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SOME ENZYMES OF GENERAL METABOLISM IN THE LATEX OF *PAPAVER SOMNIFERUM**

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Abstract—Enzymes of general metabolism have been determined in the latex of *Papaver somniferum* in an attempt to elucidate further the nature of the 1000 *g*/30 min organelles and their role in alkaloid biogenesis. A number of enzymes involved in the glyoxylic acid and tricarboxylic acid cycles have been found, namely, aconitase, isocitrate dehydrogenase, succinate dehydrogenase, fumarase, malate dehydrogenase and isocitrate lyase. Two enzymes of glycolysis, namely, pyruvate kinase and lactate dehydrogenase, as well as enzymes associated with peroxisomes (glyoxylate reductase, catalase) and lysosomes (arylesterase, acid phosphatase) have been studied. Finally, some enzymes previously reported as occurring in poppy seedlings have been investigated, namely peroxidase, glutamate–oxaloacetate and glutamate–pyruvate transaminases, together with phenylalanine, tyrosine, DOPA and glutamic acid decarboxylases.

INTRODUCTION

Preliminary light microscopic studies of *Papaver somniferum* laticifers showed that their contents consist of a multitude of particles suspended in a large central vacuole [2] and this work together with the use of electron microscopy [3–5] has indicated the presence of endoplasmic reticulum, nuclei, mitochondria, Frey–Wyssling particles and other spherical bodies or vesicles referred to previously as 1000 *g*/30 min organelles [6]. These 1000 *g*/30 min organelles may correspond to the almost circular areas in a net of protoplasm in *Chelidonium* which were described as forming a distinct vacuole [7] or they are comparable with the vacuoles of young latticifers in *Manihot* and *Hevea* as described by Milanez [8]. Recent work has shown the vacuoles in *Chelidonium* [9] and *Heavea* [10] lattices to be lysosomal.

Meissner [11, 13] studied the expelled latex of 29 species of plants. Their low protein content suggested that at best latex was an extremely diluted cytoplasmic system. He found evidence of ribosomes in poppy latex and was able to report a high rate of gaseous exchange which was not due to micro-organisms. More recently [14] poppy latex has yielded a fraction with a P/O ratio characteristic of mitochondria. The inhibition of the oxygen uptake by malonate, an inhibitor of succinate dehydrogenase, indicated that at least part of the respiration observed in the latex resulted from Krebs cycle activity. The polyphenolase observed by Meissner [11] was found to be entirely confined to the 1000 *g*/30 min fraction of the latex [6] which also contained the alkaloids found in the latex [15]. Furthermore it was also shown that these organelles will utilize DOPA in the formation of morphine [15].

The present work attempts to investigate the extent to which enzymes of general metabolism may be detected in poppy latex in an attempt to elucidate further the nature of the 1000 *g*/30 min organelles.

RESULTS

The limited period of time (8 weeks) during which fresh latex was available proved to be a problem and a technique was therefore devised by

* Part III in the series "Enzymic Studies with *Papaver somniferum*". For Part II see Ref. [1].

Table 1. Occurrence and distribution in *P. somniferum* of some enzymes of the tricarboxylic acid and glyoxalic acid cycles and of glycolysis

Enzyme	Activity μmol/min/ml/ latex 1000 *g*	Supernatant	
Aconitase	00	0·6	F.L.
Isocitrate dehydrogenase (NAD specific)	00	00	F.L.
Isocitrate dehydrogenase (NADP specific)	00	1·4	F.L.
Succinate dehydrogenase	00	0·044	F.L.*
Fumarase	00	00	F.L.
Malate dehydrogenase (malate formation)	00	87	S.L.
Malate dehydrogenase (oxaloacetate formation)	00	0·8	F.L.
Pyruvate kinase	00	0·35	S.L.
Lactate dehydrogenase	00	2·0	F.L.

S.L. Stored latex (processed 2–9 months after collection).
F.L. Fresh latex (processed 4–6 hr after collection).
N.D. Not done.
Incubation at 25° except * 30°; † 37°.
Activity: μmol of substrate utilized/min/ml of latex.

which latex was stored at −20° after separation into the various particulate fractions and supernatant. Where possible, experiments carried out with stored latex were also checked with fresh latex, but in a number of cases this was not possible and the activities given for the enzymes considered may not as a result be maximal or necessarily comparable one with another, and therefoe can only be considered to indicate the presence of any given enzyme. Numerous experiments indicated that the metabolic activity of the latex in respect of individual enzymes could be very variable but, in general, maximal activity in the latex was observed at around 7 days from flowering [16] with the majority of enzymes investigated.

In the experiments shown in Tables 1–3 the latex was separated by centrifugation into a 1000 *g*/30 min fraction and supernatant. The latex used for the experiments given in Table 4 was separated to given 1000 *g*/30 min, 25000 *g*/2 hr fraction and the supernatant.

Occurrence and distribution of some enzymes of the tricarboxylic and glyoxylic acid cycles

The results in Table 1, which are representative of several experiments made with latex samples over 3 yr, show that a number of the enzymes of the tricarboxylic and glyoxylic acid cycles are readily detectable in poppy latex and that these occur exclusively in the supernatant of the latex.

Table 2. Occurrence and distribution in poppy latex of enzymes associated with lysosomes, peroxisomes and glyoxosomes

Enzyme	Activity μmol/min/ml latex 1000 *g*	Supernatant	
Glyoxylate reductase	00	0·3	S.L.
Catalase	00	00	F.L.
Isocitrate lyase	00	00	F.L.
Arylesterase (pH 6·0)	N.D.	00	S.L.
Arylesterase (pH 7·4)	N.D.	0·9	S.L.
Phosphatase (pH 5·0)	$1{\cdot}3 \times 10^{-2}$	$4{\cdot}0 \times 10^{-2}$	S.L.†
Phosphatase (pH 8·0)	N.D.	$1{\cdot}5 \times 10^{-2}$	S.L.†
Peroxidase	00	00	F.L.

For footnotes, see under Table 1.

Aconitase activity, although not detectable in stored supernatant was found in fresh supernatant. Both reactions catalysed by malate dehydrogenase, namely, malate formation and oxaloacetate formation, were observed to be present in fresh supernatant; however, only the reduction of oxaloacetate to malate was detectable in stored supernatant. NADP-isocitrate dehydrogenase was initially detected in stored supernatant but was found to have activity greater by a factor of two in fresh supernatant. The presence of Mn^{2+} (0·002 mmol) gave a two-fold enhancement of activity with stored supernatant. Attempts to demonstrate NAD-isocitrate dehydrogenase activity were unsuccessful. Succinate dehydrogenase, although not detectable in stored supernatant, was found to be present in fresh samples. The present methods could not detect fumarase or isocitrate lyase despite a series of experiments carried out over several poppy growing seasons.

Table 3. Occurrence and distribution of amino acid, decarboxylases and transaminases in poppy latex

Enzyme	Activity μmol/min/ml latex 1000 *g*	Supernatant	
Glutamate: oxaloacetate transaminase	00	2·2	S.L.
Glutamate: pyruvate transaminase	00	1·9	F.L.
Glutamate decarboxylase	00	3·0	S.L.*
Phenylalanine decarboxylase	00	00	S.L.
Tyrosine decarboxylase	00	00	S.L.
Dopa decarboxylase	00	00	S.L.

For footnotes, see under Table 1.

Table 4. Distribution of activity between 1000 *g*, 25000 *g* × 2 hr and supernatant fractions of poppy latex

Enzyme	Activity (μmol/min/ml of latex) 1000 *g*	25000 *g*	Supernatant
Glutamate decarboxylase	00	00	7·0
Arylesterase (pH 7·4)	00	00	1·7
Acid phosphatase (pH 5·0)	00	2·3*	3·4*
Malate dehydrogenase (malate formation)	00	12	75
Lactate dehydrogenase	00	00	2·0

Assays were carried out at pH 7·0 except where indicated and 25° except for glutamate decarboxylase which was carried out at 30°.

* When calculated per mg of protein the activity for 25000 *g* fraction was 3·3 μmol/hr and for the supernatant 0·3 μmol/hr.

The occurrence of enzymes of glycolysis

Lactate dehydrogenase was found in the supernatant of the stored latex. Since NADH oxidation could be due to the action of an alcohol dehydrogenase acting on acetaldehyde, resulting from the action of pyruvate decarboxylase, the absence of pyruvate decarboxylase was first confirmed (Table 1). Pyruvate kinase was also found to be present in the supernatant (Table 2). The reaction required KCl (0·225 mmol). The activity observed, 0·04 μmol of substrate utilized/min/ml of latex was increased nine-fold to 0·35 μmol the addition of $MgCl_2$ (0·012 mmol) but was reduced to 0·08 μmol if $CaCl_2$ (0·10 mmol) was also included in the assay mixture.

Occurrence of enzymes associated with lysosomes, glyoxysomes and peroxisomes

Glyoxalate reductase activity was found to be present in the latex supernatant (Table 2). Despite repeated attempts with 1000 g organelles and supernatant latex over three seasons, it was not possible to detect the presence of catalase although this enzyme has been found active in enzyme preparations from young seedlings [17]. Phosphatase activity was initially demonstrated at pH 5·0 and 8·0 in the supernatant. Although in initial experiments at pH 6·0 with arylesterase no activity was observed, activity was detected in experiments using supernatant at pH 7·4 (Table 2).

Occurrence of peroxidase transaminases and decarboxylases

The investigation of a number of enzymes previously isolated from young poppy seedlings [18] (Table 3) gave some interesting results. Somewhat surprisingly, peroxidase activity was not detected in poppy latex despite numerous experiments. Aspartate–α-ketoglutarate transaminase activity was found to be present in latex supernatant and was still detectable after 3 months storage, although the loss of activity over the period was 38%. The presence of glutamate–pyruvate transaminase activity was also detected in latex supernatant although activity was only one-third of that observed for aspartate–α-ketoglutarate transaminase.

Of all the amino acids examined using a standard manometric technique, only glutamic acid appeared to be decarboxylated by the latex supernatant fraction. In parallel experiments the activity was reduced by 84% in the presence of hydroxylamine (8×10^{-5} mmol).

Distribution of glutamate decarboxylase, arylesterase, acid phosphatase, malate dehydrogenase and lactate dehydrogenase

These five enzymes were investigated in a series of experiments in which the latex was fractionated into 1000 *g*/30 min 25000 *g*/2 hr and supernatant latex. The results (Table 4) indicate that only acid phosphatase and a part of the malate dehydrogenase which sedimented at 25000 *g* appear to be associated with material in the latex which can be sedimented.

DISCUSSION

The results indicate that the latex of *Papaver somniferum* contains many of the enzymes normally associated with cellular metabolism. Of the enzymes detected all were found in the supernatant as opposed to the 1000 *g*/30 min organelles and the fact that acid phosphatase and arylesterase are absent from the latter fraction would suggest that the 1000 *g*/30 min organelles are not lysosomes, which contrasts with the findings for particles of similar density in the latex of *Hevea brasiliensis* [10] and *Chelidonium majus*. The fact that phosphatase activity was exhibited at both acid and alkaline pH and that on further centrifugation of the latex at 25000 *g*/2 hr, a high concentration of acid phosphatase was found in this fraction deserves further investigation.

A consideration of the enzymes investigated which are associated with the tricarboxylic and

glyoxylic acid cycles presented a number of problems. NADP-isocitrate dehydrogenase appeared to be relatively stable and could be detected in latex stored at −20° for some months; however, it was not possible to detect NAD-isocitrate dehydrogenase which might also have been expected to occur. This enzyme may prove to be unstable [19], rapidly losing its activity in the first few hours after latex collection. It is also possible that this enzyme is not in contact with the substrate since this enzyme has been reported as mitochondrial in plants, [20], and therefore firmly membrane-bound. Aconitase, reported to be a mitochondrial enzyme [21], was apparently more readily soluble since this enzyme was readily detected, and a similar consideration would also explain the ease of detection of succinate dehydrogenase and oxaloacetate formation by malate dehydrogenase. The observations of Fairbairn *et al.* [14] suggested the existence of mitochondria within the latex and these enzymes could well be associated with these organelles. The apparent failure to detect fumarase which, in the light of the other enzymes found, should be present was unexpected and may result from lack of contact with the substrate or too low a concentration of the enzyme to make detection by the present methods possible. The fact that isocitrate lyase, a key enzyme in the glyoxylate cycle, was not detected may well mean that this particular pathway is not operative in poppy latex.

The presence of pyruvate kinase and lactate dehydrogenase is of interest since it is quite possible that the whole system of glycolysis may operate in the poppy latex in a manner analogous to that found in rubber latex [22]. The presence of lactate dehydrogenase deserves further study since the usual method for the generation of ATP anaerobically in plants involves pyruvate decarboxylase and alcohol dehydrogenase. Pyruvate decarboxylase was not found in the stored latex and therefore the reduction of pyruvate by the latex in the presence of NADH must have been due to the action of lactate dehydrogenase resulting in the formation of lactate. However, Archer and Audley [22] have reported that when glucose was used as a substrate with *Hevea* latex, radioactive ethanol and lactate were detected and it would be of interest to determine whether in *P. somniferum* latex the alternative pathway leading to the formation of ethanol from pyruvate exists or not. It is not certain from the current experiments whether the reduction of glyoxylate is due to the presence of lactate dehydrogenase or to a genuine reductase in the latex.

An outstanding difference between the latex of *P. somniferum* and that of *Hevea* [23] is the apparent absence in poppy latex of both peroxidase [24] and catalase [17] which is the more surprising since both enzymes are present in poppy seedlings. Of the remaining enzymes recorded by Jindra [18] as being present in poppy seedlings aspartate–α-ketoglutarate, alanine–α-ketoglutarate transaminases, as well as glutamate decarboxylase, were also found in the latex supernatant. The decarboxylation of other amino acids, phenylalanine, tyrosine and DOPA, is of interest in alkaloid biosynthesis, was not initially detected in the latex. However, recent experiments with L-DOPA-1-[^{14}C] have indicated that a DOPA decarboxylase exists in the latex [24].

This study of some of the enzymes of general metabolism has shown that, contrary to the claims of Meissner [11,13], poppy latex is metabolically very active. However, the only enzyme which has been shown clearly to be associated with the 1000 *g*/30 min organelles or alkaloid-containing vesicles is polyphenolase and this contrasts with the alkaloid-containing vesicles in *Chelidonium* which has also been shown to be lysosomal. In general, it is considered that the 1000 *g*/30 min fraction in *P. somniferum* latex is not homogeneous and further work is in progress since the polyphenolase in *Hevea* latex appears to be associated with the Frey–Wyssling particles [23]. The association of acid phosphatase with a light particle in the 25000 *g*/2 hr fraction was unexpected in view of the work with *Chelidonium* latex [9], and therefore further work to determine whether this fraction contains hydrolytic enzymes would be of interest.

EXPERIMENTAL

Preparation of latex. Latex (1–3 ml) from stem and capsules of *P. somniferum* cv. Halle was collected into ice cold mannitol 500 mM/K_2HPO_4 100 mM buffer pH 7·0. The vol. collected was in the ratio (1:1) latex–buffer. All subsequent manipulations were carried out at 2–6° unless otherwise stated.

Removal of 1000 g *organelles.* (1:1) Latex–buffer was centrifuged at 1000 *g* for 30 min. The sediment obtained is referred to as the 1000 *g*/30 min organelles and the rest of the latex, unless stated otherwise, will be referred to as the supernatant. Fractions not used immediately were stored at −20°.

Further fractionation of the supernatant. For some experiments the supernatant from latex–buffer (3 ml) was further fractionated to give a 25000 *g* 2 hr fraction which was resuspended in buffer pH 7·0 (1 ml). For phosphatase determinations the fractions were dialysed overnight against dist. H_2O to remove inorganic phosphate.

Enzyme assay procedures. All spectroscopic assay procedures were performed on a continuous recording spectrophotometer with temp. control. In all spectrophotometric assays activity was calculated as μmol of substrate utilized/min/ml latex. Unless otherwise stated assays were peformed at 25°.

Aconitase (E.C. 4.2.1.3). For assay the method of Cooper and Beevers [26] was used. The reaction mixture contained in a total vol. of 3 ml; phosphate buffer pH 7·5 80 mM; DTT 3 mM; NADP 0·03 mM; isocitrate dehydrogenase (NADP-specific) 100 μg; $MnCl_2$ 3 mM; *cis*-aconitate 5 mM and latex (0·05–0·1 ml). The reaction was initiated by the addition of *cis*-aconitate and the reduction of NADP followed at 340 nm. Controls were minus *cis*-aconitate.

NAD-NADP-isocitrate dehydrogenase (E.C. 1.1.41 and E.C. 1.1.1.42). The assay method used was that of Cooper and Beevers [26]. The assay mixture was contained in a total vol. of 3 ml *N*-2-hydroxyethylpiperazine-*N'* 2-ethane sulphonate pH 7·5, 67 mM; NADP 0·03 mM or NAD 0·1 mM; $MnCl_2$ 4 mM; DTT 1·3 mM; isocitrate 3·5 mM and latex 0·05–0·1 ml.

The reaction was initiated with isocitrate and controls were minus latex, and the reduction of NAD or NADP was followed at 340 nm.

Succinate dehydrogenase (E.C. 1.3.99.1). For the assay the method of Cooper and Beevers [26] was used. The assay mixture contained in a total volume of 3 ml phosphate buffer pH 7·5 80 mM; phenazine methosulphate 0·6 mM; potassium cyanide 19 mM; dichloroindophenol 0·07 mM; succinate 12 mM and latex 0·05–0·1 ml.

The reaction at 30° was initiated with succinate, which was omitted in control samples, and the reduction of dichloroindophenol was followed at 600 nm.

Fumarase (E.C. 4.2.1.2). Following the method of Cooper and Beevers [26], the assay mixture contained in a total vol. of 3 ml either phosphate buffer pH 7·5 50 mM or Tris–HCl buffer pH 8·0 100 mM and DTT 1·3 mM; malate 2·3 mM and latex 0·1 ml. The reaction was initiated with malate which was omitted from the controls and the formation of fumarate was followed for 2 hrs at a wavelength of 240–300 nm.

Malate dehydrogenase (E.C. 1.1.1.37). A. Malate formation. The assay mixture [27] contained in a total vol. of 3 ml, phosphate buffer pH 7·4 90 mM oxaloacetate 0·5 mM; NADH 0·23 mM and latex 0·02 ml. The reaction was initiated by oxaloacetate which was omitted from the control samples. Activity was measured by observing the oxidation of NADH at 340 nm. B. Oxaloacetate formation. The assay mixture contained in a total vol. of 3 ml Glycine–NaOH buffer pH 10 120 mM; L-malate 28 mM; NAD 1·3 mM and latex 0·05 ml. The reaction was initiated by the addition of L-malate which was omitted from control samples and the reduction of NAD followed at 340 nm.

Isocitrate lyase (E.C. 4.1.3.1). The assay mixture [28] contained in a total of 3 ml, phosphate buffer pH 6·4 41 mM; phenylhydrazine HCl 5 mM; isocitrate 2·6 mM; $MgCl_2$ 2·3 mM and latex 0·05–0·1 ml. The change in absorbance at 324 nm resulting from the formation of glyoxalic phenylhydrazone observed over a period of 1 hr.

Pyruvate kinase (E.C. 2.7.1.40). The procedure followed was that of Tietz and Ochoa [29] in which enzyme activity was linked to the reduction of pyruvate to lactate. Pyruvate kinase has an absolute requirement for monovalent ions and in particular for K^+ [30] and therefore this assay was considered adequate providing the activity observed was dependent on the addition of K^+. Under these conditions, simulation of pyruvate kinase activity by either PEP carboxylase or a phosphatase is avoided. Since a highly active lactate dehydrogenase was present in latex supernatant the addition of a commercial preparation was not required. The assay mixture contained in a total vol. of 3 ml Tris–HCl buffer pH 7·3 50 mM; $MgCl_2$ 4 mM; KCl 75 mM; ADP 4 mM; NADH 0·2 mM; phosphoenolpyruvate 1 mM and latex 0·1 ml. Control samples were minus (a) KCl, (b) $MgCl_2$ or (c) phosphoenol–pyruvate. The reaction was initiated by addition of phosphoenolpyruvate and the oxidation of NADH followed at 340 nm.

Lactate dehydrogenase (E.C. 1.1.1.27). The method used by Reeves and Fimognari [31] was followed. The assay mixture contained in a total vol. of 3 ml phosphate buffer pH 7·0 100 mM; sodium pyruvate 0·8 mM; NADH 0·25 mM and latex 0·05 ml. The reaction was initiated with pyruvate which was omitted from the control samples and the oxidation of NADH was followed at 340 nm.

Glyoxylate reductase (E.C. 1.1.1.26). The assay [32] mixture contained in a total of 3 ml, phosphate buffer pH 7·4, 85 mM; NADH 0·5 mM; glyoxylate 3 mM and latex 0·05–1·0 ml. The reaction was initiated either with latex or with glyoxylate and the oxidation of NADH followed at 340 nM. Latex was omitted in control samples.

Catalase (E.C. 1.11.1.6). H_2O_2 soln was prepared by diluting 0·1 ml 30% v/v H_2O_2 to 50 ml with 0·05 M phosphate buffer pH 7·4 so that the absorbance of the soln was about 0·50. The assay mixture contained H_2O_2 soln (2·9 ml) and latex 0·1 ml, the latex being omitted from the controls [33]. The utilization of H_2O_2 over a maximum period of 30 min was recorded by observing the change in A at 240 nm.

Arylesterase (E.C. 3.1.1.2). The assay method used the hydrolysis of an artificial substrate *p*-nitrophenyl acetate [34]. The assay mixture contained in a total vol. of 3 ml. Tris–HCl buffer pH 7·4, 40 mM; *p*-nitrophenyl acetate 1 mM and latex 0·1 ml. Latex was omitted from control samples and the reaction was initiated by the addition of latex and the formation of *p*-nitrophenol followed at 348 nm.

Alkaline and acid phosphatase (E.C. 3.1.3.1 and E.C. 3.1.3.2). The assay method of Bessey *et al.* [35] was used. The assay mixture contained *p*-nitrophenyl phosphate 1·1 mM; citric acid–NaOH buffer pH 5·0 or Tris–HCl buffer pH 8·0 32 mM and latex 0·05 ml. The reaction was stopped with 1 M NaOH 0·4 ml and the total vol. made to 5 ml with dist. H_2O. The activity was determined by measuring the change in absorbance at 400 nm due to *p*-nitrophenol formation. In control samples the latex was added after the addition of 1 M NaOH.

Peroxidase (E.C. 1.11.1.7). The assay mixture contained guaiacol 20 μmol; peroxide soln (as prepared for catalase assay) 0·10 ml and latex 0·05–0·1 ml. Controls omitted latex and the total vol. of the assay mixture was made to 3 ml with 0·1 M phosphate buffer pH 7·0. Activity was determined by observing the change in absorbance at 436 nm due to H_2O_2 utilization [36].

Glutamate–oxaloacetate transaminase (E.C. 2.6.1.1). A modification of the Bergmeyer [33] method was used. The assay mixture contained aspartate 42 mM; α-ketoglutarate 1 mM; NADH 0·2 mM and latex 0·1 ml made to 3 ml with 0·1 M phosphate buffer pH 7·4. The reaction was initiated with α-ketoglutarate which was omitted from control samples and oxidation of NADH was followed at 340 nm. The activity of malate dehydrogenase in the latex samples was such that the addition of commercial enzymes was not required. Activity was estimated by measuring the oxidation of NADH at 340 nm.

Glutamate–pyruvate transaminase (E.C. 2.6.1.2). The Bergmeyer [33] assay method was used. The assay mixture contained in a total of 3 ml, phosphate buffer pH 7·4 90 mM; L-alanine 336 mM; NADH 0·23 mM; α-ketoglutarate 7 mM; lactate dehydrogenase 100 μg and latex 0·05 ml. The reaction was initiated with L-alanine which was omitted from the control samples and followed by observation at 340 nm of the oxidation of NADH.

Amino acid decarboxylase. The standard manometric method of Bergmeyer [33] was used to determine the decarboxylation of glutamate (E.C. 4.1.1.15), tyrosine phenylalanine and DOPA (E.C. 4.1.1.26). The assay mixture contained amino acid 21 mM; hydroxylamine hydrochloride 0·026 mM; where necessary latex 0·1 ml and 0·1 M phosphate buffer pH 6·3 to a total vol. of 3 ml. Latex was omitted from control samples and a reaction time of 30 min at 30°C used. The CO_2 released was determined and activity expressed as μmol of CO_2 liberated/min/ml of latex.

Protein determinations. The method of Lowry *et al.* [37] was used.

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