Use of an "acetaldehyde clamp" in the determination of low-K_M aldehyde dehydrogenase activity in H4-II-E-C3 rat hepatoma cells

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Abstract

The high-affinity (K_M <1 μM) mitochondrial class 2 aldehyde dehydrogenase (ALDH2) metabolizes most of the acetaldehyde generated in the hepatic oxidation of ethanol. H4-II-E-C3 rat hepatoma cells have been found to express ALDH2. We report a method to assess ALDH2 activity in intact hepatoma cells that does not require mitochondrial isolation. To determine only the high-affinity ALDH2 activity it is necessary to keep constant low concentrations of acetaldehyde in the cells to minimize its metabolism by high-K_M aldehyde dehydrogenases. To maintain both low and constant concentrations of acetaldehyde we used an "acetaldehyde clamp," which keeps acetaldehyde at a concentration of 4.2 ± 0.4 μM. The clamp is attained by addition of excess yeast alcohol dehydrogenase, ^{14}C-ethanol, and oxidized form of nicotinamide adenine dinucleotide (NAD^+) to the hepatoma cell culture medium. The concentration of ^{14}C-acetaldehyde attained follows the equilibrium constant of the alcohol dehydrogenase reaction. Thus, ^{14}C-acetate is generated virtually by the low-K_M aldehyde dehydrogenase activity. ^{14}C-acetate is separated from the culture medium by an anionic resin and its radioactivity is determined. We showed that (1) acetate production is linear for 120 min, (2) addition of 160 μM cyanamide to the culture medium leads to a 75%–80% reduction of acetate generated, and (3) ALDH2 activity is dependent on cell-to-cell contact and increases after cells reach confluence. The clamp system allows the determination of ALDH2 activity in less than one million H4-II-E-C3 rat hepatoma cells. The specificity and sensitivity of the "acetaldehyde clamp" assay should be of value in evaluation of the effects of new agents that modify *Aldh2* gene expression, as well as in the study of ALDH2 regulation in intact cells.

Keywords: ALDH2; Ethanol; Acetate; H4-II-E-C3; Hepatoma

1. Introduction

Mitochondrial class 2 aldehyde dehydrogenase (ALDH2) metabolizes most of the acetaldehyde generated in the metabolism of ethanol in the liver (Klyosov et al., 1996; Parrilla et al., 1974; Tank et al., 1981). Inhibition of ALDH2 after administration of the drug disulfiram leads to acetaldehyde accumulation (Garver et al., 2000; Jensen & Faiman, 1986) and to symptoms, such as a flushing, tachycardia, and nausea, which constitute the basis for the aversion to ethanol produced by this drug (Peachey et al., 1983). A mutation in the *Aldh2* gene present in some individuals of the Asian population (Yoshida et al., 1985; Yoshida & Dave, 1985) generates a virtually inactive enzyme (*Aldh2*2*), which mimics these symptoms when individuals consume alcohol (Mizoi et al., 1994). These effects largely protect *Aldh2*2* carriers from the development of alcoholism (Chen et al., 1999; Harada et al., 1982; Higuchi, 1994; Thomasson et al., 1991; Tu & Israel, 1995).

H4-II-E-C3 rat hepatoma cells have been found to express the *Aldh2* gene (Garver et al., 2001; Huang & Lindahl, 1990). It has been shown that antisense oligonucleotides inhibit *Aldh2* gene expression in this cell system (Garver et al., 2001). Measurement of ALDH2 activity in these studies required isolation of mitochondria from the cultured cells, which (1) has variable yields, (2) requires assessment of mitochondrial integrity, and (3) requires a large number of hepatoma cells. These difficulties support the idea that future studies, requiring ALDH2 activity measurements, would be more reproducibly carried out in intact cells, without the need of isolating mitochondria.

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The main purpose of the current study was to measure ALDH2 activity in intact cells in culture, keeping the acetaldehyde concentration high enough to make this enzyme function at its maximal activity and low enough to minimize cytosolic class 1 aldehyde dehydrogenase (ALDH1) contribution to its metabolism. Accordingly, we have developed a simple and highly specific method that "clamps" the acetaldehyde concentration at about 4 μM in a cell-incubation medium, allowing quantitation of virtually the low-K_M ALDH2 activity alone. Consequently, aldehyde dehydrogenase activity was determined in intact cells by measurement of ^{14}C-acetate released into the incubation medium that contained the "clamped" concentrations of ^{14}C-acetaldehyde.

A number of investigators have evaluated the rate of ethanol metabolism in various cell cultures by measuring ^{14}C-acetate production from ^{14}C-ethanol (Bond et al., 1983; Haber et al., 1998; Nakamura et al., 1999). However, these methods measure the combined activity of alcohol dehydrogenase and aldehyde dehydrogenase and also require that the conversion of acetate into $^{14}CO_2$ be quantitated (Bond et al., 1983). In the new method presented in this article, alcohol dehydrogenase is added in excess, and the conversion of ^{14}C-acetate into $^{14}CO_2$ is blunted by addition of unlabeled acetate. Because acetaldehyde generated by an external alcohol dehydrogenase system readily diffuses across membranes, an alcohol dehydrogenase system in equilibrium can maintain low concentrations of ^{14}C-acetaldehyde in hepatoma cells, allowing the conversion of ^{14}C-acetaldehyde into ^{14}C-acetate by ALDH2 activity. Such a system was developed in the current study.

Cell cultures can display different phenotypes on the basis of cell confluence. It has been reported that, in rats, the ALDH2 activity per unit of liver weight is reduced after partial hepatectomy, when cells undergo active division, and that ALDH2 activity returns to normal levels after the original liver mass has been restored (Watanabe et al., 1985). Therefore, we have also studied whether ALDH2 activity is increased in rat hepatoma cell cultures after they reach confluence, a condition in which cell division is halted. Our study findings support the observation that ALDH2 activity is low in actively dividing cells and increases after the rate of cell division is halted.

2. Materials and methods

Ethanol [1-^{14}C] was purchased from New England Nuclear (Boston, MA, USA). Dowex 1 × 8-400 resin; cyanamide; Triton X-100; 2,5-diphenyl-oxazole (PPO); 1,4-bis(4-methyl-5-phenyl-2-oxazolyl)benzene (dimethyl POPOP); alcohol dehydrogenase (EC 1.1.1.1) from bakers' yeast; aldehyde dehydrogenase (EC 1.2.1.5) from bakers' yeast; acetaldehyde; crotonaldehyde; and 2,4-dinitrophenylhydrazine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.1. Cell culture

H4-II-E-C3 rat hepatoma cells, purchased from ATCC (Rockville, MD, USA), were cultured in Dulbecco's modified Eagle's medium [(DMEM); Gibco BRL, Grand Island, NY, USA]; supplemented with 5% fetal bovine serum, 10% equine serum (Hyclone Lab Inc., Logan, UT, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Gibco BRL); and maintained in 100-mm plates (Nunc Inc., Napierville, IL, USA) at 37ºC under 5% CO_2 and 95% air. Unless otherwise indicated, studies were performed by using confluent cells. Cells were detached with 0.25% Trypsin-EDTA (Gibco BRL) and transferred to an O-ring screw-capped 1.5-ml microcentrifuge tube and suspended in DMEM for incubation in the presence of ^{14}C-ethanol (at the concentration indicated), oxidized form of nicotinamide adenine dinucleotide [(NAD^+); 400 μM], and yeast alcohol dehydrogenase (50 U/ml). In contrast, when the effect of cell confluence on ALDH2 activity was studied cells were cultured in 6-well plates and kept attached to the wells. In these studies, the serum-containing medium was changed to a serum-free DMEM, containing ^{14}C-ethanol, NAD^+, and yeast alcohol dehydrogenase to measure ALDH2 activity in the attached cells. To prevent ^{14}C-ethanol and ^{14}C-acetaldehyde evaporation during the ALDH2 determination, wells were tightly stoppered. The number of cells was quantitated after trypsinization by using a hemocytometer (Fisher, Pittsburgh, PA, USA).

2.2. Production and determination of acetate

Excess yeast alcohol dehydrogenase, as indicated above, was added to the medium (0.6 ml) in the presence of NAD^+ to transform ^{14}C-ethanol (60–120 μM) into acetaldehyde, thus reaching equilibrium and maintaining the acetaldehyde at a "buffered" concentration according to its equilibrium constant (Speisky et al., 1985). Cells were incubated under these conditions up to 120 min at 37ºC. Addition of unlabelled sodium acetate (10 mM) prevents ^{14}C-acetate conversion into $^{14}CO_2$ by the tricarboxylic acid cycle. In some studies, cyanamide—an inhibitor of aldehyde dehydrogenases (Pruñonosa et al., 1991; Svanas & Weiner, 1985b)—was used as a control. At the end of the incubation period, the medium was acidified with 0.1 ml of 1 M H_2SO_4, and the samples were rapidly frozen. For acetate determination, a Dowex 1 × 8-400 resin was thoroughly washed with water and subsequently with 0.1 M sodium phosphate (pH 6.8). Subsequently, 0.5 ml of the slurry was loaded into a 1-ml syringe, which served as a column, and further washed with 5 ml of the same buffer. Samples were thawed, neutralized with 5 M sodium hydroxide, and slowly passed through the column. Unbound ^{14}C-ethanol was removed from the column by washing with 5 ml of water. To ensure complete removal of ^{14}C-ethanol, the radioactivity of the last milliliter of effluent was measured. Subsequently, ^{14}C-acetate bound to the column was eluted by using 5 ml of 0.05 M HCl, and the radioactivity in an aliquot of the combined

eluate fractions was determined in a liquid scintillation counter (Tri-Carb 4000 Packard, Downers Grove, IL, USA). The amount of ^{14}C-acetate formed was calculated from the specific activity of the ^{14}C-ethanol used as substrate and expressed in nanomoles per hour per million cells.

2.3. Determination of acetaldehyde

Acetaldehyde was determined by high-performance liquid chromatography (HPLC), as described by Lucas et al. (1986). Cells were incubated (as previously described in Section 2.1.) in tightly sealed O-ring screw-capped tubes in the presence of NAD^+ (400 μM), yeast alcohol dehydrogenase (50 U/ml), and ethanol (100 μM) at 37ºC. Samples were obtained at 30, 60, and 120 min and treated immediately with 0.6 M ice-cold perchloric acid in saline. After centrifugation at 4ºC, the supernatants were treated with 2,4-dinitrophenylhydrazine reagent. After addition of the internal standard (crotonaldehyde-2,4-dinitrophenylhydrazone) and 3 M sodium acetate, the derivatives were extracted and analyzed by HPLC by using 35:65 water:acetonitrile as a mobile phase in a Supelcosil LC18 column with optical density (OD) detector at 356 nm. Acetaldehyde concentration is presented as mean ± S.E.M. for three independent experiments at each time studied.

2.4. Determination of mRNA levels

Total cell RNA from H4-II-E-C3 rat hepatoma cells at different degrees of confluence (60%, 95%–100%, and 100% at 24 and 48 h postconfluence) was prepared by using 1 ml of Trizol (Gibco BRL) per 30-mm well according to the supplier's instructions. Reverse transcriptase reactions on total RNA were conducted by using 2 μg of RNA, 225 pmol of poly-dT, 200 pmol of each dNTP, and 200 U of M-MLV reverse transcriptase (Promega) in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol for 1 h at 37ºC. Independent polymerase chain reactions were then performed for both ALDH2 and β-actin by using 2 mM $MgCl_2$, 200 μM of each dNTP, 2 μM of direct primer (5′CTGCAGAGCTTGGGACAGG3′ for ALDH2 and 5′CGATTGTAACAAACTGGGAGC3′ for β-actin), 2 μM of reverse primer (5′TAAGCACTGAGGGTGGAACC3′ for ALDH2 and 5′CATGAGGTAGTCTGTCAGCTC3′ for β-actin), and 0.5 U of *Taq* DNA polymerase (Gibco BRL) in 20 mM Tris-HCl and 50 mM KCl (pH 8.4). After 25 amplification cycles (thermal-cycling program: 3 min at 94ºC; 25 cycles of 1 min at 94ºC, 1 min at 60ºC, and 1 min at 72ºC; and 10 min at 72ºC), the samples were subjected to electrophoresis on 2% agarose gels and stained with ethidium bromide. Gels were photographed and scanned within a linear range for densitometric analysis and analyzed with the use of Scion Image software.

2.5. Statistical analyses

Unless otherwise indicated, values are expressed as average ± S.E.M. of at least four experiences. When ALDH2 activity in H4-II-E-C3 rat hepatoma cells at different degrees of confluence was studied, the level of significance was calculated with the Student *t* test by using the SISA (Simple Interactive Statistical Analysis) program: http://home.clara.net/sisa.

3. Results and discussion

Fig. 1 shows the change in absorbance at 340 nm of an in vitro reaction containing excess yeast alcohol dehydrogenase, ethanol (100 μM), and NAD^+ (400 μM). As can be seen, the absorbance, owing to reduced nicotinamide adenine dinucleotide (NADH), reached a plateau in less than 1 min, indicating an equilibrium of the reaction

$$\text{ethanol} + NAD^+ \leftrightarrows \text{acetaldehyde} + NADH + H^+.$$

A concentration of approximately 4 μM can be calculated for acetaldehyde from the concentration of NADH at pH 7.4. Subsequent addition of yeast aldehyde dehydrogenase (arrow) increased the amount of NADH produced, owing to the metabolism of acetaldehyde. It can be calculated, provided only a minor fraction of ethanol is oxidized, that the levels of acetaldehyde remain virtually constant. Furthermore, the equilibrium constant for this reaction averages 1.55×10^{-11} M (Speisky et al., 1985), and, considering it occurs at a constant pH of 7.4, it can be calculated at an equilibrium constant of 3.9×10^{-4} for this pH. Because acetaldehyde and NADH are generated equimolarly, such equilibrium constant K has the acetaldehyde concentration to the second power as its numerator and the concentration of ethanol times the concentration of NAD^+ as its denominator; that is,

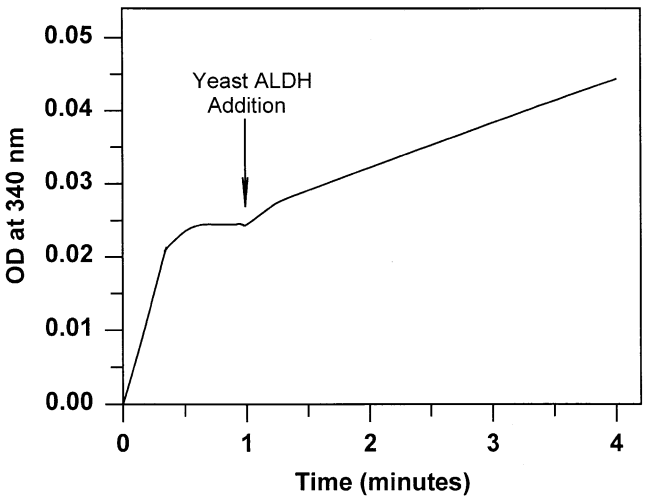

Fig. 1. Absorbance of reduced nicotinamide adenine dinucleotide (NADH) at 340 nm, recorded along time, when ethanol (100 μM) was mixed with yeast alcohol dehydrogenase (50 U) and oxidized form of nicotinamide adenine dinucleotide [(NAD^+); 400 μM]. Arrow indicates addition of yeast aldehyde dehydrogenase [(ALDH); 1 U)].

$K = [\text{acetaldehyde}]^2/([\text{ethanol}] \times [NAD^+])$.

It is therefore possible, in theory, to clamp the acetaldehyde concentration at any given value for a period by means of controlling the concentrations of ethanol and NAD^+ and allowing the equilibrium to be reached.

Because the K_M of ALDH2 is <1 μM (Garver et al., 2001; Klyosov et al., 1996), the near-saturating levels of acetaldehyde clamped in the above reaction are expected to generate acetate at a constant rate. This prediction was confirmed experimentally, as seen in Fig. 2 in which an excellent linearity of ^{14}C-acetate production was obtained for 2 h. When the actual concentration of acetaldehyde in this "clamped system" was measured by HPLC at 30, 60, and 120 min, an average value of 4.2 ± 0.4 μM was found throughout the 2 h of the assay. No significant differences were found between the three times studied, showing that the acetaldehyde concentration is indeed clamped at that range.

Subsequent experiments were conducted to determine whether the elution profile generated from ^{14}C-ethanol in the hepatoma cell system replicated the elution characteristics of ^{14}C-acetate generated by oxidizing ^{14}C-ethanol with $KMnO_4$. Fig. 3 shows that the products arising from the metabolism of ^{14}C-ethanol by H4-II-E-C3 rat hepatoma cells and ^{14}C-acetate generated chemically are eluted from the column in virtually the same fractions, thus implying the identity of the ^{14}C-product formed by the two methods.

The method used to determine ALDH2 activity was subjected to an additional test in which the production of acetate was measured in the presence and absence of cyanamide, an inhibitor of aldehyde dehydrogenases (Fig. 4). Cyanamide strongly inhibited the generation of acetate from ^{14}C-ethanol in the H4-II-E-C3 rat hepatoma cell system, with a K_I of approximately 30 μM. With a concentration of cyanamide of 160 μM, the generation of ^{14}C-acetate was inhibited by 75%–80%. Because, at this concentration, a plateau has not been reached, higher concentrations of cyanamide should increase such inhibition. Moreover, it should be noted that the hepatoma cells used are derived from the rat, a species that presents very low levels of ALDH1 activity because it lacks 13 nucleotides in the *Aldh1* promoter region (Chen et al., 1996). Furthermore, Svanas and Weiner (1985a) reported that only 6% of acetaldehyde was metabolized by ALDH1 at an acetaldehyde concentration of 200 μM. Therefore, a very low specific activity and total activity of ALDH1 should be expected and, because of that, a very low contribution of ALDH1 to the metabolism of acetaldehyde at a

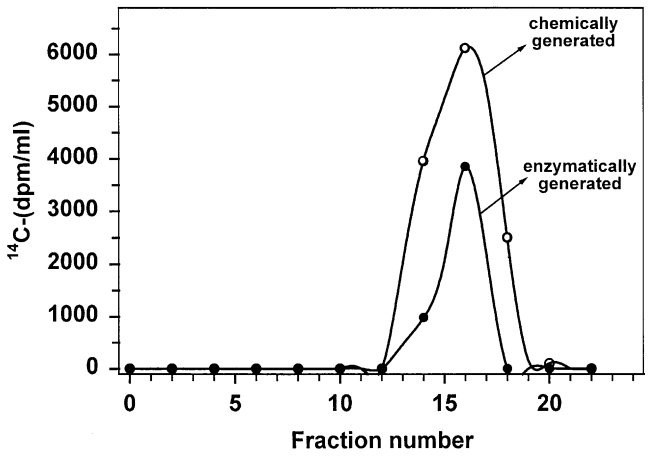

Fig. 3. Elution pattern of ^{14}C-acetate produced enzymatically in rat hepatoma cells from ^{14}C-ethanol (closed circles) or chemically after oxidation with $KMnO_4$ (open circles). Radioactivity (disintegrations per minute) measured in each 0.5-ml fraction, eluted with 0.05 M HCl from a Dowex 1 × 8-400 anion-exchange column, is represented.

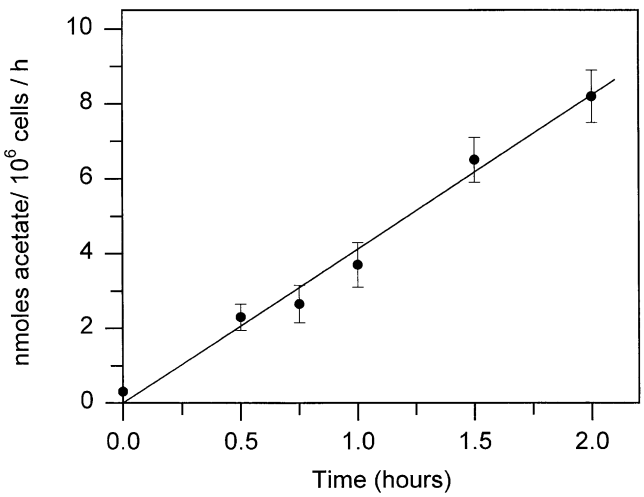

Fig. 2. Time course of acetate production by H4-II-E-C3 rat hepatoma cells incubated with ^{14}C-ethanol (100 μM), yeast alcohol dehydrogenase (50 U), and oxidized form of nicotinamide adenine dinucleotide [(NAD^+); 400 μM]. Each point represents the average ± S.E.M. of four incubations.

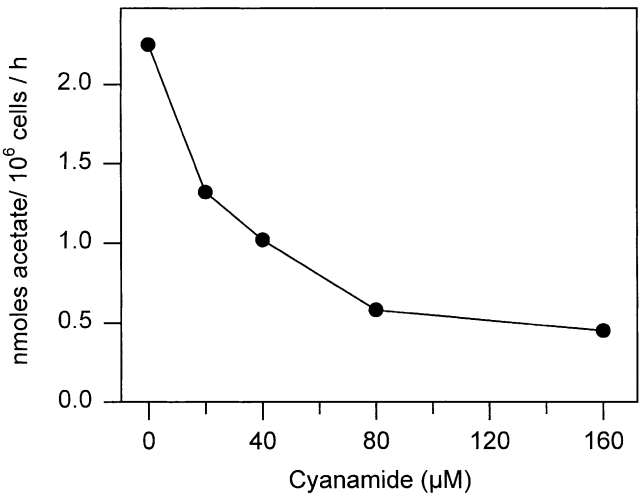

Fig. 4. Inhibition of aldehyde dehydrogenase activity by cyanamide in H4-II-E-C3 rat hepatoma cells in an acetaldehyde clamp system. Each point represents the average of four incubations. Standard error never exceeded 12% and for better clarity is not represented.

concentration of 4 μM is envisioned. These strengthen the conclusion that ^{14}C-acetate, measured in the current study, is generated by the low-K_M ALDH2 activity.

In addition, the "clamp system" presents clear advantages when compared with the spectrophotometric method. The latter method determines mitochondrial aldehyde dehydrogenase activity in total cell lysates or mitochondrial fractions by incubating them in the presence of 25 μM of propionaldehyde as a substrate and measuring the formation of NADH after the change of OD at 340 nm (Cao et al., 1989). Nevertheless, the K_M for acetaldehyde of rat ALDH1 has been reported to be in the order of 15–17 μM (Klyosov et al., 1996). Therefore, the use of acetaldehyde or propionaldehyde at 15–25 μM implies a somewhat important contribution of the ALDH1 activity on the aldehyde metabolism, a major set back in relation to specificity of the method. Notwithstanding, the use of the propionaldehyde method is possible in rats of the Fischer, Sprague–Dawley, Wistar, or Long–Evans strain because, as previously explained, these strains possess a deletion in the promoter area of the *Aldh1* gene (vide supra), which greatly reduces its expression. However, detailed studies of ALDH1 have not been performed for the hepatoma EC3 cells that we used in the current study. Another important disadvantage of the propionaldehyde method that should be emphasized is the low spectrophotometric absorbance for NADH that is reached by using concentrations of propionaldehyde such as 15–25 μM (about 0.045 when half the substrate is exhausted), because it results in a low signal-to-noise ratio in most spectrophotometers.

It should also be noted that most hepatoma cell lines display low alcohol dehydrogenase activities. Crabb et al. (1995) reported that H4-II-E-C3 rat hepatoma cells have only 30% of the alcohol dehydrogenase activity found in rat liver. Thus, measurement of acetate generated without addition of an external system of acetaldehyde production (the yeast alcohol dehydrogenase and the acetaldehyde clamp system) might underestimate the metabolic capacity of ALDH2 in these cells.

It has been reported that ALDH2 activity per unit of liver weight is reduced in hepatectomized animals and that this activity returns to normal levels only after the original liver mass has been recovered (Watanabe et al., 1985). We sought to determine whether ALDH2 activity was affected in hepatoma cell cultures after reaching confluence, a condition in which cell division is largely halted. As shown in Fig. 5, ALDH2 activity was lower in cells that were undergoing active division and increased 150% after a 48-h confluence period, in general agreement with the in vivo findings of Watanabe et al. (1985). Unexpectedly, such an increase was not accompanied by increases in ALDH2 mRNA. Rather, mRNA levels were reduced after 48 h of confluence (Fig. 6). This finding seems to indicate that an increased enzyme stability or an increased translation rate, rather than an increased transcript level, is responsible for the higher ALDH2 activity observed after confluence. Because ALDH2 needs

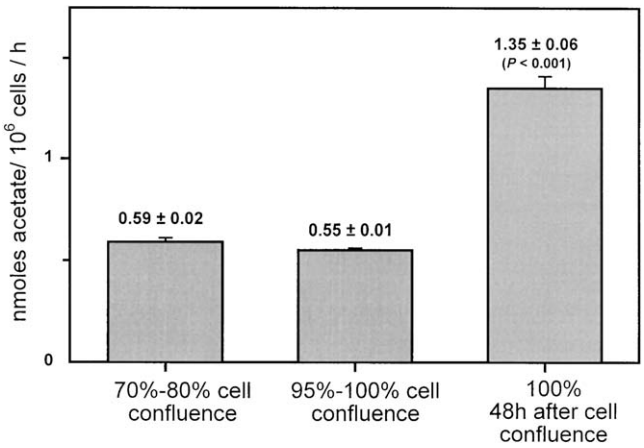

Fig. 5. Mitochondrial class 2 aldehyde dehydrogenase (ALDH2) activity in H4-II-E-C3 rat hepatoma cells at different degrees of confluence. Each point represents mean ± S.E.M. of three incubations.

to be transported into the mitochondrial matrix, an alternative explanation would consider a change in mitochondrial function, as the cells become confluents affecting the level of enzyme.

Overall, we report a method to determine the low-K_M ALDH2 activity in intact rat hepatoma cells by using an acetaldehyde clamp system that keeps acetaldehyde concentrations low and constant in the cell-incubation medium. The generation of acetate is linear with time and is inhibited by cyanamide, an aldehyde dehydrogenase inhibitor. We also report that rat hepatoma cells undergoing active division present a low ALDH2 activity, behaving similarly to liver after partial hepatectomy. Both hepatoma cells and liver in vivo increase their ALDH2 activity after the cells cease dividing.

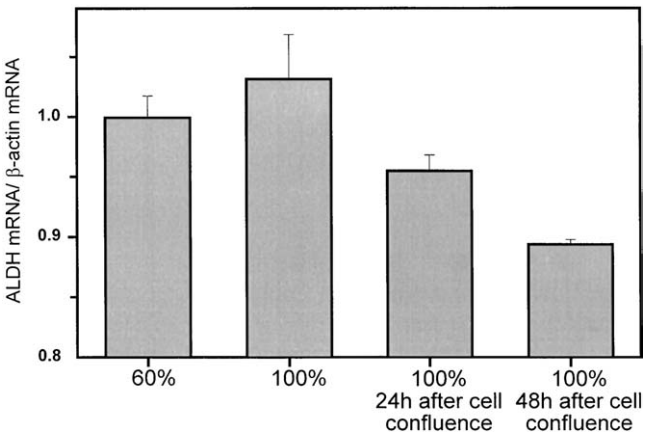

Fig. 6. Levels of mitochondrial class 2 aldehyde dehydrogenase (ALDH2) mRNA at different degrees of confluence in H4-II-E-C3 rat hepatoma cells. Values plotted represent the ratio of ALDH2 mRNA/β-actin mRNA expressed as average ± S.E.M. for three experiences.

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