determined areas of the lysozyme three-dimensional network.

In order to elucidate the role performed by the aforesaid ground state complex, we carried out some irradiation experiments at 367 nm, where only uncomplexed hematoporphyrin is involved in the light absorption process, and at 435 nm, where the incident light is predominantly absorbed by the complex. The amino acid analyses of lysozyme after 30 min irradiation in aqueous solution are reported in the Table. Apparently, the 367 nm light brought about no change in the amino acid composition of the protein; moreover, the isolated product possessed enzymic activity, UV-absorption spectrum, and chromatographic behaviour on Amberlite CG-50 coincident with those of unirradiated lysozyme. On the other hand, when the 435 nm light was used, one Met residue was converted to the sulfoxide: the enzymic activity (52%), as well as the spectral and chromatographic parameters, measured for this sample were identical with those previously determined for Met-12 sulfoxide-lysozyme, prepared by irradiation of the hematoporphyrin-lysozyme system with white light[1-3].

Now, when free Met or the dipeptide Z-Met-Asp were exposed to 367 nm light in the presence of hematoporphyrin, a fast and quantitative conversion of the thioether function to the sulfoxide was achieved. Furthermore, on illumination of lysozyme plus hematoporphyrin at 367 nm in 30% acetic acid solution, both the Met residues were photooxidized. It is apparent that the 367 nm light can promote the transition of free hematoporphyrin to the excited state which is responsible for photoactivating molecular oxygen to attack the methionine sulfur. Therefore, the lack of photooxidation of Met-12 by free hematoporphyrin, when lysozyme retains its native conformation, is to be ascribed to the burial of this residue inside the protein molecule which prevents direct interaction between the Met side chain and the dye dissolved in the aqueous solvent. On the other hand, the fact that only light absorbed by complexed hematoporphyrin is effective in inducing the photooxidation of Met-12 can be easily interpreted if the region involving this amino acid is assumed to be one of the two binding sites of the dye. In this case, the protein conformation exerts no shielding to the contact between the Met side chain and the photooxidizing agent; moreover, the high local concentration of sensitizer should greatly enhance the efficiency of the photooxidative reaction, as it has been actually observed. The second molecule of hematoporphyrin bound per molecule of lysozyme appears to interact with a site (or, possibly, to be distributed among sites) not containing any of the amino acids photooxidizable under our conditions.

These findings point out that caution must be exercised in drawing conclusions about the degree of exposure of amino acid residues in proteins from photooxidative investigation, before the detailed mechanism of the photoreaction has been cleared up. Our results open interesting prospects in the field of the dye-sensitized photooxidation of proteins, since by a procedure similar to that employed in the present study, the photodynamic action of dyes can be restricted within selected areas of protein molecules[9]; this should allow one selectively to modify a limited number of amino acid residues and to obtain some information about the nature of the binding sites of dyes[10].

Amino acid analyses of 30 min irradiated lysozyme

Amino acid	Irradiation wavelength		
	367 nm (water, pH 5.9)	435 nm (water, pH 5.9)	367 nm (30% acetic acid)
Tryptophan	6.0	5.9	5.9
Histidine	0.8	0.9	0.8
Tyrosine	3.1	2.9	3.0
Methionine	2.1	1.0	0.0
Methionine sulfoxide	0.0	0.9	2.0

All the amino acids which are present in lysozyme were examined, but the table reports only those which are known to be susceptible of photooxidation[1]. No change was found in the content of the other amino acid residues.

Riassunto. L'ematoporfirina forma con il lisozima un complesso in rapporto molare 2:1. Uno dei siti di legame è rappresentato dalla regione della molecola proteica contenente la metionina-12. L'irradiamento con luce assorbita solo dall'ematoporfirina complessata consente, quindi, la fotoossidazione selettiva di tale amminoacido.

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On the Origin of D-Aminoacid Residues in Microbial Peptides

Peptides isolated from micro-organisms frequently contain residues of D-aminoacids. Several studies have shown that these residues are derived biogenetically from free L-aminoacids, the free D-enantiomers being generally poor precursors[1]. Accordingly, inversion must occur after the L-aminoacid has been converted to a derivative. Two possibilities may be envisaged. Firstly, derivatization of the carboxyl group to form an activated compound may be accompanied or followed by racemization or inversion, the resulting activated D-aminoacid then being used to synthesize the peptide. Alternatively, the L-aminoacid may be incorporated as an L-residue which later undergoes inversion. We here present evidence for operation of the latter mechanism during biosynthesis of analogues of the cyclotetradepsipeptide, angolide, by the fungus *Pithomyces sacchari* (Speg.) M. B. Ellis, IMI 120725.

The organism was grown in surface culture on a glucose-salts-peptone-agar medium containing L-valine (12.5 mg/ml). After incubation for 2 weeks at 25 °C the angolide fraction[2] was isolated, and its properties were compared with those of authentic angolide[3]. The results

(Table I) showed that although extensive incorporation of valine had occurred, the physical properties were very similar to those of the pure compound. We interpret this as indicating that the integrity, size and stereochemistry of the angolide macrocycle were retained, and therefore that the only alteration was the partial replacement of isoleucine residues by residues of valine with the same stereochemical configuration.

The ratio of *erythro*- to *threo*-isoleucine (Ile:*a*Ile) in an acid hydrolysate of the mixture was very close to unity. The simplest interpretation is that the mixture contained

cyclo-L-Hiv-L-Ile-L-Hiv-D-*a*Ile-
1 2 3 4

Angolide (Hiv = α-hydroxyisovaleric acid)

only 2 components, namely angolide and L-Val[2], D-Val[4]-angolide. The mixture was eluted from a silicic acid column by chloroform-ethyl acetate (9:1 v/v) as a single but trailing peak. The materials present in the peak and one tail fraction were submitted to aminoacid analysis. On a molar basis, valine accounted for 14.5% of the aminoacids in the former and 32.5% in the latter. The composition of the cyclodepsipeptides present in these 2 fractions was investigated by mass spectrometry.

The mass spectrometric fragmentation of angolide and its analogues has been studied in detail[4,5]. The molecular ion peak is small. The most prominent peak in the spectrum occurs at M-44, and is due to a radical-ion formed from the molecular ion by loss of CO_2 with charge retention on the larger fragment. Accordingly, if the 2 chromatographic fractions contained only angolide and L-Val[2], D-Val[4]-angolide, the mass spectra should show small peaks at *m/e* 426 and 398 due to the respective molecular ions, and major M-44 peaks at 382 and 354, the latter being stronger in the spectrum of the valine-rich fraction than in that of the valine-poor fraction. Table II shows the results which were actually observed. The spectra were consistent with the presence not of 2 homologues, but of 3 adjacent members of an homologous series. For 3 major peaks in the spectrum of the valine-rich fraction, homology with the corresponding angolide peaks was established by precise mass measurement, which was also used to confirm the presence of angolide itself.

Therefore *P. sacchari*, growing in the presence of exogenous L-valine, produces not only angolide and L-Val[2], D-Val[4]-angolide, but also one or both of the homologues in which only one isoleucine residue is replaced by valine. The relative intensities of the mass spectral peaks suggest that 'mono-valine angolide' constitutes at least one-quarter of the valine-poor fraction, and is thus a major metabolic product under these conditions.

The mass spectra can give no indication of the stereochemistry of the 'mono-valine angolide', but evidence

Table I. Properties of an angolide fraction containing valine residues

Property measured	Result observed
$[\alpha]_D^{22}$ (*c*, 0.32 in $CHCl_3$)	−80° (value for angolide, −83°)
Melting point	255–257° (value for angolide, 261–262°)
Infrared spectrum (Nujol, Infracord 137)	Indistinguishable from that of angolide except for small differences near 10 μm.
TLC (silica gel G in $CHCl_3$:EtOAc 4:1)	Not resolved from angolide, but some tailing.
Aminoacid analysis (μmoles released by acid hydrolysis of 426 μg)	*Erythro*-isoleucine 0.81 *Threo*-isoleucine 0.82 Valine 0.60
Hydroxyacid components of acid hydrolysate (by paper chromatography)	Only α-hydroxyisovaleric acid detected.

1 E. P. Abraham, G. G. F. Newton and S. C. Warren, in *Biogenesis of Antibiotic Substances* (Academic Press, New York 1965), p. 175.

2 P. Riches, A. Rothwell and D. W. Russell, J. gen. Microbiol. *46*, 77 (1967).

3 D. W. Russell, J. chem. Soc. *1965*, 4664.

4 N. S. Wulfson, V. A. Puchkov, V. N. Bokharev, B. V. Rozinov, A. M. Zyakoon, M. M. Shemyakin, Yu. A. Ovchinnikov, A. A. Kiryushkin, E. I. Vinogradova, M. Yu. Feigina and N. A. Aldonova, Tetrahedron Lett. *17*, 951 (1964).

5 C. G. MacDonald and J. S. Shannon, Tetrahedron Lett. *42*, 3113 (1964).

Table II. Mass spectral assignments for valine-poor (A) and valine-rich (B) angolide fractions

m/e	Relative intensity A	Relative intensity B	Assignment	Precise *m/e*	Formula
426	1.8	0.9	Homologous	426.2732 ± 0.0012	$C_{22}H_{38}N_2O_6$ [a]
412	1.2	1.6	molecular ion	–	–
398	0.6	0.9	peaks	–	–
382	100	56	Homologous	–	–
368	40	100	M-44	368.2671 ± 0.0011	$C_{20}H_{36}N_2O_4$
354	4.6	47	peaks	–	–
297	42	34	$[C_{16}H_{27}NO_4]^{+\cdot}$ [b]	–	–
283	16	44	and homologues	283.1783 ± 0.0008	$C_{15}H_{25}NO_4$
269	2.8	9.3		–	–
282	44	30	$[C_{15}H_{24}NO_4]^{+\cdot}$ [b]	–	–
268	10	44	and homologues	268.1557 ± 0.0008	$C_{14}H_{22}NO_4$
254	–	1.8		–	–
197	54	41	$[C_{11}H_{19}NO_2]^{+\cdot}$ [b]	–	–
183	20	57	and homologues	–	–
169	40	37		–	–

[a] Or $C_{23}H_{34}N_6O_2$, but this is excluded by the results given in Table 1. [b] See ref. No.[5].

can be adduced from the ratio of *erythro*- to *threo*-isoleucine. Preponderance of either L-Val[2]- or D-Val[4]-angolide must cause this ratio to depart from unity. Angolide fractions were isolated from *P. sacchari* grown in the presence of L-valine at 8 different concentrations in the range 2.5–20 µg/ml. In acid hydrolysates of these fractions the mean Ile:*a*Ile ratio was 1.03, with a range 0.97–1.18. Previous work with the 2 epimeric isoleucine derivatives produced by mild hydrazinolysis of angolide has shown that little if any epimerization of isoleucines occurs during acid hydrolysis[3]. We therefore conclude that the ratio of *erythro*- to *threo*-isoleucine residues in the angolide fractions is close to unity. It follows that exogenous L-valine promotes the biosynthesis of L-Val[2]- and D-Val[4]-angolide in equal amount.

This conclusion has interesting biochemical implications. Formation of the 2 stereoisomers in equal amount is plausibly explained only if both arise from a common precursor. This precursor must contain one residue of valine and one of isoleucine, with the added proviso that both residues are biochemically equivalent, so that which one undergoes inversion is entirely a matter of chance. This supposes a high degree of symmetry in the precursor molecule, and excludes from consideration any open-chain structure, which must necessarily contain 2 aminoacid residues in non-equivalent environments.

We therefore propose that biosynthesis of the 'monovaline angolides', and hence by implication of angolide itself, proceeds by way of an all-L-cyclotetradepsipeptide precursor, the single D-residue being introduced by random inversion of one or other of the two environmentally and stereochemically equivalent L-aminoacid residues. It seems to us likely that a similar mechanism may operate in the biosynthesis of other microbial peptides, such as sporidesmolide I, valinomycin, gramicidin S, and fungisporin, for which essentially symmetrical, all-L cyclic precursors may plausibly be formulated. These in turn may be special cases of a general mechanism, by which D-aminoacid residues in microbial peptides originate by inversion of previously incorporated L-residues. An enzyme-catalysed dehydrogenation/hydrogenation mechanism by which such inversion may occur has recently been proposed[6].

A detailed account of this work will be published elsewhere.

Zusammenfassung. Ein Gemisch von Angolid und seinen drei valinhaltigen Homologen aus L-valinhaltiger Kultur von *Pithomyces sacchari* enthielt gleiche Mengen zweier Isomere mit nur einem Valinrest, was wahrscheinlich macht, dass Angolid durch Umkehrung eines L-Rests aus einem zyklisch-symmetrischen Vorläufer entsteht.

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[6] B. N. BYCROFT, Nature, Lond. *224*, 595 (1969).

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Xanthine Dehydrogenase in the Biosynthesis of the Eye Pterin Pigments of *Drosophila melanogaster*

It has been established in previous studies[1,2] that the wild type of *Drosophila melanogaster*, grown on the KELLER and GLASSMAN[3] culture medium, with 4-hydroxypyrazole (3,4-D) pyrimidine (or HPP) at 0.08 g/100 ml concentration, originates phenocopies of the 'maroon-like' (MAL) mutant. It is known that this mutant lacks the xantine deydrogenase enzyme (XDH), and therefore in the males there is no isoxanthopterin in any measurable amount, while there is an increase of its precursor 2-amino-4-hydroxypterin[3]. Furthermore, the MAL mutant shows a remarkable decrease of the amount of the eye pigment drosopterin.

Similar experiments performed with HPP on the 'sepia' (SE) mutant[4] have shown the disappearance of the isoxanthopterin in the males, an increase of the level of 2-amino-4-hydroxypterin and a decrease of sepiapterin which is the eye pigment of this mutant. This decrease reaches a certain level that cannot be lowered, even if the HPP concentration in the culture medium is increased. The effects of the addition of HPP to the wild type and to the SE mutant are obviously similar. While the wild type treated with HPP originates the phenocopy of the MAL mutant, the SE mutant treated does not have a corresponding biochemical phenotype in any *Drosophila melanogaster* single mutant.

We have therefore tried to isolate a mutant strain of which the SE mutant treated with HPP is the phenocopy. By crossing the SE with the MAL mutant, it has been possible to isolate a double mutant strain which, when analyzed by spectrophotometry and chromatography, shows the same biochemical phenotype of the SE treated with HPP. In fact, because of the SE mutation, sepiapterin is the eye pigment while, because of the MAL mutation, there is no XDH enzyme and accordingly no isoxanthopterin. Since it is impossible to distinguish by examination between the SE and the double mutant,

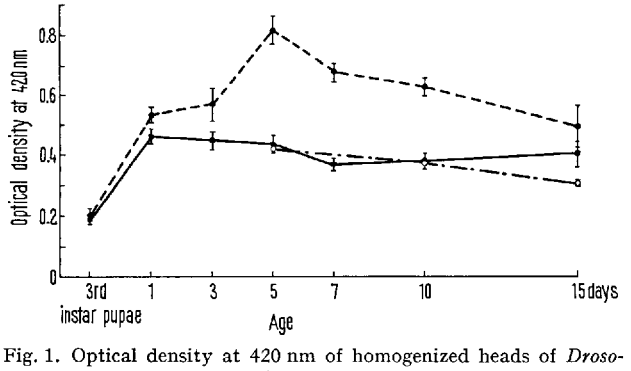

Fig. 1. Optical density at 420 nm of homogenized heads of *Drosophila melanogaster* males SE (----), MAL/SE (——) and SE treated with HPP (-·-·-). Means of 5 experiments for each different age. Vertical lines indicate S.E.

[1] P. BONI, B. DE LERMA and G. PARISI, Experientia *23*, 186 (1967).
[2] B. DE LERMA, P. BONI and G. PARISI, Boll. Soc. ital. Biol. Sper. *42*, 1764 (1966).
[3] E. C. KELLER and E. GLASSMAN, Nature, Lond. *208*, 202 (1965).
[4] P. BONI and G. PARISI, Experientia *23*, 1020 (1967).