vitamin was similar, although values per g of whole liver were lower than in previous experiments. (This reduction was expected since the retinoic acid fed is not stored (6) and the only vitamin A found was attributed to the vitamin which had been injected.)

In the light of these findings the results of Popper and Greenberg (1) must be re-evaluated. There are several reasons why their histological observations might lead to erroneous conclusions with regard to the quantitative distribution of vitamin A. First, Kupffer cells are so much smaller than hepatocytes that even though they are a large fraction of the liver cell population they represent only a tiny fraction of the liver volume (9) and liver protein content (2). Thus, while the concentration of the vitamin in Kupffer cells may be similar to or even greater than that in the cytoplasm of hepatocytes and may appear so under the microscope, its presence in the Kupffer cell cytoplasm is quantitatively unimportant. The bias of light microscopic work toward the detection of concentrated rather than diffuse substances is a second problem, since it is a particular characteristic of Kupffer cells and other macrophages to hold substances in concentrated form (2, 11). Indeed, Popper and Greenberg observed vitamin A fluorescence primarily in lipid droplets (1) while Shorrman (12) had just shown with autoradiography that vitamin A label has a diffuse distribution in the cytoplasm of hepatocytes. Finally, we cannot exclude the possibility that the Kupffer cells are the initial recipients of vitamin A, subsequently transferring it to the hepatocytes. But if so, their transferring capacity must be great, since only 1 hour after vitamin A administration we found that it had been transferred, and, when large doses of it were ingested over 1 week, the vitamin did not accumulate in the Kupffer cells. Indeed, the concentration in Kupffer cells increased only 7-fold whereas that in hepatocytes increased 50-fold.

Our procedures produced Kupffer cells of normal morphology (3) which do not leak (2), and vitamin A was not destroyed in the process. Our results thus lead to the conclusion that, although vitamin A may be found in both Kupffer and parenchymal liver cells, quantitatively almost all of it is in the hepatocytes and only a very small portion is in the Kupffer cell population.

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REFERENCES
1. Popper, H., and Greenberg, R., Arch. Pathol., 32, 11 (1941).
2. van Wyk, C. F., Linder-Horowitz, M., and Munro, H. N., J. Biol. Chem., 246, 1029 (1971).
3. Mills, D. M., and Zucker-Franklin, D., Amer. J. Pathol., 54, 300 (1969).
4. Wolfe, G., Lane, M. D., and Johnson, B. C., J. Biol. Chem., 225, 965 (1957).
5. Nisonoff, A., and Frieden, W. N., J. Natl. Cancer Inst., 79, 454 (1966).
6. Daugve, R., and Cantinho, A., Cancer Res., 19, 757 (1959).
7. Moore, T., Vitamin A, American Elsevier Publishing Company, New York, 1957, Chapter 18.
8. Arrighi, M., and Harsh, R. D., Proc. Roy. Soc. Ser. B Biol. Sci., 138, 544 (1951).
9. Weirich, F. R., Spather, W., Gnaik, H. R., and Hess, F., J. Cell Biol., 42, 68 (1969).
10. Akins, J. F., and Van Dorn, D. A., Nature, 158, 622 (1946).
11. Bloom, W., and Fawcett, D. W. (Editors), A textbook of histology, W. B. Saunders Company, Philadelphia, 1968, p. 588.
12. Sherman, B. S., Int. J. Vitamin Res., 39, 111 (1969).

New Spectral Species of L-Lysine Monoxygenase, a Flavoprotein

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SUMMARY

Several new spectral species of the prosthetic flavin were found during reactions of L-lysine monoxygenase. Immediately after the addition of lysine to the enzyme, the absorption of the enzyme-bound FAD increased slightly around 375 to 385 nm either in the presence of oxygen or in its absence. During anaerobic reduction of FAD with lysine, a short lived broad absorption band appeared in the long wave length region centered around 550 nm. In contrast, under aerobic conditions, another new spectral species appeared in the steady state of reaction, which, unlike the oxidized or reduced form of the enzyme, exhibited a long wave length absorption centering around 575 nm. This species was distinct from that observed in the anaerobic reaction and was also characterized by an absorption peak at about 455 nm, shifted from 460 nm. The presence of both oxygen and lysine was required for the appearance of this spectral species.

L-Lysine monoxygenase, a flavoprotein, catalyzes the incorporation of 1 atom of molecular oxygen into lysine to produce δ-amino-δ-valeramide and carbon dioxide (1, 2). Under anaerobic conditions full reduction of the enzyme-bound FAD with lysine resulted in the formation of α-keto acid in an amount comparable to FAD reduction (3). Studies on the spectral change of the prosthetic flavin were undertaken in order to obtain better understanding of the mechanism of the monooxygenation reaction catalyzed by this enzyme. In the present communication, we describe results of spectrophotometric studies to demonstrate several new spectral species participating in anaerobic and aerobic reactions.

Preparation and assay of the crystalline L-lysine monooxygenase were carried out as described previously (1). Since 1 mole of enzyme contains 2 moles of FAD with absorption maxima at 385 and 460 nm, the concentration of enzyme was expressed in terms of the FAD content using the molar extinction coefficient of 11,300 M⁻¹ cm⁻¹ at 460 nm (1). The reaction mixture was made anaerobic by bubbling argon gas through it. Spectra of the enzyme were recorded with a Shimadzu recording spectrophotometer MPS-50L. Fast reactions of the enzyme were followed with a Yanaco stopped flow spectrophotometer SFS-1.

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and a Hitachi rapid scan spectrophotometer RSP-2. Rate constants were calculated from plots from tracings of stopped flow experiments using Guggenheim's method (4), which is applicable to a first order reaction even when the concentrations of reactants are not known at infinite time.

Under anaerobic conditions the enzyme was reacted with lysine, and the spectral change of the enzyme-bound FAD was followed by the stopped flow apparatus with wave length varied. When the flow stopped, a spectrum was observed which was almost identical with that of the oxidized enzyme except for a slight but reproducible increase in the absorption around 375 to 385 nm (Fig. 1, O--O). Subsequently, as the absorption at both 385 and 460 nm diminished, a broad absorption band around 550 nm developed transiently. An identical spectrum was also obtained with a rapid scanning spectrophotometer. In order to elucidate the kinetic nature of this species, the absorption change was followed at 460 and 550 nm, respectively, by the stopped flow method. Semilogarithmic plots of absorption change at 460 nm indicated that at least three phases of pseudo-first order reaction were involved. In the initial phase of the reaction the absorption at 460 nm decreased very rapidly (Fig. 1, X---X) and then slowly, followed by an extremely slow process, until the enzyme was fully reduced. Concomitant with the initial drop of the 460 nm absorption peak, the absorption at 550 nm increased rapidly (Fig. 1, X---X) and, after reaching a maximum (Fig. 1, O---O), disappeared gradually. As shown in Table 1, rate constant for the initial rapid absorption changes at both 460 and 550 nm increased in a parallel fashion as lysine concentration was raised, indicating the formation of a single intermediate which alone could account for the changes of the two absorption bands. These values were not lower than the turnover number of the over-all aerobic reaction (23 sec⁻¹ per mole of FAD in the presence of air at 24°). The full reduction following the initial rapid phase involved an extremely slow step, whose rate constant was not dependent on lysine concentration over the range studied.

In the aerobic reaction of the enzyme with lysine, stopped flow studies also revealed a slight increase of absorption around 375 to 385 nm at the very early stage of reaction, which was similar to, if not identical with, that observed in the anaerobic reaction. Furthermore, another new spectral species was found in the steady state of the aerobic reaction. The incubation of the enzyme with lysine was carried out in a wide cuvette by the method of Yamashita and Yokota (5) so that the continuous supply of oxygen by bubbling did not interfere with optical measurements. As shown with a dotted line in Fig. 2, a spectrum, clearly distinguishable from that of the oxidized or reduced enzyme or the mixture of both, was observed exhibiting an absorption peak around 455 nm, shifted by approximately 5 nm from 400 nm, and a broad absorption band centered around 575 nm. The presence of both lysine and oxygen was required for the appearance of this spectrum, which was converted to the spectrum of the oxidized enzyme upon the exhaustion of lysine.

**TABLE I**

| Oxygen | Lysine | Pseudo-first-order rate constant |
|--------|--------|--------------------------------|
| %      | mM     | Minus OD₅₀₀ Plus OD₅₀₀ |
| 0      | 1      | 1.2                      |
| 0      | 10     | 20                       |
| 0      | 50     | 50                       |
| 10     | 1      | 13.8                     |
| 10     | 10     | 30.1                     |
| 25     | 1      | 20.2                     |
| 25     | 10     | 35.8                     |
| 50     | 1      | 39.0                     |
| 50     | 10     | 58.0                     |

**Fig. 1.** Spectral changes of L-lysine monooxygenase upon anaerobic incubation with lysine. The reaction mixture contained 0.1 mM glycine-KOH buffer (pH 9.5), 0.1 M KCl, 12 μM enzyme (as FAD), and 10 mM lysine. O--O represents oxidized enzyme, and - - -, reduced enzyme; both of these spectra were recorded with a MPS-50L model spectrophotometer. The remaining three curves were obtained by measuring the absorption change at each wave length in a stopped flow apparatus, and recording the difference spectra against the fully reduced enzyme at each reaction time after the flow stopped. The plotted spectra are not difference spectra, and were reconstructed from these stopped flow data and the spectrum of the reduced enzyme: •---• 0 sec; X---X, 0.1 sec; O---O, 1.1 sec.

**Fig. 2.** Absorption spectrum of L-lysine monooxygenase in the steady state of the aerobic reaction. The reaction mixture contained 0.1 mM glycine-KOH buffer (pH 9.5), 16 μM enzyme (as FAD), and 1 mM lysine in a 2-cm wide cuvette with 1-cm light path. ---, oxidized enzyme; ---, reduced enzyme; ••••, the spectrum in the steady state, in which case a gas mixture of oxygen and nitrogen (80:20) was bubbled through it continuously. Since the spectrum was present only for about 10 sec at 24° under the conditions described, the spectrum presented was composed from spectra which were recorded in four separate experiments conducted under identical conditions. Each spectrum was scanned in the range between two adjacent arrows.
An identical spectrum was obtained by the stopped flow method. The rate constant for the formation of the species with such a spectrum was determined by following initial changes of absorption at 460 and 550 nm, and was shown to be dependent on the concentration of lysine and oxygen (Table I). These values listed are well above the turnover number of the overall aerobic reaction. When the enzyme was reduced completely under anaerobic conditions and then air was admitted to the cuvette, a spectrum similar to that observed in the steady state appeared rapidly. The rate of its appearance was affected by the concentration of oxygen, but not by lysine concentration.

Spectrophotometric studies of L-lysine monooxygenase, especially by rapid reaction spectrophotometry, revealed several characteristic spectral species of the enzyme-bound FAD, some of which have never been reported with other flavoprotein oxygenases. A slight increase of absorption around 375 to 385 nm was observed immediately after the addition of lysine to the enzyme. This spectral change caused by lysine was observed either in the presence of oxygen or in its absence, and, therefore, could be assumed to reflect the interaction of lysine and the oxidized form of enzyme. A short lived species with a long wave length absorption band then appeared in the anaerobic reaction. It is not conclusive at present whether this species also participates in the aerobic reaction although the observed rate of its formation could account for its involvement in the aerobic reaction. With lactic acid oxidative decarboxylase, a similar spectrum for enzyme-bound FMN was observed earlier by Beinert and Sands (6) and reinvestigated in detail recently by Takemori et al. (7).

When oxygen was present and the aerobic reaction was actively proceeding, a broad absorption band was also observed in the long wave length region. It was clearly distinct from the anaerobic transient species with a broad long wave length absorption since the absorption peak was shifted from 460 nm to around 455 nm in the former case. The appearance of such a species in the steady state of aerobic reaction was in contrast to the case of lactic acid oxidative decarboxylase which was reported to show a spectrum identical with that of the oxidized enzyme (7).

The formation of a ternary complex of enzyme, substrate, and oxygen as an obligatory intermediate has recently been reported with several oxygenases (8-11). These oxygenases show a characteristic spectrum in the steady state of reaction depending on the presence of both substrate and oxygen. It remains to be seen whether the characteristic spectrum observed in the aerobic reaction is related to the formation of a ternary complex of enzyme, lysine, and oxygen.

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REFERENCES

1. TAKEDA, H., AND HAYAISHI, O., J. Biol. Chem., 241, 2733 (1966).
2. TAKEDA, H., YAMAMOTO, S., KONDA, Y., AND HAYAISHI, O., J. Biol. Chem., 244, 2935 (1969).
3. YAMAMOTO, S., MAKI, Y., NARAZAWA, T., KAJITA, Y., TAKEDA, H., NOZAKI, M., AND HAYAISHI, O., in R. E. COOKE (ED.), Advances in chemistry, Series 77, American Chemical Society, Washington, D. C., 1968, p. 177.
4. GUGGENHEIM, E. A., Phil. Mag., 2, 538 (1926).
5. YAMAZAKI, I., AND YOKOTA, K., Biochem. Biophys. Acta, 132, 310 (1967).
6. BEINERT, H., AND SANDS, R. H., in M. S. BLOIS, JR., H. W. BROWN, R. M. LEMMON, R. O. LINDHOLM, AND M. WEISSBLUTH (EDS.), Free radicals in biological systems, Academic Press, New York, 1961, p. 35.
7. TAKEMORI, S., NAKAI, Y., KATAGIRI, M., AND NAKAMURA, T., Fed. Eur. Biochem. Soc. Lett., 3, 214 (1969).
8. ISHIMURA, Y., NOZAKI, M., HAYAISHI, O., TAMURA, M., AND YAMAZAKI, I., J. Biol. Chem., 243, 2974 (1968).
9. ISHIMURA, Y., UNILICH, V., AND PETERSON, J. A., Biochem. Biophys. Res. Commun., 42, 140 (1971).
10. ESTABROOK, R. W., HILGERANDT, A. G., BARRON, J., NETTER, K. J., AND LEIBERMAN, R., Biochem. Biophys. Res. Commun., 42, 132 (1971).
11. FUJISAWA, H., HIROMI, K., UYEDA, M., NOZAKI, M., AND HAYAISHI, O., J. Biol. Chem., 246, 2520 (1971).
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