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*FREE RADICAL FORMATION IN RIBOFLAVIN COMPLEXES\**

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INTRODUCTION

If an aqueous solution containing  $10^{-3}$  *M* tryptophan and  $10^{-3}$  *M* riboflavin-5' phosphate is frozen, the resultant sample has a red appearance instead of the yellow shown by riboflavin alone. This red form can also be seen at room temperature if more concentrated solutions are used—for example,  $10^{-2}$  *M* tryptophan

and  $10^{-3}$  *M* riboflavin. Proteins that contain tryptophan, as well as certain tryptophan derivatives, also form red samples with riboflavin.

This report will present evidence that this red form is a tryptophan-riboflavin complex in which a riboflavin molecule has taken up one electron from the tryptophan. The riboflavin is then in a semiquinoid form, a free radical. This form of riboflavin was first studied by Kuhn and Wagner-Jauregg<sup>1</sup> and afterward by Michaelis and his co-workers.<sup>2-4</sup>

Since the work of Michaelis, the reduction of riboflavin to its semiquinone form has hardly been studied. Haas<sup>5</sup> reported that when old yellow enzyme was reduced by hydrosulfite in the presence of TPN, a red color appeared, and he attributed this red color to free radical formation. More recently, Beinert<sup>6</sup> has studied the spectral properties of the semiquinoid form of riboflavin, and Beinert<sup>7, 8</sup> and Ehrenberg and Ludwig<sup>9</sup> have further studied the occurrence of free radicals in flavo-protein catalysis.

#### MATERIALS AND METHODS

We have found it convenient to study the red riboflavin complex in two ways. For qualitative visual observations, aqueous solutions were frozen in dry ice. In this form, complex formation is greatly enhanced over room-temperature conditions, and direct and easy observations may be made as to which samples do contain complexes and which samples do not.

For quantitative studies the samples were studied at room temperature in phosphate buffer, pH 6.90, by means of a Beckman DKI recording spectrophotometer. The sample compartment cuvette contained the mixture to be studied—say, riboflavin and tryptophan—while the reference cuvette contained riboflavin of a molarity equal to the sample. In this way, by balancing out most of the riboflavin absorption, the complex absorption becomes a major component of what is recorded by the instrument. It should be noted that this technique does not lead to an exact balancing-out of the riboflavin. To achieve an exact balance, one would need to use a riboflavin concentration equal to the *free* riboflavin concentration in the sample cell rather than the *total* concentration there. It should therefore be recognized that the peak at 500  $m\mu$  reported below is not the true peak for absorption by the complex. The true peak will lie at somewhat shorter wave lengths. It also follows that the molar extinction at the peak for the complex absorption will be somewhat *higher* than that at 500  $m\mu$  reported below.

#### RESULTS

a) *Qualitative Observations.*— $10^{-3}$  *M* riboflavin-5'-phosphate was frozen in the presence of a number of amino acids, tryptophan derivatives, and several tryptophan-containing proteins. The results are shown in Table 1. A minus sign indicates no shift in color from a riboflavin control, while a plus sign means a shift toward the red.

b) *Quantitative Observations.*—Upon balancing  $10^{-4}$  *M* riboflavin-5'-phosphate with varying concentrations of tryptophan against  $10^{-4}$  *M* riboflavin-5'-phosphate, all in phosphate buffer pH 6.90, a peak at 500  $m\mu$  was obtained. Riboflavin-5'-phosphate in 10 per cent HCl, reduced to a red form by sodium hydrosulfite, also yielded a peak at 500  $m\mu$  when balanced against unreduced riboflavin-5'-phosphate.

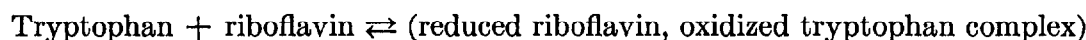
TABLE 1

Partner of $10^{-3}$ M Riboflavin-5'-Phosphate	Appearance at $-78^{\circ}$ C.
$10^{-3}$ M tryptophan	+
$10^{-3}$ M tyrosine	-
$10^{-3}$ M phenylalanine	-
$10^{-3}$ M histidine	-
$10^{-3}$ M 5-hydroxytryptamine (serotonin)	+
1-Benzyl-2-methyl-5-methoxy-N,N-dimethyl tryptamine hydrochloride*	+
1-Benzyl-2-methyl-5-methoxy-tryptamine hydrochloride*	+
1-Benzyl-2,5-dimethyl serotonin	+
1-Benzyl-2,5-dimethyl bufotenine	+
1-Methyl medmain	+
<i>d</i> -Lysergic acid diethylamide tartrate (LSD-25)†	+
1 Per cent bovine plasma albumin	+
1 Per cent myosin	+
0.3 Per cent actomyosin	+
3 Per cent Casein	+

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† The authors thank the Sandoz Pharmaceutical Company, Hanover, New Jersey, for a sample of LSD-25.

This agrees with the value of  $503 \text{ m}\mu$  reported by Beinert<sup>6</sup> for the difference maximum of the semiquinoid form in  $1 \text{ M HCl}$ . Since the red form is stable only in strong acid solution,<sup>2</sup> the tryptophan must, in some way, stabilize this semiquinoid form at neutral pH. The most obvious way to do this is by complexing. It will be assumed that a complex forms in a one-to-one fashion, so that



Let  $t$  = total tryptophan concentration in the sample;  $r$  = total riboflavin concentration in the sample;  $c$  = concentration of the complex;  $k$  = dissociation constant for the complex; and  $K = 1/k$ .

It will be assumed that the extinction of the complex bears a simple Beer's law relationship to the complex concentration. Since we are measuring a difference spectrum and since we have assumed that one molecule of semiquinoid riboflavin forms for every molecule of riboflavin that reacts, we have

$$E = \Delta\epsilon Cl,$$

where  $E$  = recorded extinction at  $500 \text{ m}\mu$ ;  $\Delta\epsilon$  = molar extinction of the complex at  $500 \text{ m}\mu$  minus the molar extinction of oxidized riboflavin at  $500 \text{ m}\mu$ ;  $C$  = concentration of the complex;  $l$  = cell path length, in our case  $1.000 \text{ cm}$ .

The tryptophan concentrations used were always at least twenty times that of the riboflavin concentrations, and therefore they necessarily greatly exceeded the complex concentrations. Under these conditions

$$\frac{t(r - c)}{C} = k = \frac{1}{K}$$

or

$$\frac{1}{t} = lrK \frac{1}{E} - K.$$

Thus a plot of  $1/t$  versus  $1/E$  should yield a straight line. The intercept on the abscissa yields  $1/\Delta\epsilon lr$  while the intercept on the ordinate yields  $-K$ .

Figure 1 shows some typical data for tryptophan and also typical data for sero-

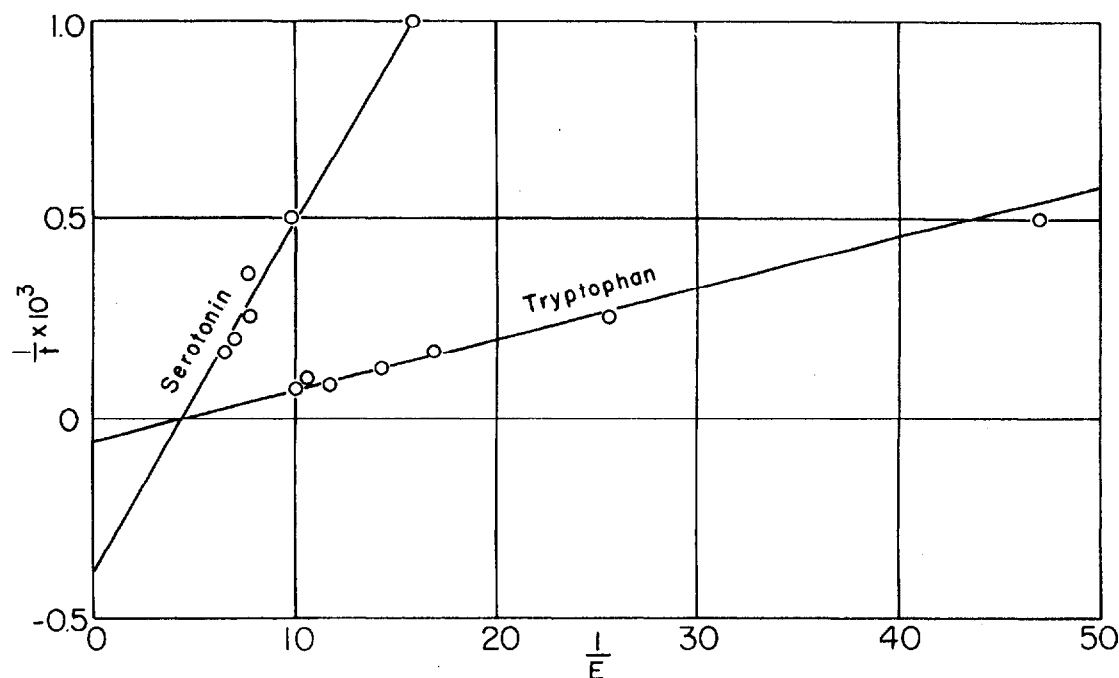


FIG. 1.—Plot of inverse concentration of riboflavin-5-phosphate partner,  $1/t$ , versus inverse extinction,  $1/E$ . The riboflavin-5'-phosphate concentration was  $10^{-4} M$  in both the sample and the reference cell.

tonin. It can be seen that straight-line plots do give reasonable representations of the data. A more striking result, however, is that, within experimental error, both plots have the same intercept on the  $1/E$  axis. This is further confirmation that the measured absorption is due to a riboflavin semiquinoid, the semiquinoid having a unique molar extinction coefficient whether in the presence of tryptophan or of serotonin. On the other hand, the two plots have different  $1/t$  intercepts. This means that serotonin and tryptophan complex with the riboflavin semiquinone with different strengths, serotonin complexing about seven times as strongly as riboflavin. The data yield  $\Delta\epsilon = 2300$  liters per mole cm.;  $K$  for tryptophan equals 60 liters per mole, while  $K$  for serotonin equals 400 liters per mole.

Since  $\epsilon$  for oxidized riboflavin at  $500 m\mu$  is approximately 2,200, we obtain an approximate value of 4,500 for the molar extinction coefficient of the complex.

It is noteworthy that solutions of riboflavin that contain the complex appear to be less fluorescent than pure riboflavin solution.

#### DISCUSSION

There has been a recent upsurge of interest in free radical formation in biological systems, due, in part, to the development and application of the technique of paramagnetic resonance.<sup>9, 10</sup> It is therefore of some interest that a semiquinoid form of riboflavin can be produced and stabilized at neutral pH by the simple addition of tryptophan or of proteins containing tryptophan or of molecules resembling tryptophan. Indeed, as shown by serotonin, other molecules may reduce and form complexes with riboflavin much more strongly than tryptophan itself.

It is clear that some of the work that has been done on free radical formation in flavoproteins may be subject to reinterpretation. For the tacit assumption has

always been made that the flavoprotein itself contained no semiquinoid form before the addition of a reducer. This now appears unjustified. This realization may also clarify the absorption spectra of certain flavoproteins. Consider the absorption spectrum of old yellow enzyme, as given, for example, by Ehrenberg and Ludwig.<sup>9</sup> The spectrum has two peaks that appear to be similar to the absorption peaks of riboflavin. In addition, there is a marked shoulder at about 490  $m\mu$ . The work reported here suggests that this shoulder results from the absorption of riboflavin-5'-phosphate in a semiquinoid form stabilized by the tryptophan in the protein.

It is possible that complex formation may be much stronger in tissue than in vitro. Just as freezing enhances complex formation in a test tube, so it is possible that in closely packed tissues constituents, such as mitochondria, a similar lattice-ordered structure of the water favors association.<sup>11</sup> Within the cell the complex formation might also be favored by additional links, as assumed by Nygaard and Theorell<sup>12</sup> for the binding of the flavin adenine nucleotide in the old yellow enzyme. So it seems possible that the complex formed by flavins with the proteins, via their tryptophan, has a major biological importance. This assumption is supported by the fact that various tissues contain strongly bound flavins in high concentration. The authors are impressed by the very great quantity of strongly bound flavin in the liver. In fact, the brown color of this organ seems to be due to the flavin radical formed in the charge transfer with the protein. The strong brown color of the liver suggests that a considerable part of the structural proteins is present as a flavin complex, containing the flavin in a free radical condition. It also follows that the tryptophan or protein, complexing with the riboflavin and donating an electron to it, is also present as a free radical.

The charge transfer between protein and riboflavin might have various biological consequences. So, for instance, if the electron, donated by the protein to the flavin, is drawn from an energy band,<sup>13</sup> the resulting hole might make the band conductant. This change would be accentuated if the flavins passed the electron thus accepted to the oxidation system. If the electrons thus lost by the protein were replaced by electrons given off by the dehydrogenated metabolites, DPN or TPN, this would mean that the electron transport goes *through* the protein molecule itself and does not take place merely on the surface of the protein, as hitherto believed.

In any case, if complex formation between tryptophan and flavin plays a major biological role, then one would expect that substances which form a similar complex with flavins and have a relatively high affinity to it interfere with the normal course of reactions and thus show a definite pharmacological effect. It is interesting to note that serotonin has a high affinity for riboflavin, and so has lysergic acid and bufotenine. These substances, which form similar complexes, have been implicated in normal and pathological mental activity. Possibly the described reactions might give an explanation of their mechanism of action on the molecular level. In this connection it is interesting that the other drugs listed in Table 1 have also been implicated in the problems of the activity of the central nervous system.

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