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DICHLORO-*P*-NITROANISOLE *O*-DEMETHYLASE—A CONVENIENT ASSAY FOR MICROSOMAL MIXED FUNCTION OXIDASE IN ISOLATED RAT HEPATOCYTES

DAN HULTMARK, KARIN SUNDH, CARL AXEL WACHTMEISTER and ERIK ARRHENIUS*
Division of Cellular Toxicology and Organic Chemistry, Environmental Toxicology Unit, Wallenberg Laboratory, Stockholm University, S-106 91 Stockholm, Sweden

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Abstract—A new assay for drug metabolism in isolated rat hepatocytes is described. 2,6-Dichloro-4-nitroanisole is *O*-demethylated to the corresponding phenol. The product, 2,6-dichloro-4-nitrophenol is not further metabolized by the hepatocytes, but accumulates in the extracellular medium. It has a low pK_a; thus the strong colour is pH-insensitive in physiological solutions. The assay involves direct spectrophotometric determination of the product in the medium. The reaction is catalyzed by enzymes present in the microsomal fraction, is dependent on oxygen and NADPH, and it is inhibited by carbon monoxide. The equimolar production of formaldehyde is demonstrated. The results indicate that cytochrome P450 is involved in the reaction.

One important function of the liver is to metabolize foreign compounds. A convenient system for studying drug metabolism in intact cells has been available since Berry and Friend [1] developed a technique for producing a high yield of viable isolated hepatocytes. Seglen made a systematic study of the technique, cf. [2], thereby considerably improving the quality of the cells obtained [2, 3]. Other workers also have contributed with important improvements [4–8].

Isolated hepatocytes are now used in many laboratories in studies on drug metabolism [9–14] and hepatotoxicity [15]. Many attempts have been made to use isolated hepatocytes for prolonged times in tissue culture [16–26]. If it is possible to maintain hepatocyte function in such a system, it could be used for *in vitro* mutagenicity and carcinogenicity screening [27, 28].

In the development of such systems, however, there is a need for simple and quantitative assays of the activity of the different drug metabolizing enzymes in the hepatocyte. Such assays can in principle be done by measuring either the accumulation of a product of an enzymatic reaction, or the disappearance of the substrate. These measurements are often difficult in systems as complex as isolated hepatocyte suspensions. The products are usually further metabolized instead of being accumulated. The measuring of substrate disappearance has other limitations. Such assays are less sensitive since one has to determine the difference between two large concentrations, and often the existence of many parallel reactions makes interpretation difficult. In the ideal assay a substrate is converted, by the enzyme to be studied, to a product which is not further metabolized. The analysis of the product should be simple.

The *p*-nitroanisole *O*-demethylase assay [29] makes possible the direct measurement of the coloured product, *p*-nitrophenol, in a microsomal suspension. The colour of a *p*-nitrophenol solution, however, is strongly influenced by pH in the physiological range. Furthermore, preliminary investigations in this laboratory showed that in the intact hepatocyte *p*-nitrophenol is rapidly conjugated to sulphate and glucuronide, in agreement with the results of Moldéus *et al.* [12].

This study was initiated to investigate if the chlorinated analogue, 2,6-dichloro-4-nitroanisole† (Fig. 1), would be a more suitable substrate for the *O*-demethylase assay. The anticipated product, dichloro-*p*-nitrophenol, is very acidic [30], giving complete dissociation at physiological pH. The conjugation of the phenol was expected to be low due to steric hindrance [31].

MATERIALS AND METHODS

Chemicals. Collagenase was obtained from Worthington Biochemical Corp.; bovine serum albumin (fraction V), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide, NADP, and *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid (HEPES) were obtained from Sigma, and Ham's F10 (Nutrient mixture F 10 with 25 mM HEPES buffer) from Gibco Bio-cult. The serum albumin was dialysed against water, and stored frozen as a concentrated solution.

2,6-Dichloro-4-nitroanisole. 2,6-Dichloroanisole was prepared from 2,6-dichlorophenol (m.p. 66–68°, Merck–Schuchardt) by methylation using standard techniques. The anisole was nitrated under controlled conditions with a large excess of nitric acid ($d = 1.40$) in acetic anhydride. The crude reaction

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† In this paper usually called dichloro-*p*-nitroanisole.

product was crystallized from methanol to give 2,6-dichloro-4-nitroanisole, m.p. 98–99° [32] free from dinitroderivative and 2,6-dichloroquinone (TLC, Silicagel HF (Merck), hexane–chloroform (1:1 v/v)).

2,6-Dichloro-4-nitrophenol. The 2,6-dichloro-4-nitroanisole was demethylated with hydrobromic acid ($d = 1.49$) in acetic acid. 2,6-Dichloro-4-nitrophenol [33] was crystallised from hexane, m.p. 124–125°. This phenol is also commercially available (Fluka AG). All other chemicals were of the highest purity available, and used without further purification.

Animals. Male rats of a Wistar strain, strain R, weighing about 250 g were used. They were bred in our laboratory, given free access to Fors R3 pellets and water.

Hepatocytes. Hepatocytes were prepared essentially by the technique of Seglen [2], with the following modifications: The animals were injected before perfusion with heparin, 0.1 ml, 5000 I U/ml intravenously. The perfusion apparatus consisted of a peristaltic pump (Multiperpex, LKB) connected via a 0.45 μ Millipore filter to the liver. The liver was immersed in the perfusion medium in a 100 ml water-jacketed glass beaker, maintained at 37°. This allows the liver to hang freely, and minimizes the mechanical stress on the liver. Oxygenation of the medium was accomplished by simply bubbling humified and filtered air through the beaker. The medium was directly recirculated from the beaker to the pump. The media used were as described by Seglen [2], but collagenase buffer and washing buffer contained 1% (w/v) bovine serum albumin to protect the cell membrane from proteolytic enzymes in the crude collagenase. The cell pellet obtained after washing was weighed and finally suspended at a concentration of 30 mg wet wt/ml* in Ham's F10 with 25 mM HEPES buffer, pH 7.2, containing 1% bovine serum albumin. The addition of serum albumin to the suspension prolonged the lifetime of the hepatocytes, and helped dissolve the dichloro-*p*-nitroanisole. The suspension was stored at 0–4° until used (usually within 1 hr). The yield of hepatocytes varied from 3 to 6 g wet wt/liver. The viability was 90–95 per cent or better, as judged by exclusion of 0.25% Trypan Blue.

Microsomes. Microsomes were prepared by the routine method used in this laboratory [34]. Livers were homogenized in 0.2 M potassium phosphate buffer, pH 7.5. The microsomes were washed by layering the post-mitochondrial supernatant on 0.3 M sucrose in 0.2 M phosphate buffer, and centrifuging at 115,000 *g* for 60 min. The microsomal pellets were suspended in 0.25 M sucrose, 1 ml suspension corresponding to 0.43 g liver. In the investigation of the subcellular distribution of the enzymatic activity (Fig. 7), the cell fractionation was performed according to Zannoni [35].

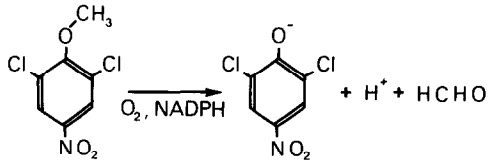
Incubations and analyses. Hepatocytes and microsomes were incubated at 37° with vigorous shaking (approximately 200 strokes/min.). Dichloro-*p*-nitroanisole was added to the tubes beforehand, as an ethanol solution, and the solvent was allowed to evaporate. In incubations with hepatocytes, prewarmed cell suspension, 1 ml, was added to 0.4 μmoles of substrate, to start the reaction. Incubations were stopped by transferring the tubes to an ice bath; the cells were then spun down in the cold, and the supernatant was assayed at 390 nm and 450 nm. Blanks without substrate, and blanks without hepatocytes were included in the assay, as were standards containing dichloro-*p*-nitrophenol. Attempts to stop the reaction by adding trichloroacetic acid or perchloric acid were not successful, since much of the dichloro-*p*-nitrophenol that was added in standards was lost this way, probably bound to the precipitate.

For the microsomal incubations dichloro-*p*-nitroanisole, 1.0 μmole in ethanol was added, and the solvent evaporated as above. Then 1.9 ml was added of a solution containing serum albumin in potassium phosphate buffer, pH 7.0, and the tubes were shaken for approximately 30 min at 37 ° in a water bath to dissolve the substrate. A "mix" was added, containing a NADPH-regenerating system (6 μmoles nicotinamide, 15 μmoles $MgCl_2$, 0.3 μmoles NADP, 15 μmoles glucose-6-phosphate, and one Kornberg unit glucose-6-phosphate dehydrogenase in 0.4 ml phosphate buffer), and the reaction was started by the addition of microsomal suspension, 0.2 ml, giving a final concentration of 1% bovine serum albumin (w/v), 0.07 M phosphate and about 1 mg microsomal protein in a total volume of 2.5 ml.

It is possible to measure the yellow colour of the phenol directly in the microsomal suspension, but as in the *p*-nitrophenol assay, a lower background and greater sensitivity were reached with an extraction method. The reaction was stopped by cooling to 0° and rapidly adding conc. HCl, 200 μl. The substrate and the formed phenolic product were extracted with chloroform, 2.5 ml, and 2 ml of the organic phase was extracted with 0.2 M potassium phosphate buffer, 1 ml, pH 7.5. Dichloro-*p*-nitrophenol was measured at 400 nm in the aqueous phase, and the unchanged anisole could be measured in the organic phase at 281 nm. For the simultaneous determination of dichloro-*p*-nitrophenol and formaldehyde the above procedure was modified. The reaction was stopped by cooling and adding chloroform, 2.5 ml. Trichloroacetic acid (30% v/v) 0.5 ml, was added under agitation. After centrifugation 2 ml of the clear aqueous phase was analysed for formaldehyde with the Nash procedure as described by Arrhenius [36]. The chloroform phase was analysed for the anisole and the phenol as described above. These analytical techniques all gave a linear response to concentration of added standard solutions, and the extractions were 96–100 per cent effective. Standards containing dichloro-*p*-nitrophenol instead of substrate, and blanks without NADPH-regenerating system were always included in the determination.

Protein concentration was determined with the method of Lowry *et al.* [37], using crystalline bovine serum albumin as reference. In some experiments the leakage of glutamic–oxalacetic transaminase

* One g was found to correspond to 10^8 cells ($0.99–1.02 \cdot 10^8$) giving a cell density of $3 \cdot 10^6$ cells/ml in the routine incubations.



(I) (II)

Fig. 1. Oxidative demethylation of 2,6-dichloro-4-nitroanisole (I) to 2,6-dichloro-4-nitrophenol (II).

(GOT) was measured, as an indicator of cell damage. After spinning down the cells, the supernatant was assayed for GOT, using the Sigma analytical kit. The results are expressed as the percent of the total activity liberated by sonicating the cells.

RESULTS

The substrate and the product. Dichloro-*p*-nitroanisole ((I) Fig. 1) is a lipophilic compound with a low solubility in water. Concentrations of 400 μM and higher could be reached, however, by adding 1% serum albumin (w/v). Extracted to chloroform the anisole has an UV absorbance maximum of 281 nm.

Dichloro-*p*-nitrophenol ((II) Fig. 1) is a highly acidic phenol. Spectrophotometric titration of an aqueous solution gave an approximate pK_a of 3.4, in agreement with the reported value of 3.54 [30, 31]. It is therefore completely dissociated in neutral aqueous solutions. It is strongly coloured in its dissociated form, having an absorbance maximum at 400 nm. Addition of serum albumin gave a complex, absorbing at 390 nm, and with a further increased absorbance (Fig. 2).

The assay. When cells were incubated with dichloro-*p*-nitroanisole the formation of dichloro-*p*-nitrophenol could be detected as an increased absorbance at 390 nm. A lower and more constant background was obtained by measuring the difference between the absorbance at 390 and 450 nm

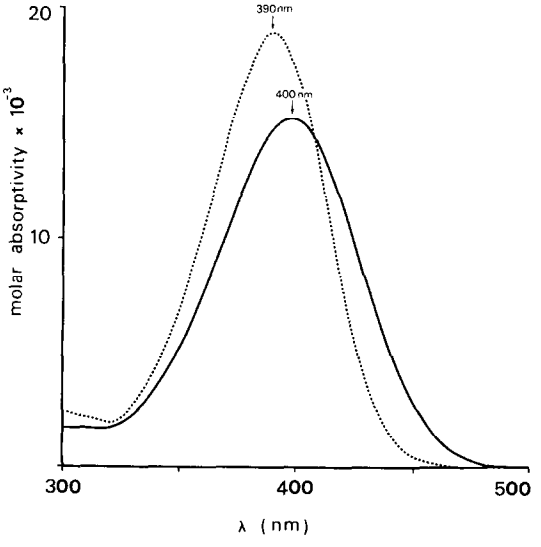


Fig. 2. Optical spectra of 2,6-dichloro-4-nitrophenol in phosphate buffer, pH 7.5, without (unbroken line), or with 1% bovine serum albumin (broken line).

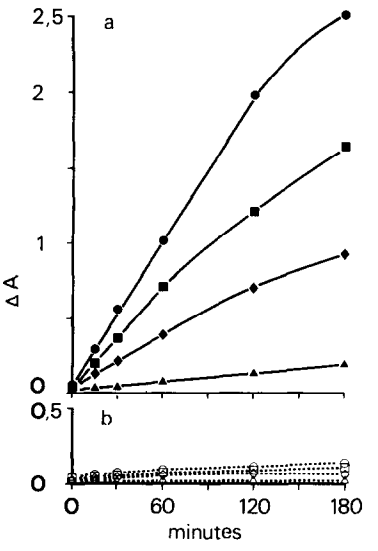


Fig. 3. Increase in absorbance at 390 nm minus absorbance at 450 nm in 1 ml hepatocyte suspensions with cell concentrations 0 mg/ml (▲ △), 15 mg/ml (◆ ◇), 30 mg/ml (■ □) or 45 mg/ml (● ○), incubated (a) with 400 μM dichloro-*p*-nitroanisole, (b) without substrate. One representative experiment of four. Cells (30 mg) were incubated at 37° in Ham's F10, pH 7.2, with 1% bovine serum albumin.

(Fig. 3). The identity of the product was confirmed by its visual spectrum in the medium, its extraction behaviour when extracted as described for microsomal suspensions, its visual spectrum in the final aqueous phase, and by thin layer chromatography of the chloroform extract in two systems (chloroform; or methanol–ethyl acetate, (1:4, v/v), on silica gel).

That no further metabolism of the phenol occurred was also tested. Hepatocytes were incubated for different times with 55 μM dichloro-*p*-nitrophenol, and no disappearance of this substance could be detected (data not shown).

The formation of dichloro-*p*-nitrophenol was linear with time and cell concentration for at least 60 min (Fig. 3). There was some variation in activity between different cell preparations, the mean from nine experiments being 18.1 ± 2.7 (S.E.) nmoles product formed/min/g hepatocytes. Evident in Fig. 3 is also a slow but significant formation of dichloro-*p*-nitrophenol in the total absence of hepatocytes. This non-enzymatic reaction is strongly pH-dependent, and is further discussed below (cf. Fig. 7). In all experiments a blank without metabolizing system was included, and the non-enzymatic reaction rate was subtracted.

Figure 4 shows the release of transaminase from the hepatocytes during the incubation. The relative release appeared to be somewhat lower at higher cell densities. No statistically significant difference in the release was seen when cells were incubated without substrate, though there was a tendency to lower values (data not shown), indicating a possible hepatotoxic effect of dichloro-*p*-nitroanisole or its products. Under all conditions the release of transaminase was low during the first 60 min.

The dependence of the reaction rate on substrate concentration is shown in Fig. 5. In a system that

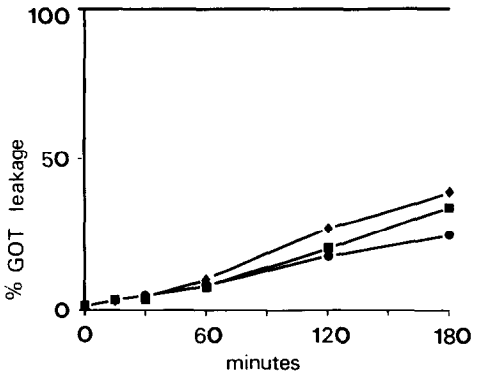


Fig. 4. Leakage of glutamic-oxalacetic transaminase from cell suspensions at different cell concentrations: 15 mg/ml (◆), 30 mg/ml (■) or 45 mg/ml (●). Expressed as per cent of total activity in sonicated suspensions. Mean values from four experiments. Standard error values are left out for clarity, S.E.M. being around 20 per cent of the experimental values. Incubations were performed as described in Fig. 3 and Materials and Methods.

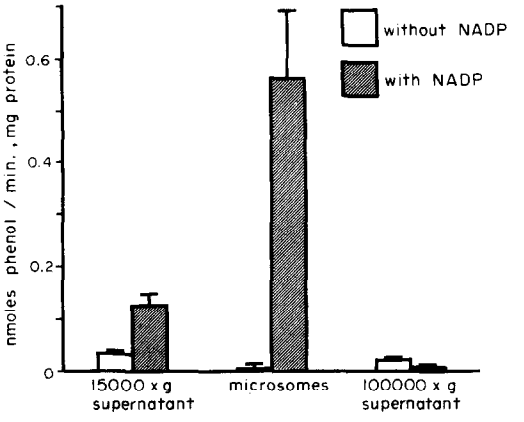


Fig. 6. *O*-demethylase activity in subcellular fractions from rat liver, prepared according to Zannoni [35], and incubated with dichloro-*p*-nitroanisole as described in Materials and Methods. The volume of the fractions that was added was: 15,000 *g* fraction, 0.4 ml; microsomes, 0.2 ml; microsomal supernatant, 0.4 ml. "TV-antennas" indicate S.E.M. from four experiments.

is as complex as intact hepatocytes the assumptions made in Michaelis–Menten kinetics are hardly valid, and the Lineweaver–Burk plot is of doubtful significance. In this case no reproducible K_M values were obtained. For routine experiments a substrate concentration of 400 μM was used. This is near the solubility limit with 1% albumin, and further addition did little to increase the reaction rate.

In the described assay only the extracellular concentration of product is measured. To determine the possible error in this method due to retention of product in the cell, the amount of dichloro-*p*-nitrophenol in the cell pellet was measured. Cell pellets were extracted after different incubation times as described for microsomal suspensions. The amounts present were barely detectable (data not shown), and after 1 hr less than 2 per cent of the phenol was found in the cell pellet (which represents 3% (w/v) of the suspension).

The enzyme—properties of the metabolizing system. The subcellular localization and NADPH-dependence of the dichloro-*p*-nitroanisole O-demethylase is shown in Fig. 6. In these experiments the 15000 *g* supernatant, the 100,000 *g* supernatant, and the washed microsomes were prepared according to Zannoni [35]. Under these conditions the assay was linear with time and amount of microsomal protein added. The NADPH-dependence was tested by adding, or not adding NADP to the NADPH-generating system. NADP itself did not stimulate microsomal metabolism of dichloro-*p*-nitroanisole in the absence of NADPH-generating system (data not shown).

Most of the activity is found in the microsomal fraction, being strictly NADPH-dependent. Considering the loss of microsomal protein during the washing procedure, all activity found in the original

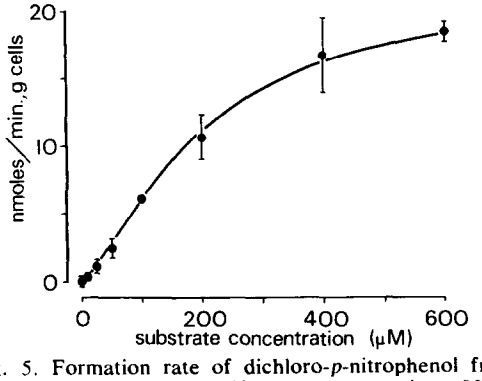


Fig. 5. Formation rate of dichloro-*p*-nitrophenol from dichloro-*p*-nitroanisole at different concentrations. 30 mg cells were incubated in 1 ml at 37° as described in Fig. 3 and Materials and Methods. Mean ± S.E. from four experiments.

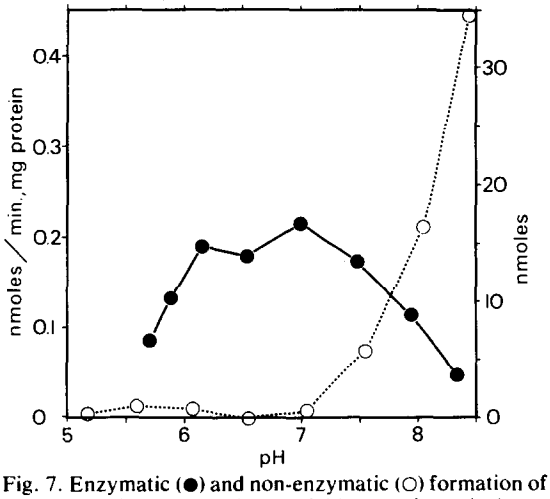


Fig. 7. Enzymatic (●) and non-enzymatic (○) formation of dichloro-*p*-nitrophenol from dichloro-*p*-nitroanisole at different pH values. Microsomes were incubated with dichloro-*p*-nitroanisole as described in experimental, but using buffers with different pH values. Final pH values were measured in extra samples, treated identically to the others. Non-enzymatic formation of product (without NADP) was subtracted from total to obtain enzymatic reaction rate. *Right scale*: total nmoles product formed. *Left scale*: specific activity (applies to enzymatic reaction only). Mean values from double determinations in two separate experiments. Note: enzyme activity lower than normal in these experiments.

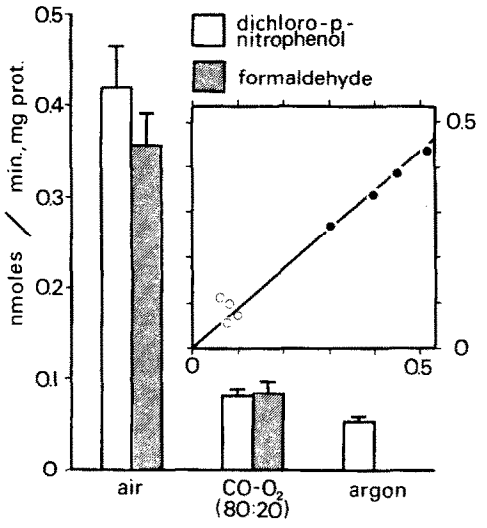


Fig. 8. Formation of dichloro-*p*-nitrophenol and formaldehyde from dichloro-*p*-nitroanisole in microsomal suspensions bubbled with different gas mixtures, and incubated as described in experimental. "TV-antennas" indicate S.E.M. from four experiments.
Inset shows the covariation of the formation rate for the two products in the four experiments. *Ordinate*: nmoles formaldehyde formed per min. and mg protein. *Abscissa*: nmoles dichloro-*p*-nitrophenol formed per min. and mg protein. Samples bubbled with air (●); bubbled with $CO–O_2$ (80:20) (○).

15,000 *g* supernatant can be accounted for as microsomal activity. There is, however, also a small NADPH-independent activity in the 100,000 *g* supernatant. Some NADP is probably present in the 15,000 *g* supernatant, and this might explain the apparent NADPH-independent activity in this fraction (being about one quarter of the total activity).

When assayed as described in Materials and Methods specific activity in microsomal suspensions was 0.74 ± 0.27 nmoles product formed/min and mg microsomal protein (mean ± S.E. from seventeen experiments performed during 6 months). Blank values were very low, A_{400} being 0.000–0.002 when microsomes were incubated without substrate.

The pH-dependence of the enzyme is shown in Fig. 7. It has a broad optimum around pH 7. Fig. 7 also shows the production of dichloro-*p*-nitrophenol in the absence of NADP. The same reaction occurred in buffers with 1% serum albumin, and containing no enzymes at all. This non-enzymatic reaction is probably due to hydrolysis of the substrate. It is strongly pH-dependent, and could be reduced virtually to zero by lowering pH to 7.0.

Figure 8 shows the simultaneous formation of dichloro-*p*-nitrophenol and formaldehyde under different conditions. It is evident that the two products are formed in approximately equimolar amounts, as expected from the reaction scheme in Fig. 1. It can also be seen that the reaction is inhibited by carbon monoxide, and that under the relatively anaerobic conditions after bubbling argon through the suspension, most of the activity is abolished. In the samples bubbled with argon the formaldehyde assay did not work well. The blank values were reduced, and the assay gave variable results, so these values are omitted.

DISCUSSION

From the results presented in this paper it can be concluded that dichloro-*p*-nitroanisole is indeed *O*-demethylated to the corresponding phenol in isolated hepatocytes. The phenol is not further metabolized, at least not when added extracellularly, and practically all the formed phenol appears rapidly in the extracellular medium. The glucuronidation of dichloro-*p*-nitrophenol has also been shown to be very low, or non-existent in microsomal systems fortified with cofactors for glucuronyl transferase [31].

The cell subfractionation experiments show that the enzymes responsible for the reaction are situated in the microsomal fraction. The parallel production of formaldehyde, and the dependence on oxygen and NADPH show that the reaction is catalysed by a mixed function oxidase. The sensitivity to carbon monoxide indicates that cytochrome P450 is involved. This conclusion is further strengthened by the fact that metabolizing activity is increased considerably by treating the animals with phenobarbital (to be published). This reaction obviously has many characteristics in common with other described pathways of xenobiotic metabolism, and should thus be a good model for these.

The low solubility of the substrate is a disadvantage that this assay shares with many other published methods. These problems are usually dealt with by dissolving the substrate in ethanol or similar solvents. The *O*-demethylation of dichloro-*p*-nitroanisole, however, is strongly inhibited by such solvents (to be published), and so all traces of solvent must be removed before starting the reaction. One further complication is the non-enzymic production of dichloro-*p*-nitrophenol at pH values higher than 7.0, and especially when low metabolizing activities are to be measured, proper blanks must be included.

The facts that the product is stable and accumulates in the medium, that it has a strong colour which is pH-independent around pH 7, and that the assay is simple should make this assay a convenient tool for assessing drug metabolizing activity in hepatocyte suspensions and cultures. The reaction has also been used successfully in this laboratory, (to be published) to measure the activity of the microsomal metabolizing system used in mutagenicity testing on *Salmonella* as described by Ames *et al.* [38]. The substrate was dissolved by autoclaving the soft agar. The reaction can be followed directly in the agar gel with a spectrophotometer, or after extraction of the gel as described for microsomal suspensions. The yellow colour can also be seen directly in the gel.

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