a lower level of circulating androgen in hyperthyroid rats.

Summary. Maintenance of elevated levels of thyroxine in rats from birth to 36 days of age accelerates the maturation of the seminiferous epithelium of the testis of the rat but at the same time reduces the rate of increase in weight of the testes and the ventral prostate gland.

1. Hoskins, M. M., *J. Exp. Zool.*, 1927, v48, 373.
2. Karnofsky, D., Cronkite, E., PROC. SOC. EXP. BIOL. AND MED., 1939, v40, 568.
3. Baume, L. J., Becks, H., Evans, H. M., *J. Dent. Res.*, 1954, v33, 80.
4. DaCosta, E., Carlson, A. J., *Am. J. Physiol.*, 1933, v104, 247.
5. Cohen, R. S., *Am. J. Anat.*, 1935, v56, 143.
6. Smelser, G. K., *Anat. Rec.*, 1939, v73, 273.
7. Allen, E., *Biol. Bull.*, 1949, v97, 230.
8. Clermont, Y., Perey, B., *Am. J. Anat.*, 1957, v100, 241.
9. Leblond, C. P., Clermont, Y., *ibid.*, 1952, v90, 167.
10. Clermont, Y., Perey, B., *Rev. Canad. de Biol.*, 1957, v16, 451.

Received July 9, 1962. P.S.E.B.M., 1962, v111.

Some Properties of the Platelet-Connective Tissue Mixed Agglutination Reaction. (27771)

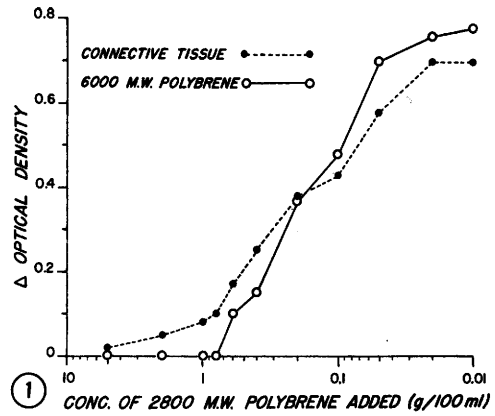
THEODORE H. SPAET, JOSE CINTRON, AND MORTON SPIVACK
Department of Hematology, Laboratory Division, Montefiore Hospital, New York

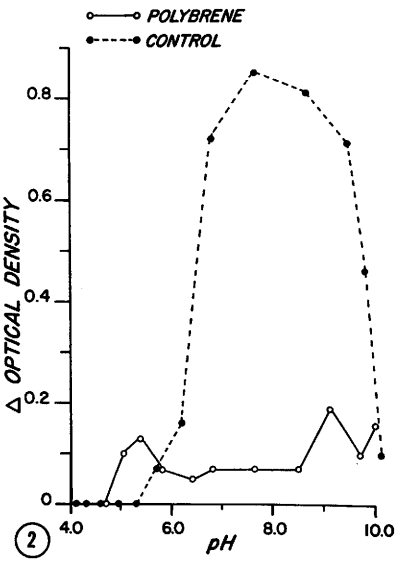
Among the earliest events in hemostasis is adherence of platelets to the site of vascular injury. It has been suggested that the effective surface is damaged cells(1), or connective tissue fibers(2-4). Zucker and Borrelli (5) have shown that suspensions of connective tissue fragments from many sources and purified collagen agglutinate platelets of plasma anticoagulated with citrate. Platelets carry a strong electronegative charge(6), and the present study explores the possibility that platelet-connective tissue mixed agglutination represents reaction with a particle possibly carrying an opposite charge.

Materials and methods. Biological reagents were of human origin. Subcutaneous fat, found to be a convenient source of connective tissue(5), was obtained during surgery from the anterior abdominal wall,* and was kept frozen until ready for use. Preparation of suspended connective tissue fragments was modified from the method of Zucker and Borrelli(5). The fat was cut into small pieces, and homogenized for about 5 minutes in a Waring Blendor with about 5 volumes of isotonic saline. The resulting material was centrifuged for 30 minutes at about 3,000 rpm in an International Clinical Centrifuge, and the middle turbid layer was used. Unless otherwise noted, it was diluted to twice the lowest effective concentration for maximal agglutination. Blood was collected into siliconized containers and anticoagulated with one-tenth volume of 3.8% sodium citrate. Platelet-rich plasma was collected following centrifugation at about 600 rpm for 15 minutes. Washed platelets(7) were trypsinized as previously described for red cells(8) with crystalline trypsin (Armour) except that the trypsin solution was diluted to 250 NF units/ml. These trypsinized platelets still gave clot retraction when one-tenth volume containing about 500,000 platelets/ml[3] was added to platelet-poor recalcified plasma. Polybrene (hexadimethrine bromide polymers) with average molecular weights (MW) of 6,000 and 2,800 respectively were supplied by Abbott Laboratories.

Agglutination of platelets was estimated semiquantitatively in a Beckman DB spectrophotometer at wavelength 610. A mixture was made containing 1 cc each of platelet-rich plasma, the agglutinating reagent, and test inhibitor. A reading was taken immediately, against platelet-poor plasma as the reference

*Kindly provided by Dr. Arthur Aufses.





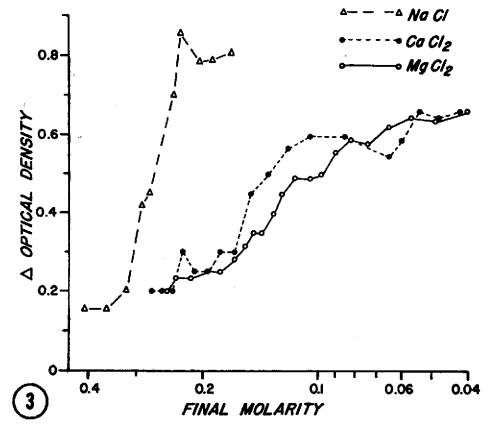


FIG. 1. Inhibition of platelet agglutination by 2,800 MW Polybrene.

FIG. 2. Effect of pH on platelet-connective tissue mixed agglutination.

FIG. 3. Inhibition of platelet-connective tissue mixed agglutination by hypertonic electrolyte solutions.

standard. The mixture was then tumbled for exactly 10 minutes at room temperature in an upright circular rotator (Baltimore Biological Laboratory), and a second reading taken. The drop in optical density correlated well with visible agglutination by phase microscopy, and was taken as a measure of agglutination intensity. Agglutination became grossly visible within 1 minute.

Unless otherwise stated, all experiments were carried out at pH of about 7.5, reagents having been adjusted with dilute HCl or NaOH. No significant pH change followed the tumbling procedure.

Results. Previous studies(9) have shown that 6,000 MW Polybrene is a potent platelet agglutinin, whereas the lower MW polymer has no such action despite its similarly positive charge. To determine whether preliminary exposure of platelets to low MW Polybrene would inhibit the agglutinating effect of the 6,000 MW compound, various concentrations of 2,800 MW Polybrene were added to platelet-rich plasma. Six thousand MW Polybrene was then added at a concentration twice that of the lowest effective concentration. Fig. 1 shows that 2,800 MW Polybrene effectively inhibits agglutination by the higher MW compound. Similar inhibition was obtained when connective tissue extract was used instead of 6,000 MW Polybrene. Concentrations of 2,800 MW Polybrene which were inhibitory to agglutination were effectively anticoagulant when added to the generating mixture in the thromboplastin generation test with human brain cephalin as the source of phosphatide.

The effect of pH and cations was studied by adding different dilutions of HCl, NaOH, or solutions of metallic chloride to connective tissue fragments and platelet-rich plasma. When the cations were used, heparin was added to the plasma at a concentration of 20 mg/100 cc, to prevent thrombin formation. An additional experiment with NaCl but without heparin gave a virtually identical curve. Fig. 2 and 3 present the results in terms of

TABLE I. Effect of Trypsinization on Platelet Agglutination.

	Buffer	Buffer + connective tissue	Plasma + connective tissue	Buffer + 6,000 MW Polybrene	Plasma + 6,000 MW polybrene
Control platelets	0	4+	4+	4+	4+
Trypsinized platelets	0	0	0	1+	1+

final mixture concentrations. Optimal agglutination occurred in the pH range of 6.5-9.5, and inhibition of agglutination by added 1% 2,800 MW Polybrene was evident throughout this range. Hypertonic electrolyte solutions were also inhibitory, and the divalent cations were more effective than NaCl at equimolar concentrations.

It has been suggested that the agglutination of platelets by thrombin is related to platelet fibrinogen, and that this effect can be abolished by prior incubation of platelets with trypsin(10). Table I shows that trypsinized platelets were not agglutinated by connective tissue fragments; but in contrast to the thrombin reaction, exposure to plasma for 15 minutes failed to restore agglutinability. Trypsinized platelets reacted less intensely with 6,000 MW Polybrene. Control platelets similarly processed, but with trypsin omitted, were normally agglutinated by both Polybrene and connective tissue.

Discussion. Agglutination of platelets by connective tissue probably is a mixed agglutination reaction, since free platelets and connective tissue fragments are both greatly diminished in agglutinated specimens observed under phase microscopy. The present studies and *in vivo* observations, although not conclusive, are compatible with the hypothesis that platelet-connective tissue mixed agglutination represents an electrostatic charge reaction. 1) Inhibition of the reaction by hypertonic electrolyte solutions and pH extremes are in line with electrostatic phenomena. These observations contrast somewhat with those of Bounameaux(4) and Hugues(3) who found that agglutinates were not dispersed by subsequent addition of hypertonic saline. 2) The agglutination reaction by 6,000 MW Polybrene is probably a charge reaction(9), and it is reasonable to suppose that its inhibition by the 2,800 MW polymer results from competition for the charged sites on the electronegative platelets between these strongly electropositive compounds. 3) Application of an electrical potential to an intact blood vessel is accompanied by development of a platelet thrombus only at the site of the anode (11). 4) The virtually instantaneous adherence of platelets to damaged endothelium(12) fits best with a physical rather than a chemical reaction.

Agglutination of platelets by connective tissue is evidently not a thrombin-fibrinogen reaction of the type described by Schmid *et al.* (10). Platelet-connective tissue agglutination occurs in an anticoagulated system; it is not inhibited by diisopropyl fluorophosphate (DFP), an agent that destroys thrombin activity(5); and platelets rendered non-agglutinable by trypsinization do not have agglutinability restored following incubation in normal plasma, a manipulation which appears to restore platelet fibrinogen(10).

It remains to be determined whether the apparent charge reaction which occurs between platelets and connective tissue *in vitro* has relevance to the essentially instantaneous formation of a platelet plug at the site of *in vivo* vascular injury.

Summary. Platelets were agglutinated by a suspension of connective tissue fragments or by 6,000 MW Polybrene. In each case agglutination was inhibited by 2,800 MW Polybrene. The agglutination reaction with connective tissue was also inhibited by hypertonic electrolyte solutions, pH extremes, and trypsinization of the platelets. Incubation of trypsinized platelets with plasma failed to restore their ability to agglutinate with connective tissue. It is suggested that platelet-connective tissue mixed agglutination is an electrostatic charge reaction.

1. O'Brien, J. R., *J. Clin. Path.*, 1961, v14, 140.
2. Bounameaux, Y., *Compt. Rend. Soc. Biol.*, 1959,

v153, 865.
3. Hugues, J., *ibid.*, 1960, v154, 866.
4. Bounameaux, Y., *Thromb. Diath. Haem.*, 1961, v6, 504.
5. Zucker, M. B., Borrelli, J., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 779.
6. Creger, W. P., Thomas, P. M., *Nature,* 1960, v186, 171.
7. Spaet, T. H., Mednicoff, I., *Bull. New England Med. Center,* 1951, v13, 201.
8. Payne, R., Spaet, T. H., Aggeler, P. M., *J. Lab. and Clin. Med.,* 1955, v46, 245.
9. Lalezari, P., Spaet, T. H., *ibid.,* 1961, v57, 868.
10. Schmid, H. J., Jackson, D. P., Conley, C. L., *J. Clin. Invest.,* 1962, v41, 543.
11. Sawyer, P. N., Pate, J. W., *U. S. Armed Forces Med. J.,* 1953, v4, 23.
12. Roskam, J., in *Blood Platelets,* ed., S. A. Johnson, R. W. Monto, J. W. Rebuck, R. C. Horn, Little, Brown & Co., Boston, 1961, p154.

Received July 11, 1962. P.S.E.B.M., 1962, v111.

Effect of Ethyl Alcohol upon Splanchnic Hemodynamics. (27772)

STEVEN M. HORVATH AND PAUL W. WILLARD
Department of Physical Education, University of California, Goleta, and Department of Pharmacodynamics, Eli Lilly and Co., Indianapolis, Ind.

Various investigators have studied the effect of ethyl alcohol infusion upon hepatic circulation(1,2,3) with conflicting results. Some investigators have reported increases, others no change in hepatic blood flow. This study was undertaken to determine the effect of slow intravenous infusion of ethanol on estimated splanchnic blood flow and cardiac output in an attempt to define more clearly the parameters by which ethyl alcohol alters blood flow through the splanchnic vascular bed.

Methods. Adult mongrel dogs (15-20 kg) were anesthetized with sodium pentobarbital (30 mg/kg body wt). Under fluoroscopic guidance a catheter was placed in a hepatic vein. A second catheter was placed in the pulmonary artery to obtain mixed venous blood. A branch of the femoral artery was cannulated for recording of pressure and an infusion cannula was placed in the saphenous vein. Heparin was given to all animals (5 mg/kg body wt) during catheterization. Hepatic vein and femoral artery pressures were measured with Statham strain gauges, appropriate amplifying and recording equipment being used simultaneously and continuously throughout the experiment. Total oxygen consumption was determined from aliquots of expired air, collected by either a Tissot or Benedict-Roth spirometer. Respiratory gas samples were analyzed for O_2 and for CO_2 where possible by a Beckman oxygen and a Liston-Becker carbon dioxide analyzer. All blood samples were analyzed for oxygen and carbon dioxide content by a modification of the Van Slyke and Neill method(4). Hematocrit values were obtained by the Wintrobe tube technic. Hemoglobins were determined by the cyanemethemoglobin method.

Hepatic blood flow was measured by means of the bromsulphalein method(5). At 10-minute intervals 3 sets of samples were drawn for bromsulphalein (BSP) analyses and determinations of control splanchnic blood flow. Following the control period a total of 500 mg of 95% alcohol/kg body weight diluted with BSP was infused over a 60-minute period. During infusion samples were drawn at 15-minute intervals for BSP and alcohol analyses.

Alcohol concentrations were determined by a modification of the method of Westerfeld *et al.*(6) and Fleming and Stotz(7) on samples obtained from the femoral artery and hepatic vein.

The amount of alcohol metabolized per minute by the liver was calculated by the formula: (Art. alcohol-Hep. vein alcohol conc.) $\times$ EHBF (ml/min). Cardiac output was calculated by the direct Fick principle.

Splanchnic vascular and total peripheral re-