activity were also noted at this time. Within a few hours the animal was lying on its side and severe dyspnea was observed. This condition became steadily worse, and no food or water was ingested. This state continued during the second and the third day, the animal becoming progressively weaker until it was unable to rise. Seventy-six hours after withdrawal, when the monkey was removed from its cage to be photographed, excitement and struggling led to further exhaustion and extreme weakness. Respiration was irregular, the animal was cyanotic, and it appeared to be moribund. Emergency measures such as intravenous glucose, injection of caffeine, etc., were attempted in an effort to save the animal, but they were without avail. Postmortem examination indicated an acutely dilated heart but no other significant changes were noted. The animal was generally in a good state of nutrition. Withdrawal signs in the other morphine animal were also severe, but not as striking as those just described.

Since these results are in essential agreement with those previously described by us,[1] it seems apparent that the normal monkey differs from the dog and from the former human morphine addict in response to this compound. The question must naturally be raised as to whether prolonged poisoning with morphine modifies or conditions the response of the human individual to subsequently administered methadon and/or other compounds and if so whether normal human subjects, previously not addicted to morphine react like the monkey or like the dog. In view of the fact that no primary case of methadon addiction has thus far been described in man, the evidence to date suggests that the response of the normal monkey is similar to that of the non-addict.

Summary. Racemic methadon administered thrice daily in maximal tolerated dose, does not induce a significant degree of physical dependence in the monkey (*Macaca mulatta*) confirming previous experiments involving single daily administration.

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Activity Curves of Crude and Purified Inhibitors and Accelerators of Blood Coagulation.*

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Most crude extracts of tissues possess in a variable proportion, clot inhibiting as well as clot accelerating substances.[1] Ordinarily, the clot accelerators are in dominance, and their action tends to mask the presence of the inhibitors. It seems from the evidence here presented that testing the activity of these extracts or their purified derivatives at different concentrations makes it possible to tell if they consist of such mixtures and, if so, whether they are *predominantly* clot accelerators or clot inhibitors. Moreover, the relative degree of purity (*i.e.* freedom from the antagonist) of the extracts is reflected by the character of their own activity curves. An extension of this concept has been made to the examination of the coagulant and anticoagulant content of other complex mixtures such as blood, plasma, and plasma fractions.

1. Activity of crude cephalin mixtures. Cephalin was prepared from acetone dried human brain by a method previously described.[2] *Crude cephalin* refers to the prod-

* Aided by a grant from the U. S. Public Health Service.

1 Tocantins, L. M., Carroll, R. T., and McBride, T. J., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 110.

2 Tocantins, L. M., *Am. J. Physiol.*, 1945, **143**, 67.

uct of only one precipitation of the ether-soluble lipid with cold absolute ethanol. This precipitate was washed once with acetone, the acetone removed and the waxy material well homogenized in a 1.4 g% solution in 0.85% NaCl, the pH being adjusted to 7.2 - 7.4. The activity of the preparation was then tested against citrated normal human plasma, collected and preserved with special precautions[3] and measured with collodion coated pipettes.[2] The clotting time of such plasma in collodion coated 13 mm wide tubes, at 38° (0.1 ml plasma, 0.1 ml 0.85% NaCl, 0.1 ml 0.02 M $CaCl_2$) ranges between 500 and 700 seconds.

Testing the activity of this crude cephalin at decreasing concentrations yields a curve with a biphasic course (Chart 1). The greatest clot accelerating effect was obtained at a concentration of the lipid extract in the plasma of 0.35 g%, which corresponds well with the optimum range of concentration (0.06 - 1.0%) for the activity of crude cephalin observed by Hanzlik and Weidenthal.[4] The crude cephalin at first gains clot accelerating power on dilution, then reverses itself and progressively becomes less potent. Such biphasic activity curves seem to be an expression of the coexistence of coagulants and their antagonists.

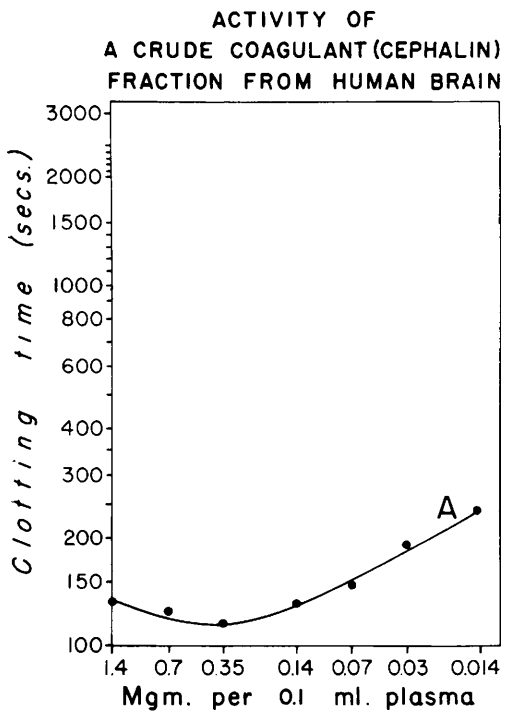

CHART 1.

Cephalin obtained from a single precipitation with absolute ethanol from an ether extract of human brain. In this and subsequent similar charts, the abscissa refers to total mg of the lipid coagulant or anticoagulant (or a mixture of both) added to 0.1 ml of recalcified citrated plasma.

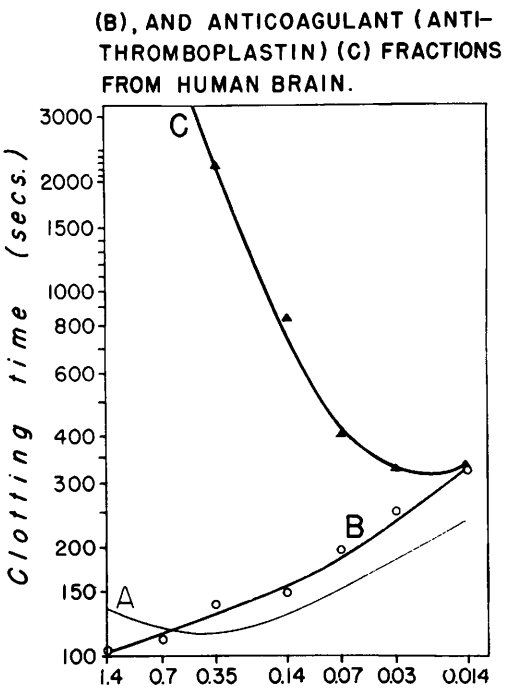

CHART 2.

2. Activity of purified extracts. The crude cephalin obtained by a single precipitation of the ether-soluble lipid was then purified by repeated precipitations with cold absolute ethanol. "Purified cephalin" is used to designate the ethanol insoluble lipid obtained after six successive precipitations. The mother liquors treated as described in method 1, elsewhere[1] yielded a clot-delaying lipid, here designated as "purified anticoagulant" (or antithromboplastin).

As shown in Chart 2, the purified coagulant

[3] Tocantins, L. M., *Am. J. Physiol.*, 1943, **139**, 265.

[4] Hanzlik, P. J., and Weidenthal, C. M., *J. Phar. and Exp. Ther.*, 1919, **14**, 157.

and anticoagulant fractions of the crude extracts have antagonistic actions, and moreover, their curves of activity (Curves B and C) no longer have the biphasic character of the crude preparation (Curve A, Chart 1). Freed of the inhibitor, the purified cephalin at 1.4 percent concentration is a more potent coagulant than the crude cephalin. The monophasic curves seem to represent the activity of preparations fairly free of their respective antagonists.

3. Activity of artificial coagulant-anticoagulant mixtures. These were prepared by mixing different proportions of solutions of the purified coagulant and anticoagulant fractions of the brain. The curves of activity for these mixtures (D,E,F, Chart 3) follow a course analogous to that of crude cephalin preparations. The mixtures with more coagulant (Curve F) exhibit the biphasic behavior at the higher concentrations, while those with more anticoagulant (Curve D) display it at lower concentrations.

The foregoing led us to test the activities of various fluids such as blood, plasma and certain plasma fractions separated by the Cohn method.[5]

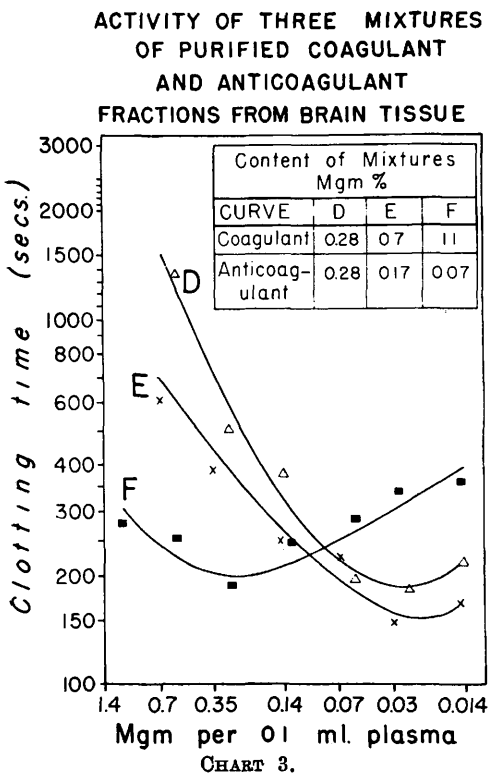

CHART 3.

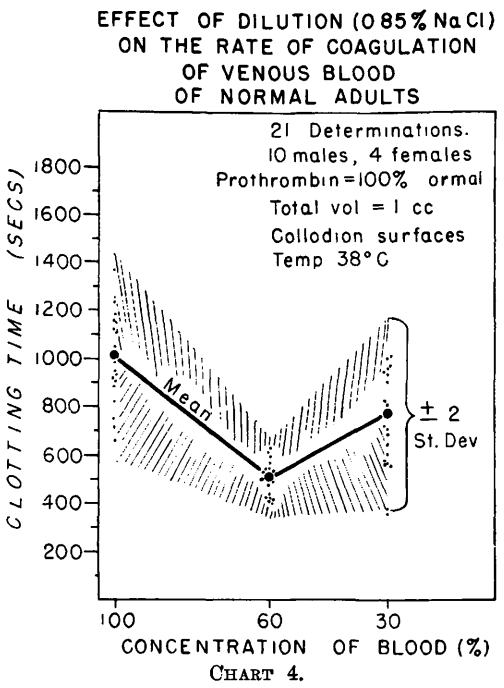

CHART 4.

Activity curves of blood and plasma. Blood was collected with special precautions[3] and placed into 3 collodion coated tubes. One ml was placed in the first tube; the amount of blood in each of the other 2 tubes was so adjusted that in the second tube the blood was diluted to 60% of its volume (0.6 ml blood, 0.4 ml 0.85% NaCl) and in the third tube to 30% (0.3 ml blood, 0.7 ml 0.85% NaCl). All three tubes were gently tilted 2 or 3 times and kept at 38°C. As shown in Chart 4, dilution of the blood to 60% of its volume accelerates its coagulation, and even when diluted to 30%, the blood still clots faster than when undiluted. Blood from hemophilics is even more strikingly affected by dilution (Chart 5).

Hypercoagulable blood, as observed after a severe hemorrhage, is little affected by contacting surfaces of different types[6] and clots nearly as rapidly in glass as in collodion tubes. Dilution of such blood prolongs its rate of coagulation from the start (Chart 6), an indi-

[5] Cohn, E. J., *Blood*, 1948, **3**, 471.

[6] Tocantins, L. M., *Blood*, 1946, **1**, 156.

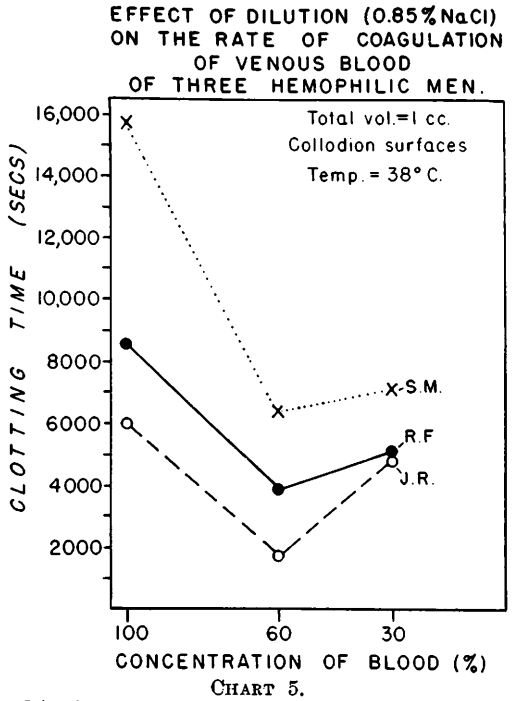

CHART 5.

Dilutions carried out as explained in text. Prothrombin concentration of undiluted blood (1-stage method) 100% of normal.

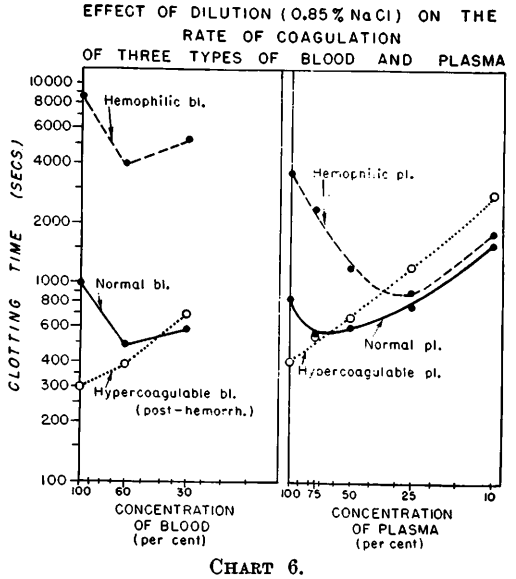

CHART 6.

Effect of dilution on the rate of coagulation of hemophilic, normal, and post-hemorrhagic blood and plasma. Blood diluted as described in text. Plasma clotting mixtures: 0.1 ml plasma, 0.1 ml 0.85% NaCl, 0.1 ml $CaCl_2$ (molar concentration adjusted to dilution of plasma); collodion coated tubes at 38°C.

cation that coagulants are predominant, the inhibitors having been reduced either by blood dilution *in vivo* during the hemorrhage, or offset by entrance of coagulants (thromboplastin) into the circulation.

The effect of dilution on hypocoagulable (hemophilic), normal and hypercoagulable (post hemorrhagic) plasmas is similar to that on whole blood (Chart 6). Hypercoagulable plasma which when undiluted clots many times faster than hemophilic plasma, actually clots slower than the latter, when both are diluted to below 20% of their original concentration. If the curves on Chart 6 are compared with those of the mixtures of purified coagulant and anticoagulant (Chart 3), it is apparent that the hemophilic plasma behaves like a predominantly anticoagulant mixture (Curve E, Chart 3) while the post hemorrhagic plasma is more like a predominantly coagulant solution (Curve B, Chart 2).

The clot-accelerating effect of dilution is best demonstrated in paraffin or collodion.[2] In glass the principal effect of dilution is to prolong the time,[7] for glass itself accelerates coagulation and dilution of the blood will only reduce that effect. Even in glass, however, the clotting time of *hemophilic* plasma is substantially reduced by dilution,[2] though the changes are not as impressive as in collodion or plastic tubes.

5. Activity Curves of Plasma Fractions. The dry plasma fractions were obtained from Dr. Cohn's laboratory and the biological laboratories of Sharp and Dohme. Three percent solutions were made in 0.85% NaCl, and the pH adjusted to between 7.0 and 7.3. The fractions were tested on recalcified citrated normal human plasma, as described before.

With the exception of fraction V all fractions had clot-accelerating activity at concentrations between 0.7 and 3%. Fraction III was the most potent due perhaps to its prothrombin and thrombin content.[5] Fraction V was apparently inert; it did not seem to have either coagulant or anticoagulant action. The curves of activity for fractions IV-1 and IV-4,

[7] Copley, A. L., and Houlihan, R. B., *Science*, 1944, **100**, 505.

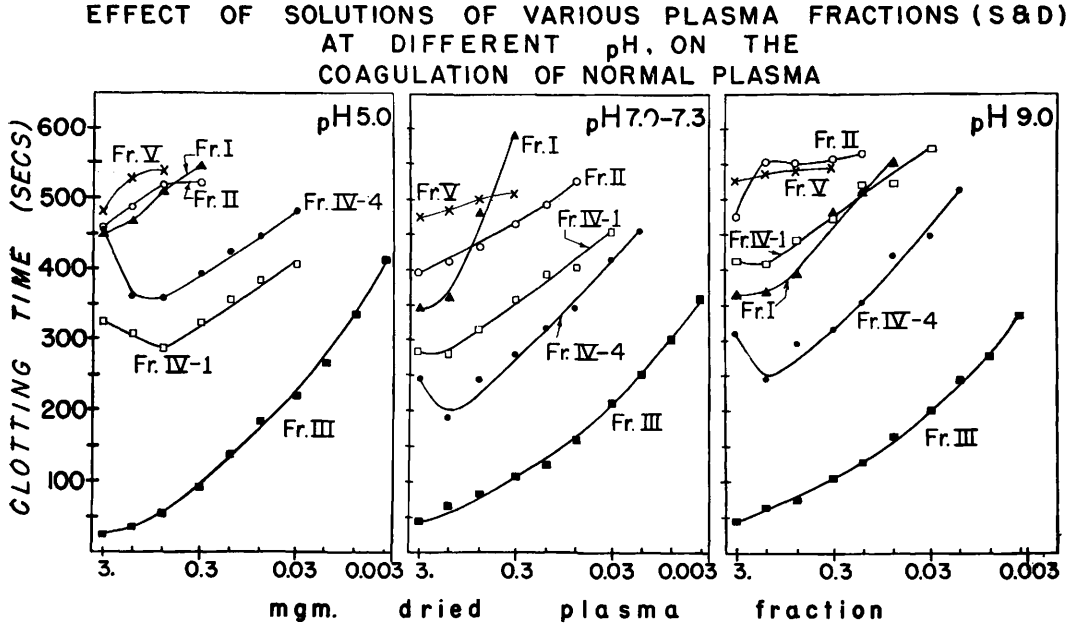

CHART 7.

The pH of the solutions was adjusted with dilute NaOH or HCl. A 3% solution of each fraction in H_2O was prepared; dilutions were made with 0.85% NaCl. 0.1 ml fraction, 0.1 ml normal citrated plasma, 0.1 ml $CaCl_2$ (optimum molar concentration for each fraction/plasma mixture.) Fraction I usually contains the largest amount of citrate.

especially the latter, were of the biphasic type. Testing the activity of the fractions in an acid solution (pH 5.) seemed to accentuate the biphasic behavior of fractions IV-1 and IV-4. Even at pH 9, however, the trend of the curves was not significantly altered (Chart 7). The course of the curves suggested that these two fractions contained anticoagulant factors. A good portion of the lipoprotein complexes of the plasma is in fraction IV,[8] and it has also yielded the highest content of the lipid antithromboplastin.[9]

Discussion. These observations seem to make it desirable that the activity of purported clot-accelerating and inhibiting substances be tested over a wide range of concentrations, using stable plasma as a substrate, held in surfaces which do not themselves accelerate coagulation. Only in this manner may it be possible to detect both coagulant and anticoagulant activity, since excess of one may mask the action of the other, when the material is tested at a single concentration. The most striking example of this behavior is found in fraction IV-4 from which an active anticoagulant may be separated,[9] yet the intact fraction is a clot accelerator.

The slope of the activity curve of the purified anticoagulant is much steeper than that of the purified coagulant. Within the range of 0.35 - 0.07 mg the relation between concentration of the material and clotting time is linear (Chart 2). The anticoagulant gains (or loses) activity in this range, at a considerably more rapid rate than the coagulant. The activity curves of the artificial mixtures (Chart 3) compromise between the steep slope of the purified inhibitor and the gradual straight rise of the purified coagulant.

The biphasic course of the activity curves of normal blood and plasma seem to indicate that these fluids themselves are complex anticoagulant-coagulant mixtures. The fact that diluted blood, though containing less pro-

[8] Mulford, D. J., *Ann. Rev. Physiol.*, 1947, **9**, 327.

[9] Carroll, R. T., and Tocantins, L. M., Separation of a Lipid Antithromboplastin from Blood, Plasma and Plasma Fractions; presented at the International Congress of Hematology, Buffalo, August 23, 1948.

thrombin, platelets, ac-globulin and fibrinogen clots faster than intact blood, is an indication of the effectiveness of inhibitors in blocking or offsetting changes in these coagulation factors. Moreover, since the coagulation of hemophilic blood or plasma is greatly shortened by dilution, it is difficult to understand how a deficiency of a plasma constituent can be responsible for the defect in this disorder. Dilution would naturally tend to accentuate the deficiency and thereby further delay coagulation.

Summary. Crude and purified cephalin and antithromboplastin extracted from brain tissue yield characteristic activity curves when tested at different concentrations, on stable citrated normal human plasma. Moderate dilution accelerates the coagulation of normal and hemophilic blood and plasma and delays that of posthemorrhagic blood and plasma. Excepting fraction V, all Cohn's plasma fractions have coagulant action; only fractions IV-1 and IV-4 display a biphasic curve of activity, an indication that they are mixtures of coagulants and anticoagulants.

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Does Administration of Diethylstilbestrol to Pregnant Women Result in Increased Output of Urinary Pregnanediol?*

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In 1946 there appeared in the literature the interesting observation that the oral administration of diethylstilbestrol to a pregnant diabetic woman resulted in an increased excretion of urinary pregnanediol as measured by the Venning method.[1] This finding was interpreted by the authors as an index of increased production of steroids by the placenta brought about by estrogenic stimulation. They suggested the oral administration of diethylstilbestrol in the prevention and treatment of the accidents of late pregnancy such as the toxemias, premature fetal death and diabetic complications associated with gestation.

In an earlier report[2] we were unable to demonstrate increased pregnanediol excretion in patients receiving large amounts of diethylstilbestrol early in pregnancy. During the past 2 years we have studied a large group of patients who presented a variety of pregnancy complications in early and late gestation. Large amounts of diethylstilbestrol (5 to 200 mg daily) were administered to these women and bi- and tri-weekly urinary pregnanediol determinations were made by a method described by us in a previous communication.[3] No obvious increase in free pregnanediol was apparent in any of our studies. The results of these observations are in the process of publication.

The apparent discrepancy in the results obtained from the administration of diethylstilbestrol to pregnant women by the Smiths and in our own laboratory led us to seek an explanation. It has been reported in numerous publications[4-7] that in the metabolism of diethylstilbestrol it is conjugated and ultimately excreted as a glucuronide. The Venning

* This work has been done under a grant from the Douglas Smith Foundation for Medical Research of the University of Chicago.

1 Smith, O. W., Smith, G. V. S., and Hurwitz, D., *Am. J. Obst. and Gynec.*, 1946, **51**, 411.

2 Davis, M. E., and Fugo, N. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **65**, 283.

3 Davis, M. E., and Fugo, N. W., PROC. SOC. EXP. BIOL. AND MED., 1947, **66**, 39.

4 Zondek, B., and Sulman, F., *Nature*, 1939, **144**, 596.

5 Stroud, S. W., *J. Endocrinology*, 1939, **1**, 201.

6 Mazur, A., and Shorr, E., *J. Biol. Chem.*, 1942, **144**, 283.