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Apparent conversion of placental cytochrome P-420 to P-450 with *p*-chloromercuribenzoate*

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SPECTROPHOTOMETRIC and biochemical studies have indicated the presence of a carbon monoxide (CO)-binding cytochrome with an absorption maximum near 450 nm in both microsomal and mitochondrial subfractions of human placental homogenates.[1-5] A degraded form of this CO-complex has been designated cytochrome P-420 and, depending on the method of analysis, reportedly is observable as an absorption maximum in the 420–430 nm region in both human placental[1,4,5] and rat hepatic[6] microsomes. The degradation of hepatic microsomal cytochrome P-450 to the inactive P-420 form also is enhanced by sulfhydryl reagents such as *p*-chloromercuribenzoate (PCMB).[7] By contrast, polyols and reduced glutathione appear to stabilize the hepatic microsomal cytochrome.[7]

During investigations of placental CO-binding cytochromes,[4] we observed that low concentrations of PCMB seemed to reconvert cytochrome P-420 to cytochrome P-450 in human placental mitochondrial fractions. Therefore, this study was designed to investigate various factors such as temperature, protein concentration, ratios of P-420 to P-450 and stabilizing agents that might be required for this apparent conversion phenomenon and to draw comparisons between the effects of such factors on human placental mitochondrial vs rat hepatic microsomal CO-binding pigments.

Human term placentas were obtained from the delivery room of the University Hospital, University of Washington, and immediately transported to our laboratory where homogenization and differential centrifugation procedures were carried out as described by Juchau and Smuckler[8] (schedule 3). Hepatic microsomes were prepared from the livers of adult male Sprague–Dawley rats according to methods described by Mazel.[9] Cytochrome b_5 was prepared and purified from New Zealand rabbit liver microsomes according to the procedure of Strittmatter.[10]

Analyses of difference spectra were performed with a model DW-2 recording spectrophotometer (American Instrument Co.). Each spectrum was calibrated against the absorption maximum of reduced

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cytochrome c at 550 nm. The methods described by Greim[11] (reduced-CO minus CO) and also by Omura and Sato[12] (reduced-CO minus reduced) both were utilized for analyses of human placental mitochondria and rat hepatic microsomes. Samples were reduced with excess sodium hydrosulfite. Protein determinations were performed by the method of Lowry *et al.*[13]

All chemicals utilized in the investigation were of analytical grade. Equine hemoglobin was purchased from CalBiochem Co., Los Angeles, Calif. Carbon monoxide (99·7 per cent pure) was obtained from the Matheson Co., Inc., Joliet, Ill.

A typical reduced-CO minus reduced difference spectrum of a preparation of human placental mitochondria (resuspended in 0·1 M potassium phosphate buffer, pH 7·4) is given in Fig. 1. The figure also illustrates the conversion of cytochrome P-450 to cytochrome P-420 as a result of thermal degradation at 37°. By this method, the absorption maximum of the cytochrome P-450–CO complex appeared at 454 nm as also was observed and discussed in earlier experiments.[4] The position of the absorption maximum at 420 nm did not change upon thermal degradation. Figure 2 illustrates the same experiments except that reduced-CO minus CO difference spectra were analyzed In these experiments, the cytochrome P-450–CO complex absorbed maximally at 450 nm and the position of the second absorption maximum shifted from 432 nm to approximately 426 nm upon thermal degradation.

A reduced-CO minus CO difference spectrum of human placental mitochondria and the apparent conversion of cytochrome P-420 to P-450 by PCMB in a concentration-dependent fashion are illustrated in Fig. 3 These mitochondria were resuspended in a 0·05 M potassium phosphate buffer solution (pH 7·34) containing 10^{-4} M dithiothreitol (final concentration) and 20% (v/v) glycerol. The cytochrome P-450 present in human placental mitochondria was stable in this solution, i.e only minimal conversion to P-420 was noted during a period of 3 days at 4 Rat hepatic microsomal cytochrome P-450 also exhibited similar stability under the same conditions. The addition of PCMB to placental mitochondrial preparations which were resuspended either in 0·05 M potassium phosphate buffer (pH 7·35) or in 0·05 M potassium phosphate buffer (pH 7·35) containing 10^{-4} M dithiothreitol did not result in the apparent conversion of cytochrome P-420 to P-450. However, this spectral shift was observed if placental mitochondria were resuspended in a 0 05 M potassium phosphate buffer solution (pH 7·35) containing only 20% glycerol (v/v), indicating that dithiothreitol was not necessary for the observation of the spectral change. A similar concentration dependency was observed with respect to PCMB Conversion could not be demonstrated unless PCMB were added to the cuvettes, i.e. glycerol alone did not appear capable of producing the spectral shift in any of these experiments The spectral shift could not be observed when concentrations of less than 1×10^{-6} M

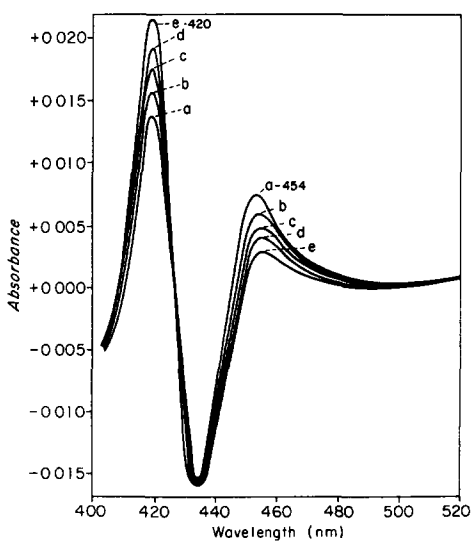

FIG. 1. CO-reduced minus reduced difference spectra of human term placental mitochondria. In curve a, the reference cuvette contained a mitochondrial suspension reduced with sodium dithionite; the sample cuvette contained the same constituents plus CO. Curves b–e illustrate the temperature degradation of "a" when incubated at 37° during a period of 2 hr Thirty min elapsed between successive spectra. The protein concentration was 2·2 mg/ml in each cuvette. Mitochondria were resuspended in 0·1 M potassium phosphate buffer solution (pH 7·4).

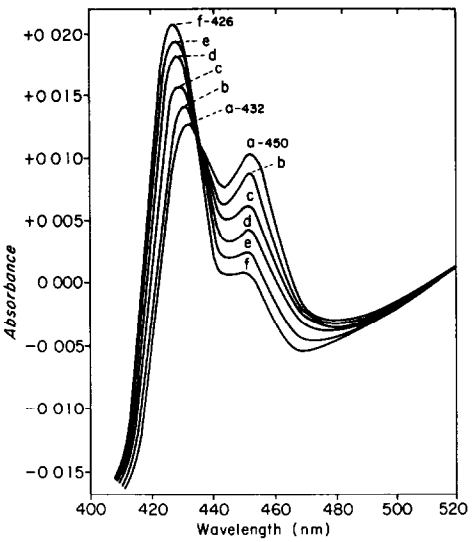

FIG. 2. CO-reduced minus CO difference spectra of human term placental mitochondria. In curve a, the reference cuvette contained a mitochondrial suspension saturated with CO. The sample cuvette contained the same constituents plus sodium dithionite. Curves b–f illustrate the temperature degradation of "a" when incubated at 37° during a period of 2·5 hr. Thirty min elapsed between successive spectra. The protein concentration was 2·2 mg/ml in each cuvette. Mitochondria were resuspended in a 0·1 M potassium phosphate buffer solution (pH 7·4).

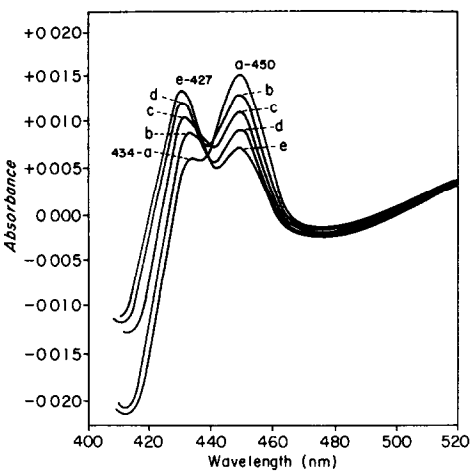

FIG. 3. CO-reduced minus CO difference spectra of human term placental mitochondria. In curve e, the reference cuvette contained a mitochondrial suspension saturated with CO. The sample cuvette contained the same constituents plus sodium dithionite. The sample was incubated (4°, 2 weeks) in order to effect a partial conversion to cytochrome P-450 to P-420 prior to spectral analysis. Curves d–a illustrate the spectral shifts produced by additions of 1, 3, 5 and 7×10^{-5} M PCMB (final concentrations) respectively. The protein concentration was 1·5 mg/ml in each cuvette. Mitochondria were resuspended in a 0·05 M potassium phosphate buffer solution (pH 7·35) containing 10^{-4} M dithiothreitol (final concentration) and 20% (v/v) glycerol.

PCMB were employed; concentrations in excess of 1×10^{-4} M PCMB produced no further changes in the spectrum. Maximal changes were observed typically at final concentrations between 1×10^{-5} M and 7×10^{-5} M PCMB. The final pH was 7·4 in all cuvettes. However, the concentration of PCMB required for the apparent conversion to cytochrome P-450 from P-420 also was shown to be dependent on the protein concentration in the cuvette and on the ratio of P-420 to P-450 absorption (420/450) Utilizing low concentrations ($< 5 \times 10^{-5}$ M) of PCMB, the maximal extent of apparent conversion of cytochrome P-450 to P-420 was observed with protein concentrations of approximately 2 mg/ml (or less) and with low absorbance ratios of P-420/P-450 (< 1).

This conversion phenomenon was observed at 5, 10, 15 and 24°. Human term placental microsomal cytochrome P-420 did not exhibit extensive spectral shifts. Some conversion was observed, but this may have been attributable to mitochondrial contamination of the microsomal fraction.

The addition of PCMB to rat hepatic microsomes, however, did not result in any observable conversion of cytochrome P-420 to P-450 under any of the experimental conditions utilized in studies of placental mitochondria. Partial conversion of rat hepatic microsomal cytochrome P-450 to P-420 was effected initially by incubating the rat liver microsomes at 37° for varying time periods depending on the composition of the resuspending solution. P-420/P-450 ratios were approximately unity in each case.

It should be emphasized that the apparent conversion of placental cytochrome P-420 to P-450 was observed utilizing either the method of Greim[11] or of Omura and Sato.[12] An absorbance maximum in the 420–430 nm range has been attributed to cytochrome b_5 when assayed by the method of Greim or to carbon monoxyhemoglobin when assayed by the method of Omura and Sato. Therefore, the effects of PCMB on carbon monoxyhemoglobin and purified rabbit liver cytochrome b_5 also were investigated (5×10^{-7} M monomer heme in 20% glycerol-phosphate buffer, pH 7·4), but no changes in the absorbance maxima within the Soret region could be observed with 10^{-6}–10^{-3} M final concentrations of PCMB. This suggested that neither hemoglobin nor cytochrome b_5 was involved in the decrease in absorbance of the maximum in the 420–430 nm wavelength range in human term placental mitochondrial preparations. Experiments were performed with an additional sulfhydryl reagent (sodium iodoacetate) in order to determine whether this reconversion of cytochrome P-420 to P-450 was dependent on the capacity of PCMB to interact with sulfhydryl groups. However, no spectral shifts could be observed with sodium iodoacetate at 10^{-6}–10^{-4} M final concentrations and under conditions in which PCMB readily produced apparent reconversion. Further studies with other sulfhydryl reagents, however, are needed to draw definite conclusions. The mechanism involved in the apparent reconversion phenomenon by PCMB in the presence of glycerol, therefore, cannot be explained at the present time.

Placental mitochondria contain an enzyme system that is responsible for the side-chain cleavage of cholesterol, a reaction that is inhibited by CO.[2] It also has been observed that this inhibition is reversible by monochromatic light near a wavelength of 450 nm, strongly suggesting that the P-450 of human placental mitochondria catalyzes the side-chain cleavage reaction. Previous preliminary investigations in this laboratory[14] also have indicated that a reciprocal relationship between rates of cholesterol side-chain cleavage and hydroxylation of 3,4-benzpyrene (also inhibited by carbon monoxide) may exist in human placental tissues at term. The results of the present investigation, which seem to indicate that PCMB is capable of effecting the conversion of human placental cytochrome P-420 to the P-450 form, appear to illustrate a major difference between human placental mitochondrial and hepatic and placental cytochrome P-450 forms. It is hoped that these observations may aid investigators in attempts to elucidate the structure and mechanisms of action of several P-450 cytochromes present in a variety of tissues

Finally, it should be emphasized that observations of spectral shifts, such as have been described here, do not provide conclusive proof regarding the interconversion of hemoprotein molecules. Extensive investigations will be required for the final interpretation of this interesting phenomenon.

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Very early effects of aminonucleoside on some rat liver enzymes

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ALTHOUGH it is well known that aminonucleoside causes nephrosis in rats after 5 days, very little information is available on the "prenephrotic" period, i.e. before the onset of nephrosis. Since in the livers of nephrotic rats increased protein synthesis, accelerated turnover of energy-rich phosphorus compounds, increase in RNA and DNA synthesis, prolongation of r-RNA half-life, as well as rise in blood glucose level and proteinuria have been observed,[1-5] the liver seems to be basically involved in the pathology of nephrosis. Many other amino-purine derivatives produce partly similar metabolic changes in the hepatocytes, aminonucleoside-puromycin (AMN) is known to result in experimental nephrosis. Our aim was to find any early influences of this compound on some enzymes in the glycogen–glucose pathway, Krebs cycle and in the nucleic acid metabolism as possible sources of metabolic changes which may be involved in the ultimate cell damage leading to nephrosis. For this study, we selected enzymes which are known to be disturbed in the nephrotic kidney or which may be involved in the prenephrotic changes in the liver.[4-10]

Male Wistar rats, weighing 200–250 g, were supplied Purina lab chow and tap water *ad lib.* The test rats were anaesthetized with ether, and 1·5 ml of a 1% solution of aminonucleoside-puromycin (AMN, Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.) in 0·9% NaCl, pH adjusted to 7·4, was injected. Because rats excrete 25–50 per cent of the AMN as unchanged compound and 90 per cent of the remainder as breakdown products within the first 8 hr,[1] we administered the compound rapidly by i v.—into the jugular vein—rather than the commonly used i.m. or s.c. route. The same number of rats used as controls against each test group received 1·5 ml physiological NaCl only. Groups of animals treated with AMN or saline were sacrificed by decapitation after different time intervals of 1–96 hr. Food was removed from each group 14–16 hr before they were killed The livers were removed immediately and placed in ice-cold 0·5 M sucrose solution containing 37·5 mM *tris* maleate, 1% Dextran-250, 5 mM $MgCl_2$, 5 mM mercaptoethanol, at pH 6·4, and homogenized. Samples of the homogenate were frozen and stored at −20 pending enzyme assay

Enzyme assays. Glucose 6-phosphate phosphohydrolase (EC 3.1.3.9) activity was determined according to the method of Swanson.[11] The amount of *p*-nitrophenol released from *p*-nitrophenyl-phosphate was used to indicate acid phosphatase (EC 3.1.3.2) activity (Sadowski and Steiner).[12] NAD-malic dehydrogenase (EC 1 1.1.37) was determined by measuring the nicotinamide adenine dinucleotide, reduced from (NADH), as described by Strominger and Lowry.[13] Urate-oxidase (EC 1.7.3.3) activity was measured by the method of Schneider and Hogeboom.[14] Protein content of the liver homogenate was determined by the method of Lowry *et al.*[15]

Results of the enzyme assays are summarized in Tables 1 and 2. Although the applied low dose of AMN does not seem to change significantly the liver protein content, it has significant influence on the activities of some enzymes shortly after its injection