thiamine but complete in every other respect show a drop in pH and an accumulation of pyruvate. Other sulfonamides gave negative results. At the present time no hypothesis is offered to account for this phenomenon pending further investigations.

1. Kratzing, C. C., and Slater, E. C., *Biochem. J.*, 1950, v47, 24.

2. Tittler, I. A., *Anat. Rec.*, 1951, v111, 113.

3. Dewey, V. C., Parks, R. E., and Kidder, G. W., *Arch. Biochem.*, v29, 281.

4. Hutner, S. H., *J. Gen. Microbiol.*, 1950, v4, 286.

5. Hall, R. P., Johnson, D. F., and Loefer, J. B., *Trans. Am. Mic. Soc.*, 1935, v54, 298.

6. Tittler, I. A., Belsky, M. M., and Hutner, S. H., *J. Gen. Microbiol.*, 1952, v6, 85.

Received March 10, 1954. P.S.E.B.M., 1954, v85.

Dicumarol and Plasma Antithrombin Activity.* (20931)

NEVENKA IVANOVIC,† J. FREDERIC JOHNSON, JOHN H. OLWIN, AND WALTER H. SEEGERS.

From the Departments of Physiology and Pharmacology, Wayne University College of Medicine, Detroit, Mich., and of Surgery, Presbyterian Hospital, Chicago, Ill.

Recent advances in the subject of prothrombin activation have brought changes in our ideas concerning the effect of dicumarol on the blood coagulation mechanisms. Dicumarol is no longer considered to act exclusively with respect to prothrombin, but also in relationship to the activation of prothrombin. The inhibitors of the blood clotting mechanisms may possibly also be involved. This paper is an attempt to contribute to the subject of antithrombin levels in dicumarol plasma which is a problem that still remains a matter of uncertainty. Hurn, Barker, and Mann(1) reported an increase of antithrombin activity in serum after dicumarol therapy. Whether this represents a real antithrombin increase or is the result of unknown factors which influenced their antithrombin test has not been completely clarified. Owen and Bollman found that dog plasma exerts about the same antithrombic activity regardless of the prothrombin concentration after dicumarolization(2). At the time their papers were written less was known about the fundamental nature of plasma antiprothrombin activity than today. The background of our paper is based on the idea that more than one mechanism is involved in antithrombin activity, and that it is possible to analyze the diverse aspects of thrombin inactivation in separate systems of study. For a better understanding of the problem the following nomenclature classifying the diverse antithrombin reactions is used in this laboratory. Antithrombin-I refers to the adsorption of thrombin on fibrin. Antithrombin-II concerns the heparin cofactor, which together with heparin interferes with the interaction of thrombin and fibrinogen. Antithrombin-III refers to the natural antithrombin, which neutralizes thrombin quantitatively as a function of the thrombin concentration, and does not require heparin. Antithrombin-IV seems to be independent of the actions cited above, and becomes manifest during the process of prothrombin conversion. It is the activity which remains after antithrombin-III activity is removed by ether extraction. We want to emphasize that this is an arbitrary nomenclature, and in part based on theory, since the experiments performed in this field do not provide conclusive support for the existence of 3 independent substances. It is still possible to consider the antithrombin-IV phenomenon as interactions of different and dissociable phenomena arising from the ar-

* This work was supported by a grant from the Michigan Heart Assn. Funds for Research Fellowships in Physiology provided by Parke, Davis and Co. We wish to thank Ray Heinrich of Harper Hospital for his advice and help.

† Dental School, University of Chile, Santiago.

TABLE I. Antithrombin-II Analysis. Clotting time (in seconds) of 0.4 ml dicumarol plasma mixed with 0.1 ml thrombin in 6 dogs.

No heparin	Heparin† (20 units)	Heparin† (2 units)
6.8*	No clot	16
6.8	" "	16
7.0	" "	14
6.0	" "	12
6.3	" "	9
6.0	" "	11

* Control without dicumarol.
† Liquaemin Roche.

tificial condition of the experimental approach. However, our experimental data allow the conclusion that an antithrombin action is closely connected with prothrombin activation. This fact leads to a reconsideration of prothrombin activation in terms of antithrombin activity.

Experimental. In this study 3 antithrombin effects were studied. Antithrombin-I was omitted because we do not consider adsorption on fibrin of appreciable importance under physiological conditions. Accordingly, all experiments were carried out on defibrinated plasmas. Prothrombin was determined by a 2-stage method(3) on all samples of blood. *Blood.* This was collected by means of 2-syringe technic. As anticoagulant 3.2% sodium citrate was mixed with blood in a 1-9 ratio; the plasma was obtained by centrifugation at 2500 g for 20 minutes. *Dicumarol administration.* Dogs were separated into 2 lots; one received a single dose of 100 mg of dicumarol, the other group was given 2 doses of 100 mg on consecutive days. On the third day they were exsanguinated after previous anesthesia. When human blood was analyzed, a single dose of dicumarol was given, and the blood was analyzed for prothrombin and antithrombin. *Antithrombin-II.* This was studied by mixing heparin (Liquaemin Roche) with purified thrombin(4). The mixture was tested for activity by adding 0.1 ml of heparin-thrombin mixture to 0.4 ml of the unknown sample. For the control the same thrombin was mixed with plasma but without heparin.

Results. Table I illustrates the antithrombin-II activity on dogs treated with dicumarol and sacrificed by exsanguination. The figures show a normal antithrombin-II activity, although in one case, in the higher dilution of heparin, a short clotting time was detected. We realized too late that it would have been better to ascertain the clotting time of the plasma before dicumarol was given, and not to rely entirely on the other kind of control.

Antithrombin-III was studied by the technic of Seegers and associates(5). The dogs were given a single dose of 100 mg dicumarol. From our extensive experience with this method we know that the antithrombin-III titer of dog plasma is subject to appreciable variations, and for this reason we consider only a variation of more than 20% of normal levels as significant. Judging by these standards the data of Table II lead us to the conclusion that no antithrombin-III changes can be detected with dogs, after the administration of dicumarol.

Antithrombin IV. As stated previously(6) there is a close relationship between antithrombin-IV and prothrombin activation. It was, therefore, necessary to devise laboratory procedures which would enable us to study both the rate of prothrombin activation and the rate of thrombin disappearance. For that purpose, a test was devised which we refer to as the thrombin-titer method of analysis(6).

TABLE II. Plasma Antithrombin-III, and Prothrombin Concentration in Dogs Given 100 mg Dose of Dicumarol.

Days after dicumarol	Dog No. Control	2	3	4	5	6
0	58 (100)*	73 (100)	75 (100)	48 (100)	68 (100)	63 (100)
1	60 (100)	70 (30)	75 (70)	48 (64)	66 (33)	69 (49)
3	66 (100)	69 (9)	76 (13)	55 (19)	54 (8)	65 (15)
8	71 (100)	68 (41)	72 (65)	48 (29)	68 (15)	71 (54)

* The figures in parentheses refer to % prothrombin conc. and the antithrombin values refer to the % of purified thrombin, 1500 units substrate, destroyed by 1 ml defibrinated plasma in 2 hr.

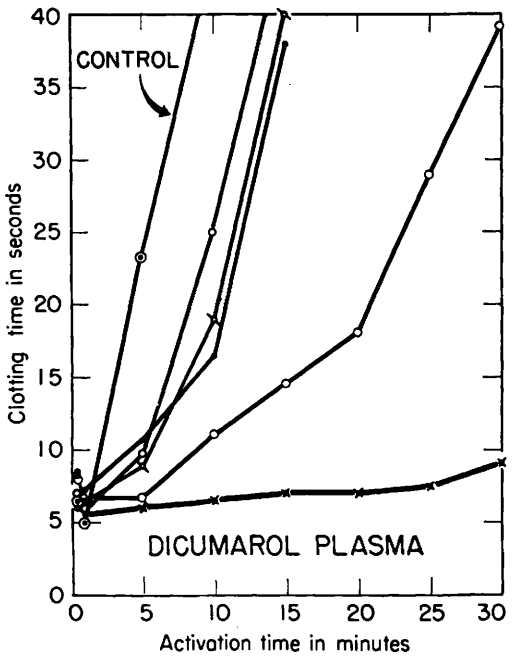

FIG. 1. Antithrombin-IV activity by thrombin-titer method. The experiment was performed at height of dicumarol effect. Prothrombin concentration of plasma was, therefore, low. Prothrombin concentration was accordingly adjusted to 300 units/ml plasma by adding purified bovine prothrombin. Each curve represents results for one dog. Control received no dicumarol.

In this method, antithrombin-III is first removed by extracting defibrinated plasma with ether. The prothrombin is then activated as one would in a one-stage prothrombin time procedure. Measured quantities of the reaction mixture are then mixed with standardized fibrinogen solution at repeated intervals to see how much thrombin is in the reaction mixture. Instead of measuring the thrombin concentration accurately one simply notes the clotting time of the fibrinogen at repeated intervals. As the clotting time becomes shorter and shorter, one observes an index of the rate of thrombin formation and as the clotting time subsequently becomes longer one observes an index of thrombin destruction. By plotting such data as activation curves it is also possible to obtain some idea of the quantity of thrombin which forms. When one wishes more accurate or detailed insight the work is supplemented with quantitative thrombin analyses as well as 2-stage prothrombin analyses.

Our work related to the study of antithrombin-IV in dogs receiving dicumarol was extensive and the results of Fig. 1 represent only one group of experimental animals. In these experiments the dogs were given 100 mg dicumarol as a single dose. The prothrombin concentration, as measured by modified 2-stage method(3), showed a decline to very low values. At about the lowest point for prothrombin activity the thrombin titer method showed that antithrombin-IV activity remained. The dog prothrombin was supplemented with purified prothrombin in order that there might be an adequate thrombin substrate that could involve antithrombin-IV action. In one dog out of the 5 antithrombin-IV activity was rather low. Nevertheless it seemed to us that although there was a tendency toward variability, antithrombin-IV activity was generally present after dicumarol administration. Accordingly, the experiments were repeated with a second group consisting of 6 dogs and there was again approximately the same kind of variation. Then another group of 6 animals was given 100 mg of dicumarol followed by a second dose of 100 mg on the second day. On the third day the dogs were sacrificed by exsanguination. The antithrombin-IV concentration of those plasma samples was again variable but in general the indications were definitely in favor of antithrombin-IV activity. In all of these experiments with dogs we found that the thrombin titer method indicated rapid activation of the prothrombin of the plasma and also of the purified prothrombin added as a supplement. In the dogs we have found no exception to this after dicumarol administration. That observation is, however, in contrast to those found in studies with human beings.

Our studies of human subjects involved the use of the thrombin-titer method and the 2-stage prothrombin analysis technic on plasma from patients under varying circumstances relative to the administration of dicumarol. Some had received the drug for years. Other cases were followed from the beginning up to a few weeks. Some were studied by adding purified prothrombin to the plasma while others were studied only in terms of their own

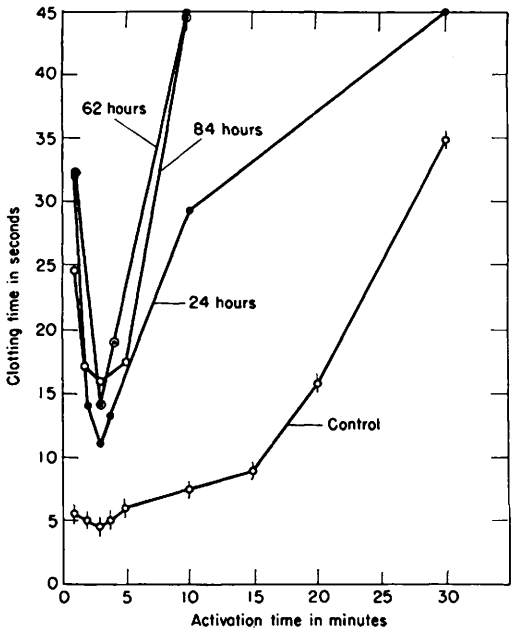

FIG. 2. Antithrombin-IV activity by thrombin-titer method. A human subject given a single 300 mg dose of dicumarol. The plasma prothrombin concentration as measured by 2-stage was: 75% of normal at 24 hr, 50% at 62 hr, and 75% at 84 hr.

prothrombin. We feel that our most relevant findings are best summarized by the illustration of Fig. 2. This illustrates the antithrombin-IV activity in plasma secured from a patient who received one dose of 300 mg of dicumarol. Antithrombin-IV activity is invariably more striking in human beings than in dogs, and dicumarol does not diminish it. The antithrombin titer method brings out a very striking change in the rate with which prothrombin becomes activated. Even within 24 hours, when the prothrombin concentration has not changed appreciably, the activation of prothrombin is very slow. Even 62 hours and 84 hours after administration of dicumarol this phenomenon is nicely illustrated by this test. In certain instances purified bovine prothrombin was added to dicumarol plasma. It also activated only very slowly and much of it was not consumed. Purified prothrombin was thus unable to supply the missing activator. Presumably the marked changes in prothrombin activation rate detected by the thrombin-titer method involve the factors referred to in the literature by the term factor VII (proconvertin, SPCA, cothromboplastin, stable conversion factor, etc.) or it is concerned with the state of prothrombin reactivity itself. We were able to establish that proconvertin-deficient plasma, from a patient known to be proconvertin-deficient, reacts in the same way as our patients given dicumarol. The thrombin-titer method thus picks up changes related to prothrombin activation in a most useful way and it may be a valuable tool for further studies of the action of dicumarol. It is also remarkable that there is a great difference in the rate of prothrombin activation under the circumstances of our test when the plasma from human patients given dicumarol is compared with the plasma of dogs given dicumarol. With the dogs there is rapid activation of prothrombin, and with the human plasma activation is slow. It is as if the factor VII effect is not demonstrable in dogs.

Miscellaneous. During the course of our studies on antithrombin we made 2 interesting observations which we do not understand and have not been able to reproduce at will. We record them on that basis. 1. Frequently when dogs had been given dicumarol and were exsanguinated they had muscle rigor at the end of the bleeding. This happened many times. Such animals became altogether rigid so that one could balance their entire weight on either the front legs or hind legs. It has not been observed with control animals similarly exsanguinated. We do not know what the requirements are for reproducing this phenomenon. 2. Sometimes it occurred that plasma samples would not destroy thrombin activity in the antithrombin-III analysis. This happened in one instance with plasma from all animals receiving dicumarol and at that time the control groups reacted in the same way. The thrombin could be mixed with the plasma, but none of the activity disappeared. This has occurred in this laboratory a number of times during the past 3 years and it is our understanding that other investigators have found the same results on certain occasions. Since this variable is not understood it means that all antithrombin studies thus far completed carry with them

a certain elementary uncertainty.

Summary. With the administration of dicumarol to dogs there are no significant changes in the activity of antithrombin-II, and -III. Antithrombin-IV activity remained but no accurate quantitative measurements were made. In human subjects antithrombin-IV activity also remains and is probably not decreased. The thrombin-titer method of analysis shows that there are very marked changes in the activation rate of prothrombin soon after dicumarol is administered to human subjects. The addition of purified prothrombin does not restore the activation rate to normal. In contrast to human subjects the activation rate of prothrombin is not reduced in dog plasma when dicumarol is administered.

1. Hurn, M., Barker, N. W., and Mann, F. D., *Am. J. Clin. Path.*, 1947, v17, 712.

2. Owen, C. A., and Boilman, J. L., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 367.

3. Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, v19, 471.

4. Seegers, W. H., McClaughry, R. I., and Fahey, J. L., *Blood*, 1950, v5, 421.

5. Seegers, W. H., Miller, K. D., Andrews, E. B., and Murphy, R. C., *Am. J. Physiol.*, 1952, v169, 700.

6. Seegers, W. H., Johnson, J. F., and Fell, C., *ibid.*, 1954, v176, 97.

Received December 22, 1953. P.S.E.B.M., 1954, v85.

Effects of 3-(o-Toloxy)-Propane-1,2-Diol (Mephenesin) and 3-(2-Methyl-6-Chlorophenoxy)-Propane-1,2-Diol (P105) on α-Excitability of Muscle (20932)

JOAN WRIGHT GOODMAN.* (Introduced by W. O. Fenn.)

From the Department of Physiology and Vital Economics, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

3-(o-toloxy)-propane-1,2-diol (Mephenesin) has gained considerable attention as a muscle relaxant in both investigative and therapeutic fields within the last 10 years; its pharmacology has been reviewed by Berger(1). Lambooy(2), seeking more potent compounds for clinical use, synthesized 3-(2-methyl-6-chlorophenoxy)-propane-1,2-diol (P105) and found that although it was more toxic and less soluble than Mephenesin, it was twice as potent a paralyzing agent.

The experiments of Smith(3) and of others (1) on mono- and multi-synaptic reflexes indicated that Mephenesin specifically depressed internuncial neurones, but reports of conduction. rheobase, and chronaxie changes in peripheral nerve have been conflicting. Berger(4) failed to find any skeletal muscle changes produced by Mephenesin even in very high concentrations. Stephen and Chandy (5), however, found cat tibialis anticus action potentials to be reduced at dosages of 50 mg/kg. Mephenesin has also been found to cause contracture of frog rectus abdominis, as well as inhibition of acetylcholine-produced contracture of this muscle(6). In view of these various findings, it appeared of interest to study the effects of Mephenesin and of its analog, P105, on the electrical excitability of muscle.

Method. Blair(7) showed that the strength-duration curve of frog's muscle is adequately represented by the equation:

$$\log \frac{V}{V-R} = kt + \log \frac{K + k\alpha}{K} \qquad (a)$$

in which V is the strength of the stimulus, t is its duration, R is the rheobase, k is the coefficient of subsidence of the excitatory state, and in which the second constant $\frac{K + k\alpha}{K}$ represents physical rather than physiological factors. It can be shown, from the derivation of equation (a), that:

$$\frac{h}{K} = \frac{R}{k} \qquad (b)$$

when h is the particular value of the excita-

* Present address: Division of Pharmacology, Dept. of Radiation Biology, University of Rochester School of Medicine and Dentistry.