of experiments, to be reported elsewhere, with anticoagulant basic dyes (toluidine blue, janus green) possessing antithromboplastic properties.

Summary. The plasma antithromboplastin seems to act on the cephalin moiety of the thromboplastic lipoprotein. Hemophilic plasma exerts a greater effect on cephalin than normal plasma. Normal plasma to which a certain amount of protamine has been added behaves towards cephalin and thromboplastin like hemophilic plasma.

[12] Ferguson, J. H., *Am. J. Physiol.*, 1940, **130**, 759.

[13] Mylon, E., Winternitz, M. C., and de Suto Nagy, G. J., *J. Biol. Chem.*, 1942, **143**, 21.