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THE RELATION BETWEEN THE SEX-DEPENDENCY OF TYPE I BINDING OF ETHYLMORPHINE AND THE 1-BUTANOL-INDUCED SPECTRAL CHANGE IN MOUSE LIVER MICROSOMES

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Abstract—1. A sex difference in the spectral interaction of 1-butanol with liver microsomes from adult mice was observed. In males a profound reverse type I spectrum was elicited, whereas only a small spectral change of irregular shape was apparent in females. This sex difference is the opposite of that observed in the type I binding of ethylmorphine. In immature animals no sex difference was found. Testosterone pretreatment of female mice increased the size of the 1-butanol spectrum concomitantly with a decrease in ethylmorphine binding. 2. Microsomes from males and females did not contain different levels of endogenous substrates. Thus, the presence or displacement of such substrates does not explain the sex differences in type I and reverse type I binding respectively. 3. 1-Butanol was found to interfere with both type II and type I binding. It is concluded that the 1-butanol-induced spectral change consists of at least two components and that the sex difference is due to a sex-dependent type I component.

Many compounds give rise to characteristic spectral changes, when added to a liver microsomal suspension. This is thought to reflect binding to cytochrome P-450 [1]. Two main types of difference spectra have been defined: type I (peak at 385–390 nm, trough at 420 nm), caused by binding to a hydrophobic part of the cytochrome, and type II (peak at 425–430 nm, trough at 390–400 nm), caused by direct binding to the heme [2]. The difference spectra elicited by 1-butanol and several other compounds did not fit this classification and were called modified type II or reverse type I [2, 3].

In this paper we describe a sex difference in the 1-butanol-induced spectral change in mouse liver microsomes. Thusfar, this spectral change has been investigated mostly in male rats. The reverse type I spectral change has been observed, which may reflect the reconversion of the (type I) substrate-bound form of cytochrome P-450 to the substrate-free form. Several authors have tried to confirm this concept, though with varying results [3–6]. Lately, some evidence has been obtained that the 1-butanol spectrum might represent a distinct type of spectral change rather than the displacement of endogenous substrates [7–9].

Previously, we have demonstrated a sex difference in type I binding in mice [10], which is opposite to that observed in rats [11]. In female mice, the maximal spectral change induced by type I substrates (ethylmorphine, hexobarbital) is much larger than in males, as the result of an inhibitory action of androgen [10, 12, 13]. The sex difference in the magnitude of the 1-butanol spectrum appeared to be the opposite of that found with the type I spectrum, suggesting the presence of different levels of endogenous substrates. On the other hand, we have obtained evidence that the sex difference in type I binding is caused by the existence of different amounts of cytochrome P-450 capable of type I binding [13]. It was, therefore, of interest to further investigate the nature of the 1-butanol-induced spectral change in mouse liver microsomes.

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MATERIALS AND METHODS

Animals. Male and female mice of the CPB-SE strain were obtained from the Central Animal Breeding Station TNO, Zeist, The Netherlands. They were kept in Makrolon cages with pinewood shavings, received standard food pellets (Hope Farms) and tap water *ad lib.*, and were used after they had reached the age of 11 weeks, unless stated otherwise.

Chemicals. 1-Butanol (p.a.) was obtained from Merck, ethylmorphine from Brocacef, and crystalline bovine serum albumin from Poviet. Testosterone propionate was a generous gift from Organon. All other chemicals used were at least reagent grade.

Treatments. In one experiment female mice were pretreated with testosterone. This was carried out by subcutaneous injection of testosterone propionate (2.5 mg in 0.1 ml arachis oil/animal) 14, 10, 7 and 3 days prior to the experiments. Control animals received an equivalent volume of oil.

Preparation of microsomes. After starving overnight, the animals were killed by a blow on the head. Livers were homogenized in 3 vol. of 0.1 M phosphate buffer (pH 7.4), using glass Potter tubes with a Teflon pestle.

The homogenate was centrifuged for 20 min at 9000 *g* and the microsomal fraction was sedimented from the resulting supernatant by centrifuging at 75,000 *g* for 90 min. The pellet was suspended in 0.1 M phosphate buffer (pH 7.4). For each determination the livers of several animals were pooled.

Organic solvent extraction. Extraction of liver microsomes was carried out essentially as described by Vore *et al.* [6]. Microsomal suspensions (7–8 mg protein/ml) were quickly frozen in liquid nitrogen, and subsequently freeze-dried. A portion of freeze-dried microsomes, containing about 36 mg protein, was homogenized in 9 ml acetone and centrifuged for 5 min at 35,000 *g* (− 20°). The pellet was resuspended in 9 ml acetone and again centrifuged. A final homogenate in 9 ml of acetone was filtered on a Büchner funnel. The filter contents were kept in a desiccator *in vacuo* for 30 min at 4°. The dried powder was homogenized in 18 ml Na/K-phosphate buffer (0.1 M, pH 7.4) and sonicated for three 5-sec intervals, in order to obtain a stable suspension. This sonication did not result in any loss of cytochrome P-450. Extraction with 1-butanol was performed by first treating the freeze-dried microsomes with 9 ml of 1-butanol, followed by two subsequent extractions with acetone, according to the above procedure. As a control, freeze-dried microsomes were directly suspended in buffer.

Spectral measurements. Difference spectra were recorded at room temperature using either an Aminco-Chance or an Amino DW-2 UV–VIS spectrophotometer in the split-beam mode. Microsomal suspensions, containing 1–2 mg protein/ml, were equally divided between sample and reference cuvettes, and a baseline of zero absorbance was established. Substrates were added as indicated in the text.

The concentration of cytochrome P-450 was estimated by gassing the microsomes for one minute with carbon monoxide, dividing them between sample and reference cuvettes, and then reducing the contents of the sample cuvette by the addition of a few crystals of sodium dithionite. The absorbance difference between 450 and 490 nm was taken as a measure of the cytochrome P-450 concentration, using an absorption coefficient of 91 $cm^{-1}mM^{-1}$ [14].

Protein determination. Microsomal protein was determined according to the method of Lowry *et al.* [15], using crystalline bovine serum albumin as a standard.

RESULTS

1-Butanol-induced spectral changes. Figure 1 shows typical recordings of the spectral changes induced by 1-butanol in male and female mice. The male spectrum is characterized by a broad trough around 385–390 nm and a peak at wavelengths varying between 416 and 420 nm. The female spectrum has a rather anomalous appearance: a slight trough around 385 nm and a peak between 410 and 420 nm. The general appearance of the spectra suggests that a different type of absorbance change interferes with that due to cytochrome P-450–substrate interaction, causing the absorbance to rise with increasing wavelength. This might represent a solvent effect of 1-butanol, causing a change in the turbidity of the microsomal suspension (it was not reversed by the addition of an equivalent volume of buffer to the reference cuvette). When the female spectrum is subtracted from the male spectrum, a spectrum is obtained which is the reverse of the type I spectral change (Fig. 1).

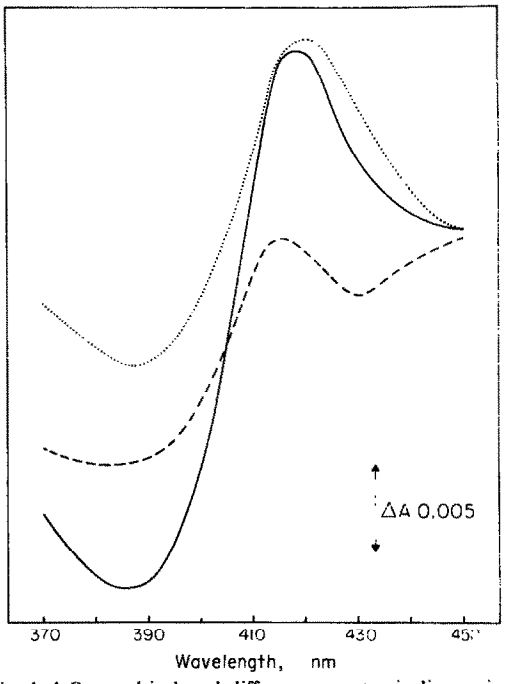

Fig. 1. 1-Butanol-induced difference spectra in liver microsomes from male and female mice. —— Males; - - - - - females; ..... difference between —— and - - - -. The cytochrome P-450 contents were: males 1.46 nmol/ml; females 1.44 nmol/ml. The 1-butanol concentration was 66 mM.

Effect of testosterone pretreatment. Both testosterone pretreatment and castration of male and female mice have previously been shown to abolish the sex difference in type I binding of ethylmorphine [12]. Testosterone pretreatment, in the present study, appeared to enlarge the 1-butanol-induced spectral change in females (Fig. 2A) together with a reduction in the size of the ethylmorphine-induced spectral change (Fig. 2B). This suggests that these effects are interrelated.

Effect of age. There was no sex difference in the type I binding ethylmorphine when 3 week old mice were used [13]. Table 1 shows that at 3 weeks the 1-butanol-induced spectral change in females is slightly larger than that observed in males, when the values are calculated on the basis of the microsomal protein content. When the magnitude of the spectral change is related to the cytochrome P-450 content, however, a sex difference is not observed. The magnitudes of the spectral changes in males are equal to those observed in adult males. This indicates that the development of the sex difference in the 1-butanol-induced spectral change results solely from a decrease in the change in females.

Extraction of microsomes. Simply washing the microsomes with 1-butanol, in a concentration used for the spectral measurements (i.e. 25 ml microsomal suspension containing approx. 2 nmole P-450/ml

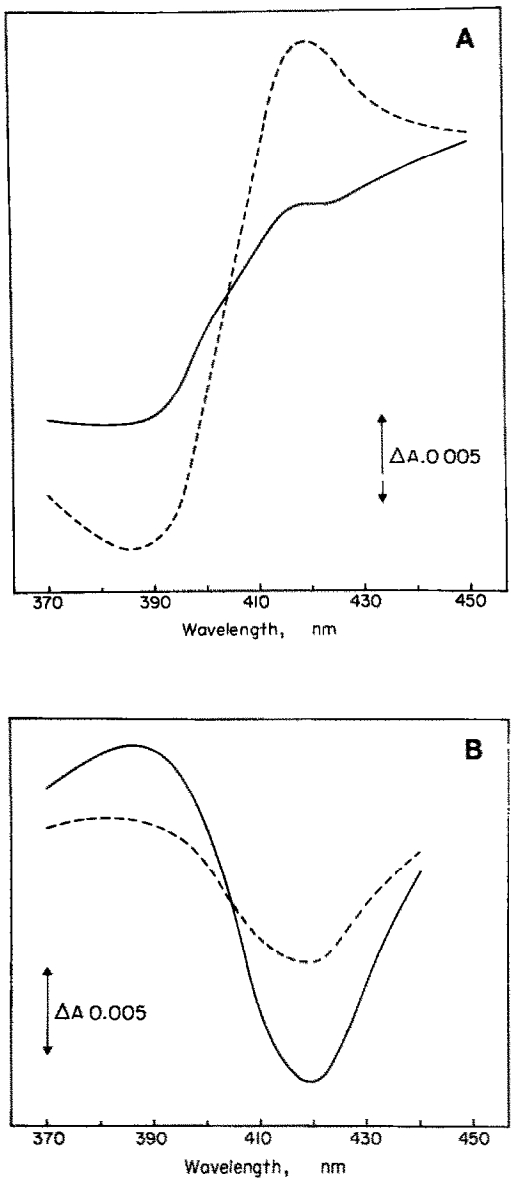

Fig. 2. Effects of testosterone pretreatment on the 1-butanol (A) and ethylmorphine (B) induced difference spectra in liver microsomes from female mice. · · Control (oil-treated); - - - - - testosterone-treated. The cytochrome P-450 contents were 1.19 and 1.23 nmol ml respectively. The substrate concentrations were: 1-butanol 88 mM; ethylmorphine 2 mM.

mixed with 200 μl 1-butanol, gently stirred for 15 min, sedimented and resuspended), did not yield significant effects. This procedure even tended to slightly decrease the type I spectrum and increase the 1-butanol spectrum, whereas the cytochrome P-450 content remained unaltered (data not shown). Extraction of freeze-dried microsomes with 1-butanol and/or acetone has been shown to increase the type I spectral change in male rats [6]. Table 2 shows that this treatment did not significantly increase the ethylmorphine binding spectrum, but decreased the 1-butanol spectrum (cytochrome P-450 was partly converted to cytochrome P-420, but the spectral size has been calculated on the basis of the amount of remaining cytochrome P-450).

Difference between the absolute absorption spectra of males and females. The absolute absorption spectra of cytochrome P-450 in microsomes from male and female mice were investigated by a direct spectrophotometric comparison (Fig. 3), in order to detect a possible sex difference in the amount of the substrate-bound form of cytochrome P-450. The amounts of cytochrome b_5 were balanced by diluting the microsomes from females by 25 per cent. Fortunately, this eliminated the difference in the cytochrome P-450 contents almost completely, so that the resulting difference spectrum reflects only differences in the composition of the male and female cytochrome P-450 populations. Figure 3 shows a decrease in absorbance with increasing wavelength, which is due to a difference in the turbidity of the two preparations, caused by the dilution of one of them. In addition to this only a small shoulder seems to be present around 410 nm.

Effects of type I and type II compounds on the 1-butanol-induced spectral change. Figure 4A shows that the presence of aniline in sample and reference cuvettes diminishes the magnitude of the 1-butanol-induced difference spectrum in males, and alters the position of the absorbance maximum, indicating the disappearance of a type II component. An analogous effect was observed in females, although the resulting spectrum is complex (Fig. 4B). This indicates either that aniline and 1-butanol compete for type II binding sites, or that 1-butanol inhibits aniline binding non-competitively.

When, in addition, ethylmorphine was added in order to saturate the type I binding sites in both cuvettes, the final spectra were the same in males and females. The female spectrum lost a large type I component, whereas the male spectrum was only slightly changed. This indicates that 1-butanol also

Table 1. The magnitude of the 1-butanol-induced spectral change in liver microsomes from immature and mature male and female mice

	$\Delta A_{420-385}$/mg protein		$\Delta A_{420-385}$/nmol P-450	
	3 weeks	Adult	3 weeks	Adult
Males	14.4 ± 0.7 (3)	21.18 ± 1.28 (14)*	19.5 ± 0.7 (3)	21.38 ± 1.01 (12)
Females	17.2 ± 0.7 (3)‡	8.24 ± 0.94 (16)†§	20.0 ± 0.9 (3)	6.93 ± 0.98 (14)†§

Values represent means ± S.E. of the number of experiments indicated in parentheses.
* Significant effect of age, $P < 0.05$ (Student's t-test).
† Significant effect of age, $P < 0.001$.
‡ Significant sex difference, $P < 0.05$.
§ Significant sex difference, $P < 0.001$.

Table 2. The effects of organic solvent extraction of freeze-dried liver microsomes from male mice on the ethylmorphine- and 1-butanol-induced spectral changes

	Spectral changes after 3 acetone extractions* ($n = 3$)		Spectral changes after 1-butanol and 2 acetone extractions* ($n = 2$)	
	Control	Control minus extracted	Control	Control minus extracted
3 mM ethylmorphine	5.2 ± 0.5	1.5 ± 1.1	5.0 ± 0.8	2.4 ± 0.7
88 mM 1-butanol	18.3 ± 0.9	− 2.3 ± 0.4†	17.5 ± 0·5	− 4.3 ± 0.0†

* Values are expressed as $\Delta A_{385\ 420}$ (ethylmorphine) or $\Delta A_{420\ 385}$ (1-butanol)/nmol P-450, and represent means ± S.E.
† Significant effect of extraction, $P < 0.05$ (paired t-test).

interferes with type I binding, which is particularly evident in females. In Fig. 5 this interference is further demonstrated by the effect of hexobarbital on the female 1-butanol spectrum. The resulting spectrum resembles the 1-butanol spectrum observed in males. These results suggest that the sex difference in the 1-butanol-induced spectral change may be due to sex-dependent type I binding.

DISCUSSION

If the 1-butanol-induced spectral change represents the displacement of endogenous substrates [4], the sex difference in this spectral change, observed in the present study (Fig. 1), indicates the existence of a high level of endogenous substrates in microsomes from male mice as compared with females. This may explain

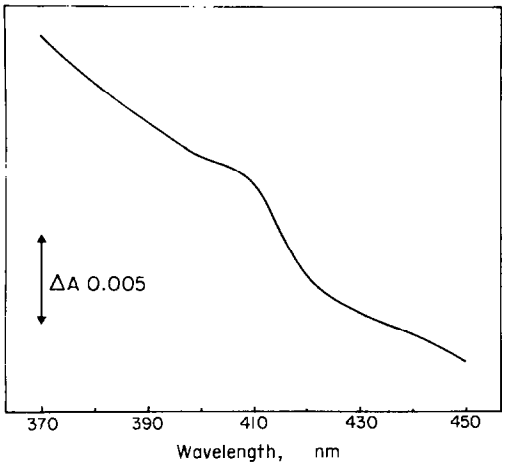

Fig. 3. Difference between the absolute absorption spectra of liver microsomes from male and female mice (males minus females). The spectrum was obtained essentially according to the method used by Kinoshita and Horie [16] for the determination of the effect of induction. The cytochrome b_5 contents of the male and female microsomal preparations (approx. 1 mg protein/ml) were estimated by reduction with NADH. The cytochrome b_5 concentrations were balanced by diluting the female preparation. The final cytochrome P-450 concentrations after this balancing were 1.05 (males) and 1.06 nmol/ml (females). A baseline of zero absorbance was established with female microsomes in both sample and reference cuvettes. The contents of the reference cuvette were then replaced by the male microsomal preparation, and the difference spectrum was recorded.

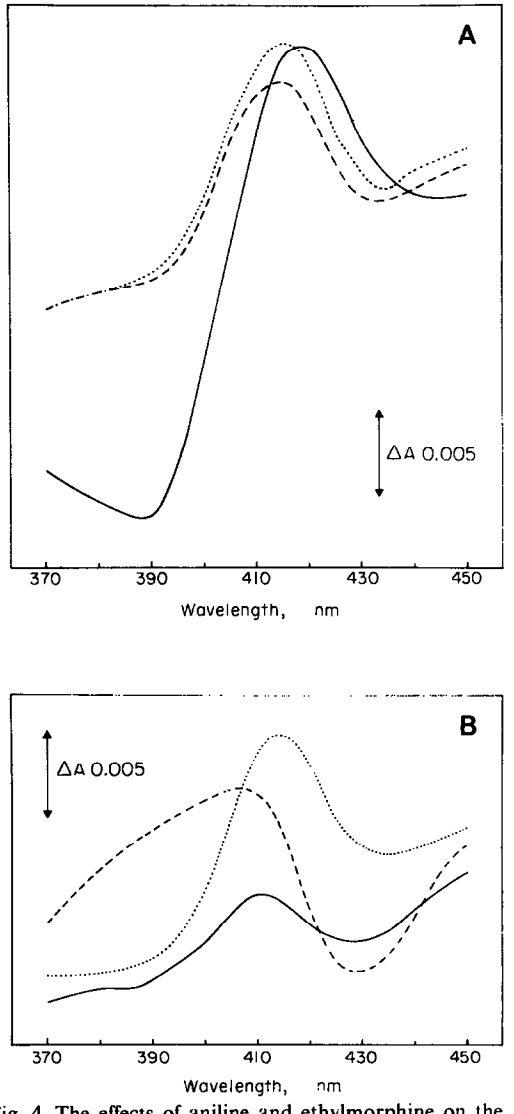

Fig. 4. The effects of aniline and ethylmorphine on the 1-butanol-induced spectral changes in liver microsomes from male (A) and female (B) mice. —— 88 mM 1-butanol in the sample cuvette; - - - - - 88 mM 1-butanol + 3 mM aniline in the sample cuvette and 3 mM aniline in the reference cuvette: ..... 88 mM 1-butanol + 3 mM aniline + 3 mM ethylmorphine in the sample cuvette and 3 mM aniline + 3 mM ethylmorphine in the reference cuvette. The cytochrome P-450 concentrations were: males 1.28 nmol/ml; females 1.22 nmol/ml.

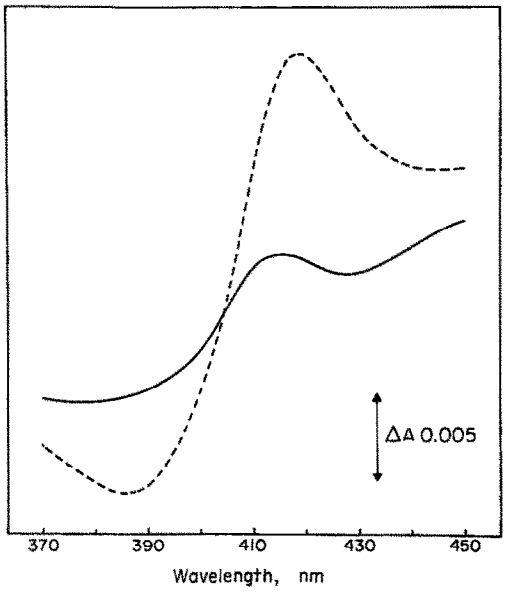

Fig. 5. The effect of hexobarbital on the 1-butanol-induced spectral change in liver microsomes from female mice. —— 88 mM 1-butanol in the sample cuvette; - - - - - 88 mM 1-butanol in the sample cuvette and 3 mM hexobarbital in both sample and reference cuvettes. The cytochrome P-450 concentration was 1.10 nmol/ml.

the sex difference in the type I binding of ethylmorphine and hexobarbital (the binding capacity in males is approximately threefold lower than in females), which is due to the unveiling of an inhibitory action of androgens during sexual maturation [10, 12, 13]. Figure 2 shows that testosterone pretreatment, in fact, caused an increase in the 1-butanol spectrum and a concomitant decrease in the ethylmorphine spectrum.

Several observations, however, make the role of endogenous substrates less obvious. (1) The sex difference in the 1-butanol spectrum is due to a developmental decrease occurring in females (Table 1). (2) The sex-dependency of type I binding is due to ΔA_{max} and not to K_s [13] (but the endogenous substrates might be bound very tightly). (3) Washing the microsomes from male mice with 1-butanol had no appreciable effect (the presumably very lipophilic compounds, however, might remain with the microsomal membrane rather than be removed with the supernatant). (4) Organic solvent extraction of freeze-dried microsomes from female mice only slightly decreased the 1-butanol spectrum, and had no effect on type I binding.

Convincing evidence that male microsomes do not contain more endogenous substrates than females was presented in Fig. 3. The binding of endogenous type I substrates should result in an increase in the amount of substrate-bound form of cytochrome P-450 at the expense of the substrate-free form. The recording of a difference spectrum with male and female microsomes (balanced with respect to the cytochrome b_5 and cytochrome P-450 contents) in sample and reference cuvettes respectively, should, therefore, simply yield a type I spectrum. No such spectrum was found, thus indicating that male microsomes do not contain more substrate-bound cytochrome P-450 than females, if it is present at all.

The reverse type I spectral change may represent a conformational change in cytochrome P-450, due to a loading of the membrane with substrate [17, 18]. Although 1-butanol affects the membrane structure [19, 20] and the microsomal membrane might have a sex-dependent lipid composition [21], this is not likely to explain the sex-dependent 1-butanol spectra. Organic solvent extraction, which removes a great deal of the lipid material [6], only slightly decreased the spectral change (Table 2).

It has been suggested that 1-butanol interacts directly with the heme-iron [7, 9], due to the nucleophilic OH-group, and thus may interfere with the binding of the type II compound aniline. Our results demonstrate that 1-butanol interferes with type II as well as type I binding. Multiple interaction between a substrate and cytochrome P-450 has been often reported [22–26]. Type I binding of 1-butanol, however, has not been observed before. Only some 1-alkanols with hydrocarbon chains longer than 5 C-atoms were shown to have a type I component in their spectra in male rats [7, 8].

The 1-butanol spectra obtained in the presence of aniline together with ethylmorphine show the same size in males and females (Fig. 4). Type II binding is not sex-dependent, at least when related to the cytochrome P-450 content, as demonstrated with aniline [10]. These observations thus indicate that the sex difference in the 1-butanol-induced spectral change in the mice we investigated is due to a sex-dependent type I binding. This explains all observed phenomena, and in particular the results presented in Table 1. Based on the total amount of cytochrome P-450, the size of the 1-butanol-induced spectrum is the same in male and female immature animals and mature males. The low spectral size observed in mature females is due to type I binding, which increases selectivity in females during sexual maturation [13]. The concept of dual binding also explains why the 1-butanol-induced spectra observed in different preparations of microsomes from female mice show some variation with regard to the absorbance maximum (Figs. 1, 2A, 4B and 5). A difference in the relative contributions of spectral components which are not exactly each others mirror image, will affect the shape of the resultant spectra to some extent.

In the accompanying paper it is shown that 1-butanol competitively inhibits the type I binding of ethylmorphine [27].

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