Fluorescence-microscopic measurement of intracellular cytochrome *P*-450 enzyme activity (ethoxyresorufin *O*-de-ethylation) in unfixed liver sections

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A fluorescence-microscopic method has been developed for measurement of the intracellular kinetics of the cytochrome *P*-450 reaction, ethoxyresorufin *O*-de-ethylation, in individual hepatocytes in unfixed non-frozen liver sections obtained from control or 3-methylcholanthrene-pretreated mice. De-ethylation was enhanced in the presence of salicylamide, but inhibited in the presence of α -naphthoflavone. A total of 21 reaction rate curves (fluorescence of the metabolite, resorufin, versus time) were constructed for different individual hepatocytes and groups of two or three hepatocytes, in a total of 12 sections from four control and eight 3-methylcholanthrene-induced mice. No two reaction curves were identical, but the curves were classified, by similarity of curve shape and fluorescence intensity, into three control categories and four 3-methylcholanthrene-induced categories. It was considered that the data represented a total of seven types of hepatocytes, which differed in their ethoxyresorufin de-ethylase reaction characteristics. Hepatocytes in the sections from 3-methylcholanthrene-pretreated mice showed higher de-ethylase activities than the hepatocytes from control mice, but the increase in activity covered a wide range of values, from 3-fold to 17-fold higher than the mean control value, suggesting that 3-methylcholanthrene did not induce ethoxyresorufin de-ethylation to the same extent in all hepatocytes.

An ability to measure the kinetics of enzyme reactions occurring within individual cells is required in order to answer questions concerning the true intracellular functions of enzymes and their distributions throughout an organ. The question of distribution is perhaps more easily investigated than that of intracellular function. Many enzymes appear to be distributed in a non-uniform manner throughout the liver (Rappaport, 1976) and included among these are several of the various enzymes that metabolize drugs and other chemicals.

It has been shown, by using microspectrophotometry (Gooding *et al.*, 1978) and immunohistochemistry (Baron *et al.*, 1981), that the concentration of cytochrome *P*-450 in hepatocytes located near the central vein is almost double that in hepatocytes nearer the portal tract. (The hepatic region nearest the central vein is designated centrilobular, pericentral or zone 3, whereas the region nearest the portal tract is termed periportal or zone 1; Rappaport, 1976.) Several other enzymes invol-

ved in drug metabolism also have a predominantly centrilobular or centrilobular-midzonal distribution, for example NADPH-cytochrome *c* reductase (Taira *et al.*, 1980), cytochrome *b*₅ (Redick *et al.*, 1980) and epoxide hydratase (Bentley *et al.*, 1979). Conversely, alcohol dehydrogenase is a mainly periportal enzyme (James *et al.*, 1981). Hepatic cytochrome *P*-450 is a family of variant enzymes differing in several characteristics, including chemical inducibility and reaction specificities (Lu & West, 1979). Two different cytochrome *P*-450 variants, induced by PB and 3-MC respectively, have each been shown by immunohistochemical means to be predominantly centrilobular, although they purportedly differ slightly in their overall patterns of hepatic distribution (Baron *et al.*, 1981, 1982).

Since the distribution of cytochrome *P*-450 probably significantly influences, through metabolism, the distribution of tissue damage caused by hepatotoxic chemicals (Gillette *et al.*, 1974; Mitchell *et al.*, 1976; Hunter *et al.*, 1977; James *et al.*, 1981), it is important that immunological analysis of the concentrations of cytochrome *P*-450 variants is matched by studies of the distributions of their enzymic activities. Histofluorimetrically-determined

Abbreviations used: PB, phenobarbitone; 3-MC, 3-methylcholanthrene; DMSO, dimethyl sulphoxide; ANF, α -naphthoflavone; PVP, polyvinylpyrrolidin-2-one; BSA, bovine serum albumin.

benzo[a]pyrene and perylene metabolism activities are primarily centrilobular (Wattenberg & Leong, 1962), whereas histochemically-measurable aniline hydroxylase is distributed uniformly throughout the liver (Gangolli & Wright, 1971). Ethoxycoumarin de-ethylase, measured on the surface of perfused livers by using fibre-optic fluorimetry, is more rapid in pericentral than in periportal regions (Sungchul *et al.*, 1981). Cytochrome *P*-450 and its benzo[a]pyrene hydroxylase activity are non-uniformly distributed among 12 subpopulations of hepatocytes separated from one liver by centrifugal sedimentation velocity analysis (Sweeney *et al.*, 1978*a,b*). Similar methods have been used to demonstrate in the lung that ethoxycoumarin de-ethylase and coumarin hydroxylase have different intercellular distributions (Urade *et al.*, 1982). An indirect approach to the question of distribution has been to administer *in vivo* chemicals that are selectively toxic to different regions of the liver and then to measure the decreases in cytochrome *P*-450 reaction in microsomes prepared from the whole livers; this showed different distributions for different reactions (James *et al.*, 1981). In the first study of the rate of a cytochrome *P*-450 reaction measured directly in individual hepatocytes, microfluorimetry was used to demonstrate a marked heterogeneity in the total population of dispersed hepatocytes with respect to ethoxyresorufin de-ethylase (Burke & Hallman, 1978).

None of the techniques used in the studies quoted above is capable, however, of measuring simultaneously both the true microanatomical distributions of cytochrome *P*-450 and their enzymic activities within the liver. The histochemical methods used did not measure reaction rates, whereas the use of isolated hepatocytes involved total disruption of the microanatomy. A method is required that can measure cytochrome *P*-450 reaction rates in individual cells retained within the matrix of a liver section. We describe here (a) the development of such a method based on microfluorimetry and (b) the use of the method to show functional heterogeneity among hepatocytes. The method measures ethoxyresorufin *O*-de-ethylation, a reaction that is highly specific to the 3-MC-induced variant(s) of cytochrome *P*-450 and which is quantified by the fluorescence of the sole metabolite, resorufin (Burke & Mayer, 1974, 1975).

Experimental

Animals

Male C57BL/10 strain mice, 12–16 weeks old and weighing approx. 30g, were used. Mice were either injected once intraperitoneally with 3-MC (80mg/kg body wt., as a 1% solution in olive oil) 3 days before use, or used untreated (controls).

Chemicals

Stock solutions of ethoxyresorufin (0.1 mM) and resorufin (0.1 mM) were prepared in DMSO. Both ethoxyresorufin and resorufin are light-sensitive and to prevent photodecomposition of the two chemicals the solutions were stored in the dark and the laboratory was illuminated with red light.

Ethoxyresorufin was both synthesized by the method of Mayer *et al.* (1977) and purchased from Molecular Probes Inc., Junction City, OR, U.S.A. Resorufin was purchased from both Molecular Probes Inc. and Matheson, Coleman and Bell (East Rutherford, NJ, U.S.A.). 3-MC was obtained from Fluka AG (Buchs, Switzerland), salicylamide from Sigma Chemical Co. (Poole, Dorset, U.K.) and ANF from Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

Vibratome sectioning of unfixed non-frozen liver

Mice were killed by cervical dislocation, their gall bladders removed and their livers excised. It was found that 60 μ m thickness cryostat liver sections were almost devoid of ethoxyresorufin de-ethylation activity and that glutaraldehyde fixation totally destroyed the activity. (To prepare the cryostat sections a small piece of liver was rapidly frozen at -10°C on the freezing stage of an MSE Base Sledge Microtome equipped with a Peltier-effect cooler. Sections were cut with a knife precooled to -20°C and transferred to a slide using a brush. The section thickness of 60 μ m was chosen to ensure a layer of unbroken hepatocytes.) Consequently, it was decided to use sections of unfixed non-frozen liver, which were cut by using a vibratome constructed from a conventional vibrating-reed veterinary hair clipper (Clukes Clippers), as described by Bazer & Knight (1980). The blade of the clippers was removed and a specially constructed razor blade brass holder was attached to the vibrator. A razor blade (double-edged cut lengthwise in half) was clamped between the two plates of the holder. The razor blade was cleaned in ethanol before mounting in its holder. The vibratome was mounted on an MSE sledge microtome in place of the knife.

A small piece (approx. 5mm cube) of freshly excised liver was quickly blotted dry and glued with cyano-acrylate glue ('Superglue') to a metal plate fixed on to the stage of the microtome sledge. Sectioning was improved when sufficient glue was used to spread some distance up the sides of the liver-cube. This portion of liver was neither cooled with the microtome cooler nor with cold buffer as each interfered with the setting of the cyano-acrylate glue and thus the quality of the sections. The sections of liver were readily removed from the razor blade using fine forceps and transferred to a glass slide covered with encapsulating resin (Sylgard; Dow Corning). The section was pinned to the resin

and superfusion of the section begun immediately as described below. Spare sections were kept unmounted in cool oxygenated Krebs solution for up to 3 h. The minimum thickness of section that could be cut using this method was approx. 200 μm , but this was sufficiently thin that individual hepatocytes could be discerned in transmitted light microscopy. Transmitted-light microscopy was used only for setting the position of the micropipette (see below), whereas epi-illumination fluorescence microscopy was used for measurement of de-ethylation activities (see below).

Quantification of the ethoxyresorufin O-deethylation reaction

The reaction rate was measured by the fluorescence of the sole metabolite, resorufin, by using a fluorescence microscope equipped with a photomultiplier tube, photometer and water immersion objective, as described below. The slide carrying the liver section was placed in a Petri dish on the stage of the microscope and the section was continuously superfused at 2 ml/min with oxygenated Krebs buffer containing 1.6 mM-salicylamide at 37°C. The superfusion solution was not recycled. The surface of the section was focused under red transmitted light and the tip of a micropipette was positioned on the section surface, as described below. After switching the microscope to the epi-illumination fluorescence mode (see below), the de-ethylation reaction was started by the pressure-ejection (described below) of a 4–8 μl portion of ethoxyresorufin solution from the micropipette on to a small area of the section surface. During the application of ethoxyresorufin and for the subsequent 4 min, superfusion of the section was stopped to allow the substrate and metabolite to accumulate in the section.

After the completion of ethoxyresorufin application, the fluorescence intensity of the whole objective field was measured at 1 min intervals. This field corresponded to a 400 μm -diameter area surrounding the point of substrate application. Any increase in the fluorescence above the background value (given by the superfused section in the absence of either ethoxyresorufin or resorufin) was assumed to be due to the metabolic production of resorufin. During a reaction, resorufin fluorescence usually increased to a maximum intensity and then declined to background levels (see the Results section). De-ethylation could be measured repeatedly in the same area of section after fresh applications of ethoxyresorufin, after first removing resorufin formed in the previous reaction by superfusing for 15 min, timed from the reattainment of background fluorescence. Alternatively the section could be repositioned and de-ethylation activity measured in a different area.

The effect of salicylamide on ethoxyresorufin de-ethylation

The effect of salicylamide was investigated by superfusing the liver sections with Krebs solution, which had 1.6 mM-salicylamide either included or omitted. The sections were first perfused with Krebs solution lacking salicylamide and fluorescence was measured at 1 min intervals after the application of 0.1 mM-ethoxyresorufin. The sections were then perfused with Krebs solution containing 1.6 mM-salicylamide and the fluorescence was again recorded after the application of 0.1 mM-ethoxyresorufin to the same area of the specimen.

Effect of ANF on ethoxyresorufin de-ethylation

A 1 mM solution of ANF in DMSO was prepared. This solution was used instead of pure DMSO to dilute the stock solution of ethoxyresorufin to 0.1 mM.

The effect of ANF on ethoxyresorufin de-ethylation was investigated by applying consecutively either 0.1 mM-ethoxyresorufin solution or 0.1 mM-ethoxyresorufin + 0.9 mM-ANF solution to the same area of tissue and measuring the increase in the tissue fluorescence. After its application ANF produced long-lasting inhibition of ethoxyresorufin de-ethylation throughout the entire section, thereby preventing any subsequent measurements of de-ethylation in that section.

Micro-pipettes, micromanipulator and pressure-ejection system

Micro-pipettes were drawn from 1.5 mm capillary glass (GC 150F-6; Clark Electromedical Instruments, Pangbourne, Berks., U.K.) by using an Industrial Science Associates micro-pipette puller M1. The micropipettes were pulled at 118 volts at heat settings of coarse 40 and fine 40, using a foil loop of diameter 2.5 mm and width 2.5 mm constructed from 0.05 mm-thick 90% platinum/10% rhodium. The tips were cut to a diameter of 5–10 μm , using microiridectomy scissors under a dissection microscope fitted with an eyepiece micrometer scale. The micro-pipettes were back-filled with ethoxyresorufin solution (0.1 mM in DMSO) using a Hamilton gas-tight syringe fitted with a gauge-26 hypodermic needle.

The micropipette was mounted in a gas-tight holder (EH-2S; Clark Electromedical Instruments) fitted on to a fine microelectrode holder (HMD-2; Narishige Scientific Instrument Laboratory, Setagaya-Ku, Tokyo, Japan), which was held in a miniature micromanipulator (MD-4; Narishige). This system enabled accurate placement of the micropipette tip on the surface of the liver section. The portion of ethoxyresorufin was ejected from the micropipette by the continuous application of N_2 gas pressure [25 lbf/in² (172 kPa)] for 1 min. The

pressure was regulated by a specially constructed system (components by Clippard Minimatic obtained from Miniature Pneumatics, Portsmouth, Hants., U.K.). The optimal conditions for substrate ejection were arrived at experimentally as follows: A drop of 1% PVP was allowed to dry on the surface of a slide. The amount of resorufin (0.2 mM in Krebs solution) ejected from a micropipette on to the PVP at different pressures was estimated visually, from the intensity of resorufin fluorescence in the PVP. [PVP was used to substitute for liver tissue, serving as a matrix to take up the resorufin. PVP is preferable to BSA for this, since it does not suffer from autofluorescence as does BSA (Rost & Ewen, 1971).] When pressure was applied in a pulsatile manner, the ejection of resorufin increased proportionately with pressure up to 50 lbf/in² (345 kPa), whereas ejection was not affected by alterations in either pulse frequency (0.25–2 Hz) or pulse width (250–750 ms). When pressure was applied continuously, maximal resorufin ejection occurred at 20–30 lbf/in² (138–207 kPa) and decreased at higher pressures. Since pulsatile pressure caused unacceptable oscillation of the micropipette tip, continuous pressure was used in all subsequent experiments.

Microscope and photometer

A Vickers M41 Photoplan microscope was used. This allowed the examination of the liver sections in either transmitted light or fluorescent light. A Zeiss $\times 40$ water-immersion objective (NA 0.75) and Vickers $\times 10$ eyepieces were used. The transmitted light source was a 15 W tungsten filament bulb, which was filtered by an Ilford glass orange filter (Delta) to produce red light. Excitation light for the specimen fluorescence was provided by epi-illumination from a mercury lamp (Osram HBO 200W). Excitation light from the 546 nm spectral line was selected with a Barr and Stroud DL8 narrow-band-pass interference filter without red suppression (maximum transmission 76.6% at 544.9 nm; bandwidth at half maximum = 3.5 nm). A TRITC (tetramethyl rhodamine isothiocyanate) dichroic mirror permitted epi-illumination of the specimen. Fluorescence from the specimen was selected with a Barr and Stroud DL75 narrow-band-pass interference filter (maximum transmission 49.2% at 590.1 nm; bandwidth at half maximum = 6.0 nm). The excitation and emission wavelength maxima of the substrate, ethoxyresorufin, are 456 nm and 570 nm respectively, whereas for the metabolite, resorufin, these wavelengths are 560 nm (excitation) and 586 nm (emission) (all wavelengths uncorrected). The filter combination was specifically assembled for this project and, using it, resorufin exhibited orange fluorescence with the surrounding medium being black, whereas ethoxy-

resorufin was only very weakly fluorescent. Resorufin fluorescence in the section was measured over the entire objective field, using an EMI 9781R photo-multiplier tube with a Vickers J37 photometer. This choice of photomultiplier and photometer is by no means optimal for this application, but was the only one available. We recommend in preference a photomultiplier tube showing lower dark current and higher sensitivity at 600 nm (e.g., Hamamatsu R928) and a digital photometer with variable zero suppression facility.

Calibration of the photometer system (resorufin fluorescence intensity versus resorufin concentration)

A series of lines was cut on a glass slide to form a graticule. This slide was placed on the microscope stage with the cut surface upwards under the $\times 40$ objective. The objective was then immersed in some resorufin solution (diluted in Krebs buffer), which was applied to the slide by using a pasteur pipette. With the specimen illuminated with transmitted red light, one of the lines cut on the slide was brought into focus. Without altering this focus the field was shifted to remove the line; this sequence ensured a constant focus for subsequent fluorescence measurement, which was important since fluorescence intensity varied with focus. The transmitted light was then extinguished, the specimen epi-illuminated with excitation light and the fluorescence of the resorufin solution measured using the photometer. The experiment was repeated with a range of resorufin concentrations from 0.01 μ M to 0.1 mM. The stock DMSO solution of resorufin was diluted with Krebs solution to prepare the range of resorufin concentrations used.

Results

Calibration of the photometer

The photomultiplier-photometer combination showed a linear response to fluorescence between 0.1 μ M- and 20 μ M-resorufin (Fig. 1). The fluorescence of ethoxyresorufin ranged from approx. 0.3% to 1.3% of the fluorescence of an equal concentration of resorufin. There was neither measurable nor observable autofluorescence of the tissue.

The lower limit of assay sensitivity was 0.1 μ M-resorufin, below which concentration the photometer registered background fluorescence ('background' was the apparent fluorescence of the graticule slide covered with buffer alone).

The concentration of resorufin corresponding to a fluorescence scale reading of 100 was 0.23 μ M, which corresponded to 1.6 pmol of resorufin actually observed. This quantity was calculated from the volume of resorufin solution observed by the objective lens (6.9 μ l). This volume was calculated

Fig. 1. The fluorescence versus-concentration response of the photomultiplier-photometer system to resorufin. Resorufin was diluted in Krebs buffer and applied to a slide without a liver section. Blank-corrected fluorescence = photometer reading minus background fluorescence. Background fluorescence (Krebs buffer alone) = 2.5×10^2 . Each concentration was measured on three separate occasions.

from the objective radius ($b = 0.175$ cm), the depth of resorufin solution ($h = 0.19$ cm) and the radius of the field of view at the microscope slide surface ($a = 0.02$ cm): volume = $\frac{1}{3} \times [\pi h(a^2 + ab + b^2)]$.

Visual estimation of ethoxyresorufin de-ethylation activity

After ethoxyresorufin application to sections from either control or 3-MC-pretreated mice, bright orange fluorescence characteristic of resorufin appeared visually to develop gradually over a small, discrete area (30–50 μm diameter) equivalent in size to one, two or three adjacent hepatocytes. Fluorescence developed in this manner only in the area directly beneath the micropipette tip resting on the section surface. The fluorescence increased to a maximum and thereafter appeared to decrease. The decreasing phase of fluorescence appeared to be due to diffusion of resorufin out of the hepatocytes, since there was a concomitant increase in fluorescence throughout the section in general. Maximum fluorescence was attained more rapidly with liver sections from 3-MC-pretreated mice than with sections from untreated mice, i.e. at approx. 10 and 45 min respectively after the application of ethoxyresorufin. Apparently the ethoxyresorufin selectively entered just the one, two or three adjacent hepatocytes immediately at the point of application and

was metabolized (more rapidly in liver sections from 3-MC-pretreated mice) to resorufin, which initially remained localized in the cells wherein it originated and then diffused out into the surrounding tissue or the superfusion buffer.

Quantitative analysis of ethoxyresorufin de-ethylation

The time that elapsed between excision of a liver and superfusion of a liver section was usually 15 to 20 min. A further 5–10 min was required to manipulate the micro-pipette over the liver section before the application of ethoxyresorufin. Surprisingly, the mounted superfused sections showed no noticeable decline in de-ethylase activity whenever the reaction was initiated during a 2.5 h period from mounting.

Before the application of ethoxyresorufin to an area of a liver section the background fluorescence of that area was recorded. Only one reading of background fluorescence was made before each application of ethoxyresorufin. Whenever this value was recorded the meter reading remained constant and the experiments performed to calibrate the photometer showed that the background fluorescence was stable and highly reproducible from one section to another. The mean background fluorescence ($\pm$ s.d.) was $(2.4 \pm 0.2) \times 10^2$ for eight areas in four sections from control mice and $(2.5 \pm 0.4) \times 10^2$ for 18 areas in 12 sections from 3-MC-pretreated mice. Neither salicylamide nor ANF (see below) had any effect on the background fluorescence. For each section the background fluorescence value was subtracted from the values obtained after the application of ethoxyresorufin, thus giving corrected values of fluorescence attributed to resorufin.

Ethoxyresorufin de-ethylase activity was measured as the rate of resorufin production successively in two different areas of each section prepared from a control mouse liver and in one, two or three different areas of each section prepared from a 3-MC-pretreated mouse. Each liver section assayed was prepared from a different mouse: these were numbered 1, 2, 3 etc. and each area was designated by a letter A, B or C. Hence, sections 2A and 2B represent two different areas of a section prepared from mouse no. 2. The corrected values of fluorescence were plotted against time to produce reaction rate curves (Figs. 2 and 3). Time zero was the beginning of the application of ethoxyresorufin and time 1 minute was the end of the application. With two exceptions, each rate curve was measured for sufficient time to follow the fluorescence past its maximum intensity. There were 21 rate curves characterizing 21 different areas of a total of 12 livers and no two curves were identical. However, we considered that the rate curves could be divided into

Fig. 2. Reaction rate curves for liver sections from control mice

The curves are classified into three groups of similar shape and height (as described in Table 1). Each curve depicts the rise and fall in resorufin fluorescence intensity (in blank-corrected arbitrary units) over a period of time after the application of ethoxyresorufin to an area of a section. Each curve relates to one area of a section. Different sections (i.e. different mice) are designated by different numbers; different areas of the same section are designated by different letters (e.g. curves 1A and 3A relate to one area in each of two different sections, each section having been taken from a different mouse, whereas curves 2A and 2B represent two different areas in the same section). See Table 1 for key to symbols.

groups on the basis of a combination of quantitative and qualitative criteria, e.g. their maximum fluorescence value, their time to reach peak fluorescence and the overall shape of their rate curve. Although this type of analysis is subjective, there were probably seven distinct sets of rate curves and the characteristics of each set are described in Table 1.

The rate curves were different for liver sections of 3-MC-pretreated mice compared with liver sections prepared from control mice. There was a much greater maximum increase in fluorescence and a generally shorter time taken to reach it in liver sections from 3-MC-pretreated mice than in liver sections from control mice. The times taken to reach peak fluorescence were much shorter for reactions measured photometrically than for reactions observed visually, but we have no explanation for this, except perhaps that the eye and photomultipliers respond differently to exponential increases in light intensity. There was also a much greater variation (6.7-fold) in maximum fluorescence among liver sections prepared from 3-MC-pretreated mice than among liver sections from control mice (2.2-fold). Two different areas measured on the same section did not in every case show similar rate curve characteristics.

Owing both to the non-linear response of the photometer to the fluorescence of resorufin concentrations less than $0.1 \mu\text{M}$ and to the low speed of data acquisition, it was not possible in these present experiments to quantify accurately the initial velocity of ethoxyresorufin de-ethylation in any of the liver sections from control mice. However, an estimate of the initial reaction rate was obtained for some of the sections from 3-MC-pretreated mice. The non-linear photometer response to low level fluorescence may be the explanation for the apparent

Table 1. Classification of reaction rate curves by shape and height

Control mice

- Fig. 2(a) Curves 1A (■) and 3A (□). A slow, steady increase in fluorescence over the 16 min period of measurement, without reaching a peak. The total increase was very small (only 40 units of fluorescence).
- Fig. 2(b) Curves 3B (■) and 4A (□). A relatively rapid increase in fluorescence, to reach a small but definite peak (60–80 units intensity) at an early time (4 min), followed by a rapid decrease.
- Fig. 2(c) Curves 1B (▲), 2A (■), 2B (□) and 4B (△). A slow increase or decrease in fluorescence from a small and indistinct peak (50–90 units intensity). The curves differed as to the time at which peak fluorescence was reached.

3-MC-pretreated mice

- Fig. 3(a) Curves 5B (△), 5C (▲), 6 (■) and 7 (□). A relatively rapid increase in fluorescence to reach a moderately high, definite peak (150–225 units intensity) at an early time (3–5 min), followed by a gradual decrease.
- Fig. 3(b) Curves 2A (●), 2B (○), 3A (□), 3B (△) and 4A (■). A rapid rise in fluorescence, to reach a high peak (500–700 units intensity) at an early time (2–6 min), followed by a rapid decrease.
- Fig. 3(c) Curves 4B (■) and 5A (□). A rapid rise in fluorescence, to reach a very high peak (800–1000 units) at an early time (3–6 min), followed by a gradual decrease.
- Fig. 3(d) Curves 1 (●) and 8 (○). A rise in fluorescence to a high, broad plateau (400–500 units intensity) maintained for several minutes. The plateau was reached at an early time in curve 8 compared with a late time in curve 1.

Fig. 3. Reaction rate curves for liver sections from 3-MC-pretreated mice

The curves are classified into four groups of similar shape and height (as described in Table 1). Other details are as in the legend to Fig. 4. See Table 1 for key to symbols.

Fig. 4. Effect of salicylamide on ethoxyresorufin de-ethylation

Each curve depicts the rise and fall in resorufin fluorescence intensity (in blank-corrected arbitrary units) over a period of time after the application of ethoxyresorufin to one area of a section. Reactions were conducted either in the presence (● ■ ▲) or absence (○ □ △) of 1.6 mM salicylamide, in two different areas of section 1 (1A and 1B) and in one area of section 2. Sections 1 and 2 were prepared from different 3-MC-pretreated mice: they are not the same sections designated 1 and 2 in Figs. 3 and 5.

lag phase of 2–4 min in the de-ethylation reaction in several sections from 3-MC-pretreated mice. In the linear region of photometer response, 100 units of fluorescence intensity was equivalent to an increase in resorufin concentration of $0.18 \mu\text{M}$. For a reaction that took place in an area corresponding to a single

hepatocyte, as was generally the case, and assuming that the hepatocyte was spheroidal with diameter $25 \mu\text{m}$ and volume $7 \times 10^{-9} \text{ ml}$, then 100 fluorescence units were equivalent to 1.3 amol ($1.3 \times 10^{-8} \text{ mol}$) of resorufin per hepatocyte. On this basis the approximate initial velocities of de-ethylation,

Fig. 5. *Effect of ANF on ethoxyresorufin de-ethylation* Each curve depicts the rise and fall in resorufin fluorescence intensity (in blank-corrected arbitrary units) over a period of time after the application of ethoxyresorufin to one area of a section. Reactions were conducted either in the presence (□ and △) or in the absence (■ and ▲) of 0.9 mM-ANF, in one area of each section (1, △ and ▲; 2, □ and ■). Sections 1 and 2 were prepared from different 3-MC-pretreated mice: they are not the same sections designated 1 and 2 in Figs. 3 and 4.

measured either from time zero or from the end of the lag phase as appropriate, were as follows (amol of resorufin formed/min per hepatocyte): section 5b, 1.0; section 2a, 7.5; section 2b, 4.5; section 3a, 9.0; section 3b, 7.0; section 4b, 2.0; section 5a, 3.0; section 8, 3.0 (Fig. 3). The reaction rate curves for the other areas of sections from 3-MC-pretreated mice did not have the initial linear segment necessary for this estimation.

Effect of salicylamide on de-ethylation

The effect of salicylamide on ethoxyresorufin de-ethylation was measured in two sections prepared from the livers of different 3-MC-pretreated mice respectively. In each area of a section, results were obtained consecutively in the absence and presence of salicylamide. Resorufin produced during the first measurement of the de-ethylation reaction was

removed from the section before initiating the reaction for the second time with the application of fresh ethoxyresorufin. Superfusion for 15 min, timed from when the fluorescence of the first reaction had returned to background levels, ensured apparently complete removal of resorufin. Additionally, two sets of results (A and B) were obtained from different areas of section 1 showing that it was possible to remove salicylamide from the liver section by superfusion after measuring area (A). Superfusion for 10 min was required to remove the salicylamide from the section. This was in contrast with ANF, which could not be superfused from the section (see below).

Omission of salicylamide from the superfusing solution during a de-ethylation reaction resulted in a lower rate of increase in fluorescence, a reduced maximum intensity of fluorescence (in sections 1B and 2) and a more rapid decrease in fluorescence (in section 1A) (Fig. 4).

Inhibition of ethoxyresorufin de-ethylation by ANF

The two liver sections used to study the effect of ANF were prepared from livers of different 3-MC-pretreated mice. In one area of each section the de-ethylation reaction was measured consecutively in the absence and presence of ANF. Resorufin was superfused out of the section between the two measurements, as described above.

The increase in section fluorescence normally seen in liver sections of 3-MC-pretreated mice after the application of 0.1 mM-ethoxyresorufin was inhibited by 0.9 mM-ANF (Fig. 5). ANF produced long-lasting inhibition of ethoxyresorufin de-ethylase, which could not be reversed by extensive superfusion (30 min).

Discussion

This study has shown that oxidative drug-metabolizing enzyme activity can be measured quantitatively in small discrete areas of unfixed non-frozen liver sections by the use of incident-light (epi-illumination) fluorescence microscopy. The intracellular kinetics of the representative cytochrome *P*-450 reaction, ethoxyresorufin *O*-de-ethylation (Burke & Mayer, 1974, 1975), were measured in areas of two or three adjacent hepatocytes, by analysis of the fluorescence of the reaction product, resorufin. (Although the fluorescence was recorded from an entire objective field, corresponding to approx. 180 hepatocytes in total, the micropipetting technique, by limiting the de-ethylation reaction to only one to three hepatocytes in the centre of the field, ensured that this fluorescence emanated from only these one to three hepatocytes).

It is indicative of the high sensitivity of our method that we were able to show clearly the

intracellular de-ethylase activity in individual hepatocytes of control mice, since in hepatic microsomes of control mice at 37°C this reaction has only a very low rate, $0.2 \text{ nmol} \cdot \text{min}^{-1} \cdot (\text{mg of protein})^{-1}$ (M. D. Burke, unpublished work).

The several different shapes of reaction rate curves described in Table 1 suggest strongly that de-ethylase activity varied among the different hepatocytes measured. The differences in the shapes of the reaction rate curves could have been due to intercellular differences in many factors, including not merely enzyme activities, but also cofactor concentrations and rates of diffusion of the substrate and metabolite into or out of the hepatocytes. In the present study the estimated initial velocity of the de-ethylation reaction in individual hepatocytes of different sections from 3-MC-pretreated mice showed a 13-fold range of values, from 1 to 13 amol of resorufin produced/min per hepatocyte. This degree of intercellular variation was similar to that observed previously with individual dispersed cells, but the mean estimated reaction rate for individual hepatocytes in sections was approx. 1000 times less than the mean rate reported previously for single hepatocytes dispersed in suspension (measured either directly for individual cells or by calculation from the rate for 1×10^6 hepatocytes together in suspension; Burke & Hallman, 1978; Burke & Orrenius, 1978). It is not known yet whether this discrepancy is due to an inherent difference in cytochrome *P*-450 activity between isolated hepatocytes and hepatocytes in a section, or whether it is due to the differences in method and equipment.

The resorufin fluorescence generated in the different liver sections from 3-MC-pretreated mice was, at the peak moment of its formation, between 1.7- and 10-fold greater than in the most active sections from control mice and between 3- and 17-fold greater than the mean peak fluorescence of all the control mouse sections. (That the greater fluorescence seen in the sections from 3MC-pretreated mice is due to enzymic activity has been confirmed by 'staining' the sections with resorufin and showing that there is no difference in binding.) It is notable that there was a much greater range in peak intracellular resorufin concentrations among areas of liver sections from 3-MC-pretreated mice than among areas of sections from control mice. The results indicate that there was induction of the de-ethylase enzyme (cytochrome *P*-450) by 3-MC, but that the degree of induction varied from cell to cell and was probably much less than the approx. 70-fold induction that has been reported for ethoxyresorufin de-ethylation by rat or C57BL/10 mouse hepatic microsomes (Burke & Mayer, 1974; M. D. Burke, G. I. Murray & G. M. Lees, unpublished work). This smaller induction may have been due to the fact that here we have been measuring a reaction within

hepatic cells as opposed to a reaction measured with isolated subcellular fragments, i.e. microsomes. However, this explanation seems unlikely, since 3-MC induction of ethoxyresorufin de-ethylation was found to be over 100-fold in experiments measuring the total resorufin production from 1×10^6 isolated hepatocytes dispersed in suspension in a fluorimeter cuvette (Burke & Orrenius, 1978). If there were regional, cell-to-cell variations in de-ethylase activity within the liver sections, we may by chance have not measured any of the regions most active in de-ethylation. Alternatively, the smaller induction measured may have been due to cellular damage incurred during the experiment.

Other studies, using microspectrophotometry or immunohistochemistry to measure the amount of cytochrome *P*-450 rather than its activity, have clearly shown that the concentration of cytochrome *P*-450 varies on a regional basis between the periportal, midzonal and centrilobular areas of liver (Gooding *et al.*, 1978; Baron *et al.*, 1981). Furthermore, induction of cytochrome *P*-450 occurs non-uniformly, with PB-induction of total cytochrome *P*-450 being mainly centrilobular (Gooding *et al.*, 1978), but 3-MC induction of a specific cytochrome *P*-450 variant being more pronounced in midzonal and periportal regions (Baron *et al.*, 1981, 1982). Although it did not prove possible in this present study to identify the region of the liver section to which ethoxyresorufin was applied each time and within which the de-ethylation reaction of cytochrome *P*-450 occurred, we believe that this will soon be achieved with our method.

The results obtained for the effects of salicylamide or ANF on the de-ethylation rate were as predicted from other studies using liver microsomes or isolated hepatocytes (Burke *et al.*, 1977; Burke & Hallman, 1978; Burke & Orrenius, 1978). This is important for three reasons: (i) it shows that the liver section cells were probably in an undamaged state and that the method measured the true characteristics of intracellular enzymes; (ii) it confirms that the presence of salicylamide is needed to measure optimal de-ethylation rates; (iii) it confirms that conjugating enzymes were also active in the liver section hepatocytes. Salicylamide inhibits enzymic sulphate- and glucuronide-conjugation (Bennet *et al.*, 1975; Heath & Dingell, 1974). In particular, it inhibits the conversion by a cytosolic sulphotransferase enzyme of resorufin to its non-fluorescent sulphate conjugate (Burke & Orrenius, 1978). Resorufin glucuronide formation also occurs (with liver microsomes) and it is to be expected that this also would be prevented by salicylamide in the liver section cells. In the absence of salicylamide, conjugation presumably removed resorufin from the hepatocyte almost as fast as it was formed by de-ethylation from ethoxyresorufin, thereby pre-

venting a build-up of resorufin fluorescence. ANF is a potent inhibitor of microsomal reactions catalysed by the 3-MC-induced form of cytochrome *P*-450 (Goujon *et al.*, 1972; Wiebel *et al.*, 1971; Ullrich *et al.*, 1975), including ethoxyresorufin de-ethylation (Burke *et al.*, 1977). Consequently, the approx. 90% ANF-inhibition of resorufin production in sections from 3-MC-pretreated mice helps to confirm that the reaction being measured was indeed cytochrome *P*-450-mediated de-ethylation.

In conclusion, we have described a method for measuring the intracellular kinetics of cytochrome *P*-450 enzyme activity with exquisite sensitivity and spatial resolution to the single cell level. Fluorogenic substrates, homologous with ethoxyresorufin, have recently been developed for the selective measurement of a variety of different variants of cytochrome *P*-450 (M. D. Burke & R. T. Mayer, unpublished work), and the use of these substrates in this method should enable investigation of the intracellular functions and microanatomical distributions of different cytochrome *P*-450 species in various tissues. These should be very useful in studying situations, such as recently reported, that ethanol induction of cytochrome *P*-450 is probably responsible for a shift in the intralobular site of hepatic necrosis caused by cocaine (Smith *et al.*, 1981). In addition, the high sensitivity of the method may recommend it for use with fine-needle liver biopsies from animals in toxicological studies (Zbinden, 1980).

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