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1 J. P. EBEL, *Bull. Soc. Chim. Biol.*, 34 (1952) 321.
2 O. H. SCHERBAUM AND E. ZEUTHEN, *Exptl. Cell. Res.*, 6 (1954) 221.
3 W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques*, Burgess Publishing Co., Minneapolis, Minn., U.S.A., 1957, p. 273.
4 P. PLESNER, *Biochim. Biophys. Acta*, 29 (1958) 462.
5 O. H. SCHERBAUM, S. C. CHOU, K. H. SERAYDARIAN AND J. E. BYFIELD, *Can. J. Microbiol.*, 8 (1962) 75.

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Fractional solubilization of haemoproteins and partial purification of carbon monoxide-binding cytochrome from liver microsomes

Recent work in this laboratory[1,2] has demonstrated that the CO-binding pigment discovered in liver microsomes[3,4] is in fact a new cytochrome of the *b* type. This cytochrome shows anomalous spectra in the microsomal bound form, but behaves as a typical haemoprotein when converted to a solubilized form. The microsomal bound and solubilized forms of the pigment have been tentatively called P-450 and P-420, respectively, to distinguish them from each other. This pigment has, however, not yet been separated from cytochrome b_5, another microsomal haemoprotein, and its properties have so far been studied only by means of difference spectrophotometry. This paper reports successful separation of the two microsomal haemoproteins by fractional solubilization and briefly describes partial purification of P-420. The microsomes used were prepared from rabbit liver by a modification[2] of the method of MITOMA *et al.*[5].

As already reported[1,2], anaerobic treatments of microsomes with heated or une heated venom of the snake, *Trimeresurus flavoviridis*[6], or with deoxycholate led to almost quantitative solubilization of both cytochrome b_5 and the CO-binding cytochrome, the latter being fully converted to the form of P-420. As can be seen from Table I, however, digestion of microsomes with crude pancreatic lipase ("steapsin") under suitable conditions resulted in the quantitative solubilization of only cytochrome b_5. Most of the CO-binding cytochrome thereby remained attached to the undigested particles and could be sedimented by centrifugation at 105000 × *g* for 60 min. The sediment, free from cytochrome b_5, represented about half of the microsomal protein and will be referred to as CO-binding particles. In these particles the CO-binding pigment existed both in the forms of P-450 and P-420 as evidenced by the appearance of two absorption maxima at 452 mμ and 421 mμ on addition of CO to dithionite-reduced particles, corresponding to the formation of CO compounds of

TABLE I

FRACTIONAL SOLUBILIZATION OF HAEMOPROTEINS FROM LIVER MICROSOMES

Rabbit-liver microsomes (final concn., 6–7 mg protein/ml) in 0.1 M phosphate buffer were treated anaerobically at 37°. Heated snake venom was prepared as described previously[6]. Per cent solubilization was estimated based on the amount not sedimentable by centrifugation at 105000 × *g* for 60 min. Protohaem and cytochrome *b*1 were determined as in a previous paper[2]. P-450 and P-420 were estimated from the CO-difference spectra of dithionite reduced samples using the molar extinction coefficients reported in the text. Protein was determined by the method of LOWRY *et al.*[8].

Solubilization treatment	*% Solubilization*				*P-450* %	*P-420* %
	Protein	*Protohaem*	*Cytochrome* b_5	*CO-binding cytochrome*		
0.07% steapsin (pH 7.5, 60 min)	46.3	31.5	79.1	8.3	48.9	51.1
0.14% steapsin (pH 7.5, 60 min)	57.1	41.7	98.3	12.5	33.5	66.5
0.1% heated snake venom (pH 8.5, 20 min)	88.0	95.0	98.0	97.0	0	100

P-450 and P-420 respectively. It was, therefore, suggested that the CO-binding cytochrome which is still bound to particulate structures can take the form of P-420 if the microsomal constitution suffers considerable alterations by enzymic digestion.

The cytochrome in the CO-binding particles was found to be labile to oxygen; P-420 was much more rapidly decomposed than P-450. The decomposition was accompanied by a corresponding decrease in protohaem content. It is likely, as has been suggested[2], that the aerobic peroxidation of microsomal lipids[7] is responsible for the degradation of protohaem. In a nitrogen atmosphere the CO-binding particles could be stored for several days without any loss of the CO-binding cytochrome.

When the CO-binding particles were treated anaerobically with 0.1% heated snake venom or 0.1% deoxycholate or both (pH 8.5, 37°, 20 min), the CO-binding cytochrome was fully converted to the form of P-420 and at the same time brought into solution. The cytochrome solubilized by the combined action of heated snake venom and deoxycholate could be purified by ammonium sulphate fractionation (precipitate between 20 and 40% saturation), gel filtration through Sephadex, and hydroxylapatite column chromatography. The preparation thus obtained represented 4- to 6-fold purification over microsomes and contained 6–7 mμmoles of protohaem/mg of protein. Unlike the CO-binding particles, the purified cytochrome was quite stable to aeration probably owing to the removal of unsaturated lipids during purification. Although this preparation behaved as a homogeneous protein in electrophoresis, it was still heterogeneous in ultracentrifugal analysis.

The absence of cytochrome b_5 in this preparation now permitted direct measurements of absolute spectra of P-420. In Fig. 1 are shown absorption spectra of the oxidized and reduced forms as well as the CO compound of purified P-420. It may be seen that the reduced form shows α, β and Soret bands at 559, 530 and 426 mμ, respectively, and the general shape of the spectrum is characteristic of a cytochrome of the *b* type. On combination with CO, the Soret peak of reduced cytochrome shifts to 422 mμ and is considerably intensified.

It was possible from these spectra to calculate molar extinction coefficients on protohaem basis. The following values (in terms of mM^{-1} cm^{-1}) were thus obtained: Oxidized form (at 414 mμ), 124; reduced form (at 426 mμ), 149; CO compound

(at 422 mμ), 213; CO compound (increment between 420 and 490 mμ), 111. From the last-mentioned value and the change in CO-difference spectrum accompanying the conversion of P-450 to P-420 by anaerobic digestion of microsomes[1,2], it was further possible to estimate the increment in molar extinction coefficient between 450 and 490 mμ of the CO compound of microsomal-bound P-450. The value thus obtained was 91 mM^{-1} cm^{-1}. Using this value, the content of P-450 in a microsomal preparation was determined to be 1.55 mμmole/mg of protein, whereas the same preparation was found to contain, per mg of protein, 1.12 and 2.55 mμmoles of cytochrome b_5 and protohaem, respectively. These data indicate that all of the microsomal haem can be accounted for by cytochrome b_5 and the CO-binding cytochrome.

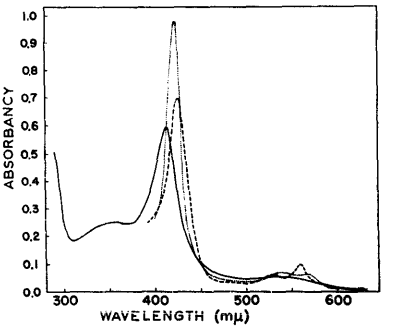

Fig. 1. Absorption spectra of purified P-420. Protein, 0.67 mg/ml. Protohaem, 4.6 mμmoles/ml 0.1 M phosphate buffer (pH 7.0). ———, oxidized form; – – –, reduced form ($Na_2S_2O_4$); ·····, CO compound of reduced cytochrome.

Although P-450 in microsomes and P-420 in anaerobically digested microsomes can be reduced nicotinamide-adenine dinucleotides, these reagents were unable to reduce the purified preparation of P-420. It is likely that a reductase or reductases catalysing these reactions have been removed during purification.

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[1] T. OMURA AND R. SATO, *J. Biol. Chem.*, 237 (1962) PC1375.
[2] T. OMURA AND R. SATO, *J. Biol. Chem.*, submitted for publication.
[3] M. KLINGENBERG, *Arch. Biochem. Biophys.*, 75 (1958) 376.
[4] D. GARFINKEL, *Arch. Biochem. Biophys.*, 77 (1958) 493.
[5] C. MITOMA, H. S. POSNER, H. C. REITZ AND S. UDENFRIEND, *Arch. Biochem. Biophys.*, 61 (1956) 431.
[6] Y. IMAI AND R. SATO, *Biochim. Biophys. Acta*, 42 (1960) 164.
[7] A. L. TAPPEL AND H. ZALKIN, *Nature*, 185 (1960) 35.
[8] O. H. LOWRY, N. J. ROSENBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

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