Proceedings of the National Academy of Sciences
Vol. 68, No. 3, pp. 609–613, March 1971

Heme Binding and Transport—A Spectrophotometric Study of Plasma Glycoglobulin Hemochromogens

DAVID L. DRABKIN

Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pa. 19104

Communicated by Britton Chance, December 15, 1970

ABSTRACT **A hitherto unreported phenomenon is the immediate production of the spectrum of ferrohemochromogens (in the presence of sodium dithionite) upon the addition *in vitro* of hydroxyhemin (pH 7.6–7.8) to the plasmas or sera, as well as to certain Cohn plasma protein fractions, of all mammalian species thus far examined. This distinctive reaction is characteristic of a coordination complex with heme iron, and is ascribed to a remarkable affinity for heme of certain plasma glycoglobulins, which include hemopexin. Spectrophotometry has permitted estimations of the specific heme-binding capacity (as ferrohemochromogen) of the plasmas, the rate of removal from plasma of injected heme, and the production of bile pigments therefrom. The study leads to a new proposal of the cooperative roles of the glycoglobulin hemochromogens and hematin-albumin in heme transport and bile pigment production.**

The finding (1, 2) that heme complexes, identifiable spectroscopically as ferrohemochromogens* in their reduced state, are formed when hydroxyhemin (hematin hydroxide at pH 7.6–7.8) is added to mammalian blood sera *in vitro* and *in vivo* is relevant to the interpretation of the results of two kinds of earlier work in our laboratory: (*a*) in investigations of lethal stress states (3–6), the blood plasma yielded a hemochromogen-like spectrum, whose origin remained unexplained (1, 6) and (*b*) in studies of bile-pigment production (7) it was found that the heme of intravenously injected hemoglobin was efficiently converted to bile pigment in the biliary fistula rat, but not in isolated rat liver perfusion, although in such perfusion experiments hematin was actively degraded to bilirubin. This report has a bearing on heme transport and the early stages of hemoprotein degradation, both of which are currently in need of elucidation (8–10).

MATERIALS AND METHODS

Only freshly collected sera and freshly made heme solutions were employed. The pH of sera was kept below 7.8 and that of Cohn fraction IV-4 did not exceed pH 8.2.

Sera and plasma protein fractions

Sera (from fasted horse, cow, man, pig, dog, rabbit, and rat) were tested. As a result of careful separation, the hemoglobin content was only 0.004–0.007 mmol/liter (or 0.04–0.07% of the hemoglobin content of whole blood). At this concentration all of the hemoglobin present was probably in the form of hemoglobin–haptoglobin (11). In most cases the serum was used directly; in some it was found desirable to dilute the serum 1:1 with 0.2 M phosphate buffer, pH 7.6, prior to the addition of heme and reductant. Plasma protein fractions IV-1, IV-4, IV-7, and VI of most of the above species [prepared by the alcohol–low temperature technique (12–15) and obtained mainly from the Nutritional Biochemical Corp.] were also examined. Fractions IV-4 (12) and IV-7 (13–15) include the low molecular weight β_1 glycoglobulins, which have a high carbohydrate content, are soluble in 0.6 M perchloric acid (16, 17), and contain a heme-binding apoprotein, hemopexin (16–26). A purified sample of the latter (6.8 mg in 2 ml of 0.1 M phosphate buffer, pH 7.6), isolated from rabbit plasma by a modified perchloric acid method, served for comparative spectrophotometric identification. It was a gift from Dr. Ursula Müller-Eberhard (Scripps Clinic and Research Foundation). The sample was diluted to 5 ml; micro-biuret analysis (27), with crystalline human serum albumin as standard (28), was 1.27 mg/ml. For the interpretation of spectrophotometric findings, under conditions in which heme in excess of that bound as glycoglobulin hemochromogen has been added to serum, data were secured on methemalbumin [hematin–albumin (29)], prepared according to Rosenfeld and Surgenor (30) from crystalline human serum albumin. Ancillary data on protein content, with the exception of the hemopexin sample, were obtained by a standard biuret technique (31), and carbohydrate was estimated (as neutral hexose) by the method of Dubois *et al.* (32) after hydrolysis for 20 hr in 1 N HCl, with fetuin (Nutritional Biochemical Corp.), a low molecular weight glycoglobulin (33) that does not form a hemochromogen, as a secondary standard.

Preparation of chlorohemin, [^{14}C]chlorohemin, and hydroxyhemin

Crystalline chlorohemin was prepared by the method of Drabkin and Austin (34, 35). [^{14}C]chlorohemin was similarly prepared from dog [^{14}C]hemoglobin, labeled *in vivo* by means of [2-^{14}C]glycine. Fresh stock solutions of hydroxyhemin with a concentration of about 2 mmol/liter (referable to a molecular weight of 651.6 for chlorohemin) were made as follows. A weighed quantity of chlorohemin (usually about 6.5 mg) was dissolved in 1 ml of 0.1 N NaOH. The solution was partially neutralized by the careful addition of 0.1 N HCl, and diluted to 5 ml with 0.1 M phosphate buffer, pH 7.6, or, in some cases, with 0.2 M phosphate buffer. Before use the solu-

* In the past, the term hemochromogen (synonym, *hemochrome*) has been most frequently applied to artifactual products such as denatured globin hemochromogen, and examples of naturally occurring ferrohemochromogens have been confined to certain ferrocytochromes, as *c* and b_5. Since this report discloses a new class of apoproteins capable of hemochromogen formation, it was obligatory to use conditions that should exclude or minimize protein denaturation.

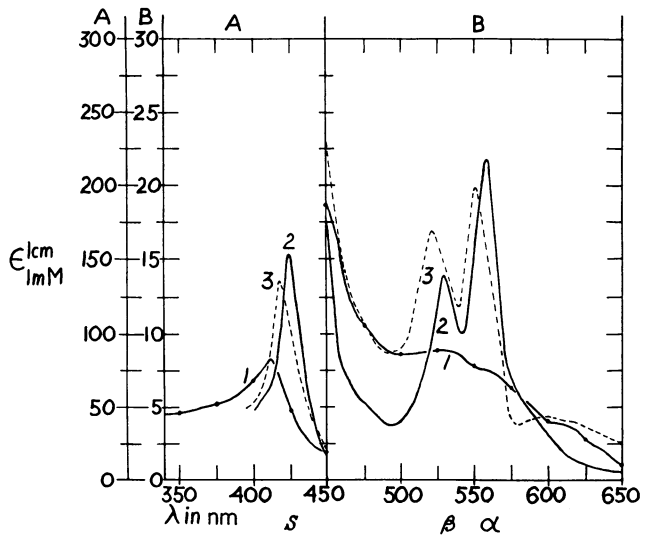

FIG. 1. Typical spectral absorption patterns for ferri- and ferrohydroxyhemin–glycoglobulin hemochromogens. Curves 1 and 2 were plotted from the means of satisfactorily concordant data obtained from the *in vitro* titration of sera from four dogs with ferrihydroxyhemin in 0.1 M phosphate buffer, pH 7.6. The heme titers were between 0.01195 and 0.020 mmol/liter. Curve 1, ferriglycoglobulin hemochromogen. Curve 2, ferroglycoglobulin hemochromogen. Curve 3, a sample of ferroglycoglobulin hemochromogen after storage at refrigerator temperature for 48 hr, with more $Na_2S_2O_4$ added before measurement.

tion was centrifuged at 8000 rpm in a refrigerated centrifuge to remove undissolved material, and its heme content was determined spectrophotometrically as dicyano-hematin, $Hm(CN)_2$. To secure well-defined spectra of the serum ferrohemochromogen, particularly in the spectrophotometric titration of the serum heme-binding capacity as hemochromogen, we found it essential to employ low concentrations of heme. Thus, 0.01–0.05 ml of the above stock was added per 5 ml of serum, yielding heme concentrations in the sera of 0.004–0.02 mmol/liter.

Spectrophotometry

For rapid and accurate recording of the visible and ultraviolet spectral pattern a thoroughly calibrated Unicam model SP 800A spectrophotometer was used. At fixed wavelengths (as in determining the concentration of heme) the Beckman DU spectrophotometer was employed. To conserve serum, we used semimicro cuvettes of 5 and 10 mm depth (Precision Cells, Inc., Type 9). The spectral data were obtained promptly after the preparation of the sample. Reduction was accomplished by the addition of solid sodium dithionite, $Na_2S_2O_4$ (low in iron, Fisher Scientific Co.), to both the sample and balancing solution within the cuvettes. In the analysis of mixed pigment species (for example, serum ferrohemochromogen and ferrohematin–albumin) the method of Austin and Drabkin (36, 37) was applied†. The titration of the serum heme-binding capacity as ferrohemochromogen was based on the prominence of the α and β bands and especially on the ratio of the α maximum to the minimum between α and β (34, 38–42), since each would be appreciably depressed by the presence of a component with the spectral characteristics of ferrohematin–albumin. In quantitation the following constants (ϵ, 1 mM, 1 cm) were employed: heme concentration, as $Hm(CN)_2$, 11.3 at 545 mm; biliverdin · HCl, 28.7 at 683 nm and 61.9 at 377 nm; bilirubin, 57.0 at 454 nm; degree of hemolysis, as oxyhemoglobin, 130.7 at 413 nm, or as cyanmethemoglobin, 110.8 at 417.5 nm.

† Spectrophotometric details will be published elsewhere.

RESULTS

The unmistakable spectrum of ferrohemochromogen (34, 38–42) was obtained almost immediately upon the addition of hydroxyhemin, followed by reduction with $Na_2S_2O_4$, in all the sera examined. Of the serum protein fractions only fraction IV-4 was consistent in yielding the ferrohemochromogen spectrum after the addition of heme and reductant. Attention may be directed to the following:

1. The titration of the serum-heme binding capacity as ferrohemochromogen (Fig. 1) was in a similar range in the different mammalian species, with an overall mean of 0.01244 mmol/liter. If we assume a molecular weight for the specific heme-binding apoprotein of 64,000 (ref. 21, and U. Müller-Eberhard, personal communication), values in mg/100 ml serum are 76–128 (dog), 55–95 (man), 48–71 (rabbit) and 63–82 (rat). These are in reasonable agreement with those given

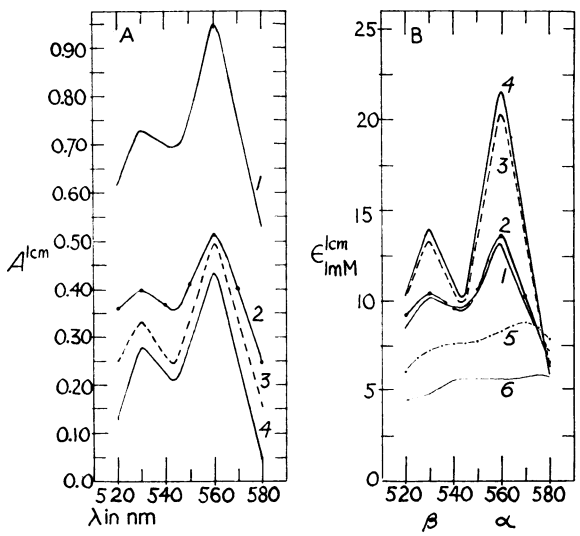

FIG. 2. Removal of intravenously injected heme from plasma. At zero time, 20 ml of 2 mmol/liter hydroxyhemin (pH 7.6) were injected into a 10-kg dog, with an estimated plasma volume of 450 ml. In *A*, absorbance values; in *B*, corresponding millimolar extinction coefficients (ϵ values), referred to heme concentration. Serum samples were balanced against serum obtained prior to injection of heme. Curves 1 (*A* and *B*), sample (with added $Na_2S_2O_4$) 10 min after injection. Heme concentration, 0.072 mmol/liter. Curves 2 (*A* and *B*), sample (with added $Na_2S_2O_4$) 40 min after injection. Heme concentration, 0.038 mmol/liter. Curves 3 (*A* and *B*), sample (with added $Na_2S_2O_4$) 120 min after first heme injection and 10 min after second injection of 6 ml of 2 mmol/liter hydroxyhemin. Heme concentration, 0.0244 mmol/liter. Curves 4 (*A* and *B*), ferroglycoglobulin hemochromogen, obtained by heme titration *in vitro* of the serum prior to the intravenous injection of heme. Heme concentration, 0.02 mmol/liter. Curve 5, ferrohematin–albumin in 0.1 M phosphate buffer, pH 7.6; molar ratio of heme to alubmin, 1:1. Curve 6, ferrohydroxyhemin in 0.1 M phosphate buffer, pH 7.6.

(for man) by such techniques as radial immunodiffusion (23), and are consistent with a 1:1 molar ratio of heme to binding serum protein (Fig. 2). It may be emphasized that under the conditions of the titration with the above low final concentrations of heme, only minute amounts of heme (not measurable by the present technique) can be associated with albumin, despite the fact that the molar ratio, in the sera, of albumin to heme-binding protein is 47:1 (from 0.58:0.0124 mmol/liter).

2. The data in Fig. 2 support the deductions that the affinity of the heme-binding protein for heme is very high in comparison with that of albumin, and that the removal of any heme that may enter the serum will be very rapid. After the intravenous injection of hydroxyhemin in an amount seven times as much as the heme-binding capacity (as hemochromogen), the data obtained at 560, 543, and 530 nm were compatible with a two-component mixture (see ref. 36) of ferrohemochromogen and ferrohematin–albumin. After injection, the ratios of ferrohemochromogen to ferrohematin–albumin were 0.429:0.571 at 10 min, 0.455:0.545 at 40 min, and 0.905:0.095 at 130 min (and 10 min after a second injection). In a 10-kg dog, with a plasma volume of 450 ml, 49 mg of bile pigment is produced per day, or 2.04 mg/hr (1). This corresponds to 2.4 mg/hr of heme degraded to bile pigment. At a titer of 0.02 mmol/liter heme bound as hemochromogen, a total of 5.87 mg of heme would be so bound in 450 ml of plasma. This is 2.45 times as much as the heme normally converted to bile pigment per hour. The heme concentration in the plasma was reduced by 0.034 mmol/liter per 30 min, or 0.068 mmol/liter per hour (from values at 10 and 40 min after injection). This is calculated to correspond to a clearance rate of 19.93 mg of heme per hour (from 450 ml of plasma), a rapid rate indeed. Moreover, the clearance in the dog is 8 times as great as the heme-binding capacity as hemochromogen.

3. 10 min after the addition of ferrihematin–albumin to serum, the analytical ratio of ferrohemochromogen to ferrohematin-albumin was found to be 0.68:0.32. This rapid transfer of heme from albumin to the specific binding protein is consistent with the relative affinities of the two proteins for heme, preponderantly favorable for heme binding as hemochromogen. It permits the deduction that, in contrast to the tight bonding of heme to the specific carrier or binding protein, the association complex of heme and albumin is loose and easily dissociated.

4. The data in Fig. 3 are typical of those obtained with the plasma protein fraction IV-4 of the different mammalian species studied. To obtain adequate and rapid reduction of dissolved fraction IV-4 with $Na_2S_2O_4$ it proved desirable to adjust the pH to 8.2 (compare Curves 1 and 2). In this fraction the concentration of the heme-binding protein, in terms of total protein present, is calculated to be 12.8% (compare 1.14% in whole serum). Relevant to the identification of the heme-binding protein as a low molecular weight β_1 glycoglobulin are the rather high value of 4.0% (referable to protein content) obtained for neutral hexose of this crude fraction, the solubility of the heme-binding protein in 0.6 M perchloric acid (15–17) (Curve 3), with an increase of the neutral hexose content to 7.2%, and the recovery of 99% of the heme-binding protein in the albumin fraction after molecular sieve chromatography on Bio-Gel P-200, 100–200 mesh (Bio-Rad Laboratories), equilibrated with 0.01 M phosphate (pH 7.4)–0.15 M NaCl.

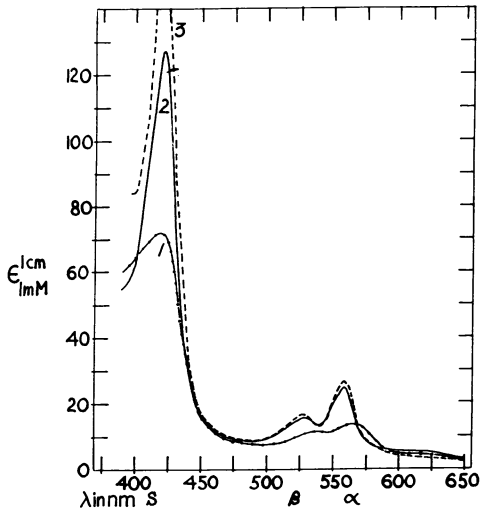

FIG. 3. Heme-binding glycoglobulin in Cohn protein fraction IV-4 of human serum. The samples were made up in phosphate buffer to a protein concentration of 6.22 mg/ml, or to about 8.9% of the total protein present in serum. pH was adjusted to 7.8 and 8.2, and hydroxyhemin was added to a final concentration, in Curves 1 and 2, of 0.0148 mmol/liter. Reductant was added in all cases. Curve 1, at pH 7.8. Curve 2, at pH adjusted to 8.2. Curve 3, an aliquot of sample as in Curve 2, extracted with 0.6 M perchloric acid (refs. 15–17) prior to addition of heme; centrifuged for recovery of supernatant fluid and neutralized, and hydroxyhemin added. Final concentration of heme = 0.00295 mmol/liter.

5. Because of their common localization in the β_1 glycoglobulins in fraction IV-4 and their extractability with 0.6 M perchloric acid, it appears very probable that hemopexin and the heme-binding protein disclosed by spectrophotometry are identical or closely related. This is verified by the typical ferrohemochromogen spectrum yielded by ferrohydroxyhemin–hemopexin (Curve 4, Fig. 4). My analysis for neutral hexose was 10.6%, referable to protein content. This is in reasonable accord with the available literature (15, 17). On the other hand, reservations remain as to the unaltered or native state of the isolated purified hemopexin sample. The spectral pattern of ferrihydroxyhemin-hemopexin is not that of a typical ferrihemochromogen, particularly in the Soret region (compare Curve 1, Fig. 1 of ferriglycoglobulin hemochromogen with Curve 2, Fig. 4). The latter curve suggests that there was little reactivity with heme prior to addition of reductant.

6. An interesting phenomenon is the spectral shift of 8–10 nm towards the shorter wavelengths (Curve 3, Fig. 1 and Curve 5, Fig. 4). These pronounced spectral shifts occurred in samples containing added $Na_2S_2O_4$ and kept at refrigerator temperature for 48 hr. At room temperature, in the presence of reductant, a progressive wavelength shift was observed, which could be detected as early as 2 hr after addition of heme and $Na_2S_2O_4$. No spectral shift was evident upon the addition of $Na_2S_2O_4$ to ferriglycoglobulin hemochromogens kept at refrigerator temperature for 48 hr.

In the perfusion of isolated rat liver (7, 43), both [^{14}C]ferrihydroxyhemin and [^{14}C]ferrihydroxyhemin–glycoglobulin hemochromogen (prepared by addition of 20,900 cpm/mg [^{14}C]ferrihydroxyhemin to fraction IV-4 of rat plasma) were found to be excellent substrates in the production of bile pigments. At a concentration of [^{14}C]heme in the perfusion medium of 0.08 mg/ml, the mean output of bile pigment dur-

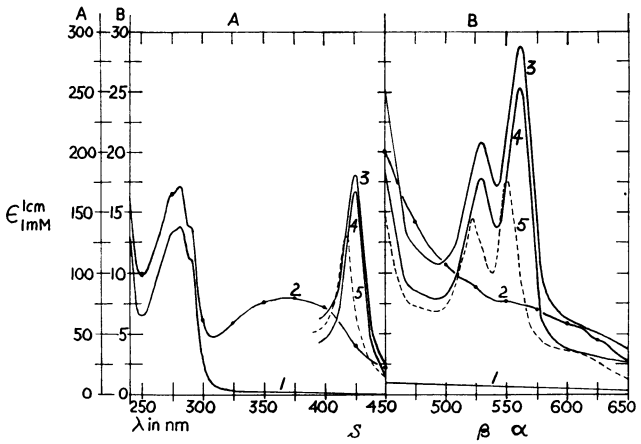

FIG. 4. Spectra of ferri- and ferrohydroxyhemin complexes with hemopexin. The ϵ values are based on a molecular weight of 64,000 for hemopexin. Curve 1, hemopexin in 0.1 M phosphate buffer, pH 7.6. Original data at 0.0661 mmol/liter. Curve 2, ferrihydroxyhemin–hemopexin in 0.1 M phosphate buffer, pH 7.6. Original data at 0.00665 and 0.0131 mmol/liter respectively for the ultraviolet (*A*) and visible (*B*) regions. Heme to hemopexin ratio, 1.086:1.0. Curve 3, ferrohydroxyhemin–hemopexin in 0.1 M phosphate buffer, pH 7.6. Original data at 0.0110 mmol/liter in Soret region (*A*) and 0.0195 mmol/liter in visible region (*B*). Heme to hemopexin ratio, 1.6:1.0; $Na_2S_2O_4$ added. Curve 4, ferrohydroxyhemin–hemopexin in 0.1 M phosphate buffer, pH 7.6. Data in Curve 3 adjusted for excess heme above 1:1 molar ratio. Curve 5, ferrohydroxyhemin–hemopexin in 0.1 M phosphate buffer, pH 7.6, kept at refrigerator temperature for 48 hr. Solution (0.01093 mmol/liter) read after addition of more $Na_2S_2O_4$.

ing a period of 3 hr, in mg/hr, was 0.055 (no substrate addition), 0.200 (with ferrihydroxyhemin), and 0.263 (with ferrihydroxyhemin–glycoglobulin hemochromogen). The specific activity of the recovered bile pigment, measured spectrophotometrically as biliverdin (1,7) was 18,900 cpm/mg. Hemoglobin, added even at concentrations of 7 mg/ml, and undenatured ferrihematin–albumin (ref. 44) produced no significant increase in bile-pigment output. The ferrihydroxyheme–glycoglobulin hemochromogen was also found to be a very efficient substrate in bile-pigment formation by our cell-free enzymic system prepared from the hemophagous organs of dog placenta (1, 7, 45, 46). In a recent report, Tenhunen, Marver, and Schmid (47) have stated that in one experiment hemopexin-bound hemin served as a substrate in their hepatic microsomal system for producing bile pigment in the presence of heme oxygenase, but with only 49% of the activity of protohemin IX as substrate.

DISCUSSION

The serum glycoglobulin hemochromogens represent a new type of mammalian hemoproteins, postulated, on the basis of present findings, to be functionally important in heme transport and in the early events of bile-pigment production. The disclosure and measurement of this heme–(serum protein) complex by direct spectrophotometry in the presence of high concentrations of albumin, with its potential for association with heme as hematin–albumin, was possible because of the extraordinarily high affinity of the specific heme-binding protein for heme and because of the character of the respective spectra in the reduced state. The binding constant (yet to be determined) of heme and its specific carrier protein is presumably very high. In past studies of hemopexin, spectrophotometry has been erroneously assessed as unfeasible or ineffective (23).

The rapid rate of clearance of heme from the plasma *in vivo*, and the unimpaired formation of the glycoglobulin ferrohemochromogen after a second injection of heme (Fig. 2) suggest either a rapid turnover for the specific heme-binding protein or its reutilization or recycling after it delivers its bound hydroxyhemin to a tissue such as the liver. Decisive evidence as to these alternatives has not yet been furnished. At this stage, speculation (25) upon the role of hemopexin in the enzymic regulation of heme synthesis appears to be premature.

The great instability (U. Müller-Eberhard, personal communication) of the purified hemopexin, isolated by means of perchloric acid, in itself may point to a possible alteration in the protein structure in the preparative procedure. Indeed, Müller-Eberhard and English (22) criticized the use of perchloric acid earlier on these very grounds. In its native or near-native state in serum and in Cohn fraction IV-4, the heme-binding glycoglobulin appears to be quite stable. The inferred lack of reactivity of ferriheme with the isolated hemopexin (Curve 2, Fig. 4) may be due to the oxidation of a sulfhydryl group in the vicinity of the heme-binding groups in the apoprotein. The spectral shift towards shorter wavelengths of the ferroglycoglobulin hemochromogens (Curve 3, Fig. 1 and Curve 5, Fig. 4) may be due to a secondary binding with the apoprotein, involving the vinyl groups of the heme moiety (compare 40, 42). The validity of these interpretations is being tested.

In accordance with our experimental findings, a new proposal is made. The specific heme-binding plasma β_1 glycoglobulin and plasma albumin have cooperative, physiologically functional roles in heme transport and bile-pigment production. The albumin, present in relative abundance, forms an easily dissociable complex with heme and serves as a reservoir for excess heme that may enter the plasma. The heme–albumin replenishes or transfers the heme to the specific heme-binding protein, which carries it to the tissues (such as liver) and permits its passage across the membrane barriers into tissue cells, where heme may be degraded to bile pigment or serve other metabolic purposes.

This work has been partially supported by grants, NONR-551(45), NSF-G-19785, USPHS-HE-10147, and USPHS-AM-00831.

1. Drabkin, D. L., in "Biological Effects of Carbon Monoxide," ed. R. F. Coburn and P. J. Lawther, *Ann. N.Y. Acad. Sci.*, **174**, 49 (1970).
2. Drabkin, D. L., *Fed. Proc.*, **29**, 800 (1970).
3. Nemir, P., Jr., H. R. Hawthorne, I. Cohn, Jr., and D. L. Drabkin, *Ann. Surg.*, **130**, 857 (1949).
4. Nemir, P., Jr., and D. L. Drabkin, *Surgery*, **40**, 171 (1956).
5. Nemir, P., Jr., A. Ahmadi, and D. L. Drabkin, *Ann. Surg.*, **165**, 135 (1967).
6. Nemir, P., Jr., A. Ahmadi, A. Fronek, and D. L. Drabkin, *Ann. Surg.*, **166**, 919 (1967).
7. Wise, C. D., *Studies on the Degradation of Hemoglobin to Bile Pigment*, Ph.D. thesis, University of Pennsylvania (1963).
8. Lemberg, R., and J. W. Legge, in *Haematin Compounds and Bile Pigments* (Interscience Publishers, Inc., New York, 1949), p. 453.
9. London, I. M., *J. Biol. Chem.*, **184**, 373 (1950).

10. Gray, C. H., in *The Bile Pigments* (Methuen and Co., Ltd., London, 1953), p. 37.
11. Drabkin, D. L., and R. Barcelo, *Fed. Proc.*, **22**, 597 (1963).
12. Cohn, E. J., L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. N. Ashworth, M. Melin, and H. L. Taylor, *J. Amer. Chem. Soc.*, **68**, 459 (1946).
13. Surgenor, D. M., B. A. Koechlin, and L. E. Strong, *J. Clin. Invest.*, **28**, 73 (1949).
14. Cohn, E. J., F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid, and E. Uroma, *J. Amer. Chem. Soc.*, **72**, 465 (1950).
15. Putnam, F. W., "Structure and Function of the Plasma Proteins," in *The Proteins—Composition, Structure and Function*, ed. H. Neurath (Academic Press, New York and London, 1965), 2nd ed., vol. 3, pp. 153–267.
16. Grabar, P., C. De Vaux St. Cyr, and H. Cleve, *Bull. Soc. Chim. Biol.*, **42**, 853 (1960).
17. Schultze, H. E., K. Heide, and H. Haupt, *Clin. Chim. Acta*, **7**, 854 (1962).
18. Aber, G. M., and D. S. Rowe, *Brit. J. Haematol.*, **6**, 160 (1960).
19. Nyman, M., *Scand. J. Clin. Lab. Invest.*, **12**, 121 (1960).
20. Müller-Eberhard, U., and H. Cleve, *Nature*, **197**, 602 (1963).
21. Witz, I., and J. Gross, *Proc. Soc. Exp. Biol. Med.*, **118**, 1003 (1965).
22. Müller-Eberhard, U., and E. C. English, *J. Lab. Clin. Med.*, **70**, 619 (1967).
23. Hanstein, A., and U. Müller-Eberhard, *J. Lab. Clin. Med.*, **71**, 232 (1968).
24. Müller-Eberhard, U., H. H. Liem, C. A. Yu, and I. C. Gunsalus, *Biochem. Biophys. Res. Commun.*, **35**, 229 (1969).
25. Ross, J. D., U. Müller-Eberhard, and L. A. Carroll, *J. Lab. Clin. Med.*, **75**, 694 (1970).
26. Müller-Eberhard, U., C. Bosman, and H. H. Liem, *Fed. Proc.*, **29**, 624 (1970).
27. Itzhaki, R. F., and D. M. Gill, *Anal. Biochem.*, **9**, 401 (1964).
28. Cohn, E. J., W. L. Hughes, Jr., and J. H. Weare, *J. Amer. Chem. Soc.*, **69**, 1753 (1947).
29. Keilin, J., *Nature*, **154**, 120 (1944).
30. Rosenfeld, M., and D. M. Surgenor, *J. Biol. Chem.*, **183**, 663 (1950).
31. Gornall, A. G., C. J. Bardawill, and M. M. David, *J. Biol. Chem.*, **177**, 751 (1949).
32. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
33. Spiro, R. G., *J. Biol. Chem.*, **235**, 2860 (1960).
34. Drabkin, D. L., and J. H. Austin, *J. Biol. Chem.*, **112**, 89 (1935–36).
35. Drabkin, D. L., *J. Biol. Chem.*, **140**, 387 (1941).
36. Austin, J. H., and D. L. Drabkin, *J. Biol. Chem.*, **112**, 67 (1935–36).
37. Drabkin, D. L., in *Haemoglobin [Joseph Barcroft Memorial Conference]*, ed. F. J. W. Roughton, and J. C. Kendrew (Butterworths Scientific Publications, London, 1949), p. 35.
38. Drabkin, D. L., *Proc. Soc. Exp. Biol. Med.*, **41**, 225 (1939).
39. Drabkin, D. L., *J. Biol. Chem.*, **140**, 373 (1941).
40. Drabkin, D. L., *J. Biol. Chem.*, **146**, 605 (1942).
41. Drabkin, D. L., *Fed. Proc.*, **2**, 61 (1943).
42. Drabkin, D. L., in *Haematin Enzymes*, ed. J. E. Falk, R. Lemberg, and R. K. Morton (Pergamon Press, London, 1961), vol. 1, p. 142.
43. Miller, L. L., C. G. Bly, M. T. Watson, and W. F. Bale, *J. Exp. Med.*, **94**, 431 (1951).
44. Cohen, S., and A. H. Gordon, *Biochem. J.*, **70**, 544 (1958).
45. Wise, C. D., and D. L. Drabkin, *Fed. Proc.*, **23**, 323 (1964).
46. Wise, C. D., and D. L. Drabkin, *Fed. Proc.*, **24**, 222 (1965).
47. Tenhunen, R., H. S. Marver, and R. Schmid, *J. Biol. Chem.*, **344**, 6388 (1969).