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FURTHER STUDIES ON THE INCORPORATION OF [^{32}P] PHOSPHATE INTO NUCLEIC ACIDS OF NORMAL AND BORON-DEFICIENT TISSUE

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Abstract—The previously observed increased incorporation of labelled phosphate into the nucleic acids of boron-deficient bean radicles has been shown to affect soluble RNA, DNA and particularly ribosomal RNA. The results cannot be accounted for in terms of changes in the specific activity of 5'- phosphate in the total acid-soluble nucleotide pool of the tissue.

INTRODUCTION

RECENT studies[1] have demonstrated an increased incorporation of labelled precursors into the nucleic acids of radicles of boron-deficient "tick" beans (*Vicia faba* var. *minor*). Subsequent work to characterize further the effects involved have followed two lines. (1) To eliminate the possibility that the increased incorporation of [^{32}P] phosphate ($^{32}P_i$), observed by use of hot perchloric acid extraction, might be merely due to artefactual association of radioactivity with the nucleic acid fraction, nucleic acids were isolated by phenol treatment of the tissue and subsequently fractionated by chromatography on methylated serum albumin kieselguhr (MSA-K) columns. These fractionations were also used to compare the specific activities of the various types of nucleic acid. (2) On the hypothesis that the effect might be due to increased specific activity of nucleic acid precursors, acid-soluble nucleotides were extracted for determination of the specific activity of the 5'-phosphate moieties.

RESULTS AND DISCUSSION

Comparison of the Specific Activities of Nucleic Acid Fractions from Normal (B+) and Boron-deficient (B−) Root Tissue

Using extraction by phenol treatment followed by chromatography on MSA-K columns, comparisons were made of the specific activities of nucleic acid fractions from bean radicles which had been allowed to grow for 48, 24, 12 and 6 hr in a liquid nutrient medium in the presence or absence of boron (0·5 ppm) before being labelled with $^{32}P_i$. The fractionation yielded three nucleic acid peaks characterized as containing soluble RNA, DNA and ribosomal RNA, respectively, in agreement with the observations of other workers.[2, 3] It was

[1] S. CORY, L. R. FINCH and R. W. HINDE, *Phytochem.* **5**, 625 (1966).
[2] J. L. KEY and J. INGLE, *Proc. Nat. Acad. Sci. U.S.* **52**, 1382 (1964).
[3] J. INGLE, J. L. KEY and R. H. HOLM, *J. Mol. Biol.* **11**, 730 (1965).

observed that the specific activities of soluble RNA, DNA and particularly ribosomal RNA are greater in boron-deficient than in normal tissue. Table 1 presents results for the specific activities of DNA and ribosomal RNA in a number of experiments.

The general results of these fractionation studies confirmed the observations made by hot perchloric acid extractions.[1] The specific activity of nucleic acids increased with the distance of the tissue from the tip and with the time of growth in liquid medium before labelling, and was higher in deficient tissue than in normal tissue. The additional information obtained was that the increase in specific activity occurred for the three nucleic acid fractions from boron-deficient tissue, the most marked effect being for ribosomal RNA.

TABLE 1. COMPARISON OF SPECIFIC ACTIVITIES OF NUCLEIC ACID FRACTIONS FROM SECTIONS OF ROOTS LABELLED WITH $^{32}P_i$ AFTER VARIOUS TIMES OF GROWTH IN NORMAL (B+) AND BORON-DEFICIENT (B−) MEDIA

Section and time of growth in liquid medium	Nucleic acid fraction			
	DNA		Ribosomal RNA	
	B+	B−	B+	B−
	(Counts/min/ml/absorptivity unit at 260 nm)			
Second section (24 hr)	750	1180	1070	3300
Tip (12 hr)*	458	272	755	510
Second section (12 hr)*	1325	1630	1535	2190
Second section (6 hr)	372	418	790	800
Second section (6 hr)	200	251	394	480

* Both sections taken from the same radicles.

After the beans had grown for 12 hr in normal (B+) or boron-deficient (B−) liquid medium, the radicles were labelled by immersion for 50 min in 650 ml of dilute P_i solution (10^{-6} M) containing 1·0 mc of $^{32}P_i$. The radicles were sectioned into 2 mm tips and 3 mm second sections and extracts were prepared by phenol treatment and chromatographed on MSA-K columns as described in the text. The radioactivity per ml and the absorptivity (at 260 nm) of the fractions were determined. Radioactivity was corrected for decay to the time of labelling. Specific activities for the peaks were calculated by dividing the sum of the optical densities into the sum of the radioactivities for the fractions composing the peak.

Specific Activities of 5′-phosphate in Acid-soluble Nucleotides from Normal and Boron-deficient Radicles

The specific activities of 5′-monophosphate in acid-soluble nucleotides were compared for three sections from $^{32}P_i$-labelled radicles of boron-deficient and normal beans which had been grown for varying lengths of time in liquid medium. The results are presented in Table 2.

No difference could be detected between the specific activities of 5′-phosphate in nucleotides extracted from normal and boron-deficient tissue. In addition, although there is an increased incorporation up the root in both types of bean, the magnitude of this increase is not sufficient to account for the similar increase up the root in the incorporation of $^{32}P_i$ into nucleic acids.[1] It would thus appear that, either the increase in the labelling of nucleic acids is not a consequence of increased specific activity of precursor nucleoside 5′-monophosphates and must rather be due to an increased *incorporation* of nucleotides; or that such precursors form only a small and separate compartment of the total nucleotide pool being examined by the experimental procedures used. To investigate the latter possibility further would involve the isolation and examination of distinct nucleotide pools, particularly of the nucleus. Since all attempts to isolate intact nuclei from this tissue have so far been unsuccessful, including the

use of the homogenizing techniques of Chayen and Benfield,[4] further investigations of nucleotide labelling have not been attempted.

The nucleotide data would seem to preclude the possibility that the increased incorporation of $^{32}P_i$ into nucleic acids of deficient tissue is the result of a generalized increased permeability of deficient radicles to the radioisotope.

Since the increased incorporation has been found to occur as early as 3–4 hr after boron is removed from the growth medium,[1] it would appear that a lack of boron has an almost

TABLE 2. COMPARISON OF SPECIFIC ACTIVITIES OF NUCLEOSIDE 5′– MONOPHOSPHATE FROM SECTIONS OF ROOTS LABELLED WITH $^{32}P_i$ AFTER VARIOUS TIMES OF GROWTH IN NORMAL AND BORON-DEFICIENT MEDIA

Time of growth in liquid medium		Cpm/absorptivity at 315 nm: Tip (2 mm)	Second section (3 mm)	Third section (5 mm)
5 hr	β^{B-}	40 31	61 61	99 113
	β^{B+}	36 38	46 66	105 132
	δ	1·13 0·82	1·33 0·93	0·94 0·86
	Mean δ	0·96	1·13	0·90
10 hr	β^{B-}	42 46	75 63	154 143
	β^{B+}	46 45	61 74	158 151
	δ	0·91 1·02	1·24 0·85	0·97 0·95
	Mean β	0·97	1·05	0·96
15·25 hr	β^{B-}	50 49	93 90	174 158
	β^{B+}	74 64	91 90	204 157
	δ	0·68 0·77	1·02 1·00	0·85 1·01
	Mean δ	0·72	1·01	0·93

The beans were grown in liquid media for various times. 100 normal and 100 boron-deficient beans were labelled with 0·25 mc of $^{32}P_i$ for 35 min, there being duplicate sets each comparing fifty normal with fifty deficient beans. The radicles were then sectioned and the nucleoside monophosphates extracted and digested to P_i as described in the text. Specific activities were calculated from the ratios of radioactivity (counts/min $\times$ 10^{-3})—corrected for decay to the time of labelling—to phosphate content (optical density at 315 nm). The specific activities are paired vertically to correspond with the pairing during extraction. The ratio (δ) of the specific activity of deficient tissue (β^{B-}) to that of normal tissue (β^{B+}) was calculated for each pair and the mean taken. The results presented are from two separate experiments, one including labellings at 5 and 10 hr, the other a labelling at 15·25 hr.

immediate effect. Such a conclusion is in direct contrast to the usual assumption that a deficiency of boron does not exist until growth ceases, and that the delay in cessation of growth of the radicles is the result of the gradual depletion of an internal reserve of boron in the plant tissue (cf. Neales).[5] Further, the evidence of this and earlier studies[1, 6, 7] suggests that, in deficient radicles, a given section behaves more like a section found further from the tip in the normal root. Consideration of these facts in relation to the morphology of the

[4] J. CHAYEN and A. H. BENFIELD, *Exp. Cell Res.* **20**, 172 (1960).
[5] T. F. NEALES, *Aust. J. Biol. Sci.* **13**, 232 (1960).
[6] R. W. HINDE and L. R. FINCH, *Phytochem.* **5**, 619 (1966).
[7] R. W. HINDE, L. R. FINCH and S. CORY, *Phytochem.* **5**, 609 (1966).

tissues involved[5, 7, 8] has led to the conclusion that all the changes so far observed to accompany boron deficiency can be accounted for in terms of the degree of maturity, or differentiation, of the tissues. Thus, any investigation of the primary effect of boron deficiency in higher plants should be made very early after the removal of boron, in order to reduce the likelihood that any changes observed are only secondary effects.

EXPERIMENTAL

Cultivation, Labelling and Harvesting

Planting, labelling and harvesting procedures were as described previously.[1]

Extraction and Fractionation of Nucleic Acids

For extraction after the manner of Kirby,[9] 100 tissue sections were ground with 0·3 ml sodium dodecyl sulphate solution (10% w/v) in chilled mortars in the cold. The pulp was then transferred to a chilled glass homogenizer where it was dispersed in 1 ml of cold buffer (50 mM Tris pH 7·5, 5 mM $MgCl_2$, 0·5 mM EDTA, 0·1 M NaCl) by twelve passes with a loose-fitting nylon pestle. The homogenate was transferred to a Quickfit centrifuge tube together with 6 ml of buffer used to rinse the pestle and homogenizing vessel. 7 ml of cold aqueous phenol (equilibrated with buffer and containing 0·1% 8-OH quinoline) was added and the mixture shaken for 20 min at 0–4°. After centrifugation, the aqueous phase was largely freed of phenol and $^{32}P_i$ by dialysis against three changes of 3 l. of 5 mM EDTA which contained 0·1 M NaCl.

Subsequent chromatography was carried out on MSA-K columns (10 cm length × 2·2 cm dia.) prepared by the method of Monier *et al.*[10] The nucleic acids were eluted with a linear gradient from 0·3 M NaCl (300 ml) to 1·5 M NaCl (300 ml) containing 25 mM Tris, pH 7·5. Fractions (4 or 5 ml) were collected on an automatic fraction collector (Paton. Absorptivity measurements were made at 260 nm, with a Zeiss PMQ II spectrophotometer. Portions of the fractions were plated, dried and counted by a gas-flow counter equipped with micromil window (Nuclear-Chicago).

Extraction of Nucleotides followed by Hydrolysis to Nucleoside 5'-Monophosphates

The frozen sections were homogenized in 1 ml of cold methanol at 0° by fifteen passes with a loose-fitting nylon pestle, which was then rinsed with a further 2 ml of methanol. The extraction with methanol was allowed to continue for 40 min at 0°. After centrifugation, the supernatant was discarded. The procedure was repeated twice more, replacing the absolute methanol with cold methanol containing 0·05 M formic acid.

For extraction with cold perchloric acid, carried out in the cold room at 0–4° observing the precautions described previously,[1] the residue was suspended by homogenization in 1 ml of methanol–water (1:2 v/v). After washing the pestle with 2 ml of water, 2 ml of ice-cold 0·5 M perchloric acid was added to the homogenate, with stirring, the suspension being centrifuged immediately at 2000 *g*. The extract was retained and the procedure repeated a further three times with the modification that water (1 ml) replaced the methanol–water mixture. To the combined supernatants was added a heaped spatula of acid-washed kieselguhr and, after shaking for a few minutes, the mixture was allowed to stand for 1 hr at 0°.

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[9] K. S. KIRBY, *Biochem. J.* **64**, 405 (1956).
[10] R. MONIER, S. NAONO, D. HAYES, F. HAYES and F. GROS, *J. Mol. Biol.* **5**, 311 (1962).

The kieselguhr was then removed by filtration under reduced pressure in a microfilter tube[11] at room temperature. The filtered perchloric acid extracts were heated in a boiling water bath for 45 min, under which conditions there is complete hydrolysis of all pyrophosphate bonds. Some depurination occurs under these conditions, but any effect of this on the comparison between tissues was minimized by rigid standardization of the procedure and pairing of the corresponding B+ and B− samples. After cooling, the hydrolysed samples were shaken at room temperature with 0·1 ml packed volume of moist charcoal (approximately 70 mg dry wt.), prepared according to Plaisted and Reggio[12] and dispensed from a cut-off, 0·5 ml syringe. Two additional 0·1 ml aliquots of charcoal were added at 20 min intervals. After 1 hr the extracts were filtered under reduced pressure in micro-filter tubes. The charcoal was washed with distilled water until the effluent had a pH of about 5. The phosphate compounds retained on the charcoal after acid treatment were eluted by five washes with 1 ml lots of cold 0·5% NH_3 in 20% ethanol (v/v). (Identification of nucleotides in this material was made by high voltage co-electrophoresis with carrier AMP, UMP, GMP and CMP. The radioactivity was coincident with the u.v. absorbing spots of these nucleotides, with the major portion being in AMP.) The eluates were transferred to micro-Kjeldahl digestion flasks, in which they were evacuated to dryness in a water bath under a stream of air, drawn in through a capillary by reduced pressure. P_i was determined by the method of Michelson[13] as adapted by Collins,[14] the only difference being that the perchloric acid digestion was with 0·1 ml of 72% (w/v) perchloric acid; thus to establish the required pH, 5 ml of 0·15 M NaOH was used instead of 5 ml of water in the subsequent step. After determination of P_i content by the absorptivity at 315 nm, the colorimetric mixture was counted for ^{32}P.

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