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MICROFLUORIMETRIC ANALYSIS OF THE CYTOCHROME P448 ASSOCIATED, ETHOXYRESORUFIN *O*-DEETHYLASE ACTIVITIES OF INDIVIDUAL ISOLATED RAT HEPATOCYTES*

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Abstract—Ethoxyresorufin *O*-deethylase activities of individual isolated hepatocytes, from control or 3-methylcholanthrene pretreated rats, were measured by a new direct microfluorimetric method. There was a wide inter-individual variation in ethoxyresorufin *O*-deethylase activity among the hepatocytes isolated from a 3-methylcholanthrene pretreated rat. The mean activity for a sample of eighty individual hepatocytes from a 3-methylcholanthrene pretreated rat was 0.8×10^{-3} pmoles/min/cell, with a spread of activities of approximately $0.2–2.7 \times 10^{-3}$ pmoles/min/cell. In contrast, there was only a very small intercellular variation in the much lower deethylase activities of hepatocytes from a control rat, with a mean activity of 0.01×10^{-3} pmoles/min/cell. The mean ethoxyresorufin *O*-deethylase activity of a sample of individual hepatocytes isolated from a 3-methylcholanthrene pretreated rat was inhibited approximately 60 per cent by 12.5 μM α-naphthoflavone but was inhibited only approximately 22 per cent by 125 μM metyrapone.

On the assumption that suspensions of isolated hepatocytes are a more physiological system than liver microsomal preparations, there is an expanding interest in the investigation of drug metabolism using isolated hepatocytes [1]. Dealkylation and hydroxylation reactions [1–8], conjugations [5, 7, 8], cytochrome P450 [9], and its Type 1 binding of drug substrates [2, 9, 10], have all been measured with isolated hepatocytes.

Studies of drug metabolism with isolated hepatocyte suspensions have usually measured the mean enzyme activities for samples of "bulked" hepatocytes, containing generally about 1×10^6 cells per sample. However, because most tissues contain a heterogeneous cell population, accurate biochemical assessment requires the measurement of enzyme activities in individual cells. Measurement of enzymes and cofactors asssociated with drug- and steroid-metabolism has been less satisfactory for individual cells than for suspensions of bulked isolated hepatocytes. Semi-quantitative histochemical methods have been developed for detecting aniline hydroxylase [11], glucose-6-phosphate dehydrogenase and NADPH-diaphorase [12], in tissue microtome sections. Aryl hydrocarbon hydroxylase can be estimated histofluorimetrically in tissue sections containing damaged cells [13], while there is a sophisticated microfluorimetric technique for the semi-quantitative analysis of the related enzyme, benzo(a)pyrene hydroxylase, and of NADPH concentrations in ascites EL 2 cells [14, 15].

We report here a quantitative microfluorimetric study of the activity of ethoxyresorufin *O*-deethylase in individual isolated rat hepatocytes. This enzyme is identifiable with cytochrome P448 (or P_1450) [16, 17].

MATERIALS AND METHODS

Animals. Male Sprague–Dawley rats, 200 g were used. They were allowed food (pellet diet "R3", manufactured by Astra-Ewas, Södertälje, Sweden) and water *ad libitum*. Some rats were injected with 3-methylcholanthrene (20 mg/kg body wt, as a 5 mg/ml solution in corn oil; i.p., daily for 3 days), while others were injected with corn oil (0.5 ml i.p., daily for 3 days).

Chemicals. Ethoxyresorufin (99 per cent pure by high pressure liquid chromatography) was synthesised as described elsewhere [16]. Resorufin was obtained from Matheson, Coleman and Bell (East Rutherford, NJ, U.S.A.).

Preparation of isolated hepatocytes. Hepatocytes were isolated by a modification of the method of Berry and Friend [18], as described elsewhere [19]. The freshly isolated hepatocytes were of good condition, with more than 90 per cent of the sample excluding NADH [20], or Trypan Blue.

Microfluorimetric measurement of ethoxyresorufin deethylase activity with individual hepatocytes. A modified Leitz microspectrofluorimeter, as previously described was used [21]. A mercury lamp (Philips CS 100 W-2) was employed to excite the sample with light from the 492 nm (minor) and 546 nm (major) mercury lines, selected with an

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interference filter (50 per cent transmission at 527 nm; half width ± 13 nm). Epi-illumination and a × 25 Fluorite oil-immersion objective were used to excite and observe the sample. Photodecomposition of ethyoxyresorufin or resorufin did not occur, as evidenced by the stable fluorescence of solutions of either compound when excited in the microfluorimeter. Scattered excitation light was excluded from the sample fluorescence by means of a barrier filter in the fluorescence light path. Fluorescence spectra were recorded using a barrier filter with 50 per cent transmission at 550 nm (half width ± 10 nm). The fluorescence was analysed with a scanning monochromator and a photomultiplier (EMI 9590 AQ), the signal from which was passed to an X-Y recorder. All fluorescence spectra were uncorrected. Fluorescence intensity measurements for reaction rates were recorded at 595 nm (selected with the monochromator) and using a barrier filter with 50 per cent transmission at 580 nm (half width ± 10 nm). The photomultiplier signal was fed to a digital voltmeter, which automatically recorded on a magnetic tape a measurement every 2.5 sec. For both spectra and rate measurements the diameter of the circular sample-fluorescence measuring area was set at 40 μ with a variable diaphragm. Forty μ was slightly less than the average diameter of the hepatocytes, which were not exactly round. Instrument stability was determined with the fluorescence of a solution of rhodamine B (1 μg/ml in ethylene glycol).

A 0.1 mm depth dry glass Bürker chamber with cover-slide in place, was placed under the microfluorimeter objective and the counting grids were focussed. The optimum reaction conditions for deethylation of ethoxyresorufin were determined previously with bulked isolated hepatocytes [19]. To a 0.2 ml suspension of hepatocytes in well-oxygenated, albumin-free Krebs–Henseleit buffer containing 4 mM salicylamide (in a small tube) was added 0.3 ml of ethoxyresorufin solution (in Krebs–Henseleit buffer, giving a final concentration in the reaction mixture of 6 μM). This mixture was rapidly mixed in the tube and a sample was transferred with a Pasteur pipette to the Bürker chamber. The chamber filled instantaneously by capillary action and fluorescence readings were taken as soon as an apparently undamaged single hepatocyte was focussed in the centre of the objective field. The variable diaphragm, set at 40 μ, allowed the fluorescence from within an individual hepatocyte to be measured exclusive of fluorescence from the extracellular medium. When, alternatively, 40 μ-diameter areas of extracellular medium were measured for resorufin, these areas were chosen to be at least 100 μ distant from a hepatocyte. Fluorescence readings could be routinely taken within 30 sec of mixing cells and substrate. A concentration of 500 hepatocytes/0.5 ml of total reaction mixture gave a high enough cell density in the chamber to facilitate rapid choosing of a suitable hepatocyte, yet not so high a density as to cause difficulty in finding single as opposed to grouped cells. Salicylamide (1.6 mM) was added to prevent conjugation of the resorufin formed by the deethylation reaction [19]. BSA was omitted because it interfered with the deethylation,

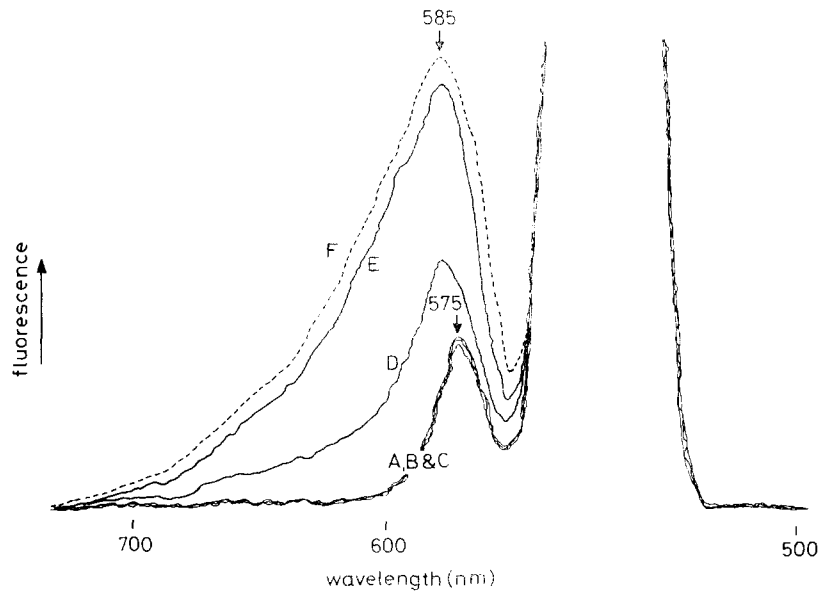

Fig. 1. Fluorescence spectrum of an individual hepatocyte, which is active in ethoxyresorufin *O*-deethylation

An individual hepatocyte from a 3-methylcholanthrene pretreated rat was incubated with ethoxyresorufin (6 μM) and salicylamide (1.6 mM) in a Bürker chamber and observed with a microfluorimeter, see Methods. The figure shows the fluorescence spectrum of the hepatocyte at 1 min (D) and 3 min (E) after mixing it with ethoxyresorufin. It also shows the fluorescence spectra of buffer alone (A), ethoxyresorufin (6 μM) in buffer (without hepatocytes) (B) or a hepatocyte from a control rat in the presence of ethoxyresorufin (C). The construction of the scanning monochromator resulted in a non-linear wavelength scale. Curve (F) is the fluorescence spectrum of a solution of authentic resorufin in buffer.

bound resorufin and quenched its fluorescence [19]. The temperature of the Bürker chamber in the microfluorimeter was 29–31°.

RESULTS

Qualitative identification of ethoxyresorufin deethylation. When hepatocytes from 3-methylcholanthrene pretreated rats were mixed with ethoxyresorufin in Krebs–Henseleit buffer containing 1.5 μM salicylamide and observed by eye in a microfluorimeter (see Methods), most of the cells showed a bright pink fluorescence that increased in intensity during the first 2–3 min after mixing with substrate. The fluorescence of the extracellular medium remained green. The pink fluorescence was characteristic of the single product of ethoxyresorufin deethylation, resorufin [16]. This was confirmed by the intracellular microfluorescence spectrum of a single pink-fluorescing hepatocyte, which showed a peak at 585 nm (Fig. 1E) and was identical with the spectrum of a solution of authentic resorufin in the Bürker chamber (Fig. 1F). Spectrum E in fig. 1 was recorded 3 min after mixing hepatocytes with ethoxyresorufin. The same hepatocyte observed 1 min after mixing showed appropriately less fluorescence (Fig. 1D). The assymetric shapes of the microfluorescence in Fig. 1 are due to a sharp cut-off below 570 nm, caused by the 550 nm (50% T) barrier filter. The broad intense peak from approximately 520–550 nm was residual scattered excitation light. The microfluorimeter itself showed an inherent small fluorescence peak at 575 nm, which was probably due to excitation light from the mercury line at 577 nm, and which was apparent with either just air, buffer, ethoxyresorufin solution or inactive hepatocytes (from a control rat) (Fig. 1A–C). An excitation wavelength of 595 nm gave the best ratio of resorufin-to-background fluorescence and was therefore used for subsequent quantitative measurements of resorufin formed during the deethylation reaction. A 580 nm (50% T) barrier filter was used for the quantitative measurements, because it ensured a more complete removal of scattered excitation light with little loss of 595 nm fluorescence, compared to the use of a 550 nm barrier filter.

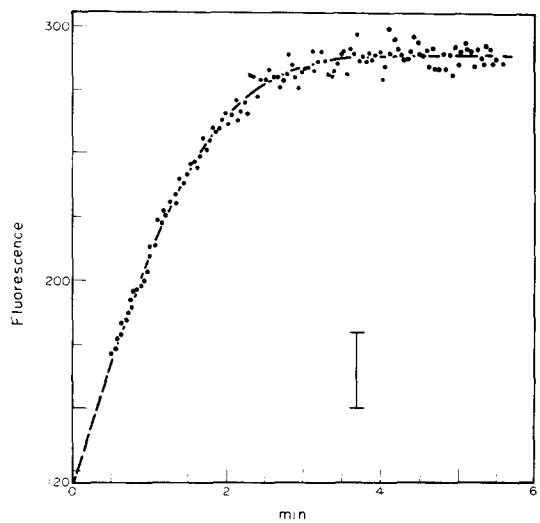

Fig. 2. Deethylation of ethoxyresorufin with an individual hepatocyte from a 3-methylcholanthrene pretreated rat

An individual hepatocyte in the presence of ethoxyresorufin (6 μM) and salicylamide (1.6 mM) was observed with a microfluorimeter, see Methods. The Fig. shows the change in the fluorescence at 595 nm of the hepatocyte after its mixing with ethoxyresorufin: a fluorescence reading was taken automatically every 2.5 sec. The vertical bar indicates the fluorescence of 6.2×10^{-4} pmoles of resorufin measured in the buffer in the same way.

Quantitative determination of ethoxyresorufin deethylase activity. Direct kinetic analysis of ethoxyresorufin *O*-deethylase activity within one hepatocyte is shown in Fig. 2. Each datum-point represents this hepatocyte's intracellular resorufin (595 nm) fluorescence at the indicated times after mixing a population of isolated hepatocytes with ethoxyresorufin. It was not possible to measure the fluorescence sooner than 30 sec after the mixing. The fluorescence at zero time was taken as being equal to that of the substrate solution itself (Fig. 1A–C). The reason for the apparent cessation of deethylase activity after 3 min is discussed later.

If salicylamide was omitted from the reaction mixture, then there was only a small increase in intra- or extra-cellular resorufin fluorescence. Salicylamide inhibited the conversion of resorufin to non-fluorescent conjugates [19]. The intracellular deethylation reaction was slightly inhibited by the presence of BSA (2% w/v) [19], (data not shown).

Microfluorescence determination of ethoxyresorufin deethylase activity was calibrated by subsequent comparison with the fluorescence of a solution of authentic resorufin in buffer, measured separately but using the same Bürker chamber. The concentration of standard resorufin was routinely 5 μM, which corresponded to 6.2×10^{-4} pmoles of resorufin actually observed (the volume in the objective field, with a 40 μ diameter measuring area and a 0.1 mm depth Bürker chamber, = 126×10^{-9} ml). The fluorescence due to 6.2×10^{-4} pmoles of resorufin is shown by a vertical bar in each of Figs. 2–4. Resorufin fluorescence measured in the microfluorimeter was linearly related to resorufin concentration up to 12.5 μM. For comparison, when using a standard 1 cm-square cuvette in a normal fluorimeter, linearity is lost above 400 nM [16]. The extended linear response of the microfluorimeter was probably due to the very short light path (0.1 mm) through the fluorescent solution in the Bürker chamber, which greatly reduced the inner filter effect. When hepatocytes were suspended in resorufin solution, in the presence of salicylamide, the resorufin rapidly entered the cells, since its fluorescence was found to be the same both inside and outside the individual hepatocytes. In the presence of salicylamide, the intracellular fluorescence of authentic resorufin was identical with all the cells of a sample of hepatocytes, and it was not influenced by the presence of ethoxyresorufin. This was observed at any resorufin concentration up to 12.5 μM.

Inter-individual variation between isolated hepatocytes in their ethoxyresorufin deethylase activities. Some of the hepatocytes in a sample mixed with ethoxyresorufin showed much less pink fluor-

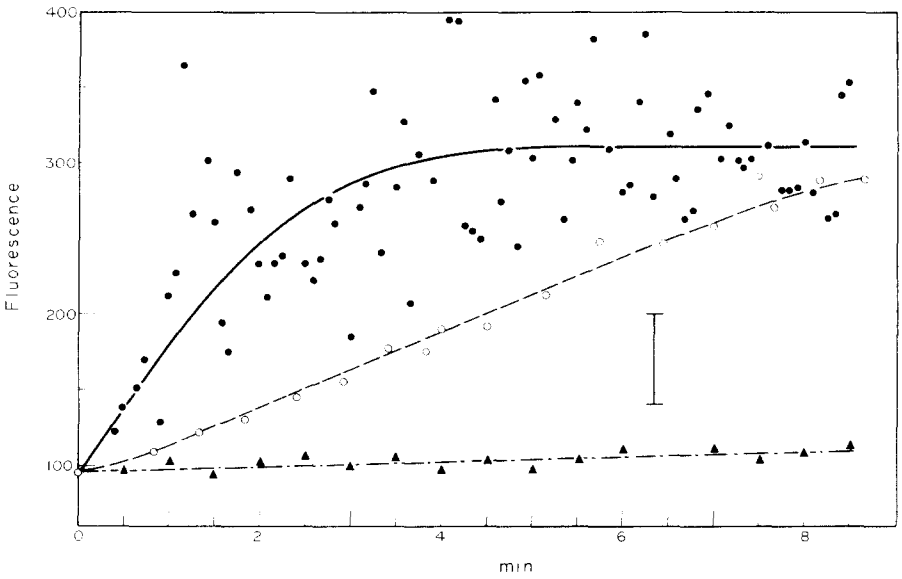

Fig. 3. Ethoxyresorufin deethylase activities of different individual hepatocytes from control or 3-methylcholanthrene pretreated rats

A sample of isolated hepatocytes, from either a control (▲) or a 3-methylcholanthrene pretreated (●) rat, in the presence of ethoxyresorufin (6 μM) and salicylamide (1.6 mM), was observed with a microfluorimeter, see Methods. The Fig. shows the fluorescence at 595 nm of different individual cells in the samples after mixing with ethoxyresorufin. A different cell, chosen by systematic searching of the Bürker chamber, was measured every 10 sec. The fluorescence is also shown of different areas of the extracellular medium (○) surrounding a sample of hepatocytes from a 3-methylcholanthrene pretreated rat in the presence of ethoxyresorufin. The vertical bar indicates the fluorescence of 6.2×10^{-4} pmoles of resorufin measured in the same way. The curve for 3-methylcholanthrene pretreated rats was fitted to the mean fluorescence values for all the hepatocytes measured within successive minute intervals, i.e. the mean for the 0–1 min group, the mean for the 1–2 min group, etc. This curve is shown horizontal from 5 min, since data gathered up to 12 min (but not shown) indicated that the mean intracellular fluorescence remained constant.

escence than others. A broad spread of ethoxy-resorufin deethylase activities was obvious when quantitative measurements were made. Figure 3 is a "field-plot" of intracellular resorufin (595 nm) fluorescence for individual hepatocytes after mixing a population with ethoxyresorufin. In this plot each datum-point represents the fluorescence of a different individual hepatocyte, selected every 10 sec by systematic searching of the Bürker chamber counting-grid. A curve has been fitted to the data, to indicate the mean, increasing resorufin fluorescence in the total cell population. During the period 4–8 min, when there was no further increase in mean intracellular resorufin fluorescence, there was an approximately symmetrical distribution of individual intracellular fluorescence about the mean for the population.

The extracellular resorufin fluorescence, of a sample of hepatocytes mixed with ethoxyresorufin, increased more slowly than the intracellular fluorescence (Fig. 3). The extracellular fluorescence eventually reached the mean intracellular value some 10 min after this had reached a constant level. Presumably, resorufin formed during the intracellular deethylation reaction was passing out of the cells.

The measured initial rate of ethoxyresorufin deethylation (i.e. intracellular resorufin accumulation) was very probably influenced by the rate of resorufin loss from the cells. Thus, the true deethylation rate was probably faster than the measured rate. The cessation of deethylation activity after 3–5 min (Figs. 2 and 3) was not due to depletion of substrate (higher ethoxyresorufin concentrations did not prolong the reaction), nor was it because the intracellular resorufin concentration became high enough to evince fluorescence self-quenching effects (these did not become apparent below 12.5 μM resorufin). The fact that the extracellular resorufin concentration continued rising for several minutes after the mean intracellular concentration had stabilised, suggests That deethylation (accompanied by resorufin leakage) continued for several minutes longer in some cells than in others. The mean intracellular fluorescence would have fallen if the continued extracellular fluorescence increase had been entirely due to leakage of accumulated resorufin from cells which had ceased metabolising ethoxyresorufin. Possibly, the cessation of deethylation activity was due to product-inhibition, arising from high concentrations of accumulated intracellular resorufin. This might have occurred if passage of resorufin out of the hepatocytes was a non-active process, dependent on the resorufin concentration gradient across the cell membrane, so that loss of resorufin from the cells would have become slower as the extracellular concentration rose. Product-inhibition of ethoxyresorufin deethylation has been noted with liver microsomal preparations (M. D. Burke, unpublished observations). A further cause for the

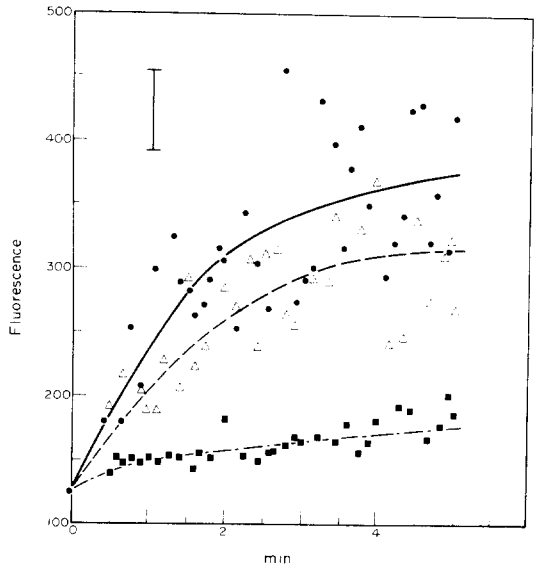

Fig. 4. The effect of α-naphthoflavone or metyrapone on the ethoxyresorufin *O*-deethylase activities of individual hepatocytes from a 3-methylcholanthrene pretreated rat. A sample of isolated hepatocytes in the presence of ethoxyresorufin (6 μM) and salicylamide (1.6 mM) either alone (—●—) or the presence of either ANF (12.5 μM) (■) or metyrapone (125 μM) (–△–), was observed with a microfluorimeter, see Methods. The fluorescence of a different hepatocyte was measured every 10 sec. The vertical bar indicates the fluoresence of 6.2×10^{-4} pmoles of resorufin measured in the same way. The curves were fitted to the data as described in Fig. 3.

cessation of deethylation activity might have been anaerobiosis of the hepatocytes.

When the hepatocytes in ethoxyresorufin were observed by eye in the microfluorimeter, the pink fluorescence of the cells faded after about 4 min of reaction. This apparent fading was probably due to a gradual loss of contrast between the cell and the medium as the fluorescence of the latter increased. It was probably not due to any loss of resorufin from the hepatocytes.

The increase in intracellular fluorescence was much slower when ethoxyresorufin was mixed with hepatocytes isolated from a control rat, compared to hepatocytes from a 3-methylcholanthrene pretreated rat (Fig. 3). The inter-individual variation in ethoxyresorufin deethylase activity was also very much less with hepatocytes from control compared to 3-methylcholanthrene pretreated rats.

Inhibition of deethylation by α-naphthoflavone or metyrapone. Liver microsomal reactions catalysed by 3-methylcholanthrene induced cytochrome P448 are generally more strongly inhibited by ANF than by metyrapone [22–24]. Liver microsomal ethoxyresorufin *O*-deethylation follows this trend [25]. Similarly, the increase in intracellular resorufin fluorescence after mixing ethoxyresorufin with hepatocytes from 3-methylcholanthrene pretreated rats was approximately 60 per cent inhibited by ANF (12.5 μM), but was hardly affected by a 10-fold higher concentration of metyrapone (125 μM) (Fig. 4). ANF-inhibition also markedly decreased the inter-individual variation in the ethoxyresorufin deethylase activity of the hepatocytes. Metyrapone possibly inhibited the deethylase to a greater extent in some hepatocytes than in others, but this was not investigated further. Neither ANF nor metyrapone directly affected the fluorescence of resorufin.

DISCUSSION

Studies of the activities of the monooxygenase enzymes responsible for metabolising drugs have been reported for samples of bulked isolated hepatocytes in suspension [1–10]. Methods currently available are unsuitable for the quantitative measurement of drug metabolising reactions with individual isolated cells, whereas inter-individual differences in enzyme activities between cells may be highly important for tissues comprised of several different cell types. We describe here the first direct kinetic measurement of a cytochrome P450 type monooxygenase enzyme activity, ethoxyresorufin *O*-deethylase, within individual isolated hepatocytes.

The initial ethoxyresorufin *O*-deethylase activity in the single hepatocyte (from a 3-methylcholanthrene pretreated rat) measured for Fig. 2 was 2×10^{-3} pmoles/min, which is equivalent to 2 nmoles/min/10^6 cells. This value, determined using an enzyme-saturating substrate concentration, was similar but not identical to the apparent V_{max} determined for a mixture of 1×10^6 hepatocytes (10 nmoles/min/10^6 cells) [19]. However, as shown in Figs. 3 and 4, there was a very great variation in the deethylase activity between the individual cells of a sample of hepatocytes isolated from the same rat liver. The mean initial activity for the sample of 80 hepatocytes (from a 3-methylcholanthrene pretreated rat) measured for Fig. 3 was approximately 0.8×10^{-3} pmoles/min/cell, with a spread of activities of approximately $0.2–2.7 \times 10^{-3}$ pmoles/min/cell. The intercellular variations in apparent rates of deethylation were very probably due to real differences in deethylase activity, rather than to differences in rates of resorufin breakdown or loss from the cells, or to differences in cell-protein binding and fluorescence quenching of resorufin. Thus, the differences were largely abolished by ANF inhibition of the deethylase, while hepatocytes from a control rat showed uniformly low deethylase activities (mean activity = 0.01×10^{-3} pmoles/min/cell). Cellular uptake of trypan blue in the absence of ethoxyresorufin indicated that there was no significant intercellular variation in cell viability. Resorufin conjugation was totally inhibited in these experiments by salicylamide [19], and there was no net disappearance of resorufin from the hepatocytes, due to either metabolism or leakage, as measured after the active period of deethylation. The fluorescence vs concentration curve for authentic intracellular resorufin, measured in the presence of salicylamide to inhibit conjugation, indicated that resorufin was not bound or quenched more strongly by some hepatocytes than by others. Neither was the fluorescence of resorufin different when measured in different parts of the Bürker chamber. It is possible that salicylamide inhibited the ethoxyresorufin deethylase to different extents with different cells, but the concentration used did not inhibit the

reaction when measured with samples of 1×10^6 bulked hepatocytes in the standard fluorimeter [19]. The areas of extracellular medium measured were at least 100 μ distant from any hepatocyte. This would probably have allowed the different resorufin concentrations put out by each hepatocyte to have completely equilibrated in the medium, and would account for the lack of variation in resorufin concentration between different areas of the extracellular medium. A similar intercellular variation in aniline hydroxylase activity has been reported for histochemically analysed liver sections from phenobarbital pretreated rats [26], and occurs also with glucose-6-phosphate dehydrogenase and succinate dehydrogenase in normal rat liver.‡

The sensitivity of ethoxyresorufin deethylase in the individual isolated hepatocytes to inhibition by ANF was in clear contrast to the lack of inhibition by metyrapone. This difference in response to the two agents showed that a similar effect seen, for ethoxyresorufin *O*-deethylation [25] and other cytochrome P448 mediated reactions [23], with liver microsomal preparations was not an artifact of cellular subfractionation. It was remarkable that after ANF inhibition there was very little intercellular variation in deethylase activity. A gradual diminution of the intercellular variation occurred concomittantly with an increasing inhibition as higher ANF concentrations were used (data not shown).

These experiments also provided a direct measurement of the movement of the deethylation reaction product, resorufin, into or out of hepatocytes. Previous studies have necessitated removal of hepatocytes from their extracellular medium after a period of intracellular reaction [3]. Exogenous resorufin equilibrated itself rapidly (within 30 sec, the dead-response time of the method) on either side of the hepatocyte cell membrane. It was equally able to move out from the hepatocytes. In contrast, some hydroxylated benzo(a)pyrene metabolites are preferentially either retained or excluded from hepatocytes [3].

In conclusion, we have reported here a microfluorimetric analysis of ethoxyresorufin *O*-deethylase activity in individual isolated rat hepatocytes. There was a wide inter-individual variation in the deethylase activity among the liver cells of a 3-methylcholanthrene pretreated rat. It is clear that measurement of monooxygenase activities with both isolated bulked hepatocytes and individual cells is necessary for a true description of the activity of a whole liver.

The abbreviations used are: BSA—bovine serum albumin; ANF—α-naphthoflavone.

‡ J. R. Fry, personal communication.

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