Energy Transfer in Protein Pyridoxamine-5-phosphate Conjugates*

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ABSTRACT: The fluorescence spectra, quantum yield, and polarization of fluorescence of several protein-pyridoxamine-5-phosphate conjugates are reported. Energy transfer from the tryptophan residues to the pyridoxamine-5-phosphate chromophores was studied in human serum albumin, carboxypeptidase, and lysozyme, and the results are in good agreement with the predictions of Forster's theory of radiationless energy transfer.

The fluorescence as well as the energy-transfer properties of glutamic-oxaloacetic transaminase are also discussed.

Previous studies on protein–5-dimethylamino-1-naphthalenesulfonyl chloride conjugates (Shore and Pardee, 1956; Stryer, 1959; Weber and Teale, 1959; Slayter and Hall, 1964) have indicated that migration of electronic energy from the aromatic residues to dye molecules can be explained by the long-range transfer mechanism as proposed by Forster's theory (Forster, 1948; Duysens, 1964). It is the purpose of the present paper to investigate the energy-transfer properties of several protein-PMP[1] conjugates and to discuss the mechanism involved as well as the various parameters influencing the efficiency of the energy-transfer process. Evidence supporting the predominant role of Forster's mechanism is also presented.

Experimental

Methods. Fluorescence emission spectra were recorded in an automatic recording spectrofluorometer (Weber and Young, 1964). The quantum yield of fluorescence of the proteins was measured by comparing the area of their fluorescence spectra to that of a solution of tryptophan in 0.05 M potassium phosphate buffer, *p*H 7.2, made up to an equal OD = 0.1 at the exciting wavelength (280 mμ). The quantum yield of tryptophan is 0.20 (Teale and Weber, 1957).

PMP quantum yield was determined in the same way using 1-dimethylaminonaphthalene-5-sulfonate as standard (Weber and Teale, 1957). For energy-transfer experiments, the optical densities of the protein solutions were always of the order of 0.6 (1 cm quartz cuvet) at the exciting light (280 mμ). The probable effect of absorption and reemission of fluorescence by the acceptor, "trivial process," was taken into account by repeating the fluorescence measurements in 0.2-cm quartz cuvets. It was found that under our experimental conditions the "trivial process" made a negligible contribution to the sensitized fluorescence. Polarization of fluorescence measurements were conducted in a double-beam null-point polarization photometer (Weber, 1956) equipped with a mercury lamp as light source. The exciting beam was passed through a combination of Corning CS-7.54 and Baird Atomic filters to select light of 313 mμ. A Corning CS-5-61 filter was used as fluorescent filter. Temperature was controlled by circulating water from a Haake ultrathermostat through a hollow copper block which surrounded the sample cuvet.

Materials. Pyridoxal-5-phosphate (Sigma), human serum albumin (Pentex), carboxypeptidase (Pentex), lysozyme (Sigma), and polylysine (Yeda) were used without further purification. The sample of glutamic-oxaloacetic transaminase (pig heart), purchased from Calbiochem (lot 46058), was purified by hydroxylapatite chromatography as described by Jenkins *et al.* (1959). The purified enzyme shows a sedimentation constant of $s_{20} = 5.4$ S when analyzed in the Spinco analytical ultracentrifuge at 56,100 rpm.

The protein-PMP conjugates were prepared by the following procedure: Protein (50 mg) dissolved in 20 ml of 0.05 M phosphate buffer, *p*H 7.5, was allowed to react with 5 mg of pyridoxal-5-phosphate at 37° for 30 minutes. Then the solution cooled at 4° was treated with 5 mg of $NaBH_4$ for 10 minutes. The sample was acidified to *p*H 5 with acetic acid in order to eliminate excess of $NaBH_4$. The protein conjugate was dialyzed at 5° against several changes of 0.05 M phosphate buffer, *p*H 7.5. Treatment of an aliquot of the solution with 10% trichloroacetic acid, followed by centrifugation, indicated no detectable release of PMP in the supernatant. The extents of labeling of the PMP conjugates were determined from the absorbancy at 325 mμ. The molar extinction coefficient at this wavelength is 8.300 cm^2/mmole. Protein concentrations were determined from the ultraviolet absorbancy at 280 mμ. The extinction coefficients of lysozyme, carboxypep-

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[1] Abbreviation used in this work: PMP, pyridoxamine-5-phosphate.

TABLE I: Fluorescence Properties of Protein-PMP Conjugates.

Sample	QPMP[a]	λF (mμ)[b]	λA (mμ)[c]	P[d]	t (°C)
PMP	0.55	392	327	0.06	19
PMP (90 glycerol)	0.54	392	327	0.37	20
Human serum albumin–2 PMP	0.54	392	327	0.14	20
Lysozyme–2 PMP	0.54	392	327	0.13	20
Carboxypeptidase–2 PMP	0.54	392	327	0.14	20
Reduced glutamic-oxaloacetic transaminase–2 PMP	0.03	393	330	0.36	20
Reduced-glutamic-oxaloacetic transaminase–2 PMP[e]	0.20	393	330	0.13	21
Reduced glutamic-oxaloacetic transaminase–6 PMP	0.53	392	327	0.13	20
Polylysine–2 PMP	0.53	392	327	0.12	20
Polylysine–50 PMP	0.53	392	327	0.11	20

[a] QPMP, quantum yield of PMP fluorescence. Excited at 330 mμ. [b] λF, fluorescence maximum. [c] λA, absorption maximum. [d] P, polarization of fluorescence. [e] Enzyme heated at 50° for 2 minutes in the presence of 8 M urea.

tidase, and human serum albumin correspond to values published in the literature (Sophianopoulos *et al.*, 1962; Simpson *et al.*, 1963; Weber and Young, 1964). Glutamic-oxaloacetic transaminase apoenzyme was prepared by a method similar to that described by Scardi *et al.* (1963), and protein concentration was determined by Lowry's method (Lowry *et al.*, 1951). Reduced glutamic-oxaloacetic transaminase enzyme was prepared according to Hughes *et al.*, 1962. The preparation exhibits an absorption band at 330 mμ corresponding to the PMP derivative.

Results

The absorption and fluorescence spectra of the conjugates were examined as outlined under Methods, and Table I includes the pertinent results. All the conjugates exhibit an absorption maximum at 325 mμ, and their fluorescence spectra are also similar in profile and position of band maxima (λ_{max} = 392 mμ). Interestingly, the $NaBH_4$-reduced enzyme (glutamic-oxaloacetic transaminase) is characterized by a low fluorescence yield (0.03) which differs from the rest of the conjugates (0.54).

This abnormal fluorescence yield of the PMP molecules bound to the enzyme appears to be owing to specific interactions with amino acids residues of the protein moiety, since unfolding of the protein configuration by 8 M urea (Fasella and Hammes, 1964) brings about a concomitant enhancement of PMP fluorescence yield. Further evidence supporting the existence of "quenched PMP groups" in the enzyme appeared in the polarization of fluorescence experiments, as discussed in succeeding paragraphs.

Polarization of Fluorescence. As illustrated in Table I, most of the conjugates exhibit similar polarization values (0.13–0.14). In marked contrast, the polarization of the reduced glutamic-oxaloacetic transaminase enzyme approaches the limiting polarization (p_o = 0.41) of free PMP and remains constant over the temperature range 5–35°.

A behavior of this kind indicates either that τ, the actual lifetime of the excited state, has decreased, or that the Brownian rotation of the fluorescent molecule is influenced by strong interactions with the enzyme. Since the actual lifetime of the excited state, τ, is proportional to the quantum yield, q,

$$\tau = q\tau_o \tag{1}$$

(τ_o is the natural lifetime of the excited state) it is clear that a decrease of q results in a shorter lifetime of the excited state. For the two PMP molecules bound to the glutamic-oxaloacetic transaminase enzyme, q = 0.03 and $\tau_o = 8 \times 10^{-8}$ second (Churchich; 1964); the actual lifetime is $\tau < 10^{-9}$ second. In addition, if internal degrees of rotational freedom are absent, the observed rotational relaxation time corresponds to the whole macromolecule (Weber, 1952; Churchich, 1962). It follows, therefore, that for a protein of 110,000 mw (Jenkins *et al.*, 1959), the relaxation time ρ_h should be of the order of 2×10^{-7} second (Churchich, 1961). From Perrin's equation (equation 2), which relates p to the parameters τ and ρ_h, it becomes evident that for $\tau < 10^{-9}$, which is considerably smaller than $\rho_h = 2 \times 10^{-7}$ second, the observed polarization (P = 0.36) tends to the limiting polarization of fluorescence (P_o = 0.41).

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right) \tag{2}$$

TABLE II: Energy-Transfer Properties of Protein-PMP Conjugates.[a]

Sample	R_0 (A)	T	PMP	Q_t	Q_p	Q_p*	Q_A	E
Human serum albumin	24	1	3	0.38	0.074	0.006	0.037	50
Carboxypeptidase	20	6	2.5	0.22	0.12	0.07	0.020	40
Lysozyme	16	6	2	0.065	0.06	0.05	0.003	30
Reduced glutamic-oxaloacetic transaminase enzyme	18	28	2	0.12	0.09	0.09	0	0
Reduced glutamic-oxaloacetic transaminase enzyme	18	28	6	0.12	0.09	0.05	0.020	50

[a] R_0, critical transfer distance; T, number of tryptophan residues per mole of protein; PMP, number of PMP residues per mole of protein; Q_t, quantum yield of tryptophan residues; Q_p, quantum yield of protein fluorescence excited at 280 mμ; Q_p*, quantum yield of protein PMP conjugates excited at 280 mμ; Q_A, quantum yield of sensitized fluorescence excited at 280 mμ; E, efficiency of transfer defined by equation $E = (Q_A/Q_p - Q_p^*) \times 100$.

Denaturation of the enzyme by 8 M urea results in a remarkable change of both q and p (Table I). Two factors to consider which may account for this change are: (1) the enhancement of fluorescence yield which influences τ and (2) the loss of internal rigidity of the protein molecule in the presence of the denaturing agent (Steiner and Edelhoch, 1962). The two effects are not easily distinguished experimentally and it is likely that both are responsible for the abrupt change of polarization of fluorescence. These experimental results, along with the fluorescence-yield measurements, led to the conclusion that 8 M urea disrupts the protein moieties which provide the specific quenching environment of the PMP residues bound to the reduced glutamic-oxaloacetic transaminase enzyme.

Energy Transfer between Unlike Molecules in Protein Conjugates. The theory of energy transfer between unlike molecules owing to resonance interaction has been formulated by Forster (1948) in a manner that is amenable to experimental observation. A necessary condition for this mechanism to be operative is extensive overlap between the emission band of the donor (sensitizer) and the absorption spectra of the acceptor ($J_{\bar{\nu}}$). This requirement is met by the tryptophan PMP pair, since the overlap integral $J_{\bar{\nu}} = 9.8 \times 10^{10}$ cm³/mmole² indicates the possibility of energy exchange (Figure 1).

According to Forster's theory the strength of the interaction is conveniently expressed in terms of R_0, the distance of separation between the donor-acceptor pair for which the probability of dipole-dipole energy exchange is 50%. This critical transfer distance, R_0, is related to several parameters by equation (3).

$$R_0^6 = \frac{9 \times 10^6 \times (\ln 10)^2 \times k^2 \times c \times \tau_s \times J_{\bar{\nu}}}{16\pi^4 n^2 N^2 \bar{\nu}_{0,0}^2} = \frac{1.69 \times 10^{-33} \times \tau_s \times J_{\bar{\nu}}}{n^2 \times \bar{\nu}_{0,0}^2} \quad (3)$$

where n = refractive index, τ_s = the actual mean lifetime of the energy donor (tryptophan), c = velocity of light, $\bar{\nu}_{0,0}$ = the 0,0 wave number for the emission of the donor, k^2 = orientation factor, N = Avogadro's number, $J_{\bar{\nu}} = \int \epsilon_a^A(\bar{\nu}) \cdot \epsilon_e^D(\bar{\nu}) d\bar{\nu}, \epsilon_a^A(\bar{\nu})$ represents the molar extinction coefficient of the acceptor, and $\epsilon_e^D(\bar{\nu})$ the intensity of the emission of the donor measured in the same units as the extinction coefficient.

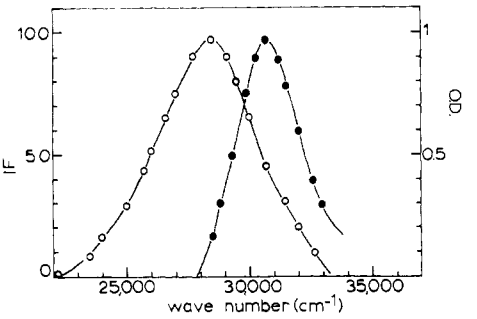

FIGURE 1: Degree of overlap between the emission spectra of tryptophan (○) (excited at 280 mμ) and the absorption spectra of pyridoxamine-5-phosphate (●). The abbreviation I.F. = intensity of fluorescence.

The critical distances corresponding to the protein conjugates were determined as follows. The integral $J_{\bar{\nu}}$ as well as $\bar{\nu}_{0,0}$ were obtained from spectral data. The refractive index was taken as the refractive index of water (n = 1.33). Lifetime of the excited state was calculated by means of equation (1), where $\tau_0 = 2 \times 10^{-9}$ second (Karreman *et al.*, 1958) and q corresponds to values reported in the literature (Teale, 1960). The values of R_0 (Table II) for the different conjugates indicate that the distances of separation between an acceptor-donor pair are compatible with the protein dimensions. On the other hand, the experimental results show (Table II) that migration of excitation energy occurs in human serum albumin, lysozyme, and carboxypeptidase, and in every case it is easily detected by measuring either Q_p* (quantum yield of protein fluorescence) or Q_a (quantum yield of sensitized

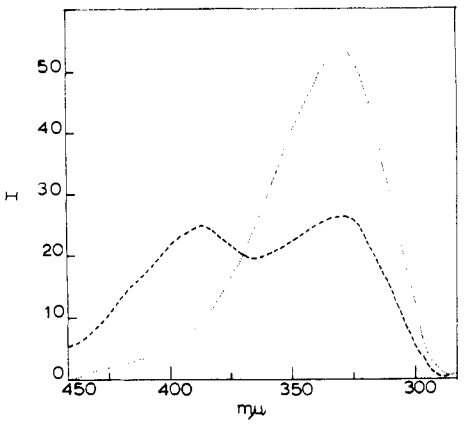

FIGURE 2: Emission spectra of glutamic-oxaloacetic transaminase–2 PMP (2 moles of PMP per 110,000 mw) (· · · · ·) and glutamic-oxaloacetic transaminase–6 PMP (6 moles of PMP per 110,000 mw) (– – – –) in 0.05 M phosphate buffer (*p*H 7.2). Both species have the same OD = 0.5 (1-cm cuvet) at the exciting wavelength (280 mμ).

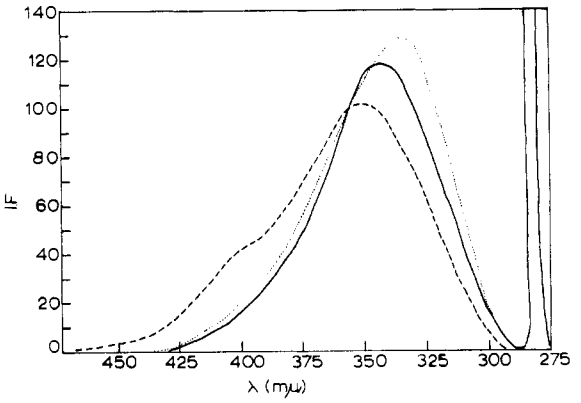

FIGURE 3: Emission spectra of glutamic-oxaloacetic transaminase–2 PMP (· · · · ·) in 0.05 M phosphate buffer (*p*H 7.2), denatured glutamic-oxaloacetic transaminase epoenzyme (———) in 8 M urea, and denatured glutamic-oxaloacetic transaminase–2 PMP (– – – –) in 8 M urea. The wavelength of exciting light was 280 mμ. Abbreviation as in Figure 1.

fluorescence). The general trend of the experimental data indicates a correlation between Q_t (quantum yield of the emitting chromophores) and the efficiency of the energy transfer process. Thus the tryptophan residues of both human serum albumin and carboxypeptidase behave as good donors, since the fluorescence yields of both proteins (Q_p*) are extensively quenched by few molecules of PMP (3 moles). Conversely, the lowest efficiency of transfer is found in lysozyme (Q_t = 0.065) where the addition of 2 moles of PMP per 14,000 mw results in a weak quenching of the protein fluorescence. An interesting feature of these results is the absence of

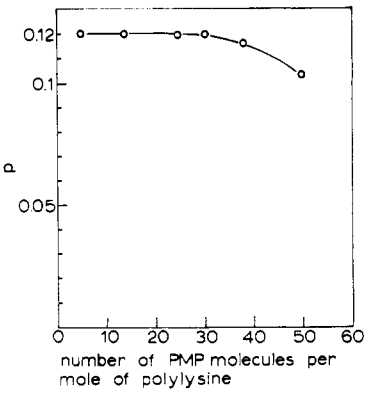

FIGURE 4: Plot of polarization of fluorescence versus number of PMP molecules per mole of polylysine (110,000 mw). Experiments were conducted in NaCl 0.1 M, 0.05 M phosphate buffer (*p*H 7.2) at a polylysine concentration of the order of 0.5 mg/ml.

sensitized fluorescence in the reduced glutamic-oxaloacetic transaminase enzyme. It is rather surprising that no migration of excitation energy could be detected even when the calculated value of R_0 = 18 A for a molecule containing twenty-eight tryptophan residues per 110,000 mw (Turano *et al.*, 1963) would predict the occurrence of the phenomenon. It is possible that this divergence from the theory resides in the assumption that the oscillators are characterized by a random directional distribution (K^2 = 2/3) which is the value of freely rotating molecules (Forster, 1959). It must be noted, however, that the energy-transfer rate constant is determined by the mutual orientation of the oscillators (Forster, 1960). Thus for molecules with fixed orientation ($K^2 < {}^2/_3$) the efficiency of transfer is reduced (Forster, 1960). This raises the possibility that the first two PMP molecules bound to the enzyme are less favorably oriented to interact with the tryptophan chromophores. In this connection, it is significant that further addition of PMP molecules to the glutamic-oxaloacetic transaminase enzyme results in the appearance of sensitized fluorescence (Table II, Figure 2). As shown in Figure 2, an emission band corresponding to the sensitized fluorescence, such as is observed in the artificial conjugates, appears at longer wavelengths when the protein is excited at 280 mμ, and, as expected, migration of electronic energy from the tryptophan chromophores to the PMP residues results in a decrease of Q_p. It is concluded, therefore, that the PMP molecules, which are characterized by q and p values similar to the artificial conjugates (Table I), are capable of quenching tryptophan fluorescence. A similar effect is observed when the reduced glutamic-oxaloacetic transaminase enzyme, containing 2 moles of PMP per 110,000 mw, is subjected to urea denaturation (Figure 3). This finding is consistent with the hypothesis that a drastic change in the molecular organization of glutamic-oxaloacetic transaminase caused by 8 M urea results in a rearrangement of the orientation of the

oscillators, which become aligned in directions more favorable for energy exchange.

Sensitized Fluorescence between Like Molecules. Closely related to these studies of energy exchange is the problem of sensitized fluorescence between like molecules bound to the protein. Since the occurrence of sensitized fluorescence is accompanied by a decrease of P, it is evident that the depolarization of fluorescence method is a powerful tool to detect radiationless transfer between like molecules (Feofilov, 1961). To investigate this problem, the following experimental approach was chosen. Polylysine conjugates of different PMP composition were prepared and their polarization values were measured in 0.1 M NaCl, 0.05 M phosphate buffer, *p*H 7.2, at 25°. As illustrated in Figure 4, the parameter P remains constant ($P = 0.12$) over the range 5–30 moles of PMP per mole of polylysine (110,000 mw). Further increase in the number of PMP molecules (one PMP molecule per ten lysine residues) results in a change of the polarization of fluorescence ($p = 0.11$) as a consequence of their accumulation along the polymer chain. Since the fluorescence quantum yield of the polylysine conjugates is independent of the PMP composition (Table I) this variation in polarization is attributable to radiationless energy transfer. To test this point further, the critical transfer distance $R_0 = 7$ A was calculated by substituting in equation (3) the numerical values

$$J_{\bar{\nu}} = 3 \times 10^7 \frac{\text{cm}^3}{\text{mmole}^2}$$

$$\tau = 5 \times 10^{-9} \text{ sec}$$

$$\bar{\nu}_{0,0} = 27.777 \text{ cm}^{-1}$$

corresponding to $J_{\bar{\nu}}$, τ, and $\bar{\nu}_{0,0}$, respectively. This result, together with the depolarization of fluorescence data, strongly indicates that energy exchange between PMP molecules should be operative if these molecules are in close contact (7 A apart). It is unlikely, therefore, that this type of exchange could be detected in protein conjugates containing few molecules of PMP, since their interaction distances are expected to be greater than 7 A.

Discussion

The energy-transfer experiments presented in this paper were analyzed in terms of energy exchange (Forster theory) between unlike molecules, which implies that migration of electronic energy via a single step from sensitizer to acceptor is the predominant process in protein-PMP conjugates. This assumption is fully supported by the observation that multistage transfer between sensitizers (tryptophan residues in proteins) plays a minor role in protein conjugates (Forster, 1960). The results of this investigation indicate that numerical calculations based on Forster's equation can predict sensitized fluorescence in most of the protein-PMP conjugates. A notable exception is the reduced glutamic-oxaloacetic transaminase enzyme, where the absence of sensitized fluorescence may be attributed to unfavorable orientation of the oscillators involved in energy exchange ($K^2 < {}^2/_3$). In addition it was shown that the first two PMP molecules which appear to be bound to the active site of the glutamic-oxaloacetic transaminase enzyme (Hughes *et al.*, 1962) are characterized by p and q values ($p = 0.36$, $q = 0.03$) completely different from the other conjugates. This finding lends support to the hypothesis that the two PMP molecules are interacting with amino acids residues of the polypeptide chains. Finally, the process of sensitized luminescence between PMP molecules covalently attached to a macromolecule (polylysine) was analyzed by two independent methods. The results demonstrate the adequacy of Forster's theory to analyze the phenomena of energy transfer.

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Isolation, Characterization, and Properties of Fusarinine, a δ-Hydroxamic Acid Derivative of Ornithine*

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ABSTRACT: Several previously undescribed hydroxamic acids have been isolated from *Fusarium* sp. and *Fusarium roseum* (ATCC 12822). Iron at concentrations as low as 4×10^{-7} M severely depresses the hydroxamate formation. The amount of hydroxamic acids reaches a maximum after 5–6 days of growth and then rapidly declines. All of the hydroxamates appear to contain δ-*N*-hydroxyornithine as the hydroxylamino moiety. No other amino acids were found. The compounds are all intensely ninhydrin positive. The most abundantly produced of these substances, fusarinine, has been characterized as δ-*N*-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-δ-*N*-hydroxy-L-ornithine. No growth factor or antibiotic properties could be demonstrated for this compound. The finding that *N*-hydroxyornithine-δ-hydroxamates can occur at the amino acid level suggests that such hydroxamates are precursors of the hydroxamate functions of the ferrichrome compounds.

Approximately two dozen hydroxamic acids of microbial origin have now been described, and there is increasing evidence that hydroxamic acids are common to many, if not most, microorganisms. Although the structural diversity of these compounds is enormous these substances all have a common feature: the hydroxylamino group participating in the hydroxamate linkage is always donated by an hydroxylamino acid or a close derivative thereof. The most commonly found hydroxylamino acid is δ-*N*-hydroxyornithine, $HONH(CH_2)_3CH(NH_2)COOH$. Although this amino acid derivative has not been found as such in nature, it is the fundamental subunit of albomycin (Turková *et al.*, 1962), the ferrichromes (Emery and Neilands, 1961), ferrichrysin, ferrirhodin, ferrirubin, and ferricrocin (Zähner *et al.*, 1963). In all of these compounds the α-amino and α-carboxyl groups of the ornithine residues are linked by amide bonds in a cyclic hexapeptide, and the hydroxylamino groups are acylated to yield the hydroxamic acids.

This paper describes the occurrence of a number of new hydroxamic acids synthesized by the fungus *Fusarium*. All of these compounds appear to contain δ-*N*-hydroxyornithine. One of these compounds, fusarinine, has been identified as δ-*N*-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-δ-*N*-hydroxy-L-ornithine and is thus the first ornithine hydroxamate described in which the amino and carboxyl groups of the ornithine moiety are not bound in peptide linkage. The significance of this finding to the problem of the biosynthesis of hydroxamic acids is discussed.

Experimental

Isolation and Purification of Hydroxamic Acids of Fusaria. Fusarium roseum (ATCC 12822) or *Fusarium* sp., isolated as a laboratory contaminant, was grown in 2-liter Fernbach flasks containing 500 ml of modified Grimm-Allen medium (Garibaldi and Neilands, 1955). The inoculum was 5–10 ml of a 48-hour culture of the organism grown on the same medium. The flasks were incubated at 26° on a reciprocal shaker with a 3.8-cm stroke at 80 strokes per minute. After 5–6 days the mycelia were removed by suction filtration and the clear yellowish filtrate was concentrated to dryness *in vacuo* at 40°. The residue was triturated several times with methanol. The combined methanol extracts

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