Cold-Adapted Animals. I. Changes in Blood Clotting and Electrophoretic Properties of Rabbit Plasma.* (22170)

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Changes in the clotting mechanism as a result of prolonged exposure to low temperatures, "cold adaption," have occasionally been noted(1) for non-hiberating mammals and people. More marked changes have been observed during hibernation or aestivation in mammals such as ground squirrel or hamster (1-4). During preliminary studies on cold adaption in rabbits, it was observed that plasma clotting times were prolonged, prothrombin times decreased, platelet material increased, and fibrin yield was greater. The present studies were undertaken to extend these experiments. Some changes in technic were made and earlier results, in qualitative agreement with the present ones are not included in the Tables. Electrophoretic studies, initially made to determine if abnormalities occurred in the fibrinogen component, have been included.

Methods. Conditioning of rabbits. Six young healthy male rabbits, about 3 kg, were placed in a cold room at 4°C for 2 months. During the first month animals were progressively clipped until hair remained only on the extremities. Animals were maintained in this condition by regular clippings for the last month. Animals gained weight at a slightly higher rate than controls and appeared healthy throughout the experiment. A similar number of controls were kept, unclipped, in the usual animal quarters. *Hematological technics.* 1. *Bleeding.* All glassware and needles were freshly siliconed before use (Desicote, Beckman Instrument Co.). Three ml of blood were drawn by cardiac puncture and discarded. A 50-ml syringe was placed on the needle, temporarily left *in situ,* and 40 ml blood was rapidly withdrawn. Thirteen ml of this blood was immediately transferred to paraffined 15-ml conical centrifuge tube, the remaining 27 ml transferred to 50-ml centrifuge tube containing 3 ml of 0.1 M sodium oxalate, and stirred by gentle inversion, using Parafilm as cover. 2. *Blood clotting times.* The 15-ml centrifuge tubes stood at room temperature for 10 minutes, and then tilted every 30 seconds until no flow occurred on complete inversion. 3. *Erythrocyte and platelet counts.* Portions of oxalated blood were diluted in Rees-Ecker solution and counted in a Neubauer counting chamber. 4. *Plasma clotting times.* The oxalated blood was spun for 5 minutes at 1500 rpm (133 g) and a portion of platelet-containing plasma withdrawn (samples "A" in Table II). Blood was then spun for 45 minutes more at 3000 rpm (1600 g) and platelet-poor plasma removed, (samples "B" in Table II). A portion of each sample was diluted with equal volume of 1% saline, and 0.1 ml transferred to 13 x 100 mm test tube and incubated for 150 seconds at 37°C. An equal volume of 0.0139 M $CaCl_2$† at 37°C was then added and the tube sat for

TABLE I. Platelet and Erythrocyte Counts, and Hematocrits of Normal and Cold-Adapted Rabbits.

Rabbit No.	Platelet counts (× 1000)	Erythrocyte counts (× 1000)	Hematocrits, %
	Normal rabbits		
993	322	5170	43.2
994	317	5930	44.5
995	417	5830	41.0
996	383	5960	41.6
997	389	4960	39.2
Mean	366	5570	41.9
	Cold-adapted rabbits		
976	395	5790	41.0
977	382	6730	47.4
978	485	6340	45.0
979	399	5210	43.9
980	487	5720	47.4
982	348	5620	45.5
Mean	416	5900	45.0
Mean % change in cold	+13.7	+5.9	+7.4

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TABLE II. Clotting Data on Blood from Normal and Cold-Adapted Rabbits.

Rabbit No.	Blood clotting time (min.)	Plasma clotting time (sec) A*	Plasma clotting time (sec) B*	Prothrombin time (sec)	Fibrin yield (g/100 ml)
		Normal rabbits			
993	26.5	87	173	66.0	.44
994	29.5	87	170	58.5	.52
995	24.5	77	150	47.0	.44
996	10.0	67	160	—	.43
997	25.0	77	400	57.5	.37
Mean	23.1	79.0	210.6	57.3	.44
		Cold-adapted rabbits			
976	29.0	87	273	—	.47
977	24.0	77	173	36	.59
978	21.5	117	733	38	.59
979	32.0	77	143	39	.60
980	38.0	93	263	32	.81
982	50.0	67	113	40	.66
Mean	32.4	86.3	283.0	37.0	.62
Mean % change in cold	+40.2	+9.3	+34.3	-35.4	+41.0

* Samples "A" spun at 1500 rpm (133 g) for 5 min.
" "B" " " 3000 " (1600 g) " 45 " .

50 seconds. It was tipped thereafter every 10 seconds, and the time for a firm clot to appear was taken as the end point. 5. *Fibrin determinations.* Clots from plasma clotting time determinations sat at room temperature for 16 hours, and then shaken free from walls of tubes, and the tubes filled with 1% saline. They were spun at 3000 rpm (1600 g) for 45 minutes and fluid above the collapsed clot drawn off through a capillary by suction. The fibrin mat was washed in this fashion 3 more times, and then analyzed for nitrogen by Micro-Nessler technic of Lanni(5). Conversion to protein was made by using the 6.25 factor. 6. *Prothrombin time.* These values were obtained in triplicate according to the one-stage method of Campbell, Smith, Roberts, and Link, as adapted by Holburn(6). Dilutions of platelet-free plasma were 5.0, 8.34, 12.5 and 20%, and values presented were taken from the log log plots of clotting time vs plasma concentration at the 10% plasma point. 7. *Total protein determinations* were done by the Micro-Nessler method of Lanni(5) on triplicate 0.2 samples of 1:100 dilution of plasma. 8. *Electrophoretic analyses* were made with a Perkin-Elmer apparatus using pH 8.6 Veronal buffer, ionic strength 0.1.

Results. From the data presented in Table I it appears that cold-adapted animals develop an increase in platelet‡ and erythrocyte counts and in hematocrit.

Table II presents results of clotting studies. It can be seen that in cold adapted rabbits blood and plasma clotting times are increased, while prothrombin time decreased. There is also an increase in the fibrin yield.

The results of electrophoretic analyses are presented in Table III. Cold adaption resulted in decrease in albumin, and increase in β globulin and fibrinogen fractions. There also appears to be an overall increase in total protein under these conditions which although not statistically significant by these rigorous standards, was also present in preliminary studies. None of the serum patterns (results not presented) showed presence of any residual fibrinogen after clotting in either cold-adapted or normal animals.

† This concentration of $CaCl_2$ was used since it gave the shortest clotting time as determined in previous studies with rabbits.

‡ Although the present data definitely suggests an increase in platelet count in cold-adapted animals, the statistical significance of the results might be questioned because of the poor reproducibility of this type of determination.

TABLE III. Relative Concentration of Proteins in Normal and Cold-Adapted Rabbits Based on Electrophoretic Data.

Rabbit No.	Albumin	α-Globulin	β-Globulin	Fibrinogen	γ-Globulin	Total protein, g/100 ml
			%			
			Normal rabbits			
993	68.8	8.8	8.3	9.0	5.1	7.01
994	64.4	9.4	10.0	8.6	5.6	7.30
995	66.4	8.5	12.0	7.6	5.7	7.48
997	65.2	9.0	10.0	8.5	7.5	7.20
Mean	66.2	8.9	10.1	8.4	6.0	7.25
			Cold-adapted rabbits			
977	58.6	8.4	14.8	11.8	6.3	8.03
978	53.7	8.0	15.8	14.0	8.7	7.18
979	61.0	6.6	13.4	12.5	6.6	7.30
980	56.0	8.2	13.6	15.0	7.2	7.93
982	57.6	9.5	13.5	11.6	7.9	7.38
Mean	57.4	8.1	14.2	13.0	7.3	7.56
$\frac{\Delta\ \%^*}{3S_D}$	2.08	.35	1.55	2.16	.33	.52

* Critical ratio—a value of 1 or greater indicates a probability of at least 99.7 that the means are significantly different.

Discussion. The observed increase in total protein (4.3%), erythrocyte count (5.9%), and hematocrit (7.4%) suggests that hemoconcentration occurred as a result of cold adaption. This effect has been observed by others(7,8) and evidence was presented which indicated that this phenomenon was of rapid development and short duration. Unfortunately we have no determinations of blood volume which would indicate whether the observed hemoconcentration was the result of reduced volume, or of increased quantities of the factors mentioned. Hemoconcentration, however, would not be expected to produce the observed changes in the clotting mechanism.

Increase in blood and plasma clotting time in the presence of decreased prothrombin time suggests that thromboplastin is less available in cold-adapted rabbits. Since there is an increase in platelet count it appears that platelets in these animals may be more resistant, although it is possible that thromboplastin is itself less effective for chemical reasons.

The decrease in prothrombin times in plasma from cold-adapted rabbits could be attributable to a number of factors, but may possibly be explained by increased fibrinogen shown by electrophoresis and increased fibrin yield. The prothrombin times are plotted in Fig. 1 as a function of fibrin yield. Sufficient correlation exists between these parameters to indicate that at least part of the observed change is due to the increased fibrinogen.

Summary. Rabbits maintained for 2 months at 4°C, developed increases in platelet and erythrocyte count, hematocrit, plasma protein concentration, β-globulins and fibrinogen and in whole blood and plasma clotting times. Decreases occurred in serum albumin concentration and prothrombin time. It was suggested that retardation of clotting in spite of decreased prothrombin time might be due

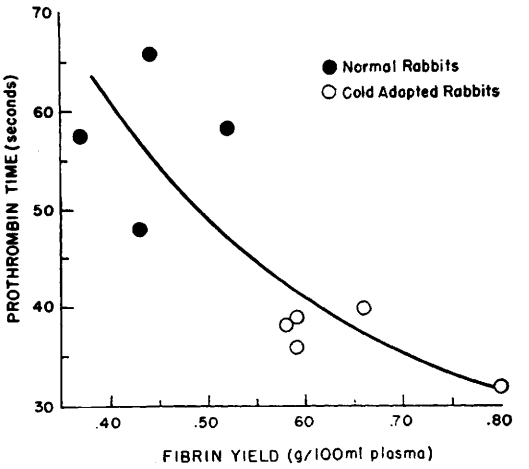

FIG. 1. Prothrombin times as a function of fibrin yield in all rabbits studied.

to an increase in resistance of platelets or to some chemical alteration of thromboplastin.

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Adrenal Corticoid Activities of 2-Methylhydrocortisone Acetate and 2-Methyl-9α-Fluorohydrocortisone Acetate. (22171)

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Biological activity of hydrocortisone is markedly increased by halogenation of C-9 (1-5) and by dehydrogenation at positions C-1 and C-2(6,7). When both modifications are present in the molecule, glucocorticoid, but not mineralocorticoid, activity of the steroid is further potentiated(8-10). This paper reports the results from biological testing of 2 new analogues of hydrocortisone: 2-methylhydrocortisone and 2-methyl-9 α-fluorohydrocortisone. These compounds have greatly enhanced gluco- and mineralocorticoid activities, as determined by subcutaneous administration on four different assays.

Materials and methods. Steroids. Steroid suspensions were made by grinding the compounds with carboxymethyl cellulose vehicle (8) in a ground glass tissue homogenizer. Compounds used were: 11 β, 17 α, 21-trihydroxy-4-pregnene-3,20-dione(F), 21-hydroxy-4-pregnene-3,20-dione, 21 acetate (DOCA), 9α-fluoro-11 β, 17α, 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (FF ac), 2-methyl-11 β, 17 α, 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (methyl F ac), and 2-methyl-9 α-fluoro-11 β, 17α, 21-trihydroxy-4-pregnene-3,20-dione, 21-acetate (methyl FF ac). The latter 2 new steroids were recently reported by Hogg, Lincoln, Jackson and Schneider(11). *Anti-Inflammatory Test.* This test measures the effect of steroids on granuloma formation in the rat by the cotton pellet assay described by Meier, Schuler and Desaulles(12) as modified by Dulin(9).

TABLE I. Anti-Inflammatory Assays Comparing Methylhydrocortisone Acetate, Methylfluorohydrocortisone Acetate, Hydrocortisone, and Hydrocortisone Acetate.

Compound*	Dose (mg/day)	No. rats	Mean granuloma dry wt (mg)	Potency ratio†
Vehicle	—	10	15	—
HA	.3	10	13.9	MA is
	.9	9	11.9	6.4 × HA
MA	.2	10	10.6	(3.4-10.7)
	.6	10	9.4	
Vehicle	—	8	14.2	
H	.3	8	11.7	MA is
	.9	8	8.4	4.5 × H
MA	.1	8	11.0	(2.6-7.2)
	.3	8	7.1	
Vehicle	—	10	15.3	
H	.2	9	10.6	MFA is
	1.0	8	6.3	9 × H
MFA	.02	9	9.4	(7.1-11.4)
	.1	8	7.3	

* HA = Hydrocortisone acetate; MA = Methylhydrocortisone acetate; H = Hydrocortisone; and MFA = Methylfluorohydrocortisone acetate.

† Figures in parentheses indicate limits of potency ratio at P = .95.