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Optimal Conditions for Amino Acid Incorporation by Isolated Rat Liver Mitochondria. Stimulation by Valinomycin and Other Agents†

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ABSTRACT: Amino acid incorporation by isolated rat liver mitochondria proceeded at the same rate when either succinate plus ATP or an ATP generating system containing phosphoenolpyruvate was used as an energy source. Antimycin A caused a 90% inhibition of the incorporation rate when succinate was present suggesting that ATP was largely synthesized by respiratory chain-linked phosphorylations. In contrast, atractyloside caused a 70% inhibition of the incorporation rate, when ATP and phosphoenolpyruvate were present, suggesting that the ATP necessary for protein synthesis under these conditions had been previously transported across the mitochondrial membrane by the atractyloside-sensitive adenine translocase. The inhibitory effects of atractyloside in the ATP–P-enolpyruvate system could be completely reversed by the addition of glutamate or succinate. Substances which affect the adenine translocase such that the transport of ATP into the mitochondria is increased also stimulated the rates of leucine incorporation. Addition of low concentrations of the ionophore valinomycin (1 μg/mg of protein) stimulated amino acid incorporation two- to threefold provided that KCl was present. Phosphate was not required for the valinomycin stimulation. Valinomycin caused identical or even greater stimulations of the incorporation rate when antimycin A or the uncoupler, carbonyl cyanide phenylhydrazone (CCP), was also present. Low concentrations of CCP doubled the rate of amino acid incorporation provided that oligomycin was also present. Calcium ions also stimulated the incorporation rate when the ATP concentration was lowered to 0.2 mM. Atractyloside, the inhibitor of the adenine translocase, prevented the stimulation of amino acid incorporation by all these substances. These results suggest that ATP present in the matrix of the mitochondria whether generated by respiration or previously transported across the membrane is necessary for optimal rates of amino acid incorporation in rat liver mitochondria. Any substances which affect the intramitochondrial ATP level will increase the incorporation rate.

Investigators in many laboratories have established the conditions necessary to study amino acid incorporation into protein by isolated mitochondria *in vitro* (Beattie, 1971). The many different, and often, conflicting factors which have been reported to influence the rate of incorporation may be a reflection of the different mitochondrial preparations used by the various groups rather than any intrinsic differences in the incorporation mechanism. The recent publications (Hamberger *et al.*, 1969; Coote and Work, 1971; Williams and Birt, 1971a, 1971b) of incorporation media which varied significantly from that defined in our previous studies (Beattie *et al.*, 1967a) prompted us to reinvestigate the conditions necessary to achieve optimal rates of amino acid incorporation with isolated mitochondria.

The results obtained suggest that the ionic composition of the medium is critical for optimal rates of amino acid incorporation by either intact mitochondria or the isolated inner membrane–matrix fraction. The substitution of sodium or ammonium ions for potassium ion in the medium resulted in greatly lowered rates of incorporation. A need for a source of energy to support amino acid incorporation has been generally accepted by workers in this field (Beattie, 1971). In the present study, we observed that nearly identical rates of incorporation could be obtained if the necessary ATP was either generated by respiratory chain-linked phosphorylations or by the addition of an external ATP-generating system. In the latter case the exogenous ATP had to be first transported across the inner mitochondrial membrane by the adenine translocase (Klingenberg, 1970). The addition of substances such as valinomycin (plus potassium), low concentrations of calcium, or uncouplers of oxidative phosphorylation which affect the adenine translocase such that a higher intramitochondrial concentration of ATP results caused a large increase in the rate of amino acid incorporation.

Methods

Preparation of Mitochondria. Liver mitochondria were prepared under sterile conditions in a medium containing 0.25 M sucrose, 0.01 M Tris-chloride, pH 7.8, and 0.001 M EDTA (sodium salt) by previously described methods (Beattie, 1968) which yield a mitochondrial pellet which is 3% contaminated

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TABLE I: Effect of Valinomycin on Amino Acid Incorporation by Liver Mitochondria.[a]

Conditions	Cpm/mg Control	Cpm/mg Valinomycin	% Change
90 mM KCl	2280	4850	+113
− phosphate	1660	4430	+166
30 mM KCl + 70 mM NH_4Cl	1560	3900	+148
90 mM NH_4Cl	1400	938	−33

[a] Liver mitochondria were incubated in the medium described under Materials and Methods. Where indicated, 90 mM KCl was replaced with 30 mM KCl + 70 mM NH_4Cl or 90 mM NH_4Cl. Valinomycin was added to a final concentration of 0.5 μg/ml.

with microsomal protein. Inner membrane–matrix fractions were prepared with digitonin as described by Schnaitman and Greenwalt (1968). The pellet obtained by centrifuging the digitonin-treated mitochondria was washed twice by resuspending in the same volume of sucrose–Tris–EDTA and centrifuging at 9000*g*.

Amino Acid Incorporation Studies. Amino acid incorporation into mitochondrial protein *in vitro* was determined using mitochondria obtained under sterile conditions (Beattie *et al.*, 1967b). The four-times washed liver mitochondrial pellet and the twice-washed inner membrane–matrix fraction were resuspended in 0.25 M sucrose to a final concentration of 6–8 mg/ml of protein and incubated in a medium containing 50 mM Bicine buffer, pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM phosphate, pH 7.6, 90 mM KCl, 2.0 mM ATP, 5 mM P-enolpyruvate,[1] 10 μg/ml of pyruvic kinase, 22.5 μg of a complete amino acid mixture minus leucine, as described by Roodyn *et al.* (1961), 0.25 μCi/ml of uniformly labeled [^{14}C]L-leucine, and 2.0–3.0 mg of mitochondrial protein in a final volume of 2.0 ml. Respiratory substrates were added in the concentrations indicated in the legends to Tables I–VI. After 30 min at 30° in a metabolic shaker, the incubation was terminated by the addition of 10 mM unlabeled leucine followed by precipitation with 5% CCl_3COOH. The labeled proteins were prepared for counting by previously described methods (Beattie *et al.*, 1967b), and counted in a scintillation counter with an efficiency for ^{14}C of 90%. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Amino Acid Uptake. The uptake of radioactive leucine was studied at 20° in the same medium as used for incorporation with the further addition of sufficient chloramphenicol (50 μg/ml) to block completely protein synthesis. Aliquots containing less than 1 mg of mitochondrial protein were removed at various time intervals and immediately filtered on Millipore filters (0.45 μ). The entire sampling process took no more than 5 sec. The filters were immediately washed three times with ice-cold sucrose. The filters were air-dried, placed in the solution described by Bray (1960), and counted.

Materials

Nucleotides, oligomycin, antimycin A, and atractyloside were obtained from Sigma, carbonyl cyanide *m*-chlorophenylhydrazone (CCP)[1] and valinomycin from Calbiochem, and uniformly labeled L-leucine (250 μCi/μmol) from New England Nuclear.

[1] Abbreviation used: CCP, carbonyl cyanide *m*-chlorophenylhydrazone. P-enolpyruvate, phosphoenolpyruvate.

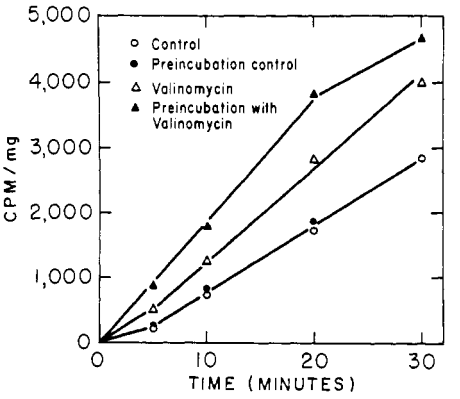

FIGURE 1: Time curve of amino acid incorporation by rat liver mitochondria with and without valinomycin. Mitochondria were incubated as described in Materials and Methods with the addition of 0.5 μg/ml of valinomycin where indicated. Mitochondria were also preincubated for 10 min with or without valinomycin prior to the addition of [^{14}C]leucine: (●) control; (○) preincubated control; (▲) valinomycin; (△) preincubation with valinomycin.

Results

Ionic Composition of the Medium. The presence of 90 mM KCl in the incubation medium resulted in optimal rates of amino acid incorporation by isolated rat liver mitochondria. The substitution of NH_4Cl or NaCl for part or all of the KCl in the incubation medium caused a 40–60% decrease in the incorporation rate (Table I).

Effect of Valinomycin. The addition of 1 μg of valinomycin to the incubation medium resulted in a nearly twofold stimulation in the incorporation rate throughout a 30-min incubation period (Figure 1). The rate was stimulated at the earliest time point, 5 min, where a definite lag was observed in the control. Preincubation of the mitochondria for 10 min at 30° prior to the addition of radioactive leucine had no effect on the kinetics. Preincubation with valinomycin under identical conditions, however, resulted in even greater stimulation of the incorporation rate and eliminated the lag observed in the control.

Valinomycin also stimulated the rate of amino acid incorporation when added during incubation at 5, 10, and 20 min (Figure 2). The incorporation observed after 30 min was almost identical whether the valinomycin was initially present in the incubation mixture or added at any time during the incubation.

The stimulation of incorporation by valinomycin did not require the presence of phosphate (Table I). Addition of valinomycin also caused a stimulation when the KCl concentration was lowered to 30 mM and the ionic strength maintained by substitution of NH_4Cl for the remaining KCl. However, when the KCl was completely omitted, the addition of valinomycin caused an inhibition of the incorporation rate.

Stimulation by Valinomycin in the Presence of Inhibitors. In an attempt to clarify the mechanism by which valinomycin stimulates amino acid incorporation, the effects of valinomycin were studied in the presence of various inhibitors and uncouplers of oxidative phosphorylation. The addition of CCP and antimycin A to the incubation medium caused a 50–60% inhibition of incorporation (Table II). The further addition of valinomycin in the presence of these inhibitors

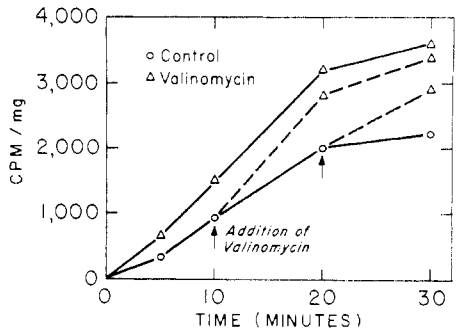

FIGURE 2: Effect on incorporation rate of addition of valinomycin at various times during the incubation. Valinomycin was added to a final concentration of 0.5 μg/ml: (○) control; (Δ) plus valinomycin.

stimulated the incorporation rate to a greater extent than that observed in the control.

The rate of amino acid incorporation in the presence of ATP and P-enolpyruvate was also inhibited by atractyloside (Table II), the inhibitor of the adenine translocase (Klingenberg, 1970). A similar inhibition by atractyloside was also observed when antimycin A and CCP were present. The stimulation of amino acid incorporation by valinomycin was partially prevented by atractyloside (Table II). The rates in the presence of atractyloside plus valinomycin, however, were higher than those observed with atractyloside alone except when antimycin A was also present. The 2.5-fold stimulation of the incorporation rate by valinomycin in the presence of antimycin A was completely blocked by atractyloside.

As seen in Table III, almost identical rates of amino acid incorporation were observed when ATP and the respiratory substrate, succinate, were substituted for ATP and the regenerating system. The addition of antimycin A caused a 93% inhibition of the incorporation rate when succinate was present and a 37% inhibition where ATP and P-enolpyruvate were present. Likewise, oligomycin caused a 43% inhibition in the former system and no inhibition in the latter system. In contrast, atractyloside caused a 60% inhibition of amino acid incorporation in the ATP–P-enolpyruvate system but only a 34% inhibition when succinate was present. Valinomycin only caused a significant stimulation of the incorporation rate when ATP and P-enolpyruvate were present.

Klingenberg (1970) has demonstrated that the addition of valinomycin plus potassium ions causes a change in the adenine translocase such that the exchange with exogenous ATP becomes more rapid. Hence, it seemed possible that the observed stimulation of amino acid incorporation by valinomycin resulted from the presence of higher intramitochondrial concentrations of ATP which might result from such a change.

Several other conditions which have been reported to stimulate the adenine translocase were also tested for their effect on amino acid incorporation. As seen in Table IV, the addition of 0.2 mM Ca^{2+} was inhibitory when 2.0 mM ATP was present. However, the same concentration of Ca^{2+} almost doubled the extent of amino acid incorporation when the ATP concentration was lowered to 0.2 mM. This stimulation was completely blocked by atractyloside. A recent report (Spencer and Bygrave, 1971) has suggested that 0.2 mM concentrations of Ca^{2+} stimulate the adenine translocase provided that the concentration of exogenous ATP is 0.1–0.2 mM.

The addition of uncouplers such as CCP or dinitrophenol also changes the direction of the adenine translocase such that there is a more rapid transport of ATP into the mitochondria (Klingenberg, 1970). The addition of CCP by itself, however, caused a significant inhibition of the rate of amino acid incorporation (Table IV). This result may mean that this concentration of CCP (0.1 μM) induces sufficient ATPase activity to lower the intramitochondrial ATP concentration necessary to support amino acid incorporation. The subsequent addition of oligomycin to block the CCP-stimulated ATPase activity caused a 60% stimulation of the incorporation rate.

TABLE II: Effect of Valinomycin on Amino Acid Incorporation in the Presence of Inhibitors of Oxidative Phosphorylation and Atractyloside.[a]

Conditions	cpm/mg		
	None	Antimycin A	CCP
Control	2150	1060	813
+ valinomycin	3880	2690	1810
+ atractyloside	572	247	347
+ valinomycin and atractyloside	1160	238	648

[a] Liver mitochondria were incubated as described under Materials and Methods. Additions were made to a final concentration of 0.5 μg/ml of valinomycin, 50 μM atractyloside, 2.5 μg/ml of antimycin A, and 1 μM CCP.

TABLE III: Effect of Valinomycin and Inhibitors on Amino Acid Incorporation when ATP Is Generated under Different Conditions.[a]

Additions	ATP–P-enolpyruvate		ATP–Succinate	
	cpm/mg	% Change	cpm/mg	% Change
None	2170		1920	
+ valinomycin (0.5 μg/ml)	4300	+98	2150	+12
+ atractyloside (50 μM)	883	−60	1270	−34
+ oligomycin (5 μg/ml)	2260	No	1090	−43
+ antimycin A (2.5 μg/ml)	1360	−37	131	−93

[a] Mitochondria were incubated as described under Materials and Methods. Final concentration of succinate was 10 mM.

TABLE IV: Effect of Other Conditions Which Stimulate the Adenine Translocase on Amino Acid Incorporation.[a]

Conditions	cpm/mg
Control (2.0 mM ATP)	2280
+ $CaCl_2$ (0.2 mM)	1410
+ CCP (0.1 μM)	825
+ oligomycin (5 μg/ml)	2150
+ CCP and oligomycin	3620
+ CCP, oligomycin, and atractyloside (50 μM)	2500
Control (0.2 mM ATP)	491
+ $CaCl_2$	994
+ $CaCl_2$ and atractyloside	166

[a] Liver mitochondria were incubated as described under Materials and Methods.

TABLE V: Effect of Substrates and Actractyloside on Amino Acid Incorporation.[a]

Additions	cpm/mg
ATP–P-enolpyruvate–pyruvate kinase	1560
+ atractyloside	474
+ atractyloside and succinate	2100
+ atractyloside and glutamate	1400
Succinate (no ATP)	1600
+ atractyloside	3020
Glutamate (no ATP)	1350
+ atractyloside	2210

[a] Liver mitochondria were incubated as described under Materials and Methods. No exogenous adenine nucleotides were added where it states no ATP. Atractyloside was added to a concentration of 50 μM and respiratory substrates to a concentration of 10 mM.

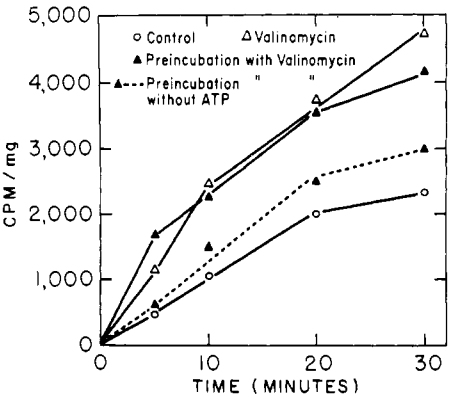

FIGURE 3: Effect of preincubation with valinomycin in the presence or absence of ATP. Mitochondria were incubated as described in Materials and Methods with the addition of valinomycin at 0.5 μg/ml: (●) control; (▲—▲) valinomycin; (○) preincubation with valinomycin and ATP for 10 min prior to addition of [^{14}C]leucine; (▲- - -▲) preincubation with valinomycin minus ATP prior to addition of [^{14}C]leucine plus ATP.

Atractyloside completely inhibited the stimulation of incorporation by CCP and oligomycin.

Effect of Substrate Addition. The addition of a respiratory substrate, either succinate or glutamate, to the incubation medium containing ATP and P-enolpyruvate reversed the atractyloside inhibition of amino acid incorporation (Table V). When the substrates were added without exogenous adenine nucleotides, the addition of atractyloside increased the incorporation rate nearly twofold.

Preincubation without ATP. As described above (Figure 1), preincubation with valinomycin prior to addition of radioactive leucine resulted in an enhanced stimulation of the incorporation rate. If ATP were omitted during the preincubation with valinomycin, no increase in the rate of amino acid incorporation was observed during the first 5 min of the incubation (Figure 3). After that time, however, the incorporation increased at a rapid rate comparable to that observed when ATP was present during the preincubation with valinomycin.

Inner Membrane–Matrix Fraction. Amino acid incorporation by the digitonin inner membrane fraction can only be supported by exogenous ATP and the regenerating system (Table VI). Very low incorporation rates were observed when succinate and either ATP or ADP were added. Valinomycin caused a stimulation of the incorporation rate which was completely blocked by atractyloside. This result was anticipated since Winkler *et al.* (1968) reported that the atractyloside-sensitive adenine translocase was localized in the inner membrane.

Amino Acid Uptake. Amino acid uptake into mitochondria when protein synthesis was blocked with chloramphenicol proceeded at a linear rate after a short lag at 1.5 min (Figure 4). This lag may represent the time for the incubation medium to reach 20° after removal from the ice bath. Maximum uptake was achieved between 5 and 7 min as was also reported by Buchanan *et al.* (1969). No further uptake of leucine was observed throughout a 30-min incubation nor was any decrease in the amount of labeled intramitochondrial leucine observed as described by Wheeldon and Lehninger (1966). The addition of valinomycin had no effect on the uptake of leucine.

Discussion

Rat liver mitochondria prepared by extensive washings with sucrose can incorporate amino acids into protein at approximately the same rate when either an external source of ATP plus an ATP-generating system is present or when ATP is synthesized by the respiratory chain using succinate as substrate. The inhibitors atractyloside to block the transport of ATP into the mitochondria and antimycin A to block electron transport were used to determine the source of ATP used for the amino acid incorporation. In the presence of succinate and either ATP or ADP the necessary ATP was largely generated by the respiratory chain as indicated by the 90% inhibition by antimycin A. In the presence of the ATP-regenerating system, the majority of the ATP must have been transported across the mitochondrial membrane as indicated by the 70% inhibition by atractyloside. However, some of the incorporation in the presence of exogenous ATP and the regenerating system was still dependent on oxidative phosphorylation as indicated by the 30–40% inhibitions by antimycin A. It thus appears that mitochondrial protein synthesis occurs in the matrix of the mitochondria and that intramitochondrial ATP, either generated by respiratory chain-linked phosphorylation or transported across the inner membrane, provides the energy necessary for amino acid incorporation.

The inhibitory effects of atractyloside in the presence of ATP and the regenerating system do not agree with previous studies which indicated that atractyloside stimulated amino acid incorporation (Gangal and Bessman, 1968) or had no

TABLE VI: Amino Acid Incorporation by the Digitonin Inner Membrane Fraction.[a]

Conditions	cpm/mg
ATP–P-enolpyruvate	2990
+ valinomycin	5220
+ atractyloside	1820
+ valinomycin and atractyloside	2050
ATP–succinate	755
ADP–succinate	200

[a] The inner membrane–matrix fraction prepared by the method of Schnaitman and Greenawalt (1968) was incubated as described in Materials and Methods. The concentrations of various substances were: valinomycin, 0.5 μg/ml; atractyloside, 50 μM; and succinate, 10 mM.

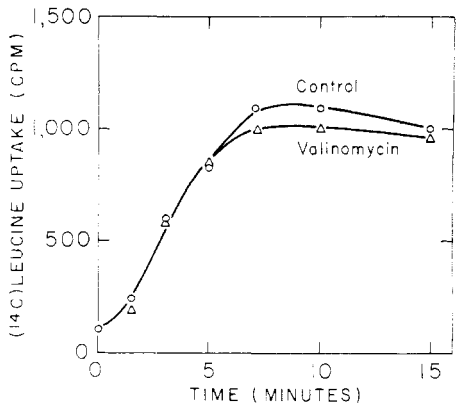

FIGURE 4: Time course of uptake [^{14}C]leucine into mitochondria in the presence (0.5 μg/ml) and absence of valinomycin. Mitochondria were incubated at 20° as described in Materials and Methods with the addition of chloramphenicol (50 μg/ml). The mitochondria were rapidly isolated on Millipore filters prior to counting as described under Materials and Methods.

effect (Mockel, 1972). Perhaps the incorporation in these studies was solely dependent on ATP synthesized within the mitochondria and not by the transport of exogenous ATP into the mitochondria. It is interesting in this context that addition of respiratory substrates reversed the atractyloside inhibition observed in the ATP–PEP system. Presumably these additional substrates might generate sufficient ATP by respiration using the endogenous adenine nucleotide pools.

The adenine translocase in coupled mitochondria reacts nearly five times more rapidly with exogenous ADP than ATP (Klingenberg, 1970). As a result, the intramitochondrial concentration of ADP is correspondingly greater than that of ATP. The addition of either uncouplers or ionophores plus potassium ions causes the exchanges of ADP and ATP to become equal. Hence, the intramitochondrial ratio of ADP to ATP becomes 1:1, an effective increase in the intramitochondrial ATP concentration assuming that the intramitochondrial adenine nucleotide pool remains constant. The presence of potassium ions also acts to control the adenine nucleotide exchange in the same direction (Meisner, 1971). Calcium ions have also been shown to stimulate the adenine translocase when the exogenous ATP concentration is approximately 0.1 mM (Spencer and Bygrave, 1971).

The present study indicates that amino acid incorporation in rat liver mitochondria requires potassium ion for maximum activity. In addition, the incorporation rate is stimulated by all the conditions which change the direction of the adenine translocase as outlined above, *e.g.*, valinomycin plus potassium, the uncoupler CCP provided that oligomycin is also present, and by calcium in the presence of low concentrations of ATP. The fact that the stimulation of incorporation by valinomycin, CCP, or calcium is blocked by atractyloside, the inhibitor of the adenine translocase, provides further substantiation for the suggestion that these conditions stimulate amino acid incorporation by their effects on the adenine translocase.

It was puzzling that atractyloside could not completely prevent the stimulation of amino acid incorporation by valinomycin. One explanation is that when atractyloside is present, a greater amount of amino acid incorporation might become dependent on respiratory chain-linked synthesis of ATP. Presumably, atractyloside might prevent the efflux of newly synthesized ATP, thus making it more available to support amino acid incorporation. This explanation appears likely since the addition of atractyloside stimulated the incorporation rate when glutamate or succinate were present without exogenous adenine nucleotides (Table V). In addition, atractyloside completely blocked the valinomycin stimulation of incorporation when antimycin A was also present to block any respiratory chain-linked phosphorylation.

The studies of amino acid uptake into mitochondria when protein synthesis was blocked with chloramphenicol suggest that the transport of leucine into the mitochondria is not the rate-limiting step in the overall incorporation. The uptake of leucine proceeded rapidly reaching a maximum within 5–7 min. The addition of valinomycin had no stimulatory effect on leucine uptake at any time.

Another mitochondrial process requiring ATP is the synthesis of citrulline. Exogenous ATP was shown to be a less effective energy donor than ATP synthesized by the respiratory chain (Graafsmans *et al.*, 1968; Charles and van den Bergh, 1967). It was observed that uncouplers plus oligomycin stimulated citrulline synthesis and that the further addition of atractyloside prevented this stimulation. The explanation for the stimulatory effects was that uncouplers affected the adenine translocase such that more ATP was transported into the mitochondria.

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