lenelanosterol, and also the evidence for the presence of 4 α -methyl sterols containing a methylene group at C-24, is of considerable significance. The occurrence of such compounds in fungi can be postulated on biosynthetic grounds (Akhtar *et al.* 1966b; Barton *et al.* 1966; Goad, 1966), and we believe that trans-methylation does occur at the lanosterol level to produce 24-methylenelanosterol, which is subsequently modified by loss of the 4 α -, 4 β - and 14 α -methyl groups and double-bond rearrangements to give ergosterol.

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The Iodination of Super-reactive and Essential Tyrosine Residues in a Microbial Aminopeptidase

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Uhlig, Lehmann, Salmon, Jollès & Jollès (1965) isolated an aminopeptidase from micro-organisms that has mol.wt. 60 000. The present communication reports on an investigation of the role of tyrosine residues in the catalytic properties of this enzyme by the active-site labelling technique. It has been previously discovered that the aminopeptidase isolated from pig-kidney particles was inactivated when some of its tyrosine residues were iodinated (Wachsmuth, 1966b, 1967), and it was considered that the microbial aminopeptidase might behave similarly. This investigation seemed more worthwhile since the tyrosine residues of chymotrypsin do not appear to be important for the

catalysis (Filmer & Koshland, 1964) whereas those of carboxypeptidase A do appear to be involved (Roholt, Radzinski & Pressman, 1965).

It was shown that the enzymic activity of microbial aminopeptidase was decreased by incubation with iodine. At first therefore it was necessary to demonstrate whether the enzyme contains free SH groups, which could be oxidized by the iodine. For this purpose 3mg. of aminopeptidase was dissolved in 2ml. of 10M-urea in 0.6M-phosphate buffer, pH 7.2, and 0.05ml. of 15mM-5,5'-dithiobis-(p-nitrobenzoic acid) was added. The difference in E_{412} (Ellman, 1959) between this solution and control, containing no enzyme, was measured. The increase in extinction (0.004) corresponded to only 0.01 SH group/protein unit of mol.wt. 60 000. Thus

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the microbial aminopeptidase, like other aminopeptidases (Wachsmuth, 1966a), does not contain a significant amount of free SH groups.

To detect a relationship between enzymic activity and the incorporation of iodine into the protein, the enzyme was labelled with different amounts of ^{131}I . From 0.01 to 0.1 ml. of 10 mM- KI_3 in 67 mM-phosphate buffer, pH 7, was added to a solution of 1.6 mg. of aminopeptidase in 1 ml. of 0.5 M-glycine-NaOH buffer, pH 9.0 (17–170-fold excess of iodine over the enzyme, but the effective concentration of iodine after adding at pH 9.0 is considerably lower because of the disproportionation of iodine). Then 20 sec. later the iodination was terminated by the addition of 0.05 ml. of 0.1 N- $\text{Na}_2\text{S}_2\text{O}_3$. The KI_3 solution had been previously mixed with small amounts of carrier-free Na^{131}I . The reaction mixture was filtered through a column (1 cm. diam. $\times$ 25 cm. long) of Sephadex G-25 (fine grade) that had been equilibrated with 20 mM-phosphate buffer, pH 7.2. The protein was eluted in 5 ml. and was well separated from the excess of radioactive material. The enzymic activity of the protein eluate was measured with leucine *p*-nitroanilide as substrate (Wachsmuth, Fritze & Pfeleiderer, 1966) and the protein concentration with biuret reagent (Beisenherz *et al.* 1953) by using the conversion factor 17. The radioactivity in the protein was estimated in a methane flow-through counter and compared with that of a sample of K^{131}I_3 standard that had been treated with $\text{Na}_2\text{S}_2\text{O}_3$. A linear relationship was found between specific enzymic activity of the inhibited enzyme and the specific radioactivity, which extrapolates to an incorporation of 2.7 atoms of I/mol. of aminopeptidase at 100% inactivation (Fig. 1).

After this finding it was decided to investigate which amino acids had been labelled with ^{131}I , as these seem to be involved in the activity of the enzyme. To do this total hydrolysis of the ^{131}I -labelled enzyme was carried out. Two 0.16 mg. samples of iodoaminopeptidase (13% and 30%

inhibited) were digested with 1 mg. of Pronase (B grade; Calbiochem, Los Angeles, Calif., U.S.A.) in 1 ml. of 10 mM-tris-HCl buffer, pH 8.0, for 24 hr. at 37°. Samples of the hydrolysates were examined by electrophoresis at pH 1.9 for 1 hr. at 50 v/cm. and by chromatography (butan-2-ol-formic acid-water, 15:3:2, by vol.) on thin layers of Kieselgel-starch (Macherey, Nagel and Co., Düren, Germany). Both methods showed that the radioactivity was only associated with substances that had the same migration as di- and mono-iodotyrosine. About 32% of the total radioactivity was associated with di-iodotyrosine and 68% with monoiodotyrosine. If it is assumed that the moniodination of tyrosine is sufficient to destroy the catalytic properties of the protein, then it may be calculated, from the 2.7 atoms of I incorporated/mol., that only 2.25 tyrosine residues/mol. of aminopeptidase must be modified for total inhibition.

To find out how many peptides contain these labelled tyrosines, 'fingerprinting' of ^{131}I -labelled aminopeptidase was used. A 5 mg. sample of 20%-inhibited enzyme in 5 ml. of 10 mM- NH_4HCO_3 was heated at 100° for 3 min. and concentrated by rotary evaporation at 40°. Two samples of trypsin (0.1 mg.) were added to 0.3 ml. of denatured iodoaminopeptidase solution after 0 and 6 hr. incubation at room temperature. After 25 hr. samples of the tryptic hydrolysates were examined by two-dimensional thin-layer chromatography [first solvent chloroform-methanol-aq. ammonia (sp.gr. 0.88) (2:2:1, by vol.); second solvent butan-2-ol-formic acid-water (15:3:2, by vol.)] on thin layers of Kieselgel-starch. Only one radioactive spot was found (R_{F1} 0.68; R_{F2} 0.4), but it is not yet known whether this spot contains one unique peptide with one or two tyrosine residues or two peptides.

The only amino acids in proteins that are known to be modified by iodine are those of cysteine, tyrosine, methionine, tryptophan and histidine (Cha & Scheraga, 1963; Filmer & Koshland, 1964; Covelli & Wolff, 1966). This aminopeptidase does not contain significant amounts of cysteine. The total ^{131}I of iodinated microbial aminopeptidase was accounted for as mono- and di-iodotyrosine. The ratio of mono- to di-iodotyrosine found in the labelled protein indicates that moniodination is sufficient for inactivation. Histidine was not modified by iodination, but no direct information on the possible oxidative modification of methionine or tryptophan (or both) was obtained. However, since the iodination reaction was carried out for a short time and at pH 9.0, at the pH optimum of the enzyme, it is reasonable to assume that the observed inactivation is due directly to the iodination of two tyrosine residues/mol. of enzyme. Moreover, by 'fingerprint' analysis the total radioactivity incorporated into the enzyme was found to be

Fig. 1. Relationship between aminopeptidase enzymic activity and the incorporation of iodine. For conditions see the text.

associated with only one of the peptides produced by tryptic digestion of the inactivated enzyme protein.

These results suggest that the microbial aminopeptidase may be composed of two similar, if not identical, chains, each containing 1 super-reactive and essential tyrosine residue/protein unit of mol.wt. 30 000. A similar result was obtained with aminopeptidase from pig-kidney particles (Wachsmuth, 1967), which was found to contain 10 essential and super-reactive tyrosine residues/mol. (mol.wt. 280 000) or only 1 tyrosine residue/sub-unit. Additional experiments, involving in each case the isolation and characterization of the radioactive iodinated tyrosine peptide, are clearly necessary before the sub-unit structure of the aminopeptidases is further established.

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Polarographic Determination of Dipicolinic Acid in the Presence of Bacterial Spores and Vegetative Cells

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DPA* is a major and characteristic component of bacterial spores (Powell, 1953). The determination of DPA released from spores is important in studies of heat-resistance (Church & Halvorson, 1959) and germination (Levinson & Hyatt, 1966). Methods available for estimation of DPA involve separation of the spores from the assay system and are time-consuming (Janssen, Lund & Anderson, 1958).

DPA gives a cathodic polarographic wave in acid solution (Volke & Volkova, 1954). A polarographic procedure has therefore been devised that affords a rapid and sensitive method of determining DPA in the presence of nutrient broth, vegetative cells and spores.

Experimental procedure. A Davis differential cathode-ray polarograph (Southern Analytical A1660) was used with mercury-pool anodes. All determinations were made at 25° with 5 ml. samples

* Abbreviation: DPA, dipicolinic acid.

and each solution was flushed with N₂ (5 min.) before polarography. Peak current readings were corrected to maximum instrument sensitivity.

Method 1. Direct polarographic determinations were made with DPA solutions (100 ml.) containing 16 ml. of 0.2 M-citric acid and 4 ml. of M-Na₂HPO₄ at pH 3.3. The start potential was -1.0 v.

Method 2. DPA solutions (100 ml.) containing 5 N-HCl (10 ml.) and nutrient broth (50 ml.) were prepared and polarographed by using a start potential of -0.50 v and the 50 msec. derivative circuit.

The peak potentials occurred at about -1.3 v with method 1 and -0.8 v with method 2. Concentrations of DPA between 0.25 and 80 µg./ml. were used. Calibration curves (five points) for concentrations between 2 and 32 µg./ml. were linear with both methods (method 1: $r = 0.998$, $P > 0.999$; method 2: $r = 0.988$, $P > 0.999$). Each calibration point represented the mean of five