

REGULATION OF FAT BODY PYRUVATE DEHYDROGENASE COMPLEX IN THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.) (LEPIDOPTERA: SPHINGIDAE)*

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Abstract—Fat body pyruvate dehydrogenase complex (PDC) from *Manduca sexta* undergoes interconversion between an active, nonphosphorylated form and an inactive, phosphorylated form. Inactivation is catalyzed by a Mg^{2+} -ATP-requiring kinase, and reactivation is catalyzed by a Ca^{2+} - Mg^{2+} -requiring phosphatase. By altering the kinase activity, NAD^+ and CoA activate the PDC while NADH and acetyl-CoA facilitate inactivation of the complex. Levels of active enzyme are highest during larval development and in the fed state. Injection of glucose into mature larvae, starved for 48 hr, produces a threefold increase in total PDC activity with a corresponding increase in the fraction in the active form. Experiments conducted with ligated larvae provide evidence that a neuroendocrine factor may facilitate activation of the fat body complex.

Key Word Index: *Manduca sexta*, fat body, pyruvate dehydrogenase complex, activation, interconversion

INTRODUCTION

THE PYRUVATE dehydrogenase multienzyme complex (PDC)† catalyzes the oxidation of pyruvate to acetyl-CoA. This essentially irreversible reaction results in a net loss of body carbohydrate reserves. Thus regulation of this step is of central importance to the general energy balance and fuel economy of animal cells.

PDC has been purified from bacteria (KOIKE *et al.*, 1960; BRESTERS *et al.*, 1975), fungi (HARDING *et al.*, 1970), yeast (WAIS *et al.*, 1973), peas (THOMPSON *et al.*, 1975), broccoli (RUBIN and RANDALL, 1977), avian muscle (JAGANNATHAN and SCHWERT, 1952), and

mammalian tissues (HAYAKAWA *et al.*, 1966, ISHIKAWA *et al.*, 1966; LINN *et al.*, 1969, 1972; ROCHE and CATE, 1977). Only recently have results been published on PDC from an invertebrate, *Ascaris lumbricoides* (KOMUNIECKI *et al.*, 1979), probably because of the difficulty in obtaining a sufficient quantity of this complex for analysis. The present study has been conducted on PDC from the tobacco hornworm, *Manduca sexta*, because mature larvae reach a large size (~10 g), large numbers can be raised on an artificial diet, and specific developmental stages can be determined with accuracy. The specific tissues of interest are the fat body and the cerebral neurosecretory system. The former stores and metabolizes carbohydrate and lipid; the latter is the source of neuroendocrine factors that modulate metabolism (WYATT, 1967).

In higher animals, PDC is regulated by a reversible covalent phosphorylation-dephosphorylation mechanism. The interconversion between active, nonphosphorylated and inactive, phosphorylated complex is catalyzed by a protein kinase and phosphatase, respectively (LINN *et al.*, 1969; DENTON *et al.*, 1975). Insect fat body PDC has been investigated in order to determine whether it is regulated by interconversion and to determine how the activity of this enzyme complex responds to changes in nutritional state. The present paper presents evidence that interconversion does occur and that enzyme levels change significantly in response to both nutritional state and stage of development. A preliminary report of these results has previously appeared (ROCHE *et al.*, 1978).

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†Abbreviations: PDC, pyruvate dehydrogenase complex; PDC_t, total pyruvate dehydrogenase complex activity; PDC_a, portion of PDC in active form; PDH, pyruvate dehydrogenase component; PDH_a, active nonphosphorylated form of PDH; PDH_i, inactive phosphorylated form of PDH; MOPS, 3-(N-morpholino) propane sulfonate; PEG, polyethylene glycol; DTT, dithiothreitol; EGTA, ethylene glycol bis(2-aminoethyl)-N,N'-tetraacetate; EDTA, ethylenediamine tetraacetate; PEG, polyethylene glycol; TPP, thiamin pyrophosphate; NADH, NAD⁺, nicotinamide-adenine dinucleotide in its reduced and oxidized forms, respectively.

MATERIALS AND METHODS

Insects and mitochondria preparation

M. sexta eggs were obtained from Dr. J. P. REINECKE (Metabolism and Radiation Research Laboratory, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Fargo, ND). Larvae were reared at 28°C and 60% relative humidity with a 16 hr light photoperiod and standard diet (BELL and JOACHIM, 1976). Fat body from fifth instar larvae was dissected using standard techniques (SCHNEIDERMAN, 1967). For preparation of mitochondria, the tissue was placed in a solution of 225 mM mannitol, 7.5 mM sucrose, 0.5 mM EDTA (about one ml per fat body). The pH of this solution was maintained at 7.2–8.5 by addition of 1 M NaOH. After thirty organs (~8 g) were collected, they were homogenized with a Tekmar Tissumizer and centrifuged at 4°C for 4 min at 500 g. The supernatant was collected and then centrifuged at 17,000 g for 10 min at 4°C. The pellet was resuspended and then collected by centrifugation at 17,000 g under the following conditions: (1) twice with 6 ml of mannitol buffer, pH 7.4; (2) once with 6 ml of 0.5 mM EDTA, pH 7; and (3) once with 6 ml of 20 mM Na₂HPO₄, 0.5 mM EDTA, pH 7. A firm mitochondrial pellet, essentially free of lipid material and constituting about 70 mg of mitochondrial protein, was obtained in the final pellet that was stored at -70°C.

Purification of insect fat body pyruvate dehydrogenase complex

Mitochondria from three or four of the above preparations (i.e. from 24–33 g fat body tissue) were thawed and 20 mM potassium phosphate, pH 6.5, was added to give a volume of 12 ml to which the following were added: 0.24 ml rabbit serum (added to protect fat body protein from proteolytic degradation), 1.2 ml 20% (v/v) Triton X-100, and 0.12 ml 5 M NaCl. This and all subsequent procedures were conducted at 4°C. The suspension was centrifuged at 30,000 g for 10 min and this was repeated on the supernatant to obtain a mitochondrial extract that was almost clear. Following assays for protein and enzyme activity, extracts were diluted to 10 mg protein/ml, the pH was adjusted to pH 6.5, and MgCl₂ was added to give a final concentration of 10 mM. A 50% (w/v) polyethylene glycol 6000 solution was added at 6% (v/v) to the mitochondrial extract. The precipitate was collected by centrifugation for 10 min at 20,000 g. The pellet was suspended in 0.5 ml of 50 mM MOPS-Na, pH 7.2, 0.5 mM DTT, 0.2 mM EDTA and allowed to stand 1 hr with occasional stirring. A major portion of the pellet failed to dissolve and was removed by centrifugation for 10 min at 20,000 g. The clear supernatant was then assayed for protein and PDC activity. This preparation was the source of enzyme for subsequent experiments. Bovine kidney PDC was prepared as described previously (ROCHE and CATE, 1977).

Enzyme assays

The activity of partially purified PDC was assayed in triplicate as the rate of NAD⁺ reduction by the procedure of LINN *et al.* (1972). Assays of the rate of inactivation by the kinase and activation by the phosphatase were carried out by modification of the procedures of HUCHO *et al.* (1972). Endogenous kinase activity was measured as the initial rate of inactivation of the PDC in the presence of ATP. Standard reaction mixtures contained 0.07 units (one unit = 1 μmol/min) of PDC activity and 40 mM MOPS-imidazole buffer (pH 7.2), 60 mM KCl, 1.0 mM MgCl₂, 0.1 mM ATP or a mixture of 0.5 mM ADP plus 0.1 mM ATP. Mixtures were incubated for 1 min at 30° prior to the addition of ATP or the mixture of ATP and ADP. Aliquots (40 μl) were removed and diluted at 60 sec or 120 sec into NAD-reduction assay mixtures. PDH_b phosphatase assays were conducted in 40 mM MOPS-imidazole buffer (pH 7.2) under conditions described in the legend to Table 2. Typical data from one enzyme preparation are presented with S.E.M. < ± 10%.

Preparation of tissue samples to determine PDC_a or PDC_k activity

For determination of the level of PDC_a, a portion of the fat body (0.08–0.25 g wet weight) was added to 1.0 ml of the following buffer at 2°C: 40 mM MOPS-Na (pH 7.5), 5 mM potassium phosphate (pH 7.5), 5 mM EDTA, 12.5 mM NaF, 2.5 mM dichloroacetate, 0.25% (v/v) Triton, 2% (v/v) rabbit serum, and 0.01% (w/v) trasylol. NaF and EDTA prevented PDH_b phosphatase activity while EDTA and the combination of phosphate and dichloroacetate strongly inhibited PDH_a kinase activity. Rabbit serum (ROCHE and CATE, 1977) and trasylol (EISENTRAUT *et al.*, 1968) were added to prevent proteolysis. Samples were immediately homogenized for 5 sec with a Tekmar Tissumizer (10 EN probe at a speed setting of 15) and shell-frozen with a dry ice-isopropanol bath. For determination of PDC_k activity an equivalent size sample of fat body was added to 1.0 ml of the following buffer: 40 mM MOPS-Na, 10 mM MgCl₂, 0.1 mM CaCl₂, 5 mM glucose, 10 units hexokinase, 1% (v/v) Triton, 0.01% (w/v) trasylol, 2% (v/v) rabbit serum and homogenized as above. The hexokinase treatment depleted tissue ATP. These samples were incubated for 10 min at 30°C and then shell-frozen. Control experiments established that there was complete activation of PDC in 5 min and no activity loss at 15 min.

Determination of PDC activity in tissue samples

Each of the above samples was thawed and immediately assayed in triplicate by addition of 0.2 ml of homogenized tissue to a reaction mixture containing 0.21 mM [1-¹⁴C]-pyruvate, 1.0 mM NAD⁺, 0.1 mM thiamin pyrophosphate, 0.18 mM CoA, 1.0 mM DTT, 8 mg/ml BSA, 1.0 mM MgCl₂, and 20 mM potassium phosphate (pH 7.2) in a final volume of 0.55 ml. The reaction was carried out for 2.5 min at 30°C in a serum bottle capped with a rubber stopper and was terminated by addition of 0.2 ml of 0.2 M H₂SO₄. Radioactive CO₂ released during 1 hr incubation at 25°C was absorbed by 0.2 ml phenethylamine that was contained in a gelatin capsule which was inserted in a small test tube inside the serum bottle.

Quantitation of amount of tissue

A turbidometric assay was developed to determine the weight of fat body. Following the above assays, tissue samples that had been kept on ice were again homogenized with the Tekmar Tissumizer for 3 sec. A portion was diluted in the range of 1:40 to 1:100 into 20 mM potassium phosphate buffer, pH 7.5, and the absorbance at 450 nm was determined. Control experiments established that, for absorbance readings in the range of 0.45–0.95 and for different samples in which wet weight was carefully determined before homogenization, the reading was proportional to the amount of fat body. Use of this procedure allowed utilization of tissues for enzyme assays more quickly without having to determine directly the wet weight of tissues. For experiments comparing fat body PDC from different developmental stages, tissue wet weights were determined with an analytical balance.

RESULTS AND DISCUSSION

Partially purified PDC

In mammalian cells PDC is found in mitochondria, apparently in the matrix space (ADDINK *et al.*, 1972). Mitochondria from hornworm fat body were prepared from about 25 g of wet tissue, and insect PDC was partially purified by procedures adapted from mammalian tissue methods. Table 1 shows the averaged results obtained from three of our more active preparations of fat body PDC. Complex was

Table 1. Partial purification of *Manduca sexta* fat body PDC*

Fraction	Protein (mg)	Units (μ moles/min)	Specific activity	Recovery (%)	Purification
Mitochondrial extract	111	2.68	0.025 \pm 0.007	100	1
PEG-MgCl ₂ fractionation	7.25	3.00	0.43 \pm 0.11	112	17.2

* Values shown are average of three preparations from about 25 g of wet tissue each.

prepared from mitochondria by extraction with Triton X-100 buffer and purified 17-fold by precipitation with polyethylene glycol as described in Materials and Methods. Partially purified fat body PDC had a specific activity of 0.43 μ mol/min/mg which was approximately 25 times lower than that for complex purified to homogeneity from vertebrate tissues (ROCHE and CATE, 1977). Variable success was achieved in purifying the insect complex, and it is not understood completely what deleterious factors cause low recovery of enzyme activity in some preparations. Some of the decrease in activity was due to tissue proteases, and this degradation was prevented by addition of rabbit serum (2% v/v) and trasylol (0.01% w/v).

Studies on regulation of fat body PDC

Table 2 shows that the activity of insect PDC is strongly affected by the presence of ATP and divalent metal ions. The complex was inactivated more than four-fold by incubation for 1 min with ATP and Mg²⁺. Further inactivation occurred with longer periods of incubation. This effect probably resulted from phosphorylation of PDH_a catalyzed by a Mg²⁺-ATP-requiring kinase. In contrast, a nearly two-fold activation occurred during a 5 min incubation in the presence of millimolar levels of Mg²⁺ and micromolar levels of Ca²⁺. This result suggested that fat body PDC can be reactivated by dephosphorylation of PDH_a by a Mg²⁺-Ca²⁺-requiring phosphatase. Mg²⁺ or Ca²⁺ alone produced no change in the activity of the complex. Overall, these results indicated that, in insect fat body, interconversion occurred between an active, nonphosphorylated protein (PDC_a) and an inactive, phosphorylated form (PDC_b). This is in the first report of interconversion of PDC from an

invertebrate tissue. KOMUNIECKI *et al.* (1979) were unable to demonstrate a phospho-dephospho form of regulation with muscle PDC complex obtained from a nematode.

Attempts to study further the two regulatory enzymes in the purified insect complex were met with mixed success. The phosphatase could not be characterized probably because, as was the case with vertebrate PDC, a major portion of this regulatory enzyme dissociated from the complex during purification. Apparently this component was quite unstable once dissociated. In contrast, properties of the kinase were more easily determined. Table 3 shows that the activity of the Mg²⁺-ATP-dependent PDH_a kinase was modulated by a number of effectors that have been shown previously to affect PDH_a kinase from mammalian tissues (HUCHO *et al.*, 1972; ROCHE and REED, 1972; PETTIT *et al.*, 1975). PDH_a kinase was activated by NADH or acetyl-CoA, in the presence of a reductant such as DTT, and was inhibited by TPP, NAD⁺ and CoA.

From the foregoing experiments, it appeared that ratios of metabolites and cofactors would be important factors in altering the activity of PDC. In Table 4 the influence of two effector ratios (NADH:NAD⁺ and acetyl-CoA:CoA) on fat body PDH_a kinase activity is shown and compared with those obtained with purified bovine kidney PDH_a kinase. NADH was more effective in stimulating PDH_a kinase than NAD⁺ was in inhibiting it. Bovine kidney PDH_a kinase exhibited a similar behaviour. However, insect PDH_a kinase was inhibited by CoA at much higher ratios of acetyl-CoA:CoA than the mammalian PDH_a kinase. This result showed that insect PDC would be activated at higher acetyl-CoA:CoA ratios than vertebrate PDC.

Table 2. Metal requirements for fat body PDC activity*

Addition	PDC activity (nmol/min/mg protein)
None	196
0.5 mM ATP, 0.5 mM MgCl ₂	45
10 mM MgCl ₂ , 0.1 mM CaCl ₂	358
10 mM MgCl ₂ , 1.0 mM EGTA	200
0.1 mM CaCl ₂	219

* The sample to which ATP was added was incubated for 1 min prior to addition of ATP and then for 1 min in the presence of ATP. All other samples were incubated for 5 min at 28°C in the presence of indicated ligands. The control sample (no additions) was assayed at 2 and 5 min and did not change. Other conditions were as described in Materials and Methods.

Table 3. Effectors of fat body PDH_a kinase

Addition (mM)	Decrease in PDC activity (nmol/min)	% Control
None*	8.1	100
NADH (0.5)	11.9	147
Acetyl-CoA (0.5), DTT (2.0)	13.1	162
Acetyl-CoA (0.5)	6.6	81
DTT (2.0)	7.5	93
TPP (0.1)	2.1	26
NAD ⁺ (0.5)	5.4	67
CoA (0.5)	6.1	75

* PDA_a kinase assays were conducted as described in Materials and Methods with all activity measurements made 60 sec following the addition of a mixture of 0.15 mM ATP and 0.45 mM ADP.

Table 4. Effects of acetyl-CoA:CoA ratio and NADH:NAD ratio on PDH_a kinase activity from insect fat body and bovine kidney*

Addition (concentration or ratio†)	PDH _a kinase activity (%)	
	Insect fat body	Bovine kidney
None*	100	100
NADH (0.5 mM)	150	140
NADH:NAD ⁺ (0.2)	158	148
NADH:NAD ⁺ (0.1)	133	143
Acetyl-CoA (0.5 mM)	176	180
Acetyl-CoA (0.05 mM)	166	178
Acetyl-CoA:CoA (2.0)	154	177
Acetyl-CoA:CoA (0.5)	74	163
Acetyl-CoA:CoA (0.2)	62	140
Acetyl-CoA:CoA (0.1)	61	123
CoA (0.5 mM)	61	85

* Assays were conducted as described for Table 3.

† The total pool for various ratios of NAD⁺ and NADH or CoA and acetyl CoA was 0.5 mM.

Ontogenetic studies

During development of *M. sexta*, the level of PDH_a activity changed dramatically (Fig. 1). Fifth instar larvae that weighed 2–5 g showed the highest activity (45–75 nmol/min/g wet tissue). These rapidly growing larvae are apparently metabolizing carbohydrate and accumulating fatty acid reserves in preparation for metamorphosis (BAILEY, 1975) because thereafter activity decreased by nearly 10-fold in fully grown

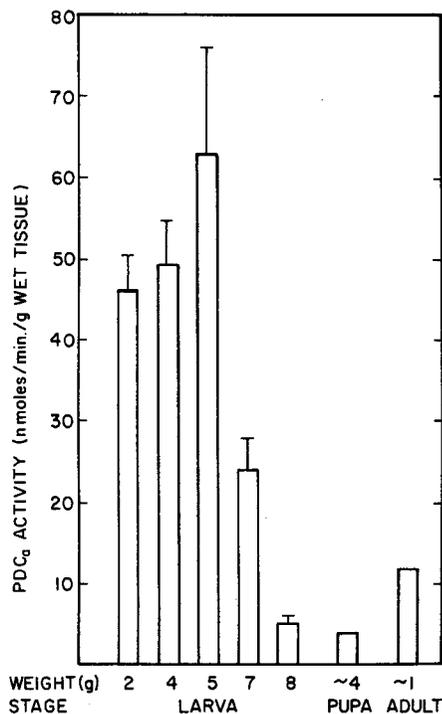


Fig. 1. Level of PDH_a activity in *M. sexta* fat body at different stages of development. For fifth instar larvae three insects of each weight were used; all samples were assayed in triplicate. Bars = S.E.M.

Table 5. Levels of fat body PDC_a and PDC_t for starved and fed *M. sexta* larvae*

Nutritional state	Activity (nmol/min/g)		% Active form
	PDC _a	PDC _t	
Starved (48 hr)	2.9–5.7	27.2–41.6	8.5–16.5
Fed	12.0–245	83.7–311	26–87

* Fifth instar larvae weighing 5 g.

larvae (8–9 g). Pupal and adult fat body also contained low levels of active PDC.

Nutritional studies

The effect of nutritional state on PDC activity was also investigated. Activities in larvae (~4–5 g) that had fed *ad libitum* and that had been starved for 48 hr were compared (Table 5). Starved animals exhibited consistently low levels of both PDC_a and total PDC (attained by complete activation). Only about 12% of the enzyme was in the active form. A much higher yet variable activity was observed with fed animals (3–60-fold higher), and the proportion of active enzyme ranged from about 30 to 90%. These results demonstrated that both the total level of PDC and the fraction of enzyme in the activated form varied greatly and were influenced by nutritional state. In mammalian tissues, the variation in active enzyme with nutritional state has been observed previously

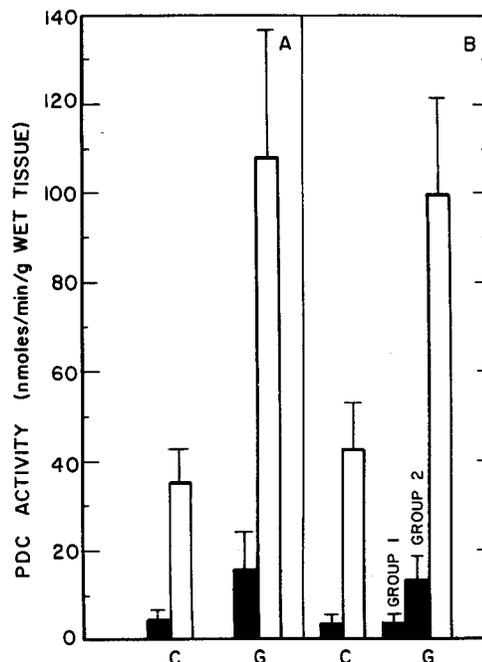


Fig. 2. Effect of glucose injection on PDC_a and total PDC activity in starved (A) and ligated (B) hornworms. All insects were starved for 48 hr. Glucose (200 mM), dissolved in 50 μ l of insect saline, was injected through an abdominal proleg into the haemocoel. For each block shown, fat body from at least six larvae were assayed in triplicate. Bar = S.E.M.; C = control; G = glucose treated; closed bar = PDC_a level; open bar = total PDC activity level.

(WIELAND *et al.*, 1971). However, the total amount of enzyme did not significantly change.

The effect of carbohydrate on PDC levels in *M. sexta* was also determined. As shown in Fig. 2A, glucose injection into 4.5 g larvae that had been starved for 48 hr produced a threefold increase in total PDC activity. Time course experiments established that this increase was rapid and complete 45 min after injection (data not shown). Thus, there is a net synthesis of enzyme and/or a decrease in enzyme turnover induced by glucose. A corresponding increase in PDC_a also occurred; however, the fractional level of PDC_a remained about the same in control and glucose-treated animals (~15% in the active form). Other sugars such as sucrose and trehalose were not similarly effective. Such rapid and extensive changes in total PDC activity have not been observed in mammalian tissues.

Effect of ligation on PDC level

An attempt was made to show that the level of PDC activity in insect fat body is regulated by the cerebral neuroendocrine system. Insects were ligated behind the head to prevent any neurosecretory products from reaching the fat body. After this treatment, larvae remained viable for about two days; experiments were completed within two hours. Injection of glucose into ligated animals produced about a three-fold increase in total PDC activity (Fig. 2B). However, the level of PDC_a was unchanged in most animals. Approximately 60% of the ligated animals (group 1) failed to show a significant increase in PDC_a; the other 40% (group 2) exhibited only a two-fold increase. These results suggested that, although not required for glucose-induced PDC synthesis, a factor in the cerebral neuroendocrine system may facilitate activation of the complex. The nature of this factor is unknown. In vertebrate fat cells PDC is an insulin-sensitive enzyme that is regulated by a cyclic nucleotide-independent phosphorylation-dephosphorylation cycle. Exposure to hormone rapidly activated PDC as a result of dephosphorylation (JUNGAS, 1970; WEISS *et al.*, 1971; COORE *et al.*, 1971). Experiments are in progress in our laboratory to determine whether the insulin-like peptide found in the cerebral neurosecretory system and haemolymph of insects has a similar effect on fat body PDC (TAGER *et al.*, 1976; DUVE *et al.*, 1979; KRAMER *et al.*, 1980).

Figure 3 shows a reaction scheme for the

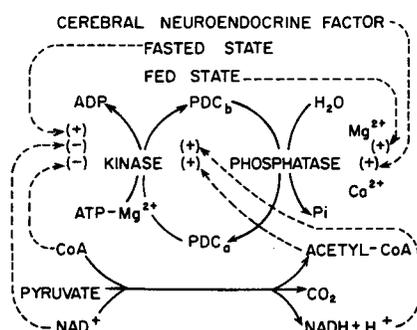


Fig. 3. Scheme for the regulation of fat body pyruvate dehydrogenase for *M. sexta*.

interconversion of insect PDC. A Mg²⁺-ATP-requiring kinase inactivates the complex via phosphorylation. NAD⁺ and CoA inhibit the kinase while NADH and acetyl-CoA activate it. A Ca²⁺-Mg²⁺-requiring phosphatase activates the complex via dephosphorylation. The latter regulatory enzyme may be stimulated by a cerebral neuroendocrine factor (or, alternatively, the kinase may be inhibited). The degree of phosphorylation is under dietary control. During starvation phosphorylation is increased. Dephosphorylation occurs under fed conditions where nearly complete activation of the complex may occur.

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