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Some physical and immunological properties of ox kidney biliverdin reductase

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The liver, kidney and spleen of the mouse and rat and the kidney and spleen of the ox express a monomeric form of biliverdin reductase (M_r 34000), which in the case of the ox kidney enzyme exists in two forms (pI 5.4 and 5.2) that are probably charge isomers. The livers of the mouse and rat express, in addition, a protein (M_r 46000) that cross-reacts with antibodies raised against the ox kidney enzyme and may be related to form 2 described by Frydman, Tomaro, Awruch & Frydman [(1983) Biochim. Biophys. Acta **759**, 257–263]. Higher-M_r forms appear to exist in the guinea pig and hamster. The ox kidney enzyme has three thiol groups, of which two are accessible to 5,5′-dithiobis-(2-nitrobenzoate) in the native enzyme. Immunocytochemical analysis reveals that biliverdin reductase is localized in proximal tubules of the inner cortex of the rat kidney. Biliverdin reductase antiserum also stains proximal tubules in human and ox kidney. The staining of podocytes in glomeruli of ox kidney with antiserum to aldose reductase is particularly prominent. The localization of biliverdin reductase in the inner cortical zone of rat kidney is similar to that described for glutathione *S*-transferase YfYf, and it is suggested that one function of this 'intracellular binding protein' may be to maintain a low free concentration of biliverdin to allow biliverdin reductase to operate efficiently.

INTRODUCTION

Biliverdin reductase catalyses the NAD(P)H-dependent reduction of biliverdin to produce the bile pigment bilirubin. The enzyme has been purified from rat liver, pig spleen and ox kidney (Kutty & Maines, 1981; Noguchi *et al.*, 1979; Phillips & Mantle, 1981) and in each case has been reported to exist as a monomer of M_r 34000-36000. The enzyme has been reported to occur only in mammals (Colleran & O'Carra, 1977), where it is believed to play a role in clearing biliverdin from the foetus (McDonagh *et al.*, 1981). Biliverdin is not known to exhibit any toxicity, and although Okazaki *et al.* (1978) suggested that it might play a role in liver regeneration we have been unable to confirm this suggestion (Phillips *et al.*, 1984). The enzyme is subject to potent substrate inhibition by biliverdin (O'Carra & Colleran, 1971; Phillips & Mantle, 1981), although this effect is probably modulated *in vivo* by intracellular binding proteins (Phillips *et al.*, 1984).

Most studies on the kinetics of this enzyme have included serum albumin in the assay mixture, and as this binds biliverdin it is necessary to apply a correction factor to obtain the free concentration of biliverdin (Phillips & Mantle, 1981). In addition, albumin may have direct effects on the activity of biliverdin reductase (Phillips, 1981; O. Phillips & T. J. Mantle, unpublished work). In order to conduct a detailed kinetic study of this enzyme we have used a slight modification of our original preparation (Phillips & Mantle, 1981) as described elsewhere (Phillips, 1981; Daly & Mantle, 1982) to obtain sufficient quantities of the enzyme. This has allowed us to investigate certain physical and immunological properties of the enzyme, which are described in the present paper.

MATERIALS AND METHODS

Materials

Biliverdin was synthesized by the method of McDonagh (1979). Procion Blue MX-R–Sepharose 4B was prepared as described by Baird *et al.* (1976). NADPH was purchased from Boehringer, and 2′,5′-bisphosphoadenosine–Sepharose and Sephadex G-100 were obtained from Pharmacia.

Purification of biliverdin reductase and aldose reductase

Our current procedure represent minor variations to those described in earlier work (Phillips & Mantle, 1981; Daly & Mantle, 1982). Briefly, ox kidney cytosol is fractionated by $(NH_4)_2SO_4$ fractionation (40–65% saturation), DEAE-cellulose chromatography and chromatography on Procion Blue MX-R–Sepharose. After this step fractions with a specific activity of more than 550 nmol/min per mg were pooled and gel-filtered on Sephadex G-100. After this step the preparation contained two polypeptides of M_r 34000 (biliverdin reductase) and 32000 (aldose reductase). These two proteins were separated on 2′,5′-bisphosphoadenosine–Sepharose as described previously (Phillips, 1981; Daly & Mantle, 1982).

Details of the assay methods for biliverdin reductase and aldose reductase can be found in Phillips *et al.* (1984) and Daly & Mantle (1982).

Electrophoretic methods

SDS/polyacrylamide-gel electrophoresis was carried out according to the method of Laemmli (1970). Isoelectric focusing was performed in flat-bed polyacrylamide gels in an LKB Multiphor apparatus under

native and denaturing (8 M-urea) conditions. Immunoblotting was carried out according to the method of Towbin *et al.* (1979).

Activity stain for biliverdin reductase

After native isoelectric focusing the polyacrylamide gel was overlaid with nitrocellulose paper that had been soaked in 30 μM-biliverdin/100 μM-NADH/50 μM-bovine serum albumin/100 mM-sodium phosphate buffer, pH 7.2, and then blotted dry. Enzyme activity was easily located as yellow bands (bilirubin) against a pale-green background.

Preparation of antisera

Purified preparations of biliverdin reductase and aldose reductase (100 μg of each) were emulsified with Freund's complete adjuvant and injected into multiple sites on the backs of New Zealand White rabbits. Then 2 weeks later the animals were boosted with a further 100 μg of antigen (emulsified with Freund's incomplete adjuvant) by intramuscular injection into the hind leg. After a further 2 weeks the animals were exsanguinated, and the blood was allowed to clot at 4 °C overnight and the serum was stored at −20 °C.

Preparation of cytosols

Animal tissues were obtained from local suppliers with the exception of those from the wallaby, which were kindly supplied by Dr. C. H. Tyndale-Biscoe, C.S.I.R.O., Canberra, A.C.T., Australia. Liver, kidney and spleen from the rat, mouse, hamster, guinea pig, fox and wallaby and human liver were homogenized in 0.25 M-sucrose/10 mM-Tris/HCl/1 mM-EDTA buffer, pH 7.2, in a Polytron homogenizer and centrifuged at 48000 ***g*** for 20 min. The supernatants were stored at −20 °C at a protein concentration in the range 10–50 mg/ml.

Immunocytochemical analysis

Sections (5 μm) of liver, kidney and spleen from the rat, human and ox that had been fixed in Bouin's fluid were stained by the peroxidase–anti-peroxidase technique of Sternberger *et al.* (1970).

RESULTS AND DISCUSSION

By substituting one Procion dye column instead of two (Phillips & Mantle, 1981) and by including 2′,5′-bisphosphoadenosine–Sepharose (Phillips, 1981; Daly & Mantle, 1982) we can now prepare 1–2 mg of electrophoretically homogeneous material in 3 days. The purified enzyme focuses as a single band with a pI of 6.2 under denaturing conditions. However, when the enzyme is focused under native conditions two bands are resolved with isoelectric points of 5.4 and 5.2 (Fig. 1). Both bands are active, as demonstrated by activity staining on nitrocellulose paper (results not shown), demonstrating the existence of two molecular forms of biliverdin reductase, which we term BVR I (pI 5.4) and BVR II (PI 5.2). The separation of two major bands was quite reproducible. However, in some preparations a third band (pI 5.1) was also observed (see, e.g., track 3 in Fig. 1). BVR I and BVR II have identical mobilities on SDS/polyacrylamide-gel electrophoresis, as shown in Fig. 2. The structural relationship between the two forms was examined by peptide mapping with the use of *Staphylococcus aureus* V8 proteinase. No difference between the

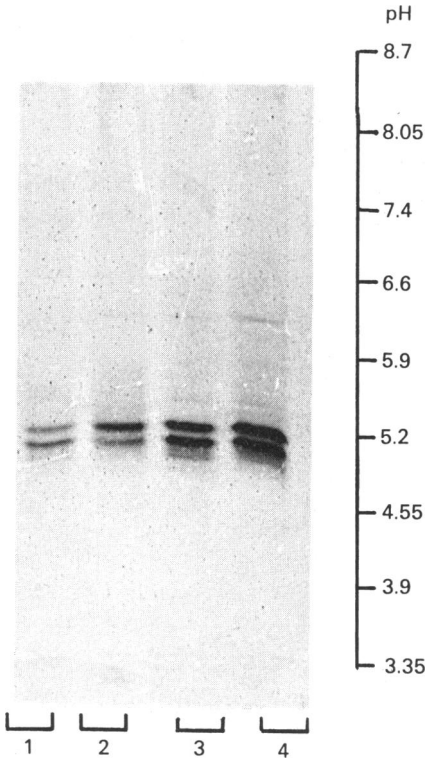

Fig. 1. Isoelectric focusing of ox kidney biliverdin reductase

Tracks 1, 2, 3 and 4 contain 6 μg of ox kidney biliverdin reductase from four preparations.

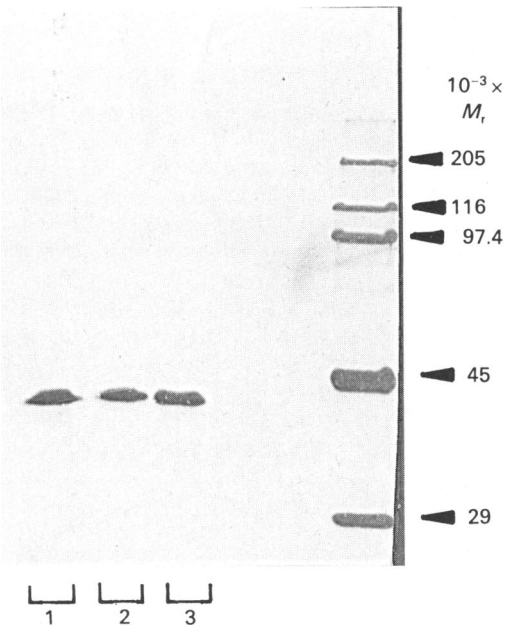

Fig. 2. SDS/polyacrylamide-gel electrophoresis of biliverdin reductase

The two forms of biliverdin reductase (BVR I and BVR II) were excised from the native isoelectric-focusing gel and incubated in 0.125 M-Tris/HCl/1 mM-EDTA/0.1% SDS for 4 h at 4 °C before SDS/polyacrylamide-gel electrophoresis. Tracks 1, 2 and 3 contain 6 μg of BVR I, BVR II and a mixture of BVR I and BVR II respectively. The mobilities of the M_r markers are indicated. The protein bands were detected by silver staining.

Table 1. Effect of 5,5′-dithiobis-(2-nitrobenzoate) on biliverdin reductase

Reaction of 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) at a final concentration of 25 μM with 2.5 μM enzyme was monitored at 412 nm in 0.1 M-sodium phosphate buffer, pH 8, containing 10 mM-EDTA. Results are shown for a single experiment as a relatively high concentration of enzyme was used. However, the values obtained are in good agreement with results obtained in a separate experiment conducted with 1.47 μM enzyme. The 'protecting' concentrations of substrates were 22 μM and 200 μM for biliverdin and $NADP^+$ respectively.

Substrate present before addition of DTNB	Thiol groups modified (mol/mol of enzyme)		Activity remaining (%)
	No SDS	SDS added	
None	1.66	2.70	0
Biliverdin	0.86	2.82	0
NADP	1.78	3.30	0
Biliverdin plus $NADP^+$	1.10	3.20	30

two forms could be detected by this technique (results not shown). In addition, we have been unable to detect any evidence for kinetic heterogeneity in our steady-state studies (E. M. Rigney & T. J. Mantle, unpublished work). Further attempts to separate BVR I and BVR II by chromatofocusing were not successful; all the activity was eluted in a symmetrical peak at pH 4.6 (results not shown). The two molecular forms of biliverdin reductase are clearly closely related, and we assume that they represent charge isomers. We have not attempted in the present work to define further the relationship between BVR I and BVR II and have conducted the following experiments with preparations containing both forms.

The amino acid composition of biliverdin reductase has been reported previously (Phillips, 1981), but unfortunately no data were presented on the number of thiol groups reactive with 5,5′-dithiobis-(2-nitrobenzoate). Table 1 shows the effect of 5,5′-dithiobis-(2-nitrobenzoate) on biliverdin reductase activity in the presence and in the absence of substrate and also the number of 5,5′-dithiobis-(2-nitrobenzoate) reactive thiol groups per subunit in the absence and in the presence of 1% SDS. It is clear that the polypeptide has three reactive thiol groups of which two are available in the native enzyme. $NADP^+$ did not protect either of the two thiol groups in the native enzyme, whereas biliverdin, either alone or in the presence of $NADP^+$, does appear to protect one of the thiol groups. Neither substrate alone was able to protect enzyme activity. However, 30% activity remained when the enzyme was incubated with 5,5′-dithiobis-(2-nitrobenzoate) in the presence of both substrates. It is unclear why biliverdin, either in the presence or in the absence of $NADP^+$, apparently protects the same thiol group but with different amounts of activity remaining. Ox kidney biliverdin reductase thus resembles the rat liver enzyme, which is also reported to contain three cysteine residues (Kutty & Maines, 1981).

The ability of biliverdin to protect one of the three thiol groups suggests the existence of an enzyme–biliverdin complex. To examine this possibility further we have utilized an earlier observation that when biliverdin is dissolved in a non-aqueous solvent, such as ethyl acetate, in the presence of a thiol a change in colour

Table 2. Species and tissue distribution of biliverdin reductase

Immunoprecipitation was carried out by incubating the respective cytosols with 10 μl of Protein A–Sepharose and 50 μl of antiserum for 6 h at 4 °C and then centrifuging the slurry in a bench centrifuge for 3 min. The supernatants were then assayed for biliverdin reductase activity. Controls in which either the antibody or Protein A–Sepharose had been omitted did not precipitate any biliverdin reductase activity. Abbreviation: N.D., not determined.

Tissue	Specific activity (nmol/min per mg)	Activity remaining after immunoprecipitation (%)
Ox kidney	0.790	0
Ox liver	0.002	0
Pig liver	0.134	0
Guinea-pig liver	0.116	0
Guinea-pig spleen	0.545	0
Guinea-pig kidney	0.401	0
Mouse liver	0.156	0
Mouse spleen	0.585	0
Mouse kidney	0.584	10
Rat liver	0.167	20
Rat spleen	0.436	N.D.
Rat kidney	0.525	19
Hamster liver	0.162	0
Hamster spleen	0.080	0
Hamster kidney	0.060	0
Fox liver	0.144	0
Fox spleen	0.335	0
Fox kidney	0.333	0
Wallaby kidney	0.275	N.D.
Wallaby liver	0.084	N.D.
Wallaby spleen	1.003	N.D.
Human liver	0.053	0

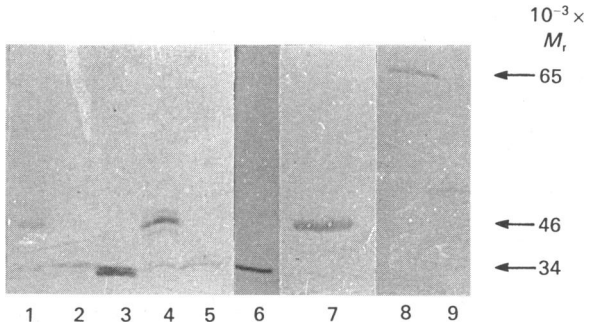

Fig. 3. Immunoblotting cytosols from different species

Cytosols from the different species listed below were immunoblotted with the use of antiserum against the ox kidney enzyme. Tracks 1–9 contained mouse liver (300 μg), mouse spleen (320 μg), mouse kidney (320 μg), rat liver (350 μg), rat spleen (350 μg), rat kidney (350 μg), human liver (350 μg), hamster kidney (160 μg) and fox liver (350 μg) respectively.

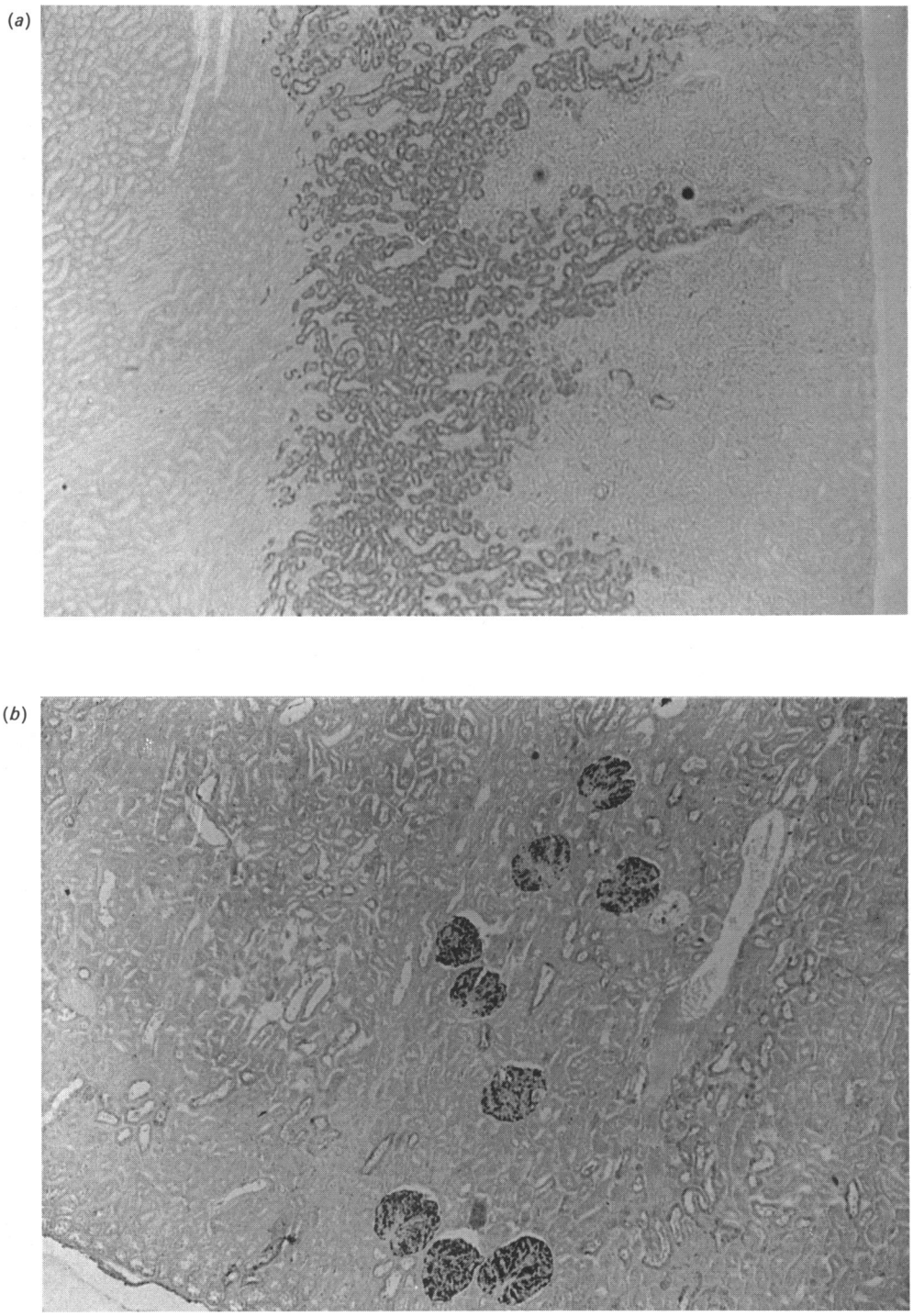

Fig. 4. Immunocytochemical staining of rat kidney and ox kidney

Staining was conducted as described in the text with the use of (*a*) anti-(biliverdin reductase) serum with rat kidney and (*b*) anti-(aldose reductase) serum with ox kidney (*b*).

from green to yellow is observed, which is due to the formation of thioether adduct at the central methylene bridge (Manitto & Monti, 1979). This colour change also occurs when biliverdin is dissolved in aqueous solution in the presence of serum albumin and a mercaptan. We have obtained similar results with dithiothreitol and GSH as mercaptans and with glutathione *S*-transferase YaYc as the biliverdin-binding protein (A. Johns, O. Phillips & T. J. Mantle, unpublished work). We attempted to obtain further evidence for biliverdin binding to the free enzyme by using biliverdin reductase as the 'biliverdin-binding protein' in similar experiments. Unfortunately when biliverdin reductase was used as the 'biliverdin-binding protein' no such effect could be

demonstrated, even in the presence of $NADP^+$. As biliverdin reductase binds biliverdin under these conditions in a ternary enzyme–$NADP^+$–biliverdin complex (Colleran & O'Carra, 1977; Phillips & Mantle, 1981), this suggests that even the smallest mercaptan we used, 2-mercaptoethanol, cannot gain access to the biliverdin-binding site. Clearly no such hindrance exists in the biliverdin-binding proteins such as serum albumin and glutathione *S*-transferase YaYc, which are known to display less selectivity towards hydrophobic ligands and can obviously accommodate the various thioether adducts.

Although biliverdin reductase is believed to be a 'mammalian' enzyme, there have been few surveys to extend the range of species studied. With the availability of antibodies to the ox kidney enzyme we have examined the distribution of this enzyme and the immunological cross-reactivity in several species. Biliverdin reductase activity was present in the kidney, spleen and liver cytosol of all species studied (Table 2), although very low activities were observed in ox liver (see also Colleran & O'Carra, 1977). Biliverdin reductase activity from cytosols of liver, kidney and spleen of the species listed in Table 2 was immunoprecipitated by antibodies to the ox kidney enzyme with the use of Protein A–Sepharose 6B. Under the conditions used 100% immunoprecipitation was achieved in all of the samples with the exception of mouse kidney, rat liver and rat kidney, where 10–20% of the activity remained in the supernatant. Possibly a fraction of biliverdin reductase in mouse kidney, rat liver and rat kidney is immunologically unrelated to the ox kidney enzyme.

Cytosols from the various tissues were also analysed by immunoblotting. From most species cross-reacting material with a subunit M_r of 34000–46000 is clearly seen (Fig. 3). However, hamster kidney (track 7 in Fig. 3) and guinea-pig liver (results not shown) express cross-reacting material with significantly larger M_r values (65000 and 85000 respectively). O'Carra & Colleran (1977) have reported that the guinea-pig enzyme has an M_r of 70000, and the present result is consistent with that observation. Interestingly mouse and rat tissues behaved identically in that liver from both these species contain two cross-reactive proteins with M_r values of 34000 and 46000. Spleen and kidney from both these tissues express only the protein of M_r 34000. It is unclear at the present time whether the proteins of M_r 34000 and 46000 correspond to forms 1 and 2 described by Frydman *et al.* (1983), although in support of this these workers have reported that only their form 1 (M_r 34000) is present in kidney and spleen.

We have studied the localization of biliverdin reductase and aldose reductase in the kidney, liver and spleen of the ox, rat and human. In the rat only the proximal tubules of the inner cortex are stained with anti-(biliverdin reductase) serum (Fig. 4*a*). Interestingly a similar distribution has been noted for glutathione *S*-transferase YfYf and glutathione *S*-transferase YbYb in rat and mouse kidney respectively (Boyce *et al.*, 1987). It is tempting to speculate that these intracellular binding proteins play a functional role with regard to maintaining a low free concentration of biliverdin (Phillips & Mantle, 1981) to allow biliverdin reductase to operate efficiently in these tubules. Biliverdin reductase antiserum also stains proximal tubules in human and ox kidney (results not shown). With antisera to aldose reductase with rat kidney we obtained intense staining of the inner medulla (thin limbs of the loop of Henle and collecting tubules) and weak staining of the inner cortex and glomerular podocytes. A similar finding has been reported by Ludvigson & Sorenson (1980). Similar results were obtained with anti-(aldose reductase) serum staining of human kidney, although there is no precise counterpart of the rat/mouse inner cortical zone in this case. Interestingly the glomerular podocytes of the ox kidney stained very intensely with anti-(aldose reductase) serum (Fig. 4*b*), in contrast with the very weak staining seen with this structure in the rat and human. In the spleen the red pulp but not the splenic nodules stained with biliverdin reductase antiserum (results not shown). This is not surprising as the nodules are not recognized sites of erythrocyte degradation.

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REFERENCES

Baird, J. K., Sherwood, R. F., Carr, R. J. C. & Atkinson, A. A. (1976) FEBS Lett. **70**, 61–68

Boyce, S. J., McBennett, S. M., Mantle, T. J. & Hayes, J. D. (1987) in Glutathione *S*-Transferases and Carcinogenesis (Mantle, T. J., Pickett, C. B. & Hayes, J. D., eds.), pp. 69–72, Taylor and Francis, London, New York and Philadelphia

Colleran, E. & O'Carra, P. (1977) in DHEW Publ. (NIH) U.S. no. 77-1100: Chemistry and Physiology of Bile Pigments (Berk, P. D. & Berlin, N. I., eds.), pp. 69–80, U.S. Department of Health, Education and Welfare, Washington

Daly, A. K. & Mantle, T. J. (1982) Biochem. J. **205**, 373–380

Frydman, R. B., Tomaro, M. L., Awruch, J. & Frydman, B. (1983) Biochim. Biophys. Acta **759**, 257–263

Kutty, R. K. & Maines, M. D. (1981) J. Biol. Chem. **256**, 3956–3962

Laemmli, U. K. (1970) Nature (London) **227**, 680–685

Ludvigson, M. A. & Sorenson, R. L. (1980) Diabetes **29**, 450–459

Manitto, P. & Monti, D. (1979) Experientia **35**, 1418–1420

McDonagh, A. F. (1979) in The Porphyrins (Dolphin, D., ed.), vol. 6, pp. 453–455, Academic Press, London

McDonagh, A. F., Palma, L. A. & Schmid, R. (1981) Biochem. J. **194**, 273–282

Noguchi, M., Yoshida, T. & Kikuchi, G. (1979) J. Biochem. (Tokyo) **86**, 833–848

O'Carra, P. & Colleran, E. (1971) Biochem. J. **125**, 110P

Okazaki, K., Nishimura, H., Arizono, H., Nishimura, N. & Suzuki, Y. (1978) Biochem. Biophys. Res. Commun. **81**, 512–520

Phillips, O. (1981) Ph.D. Thesis, University of Dublin

Phillips, O. & Mantle, T. J. (1981) Biochem. Soc. Trans. **9**, 275–278

Phillips, O., Mantle, T. J., Tuffery, A. R., Heyworth, C. M., Wilson, S. R. & Houslay, M. D. (1984) Biochem. Pharmacol. **33**, 1963–1967

Sternberger, L. A., Hardy, P. H., Cuculis, J. J. & Meyer, H. G. (1970) J. Histochem. Cytochem. **18**, 315–333

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

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