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.

We are indebted to Dr. J. H. Lawrence and Dr. D. C. Van Dyke for reading the manuscript and for suggestions and helpful criticisms.

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Received September 14, 1959. P.S.E.B.M., 1960, v103.

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

* Paper of the Journal Series, N. J. Agricultural Experiment Station, Rutgers, State University, New Brunswick, N. J. Supported in part by P.H.S. grant.

TABLE I. Effect of Feeding Nicarbazin on the Plasma Cholesterol of the Chicken.

Sex→	Females										Males					
Age at start→	6-8 mo					15-18 mo					6-12 mo					
% Nicarbazin→	.0 (control)			.04		.06			.04-.07		.0 (control)			.04-.06		
Wk after start	Body wt, kg	Eggs/ bird/ day*	Plas. chol., mg %	Body wt, kg	Eggs/ bird/ day*	Plas. chol., mg %	Body wt, kg	Eggs/ bird/ day*	Plas. chol., mg %	Body wt, kg	Eggs/ bird/ day*	Plas. chol., mg %	Body wt, kg	Plasma chol., mg %	Body wt, kg	Plasma chol., mg %
0	1.95	.75	211	1.88	.64	195	1.86	.72	199	1.62	.69	213	2.46	159	2.34	148
1				1.82	.67	263	1.77	.32	467	1.58	.27	401	2.42	164	2.17	203
2	2.04	.70	205	1.83	.22	507	1.72	.11	425	1.51	.03	238	2.44	177	2.12	206
4	2.04	.71	203	1.82	.00	652	1.68	.03	478	1.43	.00	189	2.43	180	2.05	207
8	2.05	.70	169	1.90	.00	961										
16	2.04	.63	227	1.87	.00	888										
32	1.90	.59	221	1.94	.00	738										
Avg No. of birds/measurement	23.8	23.8	23.8	15.3	17.0	9.2	18.8	15.0	14.2	7.2	7.2	7.0	5.4	5.4	6.2	6.2
Pooled S.E.	.060	.050	11.7	.062	.049	95.1	.035	.058	55.4	.076	.042	66.4	.076	11.1	.036	11.1

* Computed for the 7 days preceding body wt and plasma cholesterol measurements.

were also elevated as indicated by the extremely milky appearance of the plasma.

Rate of egg laying decreased even more rapidly in both younger hens on the 0.06% level, and older hens (15-18 months) on Nicarbazin levels between 0.04 and 0.07%, but plasma cholesterol did not reach as high a concentration as in the younger hens on the 0.04% dose. In fact plasma cholesterol of older hens declined by the second week and was near control levels by the 4th week. In both younger hens at the 0.06% level and in older hens, body weight fell steadily over the test period, in contrast to comparatively small changes in body weight of younger hens on 0.04% Nicarbazin.

Despite their failure to lay eggs, those birds that maintained elevated plasma cholesterol levels had normal plumage and large bright red combs, generally indicative in this breed of active ovarian function. As shown in Table II, their comb weight was equal to that of the controls. Similar observations have been made by Sherwood *et al.*(7) and Baker *et al.* (8) with respect to the appearance of hens on Nicarbazin. On the other hand there were often in these same birds external indications of non-laying such as increased yellow pigment in the beak, ear lobes, and shanks, and decreased spread between the pelvic bones. It was further observed that those Nicarbazin-treated birds in which plasma cholesterol never rose or in which it fell after an initial rise showed external signs of reproductive inactivity such as molting of plumage, decrease in size of combs (Table II) and loss of red color of comb.

In a few instances, a Nicarbazin-fed bird began to lay eggs at a slow rate after having ceased production and having maintained a high plasma cholesterol level for several weeks. In such cases plasma cholesterol always fell with the onset of egg-laying. Some birds in the 32-week-test passed through several such cycles of high and low plasma cholesterol with concomitant signs of reproductive activity and inactivity. After feeding Nicarbazin for 4-6 weeks, replacement of the drug-containing-ration by control feed resulted in resumed egg-laying(7,8) and return of plasma cholesterol to control levels in ap-

TABLE II. Effect of Injecting PMS into Nicarbazin-Fed Hens.

Treatment	Body wt, kg	Eggs/bird /day*	Plas. chol., mg %	Comb wt, g	Oviduct wt, g	Largest ova, in decreasing order, g			
						No. 1	No. 2	No. 3	No. 4
Controls	1.88	.76	172	21.4	51.6	12.3	9.6	7.1	5.0
Nicarbazin† (active ovaries)	1.77	.00	853	23.5	29.1	1.7	1.1	1.0	
Controls + PMS‡	1.90	.09	241	26.2	55.9	15.3	14.9	13.7	12.9
Nicarbazin† + PMS‡	1.69	.00	663	20.2	31.4	1.0	.8	.6	
Nicarbazin† (inactive ovaries)	1.41	.00	228	3.1	16.0	<.1	<.1	<.1	<.1
Avg No. birds/measurement§	3.3	3.0	3.3	2.0	3.5			3.9 ‖	
Pooled stand. error§	.116	.076	63.9	1.70	4.69			1.98‖	
F ratio§	2.04	38.92¶	10.01¶	31.19¶	10.44¶			10.08¶	

* For 7 days preceding sacrifice. † .04% for minimum of 6 wk. ‡ Eight IM inj. of 50-100 units (1-2 cc) each of Gonadin Serum (Cutter Laboratories) over a 10-14 day interval. § From analysis of variance. ‖ Inactive ovary group excluded in computation. ¶ Significant at 1% level.

proximately 2-3 weeks. This was not tried on birds which had been on treatment for longer than 6 weeks.

On post mortem examination, the non-laying, high-cholesterol, Nicarbazin-fed bird presented a picture of partial reproductive activity similar to that described by Baker *et al.* (8). Yolk formation was obviously continuing, since many follicles were present which were at least 10 times heavier than those in an inactive ovary, but the largest follicles were still only about 1/10 the weight of normal ova approaching maturity (Table II). There were always present a number of small, flabby ova suggestive of loss of yolk material, although there was no free yolk in the body cavity. The oviducts of these birds looked comparatively normal, but were about half normal weight, although still twice as heavy as in birds with completely inactive ovaries (Table II).

Eight intramuscular injections of 50-100 units each of PMS over a 2-week interval did not cause eggs to be laid by the Nicarbazin-fed birds (Table II). This in itself was of little significance, inasmuch as the same PMS treatment actually inhibited egg-laying in control hens. However, on post mortem, it was apparent that PMS had induced yolk deposition in the control hens, since the usual sequential decrease in size of maturing ova was for the most part obscured. The ova of the Nicarbazin + PMS group, on the other hand, were no different in appearance or weight than those of birds treated with Nicarbazin alone (Table II).

Discussion. The accumulated evidence suggests strongly that the hypercholesterolemia induced in the hen by Nicarbazin is the result of resorption of yolk material before the ova reach ovulatory size and while they are still attached to the ovary. Further, in order to sustain the hypercholesterolemia, the reproductive system must remain functional to the extent that new ova are continulaly developing at least through the initial stages of yolk deposition. These conclusions are substantiated by the observations (a) that Nicarbazin does not induce equivalent cholesterolemia in males or in females with inactive reproductive systems; (b) that the hypercholesterolemia develops in inverse relationship to egg-laying, and once induced cannot be sustained if egg-laying recommences or the reproductive system becomes inactive; (c) that active yolk deposition through the initial stages of ova growth is seen in birds with Nicarbazin-induced hypercholesterolemia; (d) that resorption of yolk is indicated by absence of any ova approaching mature size and by presence of flabby ova.

Nicarbazin-induced hypercholesterolemia thus bears some resemblance to the heightened lipemic response Stamler *et al.*(9) obtained to dietary cholesterol in hens with ligated oviducts. These authors presumed that not only was excretion of lipoprotein through the egg prevented, but that mature yolks were being

ovulated into and absorbed from the body cavity. However, the hypercholesterolemia observed with Nicarbazin is several times greater than in the ligated-oviduct birds(9) despite a far smaller dietary cholesterol intake. In view of their low dietary cholesterol intake, the high blood cholesterol of the Nicarbazin birds must be primarily of endogenous origin, and therefore may raise some question as to the functioning of homeostatic mechanisms in the regulation of cholesterol biosynthesis in hens. If resorption of ova occurs sporadically in normal birds or can be stimulated by dietary fat manipulations, it may account for the great variability in the laying hens' palsma cholesterol, particularly the very high values sometimes encountered, (10,11), and may also explain the negative correlation reported occasionally between plasma cholesterol and egg production(3).

The failure of ova to mature under Nicarbazin treatment does not appear to be due to a relative lack of pituitary gonadotrophins (8). Exogenous gonadotrophins (PMS), while inducing growth in control ova, failed to increase the size of ova of Nicarbazin-treated birds (Table II). Further, no consistent effect on male gonads or fertility has been observed with Nicarbazin to suggest any specific effect on the pituitary(7,12,2), and in fact other than effects due to inanition no general tissue changes have been noted until feed levels reach 0.16%(12). The action of Nicarbazin at lower doses would seem to be specifically on the ovary and ova, and there are several reports that indicate that the DNC component of the drug is deposited in the yolk, and that the permeability of the vitelline membrane is altered(13,14,15).

Production of sustained hypercholesterolemia without changes in dietary lipid or protein may provide another means of studying cholesterol metabolism and the relationship of plasma cholesterol to atherogenesis. The optimum level of Nicarbazin to induce hypercholesterolemia in hens should be the lowest level which causes involution of ova without affecting other functions. Since egg production, while severely depressed, may still occur at drug concentrations between 0.02 to 0.04% of the feed(16), this range may represent a minimum. However, the age of the bird seems to be important in these responses, for the 0.04% level, which was effective in young hens appeared to depress body weight in older hens. For young hens the 0.06% level appears to be excessive as indicated by the loss of body weight.

Summary. The equimolecular complex of 4,4′ dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine (Nicarbazin, Merck), when fed at 0.04% of a low-fat, low-cholesterol diet to 6-8 month-old laying hens, doubled plasma cholesterol within 2 weeks and tripled it within 4 weeks. Thereafter the hypercholesterolemia was maintained at levels between 600 and 900 mg % for as long as 32 weeks. The hypercholesterolemia appears to be induced and sustained by continuous resorption of yolk from partially developed ova, while yolk material continues to be deposited in other ova just beginning the growth cycle.

Technical assistance of Kurt Textor and Eugene Borbely is gratefully acknowledged. Nicarbazin was supplied as 25% premix through courtesy of Dr. A. Zeissig, Merck Sharp & Dohme.

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Received September 16, 1959. P.S.E.B.M., 1960, v103.

Thromboplastin. I. Phospholipid Moiety of Thromboplastin.* (25409)

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Howell(1) and McLean(2) established that the phospholipid factor necessary for formation of thromboplastin is an unsaturated cephalin. The question, however, remained whether PE† by itself, or PS by itself, their mixtures, or mixtures with lecithin are necessary. Poole and Robinson(3), O'Brien (4), and Rouser, White and Schloredt(5) maintain that PE is the active substance. Fischer and Hecht(6), however, showed that the purest PE is actually inactive. Even more confusing are opinions on activity of PS. According to Maltaner and Rapport(7), PS is active if rabbit, not avian plasma, is used in the test. Barkhan, Newlands and Wild(8) concluded from experiments on human brain PLs that PS is probably an inhibitor. Barkhan, Silva da Costa and Tocantins(9) found that PS is clearly inhibitory in buffered, saline suspension. Silva, Turner and Tocantins(10) assayed PS as an anticoagulant by using Na deoxycholate in the test suspension. Contrary to these results, Troup and Reed (11) found that of various PLs only PS gives maximal thromboplastic effect. Marcus and Spaet(12) confirmed this, stating that PS isolated from platelets could replace the platelets in thromboplastin generation. Several authors claimed that the coagulation activity is not exercised by a single PL, but by interaction of at least 2. The following authors state that either presence of lecithin is indispensable, or that lecithin increases activity of the corresponding cephalin fractions: Zunz and La Barre(13) "cytozymin"; Gracia and Levene(14) cephalin; Rapport(15) a cephalin fraction consisting mostly of PS; Therriault, Nichols and Jensen(16) very pure PS.

Methods and materials. Testing. One reason for the diametrically opposite results of different authors is the fact that they used entirely different and often dubious test methods. We found that the original Thromboplastin Generation Test of Biggs, Douglas and McFarlane(17) gives a reliable picture of activity of PL preparations, and this method was, therefore, used exclusively. PLs used, raw lecithin, protagon, and cephalin were prepared from fresh calf brain according to Klenk and Böhm(18) and Debuch(19). Lecithin was purified by chromatography on alumina(20), and cephalin was separated into 5 fractions by the method of Folch(21). Fraction III (molar ratio N : P = 0.95) contained not only PS, but also small amounts of PE, lyso-PE, mono phosphatidyl inositol, and diphosphatidyl inositol, as described by Therriault, Nichols and Jensen(16). Fraction IV (molar ratio N : P = 1.1) contained PE and PS in the proportion of 4 : 1, whereas fraction V (molar ratio N : P = 1.1) was almost pure PE with a trace of PS. Total PLs from egg were prepared from fresh egg yolks by the method of Rhodes and Lea(20). The PLs were dialyzed, and the dialyzate contained comparatively large quantities of free ethanolamine and O-phosphoryl serine, but no serine. Upon evaporation, the dialyzate left a residue which was chromatographed on silicic acid paper with phenol-water. Three ninhydrin-positive spots with Rf = 0.05 (= O-phosphoryl serine), 0.16 (= glyceryl phosphoryl serine?) and 0.73 (= ethanolamine) were obtained. We found in the total egg, PLs

* This work was supported by grant from N.I.H., Bethesda, Md.

† Abbreviations used are: PL, phospholipid; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine.