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THE INCORPORATION OF [^{32}P]PHOSPHATE INTO NUCLEIC ACIDS OF NORMAL AND BORON-DEFICIENT BEAN ROOTS

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Abstract—An increased ability to incorporate [^{32}P]phosphate into nucleic acids has been observed in the radicles of beans (*Vicia faba* var. *minor*) grown in a boron-deficient liquid medium. This effect is most marked in the second section of the radicle (2–5 mm from the tip) where it is observable after as little as 4 hr in the deficient medium. This is an earlier effect of boron deficiency than any previously reported. In each section examined, both normal and deficient, the incorporation increases with the period of growth in the liquid medium before labelling and with the distance of the sections from the tip.

INTRODUCTION

MANY differences between normal and boron-deficient tissues have been observed, in both morphological and biochemical characteristics (see reviews[1, 2]). In the previous two papers[3, 4] it has been shown that such differences can be detected by changes in a number of biochemical characteristics before deficiency is manifested as a cessation of elongation. Thus, alterations in biochemical characteristics, observable near the point of cessation of growth, are likely to be only indirect symptoms of the deficiency. Alterations more fundamental to the process of deficiency, and thus more relevant to an understanding of the function of boron in plant growth, must be sought at earlier times after the removal of boron from the nutrient medium. The earlier such alterations can be detected, the more fundamental they are likely to be.

On the hypothesis that proteins are the effective agents of cellular function, it might be expected that changes in protein components would precede and underlie the morphological and physiological changes in deficiency. Before deficiency is otherwise observable, variations in levels of proteins might be detected by seeking evidence of changes in enzymic activities, in the antigenic patterns of the tissue or in components characterized by the techniques of protein fractionation. The search for any such change would be greatly assisted by some definition of its likely time of appearance.

The synthesis of new messenger RNA must presumably precede that of new proteins, so the earliest time at which a lack of boron affects the tissue could perhaps be delineated by seeking an alteration in the RNA fractions. The present studies of the labelling of nucleic acids in normal (B_+) and deficient (B_-) bean roots using [^{32}P]orthophosphate ($^{32}P_i$) were commenced to further this approach.

[1] H. G. GAUCH and W. M. DUGGAR, Jr., *Maryland Univ. Agr. Exp. Sta. Tech. Bull* A-80 (1954).
[2] J. SKOK, In *Trace Elements* (Edited by C. A. LAMB, O. G. BENTLEY and J. M. BEATTIE) p. 227. Academic Press, London (1958).
[3] R. W. HINDE and L. R. FINCH, *Phytochem.* **5**, 619 (1966).
[4] R. W. HINDE, L. R. FINCH and S. CORY, *Phytochem.* **5**, 609 (1966).

RESULTS AND DISCUSSION

Initially, several experiments with samples of ten roots of *Vicia faba* var. *minor* indicated that boron deficiency is accompanied by an increased incorporation of exogenous P_i into nucleic acids. This effect was apparent in each radicle section examined, but was rather variable. Since light is known to inhibit the growth of the radicle and the use of light could not be avoided during the procedures of planting, labelling and harvesting, it was thought that the lack of reproducibility might have resulted from changes in the conditions of illumination of the beans from one experiment to the next. An alternative explanation was that the sample of ten beans was not sufficiently large to overcome biological variation. To test these possibilities experiments were designed in which a larger number of beans was labelled and the conditions of illumination were varied.

Two experiments were carried out, one on normal tissue after 29·5 hr in liquid medium, the other on deficient tissue after 31 hr. The beans on one side of the labelling vessel were exposed to the incandescent light source and those on the other side were shielded. Each treatment was done in duplicate with a sample size of thirty or thirty-five beans. Table 1 presents the results of the incorporation as the specific activities (α) of the nucleic acid fractions for three sections of the radicles.

The specific activity of the nucleic acid fractions, from either normal or deficient tissue, was not affected by the different conditions of illumination. Thus the variability observed in the initial experiments is unlikely to have resulted from the slight variations in lighting conditions. However, the increased sample size greatly improved reproducibility. That the third section shows greatest variability may be related to the fact that this section is the one in which there is most variation in morphological appearance according to the length of the roots. Subsequent experiments were done in duplicate comparing fifty bean samples of normal and boron-deficient beans, under which conditions the difference between duplicates was found to be small compared to the difference between treatments.

To detect the earliest time of appearance of the increased incorporation of $^{32}P_i$ into the nucleic acids of boron-deficient tissue, the prior period of growth in liquid medium was successively reduced in three experiments (A, B and C) from 30 hr down to 1 hr. Each experiment consisted of labellings at three different times of growth, one of which overlapped with the labelling at the shortest time in the previous experiment. The growth curves determined for each of the experiments A, B and C by the method of Neales[5] are shown in Fig. 1.

The resultant specific activities of the nucleic acid fractions have been summarized in Table 2. It should be noted that each experiment contained within it a duplicate determination of specific activity since at each sampling time 200 roots were labelled, there being two duplicate sets of 50 B_+ being compared with 50 B_- (50 B_+/50 B_-). All extractions were done two at a time, pairing the corresponding B_+ and B_- samples, this being particularly important during the cold acid extraction (see Experimental). Within these pairs, it can be expected that the differences in α values are due to the effect of boron. The conditions for the duplicate B_+/B_- pair were as equivalent as possible, but allowance must also be made for the experimental error introduced by slight variation in the length of the cold acid extraction. In the experiments at less than 4 hr, it must be also borne in mind that the second set of 50 B_+/50 B_- roots could have been grown in a more deficient medium than the first set, if the boron level in the medium were still being reduced over the period of planting (see Experimental).

[5] T. F. NEALES, *Australian J. Biol. Sci.* **13**, 232 (1960).

TABLE 1. THE EFFECT OF LIGHT ON THE SPECIFIC ACTIVITY OF THE NUCLEIC ACIDS OF NORMAL AND BORON-DEFICIENT TISSUE

Treatment	Specific activity (α)					
	Tip (2 mm)		Second section (3 mm)		Third section (5 mm)	
Normal						
− light	2·4	2·8	6·2	7·1	19	36
+ light	2·6	3·5	6·8	9·1	19	20
Boron deficient						
− light	3·7	3·1	7·2	12	15	21
+ light	3·0	4·1	13	12	26	22

The beans were grown for 29·5 and 31 hr in B_+ and B_- liquid media respectively. In each case, 110 radicles were labelled with $^{32}P_i$ in boron-free water for 50 min. One half of the beans was exposed to an incandescent light source, while the other half was shielded. The radicles were then sectioned and the nucleic acids extracted as described in the text. Duplicate determinations were made. Specific activities were calculated from the ratio of counts/min to optical density at 260 nm, the values being normalized to a labelling solution giving 13,000 counts/min at the time of labelling. The specific activities are paired vertically according to the pairing during the experimental procedure (see p. 641).

In Table 2, three effects of the growth conditions are discernible. Firstly, there is an increased incorporation of $^{32}P_i$ into the nucleic acids of boron-deficient tissue. Secondly, the magnitude of the specific activities increases with the time the beans have been growing in liquid medium. Thirdly, the magnitude of the specific activity varies with the position of the section in the radicle.

The first effect is more obvious in the graphical presentation in Fig. 2, where the average ratio of the specific activity of the B_- fraction over the specific activity of the corresponding B_+ fraction has been plotted against the number of hours' growth in the liquid medium. The ratio (γ) was determined separately for each of the pairs of B_+ and B_- samples and then averaged. The graph thus shows the magnitude of the increased incorporation of ^{32}P into B_- tissue for the three sections after various times in the liquid medium.

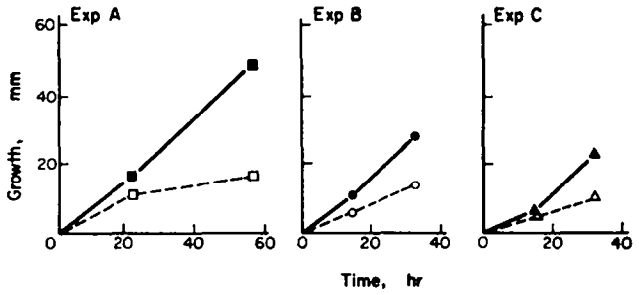

FIG. 1. GROWTH RATES OF NORMAL AND BORON-DEFICIENT *Vicia faba* var. *minor*.

There were approximately 250 beans in each vessel containing the liquid nutrient medium; of these, 16 radicles were measured at various time intervals. The sum of the growth increments for the several vessels was averaged and the resultant values plotted against time. Open symbols, boron-deficient tissue; closed symbols, normal tissue.

TABLE 2. COMPARISON OF THE SPECIFIC ACTIVITIES OF NUCLEIC ACIDS 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 (hr)		Tip (2 mm)	Second section (3 mm)	Third section (5 mm)
Experiment A				
9·25	α^{B-}	9·7 8·8	42 44	172 189
	α^{B+}	11 14	28 28	170 187
	Mean γ	0·7±0·1	1·5±0·1	1·0±0
14·5	α^{B-}	7·7 9·3	61 76	306 229
	α^{B+}	10 9·8	35 37	174 194
	Mean γ	0·85±0·1	1·9±0·2	1·5±0·3
30·3	α^{B-}	22 21	99 87	261 282
	α^{B+}	11 15	25 43	210 166
	Mean γ	1·7±0·3	3±1	1·5±0·2
Experiment B				
4·25	α^{B-}	6·4 5·5	18 19	84 106
	α^{B+}	5·4 6·4	12 11	80 85
	Mean γ	1·0±0·2	1·6±0·1	1·2±0·1
7	α^{B-}	7·7 7·9	21 27	122
	α^{B+}	7·3 6·9	16 15	113
	Mean γ	1·1±0	1·5±0·3	1·1
15	α^{B-}	5·6 7·7	49 40	
	α^{B-}	5·6 7·8	19 21	
	Mean γ	0·99±0	2·2±0·4	
Experiment C				
1	α^{B-}	8·3 6·0	6·1 7·7	29 39
	α^{B+}	8·3 6·5	6·5 8·8	21 38
	Mean γ	0·96±0·04	0·90±0·04	1·2±0·2
3	α^{B-}	9·0 11	9·9 14	64 46
	α^{B+}	10 11	9·7 12	50 65
	Mean γ	0·98±0·09	1·1±0·1	1·0±0·3
6·17	α^{B-}	15 13	15 17	47 52
	α^{B+}	16 14	12 12	37 49
	Mean γ	0·93±0	1·3±0·1	1·2±0·1

The beans were grown in B_+ and B_- liquid media for various times. 100 normal and 100 boron-deficient beans were labelled in the same vessel with $^{32}P_i$ in boron-free water for 50 min, there being duplicate sets each comparing 50 B_+ with 50 B_- beans. The radicles were then sectioned and the nucleic acids extracted as described in the text. Specific activities were calculated from the ratio of counts/min to absorptivity at 260 nm, the values being normalized to a labelling solution giving 13,000 counts/min at the time of labelling. The specific activities are paired vertically to correspond with the pairing during extraction. The ratio (γ) of the specific activity of boron-deficient tissue (α^{B-}) to that of normal tissue (α^{B+}) was calculated for each pair and the mean taken. Three separate experiments, A, B and C were carried out over a time period of 30 hr down to 1 hr.

The most marked effect on the incorporation of $^{32}P_i$ into the deficient tissue is in the second section where an increase is observed as early as 3–4 hr. This effect becomes more marked with longer times of growth in the deficient medium. A similar increase is observed in the third (5 mm) section, but here it appears later (12–15 hr) and does not continue to increase with time to the same extent. In the tip, the increase is not observed until well after 15 hr in deficient medium and it is possible that there may have been an inhibition of labelling at earlier times. In the preliminary experiments, on smaller numbers of beans, there was an

increased incorporation for the deficient tissue at least up to 52 hr, which was the latest time examined.

In both types of tissue, the incorporation of exogenous P_i into nucleic acids becomes greater the longer the beans are allowed to grow in the liquid medium. Figure 3 presents the increase in the magnitude of the specific activities plotted against the time of growth in liquid medium. Each average specific activity was divided by that of the first B_+ value in each series, which gave a comparison of the increase for tissues *within* each labelling series. To attempt to visualize the changes over the full period of 30 hr in liquid medium, it was assumed

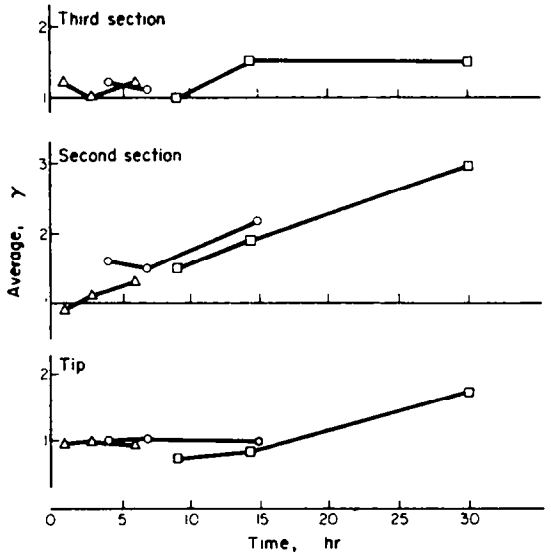

FIG. 2. COMPARISON OF THE INCORPORATION OF $^{32}P_i$ INTO THE NUCLEIC ACIDS OF BORON DEFICIENT AND NORMAL TISSUE.

The ratio (γ) of the specific activity of the B- nucleic acid fraction to the corresponding B_+ fraction was determined separately for each of the pairs of B_+ and B- samples and then averaged. The average ratio has been plotted against the number of hours' growth in the liquid medium. The three experiments, A, B and C are indicated by the symbols □, ○ and △ respectively.

that the first value in the later series would lie on the curve for the preceding series. The resultant corrected specific activities have been plotted in Fig. 3 against the time of growth in liquid medium.

It can be seen from the graph, that the specific activity of the nucleic acid fraction increases with the period of growth in liquid medium from 1 hr onwards. The effect occurs in both normal and deficient tissue, but is of greater magnitude in deficient tissue. In the second and third sections, the increase is fairly rapid up to approximately 15 hr, then slows down, particularly in the third section. The most marked increase occurs in the second section.

The third effect of the growth conditions in liquid medium, that the magnitude of the specific activity varies with the position in the radicle, can be seen by examination of Table 2. This has been illustrated in Fig. 4 by graphing the values obtained for 14·5 hr growth in liquid medium. The height of each column was calculated by dividing the average specific activity of the section by the average specific activity of the corresponding 2 mm tip. It can be seen that the specific activity increases on proceeding up the root. In general, the difference

between the specific activity of the tip and the third section of the same tissue increases with the time the roots have been allowed to grow in liquid medium. It can be seen that, under these conditions, the increase up the root occurs for both normal and deficient tissue, but is greater in the case of boron deficiency.

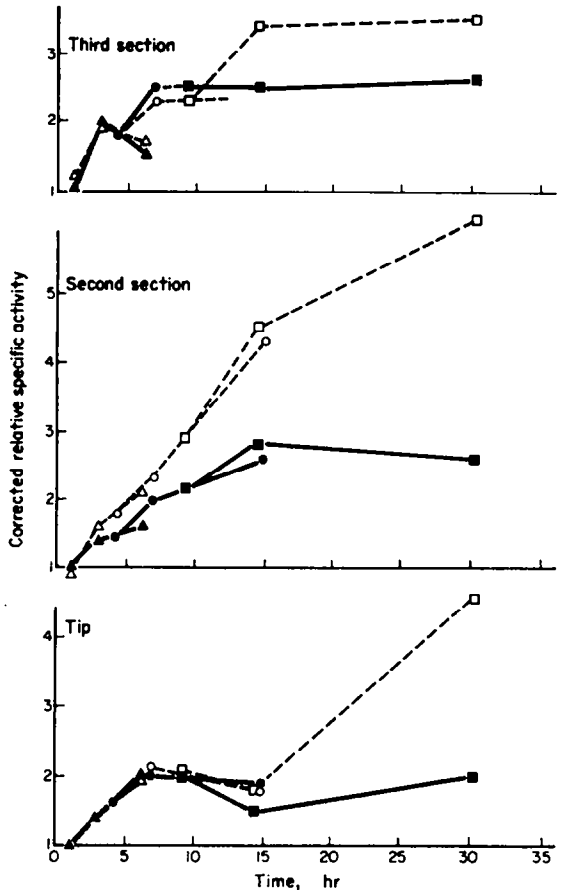

FIG. 3. EFFECT OF TIME OF GROWTH IN LIQUID MEDIUM ON THE INCORPORATION OF $^{32}P_i$ INTO THE NUCLEIC ACIDS OF NORMAL AND BORON-DEFICIENT TISSUE.

The magnitude of the mean specific activities from Table 2 has been plotted against the time of growth in liquid medium. Each average specific activity was divided by that of the first α^{B+} in each series, giving a comparison of the increase for tissues *within* each labelling series. To visualize the changes over the full period of 30 hr in liquid medium, it was assumed that the first value in the later series would lie on the curve for the preceding series. This corrected relative specific activity was the value used to plot against the time of growth in liquid medium. Results from the three separate experiments A, B and C are indicated by the symbols ■, ● and ▲ respectively for normal tissue and □, ○ and △ respectively for boron-deficient tissue.

Consideration was given to the possibility that the increased incorporation of $^{32}P_i$ into nucleic acids might be due to contamination by inorganic phosphate and nucleotides carrying over from the cold acid extraction of the tougher, deficient radicles. However, this appears unlikely. Any noticeable resistance of the deficient tissue to homogenization does not appear before about 48 hr in a B– medium, whereas increased incorporation is noted after about

4 hr. Furthermore, preliminary experiments have shown that the increased specific activity of the nucleic acids from deficient tissue is also observed in nucleic acid fractions obtained by extraction with phenol[6] followed by chromatography on methylated serum albumin columns.[7] When plant nucleic acids are extracted by the phenol method and fractionated on methylated serum albumin, they are clearly separated from nucleotides and inorganic phosphate (Finch, unpublished results). Thus the observations of an increased specific activity for the nucleic acid peaks from B– roots labelled with $^{32}P_i$, after 24 and 48 hr growth in liquid medium, are not due to contamination.

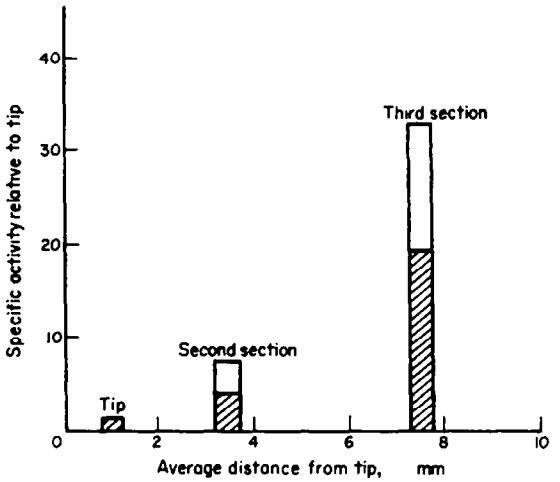

FIG. 4. VARIATION OF SPECIFIC ACTIVITY WITH POSITION OF THE SECTION IN THE ROOT.
■ Normal tissue □ Boron deficient tissue

The values obtained by dividing the average specific activity for the section by the average specific activity of the corresponding 2 mm tip have been plotted against the average position of the section in the root. The roots were those labelled after 14·5 hr growth in liquid medium.

The question then arises as to whether the differences in incorporation are the result of greater uptake of $^{32}P_i$ from the medium by the deficient radicles. The likelihood of incomplete recovery makes comparisons of the total counts extracted somewhat unreliable. However, such comparisons gave no consistent indication of greater uptake by the deficient tissue so that this possibility seems unlikely.

The other possibility, that the increased specific activity of the nucleic acids of deficient tissue is simply due to a decreased nucleic acid fraction, was not borne out by the observed patterns in the absolute amounts of nucleic acid present in each section. There is a decrease up the root, paralleling the tendency for the cells to be larger and therefore fewer. This could contribute to the increase in specific activity up the root. With longer periods of growth in the deficient medium, there is more nucleic acid in the first section, as would be expected from the thickening of the tip, but at the same time the α values *increase*. After 30 hr in liquid medium, the boron-deficient third section is thicker than the corresponding normal section so more nucleic acid is extracted from the former, and it is despite this that the specific activity is greater in the case of deficiency.

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

To the extent to which it might be composed of nucleotides, the "cold acid extract" could represent nucleic acid precursors and so its specific activity could be relevant. However, investigation by the method of Berenblum and Chain [8] showed that approximately 50 per cent of the counts were derived from P_i so that the specific activity of the "cold acid extract" is not representative of that of the nucleotides and these determinations were discarded. It is proposed to investigate the specific activity of the nucleotide precursors by an alternative method.

CONCLUSIONS

It has been shown that there is an increased incorporation of labelled precursors into the nucleic acids of the radicles of boron-deficient beans (*Vicia faba* var. *minor*) and in the section immediately behind the tip this effect has been observed as early as 4 hr after placing the seedlings in a deficient medium. In each section examined, the specific activity resulting from a 50-min labelling increases with the time the radicles have been allowed to grow in liquid medium. In both normal and deficient tissue the specific activity increases with the distance of the sections from the tip.

These results are of interest because they represent an observation of earlier effects of boron deficiency than any that has been reported previously. In part, the pattern of behaviour is similar to that already described for enzymic activities at later times [2, 3] in that the tissue of a given section in the boron-deficient root is becoming more like that of a higher section in the normal root. The tip of the root is slower in giving this response than is the second section. Examination of the basis of the effects is in progress.

MATERIALS AND METHODS

Cultivation

"Tick", or field, beans (*Vicia faba* var. *minor*) were germinated and cultured as described in the preceding papers.[3, 4]

Labelling and Harvesting

The labelling vessel was a square, 2·5 cm deep polythene container of 650 ml volume fitted with a polythene lid. Forty slots (3·2 × 19·0 mm), each able to hold five beans, were cut in the lid and were grouped into four sets of ten, two of which were for B_+ roots and two for B_- roots. In this way, 200 roots could be labelled in duplicate experiments comparing fifty normal and fifty deficient beans under equivalent conditions. The labelling solution, made up with metal-distilled water, contained 0·25–1·0 mc of $^{32}P_i$ with carrier added to give a final P_i concentration of less than 10^{-6} M.

When labelling the beans, five beans/min were removed at random from the liquid nutrient medium and their radicles washed and introduced into the radioactive solution through the appropriate slot of the lid. Groups of five B_- and five B_+ beans were taken alternately. After labelling for 50 min, the beans were removed five/min in the same order as they had been inserted, washed carefully and sectioned on an appropriately marked "Perspex" sheet. The sections used were tip (2 mm), second section (3 mm) and third section (5 mm). The sections were immediately placed in chilled beakers in ice, frozen with liquid nitrogen and stored at −15° until analysis.

[8] I. BERENBLUM and E. CHAIN, *Biochem. J.* **32**, 295 (1938).

The planting and labelling procedures were modified in experiments with roots planted in liquid medium for less than 4 hr. For these short periods in liquid medium, the 45 min required, for planting into the culture buckets, would lead to considerable variation in the time of growth in liquid medium and thus possibly in the extent of boron deficiency, if the beans were randomly selected for labelling. In these experiments, beans were rapidly planted into liquid medium through all holes in the "Perspex" sheet[5] except those marked for the labellings at 1 and 3 hr. The time was noted, then groups of ten beans/min were alternately planted in B_+ and B_- media through the holes in the marked areas. The areas that stretched across a measuring row[3,4] took twelve beans instead of ten, so that the measuring row contained beans representing all stages of the planting out.

One hour after the noted time, the beans were removed for labelling, five/min, in the same order as they had been planted. Thus, all the beans had had exactly 1 hr in the liquid medium before labelling. The remaining five beans from each group were used similarly for the labelling at 3 hr. The beans for the third labelling at 6 hr were those planted first, their period in liquid medium being assessed from the mean time of their planting.

In experiments to test the effect of light on the incorporation of $^{32}P_i$ into nucleic acids, one-half of the beans was subjected to incandescent light (50 W at 25 cm), whilst the other half was shielded from light (as far as possible) through the labelling and harvesting procedures.

Extraction and Estimation

The method of Finch and Carr[9] was modified for the extraction and estimation of nucleic acids. The extractions were carried out in 10 × 1·25 cm pyrex tubes suitable both for homogenization and centrifugation. The frozen sections were homogenized in 1 ml 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 30 min at 0°, then for 5 min at 37°. Next, 3 ml of chloroform was added, with stirring, and a further 5 min extraction at 37° allowed. After centrifugation, the supernatant was discarded. The procedure was then repeated, reducing the methanol extraction to 5 min at 37°. After a third homogenization in methanol, the chloroform was immediately added to the suspension and the extraction continued for a final 5 min. After centrifugation the supernatant was again discarded; the residue was washed twice with 6 ml of ether and dried at 37°.

For extraction with cold $HClO_4$, carried out at 0–4°, the residue was suspended by homogenization in 1 ml ethanol–water (1:2 v/v), the pestle being washed with 2 ml water. Then 2 ml of ice-cold 0·5 M $HClO_4$ was added, with stirring, and the suspension centrifuged immediately at 2000 *g*. The extract was retained and the procedure repeated a further three times with the modification that water replaced the ethanol–water mixture. The combined "cold acid extracts" were made up to 20 ml. For reproducible results, it was essential to carry out this cold acid extraction as rapidly as possible, the time required being approximately 10 min in all in these experiments. To minimize the effect of any variations in the conditions of extraction on the comparison between tissues, the entire procedure was carried out on only two samples at a time, with pairing of the corresponding B_+ and B_- samples.

The residue was next extracted by suspension in 3 ml of 0·5 M $HClO_4$ for 15 min at 70°. followed by centrifugation and collection of the supernatant. This procedure was repeated three times, the combined extracts being made up to 13 ml and referred to as the "hot acid extract".

[9] L. R. Finch and D. J. Carr, *Australian J. Biol. Sci.* **9**, 355 (1956).

The residues remaining after hot acid treatment were suspended in 10 ml of water for counting.

$^{32}P_i$ was counted in an M6 liquid counter of 9 ml capacity (20th Century Electronics).

Absorptivity was measured at 260 nm in a Zeiss PMQ II spectrophotometer.

The method of Berenblum and Chain [8] was used to determine P_i in the cold acid extracts.

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