METABOLISM OF BENZO[a]PYRENE-3,6-QUINONE BY ISOLATED HEPATOCYTES

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1. Introduction

Quinones are almost invariably found as products of the oxidative metabolism of benzo[a]pyrene supported by rat liver microsomes [1,2] or isolated hepatocytes [3,4]. Quinones are redox-active compounds which can enzymatically accept electrons from NADPH to form hydroquinone derivatives [5–7], or, when, reduced, can donate electrons to molecular oxygen to generate reactive species of oxygen [5]. During this redox cycle they compete for oxygen and reducing equivalents and may thus affect the rate of overall metabolism of B[a]P [8].

The disposition of B[a]P quinones by the cell seems to require that they are first reduced and then conjugated prior to being eliminated as water-soluble products [5–7]. Hence the addition of uridine-5′-diphosphate glucuronic acid to incubation mixtures containing rat liver microsomes, B[a]P and NADPH has a stimulatory effect on the rate of B[a]P metabolism [8–10] and also results in the conversion of most of the B[a]P quinones to water-soluble conjugates [7–11]. The microsomal conversion of B[a]P quinones to glucuronides is NADPH-dependent and has been reported to be dicoumarol-sensitive suggesting a role for DT-diaphorase in this process [7]. On the other hand, the microsomal cytochrome P450 reductase is a very active B[a]P quinone reductase which catalyzes the formation of hydroquinones at substantially higher rates than DT-diaphorase [6]; this enzyme is NADPH-dependent and is not inhibited by dicoumarol [6].

Abbreviations: B[a]P, benzo(a)pyrene; UDPGA, uridine-5′-diphosphate glucuronic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; GSH, glutathione, reduced form

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Here we have used isolated hepatocytes and the liver-microsomal fraction obtained from phenobarbital-treated rats to study the reduction of B[a]P-3,6-quinone, the sensitivity of this reaction to dicoumarol, and the role of glucuronide formation in quinone disposition by the cell.

2. Materials and methods

B[a]P-3,6-quinone was obtained from the NCI Carcinogenesis Research Program, Bethesda, MD. It was dissolved in dimethylsulfoxide and its concentration determined by spectrophotometry. Other chemicals used were obtained at the highest purity available commercially.

Microsomal fraction and hepatocytes were isolated from the livers of phenobarbital-treated male Sprague-Dawley rats (200–250 g) as in [4,12].

NADPH-dependent, microsomal reduction of B[a]P-3,6-quinone was assayed as in [6], except that Krebs-Henseleit buffer (pH 7.4) containing 25 mM Hepes was used instead of Tris–HCl buffer. The metabolism of B[a]P-3,6-quinone by isolated hepatocytes was studied by dividing a cell suspension ($1-3 \times 10^6$ cells/ml) between the sample and reference cuvettes of an Aminco DW-2-spectrophotometer. After recording a baseline of equal absorbance, the reaction was initiated by adding the B[a]P-3,6-quinone to the sample cuvette. Repetitive scanning was performed between 380 and 680 nm with continuous mixing of both sample and reference cuvettes to avoid cell sedimentation and anaerobiosis.

The rate of quinone reduction catalyzed by isolated hepatocytes was determined spectrophotometrically in the dual wavelength mode, monitoring the time-dependent change in absorbance for the wave-

length pair 458–485 nm. A 20 ml cell suspension in Krebs-Henseleit buffer (pH 7.4) containing 25 mM Hepes, and 2 mM salicylamide, with or without the addition of dicoumarol (10 μM final conc.), was kept in a pyrex beaker (3.7 cm lightpath) placed in the beampath of the spectrophotometer [13]. The suspension was mixed with the aid of a magnetic stirrer. After balancing the absorbance difference between 458 and 485 nm, the reaction was started by adding the B[a]P-3,6-quinone.

All the results reported here were obtained at 25°C.

3. Results and discussion

The time-dependent changes in light absorbance upon addition of B[a]P-3,6-quinone to a suspension of isolated hepatocytes are shown in fig.1. The rapid reduction of the quinone to its hydroquinone form is indicated by the loss of absorbance at ~480 nm and the appearance of a transient shoulder at ~448 nm (fig.1, line 2; [6]). As the reaction proceeded, there was a continuous decrease in quinone concentration (480 nm) and, at the same time, the absorbance band at 448 nm progressively shifted towards lower wavelengths (fig.1, lines 3–5). The system reached a steady-state at ~11 min after addition of B[a]P-3,6-quinone, with the resulting spectrum showing maxima at 390 and 437 nm (fig.1, line 6). This was not changed even after 40 min incubation and was unaltered when $Na_2S_2O_4$ was added to the contents of both sample and reference cuvettes. The latter indicates that under these conditions essentially all of the added quinone had been metabolized to originate the spectral intermediate above.

As described [6], the NADPH-dependent reduction of B[a]P-3,6-quinone catalyzed by liver microsomes obtained from phenobarbital-treated rats resulted in a loss of absorbance at ~480 nm and the concomitant appearance of a typical hydroquinone spectrum with absorption maxima at 401 and 448 nm. Under conditions of limiting NADPH, these spectral changes were reversed upon depletion of reducing equivalents and the spectrum of the fully oxidized quinone was recovered. The addition of GSH (5 mM final conc.) to the incubation had no apparent effect on the shape of the spectral changes described. On the other hand, and as shown in fig.2,

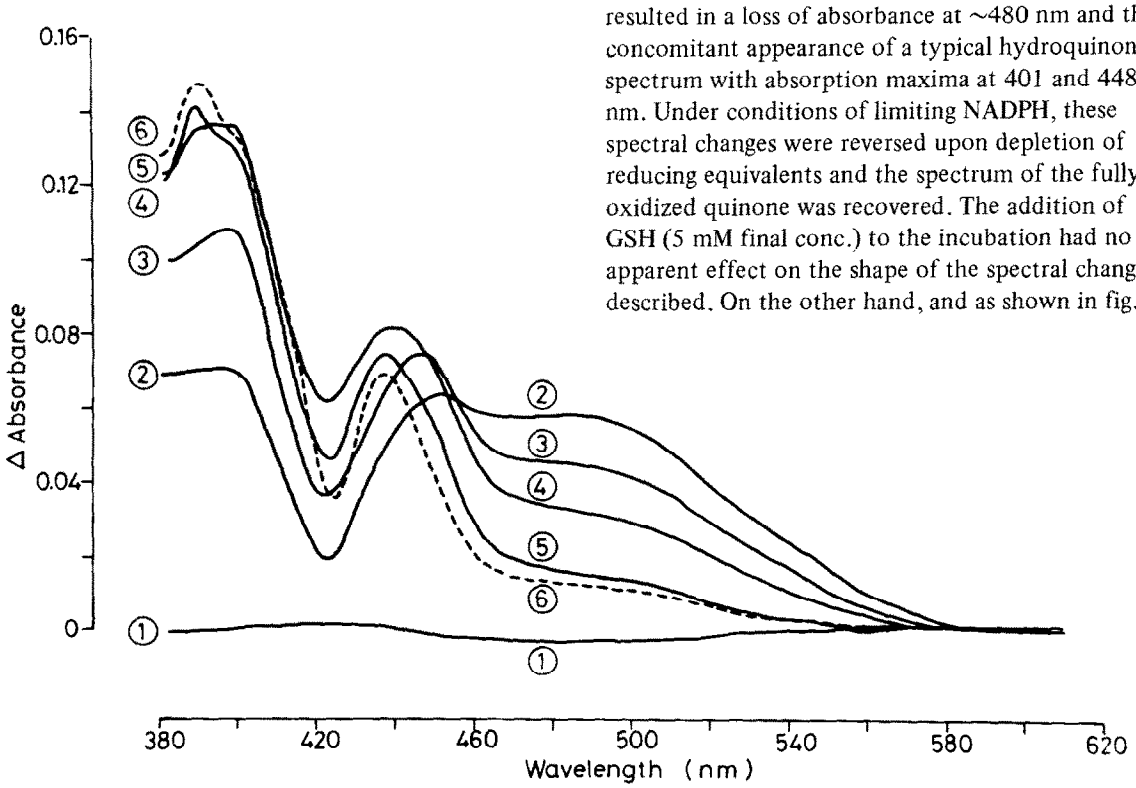

Fig.1. Spectral analysis of the metabolism of B[a]P-3,6-quinone by isolated hepatocytes. A cell suspension (1.8×10^6 cells/ml) was placed in the sample and reference cuvettes of the spectrophotometer and a baseline of equal absorbance was recorded (line 1). The reaction was then initiated by the addition of B[a]P-3,6-quinone (7.3 μM) to the sample cuvette, and immediately thereafter the spectrum shown by line 2 was obtained. Scanning of the reaction mixture was then repeated at 3,6,9 and 12 min (lines 3,4,5 and 6, respectively).

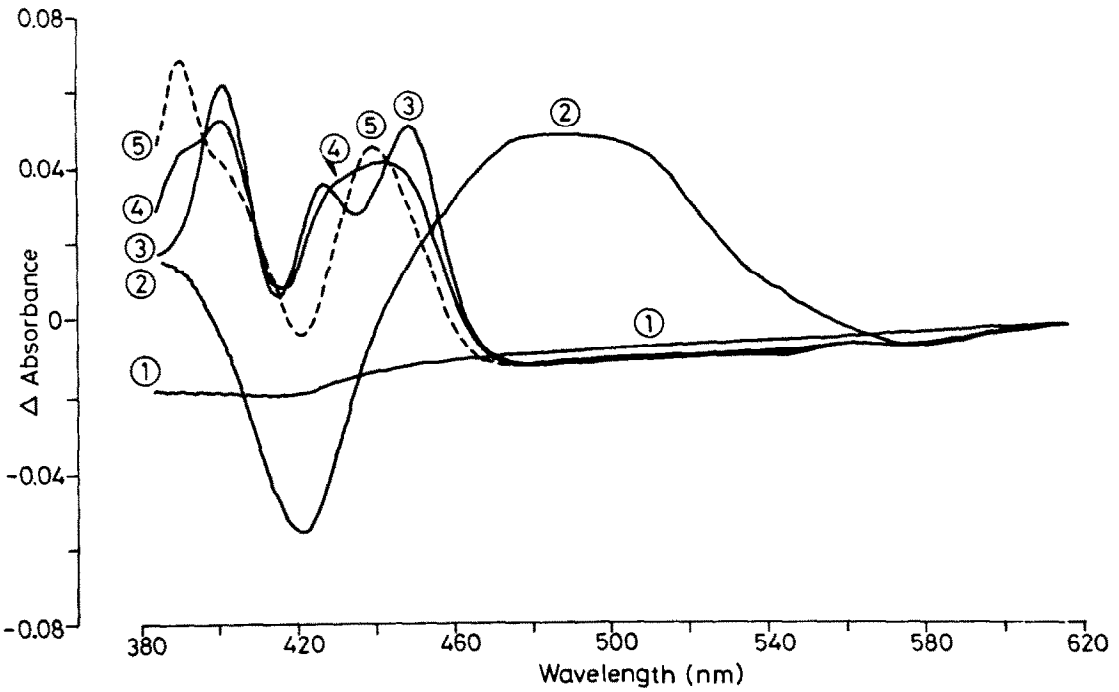

Fig.2. Spectral analysis of the microsomal metabolism of B[a]P-3,6-quinone in the presence of UDPGA. A suspension of liver microsomes (0.8 mg protein/ml) containing UDPGA (5 mM final conc.) and an NADPH-regenerating system (except NADPH), was placed in the sample and reference cuvettes of the spectrophotometer and the baseline (line 1) was recorded. After addition of B[a]P-3,6-quinone (7.3 μM) to the sample cuvette the spectrum shown by line 2 was recorded. The reaction was then initiated by the addition of NADPH (0.4 mM) to the contents of both sample and reference cuvettes. Spectra shown by lines 3,4 and 5 were recorded at 4,20 and 45 min after initiation of the reaction, respectively.

when the microsome-catalyzed reaction was assayed in the presence of UDPGA (5 mM final conc.) a spectral intermediate similar to that obtained with the isolated hepatocytes was formed. For the experiment illustrated in fig.2, we divided a microsomal suspension containing an NADPH-regenerating system and 5 mM UDPGA between the sample and reference cuvettes of the spectrophotometer. After establishing a baseline of equal absorbance (fig.2, line 1), the B[a]P-3,6-quinone was added to the contents of the sample cuvette and the spectrum shown by line 2 (fig.2) was recorded. This contains superimposed to the typical quinone band, the contribution of a type I complex formed between the quinone and ferric cytochrome P450 [6]. Upon addition of NADPH to the sample and reference cuvettes, the rapid reduction of the quinone resulted in a loss of absorbance at ~480 nm and the appearance of the bands at 401 and 448 nm. An apparent steady-state of reduction was obtained after 4 min incubation (fig.2, line 3). However, as the reaction proceeded with time, there was a gradual shift of the absorbance maxima at 401 and 448 nm towards lower wavelengths; this is clearly shown by the absorbance of the incubation mixture registered 20 min after initiation of the reaction with NADPH (fig.2, line 4). Finally, after 45 min incubation under continuous mixing, the reaction reached a new steady-state which is illustrated by line 5 (fig.2). This spectral species remained unchanged even after 60 min and was not affected when $Na_2S_2O_4$ was added to the contents of both sample and reference cuvettes.

The products of the hepatocyte-supported metabolism of B[a]P-3,6-quinone and of that catalyzed by the microsomal fraction fortified with NADPH and UDPGA are spectrally almost identical (compare lines 6 and 5 of fig.1 and 2), the major difference being that formation of the ultimate spectral species seems to occur more rapidly with hepatocytes than with the microsomal fraction. Thus, it appears that hepatocytes

do reduce B[a]P-3,6-quinone and that the hydroquinone is subsequently rapidly conjugated with glucuronic acid. This is further substantiated by the results shown in fig.3. In this experiment the hepatocytes were preincubated for 2 min with salicylamide prior to the addition of the B[a]P-3,6-quinone; salicylamide is a potent inhibitor of glucuronic acid and sulfate conjugation in hepatocytes [4]. As shown in fig.3, the addition of B[a]P-3,6-quinone to the cell suspension in the presence of salicylamide resulted in the rapid conversion of the quinone to its hydroquinone form (fig.3, lines 2,3). At ~6 min after initiation of the reaction, the system reached a steady-state in which ~60–70% of the added quinone was present as hydroquinone (fig.3, line 4). This remained constant for at least 15 min and upon addition of $Na_2S_2O_4$ to both the sample and reference cuvettes, the spectrum of the fully reduced hydroquinone was obtained (fig.3, line 5).

The results shown so far indicate that in isolated hepatocytes the B[a]P-3,6-quinone is rapidly reduced to a hydroquinone which is then efficiently conjugated with UDPGA. It must be noted, however, that a sulfate conjugate would not be spectrally distinguishable from a glucuronide under our conditions of analysis, since the bands obtained at 390 and 437 nm are mainly due to the aromatic residue of the polycyclic hydrocarbon and its resonance properties are very similar for both types of conjugates. In an experiment not shown here, we therefore extracted the water-soluble products of a reaction mixture containing isolated hepatocytes (2×10^6 cells/ml) and B[a]P-3,6-quinone (15 μM), incubated at 25°C for 50 min, which were then hydrolyzed with β-glucuronidase as in [4]. After extraction of the hydrolysate with ethylacetate, B[a]P-3,6-quinone was recovered in the organic phase suggesting that, indeed, the glucuronic acid conjugate of the hydroquinone is a major water-soluble metabolite of B[a]P-3,6-quinone in hepatocytes.

Table 1 compares the rates of B[a]P-3,6-quinone reduction with NADPH-fortified microsomal fraction and isolated hepatocytes; quinone reduction in isolated hepatocytes was assayed in the presence of sali-

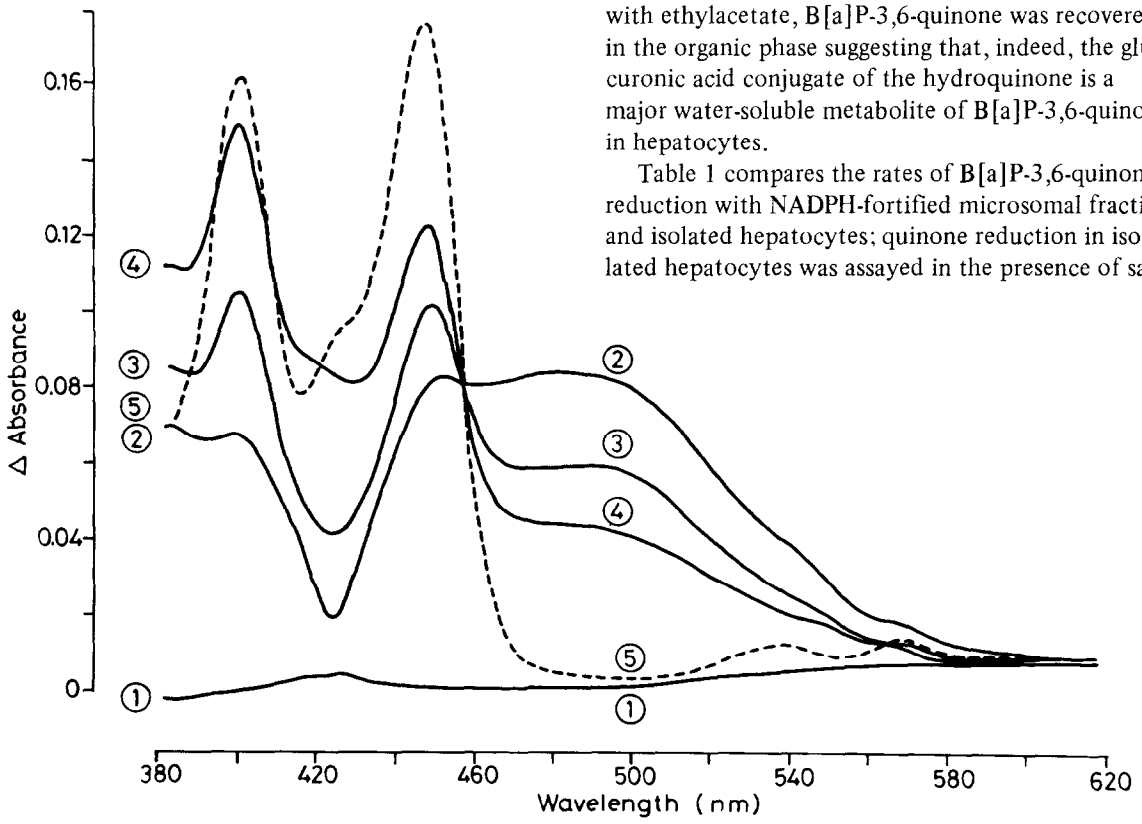

Fig.3. Spectral analysis of the metabolism of B[a]P-3,6-quinone by isolated hepatocytes in the presence of salicylamide. A cell suspension (1.8×10^6 cells/ml) was preincubated with salicylamide (2 mM final conc.) for 2 min prior to addition to the sample and reference cuvettes of the spectrophotometer. A baseline of equal absorbance (line 1) was recorded. After initiation of the reaction by adding B[a]P-3,6-quinone (7.3 μM) to the sample cuvette, spectra shown by lines 2,3 and 4 were recorded at 0, 3 and 6 min after initiation of the reaction, respectively. When a spectral steady-state was achieved, $Na_2S_2O_4$ was added to the contents of both sample and reference cuvettes and the spectrum shown by line 5 was recorded.

Table 1
Rate of reduction of benzo[a]pyrene-3,6-quinone catalyzed by liver microsomes and isolated hepatocytes

Experimental system	Control	+ Dicoumarol (10 μM)
Microsomes (nmol . min^{-1} . mg $prot.^{-1}$)[a]	168	165
Hepatocytes (nmol . min^{-1} . 10^6 $cells^{-1}$)[b]	14.6	15.3

[a] Values given for the microsomal reaction were obtained from [6]

[b] Values shown correspond to av. 3 determinations performed in the presence of 2 mM salicylamide. The reaction was initiated by addition of B[a]P-3,6-quinone to 15 μM final conc.

cylamide (2 mM) as in section 2. The rate of reduction catalyzed by the microsomal fraction was found to be 3–4-fold higher than that observed with the cell preparation (10^6 hepatocytes correspond to ~0.35 mg microsomal protein). Several factors may have contributed to the observed difference in rates of quinone reduction in the two experimental systems including possible differences in availability of quinone and NADPH at saturating concentrations at the metabolic sites. The presence of dicoumarol (10 μM) in the reaction mixture containing either microsomal fraction or isolated hepatocytes had no apparent effect on the rate of B[a]P-3,6-quinone reduction (table 1), indicating that DT-diaphorase may in fact play a minor role, if any, in the reduction of the quinone by hepatocytes as well as by the microsomal fraction [6].

We have provided evidence that isolated hepatocytes catalyze the reduction of B[a]P-3,6-quinone to its hydroquinone form, and that the hydroquinone is subsequently conjugated, mainly with glucuronic acid, prior to its disposition as a water-soluble product. Moreover, our results suggest that reduction of the quinone in the intact cell is catalyzed primarily by cytochrome P450 reductase, and not by DT-diaphorase, in accordance with findings with microsomes [6]. Our results reveal a high capacity of the hepatocytes to rapidly conjugate the hydroquinone formed, a mechanism which may protect the cell from formation of reactive species of oxygen as the result of autooxidation of accumulating hydroquinone [5].

Acknowledgements

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