which then underwent decarboxylation and recarboxylation, losing the label in the process. In addition to absence of known metabolic transformations of this type, however, lack of incorporation of C^{14} from C^{14}-labelled glucose into the hippuric acid renders this possibility unlikely.

Summary. 1. Humans and rats receiving a purified laboratory diet excrete hippuric acid, 1 to 3 mg/kg/day. This amount appears to be formed endogenously and its excretion in the rat is not affected by fasting, by inclusion of sulfasuxidine in the diet, or by injection of phenylalanine. 2. 0.05% of the radioactivity of 3-C^{14}-L-phenylalanine injected into a rat was excreted in 24 hours in the form of hippuric acid. Further experiments suggested that endogenous hippuric acid could not be derived from phenylalanine in any direct manner.

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Studies on Thrombin Formation. (22135)

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Treatment of plasma with barium sulfate has been reported to remove the following clotting factors: Prothrombin, the serum prothrombin accelerator system*(1), plasma thromboplastin component*(2) and plasma thromboplastin*(3). However, antihemophilic factor* (AHF) and plasma-accelerator-globulin* (PacG) are supposedly not removed by the adsorbent(4). The present study is concerned with the influence of AHF and of PAcG on the rate of thrombin formation in a reaction mixture containing calcium ions and platelets as well as factors removed from bovine plasma by barium sulfate (plasma eluate). Concomitant investigations were made on the influence of a barium sulfate eluate from rabbit serum (serum eluate) on the rate of thrombin formation.

Materials. Bovine plasma: Oxalated bovine blood (9 parts of blood and 1 part of 3.7% potassium oxalate in 1% oxalic acid) was collected at the slaughter house and centrifuged at 4°C. *Veronal buffer*: A stock solution was made by dissolving 19.428 g sodium acetate • $3H_2O$ and 29.428 g sodium diethylbarbiturate in one l of distilled water. Veronal buffer of pH 7.2 was used throughout and consisted of 100 ml of the stock solution, 40 ml of 8.5% sodium chloride, 100 ml of 0.1 M hydrochloric acid and 260 ml of distilled water. *Bovine platelets* were obtained from oxalated bovine plasma by differential centrifugation according to the procedure described by Seegers and his associates(5). After the final washing with 0.9% NaCl, the platelets were dried with acetone and then pulverized in a mortar. Suspensions of the dried platelet material were made in veronal buffer. *Bovine plasma eluate*: Oxalated bovine plasma was stirred at room temperature for 10 minutes with $BaSO_4$ (50 mg/ml), centrifuged at 4°C and the supernatant plasma decanted. The $BaSO_4$ was washed successively with 0.9% NaCl, 0.1 M sodium oxal-

* For a different terminology of these factors reference is made to Appendix, The Coagulation of Blood. Method of Study. Edited by L. M. Tocantins, Grune and Stratton, N. Y. City, 1955.

ate, and 0.9% NaCl, the volumes of each washing being equal to the original plasma volume. The $BaSO_4$ was then eluted twice with 0.1 M sodium citrate solution (1/10 of the original plasma volume) by stirring for 10 minutes at room temperature, and then centrifuging and decanting. The two citrate eluates were combined and dialyzed against distilled water at 4°C until free of citrate and then lyophilized. A given amount of the dried preparation was dissolved in veronal buffer, corresponding to definite plasma concentrations. *Rabbit serum eluate* was prepared from stored aged rabbit serum of low prothrombin content according to the procedure described for the plasma eluate. An amount of the dried material was extracted with veronal buffer equivalent to serum concentration, centrifuged for 20 minutes at 4°C and the clear supernatant was employed in the various tests. *Plasma accelerator globulin (PAcG)* was prepared according to the method of Lewis and Ware(6) from bovine oxalated plasma after treatment with $BaSO_4$. The final preparation was dissolved in a volume of 0.9% NaCl, corresponding approximately to 1/5 of the original plasma volume, and kept frozen in small aliquots until used. *Antihemophilic globulin factor (AHF)* was prepared according to the procedure described by Brinkhous and his associates(7,8). $BaSO_4$-treated oxalated bovine plasma was heated for 10 minutes at 52°C to remove fibrinogen. After centrifugation, ammonium sulfate was added to the supernatant to 25% saturation. After one hour at 4°C, the resulting precipitate was removed by centrifugation, dissolved in distilled water and dialyzed at 4°C against 0.9% NaCl. Final volume of the AHF solution was adjusted to the original plasma volume, and kept frozen in small aliquots until used. *Fibrinogen* was prepared from oxalated bovine plasma according to the method of Seegers and his associates(9). Before dilution, the fibrinogen solution was treated with $BaSO_4$ (100 mg/ml) to remove any absorbable clotting factors which may have been present. For the test, the fibrinogen was diluted with 0.9% NaCl to a concentration of 0.4%. The fibrinogen preparation contained 89% clottable protein.

Methods. A. One-stage thrombin formation procedure: The total incubation mixture consisted of 1.25 ml plasma eluate (2 times plasma concentration) in veronal buffer, 0.6 ml AHF solution, 0.6 ml PAcG solution, 0.55 ml serum eluate (serum concentration) in veronal buffer, 0.15 ml platelets suspension (1.5 mg/ml veronal buffer), and 0.3 ml $CaCl_2$ (0.125 M). Veronal buffer was substituted for one or more of the above reagents in the various experiments. On the addition of the $CaCl_2$ to the other factors and agitation of the mixture, a stop watch was started and the reaction mixture was incubated at 26°C. Thrombin formation was determined at timed intervals by the addition of 0.1 ml of the reaction mixture to a fibrinogen solution (0.3 ml veronal buffer and 0.1 ml fibrinogen) at 37°C. When the clotting times (as obtained by the tilt method) were below 12 seconds, the 0.1 ml portions of the reaction mixture were suitably diluted with veronal buffer before addition to the fibrinogen solution. The recorded clotting times were interpreted in terms of National Institutes of Health (NIH) thrombin units from a curve prepared with a standard NIH thrombin preparation.† *B. Two-stage thrombin formation procedure*: In

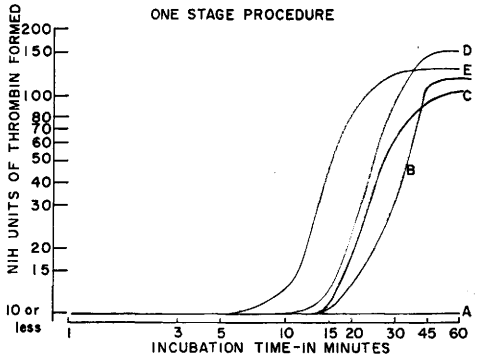

FIG. 1. Formation of thrombin in bovine plasma eluate, platelets and $CaCl_2$ in the presence of serum eluate, AHF, PAcG and AHF plus PAcG. A, plasma eluate, platelets and $CaCl_2$; B, plasma eluate, platelets, $CaCl_2$, and serum eluate; C, plasma eluate, platelets, $CaCl_2$ and AHF; D, plasma eluate, platelets, $CaCl_2$ and PAcG; E, plasma eluate, platelets, $CaCl_2$, AHF and PAcG.

† Kindly supplied by Biologics Control Laboratory, National Institutes of Health, Bethesda, Md.

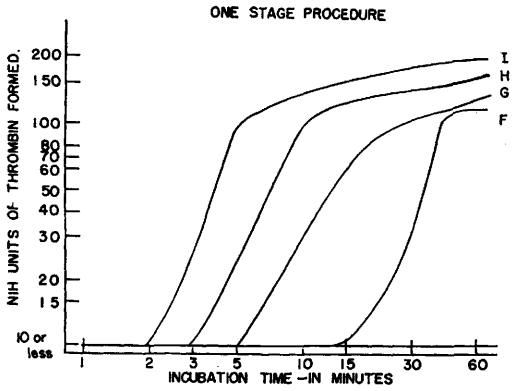

FIG. 2. Formation of thrombin in plasma eluate, serum eluate, platelets and $CaCl_2$ in the presence of PAcG, AHF and PAcG plus AHF. F, plasma eluate, serum eluate, platelets and $CaCl_2$; G, plasma eluate, serum eluate, platelets, $CaCl_2$ and PAcG; H, plasma eluate, serum eluate, platelets, $CaCl_2$ and AHF; I, plasma eluate, serum eluate, platelets, $CaCl_2$, AHF and PAcG.

this procedure serum eluate, platelets and $CaCl_2$ with and without AHF or PAcG were incubated for 20 minutes at 26°C and the plasma eluate was then added to the incubation mixture. The volumes for the different reagents in this procedure were the same as in the one-stage thrombin activation procedure. The prothrombin converting activity of the incubation mixture was assessed by recording the ability and the velocity of the reaction mixture to form thrombin upon addition of a plasma eluate and incubation at 26°C. The rate of thrombin formation was followed in the same manner as in the one-stage thrombin formation procedure. Details of representative individual experiments are included in Fig. 1, 2 and 3.

Results. In any interpretation of the various reaction rates of the different factors controlling the formation of thrombin, consideration must be given to the law of mass action. Cognizance must also be taken of the possible presence of other clotting factors or inhibitors as impurities in the preparations employed, however small these contaminations may be. Nevertheless, it is felt that only quantitative and not qualitative differences may occur when corresponding reactions are executed with more purified preparations. Data presented in the various figures constitute mean values of 3 or 4 identical experiments. The clotting times did not differ by more than 10% for each group of identical experiments.

In the curves of Fig. 1 and 2, depicting simultaneous mixing of the reactants, the rate of thrombin formation represents the summation of all intermediate interactions involved in the elaboration of thrombin. As can be seen from curve A of Fig. 1, incubation of the bovine plasma eluate with platelets and $CaCl_2$ yielded only traces of thrombin. However, in the presence of either serum eluate (curve B of Fig. 1), or AHF (curve C of Fig. 1), or PAcG (curve D of Fig. 1), prothrombin conversion became more pronounced. As can be seen from Fig. 1 marked thrombin formation did not commence until after 10 to 15 minutes of incubation. Once thrombin formation began, the rate of conversion of prothrombin to thrombin increased rapidly. In the presence of both AHF and PAcG (curve E of Fig. 1), thrombin evolution was relatively fast: 80 NIH units were elaborated after 20 minutes compared with 29 units with PAcG, 20 NIH units with AHF and 15 NIH units with serum eluate. Addition of either serum eluate, or of PAcG, or of AHF, or of AHF and PAcG seemed to influence mainly the rate but not the amount of thrombin formation.

The data in Fig. 2 indicate that addition of

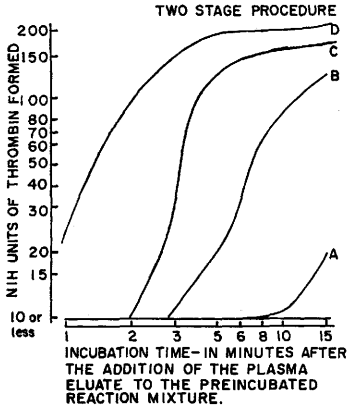

FIG. 3. Formation of prothrombin converting activity on 20 min. incubation of serum eluate, platelets and $CaCl_2$ in the presence of PAcG, AHF and PAcG plus AHF. A, serum eluate, platelets and $CaCl_2$; B, serum eluate, platelets, $CaCl_2$ and PAcG; C, serum eluate, platelets, $CaCl_2$ and AHF; D, serum eluate, platelets, $CaCl_2$, PAcG and AHF.

serum eluate to the plasma eluate enhanced the accelerating influence of either PAcG or AHF in the utilization of prothrombin. In the presence of AHF (curve H of Fig. 2) there was both an increase in the yield and the rate of thrombin formation after 10 minutes of incubation (112 NIH units) compared with PAcG (30 NIH units, curve G of Fig. 2). These observations substantiate the concept of Brinkhous and his associates(4) that AHF augments the utilization of prothrombin. In the presence of both AHF and PAcG (curve I of Fig. 2), the lag period of prothrombin conversion was further shortened; about 26 NIH thrombin units were formed within 3 minutes, while only traces of thrombin were elaborated during the same period of incubation with either AHF or PAcG alone. Comparison of the data of Fig. 1 and 2 indicate that AHF as well as PAcG in the presence of serum eluate caused a more pronounced shortening in the prothrombin utilization than in the absence of serum eluate. The quantity of thrombin formed in a mixture of plasma eluate, platelets and calcium at any given time not only was dependent on the amount of prothrombin present but also on the rate of its conversion controlled by the presence and quantity of such factors as AHF, PAcG and those present in serum eluate. Replacing either platelets or $CaCl_2$ with veronal buffer in the experiments did not yield any measurable thrombin formation.

In an attempt to reduce further the lag period observed in the one-stage thrombin formation procedure, those factors which apparently participate in the conversion of prothrombin to thrombin were preincubated for 20 minutes at 26°C before addition of the plasma eluate. The results illustrated in Fig. 2 and 3 show that preincubation of serum eluate, platelets and $CaCl_2$ in the presence of PAcG or AHF or both PAcG and AHF before addition of the plasma eluate to the incubation mixture, resulted in a marked enhancement in the prothrombin conversion compared with the one-stage thrombin formation procedure. It appears from these data that AHF as well as PAcG interact with factor(s) in serum eluate in the presence of platelets and calcium prior to the activation of prothrombin. In the presence of AHF (curve C of Fig. 3) a more potent prothrombin activator seemed to evolve than in the presence of PAcG (curve B of Fig. 3). This is in further agreement with the finding of Brinkhous and his associates(4) that AHF greatly enhances the utilization of prothrombin. Jensen, Gray and Schaefer(10) have presented evidence that PAcG accelerates the interaction of platelets and factor(s) in a serum eluate in the presence of $CaCl_2$ yielding a prothrombin conversion factor. With both AHF and PAcG in the preincubation reaction mixture, (curve D of Fig. 3), 24 NIH thrombin units were already formed within 1 minute after addition of the plasma eluate. In the presence of AHF only (curve C of Fig. 3) and PAcG only (curve B of Fig. 3) 2.5 minutes and 5 minutes respectively were required to yield the same amount of thrombin. Replacement of platelets or of $CaCl_2$ with veronal buffer did not yield any prothrombin converting activity. Serum eluate, AHF, PAcG, platelets and $CaCl_2$ after incubation for 20 minutes at 26°C produced a clot with fibrinogen in 9 minutes, indicating the presence of only traces of prothrombin in the above preparations.

A comparison of the rates of thrombin formation (Fig. 2 and 3) shows that preincubation of AHF or PAcG or both with serum eluate, platelets and $CaCl_2$ greatly reduced the lag period in the conversion of prothrombin to thrombin. It appears from the data in Fig. 2 and 3 that regardless of whether the prothrombin converting complex was formed in one stage or two, approximately the same amount of thrombin was formed. The main difference between the one and 2-stage reaction was in the rate of prothrombin conversion. The greater velocity of thrombin formation in the 2-stage procedure indicates that during preincubation precursory interactions of certain factors had been initiated or completed.

Preincubation of either platelets, AHF and $CaCl_2$ or of platelets, PAcG and $CaCl_2$ or of platelets, AHF, PAcG and $CaCl_2$ before addition of either plasma eluate or of plasma

eluate plus serum eluate to the preincubation mixture resulted in approximately the same rates of thrombin elaboration as was found in the one-stage thrombin procedure (Fig. 1 and 2). These findings indicate that no interaction between the various factors had taken place during the preincubation period under these conditions. It appears from these observations that AHF as well as PAcG will interact with platelets and calcium ions only in the presence of factor(s) in serum or plasma eluate yielding prothrombin converting activity.

Discussion. The data presented point to the existence of several factors in blood which react, perhaps in stages, to produce a powerful activator of prothrombin, called the thrombokinase-complex.‡ Factors concerned in the thrombokinase forming system are AHF, PAcG, factor(s) in serum or plasma eluate, platelets and calcium ions. Apparently PAcG and AHF will react with platelets and calcium only in the presence of factor(s) in serum or plasma eluate yielding prothrombin converting activity. Whether the activities of the thrombokinase generated with and without AHF or PAcG differ qualitatively as well as quantitatively remains to be determined. Applying these results to the mechanism of fibrin formation in blood, the role of the different natural anticoagulants must be taken into account.

The findings reported are in agreement with the concept of Biggs(12) that the Christmas factor* (present in the eluate of plasma or serum), AHF, factor V* (PAcG), factor VII* (present in the eluate of plasma or serum), platelets and calcium ions are all required for the generation of a "complete" thromboplastin§ (thrombokinase). According to Spurling and King(13), plasma thromboplastin component‖ (present in plasma or serum eluate), AHF, platelets, rough surfaces and calcium are required for maximum thromboplastin (thrombokinase) production *in vitro*. These investigators, apparently, did not include PAcG in their concept of thrombokinase formation. Flynn and Coon(14) have presented evidence that brain thromboplastin extract, stable factor* (present in plasma or serum eluate) and labile factor* (PAcG) react together prior to the activation of prothrombin. According to Seegers(15), the plasma platelet cofactor 1* (AHF) reacts with platelet factor 3* to form threone which in combination with serum accelerator globulin* (derived from PAcG) will convert prothrombin to thrombin. Mann and Hurn (16) observed that platelets interact with a plasma—or serum factor (cothromboplastin)* before interaction with prothrombin takes place.

The data presented here as well as the observations of others already cited are in harmony with the 3 stage concept of fibrin formation as formulated by Milstone(11).

Prothrombokinase-complex $\longrightarrow$ Thrombokinase complex

Prothrombin $\xrightarrow{\text{Thrombokinase complex}}$ Thrombin

Fibrinogen $\xrightarrow{\text{Thrombin}}$ Fibrin

The prothrombokinase complex would include such factors as platelets, AHF, PAcG, factor(s) in serum or plasma eluate and calcium. For the thrombokinase complex formation, platelets, factor(s) in serum or plasma eluate and calcium seem to be essential, while AHF as well as PAcG will enhance its formation. The fundamental phase of the blood clotting mechanism seems to involve the generation of a thrombokinase. Once sufficient thrombokinase is formed, the conversion of prothrombin to thrombin by thrombokinase and the conversion of fibrinogen to fibrin by thrombin proceed comparatively rapidly. The time required for the thrombokinase-forming reactions determines the blood coagulation time. The nature and sequences in the interaction between the various factors, yielding thrombokinase, have yet to be elucidated. It is known that AHF and PAcG are

‡ Following the proposal of Milstone(11), thrombokinase is defined as an agent which has the capacity to convert prothrombin to thrombin directly.

§ Biggs defines thromboplastin as a substance which is directly responsible for conversion of prothrombin to thrombin in the presence of calcium ions.

‖ Plasma thromboplastin component is assumed to be identical with the Christmas factor(12).

utilized during the clotting of blood. Whether they become a component part of the thrombokinase complex is at present not known. According to Owren and his associates(17) convertin (obtained by the interaction of brain extract and proconvertin, present in serum or plasma eluate) and accelerin¶ combine to form prothrombinase (thrombokinase) which in the presence of calcium produces rapid conversion of prothrombin to thrombin. Whether the calcium necessary for the formation of thrombokinase becomes an integral component of the thrombokinase complex or whether it serves merely as a catalyst is not known.

Summary. Plasma accelerator globulin, antihemophilic factor as well as serum eluate were found to produce increased thrombin elaboration in a reaction mixture consisting of bovine plasma eluate, platelets and calcium ions. In the presence of both plasma accelerator globulin and antihemophilic factor, conversion of prothrombin to thrombin became accelerated. Addition of serum eluate to the plasma eluate enhanced the accelerating influence of either plasma accelerator globulin, of antihemophilic factor or of both in the utilization of prothrombin. Upon preincubation of those factors which are supposed to participate in the conversion of prothrombin, before addition of a plasma eluate to the incubation mixture, it was found that more prothrombin was converted in a shorter period of time than in the one-stage thrombin formation procedure. The greater velocity of thrombin formation in the two-stage procedure indicated that precursory interactions of certain factors had taken place during the initial incubation. Evidence has been obtained that antihemophilic factor as well as plasma accelerator globulin will interact with platelets and calcium ions only in the presence of factor(s) in serum or plasma eluate yielding prothrombin converting activity.

¶ Obtained by addition of thrombin to prothrombin and proconvertin free bovine plasma. Accelerin was then precipitated by dilution and acidification. The preparation may have been contaminated with AHF.

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