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Secretion of Haem by Hepatic Parenchymal Cells

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Hepatic parenchymal cells in primary culture, and also the intact perfused liver, secrete newly synthesized haem into extracellular fluids. In cultures incubated with the haem precursor δ-amino[4-^{14}C]laevulinate, labelled haem was formed at a linear rate for at least 8h, and 10–20% of the total labelled haem was present in the culture medium. The appearance of labelled extracellular haem was proportional both to the concentration of labelled precursor offered to the cells and to the time of incubation. Similar results were obtained when [2-^{14}C]glycine was added as haem precursor. Studies with the isolated perfused liver indicated that newly synthesized haem is secreted also by the intact liver. Approximately equal amounts of haem appeared in bile and in perfusate. The findings are discussed in relation to the pathogenesis of symptoms in the hereditary hepatic porphyrias.

Synthesis of haem is a prominent liver function, subserving formation of cellular cytochromes and other haemoproteins. Quantitatively, the most important of these hepatic haemoproteins are the microsomal cytochromes known collectively as *P*-450, which appear to account for at least 50% of total hepatic haem synthesis (Meyer & Schmid, 1978). It is also established that a proportion of newly synthesized hepatic haem is degraded rapidly to bile pigment, appearing 15–20min after administration of a radioactive haem precursor to experimental animals (Robinson, 1968). On the basis of these findings, it has generally been assumed that hepatic haem either is incorporated into endogenous haem proteins or is converted into bile pigment. The possibility of still other routes of metabolism for newly synthesized haem has not been raised.

The present studies document that a significant fraction of endogenous hepatic haem is exported to extracellular fluid, on the basis of two experimental approaches. In studies with hepatocytes in primary culture incubated with a labelled haem precursor, the amount of haem secreted into the medium was determined as a fraction of newly synthesized tissue haem present after a given time period. Studies exploring the mechanism of haem secretion revealed that a sizeable fraction of the extracellular haem was associated with newly synthesized haemopexin. Studies with the isolated perfused liver confirmed the finding of haem secretion by hepatocytes and permitted quantification of the proportion of haem exported across the sinusoidal or canalicular faces of the cell. This work has been presented, in part, as a preliminary communication (Bissell *et al.*, 1977).

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Experimental

Primary hepatocyte culture

Hepatic parenchymal cells were prepared from male rats subjected to a prior partial hepatectomy and cultured in a fully defined serum-free medium, as described elsewhere (Bissell *et al.*, 1973). Alternatively, cells from resting rat liver were prepared and cultured in medium (see below) containing crystalline insulin (4munits/ml) and 1% (v/v) normal rat serum. Comparable results were obtained with either type of initial cell material. Approx. 4×10^6 cells were plated in 60mm-diam. plastic Petri dishes coated with 20–30μg of reconstituted rat tail collagen (Bissell *et al.*, 1978). The medium consisted of a modified Medium 199 supplemented with ascorbic acid (0.3mM) (Bissell & Guzelian, 1979) in an atmosphere of humidified air with 2% CO_2 at 37°C (pH7.3–7.4). After the initial 4h of incubation, and again after 20h, the medium was renewed. Cells were studied at the time of cell plating and after 4h or 20h of incubation, as noted for the individual studies. The principal reason for studying cells during the first 24h of culture was to monitor the synthesis of haemopexin and albumin, which was greatest in young cultures. With regard to haem metabolism, qualitatively similar results were obtained with cells that were freshly plated or studied after 20h of culture. δ-Amino-[4-^{14}C]laevulinate (50Ci/mol; New England Nuclear Corp., Boston, MA, U.S.A) was introduced at various concentrations, as noted in the Results section; [2-^{14}C]glycine (New England Nuclear) was

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added to complete medium at a final specific radioactivity of 2.9 Ci/mol (final concn. 0.7 mM-glycine).

Isolated perfused rat liver

This procedure was carried out as previously published (Liem *et al.*, 1977). For studies of haem synthesis, δ-amino[4-^{14}C]laevulinate was added to 120 ml of perfusate at the start of the study; 20 ml samples were taken at the indicated time intervals and freeze-dried. Before measurement of [^{14}C]haem, the freeze-dried material was reconstituted in 3 ml of 0.9% NaCl. The bile duct of the perfused liver was cannulated and bile collected for the duration of each study. After 5 h, the perfusion was terminated with homogenization of the liver in 1 vol. of 0.25 M-sucrose.

Measurement of [^{14}C]haem

Samples from hepatocyte culture or from the studies with the isolated perfused liver were mixed with 6–10 mg of carrier haem in the form of haemoglobin, and labelled haem was extracted and determined by two methods (Bissell & Hammaker, 1976; Vogel *et al.*, 1960), which yielded comparable results; the efficiency of extraction was approx. 70%. Radioactivity was measured by liquid-scintillation spectrometry with an efficiency of 85%. Quench correction was estimated by subsequent addition of a ^{14}C-labelled standard to the samples. The specific radioactivity of extracted haem samples was unchanged after recrystallization from pyridine (Labbe & Nishida, 1957), and radiochemical purity was 99% on analysis by paper chromatography with a descending 2,6-dimethylpyridine/water (10:7, v/v) solvent system (Falk, 1964) (δ-aminolaevulinate migrates in this chromatographic system as a discrete spot, with an R_F value approximately one-half that of haem). The impurity appeared to be largely labelled δ-aminolaevulinate, representing approx. 0.003% of the label added to culture media or perfusate. Its carry-over in the haem extracts led to a small but measurable 'zero-time' radioactivity, and the experimental results reported below have been corrected for this amount. Our data on the specificity of these methods for haem extraction and the purity of the product are similar to those reported by Bonkowsky *et al.* (1978).

Measurement of rat albumin and haemopexin

The concentration of these proteins in culture media was determined by radioimmunoassay (Kida & Muller-Eberhard, 1974). For measurement of labelled haem bound to albumin or haemopexin, washed immunoprecipitates were mixed with carrier haem and extracted, as described above. Control studies included immunoprecipitation of known amounts of albumin- or haemopexin-bound [^{14}C]-haem. These indicated that approx. 50% of albumin-bound haem and 90–100% of haemopexin-bound haem were retained in the precipitates.

Miscellaneous procedures

Protein (Lowry *et al.*, 1951) and lactate dehydrogenase (Beutler, 1971) were measured by the methods referenced. Cycloheximide (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added at 50 μM, a concentration sufficient to inhibit by 90–95% the incorporation of radioactive leucine into trichloroacetic acid-precipitable material.

Results

Distribution of newly synthesized hepatic haem between cells and extracellular fluid of cultured hepatocytes

Hepatocytes in monolayer culture convert δ-amino[4-^{14}C]laevulinate into haem at linear rates (Fig. 1). A separate analysis of cells and culture medium revealed that a substantial amount of [^{14}C]-haem appeared extracellularly under these experimental conditions (Fig. 2). In a typical study, the proportion of radioactive haem in the medium was 10% of the total [^{14}C]haem formed in the culture. The amount of labelled haem in both cells and medium was directly proportional to the concentration of labelled δ-aminolaevulinate introduced into the incubation (Table 1).

Because administration of δ-aminolaevulinate bypasses the rate-determining step in the haem-synthetic pathway (Meyer & Schmid, 1978), studies similar to those shown in Fig. 2 were conducted with [2-^{14}C]-glycine as haem precursor. With this compound, the ratio of labelled haem in the cells to that in the medium was comparable with the ratio obtained with δ-

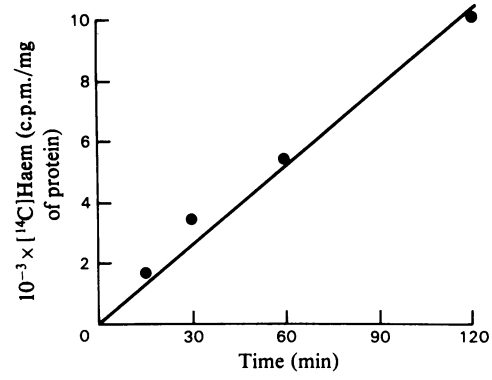

Fig. 1. *Time-course for the conversion of labelled δ-aminolaevulinate into labelled haem by cultured rat hepatocytes*

Cells were prepared and incubated from the time of plating in a medium containing δ-amino[4-^{14}C]-laevulinate (1 μCi/ml) at a concentration of 10 μM. At the time points shown, cells and media from two plates were combined and analysed for [^{14}C]haem.

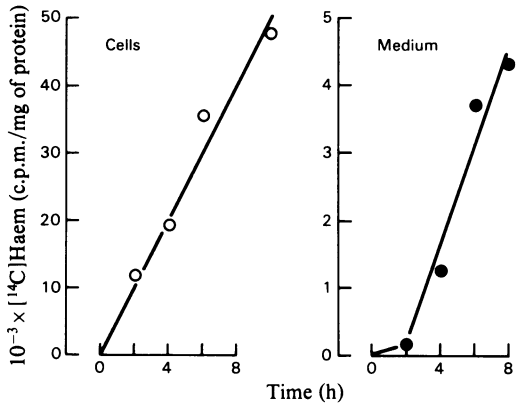

Fig. 2. *Time-course for the appearance of [^{14}C]haem in cells or incubation medium of hepatocytes cultured with δ-amino-[4-^{14}C]laevulinate*

The study was conducted as in Fig. 1. At the indicated time points, the medium was removed and centrifuged, to eliminate intact hepatocytes from the medium; pelleted cells (if any) were combined with cells removed from the culture plate by mechanical scraping. After addition of carrier haemoglobin, labelled haem was isolated as described in the Experimental section.

Table 1. *Formation and secretion of [^{14}C]haem in hepatocyte cultures incubated with various concentrations of δ-amino-[4-^{14}C]laevulinate*

Cultures were incubated from the time of cell plating with labelled δ-aminolaevulinate (15 Ci/mol) at the indicated concentrations. After 4h, cells and medium were analysed individually for total extractable [^{14}C]-haem, as described in the Experimental section.

Concn. of δ-amino-[4-^{14}C]laevulinate (μM)	[^{14}C]Haem (c.p.m./mg of cell protein)		100 × Ratio (medium/cells)
	Cells	Medium	
65	12700	2100	16.5
130	21700	4000	18.4
390	63000	10500	16.7

aminolaevulinate as haem precursor (results not shown).

Haem secretion by the isolated perfused liver

The intact perfused liver preserves the anatomical compartmentation of bile and plasma, and therefore provides a means for distinguishing between movement of substances across the plasma and the canalicular membranes of the cells. When δ-amino-[4-^{14}C]laevulinate was added to the perfusate, labelled haem appeared in both the circulating medium and in bile. As shown in Table 2, after 2h of perfusion the amounts of [^{14}C]haem in the circulating medium and in bile were similar; after 5h, the amount in bile exceeded that in the perfusate, probably because the perfusate was re-circulated, which would allow for re-uptake and degradation of haem by the liver. Overall, the total extra-hepatic haem (bile and perfusate) comprised 15% of the labelled haem in the liver, a value comparable with that obtained in the studies of cultured hepatocytes.

Possible routes of secretion by the liver

The mechanisms for movement of haem from the hepatic parenchymal cells to the surrounding plasma space include leakage through the membrane (secondary in the cultures, perhaps, to transient damage sustained during the preparation of hepatocytes), passive diffusion from a cellular protein to a protein acceptor in the extracellular fluid, or transfer by a specific transport process. Leakage of cell contents across the plasma membrane may be conveniently assessed by the appearance of a cytoplasmic enzyme activity in extracellular fluids. Lactate dehydrogenase was chosen for this purpose, because it is stable in culture media (at 37°C) for at least 24h (D. M. Bissell, unpublished work) and because the sensitivity of its assay is sufficient for monitoring a low extent of leakage from hepatocytes placed in culture. In freshly plated cells, lactate dehydrogenase was detectable, but, over the first 4h of incubation, its rate of release (per h) from the cultured hepatocytes declined progressively from 0.14 to 0.03% of the total activity in the culture, whereas the cell-associated activity remained constant. This observation is consistent with other evidence that collagenase-prepared hepatocytes sustain damage to surface structures, but undergo repair after the cells are placed in culture (Bissell & Guzelian, 1979). By contrast, during the first hours of incubation, the amount of haem in the culture medium increased progressively (Fig. 2), and thus appeared by a mechanism other than leakage.

The possibility of passive diffusion of haem from cells to a protein acceptor in the culture medium or perfusate was tested as follows. Cells were incubated with labelled δ-aminolaevulinate for 2–3h until the accumulation of intracellular [^{14}C]haem was significant (cf. Fig. 1). The incubation medium was then changed to one with or without added protein in the form of rat serum albumin (1%, w/v). This manipulation had no effect on the subsequent transfer of haem from cells to medium (results not shown).

Among specific transport processes, one involving secretion of haem carried on newly synthesized albumin or haemopexin was considered. To explore this possibility, the rate of secretion of these haem-binding proteins by cultured hepatocytes was

Table 2. *Haem secretion by the isolated perfused rat liver*
Isolated-liver perfusion was carried out as described in the Experimental section with δ-amino[4-^{14}C]-laevulinic acid added at a final concentration of 12.4μM (0.4μCi/ml of perfusate). At 2 and 5h, samples of perfusate were taken for determination of [^{14}C]haem; the data are corrected for the amount of haem withdrawn at each time point and for the total volume of perfusate. Total bile output was collected over the same time periods and was similarly analysed. Repeat studies (two) gave similar results.

Time (h)	10^{-3} × Cumulative [^{14}C]haem (c.p.m.)		
	Perfusate	Bile	Liver
2	417	432	—
5	722	1601	15737

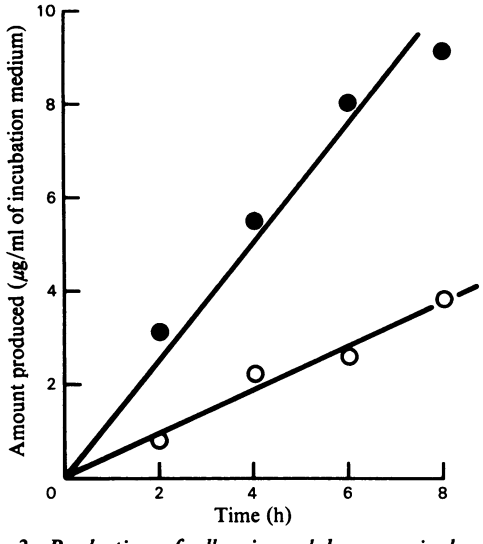

Fig. 3. *Production of albumin and haemopexin by rat hepatocytes in primary culture*
Cells were studied from the time of plating. At the indicated time points, medium was removed, centrifuged and the supernatant assayed for albumin (●) and haemopexin (○). Each plate contained 2ml of culture medium, and the cell protein content per plate averaged 3.5mg.

determined (Fig. 3). Both were excreted at linear rates; quantitatively, the production of albumin was approximately twice that of haemopexin (the two proteins have similar molecular weights), and their secretion was inhibited nearly completely by cycloheximide (Fig. 4; results for albumin were obtained from a separate study, not shown). In parallel studies of cultures incubated with labelled δ-aminolaevulinate, secretion of [^{14}C]haem into the culture

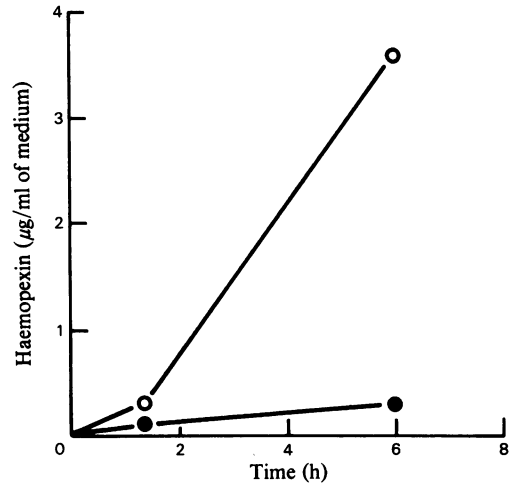

Fig. 4. *Effect of cycloheximide on haemopexin secretion by cultured rat hepatocytes*
Freshly prepared hepatocytes were plated in the presence (●) or absence (○) of 50μM-cycloheximide, and the appearance of haemopexin in the culture medium was monitored as in Fig. 3.

Table 3. *Effect of cyclohexinide on haem synthesis and secretion by rat hepatocytes in primary culture*
Cells were prepared and cultured as described in the Experimental section. After 4h, the medium was renewed; δ-amino[4-^{14}C]laevulinic acid (1μCi/ml of culture medium) was added to all plates and cycloheximide (50μM) to a portion. At the time points shown, cells and media were assayed individually for [^{14}C]haem, as described in the legend to Fig. 2. The data are from a typical study and represent the average of two individual determinations. Protein content per plate between control and cycloheximide-treated cultures varied less than 5%. The values in parentheses indicate the effect of cycloheximide, as a percentage of the value in the corresponding control culture.

	10^{-2} × [^{14}C]Haem per culture plate (c.p.m.)			
	Cells		Medium	
Time (h)	Control	+Cyclo-heximide	Control	+Cyclo-heximide
2	567	520 (92%)	43	22 (51%)
5	1079	1145 (106%)	135	73 (54%)

medium was inhibited by approx. 50% by cycloheximide, whereas formation of cell-associated labelled haem was decreased by less than 10% (Table 3). These data were compatible with participation of secretory proteins in the export of haem. To

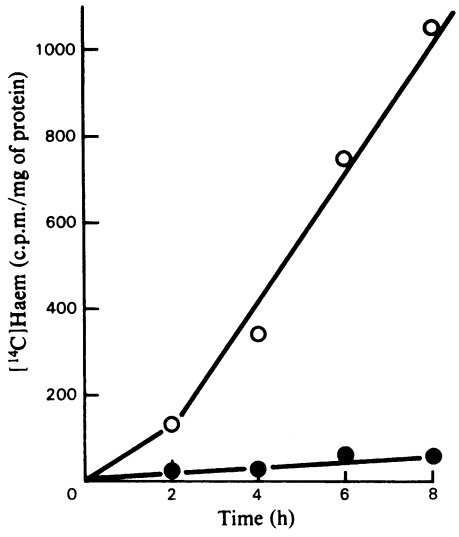

Fig. 5. *Association of secreted* [^{14}C]*haem with albumin* (●) *or haemopexin* (○) *in the incubation medium of cultured rat hepatocytes*

Freshly prepared hepatocytes were plated in culture in a medium containing δ-amino[4-^{14}C]laevulinate (2μCi/ml). At the time points shown, medium was removed, centrifuged and incubated with anti-(rat albumin) or anti-(rat haemopexin) serum, for preparation of immunoprecipitates. Washed immunoprecipitates were mixed with carrier haemoglobin and assayed for associated [^{14}C]haem, as described in the Experimental section.

test whether haem is transported out of the cell bound to secreted proteins, cultures were incubated with labelled δ-aminolaevulinate, albumin and haemopexin were isolated from the medium by specific immunoprecipitation, and the amount of [^{14}C]haem associated with the immunoprecipitates was determined. As shown in Fig. 5, approx. 30% of the total labelled haem in the culture medium was associated with haemopexin, whereas the amount bound to albumin was negligible.

Discussion

These studies document that a portion of newly synthesized hepatic haem undergoes export both to the sinusoidal circulation and to bile. The precise amount of secreted haem (as a fraction of total haem synthesis) is conjectural. We have expressed secretion as a fraction of the labelled haem present in the liver after a specified interval of incubation with δ-amino[4-^{14}C]laevulinate, showing that hepatocytes in primary culture and the intact perfused liver export comparable amounts. However, an estimate of total hepatic haem synthesis must consider not only the total accumulated labelled haem but also the amount of haem metabolized to other products, which may include both bilirubin and compounds other than bile pigments (D. M. Bissell & P. S. Guzelian, unpublished work). Hepatocytes in culture, after 2h of incubation with δ-amino[4-^{14}C]laevulinate, produce approximately twice as much labelled bilirubin as labelled haem (D. M. Bissell, unpublished work). Allowing for these factors, it may be estimated that secreted haem constitutes 2–4% of total hepatic haem synthesis.

The mechanism by which haem is secreted by the liver remains to be established. The present findings indicate formation of a complex between newly secreted haem and haemopexin, raising the intriguing possibility that haem is carried out of the cell bound to newly formed haemopexin. However, we cannot exclude that haem was secreted by another route, its binding to haemopexin occurring secondarily in the culture medium. If haem were secreted bound to albumin, its transfer to haemopexin in the medium would be expected on the basis of the greater affinity of the latter protein for haem in virtually all animal species studied to date (Muller-Eberhard & Liem, 1974). On the other hand, it may be noted that less than half of the labelled haem in the culture medium was associated with haemopexin, implying the presence of other haem-binding moieties produced by hepatic parenchymal cells. Although these are a subject for further investigation, it is conceivable that cytosolic haem-binding proteins, such as ligandin (Tipping *et al.*, 1976; Arias, 1977), in fact are secreted by the liver and thus effect the export of haem. Ligandin is readily detected in normal rat plasma, although it remains to be shown that its source is the liver (Bass *et al.*, 1978).

Interesting questions raised by these data concern the fate of the secreted haem. Haem mixed with serum or plasma binds to haemopexin and is transported mainly to the liver, where haemopexin cycles, delivering its bound haem and then returning to the plasma (Smith & Morgan, 1978). Haem circulates only briefly, with a half-life of 1–2h; however, 24–36h are required for recovery of 90% of the administered label in bile (Snyder & Schmid, 1965). These data leave open the possibility that some of the circulating haem is taken up by tissues and incorporated into cellular haemoproteins before its degradation. Snyder & Schmid (1965) examined various tissues, including brain, and failed to find significant extrahepatic accumulation of label. However, the method was relatively insensitive because of the low specific radioactivity of the haem utilized.

The possibility that extrahepatic tissues, notably neural cells, may take up small amounts of circulating haem is of interest with regard to the pathogenesis of symptoms in the hereditary hepatic porphyrias.

The most serious manifestation of these disorders, neuropsychiatric dysfunction, is dramatically alleviated by intravenous administration of haem (Bonkowksy *et al.*, 1971; Watson *et al.*, 1977). The latter observation has introduced the possibility that normal function of the nervous system may depend on exogenous haem (Shanley *et al.*, 1977), and the present findings provide strongly suggestive evidence for this hypothesis. Hepatic haem released into the circulation presumably is available for uptake and utilization by the nervous system and, indeed, the liver may be the only source of such haem. Haemoglobin haem would not be similarly available, since free haemoglobin in the circulation binds rapidly (and probably irreversibly) to haptoglobin, is sequestered in sites of degradation and converted into bile pigment (Ostrow *et al.*, 1962, Bissell *et al.*, 1972). Moreover, the rate of hepatic haem secretion would be expected to decline during acute porphyric attacks. On the basis of numerous clinical and experimental observations, hepatic porphyrias with neurological symptoms have been characterized as 'haem-deficient' conditions, in which hepatic haem production fails to meet the demand for haem-protein synthesis (Watson *et al.*, 1977; Meyer & Schmid, 1978; Bissell, 1979). A relative deficiency of haem production during acute porphyric attacks presumably would be associated with diminished secretion of haem by the liver. According to the above hypothesis, this would be responsible for neuronal haem deficiency and neurological symptoms. Although speculative at present, the question of decreased hepatic haem secretion in acute porphyria and utilization of circulating haem by neuronal tissues are amenable to study in experimental animals, and the findings reported here clearly suggest that this line of investigation should be pursued.

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References

Arias, I. M. (1977) in *Chemistry and Physiology of Bile Pigments* (Berk, P. D. & Berlin, N. I., eds.), DHEW Publication No. (NIH) 77-1100, pp. 306–314, U.S. Government Printing Office, Washington D.C.

Bass, N. M., Kirsch, R. E., Tuff, S. A. & Saunders, S. J. (1978) *Gastroenterology* **75**, 589–594

Beutler, E. (1971) *Red Cell Metabolism: A Manual of Biochemical Methods*, pp. 58–59, Grune and Stratton, New York

Bissell, D. M. (1979) in *Liver and Biliary Disease: A Pathophysiological Approach* (Wright, R., Alberti, K. G. M. M., Karran, S. & Millward-Sadler, H., eds.), W. B. Saunders Co., London, in the press

Bissell, D. M. & Guzelian, P. S. (1979) *Arch. Biochem. Biophys.* **192**, 569–576

Bissell, D. M. & Hammaker, L. E. (1976) *Arch. Biochem. Biophys.* **176**, 91–102

Bissell, D. M., Hammaker, L. & Schmid, R. (1972) *Blood* **40**, 812–822

Bissell, D. M., Hammaker, L. & Meyer, U. A. (1973) *J. Cell Biol.* **59**, 722–734

Bissell, D. M., Liem, H. H. & Muller-Eberhard, U. (1977) *Clin. Res.* **25**, 307A

Bissell, D. M., Levine, G. A. & Bissell, M. J. (1978) *Am. J. Physiol.* **234**, C122–C130

Bonkowsky, H. L., Tschudy, D. R., Collins, A., Doherty, J., Bossenmaier, I., Cardinal, R. & Watson, C. J. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2725–2729

Bonkowsky, H. L., Bement, W. J. & Erny, R. (1978) *Biochim. Biophys. Acta* **541**, 119–123

Falk, J. E. (1964) *Porphyrins and Metalloporphyrins*, p. 184, Elsevier, Amsterdam

Kida, S. & Muller-Eberhard, U. (1974) *Immunochemistry* **12**, 97–99

Labbe, R. F. & Nishida, G. (1957) *Biochim. Biophys. Acta* **26**, 437

Liem, H. H., Miyai, K. & Muller-Eberhard, U. (1977) *Biochim. Biophys. Acta* **496**, 52–64

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Meyer, U. A. & Schmid, R. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B. & Fredrickson, D. S., eds.), 4th edn., pp. 1166–1220, McGraw-Hill, New York

Muller-Eberhard, U. & Liem, H. H. (1974) in *Structure and Function of Plasma Proteins* (Allison, A. C., ed.), vol. 1, pp. 35–53, Plenum Press, London

Ostrow, J. D., Jandl, J. H. & Schmid, R. (1962) *J. Clin. Invest.* **41**, 1628–1637

Robinson, S. H. (1968) *N. Engl. J. Med.* **279**, 143–149

Shanley, B. C., Percy, V. A. & Neethling, A. C. (1977) *S. Afr. Med. J.* **51**, 458–460

Smith, A. & Morgan, W. T. (1978) *Biochem. Biophys. Res. Commun.* **84**, 151–157

Snyder, A. L. & Schmid, R. (1965) *J. Lab. Clin. Med.* **65**, 817–824

Tipping, E., Ketterer, B., Christodoulides, L. & Enderby, G. (1976) *Biochem. J.* **157**, 461–467

Vogel, W., Richert, D. A., Pixley, B. Q. & Schylman, M. P. (1960) *J. Biol. Chem.* **235**, 1769–1775

Watson, C. J., Pierach, C. A., Bossenmaier, I. & Cardinal, R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2118–2120