TABLE I. Teratogenic Effect of Methylazoxymethanol in Golden Hamsters.

No. of litters	Treatment Type	Dose, mg/kg	No. of implantations	No. of alive fetuses	No. of malformations	No. of dead fetuses	No. of resorptions
4	Saline		41	39	0	0	2
4	MAM	12	52	49	"	"	3
10	"	20	123	111	111	"	12
6	"	23	80	53	53	17	10
3	"	25	36	1	1	0	35

lished after completion of microscopic study of the fetuses.

Discussion. Although the mechanisms through which the malformations are induced are presently obscure, observations using thin-layer chromatography have established the presence of MAM in embryos following intragastric administration of cycasin to mothers. This finding is also of considerable significance in view of the recent observations that tumors can be induced with cycad material by the transplacental route(9). A combined teratogenic and carcinogenic effect of a single compound was recently reported in rats by Druckrey, Ivancovic and Preussmann(10) following an intravenous injection of ethylnitrosourea on day 15 of pregnancy. The animals had malformed paws at birth and developed tumors of brain and peripheral nerves by the 160th day.

Summary. Methylazoxymethanol (MAM), the aglycone of cycasin, a naturally occurring glucoside of cycad plants, causes retardation of growth and a variety of malformations in embryos of the golden hamster. This same substance was previously shown to be carcinogenic, hepatotoxic and to cause mutations, methylation of nucleic acids and chromosome breakage.

We thank Dr. G. L. Laqueur for helpful discussions and Miss JoAnn Holmes for technical assistance.

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Received October 26, 1966. P.S.E.B.M., 1967, v124.

Interaction of Progesterone and Aldosterone with Red Blood Cells of The Rat. (31768)

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Interactions between steroid hormones and proteins have been actively investigated(1-3). However, very little is known regarding the binding of steroids to red blood cells (RBC) and the possible role of RBC in the metabolism and transport of steroids. Some investigators(4) have reported that erythrocytes play a minor role in the transport of steroids; others(5,6) suggest that red blood cells might have an important function in the mechanism of transport of steroids. Previous investigations(7,8) on the binding of aldosterone and

progesterone have demonstrated that these steroids show a binding affinity for plasma proteins and for subcellular fractions of liver and kidney. In the present studies, the binding of progesterone and aldosterone to red blood cells was investigated in order to elucidate the role played by erythrocytes in steroid transport. The metabolism of the above steroids by erythrocytes was also studied.

Materials and methods. The following materials were used: seamless cellulose casings of 36/32″ inflated diameter (Visking Corp.); d-aldosterone-7-H^3, specific activity 20 $\mu c/\mu g$; unlabeled d-aldosterone; progesterone-16-H^3, specific activity 1.8 $\mu c/\mu g$ (New England Nuclear Corp.) and unlabeled progesterone.* The chemical purity of the steroids, checked by paper chromatography, was found to be higher than 96%.

Male Sprague-Dawley rats of 250-300 g body weight were used in these experiments. Blood was obtained by puncture of the abdominal aorta under ether anesthesia and was collected using a heparinized syringe. After centrifugation at 400 $\times$ *g* for 15 minutes, plasma and white blood cells were removed by aspiration through a capillary tube; the precipitate, consisting of red blood cells, was washed with Krebs-Ringer phosphate buffer, pH 7.4, until buffer was clear. In every washing, the buffer was removed by aspiration after centrifugation at 400 $\times$ *g* for 10 minutes. The final washings were tested with a 10% aqueous solution of sulphosalicylic acid to assure that no protein was present. The washed RBC were suspended in 25 ml Krebs-Ringer solution. The concentration of hemoglobin was determined colorimetrically at 540 mμ by the cyanmethemoglobin method as described by Crosby and coworkers(9). The hematocrit was determined after centrifugation for 30 minutes at 500 $\times$ *g* using 1.0 ml of diluted RBC suspension in hematocrit tubes.

The method of equilibrium dialysis was used for the interaction studies of erythrocytes with progesterone or aldosterone. An aliquot of 10 ml of RBC suspension or smaller aliquots diluted to 10 ml with buffer were enclosed in a dialysis bag and dialyzed against 20 ml of Krebs-Ringer phosphate buffer containing the dissolved steroid. Methanolic solutions of the labeled steroids were evaporated at room temperature under nitrogen and the dry residue was dissolved in buffer; aliquots were taken from these solutions for the various dialysis systems. The percent hematocrit, the concentration of hemoglobin in the suspension inside the dialysis bag, the concentration of labeled steroids used and the conditions of dialysis are given in the Tables. The dialysis systems were gently agitated for 24 hours at 4°C. This equilibration period is sufficient since it was found in dialysis experiments at 4°C, analyzed after 24, 48 and 72 hours, that, in a system containing diluted normal serum and aldosterone, equilibrium was reached within 24 hours; this has also been found true for similar dialysis systems (10).

At the end of the dialysis period, the inside and outside solutions were sampled using 3 and 5 ml duplicates, respectively. Each sample was extracted 6 times with ethyl acetate, and the combined extracts were dried and then dissolved in 10 ml of a phosphor solution consisting of 4 g of 2,5-diphenyloxide and 0.4 g of 1,4-bis-2(5-phenyloxazolyl)- benzene dissolved in 1 liter of redistilled toluene and counted in a Packard Tricarb automatic scintillation spectrophotometer using four 10-minute counts per sample. The standard deviation for the counting rate was less than $\pm$ 0.8%. Percent hematocrit and hemoglobin concentration in the solution inside the dialysis bag was determined also after dialysis. In all dialysis studies the recovery of radioactive material was checked and reported in the tables. The percent binding was determined by the following formula: % binding $= \frac{A-B}{A} \times 100$ where A represents the concentration of radioactive steroid inside the dialysis bag at equilibrium and indicates the total (bound plus unbound) steroid. B represents concentration of radioactive steroid in the outside solution at equilibrium and indicates the unbound steroid. All values were

* The author is greatly indebted to Dr. J. F. Tait, Worcester Foundation for Experimental Biology, for aldosterone-7-H^3; to Dr. R. Gaunt, Ciba Pharmaceutical Products, Inc., for d-aldosterone, free alcohol.

TABLE I. Interaction of Progesterone with Red Blood Cells as Determined by Equilibrium Dialysis.

Experiment number		1*	2*	3	4
Progesterone concentration		.0047 μg/ml	.0047 μg/ml	.052 μg/ml	.43 μg/ml
HB† (mg/ml)	start	47.5	47.5	90.0	127.0
	end	43.0	47.0	86.0	123.0
HT‡ (%)	start	15.5	15.5	30.0	42.0
	end	12.0	12.0	32.0	37.0
Total cpm	start	29,940	29,480	36,420	35,420
Total cpm	end inside	17,470	17,440	29,270	29,150
	outside	10,440	10,120	8,940	9,120
Recovery (percent)		93.2	93.5	104.9	108.0
Progesterone bound (percent)		70.1	71.0	84.7	84.4

* Exp 1 and 2 are duplicates.

† HB = Hemoglobin concentration in red blood cells suspension at the beginning (start) and at termination of dialysis (end).

‡ HT = % of hematocrit in red blood cells suspension at beginning (start) and at termination of dialysis (end).

determined at least in duplicate and are based on direct measurement of radioactivity in the inside and outside solutions.

The metabolism of steroids by red blood cells was studied by placing in a flask 25 μg of aldosterone-7-H^3 or progesterone-16-H^3 and 20 ml of the suspension of washed red blood cells corresponding to 250 mg hemoglobin and incubating in air for 2 hours at 37°C with constant agitation. In a control incubation, the labeled steroid was dissolved in 20 ml of Krebs-Ringer buffer but no RBC were present. Similar incubations were also carried out with water-hemolyzed RBC. After incubation the contents of each flask were extrated with ethyl acetate 6 times, using 3 volumes of ethyl acetate each time. The ethyl acetate extracts were separated by centrifugation and combined for each incubation; they were reduced to a small volume under nitrogen, filtered and used for analysis of steroid metabolites by paper chromatographic techniques.

Results. The results of the interaction of progesterone with red blood cells using different hemoglobin and steroid concentrations are shown in Table I. With hemoglobin concentration of 47.5 mg/ml and a progesterone concentration of 4.7×10^{-3} μg/ml, which corresponds to a ratio μg hemoglobin/μg progesterone of approximately 10^7 to 1, the binding is 70.5% (Exp. 1 and 2). If the concentration of the hemoglobin is increased by a factor of about 2 and that of the steroid by a factor of 10 (Exp. 3), determining thus a ratio of approximately 1.7×10^6 to 1 in μg hemoglobin to μg progesterone, the binding increases to a value of 84.7%. Further increase of the hemoglobin concentration to 127 mg/ml and of the steroid concentration to 0.43 μg/ml, lowering the hemoglobin/progesterone ratio to a value of 3×10^5 to 1, the percent binding remains unchanged. Fig. 1 shows the percent binding of progesterone in relation to the hemoglobin and to the progesterone concentration in the dialysis system. It can be seen that maximum binding occurs with hemoglobin concentration at or above 90 mg/ml and with progesterone concentration at or above 0.05 μg/ml. The results of the interaction of aldosterone with red blood cells are shown in Table II. Whereas progesterone shows a high percent binding to erythrocytes, the percent binding demonstrated by aldosterone is very low in all 3 experiments made using 2 different concentrations of hemoglobin. The data in Table I and II indicate that the recovery of the radioactive material in all the incubation systems was quantitative within the experimental error inherent in the analysis techniques. The study of the metabolism of progesterone and aldosterone by intact or hemolyzed red blood cell after *in vitro* incubation showed no evidence of metabolites of these two steroids. The extracted radioactivity moved

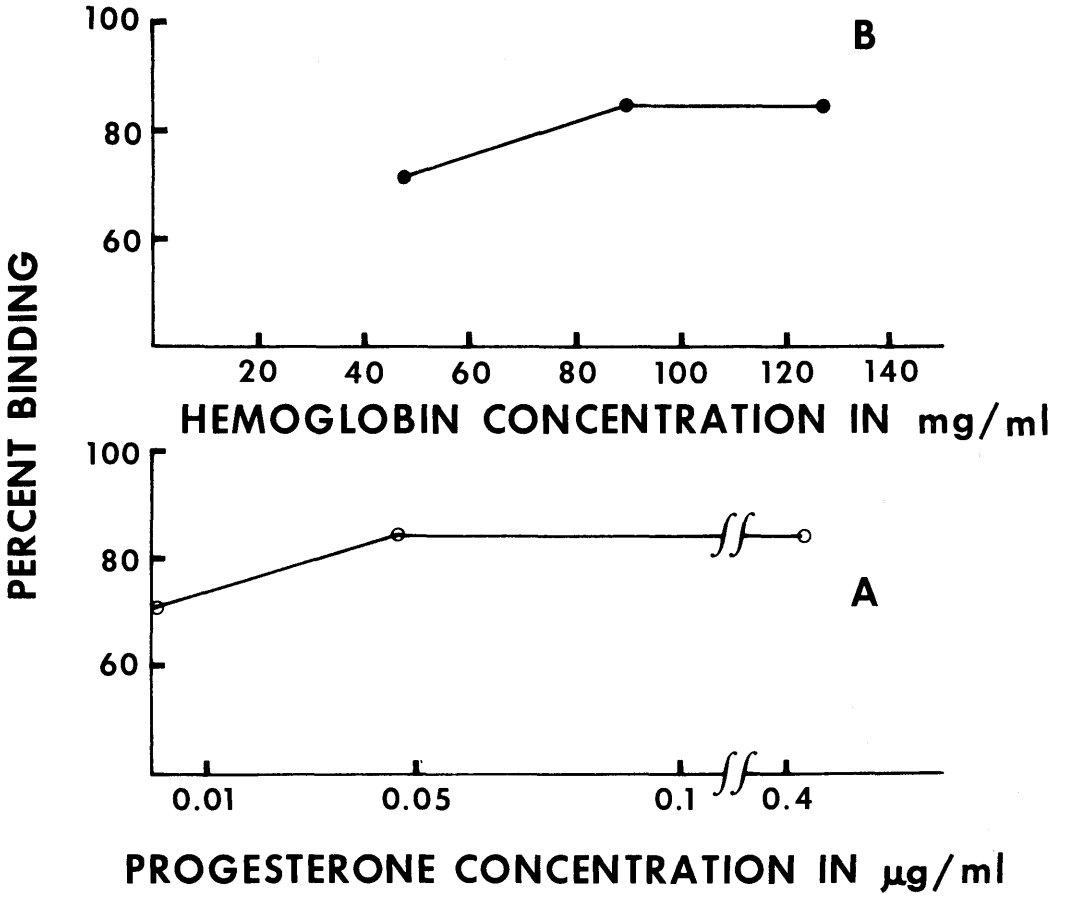

FIG. 1. Percent binding of progesterone-16-H^3 to red blood cells as a function of progesterone (A) and hemoglobin (B) concentration.

TABLE II. Interaction of Aldosterone with Red Blood Cells as Determined by Equilibrium Dialysis.

Experiment number		1*	2*	3
Aldosterone concentration		.024 µg/ml	.024 µg/ml	.024 µg/ml
HB† (mg/ml)	start	3.6	3.7	26.5
	end	3.2	3.5	21.0
HT‡ (%)	start	—	—	—
	end	1.0	1.0	6.5
Total cpm	start	26,540	25,445	26,490
Total cpm	end inside	8,790	8,770	9,210
	outside	16,060	16,640	16,680
Recovery (%)		93.6	99.9	97.7
Aldosterone bound (%)		3.0	5.1	9.4

* Exp 1 and 2 are duplicates.

† HB = Hemoglobin concentration in red blood cells suspension at beginning (start) and at termination of dialysis (end).

‡ HT = % of hematocrit in red blood cells suspension at beginning (start) and at termination of dialysis (end).

as a single peak on paper chromatographic analysis and showed a migration rate identical with that of the authentic steroid, progesterone or aldosterone, and also identical with the migration rate of the radioactive material extracted from the incubated control. The recovery of the steroid in the radioactive peak on the chromatogram was between 95 and 97% of the total radioactivity applied to the chromatogram and between 93 and 95% of the radioactivity added to the incubation flask.

Discussion. It has been reported by Agarwal and Garby(11) that a number of various corticosteroids have an inhibitory action on the lysis of human red blood cells *in vitro* and that they protect these cells against certain hemolytic processes. Agarwal and Carstensen (12) postulated that the differences observed in the inhibitory activity of several steroids could be due to differences in uptake on or in the red cells from the medium. The results in Table I and II show that, indeeed, two different steroids demonstrate different interactions with red blood cells. The uptake of progesterone is of the order of 70-85% of the total steroid in contact with red blood cells whereas the uptake of aldosterone is insignificant. It was found(11) that aldosterone was inactive in the inhibition of the lysis of erythrocytes caused by mechanical stress and by cold storage in acid-citrate dextrose solution; it is possible that the absence of antihemolytic activity is related to the very low uptake of the steroid by the erythrocytes. Maximum binding of progesterone (Exp. 3, Table I) is obtained when the ratio μg hemoglobin/μg progesterone is of the order of 1.7×10^6. Assuming for hemoglobin a molecular weight of 67,000, the molar ratio hemoglobin/progesterone is approximately 8,000. In Experiment 4, Table I, the concentration of progesterone is increased and the molar ratio hemoglobin/progesterone is a little over 1,000; however, in this case in which the excess of hemoglobin is not as great as in Experiment 3, the uptake of progesterone remains the same. To obtain an indication of the binding affinity of progesterone to hemoglobin the C-value can be calculated by the formula C = percent steroid bound/percent steroid unbound $\times$ [HB] where [HB] represents the hemoglobin concentration in g/liter. The values calculated for the experiments in Table II range between 4.5×10^{-2} and 6.0×10^{-2}; these values indicate a very low combining affinity. These results would suggest that the steroid does not penetrate into the erythrocytes, but is adsorbed at the red cell surface. The greater uptake of progesterone as compared to that of aldosterone could be explained by the fact that progesterone is more lipophilic and contains less polar groups, qualities which generally result in a strong affinity to serum proteins and cell constituents(10). Vermeulen (6) in his studies on the transport of 4-^{14}C-cortisol by erythrocytes also came to the conclusion that the steroid is adsorbed to the red cell surface, whereas Bischoff and Bryson (13) in their investigations on estradiol transport by human red cells present evidence that estradiol penetrates into the intact cell. Willmer(14) speculates that corticosteroids become attached or packed into the phospholipid layer of cell membranes.

The chromatographic analysis of extracts obtained after incubation of erythrocytes and progesterone or aldosterone demonstrated that the red blood cells do not metabolize these two steroids in an *in vitro* system. These findings are in agreement with the results of other investigators (6,12) who reported the inability of the erythrocytes to metabolize other glucocorticoids.

Summary. The interaction of progesterone and aldosterone with red blood cells was studied in an *in vitro* system by equilibrium dialysis technique. The uptake of progesterone by the erythrocytes is of the order of 70-85% of the total steroid in contact with the red blood cell, whereas the uptake of aldosterone is insignificant. Considerations of molar ratios of hemoglobin and steroid and calculations of binding affinity of steroid to hemoglobin suggest that the steroid does not enter into the red blood cells, but could be adsorbed at the cells' surface. Studies on the metabolism of progesterone and aldosterone by the red blood cells *in vitro* show that no metabolites of the two steroids occur.

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Received August 24, 1966. P.S.E.B.M., 1967, v124.

Absence of Effect of Tocopherol on Acute Oral Toxicity of Sodium Selenite in the Rat. (31769)

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Selenium is now recognized to play two different roles in nutrition. At low levels it is an essential element whose function is closely related to that of vitamin E, although the exact mechanism is not thoroughly understood. At higher levels it is toxic. Evidence is accumulating that selenium and tocopherol administered together may be more effective in treating such disturbances as "white muscle disease" or "nutritional muscular dystrophy" than is either alone (1,2). The statement has been made that tocopherol also reduces the toxicity of selenium(3). The following experiments were undertaken to determine whether the administration of tocopherol with selenium did, in fact, alter the toxicity of selenium when it was provided to rats in a single dose orally as sodium selenite. The results indicated that it did not. Previous studies on the toxicity of selenium have been reviewed by Smith *et al*(4), Painter(5), Moxon and Rhian(6), and Rosenfeld and Beath (7), but the acute oral toxicity of sodium selenite in the rat seems not to have been investigated.

Experimental. Sodium selenite was dissolved in physiological saline to give a solution of final concentration 1.0 mg selenium per ml (2.19 mg sodium selenite per ml). The selenite-tocopherol combination was supplied by H. C. Burns Co.*

Male and female Long-Evans rats were maintained on a diet of laboratory chow (Purina Labena) and water, *ad libitum*. They were starved for 24 hours before administration of the selenite solutions by stomach tube. Dose was based on body weight of the animals (70 to 380 g range). Food was then made available and the rats were inspected frequently to note symptoms, weight changes, and time of death; autopsies were performed immediately thereafter. Control animals received physiological saline equal in volume to the selenite solutions. None of these died.

Results and discussion. Within 15 minutes after administration of selenite, with or without tocopherol, most of the rats became subdued, and developed a "garlicky odor." This odor is often used to identify selenium poisoning and is presumed to be due to di-

* Seletoc®, H. C. Burns Co., Oakland, Calif., reported to contain per ml: selenium (as sodium selenite), 1 mg; vit. E (as d-α-tocopheryl acetate), 50 mg; polyoxysorbitan mono-oleate, 10 mg; propyl p-hydroxybenzoate, 1 mg.