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TRIACETYLOLEANDOMYCIN AS INDUCER OF CYTOCHROME P-450 LM_{3c} FROM RABBIT LIVER MICROSOMES

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Abstract—A cytochrome P-450 LM_3 isozyme has been isolated and purified to electrophoretic homogeneity from liver microsomes of New Zealand white rabbits treated with TAO. On the basis of *N*-terminal sequence analysis and Ouchterlony double diffusion experiments, this isozyme appeared to be closely related to P-450 LM_{3c} isolated from control animals and was designated LM_{3c} (TAO). Anti LM_{3c} (TAO) IgG totally inhibited both erythromycin demethylase and P-450-TAO metabolite complex formation, two monooxygenase activities specifically stimulated by TAO in liver microsomes from male and female rabbits. Moreover, immunoquantitation experiments showed that the level of LM_{3c} (TAO) was increased 10–15 times above control values in liver microsomes from TAO treated male and female rabbits. We conclude that an isozyme identical or closely related to LM_{3c} is the major form of P-450 induced by TAO in rabbit liver microsomes.

TAO (triacetyloleandomycin) is known as a strong inducer of liver microsomal cytochrome (P-450) in rat [1], rabbit [2] and man [3]. Recently, we reported that a P-450 LM_3 form was induced by TAO in rabbit liver microsomes [2, 4]. We showed that P-450 LM_{3b} isolated from these microsomes comigrated, in SDS–PAGE, with the induced LM_3 form present in the parent microsomes and exhibited, in a reconstituted system, a high turnover for erythromycin, a microsomal activity specifically stimulated by TAO. Further experiments which will be described in this paper, demonstrated that, although its level was elevated in liver microsomes from TAO treated rabbits, cytochrome P-450 LM_{3b} was not the major form induced by TAO. We therefore decided to reinvestigate the effect of TAO in P-450 LM_3 forms from rabbit liver microsomes.

We report in this paper the isolation of another LM_3 form from liver microsomes of TAO treated rabbits. This form, which is biochemically and immunologically similar (or identical) to P-450 LM_{3c} from uninduced animals, appears to be the major form of cytochrome P-450 induced by TAO.

MATERIALS AND METHODS

Sexually mature male and female New Zealand white rabbits (2.5–3.0 kg) were used in this work. TAO (2–3 g depending on the rabbit weight), was thoroughly mixed with 100 g of Purina rabbit chow, so that the theoretical dose was 1 mmole/kg/day. From a number of experiments we found that maximal induction of LM_{3c} occurs after 7 days of such a treatment.

LM_{3c} was prepared from TAO treated microsomes by a slight modification of the procedure described by Koop *et al.* [5] for the purification of LM_{3c} from untreated animals. Microsomes were solubilized with cholate and precipitated with polyethylene glycol PEG (8–12%). The PEG fraction was chromatographed on a DEAE cellulose column equilibrated against 0.01 M Tris acetate pH:7.4, 20% glycerol, 0.5% tergitol and 1 mM EDTA. The column was washed with this buffer and the first eluate, fraction I containing most LM_{3c} (TAO), was used for further purification. LM_4 eluted slightly later as a second fraction. The column was then eluted with 10 and 100 mM K_2SO_4; LM_{3b} (TAO) was eluted in fraction III (100 mM K_2SO_4). The DEAE fraction I was equilibrated against 0.01 M phosphate, pH: 7.4, 20% glycerol, 0.2% tergitol and 1 mM EDTA, by gel filtration on a Biogel P10 column. The solution was loaded on a CM cellulose column equilibrated against the same buffer. The column was washed first with 0.01 M phosphate pH: 7.4, then with a linear gradient from 0.01 to 0.08 M phosphate pH: 7.4. Fraction eluting between 0.015 and 0.02 M phosphate was loaded on a hydroxylapatite column equilibrated against 0.01 M phosphate, pH: 7.4, 20% glycerol, 0.2% tergitol, 1 mM EDTA. The column was washed with each of the following buffers: 0.05, 0.10, 0.15 and 0.20 M, phosphate pH: 7.4. The latter fraction contained LM_{3c} (TAO) and had a specific content of 16 nmoles/mg/protein. Non-ionic tergitol detergent was removed as published elsewhere [4]. LM_{3c} (TAO) was finally dialyzed against 0.10 M phosphate, pH: 7.4, 20% glycerol, 1 mM EDTA and stored at −20°.

Isozymes LM_2, LM_{3b} (TAO) and LM_4 and NADPH cytochrome P-450 reductase were purified according to previously published procedures [4–8]. Isozymes LM_{3b} and LM_{3c} from uninduced animals were kindly provided by Professor M. J. Coon and Dr. D. Koop (Michigan University). Anti LM_{3c} (TAO) IgG was purified from sera of guinea pigs immunized by four subcutaneous multisites injec-

tions on the back, at 3 weeks interval, of 20 μg of electrophoretically homogeneous cytochrome P-450 LM_{3c} (TAO) in 200 μl of 50% Freund complete adjuvant.

Polyacrylamide slab gel electrophoresis, spectral analysis, *N*-terminal sequence, Ouchterlony double diffusion analysis and measurements of monooxygenase activities (erythromycin, TAO, chlorcyclizine and aminopyrine demethylase) were carried out as described in a previous paper [4]. Formation of the P-450-TAO metabolite complex, absorbing at 457 nm, was followed by repetitive spectral recording after initiation of the reaction by addition of 1 mM NADPH. Isozymes LM_2, LM_{3b}, LM_{3c} (TAO) and LM_4 were quantified by radial immunodiffusion assay as described by Thomas *et al.* [9]. Standard plots were obtained for each isozyme by plotting the square of diffusion ring diameter vs the P-450 concentration.

RESULTS AND DISCUSSION

In our previous work [4], we used erythromycin demethylase as a characteristic activity induced by TAO in rabbit liver microsomes to screen the LM_3 enriched fractions during the purification procedure. We thus purified a LM_3 (TAO) form which was indistinguishable from LM_{3b} purified from uninduced animals as judged by a battery of biochemical tests. Both the LM_{3b} purified from untreated animals, and the LM_{3b} (TAO) purified from TAO treated rabbits exhibited a high turnover for erythromycin demethylase, when compared to isozymes LM_2, LM_{3c} and LM_4 in the reconstituted system. However, we recently found that antibodies directed against LM_{3b} (TAO) failed to inhibit significantly erythromycin demethylase in microsomes from TAO treated animals (see for example results presented in Fig. 4); it appeared therefore likely that LM_{3b} was not the major form induced by TAO. In order to isolate and purify the putative induced LM_3 form, we decided to adapt the procedure recently used by Koop *et al.* [5] to purify constitutive LM_3 isozymes from uninduced rabbit.

The LM_3 fractions, eluting from the DEAE cellulose column and comigrating with the LM_3 band present in the microsomes from TAO treated animals, were routinely tested by Ouchterlony double diffusion analysis with anti P-450 LM_{3b} (TAO) IgG in order to identify any P-450 LM_3 isozyme possibly induced by TAO, but different from LM_{3b}. We thus isolated from fraction I (see Material and Methods) strongly enriched in P-450, a LM_3 form immunochemically different from LM_{3b} and which in contrast to LM_{3b} did not bind to the gel. It must be emphasized here that the elution profile obtained from the DEAE cellulose column was identical to the one observed by Koop *et al.* [5]. However, the yield of fraction I increased from 10 to 24% when TAO microsomes instead of control microsomes were used as the starting material. In contrast, no apparent increase was found in the yield

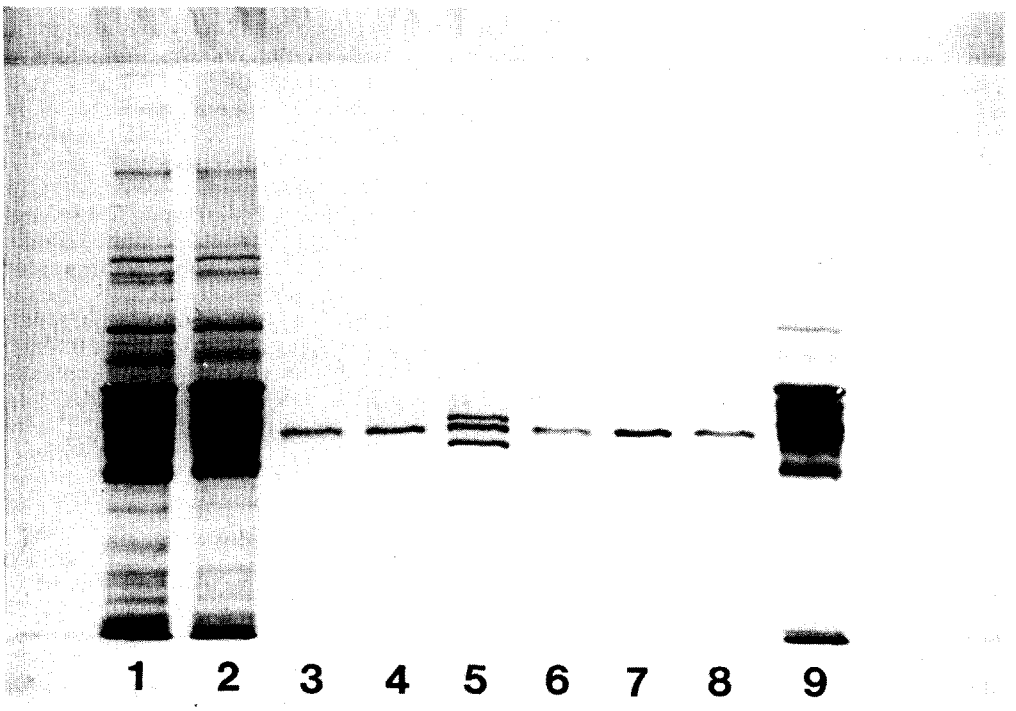

Fig. 1. SDS–PAGE of rabbit liver microsomes and purified P-450 isozymes. Experimental conditions as in [4]. Lane 1: microsomes from untreated male rabbits; Lanes 2 and 9: microsomes from TAO treated male rabbit; Lanes 3 and 4: isozymes LM_{3b} and LM_{3c} from uninduced animals; Lane 5: mixture of isozymes LM_2, LM_{3b}, LM_{3c} and LM_4; Lanes 6 and 7: isozymes LM_{3b} (TAO) and LM_{3c} (TAO) from TAO treated animals; Lane 8: mixture of isozymes LM_{3b} (TAO) and LM_{3c} (TAO).

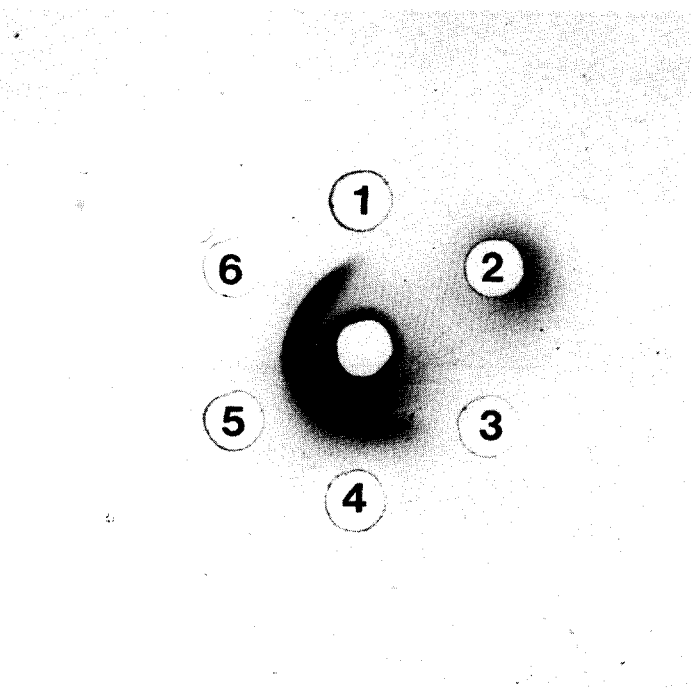

Fig. 2. Ouchterlony double diffusion analysis of highly purified P-450 isozymes. Central well contains IgG anti LM_{3c} (TAO); peripheral wells contain: 1, LM_2; 2, LM_4; 3, LM_{3b}; 4, LM_{3c}; 5, LM_{3c} (TAO); 6, LM_{3c} (TAO). Forty pmoles of P-450 in each well, except well 6, 80 pmoles.

of the other DEAE fractions. The LM_3 form induced by TAO was purified to electrophoretic homogeneity (Fig. 1) with a specific content of 16 nmoles/mg and a final yield of 4–7%.

Figure 1 shows that both LM_{3b} and LM_{3c} isolated from control animals, and LM_{3b} (TAO) and the new form LM_3 isolated from TAO treated rabbits exhibit the same mobility on SDS–PAGE and cannot be readily distinguished from the mobility of the broad LM_3 band present in the parent microsomes. Spectral characterization of this purified LM_3 form showed that it is low spin in its native state and exhibits Soret maxima at 418, 413 and 450 nm, in the oxidized, ferrous and ferrous carbonyl states, respectively. The absorbance ratio between 418 and 280 nm (in the oxidized state) was 1.04 in agreement with the high specific content of the preparation.

The purified LM_3 isozyme was injected into guinea pigs to raise specific antibodies and its immunologic reactivity was compared to that of other P-450 forms LM_2, LM_{3b} (TAO), LM_{3c} (TAO), LM_{3c} and LM_4 by Ouchterlony double diffusion analysis (Fig. 2). The LM_3 form purified from microsomes of TAO treated rabbits, cross-reacted only with LM_{3c} purified from control animals. Next we carried out *N*-terminal sequence analysis on the first 15 amino acids of the cytochrome. As it appears from Table 1 the sequence was identical to that published by Coon *et al.* for LM_{3c} purified from uninduced animals [10] but different from that of LM_{3b}. We conclude from these experiments that the LM_3 form is closely related (or identical) to P-450 LM_{3c} from control animals. This form is designated LM_{3c} (TAO).

LM_{3c} (TAO) specifically binds TAO (Fig. 3): type I spectra of increasing intensity were generated following the addition of increasing amounts of TAO to the oxidized cytochrome. The apparent dissociation constant deduced from these experiments was 12 μM. No type I difference spectrum could be detected in similar experiments with LM_2, LM_{3b} (TAO), LM_4 or LM_6. A type I binding spectrum was also observed when microsomes from TAO treated animals (but not from control, phenobarbital or β-naphthoflavone treated animals) were incubated in the presence of TAO (data not shown). These observations suggest that LM_{3c} (TAO) retains its ability

Table 1. *N*-terminal sequence of cytochrome P-450 forms

P-450 LM	*N*-terminal sequence															Refs
LM_{3b}	Met	Asp	Leu	Leu	Ile	Ile	Leu	Gly	Ile	?	Leu	Ser	Glu	Val	Leu	[10]
LM_{3b} (TAO)	Met	Asp	Leu	Leu	Ile	Ile	Leu	Gly	Ile							[4]
LM_{3c}	Met	Asp	Leu	Ile	Phe	Ser	Leu	Glu	Thr	Trp	Val	Leu	Leu	Ala	Ala	[10]
LM_{3c} (TAO)	Met	Asp	Leu	Ile	Phe	Ser	Leu	Glu	Thr	Trp	Val	Leu	Leu	Ala	Ala	This work

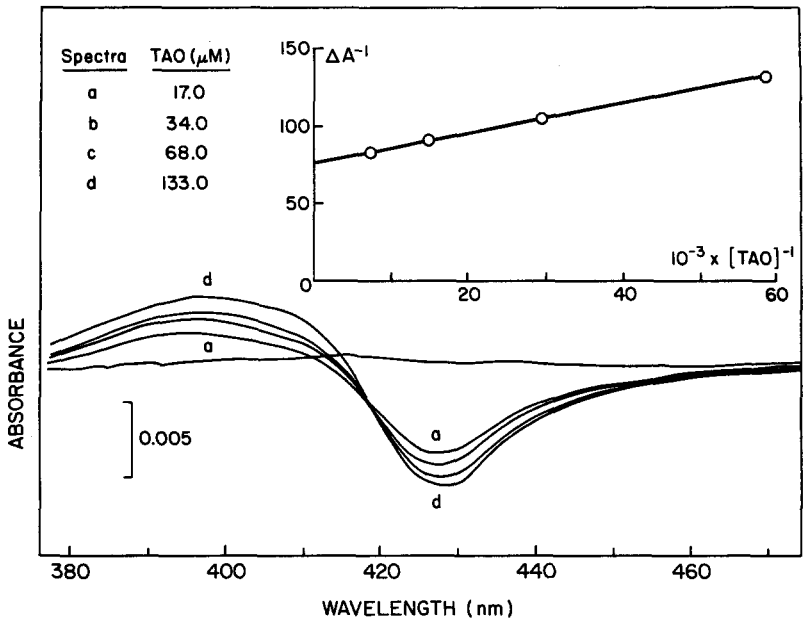

Fig. 3. Difference spectroscopy analysis of interaction between TAO and P-450 LM_{3c} (TAO). P-450 LM_{3c} (TAO) was diluted to 1 μM in 100 mM potassium phosphate buffer pH 7.4, in the presence of 0.1 mM EDTA and 20% glycerol. Seven hundred microliters of solution were placed in both sample and reference cuvettes and the base line was recorded. Increasing amounts of 5 mM TAO in DMSO were added to the sample cuvette, while the same volume of DMSO was added to the reference cuvette; difference spectrum was recorded after each successive addition. Insert: double reciprocal plot of absorbance change between 394 and 426 nm vs TAO concentration.

to specifically bind TAO, although in a reconstituted system, it was unable to produce the P-450-TAO metabolite complex absorbing at 457 nm, routinely observed upon incubation of microsomes from TAO treated animals with TAO and NADPH. Recent work has shown that the P-450 form induced by TAO in rat liver microsomes is similarly unable to generate the P-450-TAO metabolite complex in a reconstituted system†.

Monooxygenase activities of LM_{3c} (TAO) were determined in a reconstituted system consisting of 1 μM P-450, 3 μM NADPH cytochrome P-450 reductase, 35 μg/ml dilauroylphosphorylcholine and erythromycin, TAO, chlorcyclizine and aminopyrine as substrates. Turnover rates for demethylase activities are reported in Table 2, where they are compared with those obtained under similar conditions with other isozymes including LM_2, LM_{3b}, LM_{3b} (TAO), LM_{3c} and LM_4. It appears that, with the exception of chlorcyclizine, LM_{3c} (TAO) does not actively support those monooxygenase activities stimulated in the microsomes from TAO treated animals. Specially striking is the finding that LM_{3b} and LM_{3b} (TAO) are more active in erythromycin and TAO demethylase activities than LM_{3c} or LM_{3c} (TAO). One likely explanation for these results is that, upon purification, the enzyme could become partially inactivated as a consequence of the removal of some essential component required for its structural integrity and functional activity.

Even though there appears to be a functional deficiency of purified LM_{3c} (TAO), the native isozyme in TAO microsomes appears to be directly involved in both erythromycin demethylase and P-

† S. A. Wrighton, P. Maurel, E. G. Schuetz, P. B. Watkins, B. Whitley and P. S. Guzelian, *Biochemistry*, in press.

Table 2. Monooxygenase activities of the reconstituted system. Data concerning LM_2, LM_{3b}, LM_{3b} (TAO) and LM_4 are from [4]

	P-450 LM					
Substrates	LM_2	LM_{3b}	LM_{3b} (TAO)	LM_{3c}	LM_{3c} (TAO)	LM_4
Aminopyrine	9.7	22.0	22.2	1.2	3.3	9.3
Chlorcyclizine	3.4		6.6		8.5	3.6
Erythromycin	1.8	8.2	9.9	3.7	3.5	3.4
TAO	1.8		7.3		2.0	2.3

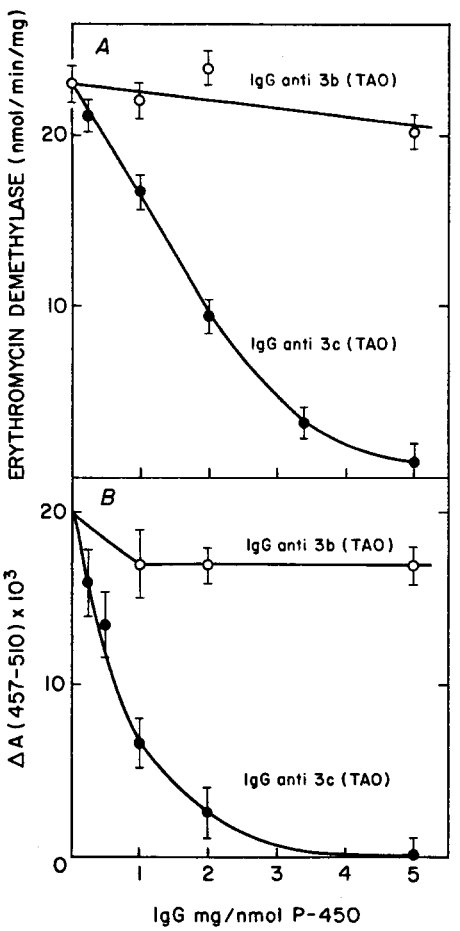

Fig. 4. Effect of IgG anti LM_{3b} (TAO) and anti LM_{3c} (TAO) on monooxygenase activities catalyzed by liver microsomes from TAO treated rabbits. Microsomes were diluted (P-450 concentration 1 μM) in 0.1 M potassium phosphate buffer pH 7.4 in the presence of 13 μM K_3FeCN_6, in order to destroy the P-450 TAO metabolite complex formed *in vivo*. The solution was then incubated 15 min at room temperature, in the presence of variable amounts of IgG anti LM_{3b} (TAO) or anti LM_{3c} (TAO). The specific assay was then carried out at 35° in the presence of 1 mM erythromycin (A) or 21 μM TAO (B), the reaction being initiated by the addition of 0.8 mM NADPH. Erythromycin demethylase activity (A) was determined as in [4]. *In vitro* formation of the P-450 TAO metabolite complex (B) was carried out as indicated under Materials and Methods; in that case the absorbance change between 457 and 510 nm proportional to the amount of complex, was plotted against IgG concentration.

450-TAO metabolite complex formation. Anti P-450 LM_{3c} (TAO) IgG blocked more than 95% of both activities (Fig. 4, A, B). In contrast similar amounts of anti LM_{3b} (TAO) IgG (Fig. 4) or nonimmune globulin (data not shown) inhibited no more than 5% of erythromycin demethylase and 15% of the P-450-TAO metabolite complex formation. Thus, although LM_{3b} exhibits higher turnover than LM_{3c} for erythromycin demethylation in the reconstituted system, the reverse appears to be true in the microsomes. This emphasizes the point that activity of a given form of P-450 in the reconstituted system does not necessarily reflect its actual activity in the microsomal membrane.

Immunoquantitation experiments provided additional evidence that LM_{3c} or a closely related form is the major form induced by TAO. Liver microsomes from both male and female control and TAO treated animals were assayed for concentrations of several forms of P-450 including LM_2, LM_{3b}, LM_{3c} and LM_4 by radial immunodiffusion according to the method recommended by Thomas *et al.* [9]. The concentration of LM_{3c} (Table 3) accounts for 4–7% of the total spectral P-450 in untreated animals. In microsomes from both male and female rabbits treated with TAO, the concentration of LM_{3c} increased by 10–15-fold accounting for up to 60% of spectral P-450. Similar results were obtained when LM_{3c} was immunoquantitated in immunoblots (data not shown). Form LM_{3b} appears to be either unaffected or slightly induced—especially in females—in the liver microsomes of TAO treated animals. The concentration of LM_2 and LM_4 was not affected by TAO treatment although LM_4 could be slightly repressed in female. In both male and female animals LM_{3b} and LM_4 represent 35 and 50% of spectral P-450, respectively, whereas LM_2 only accounts for less than 5%. As has been noted for other forms in outbred rabbits [11] we found that the specific contents of LM_4 in control or treated animals as well as that of LM_{3c} in treated animals varied widely (Table 3). We also found, as have others [12], that the specific content of total P-450 determined spectrally is always lower than the sum of the specific content of LM_2, LM_{3b}, LM_{3c} and LM_4, determined from immunoreaction. This could reflect cross reaction of one 'specific' antibody for several different forms or the presence of apocytochrome in the microsomal membrane. From the results reported in Table 3 it appears that the TAO induced increase in LM_{3c} content, determined by radialimmunodiffusion, reasonably accounts for—and actually exceeds in some occasions—the increase in total P-450 determined from spectral data. It is therefore suggested that TAO mediated induction of any other form of P-450 represents, at most, a minor contribution in terms of apoprotein P-450 synthesis.

Some of the reasons for the disagreement between the present and our previous report [4], clearly appear from the results presented in this paper. Our conclusion [4], that LM_{3b} was induced by TAO was based on two essential observations: first, isolation of that isozyme with a yield (3–4%) compatible with a possible induction; second, the high turnover exhibited by that isozyme (either purified from TAO or control rabbits) in erythromycin demethylase activity. Our results show here that, indeed the level of LM_{3b} is elevated in both control and treated animals (Table 3), and in some occasions appears to be comparable to that of LM_{3c} (in TAO treated animals). On the other hand, isozyme LM_{3c} highly purified either from control or TAO treated animals appears relatively inefficient in erythromycin demethylase when compared to LM_{3b} and LM_{3b} (TAO). Whether this observation results from a partial denaturation of isozyme LM_{3c} under purific-

Table 3. Immunological estimation of four P-450 forms in liver microsomes from untreated male and female rabbits, from radial immunodiffusion experiments according to the method of Thomas *et al.* [9]

Microsomes	N	Spectral P-450 (nmoles/mg)	P-450 (nmoles/mg)			
			LM_2	LM_{3b}	LM_{3c}	LM_4
UT male	4	1.77	0.074	0.96	0.07	0.94
	11	1.72	nd	0.58	0.13	0.89
TAO male	4	2.20	0.070	0.80	0.70	1.40
	6	2.41	nd	0.58	1.43	0.74
UT female	2	1.89	0.074	0.57	0.13	1.61
	2	1.43	nd	0.40	0.09	0.96
TAO female	2	2.90	0.069	0.84	1.72	0.77
	2	1.93	nd	0.53	0.81	0.67

nd: not determined. N: number of animals in each series.

ation, or reflects the actual specificity of LM_{3b} and LM_{3c} in the reconstituted system remains to be determined.

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