chromatography. Further purification is required to determine whether the biological effects of atrial extract are elicited by a single substance. The isolated smooth muscle preparations used in this study provide a rapid and simple in vitro bioassay for the quantitative detection of bioactive substances derived from the atria during such purification efforts. Such a volume-regulating system in the atria could explain the decrease in vascular resistance and the natriuresis and diuresis caused by distention of the atria (*3*, *6*).

The relation of the bioactive atrial substance to other reported natriuretic factors derived from plasma and urine extracts is uncertain. The "natriuretic hormone" described by de Wardener (*7*) has a molecular weight of less than 500, is not sensitive to proteolytic digestion, and lacks amino acids. An apparently different natriuretic factor, purified from the plasma of salt-loaded dogs, is a heat-stable, acidic peptide of low molecular weight that appears to be derived from a larger precursor (*8*). This natriuretic factor, however, has been shown to potentiate vasoconstriction by norepinephrine, whereas our rat atrial extract actually reversed norepinephrine-induced contraction of the isolated blood vessel segment (Fig. 1).

We propose that mammalian atria have the potential to regulate fluid volume. The atria are in an ideal location to detect volume changes and appear to be the source of substances that influence renal function and vascular tone (Fig. 3). We envision that the atria detect an increase in the extracellular fluid volume and respond by releasing bioactive substances into the bloodstream that stimulate a rapid loss of fluid through the urine and peripheral vasodilation.

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Dicarboxylic Aciduria: Deficient [1-^{14}C]Octanoate Oxidation and Medium-Chain Acyl-CoA Dehydrogenase in Fibroblasts

Abstract. *Dicarboxylic aciduria, an inborn error of metabolism in man, is thought to be caused by defective β-oxidation of six-carbon to ten-carbon fatty acids. Oxidation of [1-^{14}C]octanoate was impaired in intact fibroblasts from three unrelated patients with dicarboxylic aciduria (19 percent of control), as was the activity of medium-chain (octanoyl-)acyl-CoA dehydrogenase in the supernatants of sonicated fibroblast mitochondria (5 percent of control). These data confirm that dicarboxylic aciduria is caused by an enzyme defect in the β-oxidation cycle.*

Dicarboxylic aciduria (DCA) is a metabolic disorder of man characterized by episodic hypoglycemia without accompanying ketosis and the excretion of elevated amounts of C_6–C_{10} ω-dicarboxylic acids in the urine (*1–3*). These dicarboxylic acids, primarily adipic (hexanedioic), suberic (octanedioic), and sebacic (decanedioic), are formed by microsomal ω-oxidation of fatty acids of either medium- or long-chain length which are then shortened to medium-chain dicarboxylic acids by mitochondrial β-oxidation (*4*). Although excretion of these dicarboxylic acids has been found in patients with other metabolic derangements (*5*, *6*), nine to ten patients with DCA have been identified in whom other entities can be excluded on clinical and biochemical grounds (*1–3*). The presence of C_6–C_{10} DCA without associated ketosis has suggested that mitochondrial β-oxidation of fatty acids is impaired in these patients, since this catabolic pathway is responsible for the stepwise degradation of C_4–C_{18} fatty acids to acetyl coenzyme A (acetyl-CoA) in mammals, as well as the production of acetoacetate and β-hydroxybutyrate. Increased fatty acid ω-oxidation and the resultant formation of dicarboxylic acids has been observed both in vivo and in vitro when the catabolism of fatty acids is impaired either by toxins or by inherited enzymatic deficiencies (*6*). The most important enzyme of β-oxidation with activity toward fatty acids of medium-chain length is the mitochondrial acyl-CoA dehydrogenase (E.C. 1.3.99.3) that demonstrates specificity for C_4–C_{14} acyl-CoA's with maximal activity toward C_6–C_{10} acyl-CoA's, with separate enzymes showing maximal activities toward the analogous short-chain (E.C. 1.3.99.2) and long-chain (E.C. 1.3.99.4) substrates (*7*). This enzyme, termed Y_1-fatty acyl-CoA, general acyl-CoA, or medium-chain acyl-CoA dehydrogenase (MCADH) is probably essential for normal functioning of the β-oxidation pathway (*7*); deficient activity

Table 1. The oxidation of ^{14}C-labeled substrates by human diploid skin fibroblasts. Culture of normal diploid skin fibroblasts and ^{14}C-substrate oxidation studies were performed as described (*15*), with the cells (passages 7 to 12) being harvested 8 days after subculture (1:4). All assays were done in triplicate and all values are expressed as means ± standard error of the mean. The final concentration and specific activity of each substrate were as follows: [1-^{14}C]butyrate, 1 m*M*, 10 mCi/mmole; [1-^{14}C]octanoate, 1 m*M*, 4.7 mCi/mmole; [1-^{14}C]palmitate, 0.2 m*M*, 5.0 mCi/mmole; [1,4-^{14}C]succinate, 2.0 m*M*, 1.0 mCi/mmole; [2-^{14}C]leucine, 1.5 m*M*, 0.5 mCi/mmole. The results obtained with five normal cell lines were compared to those from the three DCA cell lines.

Cell line	$^{14}CO_2$ production by intact fibroblasts per hour (pmole/mg protein)				
	[1-^{14}C]-butyrate	[1-^{14}C]-octanoate	[1-^{14}C]-palmitate	[2-^{14}C]-leucine	[1,4-^{14}C]-succinate
Normal control	2450 ± 143	2070 ± 204	102 ± 15	779 ± 67	945 ± 71
DCA	1760 ± 150	386 ± 69	59 ± 12	736 ± 83	979 ± 128
Percent of control	72	19	58	94	116

Table 2. The oxidation of ^{14}C-labeled substrates by isolated fibroblast mitochondria. Fibroblast mitochondria were isolated precisely as described (*12*), except that the digitonin step was omitted. Reaction conditions were as noted in Table 1, with the following differences: 250 to 500 μg of mitochondrial protein was used per assay, the buffer utilized consisted of 100 m*M* KCl, 50 m*M* tris-HCl, *p*H 7.5, 5 m*M* $MgCl_2$, 1 m*M* ATP, 1 m*M* EGTA, and the incubation time was 1/2 hour. The concentration and specific activity of the substrates were as noted in Table 1. When [1-^{14}C]butyrate and [1-^{14}C]octanoate were used, 2.5 m*M* ATP, 0.5 m*M* carnitine, and 0.1 m*M* CoA were added to incubation medium. With [1-^{14}C]palmityl-CoA (0.1 m*M* and 1.0 mCi/mmole) as substrate, 2.5 m*M* ATP and 0.5 m*M* carnitine were added. All assays were done in triplicate and all values are expressed as means ± standard error of the mean.

Cell line	$^{14}CO_2$ production by intact mitochondria per hour (pmole/mg protein)		
	[1-^{14}C]butyrate	[1-^{14}C]octanoate	[1-^{14}C]palmityl-CoA
Normal control	9700 ± 3300	3210 ± 1200	590 ± 60
DCA	4350 ± 2670	158 ± 114	190
Percent of control	45	5	32

of MCADH could produce the defective ketogenesis, hypoglycemia, and organic aciduria observed in DCA.

The oxidation of ^{14}C-labeled substrates in vitro by intact diploid fibroblasts to $^{14}CO_2$ has been used successfully to demonstrate oxidative defects in many inborn errors of amino acid and fatty acid metabolism (*8*); this approach avoids the potential medical complications involved in performing metabolic studies with either the patients themselves or surgically obtained biopsy specimens of liver and muscle. Intact skin fibroblasts from three unrelated patients with well-documented DCA (*1, 9*) demonstrate markedly impaired oxidation of [1-^{14}C]octanoate (19 percent of control levels; $N = 22$; $P < .05$), whereas the oxidation of [1-^{14}C]butyrate and [1-^{14}C]palmitate is mildly decreased (72 and 58 percent of control, respectively; $P < .05$; Table 1). Very few comparable data are available in the literature; Naylor *et al.* (*2*) stated that fibroblasts from another child with DCA oxidized [1-^{14}C]palmitate normally but oxidized [U-^{14}C]palmitate at 50 percent of control levels (*10, 11*). Our data show that DCA cells are most defective in the oxidation of fatty acids of medium-chain length, as evidenced by the severe and fairly isolated deficiency in [1-^{14}C]octanoate oxidation. The normal rates of [1,4-^{14}C]succinate and [2-^{14}C]leucine oxidation by DCA fibroblasts suggest that other oxidative pathways are intact in these cells (*8*).

Since the β-oxidation of fatty acids occurs in the mitochondrial matrix, we have studied the oxidation of ^{14}C-labeled fatty acids in fibroblast mitochondria isolated from both normal controls and one of the DCA cell lines. We have previously used similar mitochondrial preparations to demonstrate that isovaleric acidemia is due to an isolated deficiency of a separate, branched-chain acyl-CoA dehydrogenase, isovaleryl-CoA dehydrogenase (*12*), and that mitochondrial oxidation of both straight- and branched-chain fatty acids is defective in glutaric aciduria type II (*13*). Intact mitochondria from DCA fibroblasts oxidize [1-^{14}C]octanoate very poorly (Table 2), at only 5 percent of control levels ($N = 4$; $P < .05$); the oxidation of [1-^{14}C]butyrate and [1-^{14}C]palmitoyl-CoA by these same DCA mitochondrial preparations is less severely impaired (45 and 32 percent of control, respectively; $P > .2$). Thus the oxidative defects in intact fibroblasts (Table 1) are also present in the isolated mitochondria. The higher rates of [1-^{14}C]octanoate oxidation in intact fibroblasts, compared to isolated mitochondria, may reflect the contribution of peroxisomal β-oxidation toward catabolism of this substrate in the intact cell. This oxidative system demonstrates some activity toward C_7–C_8 acyl-CoA's, although it is most active with C_{14}–C_{22} substrates (*14*).

Table 3. Acyl-CoA dehydrogenase activity measured with three different substrates. The activity was determined as described (*12*) by using the supernatants of sonicated mitochondria to which was added 7.8 μg of electron-transferring flavoprotein from pig liver. Concentrations of all acyl-CoA's were 50 μ*M*, except palmityl-CoA (25 μ*M*). The rate of reduction of dichlorophenolindophenol was calculated from the change in absorbance (OD_{600}) during the first minute of reaction in the presence of 1 m*M* *N*-ethylmaleimide.

Cell line	Acyl-CoA dehydrogenase activity per minute (pmole/mg protein)		
	Butyryl-CoA	Octanoyl-CoA	Palmityl-CoA
Normal control	379 ± 65	660 ± 151	545 ± 74
DCA	209 ± 36	34 ± 15	432 ± 38
Percent of control	55	5	79

We used the widely accepted dichlorophenolindophenol (DCIP) dye reduction method to assay the three straight-chain acyl-CoA dehydrogenases in both DCA and normal control fibroblast mitochondria (Table 3). Initially, apparent MCADH activity in the normal controls as assayed with octanoyl-CoA was much higher than was predicted (*7*) from previous measurements of butyryl-CoA dehydrogenase activity in similar preparations (*12, 15*). Furthermore, apparent MCADH activity in the DCA mitochondrial supernatants was distinctly elevated (38 ± 17 percent of control; $N = 4$; data not shown) when compared to the oxidation of [1-^{14}C]octanoate by intact DCA cells and mitochondria. Elevation of apparent acyl-CoA dehydrogenase activity in mitochondrial sonic supernatants, as measured by the DCIP dye reduction assay, can be caused by hydrolysis of the acyl-CoA substrates by acyl-CoA thioesterases (E.C. 3.1.2.2) and subsequent factitious reduction of DCIP by free CoA sulfhydryl groups (CoASH) (*7*). Thioesterases are found in microsomes, mitochondria, and the cytosol; demonstrate maximal hydrolytic activity toward C_8–C_{20} acyl-CoA's; and, in general, are inhibited by *N*-ethylmaleimide [NEM (*16*)]. Since NEM also acylates free sulfhydryls, which could also reduce DCIP, and does not inhibit purified acyl-CoA dehydrogenases from rat, pig, and bovine liver (*7, 17*), this reagent can be added to crude cellular and mitochondrial extracts to permit accurate assay of acyl-CoA dehydrogenase activity by the dye reduction method. Addition of 1 m*M* NEM to the supernatants of sonicated mitochondria lowered the apparent MCADH activity in control mitochondria by ten times, and showed that the residual MCADH activity in the mitochondrial preparations from all three DCA cell lines was profoundly decreased (5 percent of control; $P < .02$; $N = 13$). Apparent short-chain acyl-CoA dehydrogenase activity, assayed with butyryl-CoA, was much nearer normal (55 percent of control; $P < .05$) in DCA mitochondria, as was long-chain acyl-CoA dehydrogenase activity measured with palmitoyl-CoA as substrate (79 percent of control; $P > .2$). The reduced butyryl- and palmitoyl-CoA dehydrogenase activities in sonicated supernatants of DCA mitochondria paralleled the results obtained in intact cells and whole mitochondria and probably represent the effect of deficient MCADH activity on the mitochondrial oxidation of short- and long-chain acyl-CoA substrates.

These results strongly suggest that

DCA is due to deficient activity of the medium-chain acyl-CoA dehydrogenase, the enzyme responsible for initiating the β-oxidation of C_6–C_{10} fatty acyl-CoA's. To our knowledge, DCA is the first inborn error of metabolism in man that has been found to be caused by a deficiency of one of the enzymes of the β-oxidation cycle. This study underscores the utility of using purified mitochondrial preparations from normal diploid fibroblasts to study the biochemistry of human metabolic diseases. The use of such mitochondrial preparations from fibroblasts or other cultured human cells should allow us to pursue detailed investigations of the biochemistry of DCA, as well as other human disorders involving mitochondrial function, such as glutaric aciduria type II, ethylmalonic-adipic aciduria (*13, 15*), and the etiologically heterogeneous group of human mitochondrial myopathies (*18*).

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Inhibition of Tumor Promotion by a Biomimetic Superoxide Dismutase

Abstract. *A low molecular weight, lipophilic, copper coordination complex with superoxide dismutase-mimetic activity inhibited biochemical and biological actions of a tumor promoter in mouse epidermis. Such inhibitory effects implicate reactive oxygen species in the tumor promotion process.*

Tumor promoters are noncarcinogenic compounds that cause the development of tumors when applied repeatedly after initiation with a single subthreshold dose of a carcinogen. The phenomenon of two-stage carcinogenesis (initiation followed by promotion) has been studied most thoroughly in mouse skin, where esters of the tetracyclic diterpene, phorbol, are the most potent promoters. Topical application of phorbol esters to mouse skin results in numerous biochemical changes that lead to enhanced cell proliferation and that are associated with the promotion process (*1*). Reactive oxygen, that is, free oxygen radicals may play important roles as mediators of these pleiotropic responses.

Respiring cells produce free radicals from molecular oxygen through enzymatic and nonenzymatic reactions. The univalent pathway of oxygen reduction generates, in turn, the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), the hydroxyl radical (OH·), and finally water. The intermediates of oxygen reduction to water are highly reactive and present a challenge to the integrity of cells. As either an oxidant or a reductant O_2^- can, directly or indirectly as a precursor for more potent radicals, modify a variety of biologically important molecules. For example, fluxes of O_2^- generated enzymatically, photochemically, or radiochemically have been shown to peroxidize lipids, depolymerize polysaccharides, cleave DNA, and kill cells (*2*). In peripheral white cells, the formation of O_2^-, triggered by phorbol esters, produces extensive single-strand breakage of DNA (*3*). However, not all reactions of oxygen radicals are necessarily destructive to biological macromolecules. H_2O_2 in particular appears to be involved in the activation of guanylate cyclase and glucose transport (*4*). The possible role of O_2^- and H_2O_2 in the regulation of cell division has been recently reviewed by Oberley *et al.* (*5*).

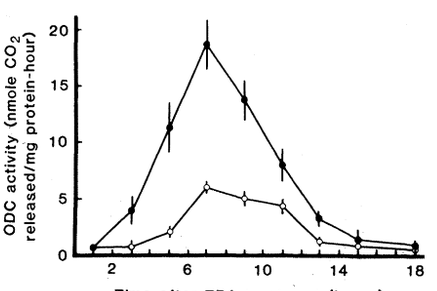

Fig. 1. Effect of treatment with CuDIPS on the induction of epidermal ODC activity by TPA. Mice were shaved with surgical clippers 2 days before use and only those in the resting phase of the hair growth cycle were used. Animals were treated topically with 5 μmole of CuDIPS (in 0.2 ml of diethyl ether) (●) 30 minutes before the application of 17 nmole of TPA (in 0.2 ml of acetone) and killed at the indicated times after TPA treatment. Epidermis was isolated and ODC activity determined in the 12,000*g* supernatants of epidermal homogenates (*13*). Each point represents the mean ± standard error of triplicate determinations done on each of three mice.

The involvement of reactive oxygen in tumor promotion is suggested by several recent observations. Leukocytes, macrophages, and lymphocytes respond to promoting agents, such as phorbol esters, by generating O_2^- and chemiluminescense, and antipromoting agents such as retinoids, protease inhibitors, and anti-inflammatory drugs inhibit these oxygen-related responses (*6*). Furthermore, benzoyl peroxide, lauroyl peroxide, and *m*-chlorobenzoic acid, all free radical–generating compounds, promote skin tumors in mice (*7*), whereas antioxi-