Pharmacology Biochemistry and Behavior, Vol. 2, pp. 817–826. ANKHO International Inc., 1974. Printed in the U.S.A.

Anemia from Sleep Deprivation with Anticoagulants in Rats with Enhancement by PCPA[1]

R. R. DRUCKER-COLÍN[2] L. B. JAQUES AND T. A. CUNNINGHAM

Hemostasis-Thrombosis Research Unit, Department of Physiology and Department of Pathology College of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N OWO

(Received 27 November 1972)

DRUCKER-COLÍN, R. R., L. B. JAQUES AND T. A. CUNNINGHAM. *Anemia from sleep deprivation with anticoagulants in rats with enhancement by PCPA.* PHARMAC. BIOCHEM. BEHAV. 2(6) 817–826, 1974. – Profound anemia was observed to occur in 5 to 8 days in rats fed the anticoagulants phenylindanedione and dicumarol, when fast wave sleep (FWS) was prevented by the inverted flower pot technique. No anemia occurred in groups of rats deprived of FWS or receiving anticoagulants only. Anemia was accelerated by parachlorophenylalanine (PCPA). Anemia was slight when limited FWS was allowed by use of a larger platform or a continuous feeding period. Red blood cell, hemoglobin and hematocrit values all fell dramatically. There was little evidence of hemorrhage grossly, such as occurs in anticoagulant-treated rats with stress. Histologic study failed to display bleeding or stress involution of lymphoid tissue but did show diminished splenic iron depots and diminished haemopoietic activity in the splenic red pulp. Electroencephalography showed with sleep deprivation, a greater amount of brain waves of the type normally associated with slow wave sleep. This effect was partially blocked by serotonin depletion through PCPA injections. It is suggested that the development of anemia is due to the combined effects of several mechanisms. The results support the view that FWS-deprivation is not a stress.

Anemia Anticoagulants Sleep-deprivation PCPA

IN investigations for the past twenty years, Jaques and co-workers [5,6] have shown that when anticoagulant drugs are administered in conjunction with stress conditions, the hemostatic properties of the circulatory system can be severely altered to the point of causing death from acute hemmorrhage. A great variety of stress conditions have been tested in conjunction with anticoagulant drugs and these consistently lead to the same effect, the spontaneous hemorrhage syndrome. This refers to the finding of extensive internal hemorrhage in the animals. If the stress condition is severe, the hemorrhage may be so great as to cause the death of the animal in shock. With less severe stress, considerable extravascular blood is seen on autopsy. In addition to such stressors as intraperitoneal 10% sodium chloride, insulin convulsions, Formalin subcutaneously, electroshock, electric shock to the cage floor, restraint, limitation of movement, etc., the phenomenon is produced by anticoagulants in adrenalectomized rats and by anticoagulants given with ACTH and with certain steroids. One experimental condition which has not been tested in relation to spontaneous hemorrhage is sleep deprivation. Experiments were designed to determine whether deprivation of paradoxical sleep (Fast Wave Sleep or FWS) when combined with anticoagulant drugs would produce the same spontaneous hemmorrhagic effects as did the stressors previously used in our laboratory. While determining the adequacy of the procedure the first pilot experiments demonstrated a new and different phenomenon [3]. The rats in which FWS was limited for a period of 8 days while receiving an indirect anticoagulant, developed an extremely severe anemia with hardly any visible signs of external or internal hemorrhage. The results of the investigation of this unexpected and novel finding provide the subject of this communication. The following questions were examined – the onset and nature of the anemia produced, its underlying pathology, the relation of the phenomenon to spontaneous hemorrhage produced in anticoagulated animals by stress, the nature and effect of changes in the sleep deprivation in production of the anemia.

METHOD

Animals

Wistar strain, male rats were used. In caring for them the

[1] Complete data and experimental details have been reported by Drucker-Colín [2] together with discussion of suggestions in the literature relating to the phenomenon.

[2] Present Address: Instituto de Biologia, Universidad Nacional Autónoma de México, México, D.F., México.

principles of the Canadian Federation of Biological Societies were observed. When received, the animals weighed between 220–280 g and were allowed 15 days of acclimatization. Upon arrival the rats were randomly placed in individual cages housed in isolated airconditioned rooms at 70° to 72°F, with fluorescent lighting and with controlled 12 hr day–night cycles. During the acclimatization period, rats were fed powdered food ad lib and water containing 100 p.p.m. oxytetracycline. During an experiment, the animals were allowed to eat and drink a total of 8 hr a day, divided into three equal periods of 2 hr, 40 min in the morning, afternoon and evening.

Body weights before and after the experiments were determined. At the end of the experiments, the rats were administered a lethal dose of Nembutal. Two samples of 1.8 cc of blood each were drawn by cardiac puncture with disposable plastic syringes containing 0.2 cc of 3.8% sodium citrate. Organs and body cavities were examined carefully for signs of gross hemorrhage. Degree of hemorrhage was scored 0,1,2,3, or 4 as described previously [5,6] and the mean score calculated for each group of animals.

Fast Wave Sleep Deprivation

The sleep deprivation procedure consisted of placing the animals on a small platform (dia. 4.5 cm) surrounded by water inside a plastic container. This appeared to prevent total relaxation of the body musculature normally associated with fast wave sleep (FWS) but did not prevent slow wave sleep (SWS). In a corollary experiment, the platform size was changed to 11 cm in diameter, which allowed the animals to relax.

Drugs

Two anticoagulants were used, 2-phenylindanedione (phenindione, danilone (Frosst)) and 3:3'-methylenebis-(4-hydroxycoumarin) (dicoumarol, dicumarol (Abbott)). These were individually mixed with the powdered food, in the proportions of 800 p.p.m. and 200 p.p.m. respectively. The animals were fed this mixture during their 8 hours of feeding time. Water was available at all times. The amount of food eaten each day was determined in some groups of animals.

Para-chlorophenylalanine (PCPA) from Nutritional Biochemical Corporation, Cleveland, Ohio (316 mg) was suspended in 10 cc of saline and a few drops of Tween 80 and 316 mg/kg given i.p. Animals whose sleep deprivation periods extended beyond this received further injections at 72 hr intervals. Injections of 0.9% saline were used as controls for the effects of PCPA.

Blood Tests

Blood samples from each rat were measured by the hematology section of the University Hospital with the aid of a model S Coulter Counter, for WBC, RBC, hemoglobin (HGB), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC). Prothrombin time was measured in duplicate by the Quick modified one-stage method, using commercial thromboplastins (Baltimore Biological Lab.) and Fibroplastin (Beckton and Dickinson Co.). Times were measured to 200 sec which is about 20 times the normal values. Values are recorded as geometric means, standard deviation, standard error.

Histology

Pancreas, adrenals, kidneys, spleen, liver, testes, small intestine, large intestine, lung and heart from at least two rats from each experiment were removed immediately after the blood samples were taken and fixed in Susa for 16 hr following which the fixative was drained off and replaced with 80% alcohol. The tissues were processed in an automatic tissue processor and embedded in paraplast compound (Fisher Scientific Co.). Random sections of 8 μ thickness were cut and stained. Four staining procedures were used: hematoxylin and eosin (H & E), H.P.S. trichrome, polychrome, Prussian blue.

EEG Recording

Under semi-aseptic conditions and pentobarbital anesthesia (50 mg/kg) with the stereotaxic technique, stainless steel jeweler's screws were implanted over the cortical surface of the rat's skull 2 mm lateral to the midline, 2 mm posterior and 2 mm anterior to the bregma. The electrodes were joined to a 9 pin miniature Cannon Connector and the entire assembly was fixed to the skull with dental acrylic (Nu-Weld). After a minimum 15 days of postoperative recovery in their home cage the rats were placed inside the sleep deprivation containers in a radio frequency shielded room and EEG recordings made with a Grass Model 5 D, 4 Channel Polygraph with one of the channels integrated with a Grass Model U1-1 Unit Integrator. The animals were recorded for a period of 4 hr daily between 8:00 p.m. and midnight. Recordings from all rats and integrations were made with the same sensitivity settings. The EEG was analyzed by visual inspection and the occurrence of slow waves over time was calculated.

RESULTS

Anemia Produced by FWS – Deprivation With Anticoagulants

Independent groups of 10 rats were FWS-deprived from between 1 to 8 days with and without anticoagulants, while at the same time separate groups of rats were either treated with anticoagulant only, or given no treatment at all. On the final day of treatment, the rats were killed for determination of hematological values. The values as mean and standard deviation for all normal animals and animals with single treatments are shown in Table 1. Tests for significance (t-tests) showed no difference in these values for the normal and single treatment groups (with the exception of values for prothrombin time). There is great uniformity in values as shown by the small values for the standard deviation, indicating uniformity in the rat population even with these treatments.

In the lower part of Table 1 are shown the values for individual rats FWS-deprived and receiving dicumarol for 8 days. In this group of 10 rats, 3 showed hematocrit values below 10%, 2 more below 20%. Hematologic values for one of these were RBC – 0.71 × 10^6/mm^3, hemoglobin, 1.7 g %, hematocrit – 4.4%. In spite of these very low values, values for mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration of these animals were still in the range of normal. This shows that a normocytic anemia was produced. Three rats gave values within the normal range and one (Nr. 8-7) was close to normal. In spite of the dilution effect of these

TABLE 1

HEMATOLOGIC VALUES AND HEMORRHAGE SCORE FOR RATS RECEIVING ANTICOAGULANTS WITH AND WITHOUT SLEEP-DEPRIVATION

Rat No.	WBC × 10^3/mm^3	RBC × 10^6/mm^3	Hgb gm%	Hct %	MCV μ^3	MCH $\mu\mu$g	MCHC %	Proth. T. (sec)	Hem. Score
				Mean Values for 79 Control Rats (No Treatment)					
Mean	5.99	6.60	13.50	37.36	52.95	19.82	36.58	18.86	0
S.D.	2.84	0.51	1.00	2.95	2.85	1.03	1.62	0.11	
				Mean Values for 70 Rats on Sleep-Deprivation					
Mean	7.48	6.60	14.36	38.32	55.20	21.66	37.61	18.43	0
S.D.	1.18	0.29	0.56	1.29	2.65	0.90	0.75	0.10	
				Mean Values for 80 Rats Treated with Dicoumarol					
Mean	10.59	6.51	12.40	35.29	53.1	19.08	34.95	–	0.5
S.D.	2.59	0.44	0.55	2.06	1.59	0.59	0.64	–	
				Mean Values for 80 Rats Treated with Phenindione					
Mean	5.94	6.60	13.38	35.98	53.56	19.79	35.97	–	0.1
S.D.	2.71	0.47	0.80	2.42	2.93	1.02	3.62	–	0.4
				Individual Values for 10 Rats on Day 8 of Combined Treatment (Dicoumarol and Sleep-Deprivation)					
8–0	1.7	2.90	5.9	16.7	51.	17.8	36.6	200	1
8–1	4.1	3.43	6.9	20.8	54.	17.8	34.7	200	1
8–2	3.2	6.04	13.1	37.3	55.	19.2	36.7	125	1
8–3	3.8	6.03	12.5	35.5	52.	18.3	36.8	104	0
8–4	4.8	5.95	13.1	36.6	54.	19.4	37.5	172	0
8–5	29.8	1.76	4.6	14.0	71.	23.2	34.3	151	0
8–6	27.5	1.08	2.9	8.7	71.	23.2	33.8	200	1
8–7	6.5	5.34	11.6	32.3	53.	19.2	37.7	200	0
8–8	3.2	0.71	1.7	4.4	56.	21.2	38.6	163	1
8–9	7.0	0.83	2.2	5.6	61.	23.1	38.7	182	3
Mean	9.16	3.40	7.4	21.19	57.8	20.24	36.54	166.2	0.8
S.D.	10.40	2.26	4.69	13.24	7.46	2.24	1.73	1.25	

normal values, mean values for the group were in the anemic range. Identical results were obtained with phenindione and with dicumarol. Mean values for RBC count, hematocrit and hemoglobin for each group of 10 rats at daily intervals are shown in Fig. 1 for animals subjected to FWS-deprivation while receiving phenindione. All three values were in the anemic range on the sixth and seventh day and were half of normal mean values on the eighth day. On Day 6, one, Day 7, three, and Day 8, seven rats showed severe anemia (50% reduction in hemoglobin). Both the individual and mean values showed a parallel reduction in values for RBC's, hemoglobin, hematocrit, again indicating a normocytic anemia. Prothrombin times increased with the anticoagulants from the fourth day. Comparison of the values for hemoglobin, hematocrit and RBC's of continued control groups versus combined treatment groups for each day were made with t-tests. Values of p for Days 6, 7 and 8 in all cases were <0.001.

Figure 2a shows the appearance of a normal control rat and Fig. 2b, a sleep-deprived anticoagulated rat after 8

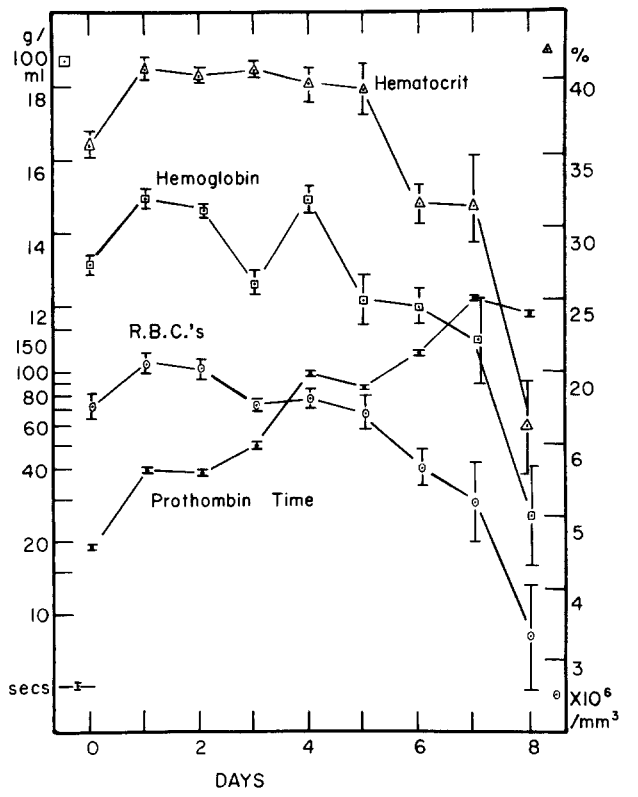

FIG. 1. Hematological values for sleep-deprived rats with phenylindanedione. Mean hematocrit, hemoglobin concentration, RBC count and prothrombin time for each day of treatment. Each point represents mean values for 10 rats with S.E. □ – □ Hemoglobin (Hgb.) △ – △ Hematocrit value. 0 – 0 Red blood cell count. ● – ● Prothrombin time.

days. It is evident that the anemia developed in these animals could even be seen on gross examination. The animal in Fig. 2b has very little blood as judged by the paleness of the tissues such as liver, kidneys, skin and lungs, when compared to the colour of tissues of the control animal. At times it was even possible to detect an anemic rat by external examination. The ears were very pale, the eyes were barely pink, and their feet seemed to have lost blood circulation. Hence, the severity of the anemia was obvious on gross inspection by Day 8 and could be observed as early as Day 5.

On visual inspection of each animal's internal organs, there was no blood or only a slight indication of free blood in the cavities or of hemorrhagic patches in the organs. In the few exceptions where some hemorrhage was present it was felt that the degree of anemia did not correspond with the degree of hemorrhage. This, as we will see later, was corroborated by the histological material. The hemorrhage scores on Day 8 for sleep-deprived rats receiving dicumarol are also reported in Table 1. The mean score was 0.8, close to that for dicoumarol alone, 0.5. In experiments on rats subjected to severe stress and then receiving anticoagulants the mean hemorrhage score on Days 6 to 10 is about 3.5 [5,6]. The mean hemorrhage score did not even reach the score of 1 and was not related to that degree of anemia. Thus the same degree of hemorrhage was observed in Rat 8-8 (Table 1) with severe anemia as in Rat 8-2 with essentially normal hematological values. Thus, when animals showed signs of hemorrhage on gross examination, these signs were minor in comparison to the magnitude of the anemia.

Histological material was studied for possible information as to the cause of the anemia. Microscopically, no evidence of hemorrhage was found. The spleen gave the greatest amount of information. The red pulp of the control animals (shown in Fig. 2c) contained active erythroid colonies and easily identifiable megakaryocytes. The numbers of both of these were markedly reduced in the sleep-deprived animals receiving anticoagulant (Fig. 2d). In contrast, the lymphoid apparatus of these spleens showed no evidence of stress involution (Fig. 2d). The control animal spleens contained easily visible iron stores (Fig. 2e). In the animals which were anemic this was considerably reduced (Fig. 2f). A few examples of erythrophagocytosis were found in the histiocytes of the red pulp in the anemic animals.

Effect of PCPA on Production of Anemia by FWS-deprivation with Anticoagulant

Independent groups of 10 rats were sleep-deprived and administered phenindione from between 1 to 8 days. In addition they were given i.p. injections of 316 mg/kg of PCPA 24 hr prior to the beginning of the sleep deprivation with phenindione, and repeated every 72 hr. Ten cc/kg of isotonic saline was injected intraperitoneally on the same days to rats of control groups. It was observed that PCPA added to FWS-deprivation and phenindione has an accelerating effect on the anemic process. This is demonstrated in Table 2 in which values are compared for Days 3, 4 and 5. Compared to the values for RBC counts for FWS-deprived + danilone without PCPA, the decrease in number of red cells when PCPA had been administered was highly significant on Day 4 ($p<0.001$) and Day 5 ($p<0.001$). The effect of PCPA was even more striking from the original records. One rat after one day of treatments had a hemoglobin value of 5.6 g, two rats on Day 3, 5.8 and 8.7. Normal hemoglobin values were found in only 1 rat on Day 5, 2 on Day 6 and 2 on Day 8, indicating that PCPA had increased the incidence of rats developing anemia. Comparing the incidence of anemia on Days 5, 6 and 8 with and without PCPA, the probability (p) of finding the increased incidence by chance alone was <0.001. The other hematological parameters for each rat changed parallel with the hemoglobin value.

Weight Loss and Production of Anemia

All animals were weighed at the beginning and at the end of their respective experimental program. It was noticed that most animals were losing weight. A close look at this loss of weight, however indicated that the anemia could not be attributed to loss of weight. A twoway histogram (Fig. 3) clearly shows that the nonanemic FWS-deprived group lost almost the same percent of weight as did the anemic animals (FWS-deprived with phenindione). None the less, hemoglobin values remained practically the same for the former and decreased for the latter. Furthermore, the animals were eating their food since all anticoagulant-treated rats were hypoprothrombinemic and daily records of food intake shows all rats ate approximately the same amount of food. Hence, decreased food intake cannot be

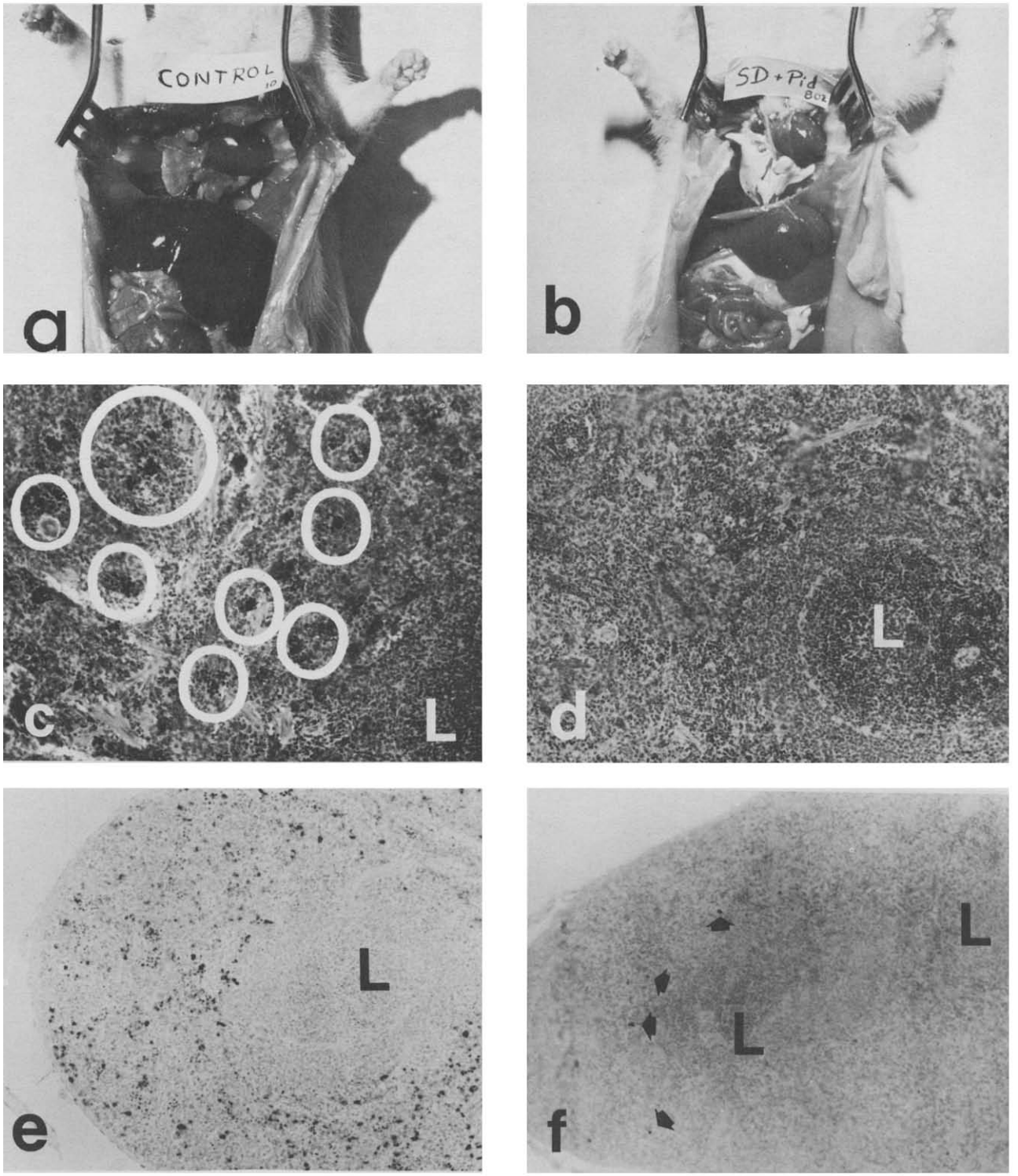

FIG. 2. Photographs of anemia in rats produced by sleep-deprivation with anticoagulants. a. Control rat. b. Anemic rat after 8 days of combined treatment by sleep-deprivation and phenindione. (Note paleness of tissue and absence of hemorrhage). c. and d. - Photomicrographs of spleen, stained with H & E. Magnificantion = 265.6 X. c = Control animal, d = Anemic, sleep-deprived rat receiving phenindione. Note normoblast colonies inside circles in red pulp of control animal and lack of it in d. Also normal lymphoid follicle, L, in d, demonstrating lack of stress effect in the experimental animal; e. and f. - Photomicrographs of spleens from anemic rats stained with Prussian blue/Neutral red. Magnification = 252 X, photographed with orange filter, Zeiss No. 54. e = Control; f = sleep-deprived treated with phenindione. Note diminished number of metallophil cells with treatment.

TABLE 2

EFFECT OF PCPA ON THE DEVELOPMENT OF ANEMIA WITH FWS-DEPRIVATION AND INDIRECT ANTICOAGULANT

Treatment	RBC count X 10^6/mm^3. Mean ± s.e.m. (n = 10) Day 3	Day 4	Day 5
FWS-deprived + danilone	6.60 ± 0.07 (0)	6.67 ± 0.14 (0)	6.47 ± 0.07 (4)
Above + Saline	6.45 ± 0.19 (0)	6.99 ± 0.11 (1)	7.35 ± 0.11 (0)
Above + PCPA	5.65 ± 0.47 (1)	4.99 ± 0.39 (7)	2.70 ± 0.64 (8)

( ) indicates number of rats with Hemoglobin <50% of normal (i.e. very anemic).

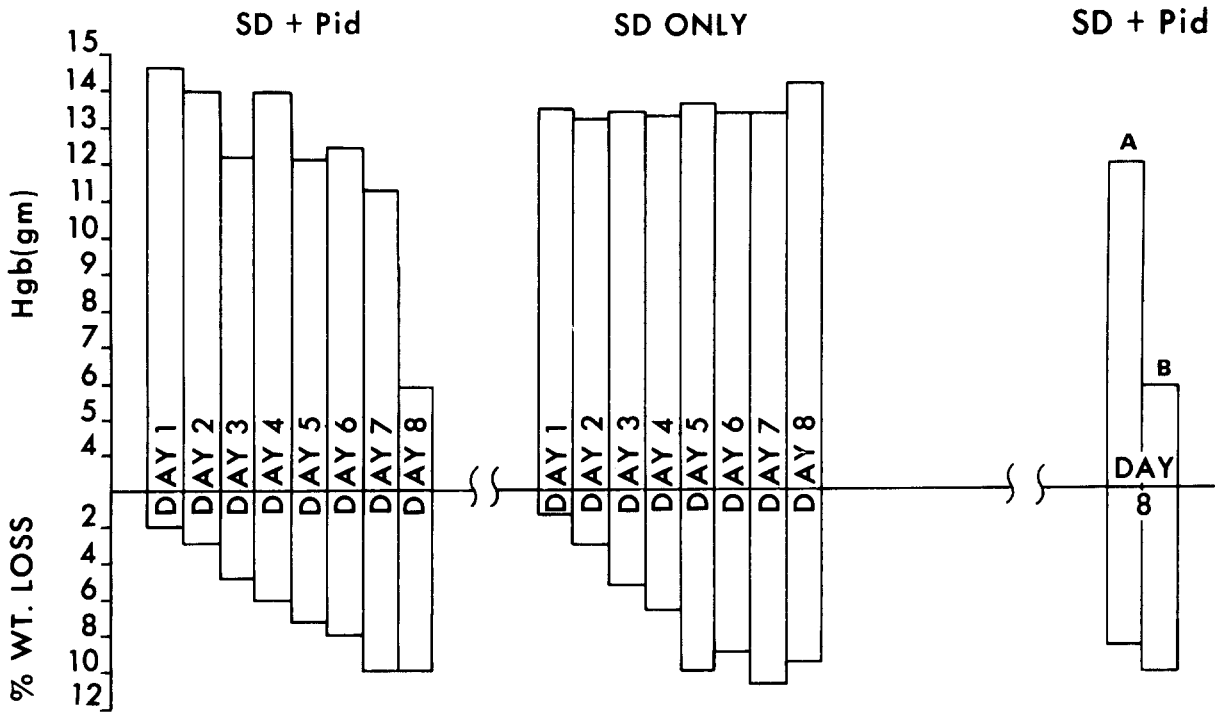

FIG. 3. Hemoglobin and weight loss of sleep-deprived rats. Twoway histogram showing mean hemoglobin and percent weight loss for sleep-deprived phenindione-treated groups (SD + Pid) and sleep-deprived (SD only) groups. On extreme right-hand side, comparison is made of values for 8th day between: A – rats fed (with rest) eight hours continuous and B – those fed for 2 hr 40 min three times a day. Rats in Group A showed normal mean hemoglobin values and the same weight change.

responsible for the anemia. The sleep deprivation + phenindione + PCPA group showed the greatest weight loss, as well as the most consistent rise in average hemorrhage score but the maximum values remained within close limits of the maximum values shown by other groups.

Also shown in Fig. 3 are the results with a group of 10 rats sleep-deprived for 8 days while receiving phenindione but fed in their home cages for 8 continuous hours as opposed to those of the previous groups which were also fed for 8 hours, but on a schedule of 2 hr 40 min 3 times a day. The difference between these groups was that the animals in the continuously fed group could not only eat, but were observed to sleep a certain amount of this time. The weight loss was similar. The animals allowed 8 consecutive hr in their cages showed hematological values within normal limits (RBC, 5.90 ± 0.21/min^3, Hgb, 12.4 ± 0.4/g,

hematocrit 35.2 ± 1.4%, MCV, 56.1/μ^3, MCH, 20.3 ± 0.35 $\mu\mu$g, MCHC 36.1 ± 0.56%, prothrombin time, 92.6 ± 1.18 sec, hemmorrhage score, 0.1).

Environment and Development of Anemia

To test the influence of the platform on the development of anemia, 10 rats were placed for 8 days in the containers with large platforms (11 cm in dia.) and fed phenindione in the usual schedule. This size of platform allowed rats to sleep inside the containers, yet the situational surroundings were exactly the same. At the end of the experiment, hemorrhage score was recorded and blood removed and analyzed. Mean values (with standard error) for the 10 rats were: initial weight 319.4 g, final weight 300.6 g, RBC's 7.03 ± 0.14, hemoglobin 14.12 ± 0.33, hematocrit 37.21 ± 0.86, prothrombin tine, 101.3 ± 1.25. The blood parameters in the group on the larger platform were completely normal. There was slight hemorrhage in only one of the animals around the testicular area.

Nature of Sleep Deprivation in These Experiments

The inverted flower pot technique of sleep-deprivation dissociates the two state of sleep, so that animals under these conditions cannot fall into FWS, but only into SWS [13]. In this manner and under these conditions, any sleep that occurs in the animal is perforce, SWS. To examine the sleep patterns of the animals under the conditions used, a separate series of rats were studied by electroencephalography.

Four groups of 12 rats were used in these experiments. They consisted of: 1 – control group, 2 – sleep deprived group, 3 – sleep deprived + PCPA group, 4 – sleep deprived + saline group. Each rat within its respective group was recorded for a period of 8 days. Prior to the sleep deprivation experiments 2 rats were recorded continuously for 18 hr during wakefulness, slow wave sleep. These recordings confirmed the adequacy of our EEG recording set up and agreed with known electrophysiological characteristics of wakefulness and sleep. When sleep and wakefulness were broken down into percentage of total recording time, we obtained 53% wakefulness, 34% SWS and 13% FWS. EEG's were then recorded from rats in the sleep-deprivation chambers for 4 hr a day. The results of the examination of the EEG records are reported in Fig. 4 as the percent of time in SW sleep. There is no doubt from behavioral observation of the rats that they would attempt to sleep and at times even begin to go into FWS, this being prevented immediately by the fall of animal's head or body into the water. The figure shows there was a marked increase in the amount of SWS in the animals subjected to sleep-deprivation. Injections of saline had no effect on this but injection of PCPA reduced the amount of SWS but not entirely. The results of analysis of these records through an integrated EEG have been reported by Drucker-Colín, *et al.* [3].

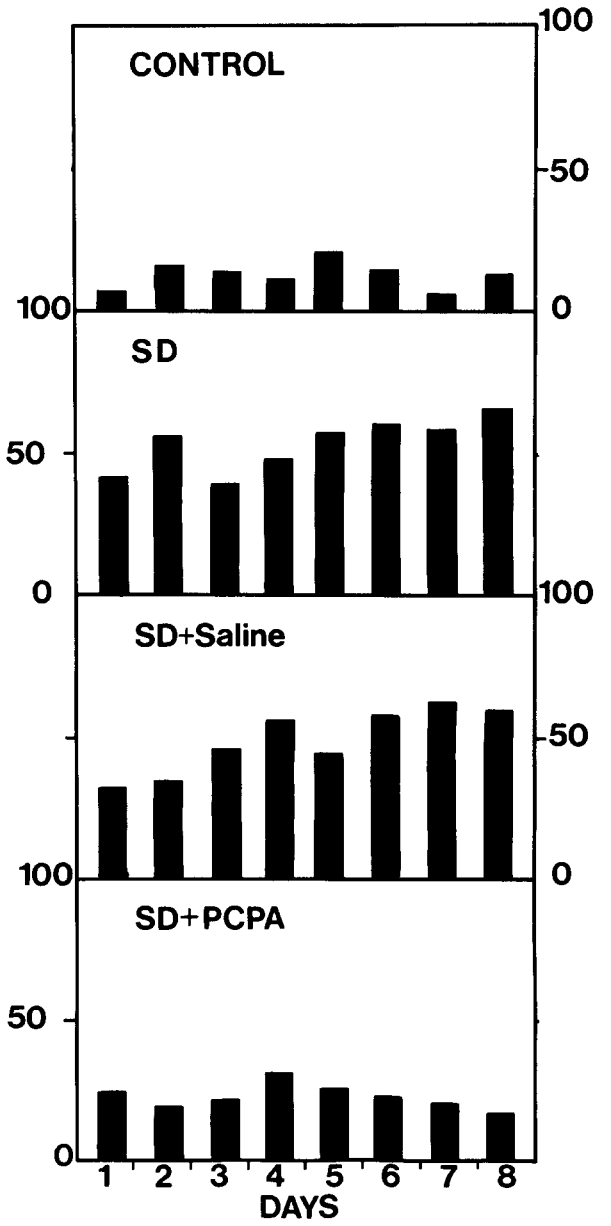

FIG. 4. Relative periods of wakefulness and slow wave sleep in rats. Mean percent of time (minutes) spent in slow wave sleep (SWS) by ECoG recording during test period for groups of 12 rats on Days 1 to 8. Control, SD = Sleep-deprived, SD + Saline = Sleep-deprived and injected with saline, SD + PCPA = Sleep-deprived and injected with PCPA 24 hr before beginning sleep deprivation and then at 72 hr intervals.

DISCUSSION

The present investigations were carried out as a result of the observation that a very severe anemia developed with the administration of anticoagulant drugs during sleep deprivation of rats. As this phenomenon has not been described previously, it requires investigation as a new phenomenon with drugs and one of possible clinical significance. Investigation is required of the nature of the anemia, its etiology and of the conditions which in combination produce it.

The severity of the anemia produced was most striking in every series of rats investigated. Many readers will wonder how the 3 rats in Table 1 with a hematocrit value less than 10% could survive. This in itself indicates that in the development of the anemia, there was time for circulatory adjustments. Further, as the table indicates, development of anemia appeared to be an all-or-nothing phenome-

non. In each group of 10 rats, about 5 rats developed anemia and 5 rats maintained normal blood values.

A severe anemia developing after 6 days of treatment without signs of external blood loss might be due to internal hemorrhage, occult blood in urine and feces, or hemolysis, and would be accelerated by suppression of hemopoiesis. In an initial investigation, it has not been possible to examine all these various possibilities but there are suggestions from the results obtained that the anemia is produced by a combination of these developments. The identification of the anemia as normocytic is in agreement with this conclusion.

Internal hemorrhage was present to a slight degree in the animals as can be seen from Table 1. A small but consistent elevation of the degree of hemorrhage occurred in those groups having combined treatments. However, the picture was quite different from that seen when stressed rats are given anticoagulants [5,6]. In these, considerable free blood in body cavities and/or extensive hematomata are found and in those animals which develop anemia with little evidence of gross bleeding, histological examination demonstrates widespread microscopic bleeding. As these symptoms were not found in FWS-deprived rats on anticoagulants, we must conclude that internal hemorrhage was not an obvious contributor to the development of anemia and that the hemorrhage that did occur was not produced by stress affecting the vascular component of hemostasis. The latter conclusion indicates that the small amount of hemorrhage observed was due to other defects in hemostasis. Such a defect could be a decreased effectiveness of platelets in hemostasis. Jaques and Fisher [7] showed that the administration of a serotonin depletor will disturb hemostasis through its effect on platelet function. In thrombasthenia with a lower level of platelet ATP, hemorrhages are observed. Ninety percent of adenine nucleotides in platelets originate from the red cell [9]. Luby *et al.* [13] reported that in man, the effort to stay awake stimulated high energy turnover in the red blood cell, depleting levels of adenine phosphates, after only 48 to 96 hr of sleep deprivation. It is possible that in our experimental animal a decrease in ADP from red cells resulted in a failure of platelets to aggregate. That the anemia resulted from an additional effect on hemostasis at the level of platelets and vessel wall is further suggested by the fact that the anemia accompanied by small hemorrhages developed faster in those animals which were depleted of serotonin by PCPA. The observation that slight hemorrhage appeared in all animals at approximately the same time as the anemia, favors the view that the hemorrhage resulted from lowered levels of adenine nucleotides in the red cell.

Evaluation of blood loss through occult blood in urine and feces requires sophisticated iron balance studies not practical in an initial investigation. Daily fecal examination of sleep-deprived rats receiving dicumarol (after the main study was completed) showed some occult blood in feces of three of ten rats on the sixth day only, so that this was not a prominent feature in the symptomatology.

Hemolysis of red cells might be expected following sleep-deprivation. The decrease in ATP observed by Luby *et al.* [13] will cause sphering of red cells and this will lead to their rupture in capillaries. This series of events occurring in the rats in our experiments could result in hemolysis. No direct tests were made of red cell fragility. Evidence for a hemolytic process was the occurrence of slight erythrophagocytosis in the spleens of the anemic animals. This was accompanied by the paradoxical disappearance of depot iron since much greater amounts of this were observed in the control animals. Disturbance of iron metabolism has been reported with total sleep deprivation in man by Kuhn *et al.* [11] who observed a marked drop in plasma iron with decrease in the daily rhythm. One would expect an increase in plasma iron with decreased iron storage so that changes in iron metabolism may not be comparable in these experiments with those of Kuhn *et al.* [11] with quite different sleep-deprivation. It is not clear why the splenic hemopioetic activity was diminished. One possibility is through the diet, since dietary protein deficiency in rats decreases hemoglobin production within a few days through decreased formation of erythropoietin. It will be noticed from our results that all animals except in the untreated control group lost a great deal of weight. This was particularly noticeable in the FWS-deprived animals. As food intake was similar for all groups (Fig. 3), then protein deficiency as a factor in increased erythropoiesis can probably be discarded except for one obvious fact. The maintenance of a prolonged period of wakefulness seems to require a great deal of energy (Luby *et al.* [13]).This is accompanied by significant changes in carbohydrate and fat metabolism (Kuhn *et al.* [12]). It is therefore possible that even though FWS-deprived animals eat the same quantities of food as control animals, their energy requirements are much greater and they need to ingest greater amounts of food to maintain an adequate intake of dietary proteins. Another possibility is that indirect anticoagulants have some effect on splenic erythropoiesis. However, no such pharmacologic effects as a general rule have been reported.

From various clinical observations it is possible to speculate that there may be relationships between sleep rhythms, growth hormone and erythropoiesis either directly or through effects on erythropoietin. Therefore we suggest that the anemia is produced by a combination of minor hemorrhages due to platelet defects in the presence of anticoagulant, diminished haemopoiesis and perhaps haemolysis. The suggestions for the relation of FWS-deprivation to these events indicate that FWS-deprivation would seem to affect blood in a very roundabout way, by having some very severe metabolic and/or physiological effects on all systems of the organism, which become obvious only when such systems are further tampered with pharmacologically.

Examination of the conditions which produced this new phenomenon refers particularly to the anticoagulants and to the FWS-deprivation. The anticoagulant effect was measured by determination of prothrombin times. The mean prothrombin time response of groups of animals are reported in Fig. 1. This increased over the eight days of treatment. While differences were observed in the prothrombin time response for phenindione and phenindione-FWS-deprived animals, this was not seen in the dicumarol series and this did not seem to be related to the development of anemia.

The contribution of the sleep-deprivation procedures to the development of anemia was examined by altering the platform size, by changing the schedule and by the parallel study of electrocorticograms. Drucker-Colín *et al.* [3] have already reported that behavioural studies and the electrocorticogram records indicated suppression of FWS. This is supported by Fig. 4 which reports the relative periods in slow wave sleep (SWS). PCPA which reduced the percent in

SWS, as shown in Table 2 accelerated the onset and increased the incidence of anemia. This again points to an association between sleep deprivation (in this case a depression of slow-wave sleep) and the development of anemia in the presence of anticoagulation. It was seen by direct observation that increasing the platform size allowed more normal sleep. As these rats did not develop anemia, this also supports the conclusion that the production of anemia is related to the interference of sleep in the procedure used.

It is likely that the anemia is produced only by a reduction in FWS to a critical level judging by the results of changing the feeding schedule. With the total feeding time in the home cages the same, anemia was not observed when this time was continuous so that the animal used part of it for sleeping as well as eating.

An important question is the relation of the procedures used to stress. As pointed out above, the origin of the investigation was the observation that FWS-deprived rats receiving an anticoagulant did not exhibit the symptoms (spontaneous hemorrhage) seen when rats exposed to various well established stressors were given anticoagulants but showed a different phenomenon (anemia). The criterion that a particular symptom is due to stress is that this symptom is not specific to the treatment and conversely that the symptom can be elicited by other nonspecific treatments. In the words of Heiner *et al.* [4], "it is important to distinguish the direct effects of paradoxical sleep deprivation from other changes not directly related to sleep." Differences in the technique used for producing FWS-deprivation may introduce stress as an accompaniment of the sleep-deprivation. It may be that the conditions of feeding (fed in the tank) used by Stern and Hartmann [18] to demonstrate adrenal gland hypertrophy and thymus gland atrophy with FWS-deprivation introduced a stress which was not present in our experiment where the animals were fed in their cages. Stern *et al.* [19] concluded that the enhanced biogenic amine synthesis observed under their conditions was due to nonspecific stress rather than to loss of FW-sleep per se. Direct histological examination of the animals in this study (see Fig. 2d) demonstrated normal lymphoid tissue with no evidence of stress effects. The changes in the spleens of the sleep-deprived-PCPA-phenindione-treated animals which were anemic were similar to those of the other anemic animals with no evidence of stress changes.

Our conclusion is that the appearance of profound anemia in anticoagulated rats when deprived of FWS is due to complex effects of these treatments in combination on hemostatic and hemopioetic mechanisms. As previously seen with the spontaneous hemorrhage syndrome [5,6], symptoms result only when exhaustion of several physiological mechanisms has occurred. The symptoms produced with FWS-deprivation are different from those produced with stressors, indicating that stress effects produced by FWS-deprivation are minor compared to the direct effects of FWS-deprivation. This conclusion is similar to that of Stern [17] from a behavioural study of FWS-deprivation in rats. The experiment with PCPA which shows that that addition of SWS-deprivation does not change the symptomatology means that this conclusion can be extended to SWS-deprivation. The similar observations of Kuhn *et al.* [12] on metabolic changes on total sleep-deprivation, the observation of no change in 17-hydroxycorticosteroids [1, 10, 20] or a slight increase [15] in man and of a lack of significant change in plasma corticosterone in animals [14] with total sleep-deprivation, further extends this conclusion to total sleep-deprivation. Since "stress may be defined as the sum of all nonspecific changes caused by function or damage" [16], while the changes observed with sleep-deprivation are largely specific, sleep-deprivation does not qualify as a stressor by Selye's classical definition of stress. The causes of its marked effects on physiological mechanisms must be sought along other lines [8].

ACKNOWLEDGEMENTS

The authors express their appreciation to Dr. Gordon McMurray and Dr. Gordon Winocur, Department of Psychology, for their interest and support and the use of the isolation chambers for the rats; to Dr. G. J. Millar, Department of Physiology and Dr. C. T. Bolton, Department of Neurology, University of Saskatchewan, for their advice and critical suggestions as the problem progressed; and to the technicians of the Department of Physiology and of the Hematology Section of the University Hospital, Saskatoon for skilled technical assistance. Phenylindanedione (danilone) was kindly given by Chas. E. Frosst and Co., Montreal, P. Q., dicumarol by Abbott Laboratories, Montreal, P. Q.

REFERENCES

1. Bliss, E. L., L. D. Clark and C. D. West. Studies of sleep deprivation-relationship to schizophrenia. *Archs Neurol. Psychiat.* **81:** 348–359, 1959.
2. Drucker-Colín, R. R. Sleep Deprivation: Effects on the EEG and on the blood of Anticoagulant Treated Rats. Ph.D. Thesis, University of Saskatchewan, 1971. Copies may be obtained from University Microfilming, University of Michigan, Ann Arbor, Michigan 48704, U.S.A. *Diss. Abstr.* **32:** 1170-B, 1971-2.
3. Drucker-Colín, R. R., L. B. Jaques and G. Winocur. Anemia in sleep-deprived anticoagulanted rats. *Science* **174:** 505–507, 1971.
4. Heiner, L., Y. Godin, J. Mark, P. Mandel. Electrolyte content of brain and blood after deprivation of paradoxical sleep. *J. Neurochem.* **15:** 150–151, 1968.
5. Jaques, L. B. *Anticoagulant Therapy: Pharmacological Principles.* Springfield, Illinois: C. C. Thomas, 1965, p. 156.
6. Jaques, L. B. Effects of hormones and drugs on hemostasis. *Proc. of the 3rd International Pharmacological Meeting, July 24–30, 1966, Volume 6.* Drugs in relation to blood coagulation, hemostasis and thrombosis. Oxford and New York: Pergamon Press, 1968, pp. 22–55.
7. Jaques, L. B. and L. M. Fisher. Platelet serotonin as a factor in hemostasis. *Archs Int. Pharmacodyn. Thér.* **123:** 325–333, 1960.
8. Johnson, L. C. Discussion 3. In: *Sleep: Physiology and Pathology,* edited by A. Kales. Philadelphia: Lippincott, 1969, p. 281.
9. Johnson, S. A., L. Fredell, J. Shepard, T. H. Tebo, C. Chang, H. J. Pederson and D. L. Van Horn. Red blood cells, fibrin and platelets in hemostasis. In: *Physiology of Hemostasis and Thrombosis,* edited by S. A. Johnson and W. H. Seegers. Springfield: C. C. Thomas, 1967, pp. 44–70.
10. Kollar, E. J., G. R. Slater, J. O. Palmer, R. F. Docter and A. L. Mandell. Stress in subjects undergoing sleep deprivation. *Psychosom. Med.* **28:** 101–113, 1966.
11. Kuhn, E., V. Brodan, M. Brodanova and V. Kordac. The influence of sleep deprivation on the daily rhythm of plasmatic iron. *Čas. Lék. česk.* **106:** 1342–1343, 1967.
12. Kuhn, E., V. Brodan, M. Brodanova and K. Rysanek. Metabolic reflection of sleep deprivation. *Activitas nerv. sup.* **11:** 165–174, 1969.

13. Luby, E. D., J. L. Grisell, G. E. Frohman, H. Lee, B. D. Cohen and J. S. Gottlieb. Biochemical, psychological and behaviour responses to sleep deprivation. *Ann. N.Y. Acad. Sci.* **196:** 71–79, 1962.
14. Morden, B., R. Conner, G. Mitchell, W. Dement and S. Levine. Effects of REM sleep deprivation in shock induced fighting. *Physiol. Behav.* **2:** 21–28, 1967.
15. Rubin, R. T., E. J. Kollar, G. G. Slater and B. R. Clark. Excretion of 17-hydroxycorticosteroids and vanillylmandelic acid during 205 hours of sleep deprivation in man. *Psychosom. Med.* **31:** 68–79, 1969.
16. Selye, H. and G. Heuser. *Fifth Annual Report on Stress 1955–6.* New York: MD Publications Inc., 1956.
17. Stern, W. C. Behavioural and biochemical aspects of rapid eye movement sleep deprivation in the rat. *Diss. Abstr.* **31:** 1574-B, 1970–71.
18. Stern, W. C. and E. L. Hartmann. Reduced amphetamine lethality following chronic stress. *Psychopharmacologia* **23:** 167–170, 1972.
19. Stern, W. C., F. P. Miller, R. H. Cox and R. P. Maickel. Brain norepinephrine and serotonin levels following R.E.M. sleep deprivation in the rat. *Psychopharmacologia* **22:** 50–55, 1971.
20. Tyler, D. B., W. Marx and J. Goodman. Effect of prolonged wakefulness on the urinary excretion of 17-ketosteroids. *Proc. Soc. exp. Biol. Med.* **62:** 68–73, 1946.