[10] J. J. B. CANNATA AND A. O. M. STOPPANI, *J. Biol. Chem.*, in the press.
[11] D. T. O. WONG AND S. J. AJL, *J. Am. Chem. Soc.*, 78 (1956) 3230.
[12] J. A. OLSON, *J. Biol. Chem.*, 234 (1959) 5.
[13] G. FAVELUKES AND A. O. M. STOPPANI, *Biochim. Biophys. Acta*, 28 (1958) 654.
[14] V. BOLCATO, B. DE BENARD AND G. LEGGIERO, *Arch. Biochem. Biophys.*, 69 (1957) 372.

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SC 2269

Phosphatase reactions during tissue extractions

In a recent paper, ULLRICH AND CALVIN[1] showed that phosphatase in a spinach-chloroplast preparation was not immediately inactivated by cold methanol, and could catalyse the formation of methyl phosphate when inorganic phosphate was present. In this laboratory, methyl and ethyl phosphates were detected in extracts from plant tissues (*e.g.* potato-tuber slices) killed in boiling 80% methanol or 80% ethanol, and it appeared likely that these esters had also resulted from phosphatase-catalysed reactions. A partly purified potato phosphatase was prepared in order to test this. 50 g potato-tuber tissue was homogenized in 150 ml 0.03 M acetate buffer (pH 4.9) plus 0.005 M cysteine, and centrifuged (3000 × *g*, 15 min, 0–5°). The protein precipitating from the supernatant between 33% and 66% saturation with $(NH_4)_2SO_4$ at pH 6.5 was dissolved in distilled water, and from this solution was collected the protein fraction which precipitated between 40 and 60% saturation with $(NH_4)_2SO_4$ at pH 6.5. This precipitate was dissolved in distilled water and dialysed against several changes of distilled water and then, for 60 h, against 0.01 M acetate buffer (pH 4.9) plus 0.001 M $MgSO_4$. The dialysate was centrifuged (3000 × *g*, 15 min) and the supernatant (20 ml) frozen till required. It hydrolysed 83 μmoles *p*-nitrophenylphosphate per h per ml enzyme, at pH 4.9 and 22°. This represented 15% of the phosphatase activity of the original homogenate. In the presence of chloroform as a bacteriocide, this phosphatase lost less than 25% of its activity during 5 days at room temperature. The phosphatase was equally stable in 20% methanol.

The phosphatase synthesized a range of alkyl phosphates, including methyl-, ethyl-, *n*-propyl- and *n*-butyl- (but not *tert.*-butyl-) phosphates from the corresponding alcohols plus inorganic phosphate. Similar findings have been reported for other phosphatases[2,3]. Similarly, the phosphatase synthesized a mixture of glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate and mannose 6-phosphate (ratio 72:17:6:5) when in the presence of glucose and inorganic phosphate, and synthesized α-glycerophosphate and β-glycerophosphate (ratio 8:1) from glycerol and inorganic phosphate. All of the above reactions were also catalysed by a commercial alkaline phosphatase (EC 3.1.3.1) (*cf.* MORTON[4]).

When the partly purified potato phosphatase solution was dripped into boiling 80% methanol, inactivation was complete in less than 10 sec, and no methyl phosphate could be detected. However, when disks of potato-tuber tissue, 1 mm thick by 1 cm diameter, were plunged into the boiling 80% methanol and boiled for 3 min, a moderate amount of methyl phosphate was formed. Killing the tissue in 80% ethanol likewise produced ethyl phosphate. All this can be taken as evidence that

the phosphatase had been active for some time after the tissue slices had been immersed in the boiling alcohol. Apparently, in parts of the tissue slice alcohol penetrates and disrupts the cell membranes before the inactivation temperature for the phosphatase, about 50°, is reached. If methyl phosphate synthesis occurred during such tissue extractions, the hydrolysis of other phosphate esters could also have taken place.

Alternative methods of inactivating the tissue phosphatase at room temperature were sought, using the phosphatase extract as a model for study. Whereas 80% methanol gave only 70% inactivation at room temperature in 1 h, and 4% formaldehyde gave only 25% inactivation, 1% formic acid gave 75% inactivation in 1 min and 95% inactivation in 10 min. Formic acid at 1% in 80% methanol gave 100% inactivation in 1 min at room temperature. Formic acid in methanol was subsequently found to inactivate phosphatase in tissue slices at temperatures below 0°. This is illustrated in the following experiment. Potato-tuber disks, 1 mm thick by 1 cm diameter, were preincubated 45 min in [^{32}P]phosphate, washed 15 min in distilled water, and blotted dry. One 2-g sample of the tissue was killed by boiling 3 min in 50 ml methanol–chloroform–water (12:5:3, v/v) and then cooled to room temperature during 5 min (boil-killing). A second 2-g sample was simultaneously killed by plunging it into 50 ml methanol–chloroform–2.0 M formic acid (12:5:3, v/v), and held in a dry ice–ethanol bath (freeze-killing). The samples were then held at −15° for 4 h. Phosphate esters from each sample were subsequently extracted as described elsewhere[5], separated by two-dimensional chromatography and their radioactivity measured (Table I).

TABLE I

RADIOACTIVITY IN COUNTS/min OF PHOSPHATE ESTERS IN EXTRACT EQUIVALENT TO 0.20 g POTATO TISSUE

Compound	*Boil-killing*	*Freeze-killing*
Methyl phosphate	105	4
P_i	7600	4800
ATP	710	1740
UTP	185	370
GTP	95	250
ADP	960	215
UDP	100	120
GDP	210	230
AMP	110	110
UMP	125	110
Hexose phosphate	7870	8040
DPN	60	60
UDPG	445	49

The amount of methyl phosphate present in each extract can be taken as representing the extent of the phosphatase-catalysed reactions. Phosphatase action during freeze-killing must therefore have been less than 5% of that occurring during boil-killing. The slow phosphatase inactivation produced by boil-killing affected the distribution of ^{32}P in the endogenous phosphate esters, allowing a marked hydrolysis of nucleotide triphosphates to the corresponding diphosphates. The monophosphate esters were not affected. It is not likely that the results were caused by any heat-accelerated, non-enzymic hydrolysis, as two notably heat-labile compounds (DPN

and UDPG) were not altered in amount. These compounds might be expected to be relatively slowly attacked by a phosphatase.

On the basis of these experiments, a procedure has been selected which allows intact tissues to be killed and phosphate esters extracted with a minimum of phosphatase-catalysed hydrolysis[5].

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Fruit Research Division, D.S.I.R., Private Bag, Auckland (New Zealand) R. L. BIELESKI

[1] J. ULLRICH AND M. CALVIN, *Biochim. Biophys. Acta*, 57 (1962) 190.
[2] H. D. KAY, *Biochem. J.*, 22 (1928) 855.
[3] J. ULLRICH AND M. CALVIN, *Biochim. Biophys. Acta*, 63 (1962) 1.
[4] R. K. MORTON, *Biochem. J.*, 70 (1958) 139.
[5] R. L. BIELESKI AND R. E. YOUNG, *Anal. Biochem.*, in the press.

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D-Ascorbic acid and collagen synthesis

Although D-ascorbic acid has long been considered ineffective as an anti-scorbutic agent[1], BURNS *et al.*[2] reported that life and weight of guinea-pigs could be maintained and some scorbutic symptomatology prevented by giving D-ascorbic acid frequently enough to compensate for its rapid excretion. Since the animals still had hemorrhagic joints and abnormal predentin, these investigators concluded that D-ascorbic acid can replace some of the non-specific, anti-scorbutic activity of L-ascorbic acid, if its concentration in the tissues is maintained but that some activities of L-ascorbic acid are specific. The determination of whether larger or more frequent dosage of the D-isomer could prevent the characteristic and classical symptom of vitamin C deficiency, namely, an inability to form collagen during tissue repair[3,4], might help to delimit the possible mechanisms of ascorbic acid activity in connective-tissue metabolism. This paper reports the ability of D-ascorbic acid to promote collagen synthesis in the scorbutic carrageenan granuloma in guinea-pigs when administered in large doses twice daily.

The subcutaneous injection of carrageenan into guinea-pigs induces within 14 days formation of a massive granuloma. About 12% of the dry weight of the granuloma is collagen if the animals receive more than 5 mg of vitamin C daily; however, if the animals are fed a scorbutigenic diet for the 14 days, then a "scorbutic" granuloma is obtained containing about 2% collagen. Administration of L-ascorbic acid to the guinea-pigs at this time initiates rapid collagen synthesis and within 3 days the concentration in these "recovery" granulomas approaches 12% (see ref. 5). A direct proportionality between collagen and ascorbic acid concentration of granulomas has been demonstrated up to an ascorbic acid concentration of 50 μg/g (see ref. 4).

Since large amounts of D-ascorbic acid were to be used in this system, it was