Phytochemistry, 1974, Vol. 13, pp. 763 to 777. Pergamon Press. Printed in England.

MEVALONATE ACTIVATING ENZYMES AND PHOSPHATASES IN HIGHER PLANTS

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(*Revised received* 15 *October* 1973. *Accepted* 1 *November* 1973)

Key Word Index—*Phaseolus vulgaris*; Leguminosae; French bean; *Zea mays*; Gramineae; maize, mevalonate kinase; acid phosphatases; ATPase; pH optima.

Abstract—The pH optima of mevalonate kinase and phosphatases in green leaves, cotyledons and chloroplasts of French bean, and in green leaves and chloroplasts of maize, have been studied. Whereas in chloroplasts the pH optimum for mevalonate kinase is at pH 7·5 with little or no activity at pH 5·5, there is with leaf and cotyledon preparations appreciable activity at the lower pH. Under some circumstances isoelectric focusing studies have given fractions showing mevalonate kinase activity at only pH 7·5 or 5·5. Acid phosphatase and ATPase activity in preparations is maximal at pH 5·5 and is much reduced in the presence of high levels of phosphate. Other investigations reported concern the stability of mevalonate kinase and phosphatase activity at pH 5·5 and 7·5 on ageing of extracts, and the activity of mevalonate kinase on greening of etiolated French bean cotyledons. The influence of metal cofactors and fluoride on mevalonate kinase and phosphatase are reported.

INTRODUCTION

MUCH EVIDENCE on the structure, function and chemical composition of the chloroplasts of higher plants suggests that the carotenoid pigments and the phytyl side-chain of chlorophyll are synthesised *in situ* in the plastid.[1–3] Such results led to the concept of control of terpenoid synthesis in developing seedlings by a combination of intracellular segregation of enzymes and specific membrane permeability.[4] Some evidence for this proposal came from the location of mevalonate kinase (E.C. 2.7.1.36) in chloroplasts from French bean leaves,[5] and subsequently in the demonstration that isoenzymes of mevalonate kinase, differing in pH optimum, could be identified in homogenates of green and etiolated leaves.[6] Two peaks of activity were evident at *ca* pH 5·5 and 7·5, while in chloroplasts only the latter could be detected. In contrast a preparation from newly emergent etiolated cotyledons of French bean yielded a peak of activity at pH 5·5 with little activity at pH 7·5. In contrast to these findings was a preliminary communication[7] reporting inability to demonstrate the mevalonate kinase with acid pH

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[1] GOODWIN, T. W. (1958) *Biochem. J.* **70**, 612.
[2] GRIFFITHS, W. T., THRELFALL, D. R. and GOODWIN, T. W. (1964) *Biochem. J.* **90**, 40P.
[3] TREHARNE, K. J., MERCER, E. I. and GOODWIN, T. W. (1966) *Biochem. J.* **99**, 239.
[4] GOODWIN, T. W. and MERCER, E. I. (1963) *Symp. Biochem. Soc.* **24**, 37.
[5] ROGERS, L. J., SHAH, S. P. J. and GOODWIN, T. W. (1966) *Biochem. J.* **99**, 381.
[6] ROGERS, L. J., SHAH, S. P. J. and GOODWIN, T. W. (1966) *Biochem. J.* **100**, 14C.
[7] GRAY, J. C. and KEKWICK, R. G. O. (1969) *Biochem. J.* **113**, 13P.

optimum in French bean preparations. This work has recently received formal expression in detailed form.[8] Other workers[9] have reported identification of mevalonate kinase isoenzymes of optimum pH 7·9 in homogenates of *Pinus pinaster* and *Agave americana*. It is therefore pertinent to report some further studies on mevalonate kinase and of accompanying phosphatase activity in preparations from higher plants made subsequent to the first[7] of these contrasting reports.

RESULTS AND DISCUSSION*

An earlier study[6] showed that in homogenates of leaves and green cotyledons of French bean mevalonate kinase with peaks of activity at pH 5·5 and 7·5 could be demonstrated. Activity at pH 7·5 was associated with chloroplasts isolated by both aqueous and non-aqueous techniques.[5] Mevalonate kinase from *Cucurbita pepo* cotyledons,[10] *Pinus radiata* seedlings,[11] and oranges[12] have also been reported to have acid pH optima, though in general mevalonate kinases from animal sources show maximum activity at higher pHs. In contrast a recent study[7,8] has indicated insignificant mevalonate kinase activity at acid pH in both leaf and etiolated cotyledon preparations from French bean, though activity at pH 7·0 was readily demonstrated. The same workers were also unable to demonstrate activity associated with isolated chloroplasts. The earlier of these reports[7] prompted further investigation on our part and it may now be of interest to report some of the data obtained. The results reported were all obtained with crude enzyme preparations of leaves, cotyledons or chloroplasts. In such extracts in the presence of sufficient ATP and Mg^{2+} the phosphorylation of MVA is not limited to the mevalonate kinase catalysed formation of MVA-5P; further successive reactions catalysed by phosphomevalonate kinase (E.C. 2.7.4.2.) and pyrophosphomevalonate decarboxylase (E.C. 4.1.1.33) leading to formation of MVA-5PP and isopentenyl pyrophosphate, respectively, may be found. Thus at the termination of reaction in the incubation mixtures used MVA-5P, MVA-5PP and isopentenyl pyrophosphate in varying proportions might be found. As in previous work[6] all preparations were treated with polyclar-AT (insoluble polyvinylpyrrolidone) to remove phenolic compounds.[10]

pH-*Activity profiles*

The relationship of mevalonate kinase activity and pH for leaf, cotyledon and chloroplast preparations from French bean seedlings are shown in Figs. 1 and 2 and in Table 1. Rates of phosphorylation of MVA were measured by the radiochemical assay method (Figs. 1 and 2) or by spectrophotometric or chemical assay methods (Table 1) over the pH range 4·5–9·0. The data show that under the conditions used differing pH-activity profiles were obtained for mevalonate kinase activity in leaf and cotyledon preparations as contrasted to chloroplasts prepared by the non-aqueous procedure. In our hands significant activity was always detected in chloroplast preparations. With the one exception shown in Table 1, however, discrete peaks of activity at pH 5·5 and pH 7·5 in leaf and cotyledon preparations were not evident, though spectrophotometric

* *Abbreviations:* MVA—mevalonic acid; MVA-5P—mevalonic acid 5-phosphate; MVA-5PP—mevalonic acid 5-pyrophosphate; ATPase—adenosine triphosphatase.

[8] Gray, J. C. and Kekwick, R. G. O. (1973) *Biochem. J.* **133**, 335.
[9] García-Peregrín, E., Suarez, M. D. and Mayor, F. (1973) *FEBS Letters* **30**, 15.
[10] Loomis, W. D. and Battaile, J. (1963) *Biochem. Biophys. Acta* **67**, 54.
[11] Valenzuela, P., Cori, O. and Yudelevich, A. (1966) *Phytochemistry* **5**, 1005.
[12] Potty, V. H. and Bruemmer, J. H. (1970) *Phytochemistry* **9**, 99.

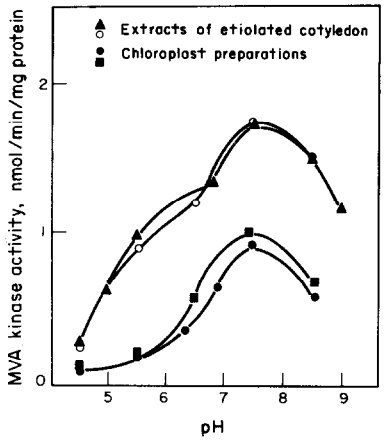

FIG. 1. EFFECT OF pH ON THE ACTIVITY OF MEVALONATE KINASE IN COTYLEDONS AND CHLOROPLASTS OF *Phaseolus vulgaris*.
Incubations contained the standard radiochemical assay mixture the buffer being: pH 4·0–5·5, acetate; pH 6·0–7·0, maleate; pH 7·0–9·0, Tris-HCl. Different symbols for the same type of preparation indicate experiments on separate occasions.

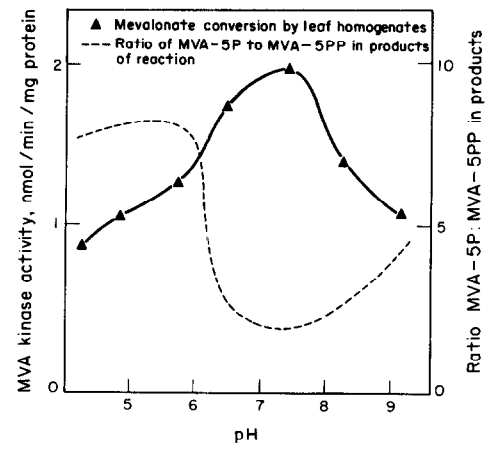

FIG. 2. EFFECT OF pH ON THE ACTIVITY OF MEVALONATE KINASE IN LEAVES OF *Phaseolus vulgaris*.
Incubations as in Fig. 1.

assays at pH 6·5 were inadvertently omitted, and in agreement with other workers[7] we did not in the present studies obtain mevalonate kinase activity almost entirely at pH 5·5 in extracts from etiolated French bean cotyledons. The quantitative difference in the present results to those reported earlier[6] may be due to two factors. In previous studies, through unidentified differences in quality of plant material or experimental method, the levels of mevalonate activating enzymes at both pH 5·5 and 7·5 were several-fold higher than those reported here or by other workers.[7] This difference is also reflected in the observation that in the present studies MVA-5P was the major reaction product in incubations whereas in the earlier study the major product was MVA-5PP. It is interesting that extracts of etiolated cotyledons of *Cucurbita pepo*, possessing high mevalonate kinase activity, also showed a pH optimum at 5·7 with much less activity at higher pHs.[10] A second equally important factor is that the present experiments, in contrast to those reported earlier,[6] were made throughout using buffer systems not containing supplemented inorganic phosphate. Later studies (Fig. 4) were to show that the presence of inorganic phosphate substantially reduces phosphatase activity. Such activity affects mevalonate kinase activity at pH 5·5 to a far greater extent than at higher pHs. Nevertheless in several experiments with leaf or cotyledon preparations by the three assay methods used mevalonate kinase activity at pH 5·5 was always substantial compared to that at pH 7·5, in marked contrast to chloroplast preparations. In the studies on extracts from green leaves there was a distinct difference in the reaction products at different pHs (Fig. 2). At the lower pHs the predominant reaction product was MVA-5P whereas at pH 7·5 amounts of MVA-5P and MVA-5PP were similar. Thus, with spectrophotometric and chemical assay methods the activity at pH 7·5 compared to that at pH 5·5 will reflect formation of ADP as the result of significant phosphomevalonate kinase activity in addition to ADP contributed by mevalonate kinase action. This factor should be taken into account when the results in Table 1 are considered.

TABLE 1. MEVALONATE KINASE ACTIVITY OF COTYLEDONS AND GREEN LEAVES

Tissue	Assay procedure	pH 4·5	5·5	6·5	7·5	9·0
Green leaf	Spectrophotometric	1·4	2·7	—	3·2	3·0
	Chemical assay	0·4	1·3	1·8	2·4	0·7
Cotyledon (green)	Spectrophotometric	2·1	4·6	—	7·3	2·4
	Chemical assay	0·8	1·4	0·9	3·0	0·6
(etiolated)	Chemical assay	1·6	2·8	4·5	5·0	3·7

Enzyme activities were measured by the spectrophotometric and chemical assay procedures, and are given as nmol MVA phosphorylated/min per mg protein.

Studies were also made on maize (Fig. 3) and in general these gave similar results to investigations on French bean. In short term (20 min) incubations (Fig. 3a) the pH-activity profiles for leaf and chloroplast preparations were quite different. Appreciable activity at pH 5·5 was evident in leaf extracts whereas little or no activity at this pH was shown by non-aqueously-prepared chloroplasts. At pH 5·5 MVA-5PP was the major reaction product while at pH 7·5 the reaction product was almost entirely MVA-5P. However, a study with the same preparation but using much longer incubation times (Fig. 3b) indicated an apparent absence of mevalonate activating enzyme activity at pH 5·5. Moreover, at pH 7·5 the conversion of MVA into phosphorylated products, which in this case included some isopentenyl pyrophosphate, was only approximately doubled though the time of incubation has been increased almost 5-fold. A similar observation has been reported elsewhere[12,13] and has been attributed to the action of phosphatases. The quantitative and qualitative nature of the reaction products identified after incubation of such extracts with MVA may therefore be dependent on a number of factors including activity of the preparation regarding mevalonate kinase, phosphomevalonate kinase, and phosphatases; the composition of the incubation medium; its pH; and the time of incubations.

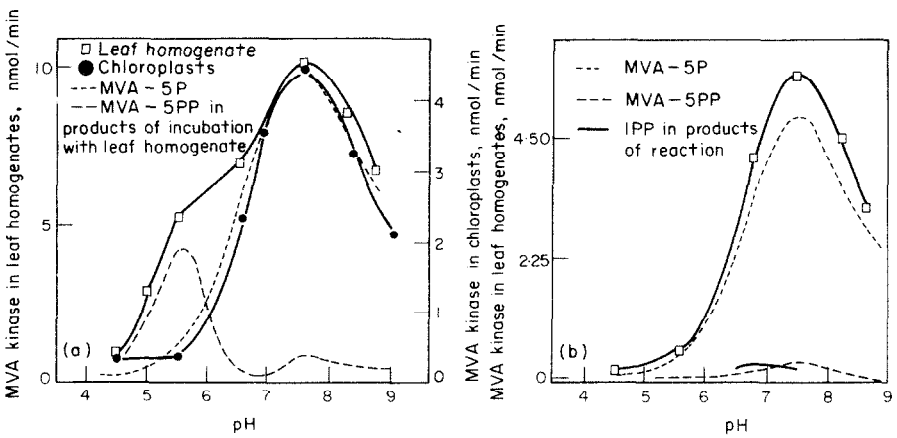

FIG. 3. EFFECT OF pH ON THE ACTIVITY OF MEVALONATE KINASE IN LEAVES AND CHLOROPLASTS OF *Zea mays*.

Incubations contained the standard radiochemical assay mixture the buffer being: pH 4·0–5·5, acetate; pH 6·0–7·0, phosphate; pH 7·0–9·0, Tris-HCl. Etiolated maize subjected to 2 days constant illumination was used. (a) The reaction was stopped after 20 min incubation. (b) The leaf homogenate was incubated for 90 min before reaction was stopped.

[13] THOMAS, D. R. and STOBART, A. K. (1970) *Phytochemistry* **9**, 1443.

The interference of phosphatases with assays of mevalonate activating enzymes in crude plant extracts was investigated by studying phosphatase activity in French bean preparations over the pH range 4–9. Some of this data are presented in Fig. 4 and shows that acid phosphatase (E.C. 3.1.3.2.) activity measured by *p*-nitrophenyl phosphate

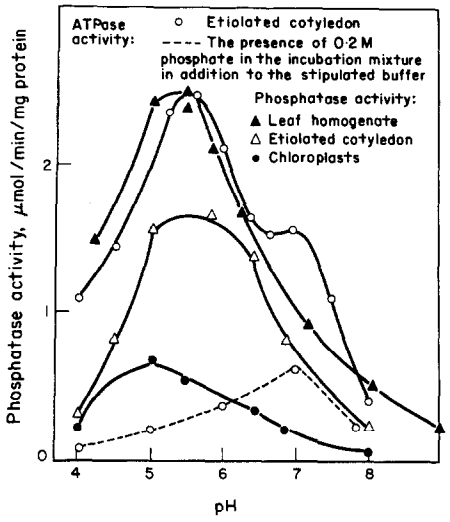

FIG. 4. EFFECT OF pH ON THE ACTIVITY OF PHOSPHATASES IN *Phaseolus vulgaris*.
Incubations contained the standard assay mixture, phosphatases being assayed by *p*-nitrophenyl phosphate hydrolysis and ATPase by ATP hydrolysis. The buffers were: pH 4·0–5·5, acetate; pH 6·0–7·0, maleate; pH 7·0 9·0, Tris-HCl.

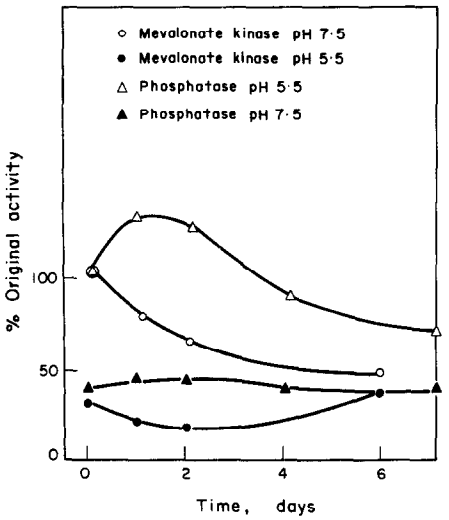

FIG. 5. STABILITY OF MEVALONATE KINASE AND PHOSPHATASE IN HOMOGENATES OF *Phaseolus vulgaris* LEAVES.
Incubations contained for mevalonate kinase the standard radiochemical assay mixture, and for phosphatase the standard assay of *p*-nitrophenyl phosphate hydrolysis. A 10 mg protein/ml extract from green leaves was maintained at 4°, activities of both enzymes being measured at pH 5·5 and pH 7·5 over a period of several days. Initial activities of the enzymes were: mevalonate kinase, 2·0 nmol/min per mg protein at pH 7·5; phosphatase, 2·5 μmol/min per mg protein at pH 5·5. The activities of mevalonate kinase at pH 5·5 and phosphatase at pH 7·5 are given relative to the activities of these enzymes at pH 7·5 and pH 5·5, respectively.

hydrolysis is some thousand-fold greater than that of mevalonate kinase in extracts of higher plant. In leaf, cotyledon and chloroplast preparations optimum activity was at *ca* pH 5·0–5·2 with much less activity above pH 7. Chloroplasts possessed much less acid phosphatase, which is thought to be largely unassociated with chloroplasts.[14] In agreement with other work[8] we found adenosine triphosphatase (ATPase E.C. 3.6.1.4) activity to be optimal at *ca* pH 5·5 but with a smaller peak of activity at *ca* pH 7·0. In the presence of high levels of phosphate ATPase activity at the lower pHs is largely eliminated, while that evidenced at pH 7·0 is reduced by some 50–60%. It was also observed that 10 mM Mg^{2+} reduced ATPase activity by some 40% though acid phosphatase activity was not significantly affected, even by 60 mM Mg^{2+}.

Investigation of the effect of high phosphate concentration on acid phosphatases indicated significant inhibition, an inhibitor constant K_i of *ca* 15 mM being obtained

[14] SLACK, C. R., HATCH, M. D. and GOODCHILD, D. J. (1969) *Biochem. J.* **114**, 489.

for phosphate levels from 0·05 to 0·2 M. At higher concentrations phosphate proved inhibitory to mevalonate kinase. The inhibition of phosphatase activity in homogenates of French bean leaves by fluoride ions was determined from a plot of reciprocal of acid phosphatase activity against fluoride concentration. This indicated non-competitive inhibition yielding an inhibitor constant, K_i, of 8×10^{-4} M for substrate concentrations of 1·65, 3·3 and 6·6 mM. Fluoride (0·6 mM) did not affect mevalonate kinase activity assayed at pH 5·5, where phosphatase activity was maximal, and activity was only slightly inhibited by 6·0 mM fluoride. Similar inhibition of phosphatases in plant extracts by fluoride has been reported elsewhere.[12]

The above data are in accord with studies by other workers. In higher plants several acid phosphatases appear to be present,[15,16] and these can differ in substrate specificity and MW, perhaps through aggregation or disaggregation of a smaller number of discrete species. Presence of such phosphatases may show seasonal dependence[17] while there may also be a diurnal variation in enzyme activity.[18] A soluble ATPase has been identified in higher plants, having a broad pH optimum at *ca* pH 5·5 and little activity at high pH.[15,19] This may originate from release and activation of a particulate phosphatase such as the ADP-kinase (ATPase) in chloroplasts, involved in photophosphorylation.[20] Chloroplasts of *Euglena* contain a highly active membrane-bound Mg^{2+}-dependent ATPase with optimum activity at pH 5·5 with a smaller peak of activity at pH 8·0.[21] The latter may be due to a Ca^{2+}-dependent soluble ATPase showing some activity with magnesium ions.[22] Similar data have been reported for chloroplasts from higher plants.[23]

Evidently presence of such phosphatases in plant extracts would adversely affect the assay of mevalonate kinase, through removal of ATP necessary for phosphorylation of MVA, and through degradation of the phosphorylated products of mevalonate metabolism. Thus isopentenyl phosphate, a product of phosphatase action on the pyrophosphate, has sometimes been found in incubation mixtures.[24]

We examined the effect of phosphatases from a French bean cotyledon extract on MVA-5P, the incubation mixture containing: MVA-5P, 80 nmol, 20000 dpm; buffer, 160 μmol; and 2 mg protein (80% $(NH_4)_2SO_4$ precipitate), in a total volume of 0·8 ml. Incubation was at 30° and after 60 min the reaction was terminated by heating at 80° for 2 min. Aliquots of the supernatant were chromatographed[5] and the conversion of MVA-5P to MVA determined. This was *ca* 0·3 nmol MVA-5P/min per mg protein and *ca* 0·15 nmol MVA-5P/min per mg protein, at pH 5·5 in acetate buffer, and pH 7·5 in Tris-HCl buffer, respectively. The conversion at pH 5·5 was inhibited some 70%, and at pH 7·5 some 50%, in the presence of 0·2 M phosphate. In extracts of germinating pea seeds little MVA was formed at pH 7·4 from MVA-5P.[25] In practice the possible degradation of MVA-5P in plant extracts may not be a serious difficulty, at least at the higher pHs, since it has been observed by other workers[13] that after long incubation times

[15] MEYER, H., MAYER, A. M. and HAREL, E. (1971) *Physiol. Plant.* **24**, 95.
[16] ROBERTS, D. W. A. (1970) *Enzymologia* **39**, 151.
[17] LYUBIMOVA, M. N. and FAIN, F. S. (1961) *Biochemistry* **31**, 915.
[18] MISHRA, D. and MOHANTY, B. (1967) *Planta* **75**, 239.
[19] WESSELS, J. S. C. and BALTSCHEFFSKY, H. (1960) *Acta Chem. Scand.* **14**, 233.
[20] LYNN, W. S. and STRAUB, K. D. (1969) *Proc. Nat. Acad. Sci. U.S.* **63**, 540.
[21] CARELL, E. F. and KAHN, J. S. (1967) *Biochem. Biophys. Acta* **131**, 571.
[22] CHANG, I. C. and KAHN, J. S. (1966) *Arch. Biochem. Biophys.* **117**, 282.
[23] CARMELI, C. (1969) *Biochem. Biophys. Acta* **189**, 256.
[24] POTTY, V. H. and BRUEMMER, J. H. (1970) *Phytochemistry* **9**, 1229.
[25] GREEN, T. R. and BAISTED, D. J. (1970) *Anal. Biochem.* **38**, 130.

the proportion of products in the form of MVA-5PP compared to MVA-5P, increases. In a number of studies[5,10,24,25,26] appreciable amounts of MVA-5PP, often exceeding levels of MVA-5P, have been found in the products of incubation with MVA. However the conversion of MVA to phosphorylated products with time may sometimes be observed to reach a maximum and then, at longer incubation times, apparently decline.[12] Initially the rate of conversion of MVA to MVA-5P may exceed the rate of breakdown of MVA-5P by phosphatases, but when available MVA is sufficiently depleted the two enzyme activities may be in balance. In other studies on French bean leaf or cotyledon extracts phosphatases hydrolysed MVA-5P, MVA-5PP and isopentenyl pyrophosphate at rates considerably in excess of MVA phosphorylation.[8]

Subcellular localisation of mevalonate kinase

In earlier studies[5] little difficulty was found in demonstrating mevalonate kinase activity in chloroplast preparations, particularly those prepared using non-aqueous methods. In contrast other workers[8] have reported very low activity of chloroplasts prepared by aqueous or non-aqueous methods. In the present studies mevalonate kinase activity in chloroplasts prepared by the non-aqueous method could be readily demonstrated (Figs. 1 and 3).

A further study was made on chloroplasts prepared from maize, following illumination of etiolated seedlings for 48 hr. The stepwise fractionation procedure of Smillie[27] was used and the results obtained are briefly summarised in Table 2.

TABLE 2. DISTRIBUTION OF MEVALONATE KINASE IN *Zea mays* LEAVES

Density (g/cc) of fraction	<1·30	1·30–1·37	>1·37
Total chlorophyll (%)	37	9	54
Mevalonate kinase (%)	62	13	25

Maize leaves were freeze-dried, homogenised in CCl_4–hexane mixture and fractionated according to density (see text for details).

Maize possesses two morphologically distinct types of chloroplast, the mesophyll cells containing chloroplasts that resemble, in size and presence of grana, those of plants utilising the Calvin cycle. These plastids do not accumulate starch in contrast to the small grana-less chloroplasts found in parenchyma sheath cells.[28] Partial separation of the two types of chloroplast from plants illuminated for some time can be made by density fractionation of freeze dried leaves in non-aqueous media.[14] The chloroplasts from mesophyll cells occur in the fraction of density less than 1·30, those from parenchyma sheath cells and thus containing starch concentrate in fractions of density greater than 1·37. It was found (Table 3) that the mevalonate kinase activity was concentrated in the fractions of highest and lowest density with little in the cellular material of intermediate density. This is taken to indicate presence of mevalonate kinase in both types of chloroplast, though perhaps predominantly in mesophyll chloroplasts since these occur to some extent also in the higher density fraction.[14] The observation, however, does not preclude the occurrence of some enzyme extrachloroplastidically. An enzyme largely or

[26] GREEN, T. R. and BAISTED, D. J. (1972) *Biochem. J.* **130**, 983.
[27] SMILLIE, R. M. (1963) *Can. J. Botany* **41**, 123.
[28] LAETSCH, W. M. (1968) *Am. J. Botany* **55**, 875.

completely unassociated with chloroplasts would be concentrated in the fraction of density greater than 1·37 with more or less equal amounts in the lower density fractions. Its occurrence in fractions would not correlate with chlorophyll distribution.[14]

TABLE 3. EFFECT ON LEAF MEVALONATE KINASE OF INTERMEDIATES OF ISOPRENOID BIOSYNTHESIS AND RELATED COMPOUNDS

	Farnesol	Geraniol	Gibberellic acid	β-Carotene	Squalene	Lanosterol	Sitosterol
pH 7·5	33	66	110	100	66	88	52
pH 5·5	130	80	135	150	100	224	100

Enzyme activities were measured at pH 7·5 and pH 5·5 by the radiochemical procedure. Each standard incubation contained the following compounds at the conc indicated, solubilised in dimethyl sulphoxide (final conc 10% v/v). Activities are given as a percentage relative to controls containing 10% dimethyl sulphoxide but with no other additions. Farnesol, geraniol and gibberellic acid (GA_3) were added to give a final concentration of 1 mM, 10 μg of the other compounds were added to the standard 1 ml incubation mixture.

Localization of mevalonate-activating enzymes in chloroplasts has also been convincingly demonstrated by independent observations that isolated French bean chloroplasts in the presence of appropriate cofactors will readily convert MVA into phytoene[29,30] and into geranylgeraniol,[30] two obligatory intermediates beyond isopentenyl pyrophosphate in the pathway of carotenoid biosynthesis from MVA.

In studies with chloroplasts prepared in aqueous media only slight mevalonate kinase activity was detected. This might be expected since soluble enzymes are readily leached out of chloroplasts isolated in aqueous media, including for example, alkaline inorganic pyrophosphatase[31] and ribulose diphosphate carboxylase,[32] but are readily demonstrated when non-aqueous isolation methods are used.[27]

It is known that during chloroplast development there is a substantial increase in the protein content of the leaf, most of the new protein being located within the chloroplasts. Thus in greening *Euglena* increases in enzymes such as $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase and fructose 1,6-diphosphatase, which are confined to chloroplasts, roughly parallel increases in chlorophyll content whereas extrachloroplastidic enzymes such as aldolase and transketolase do not show this correlation.[27] The observation that in greening French bean leaves increase in mevalonate kinase paralleled increases in chlorophyll and protein has, however, been interpreted as indicating that mevalonate kinase was not likely to be chloroplastidic.[8] In contrast, a similar observation for *Kalanchoë crenata* tissue cultures was thought to indicate the association of appreciable mevalonate kinase activity with chloroplasts.[13]

Stability of mevalonate kinase

The activities of both mevalonate kinase and acid phosphatase in French bean extracts stored for prolonged periods at 4° were studied. The results (Fig. 5) indicated that phosphatase activity at pH 5·5 increased markedly over the first 24 hr but then slowly decreased over the next 5 days to *ca* 70% the original activity. The initial increase may be due to a number of factors including release of latent enzyme from membranes.

[29] CHARLTON, J. M., TREHARNE, K. J. and GOODWIN, T. W. (1967) *Biochem. J.* **105**, 205.
[30] BUGGY, M. J., BRITTON, G. and GOODWIN, T. W. (1969) *Biochem. J.* **114**, 641.
[31] SIMMONS, S. and BUTLER, L. G. (1969) *Biochem. Biophys. Acta* **172**, 150.
[32] SMILLIE, R. M. and FULLER, R. C. (1959) *Plant Physiol.* **34**, 651.

Phosphatase activity at pH 7·5 remained more or less constant with only a slight increase in activity evident over the first 48 hr.

Mevalonate kinase activity assayed at pH 7·5 decreased markedly over the first 48 hr to some 65–70% the original activity; thereafter loss of activity was slower and after 6 days 50% of the original activity still remained. Decrease in activity over the first 48 hr of ageing was also reflected in mevalonate kinase activity assayed at pH 5·5 and presumably results from the increased phosphatase activity of the extracts over the same time period. Ageing for longer times led to recovery of mevalonate kinase activity at pH 5·5, correlating with decrease in phosphatase activity to somewhat lower levels than present in the original extract. Extracts stored at −15° for two months showed unchanged mevalonate kinase activity. Mevalonate kinase is therefore a comparatively stable enzyme.

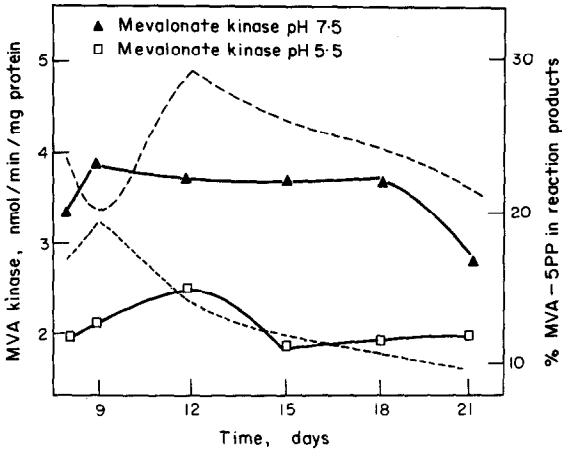

FIG. 6. MEVALONATE KINASE ACTIVITY IN ETIOLATED COTYLEDONS OF *Phaseolus vulgaris* FOLLOWING ILLUMINATION.

Incubations contained the standard radiochemical assay mixture. Plants were germinated in the dark and at day 8 were transferred to continuous light. Cotyledons were taken at the times indicated and extracts prepared by the standard method. Percentage of the reaction products as MVA-5PP are given by — —, pH 7·5; ----, pH 5·5.

It has been proposed[8] that mevalonate kinase activity at pH 5·5 may result from interaction of contaminating phenols in plant extracts with native (pH 7·0) enzyme. Thus heterogeneous mixtures of mevalonate kinase enzymes differing in charge could be identified on DEAE-cellulose chromatography of leaf extracts when polyphenols were present. However, in previous studies,[6] and those outlined here, care had been taken to minimise polyphenols present in extracts by treatment with polyclar-AT.[10] This interesting suggestion, therefore, has yet to be substantiated experimentally.

We also studied levels of mevalonate kinase at intervals over several days after etiolated French bean cotyledons had been exposed to light. The results (Fig. 6) showed that mevalonate kinase activity at pH 7·5 increased over the first 24 hr illumination but thereafter remained virtually constant until some 10 days after exposure to light when activity declined. The initial increase is emphasised if units of enzyme activity are considered since formation of the plastids will be accompanied by appreciable protein synthesis. Other workers[29] have similarly shown an enhancement in incorporation of MVA into chloroplast terpenoids following illumination of etiolated plants. Increase in

mevalonate kinase activity measurable at pH 5·5 was maintained for some 4 days after initial illumination, but then declined to and maintained the original activity until the experiment was ended.

The proportion of reaction products in the form of MVA-5PP rather than MVA-5P differed for assays at the two pHs. For mevalonate activating enzymes measured at pH 7·5 the proportion of products in the form of MVA-5PP declined over the first 24 hr, then increased over the next 72 hr. Over this time period activity measured at pH 5·5 showed the opposite behaviour. Thereafter at both pHs the proportion of MVA-5PP declined until the experiment was concluded. Throughout the experiment, however, more MVA-5PP was formed at the higher pH, in agreement with the observations shown earlier in Fig. 2.

Effect of thiol reagents

French bean cotyledon mevalonate kinase activity assayed at both pH 5·5 and 7·5 was almost completely inhibited by $HgCl_2$ or *p*-chloromercuribenzoate at 0·1 mM. Iodoacetamide was much less effective and at the 10 mM level only inhibited activity at pH 5·5 by some 20%, while not affecting activity assayed at pH 7·5. In all cases inhibitions could be alleviated by addition of excess β-mercaptoethanol. These data are in general agreement with those published elsewhere[8] though in one case iodoacetamide has been found to have a greater inhibitory effect.[10]

Metal ion activation

The effect of various metal ions on mevalonate kinase activity at pH 7·5 and acid phosphatase activity at pH 5·5 was studied. Figure 7 relates the inhibition or stimulation of enzyme activity by supplemented metal ion compared to controls in which no metal ion was included in the incubation medium. At 5·0 mM Mg^{2+} was more effective than Mn^{2+} in stimulating mevalonate kinase activity, though at 0·5 mM this situation was reversed. At levels of metal cofactor normally used in incubations we thus find Mg^{2+} to be the most effective cofactor, in agreement with Gray and Kekwick[8] but in contrast to other workers.[10] The difference may be explained by the observation that under the conditions used Mn^{2+} stimulated acid phosphatase activity. Acid phosphatase activity was not stimulated by supplemented Mg^{2+} at either concentration, though 6·6 mM Mn^{2+} increased activity by nearly 100%. The latter observation is in contrast to that reported elsewhere.[33] The most effective metal ion was Fe^{2+} and it is somewhat surprising therefore that this metal ion at 0·5 mM enhanced mevalonate kinase activity. Though not thought to be metalloenzymes it has been reported[34] that metal ions activate acid phosphatases of leaf extracts.

Mevalonate kinase activity at pH 7·5 was increasingly stimulated by Mg^{2+} until at 8 mM activity was some eight-fold that of unsupplemented extracts. Thereafter stimulation was less effective with increasing Mg^{2+} concentration and at 30 mM stimulation was only 5-fold. Mevalonate kinase activity at pH 5·5 increased with Mg^{2+} concentration to some 6-fold the activity of unsupplemented extracts with 15 mM Mg^{2+}, thereafter stimulation was less effective though activity was still some 4-fold greater than that of unsupplemented extract at 30 mM Mg^{2+}.

[33] Beytia, E., Valenzuela, P. and Cori, O. (1969) *Arch. Biochem. Biophys.* **129**, 346.
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Levels of ATP greater than 10 mM proved inhibitory to mevalonate kinase at both pH 5·5 and 7·5 presumably because of the stoichiometric combination of ATP with divalent metal ion required in mechanisms postulated for mevalonate kinase action.[35] As indicated elsewhere[10,29] the ratio of ATP/Mg^{2+} may be more important than the absolute concentrations.

Effect of terpenoid intermediates

The effect on mevalonate kinase activity of a number of terpenoid intermediates and related compounds was examined. The compounds used were those immediately obtainable from other research in progress in the laboratory and were solubilised in dimethyl sulphoxide, the concentration of the latter being adjusted to a final concentration of 10% in the incubation mixture. Activities were compared to controls containing 10% dimethyl sulphoxide but no added terpenoid. This level of dimethyl sulphoxide reduced assayable activity at pH 7·5 by some 10% and at pH 5·5 by some 50%. Iodoacetamide (10 mM) was added to incubations to block conversion of MVA-5PP to isopentenyl pyrophosphate, previous study having shown that this level of inhibitor would not affect mevalonate kinase.

The results are shown in Table 3 and indicate inhibition of mevalonate kinase at pH 7·5 by farnesol, geraniol and a number of sterols. Inhibition of mevalonate kinase from various sources by prenyl pyrophosphates has been previously reported.[8,36,37]

The effect of these additions on mevalonate kinase activity assayed at pH 5·5 was somewhat different in nearly all cases, a stimulation of activity being seen in a number of cases, in particular by lanosterol. The stimulations at pH 5·5 may, however, be due to a protective effect of the added compounds against dimethyl sulphoxide, which itself reduced mevalonate kinase activity at pH 5·5 by some 50% in controls lacking other additions. The effects of these additions on phosphatase activity were not studied. Interpretation may therefore be premature until these interesting effects have been confirmed by the continuing studies now in progress.

Isoelectric focusing studies

An attempt was made to separate molecular species of mevalonate kinase by isoelectric focusing. In a separation over the pH range 3–10 we used *ca* 80 mg protein obtained from an 80% $(NH_4)_2SO_4$ fractionation of 5-day-old French bean cotyledons. A green precipitate was observed to collect near the anode during isoelectric focusing but this did not appear to interfere with the separation or with the subsequent collection of fractions. Following separation 10 ml fractions from the column were dialysed for 24 hr against 0·01 M phosphate, pH 7·5, and were then assayed for mevalonate kinase and acid phosphatase activity at both pH 7·5 and 5·5. Because of the low levels of enzyme present the standard radiochemical assay for mevalonate kinase was modified in that DL-MVA-[1-^{14}C] of high specific activity (5 Ci/mol) was used (the results of this experiment are shown in Fig. 8).

Of particular interest is the distribution of mevalonate kinase and acid phosphatase with pH. In agreement with studies by other methods[15,16] several peaks of acid phosphatase activity could be identified. The distribution of these was the same at both

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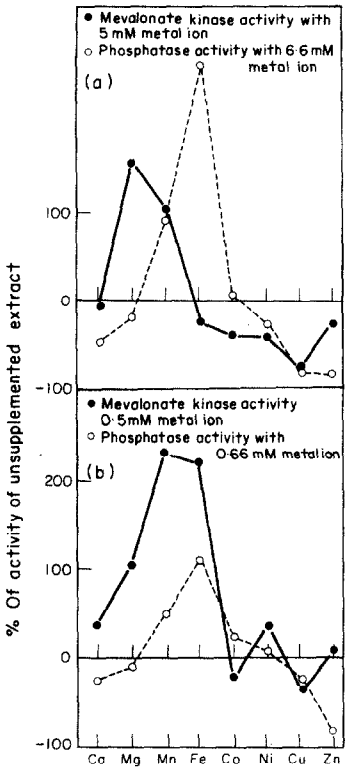

FIG. 7. EFFECT OF METAL COFACTOR ON THE ACTIVITIES OF MEVALONATE KINASE AND PHOSPHATASE.

Greened cotyledons from 5-day-old French bean seedlings were extracted and the protein fraction precipitating between 30 and 60% $(NH_4)_2SO_4$ taken for assay after removal of $(NH_4)_2SO_4$ by passage through Sephadex G25, elution being by 0·1 M Tris-HCl (pH 7·5). Mevalonate kinase and phosphatase activities were determined at pH 7·5 by the standard methods except for bivalent metal ion concentration which was varied as indicated. The activities of unsupplemented cotyledon extract were mevalonate kinase, *ca* 0·5 nmol/min per mg protein; phosphatase, *ca* 0·8 μmol/min per mg protein.

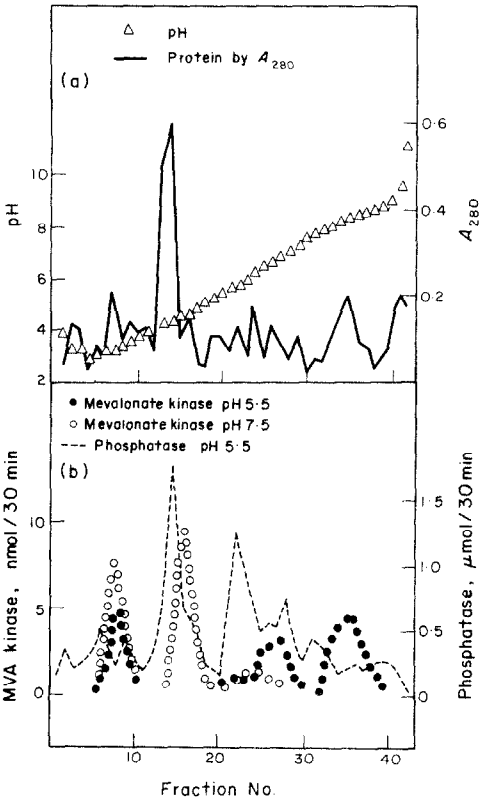

FIG. 8. ISOELECTRIC FOCUSING OF PROTEIN EXTRACTED FROM GREENED COTYLEDONS OF *Phaseolus vulgaris*.

Isoelectric focusing was made over a pH range 3–10 using 1·2% ampholine. The run was of 72 hr duration at 8° using a voltage 260–340 V. 80 mg protein precipitated from 80% $(NH_4)_2SO_4$ was applied to the column. At the end of the run 10 ml fractions were collected from the column and dialysed for 24 hr against 0·01 M phosphate, pH 7·5. 0·2 ml of each fraction was used in assay of mevalonate kinase, 0·1 ml in assay of phosphatase. Mevalonate kinase and phosphatase were assayed by the standard methods except for the former DL-MVA-[1-^{14}C] (5 Ci mol) was used. The distribution of phosphatase activity measured at pH 7·5 was similar though activity was markedly lower.

pH 5·5 and 7·5 though activities were much lower at the higher pH. Three peaks of mevalonate kinase activity at pH 7·5 could be identified at pIs of 3·3, 4·7 and 6·3. In contrast peaks of activity at pH 5·5 occurred at pIs of 3·3, (5·6), 6·9 and 8·3. Thus only one peak at each of the two pHs was coincident.

A second experiment was carried out over the pH range 3–6. On this occasion 240 mg protein was applied to the column and following isoelectric focusing fractions were directly assayed for mevalonate kinase and acid phosphatase in the presence of ampholine and sucrose, the dialysis of these being omitted. The presence of ampholine did not

significantly affect determined enzymes activities, only a 4% loss being observed at pH 7·5, while sucrose had no effect on activities though it caused streaking of MVA on subsequent chromatography.[5]

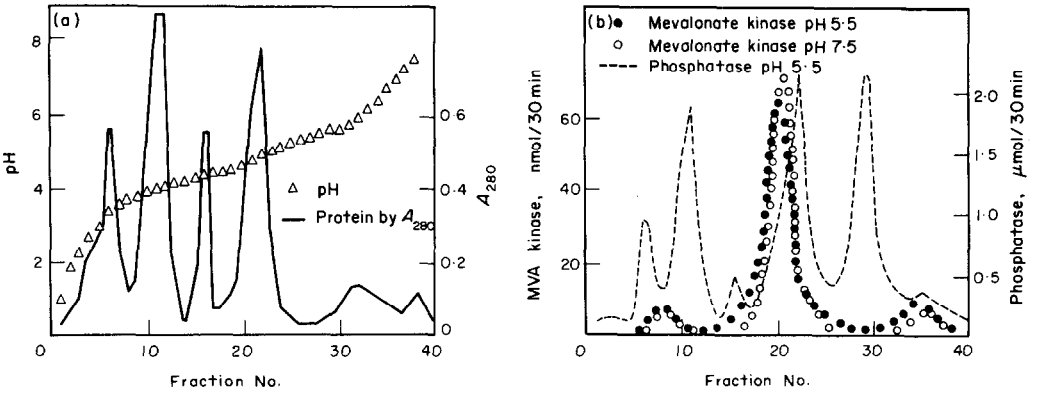

FIG. 9. ISOELECTRIC FOCUSING OF PROTEIN EXTRACTED FROM GREENED COTYLEDONS OF *Phaseolus vulgaris*. The experiment was similar to that in Fig. 8 except that isoelectric focusing of 240 mg protein was carried out over the pH range 3–6. The duration of the run was 42 hr using a voltage 260–510 V and the dialysis stage before enzyme assay was omitted.

The results of this experiment are shown in Fig. 9. Several peaks of acid phosphatase activity were again evident but a significant difference was seen in the distribution of mevalonate kinase activity three peaks being evident these being coincident for activities measured at both pH 7·5 and 5·5. The pIs for the three molecular species were 3·6, 4·7 and 6·5, in good agreement with the peaks of activity assayed at pH 7·5 in isoelectric focusing over the pH range 3–10. The major activity was focused at the intermediate pI value. The reason for the differences in distribution of mevalonate kinase assayed at pH 5·5 in the two experiments cannot be explained. Though the peaks at pIs 6·9 and 8·3 would be outside the range of isoelectric focusing at pH 3–6, mevalonate kinase activity at pH 5·5 in the first separation was not evident at pIs 4·7 and 6·3. It may be significant that mevalonate kinase activity solely at pH 5·5 in the pH 3–10 focusing was only found where phosphatase activity on the gradient was minimal. It may also be suggested that over the longer time period of the first experiment changes in mevalonate kinase occurred conferring activity at pH 5·5 though that at pH 7·5 was lost. This might be due to enzyme degradation yielding isoelectrically distinct molecular species. However, this would probably yield a heterogeneous distribution of activity at ill-defined pIs, and not the clear separation obtained. Moreover, an earlier study (Fig. 5) showed that activity at both pH 5·5 and 7·5 in extracts was stable for several days. Although cotyledon extracts contain little or no polyphenolic material[8] they were nevertheless treated with polyclar-AT.[10] The possibility of interaction of polyphenols with native enzyme to produce heterogeneous molecular species was therefore unlikely.

Two fractions with mevalonate kinase activity at pH 7·9 have been separated from *Pinus pinaster* and *Agave americana* by gel filtration on Sephadex G100 of crude extracts.[9] The larger molecular species appeared to be eluted close to the void volume of the column indicating a MW probably somewhat in excess of the 100000 reported by other workers[8] for mevalonate kinase of optimum pH 7·0 isolated from French bean. It is not

known which of the molecular species now identified by isoelectric focusing would correspond to these purified preparations.

The present studies indicate that mevalonate kinase activity at acid pHs in leaf and cotyledon preparations from higher plants is very dependent on the levels of phosphatase and mevalonate activating enzymes. In contrast mevalonate kinase activity at pH 7·5 does not appear to be significantly affected by the phosphatases present. When preparations of low mevalonate kinase activity but high phosphatase activity are used in investigations mevalonate kinase activity at acid pHs may thus be particularly difficult to demonstrate, even though activity at higher pHs is clearly evident. Isolated chloroplasts, in contrast to crude cellular extracts, evidenced a sharp optimum at pH 7·5 with no indication of significant activity at acid pHs, and substantial amounts of mevalonate kinase must be located in this intracellular compartment.

The existence of mevalonate kinase isoenzymes with pH optima at 5·5 and 7·5, respectively, therefore cannot be yet dismissed. Nevertheless the previous and present studies reported on this important enzyme are the subject of continuing investigation.

EXPERIMENTAL

Growth of plants. Seed of *Phaseolus vulgaris* cv. Lightning and *Zea Mays* cv. Rhodesian White Tooth Double Hybrid were germinated in vermiculite and grown at 25° in continuous light.

Special chemicals. DL-mevalonic acid lactone-[2-^{14}C] was obtained from the Radiochemical Centre, Amersham. The lactone was converted to the free acid by incubation with a 2% molar excess of KOH for 30 min at 40°. Polyclar-AT, before use, was boiled in 10% HCl, washed with dist. H_2O and MeOH and dried.

Preparation of MVA-5P. A soluble yeast extract was incubated with DL-MVA-[2-^{14}C] the incubation mixture at pH 7·0 containing: DL-MVA-[2-^{14}C], 8 μmol, 1 μCi; ATP, 20 μmol; $MgCl_2$, 30 μmol; phosphate, 150 μmol; iodoacetamide, 10 μmol; and yeast extract equivalent to 10 mg protein, in a final vol. of 0·5 ml. Incubation was at 30° and after 60 min reaction was terminated by heating to 80° for 2 min. The supernatant was subjected to chromatography[5] and after development and detection of radioactivity by scanning with an Actigraph the areas corresponding to phosphorylated intermediates were eluted with 60% MeOH overnight at 4°. The eluates were freeze-dried and rechromatographed until a pure sample of MVA-5P was obtained.

Buffers. The following buffers were used for the pH range indicated: sodium acetate, pH 4·0–5·5; maleate, pH 5·0–7·0; KH_2PO_4–K_2HPO_4, pH 6·0–7·5; Tris-maleate, pH 5·0–8·0; Tris-HCl, pH 7·0–9·0; Na_2CO_3–$NaHCO_3$, pH 9·0–11·0.

Radiochemical assay of mevalonate kinase. Standard incubation mixtures contained: DL-MVA-[2-^{14}C], 2 μmol, 2 Ci/mol; ATP, 10 μmol; $MgCl_2$, 15 μmol; buffer, 200 μmol; mercaptoethanol, 10 μmol; and 2–3 mg protein, in a total vol. of 1·0 ml. Incubations were normally carried out at 30° and were terminated by heating to 80° for 2 min. Chromatography of reaction products and assay of their radioactivity enabling calculation of enzyme activity were as described previously.[5]

Spectrophotometric assay of mevalonate kinase. This involved continuous assay using a coupled enzyme system for assay of ADP. The incubation mixture initially contained: NADH, 2 μmol; buffer, 0·5 mmol; and 0·2 ml mevalonate kinase preparation in a total volume of 2·7 ml. The A_{340} change was continuously monitored and assessed NADH-oxidase activity of the preparation. Then KCl, 5 μmol; $MgCl_2$, 5 μmol; phosphoenol-pyruvate, 2 μmol; lactic dehydrogenase, 30 μg protein–10 U; pyruvate kinase, 10 μg protein–1·25 U; and ATP, 3 μmol, were added. The change in A_{340} with time enabled assessment of ATPase activity in the preparation. Finally MVA, 5 μmol, was added the changed rate of A_{340} decrease enabling assessment of mevalonate kinase activity. The amounts of lactic dehydrogenase and pyruvate kinase were such that their activity was at least 100-fold greater than that of mevalonate kinase at all pHs studied.

Chemical assay of mevalonate kinase. The incubation mixture contained: $MgCl_2$, 15 μmol; ATP, 10 μmol; DL-MVA, 5 μmol; buffer, 375 μmol; and 4–6 mg protein, in a final vol. of 1·5 ml. Incubations were made at 30° and were terminated by addition of 0·5 ml cold 10% w/v trichloroacetic acid. ADP formed was assayed by a coupled enzyme system after neutralisation to pH 7·0 addition being made to the incubation mixture of: NADH, 2 μmol; $MgSO_4$, 5 μmol; phosphoenolpyruvate, 2 μmol; pyruvate kinase, 100 μg–12·5 U; and lactic dehydrogenase, 30 μg–10 U; to a final volume of 3·0 ml. The A_{340} change was followed until reaction had ceased thus enabling ADP to be determined. In all three types of assay control incubations containing no MVA were included in each experiment.

ATPase assay. Whenever feasible ATPase activity was assayed by determining inorganic phosphate released from ATP on hydrolysis. The incubation mixture contained: ATP, 10 μmol; Mg^{2+}, 5 μmol; buffer, 170 μmol;

and *ca* 1 mg protein, in a final vol. of 1·0 ml. Incubations were at 30° and reactions were terminated by addition of 1·0 ml 10% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifugation and inorganic phosphate in solution assayed by a standard procedure.[38] When phosphate buffers were used ATPase activity was assayed by measuring ADP produced in a given time. The incubation mixture was as indicated above but after termination of reaction with trichloroacetic acid the solution was neutralised to pH 7·0. Additions were then made as indicated earlier for chemical assay of mevalonate kinase. An estimate of ATPase activity could also be made from change in A_{340} before addition of MVA in the spectrophotometric assay of mevalonate kinase.

Phosphatase assay. This was made by measuring the release of *p*-nitrophenol from *p*-nitrophenyl phosphate. The incubation mixture contained: *p*-nitrophenyl phosphate, 150 μmol; buffer, 150 μmol; and the appropriate amount of protein in a total vol. of 1·5 ml. Incubations were at 30° and were terminated by heating to 80° for 2 min. 0·5 ml of the soln was then transferred to 3·0 ml 1 M Tris-HCl, 0·4 M KH_2PO_4, pH 8·5, and the A_{415} determined against a H_2O blank. The *p*-nitrophenol content of the solution was read from a standard curve relating A_{415} to *p*-nitrophenol concentration at the appropriate pH.

Protein determination. Protein was determined by the biuret method[39] or the Folin method[40] using bovine serum albumin as a standard. When presence of Tris interfered with colour development in the former method appropriate controls were included in the assay. The protein content of column eluates is given as A_{280}, expressed in enzyme activities as protein concentration through use of the relationship for a 1 cm light path cuvette; protein (mg/ml) = F × A_{280} the factor F being determined from the literature.[41]

Chlorophyll determination. The method of Arnon[42] was used.

Preparation of plant tissues. French bean and maize leaves, etiolated or green as occasion demanded, were washed with cold H_2O and then cut into small pieces which were homogenised in 0·05 M KH_2PO_4 pH 7·5 (1 ml buffer/g wet wt) at 2–4° for 12 × 15 sec periods in an Ato-mix blender. The homogenate was immediately transferred to a mortar containing 0·5 g Polyclar-AT g wet wt tissue and the two blended by pestle. The resulting suspension was squeezed through two layers of cheesecloth and the residue discarded. French bean cotyledons were homogenised in 0·05 M KH_2PO_4, pH 7·5 (1 ml/g wet wt) with a mortar and pestle at 0–4° in the presence of 0·5 g Polyclar-AT/g wet wt and 0·5 g acid-washed sand/g wet wt. The homogenate was then squeezed through two layers of cheesecloth and the residue discarded. The homogenates from both types of preparation were centrifuged at 600 *g* for 5 min, the pellet being discarded, and then at 20000 *g* for 20 min both centrifugations being at 4°. The supernatant was routinely used in assays as soluble enzyme extract.

Chloroplast isolation in aqueous media. Chloroplasts from French bean leaves were prepared by discontinuous sucrose gradient centrifugation according to Leech.[43]

Chloroplast isolation in non-aqueous media. Chloroplasts from French bean were prepared as described previously[5] or by a modification of the stepwise fractionation procedure of Smillie.[27] The latter method was also used for isolation of chloroplasts from maize leaves. Solubilisation of enzymes from non-aqueously prepared chloroplasts was achieved by suspension of 0·01 M KH_2PO_4, pH 7·5 at 4° and sonication for 30 sec.

Concentration of protein solutions. When necessary solns were concentrated by Sephadex G25 using basket centrifuge tubes, or by treatment with the calculated quantity of dehydrated polyacrylamide gel (Lyphogel, Gelman Instrument Co.).

Isoelectric focusing. Isoelectric focusing was carried out in an LKB Electrofocusing column. Gradients of pH were established by using Carrier Ampholytes of the selected pH range to a final concentration of 1·2% w/v. The pH gradient was supported in a density gradient of 0–50% sucrose prepared using an LKB gradient mixer. The anode compartment contained 50% w/v sucrose containing 8% w/v conc. H_2SO_4, and the cathode compartment 1% w/v NaOH. The sample in appropriate sucrose concentration was introduced on to the column half-way through gradient formation. Details of individual electrofocusing experiments are given in the text. The runs were made at 8° and at the conclusion of the run fractions of 10 ml each were collected at a rate of 2 ml/min. The pH of each fraction was measured with a pH meter at room temp. and protein determined by A_{280}.

Acknowledgements—We gratefully acknowledge financial support from the Science Research Council for purchase of a scintillation counter used in this and related investigations, and for a research studentship (H.M.H.).

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