For DES and EMS treatments, the cells were suspended in *tris*-maleic buffer of pH 7.0 and treated up to a duration of 1 and 4 h respectively; 0.01 ml DES or 0.1 ml EMS was added per 5 ml cell suspension. Other procedural details and the culture conditions remained the same as mentioned above. Nitrogen contents were estimated after 20 and 30 days following treatment with DES and EMS respectively.

These alkylating agents were found to inhibit the process of fixation in both the clones, presumably by switching off of the activity of enzyme nitrogenase. The effect of EMS on fixation was more lethal in comparison to other agents as it inhibited the process altogether. These chemicals altered the permeability of cell membranes, making them almost impermeable to the excretion of nitrogenous substances into the culture filtrates. The percent inhibition by the 3 chemicals was found to be dependent on duration of the treatment and is shown in the Table. However, their exact mode of action on nitrogen fixation is not clearly known.

MNNG is capable of inducing base-pair transitions[12] and frame-shift mutations, besides inducing gross chromosomal aberrations in higher organisms[13]. However, its mutagenic damage shows a high rate of repair. MNNG has been found to inhibit both DNA and ribosomal RNA synthesis in microbial cells at pH 5.5[14]. It is stable at this pH and reacts solely as a mutagenic agent.

Percent inhibition of nitrogen fixation in clones of *A. doliolum* treated with MNNG, DES and EMS

Clone	Treatment time (min)	MNNG	DES	Treatment time (h)	EMS
No. 1				1	78.71
	20	23.21	19.61	2	81.49
	40	39.81	24.08	3	99.19
	60	65.66	42.86	4	100.00
No. 4				1	75.54
	20	25.03	24.16	2	100.00
	40	32.53	26.79	3	100.00
	60	62.50	27.46	4	100.00

SHARMA and KUMAR[7] observed the inhibition of growth and nitrogen fixation in several clones of *Cylindrospermum majus* after treatment with 50 µg MNNG per ml. No mutagenic activity of MNNG was observed even at relatively a high concentration, though it induced certain reversible morphological variations including slight loss of photosynthetic pigments and excessive enlargement of cells into balloon-like structures. These changes were prominent in the beginning up to a period of 10–15 days following MNNG treatment, but disappeared gradually even without sub-culturing the clonal population.

Since GUPTA and KUMAR[4–5] were able to secure mutant strains of *Anacystis nidulans* after treatment with DES both in acidic and alkaline range, it was considered desirable to treat the selected clones of *A. doliolum* at pH 7.0. KUMAR[1] studied the effects of DES on *A. nidulans* and reported this mutagen to be strongly inhibitory to growth. The data presented here show its inhibitory action on nitrogen fixation too.

EMS has been shown to be a highly active mutagen in maize[15], *Drosophila*[16], *Neurospora*[17], bacteria[18], and phage[19]. In addition to causing chromosome breaks, it causes base substitutions in DNA leading to point mutations. KUMAR[2] studied the effects of EMS on *A. nidulans* and observed that it was more toxic in alkaline buffers than in acidic ones. The results reported here indicate that EMS treatment at pH 7.0 inhibited nitrogen fixation but did not induce mutation of any kind in clonal cells.

[12] H. V. MALLING and F. J. DE SERRES, Genetics *60*, 201 (1968).
[13] T. GICHNER, A. MICHAELIS and R. RIEGER, Biochem. biophys. Res. Commun. *11*, 120 (1963).
[14] E. CERDÁ-OLMEDO and P. C. HANAWALT, Biochim. biophys. Acta *142*, 450 (1967).
[15] E. AMANO and H. H. SMITH, Mutation Res. *2*, 344 (1965).
[16] O. G. FAHMY and M. J. FAHMY, Nature, Lond. *184*, 1927 (1959).
[17] M. WESTERGAARD, Experientia *13*, 224 (1957).
[18] A. LOVELESS and S. HOWARTH, Nature, Lond. *184*, 1780 (1959).
[19] A. LOVELESS, Nature, Lond. *181*, 1212 (1958).

Peroxidase activity in mitochondria of Prototheca moriformis

Luisa De Vecchi and Stefania Pellegrini

Istituto di Scienze Botaniche, Università degli Studi, via G. Colombo 60, I-20133 Milano (Italy), 27 July 1976

Summary. Peroxidase activity was investigated by the use of diaminobenzidine method in fixed cells of Prototheca moriformis. A strong peroxidase activity was observed in the mitochondria. DAB staining was unaffected by KCN, aminotriazole and antimycin A, but it was completely inhibited by methanol-nitroferricyanide.

During our cytochemical studies[1], aiming to detect cytochrome oxidase by means of the cytochemical reaction with 3–3′ diaminobenzidine (DAB), we found preliminary evidence that the mitochondria showed also a peroxidase activity in Prototheca, Chlorella and in its yellow mutant CM 20.

Here we present the results of a study in which the characterization of this peroxidase activity – as shown by DAB reaction in the presence of different inhibitors – was undertaken in Prototheca.

Materials and methods. Prototheca moriformis was supplied by the Culture Collection of Algae and Protozoa of Cambridge University and grown on a glucose containing medium[2] for 5 days at 25 °C. The fixation and embedding methods were the same as described in a previous paper[1]. DAB reaction was performed as described for cytochrome c peroxidase staining in baker's yeast[3]. The incubation

1 S. Pellegrini and L. De Vecchi, J. submicrosc. Cytol. *8*, 353 (1976).
2 S. A. Waksman, in: The actinomycetes, p. 193. Ed. Waltham. The Chronica Botanica Co., Mass. 1950.
3 M. M. Todd and E. L. Vigil, J. Histochem. Cytochem. *20*, 244 (1974).

medium contained 10 mg of 3–3′ DAB (Sigma Chem. Co., St. Louis, Mo., USA) dissolved in 4.9 ml of 0.05 M 2-methyl-1, 3 propandiol buffer pH 8. Just before use 0.1 ml of 2.5% H_2O_2 (freshly prepared from a 30% Merck solution) was added. Several pHs were tried from 6.5 to 8.5. To test different inhibitors, the same reaction was performed in the presence of every single inhibitor, after a preincubation of 30 min in buffer containing the same inhibitor. The inhibitor final concentration were: KCN 10^{-2} M; antimycin A 5×10^{-4} M; aminotriazole 0.2 M; methanol-nitroferricyanide which consisted of 1% sodium nitroferricyanide and 1% acetic acid in methanol.

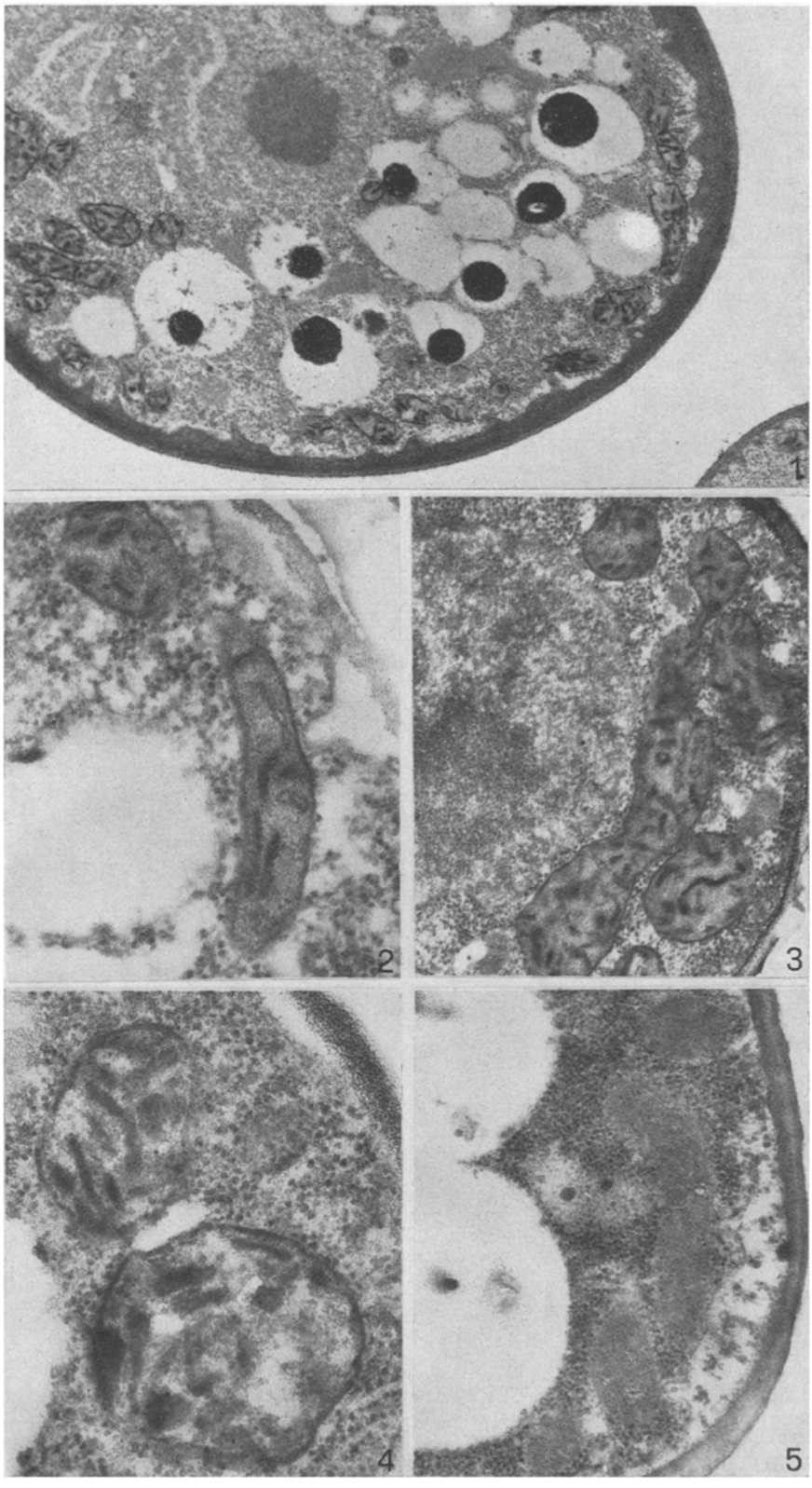

Fig. 1. Prototheca moriformis cell after incubation in BAB/H_2O_2 medium: peroxidase activity is clearly located in mitochondria only. × 13,000.

Fig. 2. Peroxidase activity after incubation in DAB/H_2O_2 medium containing 10^{-2} M KCN: mitochondria staining is little affected. × 44,000.

Fig. 3. Peroxidase activity after incubation in DAB/H_2O_2 medium containing 0.2 M aminotriazole: reaction product in mitochondria is irregular. × 25,000.

Fig. 4. Peroxidase activity after incubation in DAH/H_2O_2 medium containing 5×10^{-4} antimycin: mitochondria staining is little affected. × 51,000.

Fig. 5. Peroxidase activity after incubation in DAB/H_2O_2 medium containing 1% methanol-nitroferricyanide: no DAB oxidation product is observed in the mitochondria. × 20,000.

Results and discussion. After DAB incubation in the presence of H_2O_2, mitochondria appeared heavily stained (figure 1); maximal staining occurred when the reaction was carried out at pH near 8. The staining was localized both in the intercristal spaces and between the outer and inner membranes, while no other organelle was stained. After the incubation with KCN, aminotriazole or antimycin A, the staining was less pronounced and more irregular, but on the whole DAB deposition seemed little affected (figures 2, 3 and 4). In the presence of methanol-nitroferricyanide, no staining was observed in mitochondria (figure 5).

Our results indicate that, in Prototheca moriformis, it is possible to detect a peroxidase activity in the presence of H_2O_2 which is nearly insensitive to KCN, aminotriazole and antimycin A, and is completely inhibited by methanol-nitroferricyanide.

We have at present no biochemical information on the enzyme(s) responsable of this peroxidase activity, but a peroxidative activity of mitochondria, demonstrated by DAB method, has been described in other unicellular organisms[3-5].

In Prototheca, this peroxidase activity seems neither due to catalase, because DAB deposition is not inhibited by aminotriazole, nor to cytochrome oxidase, because DAB deposition occurs only in the presence of H_2O_2 and is insensitive to KCN.

We think that DAB deposition could not be dependent on a peroxidase reaction of cytochrome c[6], as the peroxidase activity is not inhibited by antimycin A; but we cannot exclude a participation of a haemoprotein. At present we favour the hypothesis that the peroxidase activity described in this paper could be due to a mitochondrial peroxidase similar to those described in other unicellular organisms[7-9].

4 G. E. Childs, J. Histochem. Cytochem. *21*, 26 (1973).
5 K. Hirai, J. Histochem. Cytochem. *22*, 189 (1973).
6 S. Goldfisher, C. Moore, I. Sternlieb, T. Roberts, B. Schiller and J. French, J. Histochem. Cytochem. *20*, 839 (1972).
7 H. M. Lenhoff and N. O. Kaplan, J. biol. Chem. *220*, 967 (1956).
8 T. Yonetani and T. Ohnishi, J. biol. Chem. *241*, 2983 (1966).
9 T. Yamanaka, Biochim. biophys. Acta *215*, 74 (1975).

Benzoyl cyanide in the defensive secretion of polydesmoid millipeds[1]

W. E. Conner, T. H. Jones, T. Eisner and J. Meinwald

Section of Neurobiology and Behavior, Langmuir Laboratory, and Department of Chemistry, Cornell University, Ithaca (New York 14853, USA), 28 July 1976

Summary. A novel cyanogenetic compound, benzoyl cyanide, was isolated from the defensive secretion of 3 polydesmoid millipeds (Pseudopolydesmus serratus, Apheloria corrugata and A. trimaculata). The secretion of the 3 species also contains mandelonitrile and benzaldehyde, and that of P. serratus contains mandelonitrile benzoate, benzoic acid, isovaleric acid, myristic acid and stearic acid.

One of the more striking chemical defenses of animals, remarkable in part because of its similarity to the defenses of certain plants, is the cyanogenetic glandular apparatus of millipeds of the order Polydesmida. When disturbed, these animals discharge droplets of an odorous fluid from a series of glands that open along the sides of the body, and they depend on this response for protection against predaceous enemies[2]. The 2 primary known components of these secretions are hydrogen cyanide and benzaldehyde[3-7], which are not secreted as such by the glands, but are derived, as they are in cyanogenetic plants that produce these compounds[8], from the cyanohydrin mandelonitrile[9]. The mechanism whereby the mandelonitrile stored in the glands is exposed to catalyst and forced to dissociate into benzaldehyde and hydrogen cyanide at the moment of glandular discharge has been described[9]. Polydesmoid millipeds are a diversified lot, and recent work has indicated that their secretions might be chemically more complex and variable than generally suspected[5,10,11]. We have now reinvestigated 3 polydesmoid species that had previously been shown to be cyanogenetic[6,7,12] – Pseudopolydesmus serratus, Apheloria corrugata and A. trimaculata – and found them to produce, besides mandelonitrile, a novel additional cyanogenetic compound, benzoyl cyanide. Moreover, the secretion of P. serratus was found to contain several ancillary components, which were also identified.

The millipeds stemmed from the environs of Ithaca, N.Y. Secretion was obtained by manipulating and gently tapping the animals, taking up the discharged fluid in capillary tubing, and transferring it to carbon disulfide or ether. Apparatus used in the analyses included a gas chromatograph (Varian 2100 with flame ionization detector; 2.4 m glass column, 5% OV-1 on Gaschrom Q) and a gas chromatograph/mass spectrometer (Finnigan 3300) coupled to a computer (Systems Industries 150).

Liberation of hydrogen cyanide from the discharged secretion of all 3 species was clearly indicated by the blue coloration that developed on strips of filter paper impregnated with copper acetate/benzidine acetate reagent[13] held beside the secretion. Presence of benzaldehyde was confirmed by gas chromatographic and mass spectral comparison with an authentic sample. Mandelonitrile as such could not be demonstrated by gas chromatography since it dissociated at the high instrument temperatures. However, its presence in fresh samples of secretion from all 3 species was demonstrated by thin

1 Paper No. 52 of the series Defense Mechanisms of Arthropods.
2 T. Eisner and H. E. Eisner, Nat. Hist. *74*, 30 (1965).
3 C. Guldensteeden-Egeling, Pflügers Arch. Physiol. *28*, 576 (1882).
4 M. S. Blum and J. P. Woodring, Science *138*, 512 (1962).
5 G. Casnati, A. Nencini, A. Quilico, M. Pavan, A. Rieca and T. Salvatori, Experientia *19*, 409 (1963).
6 H. E. Eisner, W. F. Wood and T. Eisner, Psyche *82*, 20 (1975).
7 H. E. Eisner, T. Eisner and J. J. Hurst, Chem. Ind. *1963*, 124 (1963).
8 J. M. Kingsbury, in: Poisonous Plants of the United States and Canada. Prentice-Hall, Englewood Cliffs, N. J. 1964.
9 T. Eisner, H. E. Eisner, J. J. Hurst, F. C. Kafatos and J. Meinwald, Science *139*, 1218 (1963).
10 H. Monteiro, Anais Ass. bras. Quím. *20*, 29 (1961).
11 R. M. Duffield, M. S. Blum, and J. M. Brand, Ann. ent. Soc. Am. *67*, 821 (1974).
12 H. E. Eisner, D. W. Alsop and T. Eisner, Psyche *74*, 107 (1967).
13 F. Feigl, in: Spot Tests in Organic Analysis. Elsevier, New York 1960.