1969

Choline-Induced Inhibition of Fibrinolysis in Normal and Hemophilic Dogs (Thrombelastography).

Wayne Frederick Brown II
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_disstheses/1642
This dissertation has been microfilmed exactly as received 70-9042

BROWN, II, Wayne Frederick, 1938-
CHOLINE-INDUCED INHIBITION OF
FIBRINOLYSIS IN NORMAL AND
HEMOPHILIC DOGS (THROMBELASTOGRAPHY).

The Louisiana State University and Agricultural
and Mechanical College, Ph.D., 1969
Physiology
University Microfilms, Inc., Ann Arbor, Michigan
Choline-induced Inhibition of Fibrinolysis in Normal and Hemophilic Dogs (Thrombelastography)

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Zoology and Physiology

by

Wayne Frederick Brown, II
B.S., Louisiana Polytechnic Institute, 1963
M.S., Louisiana Polytechnic Institute, 1967
August, 1969
ACKNOWLEDGMENTS

I would like to thank Dr. Blanche Jackson and Dr. H. Bruce Boudreaux for their continued support throughout my studies.

To Dr. Richard B. Myers, project Veterinarian, I express my warmest thanks for his services. John Thornhill, Don Kennedy and Ken Carrier are recognized for their care and feeding of the dogs. Miss Mary Harris is recognized for her work on auxiliary clotting studies of the dogs. I thank Dr. Kenneth L. Koonce for his efforts in statistically analyzing my data. I appreciate the efforts of my Committee in my behalf throughout my studies. The Graduate School, and the Departments of Zoology and Physiology, Entomology, and Veterinary Science are all recognized for the financial support provided for this project. The Louisiana Heart Association is recognized for the financial assistance it also provided.

Recognition is made to Eli Lilly Company for providing drugs used in preliminary experiments. Dr. Ralph Buckner of Oklahoma State University is acknowledged and thanked for supplying the project with hemophilic dogs to start the colony. He has also obligingly provided needed cryoprecipitate for the dogs. Finally, to my wife, Madeline, I give my love and appreciation for her supportive roles throughout my graduate work.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS.</td>
<td>i</td>
</tr>
<tr>
<td>LIST OF TABLES .</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES.</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT .</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS.</td>
<td>7</td>
</tr>
<tr>
<td>General design and plan of work</td>
<td>7</td>
</tr>
<tr>
<td>Dogs</td>
<td>8</td>
</tr>
<tr>
<td>Bleeding procedure</td>
<td>9</td>
</tr>
<tr>
<td>Platelet-poor plasma for Thrombelastographic studies</td>
<td>11</td>
</tr>
<tr>
<td>Platelet counts.</td>
<td>11</td>
</tr>
<tr>
<td>Special reagents</td>
<td>13</td>
</tr>
<tr>
<td>Owren's modified barbital buffer</td>
<td>13</td>
</tr>
<tr>
<td>Gelatin-barbital buffer</td>
<td>13</td>
</tr>
<tr>
<td>Streptokinase</td>
<td>13</td>
</tr>
<tr>
<td>Choline dihydrogen citrate</td>
<td>13</td>
</tr>
<tr>
<td>Epsilon-amino-caproic-acid</td>
<td>13</td>
</tr>
<tr>
<td>Plasma euglobulin fraction (human for use with streptokinase)</td>
<td>13</td>
</tr>
<tr>
<td>Euglobulin clot lysis times.</td>
<td>14</td>
</tr>
<tr>
<td>Preparation of clotting-lysis mixtures for study by thrombelastography</td>
<td>14</td>
</tr>
<tr>
<td>Thrombelastographic analysis of SK-activated fibrinolysis of dog plasma</td>
<td>16</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>19</td>
</tr>
<tr>
<td>RESULTS</td>
<td>21</td>
</tr>
<tr>
<td>Major characteristics of thrombelastograms obtained with plasma from dogs during the control periods</td>
<td>21</td>
</tr>
<tr>
<td>Topic</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>Effect of choline on thrombelastograms</td>
<td>25</td>
</tr>
<tr>
<td>Thrombelastograms after discontinuance of choline</td>
<td>28</td>
</tr>
<tr>
<td>Effect of platelet numbers</td>
<td>29</td>
</tr>
<tr>
<td>Comparison of fibrinolysis times for choline administered at five times the normal dietary level and ten times the normal dietary level</td>
<td>29</td>
</tr>
<tr>
<td>Effect of system used</td>
<td>30</td>
</tr>
<tr>
<td>Effect of citric acid</td>
<td>30</td>
</tr>
<tr>
<td>Effect of choline treatment of euglobulin clot lysis times</td>
<td>31</td>
</tr>
<tr>
<td>Influence of EACA on thrombelastographic patterns</td>
<td>31</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>33</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>42</td>
</tr>
<tr>
<td>VITA</td>
<td>49</td>
</tr>
</tbody>
</table>
# List of Tables

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Fibrinolysis times for dogs during control period, choline treatment, and after choline treatment.</td>
<td>23</td>
</tr>
<tr>
<td>2. Maximum amplitudes for dogs for control period, choline administration, and after choline administration</td>
<td>24</td>
</tr>
<tr>
<td>3. Statistical analysis of differences in fibrinolysis times and maximum amplitudes between control and choline periods.</td>
<td>26</td>
</tr>
<tr>
<td>4. Analysis of variance on data for fibrinolysis times and maximum amplitudes, testing for differences due to status of dog and system used</td>
<td>27</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Thrombelastograms of whole blood from hemophilic dog (G-3) and normal dog (V-1).</td>
<td>10</td>
</tr>
<tr>
<td>2.</td>
<td>Thrombelastograms of platelet-poor plasma from hemophilic dog (A-l) and normal dog (V-1).</td>
<td>12</td>
</tr>
<tr>
<td>3.</td>
<td>Correlation curve relating SK concentration and fibrinolysis times.</td>
<td>17</td>
</tr>
<tr>
<td>4.</td>
<td>Thrombelastogram illustrating SK-activated fibrinolysis.</td>
<td>18</td>
</tr>
<tr>
<td>5.</td>
<td>Relation between SK concentration and fibrinolysis times.</td>
<td>22</td>
</tr>
<tr>
<td>6.</td>
<td>Thrombelastograms demonstrating effects of choline and of SK concentration on fibrinolysis.</td>
<td>36</td>
</tr>
<tr>
<td>7.</td>
<td>Schematic representation of dynamics of SK-activated fibrinolysis.</td>
<td>40</td>
</tr>
</tbody>
</table>
ABSTRACT

Streptokinase-activated fibrinolysis of platelet-poor citrated dog plasma was studied by thrombelastography before, during, and after administration of choline. Plasma samples were tested in several clot-lysis systems containing: 1) human plasma or euglobulins for providing proactivator and needed clotting factors for hemophilic plasma; 2) varying concentrations of streptokinase; 3) calcium chloride.

Three hemophilic (Factor VIII-deficient) and four normal dogs were treated with choline for periods of 3 - 15 days during a 10-month study. The choline (as dihydrogen citrate), administered orally at 5 times normal dietary level, significantly increased fibrinolysis times within 24 hours in all of the dogs tested. Dosage with citric acid, for control purposes, did not cause prolongation of fibrinolysis.

Explanation of previously reported beneficial effects of choline-containing peanut extracts in hemophilia is discussed in terms of the effect on fibrinolysis reported here. It appears that high choline dosage should permit a more effective hemostatic clot in hemophilia. Observations on bleeding episodes of the hemophilic dogs used in this study give further support for the antifibrinolytic effect of high choline dosage.
INTRODUCTION

Hemophilia, in addition to its occurrence in humans, has been reported in horses (Sanger, Mairs, and Tropp, 1964), swine (Cornell and Merkner, 1964) and in dogs (Field, Richard, and Hutt, 1946; Mustard et al., 1960; Mustard et al., 1962; Brock et al., 1963; Didisheim and Bunting, 1964; Kaneko, Cordy, and Carlson, 1967). Clinical treatment and establishment of colonies for hemophilic dogs have been described (Graham et al., 1949; Sharp and Dike, 1964; Brock et al., 1963; Bird et al., 1964). The latter 2 reports were on the colony at Oklahoma State University, Stillwater, from which the dogs used in this research were obtained. Brinkhous and Graham (1950) described hemophilia in the female dog and Parks et al. (1964) recount laboratory detection of female carriers of canine hemophilia, in the colony at Chapel Hill, North Carolina, described by Graham et al. (1949). Canine hemophilia has been shown to have many similarities to hemophilia in man and has also been shown to be a sex-linked inherited deficiency of factor VIII activity in some of the dogs, as those in the colonies listed above at Chapel Hill and Oklahoma. Dogs from the Oklahoma colony formed the nucleus of the colony established at Louisiana State University and were used in the present study for testing the possible beneficial effect that choline may have on their bleeding tendency.
The idea of the use of choline as an aid in controlling mild bleeding episodes in hemophilia had its origin in the work by Boudreaux and Frampton (1960). Boudreaux, a factor VIII-activity deficient hemophiliac, observed that the tenderness of an acute hemarthritic knee seemed to subside after the ingestion of roasted peanuts. A series of experimental investigations followed to try to explain how peanuts could provide clinical relief from bleeding. Since no change in the coagulability of the blood could be found (Schmutzler, 1961) other explanations for the possible beneficial effect of peanuts were postulated. Ethanolic extracts of defatted peanuts (Boudreaux et al. 1960, Frampton et al. 1966) were further separated by solvent separation and by chromatography into fractions, one of which was reported to shorten bleeding time in hamsters. Astrup et al. (1960) suggested that the possible effect of peanuts is the shifting of the hemostatic balance in the organism due to protease inhibitors present. Protease inhibitors had been known to be present in peanuts since Borchers' report in 1947. Astrup et al. suggested that, acting as antifibrinolytic agents, such inhibitors could delay resolution of small fibrin deposits and thus improve hemostasis. Van Creveld and Mochtar (1961) as well as Moers and den Ottolander (1961) demonstrated shortened euglobulin clot lysis times in hemophilic patients consuming peanut products. Nilsson (1960) reported an antifibrinolytic effect using streptokinase and plasma from hemophilic patients who had ingested roasted peanuts. Schmutzler (1961) found a small amount
of antifibrinolytic activity in ethanol extract from peanuts. Brahman, Sjölin, and Astrup (1962) reported a decrease in spontaneous fibrinolytic activity with peanut administration. Bisordi (1964) reported clinical benefit in hemophiliacs treated with peanut flour. A potent antifibrinolytic substance extracted from peanut skins was claimed by Čepelák (1963) to promote hemostasis in hemophilia and other hemorrhagic states. Novak (1966) indicated that peanut extract injected intravenously inhibited fibrinolysis in dogs.

Frampton et al. (1966) processed peanuts through an extended series of extractions and chromatographic separations in an effort to isolate the active hemostatic material. Jackson, Owen, and Boudreaux (1966) on the basis of pharmacological and chemical studies on fractions derived from alcoholic extracts concluded that clinical improvement afforded hemophiliacs by consumption of peanuts or its extracts may be attributable to choline. Boudreaux is apparently controlling his bleeding tendency by using 8 grams daily of choline dihydrogen citrate.

A known inhibitor of fibrinolysis, epsilon-aminocaproic acid (EACA) has been used therapeutically in normal and hemophilic cases (Reid et al., 1964; Mainwaring and Keidan, 1965; Reid et al., 1965; Nilsson, 1966; Reid, Hodge, and Cerutti, 1967). An even more powerful inhibitor, trans-4-amino-methylcyclohexane-1-carboxylic acid (AMCHA) is currently claiming the interest of workers in this field. Results of an in vivo study using dogs have recently been published (Celander and Celander, 1968).
Although drawing general conclusions from experimental animals is difficult due to the large quantitative and qualitative differences between species (Astrup, 1969), testing the fibrinolytic inhibition of choline on hemophilic dogs seems worthwhile. Byshevsky (1965) exhibited inhibition of fibrinolysis in dogs with intravenous injections of choline. The purposes of my research was to test the antifibrinolytic effect of choline administered perorally to both hemophilic and normal dogs and to observe possible clinical benefit to the hemophilic dogs.

Thrombelastography (Hartert, 1951) was the choice for study of both the formation and dissolution of the clot formed from dogs administered choline. This method has been used to study hemophilia (de Nicola and Mazzetti, 1956; de Nicola, 1957a; de Nicola, 1957b) and shown to be a good system for studying fibrinolysis (Von Kaulla and Weiner, 1955; Von Kaulla, 1957; de Nicola and Mazzetti, 1957; Von Kaulla and Schultz, 1958; Astrup and Egeblad, 1965). The effect of EACA and the trypsin inhibitor in peanuts on fibrinolysis in vitro were demonstrated with thrombelastography (Egeblad, 1966, 1967a and 1967b). The role of fibrinolytic inhibitors on hemophilia was tested by Reid et al. (1965) also using thrombelastography. They found that EACA had a normalizing effect on the hemophilic thrombelastogram.

Normal values for the thrombelastograms in dogs were reported by Popisil (1966) but normal values for thrombelastograms of streptokinase-activated fibrinolysis in the normal or hemophilic dog could not be found in the literature. Studies on fibrinolysis in
the dog such as Holemans' study (1965) and on the effect of AMCHA on fibrinolysis by Celander and Celander (1968) were helpful. Very high platelet numbers may have either a fibrinolytic (Holemans and Gross, 1961a and 1961b) or an antifibrinolytic (Johnson, 1953 and Stefanini, 1956) action. Only small numbers of platelets were used in the present study, minimizing the effect of platelets on fibrinolysis.

Streptokinase-activated fibrinolysis by means of thrombelastography (de Nicola, 1957; Astrup and Egeblad, 1965) was the chosen method for measuring fibrinolysis after choline treatment of the dogs. Reviews (Sherry, and Alkjaersig 1958; Sherry, Fletcher, and Alkjaersig, 1959; Sherry, 1968; Astrup, 1969) suggest that SK-activated fibrinolysis is a useful system for measuring inhibitors of fibrinolysis. By using thrombelastography, other parameters of the clot, such as clot firmness (Weiner and Weisberg, 1957), could be observed simultaneously for differences that might occur due to choline.

It was kept in mind that if choline has a favorable influence in hemophilia it might have an unfavorable one in normal animals. In studies done on rats primarily to test the effect of butter and corn oil on the lytic phase of thrombelastograms, but which employed diets with high choline levels, pathological conditions associated with prolonged fibrinolysis times developed (Tillman et al., 1960; Lee et al., 1962; Kim et al., 1963; Lee, Kim, and Shêrman, 1963; Kim et al., 1964; Kim, Lee, and Thomas, 1965; Nam et al., 1965). Many suggestions are brought forth to explain the above phenomenon.
Beta-lipoproteins have been shown to have antifibrinolytic activity (Skrzydlewska and Niewiarowski, 1966; Riding and Ellis, 1964) and are elevated in atherosclerotic sera (Sarkar, 1961). Perhaps choline is associated with these two phenomena. But again one must be extremely careful in generalizing from one animal system to another.

The measurement of the beneficial effects and mechanism of action were the main aims of this research. If choline can be shown to be hemostatic in hemophilic dogs, then further studies using human subjects would be in order.
MATERIALS AND METHODS

General design and plan of work. Streptokinase (SK)-activated fibrinolysis was studied by thrombelastography in 5 normal and 3 hemophilic dogs. Blood samples were collected when the only source of choline was that provided by the diet and also while extra choline was given perorally. Six separate periods of choline treatment (3 - 15 days' duration) were used during the 10-month study. In 4 of the periods, 6 dogs were treated; in 1 case, 1 dog only; in the remaining period, 2 dogs. For choline dosage, commercially available tablets containing 600 mg choline dihydrogen citrate were used. Four times daily, each dog was made to swallow a tablet or a half-tablet, depending on body weight and desired dose. In certain test periods, doses of 300 mg choline citrate per 17 - 20 lb body weight were given (1200 mg/day for the adult dogs). In other treatments, the dosage was twice this amount.

For control purposes, 2 test periods were provided during which time the dogs were given citric acid 4 times daily, in quantities equivalent to those of the citrate provided in the tests with choline dihydrogen citrate. Also, for some comparison of the effect of choline on SK-activated fibrinolysis with that produced by a better-known inhibitor of fibrinolysis, two short experiments were run with EACA. In each of these, 2 dogs were given, perorally, single doses of
0.34 mg/kg body weight. Blood samples were taken in one experiment 3 hours after the EACA; in the other experiment, after 7 hours.

An essential part of this study was investigation of the composition of clotting-lysis mixtures suitable for thrombelastographic studies with hemophilic dog plasma. For promptness in clotting of hemophilic plasma and achievement of a satisfactory maximum amplitude, inclusion of the deficient clotting factor was essential. For SK-activation of the fibrinolytic system of the dog, a source of proactivator from human plasma was required.

**Dogs: source; status with regard to hemophilia; maintenance.**

The 8 dogs used in this work were members of an inbred colony of beagles. Three were originally from the Stillwater, Oklahoma colony of the School of Veterinary Medicine, Oklahoma State University; the remaining 5 were born in the newly established LSU colony.

Information on the individual animals is given below:

- **G-3**, hemophilic female, obtained as adult, Nov. '67
- **G-4**, hemophilic male, obtained as adult, Nov. '67
- **V-1**, normal male, obtained as adult, Aug. '67
- **K-1**, normal male's, born at LSU, Jan. '68, from mating of T-4 (carrier obtained from Stillwater) and G-4
- **K-2**, normal male's, born at LSU, Jan. '68, from mating of T-4 and G-4
- **K-9**, normal male, born at LSU, Jan. '69, from mating of T-4 and G-4
- **A-1**, hemophilic male, born at LSU, Sept. '68, from mating of G-3 and G-4

The adult beagles weighed 17-20 pounds. Three of the younger dogs weighed about half this amount when they were first used in
experimental work; when they were next studied, they had reached adult weight.

The hemophilic or normal states of the dogs furnished by Oklahoma State University and the dogs born in the LSU colony were verified by tests conducted in this laboratory. The dogs with bleeding disorders were shown to have classical canine hemophilia; i.e., deficient Factor VIII-activity, as indicated by the following tests: Lee-White clotting time, prothrombin time, partial thromboplastin time, and thromboplastin generation time. Results from thrombelastography of whole blood (following procedures according to Marchal, Leroux, and Samana, 1963) supported diagnoses of clotting defect (Fig. 1).

The dogs were housed in the LSU Canine Hemophilia Laboratory, a small air-conditioned building used exclusively for these studies. They were caged individually in the main kennel room, and each dog had its own outside runway (in an adjoining semi-enclosed area suitably protected against weather and insects). The animals were regularly attended and fed twice daily. Each adult dog received 40g ez/d Prescription Diet® and 160g f/d Prescription Diet® (Mark Morris Associates, Inc., Topeka, Kansas) per day. The quantity of ration described above provided 220 mg choline.

**Bleeding procedure; preparation of citrated samples.** The dogs were bled immediately after the morning feeding to avoid lipemia and shift of fluid from the blood into the digestive tract. The dogs were less excitable and easier to handle for venipuncture after feeding. Three to five ml of blood was carefully drawn from the jugular vein into a siliconized glass syringe equipped with a 20-gauge disposable
Fig. 1. Thrombelastograms of whole blood from hemophilic dog (G-3) and normal dog (V-1).
stainless steel needle. When the jugular vein was not entered with the first puncture through the skin, then the needle was replaced with a new one and the opposite vein used, minimizing tissue fluid contamination. Blood was withdrawn smoothly, at a rate of about 0.5 ml/sec. The needle was removed from the syringe and blood was dispensed into a siliconized calibrated test tube (12 mm diameter) containing the proper volume of ice-cold 3.8% sodium citrate solution required for a dilution of 9 parts blood with 1 part citrate solution. Blood remaining in the syringe was used for microhematocrit and some whole blood platelet counts. All citrated blood samples were kept in an ice bath until they were given special processing or directly used in tests, and every effort was made to avoid delay in the work. No samples were used that were more than 4 hours old.

**Platelet-poor plasma for Thrombelastographic studies.** Citrated blood samples were centrifuged at 3000 rpm (1500 g) for 10 min, using a Servall RC-2 centrifuge equipped with a HB-4 head and set for 4° C. Each plasma sample was promptly transferred by pipette into a cold test tube and kept in an ice bath. All glassware used in handling of plasma was siliconized. Counts were made to validate consideration of the plasma as "platelet-poor." With almost 200 samples, platelet number was not higher than 12,000/mm³ in 85% of the cases; slightly more than half of the counts were in the range of 3,000-7,000/mm³ (Fig. 2).

**Platelet counts.** The procedure used for whole blood was that described by Brecher, Schneiderman, and Cronkhite (1953). With samples of platelet-poor plasma prepared from citrated blood, counts
Fig. 2. Thrombelastograms of platelet-poor plasma from hemophilic dog (A-1) and normal dog (V-1).
were made without further dilution, and the appropriate factor used in calculating number of platelets per mm$^3$.

**Special reagents.** Owren's modified barbital buffer, pH 7.35, ionic strength of 0.154: 0.1N HCl, 43.0 ml; sodium veronal (diethylbarbital), 1.175 g; NaCl, 1.467 g; deionized water, to 200 ml (Owren, 1947).

**Gelatin-barbital buffer, 0.25%:** 0.25 g gelatin (175 Bloom, General Biochemicals) per 100 ml Owren's modified barbital buffer; stored at 4° C.

**Streptokinase** (Varidase, Lederle). Stock solutions were prepared by diluting 1 vial of SK (20,000 units) with gelatin-barbital buffer to concentrations of either 1,000 or 2,000 units/ml. The solution was sub-divided into 0.1 ml volumes and stored at -20° C. For each run a new vial was used, in some cases further dilutions being required.

**Choline dihydrogen citrate** (Eli Lilly and Co.). 600 mg tablets, No. 1697.

**Epsilon-amino-caproic-acid** (Sigma Chemical Co.). EACA dosage prepared by placing proper amount of crystals in gelatine capsule.

**Plasma euglobulin fraction (human) for use with streptokinase.** Lyophilized human plasma ("Anti-Hemophilic Plasma", Hyland Laboratories, Lot 260A168) was subdivided into 90-mg aliquots, equivalent to 1 ml original plasma plus the citrate used as anticoagulant. Weighings were made directly into small vials suitable for storage at -20° C and for reconstitution. Freshly separated euglobulins from these vials were prepared in the usual manner (see below) after
adjustment of the diluted plasma to pH 5.4. After centrifugation, the euglobulins were reconstituted to 1/5 original volume of plasma with gelatin-barbital buffer. Beginning with System 5, a more efficient method was employed, and a higher pH (5.9) for the precipitation step was used. This second lot of the Hyland material (Lot 260X25), equivalent to 82 ml original plasma, was reconstituted after precipitation and centrifugation in 4.2 ml gelatin-barbital buffer, divided into 0.1 ml aliquots, lyophilized and stored at -20° C. For use as a component in an SK-containing mixture for thrombelastography, the contents of a single vial were dissolved in 100 μl deionized water and kept in an ice bath. Desired volumes were transferred to the TE cuvettes as needed.

**Euglobulin clot lysis times.** Platelet-poor citrated plasma, 0.5 ml, was diluted 1:19 with cold deionized water, 9.5 ml, \((4{\circ}\ C)\). The pH of the diluted plasma was brought to 5.4 by addition of 1% acetic acid. The plasma was stored for 15 min at 4° C and then centrifuged for 10 min at 3,000 rpm (1,500 g). The precipitate was reconstituted to original volume of citrated plasma with Owren's modified buffer, pH 7.35. The solution was clotted by addition of 50 μl of 1.25% CaCl₂. The clotted euglobulins were put in a water bath of 37° C and observed every 5 min until the clot was totally lysed. The time necessary for dissolving of the clot was recorded.

**Preparation of clotting-lysis mixtures for study by thrombelastography.** Five different systems were used. For those containing human plasma, the procedure was the following: Into a prewarmed test tube (10 x 75 mm) kept in a 37° C water bath, desired volumes
of the components were added in rapid succession: 2 volumes of platelet-poor citrated dog plasma; 1 volume of reconstituted human plasma (Ortho-Diagnostic); 0.1 volume of SK-mixture; and 0.3 volume of 1.29% CaCl\(_2\) solution (warmed). A timer was started upon addition of the calcium chloride. The test tube was inverted several times for mixing of its contents. Immediately after, 0.35 ml of the mixture was transferred to the temperature-equilibrated TE cuvette, the appropriate pin was lowered, and the surface of the sample covered with oil. When the timer read 60 sec, the light for that cell was turned on.

Composition of the 3 mixtures containing human plasma differed in only one important respect: namely, concentration of SK in the system. These concentrations were 14.7, 38.5, and 29.1 SK units/ml final mixture in Systems 1, 2, and 3, respectively. Volumes of the several components used in System 1 were the following: 0.40 ml dog plasma, 0.20 ml human plasma, 20 \(\mu\)l SK-mixture containing 10 units SK; 60 \(\mu\)l CaCl\(_2\) solution. Systems 2 and 3 were prepared with volumes of components proportional to those given above, but total volumes were reduced slightly.

With mixtures containing human euglobulins (Systems 4 and 5), the following procedure was used. Into the thrombelastograph cuvette (37° C), the components other than calcium chloride were added in rapid succession. A stop watch was started upon transfer of the first component, 0.25 ml platelet-poor dog plasma; addition of 25 \(\mu\)l each of a human euglobulin preparation and of a diluted SK-mixture followed. At precisely 2 min after transfer of the dog plasma, 50 \(\mu\)l CaCl\(_2\)
(1.29%) was added, and the pin lowered and raised 3 times for mixing. The pin was lowered once more, and the surface of the liquid covered with oil. At 3 min after the starting of the stop watch, the light switch for the cuvette was turned on.

As indicated previously, 2 different preparations of euglobulins were used. Also, relative concentrations of the euglobulins in the clotting-lysis mixtures differed. Thus, in System 5, the second lot of euglobulins (precipitated at pH 5.9) was used in a concentration 4 times that of the first lot with System 4 (precipitated at pH 5.4). Concentrations of SK in the 2 systems differed slightly -- in System 4, final SK concentration was 6.5 units/ml; in System 5, 6.8 units/ml. Rapid lysis of the clots with such low concentration of SK is explained in large measure by the reduction in concentration of inhibitors of fibrinolysis in preparing the euglobulins from the human plasma used. Before making decisions concerning concentrations of SK to be routinely used in System 4 and in System 5, correlation curves were constructed from data on fibrinolysis times of mixtures using a given sample of dog plasma and varying concentrations of SK (Fig. 3 for correlation curve on V-1, using first euglobulin preparation).

Thrombelastographic analysis of SK-activated fibrinolysis of dog plasma. A thrombelastogram of SK-activated fibrinolysis of platelet-poor citrated dog plasma is represented in Fig. 4. Fibrinolysis time, beginning from the time of clotting to complete lysis, was measured in millimeters from the end of the reaction time, r, to a point, f, at which the light had stopped all movement, representing the complete
Fig. 3. Correlation curve relating SK concentration and fibrinolysis times. Data were obtained on tests with plasma from a normal adult dog (V-1), using human euglobulins as proactivator.
Fig. 4. Thrombelastogram illustrating SK-activated fibrinolysis. The clot-lysis mixture contained platelet-poor plasma from an adult hemophilic dog (G-3), a human euglobulin fraction, CaCl$_2$, and streptokinase. On diagram: 
$\text{r}$, reaction time; $\text{k}$, clot formation time; $\text{ma}$, maximum amplitude; $\text{f}$, end of fibrinolysis.
lysis of the clot. Since the film moved at 2 mm/min while being exposed in the thrombelastograph, the above measurement was divided by 2 to convert the fibrinolysis time measured in millimeters to fibrinolysis time expressed in minutes.

The maximum amplitude, a representation of clot firmness, is the greatest distance in millimeters between the two branches of the thrombelastogram. The maximum amplitude for normal platelet-poor citrated human plasma is considered to be 20 mm. Arbitrarily, 20 mm was chosen to be the end of k, clot formation time (deNicola, 1957).

Statistical Analysis. An analysis of variance was used to test statistically the effect of choline on fibrinolysis in the dog. The general least squares procedure was used in order to remove the effects due to disproportionate sub-class numbers as well as to remove the effects due to the co-variables, the day of choline administration and the number of platelets in the plasma. The variables subjected to this analysis were differences in control and choline test period fibrinolysis times and in control and choline test period maximum amplitudes. To obtain observations as measured for the above variables, values for the control period were averaged, and this average subtracted from the corresponding observed value for each day of choline treatment. In using the following model for determining population, all the estimates were independent. The model for the analysis of variance is as follows:

\[ Y_{ijkl} = \mu + S_i + D_{ij} + P_k + \beta_1 (x_{ijkl} - \bar{x}) + \beta_2 (x_{ijkl} - \bar{x})^2 + \beta_3 (x_{ijkl} - \bar{x})^3 + \epsilon_{ijkl} \]
where

\( Y_{ijkl} \) is observation as measured

\( \mu \) is mean of observations as measured

\( S_i \) is effect of "i" status (hemophilic or normal)

\( D_{ij} \) is effect of "j" (individual) dog within "i" status

\( P_k \) is effect of "k" system of 5 systems used

\( \beta_1 \) is partial linear regression coefficient

\( x_{ijkl} \) is day of "l" observation on "k" procedure of "j" dog in "i" status

\( \bar{x} \) is mean day of observation

\( \beta_2 \) is partial quadratic regression coefficient

\( \beta_3 \) is partial cubic regression coefficient

\( \epsilon_{ijkl} \) is random error due to all variables not accounted for previously

To test for statistically significant differences due to the status of the dog, the day of choline treatment, and the procedure used, F values were calculated from the above estimates (\( F = \text{variance ratio} \)).

To test for statistically significant differences due to the effect of choline on fibrinolysis times and maximum amplitudes, t values were calculated (\( t = \frac{\text{least squares mean}}{\text{standard error}} \)).

In all cases of statistical analysis, statistical significance was considered only at the 1% level of probability.
RESULTS

Major characteristics of thrombelastograms obtained with plasma from dogs during the control periods; influence of SK concentration and of the form in which human proactivator supplied.

With mixtures containing human plasma as source of proactivator, influence of SK concentration was conspicuous in shifting fibrinolysis times. Plasma samples from 2 dogs (V-1 and G-4) were tested with each of the 3 concentrations of SK. Plotting of log fibrinolysis time versus log concentrations of SK produces essentially a straight line (Fig. 5), as was found by Astrup (1965) using purified reagents. Mean fibrinolysis times for dog V-1 obtained with mixtures containing 14.7, 38.5, and 29.1 SK units/ml (Systems 1, 2, and 3) were 133, 12, and 51 min, respectively. For dog G-4, comparable values were 183, 25, and 73 min, respectively (Table 1). For both dogs, the maximum amplitudes developed with the lowest and the intermediate concentrations of SK were considered satisfactory; with 38.5 units/ml (System 2), however, maximum amplitudes were sometimes less than 20 mm (Table 2). Data obtained on dogs G-3, K-1, K-2, and K-3 with Systems 2 and 3 followed the trends described for V-1 and G-4. Clots prepared with plasma from the young dogs (K-1, K-2, K-3) lysed somewhat faster than those with plasma from adult dogs. Also, clots containing plasma
Fig. 5. Relation between SK concentration and fibrinolysis times. Data were obtained on tests with plasma from adult normal dog (V-1), using human plasma as proactivator. Influence of choline on SK activity is exhibited by shift in position of the curve.
Table 1. Fibrinolysis times for dogs during control period, choline treatment, and after choline treatment. Values given are means for the number of observations indicated in parentheses. Times for the period after treatment are for samples collected 5 or more days after discontinuance of choline.

<table>
<thead>
<tr>
<th>System</th>
<th>Choline</th>
<th>Period</th>
<th>G-4d</th>
<th>V-1c</th>
<th>G-3c</th>
<th>K-1c</th>
<th>K-2c</th>
<th>K-3c</th>
<th>A-1c</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>300 mg/dose</td>
<td>Control</td>
<td>183(6)</td>
<td>133(7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Choline*</td>
<td>509(6)</td>
<td>221(6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>172(3)</td>
<td>117(4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>600 mg/dose</td>
<td>Control</td>
<td>25(3)</td>
<td>16(4)</td>
<td>27(1)</td>
<td>15(3)</td>
<td>9(3)</td>
<td>14(3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Choline*</td>
<td>61(3)</td>
<td>44(3)</td>
<td>92(3)</td>
<td>33(2)</td>
<td>28(2)</td>
<td>23(2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>19(2)</td>
<td>14(2)</td>
<td>23(2)</td>
<td>21(3)</td>
<td>16(3)</td>
<td>25(3)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>300 mg/dose</td>
<td>Control</td>
<td>73(3)</td>
<td>51(3)</td>
<td>86(1)</td>
<td>36(2)</td>
<td>29(3)</td>
<td>48(3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Choline*</td>
<td>279(3)</td>
<td>155(3)</td>
<td>285(3)</td>
<td>125(2)</td>
<td>110(2)</td>
<td>75(2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>74(2)</td>
<td>55(2)</td>
<td>108(2)</td>
<td>64(3)</td>
<td>44(3)</td>
<td>58(3)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>300 mg/dose</td>
<td>Control</td>
<td>25(2)</td>
<td></td>
<td>13(1)</td>
<td>13(1)</td>
<td>10(1)</td>
<td>20(1)</td>
<td>14(1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Choline*</td>
<td>81(3)</td>
<td></td>
<td>62(3)</td>
<td>50(2)</td>
<td>36(2)</td>
<td>86(2)</td>
<td>44(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>11(1)</td>
<td></td>
<td>14(1)</td>
<td>13(1)</td>
<td>13(1)</td>
<td>13(1)</td>
<td>7(1)</td>
</tr>
<tr>
<td>5a</td>
<td>300 mg/dose</td>
<td>Control</td>
<td>54(3)</td>
<td></td>
<td>44(3)</td>
<td>45(3)</td>
<td>28(3)</td>
<td>50(3)</td>
<td>25(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Choline*</td>
<td>158(5)</td>
<td></td>
<td>113(5)</td>
<td>121(5)</td>
<td>117(5)</td>
<td>189(3)</td>
<td>88(5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>After</td>
<td>77(2)</td>
<td></td>
<td>84(2)</td>
<td>23(2)</td>
<td>21(2)</td>
<td>39(2)</td>
<td>28(2)</td>
</tr>
<tr>
<td>5b</td>
<td>600 mg/dose</td>
<td>Control</td>
<td>77(2)</td>
<td></td>
<td>23(2)</td>
<td>21(2)</td>
<td>39(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Choline*</td>
<td>180(3)</td>
<td></td>
<td>93(3)</td>
<td>48(2)</td>
<td>90(2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Statistically significant at 1% level.
Table 2. Maximum amplitudes for dogs during control period, choline treatment, and after choline treatment. Values given are means for the number of observations indicated in parentheses. Measurements for the period after treatment are for samples collected 5 days or more after discontinuance of choline.

<table>
<thead>
<tr>
<th>System</th>
<th>Choline</th>
<th>Period</th>
<th>G-4d</th>
<th>V-1o</th>
<th>G-3o</th>
<th>K-1o</th>
<th>K-2o</th>
<th>K-3o</th>
<th>A-1o</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>300 mg/dose</td>
<td>Control</td>
<td>37(6)</td>
<td>31(7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Choline</td>
<td></td>
<td>32(6)</td>
<td>30(6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>After</td>
<td></td>
<td>32(3)</td>
<td>33(4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>600 mg/dose</td>
<td>Control</td>
<td>23(3)</td>
<td>21(4)</td>
<td>26(1)</td>
<td>16(3)</td>
<td>16(3)</td>
<td>17(3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Choline*</td>
<td></td>
<td>27(3)</td>
<td>24(3)</td>
<td>34(1)</td>
<td>24(2)</td>
<td>20(2)</td>
<td>20(2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After</td>
<td></td>
<td>20(3)</td>
<td>19(2)</td>
<td>25(2)</td>
<td>23(3)</td>
<td>19(3)</td>
<td>22(3)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>600 mg/dose</td>
<td>Control</td>
<td>32(3)</td>
<td>21(3)</td>
<td>33(1)</td>
<td>22(2)</td>
<td>19(3)</td>
<td>20(3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Choline</td>
<td></td>
<td>30(3)</td>
<td>27(3)</td>
<td>37(3)</td>
<td>28(2)</td>
<td>26(2)</td>
<td>27(2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>After</td>
<td></td>
<td>28(2)</td>
<td>27(2)</td>
<td>37(2)</td>
<td>28(2)</td>
<td>25(3)</td>
<td>29(3)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>300 mg/dose</td>
<td>Control</td>
<td>15(2)</td>
<td></td>
<td>8(1)</td>
<td>12(1)</td>
<td>11(1)</td>
<td>11(1)</td>
<td>11(2)</td>
</tr>
<tr>
<td></td>
<td>Choline*</td>
<td></td>
<td>21(3)</td>
<td></td>
<td>15(3)</td>
<td>23(2)</td>
<td>20(2)</td>
<td>17(2)</td>
<td>16(3)</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td></td>
<td>11(1)</td>
<td></td>
<td>10(1)</td>
<td>17(1)</td>
<td>14(1)</td>
<td>9(1)</td>
<td>5(1)</td>
</tr>
<tr>
<td>5a</td>
<td>300 mg/dose</td>
<td>Control</td>
<td>34(3)</td>
<td></td>
<td>27(3)</td>
<td>35(3)</td>
<td>33(3)</td>
<td>34(3)</td>
<td>29(3)</td>
</tr>
<tr>
<td></td>
<td>Choline*</td>
<td></td>
<td>47(5)</td>
<td></td>
<td>47(5)</td>
<td>52(5)</td>
<td>44(5)</td>
<td>35(5)</td>
<td>38(5)</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td></td>
<td>34(2)</td>
<td></td>
<td>35(2)</td>
<td>31(2)</td>
<td>26(2)</td>
<td>35(2)</td>
<td>35(2)</td>
</tr>
<tr>
<td>5b</td>
<td>600 mg/dose</td>
<td>Control</td>
<td>34(2)</td>
<td></td>
<td>31(2)</td>
<td>26(2)</td>
<td>35(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Choline*</td>
<td></td>
<td>38(3)</td>
<td></td>
<td>36(2)</td>
<td>28(2)</td>
<td></td>
<td>37(2)</td>
<td>32(2)</td>
</tr>
</tbody>
</table>

*Statistically significant at 1% level.
from V-1 (normal adult dog) lysed more rapidly than did those with plasma from G-3 or G-4 (hemophilic adult dogs), but whether characteristic differences in fibrinolysis times between adult normal and hemophilic dogs of this colony occur cannot be determined from the 3 dogs tested. There was no significant difference between hemophilic and normal dog fibrinolysis times and maximum amplitudes as measured in all 5 systems tested (Table 4).

With the concentration of SK chosen for use with the first euglobulin preparation (System 4), lysis times ranged from 10 - 25 min in the 6 dogs tested (G-3, G-4, A-1, K-1, K-2, K-3). Maximum amplitudes were rather low (8-15 mm).

With clot-lysis mixtures designated as System 5 (containing the second euglobulin preparation in relatively high concentration), SK was used at almost the same concentration as in the preceding series. Average maximum amplitudes attained in thrombelastograms of the 6 dogs tested ranged from 27 - 34 mm, more than twice those shown with System 4. Fibrinolysis times were also increased, average values ranging from 25 - 54 minutes.

Effect of choline on thrombelastograms. Statistical analysis combining all 5 test systems showed that choline administration significantly increased fibrinolysis times and maximum amplitudes as measured (Table 3). Only Systems 2 and 4 did not demonstrate a significant increase in fibrinolysis times even though the mean fibrinolysis times were more than or almost doubled in every case. This lack of statistical significance may be because the fibrinolysis times of Systems 2 and 4 were shorter in general than those
Table 3. Statistical analysis of differences in fibrinolysis times and maximum amplitudes between control and choline periods. Analysis includes all variations introduced while making observations.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Number of Observations</th>
<th>Least-Squares Mean</th>
<th>Standard Error</th>
<th>t Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinolysis:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline minus control</td>
<td>106</td>
<td>161.82</td>
<td>15.25</td>
<td>12.58*</td>
</tr>
<tr>
<td>Maximum Amplitude:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Choline minus control</td>
<td>106</td>
<td>4.23</td>
<td>1.11</td>
<td>3.81*</td>
</tr>
</tbody>
</table>

*Statistically significant at 1% level.
Table 4. Analysis of variance on data for fibrinolysis times and maximum amplitudes, testing for differences due to status of dog and system used.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Source of Variation (VS)</th>
<th>Degree of Freedom (DF)</th>
<th>Sum of Squares (SS)</th>
<th>Mean Squares (MS)</th>
<th>Variance Ratio (F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinolysis: Choline minus Control</td>
<td>Between Status (Hemophilic or normal)</td>
<td>1</td>
<td>126366</td>
<td>126366</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>Between Procedures</td>
<td>4</td>
<td>1424449</td>
<td>356112</td>
<td>21.89*</td>
</tr>
<tr>
<td>Maximum Amplitude: Choline minus control</td>
<td>Between Status (Hemophilic or normal)</td>
<td>1</td>
<td>17.8</td>
<td>17.8</td>
<td>0.186</td>
</tr>
<tr>
<td></td>
<td>Between Procedures</td>
<td>4</td>
<td>1025.7</td>
<td>256.4</td>
<td>5.68*</td>
</tr>
</tbody>
</table>

*Statistically significant at 1% level.
of the other systems, resulting in a smaller least squares mean while still having approximately the same standard error. Table 1 illustrates the mean fibrinolysis times and those times for choline periods found significantly different from control periods. In System 2, the maximum amplitudes are significantly increased, while Systems 1 and 3 do not demonstrate this effect. Maximum amplitudes during choline treatment are 20 mm or more in almost every case (Table 2).

In the first euglobulin mixture (System 4, 6.5 SK units/ml), mean fibrinolysis times range from 36-81 min in the same dogs previously tested without choline treatment (Table 1). Mean maximum amplitudes are all significantly increased into a range of 15-23 mm (Table 2).

System 5 (euglobulin mixture containing 6.8 SK units/ml) produced mean fibrinolysis times of dogs on choline treatment ranging from 88-159 min, and mean maximum amplitudes of 35-52 mm. Fibrinolysis times and maximum amplitudes are both significantly increased (Tables 1 and 2).

Thrombelastograms after discontinuance of choline. With samples collected 5 days or more after choline treatment, fibrinolysis times and maximum amplitudes for the majority of the dogs were in the range noted before choline treatment. System 1 produced a mean fibrinolysis time of 172 min and a mean maximum amplitude of 32 for G-4; for dog V-1, 117 min and 33 mm. Systems 2 and 3 provide data which follows the trends described for dogs G-4 and V-1.
(Tables 1 and 2). Fibrinolysis times of those samples collected 5 days or longer after choline treatment decreased significantly from those times measured during choline treatment. No significant decrease in maximum amplitudes in the plasmas collected 5 days or longer after cessation of choline was observed.

In System 4 the mean fibrinolysis times for all the dogs tested ranged from 7 - 14 min, slightly lower than the range found before choline treatment but not significantly lower than during choline treatment. Mean maximum amplitudes range from 5 - 17 mm, lower than during choline treatment but not significantly so.

Mean fibrinolysis times in System 5 after choline treatment range from 21 - 35 mm. These values represent a significant decrease from those obtained when the dogs were on choline. There are no statistically significant differences between the "before" and "after" fibrinolysis times and maximum amplitudes in any of the experiments.

Effect of platelet numbers. Although the dog platelet-poor plasma samples contained varying numbers of platelets, no effect of platelet numbers could be correlated with fibrinolysis times. A negative linear regression coefficient (-0.6057), relating differences between control and test period maximum amplitudes with platelet numbers, supported the correlation that as platelet numbers in the plasma sample increased, the differences between control and test period maximum amplitudes decreased.

Comparison of fibrinolysis times for choline administered at five times the normal dietary level and ten times the normal dietary
level. In addition to 1 period in which 6 dogs were given 5 times the normal dietary level of choline, 5 of the dogs were given twice that amount of choline while using the same system, System 5. Differences for the fibrinolysis times between the 2 treatments are not significant.

**Effect of system used.** Significant differences between both the fibrinolysis times and maximum amplitudes recorded in the various systems used were found (Table 4). That is to say, the testing system can be adjusted to provide data within desired ranges of measurement.

**Effect of citric acid.** Citric acid in amounts equivalent to the citrate in choline citrate at 10 times the normal dietary level of choline was given to 6 dogs (G-3, G-4, K-1, K-2, K-3, V-1) to test the effect of citrate ions on fibrinolysis times. Using Systems 2 and 3, an increase in fibrinolysis times was not observed. With System 2, the mean fibrinolysis time for the 6 dogs during control period was 20 min with a standard deviation of ± 6; for citric acid test period, 16 ± 5 min. With System 3, the mean fibrinolysis time for the control period was 72 ± 21 min; for citric acid test period, 50 ± 12 min. One-half of the above dose of citric acid was given to 3 dogs (A-1, K-1, K-2). The mean fibrinolysis time, using System 5, was 24 ± 7 min for the control period; 22 ± 7 min for the citric acid test period.

Of 15 observations made during citric acid treatment, an increase in fibrinolysis time occurred in 1 case, in another there
was no change, and in the remaining 13, the fibrinolysis times were less than during the control period.

**Effect of choline treatment of euglobulin clot lysis times.**

To supplement the information gathered on fibrinolysis times with the thrombelastograph, euglobulin clot lysis times were measured with some of the plasmas obtained for use on the thrombelastograph while using System 5. This supplementary information was obtained in order to have an additional measure of fibrinolytic activity or experimental inhibition of it.

Six dogs (G-4, G-3, A-1, K-1, K-2, K-3) administered choline at 5 times the normal dietary level had a mean euglobulin clot lysis time of 83 min with a standard deviation of ±11. The mean euglobulin clot lysis time for samples collected 5 days or more after stoppage of choline administration was 29 ± 8 min. Five dogs (G-4, A-1, K-1, K-2, K-3) were then given choline at twice the above dosage and had a mean euglobulin clot lysis time of 35 ± 4 min, as compared to a control period mean of 28 ± 3 min.

Euglobulin clot lysis times were significantly increased by choline in both of the above experiments.

**Influence of EACA on thrombelastographic patterns.** One dose of EACA, 0.34 mg/kg, was given to 2 normal dogs. Using System 5, fibrinolysis times during a non-treatment period for adult normal dog V-1 and young normal K-9 were 18 and 33 min, respectively. Three hours after 1 peroral dose of EACA, fibrinolysis times increased to 190 min for dog V-1 and 300 min for dog K-9. Seven hours after another dose 5 days later, the times were much shorter — V-1, 60 min; K-9, 110 min. The following day, the times were even
lower, approaching the non-treatment values. Maximum amplitudes were increased after the 3-hour dose and lessened during the following observations described above.

Epsilon-aminocaproic acid had great inhibition on fibrinolysis in vivo a short time after ingestion and absorption. Fibrinolytic inhibition by EACA diminishes markedly after 3 hours (Niewiarowski and Wolosowicz, 1966).
DISCUSSION

It is yet to be demonstrated without question that hemophilic bleeding can be partially controlled with perorally administered drugs which do not alter the clotting mechanisms. But the theoretical basis for such is at hand, and is supported by the studies here reported. The theory of Astrup et al. (1960) suggests that while there is a dynamic balance between continuous fibrin formation and fibrinolysis in normal people, in hemophilia the balance is upset by retarded fibrin formation while fibrinolysis is essentially normal but possibly acting on the slowly formed fibrin to prevent proper hemostasis. Therefore, hemophilic bleeding might be partially controlled by prolonging fibrinolysis, thus partially restoring the dynamic hemostatic balance.

Choline, shown to be a major ingredient of peanut extracts which shorten bleeding time, is here revealed as a substance which can delay fibrinolysis, both as measured by the euglobulin clot lysis method, and as measured by thrombelastographic recording of streptokinase-activated fibrinolysis of plasma clots. A possible benefit to hemophiliacs of prolonged fibrinolysis is more than retention of a slow-forming clot: Retardation of fibrinolysis is important to enable the clot to form in the first place. When fibrinolysis was quickly activated with SK mixtures in this study,
even normal dog blood failed to clot enough to enlarge the thrombelastogram. With the SK mixtures, it was necessary to cause the hemophilic dog blood to clot before full activation of fibrinolysis. Otherwise, the mixture exhibited no clotting on the thrombelastograms. It seems that as clotting began, the incipient clot was unable to form because of concurrent fibrinolysis. Support for this idea comes from observations that thrombelastograms of a sample of dog plasma without SK always indicated a more elastic clot (higher maximum amplitudes) than did a substitute sample of the same plasma treated with SK. The slightly lowered elasticity of the latter could be the result of slight early initiation of some fibrinolysis while clotting was proceeding.

Streptokinase-activated fibrinolysis parallels natural activation of fibrinolysis in the body in that the SK-proactivator complex activates plasminogen, converting it into plasmin. One may compare this type fibrinolysis with that initiated by adding plasmin directly to the system. In the latter case, the maintenance of a maximum amplitude on the thrombelastogram would not occur. The thrombelastographic patterns produced by plasmin and activators of plasminogen are distinctly different (Astrup and Egeblad, 1965).

There is slight evidence that choline treatment may indeed be hemostatic in hemophilic dogs. During 2 choline treatment periods the toenails of the adult male bleeder (G-4) were clipped closely to induce bleeding. The bleeding from toenails stopped permanently in less than 3 hours in both cases. The immature male bleeder (A-1) had his toenails clipped similarly but was not under choline
treatment in one case. His toenails bled for 10 hours before choline was finally administered late at night and the bleeding was stopped by the next morning. At another time though, when under choline treatment, this young dog did bleed intermittently from a clipped toenail for 24 hours. However, he was very active and the bleeding was more a rebleeding through disturbance of the wound. Before choline treatment, his fibrinolysis times were rather short; under treatment they became about as long as the normal times of the other dogs. On another occasion this dog had a large ecchymosis or hematoma which was progressively getting larger in his neck where a venipuncture had been performed. He was then treated with choline and after 1 day the enlargement seemed to stop, and after 3 days the blue coloration had disappeared and there was a hard thrombus where the bleeding had occurred. In another case, dog G-4, was observed to be bleeding as evidenced by the presence of blood in his feces and by a low hematocrit. Fibrinolysis times indicated enhanced fibrinolytic activity (Fig. 6). He was treated with choline and 3 days later his fibrinolysis times were greatly increased, indicating decreased fibrinolytic activity. His feces were also observed to appear normal. Eight days after the last dose of choline, fibrinolysis times had returned to a normal range, although never as short as they had been during the bleeding period. These observations, while not proving the hemostatic action of choline, do suggest that further investigations are in order.
Fig. 6. Thrombelastograms demonstrating effects of choline and of SK concentration on fibrinolysis. Patterns are for adult hemophilic dog (G-4), using human euglobulin fraction as source of proactivator and of clotting factors. In each of the three groups, the top recording is for the highest SK concentration. A. Five hours before choline. At this time dog had blood in feces and low hematocrit. Fibrinolysis time shorter than usual control valves. B. After 3 days of choline treatment. C. Eight days after last dose of choline.
While the above discussion may imply that choline treatment might relieve difficult cases of bleeding, any beneficial effect of choline may be overridden by increased fibrinolytic action caused by stress, such as excessive loss of blood, repeated blood sampling, trauma, strenuous exercise, and even possibly excessive alcoholic consumption. Fearnley, Ferguson, Chakrabarti, and Vincent (1960) reported increased fibrinolytic activity of blood after beer consumption. During the early phase of one period of choline administration, my test dogs were bled daily, and larger than usual volumes were taken. This period was not included in the results because the daily sampling caused a rapid decrease in fibrinolysis times. The same dogs were continued on choline treatment without interruption and subsequently they were bled every other day. The clot lysis times during this second period significantly became prolonged (this was System 5, reported above). Celander and Celander (1968) report increased fibrinolytic activity in dogs resulting from daily bleedings.

Another implication that might arise is that excessive choline consumption in normal subjects may result in depression of fibrinolysis so that interference with resolution of thrombotic deposits in the vascular system may occur. This interference could possibly manifest itself in resultant pathology of the cardiovascular system.

This study is a first attempt at studying fibrinolysis of hemophilic blood using thrombelastography. It was necessary to modify techniques previously used. The first problem was the
formation of a satisfactory clot before clot lysis occurred. Thrombin was not useful because a satisfactory maximum amplitude could not be obtained. My choice of the several activators of fibrinolysis was streptokinase. Urokinase proved to be unsatisfactory using whole blood because clot lysis was not uniform and high levels of urokinase were needed. With streptokinase, it was necessary to use a human source of proactivator. Human diagnostic plasma provided proactivator and clotting factors for hemophilic plasma but contained some undesirable factors (e.g., inhibitors of fibrinolysis). Some advantage was gained by selecting human euglobulins as a source of proactivator and of clotting factors for hemophilic plasma. Much less SK was needed with the euglobulins (Systems 4 and 5).

This study employed platelet-poor dog plasma because the platelet-rich clots in the thrombelastograph produced large maximum amplitudes, and lysed irregularly, in that the clots would break away from walls of the cuvettes. A level of platelets of 3000/mm$^3$ or so produced satisfactory clots that lysed smoothly. A higher platelet level of 10000 to 20000/mm$^3$ is undesirable in that it tends to mask the higher maximum amplitude values of choline treated animals. These higher maximum amplitudes are interpreted to signify that choline treatment delays fibrinolysis in the test system long enough for the maximum amplitude to be reached.

Since choline was used in the citrate form, it was necessary to explore the effect of the citrate alone on fibrinolysis.
Although the results seemed to indicate no statistical difference in fibrinolysis times between untreated and citrate-treated dogs, the times with citrate were shorter. There may be a high dosage level at which the citrate may interfere with the choline effect.

It appears that choline prolongs fibrinolysis only after it has been metabolized. Though the choline delayed fibrinolysis times, the delay appeared within 24 hours, but the effect did not manifest itself as quickly as did the effect of EACA, which was evidenced 3 hours after administration. The action of EACA is as a direct inhibition of plasminogen activation (Alkjaersig, Fletcher, and Sherry, 1959; Ablondi, Hagan, Philips and DeRenzo, 1959). Choline, like EACA, also seems to mediate inhibition of plasminogen activation. In thrombelastographic analysis, persistence of a constant maximum amplitude for a short while before visible lysis is evidence that plasminogen activation is delayed (Astrup and Egeblad, 1965). Also, a shift in the curve in the log plot of fibrinolysis time vs. SK concentration indicates that a constant percentage of SK is rendered ineffective by choline, suggesting that inhibition of fibrinolysis is acting at the plasminogen activation level. Although it seems that choline may be inhibiting the activation of plasminogen, the mechanism is unknown.

Figure 7 illustrates the difference between thrombelastograms of SK-activated clot lysis and of lack of lysis of dog plasma clot and the effect of plasmin activity on maximum amplitude. The maximum amplitude of the unlysed plasma clot is higher than that
Fig. 7. Schematic representation of dynamics of SK-activated fibrinolysis. See text for discussion.
of the clot which lyses. The difference in maximum amplitudes is a measure of the activity of plasmin, as shown below the zero line. The activity of plasmin occurs after plasminogen activation. A small initial activation probably occurs, followed by an increasing later activation which causes the clot to lyse more rapidly.

Although choline is acting as an antagonist to SK-activated fibrinolysis, observations on the bleeding episodes of the dogs mentioned previously and the prolonged euglobulin clot lysis times indicate that the choline effect is not restricted to inhibition of SK-activated fibrinolysis.

An understanding of the action of choline in fibrinolysis will likely come when it is known through which of the roles the effect is mediated: 1) methyl donor; 2) lipotropic agent; 3) precursor to phosphatidyl ethanolamine, which is a known inhibitor of fibrinolysis (Reid et al., 1965), or 4) any of the other roles choline may play in metabolism.
LITERATURE CITED


VITA

Wayne Frederick Brown, II was born July 9, 1938 in Shreveport, Louisiana. He received his elementary and secondary education in the same city, receiving a diploma from St. John's High School in 1957. He attended Louisiana Polytechnic Institute and Louisiana State University School of Medicine before serving a year's active duty in the U. S. Army. He reentered Louisiana Polytechnic Institute and received a B.S. in 1963 and a M.S. in 1967. He began study toward a Ph.D. at Louisiana State University Graduate School in 1966. He is married to Madeline Monroe Brown and they are the parents of two children - Leonda Lee, 5, and Wayne F. III, 4.
EXAMINATION AND THESIS REPORT

Candidate: Wayne Frederick Brown, II
Major Field: Zoology
Title of Thesis: Choline-Induced Inhibition of Fibrinolysis in Normal and Hemophilic Dogs (Thrombelastography)

Approved:

Blanche Jackson
Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

H. Bruce Baez

Albert H. Meigs

W. J. Karmann

Date of Examination:

21 July 1969