Phytochemistry, 1968, Vol. 7, pp. 1247 to 1251. Pergamon Press. Printed in England

PLANT ENZYME SYNTHESIS: DECAY OF MESSENGER RNA FOR PEROXIDASE IN SUGAR-CANE STEM TISSUE

K. R. GAYLER* and K. T. GLASZIOU

David North Plant Research Centre, The Colonial Sugar Refining Co. Ltd., Indooroopilly, Queensland, Australia

(*Received* 17 *January* 1968)

Abstract—The rate of decay of the messenger RNA specific for the synthesis of peroxidase has been measured in sugar-cane stalk tissue using either actinomycin D or 6-methyl purine to inhibit RNA synthesis. The half-time for peroxidase m-RNA decay was 1½–2 hr.

INTRODUCTION

IN BACTERIA, the half-life for decay of the m-RNA required for synthesis of specific enzymes has been measured by following the rate of change of enzyme-forming capacity after cessation of m-RNA synthesis.[1–8] Satisfactory estimates of the half-life cannot be made unless the rate of enzyme formation is proportional to the m-RNA concentration and m-RNA decay is first order.

Cessation of m-RNA synthesis has been effected by removal of the specific inducer,[1–4] addition of actinomycin D,[5–7] or infection with phage.[4, 8] Actinomycin D was also used to examine the stability of m-RNA for enzymes from animal tissues.[9]

Peroxidase (E.C. 1.11.1.7) levels in sugar-cane internodal tissue are very low *in vivo*. When tissue slices are incubated in water, rapid peroxidase synthesis occurs after an initial lag period of 4–5 hr. Actinomycin D completely blocks peroxidase synthesis when added during the early phase of the lag period but not when added during the phase of rapid enzyme synthesis, indicating that initially m-RNA synthesis is limiting for peroxidase formation.[10, 11] It is also possible to arrange for m-RNA to be rate limiting for invertase synthesis in this tissue.[12] However, invertase undergoes rapid turnover, so that any measurement of enzyme-forming capacity after cessation of m-RNA synthesis would need to be corrected for enzyme breakdown.

When added during the rapid phase of peroxidase synthesis, *p*-fluorophenylalanine blocks any further increase in peroxidase level. There is no subsequent loss of enzyme activity,

* Present address: Botany Department, University of Queensland.

[1] D. NAKADA and B. MAGASANIK, *Biochim. Biophys. Acta* **61**, 835 (1962).
[2] A. KEPES, *Biochim. Biophys. Acta* **76**, 293 (1963).
[3] D. NAKADA and B. MAGASANIK, *J. Mol. Biol.* **8**, 105 (1964).
[4] J. P. BILESIKIAN, R. O. R. KAEMPFER and B. MAGASANIK, *J. Mol. Biol.* **27**, 495 (1967).
[5] L. H. HARTWELL and B. MAGASANIK, *J. Mol. Biol.* 7, 401 (1963); **10**, 105 (1964).
[6] L. LEIVE, *Biochem. Biophys. Res. Commun.* **20**, 321 (1965).
[7] L. LEIVE and V. KOLLIN, *J. Mol. Biol.* **24**, 247 (1967).
[8] R. O. R. KAEMPFER and B. MAGASANIK, *J. Mol. Biol.* **27**, 453 (1967).
[9] H. C. PITOT, C. PERAINO, C. LAMAR, JR. and A. L. KENNAN, *Proc. Natl Acad. Sci. U.S.* **54**, 845 (1965).
[10] K. T. GLASZIOU, J. C. WALDRON and B. H. MOST, *Phytochem.* **6**, 769 (1967).
[11] K. T. GLASZIOU, K. R. GAYLER and J. C. WALDRON, 6th International Conference on Plant Growth Substances (1967) (in press).
[12] K. T. GLASZIOU, J. C. WALDRON and T. A. BULL, *Plant Physiol.* **41**, 282 (1966).

so the enzyme formed is quite stable.[10] Hence we have selected the peroxidase system as one apparently suitable for the measurement of rate of decay of a m-RNA species in a higher plant. In this paper we report on the use of both actinomycin D and 6-methyl purine to inhibit RNA synthesis, and to show that the half-time for decay for peroxidase m-RNA is $1\frac{1}{2}$–2 hr.

RESULTS

Tissue Penetration by Actinomycin D

The half-life for loss of ^{14}C-glucose from the free space of 0·5 mm disks of sugar-cane internodal tissue was about 0·5 min,[13] hence for the 1·0 mm disks used in the current experiments diffusion equilibrium of small solute molecules into the free space would be expected to

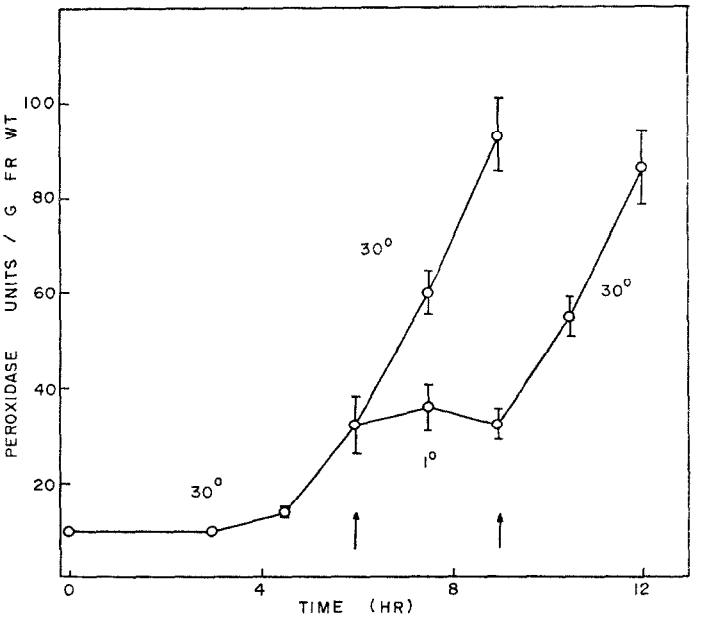

FIG. 1. EFFECT OF LOW TEMPERATURE ON PEROXIDASE SYNTHESIS.

Tissue slices were cut, washed and incubated for the periods shown at 30° and 1°. Solutions were changed at 2-hr intervals. At harvest tissues were washed, ground, squeezed through muslin and the extracts dialysed for 24 hr at 3° against water prior to enzyme assay.

take at least 6–8 min. Further penetration of relatively large molecules such as actinomycin D to its site of action may take considerably longer and thereby introduce considerable errors into the measurement of decay time for short lived m-RNA.

We found that treatment of tissue at 1° inhibited peroxidase synthesis, but had no carry-over effects on the rate of synthesis when inhibition was released by raising the temperature (Fig. 1). Further studies showed that the 1° treatment could be extended to 6 hr without adverse effects.

To find a suitable treatment time and concentration of actinomycin D which would block m-RNA synthesis with certainty, synthesis of invertase was used as a test system. The

[13] K. T. GLASZIOU, *Plant Physiol.* **35**, 895 (1960).

enzyme is situated in two cell compartments, part being associated with the cell wall and part with the vacuole. Treatment with glucose and glycine reduces the vacuolar component to virtually zero, while the cell-wall component remains almost constant. New enzyme is formed on the removal of glucose and glycine, but is dependent on prior synthesis of m-RNA.[12] Tissue was therefore pretreated in glucose and glycine, transferred to actinomycin D solutions or water at 1°, and the subsequent invertase synthesis measured after transfer to 30° (Table 1). We found that incubation of tissue for 2 hr at 1° in 50 μg/ml actinomycin D was a satisfactory method of completely blocking synthesis of new m-RNA. Inhibition of respiration by actinomycin D has been observed in leucocytes.[14] For sugar-cane tissue slices respiration rates were unaffected by actinomycin D at 50 μg/ml (Table 1).

TABLE 1. EFFECTIVENESS OF ACTINOMYCIN D TREATMENTS ON INVERTASE SYNTHESIS

Actinomycin D concentration (μg/ml)	Invertase (units/g fr. wt)	Respiration (μl O_2/hr/g fr. wt)
10	4·3	—
20	3·1	—
50	2·0	243
Control (water)	14·3	267

Level of invertase at time of transfer from glucose, glycine to the above solutions was 2·7 units.

Tissue was cut, washed and incubated in 0·11 M glucose, 0·05 M glycine for 16 hr at 30°, then in the above solutions for 2 hr at 1° and 5 hr at 30° before harvest. Solutions were changed at 2-hr intervals. Harvest and assay details as for Fig. 1.

Decay of Peroxidase m-RNA *in the Presence of Actinomycin D*

Conditions suitable for measuring decay of peroxidase m-RNA are illustrated in Fig. 2A. Tissue was first incubated for 5 hr in water by which time peroxidase synthesis had just commenced. The tissue was then chilled to 1° and held at this temperature for 2 hr in actinomycin D. After returning the temperature to 30°, tissue samples were extracted and assayed for peroxidase at various time intervals. The peroxidase-forming capacity at any time interval was obtained by subtracting the amount of peroxidase present at that time interval from the final yield of enzyme. These data are plotted in Fig. 2B and show the necessary log-linear relationship. The estimated half-time for m-RNA decay is 2·0 hr.

Decay of Peroxidase m-RNA *in the Presence of 6-Methyl purine*

Since relatively large quantities of actinomycin D are consumed in this type of experiment, we have sought for a more readily available chemical which would inhibit RNA synthesis but have no deleterious effects on the translational component of the enzyme synthesizing apparatus. 6-Methyl purine may be such an inhibitor.[15–17]

[14] J. LASLO, D. S. MILLER, K. S. MCCARTY and P. HOCHSTEIN, *Science* **151**, 1007 (1966).
[15] J. H. MILLER and E. S. KEMPNER, *Biochim. Biophys. Acta* **76**, 333 (1963).
[16] R. H. KURTZMAN, JR., A. C. HILDEBRANDT, R. H. BURRIS and A. J. RIKER, *Virology* **10**, 432 (1960).
[17] J. L. KEY, *Plant Physiol.* **41**, 1257 (1966).

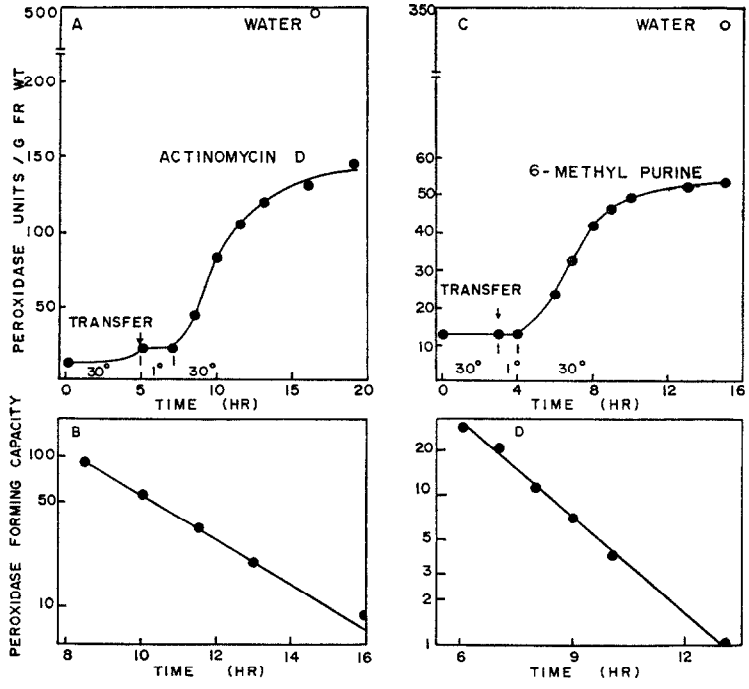

FIG. 2. DETERMINATION OF PEROXIDASE m-RNA DECAY.

Tissues were incubated in water at 30° for the periods shown, then transferred to actinomycin D (50 μg/ml) or 6-methyl purine (1×10^{-4} M) and held at 1° for 2 and 1 hr respectively. Water controls were treated similarly. The temperature was then raised to 30°, and tissue samples harvested at the intervals shown and assayed for peroxidase.

Peroxidase-forming capacity was calculated from the difference between the final level of peroxidase and intermediate levels. A and B show results with RNA synthesis blocked by actinomycin D, and C and D by 6-methyl purine.

We found that respiration rates were frequently inhibited by levels of 6-methyl purine only slightly higher than those required to inhibit peroxidase synthesis by more than 90 per cent. Since the response to 6-methyl purine varied slightly between different batches of tissue, respiration was monitored throughout all experiments using an oxygen electrode, and results rejected if respiration was affected significantly. Respiration was always affected by 6-methyl purine at or above a concentration of 5×10^{-4} M.

TABLE 2. EFFECT OF 6-METHYL PURINE ON TRANSCRIPTION AND TRANSLATION OF PEROXIDASE

State of tissue	Peroxidase synthesis (% of water control)	Respiration (% of water control)
(A) Peroxidase synthesis limited by transcription	10·4	101
(B) Peroxidase synthesis limited by translation	105	102

Tissue was cut, washed and (A) incubated immediately in either water or 5×10^{-5} M 6-methyl purine for 1 hr at 1° then 8 hr at 30°, or (B) incubated in water for 12 hr before incubation in either water or 5×10^{-5} M 6-methyl purine for 1 hr at 1° then 3 hr at 30°. Harvests and assays as for Fig. 1.

The data of Table 2 show that 6-methyl purine effectively inhibited peroxidase synthesis if added when m-RNA synthesis was limiting, but had no short-term effect if added when rapid peroxidase synthesis was proceeding and translational steps had become rate limiting.[10, 11]

When peroxidase m-RNA decay was measured using 6-methyl purine to block RNA synthesis, the results (Fig. 2C, D) were similar to those obtained with actinomycin D, the estimated half-time for m-RNA decay being 1·5 hr.

CONCLUSIONS

Bacteria contain m-RNA with a half-time for decay of only a few minutes for enzymes such as β-galactosidase,[1, 7] tryptophanase[4] and histidase,[5] and also apparently completely stable m-RNA species such as for flagellin synthesis.[18] Evidence has been obtained for the existence of both stable[19, 20] and unstable[9] forms of m-RNA species in animal tissues. This work demonstrates the presence of a short lived m-RNA specific for formation of a particular enzyme in a higher plant.

Once formed in the tissue peroxidase is very stable,[10] hence our present results indicate that the *in vivo* repression of peroxidase[11] may operate at the transcriptional level.

MATERIALS AND METHODS

Preparation, Incubation and Extraction of Tissue and Enzyme Assays

Invertase and peroxidase assays and the preparation, incubation and extraction of tissue slices were carried out as previously described.[10–12]

Measurement of Respiration of Tissue Slices

Tissue samples (2 g) were incubated at 30° in water or in the treatment medium in a closed cell stirred by a magnetic stirrer not in contact with tissue. The rate of depletion of oxygen from the water in the cell was measured continuously with a Clark Oxygen Electrode.

Chemicals

Actinomycin D was a gift from Merck, Sharp and Dohme, and 6-methyl purine was obtained from Sigma Chemical Co.

[18] R. J. MARTINEZ, *J. Mol. Biol.* **17**, 10 (1966).
[19] P. A. MARKS, E. R. BURKA and D. SCHLESSINGER, *Proc. Natl Acad. Sci. U.S.* **48**, 2163 (1962).
[20] R. W. SEED and I. H. GOLDBERG, *Proc. Natl Acad. Sci. U.S.* **50**, 275 (1963).