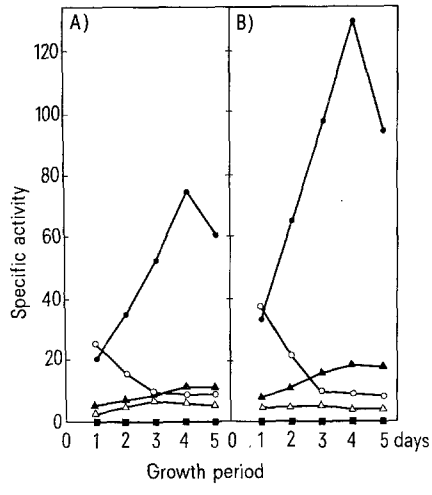
method of SKEGGS[8]. This culture is therefore referred to as 'biotin deficient' in this communication.

Cell free extracts for enzyme assays were prepared as described earlier[5]. Glutamate dehydrogenase (E.C. 1.4.1.3) and alanine dehydrogenase (E.C. 1.4.1.1) were assayed by the method described by THOMULKA and MOAT[9]. The method of ROON et al.[10] and FRY[11] was used for assay of glutamate synthase (E.C. 2.6.1.53) and glutamine synthetase (E.C. 6.3.1.2), respectively. Protein was estimated by the method of LOWRY et al.[12], using serum albumine as standard. A unit of enzyme was expressed as the amount of enzyme which causes a 0.001 change in the O.D. at 340 nm. Specific activity was expressed as units/mg of protein.

It has been demonstrated in a variety of microorganisms that inorganic nitrogen may be assimilated into amino nitrogen via glutamate, alanine, aspartate, carbamyl phosphate and glutamine, but no organism utilizes all these routes to the same degree[13,14]. Usually, a given organism will utilize one or two pathways predominantly, to the virtual exclusion of the others[15]. Since biotin deficiency in *A. nidulans* causes a marked increase in the protein content of the cells, it was of interest to study in detail the assimilation of inorganic nitrogen. Increased nitrate assimilation as a result of biotin deficiency and its regulation by ammonium ions has been demonstrated by us in this culture[5].

The results in the Figure show the specific activities of ammonia-assimilating enzymes. The data suggests that the major route of ammonia assimilation is through NADP-glutamate dehydrogenase. The NADP-linked alanine dehydrogenase and glutamine synthetase were present in both normal and biotin deficient cultures but their lower activity suggests their minor significance in ammonia assimilation. Alanine dehydrogenase was found to be NADP-linked and NAD-linked alanine dehydrogenase was not detected throughout the growth cycle. Similar results have been reported by Lamminnaki and PIERCE[16] and BURK and PATEMANN[17] in *S. cerevisiae* and *N. crassa*, respectively. Glutamate synthase was not detected in both the cultures throughout the growth cycle. This is in agreement with the results reported by BURN et al.[18], who showed absence of glutamate synthase in *N. crassa* and *A. nidulans*. Much of the information in the literature confirms the role of glutamate dehydrogenase in ammonia assimilation by microorganisms[19–23].

Thus, the results presented here suggest that the major route of ammonia assimilation in *A. nidulans* is via NADP-glutamate dehydrogenase. However, it is interesting to note the considerable increase in the activity of glutamate dehydrogenase in biotin-deficient culture as compared to that in normal culture of *A. nidulans*, which may be one of the factors for higher assimilation of ammonia[5,6]; and finally for the higher cellular synthesis as reported earlier[3,5]. However, at present it is not possible to elucidate the factor(s) responsible for the activation of this enzyme as a result of biotin deficiency in *A. nidulans*.



Specific activity of ammonia-assimilating enzymes in *Aspergillus nidulans*. Activities of NADP · glutamate dehydrogenase (●), NAD · glutamate dehydrogenase (○), NADP · alanine dehydrogenase (△), glutamine synthetase (▲), glutamate synthase (■) and NAD · alanine dehydrogenase (■) were determined on the different days of growth in normal (A) and biotin deficient (B) cultures.

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Effect of X-537A on the Phosphorylated Protein in Sarcoplasmic Reticulum Vesicles[1]

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Summary. The effect of the antibiotic X-537A on the phosphorylated ATPase (E∼P) was investigated. The results show that X-537A depresses the level of E∼P which is dependent on the Ca^{2+} gradient, while the Ca^{2+}-independent E∼P is not affected.

Sarcoplasmic reticulum (SR) regulates the concentration of free Ca^{2+} in myoplasm which controls muscle contraction and relaxation. This property of SR is governed by an ATPase which, in the presence of ATP, transports Ca^{2+} into the SR tubules with the formation of a phosphoprotein (E∼P) as an intermediate in the ATPase reaction[2–5].

In isolated preparations of SR vesicles, a Ca^{2+} gradient is generated after Ca^{2+} accumulation, and synthesis of E∼P and ATP can be coupled to the efflux of Ca^{2+} [6–13].

The ionophore X-537A abolishes the Ca^{2+} gradient in SR vesicles and stimulates the ATPase[14–17], but there are no reports on the effect of X-537A on the coupling of synthesis of E~P to Ca^{2+} efflux.

In the present report, we present data demonstrating that when the intravesicular concentration of Ca^{2+} increases in the presence of phosphate as a consequence of active transport, X-537A induces the rapid release of Ca^{2+} from SR vesicles and decreases the level of E~P. A fraction of E~P, whose formation does not depend on Ca^{2+} gradient and which is inhibited by free Ca^{2+}, is not affected by X-537A.

Material and methods. All chemicals obtained from E. Merck A. G., Darmstadt, Germany, were reagent grade. Hexokinase, ADP, acetyl phosphate were purchased from Sigma Chemical Company, St. Louis, Missouri. The antibiotic X-537A was a generous gift of Dr. JULIUS BERGER, Hoffman-La Roche, Nutley, New Jersey 07110, USA. Radioactive phosphate was purchased from Radiochemical Centre (Amersham) and purified on ionic resin columns.

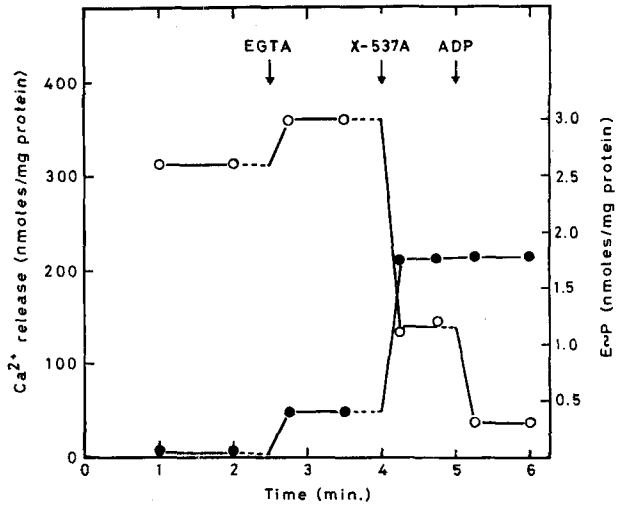

Fig. 1. Effect of X-537A on phosphoprotein in the presence of EGTA. EGTA, X-537A and ADP were added as indicated, -○-, phosphoprotein levels; -●-, calcium released.

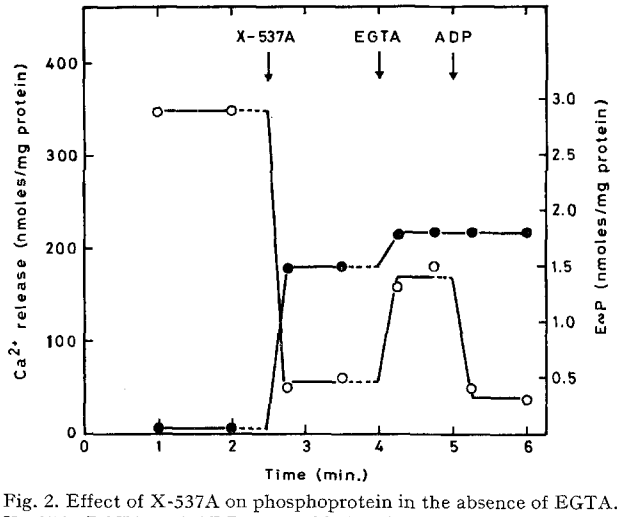

Fig. 2. Effect of X-537A on phosphoprotein in the absence of EGTA. X-537A, EGTA and ADP were added as indicated. -○-, phosphoprotein levels; -●-, calcium released.

Sarcoplasmic reticulum vesicles were prepared from rabbit white skeletal muscle by homogenization and differential centrifugation in a medium containing 0.1 *M* KCl and 10 m*M* *tris*-maleate at a pH value of 7.0. The collected microsomal fraction was washed once with a solution containing 0.6 *M* KCl and 10 m*M* *tris*-maleate at a pH value of 7.0 as described before[18]. Finally, the SR was resuspended in the isolation medium (0.1 *M* KCl – 10 m*M* *tris*-maleate, pH 7.0) and was stored at about 0 °C.

Calcium determination was carried out by taking aliquots of 0.5 mg protein, which were filtered through Millipore filters (HA 0.45 μm). The filters were washed twice by filtering each time 1,0 ml of 0.25 *M* sucrose, and they were then immersed in 2.5 ml of a solution containing 2% TCA and 0.5% La^{3+}. Finally, after vigorous agitation, Ca^{2+} analyses were performed in this solution by atomic spectroscopy in a Perkin-Elmer spectrophotometer, Model 305.

For membrane phosphorylation, sarcoplasmic reticulum vesicles (0.5 mg/ml) were first incubated at room temperature for 60 min in the presence of 40 m*M* KCl, 2 m*M* $MgCl_2$, 5 m*M* sodium orthophosphate, ^{32}P (0.24 μCi/μmole P_i), 0.2 m*M* calcium chloride, 10 m*M* *tris*-maleate and 2 m*M* acetyl phosphate at a pH value of 6.8. Aliquots of 2.0 ml were taken at several periods, as indicated in the respective figures, and added into 5.0 ml of ice cold 125 m*M* perchloric acid solution plus 20 m*M* sodium ortophosphate for phosphoprotein determination[19]. All tubes were centrifuged in the cold room in a Sorvall superspeed SS-3 automatic centrifuge at 10,000 rpm for 10 min. The supernatants were discarded and the pellets were washed 3 times with about 10 ml samples of ice-cold 125 m*M* perchloric acid solution containing 20 m*M* orthophosphate. After the washings, each pellet was resuspended in 1.0 ml of a solution composed of 0.1 *N* NaOH, 2% Na_2CO_3 and 1 m*M* orthophosphate. Dissolution was carried during 30 min in boiling water. After cooling, an aliquot of 0.5 ml was dried in a planchet and counted in a Nuclear-Chicago gas flow counter. Another aliquot of 0.3 ml was used for protein determination by the biuret method using bovine serum albumin as standard.

[1] *Acknowledgments.* This work was supported by grants from the Instituto de Alta Cultura of the Portuguese Ministry of Education (No. CB/2) and the Calouste Gulbenkian Foundation.

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In a separate and identical experiment, without radioactive phosphate, Ca^{2+} determination was also performed.

Results and discussion. The antibiotic X-537A is a carboxilic ionophore which interacts with Ca^{2+} forming a lipophilic complex[20]. It has been demonstrated that such a complex promotes a rapid Ca^{2+} efflux from Ca^{2+} loaded SR vesicles, and a subsequent disruption of the Ca^{2+} gradient created by the pump[14–17].

In Figure 1, it is shown that after the addition of X-537A in the presence of EGTA there still remains about 1.2 nmoles of E∼P/mg of protein although all Ca^{2+} has been released. This observation is consistent with results of other investigators, who have demonstrated that phosphorylation of the ATPase of SR may occur in the absence of a Ca^{2+} gradient, and that the E∼P formed under these conditions is inhibited by Ca^{2+}, but requires Mg^{2+} [21–22], whereas the presence of acetyl phosphate abolishes the Ca^{2+} inhibition[22]. It has been suggested that this phosphorylation takes place at the external surface of the membrane and that another E∼P is also formed at the internal face as soon as Ca^{2+} gradient inside-outside is increased[21].

The addition of EGTA before X-537A (Figure 1) increases the level of E∼P, probably due to the increase of the Ca^{2+} gradient and also due to the decrease of Ca^{2+} concentration outside. Once the ionophore is added, the Ca^{2+} gradient is completely destroyed and the E∼P decreases to about 1.2 nmoles/mg protein. This level of E∼P corresponds to the phosphorylated form which is Ca^{2+} gradient independent. As the presence of EGTA removes external Ca^{2+}, the remaining level of E∼P is supposed to be the form which is Mg^{2+} dependent and is inhibited by outside Ca^{2+}. ADP discharges this form which is in accordance with results of other workers[22].

If X-537A is added before EGTA, the E∼P is nearly all destroyed (Figure 2). In this case, the E∼P which is dependent on the Ca^{2+} gradient is depressed because X-537A abolishes the gradient, and the E∼P which is Mg^{2+} dependent is depressed by the extravesicular Ca^{2+}. Acetyl phosphate, which could prevent this inhibition, is in low concentration, since it was hydrolyzed during the previous Ca^{2+} uptake. The further addition of EGTA, on the other hand, decreases the outside free Ca^{2+} concentration and allows the rapid phosphorylation of the external sites to form the Mg^{2+}-dependent E∼P which is inhibited by Ca^{2+}.

We conclude that X-537A abolishes the E∼P which depends on the Ca^{2+} gradient, but has no effect on the E∼P which is Mg^{2+} dependent and inhibited by outside Ca^{2+}.

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Regulatory Properties of the Citrate Synthase from *Rhodospirillum rubrum*

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Summary. Citrate synthase, purified 600-fold from *Rhodospirillum rubrum*, is activated by KCl and inhibited by ATP and NADH; the effect of the latter inhibitor is completely counteracted by AMP and partially counteracted by KCl.

Rhodospirillum rubrum, a Gram negative, facultatively photosynthetic bacterium, contained citrate synthase (EC 4.1.3.7) both when grown anaerobically in the light or heterotrophically in the dark[2]. The enzyme in cell-free extracts was reported to be inhibited by NADH and deinhibited by AMP[2], as is the case for most citrate synthases from Gram negative bacteria which do not ferment glucose[3], including photosynthetic microorganisms like *Rhodopseudomonas capsulata*[2] and *Rhodopseudomonas spheroides*[4]. However, FLECHTNER and HANSON[5] reported that their partially purified enzyme preparation from *R. rubrum* was not inhibited by NADH, but was inhibited by ATP; they stated that the enzyme regulatory properties were similar to those of the citrate synthase from the Gram positive *Bacillus subtilis*[5,6]. Although a few possible exceptions to the regulatory pattern proposed by WEITZMAN and JONES[3] are known[7,8], we decided to re-examine the regulatory properties of the citrate synthase from *R. rubrum*, particularly with respect to the effect of NADH.

Materials and methods. The blue-green, carotenoid-less, mutant strain BG-1 of *R. rubrum*[9] was grown anaerobically in the light at 30 °C on succinate as carbon source. The cells (41 g wet weight) were disrupted by sonic disintegration, and a cell-free extract obtained by ultracentrifugation of the homogenate[9]. The crude extract was fractionated with ammonium sulphate; the 50–66% saturation fraction, dissolved in 0.05 *M Tris* HCl buffer (pH 7.6) – 1 m*M* EDTA – 0.4 *M* KCl, was subjected to gel filtration on a Sephadex G-200 column (50.5 × 1.8 cm) equilibrated with the same buffer. The elution volume of the citrate synthase was 39 ml; the void volume of the column, determined with Blue Dextran 2000, was 34.5 ml. The fractions with the higher specific activities were pooled and fractionated again with ammonium sulphate. The 50–66% saturation fraction was dissolved in 0.01 *M*

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