a failure on the part of the pituitary gland to secrete the trophic hormone.

A possible explanation for the failure of the hypophysis to produce ACTH after treatment with thiouracil may reside in the limited numbers and types of cells found in the gland of such animals. It is conceivable that when the pituitary is forced to secrete excessive amounts of TSH, as occurs after thiouracil treatment, it can only do so by a decrease in its production of ACTH. Such an explanation has been offered for the decreased release of TSH and gonadotrophins and increased production of ACTH after stress(10,11). Thus the atrophy of the adrenal gland or the failure to obtain hyperplasia after exposure to cold in thiouracil-treated rats would indicate functional damage to the pituitary part of the pituitary-adrenal axis. It is not to be denied, however, that a decreased amount of ACTH is still being secreted in view of the partial maintenance of the ascorbic acid of the adrenal gland and the depletion of the adrenal-ascorbic acid on exposure to cold(12). This would indicate that greater amounts of ACTH are needed for adrenal hyperplasia than for the adrenal-ascorbic acid depletion. The fact that the pituitary of normal animals can secrete increased amounts of both TSH and ACTH (as seen after exposure to cold) does not invalidate the above hypothesis as the situations are not comparable. In the thiouracil-treated rat the stimulus is such as to bring about an increased secretion of only one of the factors (TSH) resulting in an early appearance of ACTH deficiency. In cold stress the initially increased TSH output eventually decreases as the animal remains under the influence of the stress and as the pituitary secretes greater amounts of ACTH.

Conclusion. 1. Thiouracil produces an involution of the adrenal cortex. 2. The atrophic adrenal gland of the thiouracil-treated rat retains its sensitivity to ACTH. 3. It is suggested that the involution of the adrenal gland of the thiouracil rat is caused by a decreased ACTH secretion by the pituitary gland. This decreased ACTH level may result from the excessive TSH production in the pituitary caused by the thiouracil treatment.

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Response of Plasma to Excess of Thromboplastin as a Measure of Prothrombin Activity.* (18579)

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The one-stage method of prothrombin determination proposed by Howell(1) and the two-stage procedure employed by Bordet(2) were used for much important work on prothrombin, but the results obtained with those methods were not regarded as quantitative, since large amounts of prothrombin remained unconverted to thrombin and therefore escaped measurement. Smith and coworkers (3) introduced the two principles which subsequently proved to overcome some of the shortcomings of these two methods. They

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2. Bordet, J. and Delange, L., *Ann. Inst. Pasteur*, 1912, v26, 737.

3. Warner, E. D., Brinkhous, K. M. and Smith, H. P., *Arch. Path.*, 1934, v18, 587.

showed(3) that if plasma was sufficiently diluted and an excess of thromboplastin added, all of the prothrombin could be converted into thrombin, that the resulting clotting time varied inversely as the concentration of prothrombin and therefore could be used as a measure of the amount of prothrombin present. The Smith 2-stage method remains the standard reference method for quantitative work on prothrombin. Quick(4) modified this method by eliminating preliminary dilution of the plasma while retaining the principle of using an excess of thromboplastin. A surprisingly close agreement between this rapid convenient one-stage method and the more complex 2-stage determination was shown often to exist. The well justified popularity of this one-stage method, however, has often caused some workers to overlook its limitations and to extend its use to serious quantitative work on prothrombin. It has been claimed(5) that when an excess of thromboplastin is added to optimally recalcified "undiluted" oxalated plasma, that the clotting time is a function of the prothrombin concentration since, in a system so arranged, prothrombin is the only variable. Much of the confusion in the interpretation of one-stage prothrombin determinations stem from a too sweeping application of this concept. Aside from the fact that much of the prothrombin in the plasma remains unconverted to thrombin during the 12 to 15 second interval required for clotting to occur(6) a variously termed prothrombin convertibility factor (accelerator factor, plasma-kinin, Ac-globulin, factor V or labile factor)(7,8,8a,9,10) forecast by Smith(11) influences the rate of thrombin formation and, therefore, the speed of clotting, independently of the amount of prothrombin present. It appears from the evidence to be presented, that the rate of coagulation of plasma after adding an excess of thromboplastin is further influenced by at least 3 heretofore not sufficiently considered variables: 1. The actual concentration of the plasma in the final clotting mixture. 2. The stability of the plasma being tested. This in itself depends on: (a) the mode of collection of the blood and separation of the plasma, (b) the nature of the contacting surfaces used in the collection, storage and testing of the plasma and (c) its content of stabilizing inhibitors. 3. The source of the thromboplastin employed as activating agent.

Methods. Human blood or plasma was collected with especial precautions using siliconized surfaces throughout for collection, storage, measuring and testing. When an anticoagulant was used, it was placed in the syringe and the blood aspirated directly into it. Four types of plasma were generally employed: (a) Normal, obtained from fasting young men or women; (b) hemophilic, obtained from patients with hereditary hemophilia; (c) post-hemorrhagic, hypercoagulable plasma, drawn from patients soon after a severe hemorrhage not resulting from a systemic disorder of hemostasis; (d) asbestos plasma, *i.e.* normal plasma placed in contact with loose asbestos fibers (10 mg asbestos fibers per 1 ml plasma) for 60 minutes at 20°C, without agitation.

In the calculation of the final percentage concentration of the plasma in any given clotting mixture, any or all of the following variables were taken into account: (a) The per cent plasma volume of the sample of blood (calculated from the hematocrit); (b) the total volume of anticoagulant added to the blood; (c) the total volume of the clotting mixture and the respective volume of each of its ingredients namely, plasma, recalcifying solution, activating agent and diluting fluid. Example: 9 ml of normal blood was aspirated into a syringe containing 1.0 ml of 0.1 M sodium oxalate. The packed cell volume (hematocrit) of this mixture was 30. This meant that the 7 ml of plasma in the sample

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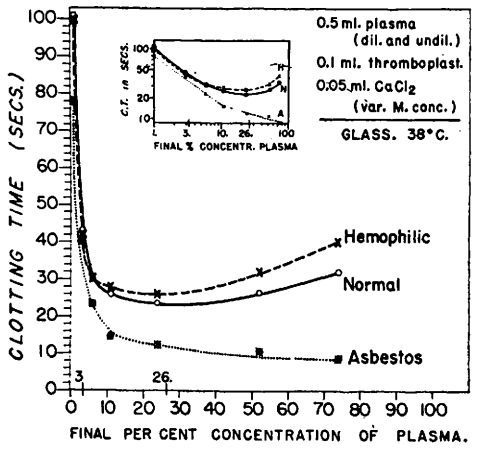

FIG. 1.

Dilution curves of 3 types of plasma activated by a strong thromboplastin. Inset: The same curves plotted on logarithm scale. Asbestos plasma obtained by allowing hemophilic plasma to stand in contact with asbestos fibers, without shaking, for 1 hr at 20°C. (10 mg asbestos fibers, 1 ml plasma). The plasmas were handled by the silicone technic until just before placing them in glass tubes. The concentrations of prothrombin and Ac-globulin of the 3 plasmas tested by the two-stage method(12) were within normal limits. Plasmas obtained from citrated blood (0.2 ml 19% citrate to 10 ml of blood, hematocrit 38). Dilution of the plasma in the first clotting mixture 60/62 × 50/65 or a starting plasma concentration of 74%.

was diluted by 1 ml of the anticoagulant (or a dilution of 6/7). In preparing the initial clotting mixture, 0.5 ml of this already diluted plasma was added to 0.05 ml of thromboplastin followed at once by 0.05 ml 0.2 M $CaCl_2$, thus involving a futher 5/6 dilution of the plasma. Therefore 6/7 x 5/6 x 100 = 71% final plasma concentration in the first clotting mixture. By using 19 or 38% citrate solution as anticoagulant and increasing the volume of plasma in the clotting mixtures, it is possible to work with higher plasma concentrations.

Results. 1. *Relation of the actual concentration of the plasma in the final clotting mixture to action of thromboplastin.* If a strong solution of an aqueous extract of human brain is added to normal, hemophilic and asbestos plamas, beginning with a concentration of plasma in the actual clotting mixture of 74% and progressing down to one per cent, curves such as those illustrated in Fig. 1 are the result. The plasmas used in this test were not allowed to come into contact with glass until the actual testing. Without these precautions the results are erratic. The greatest differences between the three types of plasma are in the clotting mixtures of high plasma concentration. High plasma concentrations slow the action of thromboplastin. Even slight dilution shortens the clotting time of hemophilic and normal plasma; little change in their rate of clotting is observable in the mixtures of plasma concentration between 10 and 30%. Asbestos plasma from which much of the action of first phase inhibitors is removed, is hypercoagulable even in high contentrations, and its clotting time gradually lengthens from the start, as the plasma is diluted. Whether on simple arithmetic or logarithmic ruled charts, (Fig. 1) the curves for hemophilic and normal plasma follow a parabolic course; the curve for asbestos plasma follows a hyperbolic course when plotted in arithmetic ruling, but rectilinear in logarithmic ruled paper. Asbestos plasma resembles in many of its characteristics, plasma that has lost much of its stability after being collected and stored in glass tubes.

The total volume of each of the clotting mixtures represented in Fig. 1 is, however, 0.65 ml. Let us see what results when the volume of the mixture is 0.3 ml (as in the one-stage method); the thromboplastin is kept constant and the amount of calcium varied to be optimal for concentrations of normal plasma from 70 to 4% (Fig. 2, Curve A). The clotting times observed may then be compared with those obtained by diluting the plasma in the conventional Quick one-stage procedure, again using the same total volume, amount of thromboplastin and optimal recalcification (Fig. 2, Curve B). When the activity of dilutions of plasma tested by the one-stage "prothrombin time" method is plotted with due attention to the final per cent plasma concentration in each clotting mixture, the curve (Curve B) almost superimposes a segment of curve A, which had its origin at high plasma concentration (70%). Curve B, therefore, when correctly plotted, is only a small part of Curve A, mainly its ascending segment; it begins at 26 to 28%

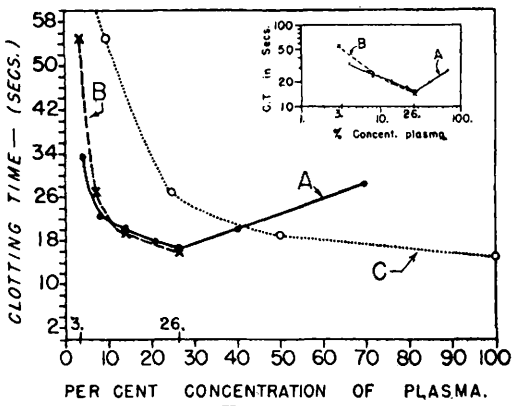

FIG. 2.

Curve A: The effect of adding a strong thromboplastin to stable normal plasma, at different concentrations of the plasma in the clotting mixture. Total volume of the clotting mixture: 0.3 ml throughout. Silicone technic used up to the point when the plasmas were placed in the glass clotting tubes. Plasmas derived from oxalated human blood (9 ml blood plus 1 ml sodium oxalate). Concentration of the thromboplastin the same throughout. *Curve B*: Curve plotted from values obtained by testing the same plasma, according to the Quick one-stage "prothrombin time" method. Total volume of the mixture 0.3 ml. Same concentration of thromboplastin as that used for values in curve A. The points on this curve as well as on curve A represent the final concentration of the plasma in the actual clotting mixture. *Curve C*: The points on curve C are the same as those of curve B, but are plotted according to the common practice of referring plasma concentration not to the actual clotting mixture, but to the alleged original concentration of the plasma without regard for dilution by the anticoagulant and the other clotting reagents. It is the familiar dilution curve used for calculation of "prothrombin concentration" by the one-stage method.

plasma concentration or at the bottom of the valley of Curve A. It can now be seen how far these two curves differ from Curve C, said to express the relation between concentration of plasma (or of prothrombin) and activated clotting time in seconds(5). The one-stage method as currently performed covers therefore the response of plasma to thromboplastin only from plasma concentrations of about 26% or below.

2. *Response of plasmas of variable stability (normal, hemophilic, posthemorrhagic) to addition of strong thromboplastin.* Various authors have stated that hemophilic plasma does not differ from normal in its response to a strong thromboplastin solution. Though such might be the case with unusually strong thromboplastins (*e.g.* placental extracts) the usually reported lack of difference in response between these two plasmas can result from tests done in clotting mixtures in which the plasma concentration is 26% or below. We have already seen how much hemophilic, normal and asbestos plasmas may differ from one another in their response to thromboplastin when tested at concentations of 74% (Fig. 1). At concentrations of about 26% the differences between hemophilic and normal plasma become non-significant, but those between normal and asbestos plasma are still substantial. When a plasma concentration of about 3% is reached (that used in the Link-Shapiro method) the differences between the three plasmas become correspondingly less.

In Fig. 3 are represented the response of 3 types of plasma to the same amounts of thromboplastin, employing 2 clotting mixtures of different plasma concentration. The hypercoagulable plasma was obtained from a pa-

RESPONSE TO THROMBOPLASTIN OF NORMAL (A), HEMOPHILIC (B) AND POST-HEMORRHAGIC (C) PLASMA, TESTED IN GLASS TUBES AT TWO PLASMA CONCENTRATIONS.

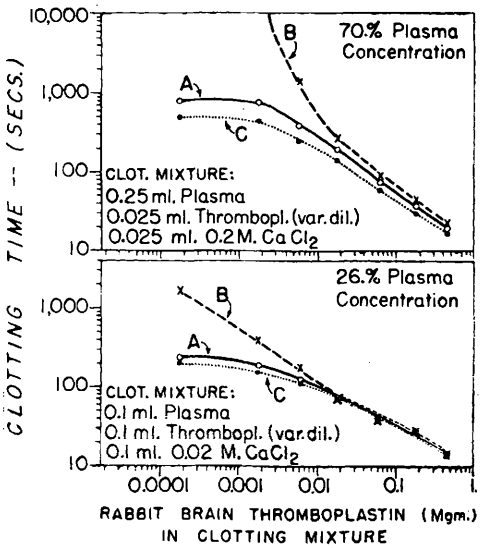

FIG. 3.

The plasma was handled by the silicone technic up to the time it was placed in glass tubes for testing. Rabbit brain thromboplastin prepared from acetone dried rabbit brain. Blood collected by the silicone technic (9 ml blood plus 1 ml 0.1 M sod. oxalate).

tient after hemorrhage and transfusion. The 3 plasmas had a normal content of prothrombin and Ac-globulin (2-stage method)(12). As shown in the chart, the clotting times were all shorter at 26 than at 70% plasma concentration, even though the latter clotting mixture had more prothrombin and Ac-globulin, and both received the same amount of thromboplastin. While no difference was demonstrable between the clotting times of the 3 plasmas at 26% plasma concentration when 0.02 mg or more thromboplastin were used, a significant difference was evident when the plasmas were tested at 70% plasma concentration. In the presence of amounts of thromboplastin (0.0018, 0.00018 mg) which easily clotted hemophilic plasma in a mixture of 26% plasma concentration, the same plasma remained incoagulable when tested in 70% concentration. Likewise at the higher plasma concentrations, differences were manifest between the normal and hypercoagulable plasma that were small or nonexistent in the 26% plasma.

3. *The rate of coagulation of stable normal plasma at various concentrations with or without the addition of an activating agent.* Citrated plasma obtained from a normal man was diluted with 0.85% NaCl in such a way that its concentration in the clotting mixture varied between 90 and 1.8%. In one series of mixtures no activating agent was added (non-activated plasma). In another series, an activating agent (thromboplastin) of uniform concentration was included in the clotting mixture (activated plasma). The results are represented in Fig. 4. Dilution of the non-activated plasma accelerated coagulation even when the plasma concentration in the clotting mixture was between 1 and 2%. When the plasma was activated by an aqueous extract of human brain, the greatest acceleration of coagulation was detected at plasma concentrations between 20 and 40%. The addition of thromboplastin depressed the curve at its descending limb, representing clotting times of mixtures of high plasma concentration (90 to 40%). It is conceivable that, if even greater increments of thromboplastin could be added to the mixture, this section of the curve could be further depressed, until it might assume a rectilinear or at least a hyperbolic course (like that of activated asbestos plasma). This was tried by adding highly concentrated brain extract, starting with a dried lyophilized aqueous brain extract. It was not possible, however, to have sufficient thromboplastin activity to accelerate the clotting time of the mixture with highest plasma concentration to the point that it would clot faster than the more dilute plasma mixtures; provided, of course, the plasma being used was stable, and had been collected and processed on silicone surfaces.

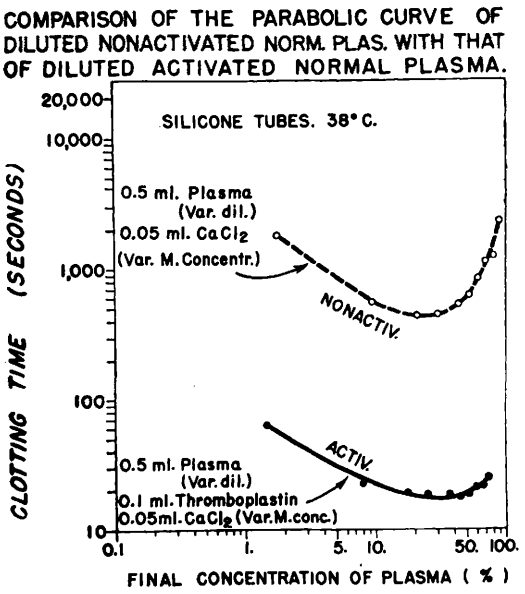

FIG. 4.

Stable plasma collected with the silicone technic and tested on silicone surfaces. 0.85% NaCl used in diluting plasma. Thromboplastin: aqueous human brain extract.

If the plasmas have been allowed to be in contact with glass surfaces, their clotting times become progressively shorter (Fig. 5). After 6 days exposure to glass there is no significant difference between the response of hemophilic and normal plasmas to thromboplastin. The dilution curves lose their parabolic character and the mixture of highest plasma concentration has the shortest clotting time.

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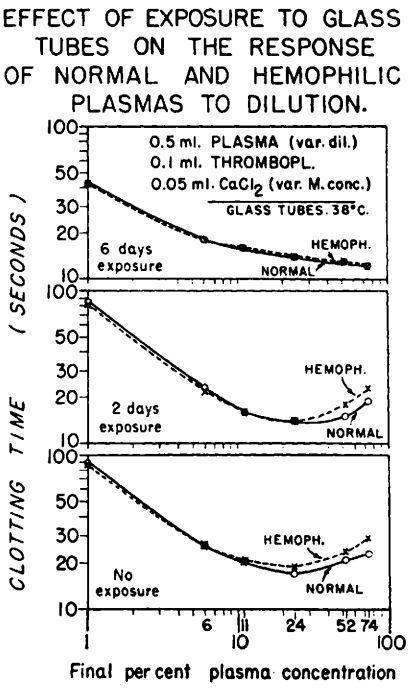

FIG. 5.
Plasmas collected with the silicone technic, then stored at 5°C after testing, in glass tubes for the periods shown.

In Table I are grouped the results of testing the prothrombin activity of 4 plasmas of varying stability using methods employing clotting mixtures from 0.5 to 70% plasma concentration. Prothrombin activity of the plasmas as measured by the 2-stage method does not differ significantly; when tested by one-stage methods at relatively higher plasma concentrations, divergent results follow. When the plasmas are tested at 70% concentration they either resist (*e.g.* hemophilic plasma) or yield too readily (asbestos plasma) to activation by thromboplastin, in either case failing to reflect quantitatively the amount of prothrombin in each plasma.

4. *Response of plasma to heterologous thromboplastins.* If dilute solutions of Russell Viper Venom are used as an activating agent, the clot accelerating effect of moderate dilution of the plasma is not observed with either normal or hemophilic plasma(13). Furthermore, regardless of how high the concentrations of plasma may be in the mixture, there is no significant difference between the response of hemophilic and normal plasma to the venom until small amounts of the venom are used. Attention was drawn previously to the fact that Russell viper venom was not vulnerable to the lipid anticephalin extracted from blood or plasma(14) and that this may underlie the lack of agreement between estimations of prothrombin activity by the one-stage method when using rabbit or human brain thromboplastin as against Russell Viper Venom(15). On Table II is shown the ineffectiveness of the lipid anticephalin extracted from human brain in a clotting system activated by Russell Viper Venom, in contrast with one activated by human brain thromboplastin. The invulnerability of the venom to the natural first phase inhibitor may explain why at low prothrombin levels, as those induced by dicumarol, the clotting time of plasma activated by brain or lung thromboplastin is proportionately longer than when activated by Russell Viper Venom. A species preferential action has been demonstrated for thromboplastin(5) as well as the plasma and tissue antithromboplastin(14,16,17). It seems important therefore, that, in studies on rates of prothrombin conversion or utilization, homologous thromboplastin be employed. Without this precaution, the full extent of a defect in prothrombin conversion may not be appreciated.

Discussion. A distinction should obviously be made between the final concentration of the plasma in the actual clotting mixture, and the original concentration of the plasma before it is added to the other clotting reagents. The one-stage methods refer to the original percent concentration of the plasma. Likewise, the familiar hyperbolic activity curves for various

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TABLE I. Results of Testing 4 Plasmas for Prothrombin Activity Using Clotting Mixtures of Various Plasma Concentration. Silicone technic for collection and storage of plasma. Clotting tested in glass tubes at 38°C.

	Prothrombin activity							
	One-stage method						Two-stage method§	
	Quick*		Link *et al.*†		Present paper‡			
Type of plasma	Time (sec.)	% of normal	Time (sec.)	% of normal	Time (sec.)	% of normal	Units per ml plasma	% of normal‖
Normal	13	100	41	100	16	100	345	111
Hereditary hemophilia	13	100	41	100	21	<100	325	104
Normal + lipid antithromboplastin (0.04 g %)	16	52	43	<100	25	<100	360	116
Asbestos plasma¶	10	>100	40	100	10	>100	375	120
Actual % conc. of plasma used in clotting mixture	26		3		70		0.4	

* 0.1 ml plasma, 0.1 ml thromboplastin, 0.1 ml 0.02 M $CaCl_2$ (PROC. SOC. EXP. BIOL. AND MED., 1939, v42, 788).

† 0.1 ml 12.5% plasma, 0.1 ml thromboplastin, 0.1 ml 0.025 M $CaCl_2$ (*J. Biol. Chem.*, 1941, v138, 1).

‡ 0.25 ml plasma, 0.025 ml thromboplastin, 0.025 ml 0.2 M $CaCl_2$.

§ Warner, Brinkhous, and Smith method as described in Ware and Seegers, *Am. J. Clin. Path.*, 1949, v19, 471.

‖ Compared with activity of frozen control normal plasma (310 units prothrombin per ml plasma).

¶ Normal plasma in contact with asbestos fibers for one hour at 20°C (10 mg asbestos per ml plasma) (Tocantins, *Am. J. Physiol.*, 1945, v143, 67).

TABLE II. Comparison of Action of Brain Lipid Anticephalin Against Two Types of Thromboplastic Agents.

	Lipid anticephalin (mg in clotting mixture)							
Activating agent	1	0.5	0.2	0.1	0.05	0.02	0.01	0.85% NaCl
	Clotting time in seconds							
Russell viper venom*	18	18	19	19	20	20	20	20
Aqueous human brain extr.†	310	247	195	128	85	49	30	15

* 0.1 ml venom solution, 0.1 ml lipid anticephalin (or 0.85% NaCl), 0.1 ml plasma, 0.1 ml 0.02 M $CaCl_2$.

† 0.1 ml brain extract, 0.1 ml lipid anticephalin (or 0.85% NaCl), 0.1 ml plasma, 0.1 ml 0.02 M $CaCl_2$.

dilutions of plasma activated by a strong thromboplastin refer to the original plasma concentration and not to that in the final clotting mixture. The greater the actual concentration of the plasma, the more it resists the action of an excess of thromboplastin. The more careful and thorough the collection and separation of the plasma, the greater is its antithromboplastin activity(16). Skillful venepunctures and protection of the blood and plasma by using silicone coated surfaces, enables the plasma to retain its property of resisting the action of thromboplastin. Such precautions are seldom taken (or indeed necessary) for the determination of prothrombin activity by the one-stage method. They are, however, along with the maintenance of a high plasma concentration in the clotting mixture, essential for the demonstration of the true response of the plasma to activation by thromboplastin.

It appears that only in sufficiently diluted plasma, can the response to an excess of thromboplastin be taken as an indication of the amount of prothrombin present. In undiluted or slightly diluted plasmas, the action of thromboplastin is resisted, prothrombin conversion is correspondingly delayed and the response of the plasma can no longer be depended upon as an indication of the amount of prothrombin present.

A clotting time of 12 to 15 seconds results

when oxalated (or citrated) normal human plasma is added to equal volumes of thromboplastin and calcium, and is said to be equivalent to 100% prothrombin activity. This does not mean, however, that the prothrombin concentration in such a clotting mixture is 100%, since it is obvious that the test itself involves diluting the plasma threefold, and this, added to the dilution introduced by the anticoagulant, gives a clotting mixture with an actual plasma concentration of about 26%. But, since both unknown and normal control plasmas are similarly diluted, it is proper to express the findings in the unknown plasma in terms of per cent of activity of the control, but incorrect to speak of them as expressing concentration of prothrombin. Prothrombin activity as currently measured is a function not only of the amount of prothrombin present but also of accompanying inhibitors and accelerators which, directly or indirectly, affect its conversion rate. Heretofore, only platelets, plasma and serum accelerators have been generally recognized to influence prothrombin conversion rate. The role of natural inhibitors in the plasma was poorly appreciated principally because, as shown above, these inhibitors were placed at a disadvantage by the use of diluted plasmas and strong heterologous thromboplastins, in tests for prothrombin activity. It may be assumed that much information may be gathered regarding the role of these inhibitors in regulating prothrombin conversion, by utilizing clotting systems with high plasma concentration, suitable contacting surfaces and moderately strong homologous thromboplastins as activating agents(18).

Summary. 1. The response of varying concentrations of stable normal plasma to strong homologous thromboplastin may be expressed by a curve of the parabolic type. Clotting mixtures of high concentration resist activation by thromboplastin, an effect which is more evident with abnormally stable plasmas like those from hemophilics. 2. All current one-stage methods for determining prothrombin activity are done on diluted plasma. The actual plasma concentration in the Quick one-stage procedure is about 26%. 3. In clotting mixtures with a high plasma concentration the action of thromboplastin is resisted, prothrombin conversion is delayed and the rate of clotting of such plasmas cannot be used as an indication of the amount of prothrombin present. Final plasma concentrations of 5% or under in the actual clotting mixture are necessary to be able to derive dependable correlations between the rate of clotting and prothrombin activity. 4. For a *full* demonstration of plasma anticephalin (antithromboplastin) activity, clotting mixtures with a high plasma concentration, a moderately strong homologous thromboplastin and inert contacting surfaces (*e.g.* silicone) are essential.

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Feeding Tests with Some Algin Products. (18580)

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Algin products are widely used in food and pharmaceutical products as stabilizing, thickening, suspending, emulsifying and gel producing agents, because of their pronounced hydrophilic, colloidal properties. They are derivatives of alginic acid which is a polymer of anhydro-β-D-mannuronic acid units extracted from the giant kelp (*Macrocystis pyrifera*). Three algin products manufactured by the Kelco Co. of San Diego, Calif.