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Studies on the Anticoagulant 3,3′-Methylene-Bis-(4-Hydroxycoumarin).

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Recent reports on experimental and clinical studies of 3,3′-methylene-bis-(4-hydroxycoumarin), (Dicumarol*), a substance originally isolated from spoiled sweet clover and capable of decreasing the prothrombin content of blood *in vivo,* have indicated the possible application of this material in clinical medicine.

The history, chemical isolation, synthesis and assay of 3,3′-methylene-bis-(4-hydroxycoumarin) have been described in a number of papers by Link and associates.[1-5] Experimental and clinical investigations have been conducted by Butt and associates,[6] as well as by Meyer and coworkers.[7, 8] Further data have appeared in the American[9] and foreign[10, 11] literature.

Unlike heparin, Dicumarol does not inhibit blood coagulation *in vitro.* It exerts its anticoagulant effect following oral or parenteral administration, but there is a considerable latent period whichever route is used. This publication deals with its efficacy in preventing thrombosis and its toxicity.

Meyer and coworkers[7] attempted to demonstrate the ability of the Dicumarol to inhibit experimental thrombosis, but they found

* Dicumarol is the collective trademark of the Wisconsin Alumni Research Foundation, which controls the use thereof.

1 Campbell, H. A., Roberts, W. L., Smith, Wm. K., and Link, K. P., *J. Biol. Chem.*, 1940, **136**, 47.

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3 Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 513.

4 Campbell, H. A., Smith W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, 1941, **138**, 1.

5 Overman, R. S., Stahmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, 1942, **142**, 941.

6 Butt, H. R., Allen, E. V., and Bollman, J. L., *Proc. Staff Meet. Mayo Clin.* 1941, **16**, 388.

7 Bingham, J. B., Meyer, O. O., and Pohle, F. J., *Am. J. Med. Sci.*, 1941, **202**, 563.

8 Meyer, O. O., Bingham, J. B., and Axelrod, V. H., *Am. J. Med. Sci.*, in press.

9 Centr. Soc. f. Clin. Res., *J. A. M. A.*, 1942, **118**, 1003.

10 Lehmann, J., *Lancet*, 1942, **1**, 318.

11 Townsend, S. R., and Mills, Edw. S., *Canad. Med. Assn. J.*, 1942, **46**, 214.

their methods inadequate. In our experiments we used a method similar to the one developed in Best's laboratory.[12] Thrombosis was produced in the peripheral veins of dogs and the prevention of thrombus formation was attempted in other dogs by premedication of the animals with Dicumarol.

Segments of about one inch in length of the radial and saphenous veins of dogs were injected through the skin with 0.15 or 0.25 cc of ethanolamine oleate (Monolate, Abbott), while they were temporarily excluded from the circulation by finger pressure applied on the proximal and distal ends of the segment. This compression was sustained for 3 minutes and then relieved, permitting restoration of circulation. Two to 4 veins were used in each of 4 animals. These segments of the veins were removed 3 to 7 days after the injection and were examined histologically. The data are assembled in the table. As the results indicate, thrombus formation follows this procedure in almost all cases, particularly when the veins were removed 6 or 7 days after injection.

In the next series of experiments, 4 dogs weighing 6 to 9 kg were given 10 or 25 mg daily of Dicumarol by mouth. Their plasma prothrombin time was determined before and during the administration by Link's[4] modification of the method described by Quick. We used the whole plasma and a 25 and 12.5% dilution, as recommended by Link and coworkers. Coagulation time of whole blood was measured by the method of Lee and White. (See table.) The prothrombin time of these dogs rose within 36-48 hours and then increased rapidly, while the coagulation time increased less markedly. Three to 5 days after the feeding with Dicumarol was started, Monolate was injected in 3 veins of each dog as described above. The administration of Dicumarol was continued at the same level. The veins were removed 3 to 6 days after the injection through a small incision which was carefully closed, in some cases with the local application of thrombin powder. In spite of these precautions the wounds oozed considerably and a blood transfusion of 100 cc given to 2 of the dogs was of temporary benefit only. All 4 died of anemia due to hemorrhage a few days after the veins were removed. Careful histological examination of all veins was made. As can be seen in the table, 8 of the 12 veins showed no change at all except a hypertrophy of the wall in one of them. A slight fibrin deposit was present in one vein and beginning or moderate thrombus formation in two. Only one vein showed an extensive thrombosis.

[12] Murray, D. W. G., Jaques, M. A., Perrett, T. S., and Best, C. H., *Surgery*, 1937, **2**, 163.

TABLE I.
Thrombus Formation by Intravenous Monolate Injection and Inhibition of Thrombosis by Dicumarol.

Dog	Daily dose, mg	No. of doses	Monolate, cc	Vein removed, days after inj.	Histological findings (thrombosis)	Prothrombin time of 12.5% plasma. Below in ( ) blood coagulation time	
						Normal	On day of monolate inj.
A	0		.25	3	++		
	0		.25	7	+++		
B	0		.15	3	+		
	0		.15	6	++		
	0		.25	3	+		
	0		.25	6	+++		
C	0		.15	3	(+)		
	0		.15	7	+++		
	0		.25	3	(+)		
	0		.25	7	+++		
D	0		.15	3	(+)		
	0		.15	7	+++		
	0		.25	3	+		
	0		.25	7	+++		
I	10	7	.25	3	Negative	40.5″	491.0″
	10	7	.25	5	″	(5′12″)	(8′0″)
	10	7	.15	5	Hypertrophy of wall; no thrombus.		
II	10	15	.25	6	Negative	33.4″	279″
	10	15	.25	6	+++	(4′20″)	(6′30″)
	10	15 2x	.25	6	Negative		
III	25	7	.25	3	″	40.9″	196.2″
	25	7	.25	5	Slight fibrin deposit	(5′0″)	(7′30″)
	25	7	.15	5	Negative		
IV	25	9	.25	6	Negative	32.6″	171″
	25	9	.25	6	+	(5′0″)	(5′0″)
	25	9	.25	6	(+)		

(+) Some fibrin deposits.
+ Intima damage and beginning thrombosis.
++ Wall adherent thrombus; lumen not completely occluded.
+++ Completely or almost completely occluding thrombus with advanced organization.

Thus, the administration of Dicumarol in the doses selected was capable of greatly reducing the incidence of thrombus formation as compared with untreated controls.

Histological examinations of the tissues of the 4 dogs dying as a result of operation during the Dicumarol administration were performed. The general picture was that of advanced anemia, and of particular interest was the occurrence of fatty infiltration of the liver and scattered zones of necrosis in this organ in 2 of these 4 dogs. While the mechanism of the prothrombin depression pro-

duced by Dicumarol is not yet understood, it is possible that the depression is a result of a direct toxic action on the liver. For this reason a study of liver pathology and liver function appeared indicated. Two dogs were given 10 or 25 mg respectively of Dicumarol for 10 days. The animal having received the 25 mg dose died on the 11th day of internal hemorrhage. Its liver showed a marked cloudy swelling, edema, and areas of beginning necrosis. The other dog was killed and autopsied. Here the liver showed a moderate degree of cloudy swelling, a vacuolization of the cells, but no signs of necrosis. Incidentally, a moderate decrease of hemoglobin and RBC count occurred in most of the animals, even when no signs of bleeding could be detected. The WBC and the urine examination did not show peculiarities.

In order to investigate the possibility of functional liver damage, bromsulfalein retention and intravenous glucose tolerance were studied in 2 dogs weighing 17 and 18 kg respectively. Each dog was injected intravenously twice in a 3-day interval with 900 mg/kg of glucose and the blood sugar determined before and 15, 30, 60, and 120 minutes after the glucose injection. Then, each of the 2 dogs was injected intramuscularly with 30 mg of Dicumarol daily for 6 days. Experiments on dogs had shown that a 2% suspension of the Dicumarol in tragacanth can be injected intramuscularly and is effective. However, it does not seem that this mode of administration greatly hastens the onset of the effect. Oral administration was resorted to for another 9 days on account of pain and swelling following the repeated injections. Prothrombin times were routinely determined. On the last day of the administrations, the glucose tolerance test was repeated but failed to show a significant deviation from the previous control tests in both dogs. Similarly, intravenous bromsulfalein injections with 5 mg/kg given before and after the administration of Dicumarol did not reveal an increased dye retention. The absence of functional liver damage in these experiments is in agreement with clinical findings published by Meyer and associates.[7]

Experiments in Monkeys (Macacus rhesus). One monkey of 4 kg weight was given 5 mg/kg of Dicumarol orally daily and another one of 3.9 kg weight, 10 mg/kg daily. Their plasma prothrombin time rose rapidly from a normal value of 50 seconds for the undiluted plasma to 600-700 seconds within 3 days. While being handled, both monkeys contracted minor external injuries and subcutaneous bruises which resulted in chronic hemorrhage. The animals finally became anemic and had to be killed 8 to 10 days after

the beginning of the experiments. The autopsies showed a picture of marked internal hemorrhage and advanced anemia. One of them showed an increased fat content of the liver and both had a few areas of necrosis in this organ. The other organs were negative except for a marked capillary dilation and a moderate degree of cloudy swelling.

Experiments in Guinea Pigs. Three groups of 6 animals each were formed. The first group was kept on a normal diet, the second one received a daily injection of 50 mg of vitamin C in addition to this diet, and the third group consisted of animals which were kept on a vitamin C-free diet for the last 2 weeks and through the duration of this experiment. All animals were given orally 25 mg of Dicumarol every second day until death occurred. The average survival times for the 3 groups were 5, 4.5, and 3.5 days respectively. While the small number of animals makes a statistical evaluation impractical, a tendency of the vitamin C-depleted animals to succumb earlier to the Dicumarol is apparent. This is in agreement with observations made by Sullivan in Link's laboratory.[13]

Histological examination of the livers in several animals of all 3 groups showed a tendency to fatty infiltration and degeneration and a few foci of early necrosis. An identical picture was observed in guinea pigs kept on a vitamin C-free diet without administration of Dicumarol. Such changes were found to be entirely reversible after normal dietary conditions were restored to the vitamin C-depleted animals. A connection between vitamin C level and sensitivity to Dicumarol must be considered possible.

Comments and Summary. 3,3'-methylene-bis-(4-hydroxycoumarin), Dicumarol, was shown in our experiments to be able to greatly reduce the incidence and degree of thrombus formation following the intravenous injection of Monolate in dogs. In dogs and monkeys some signs of necrosis of the liver were found in a number of animals, but since most of them suffered from a severe anemia, the significance of this finding is questionable. The livers of guinea pigs treated with the sweet clover factor showed changes as they occur in vitamin C-depleted animals. Intravenous glucose tolerance and bromsulfalein retention tests failed to show liver damage in animals not suffering from bleeding due to the administration of the drug. While this is in agreement with clinical findings, further studies of the physiology of this substance with particular reference to liver function appear indicated.

Our present experience shows that there is a considerable difference

[13] Sullivan, W. R., Doctor Dissertation, Univ. Wisc., Madison, May, 1942.

in tolerance between various species of animals and also among the individuals of the same species. The observation that single blood transfusions in dogs in a state of extreme depression of the prothrombin level were of only temporary benefit, is paralleled by similar clinical findings[11, 8] and calls for particular care in avoiding excessively high dosage.

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Comparative Studies of the Effect of Thiamine Deficiency in Diabetic and Non-Diabetic Rats.

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Certain workers[1, 2, 3] have claimed amelioration of human diabetes with the use of thiamine, particularly when given in combination with other vitamins. Martin[4] reported intensification of diabetes in depancreatized dogs on a vitamin B-free diet, with remarkable improvement in carbohydrate tolerance when thiamine and flavin were given. On the other hand, in normal rats, Lepkovsky, Wood and Evans[5] found the glucose tolerance impaired only in the later stages of deficiency and McIntyre and Burke[6] noted very little change in glucose tolerance, either with deficiency or excess of vitamin B.

The present study was undertaken to determine the effect of deprivation of thiamine on the diabetic condition of depancreatized rats and to ascertain whether or not diabetic animals, when deprived of thiamine, develop signs of deficiency more readily than those without diabetes. It was hoped that the results obtained might elucidate the possible rôle of vitamin B, and specifically of thiamine, in the peripheral neuritis encountered in patients with severe, uncontrolled diabetes.[7]

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