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The Effect of Atabrine and Promazine Derivatives on the Activity of the Old Yellow Enzyme

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Atabrine, chlorpromazine, and other promazine derivatives inhibited the activity in the aerobic Zwischenferment-OYE * system, but only when preincubated with the apoprotein of the OYE. The inhibition was independent of the FMN concentration. Fluorimetric studies of the apoprotein-FMN association reaction showed that compounds which inhibited the enzyme activity slowed down the reaction rate immediately after being added to the apoprotein, but did not lower the binding capacity for FMN. The mechanism of reaction between inhibitors and enzyme is discussed.

Chlorpromazine potentiated the aerobic OYE activity when added to the apoprotein at the same time as or after the FMN. Other promazine derivatives had the same ability, whereas the corresponding sulphoxides were ineffective. The anaerobic OYE activity was inhibited but not potentiated by chlorpromazine. The potentiating effect could not be explained by the experimental results, although these allowed certain conclusions.

The dissociation constants of complexes, formed between FMN and some promazines, were determined by spectrophotometric measurements.

Certain compounds with chemotherapeutical activity act as flavoprotein inhibitors. The effect of atabrine on respiration in tissue slices [1] and on oxidative phosphorylation in mitochondrial systems [2] has been explained by inhibition of flavin containing enzymes [3,4]. Studying cytochrome c reductase from yeast, Haas [3] found evidence of an irreversible reaction between the apoprotein and atabrine in competition with the coenzyme FMN. Hellerman *et al.*[5] studied the effect of this drug on D-amino acid oxidase and found a competitive inhibition, to which a simple kinetic interpretation could, however, not be applied. They concluded that the reaction between atabrine and apoprotein is complicated and reflects a general effect on proteins by the drug

* Abbreviations: OYE = Old Yellow Enzyme, FMN = flavin mononucleotide, FAD = flavin adenine dinucleotide, TPN = triphosphopyridine nucleotide, TPNH = reduced TPN, DPNH = reduced diphosphopyridine nucleotide, EDTA = ethylene diamine tetraacetic acid.

rather than a specific antagonism with flavin. Other flavoproteins are also inhibited by atabrine [6,7]. Using a fluorimetric technique, Nygaard and Theorell [8] found a reaction between the apoprotein of the OYE and atabrine, similar to that occurring between the apoprotein and FMN. Because of the strong fluorescence of atabrine its influence on the latter reaction was not studied.

Yagi *et al.*[9] showed an inhibition of D-amino acid oxidase by chlorpromazine in competition with the coenzyme FAD and calculated the dissociation constant of the enzyme-inhibitor complex. Inhibition of flavoproteins in the brain was suggested as a possible mechanism of action for the drug. Other investigators [10] found that the inhibition was due to a slow reaction between enzyme and drug and that, once established, it could not be easily reversed even by large excess FAD. The effect upon oxidative phosphorylation in mitochondria by chlorpromazine is similar to that of atabrine [11], and has been suggested to be caused by inhibition of flavoproteins [11,12]. The interaction between chlorpromazine and flavoproteins may also be influenced by the fact that a complex formation occurs between the drug and free flavin [13,14]. Yagi *et al.*[15] determined the dissociation constants for such complexes using a fluorimetric technique and concluded that, due to the high values of these constants, this type of complex formation is not responsible for the inhibition of D-amino acid oxidase by chlorpromazine.

Although many experimental data show that atabrine and chlorpromazine inhibit flavoproteins, the specificity of their activity has not been established. Both of them have also been found to cause inhibition of other isolated enzymes [5,16].

In the present work the effect of atabrine and chlorpromazine upon the activity of the OYE has been studied. Since it was found that chlorpromazine often caused a potentiation of the activity, the investigation was extended to include some other promazine derivatives and compounds of similar structure.

MATERIALS

OYE was prepared from brewer's bottom yeast (kindly supplied by Hamburgerbryggeriet, Stockholm) by the method of Theorell and Åkeson [17], and the *apoprotein* obtained according to Warburg and Christian [18]. Determination of concentration and purity was carried out as described before [19]. All enzyme preparations used in the experiments were of purity 0.75 or more.

Glucose-6-phosphate (di-sodium salt), *glucose-6-phosphate dehydrogenase (Zwischenferment)*, *TPN*, *FMN*, and *cytochrome c* (type III from horse heart) were all commercial preparations (Sigma Chemical Company).

Chlorpromazine [2-chloro-10-(3-dimethylaminopropyl)phenothiazine hydrochloride] was kindly supplied by AB Leo, Hälsingborg. The sulphoxide was obtained by oxidation with H_2O_2 in the presence of peroxidase [20]: To 2 ml of 3×10^{-3} M chlorpromazine solution were added 1 μl 3×10^{-4} M horse radish peroxidase (a gift from Dr. K. G. Paul) and 2μl 8 M H_2O_2 at +4°C. A red colour appeared. The UV-spectrum of the solution was identical with that of chlorpromazine sulphoxide [20], and paper chromatography (see Methods) showed a single spot with R_F value 0.82. *Atabrine* [6-chloro-9-((4-diethylamino-1-methylbutyl)amino)-2-methoxyacridine hydrochloride], *promazine* [10-(3-dimethylaminopropyl)phenothiazine hydrochloride], *promazine sulphoxide* oxalate, *2-methoxypromazine*, *2-methoxypromazine sulphoxide*, and *imipramine* [(3-dimethylaminopropyl)-dibenzyl amine] were all gifts from Dr. H. Löw. *Transergan* [2-diethylaminoethyl phenothiazine-10-carboxylate hydrochloride] was kindly supplied by AB. Astra, Södertälje.

METHODS

Determination of aerobic OYE activity. The same procedure as outlined in a previous publication [21] was used. The total volume of the reaction mixture was always 3.0 ml and contained 0.1 M sodium phosphate buffer pH 6.5. This pH was chosen because of the low solubility of chlorpromazine at higher pH. When not otherwise stated in the section on results, the reaction mixture contained 1.9×10^{-2} M glucose-6-phosphate, 4.5×10^{-4} M TPN, 1 Kornberg Unit Zwischenferment, and 2.7×10^{-3} M KCN. 0.1 mg OYE was added to give a final concentration of 3.2×10^{-7} M. This concentration was rate limiting and gave an oxygen uptake of approximately 100 μl per 30 min, (O_2-atmosphere). When apoprotein was used, an equivalent amount was added, and FMN was added in excess (3×10^{-6} M). All concentrations are given as final concentrations in the reaction mixture. In preincubation experiments drug and enzyme were mixed in the sidearm of the Warburg vessel and allowed to react for 30 min before being added to the other reactants. The volume in the sidearm was 0.2 ml, and thus the concentrations were here 15 times larger than in the final reaction mixture. The reaction velocity was expressed as μl oxygen consumed during a 30 min period. The activity in the vessels, where drug was added, was expressed as per cent of the activity in the control vessels. All experiments were performed at +37.0° C.

Determination of anaerobic OYE activity. The method described by Theorell [22] was used. A Thunberg cuvette contained in a total volume of 3.0 ml 0.1 M sodium phosphate pH 6.5, 9.9×10^{-3} M glucose-6-phosphate, 1 Kornberg Unit Zwischenferment, 4.0×10^{-5} M cytochrome c, and OYE. In the sidearm 0.1 ml 1.3×10^{-2} M TPN was added. The OYE concentration varied in different experiments, and in some experiments chlorpromazine was added in the main compartment. The cytochrome c stock solution was made up in 0.02 N HCl to achieve total oxidation of the cytochrome [22]. The air in the cuvette was replaced by argon, and the cuvette was carefully shaken during 5 min to obtain equilibrium. The cuvette was placed in a Beckman DU spectrophotometer, and the absorption at 550 mμ read against a buffer blank. It was approximately 0.38 in all experiments, and constant during a 10 min period. The cuvette was tilted, and the content of the sidearm mixed with that of the main compartment. The absorption at 550 mμ was measured until a constant value was obtained. The total amount cytochrome c reduced was determined, using the values $\beta = 2.20 \times 10^7$ cm$^2 \cdot$ mole^{-1} for oxidized cytochrome [23] and $\beta = 6.36 \times 10^7$ cm$^2 \cdot$ mole^{-1} for the reduced form [24]. The reaction rate was determined during the early phase of the reaction, when a rectilinear relationship was obtained between the amount of cytochrome reduced and the reaction time. The experiments were run at + 21.0°C.

Fluorimetric study of the apoprotein—FMN association reaction. The apparatus and method described by Theorell and Nygaard [25] was used. This allowed determination of the association velocity constant k_1 and the amount FMN bound by a certain amount of apoprotein. The reactions were performed in 0.1 M sodium phosphate buffer pH 6.5 at + 23.0°C.

Spectrophotometric determination of complex formation with FMN. The change in the absorption spectrum of FMN caused by chlorpromazine [15] and other compounds was used to determine the dissociation constants of the complexes. The solutions were made in 0.1 M sodium phosphate buffer pH 6.5. The experiments were performed at room temperature (+ 21°C).

Paper chromatography of chlorpromazine was carried out as described by Salzman *et al.*[26]

All experiments were done at least twice. Care was always taken to keep out light as much as possible.

RESULTS

The effect of atabrine on the aerobic OYE activity. When atabrine was mixed with FMN and other reactants in the main compartment of the Warburg flask, and the reaction was started by introducing apoprotein from the sidearm, no inhibition occurred even at the highest atabrine concentration tested,

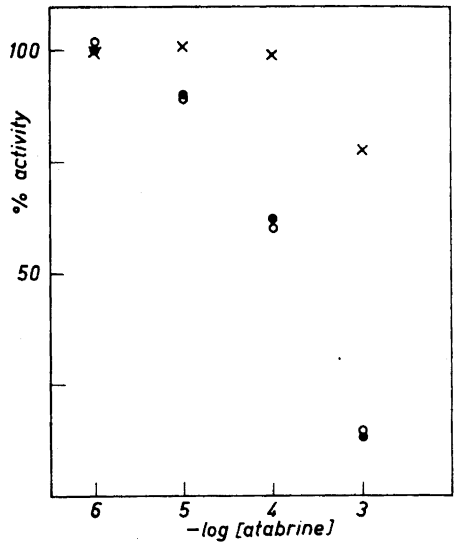


Fig. 1. The effect of atabrine on the aerobic OYE activity. The activity is expressed as per cent of the activity in control experiments, where no drug was added, and marked along the ordinate. Along the abscissa the negative logarithm of the atabrine concentration is marked. Atabrine and enzyme were preincubated in a sidearm of the Warburg flasks, where the volume was 0.2 ml. The total volume of the reaction mixture was 3.0 ml. The concentration of TPN was 4.5×10^{-4} M and of enzyme 3.2×10^{-7} M. All concentrations are expressed as final concentrations in the reaction mixture. Points marked × indicate experiments, where holoenzyme was used. O marks experiments where apoprotein was used, and 3.2×10^{-6} M FMN was added in the main compartment, ● experiments with apoprotein and 3.8×10^{-7} M FMN.

1×10^{-3} M. In different experiments the TPN or FMN concentration was lowered to 1.8×10^{-5} M or 3.8×10^{-7} M, respectively, to become rate limiting in the reaction, other experimental conditions being unchanged. 1×10^{-3} M atabrine was still without effect. However, when atabrine and apoprotein were mixed in the sidearm and thus preincubated during 30 min., inhibition occurred. It was of the same magnitude at low, rate limiting FMN concentrations as when FMN was added in excess. Some effect, although much less pronounced, was noticed also when atabrine was preincubated with the holoenzyme (Fig. 1). The inhibition of the apoprotein after preincubation was reversed by approximately 2/3 when dialysis against buffer was performed overnight at + 4°C immediately after the preincubation.

The effect of chlorpromazine on the aerobic OYE activity. Under various experimental conditions chlorpromazine caused potentiation of the enzymatic activity. This always occurred when the holoenzyme was used. It was of the same magnitude whether preincubation of OYE and chlorpromazine was performed or not. Also in experiments with apoprotein, potentiation occurred if FMN and drug were added simultaneously to the enzyme. On the other hand, if apoprotein and chlorpromazine were preincubated in the sidearm

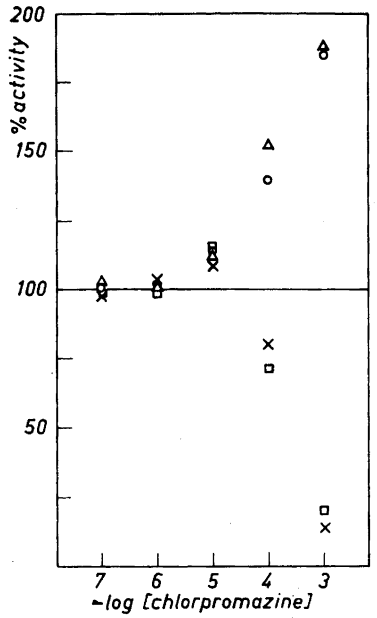


Fig. 2. The effect of chlorpromazine on the aerobic OYE activity. Activity and drug concentrations are marked as in Fig. 1. O marks experiments, where holoenzyme and chlorpromazine were preincubated in the sidearm, △ experiments, where 3.7×10^{-7} M FMN and chlorpromazine were preincubated, and apoprotein kept in the main compartment, × indicates preincubation of apoprotein and drug, when the main compartment contained 3.1×10^{-6} M FMN. □ indicates the same prodedure, but FMN concentration was 4.2×10^{-7} M. Other experimental conditions were as described under Fig. 1.

before being added to the other reactants, high concentrations of chlorpromazine caused inhibition, whereas low concentrations potentiated. Essentially the same effect was obtained at high and low FMN concentrations. In Fig. 2 some experiments are summarized to show the effect of chlorpromazine under various experimental conditions. Higher concentrations of chlorpromazine than those shown in the figure caused precipitation and lowering of the activity.

When the activity in the test system was lowered by diminishing the OYE concentration, the potentiating effect of chlorpromazine remained unchanged. When a lower activity was obtained by diminishing the TPN concentration, the effect of the drug was much less marked. This is shown in Table 1, where the OYE concentration was rate limiting in the first two groups of experiments, and the TPN concentration in the last group.

Paper chromatography was performed on reaction mixtures containing 3.2×10^{-7} M OYE, excess TPN, and 1×10^{-3} M chlorpromazine, which had caused a maximal potentiation of the OYE activity. In different experiments samples containing 10—18 μg chlorpromazine were tested. They all showed the same R_F values as the chlorpromazine blanks, 0.92, and only one spot

Table 1. The effect of chlorpromazine on the aerobic OYE activity. Chlorpromazine and OYE were preincubated in the sidearm for 30 min, then added to TPN and other reactants. In the first two groups of experiments the enzyme concentration was rate limiting in the reaction mixture without drug. In the last group TPN concentration was rate limiting.

OYE concentration	TPN concentration	Chlorpromazine concentration	O_2 uptake during 30 min	Activity in the presence of chlorpromazine
3.2×10^{-7} M	4.5×10^{-4} M	0	100.7	—
3.2×10^{-7} M	4.5×10^{-4} M	5×10^{-4} M	158.3	157.7 %
1.6×10^{-7} M	4.5×10^{-4} M	0	52.4	—
1.6×10^{-7} M	4.5×10^{-4} M	5×10^{-4} M	82.1	156.6 %
3.2×10^{-7} M	1.1×10^{-5} M	0	60.0	—
3.2×10^{-7} M	1.1×10^{-5} M	5×10^{-4} M	66.7	111.1 %

appeared in the chromatograms. Control runs of reaction mixtures without chlorpromazine did not cause any spots.

The effect of other compounds on the aerobic OYE activity. The different compounds tested were preincubated with OYE or apoprotein. The results are summarized in Table 2, where it can be seen that other promazines than the 2-chloro derivative also potentiated the OYE activity, whereas many

Table 2. The effect of various compounds on the aerobic OYE activity. The different compounds and OYE or apoprotein were preincubated for 30 min in the sidearm of the Warburg flask, then added to the other reactants in the main compartment. In experiments with apoprotein this contained also 3.0×10^{-6} M FMN. Other experimental conditions were as described under Fig. 1.

Compound	Concentration M × 10^4	Activity of OYE compared with control %	Activity of apoprotein compared with control %
Chlorpromazine	1	140	80
Daylight exposed chlorpromazine	1	108	77
UV-irradiated chlorpromazine	1	114	
Chlorpromazine sulphoxide	1	100	90
Promazine	1	135	73
Promazine sulphoxide	1	97	99
Methoxypromazine	1	120	94
Methoxypromazine sulphoxide	1	102	84
Imipramine	1	100	93
Transergan	1	98	92
Methylene blue	1	151	154
Adenine	1	98	
Caffeine	10	99	
D-Tryptophan	10	97	
L-Tryptophan	10	101	
EDTA	1	103	

structurally related compounds did not. When chlorpromazine is left in the light, the solution rapidly darkens [27] and becomes strongly fluorescent. Chlorpromazine, which had been left in daylight for 2 days at room temperature had lost most of its potentiating effect. The same change could be accomplished by irradiating the solution for 2 h with the mercury lamp of the fluorimeter.

Most of the promazines and other compounds with a similar structure caused the same degree of inhibition when preincubated with the apoprotein. The irradiated chlorpromazine, which had lost most of its potentiating activity, still caused inhibition.

Methylene blue potentiated the OYE more strongly than chlorpromazine. At 1×10^{-3} M it doubled the activity. When preincubated with the apoprotein, the potentiating effect remained unchanged.

Table 2 shows that adenine, caffeine, and tryptophan did not cause potentiation. These compounds form complexes with FMN of the same type as chlorpromazine [28,29]. EDTA was also without effect.

The effect of chlorpromazine on the anaerobic OYE activity. The results are shown in Table 3. The same amount of cytochrome c was reduced, regardless of the concentration of OYE in the reaction mixture. The variations obtained are explained by experimental error. Without OYE no reduction occurred in the absence or in the presence of chlorpromazine. The reaction rate varied with the amount OYE added, and approximately the same turnover number was obtained. The addition of chlorpromazine caused some inhibition.

Fluorimetric studies of the apoprotein-FMN association reaction. Chlorpromazine showed a slow, constant increase in fluorescence when irradiated by the lamp of the fluorimeter and was therefore excluded from these studies. Promazine, on the other hand, had a low fluorescence that remained unchanged when the solution was illuminated, at least during the period of time necessary for the experiment. Its effect on the fluorescence of FMN at the concentrations used in these experiments was negligible. When promazine was added to apoprotein solutions to give a final concentration of 1×10^{-4} M a few minutes

Table 3. The effect of chlorpromazine on the anaerobic OYE activity. The amount of cytochrome c reduced is calculated from the change in absorption at 550 mμ. The reaction rate is calculated from the beginning of the reaction (10th—30th min) when a constant amount of cytochrome c was reduced per unit time. The last column gives the turnover number of the OYE.

OYE concentration M	Chlorpromazine concentration M	Total change in absorption at 550 mμ	Absorption change at 550 mμ per min	Total amount cytochrome reduced, M	M cytochrome reduced per min	Mole cytochrome reduced per mole OYE per min
1.28×10^{-7}	0	0.719	0.0120	3.97×10^{-5}	6.96×10^{-7}	5.44
2.56×10^{-7}	0	0.760	0.0254	4.21×10^{-5}	14.04×10^{-7}	5.49
2.56×10^{-7}	1.94×10^{-4}	0.756	0.0215	4.18×10^{-5}	11.89×10^{-7}	4.64
1.66×10^{-7}	0	0.720	0.0151	3.98×10^{-5}	8.35×10^{-7}	5.03
1.66×10^{-7}	1.94×10^{-4}	0.734	0.0114	4.05×10^{-5}	6.30×10^{-7}	3.99

Table 4. The effect of promazine on the apoprotein-FMN reaction, studied by fluorimetric technique. The volume of the test solutions was 3.55 ml, containing a constant amount of apoprotein, 0.1 M sodium phosphate buffer pH 6.5 and 1×10^{-4} M promazine when added. To these solutions 2 µl aliquots of 1.17×10^{-4} M FMN were added. The temperature + 23.0°C.

Conditions	Reaction mixture	FMN coupled M	Association velocity constant, k_1 $M^{-1} \cdot sec^{-1}$
FMN added immediately to reaction mixture	Apoprotein	7.08×10^{-7}	11.17×10^4
	Apoprotein + promazine	6.90×10^{-7}	6.41×10^4
Reaction mixture dialyzed before FMN was added	Apoprotein	6.50×10^{-7}	11.97×10^4
	Apoprotein + promazine	6.68×10^{-7}	11.08×10^4

before titration with FMN was undertaken, the association velocity of the apoprotein-FMN reaction was lowered. However, the same amount of FMN was bound on each addition. Incubating promazine and apoprotein for 30 min. at +37°C before adding FMN did not alter the result. When the promazine-apoprotein mixture was dialyzed against buffer at +4°C overnight, the effect of the promazine was completely abolished. Table 4 shows a typical experiment.

Some other compounds structurally related to promazine that had been tested in the Warburg experiments showed the same fluorescence properties as promazine. They were added to apoprotein solutions a few minutes before titration with FMN was undertaken. Results from one experiment are shown in Table 5. Some compounds were much less effective than promazine, but they all influenced the reaction in a similar way.

Although atabrine is strongly fluorescent, attempts were made to study its influence on the apoprotein-FMN reaction with the fluorimetric technique. 5×10^{-6} M atabrine had the same fluorescence as 7×10^{-7} M FMN, which was half of the concentration that began to give self-quenching. Using similar experimental conditions as those, outlined under Table 4, the same increase

Table 5. The effect of various compounds on the apoprotein-FMN reaction. Their concentration was 1×10^{-4} M. FMN was added in 5 µl aliquots of an 1.55×10^{-4} M solution. Other experimental conditions as described under Table 4.

Compound added to apoprotein solution	FMN coupled M	Association velocity constant, k_1 $M^{-1} \cdot sec^{-1}$
None	9.04×10^{-7}	10.55×10^4
Promazine	9.34×10^{-7}	5.72×10^4
Promazine sulphoxide	8.99×10^{-7}	8.96×10^4
Methoxypromazine	8.87×10^{-7}	6.43×10^4
Methoxypromazine sulphoxide	8.95×10^{-7}	7.57×10^4
Imipramine	9.27×10^{-7}	6.70×10^4
Transergan	8.90×10^{-7}	8.26×10^4

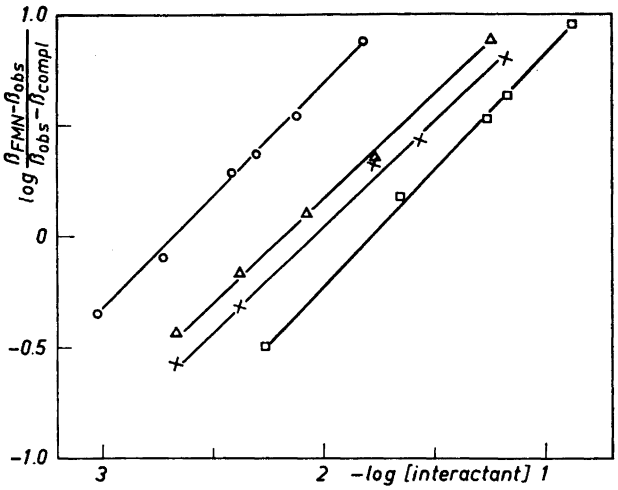


Fig. 3. Graphical determination of dissociation constants of FMN complexes after measuring the absorption at 445 mμ of FMN in the presence of various interactants. β_{FMN} is the absorption of free FMN, β_{obs} the absorption of FMN at different concentrations of interactant, shown on the abscissa, and $\beta_{compl.}$ the absorption of FMN when further addition of interactant did not cause further change in absorption. Readings were made against blanks containing the same amount of interactant as the samples. All solutions contained 0.1 M sodium phosphate pH 6.5. Temperature was + 21°C. O—O chlorpromazine, $K = 2.1 \times 10^{-3}$ M; △—△ promazine sulphoxide, $K = 6.6 \times 10^{-3}$ M; × — × promazine, $K = 9.3 \times 10^{-3}$ M; □—□ transergan, $K = 1.7 \times 10^{-2}$ M. The slopes of all curves equalled 1.

in fluorescence was caused by the FMN additions in samples containing 5×10^{-6} M atabrine as in the controls. The addition of atabrine caused a 20 % decrease of k_1, whereas the amount FMN bound was not affected.

Spectrophotometric studies of flavin complexes. All promazines as well as imipramine and transergan caused a change in the absorption spectrum of FMN, similar to that described for chlorpromazine [15], indicating the formation of a complex. Atabrine was excluded from these studies because of its strong absorption at 445 mμ. The dissociation constants of some of the complexes were determined. The results are shown in Fig. 3.

Spectrophotometric measurements were also made to determine whether complex formation occurred between chlorpromazine and FMN bound to protein in the OYE *. The absorption of 1.95×10^{-5} M OYE and 1×10^{-3} M chlorpromazine, read against a chlorpromazine blank, was compared to that of 1.95×10^{-5} M OYE alone. Certain difficulties were encountered since a slight opalescence occurred when enzyme and chlorpromazine were mixed. After centrifugation the two OYE solutions showed identical spectra in the visible region.

* Experiments concerning the OYE spectra were performed in collaboration with Dr. A. Ehrenberg. The author wishes to thank him for the right to use the results in this connection.

When OYE is reduced with excess TPNH and reoxidized with oxygen, the spectrum is shifted towards the red region [30,31]. The same shift was found in the absence and in the presence of 1×10^{-3} M chlorpromazine.

DISCUSSION

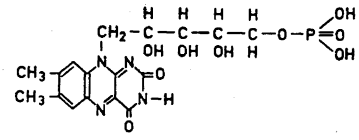
The results of the inhibition studies of the OYE give some support to the idea that atabrine and chlorpromazine, as well as other compounds of similar structure, may act as flavoprotein inhibitors. The potentiation obtained with some of the promazine derivatives is more difficult to explain. Inhibition and potentiation were in some cases caused by the same compound, depending on the experimental conditions, and thus seemed to reflect two different mechanisms of action. The effect of the drugs depended on whether they reacted with the enzyme before or after the coenzyme was bound.

Both atabrine and chlorpromazine caused inhibition of the oxygen uptake in the Zwischenferment-OYE system but, except for atabrine at high concentration, only when preincubated with the apoprotein. This leads to the conclusion that the observed inhibition was due to interference with the activity of the OYE and did not concern any of the other reactants. Also, the inhibitors interferred with the reaction between apoprotein and FMN. The effect was the same at high and low FMN concentrations. These findings bear some resemblance to results obtained with other flavoproteins [3,10], suggesting a little reversible reaction between drug and enzyme that prevents the binding of flavin. That the reaction was reversible to some extent was shown when the effect of atabrine could be partially abolished after dialysis.

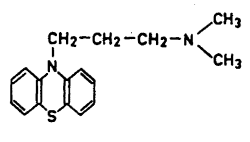
Similar results were obtained with promazine and other, structurally related compounds. The formulas of some of the compounds are shown below. They all have a certain structural resemblance to FMN but lack groups of the same type as the special combining groups [8,32] which have been suggested to participate in the binding of the coenzyme to the apoprotein. The binding of the inhibitors to the protein must therefore be supposed to be of a different character, even if they might compete for the same sites as FMN. Considering the great affinity of the apoprotein for FMN [25], one would assume that this could easily replace the inhibitors, even if added later.

Further information was obtained from the fluorimetric studies of the apoprotein-FMN reaction that showed that promazine and structurally related compounds, as well as atabrine in low concentration, slowed down the reaction rate but did not influence the binding capacity of apoprotein for FMN. It did not seem likely that this interferance was due to complex formation with FMN, because of the high values obtained for the dissociation constants of the promazine-FMN and other complexes. Thus it appeared that the inhibitors were bound to the apoprotein without occupying the binding sites of the FMN but making these less accessible. A similar effect on the reaction has been obtained by treating the apoprotein with functional group reagents, which block only some of the binding groups for each FMN molecule [8].

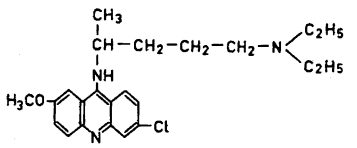
The effect upon the activity of the enzyme by atabrine and promazine derivatives is not explained by a change in the equilibrium between the apopro-



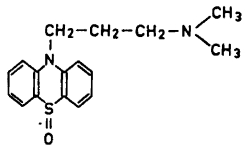
FMN.



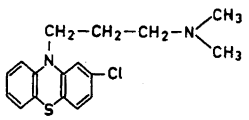
Promazine.



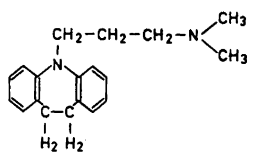
Atabrine.



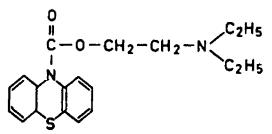
Promazine sulphoxide.



Chlorpromazine.



Imipramine.



Transergan.

tein and FMN, since the inhibition would then have depended upon the FMN concentration. To offer an explanation of the present results it might be assumed that the apoprotein of the OYE, inhibitor, and FMN form a ternary complex with low or no activity. The presence of the inhibitors disturbs but does not prevent the binding of FMN to the apoprotein. The presence of FMN completely prevents a binding of inhibitors that can reduce the activity. This suggests that FMN and the inhibitors have binding sites located close to each other. No conclusions can be drawn as to whether the inhibitors are also bound to other parts of the enzyme molecule.

The experimental results do not explain the nature of the interaction between enzyme and drug that resulted in increased activity. However, certain conclusions may be drawn that allow a discussion. It is interesting to note that potentiation by chlorpromazine has been observed in other enzyme systems [10,33].

The potentiating effect of the promazines was quite similar to that of methylene blue, which is known to oxidize reduced OYE [34]. This reaction occurs at a faster rate than the oxidation by oxygen in reaction mixtures, where the reducing system (glucose-6-phosphate, Zwischenferment, TPN) is in excess.

In such cases the velocity is determined by the oxidation of the reduced OYE [35]. On the other hand, at low concentrations of the reducing system the velocity depends on the reduction of the OYE, and the enzymatic activity is equally high with oxygen or methylene blue as electron acceptor [35]. In the present experiments it was found that the potentiating effect of chlorpromazine was much less pronounced in reaction mixtures with low concentration of TPN. It appeared that chlorpromazine interfered with the reoxidation of the reduced OYE by oxygen.

It is difficult to believe, however, that chlorpromazine, which has been characterized as an exceptionally good electron donor [14], could act as an oxidant like methylene blue. Metabolites, possibly possessing this property, were not found in paper chromatograms of reaction mixtures, where chlorpromazine had caused potentiation. A comparison between the different compounds tested gives a clue to which parts of the promazine molecule that might be responsible for the effect. Substitution in the 2-position had little influence, whereas oxidation at the sulphur atom abolished the effect. Negative results obtained with imipramine also indicate the importance of the sulphur atom, and those obtained with transergan the importance of the substitution at the nitrogen.

It may be remarked that phenothiazines are considered to form resonance stabilized free radicals [36], where the nitrogen and sulphur atoms hold key positions. Bindings to these atoms may interfere with this activity. The radical formation in chlorpromazine has been suggested as the reason for its antioxidant effect on the formation of lipid peroxides in tissue homogenates [27]. This reaction is also inhibited by methylene blue. It may be postulated that in the OYE system, the promazine radical structure may act as an intermediate in the electron transport system.

The different effect of chlorpromazine on the OYE activity in the aerobic and anaerobic system brings up the question, whether the formation of hydrogen peroxide in the former had any importance. The presence of hydrogen peroxide did not in itself influence the enzymatic activity. Hydrogen peroxide is known to oxidize phenothiazines to sulphoxides under different conditions [37]. In aqueous solutions the oxidation of chlorpromazine requires the presence of catalase or peroxidase to occur at a fast rate [20]. Possibly the nascent peroxide in the Zwischenferment-OYE system, although unable to complete the oxidation, could form a postulated intermediate radical form [38]. This is, however, supposed to appear upon UV irradiation of chlorpromazine solutions. In the present experiments such treatment lead to the disappearance of the potentiation.

Potentiation of aerobic and inhibition of anaerobic activity has also been found in microsomal cytochrome reductase [39] by substituting DPNH with TPNH, or by adding competing nucleotides when DPNH is the substrate. When oxygen is the electron acceptor, the rate limiting step is the oxidation of the reduced enzyme, and this reaction occurs faster when the oxidized pyridine nucleotides dissociate more easily from the enzyme. If the same explanation is applicable to the present experiments, it would mean that the promazines interfered with the binding of TPN, and thus potentiation and inhibition could be attributed to the same type of mechanism. Observations

recently made with horse liver alcohol dehydrogenase [40] give further support to this possibility. Some compounds that do not participate in the enzymatic reaction have been found to form a ternary complex with the enzyme. Depending upon the concentrations of the reactants this sometimes leads to inhibition and sometimes to potentiation of the activity. The potentiation is due to an easier dissociation of the ternary than the binary complex. — Nevertheless, it was possible to form the red intermediate of the OYE [30,31] in the presence of chlorpromazine.

The complex formation that occurs between chlorpromazine and flavin is supposed to involve a transfer of electrons [14]. It did not appear that such a reaction had anything to do with the potentiation. No experimental evidence was found for the formation of a complex between chlorpromazine and protein bound FMN, and other compounds able to form flavin complexes did not potentiate.

Although unexplained, the potentiation of the OYE activity is interesting, considering the exceptionally low turnover number of this enzyme. Whether this type of action has anything to do with the pharmacological properties of the promazines is completely uncertain. It may be remarked, however, that chlorpromazine and its sulphoxide, which behaved differently with regard to the potentiation, also differ to a certain extent in pharmacological activity [41].

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