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ON THE SPECTRAL INTERMEDIATE AT 440 nm FORMED DURING MIXED FUNCTION SUBSTRATE OXIDATION

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Abstract—The spectral shoulder formed at 440 nm in microsomes oxidising hexobarbital and other drugs has been investigated and some of its properties characterised. Hexobarbital, pentobarbital, ethylmorphine and barbital produce this shoulder, while acetanilide, aniline, desmethylimipramine, imipramine, metyrapone and SKF 525-A do not. The formation of the 440 nm shoulder depends on the presence of NADPH and oxygen and is reduced in size when NADH is also present. At saturating substrate concentrations the size of the 440 nm shoulder is correlated to the cytochrome P-450 content. The hexobarbital induced shoulder can be inhibited by drug metabolism inhibitors such as metyrapone, imipramine and desmethylimipramine, whereas acetanilide, aniline and SKF 525-A are indifferent. The size of the absorption at 440 nm is proportional to substrate induced oxygen consumption up to a maximum, after which the oxygen consumption alone increases with increasing substrate concentration. This may indicate a more rapid cycling within the reaction sequence caused by oversaturating substrate concentrations. The substrate induced oxygen consumption is more sensitive to low inhibitor concentrations than the peak formation, suggesting a more complex relationship between the two. The reported results lend further support for the hypothesis that the absorption at 440 nm represents an intermediary complex in the catalytic cycle of cytochrome P-450 mediated mixed function oxidation.

THE MIXED function oxidase metabolises a number of xenobiotics by hydroxylation, frequently followed by dealkylation. Substrate oxidation is closely associated with cytochrome P-450 and dependent on the presence of molecular oxygen and NADPH. Two possible spectral intermediate species have been postulated to occur during steady state oxidation of drug substrates. The first intermediate appears immediately after substrate addition as a shoulder at about 440 nm on the reduced cytochrome b_5 peak in difference spectra taken in the presence of hexobarbital,[1] ethylmorphine[2] or amphetamine.[3] The second intermediate, which absorbs at 455 nm, appears only after prolonged oxidation of SKF 525-A[4] or benzphetamine.[5]

Hildebrandt and Estabrook[2] demonstrated that the peak at 440 nm was larger if the oxidation took place solely in the presence of NADPH and smaller when NADH was also present. Evidence from EPR signals suggests that iron is present in this intermediate.[1,6] From this evidence it was concluded that the postulated intermediate is a cytochrome P-450–substrate–oxygen complex with a predilection to accept a second electron, preferentially from NADH. It then transforms possibly into the second intermediate absorbing at 455 nm and disintegrates to yield the product and cytochrome P-450. In the presence of NADPH plus NADH this process is much more rapid than with NADPH alone, thus explaining the synergistic effect of NADH

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on drug oxidations and the smaller size of the shoulder at 440 nm in the presence of NADH.

This paper reports on experiments performed to improve understanding of the nature of the substrates producing the spectral shoulder at 440 nm, the relation between absorbance size and oxygen consumption, and the effects of inhibitors on the postulated intermediate. A preliminary report has appeared already.[7]

MATERIALS AND METHODS

Substrates, chemicals and animals. Acetanilide, aniline, barbital, ethylmorphine and pentobarbital were obtained from E. Merck, Darmstadt, desmethylimipramine and imipramine from Ciba-Geigy AG, Basle; metyrapone was a gift from Ciba, Summit, N.J. and SKF 525-A (diethylaminoethyl-2,2-diphenylvalerate-HCl) from Smith, Kline and French, Philadelphia, Pa, U.S.A. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (Calf, grade 1), NADP, NADH (purest) were obtained from Boehringer, Mannheim; Tris, EDTA, sucrose (puriss), sodium dithionite and phenobarbital were from E. Merck, Darmstadt, carbon monoxide and gas mixtures were from Linde AG, Mainz, and 3-methylcholanthrene from Koch-Light, Colnbrook, Bucks, U.K. Male Sprague Dawley rats of 150–200 g, fed on a cube diet and water *ad lib* were used throughout.

Preparation of microsomes. Stimulated microsomes were prepared by injecting rats daily for 3 days with phenobarbital (normally 60 mg/kg, i.p.) or 3-methylcholanthrene (20 mg/kg in sunflower oil) and killing the rats on the fourth day. Rats were sacrificed by cervical dislocation, bled and the livers perfused with ice-cold saline for 2 min and microsomes prepared as described previously.[8] Livers from five rats were homogenised using a teflon-glass homogeniser in sucrose (0·25 M)–Tris (50 mM)–EDTA (5 mM) pH 7·4 as a 30 per cent homogenate. The cell debris and mitochondria were removed by centrifugation in a Sorvall RC 2B centrifuge at 2000 *g* for 15 min and 9000 *g* for 30 min. The supernatant was then spun in a Beckman Spinco L2B centrifuge for 60 min at 115000 *g* to precipitate the microsomes. These were resuspended in sucrose–Tris–EDTA and washed once before being dispersed into aliquots (approx. 2 ml, 20–40 mg/ml protein) and frozen (−10°). All operations were performed between 0–4°.

All microsomal preparations were normally used within two weeks of preparation; the protein content was determined using the Lowry method.[9]

Spectral methods. All measurements were performed in duplicate experiments on at least two microsomal preparations in a Perkin–Elmer Model 356 spectrometer using a modified cuvette holder maintained at 37° by a circulating water bath.

Cytochrome P-450 content was measured by the method of Omura and Sato.[10] Microsomes (0·5 mg/ml) were suspended in 66 mM Tris buffer pH 7·4, divided between two cuvettes, reduced with 2–3 mg solid Na-dithionite and the baseline was recorded between 500–300 nm. One cuvette was then gently bubbled with CO for 1 min and the new spectrum drawn. The difference in absorption between 500 and 450 nm was measured and the cytochrome P-450 content calculated using the coefficient 91 cm^{-1} mM^{-1} of Omura and Sato.[10]

For the determination of binding constants microsomes (1 mg/ml) were suspended in 66 mM Tris buffer pH 7·4, divided into two portions and scanned between 500 and 350 nm to produce a baseline using split-wavelength spectrometry. Cumula-

tive amounts of drug were added to the sample cuvette, while the reference cuvette received water or solvent in appropriate amounts.

Spectrometry of oxidising microsomes. Each cuvette contained half (3 ml) of a medium containing 50 mM Tris buffer pH 7·4, 32 mM KCl, 10 μM $MgCl_2$, 70 mM glucose-6-phosphate, 0·18 U glucose-6-phosphate dehydrogenase, 1 mM nicotinamide, various concentrations of drug substrate and 1 mg/ml protein. A baseline was drawn (500–300 nm) using split-wavelength spectrometry, and the difference spectrum on adding 40 μM NADP recorded. To monitor the velocity and extent of the absorbance formation of the spectral shoulder at 440 nm after addition of NADP dual wavelength spectrometry was employed. For practical reasons of minimizing interference from changes in the cytochrome b_5 spectrum the wavelength pair 445/490 nm was used.

Oxygen electrode measurements. Oxygen electrode measurements were performed on the oxidising microsomes in cuvettes in the spectrophotometer, the incubation system being the same as for dual and split wavelength spectrophotometric investigation. Each experiment (normally performed in duplicate incubations) was repeated in at least two microsomal preparations.

Gold[11] and reference Ag–AgCl electrodes (polarising voltage −850 mV) were placed in the cuvettes using a special stand inserted in the spectrometer. The current produced by oxygen reduction was amplified and recorded using a Transidyne "Microchemical Sensor" (Transidyne Corp., Ann Arbor, Mich., U.S.A.) amplifier and a Siemens pen-recorder. The results obtained from this system for the oxidation of hexobarbital were identical to those obtained using a conventional closed vessel Clarke type electrode (L. Eschweiler & Co., Kiel). Calibration was performed using saline solutions saturated with N_2, O_2–CO_2–N_2 (11:5:84) and air, and was linear under the conditions employed.

RESULTS

Substrates producing the spectral shoulder at 440 nm. When microsomes are incubated in the presence of a drug substrate and an NADPH regenerating system a difference spectrum, obtained by split wavelength spectrometry on adding NADP to

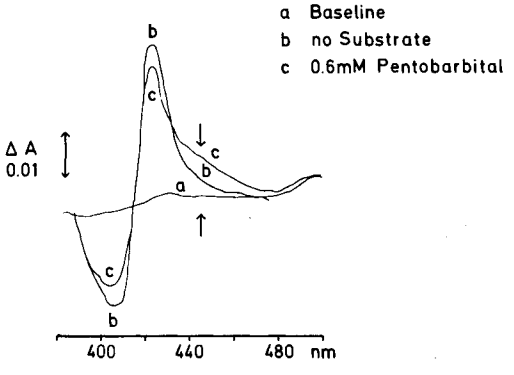

FIG. 1. Original tracing of the difference spectra obtained from oxidising microsomes. The spectra were obtained by split wavelength spectrometry of the oxidising phenobarbital stimulated microsomes in the presence and absence of pentobarbital by the procedure described in methods. The difference spectra were elicited on adding NADPH to the sample cuvette.

TABLE 1. SUBSTANCES WHICH HAVE BEEN EXAMINED FOR THE FORMATION OF A SPECTRAL SHOULDER AT 440 nm IN OXIDISING MICROSOMES.

Substance	Maximal absorption at 440 nm (Fig. 2) (A/mg/ml)	"K_{s440}" (mM)	Maximum size of binding spectrum (A/mg/ml)	K_s of binding spectrum (mM)
Hexobarbital	0·0065	0·17	0·010*	0·12† 0·08*
Pentobarbital	0·0040	0·032	—	0·055
Barbital	0·0030	1·05	0·015	2·3
Ethylmorphine	0·0075	2·0	0·007	7·6
Acetanilide, Aniline, Desmethylimipramine, Imipramine, Metyrapone, SKF 525-A	did not form a shoulder at 440 nm			

The absorption at 440 nm was measured in microsomes oxidising the substrate examined using the dual wavelength method 1 min after adding NADP. Binding of substrate to microsomes was measured as in methods. The values quoted in this table were derived from Lineweaver–Burk plots. Barbital was dissolved in dimethylformamide. The presence of a 440 nm absorption was confirmed by split wavelength scanning of the oxidising microsomes as in Methods.

* Ref. 12.

† Ref. 13.

the sample cuvette, contains a shoulder which is absent from preparations in which the drug is omitted (Fig. 1). This shoulder appears within 30 sec and can also be induced, after the microsomes have begun oxidation, by adding the substrate. It cannot be induced if sodium dithionite (2–3 mg) is substituted for NADPH as reductant, or if CO is first bubbled through the microsomal suspension. Table 1 lists substrates of the mixed function oxidase which form this peak and some which do not do so immediately. From Fig. 1 the wavelength pair 445/490 can be seen as a suitable pair

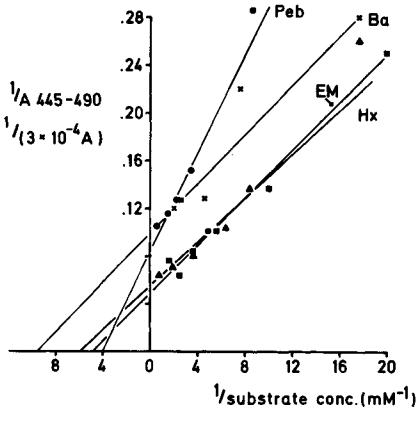

FIG. 2. Lineweaver–Burk plots of absorption size at 440 nm and substrate concentration. The absorption size was measured using dual wavelength spectrometry of phenobarbital stimulated microsomes oxidising various concentrations of the substrates barbital (Ba, ×); ethylmorphine (EM, ■); hexobarbital (Hx, ▲) and pentobarbital (Peb, ●). The difference spectra were induced by adding NADPH. The $1/S$ scale is × 100 for pentobarbital and × 10 for hexobarbital.

for measuring the peak by dual wavelength spectrometry, thus permitting more quantitative estimations on a more sensitive scale (Δ A 0·03 for full scale). The trace obtained by dual wavelength spectrometry can be seen in Fig. 8.

It was possible to obtain apparent binding constants for the suspected intermediate at 440 nm with several drugs by varying the drug substrate concentration and plotting the results on a Lineweaver–Burk plot (ref. 15; Fig. 2). The "K_{S440}" for all substrates was similar, though not identical to the K_s of the binding spectrum (as found by Estabrook *et al.*[13] for hexobarbital). However the maximum size of the binding spectrum and the shoulder at 440 nm were unrelated (Table 1).

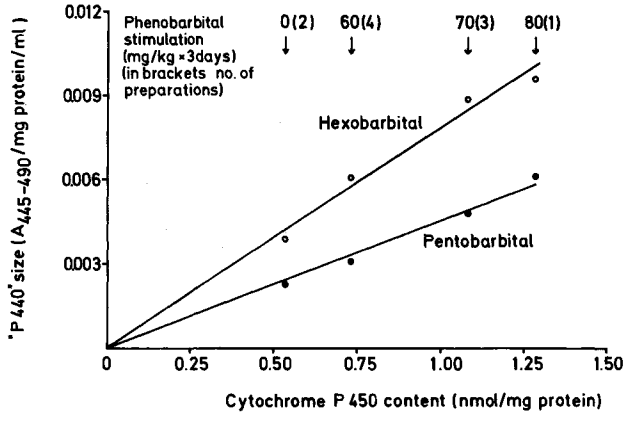

FIG. 3. Relationship of absorption at 440 nm to cytochrome P-450 content of microsomes. The optical density was measured in oxidising microsomes (1 mg/ml) by the dual wavelength spectrophotometry with 0·6 mM hexobarbital (○) or 0·6 mM pentobarbital (●) as substrate. Cytochrome P-450 was measured by its CO binding spectrum as in Methods (microsomal concn. 0·5 mg/ml). The amount of phenobarbital administered to the animals is given over each pair of points.

Relationship of substrate induced spectral intensity at 440 nm to cytochrome P-450 content. The amount of cytochrome P-450 present in the microsomes per unit of protein was varied by using unstimulated microsomes and microsomes stimulated with 60 mg/kg, 70 mg/kg and 80 mg/kg, respectively, of sodium phenobarbital. Microsomes from each group were assayed for cytochrome P-450 content by their CO binding spectrum as described in methods, and for the size of spectral absorption at 440 nm induced with hexobarbital (0·6 mM) or pentobarbital (0·6 mM) as substrate by the dual-wavelength spectrophotometric method. The results (Fig. 3) indicate that the spectral intensity of the absorption at 440 nm is correlated with the cytochrome P-450 content rather than the protein content of the microsomes.

In microsomes from 3-methylcholanthrene stimulated animals the absorbance at 440 nm does not increase even though there is an increase in the amount of cytochrome P-450 present.

Requirement for NADPH and NADH as reductants. The effect of varying the NADPH concentration in the presence and absence of NADH was studied in microsomes oxidising 0·6 mM hexobarbital using the dual wavelength spectrophotometric method as described in Methods. NADH itself was unable to elicit the peak at 440 nm in the presence of hexobarbital. When NADP and an NADPH regenerating

system were present the peak was formed maximally and measured after 30 sec in the presence of about 33 μM NADPH (Fig. 4). When 167 μM NADH was added simultaneously with the NADP then maximum peak size was elicited with only 10 μM NADP, the actual size being slightly less. Thus, NADH, though not actually forming the spectral absorption, does permit maximum peak formation at much lower concentrations of NADPH.

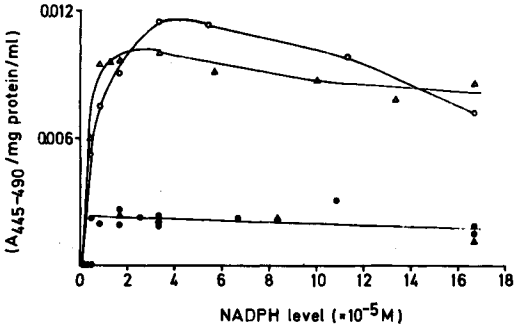

FIG. 4. The effect of NADPH and NADH on the size of spectral absorption at 440 nm induced by hexobarbital. Optical density was measured by the dual wavelength method using various concentrations of NADPH to induce the absorption at 440 nm in the presence of 0·6 mM hexobarbital. NADPH alone as reductant (○); NADPH with 167 μM NADH as reductants (△). Control experiments (no hexobarbital) were with NADPH (●) and with NADPH plus NADH as reductants (▲). Microsomes from phenobarbital stimulated rats. NADH alone had no effect.

As expected from Fig. 1 there was some increase in absorbance, even in the absence of drug substrate, from the cytochrome b_5 peak, but that interfering absorbance was equal whether both reductants or only NADH was present. The slight reduction in the overall size of the cytochrome b_5 peak (about 10 per cent), seen when only NADPH is used as reductant and drug is present, is not seen when NADH is also present.

Effect of SKF 525-A, aniline and acetanilide on peak formation at 440 nm. These three substances elicit difference spectra in microsomal suspensions, SKF 525-A a type I, aniline a type II and acetanilide a modified type II spectrum.[12] In microsomal suspensions containing all factors necessary for drug oxidation no immediate appearance of a 440 nm shoulder was noticed. When the substances were added to suspensions actively oxidising hexobarbital (0·6 mM) or pentobarbital (0·6 mM) and the effect monitored using dual wavelength spectrometry, no interference with the absorption at 440 nm was observed. Up to 3 mM acetanilide (dissolved in either acetone or dimethylformamide) and 0·6 mM of the other substances failed to inhibit the shoulder formation. The peak at 456 nm of SKF 525-A[4] was not observed within the time limit of these experiments (3–5 min), and control experiments included one containing inhibitor but no substrate at each inhibitor concentration.

Effect of desmethylimipramine, imipramine and metyrapone on peak formation at 440 nm. Desmethylimipramine, imipramine and metyrapone also fail to produce an immediate peak on adding NADP to a microsomal suspension containing buffer and a NADPH regenerating system, when measured using either dual or split-beam single wavelength spectrometry. However, if the peak is induced by 0·6 mM hexobarbital then there is a clear inhibition of this absorbance (Fig. 5). Controls were similar

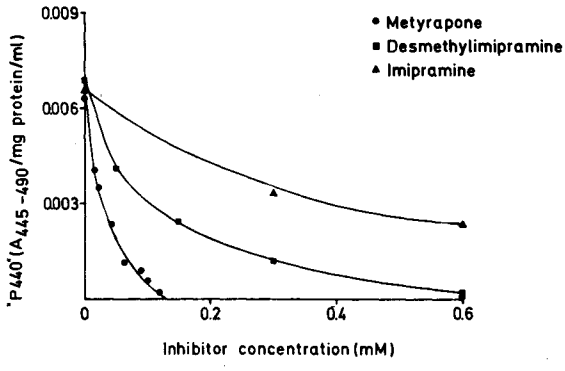

FIG. 5. Effect of varying concentrations of desmethylimipramine, imipramine and metyrapone on the absorbance at 440 nm induced by hexobarbital. The absorbance size, induced by 0·6 mM hexobarbital in phenobarbital stimulated microsomes, was measured using dual wavelength spectrometry in the presence of varying quantities of desmethylimipramine (■), imipramine (▲) or metyrapone (●). NADPH was the reductant.

to those used in the previous section. If the 440 nm absorption represents a spectral intermediate, at sub-maximal sizes the peak height should be a measure of the reaction velocity of hexobarbital oxidation. By drawing Lineweaver–Burk plots of data derived from dual-wavelength spectrometry one can see that all three substances inhibit (at about half-maximal inhibition concentrations) the production of the 440 nm shoulder from hexobarbital competitively (Fig. 6). The results in Fig. 6 are the means of three sets of duplicate experiments for each inhibitor. The apparent K_i and type of inhibition are given in Table 2.

Oxygen consumption during drug metabolism. During the oxidation of hexobarbital and other drugs there is an extra oxygen consumption by the microsomes which depends on the concentration of drug present. The oxygen consumption was always

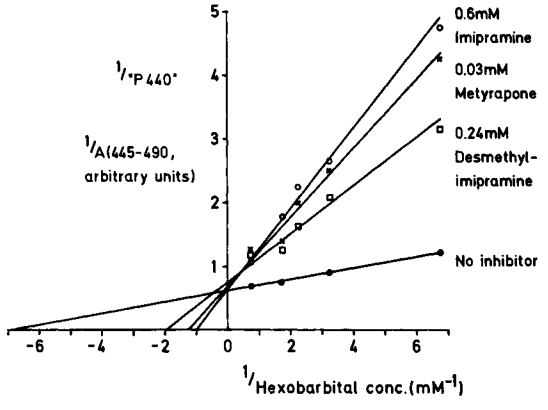

FIG. 6. Lineweaver–Burk diagram for the effects of desmethylimipramine, imipranine and metyrapone on hexobarbital-induced absorbance at 440 nm. The spectral intensity at 440 nm, induced by various concentrations of hexobarbital in phenobarbital stimulated microsomes, was measured by dual-wavelength spectrometry in the presence of 0·24 mM desmethylimipramine (□), 0·6 mM imipramine (○) or 0·03 mM metyrapone (×). Closed circles indicate controls without inhibitor. NADPH was used to induce the difference spectra.

TABLE 2. INHIBITORS OF HEXOBARBITAL-INDUCED 440 NM ABSORPTION

Inhibitor	Concentration used for Lineweaver–Burk plots (mM)	K_i (mM)	Type of Inhibition
Desmethylimipramine	0·24	0·095	Competitive
Imipramine	0·60	0·30	Competitive
Metyrapone	0·03	0·021	Competitive

The "K_i" was determined using the data in Figs. 5 and 6. The results from both figures agreed and the average value is shown above.

measured in incubations with and without substrate at every concentration of inhibitor. Metyrapone inhibits this extra oxygen consumption during substrate oxidation.[13]

In preliminary experiments with concentrations of desmethylimipramine, imipramine and metyrapone, which inhibit the spectral peak at 440 nm by about 60 per cent, it is shown that the inhibition of oxygen consumption during barbiturate oxidation was much more severe (Table 3) than that of peak formation. When the concentration of inhibitor was varied and the concentration of hexobarbital kept constant a biphasic relationship in the effect of the inhibitors could be observed in both phenobarbital simulated and unstimulated microsomes (Fig. 7, cf. Fig. 5). Low concentrations of the inhibitor caused a strong inhibition of the oxygen consumption while higher concentrations of inhibitor caused lesser increments in the inhibition.

The extent of inhibition by metyrapone or desmethylimipramine of the second phase of oxygen consumption corresponds well with the degree of inhibition of the light absorption at 440 nm (Table 4).

In experiments using acetanilide and aniline as inhibitors the inhibition of the extra oxygen consumption was much less marked (Table 3). The reason for this could be that the substrate is only partially replaced by a compound with a lower rate of metabolism.

Oxygen consumption and absorbance at 440 nm. The oxygen consumption induced by hexobarbital can be compared with the spectral intensity at 440 nm. This comparison can be carried out in the same cuvette in the spectrometer by recording both

TABLE 3. THE EFFECT OF VARIOUS SUBSTANCES ON THE EXTRA OXYGEN CONSUMPTION IN MICROSOMES CAUSED BY HEXOBARBITAL OR PENTOBARBITAL OXIDATION

Inhibitor		Extra oxygen consumption (nmoles/min; in brackets per cent of uninhibited) Hexobarbital (0·6 mM)	Pentobarbital (0·6 mM)
No inhibitor		12·40 (100)	4·60 (100)
Desmethylimipramine	(0·24)	1·68 (13)	0·16 (3)
Imipramine	(0·60)	0·32 (3)	0·16 (3)
Metyrapone	(0·03)	2·48 (20)	0·44 (10)
Acetanilide	(0·60)	11·60 (94)	4·16 (90)
Aniline	(0·60)	7·08 (57)	2·32 (51)

Oxygen consumption was measured as described in Methods. 0·6 mM of substrate was always present. The inhibitors themselves did not have a measurable oxygen consumption.

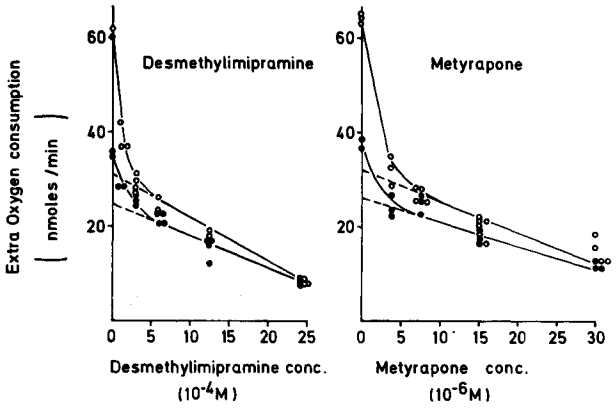

FIG. 7. The effect of desmethylimipramine and metyrapone on the extra oxygen consumption induced by hexobarbital in oxidising microsomes. The oxygen consumption of the microsomes in the presence of 0·6 mM hexobarbital was measured as described in methods. Blank incubations were performed for each inhibitor concentration. Open circles—phenobarbital stimulated microsomes, closed circles unstimulated microsomes. The reaction was started by adding NADP.

TABLE 4. COMPARISON OF THE EFFECT OF DESMETHYLIMIPRAMINE AND METYRAPONE ON THE SECOND PHASE OF OXYGEN CONSUMPTION IN OXIDISING MICROSOMES AND ON THE SIZE OF 440 nm ABSORBANCE.

Inhibitors	(mM)	% Inhibition of absorbance induced by 0·6 mM hexobarbital	% Inhibition of the second phase of extra oxygen consumption induced by 0·6 mM hexobarbital
Desmethylimipramine	(0·24)	72	73
Metyrapone	(0·03)	57	60

The data are derived from Fig. 5 and 7. The extra oxygen consumption for phase two with no inhibitor was derived by extrapolation of the line to zero inhibitor concentration (Fig. 7).

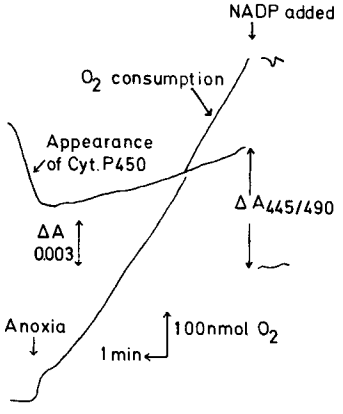

FIG. 8. Original recording of simultaneous measurement of 440 nm absorbance and oxygen consumption in an oxidising microsomal preparation. This drawing is from an incubation containing 0·6 mM hexobarbital and unstimulated microsomes. The reaction was started by adding NADP.

absorption and oxygen consumption on the same recording paper (Fig. 8). One can also adjust the size of the 440 nm absorption in the presence of a fixed concentration of hexobarbital by adding increments of desmethylimipramine and metyrapone, and again observe the oxygen consumption. The results from both these methods are plotted in Fig. 9. These experiments were performed on stimulated and unstimulated microsomes.

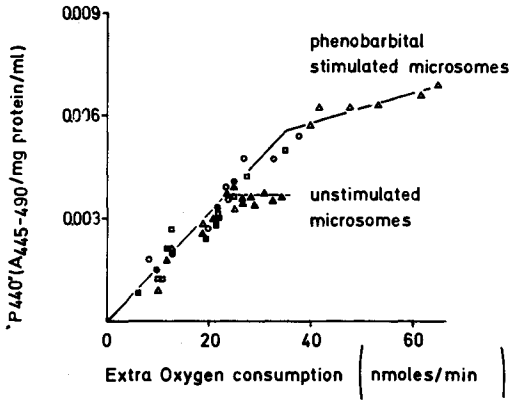

FIG. 9. Comparison of the size of 440 nm absorbance and amount of extra oxygen consumed in microsomes during substrate oxidation. Spectral absorbance ("P-440") and oxygen consumption were measured as described in methods, the absorbance by dual wavelength spectrometry at 445–490 nm. These results include those where both parameters were measured simultaneously in the same cuvette and where they were measured in separate experiments. Triangles—absorbance size and oxygen consumption varied by varying hexobarbital concentration; circles— spectral absorbance and oxygen consumption were varied by adding varying quantities of desmethylimipramine to cuvettes containing 0·6 mM hexobarbital; squares —both parameters were varied by adding various concentrations of metyrapone to cuvettes containing 0·6 mM hexobarbital. Open symbols—phenobarbital stimulated microsomes; closed symbols—unstimulated microsomes. All experiments are compared to control experiments in which the hexobarbital was omitted from the incubation medium. NADPH was used as reductant.

As can be seen in Fig. 9, there is a definite relationship between the spectral absorption ("P 440") and oxygen consumption caused by hexobarbital. The consumption of oxygen appears to be proportional to the peak size up until about the maximum size of peak (recorded in Fig. 3) for hexobarbital. Increases in oxygen consumption above this level are not accompanied by increases in absorbance.

The level at which the 440 nm absorption ceases to be proportional to the oxygen consumption is lower in unstimulated microsomes.

If one compares the points at which the spectral absorption ceases to be proportional to oxygen consumption, in stimulated and unstimulated microsomes in Fig. 9, with the lines extrapolated back to the ordinate (no inhibitor) for the phase 2 inhibition of oxygen consumption in Fig. 7, they appear very similar. For unstimulated microsomes the values are 24, 23 and 26 nmoles O_2/min, respectively for 440 nm absorption and oxygen consumption, oxygen consumption inhibited by desmethylimipramine and oxygen consumption inhibited by metyrapone. The corresponding values for stimulated microsomes are 35, 31 and 33 nmoles O_2/min. Thus the phase 1 of oxygen consumption in Fig. 7 is not dependent on the amount of spectral intermediate present.

DISCUSSION

This work investigates the spectral shoulder at 440 nm in attempt to elucidate the mechanism of substrate oxidation by the mixed function oxidase. The peak at 440 nm, produced during the oxidation of some substrates, was measured using dual and split-beam single wavelength techniques, the results from both techniques being the same. The dual wavelength couplet 445/490 nm was selected because 445 nm is less subject to the interference from the cytochrome b_5 spectrum than 440 nm. The formation of the spectral shoulder at 440 nm is also dependent on the presence of molecular oxygen. CO can prevent its formation as seen by Rösen and Stier[20] in rapid flow experiments on oxidising microsomes.

All substrates producing a peak at 440 nm during their oxidation were type I compounds (as also found in ref. 1) but not all type I compounds gave this peak. Hexobarbital,[1] ethylmorphine[2] and benzphetamine[3] were already known to form this peak; this work confirms these findings and extends them to include pentobarbital and barbital. However SKF 525-A (type I)[12] imipramine (type I),[17] desmethylimipramine (normally type I, but in phenobarbital stimulated microsomes type II)[17] and the type II or modified type II compounds acetanilide, anile[12] and metyrapone[18,19] all failed to produce a peak under our conditions. Thus not all type I compounds give the spectral intermediate, while no type II or modified type II compound yet tested elicits the peak.

If one compares the amount of 440 nm absorption induced in microsomes in the presence of saturating substrate concentrations with the amount of cytochrome P-450 present, varying the cytochrome P-450 by varying the amount of phenobarbital stimulation, then the spectral intensity at 440 nm is proportional to the presence of cytochrome P-450 rather than protein. The amount of cytochrome b_5 in the microsomes does not increase significantly when rats are pretreated with barbiturates.[21] Thus the shoulder on the cytochrome b_5 peak is formed from cytochrome P-450 rather than cytochrome b_5. Furthermore, at a given concentration of cytochrome P-450, the size of the 440 nm shoulder is, up to a maximum value, determined by the amount of oxidisable substrate present. This relationship is therefore governed by Michaelis–Menten kinetics (cf. Fig. 2). Cytochrome P-450 from 3-methylcholanthrene pretreated rats, in contrast, is unable to produce the spectral shoulder.

The size of the peak is also proportional to the oxygen consumption caused by the presence of an oxidisable substrate, which reflects the reaction rate. This is true at least up to a maximum value of the peak, after which the oxygen consumption increases without consomitant increase of peak size. Thus, at sub-saturating concentrations of substrate there is good correlation between peak size and reaction rate.

NADPH is a required cofactor for the production of the spectral intermediate, as it is for mixed function oxidation.[1] Sodium dithionite and NADH are no substitute reductants. NADH acts facilitatively on oxidation, increasing the rate only if NADPH is already present,[22–27] simultaneously reducing the size of the spectral absorption at 440 nm produced by ethylmorphine.[2] In our experiments with hexobarbital the reduction of the shoulder size at 440 nm by NADH was considerably smaller (about 0·002 OD units) than in the previous experiments with ethylmorphine,[2] where the difference in peak size without and with NADH was about 0·01 OD units. This difference might in part be due to the higher protein concentrations used by Hildebrandt and Estabrook[2] and also possibly to the different

substrates used and their concentrations. Thus, when hexobarbital is the substrate, the peak at 440 nm is not reduced so markedly, but when NADH is present much smaller quantities of NADPH are required to produce the maximum size of peak. In addition, when NADH is also present there is a slight increase in the extra oxygen consumption caused by hexobarbital (unpublished observations) suggesting that the substrate is being oxidised faster than with NADPH alone. In agreement with the lack of absorbance at 440 nm in 3-methylcholanthrene stimulated microsomes a reduction of the synergistic effect of NADH on oxidative metabolism has been found in this species.[27] Also the inability of type II compounds to accept electrons from NADH synergistically corresponds with their inability to form material absorbing at 440 nm.

These observations add further weight to the suggestion of Estabrook *et al.*,[13] that the peak is a substrate–cytochrome P 450–oxygen complex formed between the first and second electron donations, and this paper describes further properties of this intermediate.

Compounds which fail to give a shoulder at 440 nm can be divided into two groups—those which interfere with the production of the peak, desmethylimipramine, imipramine and metyrapone, and those with little interference, acetanilide, aniline and SKF 525-A. Apparent inhibitor constants for the compounds interfering with the absorbance formation can be determined using Lineweaver–Burk plots. This procedure assumes that at the concentrations at which these measurements were made the size of the peak is proportional to the velocity of the reaction. Figure 9 relates the size of peak to the oxygen consumption caused by substrate, thus validating this assumption. All the compounds gave a competitive type of inhibition, suggesting direct interference with the peak formation and consequently with hexobarbital oxidation. The compounds are known inhibitors (metyrapone; see refs. 16, 28, 29) or substrates (desmethylimipramine and imipramine; see ref. 30) of the mixed function oxidase system. Jonen *et al.*[31] have recently shown that the binding of metyrapone to microsomal cytochrome P-450 is induced by phenobarbital but not 3-methylcholanthrene. This is further evidence that the 440 nm absorption is formed using the same binding site on cytochrome P-450 as the directly-binding metyrapone.

These three compounds, desmethylimipramine, imipramine and metyrapone all strongly inhibit the oxygen consumption caused by hexobarbital and pentobarbital in microsomes. They do so in two phases, the first very strong phase (cf. Fig. 7) may be due to two substrates which form oxygen complexes on different sites being metabolised thereafter through the same second-electron donation site. The second phase might be direct interference of the second substrate with the equivalent of the 440 nm absorbance. This is confirmed as in the second phase of oxygen consumption the inhibition corresponds well with the inhibition of absorbance formation (Table 4). The maximal values for phase 2 oxygen consumption and the oxygen consumption related to absorbance size (Fig. 7 and 9) are also similar.

In the second group of inhibitors acetanilide has little effect on spectral size at 440 nm and oxygen consumption caused by hexobarbital. SKF 525-A only affects the extra oxygen consumption caused by hexobarbital in a way expected by calculation from its effect on hexobarbital sleeping time.[32] Aniline likewise inhibits oxygen consumption partially, presumably because of its own, slower, metabolism. Thus all

three compounds would never achieve the phase 2 of oxygen consumption, and thus interference with the spectral shoulder under the conditions used.

When the absorbance size is compared with the amount of extra oxygen consumed during substrate oxidation two phases clearly emerge. In the first phase both are proportional. During this phase the spectral density at 440 nm is probably also proportional to the rate of metabolism of hexobarbital as measured by oxygen consumption (Fig. 9). In the second phase, at higher substrate concentrations or with only the presence of minimal amounts of desmethylimipramine or metyrapone, the oxygen consumption is unrelated to absorbance size. Obviously there is a limit to the extent of light absorption at 440 nm that can be produced yet the metabolism rate for hexobarbital continues to rise. This might represent an increase in the rate of cycling of the suspected intermediate in the reaction cycle.

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