Hemolytic Activity of Diethylstilbestrol and Some Steroid Hormones.* (21041)

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During the course of studies of effects of steroid hormones on influenza virus increase in tissue culture, it was found that when chorioallantoic membranes of 11-day-old chick embryos were incubated with 3.88 x 10^{-4} Mol of diethylstilbestrol, progesterone, testosterone, α-estradiol, DOC, or DOCA, chick chorioallantoic membranes became completely blanched after 24-36 hours of incubation at 35°C and supernatant fluids contained significantly more hemoglobin than control media(1). This phenomenon was observed repeatedly, and suggested that the hormones cited might have hemolytic activity at these low concentrations. Experiments confirming the hemolytic effect of certain of these hormones are reported here.

Materials and methods. Red blood cell suspensions. Human type "O" erythrocytes were used throughout these experiments. Blood was obtained in syringes previously rinsed with ACD (acid citrate dextrose soluton). Whole blood was stored at 4°C in an excess of ACD prior to use—never longer than 48 hours. On the day of experiment cells were washed 3 times with 0.85% NaCl solution buffered to pH 7.14 with 0.01 M phosphate, then suspended in this solution in 10% concentration. RBC concentrations were checked by colorimetric estimation of the hemoglobin concentration of hemolyzed cells. *Hormones* were dissolved in 75% ethanol; addition of hormone solutions to test systems resulted in 10^{-2} dilution yielding final ethanol concentrations of 0.75%. If control tubes containing this concentration of ethanol showed more than 3% hemolysis after 2 hours at 37°C, experiments were discarded. *Determination of degree of hemolysis.* After appropriate incubation, tubes containing hemolytic systems were immediately centrifuged to remove unhemolyzed cells at 1500 RPM for 10 minutes at 4°C. One ml of supernatant fluid was then added to 4 ml of 0.6% NH_4OH solution and the quantity of oxyhemoglobin determined in the Coleman Jr. spectrophotometer at 540 mμ or in the Klett-Summerson Photometer using a No. 54 green filter. Control tubes for assessment of 100% hemolysis contained cell suspensions hemolyzed with distilled H_2O. *Procedure.* To tubes containing 1.8 ml of phosphate buffered saline, 0.2 ml of 10% red cell suspensions were added. Test compounds were added in 0.02 ml amounts with a micropipette.

TABLE I. Comparative Hemolytic Activities of Stilbestrol and Various Steroids.

Compound (3.88×10^{-4}M)	% hemolysis —Hr of incubation— 3	96*	120*
Ethanol control	<1	<1	1
Stilbestrol	100	—	—
Progesterone	92	—	—
DOCA	16	100	—
Testosterone	2	—	6.5
α estradiol	2	—	3.1
Cortisone	<1	—	3.0
Compounds A, B, F	<1	—	—

* Under sterile conditions with penicillin and streptomycin.

Results. Hormones inducing hemolysis. Paralleling evidences of cell toxicity manifest in tissue culture, stilbestrol and the steroids progesterone and DOCA induced hemolysis within a 3-hour period at 37°C (Table I). The sex hormones testosterone and α-estradiol induced only slight hemolysis after prolonged incubation. In the concentrations used (3.88 x 10^{-4} M), none of the corticosteroid hormones manifested hemolytic activity. However, hemolysis by stilbestrol was accelerated by the addition of corticosteroids to the hemolytic system; suggesting a possible hypolytic effect of the latter. Hypolytic concen-

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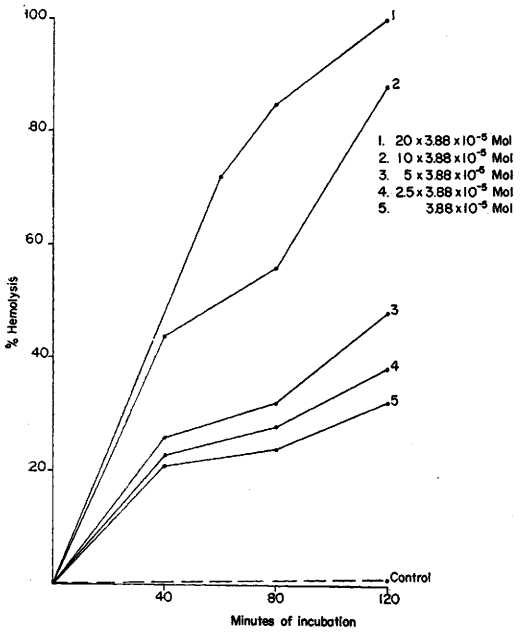

FIG. 1. Relation of stilbestrol concentration to rate of hemolysis.

trations of stilbestrol, and equivalent amounts of cortisone had no effect on the osmotic fragility of erythrocytes.

Hemolysis by stilbestrol. The hemolytic activity of stilbestrol was studied in some detail. Hemolysis was found to bear a direct but non-stoichiometric relationship to the concentration of drug employed (Fig. 1). It should be remarked that complete solution of stilbestrol was not effected at the 2 highest concentrations.

The effect of *temperature* on stilbestrol-induced hemolysis is demonstrated in Fig. 2. Maximum hemolysis was noted at 37.5°C; higher temperatures induced hemolysis in control tubes. Hemolysis was increased by hydrogen ion concentrations below neutrality, as shown in Table II, in which the influence of pH is detailed. Conversely, the addition of Ca in a final concentration of 11 mg % was found to retard the hemolysis induced by

TABLE II. Effect of pH on Stilbestrol-Induced Hemolysis.

	pH				
	5.5	6.0	6.6	7.1	8.0
% hemolysis (40′ at 37.5°C)	98	98	32	28	26

stilbestrol. The inhibitory effect of serum on several types of hemolysis is well known(2). Studies of the *effect of serum* revealed inhibition of stilbestrol hemolysis by final serum concentrations in excess of 5%. Human serum was heated at 56°C for 30 minutes for these experiments. *Prolytic potassium loss.* Prior to the appearance of detectable hemolysis, increased concentrations of potassium were detected in cell suspensions to which stilbestrol had been added. Reactions were carried out at 4°C for 20 hours. In a representative experiment, the addition of 9.7 x 10^{-5} M of stilbestrol resulted in a concentration in the suspending medium of 0.17 meq/L

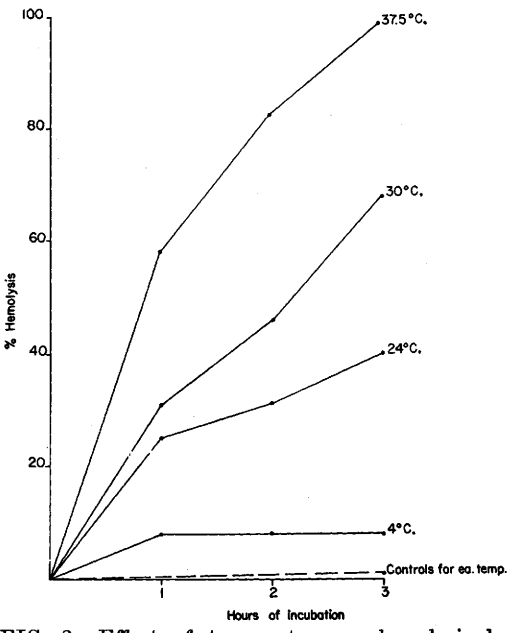

FIG. 2. Effect of temperature on hemolysis by stilbestrol (3.88×10^{-4}Mol).

of potassium compared with 0.04 meq/L in control tubes.

Red cell volume changes. During hemolysis microscopic examination of non-hemolyzed cells disclosed an increase in the size of cells in stilbestrol-treated tubes.

Summary. Diethylstilbestrol, progesterone and DOCA induced *in vitro* hemolysis of human "O" erythrocytes. The hemolytic activity of stilbestrol was modified by temperature, pH, human serum, or the addition of non-hemolytic corticosteroids. Prolytic loss

of potassium from stilbestrol-treated cells was demonstrated.

1. Tateno, I., and Kilbourne, E. D., unpublished data.

2. Ponder, E., *Hemolysis and Related Phenomena*, 1948, Grune and Stratton.

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Effects of Polyoxyethylene Sorbitan Monolaurate (Tween 20) upon Gastrointestinal Iron Absorption in Hamsters.* (21042)

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In this study iron[59] has been utilized to evaluate the effects of feeding polyoxyethylene sorbitan monolaurate (Tween 20) upon iron absorption. The experiments were performed because Eagle(1) observed that prolonged feeding of rations containing 5, 10 or 15% Tween 20 or other polyoxyethylene derivatives to golden hamsters resulted in consistent hemosiderosis of the cecum, liver and mesenteric lymph nodes and frequently in pigmentary cirrhosis.

This finding may be of some practical importance since polyoxyethylene and sorbitan compounds with fatty acids have been proposed as emulsifiers in many human foods. The theoretical implications are also evident, since investigation of the mechanism of development of these lesions may be helpful in understanding the pathogenesis of hemochromatosis and may increase knowledge of the factors regulating iron absorption from the gastrointestinal tract.

Methods and materials. Three experiments with similar plan have been performed. In each experiment adult hamsters of similar age and weight were divided into 2 groups. One group received a bread ration containing 5% Tween 20 while the second group was given a similar control ration without the Tween. In the first experiment the Tween 20 replaced some of the fat in the experimental ration while in the latter 2 experiments it was incorporated in the diet in addition to the lipid. The compositions of the rations, which were modeled after those described by Eagle are shown in Table I. The iron content of the salt mixture was such that each animal received at least 0.004 g of dietary iron (as ferric salts) per day. To prepare the rations the dry ingredients were mixed and the fat and/or Tween 20 was blended with them. The yeast was dispersed in the required amount of water and this was mixed with the other ingredients to make a dough. This was

TABLE I. Diet Compositions.

Ingredients	Tween ration Exp. 1 (g)	Tween ration Exp. 2 & 3 (g)	Control ration (g)
Vegetable fat	0	5	5
Tween 20	5	5	0
White flour	75.7	75.7	75.7
Baker's yeast	1.5	1.5	1.5
Salt mixture*	1	1	1
Lactalbumin	9	9	9
Sucrose	2	2	2
Water to make dough	56.6 ml	56.6 ml	56.6 ml
Added to ground bread			
Salt mixture*	3	3	3
Wheat germ oil	1	1	1
Vit. A & D oil	1	1	1
Vit. mixture†	0.8	0.8	0.8
Total	100	100	100

* Jones and Foster(2).

† Thiamin HCl 80 mg, riboflavin 80 mg, pyridoxine HCl 80 mg, calcium pantothenate 440 mg, niacin 400 mg, biotin 1.0 mg, folic acid 2.5 mg, 2-methyl 4-naphthoquinone 100 mg, choline chloride 10 g, para-aminobenzoic acid 1.0 g, inositol 1.5 g, Wilson 20:1, liver powder 70 g.

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