Clinical Studies on Erythropoietic Factor in Plasma. (25407)

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As the result of thorough investigations on humoral regulation of erythropoiesis in recent years, it is now widely accepted that erythropoietic factor or factors are present in plasma. In investigation of factors which influence heme synthesis, it was observed that saline suspended rabbit erythroblasts incubated with plasma from rabbits submitted to intermittent anoxia or to cobalt injections showed an accelerated heme synthesis when compared with those incubated with normal rabbit plasma (1). From these results, it was postulated that such plasma factor(s) might be increased in plasma of some patients with blood dyscrasias. In this paper this plasma factor(s) in various blood diseases was studied by measuring heme synthesis in chicken erythrocytes incubated with glycine and patient plasma.

Method. Glassware was cleaned and solutions prepared with redistilled water. Glycine and delta amino levulinic acid were supplied by Daiichi Chemical Co., and radioactive iron by Oak Ridge National Laboratory. *Incubation*: Two or 6 ml of saline suspension of chicken erythrocytes was incubated with glycine ($2 - 5 \times 10^{-2}$ M), radioactive $Fe^{59}Cl_3$ (0.5 - 1.0 microcurie), and 2 ml of patient heparinized plasma, with constant agitation for 3 hours at 37°C. The mixture was then transferred to 50 ml test tube and thoroughly washed with physiological saline. The hemin was crystallized according to the method described by Chu(2). Total activity of hemin, representing total activity of radioactive iron incorporated into heme during incubation, was counted by using a well-type scintillation counter. The cyanhematin method was employed for quantitative determination of crystallized hemin. Specific activity of hemin was calculated by dividing total radioactivity (c.p.m.) of the crystallized hemin by amount of hemin in micro-Mol. The factor(s) in plasma which accelerated incorporation of radioactive iron into heme was designated tentatively as "Heme Synthesis Accelerating Factor" (H. S. A. factor). The variable activity of chicken erythrocyte suspensions in each series of experiments necessitated strict control experiments using normal plasma.

Results. When plasma from normal human beings was used, the specific activity varied $\pm$ 15%. Table I presents results of 2 experiments in which activity of H.S.A. factor (specific activity of hemin) in various diseases, is compared with normal controls. Plasma from patients with iron deficiency anemia and hemochromatosis contained less than normal H. S. A. activity, while in hemolytic anemia, polycythemia, aplastic anemia and acute leukemia the activity was increased. The increase was especially marked in the last 2 diseases.

By using chicken erythrocyte hemolysate prepared by the method of Dresel and Falk (3), and delta amino levulinic acid ($4 - 5 \times 10^{-4}$ Mol.) instead of erythrocyte suspension and glycine, H. S. A. activity was even more distinctly demonstrable in aplastic anemia and acute leukemia (Table II).

A marked difference was observed in H. S. A. activity when heparinized plasma from a patient with aplastic anemia was assayed separately by the 2 methods.

Although it seemed possible that stable iron present in plasma might interfere with incorporation of radioactive iron into heme, there was no correlation between plasma iron levels and H. S. A. activity. A nonprotein filtrate (4) prepared from plasma which was very effective in accelerating *in vitro* radioiron incorporation into chicken erythrocyte hemoglobin (last 2 patients, Table II) did not accelerate incorporation of radioactive iron into heme in incubated chicken erythrocytes.

Discussion. Specific activity of crystallized hemin indicates the effect of heme synthesizing potency of the incubation mixture plus

TABLE I. Activity of Heme Synthesis Accelerating Factor in Various Human Diseases Assayed by Employing Chicken Erythrocyte Suspension and Glycine.

Disease	Hb, %	Blood findings, R.B.C. × million	Serum Fe, γ/100 ml	S.A. of Fe^{59} in heme, cpm/μM	% of normal
(i) Using 6 ml of chicken erythrocyte suspension					
Normal	101	4.73	151	4320	100
Iron deficiency anemia	46	3.02	48	1950	37
Idem	38	3.44	60	2340	54
Hemochromatosis	83	3.88	295	2440	57
Banti syndrome	83	4.69	65	5500	130
Physiol. saline				3820	89
(ii) Using 2 ml of chicken erythrocyte suspension					
Normal	100	4.92	135	170	100
Iron deficiency anemia	58	3.80	13	80	47
Pancreas cancer	58	3.37	45	125	74
Polycythemia vera	134	7.12	83	720	430
Hemolytic anemia	65	2.86	220	840	490
Aplastic anemia	78	3.61	255	2210	1300

that of added plasma. Using hypophysectomized rats as bioassay animals, Gurney *et al.* (5) reported a high erythropoietin level in plasma and urine from patients with aplastic anemia, acute leukemia, and pernicious anemia. A high titer of erythropoietin has been found in urine of some patients suffering from aplastic anemia(6). Elevated erythropoietin activity in plasma has been observed in patients with polycythemia(7). We observed that plasma from patients with hemolytic anemia, polycythemia, aplastic anemia and acute leukemia, accelerated *in vitro* heme synthesis in chicken erythrocytes. There is no evidence to indicate that the H.S.A. factor is the same as the erythropoietin studies by other investigators, because the methods employed to assay for erythropoietin and H.S.A. factor are essentially different. Nevertheless, it is noteworthy that the H.S.A. factor was increased in diseases in which an elevated erythropoietin level is observed. Although a decreased erythropoietin level in plasma has never been detected, activity of the H. S. A. factor was somewhat reduced in iron deficiency anemia and hemochromatosis.

Since there is no cell division in peripheral erythrocytes, the effect described here must be directly on acceleration or activation of heme synthesis. Borsook *et al.*(4) described a heat stable plasma factor which is active in accelerating labeled amino acid incorporation *in vitro* into rabbit reticulocyte protein, especially hemoglobin. The activity is not destroyed or precipitated by boiling at pH 5.5. A similarly prepared filtrate from plasma with high H. S. A. activity was completely inactive. This strongly suggests that the H. S. A. factor is different in its chemical nature from Borsook's factor.

TABLE II. Activity of Heme Synthesis Accelerating Factor in Various Human Diseases Assayed by Employing Chicken Erythrocyte Hemolysate and A. L. A.

Disease	Hb, %	Blood findings, R.B.C. × million	Serum Fe, γ/100 ml	S.A. of Fe^{59} in heme, cpm/μM		% of normal
Normal	100	4.92	135	11	Avg	100
"	95	4.87	124	8.4	9.7	100
Iron deficiency anemia	65	3.47	24	2.6		27
Idem	37	2.67	34	4.5		46
Secondary polycythemia*	112	6.12		51.4		530
Acute myeloid leukemia	32	1.48		148		1530
Idem	60	2.99	158	179		1840
"	45	2.19	176	262		2700
"	34	1.50	210	1260		13000
Aplastic anemia	78	3.61	255	1010		10400

* Secondary polycythemia due to cerebellar hemoangioma.

Summary. 1) A heme synthesis accelerating factor was detected in human plasma by employing a newly devised method of incubating chicken erythrocytes with glycine, radioactive iron and human plasma. Alternatively, one can use chicken erythrocyte hemolysate with delta amino levulinic acid. 2) This factor(s) was increased in hemolytic anemia, polycythemia, aplastic anemia and acute leukemia, and decreased in iron deficiency anemia and hemochromatosis. 3) "Heme Synthesis Accelerating Factor" and Borsook's factor are not identical. 4) Relationship between the "Heme Synthesis Accelerating Factor" and erythropoietin is discussed.

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Nicarbazin Induced Hypercholesterolemia in the Hen.* (25408)

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The coccidiostat Nicarbazin (Merck) an equimolecular complex of 4,4′ dinitrocarbanilide (DNC) and 2-hydroxy-4,6-dimethylpyrimidine (HDP)(1), may, under certain conditions, depress egg production, egg size, and hatchability; and increase yolk mottling and inhibit deposition of shell pigments(2). To this list can be added induction of hypercholesterolemia, for, as outlined in this report, such an effect was observed in hens which had been fed Nicarbazin at levels high enough to inhibit egg production, but not so high as to be otherwise toxic.

Procedure. Nicarbazin was incorporated at desired concentrations into an all mash laying ration and fed to individually caged White Leghorns. The approximate composition of the diet was 15% protein, 3-4% fat from plant sources, ½% fat from animal sources and 1/40% cholesterol(3,4). Plasma cholesterol was measured at various intervals by a slightly modified Zlatkis procedure(5,3,6). In several trials pregnant mares' serum (PMS-Veterinary Gonadin Serum, Cutter Laboratories) was injected in the leg muscles of Nicarbazin-fed birds in an attempt to induce egg formation with exogenous gonadotrophins. At least 2 replicates were run for all treatments, but the results have been pooled in the Tables.

Results. Within 1-2 weeks after feeding mature hens Nicarbazin at a level between 0.04 and 0.07% of the diet, egg production decreased drastically and plasma cholesterol rose approximately 2 fold (Table I). Only a slight rise in plasma cholesterol was observed in males. By 4-8 weeks in younger females (6-8 months old) on the 0.04% drug level, egg production ceased completely and plasma cholesterol rose to levels 3 to 4 times that existing initially or in simultaneously sampled controls. This high level of plasma cholesterol was generally maintained to the 32nd week, when the longest trial was terminated. Total plasma lipids, while not measured, probably

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