A Comparison of Porphyrin Synthesis in Certain Tissues from Normal and Anemic Dogs: Some Observations on the *in Vivo* Control of Porphyrinogeneis* (32899)

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Since all tissues and their component cells contain hemoproteins of some type, i.e., cytochromes, myoglobin, hemoglobin, etc., questions arise as to their ability to synthesize these substances from prime precursors under varying experimental conditions. The present work is concerned with the capacity of different tissues from control and anemic dogs to form the porphyrin portion of the heme moiety utilizing the quantitative methods for determining tissue porphyrins now available.

Previous studies in this laboratory (1) on fresh tissues from human subjects and dogs and on acetone powders of the tissues from different species of animals have demonstrated that all tissues studied have some capacity to synthesize porphyrins when delta-aminolevulinic acid (ALA) was used as a precursor. However, these same tissues, with the exception of nucleated erythrocytes, failed to form porphyrins when glycine plus acetate were used as precursors. A comparison made of the ability of different tissues to synthesize porphyrins from ALA showed that nucleated erythrocytes were the most active, while liver, spleen, kidney, heart, and skeletal muscle followed in that order. These results suggest either that these tissues have an extremely limited capacity for ALA synthesis or possibly that ALA may be supplied preformed. It is interesting that the porphyrin-synthesizing ability of different tissues is proportional, in general, to their mitochondrial coproporphyrinogen oxidase activity (2).

Since there is stimulation of erythropoiesis in certain types of anemia, it seemed of interest to determine porphyrinogenesis in selected tissues from anemic dogs. Two types of anemia were employed: one, that produced by phenylhydrazine administration and, the other, that produced by repeated bleedings. Certain tissues from these animals were studied for hemopoietic activity by measuring their ability to synthesize copro- and protoporphyrins from the precursors glycine plus acetate or delta-aminolevulinic acid.

Experimental. Normal mongrel dogs weighing between 10 and 12.5 kg were used in these experiments. Four groups of animals were studied: (i) A control group (12 dogs) maintained on a basal stock "dog chow" ration; (ii) ten dogs made anemic by the intravenous injection of 2.5% phenylhydrazine in 0.9% sodium chloride (40 mg of phenylhydrazine hydrochloride/kg of body wt. per day) until the hematocrit fell below 20%; (iii) six dogs made anemic by repeated bleedings, the plasma being returned intravenously, until the hematocrit fell below 20%; (iv) six dogs made anemic by the same procedure as in group iii, with the exception that iron, as ferrous gluconate, in an amount equal to that removed as hemoglobin was injected intravenously after each bleeding.

After 1 week, the dogs were anesthetized by the intravenous injection of pentothal (0.80 gm/animal), the selected tissues were quickly removed and washed with saline, and 1 g of each tissue was suspended in 10 ml of Krebs–Ringer–Phosphate (KRP) solution and homogenized in a Waring blender for 2 min. The homogenate was quantitatively transferred to a 125-ml Erlenmeyer flask and was incubated with the necessary incubation mixture including glycine (15 mg) and acetate (35 mg of sodium acetate) or delta-aminolevulinic acid (ALA) (8 mg) as precursors. The incubation procedure, extraction, and quantitative determination of copro- and protoporphyrins and their respective porphyrinogens were essentially the same as previously described (1). Each tissue from each dog was run in duplicate, together with duplicate controls. Tissue values for coproporphyrin and

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TABLE I. Biosynthesis of Porphyrins in Tissues of Control and Anemic Dogs.[a]

Group		Tissue	Copro		Proto	
			Group av	p-value[b]	Group av	p-value[b]
i	Control	Liver	18.40 ± 2.55		26.80 ± 1.74	
ii	Phenylhydrazine-treated	Liver	2.82 ± 0.56	0.000003	4.64 ± 0.56	0.000003
iii	Hemorrhagic anemia	Liver	26.54 ± 2.24	0.00010	34.76 ± 2.75	0.0009
iv	Hemorrhagic anemia + Fe	Liver	24.55 ± 2.28	0.007	32.86 ± 2.15	0.0001
i	Control	Spleen	10.80 ± 0.80		8.60 ± 0.83	
ii	Phenylhydrazine-treated	Spleen	0.20 ± 0.20	0.000003	1.82 ± 0.71	0.000003
iii	Hemorrhagic anemia	Spleen	14.23 ± 1.39	0.0001	11.90 ± 1.39	0.0009
iv	Hemorrhagic anemia + Fe	Spleen	15.44 ± 0.81	0.0001	14.76 ± 1.12	0.0001
i	Control	Kidney	5.33 ± 0.55		6.40 ± 0.54	
ii	Phenylhydrazine-treated	Kidney	2.79 ± 0.76	0.000003	3.99 ± 0.44	0.000003
iii	Hemorrhagic anemia	Kidney	7.12 ± 0.30	0.025	9.50 ± 0.64	0.0001
iv	Hemorrhagic anemia + Fe	Kidney	7.98 ± 1.00	0.0001	8.12 ± 1.00	0.0009
i	Control	Heart	1.67 ± 0.18		2.80 ± 0.29	
ii	Phenylhydrazine-treated	Heart	0.60 ± 0.39	0.00003	1.25 ± 0.27	0.000003
iii	Hemorrhagic anemia	Heart	2.54 ± 0.39	0.006	3.95 ± 0.48	0.001
iv	Hemorrhagic anemia + Fe	Heart	2.57 ± 0.35	0.001	4.23 ± 0.62	0.0002

[a] Amounts are expressed in μg of porphyrinogen-porphyrins formed per gm of tissue from ALA as a precursor. Values are corrected for the preformed porphyrins found in control tissues incubated without ALA. Values are group averages with standard deviations. Duplicate runs were made on each tissue and agreed within ± 15%.

[b] Calculated by method of Swed and Eisenhart (11).

protoporphyrin obtained after incubation were corrected for "preformed" porphyrins determined in samples incubated without added precursors, or with added precursors but without incubation.

Results. Table I presents the results obtained on the tissues from the four groups of dogs. In agreement with our earlier findings (1), none of the tissues studied from either the control or experimental groups synthesized porphyrins from glycine plus acetate. These data, therefore, are not included in the Table. The ability of the tissues from the control animals to form porphyrins from ALA, however, agreed well with those reported previously (1) for human and canine tissues and for "acetone powders" of certain species.

The data in Table I show that the tissues of the dogs made anemic by phenylhydrazine are much less active in porphyrinogenesis than are those from the control animals. This is especially true of the spleen. These results are highly significant as shown by the p-values and suggest that some inhibitory factor to porphyrin synthesis may be present in the anemic tissues. It is possible, of course, that the lowered activity of the tissue from the phenylhydrazine-treated dog may be due to some "toxic" effect of phenylhydrazine on the mechanism of porphyrinogenesis in the tissues. However, phenylhydrazine per se had no inhibitory effect on porphyrin formation as shown by six experiments (Table II) in which

TABLE II. Effect of Phenylhydrazine on Porphyrin Biosynthesis in Liver and Kidney of Control Dogs.

Tissue	Porphyrins formed[a]		
	Copro	Proto	Total
Liver	17.4	28.2	45.6
Liver + phenylhydrazine[b]	16.8	29.8	46.6
Kidney	6.8	8.2	15.0
Kidney + phenylhydrazine[b]	5.6	8.8	14.4

[a] Amounts are expressed as μg of porphyrin formed/gm of tissue. Values are averages of six experiments agreeing within ± 15%.

[b] Ten mg of phenylhydrazine hydrochloride was neutralized to pH 7.4 by sodium hydroxide and added to the incubate.

this substance was added to liver and kidney incubates.

In contrast to the results obtained on the phenylhydrazine-treated animals, tissues from the dogs of group iii made anemic by repeated bleeding (Table I) showed no inhibition of porphyrin formation. Indeed, all tissues studied showed significantly higher levels of both copro- and protoporphyrins than did those of the control group. The data obtained from the fourth group given iron are practically identical with those from group iii. This indicates that iron "overloading," which conceivably may have been a factor in the group ii dogs, is probably not responsible for the observed inhibition of porphyrinogenesis. The data from the fourth group also indicate that a lack of iron is not responsible for the elevated porphyrin values observed in the group iii animals.

Discussion. One explanation of the present results appears most plausible. In the phenylhydrazine-treated dogs, heme or possibly some related metabolite from hemolyzed erythrocytes could increase in the plasma and tissue fluids. Heme inhibits porphyrin biosynthesis, *in vitro*, apparently by a "feedback" mechanism (3–7). Thus an increased level of heme in the body fluids and tissues of the phenylhydrazine-treated group, especially in the spleen, could account for the observed inhibition of porphyrin biosynthesis. This interpretation is supported by the fact that porphyrin biosynthesis in the tissues from the animals with hemorrhagic anemia was even greater than that in the normal controls. Iron "overloading" does not appear to be a related factor as indicated by the data obtained on group iv. Phenylhydrazine per se is likewise not an inhibitory factor. The present work thus suggests that the feedback control of porphyrin-heme synthesis by heme, demonstrated in *in vitro* system by others (3–7), also may be applicable to tissues *in vivo*.

The decrease of copro- and protoporphyrin synthesis in the spleen obtained from the phenylhydrazine-treated dogs is particularly striking. This finding may be related to the fact that the spleen is a major site of erythrocyte destruction thus probably resulting in a higher content of heme, and possibly its metabolites, in this tissue.

Interesting in this connection is the observation of Zih in 1939 (8), that the intravenous administration to rats of relatively large amounts of bilirubin or certain other bile pigments to rats decreased the red cell count and blood hemoglobin level. The injection of washed, hemolyzed erythrocytes had a similar effect. Furthermore, earlier work in this laboratory (9) demonstrated the presence of a factor in the plasma of normal dogs which inhibits porphyrin formation from ALA by the liver and certain other tissues. This factor likewise could be heme or a metabolic product of heme.

The point at which heme inhibits the porphyrin-heme biosynthetic pathway has been investigated by several workers (6,7,10). Heme appears to block at the ALA-synthetase level perhaps by serving as a corepressor in the repressor mechanism which blocks the synthesis of this enzyme (10). However, other workers believe that heme may inhibit at other points in the pathway, by coordinate repression, either before the ALA-synthetase step (6) or at the level of ALA dehydrase (7). The present data appear to support the concept of coordinate repression by heme since the inhibitory effect was observed using ALA as the precursor for porphyrinogenesis.

The stimulation of porphyrin biosynthesis in the dogs with hemorrhagic anemia (groups iii and iv) could result from a general stimulation of hematopoiesis. This could conceivably be due to the hormone, erythropoietin, either directly or indirectly. Further studies will be required for the elucidation of the mechanism involved.

Summary. The ability of certain tissues from anemic dogs to form copro- and protoporphyrins from glycine and acetate or from delta-aminolevulinic acid (ALA) has been compared with that of tissues from normal control dogs. None of the tissues studied formed porphyrins from glycine and acetate. However, tissues from normal dogs formed porphyrins from ALA, and in decreasing order of activity, liver, spleen, kidney, and heart. The tissues from the phenylhydrazine-treated dogs showed significantly less copro- and protoporphyrin formation than did those of the control dogs. Porphyrin synthesis in the spleen was especially decreased. In contrast, all

tissues from the group made anemic by bleeding showed *increased* porphyrinogenesis as compared with the controls. The same results were obtained in dogs similarly treated but administered added iron. It is suggested that the decrease of porphyrin biosynthesis observed in the tissues from the phenylhydrazine-treated dogs may result from an *in vivo* "feedback" inhibition by coordinate repression by heme or possibly a related metabolite. The increased porphyrinogenesis observed in the tissues of the dogs made anemic by bleeding may be related, either directly or indirectly, to erythropoietin or to some other hemopoietic factor.

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Lack of Transfer of Morphine Tolerance by Administration of Rat Cerebral Homogenates* (32900)

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It has been reported by Ungar and Cohen (1) that tolerance to morphine can be transferred to mice by injection of homogenates of the brains of morphine-tolerant rats and dogs. They tentatively characterized the transfer factor as a small protein or peptide. Their results could prove to be very significant in explaining the mechanism of tolerance to narcotic analgesics since protein synthesis has been implicated in the development of morphine tolerance (2–4). However, recently Tirri (5) was unable to demonstrate the transfer of morphine tolerance from tolerant mice to normal mice.

The present report represents a study of the replicability of the transfer of morphine tolerance from tolerant rats to normal mice. If the development of tolerance does involve the synthesis of a "tolerance factor" which can be transferred, its transfer should have replicability and generality so this phenomenon can be more widely studied. This work was undertaken with this in mind.

Materials and Methods. The procedures followed were similar to those used by Ungar and Cohen (1); the only major modification was the use of heat, instead of pressure, as the noxious stimulus in the determination of analgesia. Male Holtzman rats weighing between 160 and 200 gm were injected subcutaneously with morphine sulfate t.i.d. at 4-hour intervals with doses increasing progressively from 10 mg/kg per injection on the first and second days to 100 mg/kg per injection on days 13 and 14. On day 15 one injection of 100 mg/kg was given 8 hours before sacrifice. Control rats received injections of 0.9% saline according to the same schedule.

All rats were sacrificed by decapitation. Cerebral hemispheres were removed and weighed rapidly, frozen on dry ice, and stored at −80°C until used. On the day of the experiment the cerebral hemispheres in each group were pooled and homogenized in 1.5

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