stripped off the nuclei during the Triton treatment, as expected[12], and after the completion of washings the preparations were essentially free of extranuclear debris (data not shown).

We propose, for use in some kinds of experiments, the incubation of nuclei in the low-salt medium, as described above, as a means for the removal of traces of cytoplasmic contaminants from nuclear preparations. Our data reveal that less than 1.8% of the total nuclear DNA is lost during the incubation. It was also demonstrated that the presence of the protease inhibitor, diisopropylfluorophosphate[13], in our incubation system did not result in any change in the amount and composition of fraction 2 liberated. Thus, the extraction is also successful under conditions of efficient inhibition of proteolysis. In our studies[14], use has been made of the described incubation in 2 systems: 1. By employing it before the phosphorylation of nuclear proteins in vitro by [γ-^{32}P]ATP, the total incorporation can be increased because the ATP-ase activity of the nuclear preparations is reduced. 2. By applying it during the purification of a nuclear protein phosphatase, the contamination of the starting material by cytoplasmic phosphatases can be reduced.

Evaluating our data from G-6-P-ase activity measurements, it is apparent that if the incubation of the nuclei in the low-salt medium is not performed, the cytoplasmic contaminants present in fraction 2 will be solubilized with the 0.14 M saline extract. This fact should be considered if one works with the latter.

1 The helpful comment of Prof. J.R. Tata in the preparation of the manuscript is kindly acknowledged.
2 C. Viticchi and F. Szeszák, Z. Alternsforsch., in press (1979).
3 F. Szeszák, Acta biochim. biophys. Acad. Sci. Hung. *11,* 113 (1976).
4 G. Blobel and V.R. Potter, Science *154,* 1662 (1966).
5 A.J. MacGillivray, A. Cameron, R.J. Krauze, D. Rickwood and J. Paul, Biochim. biophys. Acta *277,* 384 (1972).
6 U.K. Laemmli, Nature *227,* 680 (1970).
7 J.S. Bhorjee and T. Pederson, Analyt. Biochem. *71,* 393 (1976).
8 W. Schaffner and C. Weissmann, Analyt. Biochem. *56,* 502 (1973).
9 K. Burton, Biochem. J. *62,* 315 (1956).
10 W.C. Schneider, in: Methods in Enzymology, vol. III, p. 681. Academic Press, New York 1957.
11 J.B. Martin and P.M. Doty, Analyt. Chem. *21,* 965 (1949).
12 J.R. Tata, M.J. Hamilton and R.D. Cole, J. molec. Biol. *67,* 231 (1972).
13 D.B. Carter and C.B. Chae, Biochemistry *15,* 180 (1976).
14 F. Szeszák and L.A. Pinna, in preparation.

Penetration and interaction with haemoglobin of Corynebacteria-like microorganisms into erythrocytes in vitro

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Summary. Following 24 h incubation of normal blood in the presence of the microorganism, the evolution of cell wall deficient forms within the erythrocytes and a process of oxidation of the haemoglobin may be observed.

Previous research by other authors and ourselves has demonstrated within the circulating erythrocytes and platelets the presence of bacterial minimal reproductive units (MRU) and cell wall deficient (CWD) forms, which evolve in the haemocultures towards conventional forms of Corynebacteria-like microorganisms (diphtheroids) and *Staph. epidermidis.* In the majority of cases, the evolution of CWD and the reversion to conventional bacterial forms take place following prolonged periods of incubation[1-4]. No significant differences have been detected between the percentage of growth of cocci and diphteroids within the blood cultures from normal subjects and from patients in various pathological situations. Such growth, in the majority of cases, was accompanied by a more or less pronounced process of haemolysis[5].

The present research has been carried out in order to evaluate the possibility that, by adding a pure culture of a Corynebacterium to sterile blood in vitro, the bacterium penetrates the cell wall of the erythrocyte; and in order to verify the consequences of the multiplication within the erythrocytes of a bacterial strain which, in the primary haemoculture from which it had been isolated, gave rise to a notably strong process of haemolysis.

Materials and methods. The microorganism isolated from the circulating blood of a patient suffering from acute articular rheumatism in a febrile state, has been maintained in our laboratory for about 2 years. Culture media: DIFCO trypticase soy broth and agar, and DIFCO brain heart infusion. The blood agar for the tests of haemolitic activity was prepared with 5% rabbit blood whose sterility was previously checked. For the tests concerning the penetration of the bacteria into the erythrocytes and the alterations of the haemoglobin, 0.2 ml of group 0 human erythrocytes centrifuged at 250 × g 5 min, or 1 ml of the supernatant 4000 × g 10 min of haemolysate prepared from the same erythrocytes resuspended 1:5 in a NaCl 0.25% solution, were mixed with 5 ml of broth. To these preparations 0.2 ml of the bacterial growth of 24 h in broth were added. For the control of the sterility of the blood samples and for the blanks, the bacterial suspension was substituted with 0.2 ml of broth. The suspensions were placed in test-tubes and shaken only at the beginning of the incubation carried out for 24 h at 37 °C. At the end, the cultures with erythrocytes were centrifuged at 400 × g 10 min; the supernatants were discarded and with the sediments were prepared: smears which were stained with Giemsa or acridine orange to recognize the nucleic acids with UV examination; ultrathin sections for the electron microscopy by means of the same methods used for the previous research[3]. Furthermore the erythrocytes were lysed and extensively washed until the ones used as control became completely colourless. They were then suspended in 1 M phosphate buffer pH 6 and spectra were taken from 650 to 470 nm. The products of the incubation carried out in the haemolysate enriched broth were centrifuged at 4000 × g 10 min. On the clear supernatant diluted 1:5 with 1 M phosphate buffer pH 6, spectra were recorded in the visible region.

Results. The physiological characters of the micro-organism used for the present research will be described in a further

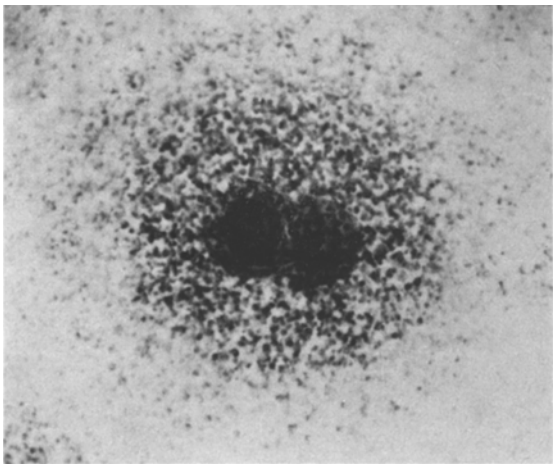

Fig. 1. Blood agar plate. Halo of non-lysed opaque erythrocytes around the bacterial colonies; the process of haemolysis is complete in the surrounding areas. × 73.

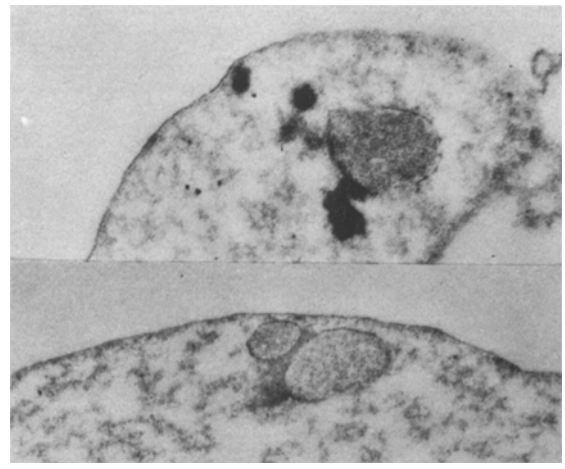

Fig. 2. Following 24 h incubation of sterile blood in the presence of the bacterium, evoluting CWD forms taking origin from MRU may be detected within the erythrocytic stroma. × 33,000.

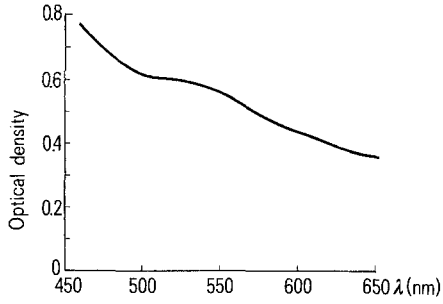

Fig. 3. Spectrum of the ghosts's suspension from normal erythrocytes incubated in the presence of the bacterium.

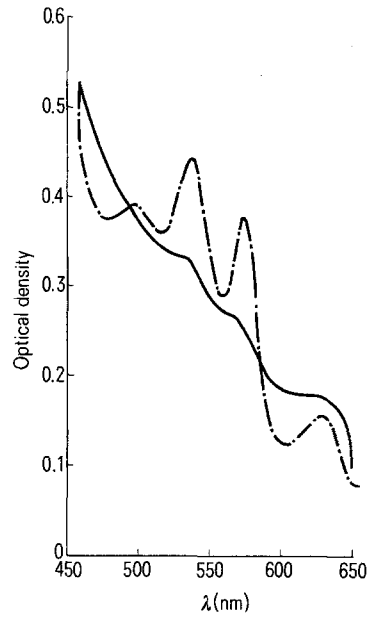

Fig. 4. Spectra of the haemolysate incubated in the presence of the bacteria (——) and in their absence (· - · - ·).

publication together with data concerning antibiotic resistance (manuscript in preparation).

In the subcultures, the bacterium grows slowly in broth and on agar: within 48–72 h it gives rise to a slight turbidity which rapidly sediments or to very small colourless lenticular colonies.

The growth is more rapid and more intense on blood agar, and a characteristic process of haemolysis is observed: the erythrocytes in extended areas far from the colonies are completely lysed, while the colonies themselves are surrounded by a halo of non-lysed but intensely opaque altered erythrocytes (figure 1). The growth in broth enriched with the suspension of erythrocytes, or with the haemolysate, provokes a rapid browning process.

The smears obtained during the incubation showed, within many erythrocytes, the presence of particulated material more evident in the erythrocytes in the course of lysis. On the basis of the affinity for Giemsa and of the fluorescence in the presence of acridine orange, many inclusion bodies were recognizable as bacterial forms in evolution.

Electron microscopy has shown the presence within the erythrocytic stroma of very small compact intensely osmiophilic particles, identifiable as MRU, which increase in volume and evolve assuming the aspect of CWD forms (figure 2).

The incubation of the erythrocytes with the bacteria produced a very massive precipitation of haemoglobin in the red blood cells: in fact the ghosts exibited a dark brown colour and their spectra are similar to the ones reported in the literature for precipitated haemoglobin within the red blood cells[6] (figure 3). At the microscopic control, these ghosts revealed the presence of a large number of Heinz bodies.

As reported in figure 4, the spectrum of the lysate, incubated with the bacterium, showed spectroscopical features compatible with the formation of hemicromes. The results of the microscopic examination have therefore completely confirmed the previous observations, concerning the stages of the intraerythrocytic evolution of the bacterial forms carried in the circulating blood of a relevant number both of normal and pathological subjects; and they also demonstrate the possibility of rapid penetration and evolution of the same bacterial forms within non-parasitized red blood cells in vitro.

The marked denaturating action exibited on the lysate by the bacterium under examination is maintained in the experiments performed with the intact erythrocytes: it is conceivable that this type of behaviour is related to the ability of the bacterium to penetrate the red cell. The characteristic positioning of the areas of lysis observed on

the blood agar plates appears indicative of the fact that with the precipitating action on the haemoglobin is associated the activity of a haemolysin which spreads in the cultural medium. The failure to recognize by naked eye or low enlargement examination the process of haemolysis near the bacterial colonies is the consequence of the strong opacity of the precipitated haemoglobin within a large number of red cells.

1 K.A. Bisset, Ann. rheum. Dis. *36*, 96 (1977).
2 G.G. Tedeschi and I. Santarelli, in: Spheroplasts, Protoplasts and L-forms of Bacteria, vol. 64, p. 341. Ed. INSERM, Paris 1977.
3 G.G. Tedeschi, A. Bondi, M. Paparelli and G. Sprovieri, Experientia *34*, 458 (1978).
4 G. Domingue, J. Schlegel and H. Woody, Microbia *3*, 3 (1976).
5 G.G. Tedeschi, G. Sprovieri and Paola Del Prete, Experientia *34*, 596 (1978).
6 E.A. Rachmilewitz, J. Peisach, T.B. Bradley Jr and W.E. Blumberg, Nature *222*, 248 (1969).

Ferns of Rajasthan – behaviour of chlorophyll and carotenoids in drought resistance

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Summary. The relationship of chlorophylls and carotenoids with drought resistance has been studied in some of the ferns found in Rajasthan, without heating as well as after heating the fronds at 60 °C. The xerophytic species showed lesser degradation of chlorophyll and exhibited higher carotenoid contents.

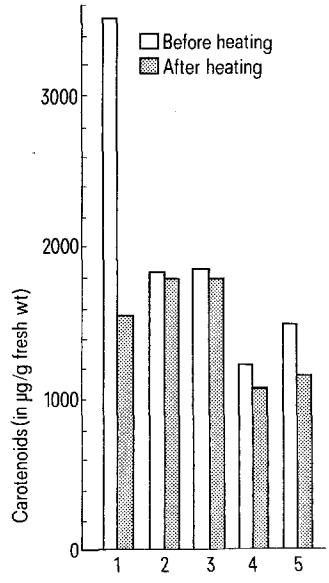
The importance of chlorophyll, proline and -SH compounds is well-known in the mechanism of drought resistance of Angiosperms[1], but their role in the lower vascular plants is yet to be studied. Pteridophytes generally grow in moist places but some of the forms also survive in comparatively drier conditions. So the study of the physiological processes involved in adaptations to such habitat proved interesting[2]. The present study furnishes information about the behaviour of chlorophyll in drought resistance of some of the ferns found growing in the arid and semi-arid regions of Rajasthan. It is also interesting to note that some of the ferns, e.g. *Actiniopteris radiata, Adiantum lunulatum,* etc, remain alive even during the months of May and June when the atmospheric temperature is very high, i.e. 44–48 °C, and relative humidity is very low: the ferns look very dry. However, with the first shower of rain these ferns become as green as fresh.

Materials and method. Fresh fern fronds were collected in the wild during the months of August and September from different places in Rajasthan e.g. Gorumghat, Mt. Abu, Sirohi and Jodhpur. Chlorophyll estimations were done by Robbelen's method[3], without heating, as well as after heating, the fronds at 60 °C (as suggested in the method by Murty and Majumdar[4]). 3 replicates were taken for each estimation.

Influence of 60 °C temperature for 2 h on chlorophyll *a*, chlorophyll *b* and total chlorophyll of some ferns collected from Rajasthan

Plants	Percentage degradation		
	Chlorophyll *a*	Chlorophyll *b*	Total chlorophyll
Actiniopteris radiata	0.00	0.00	0.50
Adiantum lunulatum	0.00	5.82	3.00
Adiantum incisum	2.14	1.60	2.00
Athyrium sp.	13.19	2.60	9.00
Cheilanthes albomarginata	4.07	0.00	2.00

Results and discussion. The degradation of chlorophyll *a*, chlorophyll *b* and total chlorophyll of various fern fronds as affected by heating are shown in the table, while the carotenoids are presented in the figure. The table indicates that in *Actiniopteris radiata* the degradation of total chlorophyll due to heating is comparatively lesser than in others ferns, which confirms the earlier work[5]. The sequence of degradation of total chlorophyll is as follows: *Actiniopteris radiata, Adiantum incisum, Cheilanthes albomarginata, Adiantum lunulatum* and *Athyrium* sp. This order of degradation is closely related to their power of drought resistance and regeneration[6]. It was also observed that the old, shrunken and almost dry leaves of *Actiniopteris radiata, Adiantum lunulatum* and *Cheilanthes albomarginata* become green and fresh just after the first shower of rain, sometimes during the months of June or July, which suggests that there is almost no degradation of chlorophyll in these plants during the scorching heat of the summer months. The present study confirms it.



Effect of 60 °C temperature for 2 h on carotenoids of some ferns collected from Rajasthan (1 *Actiniopteris radiata*, 2 *Adiantum lunulatum*, 3 *A. incisum*, 4 *Athyrium* sp., 5 *Cheilanthes albomarginata*).