Biochem ical Pharmacology, Vol. 20, pp. 15–22. Pergamon Press, 1971. Printed in Great Britain

EVIDENCE FOR A DIFFERENT CO-BINDING PIGMENT IN SOLUBILIZED RAT HEPATIC MICROSOMES*†

LORA E. RIKANS and RUSSELL A. VAN DYKE

The Dow Chemical Company, Midland, Mich. 48640, and
The Mayo Clinic, Rochester, Minn. 55901, U.S.A.

(*Received* 17 *July* 1969; *accepted* 17 *April* 1970)

Abstract—Rat liver microsomes were solubilized by treatment with deoxycholate. Chromatographic separation of the solubilized solution was accomplished on a DEAE-cellulose column eluted with a linear gradient of KCl in buffer. The effluent contained three protein peaks. The first protein peak contained hemoglobin. Cytochrome P-450 was associated with protein peak 2, cytochrome b_5 was associated with peak 3, and the cytochrome c reductase activity appeared between these two peaks. The area defined by the cytochrome c reductase activity contained a different CO-binding pigment which produced absorption maxima at 418, 522 and 552 mμ in the reduced state. This cytochrome was shown to be distinguishable from P-450, P-420, cytochrome b_5 and cytochrome c.

ENDOPLASMIC reticular membranes contain enzymes responsible for a large number of reactions involving the hydroxylation of drugs.[1] At least two components, cytochrome P-450 and NADPH cytochrome c reductase, are generally believed to be involved in the hydroxylating system. Cytochrome P-450 is the "CO-binding pigment" described by Klingenberg[2] and Garfinkel[3] in 1958 and later characterized by Omura and Sato.[4] Early studies demonstrating the inhibition of hydroxylating reactions by carbon monoxide indicated that P-450 was a component of the hydroxylating system.[5,6] Furthermore, P-450 content and NADPH cytochrome c reductase content were both increased in conjunction with an increase in overall hydroxylating activity upon treatment of animals with drugs.[7]

In addition to P-450, microsomes also contain cytochrome b_5. Cytochrome b_5 and NADH cytochrome b_5 reductase were isolated and purified from microsomes before P-450 was known,[8,9] although the biological significance of these microsomal components remains obscure.

Recent advances in solubilizing microsomes[10–13] led us to solubilize the components of the microsomal electron transport system and study them in more detail. This paper reports data on the cytochromes and cytochrome reductases found in our studies.

EXPERIMENTAL

Livers from male Sherman rats were perfused with saline and homogenized in three parts of 1·15 per cent KCl at 5°. The homogenate was centrifuged at 9000 *g* for 20 min. The 9000 *g* supernatant was then centrifuged at 105,000 *g* for 1 hr and the microsomal pellet was suspended in 0·25 M sucrose. Microsomes were solubilized according to the

* Supported in part by USPHS, NIGMS Contract No. PH 43-67-666.
† A preliminary report of these studies has appeared in *Fedn Proc.* **28**, 484 (1969).

method of Lu and Coon.[13] Centrifugation of the solubilized preparation for 2 hr at 105,000 *g* yielded a clear supernatant solution. A portion of the clear supernatant containing a total of 65 mg protein was diluted with two parts of buffer and placed on a DEAE-cellulose column (1·8 × 30 cm) which had been equilibrated with 0·05 M Tris buffer, pH 7·5, containing 10^{-4} M dithiothreitol and 0·05 per cent sodium deoxycholate. The column was eluted with a linear gradient of KCl from 0 to 0·35 M in the same buffer and 10-ml fractions were collected. Operations were carried out in the dark at 5°. Fractions collected from the column were wrapped in a dark cloth and kept refrigerated until the time of assay.

The column eluates were assayed for protein, cytochrome and cytochrome reductase concentrations. Protein was determined by measuring absorbance at 280 mμ or by the method of Lowry *et al.*[14] Cytochrome P-450 was measured in the CO difference spectrum of a dithionite-reduced sample using 91 $mM^{-1}cm^{-1}$ as the extinction coefficient for the difference in absorbance between 450 and 490 mμ.[4] Cytochrome b_5 was measured from the difference spectrum of the NADH-reduced sample using 165 $\mu M^{-1}cm^{-1}$ as the extinction coefficient for the difference in absorbance between 424 and 409 mμ.[9] The activity of NADPH cytochrome c reductase was measured by following the change in absorbance at 550 mμ as a function of time.[15] Estimation of the cytochrome in column fractions 33 through 35 was made by determining the increase in absorbance at 418 mμ upon reduction with dithionite. A Shimadzu MPS-50L spectrophotometer was used to record absolute and difference spectra.

Spectral data of cytochrome c were obtained from horse heart cytochrome c, type VI, purchased from the Sigma Chemical Company. Reduction by NADPH and reaction with aminopyrine were tested by adding 0·5 ml of the fraction containing the greatest amount of cytochrome c reductase activity to cuvettes containing cytochrome c and buffer. Either NADPH or aminopyrine was then added to the test cuvette and spectra were recorded.

RESULTS AND DISCUSSION

The pattern of the elution of the cytochromes, their reductases and proteins from the column is shown in Fig. 1. Three protein peaks were eluted from the column as determined by measuring absorbance at 280 mμ.

The Lowry method[14] of protein assay confirmed the presence of the three protein peaks, since by this method the protein concentrations per fraction at the three peaks were: 2·0–3·0, 4·0–6·0, and 1·8–2·2 mg protein respectively. Absolute spectra of the fraction in the area of the third protein peak indicated the presence of nucleic acids. Repeated experiments were run and variations in the elution patterns were seen and were the result of several factors: (1) hemoglobin was present in protein peak I and the quantity of this was dependent on the efficiency of the initial liver perfusion, since the perfusion was carried out to remove as much hemoglobin as possible; (2) the amount of protein added to the column dictated the resolution of the peaks, i.e. if the protein concentration was much above that indicated above in the methods, the resolution was very poor; (3) there were slight variations in peak heights and elution volumes, which could be traced to slight variations in the pH and KCl concentration; and (4) large amounts of P-420 tended to interfere and, therefore, care was required to keep the conversion of P-450 to P-420 at a minimum.

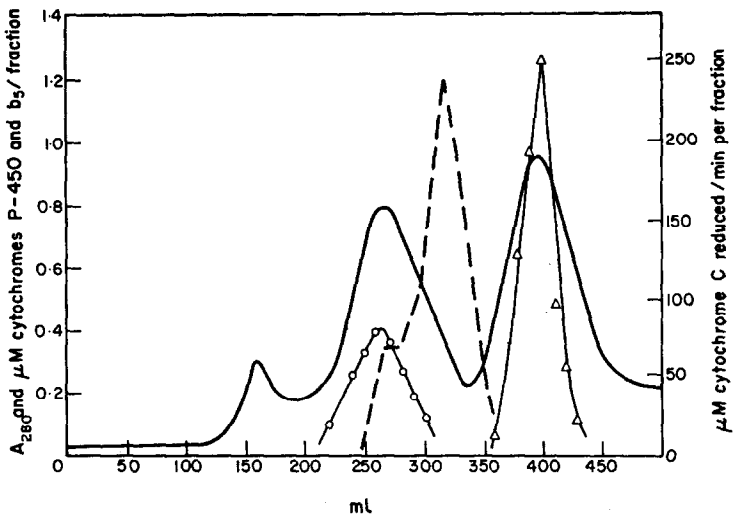

FIG. 1. Elution pattern of solubilized microsomal components separated chromatographically. A clear solution of solubilized microsomes was placed on a DEAE-cellulose column (1·8 × 30 cm). The column was eluted with a linear gradient of KCl from 0 to 0·35 M in buffer, and 10-ml fractions were collected. Protein is shown as A_{280} (——) in the undiluted eluate. Cytochromes P-450 (—o—), b_5 (—△—), and NADPH cytochrome c reductase activity (– – –) were assayed according to the methods listed in the text.

The fraction eluting at 150 ml (first protein peak) contained hemoglobin and there was no detectable reductase activity associated with this peak. The second protein peak correlated with the presence of P-450 and P-420, with P-450 eluting between 220 and 310 ml. Cytochrome b_5 was eluted in the third protein peak, between 360 and 430 ml. Assay for cytochrome b_5, using dithionite as the reducing material rather than NADH, gave an elution pattern identical to that shown in Fig. 1.

Figure 1 also shows the elution of the NADPH-cytochrome c reductase activity. A shoulder around 260 ml was consistently found for which there is no apparent explanation except that it may represent another cytochrome reductase. There was present a NADH-dependent reductase associated with the cytochrome b_5 fraction, since the cytochrome b_5 assay depends on the presence of this enzyme.

The region defined by the cytochrome c reductase activity contained another material which had the spectral properties of a cytochrome and which was distinguishable from the known cytochromes. This material, which we have tentatively considered as a cytochrome, produced absorption maxima at 410 mμ in the oxidized state and at 418, 522 and 552 mμ in the reduced state. Its spectral properties were distinctly different from those of P-450, P-420 or cytochrome b_5.[10] This cytochrome-like material was detected in fractions 33 through 35, the area of the cytochrome c reductase activity. It reacted with carbon monoxide and was reducible with NADPH as well as dithionite.

The absolute spectra of the fractions containing the greatest quantity of each cytochrome are shown in Fig. 2. Number 26 is the fraction which contained P-450. Its reduced spectrum had only one distinct maximum at 424 mμ. Fraction 34 contained the different type of cytochrome, and fraction 39 contained cytochrome b_5. Cytochrome P-420 (not shown) was eluted on the trailing edge of the P-450 peak. Its reduced

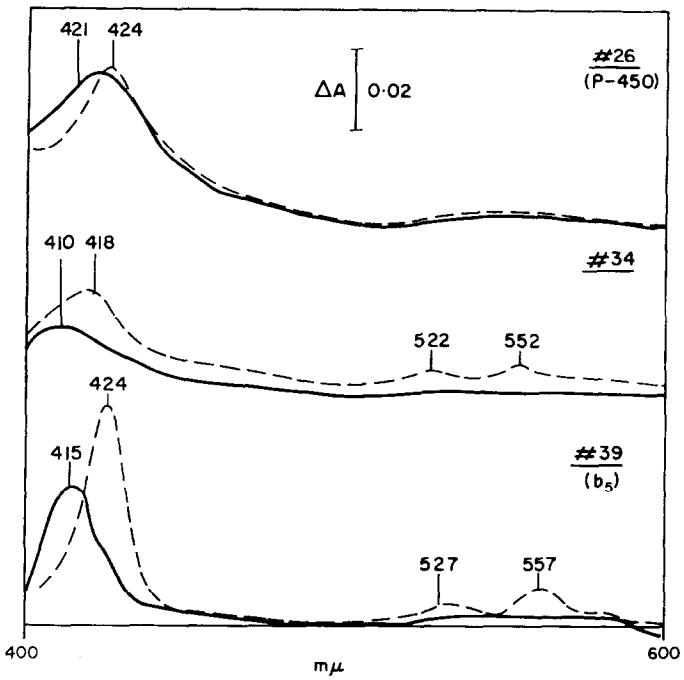

FIG. 2. Absolute spectra of column fractions containing cytochromes in the oxidized (—) and dithionite-reduced (---) states. See Fig. 1.

spectrum contained absorption maxima at 428, 530 and 559 mμ. P-420 was easily measured in the presence of P-450 and vice versa if CO was used to quantitate. However, since P-420 was eluted near fractions 33–35, if it was in high concentration, it interfered with the quantitation of the cytochrome in those fractions.

Table 1 lists results of the assays for the cytochromes versus the column fractions in which they appeared. There was apparently little overlap of these cytochromes as they came off the column. However, assay of the fraction 34 cytochrome depended on good separation from the other cytochromes (P-450, P-420 and b_5), since the presence of the other cytochromes could mask its characteristic spectrum. Masking by P-450 and b_5 also prevented the determination of the cytochrome in the original solubilized microsomes.

Figure 3 contains a difference spectrum of fraction 34 with aminopyrine in the sample cuvette and no additions to the reference cuvette. This spectrum was identical to the dithionite-reduced absolute spectrum (Fig. 2). However, neither aminopyrine nor aniline produced spectral change with the P-450 fractions in our studies. Other investigators have found conventional substrate-induced spectral changes with solubilized microsomal preparations.[12,16] At the present time, we are unable to explain the lack of similar substrate-induced spectral changes in our preparation, although it may be related to the nature of the solubilized material, that is, whether or not it is particulate and, if so, the relative size of the particles.

Since the fraction 34 cytochrome behaved very similarly to cytochrome c, attempts were made to differentiate between them. Absorption spectra from oxidized and reduced cytochrome c were recorded and compared to those of the fraction 34 cytochrome.

TABLE 1. RESULTS OF CYTOCHROME ASSAYS VERSUS COLUMN FRACTION*

Fraction	ΔA_{418}	NADH difference $A_{424-410}$	CO difference $A_{450-490}$
23			0·03
24			0·04
25			0·05
26			0·06
27			0·05
28			0·04
29			0·03
30			0·02
31			
32			
33	0·04		
34	0·06		
35	0·04		
36		0·02	
37			
38		0·18	
39		0·24	
40		0·32	
41		0·14	
42		0·06	
43		0·02	
44			

* A solution of solubilized microsomes was placed on a DEAE-cellulose column. The column was eluted with KCl in a linear gradient from 0 to 0·35 M in buffer and the 10-ml fractions collected were assayed for cytochrome content. Cytochrome P-450 was measured in the CO difference spectrum of dithionite-reduced sample and cytochrome b_5 from the difference spectrum of NADH-reduced sample. Estimation of the cytochrome in fractions 33-35 was made by determining the increase in absorbance at 418 mμ upon reduction with dithionite.

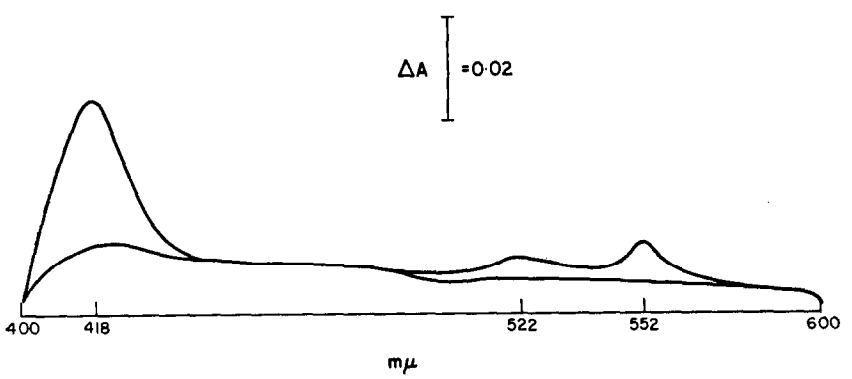

FIG. 3. Difference spectrum of column fraction 34 with aminopyrine. See Fig. 1.

The absorption maxima at 416, 521 and 550 mμ from reduced cytochrome c did not correspond to those obtained from fraction 34. Furthermore, the spectrum obtained from cytochrome c (in the presence of cytochrome c reductase) with addition oi aminopyrine was again different from that of fraction 34 with aminopyrine added.

Table 2 lists the reactions of selected column fractions and cytochrome c with carbon monoxide and aminopyrine. Fraction 34 reacted with both materials, whereas the other cytochromes reacted with only one or the other. The carbon monoxide reaction of the cytochrome from fraction 34 was different from that obtained with P-450. Carbon monoxide, when added to a cuvette containing reduced fraction 34, produced a slight spectral shift of the Soret band to around 420 mμ. Aminopyrine, when added to fraction 34, produced a spectrum identical with the reduced spectrum of the fraction 34 cytochrome. This was similar to the reaction of aminopyrine with the cytochrome b_5 fraction or with cytochrome c in the presence of reductase. The spectra obtained were the reduced spectra of the respective cytochromes.

TABLE 2. REACTIONS OF SELECTED COLUMN FRACTIONS AND CYTOCHROME c WITH CO AND AMINOPYRINE*

Column fraction	Reaction with	
	CO	Aminopyrine
26 (Cyt. P-450)	+	−
34	+	+
39 (Cyt. b_5)	−	+
Cyt. c	−	+

* A solution of solubilized microsomes was separated by column chromatography (see Table 1). The fractions listed represent those containing the greatest quantity of each cytochrome.

Further evidence that the cytochrome in fraction 34 was different from the others was its instability. Under usual assay conditions, any additions and the recording of the resulting spectrum are carried out as rapidly as possible. In one experiment, Fig. 4, the cytochrome was reduced by solid NADPH. CO and dithionite were added subsequently, and throughout the experiment, which lasted for several minutes, decreases in overall absorbance were recorded. Since decreases in absorbance of other cytochromes do not usually occur with the addition of these reagents, this indicated a rapid deterioration in this cytochrome. The preparations studied were not uniformly unstable. Figure 4 is an example of a highly unstable preparation; however, not all preparations were this unstable, but all did decay in a period of hours in spite of attempts to stabilize them. Because of this instability, not all of the various spectra of the cytochrome in fraction 34 could be obtained with the material for a single column run. The presence of this cytochrome and its instability have been confirmed by other

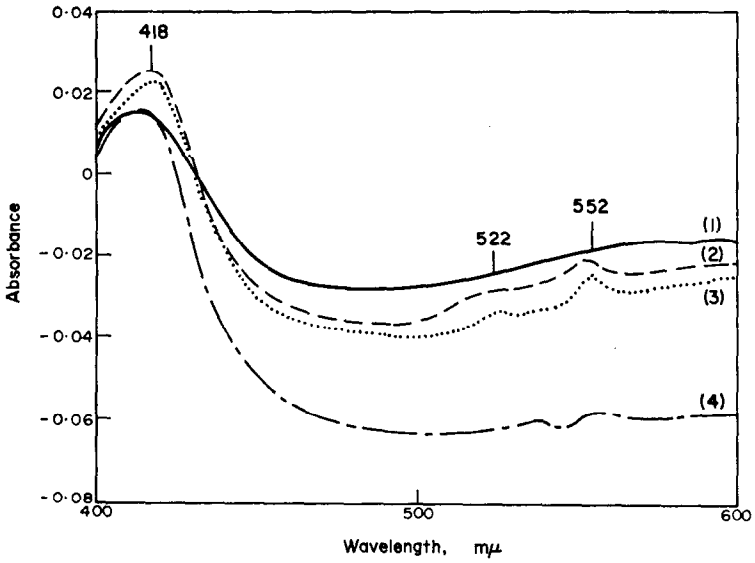

FIG. 4. Absolute spectra of column fraction 34 with (1) no additions, (2) solid NADPH, (3) CO, and (4) solid dithionite added sequentially to the sample cuvette and no additions to the reference cuvette. The difference in time between 1 and 4 exceeds by several minutes the time interval between the oxidized and reduced spectrum in Fig. 2.

investigators.* Using the same techniques, they have found a cytochrome with the same spectral characteristics as those reported in this communication and have found it to have a half-life of 3 hr after elution from the column.

The present communication suggests that there may be a cytochrome other than b_5 and P-450 present in microsomes, as have other reports which have appeared recently.[17,18] However, alternative explanations may be offered as interpretations of these data. The possibility that the data obtained in these experiments may have been the result of a contaminating hemoprotein from a nonmicrosomal source cannot be completely dismissed. For example, succinoxidase determinations revealed a mitochondrial contamination of less than 7 per cent in the microsomal preparations used. However, it was felt that any mitochondrial hemoprotein would not have been present in sufficient quantities to produce the spectra observed. Furthermore, the cytochrome described here has spectral characteristics different from any of the known mitochondrial cytochromes. In addition, when either cell supernatant or diluted rat blood was applied to the column, all the protein was found in the first protein peak and no fraction 34 cytochrome was found.

Another explanation for these data is the possibility that the cytochrome reported here is an altered form of an already known microsomal cytochrome. There is evidence that the unusual spectral properties of P-450 are maintained by hydrophobic interactions.[10,16] Thus, the solubilizing procedures may change these interactions to yield a cytochrome with characteristics which are different from those found when the cytochrome is *in situ*. If this is the case, when one attempts to isolate the cytochrome, one should use different spectral criteria in the isolated state versus those in the membrane state.

* J. L. Gaylor, personal communication.

REFERENCES

1. B. B. BRODIE, J. R. GILLETTE and B. N. LADU, *A. Rev. Biochem.* **27**, 427 (1958).
2. M. KLINGENBERG, *Archs Biochem. Biophys.* **75**, 376 (1958).
3. D. GARFINKEL, *Archs Biochem. Biophys.* **77**, 493 (1958).
4. T. OMURA and R. SATO, *J. biol. Chem.* **239**, 2379 (1964).
5. S. ORRENIUS, G. DALTNER and L. ERNSTER, *Biochem. biophys. Res. Commun.* **14**, 239 (1964).
6. T. OMURA, R. SATO, D. Y. COOPER, O. ROSENTHAL and R. W. ESTABROOK, *Fedn Proc.* **24**, 1181 (1965).
7. S. ORRENIUS, J. L. E. ERICSSON and L. ERNSTER, *J. Cell Biol.* **25**, 627 (1965).
8. C. F. STRITTMATTER and E. G. BALL, *Proc. natn. Acad. Sci. U.S.A.* **38**, 19 (1952).
9. P. STRITTMATTER and S. F. VELICK, *J. biol. Chem.* **221**, 277 (1956).
10. H. NISHIBAYASHI and R. SATO, *J. Biochem., Tokyo* **63**, 766 (1968).
11. J. MIYAKE, J. L. GAYLOR and H. S. MASON, *J. biol. Chem.* **243**, 5788 (1968).
12. M. KATAGIRI, B. N. GANGULI and I. C. GUNSALUS, *J. biol. Chem.* **243**, 3543 (1968).
13. A. Y. H. LU and M. J. COON, *J. biol. Chem.* **243**, 1331 (1968).
14. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
15. L. ERNSTER, *Acta chem. scand.* **12**, 600 (1958).
16. Y. ICHIKAWA and T. YAMANO, *Biochim. biophys. Acta* **147**, 518 (1967).
17. N. E. SLADEK and G. J. MANNERING, *Molec. Pharmac.* **5**, 186 (1969).
18. W. LEVIN and R. KUNTZMAN, *Fedn Proc.* **28**, 483 (1969).