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COMPLEXING OF RIBOFLAVIN WITH THE ENOLATE FORM OF BARBITURIC ACID

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SUMMARY

Riboflavin and the enolate form of barbituric acid form a complex at pH 7.80 as demonstrated by the appearance of two new absorption bands at 400 and 490 nm. The association constant for the complex determined from a Benesi–Hildebrand analysis of absorbances at 490 nm is 30 M^{-1}. The K evaluated from fluorescence quenching at various barbiturate: riboflavin ratios is 33 M^{-1}. The complexing of the barbiturate and riboflavin is accompanied by an inhibition of the photo-oxidation of sarcosine to formaldehyde. Under the same conditions, the barbiturate enhances the rate of photo-oxidation of NADH. The 5,5-disubstituted barbiturates do not give a new absorption band with riboflavin and do not inhibit the photo-oxidation of sarcosine, indicating that an enolate configuration of the β-diketone system is involved in complexing with riboflavin.

Previous studies in our laboratory had demonstrated that sarcosine is photo-oxidized in the presence of catalytic amounts of riboflavin, FMN, or FAD[1]:

$$H_3\overset{*}{C}\text{–}\overset{H}{N}\overset{\dagger}{}\overset{\S}{}\text{–}CH_2\text{–}COOH + O_2 + H_2O \rightarrow H_3\overset{*}{C}\text{–}\overset{H}{N}\overset{\dagger}{}\overset{\S}{} + HCHO + CO_2 + H_2O_2$$

It has been found subsequently that sodium barbiturate inhibits the reaction and forms a complex with riboflavin.

In the experiments described below, the difference absorption spectra were determined in 1-cm cells with a Cary 17 spectrophotometer and the fluorescence measurements were made with a Hitachi spectrophotofluorimeter. The photo-oxidation of sarcosine with riboflavin was carried out in quartz cuvettes placed 11 cm from a long-wave ultraviolet lamp (Model X 30, Ultra-Violet Products, Inc.). Formaldehyde was determined by the chromotropic acid procedure[2]. All reagents were of the highest quality available commercially. The amytal was a generous gift of Dr. Otto K. Behrens of the Lilly Research Laboratories.

The difference spectra of mixtures of sodium barbiturate and riboflavin, measured against riboflavin as the reference, are presented in Fig. 1. Two new absorption bands were observed at 400 and 490 nm. However, only the absorption at 490 nm could be analyzed by the BENESI–HILDEBRAND plot[3]. It should be noted

that sodium barbiturate itself has no absorption at this wavelength. Under the same conditions, no new absorption was observed with the 5,5-disubstituted derivatives, barbital and amytal.

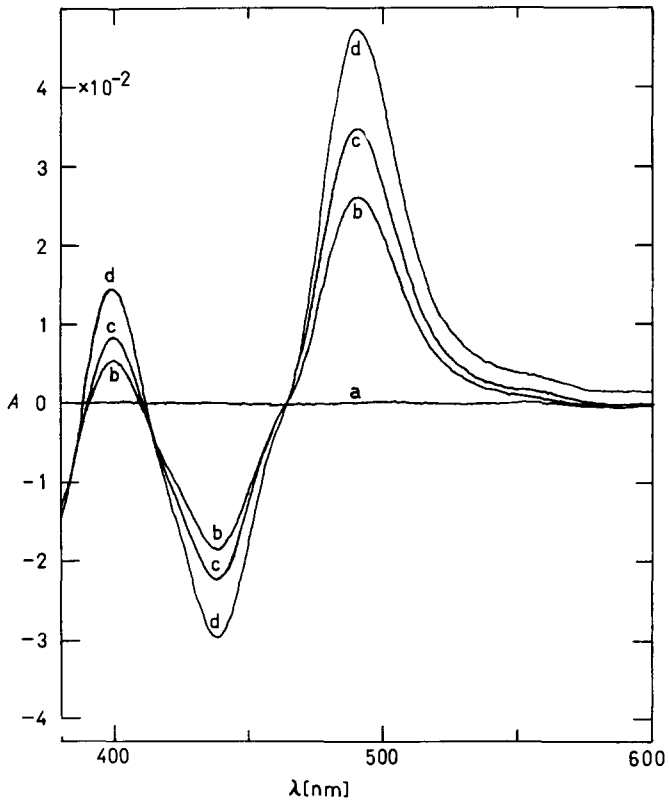
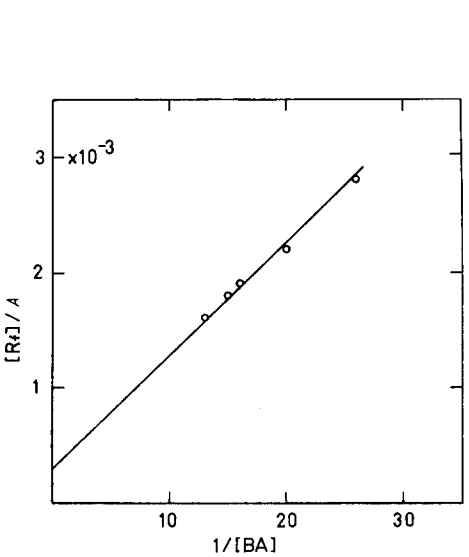


Fig. 1. Difference spectra of riboflavin *plus* sodium barbiturate *versus* riboflavin. The concentrations of the reactants in 0.075 M potassium phosphate, pH 7.80, were: riboflavin, $1.5 \cdot 10^{-5}$ M, and barbiturate, (a) ○ ; (b) $3.75 \cdot 10^{-2}$ M; (c) $5.02 \cdot 10^{-2}$ M; (d) $7.5 \cdot 10^{-2}$ M.

The association constant for the complex, determined by the data shown in Fig. 2, was found to be 30 M^{-1}. The equilibrium constant was also evaluated by measuring the degree of fluorescence quenching at various concentrations of barbiturate[4] (Fig. 3). The value of K obtained by this method was 33 M^{-1}.

Complexing of the barbiturate and riboflavin is accompanied by an inhibition of the photo-oxidation of sarcosine to formaldehyde, as demonstrated by the data in Fig. 4. Barbital and amytal do not inhibit. It may be added that under the conditions of our reactions, barbituric acid enhances the rate of oxidation of NADH[5] as first observed by GIUDITTA AND VITALE–NEUGEBAUER[6].

In the same molar ratios of flavin to barbiturate used for the analyses described in Figs. 1 and 2, new absorption bands were also detected with riboflavin and 5,5-dimethyl-1,3-cyclohexanedione ("dimedon") (490 nm), resorcinol (490 nm), acetylacetone (488 nm), and phenol (488 nm). These results, together with the inability



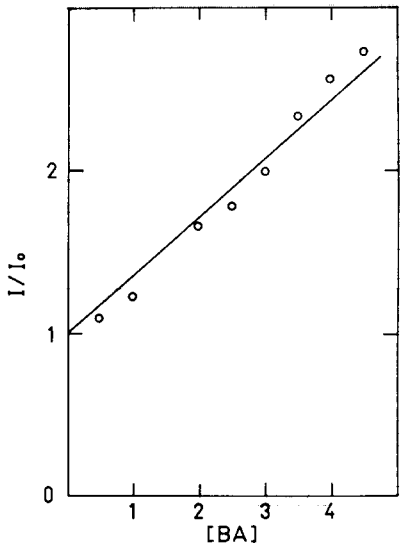


Fig. 2. The BENESI–HILDEBRAND plot[3] for the complex of riboflavin and barbiturate. The concentration of riboflavin [Rf] was $1.2 \cdot 10^{-4}$ M and that of the barbiturate [BA] was varied from $1.85 \cdot 10^{-2}$ to $7.5 \cdot 10^{-2}$ M. Appropriate amounts of sodium acetate were added to the reference solution to compensate for the barbiturate.

Fig. 3. Quenching of fluorescence of riboflavin by barbiturate. The concentration of riboflavin was $5.0 \cdot 10^{-6}$ M and that of the barbiturate [BA] was varied from $0.5 \cdot 10^{-2}$ to $4.5 \cdot 10^{-2}$M. I_0/I is the ratio of fluorescence intensities before and after addition of quencher. Sodium acetate was added to the reference solution in amounts equivalent to the barbiturate.

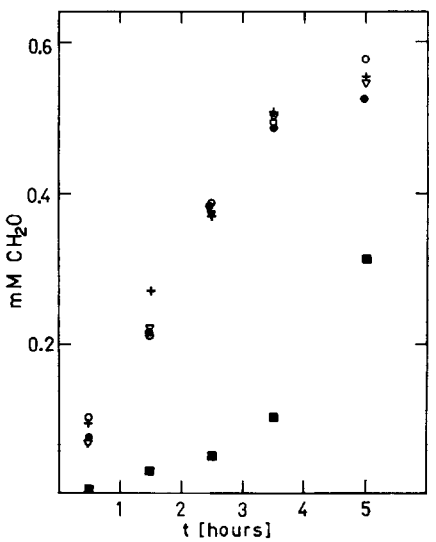


Fig. 4. Effect of barbiturate, barbital, and amytal on the photo-oxidation of sarcosine with riboflavin. The concentrations of reactants, all in 0.075 M potassium phosphate, pH 7.80, were (●) riboflavin, $1.0 \cdot 10^{-4}$ M and sarcosine, $2.0 \cdot 10^{-3}$ M; (■) same, plus barbiturate; (+) barbital; (▽) Amytal; (○) sodium acetate, each $1.0 \cdot 10^{-3}$ M.

of the 5,5-dialkyl barbiturates to react, indicate that an enolate configuration of the β-diketone system is probably involved in complexing with riboflavin.

Riboflavin and its derivatives can complex with a variety of N-heterocyclic compounds, including indoles and purines[4,7-10]. Earlier studies in our laboratory had demonstrated also that adenine derivatives inhibit the photo-oxidation of sarcosine by riboflavin[1]. The reaction of barbiturate reported in this paper provides an additional example of the diversity of heterocyclic compounds capable of complexing with flavins.

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