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Methyl-Group Donors Cannot Prevent Apoptotic Death of Rat Hepatocytes Induced by Choline-Deficiency

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Abstract Choline-deficiency causes liver cells to die by apoptosis, and it has not been clear whether the effects of choline-deficiency are mediated by methyl-deficiency or by lack of choline moieties. SV40 immortalized CWSV-1 hepatocytes were cultivated in media that were choline-sufficient, choline-deficient, choline-deficient with methyl-donors (betaine or methionine), or choline-deficient with extra folate/vitamin B_{12}. Choline-deficient CWSV-1 hepatocytes were not methyl-deficient as they had increased intracellular S-adenosylmethionine concentrations (132% of control; $P < 0.01$). Despite increased phosphatidylcholine synthesis via sequential methylation of phosphatidylethanolamine, choline-deficient hepatocytes had significantly decreased ($P < 0.01$) intracellular concentrations of choline (20% of control), phosphocholine (6% of control), glycerophosphocholine (15% of control), and phosphatidylcholine (55% of control). Methyl-supplementation in choline-deficiency enhanced intracellular methyl-group availability, but did not correct choline-deficiency induced abnormalities in either choline metabolite or phospholipid content in hepatocytes. Methyl-supplemented, choline-deficient cells died by apoptosis. In a rat study, 2 weeks of a choline-deficient diet supplemented with betaine did not prevent the occurrence of fatty liver and the increased DNA strand breakage induced by choline-deficiency. Though dietary supplementation with betaine restored hepatic betaine concentration and increased hepatic S-adenosylmethionine/S-adenosylhomocysteine ratio, it did not correct depleted choline (15% of control), phosphocholine (6% control), or phosphatidylcholine (48% of control) concentrations in deficient livers. These data show that decreased intracellular choline and/or choline metabolite concentrations, and not methyl deficiency, are associated with apoptotic death of hepatocytes. J. Cell. Biochem. 64:196–208. © 1997 Wiley-Liss, Inc.

Key words: choline; phosphatidylcholine; methionine; betaine; apoptosis

Choline is a precursor for the biosynthesis of acetylcholine and choline-phospholipids [Zeisel and Blusztajn, 1994]. It is also the major dietary source for labile methyl-groups [Zeisel et al., 1989]. Humans can become choline-deficient when fed purified diets [Chawla et al., 1989; Zeisel et al., 1991], or when fed parenterally [Buchman et al., 1992, 1993; Sheard et al., 1986]. Choline and methyl-group metabolism are closely inter-related, as either betaine (a choline metabolite) or methyl-tetrahydrofolate can donate a methyl-group to homocysteine, forming methionine [Finkelstein and Martin, 1984; Zeisel and Blusztajn, 1994]. Choline-deficient diets deplete liver of methyl-folate [Kim et al., 1995; Varela-Moreiras et al., 1992], S-adenosylmethionine (SAM) [Zeisel et al., 1989], and choline compounds including betaine and phosphatidylcholine (PtdCho) [Pomfret et al., 1990]. The choline moiety can be synthesized via sequential methylation of phosphatidylethanolamine (PtdEtn) [Gibbons et al., 1993; Ridgway and Vance, 1987]. In the liver, 30–40% of PtdCho is synthe-

Abbreviations used: SAM, S-adenosylmethionine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; TLC, thin layer chromatography; SAH, S-adenosylhomocysteine; HPLC, high performance liquid chromatography; SM, sphingomyelin; PtdSer, phosphatidylserine; PtdInt, phosphatidylinositol; TUNEL, terminal deoxynucleotidyl transferase mediated dUTP nick end labeling; PCho, phosphocholine; GPCho, glycerophosphocholine; lvsoPtdCho, lysophosphatidylcholine; CDP-choline, cytidine diphosphocholine.

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sized via sequential methylation of PtdEtn [Vance and Ridgway, 1988]. For these reasons it has been difficult to separate the effects of choline deficiency from those of methyl-group deficiency.

Livers from rats fed diets devoid of choline (1) accumulated triacylglycerol and 1,2-*sn*-diacylglycerol with sustained activation of protein kinase C [da Costa et al., 1993, 1995], (2) became depleted of labile methyl groups resulting in DNA hypomethylation [Christman, 1995a,b; Dizik et al., 1991; Locker et al., 1986; Wainfan and Poirier, 1992], (3) accumulated reactive oxygen species with associated damage to DNA [Banni et al., 1990; Perera et al., 1987], (4) had increased liver cell death and regeneration [Chandar et al., 1987; Takahashi et al., 1979], and (5) developed hepatocellular carcinoma [da Costa et al., 1995; Ghoshal and Farber, 1984; Mikol et al., 1983; Newberne and Rogers, 1986]. We found that the increased rate of cell death that occurs in early choline-deficiency is due to apoptosis [Albright et al., 1996]. Apoptosis is a regulated form of cell suicide which is involved in various physiological conditions, such as normal cell turnover, hormone induced tissue atrophy, and embryogenesis [Kerr et al., 1972]. Cells undergoing apoptosis show internucleosomal DNA fragmentation [Kokileva, 1994; Zhivotovsky et al., 1994] and characteristic morphological changes such as marked decrease of cell volume, condensation of nuclei, and formation of apoptotic bodies [Schwartzman and Cidlowski, 1993; Wyllie, 1987]. Choline-deficiency induced apoptosis, and subsequent hepatocyte regeneration, might make livers susceptible to carcinogens, shifting the homeostatic balance to favor promotion and progression resulting in hepatocellularcarcinomas [Bursch et al., 1994].

Is the apoptosis induced by choline-deficiency due to lack of choline moieties or to methyl-deficiency? Inhibition of transmethylation reactions by pharmacologic inhibitors [Endresen et al., 1993, 1994] or by folate deficiency [James et al., 1994; Koury and Horne, 1994] can induce apoptotic cell death. Many research groups have considered choline-deficiency and methyl-deficiency as the same phenomenon, and believed that the effects of choline-deficiency were due to the decrease of methyl-group availability [Christman, 1995b; Newberne and Rogers, 1986]. We hypothesized that apoptosis is a specific consequence of lack of choline moieties and not caused by methyl-deficiency, and we tested this by determining whether addition of methyl-group donors prevents apoptotic death of hepatocytes induced by choline-deficiency.

MATERIALS AND METHODS

Animals

Animal care was done in accordance with guidelines of National Institutes of Health and the University of North Carolina at Chapel Hill. Male Fischer 344 rats (40–50 g on arrival) were fed a semisynthetic control diet for 1 week, and then fed control, choline-deficient or betaine-substituted diets for 2 weeks. Water and diets were offered ad libitum. The control diet contained 0.2% cystine, 0.2% methionine, 0.05 g/kg folate, 0.003 g/kg B_{12}, and 57.2 mmol choline/kg (Lombardi diet, Dyets, Bethlehem, PA). The choline-deficient diet did not contain choline, and the betaine-substituted diet contained 57.2 mmol betaine/kg diet instead of choline. At the times indicated, animals were anesthetized with ether, and livers were collected by freeze-clamping with tongs cooled in liquid nitrogen and stored at −80°C until used for choline, betaine, triacylglycerol, and SAM assays.

Cell Culture

A SV40 large T-antigen immortalized cell line (CWSV-1; a generous gift from Dr. Harriet Isom) derived from male Fischer 344 rat hepatocytes [Woodworth and Isom, 1987; Woodworth et al., 1986, 1988] was cultivated in control defined medium (RPMI-1640 containing 11.5 μM bovine serum albumin, 2 mM L-glutamine, 20 μM oleate, 7.2 μM linolenate, 66 μM ethanolamine, 1.27 μM transferrin, 10.4 nM insulin, 1 μM dexamethasone, 10 nM glucagon, 15 mM HEPES, pH 7.4, trace metals, 100 μg/mL penicillin, 100 μg/mL streptomycin, and 70 μM choline; RPMI-1640 contains 100 μM methionine) until 50% confluent. Cells were then switched to experimental media: control (70 μM choline), choline-deficient (0 μM choline), substituted with betaine (0 μM choline with 70–1,400 μM betaine, as indicated), or methionine substituted (0 μM choline with additional 70–700 μM methionine, as indicated). Media were changed every other day and all experiments were completed before cells became confluent.

[^{3}H]Methionine or [^{3}H]Ethanolamine Incorporation Into Phosphatidylcholine

Cells were cultivated in control medium until 50% confluent, and then 5 μCi of [^{3}H]methio-

nine (70 mCi/μmol; Dupont New England Nuclear, Boston, MA) or [^{3}H]ethanolamine (28 mCi/μmol; Amersham, Arlington Heights, IL) were added to culture media with (control), or without (choline-deficient) choline. After 1 or 2 days cultivation with radiolabel, cells were washed with phosphate buffered saline and collected. Lipids were extracted from cells [Bligh and Dyer, 1959], and PtdCho was separated by thin layer chromatography (TLC; chloroform/methanol/40% methylamine, 60:20:5), and radioactivity incorporated into PtdCho was measured by liquid scintillation spectrophotometry (Pharmacia, Piscataway, NJ; Wallac 1410).

Formation of Labeled Methionine From Labeled Choline

CWSV-1 hepatocytes were cultivated in control medium until they were 50% confluent on a 100 mm culture plate. [^{14}C-methyl]-choline (1 μCi) (Dupont New England Nuclear) was added, and cells were washed and harvested at timed intervals. Cells were extracted [Bligh and Dyer, 1959] and methionine was isolated using thin layer chromatography (0.5% NaCl/ethanol/methanol/NH_4OH; 50:30:20:5 v/v) [Rydzewska et al., 1993]. Radioactivity in methionine was analyzed using liquid scintillation spectrophotometry (Pharmacia; Wallac 1410).

Betaine Uptake by CWSV-1 Hepatocytes

Cells cultivated in control medium until 50% confluent were treated with [^{14}C]choline or [^{14}C]betaine (to achieve a specific activity 1 μCi/70 μmol of choline or betaine; Dupont New England Nuclear) [Mar et al., 1995]. After 0.25, 0.5, 1, 2, and 4 h incubation, cells were washed three times with phosphate buffered saline containing 70 μM choline or 70–140 μM betaine, collected, and the radioactivity incorporated into hepatocytes was measured by liquid scintillation spectrophotometry (Pharmacia; Wallac 1410).

S-Adenosylmethionine and S-Adenosylhomocysteine Assay

SAM and S-adenosylhomocysteine (SAH) were extracted from approximately 2–4 $\times$ 10^6 cells or from 50 mg liver pulverized in liquid nitrogen, using 100 μl of 0.1M acetate buffer (pH 6.0) and 75 μl 40% TCA solution. The extracts were washed three times with ether, and stored at -80°C until assayed. SAM and SAH were separated by HPLC [Molloy et al., 1990]. The initial solvent contained 25 mM NaH_2PO_4, 10 mM 1-heptanesulfuric acid (pH 3.2), and 10% methanol. SAM and SAH were eluted from a C18 column (Beckman, Fullerton, CA Ultrasphere, 4.6 mm $\times$ 25 cm) using a linear gradient up to 25% methanol. Peak area responses were measured at 254 nm.

Choline Metabolite Assay

Choline metabolites were measured in extracts of 2–4 $\times$ 10^6 cells or from liver samples pulverized in liquid nitrogen. Briefly, samples were extracted using a modified Bligh-Dyer procedure [Bligh and Dyer, 1959], and water soluble choline metabolites were separated by HPLC after addition of ^{14}C-labeled internal standards. [^{2}H-methyl]-internal standards of each metabolite were also added to permit correction for recovery after analysis of choline moiety by a gas chromatography/mass spectrometry assay [Pomfret et al., 1989]. PtdCho was analyzed in the organic phase of the extracts using TLC (chloroform/methanol/water, 65:30:4), followed by hydrolysis and subsequent spectrometric analysis of choline [Pomfret et al., 1989].

Betaine Assay

Betaine was extracted and isolated by HPLC, as above [Bligh and Dyer, 1959], based on mobility of ^{14}C-labeled internal standard. The betaine fraction was collected and derivatized with 4′-bromo-phenacyl triflate. The betaine-triflate derivative was isolated by HPLC and quantified by peak area response at 254 nm [Mar et al., 1995].

Folate and Vitamin B_{12} Assay

To extract intracellular folate, a 2 mL aliquot of hot (90°C) 2% 2-mercaptoethanol and 2% ascorbic acid (pH 6.0) were added to 0.8 mL cell suspension, and incubated in boiling water for 1 min. The folate extract was then separated from denatured cellular debris by centrifugation (3,000 rpm for 30 min; Sorvall RC-3B Centrifuge, Sorvall, Wilmington, DE) [Allegra et al., 1986]. Cellular vitamin B_{12} was extracted from 1 mL 20% cell solution (0.2 mL packed cells, 0.2 mL 0.4 M acetate buffer [pH 4.9], 0.015 mL 0.1% sodium cyanide, and 0.585 mL double distilled water). After repeated pipetting and autoclaving (121°C for 18 min), the vitamin B_{12} extract was separated by centrifugation (8,000 rpm for 10 min) [Tisman et al.,

1993]. Total folate and vitamin B_{12} concentrations were measured from each folate or vitamin B_{12} extract using the Solid Phase No Boil Dualcount assay kit (KDSP2, Diagnostic Products Corporation, Los Angeles, CA) according to the manufacturer's instructions.

Phosphate Assay for Cellular Phospholipid Composition

Lipids were extracted from approximately 7–10 $\times$ 10^6 cells [Bligh and Dyer, 1959], and PtdCho (Rf, 0.37), phosphatidylethanolamine (PtdEtn; Rf, 0.43), sphingomyelin (SM, Rf, 0.25), phosphatidylserine (PtdSer, Rf, 0.17), and phosphatidylinositol (PtdInt, Rf, 0.09) were separated by TLC (chloroform/methanol/40% methylamine, 60:20:5), and quantified with a phosphate assay [Svanborg and Svennerholm, 1961]. Each phospholipid amount was expressed as a percentage of total phospholipid.

Lactate Dehydrogenase Assay

Release of lactate dehydrogenase into culture media [Decker and Lohmann-Matthes, 1988] was measured using an in vitro toxicology assay kit (Sigma, St. Louis, MO) according to the manufacturer's instructions.

DNA Fragmentation Analysis by Agarose Gel Electrophoresis

DNA was isolated from cells cultivated in each experimental medium, using a guanidine-detergent lysing solution (DNA zol™, Molecular Research Center, Inc., Cincinnati, OH). Briefly, 8 $\times$ 10^6 cells were homogenized with guanidine-detergent lysing solution by repeated pipetting, and incubated 30 min with agitation at room temperature. After centrifugation (10,000 rpm, 10 min), the DNA solution was then transferred to a new microcentrifuge tube. DNA was precipitated by 1/2 volume ethanol, washed 2 times using 95% ethanol solution, dried, and dissolved in Tris-EDTA buffer (pH 8.0). DNA (5–10 μg) was analyzed for fragmentation by electrophoresis on 0.8% agarose slab gel containing 1 μg/mL ethidium bromide. A 1 kb DNA ladder was included as a standard. The bands were visualized under ultraviolet light and photographed.

Terminal Deoxynucleotidyl Transferase Mediated dUTP Nick End Labeling (TUNEL)

Apoptotic cells were detected using a direct immunoperoxidase method (ApopTag™, Oncor Inc., Gaithersburg, MD) to visualize the incorporation of digoxigenin-11-dUTP into 3′-hydroxyl ends of DNA fragments [Albright et al., 1996].

Triacylglycerol Assay

Lipids were extracted from liver homogenate using a modified Bligh Dyer method [Bligh and Dyer, 1959]. Hepatic triacylglycerol was isolated by TLC (hexane/ethylether/acetic acid, 50:50:1), hydrolyzed and measured as fatty acid methyl esters using capillary gas chromatography [Tacconi and Wurtman, 1985]. Triheptadecanoin was used as an internal standard to correct for variations in recovery.

Statistics

Statistical differences were assessed using unpaired *t*-test, or for multiple comparisons, by 1-way ANOVA and Dunnett's test [Bruning and Kintz, 1987].

RESULTS

Betaine was taken up by CWSV-1 hepatocytes twenty times more slowly than choline (Fig. 1). For this reason we used several betaine concentrations in our studies. We added up to 1,400 μM (20×) betaine or 700 μM (10×) extra methionine in choline-deficient medium to provide enough methyl-group donors to the choline-deficient hepatocytes. Cells, cultivated in 20× betaine-substituted medium for 2 days, had significantly higher amounts of cellular betaine (1,783 $\pm$ 188 pmol/10^6 cells) compared to controls (60 $\pm$ 13 pmol/10^6 cells, $P < 0.01$). We did not detect betaine in the cells cultivated in choline-deficient or 10× methionine-substituted medium (less than 0.01 pmol/10^6 cells).

Cells cultivated in choline-deficient medium for 2 days increased their intracellular SAM concentrations (1,113 $\pm$ 76 pmol/10^6 cells) compared with controls (838 $\pm$ 48 pmol/106 cells, $P < 0.01$). Betaine (1–2×) or 1× extra methionine in choline-deficient medium also resulted in higher SAM concentrations compared with controls (1× betaine, 1,166 $\pm$ 19 pmol/10^6 cells; 2× betaine, 1,178 $\pm$ 46 pmol/10^6 cells; 1× extra methionine, 1,247 $\pm$ 9 pmol/10^6 cells, each $P < 0.01$).

Cells cultivated in choline-deficient medium for more than 24 h increased synthesis of choline moiety via sequential methylation of PtdEtn as measured by incorporation of [^{3}H]methio-

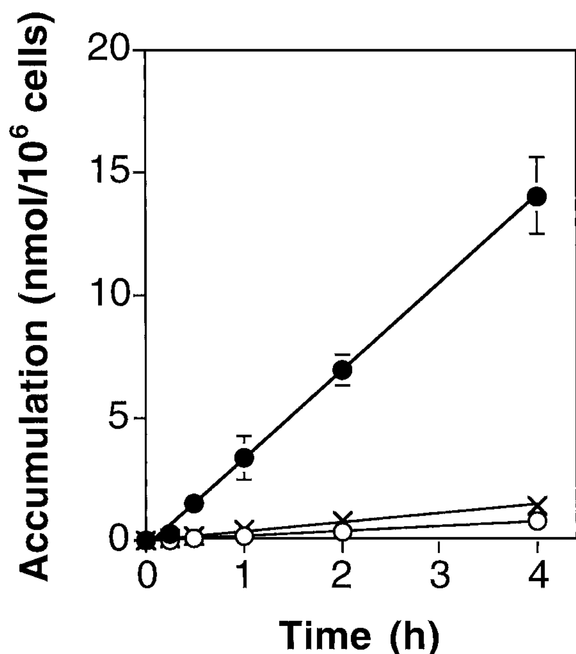

Fig. 1. Hepatocytes took up choline more readily than they did betaine. Accumulation of [^{14}C]choline or [^{14}C]betaine (specific activity: 1 μCi/70 μmol of choline or betaine) was studied in CWSV-1 hepatocytes maintained in control or betaine-substituted medium (70 μM choline, ●; 70 μM betaine, ○; or 140 μM betaine, X. Radiolabel was added to 50% confluent cell cultures and after 0.25, 0.5, 1, 2, and 4 h cultivation, cells were washed with phosphate buffered saline containing cold choline or betaine, collected, and the incorporation of radioactivity into the cells was measured using scintillation spectrophotometry.

nine or [^{3}H]ethanolamine into PtdCho (Table I). Even though choline-deficiency increased synthesis ofcholine moiety via methylation of PtdEtn, CWSV-1 cells cultivated in choline-deficient medium had decreased intracellular choline metabolite concentrations. Methyl supplementation (20× betaine or 10× extra methionine) did not correct the low choline metabolite concentrations induced by withdrawal of choline from culture medium (Table II).

Choline-deficient CWSV-1 hepatocytes had similar total phospholipid concentrations (39 ± 5 nmol/10^6 cells) compared with controls (38 ± 3 nmol/10^6 cells; not significantly different from control by unpaired *t*-test), despite reduced PtdCho concentrations (Table II). Methyl supplementation of choline-deficient medium did not prevent either the PtdCho concentration decrease or the phospholipid composition changes induced by choline-deficiency (Tables II and III). When CWSV-1 hepatocytes were incubated in control medium containing [^{14}C-methyl]-choline, they formed labeled methionine (183 ± 51 dpm/10^6 cells at 1 h, and 347 ± 30 dpm/10^6 cells at 3 h).

CWSV-1 hepatocytes cultivated in choline-deficient or methyl-substituted medium for 2 days stopped increasing cell numbers, and further cultivation in choline-deficient or methyl substituted media induced cell detachment from culture plates (Fig. 2). After 2 days, culture media from choline-deficient cell incubations also had higher lactate dehydrogenase activity compared to controls (135 ± 3% increase; $P < 0.01$). Methyl supplementation did not prevent leakage of lactate dehydrogenase into culture media that was induced by choline-deficiency (20× betaine, 141 ± 3% of control; 10× methionine, 130 ± 3% of control, $P < 0.05$).

Betaine (1,400 μM) or extra methionine (700 μM) was not toxic to the CWSV-1 hepatocytes. Cells cultivated in control medium (70 μM choline) containing 1,400 μM betaine or 700 μM extra methionine did not decrease their cell growth rate and did not detach from the plates (Fig. 2 inset). Internucleosomal DNA fragmentation, a typical biochemical marker of apoptosis, was detected as increased DNA strand breakage in choline-deficient cells, and this was not reversed by addition of betaine or methionine (Figs. 3 and 4). Betaine (20×) or extra methionine (10×) supplementation in control medium did not induce DNA strand breakage of CWSV-1 cells as measured using TUNEL method (Fig. 3).

We also added up to 50 times more folate and vitamin B_{12} in our choline-deficient medium (folate, 0.05 mg/mL; vitamin B_{12}, 0.25 μg/mL) to determine whether apoptotic death of choline-deficient CWSV-1 cells is associated with depletion of folate or vitamin B_{12}. This folate and vitamin B_{12} supplementation of choline-deficient media did not prevent apoptotic cell death induced by lack of choline (Fig. 5) even though these supplements increased intracellular folate and vitamin B_{12} concentrations to above normal in choline deficient cells (Table IV).

CWSV-1 hepatocytes cultivated in choline-deficient or methyl-substituted medium exhibited the typical apoptotic morphology with shrinkage of nuclear and cell volume, membrane blebbing, chromatin condensation, and formation of classical apoptotic bodies (Fig. 6).

In a rat study, replacement of dietary choline with betaine increased hepatic methyl-group availability. Livers from rats fed the betaine-substituted diet for 2 weeks had almost normal

TABLE I. Choline-Deficient Hepatocytes Had Increased Phosphatidylcholine Synthesis Via Methylation of Phosphatidylethanolamine*

	Label incorporated into PtdCho (cpm/10^6 cells)			
	[^{3}H]methionine		[^{3}H]ethanolamine	
	24 h	48 h	24 h	48 h
Control	1,185 ± 108	1,243 ± 62	815 ± 54	1,034 ± 74
Choline-deficiency	1,101 ± 113	1,997 ± 116**	1,025 ± 92	1,923 ± 64**

*CWSV-1 hepatocytes were cultivated in control medium until 50% confluent. [^{3}H]methionine or [^{3}H]ethanolamine (5 μCi) were added to culture media with (control), or without (choline-deficient) choline. After 1 or 2 days of cultivation, cells were collected and washed with phosphate buffered saline. Lipids were extracted from cells, and PtdCho was separated by TLC (chloroform/methanol/40% methylamine, 60:20:5). The radioactivity incorporated into PtdCho was measured by liquid scintillation spectrophotometry (Pharmacia; Wallac 1410). Data are expressed as mean ± SD (n = 3/group). Statistical difference was determined by unpaired *t*-test (**different from control, $P < 0.01$).

TABLE II. Choline-Deficiency Depleted Intracellular Choline and Choline Esters Even When Adequate Methyl-Groups Were Supplied*

	(pmol/10^6 cells)			
	Choline	PCho	GPCho	PtdCho
Control	193 ± 8	2,356 ± 281	374 ± 27	14,514 ± 2,197
Choline-deficient	39 ± 2**	146 ± 10**	56 ± 4**	7,903 ± 388**
20× betaine substitution	23 ± 4**	73 ± 9**	28 ± 5**	6,056 ± 990**
10× extra methionine	33 ± 9**	83 ± 13**	51 ± 14**	8,865 ± 1,346**

*CWSV-1 hepatocytes were cultivated for 2 days in experimental medium (Control, 70 μM choline; Choline-deficient, 0 μM choline; 20× betaine substitution, 1,400 μM betaine plus 0 μM choline; 10× extra methionine, 700 μM extra methionine plus 0 μM choline). Samples were extracted, and water soluble choline metabolites were separated by HPLC and analyzed for choline moiety by a gas chromatography/mass spectrometry assay. PtdCho was analyzed in the organic phase of the extracts, using TLC (chloroform/methanol/water, 65:30:4) and hydrolysis with subsequent spectrometric analysis of choline. Data are expressed as mean ± SD (n = 3/group). Statistical difference was determined by 1-way ANOVA and Dunnett's test (**different from control, $P < 0.01$).

TABLE III. Choline-Deficiency Depleted Hepatocytes of Phosphatidylcholine Even When Adequate Methyl-Groups Were Provided†

	Percent total phospholipid				
	PtdCho	PtdEtn	SM	PtdInt	PtdSer
Control	45.4 ± 0.9	26.3 ± 0.9	10.6 ± 0.4	9.1 ± 0.4	8.6 ± 0.4
Choline-deficiency	26.9 ± 0.9**	37.6 ± 0.5**	12.6 ± 0.4*	13.1 ± 0.2**	9.9 ± 0.3
20× betaine substitution	28.2 ± 0.5**	37.2 ± 0.6**	12.3 ± 0.3*	12.9 ± 0.2**	9.3 ± 0.7
10× extra methionine	27.1 ± 1.0**	36.7 ± 2.0**	14.1 ± 0.9*	13.0 ± 0.6**	9.1 ± 0.6

†CWSV-1 hepatocytes were cultivated as described in Table II legend. Lipids were extracted from approximately 7–10 × 10^6 cells, and the major phospholipids were separated by TLC (chloroform/methanol/40% methylamine, 60:20:5), and quantified with a phosphate assay. Each detectable phospholipid was expressed as a percentage of total phospholipid. Data are expressed as mean ± SD (n = 4/group). Statistical difference was determined by 1-way ANOVA and Dunnett's test (different from control, *$P < 0.05$, **$P < 0.01$).

hepatic betaine concentrations (Table V), and had significantly higher SAM/SAH ratios compared with controls (Table VI; $P < 0.01$). However this increased hepatic methyl group availability did not prevent fatty liver, DNA strand breakage (Table VI), or the abnormalities in hepatic choline metabolites (Table V) induced by choline-deficiency.

DISCUSSION

The major observation presented in this report is that methyl supplementation with betaine, methionine, folate, or vitamin B_{12} did not prevent apoptotic death induced by choline-deficiency in hepatocytes. Therefore, depletion of intracellular choline moieties rather than

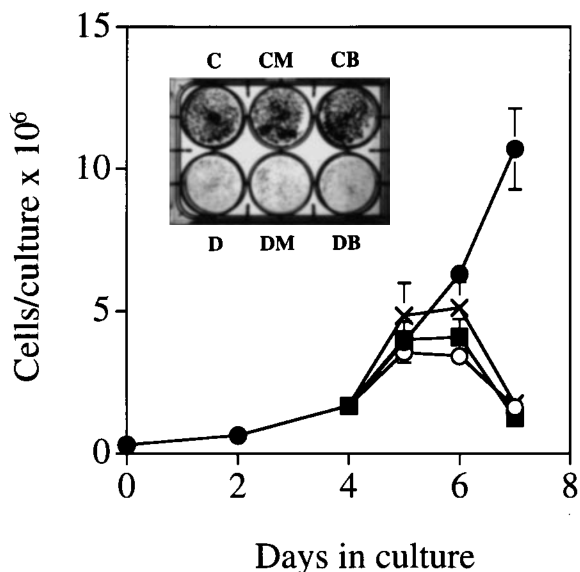

Fig. 2. Hepatocytes ceased growing when cultivated in choline-deficient media even when adequate methyl-groups were provided. CWSV-1 hepatocytes were maintained in control medium for 4 days, and then switched to control (70 μM choline, ●), choline-deficient (0 μM choline, ○), betaine substituted (0 μM choline and 1,400 μM betaine, X), or methionine substituted (0 μM choline plus 700 μM extra methionine, ■) for 3 days. Cells were trypsinized, and viable (trypan blue negative), attached cells were counted using a hemacytometer. **Inset:** CWSV-1 hepatocytes were cultivated in experimental medium (C, choline-sufficient [70 μM choline]; CB, choline-sufficient with 1,400 μM betaine; CM, choline-sufficient with 700 μM extra methionine; D, choline-deficient (0 μM choline), DB, choline-deficient with 1,400 μM betaine; DM, choline-deficient with 700 μM extra methionine) for 3 days and attached cells were fixed by 70% ethanol solution, and stained with hematoxylin. Betaine or methionine supplementation did not retard the growth of choline-sufficient cells, and did not protect cells against choline-deficiency.

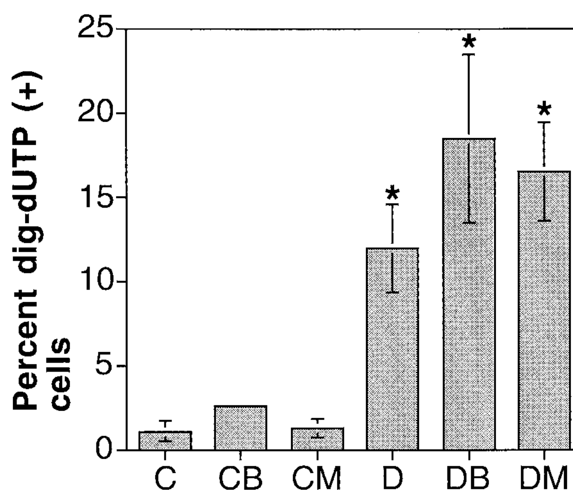

Fig. 3. Choline-deficient hepatocytes had increased DNA strand breakage even when adequate methyl-groups were provided. Incorporation of digoxigenin 11-dUTP (TUNEL method), a measure of DNA strand breakage, was measured in attached CWSV-1 hepatocytes after 3 days in experimental medium (C, choline-sufficient [70 μM choline]; CB, choline-sufficient with 1,400 μM betaine; CM, choline-sufficient with 700 μM extra methionine; D, choline-deficient (0 μM choline); DB, choline-deficient with 1,400 μM betaine; DM, choline-deficient with 700 μM extra methionine). $^*P < 0.05$, significantly different from control by 1-way ANOVA and Dunnett's test.

depletion of methyl-groups was the critical parameter involved in induction of apoptosis. This observation is important, because for the first time the effects of choline deficiency are separated from methyl-deficiency. Current theory holds that most of the consequences of choline deficient diets, including hepatocarcinogenesis, are caused by methyl-deficiency [Newberne and Rogers, 1986]. For this reason, it has been assumed that there is no dietary requirement for choline if adequate methyl-groups are provided. Our observations show, for the first time, that there are specific, and important functions for choline, that are not met when alternative methyl-donors are substituted.

We use the SV40 large T-antigen immortalized CWSV1 cell line at passage 25–34 (provided as a gift by Dr. Harriet Isom at Pennsylvania State University) as our model system for study of the effects of choline deficiency. This cell line was established from normal male (Fischer 344) hepatocytes [Woodworth and Isom, 1987; Woodworth et al., 1986, 1988]. CWSV1 hepatocytes secrete several liver-specific proteins [Woodworth et al., 1988], including transferrin, the 3rd component of complement (C3), hemopexin, glucose-6-phosphatase, tyrosine aminotransferase activity which is inducible by glucocorticoids [Woodworth and Isom, 1987], α1-antitrypsin, and phosphoenolpyruvate carboxykinase [Woodworth et al., 1988]. CWSV1 do not express α-fetoprotein (AFP) or AFP mRNA [Woodworth and Isom, 1987]. Our data show that these hepatocytes contain choline dehydrogenase as betaine is detected in cells grown in control medium, and they show that these cells form all of the intermediates formed in the Kennedy pathway for phosphatidylcholine synthesis (phosphocholine, CDP-choline, phosphatidylcholine). These cells also contain phosphatidylethanolamine-*N*-methyltransferase (Table I). These hepatocytes form SAM and SAH, and they form labeled methionine when incubated with [^{14}C-methyl]-choline, indicating they express betaine homocysteine methyltransferase activity.

Several choline compounds are likely candidates for the choline derivative that is needed

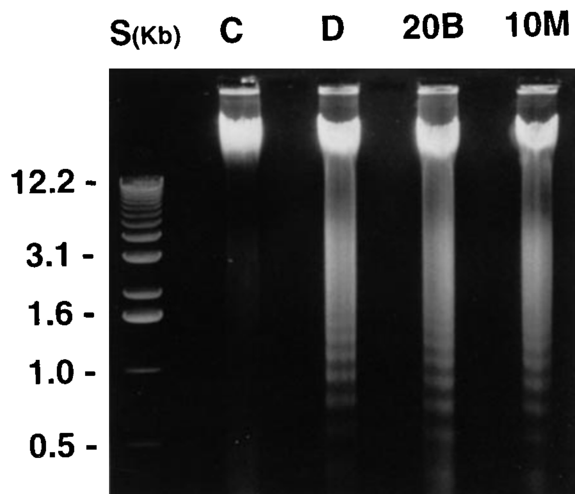

Fig. 4. DNA fragmentation occurred in choline-deficient hepatocytes even when adequate methyl-groups were provided. Genomic DNA from CWSV-1 hepatocytes, which were cultivated for 2 days in experimental medium (70 μM choline, C; 0 μM choline, D; 0 μM choline with 1,400 μM betaine, 20B; 0 μM choline with 700 μM extra methionine, 10M, was analyzed for fragmentation using electrophoresis on an 0.8% agarose slab gel. A DNA ladder consistent with internucleosomal DNA fragmentation and apoptosis is seen in 0 μM choline-treated cells with, or without, betaine or methionine supplementation, but not in control cells.

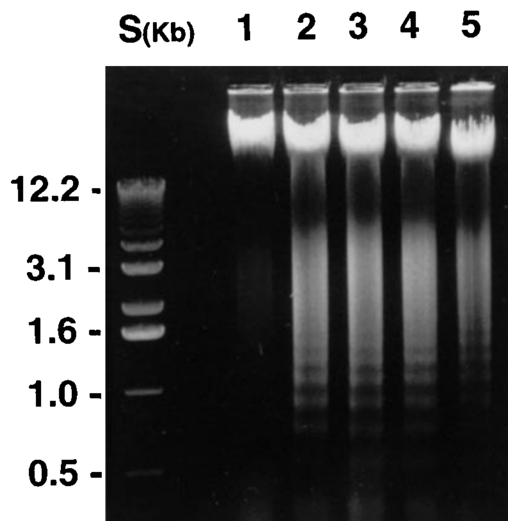

Fig. 5. DNA fragmentation occurred in choline-deficient hepatocytes even when adequate vitamin B_{12} and folate were provided. Genomic DNA, from CWSV-1 hepatocytes which were cultivated for 2 days in experimental medium, was analyzed for fragmentation using electrophoresis on an 0.8% agarose slab gel. DNA extracted from cells in control **(lane 1)**; choline-deficient (1 × F/B_{12}; 0.001 mg/mL folate and 0.005 μg/mL vitamin B_{12}, 0 μM choline; **lane 2**); 5 × F/B_{12} (0.005 mg/mL folate and 0.025 μg/mL vitamin B_{12}, 0 μM choline; **lane 3**); 10 × F/B_{12} (0.01 mg/mL folate and 0.05 μg/mL vitamin B_{12}, 0 μM choline; **lane 4**); 50 × F/B_{12} (0.05 mg/mL folate and 0.25 μg/mL vitamin B_{12}, 0 μM choline; **lane 5**). A DNA ladder consistent with internucleosomal DNA fragmentation and apoptosis was observed in 0 μM choline-treated cells with, or without, extra folate and vitamin B_{12} supplementation, but not in control cells.

to prevent apoptosis. In NIH 3T3 cells, generation of phosphocholine from PtdCho by phospholipase D and choline kinase is required for the induction of DNA synthesis as well as activation of Raf-1 and MAP kinases [Jimenez et al., 1995]. PtdCho, as a major phospholipid species in eucaryotic membranes, may be essential for the survival of hepatocytes [Vance, 1990]. The basic structure of eucaryotic membranes is a double layer of phospholipids. Protein, carbohydrate, and phospholipid components in membrane structure are asymmetrically distributed [Bretscher, 1985; Higgins and Dawson, 1977; Op den Kamp, 1979; Sanchez-Yague and Llanillo, 1986]. The outer leaflet is rich in choline-phospholipids such as PtdCho and SM whereas the inner leaflet is dominated by PtdEtn, PtdSer, and PtdInt. The most significant change in phospholipid composition in choline-deficient CWSV-1 cells was a decrease of PtdCho with a compensatory increase of PtdEtn concentration. This phospholipid composition change could alter activities of membrane bound enzymes including protein kinase C, protein kinase A, adenylyl cyclase, and lipases [Gavrilova et al., 1992; Jansson et al., 1993; Kano-Sueoka and Nicks, 1993; Tretyakov and Farber, 1993]. Synthesis of PtdCho is needed for progression of the cell cycle [Jackowski, 1994; Makarov et al., 1994; Terce et al., 1994]. Cells cultivated in choline-deficient medium are arrested in G1 phase [Terce et al., 1994] and many studies have suggested that events during G1 can trigger apoptosis [Meikrantz and Schlegel, 1995]. Inhibition of PtdCho synthesis by pharmacologic inhibitors induces apoptosis [Boggs et al., 1995a,b; Haug et al., 1994]. This apoptotic cell death could be partially prevented by PtdCho [Haug et al., 1994] or lysoPtdCho [Boggs et al., 1995b] supplementation. All of these data suggest that PtdCho is the critical molecule that is missing in choline deficient cells. However, CHO cells, with a temperature-sensitive inactivation of the CDP-choline pathway for PtdCho synthesis, could not be rescued despite complete restoration of cellular PtdCho levels by over expression of phosphatidylethanolamine methyltransferase [Houweling et al., 1995].

We observed an increase in SAM concentrations in choline deficient cells. This is very

TABLE IV. Folate and Vitamin B_{12} Supplementation of Choline Deficient Media Increased Intracellular Folate and Vitamin B_{12} Concentrations*

	Control (70 µM choline with 1 × F/B12)	Choline deficiency (0 µM choline with 1 × F/B12)	5 × F/B12 (0 µM choline)	10 × F/B12 (0 µM choline)	50 × F/B12 (0 µM choline)
Folate (pmol/10^6)	17.1 ± 2.6	12.9 ± 0.9	30.6 ± 4.9**	39.4 ± 1.4**	37.5 ± 8.9**
B_{12} (fmol/10^6)	52.7 ± 5.6	36.4 ± 9.5**	37.0 ± 3.6**	52.2 ± 2.0	45.0 ± 5.9

*Cells were cultivated in control medium until 50% confluent, and then switched to choline deficient media supplemented with 1, 5×, 10×, or 50× folate (F; 0.001–0.05 mg/mL) and 1, 5×, 10×, or 50× vitamin B_{12} (B_{12}; 0.005–0.25 µg/mL). After 2 days, cells were washed with phosphate buffered saline and harvested by brief trypsinization. Folate and vitamin B_{12} were extracted and assayed as described in Materials and Methods section. Data are presented as mean ± SD. Statistical difference was determined by 1-way ANOVA and Dunnett's test (**different from control, $P < 0.01$).

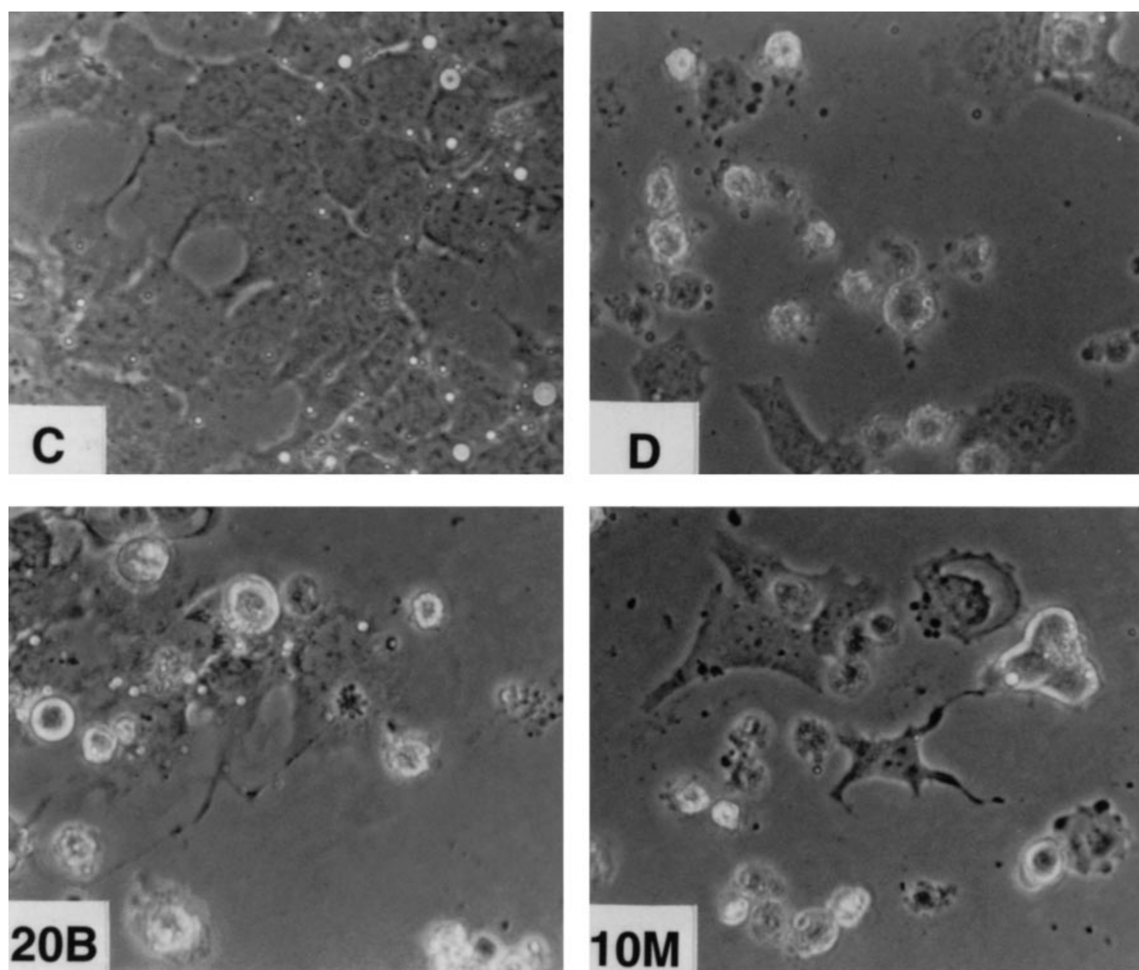

Fig. 6. Morphology of CWSV-1 hepatocytes undergoing apoptosis in cell culture. Apoptotic bodies are present in cells maintained in 0 µM choline with or without betaine or methionine supplementation. C, control (70 µM choline); D, choline deficiency (0 µM choline); 20B, 20× betaine supplementation in choline deficiency (0 µM choline with 1,400 µM betaine); 10M, 10× extra methionine supplementation in choline deficiency (0 µM choline with 700 µM extra methionine). Phase contrast, ×400.

different than what occurs in choline deficient rats, where hepatic SAM usually decreases when rats are fed a choline deficient diet [Finkelstein et al., 1982; Zeisel et al., 1989]. In our cell culture system we provide adequate sources of methionine (RPMI 1640 medium contains 100 µM methionine) while in the rat, the methionine content of the diet is limited by

TABLE V. Choline Deficiency Depleted Rat Liver of Choline, Choline Esters, and Betaine Even When Adequate Methyl Groups Were Provided*

Diets	(nmol/mg protein) Betaine	Choline	PCho	PtdCho
Control	141 ± 12	0.7 ± 0.04	5.2 ± 0.7	134 ± 16
Choline-deficiency	1.6 ± 0.4**	0.2 ± 0.04**	0.5 ± 0.04**	64 ± 3**
Betaine substitution	110 ± 13	0.1 ± 0.01**	0.3 ± 0.02**	75 ± 5**

*Fischer 344 rats were fed control, choline-deficient, or betaine substituted diet for 2 weeks. Livers, pulverized in liquid nitrogen, were used for choline metabolite assays as described in Materials and Methods section. Data are expressed as mean ± SEM (n = 6/group). Statistical difference was determined by 1-way ANOVA and Dunnett's test (different from control, $^{**}P < 0.01$).

TABLE VI. Rats Eating Diets Missing Choline (With or Without Betaine) Had Increased DNA Strand Breakage and Fatty Liver†

Diets	SAM/SAH ratio	Triacylglycerol (nmol/mg protein)	End-labeled cells (% total cells)
Control	0.72 ± 0.07	138 ± 21	0.2 ± 0.1
Choline-deficiency	0.64 ± 0.03	2,197 ± 387**	1.3 ± 0.2*
Betaine substitution	1.3 ± 0.2**	1,811 ± 164**	0.8 ± 0.2*

†Fischer 344 rats were fed control, choline-deficient, or betaine substituted diet for 2 weeks. Livers, pulverized in liquid nitrogen, were used for SAM/SAH and triacylglycerol assay as described in Materials and Methods section. Statistical difference was determined by 1-way ANOVA and Dunnett's test (different from control, $^{*}P < 0.05$, $^{**}P < 0.01$).

using soy protein which is methionine-poor compared to the casein normally used in rat diet formulations [Zeisel et al., 1989]. We observed an activation of PtdCho synthesis via phosphatidylethanolamine-*N*-methyltransferase activity in choline-deficient hepatocytes. This is consistent with earlier observations that phosphatidylethanolamine-*N*-methyltransferase activity increases in rats fed a choline deficient diet [Cui and Vance, 1996]. We believe this partially compensated for diminished PtdCho synthesis via the CDP-choline pathway, but there was still insufficient PtdCho resulting in growth arrest/apoptosis. Vance and colleagues observed that the expression of rat liver phosphatidylethanolamine-*N*-methyltransferase-2 (PEMT2) in McA-RH7777 rat hepatoma cells resulted in the unexpected inhibition of cell growth by an unknown mechanism [Cui et al., 1994]. Expression of other proteins via the same vector did not inhibit McA-RH7777 cell growth; thus, retardation of cell division was specific for the methyltransferase. Addition of 3-deazaadenosine, which caused inhibition of phosphatidylethanolamine methylation, reversed the PEMT2-mediated inhibition of cell division. Perhaps, in CWSV-1 hepatocytes, the compensatory increase in PtdCho synthesis via the methylation pathway also results in growth inhibition by a similar mechanism.

We found that rats fed a choline (and methionine and folate) deficient diet developed fatty liver and had more apoptotic cells detected by TUNEL labeling. Addition of methyl groups to the diet in the form of betaine (matching the methyl groups delivered in the control diet by choline) did not prevent fatty liver or increased apoptosis. We used a diet that is traditionally used to make rats choline deficient in studies of carcinogenesis. This restoration of choline prevents hepatocarcinogenesis [da Costa et al., 1995]. This diet, first developed by Lombardi and colleagues, contains 0.2% methionine, 0.05 g/kg folate, 0.003 g/kg B_{12}, and 57.2 mmol choline/kg. It is methionine and folate limited, as well as choline deficient (or relatively high in choline in the control group so as to provide total methyl-groups similar to the AIN recommendations). Other investigators often use a diet similar to the AIN formulation which has more methionine and folate. For example Finkelstein et al. [1983] used a diet containing 0.3% methionine and 0.5% cystine. We believe that this accounts for differences in SAM/SAH ratios reported in liver. In the higher methionine diets the ratio should be higher (approximately 3–4) while in the methionine limited diet we expect lower SAM/SAH ratios (near to 1). For example, Wainfan and colleagues [Wainfan and Poirier, 1992] using a diet lacking cho-

line and methionine, report a SAM/SAH ratio in liver of 1.4 at 4 weeks on a deficient diet. We did not see an improvement in the SAM/SAH ratio when we added choline back to the diet of rats. Perhaps this is because the methionine content of the diet remains low. Wainfan did see a correction in SAM/SAH ratio (to 4), but added methionine as well as choline back to the diet she offered her rats.

Choline-deficiency and methyl-deficiency have been thought of as similar, if not identical, phenomena [Newberne and Rogers, 1986]. Many of the theories attempting to explain the increased hepatocarcinogenesis associated with choline deficiency have focused on effects of hypomethylation [Christman, 1995a,b]. It has not been fully appreciated that choline-deficiency and methyl-deficiency can be disassociated. It is not clear why choline-deficiency induces apoptotic death of hepatocytes, but these studies show that it is the availability of choline moieties, and not of methyl-group donors, that is critical for this phenomenon.

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