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# A DIRECT TEST FOR MONO-OXYGENASE ACTIVITY OF INTACT SMALL INTESTINE USING SURFACE REFLECTANCE FLUORIMETRY*

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**Abstract**—The *O*-dealkylation activity for 7-ethoxycoumarin of mouse small intestine was determined by recording the surface fluorescence of the umbelliferone formed in everted intestinal segments. A simple holding device suitable for commercially available cuvettes has been designed. After hypotonic shocking of the mucosal cells the substrate was added in 0·1 M Tris buffer pH 7·9 causing a linear increase in fluorescence for more than 15 min. Pretreatment of mice with phenobarbital increased the activity several fold, the induction following a biphasic course. The maximal rate of *O*-dealkylation by the entire small intestine was calculated as 19 nmoles $min^{-1}$. In agreement with previous results obtained with homogenates of small intestine the highest activity was located at a distance of 8–12 cm from the pylorus. Inhibition experiments indicate the involvement of cytochrome P-450.

USING the sensitive fluorometric test for benzpyrene hydroxylation, it has been shown that the small intestine among other tissues shows mono-oxygenase activity towards drugs and foreign compounds.[1,2] This activity was increased upon pretreatment of the animals with aromatic hydrocarbons. Previously, we have reported that a phenobarbital-inducible mono-oxygenase activity in small intestine can be demonstrated with another fluorogenic substrate, 7-ethoxycoumarin.[3] A similar finding has been reported with aminopyrine as substrate.[4] It was also possible to obtain induction by phenobarbital in the isolated small intestine during a 2 hr incubation period. The increase in *O*-dealkylation activity was found to correlate with an increase in NADPH-cytochrome *c* reductase activity.[5,6]

Since the digestive tract represents one of the major portals of entry of drugs and other xenobiotics into the organism, it seemed worthwhile to study the mono-oxygenase of the small intestine and its characteristics in greater detail. This, however, requires a quick and reliable test which should reflect *in vivo* conditions as closely as possible. Subcellular fractions did not prove suitable for this purpose because of the rapid peptic degradation of the mono-oxygenase system following breakage of the mucosal cell membrane.

The present paper describes a direct test for the *O*-dealkylation activity based on the surface fluorescence of mucosal cells in the presence of the fluorogenic substrate 7-ethoxycoumarin.

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## METHODS AND MATERIALS

Male black mice (strain C57 BL/6N) of 25g body wt were kept on a commercial diet (Altromin®) and water *ad lib*. The animals were decapitated and the intestine was cut at the pylorus. The intestine was rinsed *in situ* with 20 ml of ice-cold isotonic KCl-solution. 4 cm long segments were excised and carefully moved over the tip of the stainless steel holder of the cuvette insert (Fig. 1). The intestine was everted during this procedure. Care had to be taken to avoid stretching or compressing the segment in order to make it comparable with the measurements of other segments. Excess tissue was cut off and the sample holder was mounted on a cuvette of 10 mm pathlength filled with 2 ml of oxygen-saturated incubation medium. An excess of medium was squeezed out through the two holes in the cover. A schematic drawing of the cuvette insert is shown in Fig. 1.

The cuvette was placed in a cuvette holder of a Zeiss PMQ-2 fluorimeter in the position for reflectance measurement. The excitation wavelength was set as 385 nm in order to avoid interference from the reduced pyridine nucleotide excitation. A filter with transmission above 420 nm was used instead of a second monochromator. All measurements were made at room temperature.

The substrate 7-ethoxycoumarin was synthesized as described earlier.[7] Metyrapone was a gift from Ciba, Pharmaceutical Company, Basel, Switzerland and 7,8-benzoflavone was purchased from EGA-Chemie, KG, Steinheim, W. Germany.

## RESULTS AND DISCUSSION

The device, schematically presented in Fig. 1, allows rapid mounting of intestinal segments with concomitant eversion so that the mucosal layer is exposed to the light

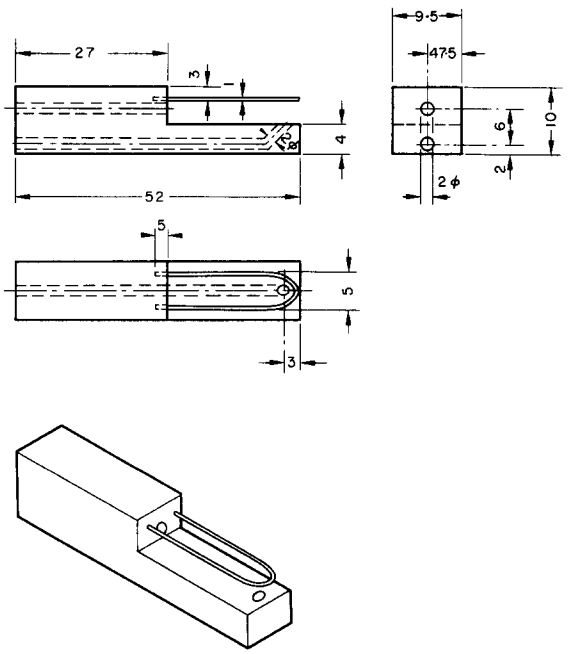


FIG. 1. Holding device for the measurement of surface fluorescence of segments of mouse small intestine. Materials used were Teflon and 1 mm stainless steel wire. Dimensions in mm.

beam. The main advantage of this technique consists in the ability to measure drug mono-oxygenase activity of small intestine close to *in vivo* conditions. Compared with homogenates the activity is stable over a longer period of time and a series of experiments can be performed with one sample. When comparing the activity between different segments care has to be taken that both samples are mounted identically.

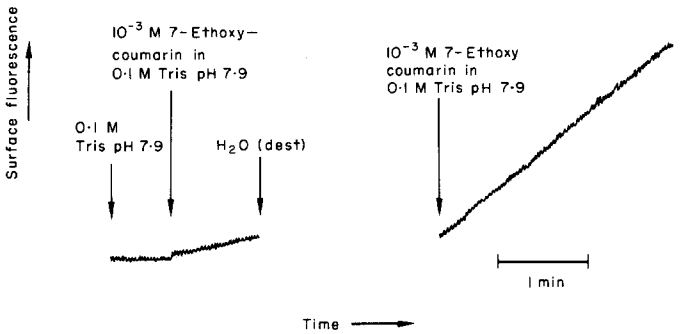


FIG. 2. Test for *O*-dealkylation activity in whole small intestine. The preparation of the sample and the fluorescence assay we described under Methods. A 3-cm segment of small intestine from a phenobarbital-pretreated mouse (1 × 100 mg phenobarbital $kg^{-1}$ orally, sacrificed after 5 hr) was mounted on the special holder depicted in Fig. 1. Changes in the medium were made as indicated by arrows. The gap indicates the time of incubation in a beaker filled with 100 ml of distilled water before the insert is replaced into the cuvette.

Unlike our previous studies with homogenates the substrate 7-ethoxycoumarin had to penetrate the mucosal cell membrane in order to be dealkylated by the mono-oxygenase system in the endoplasmic reticulum. As can be seen from Fig. 2 only weak fluorescence emerged when the substrate, dissolved in 0·1 1 M Tris buffer pH 7·9, was added to a segment of small intestine. However, when previously exposed to distilled water for about 1 min a higher and constant increase of fluorescence was recorded. Obviously, hypotonic shocking of the mucosal cells facilitated the penetration of 7-ethoxycoumarin through the cell membrane. No breakage of the cells occurred under these conditions as indicated by the linear progress of the dealkylation without additional energy sources such as glucose. Furthermore, it was established that all the fluorescent product remained within the cells and disruption of the cells with concomitant discharge of the product umbelliferone into the medium occurred only after about 1 hr of incubation. The plot of activity versus duration of hypotonic shocking shown in Fig. 3 demonstrates that 1 min is a sufficient time for the penetration of substrate not to become rate-limiting for the dealkylation reaction. Therefore, all experiments were performed after incubating the sample in distilled water for 1 min. Subsequently, the substrate was added in 0·1 M Tris buffer pH 7·9 since the impermeability of the mucosal cell membrane was partly restored in isotonic Tyrode solution.

The presence of glucose in the incubation medium did not further stimulate the mono-oxygenation rate which suggests that sufficient endogenous reducing equivalents were available for the generation of NADPH.

In order to assure a maximal rate of mono-oxygenation the dependence of the surface fluorescence on the substrate concentration was investigated. Different

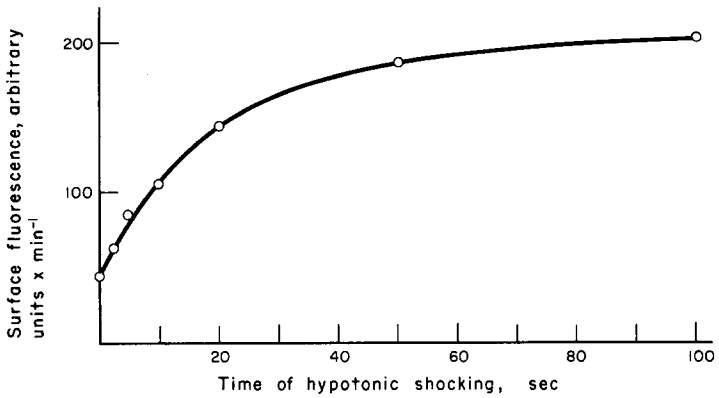


FIG. 3. Effect of hypotonic shocking on the *O*-dealkylation activity of small intestine. The test is described under Methods and in Fig. 2. Each point represents the mean from two 3-cm segments excised between 9 and 15 cm beyond the pylorus of a phenobarbital-pretreated mouse.

saturation values were obtained with small intestine segments from control mice compared with those from animals pretreated with phenobarbital as shown in Fig. 4. Saturation occurred at about $5 \times 10^{-4}$M 7-ethoxycoumarin in controls but more than 10-fold higher concentrations were necessary for the segments from phenobarbital pretreated mice. The values did not strictly obey Michaelis–Menten kinetics and therefore the calculation of a $K_m$ was not attempted.

It had already been concluded from studies of small intestine homogenates[6] that mono-oxygenase activity is not evenly distributed over the whole length of the intestine. The area of highest activity could be localized in the segments between 8 and 12 cm beyond the pylorus (Fig. 5). This was consistently found to be the case in small intestines from 25 g control mice and from mice pretreated with phenobarbital for a short time (about 3 hr). Longer treatment with intraperitoneal injections of pheno-

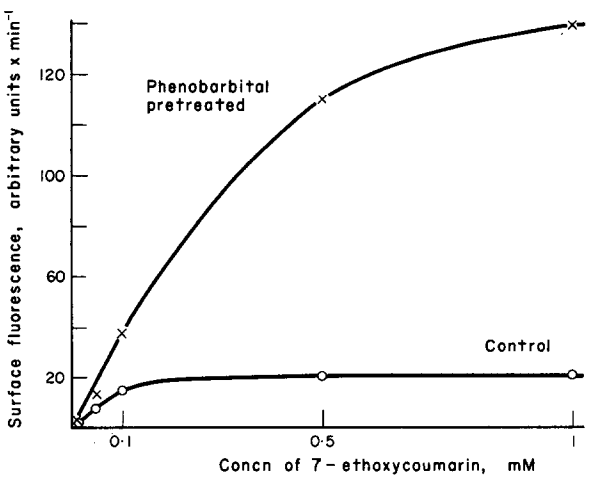


FIG. 4. Dependence on substrate concentration of the *O*-dealkylation activity for 7-ethoxycoumarin in whole mouse small intestine. The test is described under Methods and in Fig. 2. Each curve was obtained with one sample which was exposed to the various substrate concentrations for 2 min. Typical experiment from a series of four.

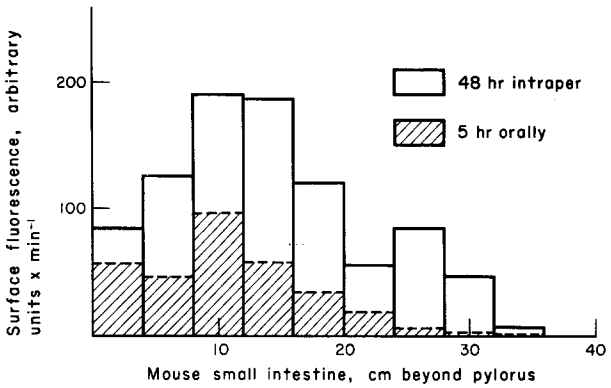


FIG. 5. Distribution of the *O*-dealkylation activity in the bowel of phenobarbital-pretreated mice. The test is described under Methods and in Fig. 2. Typical experiment from a series of five.

barbital for 2 days, resulted in a second peak between 24 and 28 cm, with a distinct minimum around 20–22 cm beyond the pylorus.

A biphasic behaviour was seen when the induction of *O*-dealkylation activity after a single dose of phenobarbital was followed over a period of time (Fig. 6). The slight decrease in activity within the first hour after the application of the barbiturate may be real but it it could also be a consequence of a competitive inhibition by phenobarbital which was probably still present in the mucosal cells. The maximum response, occurring after 3 hr, corresponds to the *in vitro* inducing effect of phenobarbital on isolated small intestine.[6] The subsequent decrease was consistently found before the activity increased again after a time interval of about 5 hr. At present no interpretation of the biphasic effects on both the localization and time course of the induced activity can be offered. This will require more biochemical studies on the nature of the induction process.

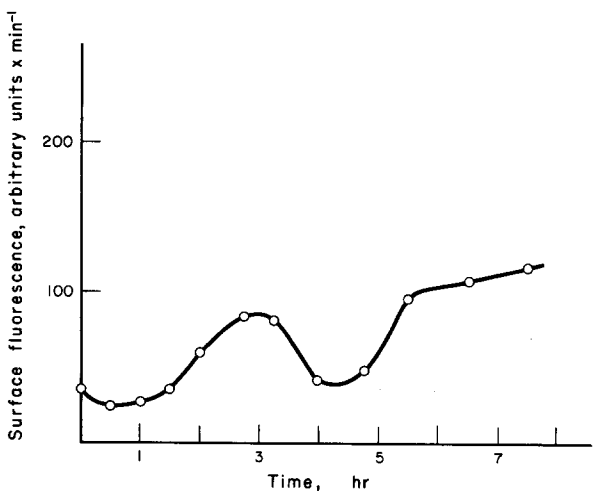


FIG. 6. Time course of *O*-dealkylation activity after a single i.p. injection of phenobarbital to 12 mice (100 mg/kg body weight). After killing at the times indicated segments of the small intestines from 8–12 cm beyond the pylorus were removed and tested for activity as described under Methods and in Fig. 2. The curve represents a typical experiment from a series of six.

TABLE 1. INHIBITION OF 7-ETHOXYCOUMARIN *O*-DEALKYLATION IN MOUSE SMALL INTESTINE

| System | % Inhibition | |
|---|---|---|
| | Controls | Phenobarbital-pretreated (24 hr with 1 × 100 mg/kg) |
| Complete system (100% $O_2$) | 0 | 0 |
| + (50% $O_2$ + 50% $N_2$) | 0 | 0 |
| + (20% $O_2$ + 80% $N_2$) | 20 | 22 |
| + (50% $O_2$ + 50% CO) | 100 | 100 |
| + Metyrapone ($5 \times 10^{-6}$M) | 52 | 43 |
| + 7,8-Benzoflavone ($1 \times 10^{-5}$M) | 77 | 35 |
| + Ethanol (0·1 M) | 48 | 10 |
| + Ethanol (0·4 M) | 100 | 55 |

The assay and test are described under Methods and in Fig. 2. When gas mixtures were used the incubation medium was pre-gassed with the mixture for 5 min at a flow rate of 40 ml/min. All activities were compared with the complete system which contained $10^{-3}$M 7-ethoxycoumarin in 0·1 M Tris buffer pH 7·9 and saturating concentrations of oxygen. Metyrapone and 7,8-benzoflavone were added as 0·1 M methanolic solutions and the inhibition was based on assays containing equal amounts of the solvent.

Some information on the mono-oxygenase system was obtained from inhibition studies (Table 1). When the oxygen concentration of the gas phase was lowered to less than 50 per cent, inhibition of the *O*-dealkylation was observed. A gas mixture of 50% oxygen and 50% carbon monoxide caused complete inhibition of the reaction, consistent with the involvement of cytochrome P-450 which is the only CO-sensitive oxygen activating component in mono-oxygenases known at present. Similarly, metyrapone as a ligand to cytochrome P-450[8] was able to block the dealkylation

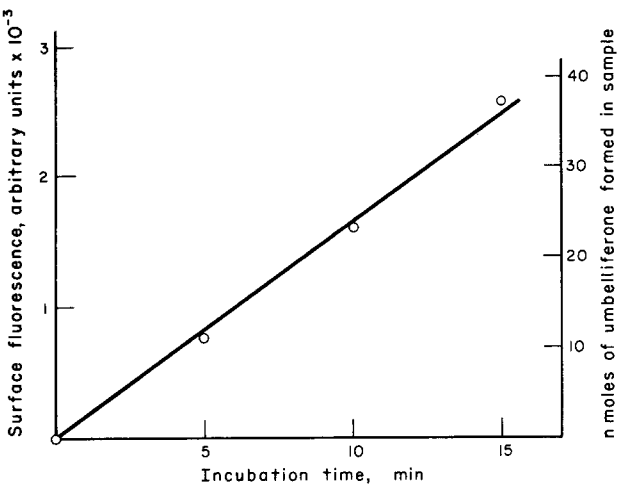


FIG. 7. Time course of umbelliferone formation as a product of 7-ethoxycoumarin dealkylation in mouse small intestine. 3-cm segments of the bowel between 9 and 12 cm beyond the pylorus were tested for surface fluorescence in the presence of $10^{-3}$ M 7-ethoxycoumarin. After the time indicated the segments were immersed in 2 ml 0·1 N $H_2SO_4$ containing 5% sodium dodecyl sulfate. 10 ml of ethyl ether were added and the mixture was shaken for 10 min. 9 ml of the ether phase was extracted with 3 ml of 0·1 N NaOH for 5 min. The fluorescence of the alkaline solution was measured as described under Methods. In order to correct for losses during the isolation various umbelliferone concentrations were added together with nonincubated segments. These assays were used for calibration. The values were obtained from triplicate experiments.

activity. The difference in the percentage of inhibition in small bowels from control mice and those pretreated with phenobarbital was not significant since the values varied over a broad range. More consistent was the higher inhibition of the activity by 7,8-benzoflavone in control mice. Methanol which was used as a solvent for 7,8-benzoflavone was slightly inhibitory only at concentrations above 0·05 M. Ethanol affected the activity in controls to a significantly higher extent than in phenobarbital-pretreated animals. Whether these differences reflect a different pattern of cytochrome P-450 species as we have shown for the *O*-dealkylation activity in liver,[9] requires further investigation.

In order to evaluate the significance of the mono-oxygenase activity in the intestine with regard to the total capacity of the drug-metabolizing system in the whole organism, we have attempted to quantify the rate of umbelliferone formation in the segments of small intestine. This required the isolation of the product from the tissue and its determination from calibration values. Fig. 7 shows the formation of umbelliferone after 5, 10 and 15 min in arbitrary units of surface fluorescence and the corresponding amounts of umbelliferone formed in 3-cm segments of the small intestine. The activity in the most active section amounts to about 1 nmole $min^{-1}$ $cm^{-1}$ of the intestine. If one integrates the total activity according to the activity distribution depicted in Fig. 4, the *O*-dealkylation rate is about 19 nmoles $min^{-1}$ for 7-ethoxycoumarin. The total activity in the liver of phenobarbital-pretreated mice was estimated to be approximately 150 nmoles $min^{-1}$ for this substrate under saturating conditions. This is considerably higher but, for a drug or a xenobiotic taken up orally in small amounts, the mono-oxygenase activity of the bowel may still be sufficient to metabolize a high percentage of the compound before it enters the liver.

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