Growth Inhibition and Leg Disorder in Chicks Following Parenteral Administration of Hemin and Non-Metalloporphyrins.* (25594)

UNABELLE B. BLACKWOOD† AND WILLIAM O. POLLARD (Introduced by M. S. Shorb)
Dept. of Poultry Husbandry, University of Maryland, College Park

The major portion of heme in the animal body is found as the iron-porphyrin portion of hemoglobin. It is also found in molecules of some very important enzymes such as catalases, peroxidases, and cytochromes. In recent years considerable information has been presented concerning synthesis of heme and its porphyrin precursors(1,2). It is known that many co-factors, such as pyridoxal phosphate (Vit. B_6), coenzyme A (thus pantothenic acid) and others are required to synthesize heme and porphyrins from succinic acid and glycine. It is also known that 2-ethyl-5-methylbenzimidazole (EMB) inhibits the synthesis of heme in chicken erythrocytes (3,4). However, very little is known about the metabolic steps or co-factors involved in the catabolism of heme or related porphyrins. This paper demonstrates that in chick, hemin (heme chloride) is unable to reverse inhibition of EMB and that hemin itself and non-metalloporphyrins have inhibitory effects resulting in growth depression and leg deformity.

Methods and materials. Day-old chicks of the strains and crosses indicated in Table I were weighed and distributed into groups of 18 or 20 of equal average weights in wire batteries (avg. starting wt. all exp. = 42.4 g) and placed on a practical starter ration. They were fed *ad libitum* and a constant light source was supplied. Materials used were 2-ethyl-5-methylbenzimidazole (supplied by Dr. Karl Folkers, Merck & Co.); hemin (recrystallized) Eastman Kodak Co.; hematoporphyrin dihydrochloride (recrystallized) and protoporphyrin (C.P.), Mann Research Laboratories, Inc. In 2 experiments hemin was dissolved in 0.1 N NaOH and EMB was dissolved in 0.07 N HCl. In the remaining experiments, hemin and the porphyrins were dissolved in 1% NH_4OH and pH of the solution adjusted to 7.5 to 8.0 with glacial acetic acid. The volume was then adjusted with distilled water so that each 0.2 cc contained either 3 or 5 mg. Controls consisted of an equal number of birds as experimental groups; one group received no treatment, and the other received solvent injections. A total of 5 daily injections of 0.2 cc were given in the area of loose connective tissue between the medial surface of the femur and body wall beginning at 2 days of age. Hematocrits were determined on individual chicks at termination of experiments using standard Wintrobe hematocrit tubes. Chicks were weighed individually at 5, 10, and 15 days after beginning of treatment in all experiments and also at 21 days in 3 of these experiments (Table I).

Results. That hemin has inhibitory activity in the chick was found when it was administered with EMB to New Hampshire-Columbian females to test its reversing ability. Table I shows that hemin not only did not reverse the small but significant weight depression of EMB, but severely depressed growth below that of EMB alone. A large proportion of chicks which survived EMB-hemin treatment developed outwardly bowed legs similar to that found in certain nutritional deficiencies such as choline, Mn, biotin and others. However, birds did not develop "slipped-tendon" as seen in classical perosis. Injection of 3 mg hemin with and without 1.5 mg EMB into White Leghorn chicks of both sexes showed that hemin alone was as effective a growth depressant as the combination of hemin-EMB (1-8 day weight gains‡ : 45.2 g solvent control; 31.7 g hemin-EMB; 31.1 g hemin). Additional experiments (Table I) confirmed the indication that subcutaneously administered hemin adversely affects chick

* Article No. A801; Contribution No. 3071 of the Maryland Agric. Exp. Station. Work supported by grant from Nat. Inst. of Arthritis and Metab. Dis.

† Present address: Poultry Science Dept., Ohio State Univ., Columbus.

‡ Results of preliminary experiment, data not included in table. Four injections given over 8 day period.

TABLE I. Average 5 Day Period Weight Gains and Leg Disorder Observed in Various Strains of Chicks Following Treatment with EMB, Hemin and Certain Non-metalloporphyrins. Mean ± S.E. of difference from control is indicated where calculated. Doses indicated are daily doses, administered for 5 days.

Treatment	Days of exp. 1-5	6-10	11-15	16-21	Bowed legs (% survivors)	Mortality, %
	g gain/period					
New Hampshire-Columbian Females (20 chicks/treatment)						
None	15.9	33.0	60.2	142.8	0	0
Solvent (HCl)	15.8	35.0	58.2	148.2	0	0
EMB, 1.5 mg	11.0 ± 1.21*	30.2 ± 2.01	59.0 ± 2.59	146.1 ± 3.84	0	5
Idem + hemin, 5 mg	4.6 ± 1.31*	22.3 ± 3.01*	51.7 ± 4.09†	161.2 ± 5.13	36.0	45
Vantress-Columbian Males (18 chicks/treatment)						
None	25.8	44.4	84.4	78.9	0	0
Solvent (NH_4OH)	27.1	42.5	82.6	80.5	0	0
Hemin, 3 mg	17.8 ± 1.2*	31.4 ± 1.9*	75.0 ± 3.2*	77.8 ± 2.6	0	0
" 5 "	13.6 ± 1.8*	31.0 ± 1.7*	74.4 ± 3.6*	74.4 ± 3.1	0	0
Vantress-Columbian Females (18 chicks/treatment)						
None	28.0	43.2	78.2	82.3	0	0
Solvent (NH_4OH)	25.9 ± 1.9	45.8	77.6	75.5	0	0
Hemin, 3 mg	18.8 ± 1.8*	33.9 ± 2.1*	73.8 ± 3.1	74.1 ± 3.4	11.1	0
" 5 "	15.8 ± 1.9*	31.4 ± 2.1*	75.9 ± 3.2	69.3 ± 3.1	35.3	0
Hematoporphyrin, 2 mg‡	24.4 ± 2.1	31.9 ± 2.7*	66.1 ± 3.8*	69.5 ± 7.2	0	0
White Rock Males (20 chicks/treatment)						
None	27.2	36.2	85.0		0	10
Hemin, 5 mg	10.3 ± 1.9*	46.3 ± 3.4*	71.7 ± 4.6*		35.3	15
White Rock Males (20 chicks/treatment)						
None	37.8	45.7	67.3		0	0
Solvent (NH_4OH)	34.3	48.1	71.0		0	5
Hemin, 5 mg	23.1 ± 2.4*	29.5 ± 4.8*	56.3 ± 6.8†		33.0	40
Hematoporphyrin, 5 mg	16.5 ± 2.9*	20.0 ± 5.4*	43.4 ± 6.2*		0	45
Protoporphyrin, 5 "	22.8 ± 2.0*	30.7 ± 3.5*	48.5 ± 5.2*		29.4	15

* 0.1%-2.0% level of significance (t test). † 2.5%-5.0% level of significance (t test).
‡ Group contained 14 chicks. Treatment started day 3.

growth and leg development.

Very little difference between 3 and 5 mg of hemin was noted in growth depressant effect, although 5 mg was considerably more effective than 3 mg in producing bowed legs. Recovery from hemin treatment, as indicated by weight gains at progressive periods, was not complete at 15 days (10 days after cessation of administration) in any experiment except one involving Vantress-Columbian females.

Experiment 5 showed that protoporphyrin has effects similar to hemin with respect to growth depression and bowing of legs, and that hematoporphyrin (Exp. 3 & 5) depresses growth to the same or greater extent than hemin and protoporphyrin, but fails to cause bowed legs. Hematoporphyrin acted over a longer period of time than hemin in each of 2 experiments in which it was tested.§ This was indicated by lower weight gains during 11-15 day period.

Bowed legs were found only in females of the Columbian crosses. However, this condition was not restricted to females since White Rock males (Fig. 1) developed bowed legs to as great an extent as Columbian cross females. Fig. 1 also shows that the left leg appears more severely involved, which was noted throughout all experiments but was most prominent in experiments with Columbian cross females in that bowing of legs was confined solely to the left leg.

Solvent control injections did not depress

§ Since manuscript was written, additional experiments (Pellard, Shorb, unpublished) indicate that hemin incites a foreign body reaction at injection site, consisting of a walling off of injected substance. This process would make toxicity of hemin of shorter duration than porphyrins which do not incite this reaction, and also account for longer period of activity of hematoporphyrin and protoporphyrin.

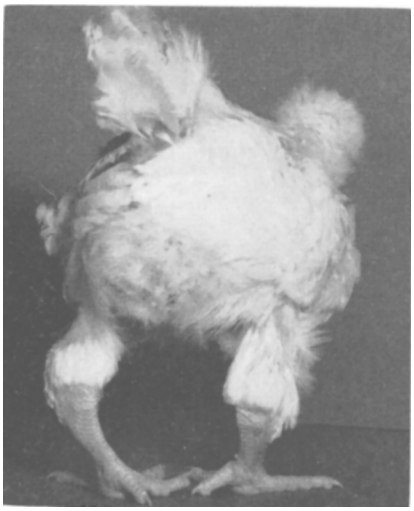

FIG. 1. White Rock male 10 days following 25 mg hemin treatment. Note outward bowing of legs, more severe in left.

chick growth substantially below that of non-treated groups (Table 1). Thus dilute HCl, NaOH and NH_4OH cannot account for the adverse effects found in the chicks receiving EMB, hemin or the porphyrins. Gains during 1-10 days with the latter compounds were lower than controls regardless of treatment.

Determinations of hematocrits at both 16 and 21 days revealed no significant effect of hemin or hematoporphyrin on the packed cell volume (29.5-32.4%).

Discussion. This study confirms conclusively our previous report(5) that parenteral administration of hemin and hematoporphyrin has adverse effects on chick growth and hemin causes an outward bowing of legs. It shows, in addition, that protoporphyrin also affects chick growth and produces a similar bowing of legs.

The mechanism of hemin, proto- and hemato-porphyrin inhibition in the chick is unknown. It is evident, however, that the effects of hemin cannot be attributed to the presence of iron since its metal-free porphyrin homologue, protoporphyrin, has similar effects. Hematoporphyrin, differing from protoporphyrin by having 2 hydroxyethyl side chains in place of 2 vinyl chains, failed to cause bowed legs. The inhibition does not appear to involve formed blood elements quantitatively as indicated by hematocrit, since packed cell volume was not affected. Although Vannotti *et al.*(6) found that protoporphyrin administered intravenously into the normal rabbit had no effect on hemoglobin synthesis, they found it caused a slight increase in liver cytochrome *c*. It is possible that the higher levels of hemin and protoporphyrin used in this study cause a metabolic imbalance in the chick by increasing cytochrome *c* and that the chick is less able to metabolize exogenous hemin and porphyrins than mammals.

Sex difference in leg bowing could not be attributed to difference in growth rate or to differences in recovery from hemin and, therefore at present, is unexplained. Since the bowed leg condition resembles that resulting from several nutrient deficiencies, it is possible that catabolic breakdown of this additional hemin and protoporphyrin requires certain metabolites to a higher degree than were supplied in the diet, thus resulting in nutrient deficiencies. Or, on the other hand, it may be that their presence blocks some metabolic step or prevents utilization of available metabolites. If these hypotheses are true, inhibitory effects of hemin and porphyrins in the chick could serve as a means for studying metabolic steps and co-factors required for their catabolisms, or for studying other metabolic processes in the chick.

Summary. In this study on 2-day old chicks the reversing effects of hemin on 2-ethyl-5-methylbenzimidazole and growth depression and leg deformity produced by hemin and certain non-metalloporphyrins were investigated. Hemin did not reverse the growth depressing action of EMB. Hemin and porphyrins, proto- and hemato-porphyrin, caused depression of growth below that of controls; also, hemin and protoporphyrin caused bowing of legs. That the basic porphyrin nucleus is the probable cause of this depression and leg bowing and not iron imbalance is demonstrated by similarity of action of protoporphyrin and hemin. Hematoporphyrin caused growth depression but did not produce leg bowing. Incidence of bowing was directly related to dose. Hematocrits determined at 16 and 21 days were not affected by treatment.

1. Wolstenholme, G. E. W., Millar, E. C. P., Eds., *Porphyrin Biosynthesis and Metabolism*, Ciba Fn.

Symposium, Little, Brown and Co., Boston, Mass., 308 pp, 1955.

2. Rimington, C., *Ann. Rev. Biochem.*, 1957, v26, 561.

3. Abbott, L. D., Dodson, M. J., PROC. SOC. EXP. BIOL. AND MED., 1954, v86, 475.

4. Abbott, L. D., Dodson, M. J., Auvil, D. K., *Fed. Proc.*, March 1956, v15, Abs. #674.

5. Blackwood, U. B., Pollard, W. O., *Poultry Sci.*, 1959, v38, #5, 1190.

6. Vannotti, A., *Porphyrin Biosynthesis and Metabolism*, 1955, 128, Little, Brown and Co., Boston, Mass.

Received September 28, 1959. P.S.E.B.M., 1960, v103.

Liver Ketogenesis. II. Role of Glucose Cycloacetoacetate on Ketogenesis in Hyper- and Hypothyroid Rats.* (25595)

M. C. NATH AND J. M. KHANADE

Dept. of Biochemistry, Nagpur University, Nagpur, India

The authors reported that hydrolyzed glucose cycloacetoacetate (GCA) when added to liver slices in presence of pyruvate and acetate enormously decreases formation of acetoacetate(1) and increases glycogen(2). Because thyroid feeding (reviewed by Barker(3)) causes enormous rise in production of acetoacetate in the liver, we attempted to find out the role of hydrolyzed GCA in preventing excess of ketogenesis in liver induced by thyroid feeding. We also studied the effect of thiouracil and the combined effect of thiouracil and GCA on acetoacetate formation from lower fatty acids in presence of liver slices. Utilization of acetoacetate by kidney slices in different groups of animals has also been measured.

Method. In view of relatively rapid response of young rats to thyroid and thiouracil feeding(4), male albino rats weighing 75 g to 100 g and growing on standard diet, were used. The animals were divided into 6 groups of 6 rats each and fed the following basal diet *ad lib.* for 6 weeks:

	%		%
Wheat flour	10	Salt mixture	5
Sucrose	49	Yeast powder	4
Casein	20	Marmite	2
Groundnut oil	10	Cod liver oil	.5

Rats in Group I received basal diet and Group II basal diet plus freshly hydrolyzed GCA, 0.8% of diet by weight. Desiccated thyroid (0.3% of diet by wt) was mixed in diet of animals in Groups III and V and thiouracil 0.3% in Groups IV and VI as these concentrations were effective(Hander,5). Rats in Groups V and VI received 0.8% GCA hydrolyzed daily in addition to thyroid and thiouracil respectively.

Tissue preparation and analytical methods. At the end of 6 weeks, animals were stunned by a blow on head and decapitated. Liver and kidneys were taken out immediately and placed in ice-cold saline. Tissue preparations were done as described by Umbreit *et al.*(6). Liver and kidney slices were cut with razor blade, suspended in conventional Warburg vessels containing 3 cc of Krebs' Ringer Phosphate buffer of pH 7.2. The vessels were gassed for 1 minute with oxygen, then incubated for 2 hrs. at 37.5°C with constant shaking. Then acetoacetate was estimated from media in vessel according to manometric method of Edson(7). Liver glycogen estimations were done by the method of Good *et al.* (8) and blood sugar by that of Hagedorn and Jensen(9). GCA, a crystalline product obtained by condensation of glucose with acetoacetate, was prepared according to the method of West(10) modified by Nath *et al.*(11). It was hydrolyzed with 2N HCl for 12 min and extracted with ether to remove resinous materials formed. Aqueous solution was adjusted to pH 7.2 by adding requisite amount of 2N NaOH, then fed to the animals. Total fat content of liver was extracted thrice with alcohol-ether mixture (1:1). The extract was

* The authors record their thanks to the Council of Scientific and Industrial Research for a Research Grant.