Importance of experimental conditions in evaluating the malonyl-CoA sensitivity of liver carnitine acyltransferase

Studies with fed and starved rats

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The experiments reconfirm the powerful inhibitory effect of malonyl-CoA on carnitine acyltransferase I and fatty acid oxidation in rat liver mitochondria (K_i 1.5 μM). Sensitivity decreased with starvation (K_i after 18 h starvation 3.0 μM , and after 42 h 5.0 μM). Observations by Cook, Otto & Cornell [Biochem. J. (1980) 192, 955–958] and Ontko & Johns [Biochem. J. (1980) 192, 959–962] have cast doubt on the physiological role of malonyl-CoA in the regulation of hepatic fatty acid oxidation and ketogenesis. The high K_i values obtained in the cited studies are shown to be due to incubation conditions that cause substrate depletion, destruction of malonyl-CoA or generation of excessively high concentrations of unbound acyl-CoA (which offsets the competitive inhibition of malonyl-CoA towards carnitine acyltransferase I). The present results are entirely consistent with the postulated role of malonyl-CoA as the primary regulator of fatty acid synthesis and oxidation in rat liver.

We have previously shown that malonyl-CoA is a potent inhibitor of carnitine acyltransferase I (EC 2.3.1.21) (K_i 1–2 μM) and have reviewed the evidence in support of its central role in the regulation of fatty acid synthesis and degradation in rat liver (McGarry & Foster, 1980a). Some groups, however, have had difficulty reproducing the suppression of mitochondrial fatty acid oxidation by malonyl-CoA (Pande, 1980; Tubbs *et al.*, 1980), whereas others (Cook *et al.*, 1980; Ontko & Johns, 1980), though confirming the effect, have reported K_i values some 10–20 times greater than that found by us.

We have now compared the inhibitory effect of malonyl-CoA obtained under our original experimental conditions and those of Cook *et al.* (1980) and Ontko & Johns (1980). The results corroborate our earlier reports and indicate that the lack of sensitivity to malonyl-CoA described in the cited studies was due to problems in experimental design. We consider the issue important in view of the concept that malonyl-CoA controls fatty acid-synthesizing and -oxidizing pathways under a variety of physiological and pathological conditions.

Methods

Animals

Male Sprague–Dawley rats weighing 130–150 g were used. They were maintained on a high-sucrose

low-fat diet (McGarry *et al.*, 1977) in a room with lighting from 15:00 to 03:00 h. Experiments began at 08:30 h. Starved animals were deprived of food for 18 or 42 h before use.

Studies of mitochondrial fatty acid oxidation

Liver mitochondria were prepared as described by McGarry *et al.* (1978a). The standard procedure for the measurement of mitochondrial fatty acid oxidation was that used in our previous studies (McGarry *et al.*, 1977, 1978a). Incubations were carried out in 25 ml Erlenmeyer flasks, which initially contained, in a volume of 2.2 ml of modified Krebs–Henseleit buffer, pH 7.4 (McGarry *et al.*, 1977): 100 nmol of sodium [$1\text{-}^{14}\text{C}$]oleate (0.4 μCi) bound to 20 mg of fatty-acid-free albumin; 10 μmol of ATP, 2.5 μmol of ADP, 125 nmol of CoASH, 625 nmol of reduced glutathione and 250 nmol of L-carnitine. At this point 0.1 ml of 150 mM-KCl containing sufficient malonyl-CoA to give the desired final concentration was added, the flasks were gassed with O_2/CO_2 (19:1), capped, and maintained at room temperature for 5–10 min. Then 0.2 ml of mitochondria (1–1.5 mg of protein) suspended in ice-cold 150 mM-KCl was added, the flasks were re-gassed with O_2/CO_2 and shaken for 5 min at 80 oscillations/min in a 30°C water bath. Final concentrations of reaction components were: oleate, 40 μM ; albumin, 0.8% (molar ratio fatty acid/

albumin = 0.35); ATP, 4 mM; ADP, 1 mM; CoASH, 50 μ M; reduced glutathione, 250 μ M; carnitine, 100 μ M. Reactions were terminated by the addition of 0.4 ml of 5% (w/v) HClO_4 and the quantity of oleic acid converted into total acid-soluble products was determined by liquid-scintillation counting of the HClO_4 extracts (McGarry *et al.*, 1978a). In the initial experiments acid extracts were neutralized and the quantity of label present in acetoacetate and β -hydroxybutyrate was measured as described by McGarry & Foster (1970). Radioactive CO_2 was not collected, since we have previously shown that this amounts to less than 1% of the label present in acid-soluble products (McGarry *et al.*, 1977). Protein was measured by the method of Lowry *et al.*, (1951).

To simulate the experimental conditions employed by Cook *et al.* (1980) and Ontko & Johns (1980), one or more of the following modifications were made to the standard incubation system described above: the quantity of mitochondrial protein and/or the concentration of oleate was increased; the concentration of CoASH was lowered; the incubation time was changed to 10 min; the temperature was raised to 37°C.

Studies of mitochondrial carnitine acyltransferase

The activity of carnitine acyltransferase in intact mitochondria was measured over a 5 min period at 30°C by the isotope-exchange assay (assay I)

described by McGarry *et al.* (1978a). This method detects both carnitine acyltransferase I and carnitine acyltransferase II activity, but only the former is sensitive to inhibition by malonyl-CoA.

Materials

The sources of all materials have been given previously (McGarry *et al.*, 1977, 1978a).

Results and discussion

Effect of malonyl-CoA on oleate oxidation under standard conditions

Fig. 1 shows the effect of malonyl-CoA on the oxidation of oleate by mitochondria from fed, 18 h- and 42 h-starved rats under our standard incubation conditions. Control rates for the conversion of oleate into total acid-soluble products (Fig. 1a) were similar in all three groups (about 5.5 nmol/5 min per mg of mitochondrial protein); the amount of oleate oxidized was usually around 8% of that initially added (100 nmol). With mitochondria from fed rats 50% inhibition (I_{50}) of oleate oxidation was achieved at a malonyl-CoA concentration of only 2 μ M, whereas the same degree of inhibition in mitochondria from 18 h- and 42 h-starved rats required 3.5 μ M- and 8 μ M-malonyl-CoA respectively. A similar pattern was seen for the conversion of oleate into ketone bodies (Fig. 1b), but in this case the

Fig. 1. *Effect of malonyl-CoA on the oxidation of oleate by liver mitochondria from fed and starved rats* Mitochondria (1–1.5 mg of protein) from (○) fed, (△) 18 h- and (□) 42 h-starved rats were incubated with [$1\text{-}^{14}\text{C}$]oleate under standard conditions as described in the Methods section. Absolute quantities of oleate converted into acid-soluble products (mainly ketone bodies) averaged 5.2, 5.3 and 5.9 nmol/5 min per mg of protein in the fed, 18 h- and 42 h-starved groups respectively. The concentrations of malonyl-CoA (Mal-CoA) required for 50% inhibition are indicated. Values are means $\pm$ S.E.M. for four experiments in each group.

sensitivity to malonyl-CoA was somewhat greater (I_{50} 1.5 μM , 3 μM and 5 μM in the fed, 18h- and 42h-starved groups respectively). Although the sensitivity of mitochondrial fatty acid oxidation to malonyl-CoA is most accurately assessed by measurement of isotope recovery in ketone bodies, the simpler procedure of measuring incorporation of radioactivity into acid-soluble products yields values closely reflecting those obtained with the longer technique and was routinely used in the remaining experiments.

Fig. 2 depicts the sensitivity of carnitine acyltransferase activity in mitochondria from fed and starved rats to inhibition by malonyl-CoA. As for oleate oxidation (Fig. 1), mitochondria from fed rats were most responsive and those from 42h-starved animals the least. Even in the latter case, however, only 3.7 μM -malonyl-CoA was needed to inhibit the activity of carnitine acyltransferase I by 50%.

The data of Figs. 1 and 2 confirmed that under our standard assay conditions a concentration of malonyl-CoA of only 1–2 μM was sufficient to

suppress by 50% both the activity of carnitine acyltransferase I and the oxidation of oleate in mitochondria from fed rats, in keeping with earlier studies (McGarry *et al.*, 1978a). [In the legend to Fig. 6 of McGarry *et al.* (1978a), the quantity of mitochondrial protein should have read 1.6 mg, not 3.6 mg as stated.] Sensitivity to malonyl-CoA decreased with starvation, as we had previously shown in studies with whole homogenates (McGarry *et al.*, 1977). To reiterate, concentrations of malonyl-CoA of only 3.7 μM and 5 μM respectively were sufficient to decrease by 50% the activity of carnitine acyltransferase I and the oxidation of oleate in mitochondria from 42h-starved rats. We thus sought the reason why both Cook *et al.* (1980) and Ontko & Johns (1980) required much higher concentrations of malonyl-CoA to achieve a similar degree of inhibition of mitochondrial palmitate oxidation (I_{50} values of about 20 μM and 100 μM for mitochondria from fed and starved rats respectively were reported).

Importance of mitochondrial concentration in demonstrating the malonyl-CoA effect

In comparing our experimental conditions with those used by Ontko & Johns (1980), two differences seemed obvious: we used 1–1.5 mg of mitochondrial protein with an incubation time of 5 min; they used 4.7–7.5 mg of mitochondrial protein and an incubation time of 10 min. The latter conditions pose two potential problems: the risk of substrate depletion [it can be calculated from the data of Ontko & Johns (1980) that in some of their experiments 90% of the added fatty acid must have been consumed] and loss of malonyl-CoA through mitochondrial degradation (McGarry *et al.*, 1978a). Both points are illustrated in the experiments of Figs. 3 and 4. When oleate oxidation was followed over a 10 min period with a low amount of mitochondria from fed rats (1.25 mg of protein), the results shown in Fig. 3(b) were obtained. The control rate of fatty acid oxidation accelerated during the first 5 min (owing to temperature equilibration); additional experiments (results not shown) established that it remained linear thereafter (between 5 and 15 min of incubation). Typical inhibition by malonyl-CoA was seen at 5 min and inhibitory effects were clearly demonstrable at 10 min even with the lowest concentration of malonyl-CoA added (2 μM). This is reflected in the data of Fig. 3(a), which reveal I_{50} values of 1.5 μM and 2.5 μM at the 5 and 10 min time points respectively. By contrast, when the concentration of mitochondria was increased 5-fold (to 6.25 mg of protein) the control rate of fatty acid oxidation decreased between 5 and 10 min of incubation (Fig. 3c). This was not surprising, since the initial concentration of oleate used (40 μM) was not saturating and 70% had been oxidized after

Fig. 2. Effect of malonyl-CoA on carnitine acyltransferase (CAT) activity in liver mitochondria from fed and starved rats

Mitochondria (0.5–0.75 mg of protein) from (○) fed, (△) 18h- and (□) 42h-starved rats were assayed for CAT activity as described in the Methods section. In the fed group maximal suppression (52%) of the total CAT activity was achieved with 20 μM -malonyl-CoA, and this portion was taken to represent CAT I (McGarry *et al.*, 1978a). The concentrations of malonyl-CoA required for 50% inhibition of CAT I are indicated. Values are means $\pm$ S.E.M. for six experiments in each group.

Fig. 3. Effect of mitochondrial concentration and incubation time on the malonyl-CoA inhibition of oleate oxidation in mitochondria from fed rats

The indicated quantities of mitochondria (O, ●, 1.25 mg of protein; Δ , $\blacktriangle$, 6.25 mg of protein) from fed rats were incubated with [$1\text{-}^{14}\text{C}$]oleate under standard conditions (O, Δ , 5 min; ●, $\blacktriangle$, 10 min) as described in the Methods section, and the production of acid-soluble radioactivity was followed. Panels (b) and (c) show the time courses of oleate oxidation with different amounts of mitochondria at various concentrations of malonyl-CoA [Mal-CoA]. The concentrations of malonyl-CoA required for 50% inhibition are shown in panel (a). A typical experiment is depicted.

10 min. Further inspection of Fig. 3(c) reveals that although malonyl-CoA was clearly inhibitory over the initial 5 min period, its effectiveness was greatly decreased in the subsequent 5 min. As a result, I_{50} values of 4 μM and 15.5 μM were obtained for the 5 and 10 min time points respectively (Fig. 3a). Thus a 10-fold difference in the potency of the malonyl-CoA effect could be obtained by switching from the standard incubation conditions (1.25 mg of protein; 5 min) to conditions equivalent to those employed by Ontko & Johns (1980) (6.25 mg of protein; 10 min). Qualitatively similar results were obtained when the above experiment was repeated with mitochondria from 18h-starved rats. In this case I_{50} values for malonyl-CoA ranged from 5.5 μM under standard conditions (1.04 mg of mitochondrial protein; 5 min) to 22 μM when 5.2 mg of mitochondrial protein and a 10 min reaction time were used (results not shown). The critical importance of choosing a low concentration of mitochondria and a short incubation time is further illustrated by the fact that if the data of Fig. 3(c) are arranged such that the absolute quantity of oleate oxidized between 5 and 10 min is plotted against the initial malonyl-CoA concentration, the latter up to 5 μM actually appears to be stimulatory for fatty acid oxidation (plot not shown). Such a conclusion would obviously be erroneous. The paradox stems from a combination of two factors: in control mitochondria under non-standard conditions the rate of oleate oxidation fell between 5 and

10 min because of substrate depletion, whereas in malonyl-CoA-treated samples the rate increased, presumably because of hydrolysis of the CoA ester (with removal of the initial inhibition).

Direct evidence for the rapid mitochondrial destruction of malonyl-CoA is shown by the experiment of Fig. 4. In this case a high concentration of mitochondria (7.4 mg of protein) was preincubated for 10 min with the indicated quantities of CoASH or malonyl-CoA before addition of the remaining components of the fatty acid oxidation system. As expected, in the absence of either compound the rate of oleate oxidation was low because CoASH was limiting and increased greatly as CoASH was added. Preincubation with malonyl-CoA up to a concentration of 10 μM was just as effective as CoASH; only at higher concentrations did the CoA ester prove inhibitory. It is thus evident that this quantity of mitochondria had the capacity to destroy at least 25 nmol of malonyl-CoA with the release of an equivalent amount of free CoASH over a 10 min period. [The mechanism of this destruction is not known. It could result either from direct deacylation of malonyl-CoA or from decarboxylation of malonyl-CoA followed by deacylation of acetyl-CoA.] Such hydrolysis would readily account for the decreased effectiveness of low concentration of malonyl-CoA in inhibiting fatty acid oxidation when high concentrations of mitochondria are incubated for longer than 5 min (Fig. 3).

Fig. 4. Effect of preincubation of mitochondria with CoASH or malonyl-CoA on the subsequent oxidation of oleate

Mitochondria from fed rats (7.4 mg of protein in 0.2 ml of 0.15 M-KCl) were preincubated for 10 min at 30°C with 1.3 ml of modified Krebs–Henseleit buffer containing 625 nmol of reduced glutathione and 0–75 nmol of CoASH (O) or malonyl-CoA (●). The remainder of the standard components of the fatty acid-oxidation system (minus CoASH) were then added in a volume of 1 ml of buffer, and the formation of acid-soluble products was measured over the subsequent 5 min period. A typical experiment is shown.

Importance of other variables in demonstrating the malonyl-CoA effect

Comparison of our standard incubation conditions with those used by Cook *et al.* (1980) reveals a number of differences. First, these investigators also used a relatively high quantity of mitochondria (5 mg of protein). Second, their concentrations of palmitate and albumin were 450 μM and 1.5% respectively (molar ratio fatty acid/albumin = 2.07); equivalent values in our system were 40 μM and 0.8% respectively (molar ratio = 0.35). Third, they used a higher temperature (37°C, as opposed to 30°C in our experiments). Fourth, their concentration of CoASH was lower than ours (20 μM versus 50 μM).

The effects of these variables on the sensitivity of oleate oxidation are shown in Fig. 5. Under standard conditions mitochondria from fed rats responded to malonyl-CoA in typical fashion, the I_{50} value in this case being 1 μM. Elevation of the oleate concentration from 40 μM to 400 μM (increase in [fatty acid]/[albumin] from 0.35 to 1.81) with all other components unchanged increased the I_{50} value for malonyl-CoA to 7 μM. When changes were also made in the quantity of mitochondria, the CoASH concentration and the temperature (to simulate the conditions of Cook *et al.*, 1980), the curve shifted further to the right (I_{50} 11 μM), the loss of malonyl-CoA sensitivity being most pronounced at the lower concentrations of the inhibitor. We interpret these results as follows. When the only modification of the standard system was elevation of the oleate concentration, the rate of fatty acid oxidation increased from 7 to 15 nmol per 5 min. This undoubtedly reflected an increased concentration of oleoyl-CoA available to carnitine acyltransferase I, probably through the combined effects of accelerated synthesis and displacement of long-chain acyl-CoA molecules from albumin by the high concentration of competing non-esterified fatty acid. Since malonyl-CoA is a competitive inhibitor towards long-chain acyl-CoA in the carnitine acyltransferase I reaction (McGarry *et al.*, 1978a,b), a loss of its inhibitory potency would be expected under such conditions. When the higher oleate concentration was combined with a 3-fold increase in mitochondria, a decrease of the CoASH concentration and an operating temperature of 37°C, there was a dramatic enhancement in the control rate of fatty acid oxidation to 109 nmol per 5 min, despite the fact that this concentration of CoASH (20 μM) was suboptimal under standard conditions. Since long-chain acyl-CoA generation is not limiting in this situation (Cook *et al.*, 1980), it is likely that the free concentration of oleoyl-CoA also increased, contributing to a further diminution in the potency of malonyl-CoA. The disproportionate fall in the effectiveness of the lowest concentrations of malonyl-CoA was probably due to the offsetting effects of malonyl-CoA destruction coupled with liberation of free CoASH, as noted in the experiment of Fig. 3. The reasons why we consider fatty acid effects to be secondary *in vivo* have been discussed elsewhere (McGarry & Foster, 1980b).

Ontko & Johns (1980) noted that the ability of malonyl-CoA to inhibit fatty acid oxidation progressively diminished as the fatty acid/albumin molar ratio was increased. They suggested that the value for this ratio used in our studies (0.35) was too low and resulted in an artificial accentuation of the effectiveness of malonyl-CoA. An operational value of 0.6–0.7, as obtains in rat plasma, was therefore recommended. However, even with a ratio of 1.81 we could obtain 50% inhibition of oleate oxidation

with $7\mu\text{M}$ -malonyl-CoA. They observed less than 10% inhibition with a lower fatty acid/albumin ratio (about 1.25) and $20\mu\text{M}$ -malonyl-CoA, probably because most of the malonyl-CoA had been destroyed by the high concentration of mitochondria used. It is inappropriate to equate the concentration of fatty acids in plasma with that present inside the liver cell, since the latter is generally much lower than the former, owing to rapid metabolism (McGarry & Foster, 1971). Moreover, the important parameter is the free concentration of long-chain acyl-CoA, not non-esterified fatty acids. This is difficult to ascertain, because of extensive binding of long-chain acyl-CoA species to intracellular proteins. However, the total content of long-chain acyl-CoA in the liver of a fed rat is about 25 nmol/g wet wt. (Guynn *et al.*, 1972). Using palmitoyl-CoA at a concentration of $40\mu\text{M}$ in the presence of 0.8% albumin, we observed 80% suppression of β -oxidation with only $10\mu\text{M}$ -malonyl-CoA (McGarry *et al.*, 1978a). We are therefore confident that the effectiveness of malonyl-CoA is blocking fatty acid oxidation in our studies with isolated mitochondria is representative of events taking place in the intact liver cell.

Fig. 5. Effect of different variables on the inhibition of mitochondrial fatty acid oxidation by malonyl-CoA. Mitochondria from the livers of fed rats were incubated with $[1-^{14}\text{C}]$ oleate for 5 min under the following conditions:

	Mitochondrial protein (mg)	[Oleate] (μM)	[Oleate] [albumin]	[CoASH] (μM)	Temp.
○	1.5	40	0.35	50	30°C
●	1.5	400	1.81	50	30°C
▲	4.5	400	1.81	20	37°C

Absolute quantities of oleate converted into acid-soluble products were: (○), 7 nmol; (●), 15 nmol; (▲), 109 nmol. The concentrations of malonyl-CoA required for 50% inhibition are indicated. A typical experiment is depicted.

Does the modest decrease in sensitivity of carnitine acyltransferase I to inhibition by malonyl-CoA induced by starvation require re-interpretation of the primary role of malonyl-CoA in regulating rates of fatty acid synthesis and oxidation, as suggested by Cook *et al.* (1980) and Ontko & Johns (1980)? We think not. We have produced abrupt shifts in the concentration of malonyl-CoA from 1 to 10 nmol per g in hepatocytes taken from 18 h-starved rats by adding substrates such as lactate, pyruvate and glutamine (Boyd *et al.*, 1981). Under these conditions overall oxidation of oleic acid fell acutely by 75%, while suppression of ketogenesis reached 95%, entirely compatible with the malonyl-CoA sensitivity of carnitine acyltransferase I in mitochondria from 18 h-starved rats shown directly in the present studies. Thus, although starvation does appear to result in decreased mitochondrial sensitivity to malonyl-CoA, the effect is not profound, and at present its physiological significance, if any, remains obscure.

The biochemical basis for altered sensitivity of carnitine acyltransferase I in starvation is not clear. Mixing experiments involving mitochondria from fed and starved rats failed to indicate the presence of a diffusible inhibitor, and rates of malonyl-CoA degradation were equivalent in the two preparations (results not shown). When palmitoyl-CoA was used as the oxidizable substrate (in place of non-esterified fatty acid), the lower sensitivity of mitochondria from starved rats to malonyl-CoA was still observed, indicating that the change was not due to enhanced formation of oleoyl-CoA during starvation. We have previously shown that the physical environment of carnitine acyltransferase I on the outer aspect of the mitochondrial inner membrane is important in conferring malonyl-CoA sensitivity to the enzyme (McGarry *et al.*, 1978a). Perhaps the membrane structure or the enzyme itself is altered in starvation.

The present studies serve to underscore the necessity for careful attention to experimental detail in evaluating the kinetics of the interaction of malonyl-CoA with the process of mitochondrial fatty acid oxidation. They confirm that in the fed

state carnitine acyltransferase I is exquisitely sensitive to the inhibitory action of this simple molecule. Although food deprivation results in some loss in the sensitivity of the enzyme to malonyl-CoA (apparently in a time-dependent manner), the magnitude of this change is not great in absolute terms. Thus, of the two variables, i.e. the concentration of malonyl-CoA and the sensitivity of carnitine acyltransferase I to the CoA ester, alterations in the former will probably be of primary importance in regulating carbon flow through the β -oxidative pathway. It must also be recognized that, in addition to the tissue content of acyl-CoA and malonyl-CoA, other factors, notably the intracellular concentration of carnitine, play important roles in determining the rate of fatty acid oxidation in liver.

In summary, we see no need to modify current concepts regarding the role of malonyl-CoA in controlling interactions between hepatic carbohydrate and lipid metabolism as discussed in detail elsewhere (McGarry *et al.*, 1978c; McGarry & Foster, 1979, 1980a).

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Since the submission of this paper, another report has appeared in which a starvation-induced decrease in malonyl-CoA sensitivity of liver mitochondrial carnitine acyltransferase was documented (Saggerson & Carpenter, 1981). In this case I_{50} values for malonyl-CoA of $5.4\mu\text{M}$ and $32\mu\text{M}$ were recorded with mitochondria from fed and 24h-starved rats respectively. However, the proportion of total transferase calculated to be inhibitable by malonyl-CoA (and assumed to be a measure of carnitine acyltransferase I) was extremely high (93% and 83% in the fed and starved groups respectively), in comparison with our value of about 50% in both nutritional states. From the limited details of the assay provided, we suspect that these high values might have stemmed from the use of an excessive ratio of [palmitoyl-CoA]/[protein] in the incubation mixture. In addition to offsetting the malonyl-CoA effect, unbound palmitoyl-CoA, by virtue of its potent detergent properties, could be expected to alter the relative sensitivities of both carnitine acyltransferase I and carnitine acyltransferase II to malonyl-CoA (McGarry *et al.*, 1978a). It is of interest that Saggerson & Carpenter (1981) found that only 50–59% of the total transferase activity could be inhibited by malonyl-CoA when palmitoyl-CoA

was replaced by octanoyl-CoA as substrate (the latter is a much weaker detergent). Moreover, under these conditions the concentrations of malonyl-CoA needed for 50% suppression of carnitine acyltransferase I were only $0.7\mu\text{M}$ and $0.9\mu\text{M}$ in mitochondria from fed and starved rats respectively. Such values are much closer to those found in our laboratory.

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