

Immunochemical Studies of Ferrochelatase Protein: Characterization of the Normal and Mutant Protein in Bovine and Human Protoporphyrin

James G. Straka, Hazel D. Hill, Joan M. Krikava, Ann M. Kools, and Joseph R. Bloomer

Department of Medicine, Division of Gastroenterology, University of Minnesota, Minneapolis

Summary

Protoporphyrin is a hereditary disorder characterized by a marked decrease in the activity of ferrochelatase, the terminal enzyme in the heme biosynthetic pathway. We have prepared specific polyvalent antibodies against bovine ferrochelatase in rabbits. The specificity of the antibody preparation against ferrochelatase was demonstrated by western blot analysis and immunoprecipitation of ferrochelatase activity. The antibody also cross-reacted weakly with ferrochelatase from human mitochondria. To quantify immunoreactive ferrochelatase in tissue samples, a kinetic-based enzyme-linked immunosorbent assay (k-ELISA) was developed. Ferrochelatase activity and the level of immunoreactive protein were measured in hepatic mitochondria isolated from six normal and nine protoporphyric (homozygous) cattle. Ferrochelatase activity was less than 10% of normal in mitochondria from protoporphyric animals; the amount of immunoreactive material was equivalent to that from normal animals. Similar studies were performed with samples from three normal and two protoporphyric (heterozygous) humans. Ferrochelatase activity was decreased in protoporphyric samples (about 17% of normal, but there was no concomitant decrease in immunoreactive material). These data demonstrate that a normal amount of ferrochelatase protein is present and suggest that bovine and human protoporphyria result from point mutations in the gene encoding ferrochelatase.

Introduction

Ferrochelatase (protoheme ferredoxin lyase; E.C. 4.99.1.1) is the final enzyme of the heme biosynthetic pathway and catalyzes the insertion of ferrous iron into protoporphyrin to form heme. It is an integral component of the inner mitochondrial membrane, the active site facing the matrix compartment (Jones and Jones 1969; Harbin and Dailey 1985). The enzyme can be brought into solution by treating a suspension of mitochondrial membranes with detergents such as Tween 20 or sodium cholate (Porra et al. 1967; Taketani and Tokunaga 1982).

Protoporphyrin is an inborn error of metabolism of humans and cattle which is associated with decreased

ferrochelatase activity in tissues of affected individuals (Bonkowsky et al. 1975; Ruth et al. 1977; Bloomer et al. 1982). Protoporphyrin is inherited as an autosomal dominant trait in humans and as an autosomal recessive trait in cattle (Bloomer et al. 1982). The disease leads to excessive accumulation and excretion of protoporphyrin and is characterized clinically by photosensitivity of exposed areas of the skin. Liver damage has occurred in a portion of humans with protoporphyria, in some cases leading to liver failure and death (Bloomer and Straka 1988). Liver injury has been associated with the presence of protoporphyrin deposits in hepatocytes, Kupffer cells, and biliary structures, and crystalline protoporphyrin has been isolated from the livers of patients at the time of transplant (Morton et al. 1988).

Ferrochelatase has been purified from hepatic mitochondria of normal and protoporphyric cattle (Bloomer et al. 1987). The molecular weight of both the normal and mutant proteins is approximately 40,000, determined by either SDS-PAGE or gel-filtration chromatography. Michaelis constants for iron and porphyrin sub-

Received May 17, 1990; revision received August 13, 1990.

Address for correspondence and reprints: James G. Straka, Ph.D., Dight Laboratories, 400 Church Street, S.E., Minneapolis, MN 55455.
© 1990 by The American Society of Human Genetics. All rights reserved.
0002-9297/90/4801-0010\$02.00

strates are approximately the same for enzymes derived from either source. To date, the single observed difference between the two proteins is that the V_{max} of the protoporphyrin enzyme is about 10% that of the normal enzyme, whether measured in crude mitochondrial extracts or in purified enzyme.

The present study was undertaken to immunochemically characterize and quantify ferrochelatase protein in tissue extracts from normal and protoporphyrin animals and humans. A kinetic-based single-tube enzyme-linked immunosorbent assay (k-ELISA) (Tsang et al. 1983) was adapted for this purpose.

Material and Methods

Material

Acrylamide N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethyl-1,2-ethylenediamine, low-molecular-weight standards, Coomassie brilliant blue R-250 and G-250, gelatin, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP color development kit, and nitrocellulose (0.45- μ m) sheets were purchased from BioRad Laboratories (Richmond, CA). Bicinchoninic acid (BCA) protein-determination reagents were from Pierce Chemical (Rockford, IL). Tris base, 2-mercaptoethanol, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), purified HRP (type II), crystalline BSA, Tween 20, sodium cholate (ox bile), and Freund's complete and incomplete adjuvant were from Sigma Chemical (St. Louis). Reactive blue and reactive red Sepharose were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Formalin-fixed protein A bearing *Staphylococcus aureus* cells, Cowan I strain (staph-A cells; Pansorbin A™), were purchased from Calbiochem-Behring (La Jolla, CA). Deuteroporphyrin dihydrochloride was from Porphyrin Products (Logan, UT). Disposable 1-ml polystyrene cuvettes (maximum volume 2.9 ml; Ultra-vu™, catalog 14-385-991) were purchased from Fisher Scientific (Pittsburgh). All other materials and chemicals were of the highest grade available.

Bovine liver was obtained at a local abattoir. Samples of normal human liver were obtained shortly after death, either at autopsy or from organ donors which could not be used for transplant. Samples of two human protoporphyrin livers were obtained at transplant (Morton et al. 1988). Mitochondria were prepared from freshly obtained tissue by differential centrifugation according to a method described elsewhere (Bloomer et al. 1987).

Methods

Ferrochelatase.—Ferrochelatase was assayed spectrophotometrically using deuteroporphyrin (125 μ M) and Fe(II) citrate (125 μ M) in the presence of 1% (w/v) Tween 20 or 1% (w/v) sodium cholate according to a method described elsewhere (Dailey and Fleming 1983; Bloomer et al. 1987). Mitochondrial suspensions were generally extracted with 1% sodium cholate prior to assay. Crude mitochondrial protein was added to a final concentration of 0.35–0.4 mg/ml.

Ferrochelatase was purified from bovine liver mitochondria according to a method described elsewhere (Taketani and Tokunaga 1982; Dailey and Fleming 1983; Bloomer et al. 1987). Ferrochelatase from the final step of purification (chromatography on reactive blue Sepharose; Taketani and Tokunaga 1982) was judged to be pure by its appearance as a single band ($M_r = 40,000$) on SDS-polyacrylamide gel electropherograms (Laemmli 1970).

Immunization.—New Zealand white rabbits obtained from a local breeder were immunized with purified native and denatured ferrochelatase. Ferrochelatase eluted from reactive blue Sepharose in Freund's adjuvant was used as immunogen (Harboe and Ingild 1973; Hurn and Chantler 1980). The resulting antiserum was denoted "anti-native ferrochelatase antiserum."

In addition, enzyme preparations were subjected to preparative SDS-PAGE (Laemmli 1970). The gels were stained, and the band corresponding to $M_r = 40,000$ was excised, minced finely, and homogenized in 0.05 M Tris HCl, pH 7.4. The resulting homogenate, without removal of the polyacrylamide, was used for immunization as described above. This antiserum was denoted "anti-denatured ferrochelatase antiserum."

SDS-PAGE.—Proteins were subjected to SDS-PAGE using the discontinuous system of Laemmli (1970). The resolving gel was 10% T, 2.74% C. The gels were stained with Coomassie brilliant blue R-250 (0.07% in methanol/acetic acid/water: 50/10/40 [v/v/v]) and were destained with the same solvent without the dye, followed by 7% acetic acid.

Immunoblotting (western blot).—Proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose sheets according to a method described by Towbin et al. (1979) and modified by Burnette (1981). Molecular-weight standards (low range) were run on either side of the gel. The lanes containing standards were cut away and stained using 0.07% Coomassie brilliant blue G-250 in 0.4% (w/v) HClO₄, destained in methanol/acetic acid/water (50/10/40 [v/v/v]), rinsed with water, and dried. Alternatively, the strips were

stained overnight with India ink (Hancock and Tsang 1983), washed with water, and dried.

Immunostaining.—Nitrocellulose sheets to which antigen was directly blotted or electrophoretically transferred were immunostained essentially as described by Towbin et al. (1989), by using 3% gelatin as blocking agent and HRP-conjugated goat anti-rabbit IgG as second antibody. The blots were developed in a freshly prepared solution of 4-chloro-1-naphthol (0.5 mg/ml) and 0.015% (w/v) H₂O₂ in Tris-buffered salt (TBS [0.5 M NaCl in 0.02 M Tris HCl, pH 7.5]) containing 17% (v/v) methanol.

Immunoprecipitation.—Ferrochelatase was immunoprecipitated and analyzed with the aid of staph-A cells according to a method described elsewhere (Bloomer et al. 1987).

k-ELISA assay, standard method.—The standard method for the k-ELISA assay was modified from that described by Tsang et al. (1983). In brief, 1-ml plastic cuvettes were prewashed with three portions of methanol. Protein (0–1.0 µg) diluted into a total volume of 0.50 ml of 0.05 M Tris HCl containing 0.3 M KCl and 2 mM EDTA, pH 8.0, was incubated in the cuvette with shaking for 1 h at 37°C. The cuvettes were rinsed with three portions of PBS-Tween (PBS [0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2] containing 0.3% [w/v] Tween 20), incubated with anti-native ferrochelatase antiserum in 1% gelatin in PBS-Tween for 1 h, rinsed, and incubated with 0.50 ml HRP-conjugated goat anti-rabbit IgG diluted 1:2,000 in PBS-Tween for 1 h. The cuvettes were rinsed as described above. HRP activity determination was initiated with the addition of 1.0 ml of 0.05 M citrate and 0.05 M sodium phosphate buffer, pH 5.0, containing 1.0 mM H₂O₂ and 2.0 mM ABTS. The contents of the cuvettes were mixed continuously. The reaction was monitored by measuring the change, with time, in absorbance at 412 nm. The rate of the HRP reaction obtained was expressed in units of change in absorbance per minute per microgram antigen initially incubated in each cuvette. Because of slight daily fluctuations (approximately ± 5%) in the values obtained for a given sample of antigen, a sample of normal bovine mitochondrial extract was run as a standard with each series of determinations, and its activity was assigned the relative value of 1 k-ELISA unit/mg protein.

Succinic dehydrogenase.—Succinic dehydrogenase was determined spectrophotometrically by the succinate-dependent reduction of ferricytochrome c in the presence of KCN (Sottocasa et al. 1967).

Lipid analysis.—Phospholipid fatty acyl composition

of isolated mitochondrial membranes was analyzed according to a method described by Kools et al. (1989). Membrane phospholipid fatty-acid profiles measured 48 fatty acids and evaluated 28 derived parameters, including fatty-acid-class totals, ratios, and metabolites. Values are expressed as percentage of total fatty acids.

Protein determination.—Protein was determined using the BCA method described by Smith et al. (1985), as modified by Hill and Straka (1988). Crystalline BSA was used as standard.

Results

Characterization of Anti-Ferrochelatase Antibody Preparations

Cross-reactivity of antibody and western blot analysis.—Anti-native ferrochelatase antisera were found to react with both native and denatured protein spotted onto nitrocellulose. Anti-denatured ferrochelatase antisera were found to react only with denatured samples of protein. Therefore, only anti-native ferrochelatase was used for the present study.

Specificities of antisera were tested by western blot analysis of crude sodium cholate extracts of bovine mitochondria. A blot showing a cleanly reacting sample of antiserum is shown in figure 1. Only anti-native ferrochelatase antisera reacting predominantly with a single protein band corresponding to $M_r = 40,000$ were selected for further study. No antisera which reacted strongly with other proteins in the blot were used.

Cross-reactivities of the antisera against extracts of human mitochondria were tested as above. Both anti-native ferrochelatase and anti-denatured ferrochelatase antisera were active against the human samples, although the reactivity was much weaker. Western blots showed reactivity toward a single protein band of about 42,000 daltons.

Immunoprecipitation.—Specificity of the antisera against ferrochelatase was further tested by immunoprecipitating enzyme activity from solution. Staph-A cell suspensions preincubated with antiserum and incubated with crude mitochondrial extracts precipitated ferrochelatase activity (2.5–6.8 nmol heme/h/ml suspension). The supernatant liquor from these suspensions showed a corresponding loss of activity. Western blot analysis of these cell suspensions confirmed the presence of a unique 40,000-dalton protein associated with the cells. Neither ferrochelatase activity nor the 40,000-dalton protein was associated with staph-A cells incubated with either antibody or enzyme alone.

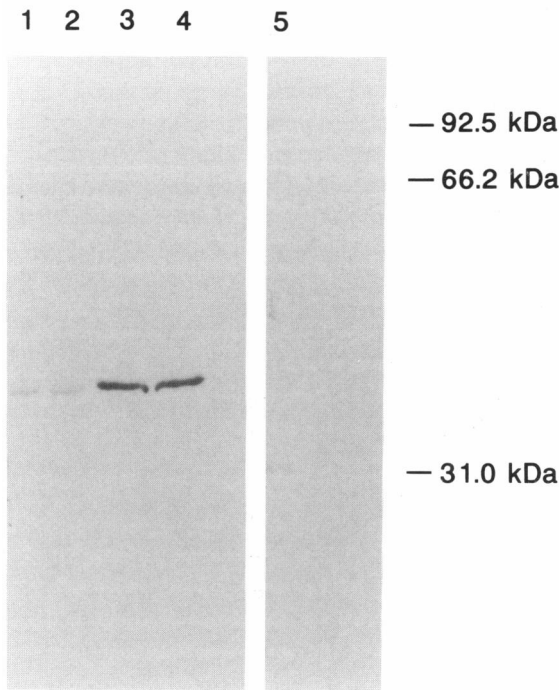


Figure 1 Western blot of crude mitochondrial extracts (lanes 1 and 2; ~30 µg protein/lane) and purified ferrochelatase (lanes 3 and 4; ~3 µg protein/lane) from normal bovine liver. First antibody was a 1:100 dilution of anti-native ferrochelatase (see text). Lane 5 contains low-molecular-weight standards stained with Coomassie brilliant blue as described in Methods.

Characterization of k-ELISA

Quantification of antigen by the k-ELISA depends both on the linearity of the HRP reaction and on the linearity of response with the amount of antigen. Preliminary experiments using purified HRP demonstrated that HRP activity was diminished at H₂O₂ concentrations greater than 5 mM, in keeping with previous observations (Kay et al. 1967). The chromophoric substrate ABTS was chosen because the colored product

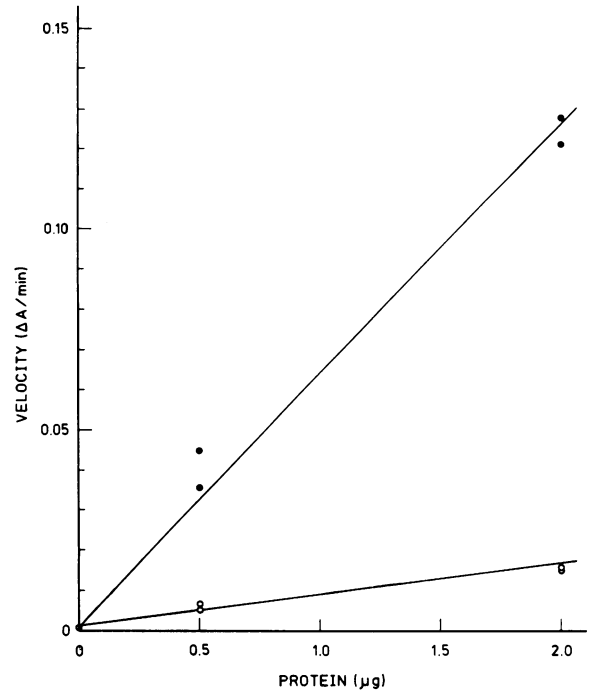


Figure 2 Comparison of normal bovine (black dots) and normal human (open circles) mitochondrial extract in k-ELISA assay system, as function of total protein incubated in cuvette.

is stable and soluble (Engvall 1980). With 1 mM H₂O₂ and 2 mM ABTS, HRP activity was linear when purified enzyme was 0–0.15 µg/ml.

The complete k-ELISA system was tested for linearity with antigen by using cholate extracts of bovine mitochondria obtained from several normal animals. The response was linear when antigen concentration was 0–3 µg/ml. When antigen concentration was above this amount, the rate of reaction leveled off, presumably reflecting saturation of protein-binding sites on the surface of the cuvette.

When tested with extracts of normal human hepatic

Table I

Mean ± SD Levels of Ferrochelatase, Immunoreactive Protein, and Succinic Dehydrogenase in Extracts of Bovine Hepatic Mitochondria

Measurement	Normal	Protoporphyrin	P ^a
Ferrochelatase (nmol heme/h/mg).....	38.8 ± 14.4 (N = 6)	2.18 ± .11 (N = 9)	<<.001
k-ELISA (relative units).....	.97 ± .25 (N = 6)	.99 ± .26 (N = 9)	>.9
Succinic dehydrogenase (nmol cytochrome c/min/mg).....	108 ± 39 (N = 4)	123 ± 7.9 (N = 4)	>.7

^a By Student *t*-statistic.

Table 2
Mean ± SD Levels Phospholipid Fatty Acyl Content of Bovine Hepatic Mitochondrial Membranes from Normal and Protoporphyrin Cattle

ANIMAL GROUP	FATTY ACID										
	16:0	18:0	18:1	18:2ω6	20:2ω6	20:4ω6	20:4ω6	20:5ω3	22:4ω6	22:5ω3	22:6ω3
Protoporphyrin	16.4 ± 7.3	25.3 ± 10	13.7 ± 4.1	13.6 ± 5.0	.21 ± .05	9.8 ± 3.6	.89 ± .29	1.14 ± 1.1	3.47 ± 2.5	2.4 ± 1.7	
Normal.....	12.3 ± 1.3	24.8 ± 2.9	13.0 ± 2.5	11.9 ± 2.0	.18 ± .04	12.8 ± 2.6	2.93 ± 1.58	1.11 ± .86	3.45 ± 2.2	1.0 ± .5	
P ^a175	.9	.7	.4	.2	.01	.003	1.0	1.0	.07	

NOTE.—All data are presented as mole-percent of total fatty acids for six animals in each group.

^a By Student *t*-statistic.

mitochondria, the k-ELISA rates were found to be about 10% of those seen when bovine mitochondria were used (fig. 2). Nevertheless, up to the point where protein binding to the cuvette was saturated, the response remained linear with antigen concentration.

A certain amount of day-to-day variation (about 5%) was observed in the rates for any given sample of antigen. However, repeated assays of antigen from several normal sources demonstrated that their relative k-ELISA rates were constant. Therefore, a single sample of antigen was selected which served as a standard against which all other samples were measured. For this reason, results from k-ELISA determinations are given in relative “k-ELISA units.”

Comparison of Normal and Protoporphyrin Mitochondria

Crude cholate extracts of bovine hepatic mitochondria obtained from six normal and nine protoporphyric animals were compared in the k-ELISA. Ferrochelatase activity was determined in the identical samples. Results are shown in table 1. Mean ferrochelatase activity in the samples from protoporphyric animals was 6% of that in samples from normal animals. However, no significant difference in the amount of immunoreactive protein was seen.

Succinic dehydrogenase and the fatty acyl composition of the phospholipid were measured in portions of the mitochondrial preparations to confirm that the difference in ferrochelatase activity was not due to a generalized defect in mitochondrial structure or function. As shown in table 1, no difference in succinic dehydrogenase was seen between normal and protoporphyric mitochondria. Membrane phospholipid fatty-acid profiles were measured in the same mitochondrial preparations (table 2). No significant differences were seen in total saturated, mono- or polyunsaturated (i.e., ω3, ω6, and ω9, respectively) fatty acids. Small but statistically significant differences between normal and protoporphyric mitochondrial membranes were seen in the relative levels of only three fatty acids: 20:4ω6 (arachidonic), 22:4ω6, and 22:6ω3 acids. The minor differences in fatty acyl content cannot account for the large differences seen in ferrochelatase activity (Kools et al. 1989).

Samples of human hepatic mitochondria were similarly compared, and the results summarized in table 3. As with the bovine samples, ferrochelatase activity was significantly decreased in the protoporphyria samples, yet there was no apparent difference in the amount of immunoreactive protein (table 2). Although succinic dehydrogenase activity was slightly different between

Table 3**Ferrochelatase, Immunoreactive Protein, and Succinic Dehydrogenase in Extracts of Human Hepatic Mitochondria**

Subject	Ferrochelatase ^a (nmol/h/mg)	k-ELISA ^b (Relative units)	Succinic Dehydrogenase ^a (nmol cytochrome c/min/mg)
Normal:			
1	65.1	.073	17.7
2	48.5	ND	14.9
3	65.9	.162	18.2
Mean	59.8	.118	17.1
Protoporphyria:			
1	13.7	.074	12.8
2	6.2	.103	11.4
Mean	9.9	.089	12.2

^a Each value represents the mean of at least two determinations.^b Each value represents the mean of four determinations. ND = not done.

normal and protoporphyria samples, the decrease was small compared with the decrease in ferrochelatase activity. None of the fatty-acid analyses showed any differences between normal and protoporphyric mitochondrial membranes.

Discussion

The present study reports the production and characterization of antibodies against the last enzyme of heme biosynthesis, ferrochelatase. Antibodies raised against SDS-denatured protein recognized only denatured protein, while those raised against native enzyme recognized both native and denatured forms. Furthermore, none of the antibody preparations either precipitated or inhibited the activity of the enzyme. Antibodies raised against bovine enzyme cross-reacted with ferrochelatase in extracts of human mitochondria. Antisera produced were specific for ferrochelatase protein and were used as the basis for a quantitative measurement of ferrochelatase protein in crude extracts of bovine and human hepatic mitochondria.

The k-ELISA described by Tsang et al. (1983) has been adapted for the present study. Conditions were defined which guaranteed that the determination was linear with HRP, linear and quantitative for immunoreactive protein, and specific for ferrochelatase. Because the k-ELISA is based on a kinetic determination, samples containing antigenic protein of either low concentration or decreased reactivity, as in the human samples, were easily measured by lengthening the time of reaction.

Bovine protoporphyria is inherited as a recessive trait, and therefore all ferrochelatase protein in affected animals will show the effects of the mutation. Comparison of ferrochelatase activity and ferrochelatase antigenic protein in samples of bovine hepatic mitochondria demonstrated that only ferrochelatase activity was decreased in the protoporphyria samples; in the amount of immunoreactive proteins, no differences were observed between normal and protoporphyria samples. Mutations of this type, where cross-reacting immunological material (CRIM) is normal despite a marked decrease in the activity of the affected protein, are referred to as CRIM-positive. Measurement of succinic dehydrogenase and fatty-acid profiles demonstrated that the differences in ferrochelatase activity were not due to a generalized defect in mitochondrial structure or function.

These results indicate that in bovine protoporphyria there is a normal amount of immunoreactive but catalytically nonfunctional protein. This, in conjunction with the kinetic behavior of the protoporphyric enzyme (Bloemer et al. 1987), is most consistent with a point mutation in the structural gene for ferrochelatase. All cattle with protoporphyria are direct descendants of a single bull (Ruth et al. 1977). Thus, animals with protoporphyria are expected to be genetically homogeneous for the mutation responsible for expression of the disease. Further work is required to fully define the nature of the enzyme defect in bovine protoporphyria.

Unlike the bovine disease, human protoporphyria is a dominant trait and is expected to show genetic heterogeneity. A decrease (about 30%) in succinic de-

hydrogenase activity was seen in liver tissue from the patients with protoporphyria, and liver disease had advanced to a point requiring liver transplantation in both. It is therefore likely that both the decrease in succinic dehydrogenase and the very low level of ferrochelatase found in these tissue samples were partially due to nonspecific effects of liver disease. Nevertheless, the amount of immunoreactive ferrochelatase protein was normal in the two cases studied, leading to the conclusion that at least some cases of human protoporphyria arise from CRIM-positive mutations.

Acknowledgments

These studies were supported by research grant DK-26466 and center grant DK-34931 from the National Institutes of Health and by a student grant to J.M.K. from the Minnesota Medical Research Foundation.

References

- Bloomer JR, Hill HD, Morton KO, Anderson-Burnham LA, Straka JG (1987) The enzyme defect in bovine protoporphyria: studies with purified ferrochelatase. *J Biol Chem* 262:667-671
- Bloomer JR, Morton KO, Reuter RJ, Ruth GR (1982) Bovine protoporphyria: documentation of autosomal recessive inheritance and comparison with the human disease through measurement of heme synthase activity. *Am J Hum Genet* 34:322-330
- Bloomer JR, Straka JG (1988) Porphyrin metabolism. In: Arias IM, Jakoby WB, Popper H, Schachter D, Shafritz DA (eds) *The liver: biology and pathobiology*, 2d ed. Raven, New York, 451-466
- Bonkowsky HL, Bloomer JR, Ebert PS, Mahoney MJ (1975) Heme synthetase deficiency in human protoporphyria: demonstration of the defect in liver and cultured skin fibroblasts. *J Clin Invest* 56:1139-1148
- Burnette, WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112:195-203
- Dailey HA, Fleming JE (1983) Bovine ferrochelatase: kinetic analysis of inhibition by N-methylprotoporphyrin, manganese and heme. *J Biol Chem* 258:11453-11459
- Engvall, E (1980) Enzyme immunoassay: ELISA and EMIT. *Methods Enzymol* 70:419-439
- Hancock K, Tsang VCW (1983) India ink staining of proteins on nitrocellulose paper. *Anal Biochem* 133:157-162
- Harbin BM, Dailey HA (1985) Orientation of ferrochelatase in bovine liver mitochondria. *Biochemistry* 24:366-370
- Harboe N, Ingild A (1973) Immunization, isolation of immunoglobulins, estimation of antibody titre. *Scand J Immunol* 2 [Suppl 1]: 161-164
- Hill HD, Straka JG (1988) Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. *Anal Biochem* 170:203-208
- Hurn BAL, Chantler SM (1980) Production of reagent antibodies. *Methods in Enzymol* 70:104-142
- Jones MS, Jones OTG (1969) The structural organization of haem synthesis in rat liver mitochondria. *Biochem J* 113: 507-514
- Kay E, Shannon LM, Lew JY (1967) Peroxidase isozymes from horseradish roots. II. Catalytic properties. *J Biol Chem* 242:2470-2473
- Kools AM, Straka JG, Hill HD, Whitmer DI, Holman RT, Bloomer JR (1989) Modulation of hepatic ferrochelatase activity by dietary manipulation of mitochondrial phospholipid fatty acyl groups. *Hepatology* 9:557-561
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685
- Morton KO, Schneider F, Weimer MK, Straka JG, Bloomer JR (1988) Hepatic and bile porphyrins in patients with protoporphyria and liver failure. *Gastroenterology* 94:1488-1492
- Porra RJ, Vitols KS, Labbe RF, Newton NA (1967) Studies on ferrochelatase: the effects of thiols and other factors on the determination of activity. *Biochem J* 104:321-327
- Ruth GR, Schwartz S, Stephenson B (1977) Bovine protoporphyria: the first non-human model of this hereditary photosensitizing disease. *Science* 198:199-201
- Smith PK, Krohn RI, Hermanson GT, Mallis AK, Gartner FH, Provenzano MD, Fujimoto EK, et al (1985) Measurement of protein using bicinchoninic acid. *Anal Biochem* 150:76-85
- Sottocasa GL, Kuylenstierna B, Ernster L, Bergstrand A (1967) An electron transport system associated with the outer membrane of liver mitochondria: a biochemical and morphological study. *J Cell Biol* 32:415-438
- Taketani S, Tokunaga R (1982) Purification and substrate specificity of bovine liver-ferrochelatase. *Eur J Biochem* 127:443-447
- Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350-4354
- Tsang VCW, Wilson BC, Peralta JM (1983) Quantitative, single-tube, kinetic-dependent enzyme-linked immunosorbent assay (k-ELISA). *Methods Enzymol* 92:391-403