FIG. 3. Embryo (P1, 8-d) treated (yolk sac) on 4th day of incubation with 5 mg of prostigmine bromide. ×2.

FIG. 4. Mid-sagittal section of control embryo (12-d) treated (yolk sac) on 4th, 5th and 8th days of incubation with 0.1, 0.5 and 0.5 cc of 0.85% saline. ×3.4.

FIG. 5. Mid-sagittal section of embryo (12-d) with extensive kyphosis showing 3 compensating S-shaped curvatures. Treated (yolk sac) on 4th, 5th and 8th days of incubation with 5 mg of prostigmine bromide; total dosage 15 mg. ×3.4.

FIG. 6. Embryo with micromelia and shortened lower beak (E-10, 13½-d). Treated (yolk sac) on 4th day of incubation with 0.05 mg of physostigmine salicylate. ×2.

ism of formation may be similar.

It is interesting that the treatment of embryos with large doses of acetylcholine during 4 to 12 days of incubation did not result in malformations similar to those obtained with cholinesterase inhibition. This would suggest the destruction of acetylcholine shortly after its administration. Since cholinesterase increases rapidly as the neuromuscular system develops this is entirely possible. Atropine, a cholinergic block, had no deleterious effect on development.

Summary. 1. Large doses of acetylcholine (1.20-2.85 mg) were injected in the yolk sac, air chamber, or directly on the embryo during the 4-12-day incubation period. These doses were well tolerated by the embryo and attended with no gross abnormalities. Atropine similarly administered produced no malformation. 2. The yolk sac of 4-day embryos was injected with weak doses (0.05 mg) of physostigmine salicylate. Shortened beaks, micromelia, and retarded development of eyelids were observed at 8 to 13 days' incubation. Inasmuch as similar anomalies were reported in sulfanilamide and insulin treated animals, these anomalies cannot be considered eserine specific. 3. With larger doses (1-15 mg) of prostigmine bromide or physostigmine salicylate, the above mentioned anomalies were generally associated with severe midline bendings of the vertebral column (Figs. 4,5), and this was interpreted as specific for cholinesterase inhibition.

1. Boell, E. J., and Shen, S. C., *J. Exp. Zool.*, 1944, v97, 21.

2. ———, *ibid.*, 1950, v113, 583.

3. Landauer, W., *J. Cell. and Comp. Physiol.*, 1954, v43, 261.

4. Nachmansohn, D., *J. Physiol.*, 1939a, v95, 29.

5. ———, *Bull. Soc. Chim. Biol.*, 1939b, v21, 761.

6. Sawyer, C. H., *J. Exp. Zool.*, 1943a, v92, 1.

7. ———, *ibid.*, 1943b, v94, 1.

8. Wenger, B. S., *Fed. Proc.*, 1951, v10, 268.

9. Youngstrom, K. A., *J. Neurophysiol.*, 1938, v1, 357.

10. Zacks, S. I., *Anat. Rec.*, 1954, v118, 509.

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Extraction and Concentration of Thromboplastic Material from Human Urine. (22321)

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Although human urine is known to have thromboplastic property(1,2), little attention has been given to the concentration or purification of the urinary component responsible for this property. There is described herein a simple method for extraction and concentration from normal urine of material with thromboplastic activity, and some characteristics of this material are discussed.

Material and methods. Pooled urine obtained without preservative from normal men is used. The urine, refrigerated not longer

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than 2 days, is filtered through nonabsorbent cotton, and the pH brought to 4.5 with 2 N HCl. Five g of $BaSO_4$ (x-ray quality) is added to each liter of urine, and the mixture is stirred for 15 min. without being allowed to foam. It is then filtered through large Berkefeld candles type N and the filtrate discarded. Thromboplastic material and a fibrinolysokinase are absorbed on the $BaSO_4$. The $BaSO_4$ is mixed with distilled water (one volume for every 20 volumes of urine), and stirred for 15 min. at a pH of 5.2. The mixture is next centrifuged (20 min. at 1060 *g*). This elution is repeated. The eluates are combined and the sediment set aside for elution of fibrinolysokinase with 3.8% Na-citrate, if desired. The clear, almost colorless eluates are then dialyzed in the cold for two days against distilled water; a slight turbidity forms. All further procedures are carried out at 5°C. The pH is adjusted by a glass electrode to 3.5, and a precipitate is formed. This is collected by centrifugation after standing for one hour and is then dissolved in 1/1,000 of original volume of urine with 1/100 M glycine buffer of pH 9, stirred for 10 min., and stored for one hour. Then the preparation is centrifuged for 30 min. at 2000 *g*. The supernatant is dialyzed by a continuous rocking device, in a cellophane (8/32 inch, Nojax Casing Visking) tubing against running CO_2-free distilled water until pH of 7 is reached. When the supernatant is lyophilized, a white powder soluble to the extent of 1% in 1/100 M glycine buffer of pH 9 is obtained. In solution, it forms a colorless, viscous, slightly opalescent fluid which can be heated to the boiling point (96.5° in Denver) (Table I) with relatively slight loss of thromboplastic activity. This substance is subsequently referred to as the "T" (thromboplastic) fraction. Yield is 2-3 mg/liter.

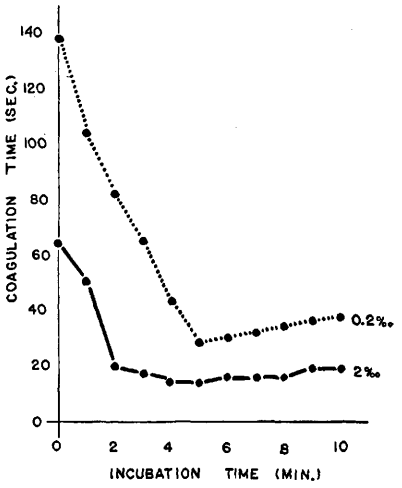

FIG. 1. Thromboplastin generation test. Platelet suspension is replaced by "T" fraction in 1/100 M glycine buffer, pH 9.

Properties. Action of the "T" fraction on hemophilic plasma under various conditions is summarized in Table I. A concentration of only a few micrograms of the "T" fraction per cc of hemophilic plasma is required to restore the clotting process to normal. Heating affects activity of the "T" fraction only to a minor degree, whereas prolonged high-speed centrifugation removes the active material.

The "T" fraction is similar in behavior to

TABLE I. Effect of "T" Fraction on Clotting of Hemophilic Plasma under Various Experimental Conditions. 6.6 μg "T" fraction added/cc plasma.*

Treatment	Recalcification time after addition of "T" fraction (min.)	Prothrombin serum time after addition of "T" fraction (sec.)
2% in glycine buffer, pH 9	9	34.5
Idem; centrifuged 90 min. at 31,000 *g*	46	20
2% in glycine buffer, pH 9	9.3	54
Idem; heated in sealed ampules at 96.5°C, 20 min.	9.5	45

* One μg corresponds to 0.3 ml urine.

Plasma from 2 different subjects has been used to obtain the data for this table. Control recalcification time and prothrombin serum time were 157 min. and 19 sec., respectively, for the first 2 experiments and 58 min. and 20.2 sec. for the last two.

brain extract since both substances contain an incompletely activated thromboplastin. The thromboplastin-like activity of the "T" fraction can be considerably enhanced by incubating it with serum and prothrombin-free plasma, as is done in the thromboplastin generation test(3). Fig. 1 shows that the "T" fraction becomes fully activated after incubation for 4 min. with serum, together with plasma treated with $BaSO_4$.

Discussion. One difference between the "T" fraction and brain thromboplastin is that the latter, when heated, has no effect on the prothrombin consumption of hemophilic blood (4). Another difference is that the "T" fraction can be centrifuged at 2000 *g* for 30 min. without an appreciable reduction in activity. A centrifugal force of 31,000 *g* for 90 min.—conditions similar to those used by Chargaff (5) to purify tissue thromboplastin—is required in order to precipitate this material. Biggs, Douglas and Macfarlane(6) have previously shown that brain extract can be activated by incubation with serum and prothrombin-free plasma—a phenomenon also demonstrable with the "T" fraction. Further studies are indicated in order to determine whether tissue thromboplastin is identical with urine thromboplastin.

Neither the physiologic significance nor the source of the urinary thromboplastic material is known. Using the present method for purification, it is possible to obtain thromboplastin from the very same individual on whom studies are to be performed. Thus, it is no longer necessary to resort to heterologous materials in studies of clotting.

Summary. A method is described whereby thromboplastic material is extracted from normal male human urine by adsorption on $BaSO_4$ and subsequent elution. The thromboplastic material is comparatively resistant to heat; its activity is enhanced by incubation with serum and prothrombin-free plasma. As little as 6 μg/cc of the thromboplastic material per cc of hemophilic plasma was found to restore the clotting properties of this plasma to normal.

1. Tocantins, L. M., Lindquist, H. J., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 44.

2. Grunke, W., *Z. ges. exp. Med.*, 1935, v96, 512.

3. Duckert, F., *et al., Acta haematol.*, 1954, v12, 197.

4. Quick, A. J., and Hussey, C. V., *Am. J. Med. Sci.*, 1952, v223, 401.

5. Chargaff, E., Bendich, A., Cohen, S. S., *J. Biol. Chem.*, 1944, v156, 161.

6. Biggs, R., Douglas, A. S., Macfarlane, R. G., *J. Physiol.*, 1953, v122, 554.

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Metabolic Interrelationship between Ascorbic Acid and Pteroylglutamic Acid. (22322)

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A close interrelationship between metabolism of ascorbic acid and pteroylglutamic acid (PGA) was reported by various workers (1-4). Schwartz and Williams(5-7) observed that in PGA deficiency produced by feeding aminopterin, rats excreted less ascorbic acid in urine, and their liver ascorbic acid contents were diminished. This indicated that biosynthesis of ascorbic acid was dependent on PGA-nutrition of animals. Chloretone enhances the biosynthesis of ascorbic acid in rats(8). It was, therefore, of interest to find out if the increased synthesis of ascorbic acid, after chloretone treatment, was interfered with in rats treated with aminopterin. Ascorbic acid helps in the conversion of PGA to citrovorum factor (CF) in rats(9). It was also of interest to find out if this conversion was further stimulated in chloretonised rats.

Methods. Biosynthesis of ascorbic acid. Male rats weighing 100-170 g were fed mineralized whole milk with 2 drops of concen-