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Synthesis of Protein in Host-Free Reticulate Bodies of *Chlamydia psittaci* and *Chlamydia trachomatis*

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Synthesis of protein by the obligate intracellular parasitic bacteria *Chlamydia psittaci* (6BC) and *Chlamydia trachomatis* (serovar L2) isolated from host cells (host-free chlamydiae) was demonstrated for the first time. Incorporation of [^{35}S]methionine and [^{35}S]cysteine into trichloroacetic acid-precipitable material by reticulate bodies of chlamydiae persisted for 2 h and was dependent upon a exogenous source of ATP, an ATP-regenerating system, and potassium or sodium ions. Magnesium ions and amino acids stimulated synthesis; chloramphenicol, rifampin, oligomycin, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (a proton ionophore) inhibited incorporation. Ribonucleoside triphosphates (other than ATP) had little stimulatory effect. The optimum pH for host-free synthesis was between 7.0 and 7.5. The molecular weights of proteins synthesized by host-free reticulate bodies closely resembled the molecular weights of proteins synthesized by reticulate bodies in an intracellular environment, and included outer membrane proteins. Elementary bodies of chlamydiae were unable to synthesize protein even when incubated in the presence of 10 mM dithiothreitol, a reducing agent which converted the highly disulfide bond cross-linked major outer membrane protein to monomeric form.

Chlamydiae are intracellular parasitic bacteria which have not been propagated outside of eucaryotic host cells. Their unique developmental cycle includes infectious elementary bodies (EBs), noninfectious reticulate bodies (RBs), and a continuous progression of intermediate forms. EBs possess extensively disulfide bond cross-linked outer membrane proteins and are metabolically inert outside of host cells (2, 5, 9). The cross-linked proteins of EBs include the 40-kilodalton (kd) major outer membrane protein (MOMP) and 60- and 12-kd cysteine-rich proteins (5). Once within host cells, EBs reorganize into RBs—a process which includes reduction of cross-linked MOMP to monomers. By 8 to 12 h postinfection, RBs are fully metabolically active and divide with an optimal generation time of 2 h. In an intracellular environment, chlamydiae synthesize their own DNA, RNA, and protein and are sensitive to typical procaryotic inhibitors such as rifampin, tetracyclines, and chloramphenicol. However, unlike other bacteria, chlamydiae are dependent on unusual host-supplied nutrients such as ATP (4). As early as 20 h postinfection, some of the intracellular RBs commence reorganization back to the metabolically inert EB form. Ultimately the host cell is destroyed and infectious EBs are released.

Although chlamydiae have not been grown outside of host cells, biosynthetic and transport activities have been detected in host-free organisms. GTP is incorporated into RNA by RBs (3, 11), UTP has been reported to be incorporated into EBs rendered permeable with mercaptoethanol (10), and ATP and lysine are transported into host-free RBs by specific transport mechanisms (4). Host-free protein synthesis by chlamydiae has not been reported, although Weiss and Wilson (13) observed the ATP-dependent incorporation of isoleucine and aspartic acid into the lipid fraction of *Chlamydia psittaci*.

The purpose of this investigation was to define the conditions under which host-free RBs of *C. psittaci* (6BC) and *Chlamydia trachomatis* (serovar L2) synthesize protein. Host-free protein synthesis was found to be short-lived and dependent on a continuous supply of exogenous ATP.

(Part of this study was reported at the 5th International Symposium on Human Chlamydial Infection, Lund, Sweden, 1982 [3]).

MATERIALS AND METHODS

Growth of organisms. *C. psittaci* (6BC) was grown in 929L cells, and *C. trachomatis* (serovar L2) was grown in 229 Hela cells (6). RBs and EBs were harvested at 20 and 48 h, respectively, and purified by Renografin (E. R. Squibb & Sons, Princeton, N.J.) density centrifugation (4).

Standard reaction mixture. Except when noted otherwise, RBs (10 to 50 μg of protein per sample) were incubated in a standard reaction mixture containing 100 mM Tris-hydrochloride (pH 7.0), 50 mM KCl, 5 mM $MgCl_2$, 7.8 mM creatine phosphate, 1.5 mg of phosphocreatine kinase per ml, 19 unlabeled amino acids (each present at a final concentration of 50 μM), 1 mM ATP, and 1 to 10 μCi of [^{35}S]methionine or [^{35}S]cysteine. Carrier methionine or cysteine was added to a final concentration of between 1 and 5 μM in most experiments. After incubation at 37°C, samples (200 μl) were treated with 5% trichloroacetic acid, and the resulting precipitate was filtered and washed on 0.45-μm microporous filters.

Host-free versus in vivo protein synthesis. A culture of *C. psittaci*-infected L cells (6×10^7 cells) was divided into two equal parts, one of which was suspended in 10 ml of Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) containing 100 μg of cycloheximide per ml for in vivo studies, and the other of which was harvested for host-free studies. The infected cells in Hanks salts and cycloheximide were divided equally, and 20 μCi of [^{35}S]methionine (1.4 Ci/μmol) and 20 μCi of [^{35}S]cysteine (1.1 Ci/μmol) were added to the separate flasks. After 2 h of incubation at 37°C, the infected cells were lysed and RBs were purified. For the

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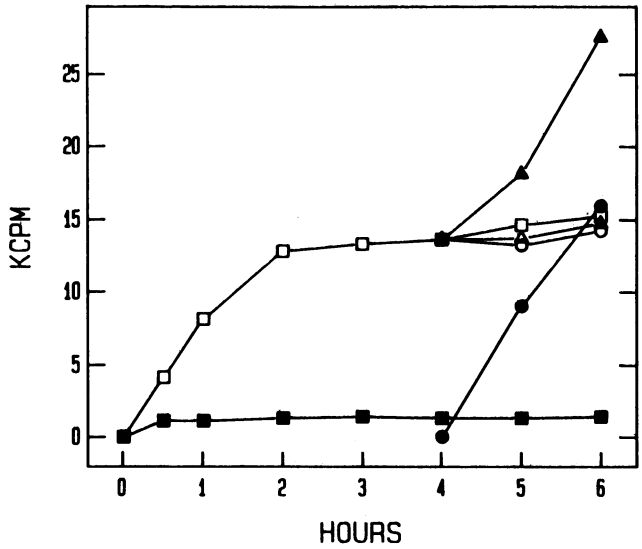

FIG. 1. Incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material of host-free RBs of *C. psittaci*. At time zero, RBs were incubated at 37°C in the standard reaction mixture with and without 1 mM ATP, and 200-μl samples were withdrawn and treated with trichloroacetic acid at the times indicated. Each sample contained 15 μg of RB protein and 2.7 μCi of [^{35}S]methionine (0.7 Ci/mmol). After 4 h of incubation, fresh RBs (maintained on ice), fresh reaction mixture (maintained on ice), or Tris-KCl was added to portions of the ATP-containing reaction (in amounts proportional to the amounts added at time zero), and samples (adjusted for the increased volume) were taken at the times indicated. The counts incorporated in the minus ATP samples were subtracted from corresponding samples incubated in the presence of ATP. Symbols: □, + ATP at time zero; ■, −ATP at time zero; ●, +ATP, incubated on ice from 0 to 4 h, and at 37°C after 4 h; ▲, additional RBs added at 4 h; ○, additional reaction mixture added at 4 h; △, additional Tris-KCl added at 4 h.

host-free study, purified RBs (424 μg of protein) were divided equally between a standard reaction mixture of 600 μl containing 5 μCi of [^{35}S]methionine (5 Ci/mmol) and one containing 5 μCi of [^{35}S]cysteine (5 Ci/mmol) and incubated for 2 h. RBs from the in vivo and the host-free reactions were divided into equal parts, one of which was retained as whole RBs, and the other of which was extracted with 2% sodium lauryl sarkosinate (Sarkosyl; Sigma Chemical Co., St. Louis, Mo.) for outer membrane preparation (5). Whole RBs and outer membranes derived from an equivalent number of whole RBs were suspended in 200 μl of solubilization buffer (5) containing 5% (vol/vol) 2-mercaptoethanol and heated to 100°C for 1 min, and 20 μl of each preparation was subjected to electrophoresis on a 7.5 to 15% gradient polyacrylamide gel (5) by the Tris-glycine buffer system of Laemmli (7).

Effect of pH. RBs of *C. psittaci* (24 μg of protein) were incubated in the standard reaction mixture for 1 h at 37°C except that the buffer at pH 6.0 and 6.5 was 100 mM 2-[(2-amino-2-oxyethyl)-amino]ethanesulfonic acid–KOH and creatine phosphate and phosphocreatine kinase were deleted from the reactions lacking the ATP-regenerating system. Two microcuries of [^{35}S]methionine (4 Ci/mmol) was added to the reactions containing the regenerating system, and 1 μCi of [^{35}S]methionine (1.4 Ci/μmol) was added to the reactions lacking the regenerating system.

Materials. [^{35}S]methionine (1.4 Ci/μmol) and [^{35}S]cysteine (1.1 Ci/μmol) were purchased from Amersham Corp., Arlington Heights, Ill. Metabolic inhibitors, amino acids, enzymes, and ribonucleoside triphosphates were obtained from Sigma. Rifampin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were dissolved in 50% dimethyl sulfoxide.

RESULTS

Host-free protein synthesis under standard conditions. Incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material by host-free RBs of *C. psittaci* in the standard incubation mixture was linear for approximately 1 h, slowed between 1 and 2 h, and almost ceased beyond 2 h (Fig. 1). Deletion of exogenous ATP or the ATP-regenerating system (creatine phosphate and phosphocreatine kinase) reduced uptake to background (heat-inactivated RBs). The addition of fresh RBs, but not the addition of other components of the standard reaction mixture, at 4 h revived incorporation. The cause of the cessation of host-free protein synthesis at 2 h has not been determined. In experiments not shown, the substitution of 50 mM NaCl for 50 mM KCl reduced incorporation by 50%, whereas deletion of both potassium and sodium resulted in background incorporation. Deletion of either exogenous amino acids or Mg^{2+} ions also resulted in a 50% reduction in incorporation. Incubation of RBs in the presence of [^{35}S]methionine (specific activity, 1.4 Ci/μmol) in the absence of carrier methionine resulted in incorporation of 40% of the added radioactivity and was complete within 5 min of incubation. Also in the absence of carrier methionine, a short burst of incorporation could be detected in the presence of 10 mM ATP without the ATP-regenerating system. RB host-free protein synthesis in both the presence and the absence of the ATP-regenerating system was optimal between pH 7.0 and 7.5 and dropped off sharply at acidic pH values (Fig. 2).

The patterns of incorporation of [^{35}S]methionine and [^{35}S]cysteine into the proteins of host-free RBs were similar, but not identical, to those of *C. psittaci* organisms growing in L cells (Fig. 3). [^{35}S]cysteine was incorporated by host-free RBs into known outer membrane proteins including the 40-kd MOMP and the 60- and 12-kd cysteine-rich proteins (Fig. 3B, lanes G and H) although incorporation was less

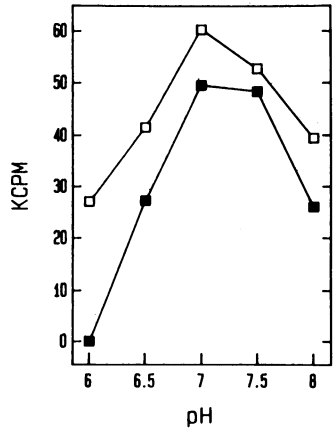

FIG. 2. Effect of pH on host-free protein synthesis in *C. psittaci*. The reactions were carried out as described in the text. No carrier L-methionine was added to the reaction mixture that lacked the ATP-regenerating system (■); thus, the short 5-min burst of ^{35}S incorporation is plotted. □, Plus ATP-regenerating system.

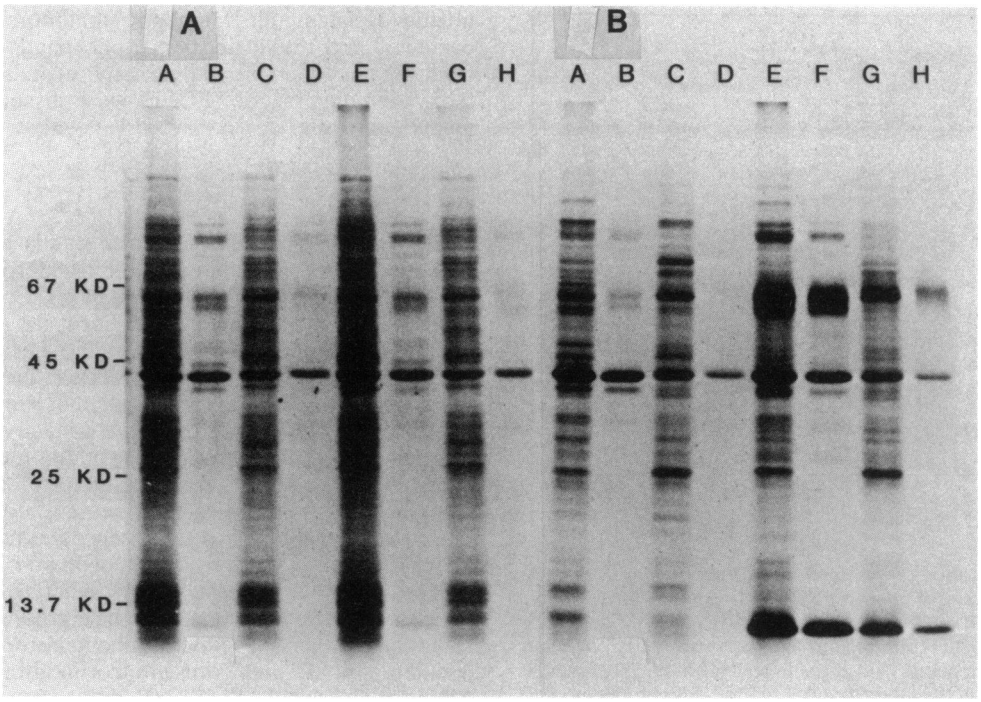

FIG. 3. Incorporation of [^{35}S]methionine and [^{35}S]cysteine into proteins of *C. psittaci*. Details of the experiment are described in the text. (A) Lanes of the Coomassie brilliant blue-stained gel. Lanes: A, whole RBs incubated in vivo with [^{35}S]methionine; B, outer membranes from RBs incubated in vivo with [^{35}S]methionine; C, host-free RBs incubated with [^{35}S]methionine; D, outer membranes from host-free RBs incubated with [^{35}S]methionine; E, whole RBs incubated in vivo with [^{35}S]cysteine; F, outer membranes from RBs incubated in vivo with [^{35}S]cysteine; G, host-free RBs incubated with [^{35}S]cysteine; H, outer membranes from host-free RBs incubated with [^{35}S]cysteine. (B) Autoradiograph of the gel.

prominent in the host-free system than in vivo. The poorer incorporation in the host-free system may be due to inefficient processing of outer membrane proteins as well as to a general loss of outer membrane material in the host-free preparations. The pattern of incorporation of [^{35}S]methionine and [^{35}S]cysteine into proteins of host-free RBs of *C. trachomatis* (serovar L2) is shown in Fig. 4. In general, optimum conditions for host-free protein synthesis were the same for both species of *Chlamydia*; however, incorporation of radioactivity was approximately 100-fold lower per microgram of RB protein added to the incubation mixture for *C. trachomatis*.

Incorporation of radioactive amino acids into protein could not be detected in host-free EBs of either *C. psittaci* or *C. trachomatis* incubating in the standard reaction mixture (data not shown). This observation was not surprising in that host-free EBs of *C. psittaci* previously have been shown to be incapable of transporting ATP (4). The outer membranes of EBs of *C. psittaci* and *C. trachomatis* are extensively disulfide bond cross-linked; those of RBs are not (2, 5, 9). Bavoil et al. (1) have presented evidence that transport in EBs of *C. trachomatis* (serovar L2) may be limited by the cross-linked nature of their MOMP. In an attempt to facilitate transport of both ATP and methionine, 10 mM dithiothreitol was added to the standard incubation mixture for host-free protein synthesis. Although treatment of both *C. psittaci* and *C. trachomatis* EBs with dithiothreitol converted cross-linked MOMP to monomers, it failed to reduce the cross-linked cysteine-rich outer membrane proteins to monomers and did not stimulate ATP transport or methionine incorporation. Dithiothreitol (10 mM) inhibited *C. psittaci* and *C. trachomatis* RB host-free protein synthesis by approximately 25% (data not shown).

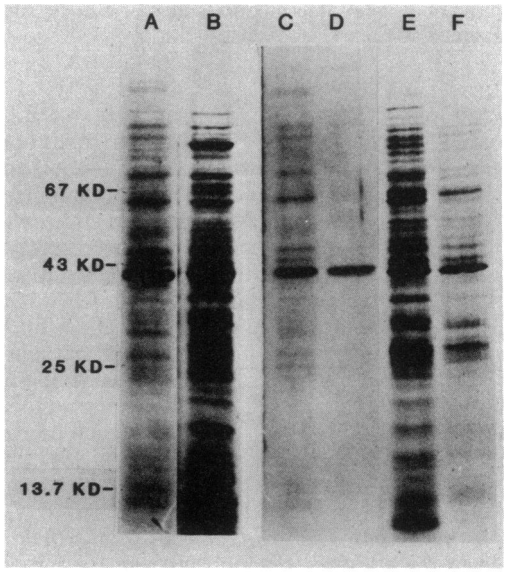

FIG. 4. Incorporation of [^{35}S]methionine and [^{35}S]cysteine into proteins of host-free RBs of *C. trachomatis* (serovar L2). In separate experiments, RBs were reacted for 1.5 h in the standard reaction mixture containing either [^{35}S]methionine (1.4 Ci/μmol) or [^{35}S]cysteine (1.1 Ci/μmol), and the whole RBs and outer membranes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Lanes: A, Coomassie brilliant blue-stained gel of whole RBs incubated with [^{35}S]methionine; B, autoradiograph of A; C, stained gel of whole RBs incubated with [^{35}S]cysteine; D, stained gel of outer membranes prepared from RBs incubated with [^{35}S]cysteine; E and F, autoradiographs of C and D.

TABLE 1. Effect of inhibitors on host-free protein synthesis[a]

Addition	cpm
None	58,792
Cycloheximide (50 μg/ml)	62,536
Chloramphenicol (50 μg/ml)	6,062
Rifampin (50 μg/ml)	16,325
FCCP (50 μM)	2,514
−ATP[b]	2,820

[a] RBs of *C. psittaci* (26 μg of protein) were preincubated at 37°C for 15 min with the additions as noted. Incorporation was for 1 h in the standard reaction mixture (2 μCi of [^{35}S]methionine; 2 Ci/mmol). Five percent dimethyl sulfoxide (the solvent in which rifampin and FCCP were dissolved) was shown to have no effect on incorporation.

[b] ATP was deleted from the incubation mixture.

TABLE 2. Effect of FCCP on [^{35}S]methionine incorporation by RBs of *C. psittaci*[a]

Samples	cpm incorporated	
	Soluble	Insoluble
Control	35,046	299,411
ΔRB[b]	2,638	6,338
−ATP	4,818	22,946
+FCCP	2,330	4,954

[a] RBs (37 μg of protein) were preincubated for 15 min at 37°C in the presence or absence of 50 μM FCCP as indicated; at time zero, the standard reaction mixture (lacking ATP where indicated) was added and the incorporation of [^{35}S]methionine (1.5×10^6 cpm; 1.4 Ci/μmol) into 2 M formic acid-soluble and -insoluble material was determined after 5 min of incubation at 37°C.

[b] ΔRBs inactivated for 3 min at 60°C.

Effects of inhibitors and nucleotides on host-free protein synthesis. Cycloheximide, an inhibitor of L-cell protein synthesis, had no inhibitory effect on host-free protein synthesis by RBs of *C. psittaci* (Table 1). Rifampin and chloramphenicol, inhibitors of bacterial transcription and translation, and FCCP, a proton ionophore, were effective inhibitors. FCCP was found to inhibit the uptake of methionine (Table 2), an observation which may account for its apparently inhibitory effect on protein synthesis. FCCP previously has been shown to have no effect on ATP transport by host-free RBs (4).

Although an exogenous supply of 1 mM ATP and an ATP-regenerating system were required for optimal host-free protein synthesis, exogenous GTP (which is required for cell-free protein synthesis in both eucaryotic and procaryotic systems) had no stimulatory effect. ATP could not be replaced by GTP; however, in the absence of an ATP-regenerating system, the combination of 10 mM ADP and 10 mM GTP was as effective as 10 mM ATP alone in promoting a short burst of protein synthesis (data not shown). This observation suggests that chlamydiae possess a nucleotide diphosphokinase that can transfer the terminal phosphate of NTP to NDP. The addition of 50 μM GTP, UTP, and CTP in concert to the standard reaction mixture stimulated the rate of host-free protein synthesis by up to 20% in some experiments but did not significantly prolong its duration (data not shown).

DISCUSSION

The conditions under which chlamydiae can be induced to synthesize protein in a host-free environment have been defined for the first time. Optimum synthesis was dependent on a continuous supply of exogenous ATP, an observation that supports Moulder's hypothesis that chlamydiae are energy parasites (8). The failure of Weiss and Wilson (13) to detect incorporation of isoleucine and aspartic acid into protein of host-free chlamydiae may be related to their failure to use an ATP-regenerating system and the use of a chlamydial population that may have consisted largely of EBs.

Although differences were noted, the molecular weight profile of proteins synthesized by host-free RBs of chlamydiae closely resembled that of RBs growing within host cells. Proteins synthesized included the MOMP and the cysteine-rich outer membrane proteins which were at least partially inserted into the outer membrane in the host-free system (Fig. 3B).

The cause of the cessation of host-free protein synthesis after a few hours was not determined, although components of the reaction mixture (other than the RBs) clearly were not limiting. The observation that rifampin inhibited the incorporation of methionine into proteins indicates that host-free protein synthesis is dependent upon host-free transcription. It is possible, therefore, that the cessation of host-free protein synthesis may reflect the inability of host-free RBs to synthesize mRNA beyond 1 or 2 h. In this respect, it was disappointing that the addition of GTP, UTP, and CTP to the reaction mixture neither enhanced the rate of protein synthesis nor prolonged its duration to a significant extent. Endogenous pools of CTP, UTP, and GTP, which may be regenerated at the expense of ATP via nucleotide diphophokinase, apparently are sufficient to generate the message needed to promote the limited host-free protein synthesis reported here. GTP, which is required by bacteria and eucaryotic cells for translation of message, also may be supplied by a similar mechanism in the host-free system.

Chlamydiae grow inside host cells within a membrane-bound vacuole that is not clathrin-coated (12). The pH of chlamydia-containing phagosomes is not known; however, host-free protein synthesis is optimal at pH 7.0 to 7.5, and it is logical to assume that a neutral pH also is required for intracellular protein synthesis and growth.

It was not surprising that host-free EBs of *C. psittaci* did not incorporate [^{35}S]methionine into protein since EBs normally are metabolically inactive and have previously been shown to be incapable of transporting ATP (4). The failure of EBs to transport ATP may be related to the extensively disulfide bond cross-linked nature of their MOMP (2, 5, 9). Bavoil et al. (1) have demonstrated that the MOMP of *C. trachomatis* has porin activity when incorporated into liposomes. This activity was enhanced approximately 10-fold if the free sulfhydryl groups of the MOMP were alkylated with iodoacetamide before the liposomes were prepared. Sarov and Becker (10) induced mercaptoethanol-treated EBs of *C. trachomatis* to incorporate UTP into RNA, and Hackstadt et al. (2) induced dithiothreitol-treated EBs of *C. trachomatis* to oxidatively decarboxylate glutamate. In the present study, the addition of 10 mM dithiothreitol to the standard reaction mixture failed to induce host-free protein synthesis or ATP transport in chlamydial EBs, although the dithiothreitol did convert cross-linked MOMP of EBs to monomers. Our inability to detect metabolic activity in host-free EBs may be related to the failure of dithiothreitol to reduce the cross-linked cysteine-rich outer membrane proteins to monomers in our studies.

ACKNOWLEDGMENT

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