the genes determining the scored traits are located on a single plasmid.

Isolation of plasmid DNA and characterization of plasmid DNA molecules, including heteroduplex molecules by electron microscopic examination, have been described (*8*). The sizes of plasmid DNA molecules in preparations of the original strain 86 and various K12 strains carrying pCG86 are listed in Table 2. Included are strains that have received pCG86 by conjugation and by transduction. In strain 86, four sizes of plasmids are seen, whereas three sizes are present in the strains which have received all the scored plasmid-determined traits. The transductant strain which is Tc^S has plasmid molecules of only one size.

In interpreting these findings we shall first consider the difference between strain 86 and the K12 strains. Plasmid molecules whose length is 62 kilobases are present in strain 86 and absent in the other strains. It is likely that the 62-kilobase plasmid carries the genes for the production of K88 antigen and raffinose fermentation. These traits are present in strain 86, but not in the other strains, and the genes determining these two traits are known to be located on a single plasmid (*9*).

To explain the presence of three plasmid sizes in strains carrying all the known pCG86 genes we may consider two possibilities: either there are three plasmids that are always transmitted together or it is only the large plasmid that is transmitted and that dissociates into two smaller ones after transfer. The latter possibility appears to be more likely, because the two smaller plasmids are present at lower frequencies in the plasmid preparations than the large one and, as pointed out above, it is unlikely that during transduction more than one plasmid molecule is transmitted. Furthermore, in physical terms, phage P1 DNA is 90 kilobases long and in transducing particles the DNA substituted for phage DNA is usually of a similar size. It is therefore most unlikely that a transducing particle would contain three DNA molecules with size 114, 84, and 32 kilobases. In fact, we think that the reason for the low transduction frequency of pCG86 is the rarity with which the 114-kilobase DNA molecule is packed into the P1 phage coat.

The transductant selected for Sm^R, which is Tc^S, contains only one size of plasmid DNA—94 kilobases. Thus, it can be concluded unambiguously that at least the genes determining Sm^R, Su^R, LT^+, ST^+, and Tra^+ are carried on a single plasmid. In heteroduplex experiments we have found complete homology between the 94-kilobase molecule and a corresponding 94-kilobase segment of the 114-kilobase molecule present in the other strains. We have also found that the 114-kilobase plasmid, but not the 94-kilobase plasmid, contains a 7.0-kilobase segment bounded by inverted repeat segments each of 1.4 kilobase. It has been shown with other plasmids that the gene for Tc^R is part of a transposon (*10*). This *tet* transposon has the same physical dimensions as the 7.0-kilobase segment bounded by the inverted segments. From these observations we conclude that the 94-kilobase plasmid was generated from the 114-kilobase plasmid, presumably during the period of phage growth after infection by phage P1.

Our results appear to be the first report of a plasmid carrying genes for drug resistance and enterotoxin production. This plasmid is readily transmissible by conjugation among strains of *E. coli*. Presumably the plasmid was formed as a result of recombination between an R factor and an Ent plasmid. The likelihood of such an event depends on the frequency with which these parent plasmids occur in nature, and it is well known that the widespread use of antibiotics has resulted in a greatly increased frequency of R factors among natural isolates. Our results show that genes for drug resistance are spread in nature not only by being part of an R factor, but also by becoming incorporated into other plasmids, in this case a plasmid carrying genes which contribute to pathogenicity.

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Bovine Protoporphyria: The First Nonhuman Model of This Hereditary Photosensitizing Disease

Abstract. *Protoporphyria, a photosensitizing disease documented only in humans, was transmitted as a recessive trait to seven female calves. Cutaneous lesions were extensive, and erythrocyte and fecal protoporphyrin concentrations exceeded by far those of human protoporphyria. Average ferrochelatase activity was decreased to one-half of normal in the liver of carriers, and to about one-tenth of normal in liver, kidney, heart, spleen, lung, and marrow of protoporphyrics.*

We discovered four calves and one adult cow with clinical and chemical findings characteristic of protoporphyria (EPP). They constitute the first known animal model for any of the hereditary human porphyrias other than so-called "erythropoietic porphyria" (EP, congenital, or Gunther's porphyria). Of special interest was the finding of a marked uniform decrease in activity of ferrochelatase (heme synthetase) in liver, heart, kidney, marrow, and other tissues studied.

Within a few days after birth, all the protoporphyric calves actively avoided sunlight, crawled under haystacks, or sought shade even behind fence posts. Within a few weeks, skin over the snout, ears, and back became edematous, erythematous, fissured, partially alopecic, and scabbed. When presented at the University of Minnesota, the four heifers ranged in age from 2 to 3 months. A tentative diagnosis of protoporphyria was made because of the apparently painful photosensitivity, without the discoloration of teeth or urine (*1*) which characterizes EP in humans (*2*), cows (*3*), pigs (*4*), cats (*5*), and squirrels (*6*).

The affected calves were sired by the

Table 1. Initial concentrations of erythrocyte (RBC) and fecal protoporphyrin (*16, 17*) and of fecal urobilinogen (*18*). Porphyrins were extracted into a mixture of ethyl acetate and glacial acetic acid (4 : 1), and thence into 3*N* HCl (*16*). After fluorimetric assay, the HCl solutions were added to a Florisil column (*17*) for fractionation of individual porphyrins. Fecal protoporphyrin was eluted with a 2 : 1 mixture of 1 percent NH_4OH and acetone (2 : 1) rather than the 1 : 2 mixture recommended earlier, since the latter mixture also elutes green (red fluorescing) chlorophyll derivatives. Plasma values of 14 to 25 μg per 100 ml were found in calves 1 to 3.

Subjects	Protoporphyrin		Urobilinogen
	RBC (μg/100 ml)	Feces (μg/g)	Feces (mg/100 g)
Protoporphyrics*			
Calves 1 to 3	35,000–46,000	1460–2650	39–51
Calf 4	35,800	998	1.6
Cow 5	42,000	21	0.5
Carriers 6 to10	170–310	2.3–3.2	0.2–1.0
Normals			
Calves 11 to 13	100–133	1.2–2.4	0.8–0.8
Adults 14 to 16	158–190	3.3–4.2	1.8–3.9

*Calves 1 to 3 were removed from pasture 2 days before obtaining these samples, while calf 4 (with less fecal pigments) was taken from pasture and sheltered from sunlight 1 month earlier. The average fecal coproporphyrin concentrations in calves 1 to 3 fell from 9 to 0.8 μg per gram while being sheltered from sunlight for about 4 months. An average of 0.5 μg per gram was found in feces of carrier and normal animals. Concentrations of urinary uro- and coproporphyrin were all normal or slightly elevated to less than 25 μg per 100 ml.

same bull, a member of an exotic European beef breed. Their clinically normal dams were sired by two related bulls mated to normal Hereford cows. All three bulls were clinically normal. The pattern of recessive inheritance distinguishes this protoporphyria from human protoporphyria which is transmitted as an autosomal dominant trait with variable penetrance. In addition, the human disease affects males and females equally, while the bovine disease has been seen, to date, only in seven females. (In two of these seven, a retrospective diagnosis was based on a history of severe photosensitivity in genetically related calves killed shortly after birth.) Photosensitivity in the bovine disease may also be a distinguishing feature since it appears during the first few days of life. The only known adult protoporphyric cow has almost no signs of photosensitivity now.

Heparinized blood samples from the photosensitive calves had extraordinarily intense red fluorescence on exposure to near-ultraviolet light. Initial chemical findings in blood and feces of the porphyric calves, their carrier dams, and six control animals are summarized in Table 1. (The three carrier bulls were not available for chemical studies.) Concentrations of 35,000 to 46,000 μg of protoporphyrin per 100 ml of cells in the porphyric animals were at least seven times higher than any we have observed in humans with protoporphyria. Fecal urobilinogen excretion was also increased markedly and proportionately more than in human EPP. This increased urobilinogen was, presumably, formed from both hemoglobin and nonhemoglobin hemes by two mechanisms: (i) increased red cell destruction due to photohemolysis, and (ii) liberation of protoporphyrin from young red cells, with subsequent conversion to hemes rapidly turning over in liver and other tissues. Such rapid conversion of injected [^{14}C]protoporphyrin to ^{14}C-labeled hepatic heme and bile bilirubin has been reported in bile fistula dogs (*7*), and in an EPP patient in whom isotopic studies indicated that more than half of the excess protoporphyrin formed was thus metabolized and excreted as urobilinogen within a few days (*8*).

Cow number 5, now 3 years of age, has lost her earlier severe photosensitivity. Although still on pasture and despite a very high concentration of erythrocyte protoporphyrin, she had only minimal skin lesions when seen in July 1976. Fecal porphyrin and urobilinogen values were essentially normal.

Table 2. Mean activity of ferrochelatase (expressed as nanomoles of mesoporphyrin utilized per gram per hour) in tissues of normal, carrier, and protoporphyric bovine animals. Ferrochelatase activity was determined by fluorimetric assay of residual mesoporphyrin in homogenates incubated for 15 to 40 minutes in a nitrogen atmosphere (*10, 15*). The number of animals included per assay is in parentheses. Tissues, including fresh samples obtained from two normal calves at an abattoir, were kept at about −60°C until assayed, several days to a few weeks later. Only liver assays were done in carrier cows. Ferrochelatase activity was negligible in marrow of porphyric as compared to normal animals. Because of the variable cellular contents, however, we are not prepared to cite marrow values in precise quantitative terms.

Tissue	Ferrochelatase activity		
	Normals	Carriers	Protoporphyrics
Liver	2807 (4)	1204 (4)	237 (4)
Kidney	2703 (3)		198 (2)
Heart	2587 (2)		132 (1)
Spleen	1760 (3)		357 (2)
Lung	523 (2)		58 (1)

Spectral analysis revealed that erythrocyte and plasma protoporphyrin of EPP calves was essentially all in the free form, as it is in human EPP, while about half was complexed with zinc in the red cells of control and carrier animals (*9*).

Red cell morphology in the protoporphyric animals was essentially normal, as were the cell indices. There was no indication of iron deficiency, with an average mean corpuscular hemoglobin concentration (four assays per animal) of 35.2 g per 100 ml of cells.

While the animals were kept in a dimly lit portion of a barn for months, the skin lesions healed but hair failed to grow back. Red cell porphyrin concentrations remained essentially unchanged, but average fecal protoporphyrin concentrations in calves 1 to 3 fell precipitously from 1850 to only 59 μg/g. Concentration of fecal urobilinogen also decreased during this period, from an initial average of 45 to 1.8 mg per 100 g.

Repeated biopsies were performed in relation to studies that tested the effect of phlebotomy or blood transfusions (or both). Calf 2 died at the time of her third surgery. Activity of ferrochelatase (*10*) was determined in several tissues of this animal and of other porphyric and normal cows. As seen in Table 2, activity of this enzyme was decreased to about 5 to 20 percent of normal in all tissues assayed from porphyric animals.

Ferrochelatase activity was comparable in liver, kidney, and heart of normal calves; except for one liver value of 3551, all individual values for these tissues fell in the range 2365 to 2788 nmole of mesoporphyrin utilized per gram per hour. The liver values for the four carriers were each about half these normal values, ranging from 1087 to 1320 nmole of mesoporphyrin utilized per gram per hour. From the markedly reduced values (averaging 58 to 357) found in all tissues studied from protoporphyric calves, the defect in ferrochelatase activity appears to be a total body one. Still uncertain is the nature of this defect, and its possible chemical manifestations (formation and accumulation of excess protoporphyrin or deficiency of hemoproteins, or both) in tissues other than erythropoietic ones.

Studies of δ-aminolevulinate synthase (ALA-S), aminolevulinate dehydratase (ALA-D), and of some hemoproteins in tissues of these animals have been made (*11*). Activity of ALA-S was increased slightly (about threefold) only in the liver of the protoporphyric calves. Activities of ALA-D were essentially normal.

Decreased activities of ferrochelatase have been reported in marrow, in cul-

tured skin fibroblasts, and in liver of some humans with protoporphyria (*12*). Cultured fibroblasts, however, had no porphyrin accumulation unless challenged by added ALA. Results with liver have been inconstant, and even where low values were found, nonspecific decreases due to tissue damage associated with chronic retention of protoporphyrin could not be ruled out. Even in marrow, all evidence points to a nonuniform manifestation of the defect, with red fluorescence being observed primarily in older normoblasts and reticulocytes. The usual finding of normal mean corpuscular hemoglobin concentrations also indicates normal hemoglobin synthesis through most of the normoblast's lifetime.

Earlier isotope studies in vitro and in vivo (*8*) demonstrated increased leakage of labeled protoporphyrin (^3H from ALA and ^{14}C from glycine) from circulating erythrocytes of a protoporphyria patient. Three protoporphyrin compartments within these cells were illustrated, with calculated protoporphyrin half-life ($T_{1/2}$) values of about 1.5 hours, 1 day, and 12.4 days, respectively. More than 95 percent of the total excess protoporphyrin synthesized was, apparently, included in the first two fractions. This leakage and the above-noted marrow findings led to the suggestion that all manifestations of this disease are consistent only with the concept that ferrochelatase activity (even if decreased in younger cells) becomes rate-limiting largely during cell aging and loss of mitochondria in late-stage normoblasts and reticulocytes. In these late-stage cells, activity of both mitochondrial enzymes, ferrochelatase, and ALA-S normally decreases rapidly. In EPP, the activity of ferrochelatase must decrease relatively faster. Persistence of high ALA-S activity (*8*) would also increase the potential upper limit of excess protoporphyrin synthesis.

Lamola and colleagues have confirmed the excessive leakage of porphyrin from red cells of EPP patients (*13*). They also reported the existence of protoporphyrin as a zinc complex in blood of subjects with lead poisoning or iron deficiency anemia, both of which are characterized by elevated red cell protoporphyrin concentrations. Only free protoporphyrin was found in EPP red cells. To explain the presence of photosensitivity and of elevated fecal protoporphyrin only in EPP patients, they proposed that the zinc complex is bound tightly to available heme binding sites of globin, while the free protoporphyrin, as in EPP, is bound more loosely to other sites on globin. This loose binding, they suggest, results in leakage, photosensitivity, and elevated fecal values.

Unpublished studies with Stadlan *et al.* (*14*) have shown that the above distinction between free and zinc protoporphyrin cannot be the critical feature. In a patient with sideroblastic anemia the red cell protoporphyrin concentration was above 3500 μg per 100 ml of cells, "all" of which was free, as in EPP. Yet, he had neither photosensitivity nor elevated fecal protoporphyrin values.

The essential common denominator, as proposed earlier, appears still to be the age of the immature red cell at which the defect occurs. If (as in lead poisoning, iron deficiency, or sideroblastic anemia) it exists while major hemoglobin synthesis is in progress, we expect (and find) hypochromasia, tight binding of excess protoporphyrin (zinc or free) to globin with its many unoccupied heme binding sites, insignificant leakage of porphyrin, lack of photosensitivity, and normal fecal protoporphyrin. If the defect is essentially limited to late normoblasts and reticulocytes where protein synthesis is also minimal, we expect (and find) no hypochromasia. Since most heme binding sites are already occupied, the excess porphyrin synthesized binds either to other sites or not at all. Photosensitivity and high fecal values follow. This formulation of absent, loose, or tight protein binding appears to be consistent, too, with the three $T_{1/2}$ values of red cell protoporphyrin compartments noted earlier in our EPP patient.

Data are not available on relative (or absolute) ferrochelatase and ALA-S activities and porphyrin content of young as compared to old normoblasts, reticulocytes, and mature cells in human or bovine protoporphyria. If the necessary cell fractionation were possible, such studies should clarify the defect and manifestations of this and related diseases. (Loss of photosensitivity in the protoporphyric cow could be due to a decrease in the relative involvement of late-stage normoblasts and reticulocytes.)

The observed "total body" defect in ferrochelatase activity in bovine protoporphyria adds to the growing evidence that different porphyrias classified as "erythropoietic" or "hepatic" include an enzyme defect common to many tissues. Implications for hemoprotein biosynthesis and metabolism in tissues of protoporphyric animals are being investigated, as are the properties of the residual ferrochelatase. The 50 percent decrease of ferrochelatase activity in liver of our carrier animals is apparently not manifest. Other illustrative examples of factors involved in the expression of reduced enzyme activity have been cited (*10, 15*) in relation to development of experimental renal porphyria in some species but not in others, even though renal ferrochelatase activity was decreased to 10 to 20 percent of normal in all.

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1. Red-brown teeth and urine are pathognomonic of congenital porphyria (EP). Photosensitivity in human protoporphyria (EPP) is more painful, while EP has more mutilating skin lesions. In protoporphyria, increased protoporphyrin is found in red cells and feces; EP subjects have major increases of uroporphyrin and coproporphyrin in normoblasts, blood, urine, and feces. Erythrocyte protoporphyrin is increased moderately in bovine, but not in human, EP. Human EP is rare; EPP may be the most common type of porphyria in humans in this country.
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