Inhibition of enzymes by Atebrin

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which has already been pointed out is obvious. It is shown that the inhibition of the enzyme by Atebrin is not due to the formation of a compound between the Atebrin and the FMN. First, the glucose-6-phosphate dehydrogenase and liver esterase, which are almost certainly not flavoproteins, were found to be inhibited by Atebrin. Glucose-6-phosphate dehydrogenase activity could not be measured in the usual way, by following directly the reduction of TPN at 340 mμ. Atebrin inhibits strongly at this wavelength. The inhibition was overcome by precipitating the Atebrin at various times by adding NaOH to pH 10. The efficiency of staining was then measured on the clear supernatant. However, less staining was observed. This occurred in the procedure. Fig. 1 shows that a 1 mM Atebrin caused considerable inhibition of this enzyme.

The enzyme was stained on a 20-40% liver microsomal preparation (fraction between 15,000 × g for 10 min and 105,000 × g for 30 min) or a 20% sucrose gradient obtained by treating an acetone powder of the microsomal pellets with ethanol, followed by extraction of the powder with 0.2 M Tris-HCl buffer, pH 7.5, 0.1 M NaHCO₃-H₂CO₃. The enzyme activity was determined microscopically in NaHCO₃-H₂CO₃ buffer. The degree of inhibition of the enzyme in microsomes at various concentrations of Atebrin is shown in Fig. 2. 50% inhibition was obtained with 3 mM Atebrin, which is similar to the concentration giving 50% inhibition of succinic oxidase in liver experiments. The soluble esterase was also inhibited to the same extent. The esterase was not inhibited by 2 mM Atebrin, 1 mM 2,4-dinitrophenol, 5 mM succinyl-CoA, 5 mM chloroform, or 5 mM acetylcholine. Chloroform (5 mM) was slightly inhibitory. 2,4-dinitrophenol inhibited quite strongly (50% inhibition by 1 mM, 100% by 5 mM).

Inhibition of enzymes by Atebrin

Inhibition of an enzymic reaction by Atebrin is frequently interpreted in the biochemical literature as a proof that a flavoprotein is involved in the reaction, especially when the inhibition is reversed by FMN or FAD. It is assumed that the drug competes with the flavin for the apo-enzyme.

It is the purpose of the present communication to draw attention to the fact,

Abbreviations: FMN, flavin mononucleotide; FAD, flavin-adenine dinucleotide; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)amino-methane.

which has already been pointed out by others^{1,2}, that Atebrin is not a specific inhibitor of flavoproteins. It is shown that reversal of inhibition by FMN is not due to competition between the flavine and Atebrin for the enzyme, but is due to the formation of a compound between the Atebrin and the FMN.

Erythrocyte glucose 6-phosphate dehydrogenase and liver esterase, which are almost certainly not flavoproteins, were found to be inhibited by Atebrin.

Glucose 6-phosphate dehydrogenase activity could not be measured in the usual way, by following directly the reduction of TPN at 340 $m\mu$, since Atebrin absorbs strongly at this wavelength. This difficulty was overcome by precipitating the Atebrin at various times by adding NaOH to pH 10. The absorbancy at 340 $m\mu$ was then measured on the clear supernatant. Recovery tests showed that no loss of TPNH occurred in this procedure. Fig. 1 shows that 5 mM Atebrin caused considerable inhibition of this enzyme.

Ali-esterase was studied either as a rat-liver microsomal preparation (fraction between 15,000 $\times g$ for 10 min and 105,000 $\times g$ for 60 min), or as a soluble preparation obtained by treating an acetone powder of the microsomal pellets with butanol, followed by extraction of the powder with 0.25 M Tris-HCl buffer, pH 7.75 (*cf.* ref. 3). The enzyme activity was determined manometrically in $\text{NaHCO}_3\text{-H}_2\text{CO}_3$ buffer. The degree of inhibition of the enzyme in microsomes at various concentrations of Atebrin is shown in Fig. 2. 50% inhibition was obtained with 5 mM Atebrin, which is similar to the concentration giving 50% inhibition of succinic oxidase in Löw's experiments⁴. The soluble ali-esterase was also inhibited to the same extent.

The ali-esterase was not inhibited by 2 $\mu\text{g/ml}$ antimycin, 4 mM 2,4-dinitrophenol, 5 mM aureomycin, 5 mM chlorpromazine, or 5 mM methylene blue. Chloramphenicol (5 mM) was slightly inhibitory. Pentachlorophenol inhibited quite strongly (50% inhibition by 1.3 mM, 100% by 5.2 mM).

Fig. 1. Inhibition of glucose 6-phosphate dehydrogenase by Atebrin. Each reaction vessel contained: 0.05 M Tris-HCl buffer, pH 7.75, 0.02 M MgCl_2 , 0.015 M glucose 6-phosphate, 0.0002 M TPN, 0.2 ml glucose 6-phosphate dehydrogenase preparation, 0.005 M Atebrin (when present), water to a final volume of 2.5 ml. The reaction was stopped by adding NaOH to pH 10 and subsequent boiling. The absorbancy found in a zero-time control was subtracted from the experimental values.

Fig. 2. Inhibition of aliesterase, microsome-bound or soluble, by Atebrin. The activity was measured in Warburg manometers, each vessel containing in the main compartment: 0.1 ml enzyme preparation, varying amounts of Atebrin, 0.025 M NaHCO_3 , water to a final volume of 2.9 ml. After temperature equilibration, the substrate, 0.1 ml 10% tributyrin, was tipped in from the side arm. Gas phase $\text{N}_2\text{-CO}_2$ (95:5).

The inhibition of the microsome-bound ali-esterase by Atebrin or pentachlorophenol was fully reversed by sedimenting the microsomes and washing with 0.5 % bovine serum albumin, pH 7.3 or $\text{NaHCO}_3\text{-H}_2\text{CO}_3$ buffer, pH 7.4.

TABLE I
REVERSAL BY FMN OF ATEBRIN INHIBITION OF ALIESTERASE

Atebrin (mM)	5	0	5	5
FMN (mM)	0	5	5	10
Inhibition (%)	55	0	37	24

Table I shows that inhibition by Atebrin was partly reversed by FMN. During the course of these experiments it was observed that FMN and Atebrin, both of which are yellow, reacted together to form a red-coloured complex. The difference spectrum of the complex is shown in Fig. 3. YAGI⁵ has shown that chloropromazine, which is chemically related to Atebrine, as well as other aromatic compounds, forms a complex with flavins.

Fig. 3. Difference spectrum: (5 mM Atebrin + 5 mM FMN) minus 5 mM Atebrin minus 5 mM FMN, in Tris-HCl buffer, pH 7.75.

These experiments strongly support HELLERMAN's view¹ that Atebrin is a relatively non-specific enzyme inhibitor, acting by binding with proteins in general.

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¹ L. HELLERMAN, A. LINDSAY AND M. R. BOVARNICK, *J. Biol. Chem.*, 163 (1946) 553.

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