The Regulation of Acetyl Coenzyme A Synthesis in Chloroplasts

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The enzymatic activities of the pyruvate dehydrogenase complex (PDC) and acetyl-CoA synthetase (ACS) have been compared in extracts of plastids isolated from spinach leaves and from both green and etiolated pea seedlings. All plastid preparations were shown to be capable of synthesizing acetyl-CoA, not only *via* acetyl-CoA synthetase, but also *via* the pyruvate dehydrogenase complex, though, with different activities. Both pathways are apparently under metabolic control. Thus, the substrate levels in photosynthetically active spinach chloroplasts appear to favor acetyl-CoA synthesis via ACS (apparent K_m for acetate of 0.1 mM), because calculated stromal pyruvate levels (0.1 mM) appear to limit its formation *via* the PDC (apparent K_m for pyruvate of 0.2–0.3 mM). In spinach chloroplasts, therefore, the PDC pathway seems to be predominantly involved in providing precursors for branched-chain amino acid biosynthesis (valine, leucine and isoleucine). Acetyl-CoA, synthesized via ACS, may additionally function as an inhibitor of the chloroplast PDC, because, as in mitochondria, relatively low concentrations of the end products NADH and acetyl-CoA strongly inhibit the PDC in chloroplast extracts. On the other hand, comparatively high concentrations of MgATP, a cofactor for ACS, inhibited the PDC complex. The pH optimum of about 8 and the high Mg-requirement distinguishes both enzymes from mitochondrial PDC and reflects an accomodation to stromal conditions in photosynthetically active chloroplasts.

Introduction

In the plastid a wide variety of products, including fatty acids [1] and isoprenoids [2–4], are ultimately derived from acetyl-CoA. For the generation of chloroplast acetyl-CoA the following alternative pathways are available [5, 6]:

I. Acetyl-CoA synthetase:

$$\text{Acetate} + \text{CoASH} + \text{ATP} \xrightarrow{\text{ACS}} \text{Acetyl-CoA} + \text{AMP} + \text{pyrophosphate}$$

In mature spinach leaves acetate [7], provided by acetyl-CoA hydrolysis in mitochondria [8], has been established as the main source of chloroplast acetyl-CoA. This pathway was further defined by the observations that, in the spinach leaf cell, acetyl-CoA synthetase is exclusively localized in the chloroplast stroma [5] and acetate diffuses freely through chloroplast membranes [9, 10]. However, the relatively high energetic cost of hydrolysis of acetyl-CoA within the mitochondria and its regeneration within the chloroplasts, as well as missing acetyl-CoA-hydrolyzing activities in pea mitochondria [11, 12], make a generalization of this pathway dubious.

II. Pyruvate dehydrogenase complex

Total reaction:

$$\text{Pyruvate} + \text{NAD}^+ + \text{CoASH} \xrightarrow{\text{PDC}} \text{Acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

Component enzyme reactions [6]:

E_1: Pyruvate decarboxylase (EC 1.2.4.1) (a+b):
a) Pyruvate + TPP-E_1 → CO_2 + hydroxyethyl-TPP-E_1
b) Hydroxyethyl-TPP-E_1 + lipoyl-E_2 → S-Acetyldihydrolipoyl-E_2 + TPP-E_2

E_2: Lipoate acyltransferase (EC 2.3.1.12) (c):
c) S-Acetyldihydrolipoyl-E_2 + CoASH → Acetyl-CoA + dihydrolipoyl-E_2

E_3: Lipoamide dehydrogenase (EC 1.6.4.3) (d+e):
d) Dihydrolipoyl-E_2 + FAD-E_3 → Lipoyl-E_2 + reduced FAD-E_3
e) Reduced FAD-E_3 + NAD^+ → NADH + H^+ + FAD-E_3

Acetyl-CoA formation from pyruvate occurs in mitochondria [13, 14] and in proplastids of plant tissue [6, 15]. However, attempts to demonstrate its

Abbreviations: ACS, acetyl-CoA synthetase; CoA, coenzyme A; CS, citrate synthetase; DTT, dithiothreitol; MDH, malate dehydrogenase; PDC, pyruvate dehydrogenase complex; TPP, thiamine pyrophosphate.

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occurrence within the chloroplast compartment have produced contradictory results [7, 16–20]. Some authors report either the absence [17, 18] or trace amounts [7] of PDC activities in spinach chloroplasts, while others were able to detect significant activities in pea chloroplasts [19, 20].

The present report investigates three possible sources for these discrepancies, namely:

1. the lability of the loosely aggregated PDC, which within chloroplast extracts appears to allow individual constituents of the complex to be measured independently from the others [21], *e.g.* pyruvate decarboxylation (E_1) [7, 17, 18] or dihydrolipoamide dehydrogenase activities (E_3) [22];

2. unfavorable assay conditions, considering that this highly regulated, multifunctional enzyme complex [7, 19–25] appears to be involved not only in acetyl-CoA but also in branched-chain amino acid synthesis *via* hydroxyethyl-TPP and acetolactate [23–26] within chloroplasts.

3. differences in the acetyl-CoA synthesizing activities in plastids from different plant materials.

Materials and Methods

Spinach cultivation and the preparation and critical examination of intact chloroplasts have been described earlier [27]. Pea seedlings (*Pisum sativum* L. var. Kleine Rheinländerin) were grown in darkness for 10 days in moist Vermiculite [28] in the greenhouse and the shoots were harvested either at the end of a dark period or following a subsequent exposure to light. In order to prevent peroxisomal contamination, the chloroplast preparations were further purified on preformed Percoll gradients according to Mourioux and Douce [29]. For etioplasts and chloroplasts from peas the gradient was formed from 40% Percoll. Chloroplast crude extracts were prepared by two different methods:

1. The chloroplasts were lysed by addition of 0,2% (w/v) Triton X-100 (for 1 min in the light) and immediately freed of their substrates by elution over a short Sephadex G25 column according to Laing and Roughan [30].

2. The chloroplasts were frozen in liquid nitrogen in the presence of DTT (8 mg/10 ml) and 2 mM $MgCl_2$, lyophilized, resuspended in buffer medium (100 mM Tricine, pH 8) and centrifuged at 30000 × *g* for 15 min until a yellowish supernatant was obtained (Randall *et al.* [14]). The chlorophyll concentration was determined in the suspension before centrifugation.

Protein concentration was determined by the method of Lowry *et al.* [31]. Aliquots of these chloroplast extracts containing 30 µg of either chlorophyll or protein were used for the following tests:

Assay of pyruvate dehydrogenase

The assay for pyruvate-dependent NAD reduction was modified after [20]. Normally, the standard reaction mixture contained: 50 mM Tricine/KOH (pH 8), 0.2 mM thiamine pyrophosphate, 5 mM $MgCl_2$, 1.5 mM NAD^+, 0.5 mM lithium-CoA, 2 mM dithiothreitol or 2.6 mM cysteine-HCI, 1.2 mM sodium pyruvate and chloroplast extract with a final chlorophyll concentration of 30 µg.

Assay of acetyl-CoA synthetase

1. Corresponding to recent investigations [27] the reaction mixture contained: 100 mM Tricine/KOH (pH 8.0), 2 mM $MgCl_2$, 1 mM ATP, 0.5 mM CoASH and 0.5 mM acetate. Acetyl-CoA formation was measured by converting acetyl-CoA (reactions a and b) to citrate. The rate of utilisation of oxaloacetate was followed by measuring NADH formation (reaction c) spectrophotometrically at 340 nm. The reaction sequence is as follows:

a) acetate + CoASH + ATP $\xrightarrow{ACS}$ acetyl-CoA + AMP + pyrophosphate

b) acetyl − CoA + oxalacetate + H_2O $\xrightarrow{CS}$ citrate + CoASH

c) malate + NAD^+ $\overset{MDH}{\rightleftharpoons}$ oxalacetate + NADH + H^+

Coupling of reaction (a) with reactions (b) and (c) was achieved by simultaneous addition of malate (10.6 mM) and 20 µl aliquots of malate dehydrogenase (21.2×10^3 U/l) and citrate synthetase (3.89×10^3 U/l) to the above standard reaction mixture.

2. The radioactivity assay by [1-^{14}C]acetate incorporation and adsorption of the ^{14}C-labelled acetyl-CoA to charcoal [5] has been described recently [27].

All the assays were performed at 20 °C.

Results and Discussion

In *E. coli*, the lipoamide dehydrogenase (E_3) activity of the PDC complex, measured by the reduction of NAD^+ by exogenous dihydrolipoamide as sub-

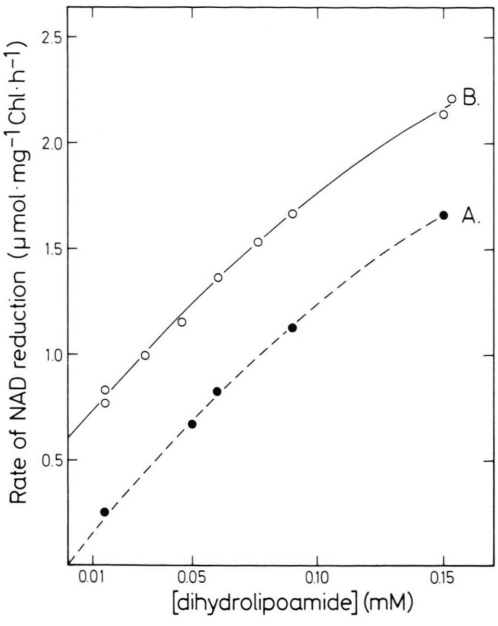

Fig. 1. Dihydrolipoamide-driven NAD reducing capacities (lipoamide dehydrogenase activities, EC 1.6.4.3) of chloroplast extracts, measured in the standard assay for pyruvate dehydrogenase (see Materials and Methods). Curve A; rate of NAD^+ reduction in the absence of pyruvate and the PDC cofactors TPP and CoASH. Curve B; enhanced rate of NAD^+ reduction after the further addition of pyruvate (0.6 mM), TPP (0.2 mM) and CoASH (0.5 mM). Any enhancement with TPP and pyruvate before CoASH addition was subtracted from the rates shown in curve B. Chloroplast extracts were prepared from lyophilized spinach and *Pisum* (from 24 h illuminated pea seedlings) chloroplasts. Data from different measurements were normalized to uniform PDC blank without dihydrolipoamide (intersection of curve B with the ordinate).

strate [21], is unaffected by the binding of the pyruvate decarboxylase (E_1). Similar lipoamide dehydrogenase (E_3) activities, indicating PDC presence, have been enriched previously from spinach chloroplast extracts [22] together with enzymes of the photosynthetic electron transport chain (*e.g.* ferredoxin-NADP-reductase). In accordance with these findings the chloroplast extracts used here showed a considerable dihydrolipoamide-driven rate of NAD reduction in the absence of pyruvate, TPP and CoASH (Fig. 1A). Dihydrolipoamide was produced from lipoamide under reducing conditions (presence of DTT) within the assay. At lower dihydrolipoamide concentrations ($\leqq$ 0.15 mM), the completion of the reaction mixture with the substrates and cofactors of PDC led to nearly stoichiometric enhancement of the dihydrolipoamide-driven rates of NAD reduction by PDC-specific ones (Fig. 1B). This supports the previous findings that the two reactions are independent. In accordance with other authors [19, 20], the CoASH-dependence of the pyruvate-driven NAD reduction was taken as an indicator of acetyl-CoA formation. In order to eliminate PDC-unspecific rates of pyruvate-dependent NAD reduction, only the CoASH-driven portion of these activities was taken into consideration throughout the present measurements, and the measured pyruvate-independent rates of NAD^+ reduction were routinely subtracted as blanks. Both pyruvate-independent and pyruvate-driven rates of NAD reduction in spinach chloroplast extracts appear to depend on the mode of extract preparation. Thus, extracts from Triton X-100 lysed chloroplasts [30], which contain a suspension of all plastid membranes and enzymes freed of their substrates, showed significantly higher rates of these PDC-unspecific activities (data not shown) than supernatants from lyophilized chloroplasts. As indicated by the substrate requirements (Table I) the latter appear to represent an enriched PDC extract, comparable to corresponding preparations from mitochondria [14].

In earlier reports the incubation of intact spinach chloroplasts with [2-^{14}C]pyruvate, purified from contaminating acetate in order to eliminate acetyl-CoA synthetase activities, has revealed significant rates of fatty acid incorporation (0.8 μmol · mg^{-1}Chl · h^{-1}) [18]. However, the identification of [^{14}C]acetyl-CoA as a labelled intermediate [7, 17] has been questioned. Evidence has been given, that the main intermediates of [2-^{14}C]pyruvate incorporation by spinach chloroplasts are precursors of branched-chain amino acid synthesis *via* hydroxyethyl-TPP [7]. The CoASH-dependent pyruvate dehydrogenase test, used in the present report, is suggested to yield stoichiometric amounts of NADH and acetyl-CoA. A second mole of NADH should be generated by reacting the freshly synthesized acetyl-CoA with citrate synthetase- and malate dehydrogenase-catalyzed reactions (see Materials and Methods). Thus, acetyl-CoA formation is indicated by an increased NAD reduction. The theoretically expected rate stimulation by a factor of 2 was, however, only observed at lower pyruvate concentrations (about 60 μM). Under normal circumstances (at half-saturating pyruvated concentrations, Table I), the observed increase was only by a factor of 1.5 (data not

Table I. Kinetic measurements of the pyruvate dehydrogenase and acetyl-CoA synthetase in extracts, prepared either from lyophilized or from Triton X-100-lysed spinach chloroplasts (see Materials and Methods). Except for the variations in metabolite concentrations, the assays were composed as described in Materials and Methods. The data result from measurements of CoASH-dependent rates of NAD reduction.

Enzyme	Kinetic constant	Substrate or inhibitor	Kinetic constants in spinach chloroplasts extracts [mM] (lyophil.)	(Triton X-100)
pyruvate dehydrogenase	app. K_m	pyruvate	0.2–0.3*	0.6
	app. K_m	NAD^+	0.1–0.3	0.6–0.7
	app. K_i	NADH (vers. pyr.)	0.02*	0.03
	app. K_i	acetyl-CoA (vers. pyr.)	0.02–0.03*	0.03
acetyl-CoA synthetase	app. K_m	acetate	0.1–0.15*	0.1

* Corresponding values have been found in extracts of lyophilized pea chloroplasts (from 3 days illuminated seedlings).

shown). These observations agree with the findings of other authors [25, 26], that under optimum conditions for fatty acid and valine synthesis in spinach chloroplast preparations, a nearly parallel incorporation of [2-^{14}C]pyruvate occurs (about 150–200 nmol·mg^{-1}Chl·h^{-1}) as both pathways compete for this substrate. The stromal pyruvate levels (0.1 mM), in chloroplasts [25, 32] appear to suffice for the synthesis of valine, leucine and isoleucine (apparent K_m for pyruvate 0.1 mM) [25]. However, the pyruvate requirement for acetyl-CoA formation within the stroma is unknown.

Substrate requirements

The pyruvate requirement of the plastid PDC, measured in extracts from purified plastids, appears to differ with the mode of extraction. The PDC in the yellowish supernatants of lyophilized pea and spinach chloroplasts was half-saturated at lower pyruvate concentrations (apparent K_m = 0.2–0.3 mM) (Table I) than in the green extracts from Triton X-100 lysed spinach chloroplasts (apparent K_m = 0.6 mM). The pyruvate requirements of the PDC suggest that the estimated stromal pyruvate level (0.1 mM) [25, 32] is sufficient for branched-chain amino acid synthesis, but not for simultaneous acetyl-CoA formation within the chloroplast. In contrast, the apparently higher level of acetate found in spinach leaf cells (1 mM) [5], which freely diffuses through chloroplast membranes [9, 10], should support substantial acetyl-CoA synthetase activity, since the K_m of this enzyme for acetate is about 0.1 mM

Table II. Maximal rates of pyruvate dehydrogenase- and acetyl-CoA synthetase activities in extracts of etioplasts and chloroplasts (of 3 days illuminated seedlings) from pea and of chloroplasts from mature spinach leaves under optimized conditions in the reaction mixture. The extracts were prepared either from lyophilized or from Triton X-100-lysed plastids. Rates are expressed either as μmol NAD reduced/mg protein·h or, in the special case of acetyl-CoA-synthetase activities, additionally as μmol [^{14}C]acetyl-CoA formed·mg^{-1} protein·h^{-1}.

Enzyme	Substrate	Activity (μmol of substrate converted·mg^{-1} protein·h^{-1}) in plastid extracts from Pea Etioplasts (lyophil.)	Pea Chloroplasts (lyophil.)	Spinach Chloroplasts (lyophil.)	Spinach Chloroplasts (Triton X-100)
pyruvate dehydrogenase*	pyruvate	2.2	1.7	1.2	0.8
acetyl-CoA synthetase	acetate	1–1.5	1.5–2	5	3–4

* Only CoASH-dependent rates of NAD reduction are given.

(Table I). Both pathways can be demonstrated in spinach chloroplast extracts under optimum conditions, where acetyl-CoA synthesis *via* pyruvate (V_{max} pyruvate = 1 μmol·mg^{-1} protein·h^{-1}) or *via* acetate (V_{max} acetate = 3–4 μmol·mg^{-1} protein·h^{-1}) [27] occurs at considerable rates. Nevertheless, the results of Table II support the idea that in photosynthetically active spinach chloroplasts acetate is the preferred substrate of acetyl-CoA formation [18], since the estimated stromal substrate levels would support 30-fold higher acetyl-CoA synthetase activities.

As further shown in Table II, the relative activities of the two reactions forming acetyl-CoA vary in the different types of plastids investigated. In spinach chloroplast extracts the ACS activity was five times higher than the PDC, while in pea plastids nearly equal amounts of both activities were present. These measurements do not reflect *"in vivo"* conditions, since the environmental conditions within the respective plastid compartments were not duplicated. These results, however, do support the proposition that the ACS is not the "typical" mechanism for the production of chloroplastic acetyl-CoA [11, 12, 19, 20].

The variation in PDC activities reported in chloroplast extracts from various leaf tissues [7, 19, 20], may be due to the cofactor requirements of this multienzyme complex, as well as to effects dependent on the species and maturity of the materials used [7]. For the PDC in pea seedlings [20] significant differences between the pH-optimum and the Mg-requirement of chloroplast and mitochondrial PDC have been shown. In the following we have compared, therefore, the pH- and Mg-requirements in pea and spinach chloroplasts.

pH Dependence

In accordance with findings in pea chloroplast extracts of other authors [20] the PDC in the plastid extracts from spinach (Fig. 2) and pea (etio- and chloroplasts; data not shown) exhibits its highest activity at a slightly alkaline pH (7.8–8.2). The pH optimum of the PDC resembles that of acetyl-CoA synthetase within the same material [27] and distinguishes the chloroplast PDC from its mitochondrial counterpart, which has a lower optimum pH within the range of 7.3 to 7.7 [14]. Lowering the pH by 1 unit from pH 8 to 7 led to a distinct inhibition of the PDC activities (Fig. 2) as reported for acetyl-CoA

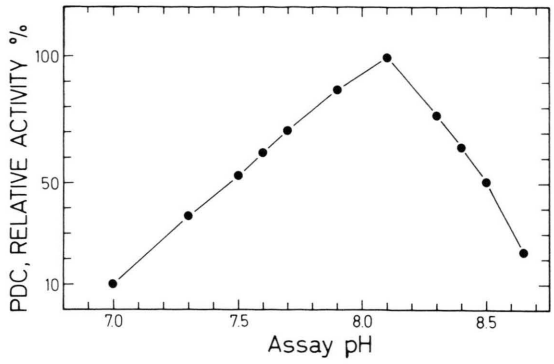

Fig. 2. Effect of pH on the pyruvate dehydrogenase (PDC) activities in spinach chloroplast extracts prepared from lyophilized spinach chloroplasts. The standard reaction mixture is described in Materials and Methods. The activities were calculated as CoASH-dependent rates of NAD reduction.

synthetase in spinach chloroplast extracts [27]. This suggests that light-induced pH changes in the chloroplast stroma may control the activities of both enzymes. This is consistent with recent findings on the pH dependence of total fatty acid synthesis from acetate in intact chloroplasts [33].

Optimal PDC activities in spinach chloroplast extracts required relatively *high Mg^{2+}-concentrations* ($\geqq$ 5 mM) (Table III), as does acetyl-CoA synthetase [27]. This has been shown by some authors [20] but apparently ignored by others [7, 17–19]. Comparable with acetyl-CoA synthetase activities within the same extracts [27], free Mg^{2+} increased the V_{max} of the oxidative decarboxylation of pyruvate to acetyl-CoA but did not change the K_m for pyruvate (data not shown). The high Mg-requirement appears to distinguish chloroplast PDC from mitochondrial activities from other sources. The mitochondrial PDC activity was unchanged or even inhibited by Mg^{2+}-concentrations higher than 1 mM [14]. This appears to reflect an accomodation to stromal conditions in illuminated chloroplasts [27, 33, 34]. The stimulatory effect of increasing Mg-concentrations could be eliminated by complexing agents such as EDTA and ATP and restored by further addition of Mg^{2+} (Table III). The observed PDC inactivation in chloroplast extracts due to increasing ATP-concentrations at fixed Mg^{2+} levels supports the recently found inhibition of the PDC from castor bean proplastids by MgATP [15]. The relation between the ATP-induced loss of

Table III. Mg-dependence of pyruvate dehydrogenase activities in spinach chloroplast extracts prepared by Triton X-100 treatment. Except for the variations of substrate additions, the basal medium contained: 50 mM Tricine (pH 8), 1.5 mM NAD^+, 2 mM DTT, 0.2 mM TPP and 0.5 mM CoASH. The activities are specified as CoASH-dependent rates of NAD reduction.

Additions to basal medium [mM]				Pyruvate dehydrogenase activity
Pyruvate	Mg^{2+}	EDTA	ATP	(nmol NADH formed x mg^{-1}Chl x h^{-1})
0.6	2.5	–	–	315
1.2	2.5	–	–	429
1.6	2.5	–	–	476
1.6	2.5	0.7	–	300
1.6	2.5	2.1	–	106
1.6	3.4	2.1	–	295
1.6	4.7	2.1	–	340
0.6	5.0	–	–	598
1.2	5.0	–	–	813
1.6	5.0	–	–	885
1.6	5.0	1.4	–	666
1.6	6.0	1.4	–	711
1.6	6.0	1.4	0.7	252
1.6	8.2	1.4	0.7	308
1.6	10.5	1.4	0.7	429

PDC-activities and the concentration of free ATP in the reaction mixture is shown in Fig. 3. These data were calculated according to Krämer [35]. In contrast to this inhibitory effect on the PDC, the presence of MgATP, required as cofactor for the acetyl-CoA synthetase [5, 27], stimulated ACS activities within the same extracts (Table I). Despite this observation, MgATP is not likely to act as fine-control modulator [13–15] between both enzyme activities, because relatively high ATP concentrations were required for the observed inhibition. In accordance with findings in plant mitochondria extracts [6, 13–15], a more effective competitive inhibition of the PDC in spinach chloroplast extracts was achieved by end products of this multienzyme complex. For acetyl-CoA, apparent K_i values between 20–30 μM and for NADH between 20–30 μM have been measured (Table I). As in the determinations of the substrate requirements, the lower values were found in the supernatants of lyophilized chloroplasts. These observations provide evidence that the inhibition of [2-^{14}C]pyruvate incorporation [18] into long-chain fatty acids, and the enhancement of branched-chain amino acid synthesis [26] in intact spinach chloroplasts by acetate addition, is due to PDC inhibition by acetyl-CoA formed by ACS. In contrast, end product control of fatty acid synthesis from acetate within the same material appeared not to occur before the stage of malonyl-CoA formation (K_i for malonyl-CoA = 0.08 mM; [36]), which has therefore been designated as the key step of acetate fixation within chloroplasts [27, 36].

Our data indicate the coexistence of both acetyl-CoA synthesizing enzymes in photosynthetically active chloroplasts (Table I), although the metabolites available [5, 25, 32] within this compartment appear to be adequate for acetyl-CoA synthetase only. The level of acetyl-CoA, formed by acetyl-CoA synthetase, may inhibit further synthesis from pyruvate and may therefore differentiate between fatty acid and branched chain amino acid synthesis at the PDC stage [26]. On the other hand the MgATP accumulation within photosynthetically active chloroplasts [37] promotes acetyl-CoA formation from acetate [5, 27] while inhibiting that from pyruvate (Table III and Fig. 3). The stromal NAD levels (0.16 mM), calcu-

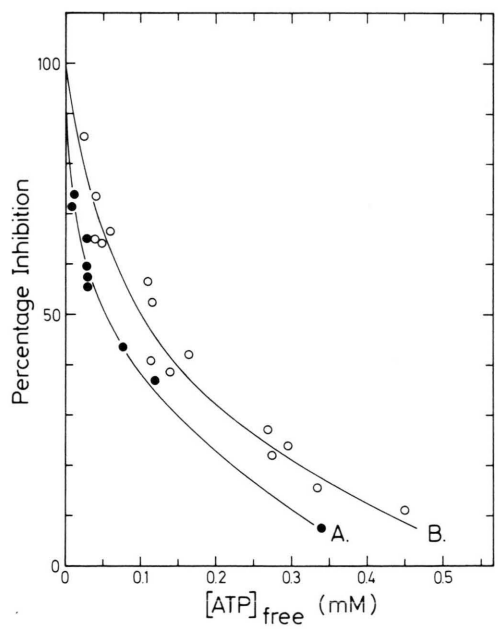

Fig. 3. Effect of ATP on the pyruvate dehydrogenase activities in extracts of lyophilized spinach (A) and pea chloroplasts (24 h illuminated) (B). The data were determined by increasing the ATP concentration at different definite Mg^{2+}-concentrations (0–6 mM) and calculating the free nucleotide concentration in the reaction mixture according to Krämer [31].

lated from the data of other authors [38] by assuming a stromal volume of about 25 $\mu l \cdot mg^{-1}$ Chl., may be compared with the NAD requirement of the PDC in chloroplast extracts (apparent K_m for NAD = 0.1–0.3 mM) (Table I). Thus the stromal NAD concentration does not seem to be a decisive limitation on the PDC. In contrast, as in mitochondria [6, 13–15], this system was apparently most effectively inhibited by traces of NADH.

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