McGiven, J. D., and Klingenberg, M. (1971), *Eur. J. Biochem. 20*, 392.
Meisner, H. (1971), *Biochemistry 10*, 3485.
Meisner, H., Palmieri, F., Peindl, P., and Quagliariello, E. (1971), *1st Eur. Biophys. Congr., Vienna, Sept 14–17, 3*, 399.
Meisner, H., and Wenner, C. (1970), *Biochim. Biophys. Acta 223*, 46.
Mitchell, P. (1966), *Biol. Rev. 41*, 445.
Mitchell, P., and Moyle, J. (1967), *Biochem. J. 105*, 1147.
Mitchell, P., and Moyle, J. (1969a), *Eur. J. Biochem. 9*, 149.
Mitchell, P., and Moyle, J. (1969b), *Eur. J. Biochem. 7*, 471.
Palmieri, F., Cisternino, M., and Quagliariello, E. (1967), *Biochim. Biophys. Acta 143*, 625.
Palmieri, F., Prezioso, G., Quagliariello, E., and Klingenberg, M. (1971), *Eur. J. Biochem. 22*, 66.
Palmieri, F., and Quagliariello, E. (1969), *Eur. J. Biochem. 8*, 473.
Palmieri, F., Quagliariello, E., and Klingenberg, M. (1970), *Eur. J. Biochem. 17*, 230.
Papa, S., Lofrumento, N. E., Loglisci, M., and Quagliariello, E. (1969), *Biochim. Biophys. Acta 189*, 311.
Pfaff, E., Heldt, H. W., and Klingenberg, M. (1969), *Eur. J. Biochem. 10*, 484.
Prezioso, G., Palmieri, F., and Quagliariello, E. J. (1972), *Bioenergetics* (in press).
Quagliariello, E., Genchi, G., and Palmieri, F. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 13*, 253.
Quagliariello, E., and Palmieri, F. (1968), *Eur. J. Biochem. 4*, 20.
Quagliariello, E., and Palmieri, F. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 8*, 105.
Quagliariello, E., and Palmieri, F. (1972), Biochemistry and Biophysics of Mitochondrial Membranes, New York, N. Y., Academic Press (in press).
Quagliariello, E., Palmieri, F., Preszioso, G., and Klingenberg, M. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 251.
Robinson, B., and Chappell, J. B. (1967), *Biochem. Biophys. Res. Commun. 28*, 249.
Robinson, B., and Williams, G. (1970), *Biochim. Biophys. Acta 216*, 63.
Tyler, D. (1969), *Biochem. J. 111*, 665.
Tyler, D., and Newton, J. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 8*, 325.
Van Breemen, C., and de Weer, P. (1970), *Nature (London) 226*, 760.

Spectral Studies of the Binding of *O,O*-Diethyl *p*-Nitrophenylphosphorothionate (Parathion) to Cytochrome P-450†

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ABSTRACT: Parathion has previously been shown to be metabolized by a microsomal mixed-function oxidase system to paraoxon and to diethylphosphorothionate plus *p*-nitrophenol. The present study shows that parathion binds to cytochrome P-450 causing a type I difference spectrum. At low concentrations of parathion the minimum absorption is shifted to lower wavelengths and a shoulder in the type I spectra is evident. These changes in the type I spectra may be explained by the presence of a type II spectrum of parathion superimposed on the type I spectrum. The inhibition of the type II spectra of aniline by parathion further suggests the presence of a type II binding component of parathion. Hexobarbital diminishes the type I spectra of parathion seen with hepatic microsomes of untreated rabbits approximately 30% and that seen with hepatic microsomes from phenobarbital-treated rabbits approximately 50%. Similarly parathion decreases the type I spectra of hexobarbital seen with microsomes from untreated and phenobarbital-treated rabbits by approximately 30 and 50%, respectively. When hexobarbital and parathion are added to the same cuvet, the resulting spectral change is equal to 70–80% of the sum of the individual type I spectral components. These data indicate there are at least three separate type I spectral binding sites for these two substrates, a hexobarbital binding site, a parathion binding site, and a binding site with affinity for both hexobarbital and parathion. Phenobarbital, aminopyrone, ethylmorphine, and benzphetamine also diminish the type I spectra of parathion. When hexobarbital was added at a concentration that gave a maximal decrease along with either phenobarbital, aminopyrone, or ethylmorphine, the total decrease was essentially the same as that of hexobarbital alone. This seems to indicate that these drugs have a common binding site on cytochrome P-450. Benzphetamine, however, completely eliminates the type I spectra of parathion. The decrease of the type I spectra of parathion by benzphetamine is biphasic in character. These data also suggest that parathion may bind to more than one type I binding site.

Cytochrome P-450 has been shown to be the terminal enzyme of a liver microsomal enzyme system which can transfer oxygen to a variety of lipophilic compounds (Cooper *et al.*, 1965). This enzyme system has been named the microsomal mixed-function oxidase enzyme system. Substrates for this enzyme system may be categorized into two groups

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according to the type of spectral changes observed on binding to cytochrome P-450. Substrates may bind to cytochrome P-450 causing a type I spectral change exemplified by an increase in absorbance at approximately 385 mμ concomitant with a decrease in absorption at 420 mμ (Remmer *et al.*, 1966; Imai and Sato, 1966).

A second group of compounds, including aliphatic and aromatic amines, has been shown to cause a type II spectral change characterized by an absorption peak at 428 mμ and a minimum at approximately 392 mμ. A modified type II spectra has also been described (Schenkman *et al.*, 1967). It differs from the type II spectra by being at fixed wavelengths. In some cases the binding constant (K_s) for the substrates, determined by the type I spectral changes, are quantitatively similar to the Michaelis constant (Schenkman *et al.*, 1967). However, current evidence indicates there is no relationship between the K_s determined from type II spectral changes and the K_m values for the compounds giving these type II spectral changes (Guarino *et al.*, 1969).

Several investigators have studied the inhibitory effect of type I and type II compounds on the metabolism of various mixed-function oxidase substrates. The type of inhibition caused by the inhibitors varies from competitive to noncompetitive and, in some cases, to uncompetitive. Nicotinamide, 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) and aminoethyldiphenylpropylacetate (SKF525A-PA), all causing type II spectral changes, inhibit ethylmorphine N-demethylation noncompetitively at low concentrations and mixed to competitively at higher concentration (Sasame and Gillette, 1970). Hexobarbital, a compound causing type I spectral change, inhibits the N-dealkylation of ethylmorphine competitively, whereas hexobarbital does not inhibit norcodeine O-dealkylation in a competitive manner (George and Tephly, 1968). The latter authors draw the conclusion that there appear to be separate rate-limiting events that account for the metabolism of ethylmorphine and norcodeine.

One of the substrates for the microsomal mixed-function oxidase enzyme system is the phosphorothionate insecticides. These phosphorothionate triesters undergo a mixed-function oxidase-catalyzed desulfuration to the phosphate triesters in various animal species. These latter compounds are potent inhibitors of various esterases. The phosphorothionate triesters also undergo a mixed-function oxidase-catalyzed loss of one of the ester groups to give the diester (Neal, 1967a; Nakatsugawa and Dahm, 1967). In the case of parathion the two mixed-function oxidase-catalyzed reactions give rise to *O,O*-diethyl *p*-nitrophenylphosphate (paraoxon) plus some as yet unidentified form of sulfur, and to *O,O*-diethylphosphorothionate plus-*p*-nitrophenol. It is quite evident that these two products are formed by either two separate mixed-function oxidase enzyme systems or by a single mixed-function oxidase enzyme system with two binding sites for parathion on the terminal cytochrome P-450 (Neal, 1967b).

The purpose of the present study was to examine if spectral changes occurring during the interaction of parathion with cytochrome P-450 or the inhibitory effect of other type I substrates on the type I spectra of parathion might provide evidence for the presence of more than one type I binding site.

Materials and Methods

New Zealand strain male rabbits weighing approximately 3 kg were used in all experiments. The rabbits were fed *ad libitum* on a stock diet. The phenobarbital-treated animals were injected with doses of 50-mg/kg daily for 5 days before sacrificing.

Microsomes were isolated in the following manner. The liver from rabbits was perfused with 1.15% KCl and homogenized for approximately 30 sec in two volumes of an 0.05 M potassium phosphate buffer (pH 7.0) in a Waring Blendor. The resulting mixture was further homogenized with a motor-driven Teflon glass homogenizer. The homogenate was centrifuged at 12,000*g* for 20 min to remove large cell debris and mitochondria. The resulting supernatant solution was recentrifuged at 105,000*g* for 60 min in a Beckman ultracentrifuge (Model L2-65B). The supernatant was discarded and the resulting pellet was resuspended in 0.05 M potassium phosphate buffer (pH 7.6) containing 50% glycerol by volume. The suspension of microsomes was stored frozen at −70°. The maximum time of storage before use was one week, unless otherwise stated.

All difference spectra were measured at 28° using a Cary 15 recording spectrophotometer equipped with a high-intensity light source. The difference spectra were measured at protein concentrations ranging from 1 to 3 mg per ml of buffer. In all experiments 2.9 ml of the microsome preparation was placed in both reference and sample cuvet. The substrates and inhibitors were added to the cells at various concentrations in 0.1 ml of methanol. The extent of the type I binding spectra was measured by the change in the absorption between 460 mμ and the wavelength of minimum absorption which varied from 414 to 420 mμ. The extent of type II binding was measured by the difference in the absorption between the wavelengths of minimum and maximum absorption. The cytochrome P-450 content of the microsomes was measured as described previously (Omura and Sato, 1964).

The parathion was synthesized according to methods previously described (Neal, 1967a). Hexobarbital was purchased from Winthrop Laboratories, phenobarbital from the Mallinckrodt Chemical Co., aminopyrine and ethylmorphine·HCl from Merck Inc., and benzphetamine from the Upjohn Co. Spectral grade glycerol was obtained from Fisher Chemical Co. All other chemicals were the purest available from commercial sources.

The K_s values were determined mathematically by the least-squares method using a computer program written for this purpose (Wolcott *et al.*, 1972). The initial estimates of K_s were obtained using the Hanes form of the Michaelis–Menten equation (Hanes, 1932). Previous work (Wilkinson, 1961) had shown, using a nonlinear least-squares method, that better estimates of K_m values were obtained using the Hanes rather than the Lineweaver–Burk (Lineweaver and Burk, 1934) form of the Michaelis–Menten equation.

Results

The difference spectrum produced by the addition of parathion to the sample cuvet containing nonphenobarbital-induced rabbit liver microsomes is illustrated in Figure 1. At high concentrations parathion displays a type I spectra having an absorption minimum at 419 mμ and an ill-defined maximum at 380–390 mμ. A more clearly defined maximum at 385–390 mμ is obtained with microsomes from phenobarbital-treated animals. At the lower concentration of parathion, however, additional differences in the shape of the curve from that of the usual type I spectrum can be seen. Firstly, the absorption minimum has been shifted to a lower wavelength (414–416 mμ). Secondly, there is a shoulder in the type I spectrum at approximately 435 mμ. As the concentration

TABLE I: Effect of Aniline on the Hexobarbital and Parathion Type I Spectra: Effect of Parathion and Hexobarbital on Aniline Type II Spectra.

Substrate	[S], mM	Modifier	[M], mM	Spectra Type	Absorbance	
					$460 - \lambda_{min}$	$\lambda_{max} - \lambda_{min}$
Phenobarbital treated[a]						
Parathion	0.034	None		I	0.0227	
Parathion	0.034	Aniline	4.0	I	0.0185	
Hexobarbital	0.68	None		I	0.0208	
Hexobarbital	0.68	Aniline	4.0	I	0.0075	
Aniline	1.62	None		II		0.0250
Aniline	1.62	Parathion	0.14	II		0.0148
Aniline	1.62	Hexobarbital	0.18	II		0.0318
Untreated[b]						
Parathion	0.069	None		I	0.0139	
Parathion	0.069	Aniline	3.24	I	0.0151	
Parathion	0.069	Aniline	6.48	I	0.0167	
Aniline	1.62	None		II		0.0233
Aniline	1.62	Parathion	0.034	II		0.0177
Aniline	1.62	Parathion	0.069	II		0.0164

[a] Absorbance change measured at a protein concentration of 1 mg of protein/ml of buffer (1.94 nmoles of P-450/mg of protein).
[b] Absorbance change measured at a protein concentration of 3 mg of protein/ml of buffer (0.61 nmoles of P-450/mg of protein).

of parathion is increased in the sample cell the shoulder at 435 mμ disappears and the absorption minimum shifts to 419 mμ. This shoulder, which varies somewhat in magnitude according to the preparation of microsomes, appears to be due to a small absorption peak at 428 mμ.

The magnitude of the absorption change in the λ_{min} at 419 mμ with increases in parathion concentration was used to determine the binding constant, K_s. Hanes plots of the increase in absorbance with increase in parathion concentration using microsomes from untreated and phenobarbital-treated rabbits are shown in Figure 2. The binding constant K_s for the normal microsomes is 6×10^{-6} M. The plot for the phenobarbital-induced microsomes shows two distinct slopes, indicating that at high concentrations of parathion the rate of change of the magnitude of the spectral absorbance at the λ_{min} is greater than that at the lower concentrations. Extrapolation of a line joining the first three points and a line joining the remaining points to the x axis gives binding constants of 5×10^{-6} M for the low concentrations of parathion and 1.3×10^{-5} M for the higher concentrations. The biphasic character of the spectral change varies somewhat with the preparation of microsomes. This variability is in the most part due to changes in the slope of the portion of the plot giving the lower K_s value.

A comparison of the effect of aniline, a compound which gives predominantly a type II spectra, on the type I spectra of parathion and hexobarbital and the effect of parathion and hexobarbital on the type II spectra of aniline using microsomes from untreated and from phenobarbital-treated animals is shown in Table I. Aniline diminishes the type I spectra of both parathion and hexobarbital using microsomes from phenobarbital-treated rabbits. These results are in agreement with previous reports which have shown that the type I spectra of hexobarbital is diminished in the presence of aniline (Schenkman, 1970). In addition, this latter report indicated that hexobarbital increased the magnitude of the type II spectra of aniline. Similar results using aniline and hexobarbital were observed with our phenobarbital-induced

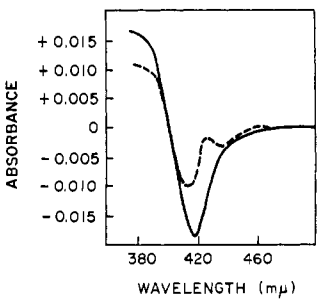

FIGURE 1: Difference spectra produced by the addition of parathion, (——) 69 μM and (- - - -) 3.4 μM, to normal rabbit liver microsomes (2 mg of protein/ml of buffer, 0.85 nmole of P-450/mg of protein).

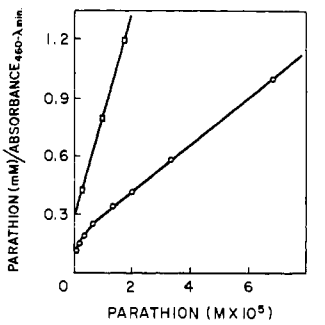

FIGURE 2: Hanes plot (Hanes, 1932) of changes in the concentration of parathion *vs.* the magnitude of the spectral absorbance at the λ_{min}. Spectra were measured with phenobarbital-induced microsomes (○) at a concentration of 1.7 mg of protein/ml of buffer (2.2 nmole of P-450/mg of protein); normal microsomes (□) at a concentration of 2 mg of protein/ml (0.85 nmole of P-450/mg of protein).

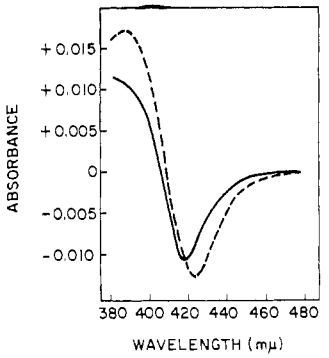

FIGURE 3: Difference spectra produced by the addition of 69 μM parathion to normal rabbit liver microsomes (—) plus the addition of 6.48 mM aniline to the reference and sample cuvets (- - - -). Microsomal protein concentration was 3 mg of protein/ml of buffer (0.61 nmole of P-450/mg of protein).

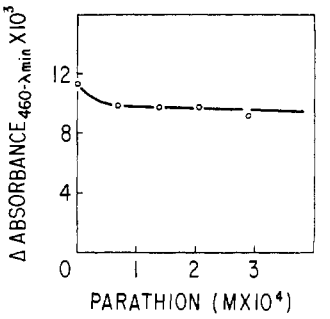

FIGURE 5: Effect of parathion on the type I spectra of hexobarbital (1.4 mM) with microsomes from untreated animals. Parathion was added to the reference and sample cuvets at the concentrations indicated. Microsome concentration was 3 mg of protein/ml (0.50 nmole of P-450/mg of protein).

microsomal preparations. However, parathion, unlike hexobarbital, decreases the magnitude of the type II spectra of aniline. The type II spectra of aniline is decreased approximately 40% in the presence of saturating amounts of the insecticide (0.14 mM). The type II spectra of aniline was also decreased at concentrations of parathion near the K_s (0.034 mM).

Microsomes from untreated animals display a similar effect to that seen with phenobarbital-induced microsomes in that parathion diminishes the type II spectra of aniline. However, with microsomes from untreated animals aniline appears to slightly increase the type I spectra of parathion. Additionally, in the presence of aniline the minimum absorption peak of the parathion type I spectra is broadened, the λ_{min} is shifted to 422 mμ, and the absorption peak at 390 mμ is more sharply defined (Figure 3).

The effect of type I spectral compounds on the type I spectra of parathion was also examined. Figure 4 shows the effect of increasing the hexobarbital concentration in both reference and sample cuvets on the magnitude of the type I spectra of parathion. With the phenobarbital-induced microsomes, the decrease of the parathion type I spectra by hexobarbital became maximal at approximately 0.3 mM. No further decrease of the type I spectra of parathion could be observed with higher concentrations of hexobarbital. In this particular experiment 50% of the original type I spectra of parathion could not be eliminated by the presence of hexobarbital. The parathion type I spectra obtained with microsomes from untreated animals was also decreased in the presence of hexobarbital. Approximately 70% of the spectra could not be eliminated by hexobarbital in this experiment. Another experiment using a different animal gave a value of approximately 80% for the parathion type I spectra not affected by hexobarbital.

The hexobarbital type I spectra can also be diminished by parathion as the data in Figure 5 indicates. Using microsomes from untreated animals, the maximum decrease of the type I spectra of hexobarbital caused by parathion is around 15–20%. Table II shows that the hexobarbital spectra seen with microsomes from phenobarbital-treated animals was decreased approximately 50% in the presence of parathion. Thus, the decrease of the hexobarbital spectra caused by parathion is quantitatively similar to the observed decrease in the parathion spectra brought about by the presence of hexobarbital. Table III shows that the type I spectra of parathion and hexobarbital are partially additive. The measured absorbance changes when parathion and hexobarbital were added together in the same cuvet are approximately 80% of the sum of the absorbance changes seen when the type I spectra of hexobarbital and parathion are measured separately.

The decrease in the parathion type I spectra brought about by the type I substrates, phenobarbital, aminopyrine, ethyl-

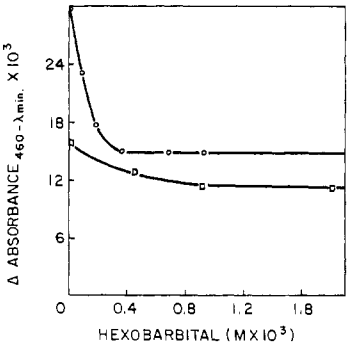

FIGURE 4: Effect of hexobarbital on the type I spectra of parathion. Hexobarbital was added to both the reference and sample cuvets at the concentrations indicated. Parathion was added to the sample cuvet at a concentration of 34 and 69 μM, respectively, with microsomes from phenobarbital-treated and untreated animals. Phenobarbital-induced microsomes (○) were used at a concentration of 1 mg of protein/ml (2.5 nmoles of P-450/mg of protein). Normal microsome (□) concentration was 3 mg of protein/ml (0.61 nmole of P-450/mg of protein).

TABLE II: Effect of Parathion on the Hexobarbital Type I Spectra.[a]

Substrate	[S], mM	Modifier	[M], mM	Absorbance 460 − λ_{min}
Hexobarbital	0.68	None		0.0215
Hexobarbital	0.68	Parathion	0.137	0.0094
Hexobarbital	0.045	None		0.0037
Hexobarbital	0.045	Parathion	0.137	0.0019

[a] Absorbance change measured with microsomes from phenobarbital-treated animals at a protein concentration of 1 mg of protein/ml of buffer (2.5 nmoles of P-450/mg of protein).

TABLE III: Additivity of the Parathion and Hexobarbital Type I Spectra.

Substrate	[S], mM	Absorbance 460 − λ_{min}	Absorbance 460 − λ_{min}
		Expt 1[a]	Expt 2[b]
Parathion	0.069	0.0154	0.0126
Hexobarbital	1.81	0.0125	0.0089
Parathion + hexobarbital	0.069 + 1.81	0.0214	0.0173

[a] Absorbance change measure at a protein concentration of 3 mg of protein/ml of buffer (0.61 nmole of P-450/mg of protein). [b] Absorbance change measured at a protein concentration of 3 mg of protein/ml of buffer (0.50 nmole of P-450/mg of protein).

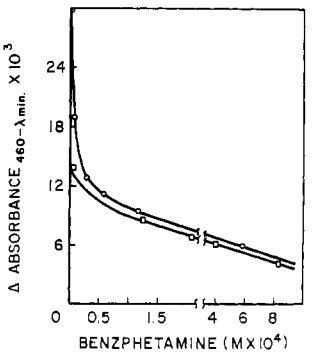

FIGURE 6: Effect of benzphetamine on the type I spectra of parathion. Benzphetamine was added to the reference and sample cuvets at concentrations indicated. Parathion was added to the sample cuvette at a concentration of 34 and 69 μM, respectively, with microsomes from phenobarbital-treated and untreated animals. Phenobarbital-induced microsomes (○) were used at a concentration of 1 mg protein/ml (2.5 nmoles of P-450/mg of protein). Normal microsome (□) concentration was 3 mg of protein/ml (0.61 nmole of P-450/mg of protein).

morphine, and benzphetamine was also examined with microsomes from phenobarbital-treated animals. The results of these experiments are listed in Table IV. The data indicate that all four drugs can diminish the type I spectra of parathion. Benzphetamine is by far the most effective of the four compounds, decreasing the spectra almost 70% at a concentration of 0.17×10^{-3} M. When hexobarbital was added at a concentration that gave maximal interference along with either phenobarbital, ethylmorphine, or aminopyrine, the total decrease was approximately equivalent to that of the hexobarbital alone. Benzphetamine, however, diminished the parathion type I spectra independently of the hexobarbital, since the same decrease of the spectra was observed with or without the addition of hexobarbital. The effect of varying the concentration of benzphetamine on the parathion spectra is shown in Figure 6. The diminution of the spectra appears to be biphasic, with an initial rapid decrease followed by a slower rate at higher concentrations of benzphetamine for microsomes from both untreated and phenobarbital-treated animals. Though not indicated in the figure, benzphetamine at a concentration of 2.1 mM can eliminate essentially all of the type I spectra of parathion.

Hexobarbital has previously been shown to competitively inhibit the N-dealkylation of ethylmorphine (George and Tephly, 1968). It may be expected, if the type I difference spectra is actually related to substrate binding at the catalytic site on cytochrome P-450, that ethylmorphine would also competitively inhibit the type I spectra of hexobarbital. This is indeed observed as shown by the Lineweaver–Burk plot in Figure 7. This suggests that these drugs compete for common binding site on cytochrome P-450.

TABLE IV: Effect of Type I Compounds on the Type I Spectra of Parathion.

Modifier	[M], mM	Absorbance 460 − λ_{min}[a]
None		0.0297
Hexobarbital	0.68	0.0149
Phenobarbital	0.17	0.0256
Phenobarbital + hexobarbital	0.17 + 0.68	0.0157
Aminopyrine	0.33	0.0246
Aminopyrine + hexobarbital	0.33 + 0.68	0.0153
Benzphetamine	0.17	0.0095
Benzphetamine	0.84	0.0050
Benzphetamine + hexobarbital	0.17 + 0.68	0.0085
Benzphetamine + hexobarbital	0.84 + 0.68	0.0057
Ethylmorphine[b]	2.3	0.0252
Ethylmorphine[b]	4.3	0.0241
Ethylmorphine + hexobarbital[b]	2.3 + 0.68	0.0172

[a] Absorbance change measured at a parathion concentration of 34 μM. [b] One-month-old microsomes were used. The magnitude of the absorbance seen on binding of parathion to this aged preparation was identical with that seen when these same microsomes were freshly prepared. Protein concentration was 1 mg of protein/ml of buffer (2.5 nmoles of P-450/mg of protein).

Discussion

The data presented in this paper indicate that the substrate, parathion, has multiple binding sites on cytochrome P-450 causing spectral changes different from those previously reported for the typical type I substrate for cytochrome P-450.

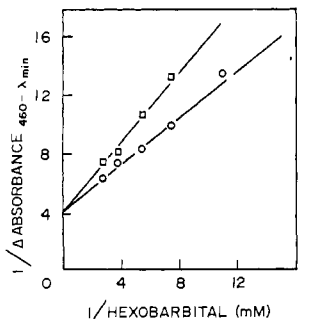

FIGURE 7: Lineweaver–Burk plot showing the inhibition of the type I spectra of hexobarbital by ethylmorphine. One-month-old phenobarbital-induced microsomes were used at a concentration of 1 mg of protein/ml. No ethylmorphine (○); 0.64 mM ethylmorphine (□) (2.5 nmoles of P-450/mg of protein).

Both the small absorption peak at 428 mμ and the shift of the wavelength of minimum absorption at low parathion concentrations may be accounted for by the presence of a type II spectra superimposed on the type I spectra. Further evidence which supports the view that parathion has a type II spectra may be seen from the data indicating that the insecticide inhibits the type II spectra of aniline. It has previously been suggested that the aniline spectra is actually a composite of a type I and a type II difference spectra (Schenkman, 1970). Thus, it might be expected that the type I binding component of parathion would inhibit the type I spectra of aniline, producing an increase in the type II spectra of aniline similar to that seen with hexobarbital (Schenkman, 1970). However, in the presence of parathion a decrease in the aniline type II spectra was seen. This strongly indicates that parathion has an affinity for the type II binding site. The actual decrease observed in the type II spectra of aniline in the presence of parathion may be greater than the measured value because of parathion inhibition of the type I spectra of aniline as well. With microsomes from untreated animals aniline causes a slight increase in the parathion type I spectra as well as a shift in the λ_{min} to a higher wavelength. These data suggest that in the normal microsomes the aniline type II binding is interfering with the type II binding of parathion, resulting in a more representative type I spectra for parathion.

The fact that parathion, a type I substrate, may also have a type II spectral component is not unique. It has previously been shown that the type I compound hexobarbital produces a type II pattern when added at high concentration to aged microsomes from 3-methylcholanthrene-treated animals (Shoeman *et al.*, 1969). Similarly, extraction of lipids from microsomes with isooctane will also cause hexobarbital at high concentration to exhibit a type II pattern (Leibman and Estabrook, 1971). At the very high concentrations needed to produce the type II spectra, hexobarbital will also decrease the spectra of aniline. However, the evidence presented in this paper is the first report of a type II component in the spectra of a type I compound used at low concentrations with freshly prepared microsomes from an untreated animal.

The data presented also suggest that parathion may have more than one type I spectral binding site on the cytochrome P-450 of rabbit liver microsomes. For example, the Hanes plot of the type I spectra of parathion with phenobarbital-induced microsomes indicates that at concentrations of the insecticide above 0.01 mM the formation of the type I spectra is facilitated over that at lower concentrations. This suggests that the type I spectrum of parathion with phenobarbital-induced microsomes is caused by binding of the insecticide to more than one type I site which have different binding constants. We thus interpret the type I spectrum resulting from the binding of parathion to microsomes from phenobarbital-treated animals to be the sum of two different type I spectra.

Additional evidence for more than one type I binding site is also given by the data concerning the diminution of the type I spectra of parathion by hexobarbital. Thus, hexobarbital can eliminate only part of the type I spectra of parathion. The part which remains unaffected by hexobarbital may reflect a binding site for parathion which has no affinity for hexobarbital. It also appears that the proportion of the type I spectra of parathion that is reduced by hexobarbital is greatly increased in the phenobarbital-treated as compared to the untreated animals. This indicates that phenobarbital treatment causes induction of the parathion type I binding component which has affinity for both hexobarbital and parathion to a greater extent than the type I spectral binding component which has affinity for parathion only.

With the microsomes from phenobarbital-treated animals phenobarbital, ethylmorphine, and aminopyrine also decrease the type I spectra of parathion. However, the decrease is not additive with that of hexobarbital. This suggests that these drugs have a common binding site on the enzyme. This is not unlikely for phenobarbital and aminopyrine since it has been shown that type I compounds will competitively inhibit the type I spectra of substrates of similar structure (Leibman *et al.*, 1969). The specificity of the hexobarbital binding site(s) appears to be quite broad since ethylmorphine, a compound of considerably different structure, will also competitively inhibit the type I spectra of hexobarbital. The fact that hexobarbital and ethylmorphine have also been shown to compete for the same metabolic binding site suggests that the spectral data may be reflecting binding of these drugs to the metabolic binding site on cytochrome P-450. Benzphetamine appears to completely eliminate the parathion spectra in contrast to phenobarbital, hexobarbital, aminopyrine, or ethylmorphine. In addition, the ability of benzphetamine and hexobarbital to diminish the type I spectra of parathion is not additive. This suggests that benzphetamine, like parathion, binds to more than one site on the cytochrome P-450. As seen in Figure 5, benzphetamine decreases the type I spectra of parathion in a biphasic manner. Other investigators have shown that Hofstee plots for the spectral changes in rat liver microsomes using benzphetamine as a substrate are also not linear (Rickert and Fouts, 1970). Unpublished experiments in this laboratory indicate that hexobarbital decreases but does not completely eliminate the benzphetamine type I spectra. This suggests that these drugs have a binding site in common. It also suggests that benzphetamine binds to a type I site which has little or no affinity for hexobarbital.

The data presented also indicate that hexobarbital has a spectral binding site which has little or no affinity for parathion. Thus, it is suggested that the microsomal mixed-function oxidase of rabbit liver must contain at least three separate spectral binding sites for hexobarbital and parathion: a hexobarbital binding site, a parathion binding site, and a binding site with affinity for both hexobarbital and parathion.

References

Cooper, D. Y., Levin, S., Narasimhulu, S., Rosenthal, O., and Estabrook, R. W. (1965), *Science 147*, 400.

George, W. J., and Tephly, T. R. (1968), *Mol. Pharmacol. 4*, 502.

Guarino, A. M., Gram, T. E., Gigon, P. L., Greene, F. E., and Gillette, J. R. (1969), *Mol. Pharmacol. 5*, 131.

Hanes, C. S. (1932), *Biochem. J. 26*, 1406.

Imai, Y., and Sato, R. (1966), *Biochem. Biophys. Res. Commun. 22*, 620.

Leibman, K. C., and Estabrook, R. W. (1971), *Mol. Pharmacol. 7*, 26.

Leibman, K. C., Hildebrandt, A. G., and Estabrook, R. W. (1969), *Biochem. Biophys. Res. Commun. 36*, 789.

Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.

Nakatsugawa, T., and Dahm, P. A. (1967), *Biochem. Pharmacol. 16*, 25.

Neal, R. A. (1967a), *Biochem. J. 103*, 183.

Neal, R. A. (1967b), *Biochem. J. 105*, 289.

Omura, T., and Sato, R. (1964), *J. Biol. Chem. 239*, 2370.
Remmer, H., Schenkman, J., Estabrook, R. W., Sasame, H., Gillette, J., Narasimhulu, S., Cooper, D. Y., and Rosenthal, O. (1966), *Mol. Pharmacol. 2*, 187.
Rickert, D. E., and Fouts, J. R. (1970), *Biochem. Pharmacol. 19*, 381.
Sasame, H. A., and Gillette, J. R. (1970), *Biochem. Pharmacol. 19*, 1025.
Schenkman, J. B. (1970), *Biochemistry 9*, 2081.
Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967), *Mol. Pharmacol. 3*, 113.
Shoeman, D. W., Chaplin, M. D., and Mannering, G. J. (1969), *Mol. Pharmacol. 5*, 412.
Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.
Wolcott, R. M., Vaughn, W. K., and Neal, R. A. (1972), *Tox. Appl. Pharmacol.* (in press).

Effect of *N,N'*-Dicyclohexylcarbodiimide and Other Carbodiimides on Electron Transfer Catalyzed by Submitochondrial Particles†

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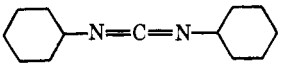
ABSTRACT: Compound I inhibited NADH oxidation in beef heart sonic submitochondrial particles (ETPH) but was not inhibitory with succinate as substrate or with electron donors which transfer electrons on the oxygen side of coenzyme Q. The oxidative phosphorylation uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) released the I-induced inhibition of NADH oxidation and thereby mimicked respiratory control observed in intact mitochondria. Inhibitor studies with a series of hydrophobic and water-soluble carbodiimides revealed that only nonpolar carbodiimides had significant inhibitory activity at reasonably low concentrations, suggesting that the inhibitory site within the mitochondrial membrane is also hydrophobic. *N,N'*-Dicyclohexylurea (hydrophobic) and 1-cyclohexyl-3-(2-morpholinoethyl)urea metho-*p*-toluenesulfonate (water soluble) were without inhibitory effect. Treatment of ETPH with I in the presence of glycine methyl ester increased the inhibitory action of I and prevented release by FCCP, suggesting the involvement of available carboxyl groups at the I-sensitive site. Gel filtration studies suggested that I and GlyOMe, reacted in the presence of I, are firmly bound to ETPH. Electron spin resonance (epr) spectrometry of I-inhibited ETPH revealed that nonheme iron of NADH dehydrogenase was reducible by NADH while other nonheme iron compounds of the electron-transfer chain giving signals at $g = 1.94$, and copper at $g = 2.00$, remained oxidized under such conditions. In addition, coenzyme Q was not reduced by NADH in I-inhibited preparations under conditions where succinate did reduce the quinone in the inhibited particle and both NADH and succinate reduced coenzyme Q in uninhibited particles. The epr and coenzyme Q data suggest that the site sensitive to hydrophobic carbodiimides lies on the oxygen side of NADH dehydrogenase nonheme iron and on the substrate (NADH) side of coenzyme Q. Experiments with submitochondrial particles from Mung bean etiolated seedlings, which are resistant to inhibition by rotenone, barbiturates, and piericidin A, as well as to I, suggested that such inhibitors all react with a common, sensitive component. A mechanism involving conformational alteration of the inhibitory site is suggested.

In addition to being a valuable reagent in the study of phosphate esters of biological importance (Khorana, 1961), compound I has received attention recently as a tool in studies of oxidative phosphorylation and respiratory metabolism. Beechey *et al.* (1966, 1967) have reported that I acts at low concentrations as an inhibitor of oxidative phosphorylation



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intact mitochondria and submitochondrialp articles. Racker in and Horstman (1967) have shown that I mimics the effects of rutamycin in that it stimulates the synthesis of ATP[1] by

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[1] Abbreviations used are: ATP and ADP, adenosine tri- and diphosphates; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HBHM, heavy-layer beef heart mitochondria; ETPH, electron-transfer particles prepared from HBHM by sonic vibrations (20 kc) in the presence of salts which are indicated in parentheses; epr, electron spin resonance; MB-ETP, electron-transfer particles prepared from Mung bean seedling mitochondria; UQ, coenzyme Q or ubiquinone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulfate; $FeNH_{N,S,\text{ or }R}$, nonheme iron proteins of the NADH dehydrogenase, succinic dehydrogenase, and cytochrome b–c_1 complex (Reiske protein), respectively.