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Note

Correlation of carbon monoxide and bilirubin production by tissue homogenates

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The production of carbon monoxide (CO) has been used as an index of bilirubin production in animals and man [1]. The validity of this concept is based on the characteristics of the heme degradation pathway. Heme oxygenase (EC 1.14.99.3), with the consumption of O_2 and reduced nicotinamide-adenine dinucleotide phosphate (NADPH), oxidizes heme into equimolar amounts of CO and biliverdin [2]. The latter is reduced directly to bilirubin with the aid of biliverdin reductase and the consumption of a second molecule of NADPH [3]. The heme oxygenase reaction is reported to be the rate-limiting step when the tissue preparations are not extensively purified [4]. Due to improved analytical techniques, we are now able to determine in vitro simultaneously the CO produced by the heme oxygenase reaction and bilirubin, the end-product of the heme degradation pathway. CO was determined in the reaction vessel by headspace gas chromatography (GC) [5, 6], and bilirubin was extracted from the reaction mixture with chloroform and quantified by high-performance liquid chromatography (HPLC) [7].

EXPERIMENTAL

Tissue preparation

Livers were removed from decapitated, mature, adult, female Wistar rats (250–300 g). The rats had been treated with 250 μmol cobalt(II) chloride per kg

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body weight, 16 h prior to sacrifice in order to increase liver heme oxygenase activity through induction [8]. The tissue was homogenized with a Biohomogenizer (Biospec Products, Bartlesville, OK, U.S.A.) in 4 volumes of 0.1 *M* potassium phosphate, pH 7.4. The homogenate was centrifuged for 15 min at 13 000 *g* and the supernatant was utilized for the reactions.

Reagents

For the preparation of 100 m*M* cobalt(II) chloride, 11 g $CoCl_2 \cdot 6H_2O$ were dissolved in distilled water and the volume was adjusted to 500 ml with distilled water. The pH of this solution was 5.3 and it could not be neutralized without precipitate formation. The heme oxygenase substrate solution, methemalbumin (2.00 m*M* heme in 0.15 m*M* albumin) [4] was prepared by dissolving 13.4 mg hemin (Sigma, St. Louis, MO, U.S.A.) in 2.5 ml of 0.4 *M* trisodium phosphate. Distilled water (5 ml) and 100 mg bovine serum albumin (Sigma) were then added. The pH was gradually adjusted to 7.4 with 1.2 *M* hydrochloric acid. The final volume was adjusted to 10 ml with distilled water. A solution of 4.5 m*M* NADPH was prepared by dissolving 4.0 mg $Na_3NADPH \cdot 3.5H_2O$ (Sigma) in 1 ml of 0.1 *M* potassium phosphate, pH 7.4 [4]. The reagents for the bilirubin determination by HPLC have been described [7].

CO determination

The heme oxygenase activity assay, described in previous reports [5, 6], was used to determine CO. Supernatants of tissue homogenate (2–20 μl) were incubated with methemalbumin in the presence (total) and absence (blank) of NADPH in septum-sealed vials at 37°C for 15 min. Reactions were terminated by transfer of the vials (2 ml) to −78°C (acetone–dry ice). The generated CO was quantitated by GC of the entire headspace volume on a 600 mm×5.3 mm I.D. stainless-steel column packed with a 5-Å molecular sieve, 60–80 mesh (Alltech Assoc., Los Altos, CA, U.S.A.) at a temperature of 125°C with CO-free air as carrier gas (50 ml/min). The column outlet was connected to a sensitive reduction gas detector (Trace Analytical, Menlo Park, CA, U.S.A.) which quantitated the CO by means of the $A_{254\,nm}$ of mercury generated from the reaction of CO with HgO at 275°C [9, 10]. The analyzer was calibrated with a mixture of CO in nitrogen (Airco Rare and Specialty Gases, Santa Clara, CA, U.S.A.). CO production is expressed as pmol CO per μl preparation per 15 min.

HPLC measurement of unconjugated bilirubin

After determination of CO in the reaction vials, the vials were refrozen immediately, shipped on dry ice and kept at −70°C until analyzed for bilirubin content within one week. Pigment extraction and analysis were performed by an established method after alkaline methanolysis and HPLC [7]. Reaction vials were washed with the methanol-containing internal standards and their contents quantitatively transferred for extraction. After evaporation under nitrogen, samples were redissolved in 100–150 μl chloroform and filtered through 0.45-μm Nylon-66 filters, using a centrifugal filter apparatus (Rainin Instrument, Woburn, MA, U.S.A.), just prior to injection. Elution from the column (250 mm×4.6 mm,

5-μm LiChrosorb, Merck, E.M. Labs., Elmsford, NY, U.S.A.) was accomplished isocratically using solvent B (acetic acid–chloroform, 0.5:99.5, v/v) [7]. Instrumentation consisted of a Waters 6000A solvent pump with a U6K injector and a 200-μl load loop (Waters Chromatography Division, Millipore, Milford, MA, U.S.A.). A Perkin-Elmer Lamda One visible spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a 3-μl flow cell was used for detection at 430 nm, and quantification was done by a Waters M730 data module.

RESULTS AND DISCUSSION

We determined the rates of production of each product as a function of added homogenate. Least-squares best-fit slopes from these curves were taken as product formation rates for that preparation. In three experiments, utilizing five different protein concentrations in duplicate, we determined the production of both CO and unconjugated bilirubin in the same reaction vial. In the three experiments described in Table I and summarized in Fig. 1, bilirubin and CO production (pmol per μl supernatant per 15 min) correlated well with the amount of enzyme as represented by the volume of incubated supernatant; the r value for least-squares regression analysis was greater than or equal to 0.98 in each case. Regression

TABLE I

DETERMINATION OF HEME OXYGENASE ACTIVITY IN THREE RAT LIVER HOMOGENATE PREPARATIONS ANALYZED BY HPLC (BILIRUBIN) AND GC (CO)

Experiment No.	Bilirubin production (pmol/μl/15 min)	r	CO production (pmol/μl/15 min)	r
1	62.0	0.98	53.2	0.99
2	41.4	0.98	32.7	0.99
3	53.5	0.98	39.7	0.99

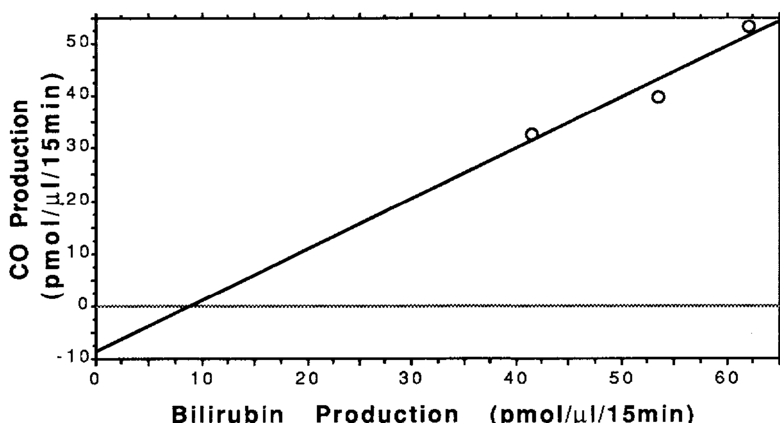

Fig. 1. Correlation of bilirubin and CO production by 13 000 g supernatants of three rat liver homogenates: $y=0.97x-8.7$; $r=0.98$.

analysis of the data in the table shows that CO production equals 0.97 times the bilirubin formation minus 8.7 pmol per μl per 15 min with a correlation coefficient of 0.96. No compounds were found to interfere with the GC determination of CO (Fig. 2A). The limit of detection for the method was 1 pmol per vial. The within-assay reproducibility of the method with 10-μl aliquots of tissue supernatant is for the total reaction 224 ± 6 pmol CO per 15 min per vial (coefficient of variation, C.V. = 3%, n = 10) and for the blank vials (no NADPH) 30 ± 5 pmol CO per 15 min per vial (C.V. = 17%, n = 10) [6]. The between-day reproducibility for the heme oxygenase activity (total minus blank) of a stable liver supernatant preparation was determined as 148 ± 18 pmol CO per 15 min per vial (C.V. = 12%, n = 5) [6]. Similarly, the HPLC method showed that there were no detectable interfering peaks present (Fig. 2B) and that the elution pattern was identical to that seen for unconjugated bilirubin and internal standard alone. The detection limit for this method was 30 pmol bilirubin per ml. The use of an internal standard, methyl ester of xanthobilirubic acid, corrected for potential incomplete extraction recovery. Precision, determined from ten extractions of a single sample and expressed as C.V., was 4.5% for day-to-day and 14% for between-day analysis at a sample bilirubin concentration of 600 pmol/ml.

We conclude that bilirubin formation, as measured by HPLC, and CO generation, as measured by GC in the same reaction vessel, are linearly correlated with

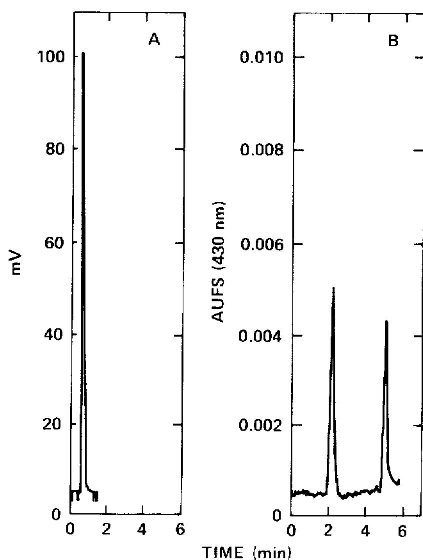

Fig. 2. Chromatograms of heme degradation products generated by rat liver homogenate in the presence of NADPH. (A) Gas chromatogram of the reactor headspace gas, containing CO (retention time = 0.58 min) generated by the reaction mixture. GC column: 600 mm × 5.4 mm I.D. 5-Å molecular sieve, 60–80 mesh at 125°C. The air carrier flow-rate was 50 ml/min. (B) HPLC pattern of the reaction mixture containing unconjugated bilirubin (peak 1, retention time = 2.12 min) and internal standard, xanthobilirubic acid methyl ester (peak 2, retention time = 5.0 min). HPLC column: 250 mm × 4.6 mm I.D. 5-μm LiChrosorb, at ambient temperature. The solvent (acetic acid–chloroform, 0.5:99.5, v/v) flow-rate was 1.50 ml/min.

the protein concentration over the range tested. The heme oxygenase activity slopes derived by the two different methods in the two separate laboratories are in close agreement. It should be noted that the sensitivity of the HPLC method is lower than that of the GC method. Thus, the measured bilirubin quantities are nearer to the lower detection limit and thus are subject to greater error. Furthermore, although bilirubin and CO are end-products of the heme degradation pathway, they are not products of the same enzymatic reaction. Therefore, comparison of the analytical amounts of the two substances are not necessarily expected to yield identical results. However, the second enzyme in the pathway, biliverdin reductase, has been reported to be in excess. We confirmed this for the rat liver preparation through the excellent correlation between CO and bilirubin formation. The GC method for heme oxygenase activity determination is simpler and more sensitive than the HPLC procedure. It has the added advantage that it measures a direct reaction product of the heme oxygenase reaction, so that sufficiency of biliverdin reductase activity or effects of inhibitors and other conditions on biliverdin reductase do not affect the heme oxygenase activity measurements. Thus, when used in combination with other heme oxygenase assays [4, 8] the GC method provides important complementary information about the heme degradation pathway. Although the heme oxygenase activities were essentially the same in these experiments, pigment extraction for the HPLC measurement of unconjugated bilirubin from this material may be variable, manifested by different intercepts. Extraneous CO production uncoupled from bilirubin production is also a possible source of error. However, this potentially confounding factor is eliminated by the use of an appropriate blank reaction (without NADPH). Overall, the data suggest stoichiometric accounting for bilirubin and CO in equimolar amounts in this system.

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