

Acetate Concentration and Chloroplast Pyruvate Dehydrogenase Complex in *Spinacia oleracea* Leaf Cells

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Acetate concentration in spinach (*Spinacia oleracea* L.) leaf tissue has been determined by direct measurement in a leaf extract to be 70 μM or less. Additional evidence for such low acetate levels came from isotope dilution experiments where the $[1\text{-}^{14}\text{C}]$ acetate incorporation into fatty acids was assayed using purified chloroplasts in the presence of tissue extracts. Pyruvate dehydrogenase complex activity was shown to be present in spinach chloroplasts and was determined by a newly developed acetyl coenzyme A trapping system (100 nmol acetyl CoA formed per mg chloroplast protein per h). The problems of acetyl CoA synthesis in both compartments, chloroplasts and cytoplasm, are discussed in this context.

Introduction

“There has been considerable uncertainty concerning the source of acetyl coenzyme A for the synthesis of fatty acids in mature higher-plant chloroplasts.” This statement starts the report of Elias and Givan [1], presenting evidence of PDHC activity in pea chloroplasts, which was further confirmed by another group in the same year [2]. Even though a number of publications have dealt with the presence of PDHC and, at least partial glycolytic path within plastids since then, the problem concerning the ultimate carbon precursor of plastidal acetyl CoA in vivo apparently continues to be a matter of debate. This is especially true when different types of plastids are considered, e.g. proplastids [3, 4], chloroplasts [5], or chromoplasts [6]. A well-balanced, comprehensive review on this topic has been presented by Givan [7].

In the case of the spinach chloroplast, a widely used experimental system, two principal metabolic schemes may be developed from the published data. One of these is based on the following facts. Acetate, the preferred substrate for FAS in isolated spinach chloroplasts [8, 9], may be formed by acetyl CoA hydrolysis, which has been shown in vitro using

isolated spinach mitochondria [5, 10]. Together with the data of Kuhn *et al.* [11], who suggested that the acetate concentration in spinach tissue is around 1.3 mM, and that the chloroplast stroma represents the sole site of ACS in the cell, a concept was possible to explain the origin of plastidal acetyl CoA by activation of endogenous acetate in the leaf cell [12–14]. Further support for this theory arose from data that PDHC seems to be absent or virtually inactive in spinach chloroplasts [5, 8].

On the other hand, another series of reports implies the presence of an active PDHC even in the case of spinach chloroplasts [15–18], though these results have not remained undisputed (for a short discussion see [7]). In any case and despite the differing reports on a partial glycolytic path leading from 3-PGA to pyruvate within plastids [3, 19–21], there are several publications dealing with the existence of a PDHC in chloroplasts (pea: [1, 2]; butter lettuce: [5]), as well as in proplastids (leukoplasts of developing castor bean endosperm: [4, 22–25]), and also in chromoplasts [6].

This report describes experiments suitable for the determination of acetate concentration in a spinach tissue extract by (i) direct measurements of acetate with the aid of a special enzymatic kit, and by (ii) incubation experiments for chloroplast FAS along the lines of isotope dilution. The second part deals with the presence of PDHC also in spinach chloroplasts which could be assayed by the use of a newly developed radiochemical method [26].

Abbreviations: ACS, acetyl coenzyme A synthetase; PDHC, pyruvate dehydrogenase complex; FAS, fatty acid synthase system; DTE, 1,4-dithioerythritol.

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Materials and Methods

The plant material used was young spinach leaves (*Spinacia oleracea* cv. "Matador") and green shoots (about 6 cm tall) of pea (*Pisum sativum* cv. "Kleine Rheinländerin") grown in a growth chamber with a 8 h light period (18°C light/16°C dark). Daffodil flowers (*Narcissus pseudonarcissus* L.) were cultivated outdoors or purchased from the local market. Procedures for chloroplast and chromoplast isolation have been described previously [27, 28].

Sources of chemicals

[2-¹⁴C]pyruvic acid (676 MBq/mmol), [1-¹⁴C]acetic acid (2.0 GBq/mmol), and [1-¹⁴C]acetyl coenzyme A (2.03 GBq/mmol) from Amersham Buchler (Braunschweig, FRG). Acetyl coenzyme A, ATP, coenzyme A, NAD, thiamine pyrophosphate, lactate dehydrogenase, and an acetate test kit (Best. Nr. 148 261) were from Boehringer Mannheim (FRG).

Protein was determined by a modified Lowry procedure [9]. Chlorophyll was measured according to Arnon [30]. With Percoll-purified chloroplasts a protein to chlorophyll ratio of 1:17 was calculated. Taking this ratio as a basis specific activities were calculated by simple chlorophyll content measurements.

Preparation of a protein free extract from spinach leaves. Young spinach leaves (size 8–12 cm; 50–80 g tissue) were treated with a juice extractor (Braun AG, Frankfurt/M., FRG). The tissue fluid was collected immediately with a simultaneous 1:1 dilution using ice-cold, double concentrated, incubation buffer [31]. Then, the buffered extract was boiled for 5 min, cooled down again and centrifuged at 8000×*g* for 10 min in a fixed angle rotor. This supernatant was used for measurements of the acetate and pyruvate concentration as well as for FAS incubation experiments using Percoll isolated spinach chloroplasts. In order to check the loss of acetate by volatilization, [1-¹⁴C]acetate was added to the buffered extract prior to the heat treatment; the recovered radioactivity accounted for 92%.

Acetate and pyruvate determination. The acetate concentration within the protein free spinach extract was determined by a special acetate kit from Boehringer Mannheim (FRG) (see also [5, 11]). Acetate activated to acetyl CoA by ACS reacts with

oxaloacetate to form citrate in the presence of citrate synthase. The oxaloacetate required for this reaction is formed from malate in the presence of malate dehydrogenase. The thereby reduced NAD is measured spectrophotometrically. Pyruvate will be reduced to L-lactate in the presence of lactate dehydrogenase. The decrease of NADH is equivalent to the amount of utilized pyruvate. In this context it is noteworthy that lactate dehydrogenase also converts β -hydroxypyruvate and glyoxalate with comparable velocity.

Other assay conditions. PDHC activity in plastids was assayed by a new radiochemical method [26] basing on acetyl CoA trapping with dithioerythritol [32]. Incubation conditions for FAS in plastids, extraction procedure and TLC of the lipids were exactly as described in [31].

Results

Acetate concentration in spinach leaf tissue

The estimation of the endogenous acetate concentration within a protein free extract of young spinach leaves was achieved by two independent methods. One of these is based on a direct determination using a special enzymatic kit, which converts acetate to citrate (see methodical part). For comparison the endogenous pyruvate concentration was also estimated via lactate dehydrogenase mediated lactate formation.

Table I summarizes the results of 12 experiments for the determination of free acetate and 19 experiments for determination of pyruvate within the tissue extract. For the endogenous acetate concentration a mean of 0.065 mM (\pm 0.048 s.d.) was calculated, and for pyruvate the mean was 0.145 mM (\pm 0.06 s.d.).

As an alternative for showing the endogenous acetate concentration, spinach chloroplasts were isolated by Percoll gradients and incubated for FAS using [1-¹⁴C]acetate as radiolabelled precursor. Thereby, the plastids were resuspended in a protein free tissue extract as a source of incubation medium, into which all of the necessary assay ingredients were added and, for a control, in normal incubation buffer. Dependent on the endogenous acetate concentration within the tissue extract the radiolabel measured in the products of FAS caused by [1-¹⁴C]-

acetate incorporation should be subject to radioisotope dilution. In Table II the results of different incubation series for FAS are listed, namely two samples which contained solely the labelled precursor [$1\text{-}^{14}\text{C}$]acetate (37 and 74 kBq, corresponding to 34 and 68 μM); two additional samples which contained 74 kBq (68 μM) [$1\text{-}^{14}\text{C}$]acetate plus unlabelled acetate at a final concentration of 0.2 mM and 1.0 mM, respectively. These dilutions of the radioactivity cause a decrease of radiolabelled fatty acids to 26% (0.2 mM) and 7% (1.0 mM) in the control series. Incubation of Percoll purified spinach chloroplasts within the tissue extract shows very similar results without any striking isotope dilution effect by the tissue extract itself. Thereby, it was without any apparent influence, whether the tissue extract was used directly after buffering as described, or after lyophilization at pH 7.8, or when the lyophilization step was carried out at pH 3.0, respectively (Table II). For clarity it has to be mentioned that lyophilization of controls containing [$1\text{-}^{14}\text{C}$]acetate at

pH 7.8 causes a loss of [$1\text{-}^{14}\text{C}$]acetate, when present in the tissue extract, by only 3%, while the loss of [$1\text{-}^{14}\text{C}$]acetate was around 75% when lyophilization was performed at pH 3.0. In this context it is noteworthy that the acetate concentration of the tissue extract used for the experiment shown in Table II was calculated by the enzymatic kit to be less than 0.05 mM (compare Table I). This correlation between the acetate concentration measured by the acetate kit and a possible reduction of [$1\text{-}^{14}\text{C}$]acetate incorporation using the tissue extract as a source of incubation medium could be observed in a number of experiments. Dilution rates effecting ca. 75% incorporation of the control corresponded to the 0.05–0.1 mM acetate concentration group (Table I). The highest dilution effect measured resulted in the conclusion of 0.1 mM acetate being present in the tissue extract. In contrast, in about 50% of all those experiments no dilution effect was detectable, but sometimes a rather pronounced stimulation effect became evident. The most likely explanation for this

Table I. Determination of acetate and pyruvate concentrations within a protein free tissue extract from spinach leaves.

	Concentration range, mM					Total	Mean \pm s.d.
	< 0.05	> 0.05 – 0.10	> 0.10 – 0.15	> 0.15 – 0.20	> 0.20 – 0.30		
Number of experiments for determination of acetate	5	4	2	1	0	12	0.065 mM \pm 0.048
pyruvate	0	4	9	3	3	19	0.145 mM \pm 0.06

Table II. [$1\text{-}^{14}\text{C}$]acetate incorporation into long-chain fatty acids by spinach chloroplast FAS under a variety of incubation conditions (cpm).

Amount of acetate administered to the assay [μM]	Incubation conditions for chloroplast FAS			
	Control	Chloroplasts in tissue extract	Chloroplasts in tissue extract after lyophilization (pH 7.8)	Chloroplasts in tissue extract after lyophilization (pH 3)
^{14}C : 34	466 040 (100%) ^a	436 400 (93.6%)	463 410 (99.4%)	482 580 (103.5%)
^{14}C : 68	738 380 (100%) ^b	692 500 (93.8%)	691 020 (93.6%)	693 980 (94.0%)
^{14}C : 68 ^{12}C : 200	195 540 (26.5%)	180 680 (24.4%)	186 780 (25.3%)	139 940 (18.9%)
^{14}C : 68 ^{12}C : 1 000	52 780 (7.1%)	51 620 (6.9%)	64 700 (8.7%)	51 720 (7.0%)

^a This 100 per cent value only refers to the upper line.

^b By this control sample 119 nmoles acetate were incorporated into long-chain fatty acids $\times \text{mg}^{-1}$ chlorophyll $\times \text{h}^{-1}$; this is the 100 per cent reference value of all other data.

stimulation effect is that metabolites of glycerolipid biosynthesis (e.g. UDP galactose, glycerol-3-phosphate) are also included in the extract and caused this increase of incorporation rates, because in all these cases the radiolabel in the fatty acid moieties of monogalactosyldiacylglycerol, phosphatidylglycerol, and mono- and diacylglycerol was enhanced. As a matter of fact, similar stimulation effects could also be achieved in control incubations by adding UDP galactose and glycerol-3-phosphate (data not shown). Certainly, stimulative effects as mentioned above were not able to compensate the result of a possible isotope dilution by endogenous acetate in the tissue extract. This was shown by the use of tissue extract solutions which are concentrated by lyophilization and then applied for chloroplast FAS at different concentration ranges. Assuming that appropriate levels of free acetate are present in the extract, its use in double to fivefold concentration should diminish the $[1-^{14}\text{C}]$ acetate label by a drastic isotope dilution effect. This, however, was not observed.

PDHC activity in spinach chloroplasts

The presence of PDHC activity in spinach chloroplasts is not unequivocal in the literature. To re-examine this question without any bias, a new radiochemical assay system has been developed for the determination of PDHC and ACS [26]. It is based on the fact that acetyl CoA, produced from $[2-^{14}\text{C}]$ pyruvate by the action of PDHC and from $[1-^{14}\text{C}]$ acetate by ACS, forms $[1-^{14}\text{C}]$ acetyl dithioerythritol (DTE) by nonenzymatic acylation, when incubated with this thiol compound [32]. $[1-^{14}\text{C}]$ acetyl DTE is easily extracted by trichloromethane and separated from unreacted labelled substrates. By this method it was possible to show the presence of PDHC activity in spinach chloroplasts (Table III).

Measurements concerning cofactor requirements of PDHC activity were done in two ways. Under standard conditions DTE was present in the assay mixture from the beginning of the reaction (Table III, left column), while a second series was incubated without DTE (Table III, right), but terminated by simultaneous addition of 20 mM DTE and 20 mM $[^{12}\text{C}]$ pyruvate in order to retard the further formation of $[1-^{14}\text{C}]$ acetyl CoA from $[2-^{14}\text{C}]$ pyruvate. Under DTE free conditions the reaction is strongly

dependent on the presence of NAD^+ , while the dependence on CoA is not that much pronounced. The absence of thiamine pyrophosphate, this cofactor being non-covalently bound to the enzyme, had a slight effect on the reaction, but omission of all cofactors (NAD^+ , CoA, TPP) reduced the activity to only 1.5% of the control (Table III, right). Under standard conditions (i.e. DTE is present in the assay) the omission of NAD^+ and CoA resulted in a decrease to only 43% and 82% compared with the control. When boiled chloroplasts, buffer alone, or

Table III. Pyruvate dehydrogenase complex activity from Percoll isolated spinach chloroplasts. 365 μg chloroplast protein was incubated for 20 min in the presence of 18.5 kBq (28 nmol) $[2-^{14}\text{C}]$ pyruvate (assay medium: N-tris(hydroxymethyl)methylglycine, 100 $\text{mmol} \cdot \text{l}^{-1}$; MgCl_2 , 2 $\text{mmol} \cdot \text{l}^{-1}$; NAD^+ , 1 $\text{mmol} \cdot \text{l}^{-1}$; thiamine pyrophosphate, 2 $\text{mmol} \cdot \text{l}^{-1}$; CoA 0.5 $\text{mmol} \cdot \text{l}^{-1}$). + DTE means 20 mM DTE was present in the assay mixture from the beginning of the reaction; – DTE means the incubation was without DTE, but the reaction was retarded by addition of 20 mM $[^{12}\text{C}]$ pyruvate and simultaneously of 20 mM DTE after the regular incubation time.

Incubation conditions	+ DTE	– DTE
Control (complete)	100% ^a	100% ^b
– NAD^+	43%	9%
– CoA	82%	24%
– TPP	84%	82%
– NAD^+ , – CoA, – TPP	9.7%	1.5%
+ 2 mM $[^{12}\text{C}]$ pyruvate	12.5% ^c	–
boiled chloroplasts	0.3%	–
no chloroplasts (buffer alone)	0.32%	–
– DTE	0.3%	–

^a 100% corresponds to 105,920 cpm extracted as $[1-^{14}\text{C}]$ acetyl DTE or 21.8 mmol per mg protein per h.

^b 100% corresponds to 51,892 cpm extracted as acetyl DTE or 10.7 mmol per mg protein per h.

^c Under saturating substrate conditions an activity of 97.5 mmol acetyl DTE per mg protein per h was calculated.

Table IV. Pyruvate dehydrogenase complex activities from organelle systems of different plant species measured by the DTE assay (assay conditions were the same as mentioned in Table III, final concentration of pyruvate was 2 mM).

Organelle system	nmol $[1-^{14}\text{C}]$ acetyl DTE formed per mg protein per h
chloroplasts	
<i>Pisum sativum</i>	150
<i>Spinacia oleracea</i>	97.5
chromoplasts	
<i>Narcissus pseudonarcissus</i>	73
mitochondria	
<i>Solanum tuberosum</i>	1250

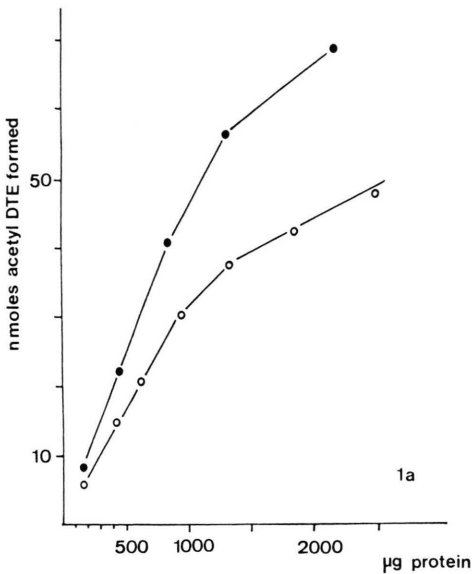


Fig. 1a. Pyruvate dehydrogenase complex activity from spinach (○—○) and pea (●—●) chloroplasts as a function of chloroplast protein (standard conditions as in Table III; concentration of pyruvate, 2 mM; incubation time was 20 min).

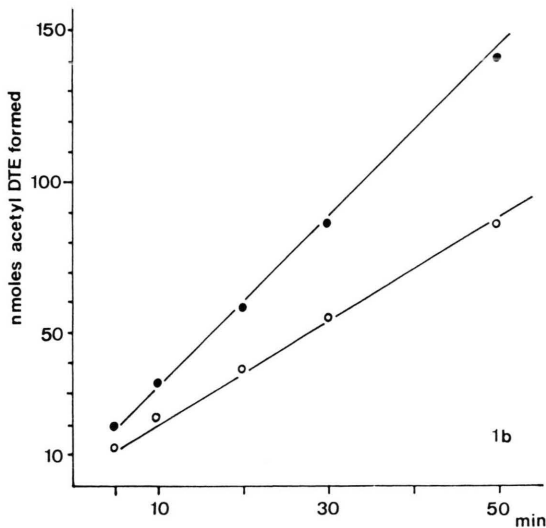


Fig. 1b. Pyruvate dehydrogenase complex activity as a function of time, when 950 µg of spinach and 980 µg of pea chloroplast protein, respectively, was used (standard conditions, concentration of pyruvate, 2 mM).

no DTE were used, no acetyl DTE was formed, but about 0.3% of the administered radioactive pyruvate was displaced by the extraction procedure. Usually, incubations in the presence of DTE are distinguished from those without the thiol compound by a higher

specific activity of about 2–3 times. Further points which are connected with the acetyl DTE assay are described elsewhere [26].

Similar results for the cofactor requirements were obtained when PDHC of pea chloroplasts and daffodil chromoplasts were assayed by this method (data not shown).

For both systems, spinach and pea chloroplasts, the dependence of PDHC activity on the amount of protein was determined (Fig. 1a). A high degree of linearity exists up to around 1 mg of protein, with a decrease above this value. A time course for both systems is given in Fig. 1b, which shows striking linearity between 5 and 50 min.

Table IV contains a survey of PDHC activities of different plastidal systems and, for comparison, of mitochondria from potato tubers. The data indicate about 1.5 times higher activities in the chloroplasts of pea in comparison with spinach.

Acetolactate synthase is a possible candidate for further pyruvate utilization in the chloroplast [5, 32]. Because this enzyme is known to be susceptible to inhibition by valine and leucine, incubation experiments were carried out for PDHC activity in the presence of both amino acids at 1 mM concentration. In these control experiments, the activity of PDHC was not influenced at all.

Discussion

Both acetate and pyruvate may serve as *in vitro* sources of plastidal acetyl CoA which starts the plastidal FAS, but for many experimental systems acetate has been proven to be the preferred substrate [9]. In search of the physiological precursor for FAS in spinach several findings favoured the conception of a carbon supply via acetate also *in vivo* [14], because chloroplast PDHC was found to be apparently inactive [5, 8]; the stroma compartment has been described as the sole site of ACS [11], acetate may be formed *in vitro* via acetyl CoA hydrolysis as has been shown using isolated mitochondria [5, 10], and acetate was measured to be present in this tissue in a range of 1 mM [11].

On the other hand, the data presented here show that the mean of free acetate concentration in tissue extracts from spinach leaves is as low as 70 µM and in a number of experiments, less than 50 µM. the resulting presumption of rather low acetate levels in the spinach cell was further confirmed by isotope

dilution experiments. During their course the tissue extract served as a source of incubation medium for Percoll isolated spinach chloroplasts, which were then assayed with $[1-^{14}\text{C}]$ acetate as a precursor. If at all, the thereby achieved isotope dilution effects – caused by endogenous acetate within the extract – were small and in agreement with the above mentioned low acetate concentration. From these results it is concluded that the acetate concentration in spinach tissue is essentially lower than so far supposed, namely in the range of 70 μM or less.

By comparison, levels of free pyruvate were also measured in the spinach extract and found to be around 145 μM . Because β -hydroxypyruvate and glyoxalate were also recorded by this lactate dehydrogenase coupled determination the true pyruvate concentration may be somewhat lower but, apparently, in a range comparable with acetate.

The idea of principally different physiological pathways for acetyl CoA synthesis in chloroplasts of different species, namely by the action of ACS or, alternatively by PDHC, looks hardly conceivable. In this context it seems to be meaningful to direct the attention to another well-known chloroplast system, the pea chloroplast. These organelles contain besides ACS activity [26, 34] an active PDHC [1, 2], but they also incorporate acetate into fatty acid moieties to a higher degree than pyruvate *in vitro* (Liedvogel, unpublished data), although in this plant cell an acetyl CoA hydrolysis in the mitochondria as a possible source for acetate seems to be absent [35]. When a newly developed radiochemical method for the determination of PDHC [26] was applied it became possible to measure this enzymatic activity in chloroplasts from both spinach and pea. With the acetyl DTE assay used in this report PDHC activity in pea chloroplasts accounted for 150 nmol acetyl DTE formed per mg protein per h, for spinach chloroplasts ca. 100 nmol per mg protein per h, and for chromoplasts from daffodil flower, also known as a PDHC active organelle [6] 73 nmol per mg protein per h (Table IV).

PDHC activities in pea chloroplasts – using other methods – were reported elsewhere to account for 350–530 nmol NADH produced per mg protein per h [2], and 1176 nmol per mg protein per h [1]. (For the conversion factor chlorophyll/protein see methodical part.)

The possible role of regulatory events influencing the PDHC activities certainly represents an impor-

tant viewpoint. Virtually nothing is known about the regulation of chloroplast PDHC, although the PDHC of castor bean proplastids has been the subject of some regulatory examination [25]. In contrast, the mitochondrial PDHC from spinach and pea has been studied in detail [36, 37] and found to be regulated by phosphorylation-dephosphorylation control. Most likely, the regulation in chloroplasts is even more complex, because already small changes of metabolite and ion concentrations or alteration of pH values exhibit rather drastic effects on the activity of chloroplast enzymes (*e.g.* [38]). Therefore, similar fluctuations of chloroplast PDHC activity are not to be excluded.

The biosynthesis of both main lipophilic compounds of chloroplasts, fatty acids and polyprenoids, starts with acetyl CoA as a precursor. Recently it has been shown that Percoll purified chloroplasts from spinach were unable to synthesize isopentenyl diphosphate, whereas the cytoplasm has proven to be the site of synthesis of this isoprenoid precursor [27]. This – together with the well-known formation of sterols at the endoplasmic reticulum – includes the necessity of a source for acetyl CoA also within the cytoplasm. By fractionation of spinach protoplasts, the chloroplast stroma has been shown to be the sole site of ACS [11]. Nevertheless, when small pieces of spinach leaves were incubated with $[1-^{14}\text{C}]$ -acetate, label was found in fatty acid moieties as expected (about two thirds of total incorporation), but the remaining label was associated with cytoplasmic sterol compounds (Lütke-Brinkhaus and Liedvogel, unpublished data). Because acetyl CoA is thought to be unable to cross the plastid envelope [39], either acetate may also be activated in the cytoplasm, or acetyl CoA, once formed within the stroma, may be discharged into the cytoplasm via a special translocator system [40].

In summary, the problem of acetyl CoA synthesis in both compartments, chloroplast and cytoplasm, does not seem to be resolved at the moment. At any rate, the proof of PDHC within spinach chloroplasts may permit a further discussion in finding the physiological pathway of acetyl CoA supply in these organelles. Furthermore, the question for the metabolic role of ACS(s) holds good although the rather low acetate concentrations in the spinach tissue as reported here possibly challenge a modified valuation of this enzymatic activity.

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