Phytochemistry, 1971, Vol. 10, pp. 1025 to 1029. Pergamon Press. Printed in England.

REQUIREMENT FOR PYRIDOXAL IN THE BIOSYNTHESIS OF 4-GLUCOSYLTRANSFERASES (PHOSPHORYLASES) IN THE ALGAE

JEROME F. FREDRICK
Research Laboratories, the Dodge Chemical Company, Bronx, New York 10469, U.S.A.

(*Received* 11 *July* 1970)

Abstract—Algal phosphorylases require pyridoxal (vitamin B_6) for the biosynthesis of active protein. When the B_6 antimetabolite, 5-hydroxy-2-(hydroxymethyl)-4H-pyridone was added to cultures of *Oscillatoria princeps*, *Chlorella pyrenoidosa* and *Cyanidium caldarium*, the phosphorylase isozymes formed were devoid of enzyme activity. Activity was not restored after incubation of the proteins with pyridoxal. However, when the algae were cultured in the presence of the antimetabolite and vitamin B_6, the isozymes were fully active. It appears that there is an absolute requirement for pyridoxal in the biosynthesis of algal phosphorylases, and that this requirement is more basic and probably occurred much earlier in the evolution of 4-glucosyltransferases than the requirement for adenosine monophosphate.

INTRODUCTION

THE ENZYME *phosphorylase* (E.C. 2.4.1.1), has been shown to exist in more than one molecular form (isozymes) in animals[1–3] and in plants.[4–7] In higher plants such as maize,[6] spinach[7] and sugar cane,[5] two phosphorylase isozymes have been detected. In lower plant forms such as algae, two phosphorylase isozymes are present in Cyanophytes and Chlorophytes.[4,8–10]

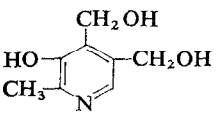
Phosphorylase was thought to be responsible for the formation of linear polyglucosides, but has subsequently, with the discovery of the *nucleoside glucosyltransferases* (E.C. 2.4.1.11) or synthetases,[11] been placed into a controversial role in metabolism of carbohydrates. After the discovery of the wide-spread distribution of the synthetases,[12] it was thought that the phosphorylases functioned only in the phosphorylative degradation of polyglucosides. Recent studies[13,14] have indicated that phosphorylase may serve a role in the synthesis of short-chain maltoglucosides necessary as substrates for the synthetases.

[1] A. A. YUNIS, E. H. FISCHER, and E. G. KREBS, *J. Biol. Chem.* **237**, 2809 (1962).
[2] E. H. FISCHER and E. G. KREBS, *Federation Proc.* **25**, 1511 (1966).
[3] A. A. YUNIS and G. K. ARIMURA, *Biochem. Biophys. Acta* **118**, 335 (1966).
[4] J. F. FREDRICK, *Phytochem.* **1**, 153 (1962).
[5] A. G. ALEXANDER, *J. Agri. Univ. Puerto Rico* **49**, 153 (1965).
[6] C. Y. TSAI and O. E. NELSON, *Plant Physiol.* **43**, 103 (1968).
[7] M. A. R. DE FEKETE, *Planta* **79**, 208 (1968).
[8] J. F. FREDRICK, *Phytochem.* **6**, 1041 (1967).
[9] J. F. FREDRICK, *Phytochem.* **7**, 931 (1968).
[10] J. F. FREDRICK, *Phytochem.* **8**, 725 (1969).
[11] L. F. LELOIR and S. M. GOLDEMBERG, *J. Biol. Chem.* **235**, 919 (1960).
[12] R. B. FRYDMAN, B. C. DESOUZA and C. F. CARDINI, *Biochim. Biophys. Acta* **113**, 620 (1966).
[13] R. B. FRYDMAN and C. F. CARDINI, *Plant Physiol.* **42**, 628 (1967).
[14] N. P. BADENHUIZEN and K. R. CHANDORKAR, *Cereal Chem.* **42**, 44 (1965).

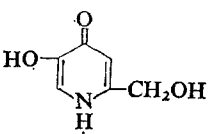
A co-factor found necessary for phosphorylase activity in animals and in plants is pyridoxal.[2,15] This vitamin (B_6) (I), although not involved directly in the active site of the phosphorylase molecule, has nonetheless been found to be an essential 'building block' for the enzyme.[2] Although it has been found that one of the two phosphorylase isozymes present in blue-green algae required adenosine monophosphate for full activity,[4,10] the role of vitamin B_6 has not been investigated in algal phosphorylases.

Studies reported previously indicated that 1,4-pyrones inhibited the action of algal phosphorylase *in vitro*.[16,17] Recently, one of these 1,4-pyrone derivatives was shown to extend the survival time of rats with the L-1210 strain of lymphoid leukemia.[18] This compound, 5-hydroxy-2-(hydroxymethyl)-4H-pyridone, *NSC-68955* (II), was shown to act as an antimetabolite to Vitamin B_6 in the synthesis of the vital nucleic acid base, thymine.[19] Current experiments indicate that *NSC-68955* is a potent inhibitor of germination and growth in the common garden pea and in lentils.[20]

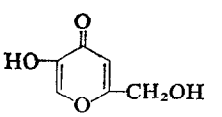
This compound afforded an opportunity, by virtue of its vitamin B_6 antimetabolite effect, to test the necessity for this vitamin as a building block for algal phosphorylases. However, since *NSC-68955* has been shown to possess chelating ability,[16] a similar chelating structure, which was not biologically active, was included in this study. This second compound, 5-hydroxy-2-(hydroxymethyl)-4H-pyrone or Kojic acid (III), *NSC-1942*, differs from *NSC-68955* in having oxygen in place of nitrogen in the ring. *NSC-1942* was found to be without effect on the L-1210 strain of leukemia by the National Cancer Institute.[21] The necessity for the inclusion of this compound in the study was dictated by the fact that the divalent



(I) Pyridoxal



(II) 5-Hydroxy-2-hydroxymethyl-4H-pyridone



(III) Kojic acid

[15] Y. P. LEE, *Biochim. Biophys. Acta* **43**, 18 (1960).
[16] J. F. FREDRICK, *Physiol. Plant.* **11**, 493 (1958).
[17] J. F. FREDRICK, *Ann. N.Y. Acad. Sci.* **88**, 385 (1960).
[18] N. C. I. *Screening Data Summary* NSC-68955 (1968).
[19] J. F. FREDRICK, *Dececo Mag.* **62**, 8 (1970).
[20] J. F. FREDRICK, in preparation.
[21] N. C. I., *Screening Data Summary* NSC-1942 (1963).

manganese ion was found to be necessary for algal phosphorylases.[22] In order to obviate chelation effects, *NSC-1942* is so structurally similar to *NSC-68955* and its chelation ability so much greater for the manganese ion,[16] that it would be a valid compound to use.

RESULTS

When the three algae, *Oscillatoria princeps*, *Chlorella pyrenoidosa* and *Cyanidium caldarium* were cultured in the presence of *NSC-68955* and the extracts subjected to polyacrylamide gel electrophoresis, proteins were detected on the amido-black stained gels in the exact positions of phosphorylase isozymes a_1 and a_2. These proteins failed to exhibit phosphorylase activity when tested in the incubation medium for the enzyme (Fig. 1). It was not possible to restore phosphorylase activity to the inactive proteins by incubation of the extracts with pyridoxal phosphate or pyridoxine hydrochloride prior to electrophoresis.

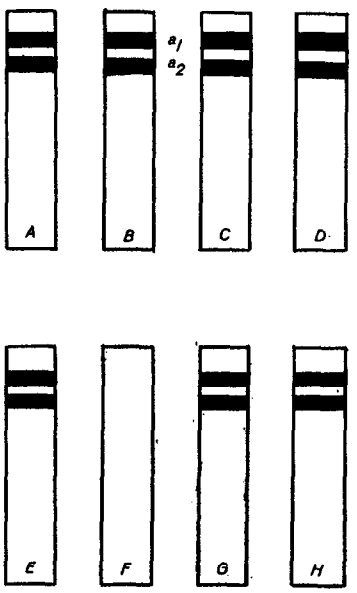


FIG. 1. EFFECT OF VITAMIN B_6 ANTIMETABOLITE ON THE SYNTHESIS OF PHOSPHORYLASE ISOZYMES IN *Oscillatoria princeps*.
A–D, stained with amido-black; E–H, histochemical phosphorylase stain. A and E, normal culture medium; B and F, *NSC-68955* added to medium; C and G, same as B and F except that vitamin B_6 was added; D and H, *NSC-1942* added to medium. a_1 and a_2 are the phosphorylase isozymes. The anode is toward the bottom of all figures. The same results were obtained with *Cyanidium caldarium* except that this alga has only the a_2 isozyme present.

The algae when cultured in normal media, and in media containing *NSC-68955* to which pyridoxine hydrochloride was added, showed the same a_1 and a_2 protein bands, but these proteins exhibited full phosphorylase activity (Fig. 1).

When the algae were cultured in the presence of *NSC-1942*, no difference was apparent in the activity of the phosphorylase isozymes from the controls.

[22] J. F. FREDRICK, *Physiol. Plantarum* **12**, 868 (1959).

Dry weight measurements of the algae showed a marked inhibition by *NSC-68955* which was effectively overcome when the algae were cultured with the vitamin. No such inhibition was obtained with *NSC-1942*. (Fig. 2).

It would appear that vitamin B_6 is a necessary co-factor for the biosynthesis of phosphorylase isozymes of algae. While a difference in the requirements for adenosine monophosphate (AMP) had been shown for phosphorylase isozymes a_1 and a_2 of blue-green and green algae,[23] the requirement for vitamin B_6 was not previously established for any of the algal phosphorylases.

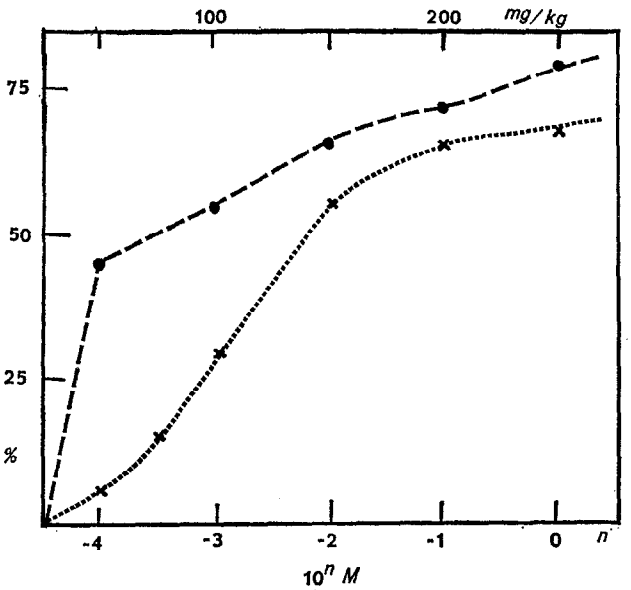


FIG. 2. INHIBITION OF GROWTH OF *Chlorella pyrenoidosa* INDUCED BY *NSC-68955*. (– – – –) Inhibition of alga follows in general the curve of (%) survival time of leukemic mice (N.C.I. data)[18] (▪▪▪▪▪). Top axis, mg/kg of *NSC-68955* injected into leukemic mice. Bottom axis, Molar concentrations of the compound added to culture medium for the alga. The ordinate axis shows either the % inhibition of algal growth or the % survival time above normal for leukemic mice receiving the chemical. The antimetabolite (vs. B_6) effects seem to be similar for both biological phenomena.

It is of interest that the mechanism involved in the action of pyridoxal in providing 'building blocks' for phosphorylase is apparently similar for both animal and plant enzymes. It was estimated that 50–80 per cent of the vitamin B_6 present in animals is bound to phosphorylase.[2] It may be that plants also bind the vitamin as part of the phosphorylase *holo*enzymes.

The apparent antimetabolite effect of *NSC-68955* (vs. vitamin B_6), indicates that this vitamin is necessary for the biosynthesis of both phosphorylase isozymes in algae. Activity cannot be restored to the deficient proteins by incubation of the protein with the vitamin after its formation.

From an evolutionary standpoint, the phosphorylase isozymes of blue-green and green algae, although differing in the need for AMP, do not differ in their absolute requirement

[23] J. F. FREDRICK, *Phytochem.* **2**, 413 (1963).

for pyridoxol. Hence, the vitamin B_6 requirement appears to be more basic and probably occurred at an earlier point in the evolution of these catalytic proteins than that for AMP. It is of interest, too, that the single phosphorylase present in *Cyanidium caldarium*, a_2, although not sensitive to AMP, nonetheless exhibited the same requirement for the vitamin as the isozymes present in *Oscillatoria princeps* and *Chorella pyrenoidosa*.

Chelation of the essential manganese ion *per se* does not appear to play a major role as witnessed by the inactivity of the analogous compound, *NSC-1942* in the biosynthesis of active phosphorylase proteins.

EXPERIMENTAL

The algae, *Oscillatoria princeps*, *Chlorella pyrenoidosa* and *Cyanidium caldarium* were cultured in media previously described.[24] Extracts were prepared and fractionated with ammonium sulfate. The phosphorylases were separated on 8% polyacrylamide gels in an E-C No. 470 Vertical Cell and protein staining and a histochemical method for the visualization of phosphorylase applied.[24,25] All techniques have been described.[24]

NSC-68955 was dissolved in the culture media to give final concentrations of 10^{-2}, 10^{-3} and 10^{-4} M. When vitamin B_6 (pyridoxine hydrochloride, Lilly) was used, it was added to the media in the same concentrations. Those cultures to which *NSC-1942* was added, did not contain the vitamin. The 5-hydroxy-2-(hydroxymethyl)-4H-pyrone (kojic acid, Matheson) was recrystallized twice before use and added in the same concentrations as *NSC-68955*.

Pyridoxal phosphate (Sigma) was added directly to the algal extracts and incubated for 1 hr at 30° at a concentration of 0·1 M.

Preparation of NSC-68955

60 g of kojic acid (Matheson) were heated in a pressure flask with 100 ml conc. NH_4OH ($d = 0{\cdot}88$) in a boiling water bath for 4 hr. The brown mixture was steamed to drive off excess ammonia and the residue was extracted several times on a boiling water bath with a total of 1 l. of methanol. The extracts were combined and treated with activated carbon (Darco). After 1 hr, the extract was filtered through iron-free paper and the filtrate evaporated to one-third its original volume on a water bath. The crystals were redissolved in methanol and recrystallized three times. They were dried over silica gel. The pale brown crystals showed a m.p. of 237–238°.

Acknowledgements—This work was supported by a research grant from the George B. Dodge Cancer Memorial Fund of the Dodge Institute for Advanced Studies. We are indebted to the National Cancer Institute of the National Institutes of Health for the screening of *NSC-68955* and the determination of its activity against lymphoid leukemia, L-1210.

[24] J. F. FREDRICK, *Phytochem.* in press (1971).
[25] C. H. DAVIS, L. H. SCHLISELFELD, D. P. WOLF, C. A. LEAVITT and E. G. KREBS, *J. Biol. Chem.* **242**, 4824 (1967).