component does not cause loss of activity(1). The fact that such fractions have anticoagulant activity *in vitro* slightly lower than PS fractions containing small amounts of LPS argues in favor of the solubilizing effect of LPS on PS.§ The inhibitory activity of the fraction III of Folch in the thromboplastin generation test reported by Barkhan, Newlands and Wild(11) can, in the light of the findings reported here for purified serine phosphatide fractions, be attributed to PS.

Summary. Serine phosphatide fractions free of lysophosphatidylserine have been prepared from brain tissue. The strong anticoagulant activity of these fractions is ascribed to a naturally occurring serine phosphatide in the diester form which has the capacity to block formation of plasma thromboplastin as well as neutralize its effect after it is formed.

§ The importance of complete solubilization of phospholipids to obtain optimal anticoagulant activity has been mentioned elsewhere(2,8,9)) and is discussed at length in a review of lipid anticoagulants (10).

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Porphyrin Formation by Tissues from Laying Hens Fed Nicarbazin.* (24747)

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The brown color of a hen's egg is due to protoporphyrin deposited on the shell while it is in the uterine portion of oviduct(1). Uterine glandular tissue from laying hens has considerable capacity for transforming δ-amino-levulenic acid (ALA), an intermediate in the porphyrin cycle(2,3), into porphyrins *in vitro*(4). It is well established that decrease in shell color can be produced by feeding the coccidiostat nicarbazin(5), a complex of 4,4′-dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine (DNC-HDP), to laying hens(6,7,8). However, a quantitative relationship between dietary level of nicarbazin and resultant decrease in shell porphyrins has not been established. Also, it is not known whether nicarbazin, of which DNC is the active component(9), prevents deposition of shell protoporphyrin by inhibiting protoporphyrin synthesis.

Methods. Hens which lay brown eggs, New Hampshires and Crossbreeds, were kept in single cages of 3-deck batteries and housed in air-conditioned room at 72 ± 2°F. Medicated rations were prepared by adding nicarbazin to open-formula breeder diet used as basal ration. All rations were fed *ad lib.* A value for egg shell porphyrin concentration was established for each hen by averaging values for all egg shells laid during 5-day period prior to feeding experimental diets. This value served as control to which subsequent values, obtained while hens were on medica-

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tion, was compared. Egg shells were prepared for porphyrin analysis by washing under tap water to remove adhering egg white, then air-drying 24 hours. The entire shell(s) was ground in mortar and 1 g samples, in duplicate, were weighed into 40 ml centrifuge tubes. Shell samples were dissolved by adding 20 ml of 2:1 5% HCl-95% ethyl alcohol. Then the tubes were centrifuged for 3-4 minutes to spin down pieces of shell membrane. Into a test tube containing 5 ml of 2:1 acid-alcohol, 5 ml of supernatant were pipetted and the contents of tube thoroughly mixed. An aliquot was read in Beckman DU spectrophotometer against a blank of acid-alcohol at 410 mμ, and at 382 and 432 mμ to obtain a corrected optical density. Protoporphyrin concentrations were calculated from corrected density values using extinction coefficient ($E^{1\%}_{1\,cm}$) of 4900(10). Nicarbazin was tested for its ability to inhibit porphyrin synthesis by determining, *in vitro*, amount of porphyrins formed in 24 hours by tissues using ALA as substrate(4), and by determining erythrocyte porphyrin formation induced by repeated bleeding(11) of normal and nicarbazin treated hens. Heparin was used as anticoagulant for blood samples. Each sample was centrifuged 15 minutes at 3000 rpm in 40 ml centrifuge tube. Plasma was removed and discarded. Red blood cells were washed twice with 15 ml of 0.9% NaCl, and then 5 ml of the cells were pipetted into 40 ml centrifuge tube. To this were added 10 ml of distilled H_2O, the tube capped, and the contents mixed by vigorous shaking. During next 30 minutes, the tube was occasionally shaken. Then porphyrins were taken up by 5 extractions with 15 ml 4:1 ethyl acetate-glacial acetic acid. Subsequent fractionation of porphyrins from ethyl acetate-glacial acetic acid was performed by the method of Dresel and Falk (10). DNC was determined in egg yolk by the method of Porter(12), and hemoglobin in red cells by the method of Schultze and Elvehjem(13), using a photovolt colorimeter.

Results. Exp. 1. The time to produce maximum depigmentation of egg shells was established by feeding 0.005 and 0.010% nicarbazin for 8 days to 2 groups of hens (5 hens group). Shell protoporphyrin concentrations decreased from 85 to 35 γ/g at 0.005% (a decrease of 58%), and from 59 to 17 γ g at 0.010% nicarbazin (a decrease of 71%) in about 5 days of medication. No further significant change in shell protoporphyrin concentration occurred after day 5.

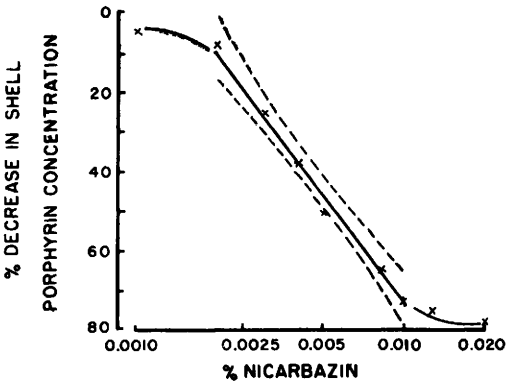

FIG. 1. Relationship between % decrease in shell porphyrin concentration, Y, and the log % nicarbazin content of feed, X, fed to hens laying brown eggs. Y = 215.95 + 85.56X. Fifty hens on experiment.

A dose-response curve was established by feeding graded levels of nicarbazin to hens for 10 days. Shell porphyrin concentrations of eggs laid during 5th to 10th days were averaged and the value compared to concentrations in shells laid prior to medication. A linear relationship, Y = 251.95 + 85.56X, was obtained between log % nicarbazin, X, and % decrease in shell porphyrin concentration, Y (Fig. 1). Confidence limits at 0.01 probability were calculated (n = 50, std. dev. = 10.24) and defined in Fig. 1 by dashed lines.

Exp. 2. Hens fed 0.0125% nicarbazin were sacrificed on 10th day of medication. Homogenates prepared from tissues of various segments in reproductive tract and from area adjoining yolk stalk in the intestine showed no significant effect from nicarbazin treatment in their capacity to form porphyrins *in vitro*. However, significant differences in rates of formation were noted among tissues (Table 1). Thus, mean rates of porphyrin formation with standard error were calculated for each tissue ignoring treatment effect, and compared using uterine rate as 100%. Only isthmian

TABLE I. Porphyrins Formed *In Vitro* from ALA at 1 × 10^{-3}M by Tissues from Laying Hens Fed Diets with or without 0.0125% Nicarbazin. Hens on medicated diets laying shells with 75% less porphyrin at time of sacrifice.

Tissue	Porphyrins formed *in vitro*—γ/100 mg tissue/24 hr						% of uterine capacity
	Control		.0125% nicarbazin		Mean ± S.E.		
Membrane of developing follicles	(2)*	45.7	(3)	59.3	(5)	53.8 ± 6.0	30.3
Ovary, less follicles >.5 g	(2)	35.6	(3)	44.0	(5)	40.7 ± 3.1	23.0
Magnum	(2)	118.2	(3)	90.9	(5)	101.8 ± 8.3	57.4
Isthmus	(2)	164.3	(3)	165.7	(5)	165.2 ± 18.8	93.2
Uterus	(2)	201.1	(3)	161.4	(5)	177.3 ± 16.8	100.0
Middle intestine	(2)	101.8	(3)	81.1	(5)	89.4 ± 6.2	50.4

Analysis of variance:

Source of variation	d.f.	Mean Sq.
Treatment	1	821.7
Tissue	5	15,856.7†
Remainder	23	648.8

* No. hens. † $P < .01$.

portion of oviduct had capacity similar to uterus. Additional data indicated that *in vitro* rate of porphyrin formation by uterine tissues from 4 hens fed 0.020% nicarbazin was similar to rate of tissues from 10 controls, 169.3 ± 14.6 *vs.* 177.9 ± 14.6 γ/100 mg tissue/24 hours, respectively, even when incubations were done in presence of DNC in suspension at 1 x 10 ^{3}M (166.1 ± 14.6).

Exp. 3. Erythropoiesis induced by repeated bleeding of laying hens results in increased formation of copro- and protoporphyrin by red cells *in vivo*(11). Hens were fed 0.020% nicarbazin for 10 days, then these hens and nonmedicated hens were bled 3 times at 72-hour intervals. At each bleeding, approximately 40 ml of blood were removed from the brachial vein. Red cells showed increase in copro- and protoporphyrin content in both treated and non-treated hens (Table II). Hemoglobin levels averaged 9.0, 7.7, and 7.5 g % for control hens at each bleeding, and 9.5, 7.5, and 8.8 g % for hens fed 0.020% nicarbazin. Feed intake was similar for both groups. Red blood cells from hens fed nicarbazin contained more DNC than plasma. Also, DNC was found in control red cells kept overnight in the cold with plasma from medicated hens (Table III). Thus, DNC entered the erythrocytes and also diffused out.

TABLE III. 4,4'-Dinitrocarbanilide (DNC) Content of Plasma and Red Blood Cells from Hens Fed Nicarbazin.

Diet	Hens	DNC—γ/100 ml*	
		Plasma	RBC
.010% nicarbazin	4	54	60
.020% "	4	107	232
Pooled samples: Overnight in cold			
Treated plasma + control RBC		54	72
Control " + treated "		82	112

* Corrected for interfering substances in control solutions.

Discussion. Egg shell protoporphyrin deposition is decreased by feeding nicarbazin to hens. Dose-response curve is linear between dose levels of 0.002 and 0.010% when data are plotted as % decrease in shell porphyrin

TABLE II. Porphyrin Content of Red Blood Cells of Hens Undergoing Repeated Bleeding at 72-Hr Intervals. Approximately 40 ml of blood removed at each bleeding. Medication initiated in treated hens 10 days prior to first bleeding.

Diet	No. hens	Coproporphyrin, γ/100 ml RBC			Protoporphyrin, γ/100 ml RBC		
		Bleeding			Bleeding		
		1st	2nd	3rd	1st	2nd	3rd
Control	3	2.7 ±.18	11.2 ± 1.4	14.6 ± 1.7	55.1 ± 7.2	76.7 ± 16.8	163.1 ± 32.3
.020% nicarbazin	3	4.6 ±.25	11.2 ± 1.4	14.7 ± 1.8	87.8 ±22.0	147.4 ± 20.9	233.5 ± 32.6

vs. log % nicarbazin in diets. Minimum concentration of the drug in the feed which produced a significant change in protoporphyrin deposition was 0.0015% (15 ppm) as compared to values reported by others(7,8) of 0.003-0.005% (30-50 ppm) when judged by perceptible change in shell color.

Glandular tissues from the isthmus and uterus of hens laying brown eggs have an equal capacity to transform ALA to porphyrins, *in vitro*, but a greater capacity than do tissues from other segments of the reproductive tract. This greater *in vitro* capacity appears to reflect the ability of these 2 segments to act as sites for protoporphyrin deposition in the shell membrane (isthmian segment) and on the shell (uterine segment). The ability of magnum and follicle tissue to form porphyrins *in vitro* requires further elucidation, particularly as protoporphyrin tissue segments (meat spots) found in egg white(14) have not been excluded as originating from the magnum.

Decreased deposition of shell porphyrin caused by feeding DNC to hens does not appear to result from inhibition of porphyrin synthesis: 1. Tissues from hens which were fed high levels of nicarbazin formed as much porphyrin from ALA, *in vitro*, as did tissues from control birds. This suggests that DNC does not inhibit porphyrin formation from ALA. 2. Of greater importance is the fact that DNC did not inhibit porphyrin formation by erythrocytes in medicated hens, even though DNC was found in these erythrocytes. Also, concentration of hemoglobin in blood of treated hens was comparable to that in control hens at the onset, and during medication. 3. Hens which lay white eggs have considerably less porphyrin in their excreta than hens which lay brown eggs, and nicarbazin does not alter the amount of either copro- or protoporphyrin in the excreta of brown egg laying hens(15).

Although it is possible that shell porphyrins may originate *via* a pathway other than the proposed glycine-succinate cycle(2), this appears unlikely in view of the large capacity of uterine glandular tissue to form porphyrins from ALA(4), and our observation that glycine-2-C^{14} injection into hens results in C^{14}-labelled shell protoporphyrin.

Summary. Feeding nicarbazin to laying hens resulted in less protoporphyrin on their egg shells. The decrease varied in proportion to dietary intake and a linear response was obtained between dietary levels of 0.002-0.010% nicarbazin. Tissue homogenates of follicular membranes, magnum, isthmus, uterus, and small intestines from laying hens catalysed the formation of porphyrins from ALA. Homogenates of isthmus and uterus were more potent than those of other tissues examined. Tissues from medicated hens formed as much porphyrin from ALA as those from nonmedicated hens. Erythrocyte-porphyrin formation induced by repeated bleeding was not inhibited in medicated hens which were laying eggs with shells containing 75% less porphyrin than controls. These data suggest that decreased deposition of shell protoporphyrin caused by feeding nicarbazin does not result from inhibition of porphyrin synthesis.

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