cytochrome P-448 have necessitated the search for investigation of benzo[alpyrene binding to cytochrome P-448. A double-cell technique has been previously used by us for spectral in rat liver microsomal fraction (Estabrook, 1980) and the binding properties of benzolalpyrene to cytochrome P-450 from yeast (Saccharomyces cerevisiae) (Wiseman, 1981) respectively. The binding properties of benzolalpyrene to cytochrome P-450 have also been studied in this laboratory, a double-cell technique was adapted to remove the interference of benzolalpyrene, which absorbs in the wavelength range used (350–500nm). Only the buffer solution was used in one of the compartments of both the reference and adjoining compartments. In order to determine the dissociation constant (Kd), a double-reciprocal plot was used to determine the dissociation constant. The absorbance changes were calculated by adding up the spectral changes at peak and trough regions of the binding spectrum (ΔA380-367 + ΔA415-500) to allow for changes in base line during the inactivation and/or activation process can be described by the equation (Rakitzis, 1977, 1980):

\[
\frac{[E]}{[E]_0} = \sum_{i=1}^{n} C_i e^{-k_it}
\]

where \([E]/[E]_0\) is fractional enzyme activity, and \(t\) is reaction time. The constants \(C_i\) and \(k_i\) were determined graphically (Defares & Sneddon, 1960). The results of the effect of diethyl pyrocarbonate on DEAE-cellulose column chromatography fractions obtained from two samples of normal gastric mucosa and three samples of stomach adenocarcinoma are presented in summary form in Table 1.

It will be seen from Table 1 that, in some cases, the value for \(C_i\) is negative and also greater than unity. This in form, eqn. (1) describes a process of enzyme activation followed by inactivation. The maximum protease activation by diethyl pyrocarbonate was 400–500% of initial protease activity. It will also be seen that the peak containing the diethyl pyrocarbonate-activated enzyme appeared, if at all, at around 580 ml of effluent produced from the column. In experiments with enzyme from normal human mucosa it was found that fractions that could be activated with diethyl pyrocarbonate could also be activated by exposure to pH 3.5 (T. B. Malliopoulou & E. T. Rakitzis, unpublished work). Proteolytic activity at pH 3.5 may be due to pepsin II produced from pepsinogen II (Becker & Rapp, 1979), and it is therefore probable that the activation effect produced by diethyl pyrocarbonate on fractions obtained from normal human gastric mucosa and stomach carcinoma is due to an effect of this compound on pepsinogen II.

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Studies on the procedure to measure accurately the binding properties of benzo[alpyrene to cytochrome P-450/P-448

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Problems in the spectral study of benzo[alpyrene binding to cytochrome P-450 (or P-448) have necessitated the search for an improved method. A single-cell method was used to measure the binding properties of benzo[alpyrene to cytochrome P-450 in rat liver microsomal fraction (Estabrook et al., 1978). The double-cell technique has been used previously by us for spectral investigation of benzo[alpyrene binding to cytochrome P-448 from yeast (Saccharomyces cerevisiae) (Woods & Wiseman, 1979).

Cytochrome P-448 was solubilized from yeast (Saccharomyces cerevisiae) microsomal fraction and purified by using methods previously described by Azari & Wiseman (1980) and Wiseman & Azari (1981) respectively. The microsomal fraction was obtained from yeast cells grown under conditions of glucose repression as before (Wiseman & Lim, 1975). When the binding spectrum of benzo[alpyrene to microsomal cytochrome P-448 from yeast was recorded previously in this laboratory, a double-cell technique was adapted to remove the interference of benzo[alpyrene, which absorbs in the wavelength range used (350–500nm). Only the buffer solution was used in one of the compartments of both the reference and the sample cuvettes to complement the enzyme solution in adjoining compartments. In order to determine the dissociation constant (Kd), the results of spectral titrations at fixed wavelength at a range of benzo[alpyrene concentrations were subjected to a double-reciprocal plot. In this method the absorbance changes were calculated by adding up the spectral changes at peak and trough regions of the binding spectrum (ΔA380-367 + ΔA415-500) to allow for changes in base line during
Table 1. Illustration of two alternative double-cell techniques for measurement of the spectral changes at different wavelength region: peak 387–377 nm and trough 415–500 nm

<table>
<thead>
<tr>
<th>Wavelength employed</th>
<th>Sample cell</th>
<th>Reference cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak and trough method</td>
<td>Cytochrome P-448 + benzo[a]pyrene</td>
<td>Denatured cytochrome P-448 + acetone</td>
</tr>
<tr>
<td></td>
<td>Cytochrome P-448 + acetone</td>
<td>Cytochrome P-448 + benzo[a]pyrene</td>
</tr>
<tr>
<td>Trough method</td>
<td>Cytochrome P-448 + benzo[a]pyrene</td>
<td>Buffer + acetone</td>
</tr>
<tr>
<td></td>
<td>Cytochrome P-448 + acetone</td>
<td>Benzo[a]pyrene</td>
</tr>
</tbody>
</table>

Therefore, if the $\Delta A_{max}$ at 415 nm is taken to determine the concentration of cytochrome P-448–benzo[a]pyrene complex by using 57 mm$^{-1}$ cm$^{-1}$ as an absorption coefficient (Estabrook & Werringloer, 1978), it is not necessary to use denatured enzyme in our double-cell technique as a reference to eliminate the effect of non-specific binding. Spectral changes at this trough wavelength (415 nm) are due only to the binding of cytochrome P-448 to benzo[a]pyrene.

The substrate dissociation constant ($K_s$) was found to be the same whether $\Delta A_{387-377} + \Delta A_{415-500}$ was used in the double-reciprocal plot (against benzo[a]pyrene concentration) or whether only the $\Delta A_{415-500}$ values were employed.

Spectral comparisons of benzo[a]pyrene binding to cytochrome P-450/P-448 in microsomal fractions and in highly purified form

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The binding of benzo[a]pyrene to cytochrome P-450 has been shown by the production of a novel type-I binding spectrum when microsomal P-450 from either rat liver (Estabrook et al., 1978) or yeast (Saccharomyces cerevisiae) (Woods & Wiseman, 1980) was used. We now report further studies on the binding properties of benzo[a]pyrene to microsomal and highly purified cytochrome P-450/P-448 from both the above-mentioned sources.

Saccharomyces cerevisiae (N.C.Y.C. 240) was grown for 44 h under glucose repression in medium containing 20% glucose and mycologepeptone and yeast extract by the method described previously (Wiseman & Lim, 1975). Yeast microsomal fraction was separated, and cytochrome P-448 was solubilized from it (Azari & Wiseman, 1980). Yeast cytochrome P-448 was purified to homogeneity (97% pure) (Wiseman & Azari, 1981).

The concentration of cytochrome P-450/P-448 was determined by a spectrophotometric method (Omura et al., 1965).

The binding spectrum of benzo[a]pyrene with microsomal and purified cytochrome P-450/P-448 was recorded by using a double-cell technique (Azari & Wiseman, 1982). Spectral titrations at fixed wavelength were subjected to a double-reciprocal plot, and the spectral dissociation constant ($K_s$) and maximum absorbance changes were determined (Fig. 1). The absorbance changes used for construction of double-reciprocal plots were those at the trough ($\Delta A_{415-500}$) (Azari & Wiseman, 1982).

Purified enzymes from both systems produced type-I spectral changes with benzo[a]pyrene, and the extra peak appeared at 367 nm instead of at 360 nm as with microsomal fractions.

The values obtained for dissociation constants were 5 $\mu$m for microsomal fraction and 31 $\mu$m for purified cytochrome P-450 from liver of phenobarbital-induced rat, and 18 $\mu$m for microsomal fraction and 50 $\mu$m for purified cytochrome P-448 from yeast (Fig. 1). These results should be compared with those reported previously, namely 0.8 $\mu$m (Estabrook et al., 1978) and 9 $\mu$m (Woods & Wiseman, 1979) for hepatic microsomal cytochrome P-450 from phenobarbital-induced rats, and 36 $\mu$m (Woods & Wiseman, 1979) for microsomal cytochrome P-448 from yeast.