The Antipsoriatic Compound Anthralin Influences Bioenergetic Parameters and Redox Properties of Energy Transducing Membranes

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Bioenergetic parameters and redox properties of energy transducing membranes in rat liver mitochondria and cyanobacteria were investigated in the presence of the antipsoriatic compound anthralin (1,8-dihydroxy-9-anthrone). Transmembrane pH and electrical gradients were determined using electron paramagnetic resonance spectroscopy. In mitochondria, ubiquinones 9,10 and other redox components of the electron transport chain are reduced by anthralin; the proton motive force is increased. In the absence of ADP, anthralin slightly stimulates mitochondrial cyanide-insensitive oxygen consumption. It is suggested that increased cyanide-insensitive respiration is due to enhanced autoxidation of mitochondrial components and/or catalyzed oxidation of anthralin. In the presence of ADP mitochondrial respiration is decreased, and ATP synthesis is inhibited. Uncoupler-induced mitochondrial respiration is also decreased by anthralin, indicating inhibition of the electron transport chain. In the cyanobacterium *Synechococcus* PCC 6311 anthralin increases the pH gradient and decreases ATP levels. Thus, anthralin acts as an electron donor to membrane associated redox components and inhibits ATP synthesis in two different biologic systems. In human keratinocytes oxygen metabolism is influenced by anthralin in a similar pattern as in isolated mitochondria, and ATP content is decreased. Because anthralin reacts with redox components in different biologic membranes, alterations of subcellular/cellular redox status and energy metabolism might contribute significantly to its antiproliferative activity. *J Invest Dermatol 94:71–76, 1990*

Anthralin (1,8-dihydroxy-9-anthrone) is a very potent antipsoriatic compound [1]. It is a strong reductant that is readily oxidized by light, trace concentrations of metal ions, and oxygen. Its pharmacodynamic mode of action is not known in detail, but it has been found that mitochondria are target organelles of its action [2]. Anthralin binds to the plasma membrane of keratinocytes and subsequently accumulates in mitochondria [3]. It inhibits oxygen consumption in keratinocytes [4], fibroblasts [5], yeast and ascites cells [6], and skin [7]. In keratinocytes respiration is inhibited at lower anthralin concentrations than thymidine incorporation. Thus, the antirespiratory effect of anthralin has been suggested as the main reason for its antipsoriatic activity [4]. However, modulation of skin phagocyte activity [8,9] and arachidonic acid metabolism [10,11] may represent further important features that contribute to its clinical efficiency. Anthralin is not an uncoupler of oxidative phosphorylation in rat liver mitochondria: ADP/O ratios remain unchanged, and the ATPase activity is not stimulated [12]. However, it has been suggested that it acts as an uncoupler of oxidative phosphorylation in plant [13] and animal [2,14] mitochondria.

In this investigation we undertook to further study the mechanism of action of anthralin. The interaction of anthralin with energy transducing membranes was studied using mammalian (rat liver) mitochondria, intact cells of the cyanobacterium (blue-green alga) *Synechococcus,* and human keratinocytes. Three different biologic systems were investigated to separate general from specific effects on energy transduction and to reveal the relevance of these effects to biochemical events in skin.

Mitochondria contain one osmotic compartment. The electrochemical proton gradient, which can be used for ATP synthesis, is generated by a respiratory electron transport chain extruding protons across the inner membrane. Cyanobacteria (with the exception of Gloeobacter) contain two osmotically independent compartments, the cytoplasmic and the thylakoid spaces. At the cytoplasmic membrane protons are expelled from the cytoplasm into the me-

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Abbreviations:

ADP: adenosine-diphosphate
ATP: adenosine-triphosphate
Bis-tris-propane: 1,3-bis-[tris(hydroxymethyl)methylamino]-propane
CCCP: carbonylcyanide-m-chlorophenylhydrazone
EPR: electron paramagnetic resonance
FAD: flavine-adeninedinucleotide
FCCP: carbonylcyanide-p-trifluoromethoxy-phenylhydrazone
Hepes: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HPLC: high performance liquid chromatography
KCN: potassium cyanide
KΦ: 2,5-dihydro-2,2,5,5-tetramethyl-3-[(triphenylphosphonio)-methyl]-1H-pyrrol-1-yloxy bromide
NAD: nicotine-adeninedinucleotide
NADP: oxidized nicotine-adenine-dinucleotide-phosphate
Ni TEPA: nickel tetraethylenepentamine sulfate
PROXAD: 3-(aminocarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy
PROXCA: 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy
PROXAM: 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy
thyl: thylakoid

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dium upon energization by respiratory electron transport and/or a reversible ATPase, whereas at the thylakoid (intracytoplasmic) membrane protons are pumped inward into the thylakoid space by respiratory or photosynthetic electron transport [15,16].

MATERIALS AND METHODS

Chemicals Anthralin (1,8-dihydroxy-9-anthrone) was obtained through Hermal Chemie (Reinbeck, FRG), kept frozen in the dark, and purified by chromatography. Anthralin was always freshly dissolved in dimethylsulfoxide, kept light protected, and used immediately. Carbonylcyanide-m-chlorophenylhydrazone (CCCP) and carbonylcyanide-p-trifluoromethoxyphenylhydrazone (FCCP) were purchased from Sigma Chemical Co. (St. Louis, MO). The spin probes 3-(aminocarbonyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PROXAD), 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PROXCA) and 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PROXAM) were purchased from Molecular Probes, Inc. (Junction City, OR). 2,5-dihydro-2,2,5,5-tetramethyl-3-[(triphenylphosphonio)methyl]-1H-pyrrol-1-yloxy (KΦ) bromide was a generous gift of Dr. K. Hideg (University of Pécs, Hungary). Nickel tetraethylenepentamine sulfate (Ni TEPA) was prepared by mixing equimolar amounts of nickel sulfate and tetraethylenepentamine and subsequent titration to pH 7.0 with sulfuric acid.

Animals Female rats, weighing about 250 to 300 g, were obtained from Simeonsen (Gilroy, CA). The animals had free access to a standard diet (Rodent Lab Chow # 5001, Purina, St. Louis, MO) and water. The environmental conditions were as follows: room temperature 20° – 22° C, light cycle 12 h on/12 h off. Student t test for paired samples was used for statistical analysis.

Mitochondria Rat liver mitochondria were prepared and suspended as described [17]. The protein concentration was determined using Folin reagent [18]. Oxygen consumption was measured by oxypolarography according to Ref 19. Respiratory state 4 is defined as oxygen consumption in nmol oxygen/min/mg mitochondrial protein in the presence of oxygen and substrate; state 3 is defined as the respiratory rate under conditions as in state 4 plus ADP. Mitochondrial ATP was assayed as described [12].

Cyanobacteria Axenic cultures of *Synechococcus* PCC 6311 were grown photoautotrophically in medium C (20) + 10 mM sodium bicarbonate + 1 mM sodium carbonate at 30 W/m², 30°C, supplied with a stream of 1% CO_2 in sterile air. Light intensities were measured on the surface of the vessel with a Li-188B photometer from Li-Cor, Inc. (Lincoln, NE). Cells were harvested during the exponential growth phase (2 d). For electron spin resonance spectroscopy, measurements of respiratory oxygen uptake, and ATP assays cells were washed and resuspended in fresh medium C (without carbonate buffer) supplemented with 50 mM Hepes/Bis-tris-propane buffer, pH 7.0, to a density of 0.25 mg chlorophyll/ml, equivalent to 6.3 mg protein/ml. Chlorophyll and protein were determined according to Mackinney [21] and Lowry [18], respectively. ATP was extracted and assayed as described [22].

Human Keratinocytes Suspensions of human keratinocytes were obtained from Dr. P. Elias (University of California, San Francisco). Keratinocytes were isolated from human newborn foreskin by a treatment with trypsin and collagenase. The cells were plated onto a feeder monolayer of mitomycin C-treated 3T3 cells in 100 mm Petri dishes and grown in Dulbecco's modified Eagle's medium containing calcium at a concentration of 1.5 mM, 5% fetal calf serum, penicillin, streptomycin, and amphotericin B. Immediately prior to experiments, any remaining fibroblasts and 3T3 cells were removed from the cultures by brief treatment with 0.1% EDTA, as assessed by differential staining properties of these cells with acridine orange. First passage cells at different times of culture were used for all of the studies. The growth rate varied slightly for each keratinocyte cell line so that the day at which they seemed to be confluent varied slightly from experiment to experiment. The monolayers were subsequently trypsinized to obtain cell suspensions and used immediately for the experiments. Oxygen consumption [19] and adenine nucleotide content [12] of keratinocyte suspensions were analyzed as described.

Tocopherol and Ubiquinol/Ubiquinone Measurements A fast single-step lipid extraction procedure and high-performance liquid chromatography with in-line UV and electrochemical detection were used for the simultaneous quantitative determination of α-tocopherol, ubiquinols 9,10, and ubiquinones 9, 10 [23]. The HPLC system consisted of a Beckmann 114 MHPLC pump, a Rheodyne injection valve, an Altex Ultrasphere ODS column (4.6 mm × 25 cm, 5 μm particle size), or an Altex Ultrasphere octyl column of equal dimension and particle size, a Waters M 490 variable wavelength detector, and a Bioanalytical System LC 4B amperometric detector.

Assay for Measuring Lipid Peroxidation The thiobarbituric acid test was used according to Ref 24.

Bioenergetic Parameters Transmembrane pH and electrical gradients of *Synechococcus* and mitochondria were calculated from the distribution ratios of a weak acid/base or a membrane permeable ion between the cell (or mitochondrial) interior and the medium, respectively [25]. The distribution ratio and the internal volume/external volume ratio have to be known for such calculations. These ratios were determined by electron paramagnetic resonance spectroscopy using nitroxide spin probes, by comparing the total signal of the whole sample with the internal signal [26]. To obtain the internal signal, the external signal was quenched by addition of the paramagnetic broadening agent nickel [26]. The following nitroxides were used: for volume determination PROXAD (uncharged), for pH determination PROXCA (weak acid, $pK_a = 3.9$) and PROXAM (weak base, $pK_a = 7.45$). For measurement of the electrical potential KΦ was used [27]. The external pH was measured with an Orion combination pH electrode 91 – 1 (Orion, Boston, MA), connected to a Corning pH meter 130. Under the experimental conditions anthralin did not significantly reduce the nitroxide spin probes.

pH and Volume Measurements Mitochondrial suspensions were kept on ice. Anthralin and CCCP were dissolved in dimethylsulfoxide, added to mitochondria in respiratory state 4 (α-ketoglutarate, pyruvate, malate, β-hydroxybutyrate, each 2.5 mM), and incubated at 25°C for 1 min. Controls were treated with dimethylsulfoxide only. Subsequently, PROXCA or PROXAD were added to a final concentration of 100 μM, KΦ to 60 μM. A 50-μl sample was then transferred into a gas-permeable tubing and rapidly placed into the EPR cavity. For determination of internal signals, Ni TEPA was added as a quencher at a final concentration of 150 mM.

To ensure viability of cyanobacteria during the entire period of the measurements cells were kept in 250-μl aliquots (in medium C + 50 mM Hepes/Bis-tris-propane buffer, pH 7.0) under illumination (50 W/m²) at 30°C in a water bath. Vials were sealed to avoid evaporation. Two-hundred-microliter aliquots of this suspension were stirred in the dark in a covered vessel under a stream of nitrogen or oxygen for 5 min at 25°C to achieve dark anaerobic or aerobic conditions. Cells were incubated for 1 min with anthralin (100 μM), freshly dissolved in dimethylsulfoxide or dimethylsulfoxide only (controls). This was followed by the addition of the nitroxide PROXCA, PROXAM, or PROXAD at a final concentration of 250 μM. Fifty microliters of the sample was then rapidly transferred into gas-permeable tubing and inserted into the EPR cavity. The cavity was flushed in the dark with either nitrogen or oxygen. Gas-permeable Teflon tubing was purchased from Zeuss Industrial Products, Inc. (Raritan, NJ) (0.032 inch inner diameter, 0.005 inch wall thickness).

Electron Paramagnetic Resonance (EPR) Spectroscopy First derivative EPR spectra were obtained at 25°C with a Varian E-109E spectrometer (X-band), operating at 100 kHz modulation frequency, 1.0 Gauss modulation, 10 mW microwave power, scan range of 20 Gauss, scan time of 4 min, and time constant of 64 ms. Because nitroxides are reduced to EPR silent hydroxylamines in

mitochondria and cyanobacteria, the highfield peak of the spectrum of both the total and the internal signal was continuously recorded to monitor the kinetics of the nitroxide reduction. The highfield peak was chosen as an indicator of the amount of nitroxide present because from the three peaks of the nitroxide spectrum it allows the best distinction between the free water signal and the membrane bound signal [28]. When necessary, the height of the internal signal was corrected graphically for an unquenched or membrane bound-signal. To obtain the true accumulation of the spin probes, signal heights were reextrapolated to the time point of addition to the sample.

Computation Concentration ratios were calculated by dividing the distribution ratio, $M_{internal}/M_{external}$, of acid, amine, and cation by the volume ratio, $V_{internal}/V_{external}$. Both ratios are given by the formula below, where V is the sample volume, H the signal height, G the gain in absence of quencher (−Q) and in presence of quencher (+Q):

$$\frac{M \text{ or } V_{internal}}{M \text{ or } V_{external}} = \frac{H_{+Q}}{H_{-Q}\left(\frac{G_{+Q} \cdot V_{-Q}}{G_{-Q} \cdot V_{+Q}}\right) - H_{+Q}}.$$

ΔpH (chemical potential) and $\Delta\psi$ (electrical potential) across the mitochondrial membrane were computed from the concentration ratios using well-established formulae [25]. For calculating the pH gradients across membranes in cyanobacteria it was necessary to measure both the distribution of an acid and an amine to solve the problem of two internal compartments in these organisms [29]. Formulae for this case have been derived [30]. A cytoplasmic volume of 90% of the total cell volume [31] was used throughout all calculations.

RESULTS

Rat Liver Mitochondria At an external pH of 6.78 the mitochondrial proton chemical potential (ΔpH) in respiratory state 4 is determined as a pH difference of −0.15 units or +9 mV. The electrical potential ($\Delta\psi$) is −109 mV. This corresponds to a proton motive force of −100 mV. After addition of the uncoupler CCCP the mitochondrial matrix becomes more acidic (ΔpH increases to −0.32 units) and more positive ($\Delta\psi$ decreases to −9 mV). Therefore, the mitochondria are de-energized by the protonophore, as indicated by a nearly abolished proton motive force of +10 mV. Conversely, with anthralin the matrix becomes more alkaline (ΔpH is +0.03 units), and $\Delta\psi$ is increased to −135 mV. Energization is therefore enhanced as demonstrated by a stronger proton motive force of −137 mV (Table I). Anthralin is a strong inhibitor of mitochondrial respiration in metabolic state 3 (substrate and ADP present). Respiration in metabolic state 4 (substrate, no ADP) is only slightly stimulated in a cyanide-insensitive manner (Fig 1). Stimulation of mitochondrial oxygen consumption by the uncoupler CCCP is abolished by anthralin if it is given before or after addition of CCCP in the presence of NAD- and FAD-dependent substrates (Table II) (data not shown for FAD dependent substrate). The redox state of mitochondrial ubiquinol/ubiquinone couples was investigated in the presence of increasing anthralin concentrations. Anthralin caused a significant reduction of mitochondrial ubiquinone 9 and 10 to the corresponding ubiquinols (Table III). Membrane concentrations of the lipophilic antioxidant α-tocopherol remained unchanged (Table III), and thiobarbituric acid reactive products, indicative of lipid peroxidation, were not formed under these conditions (data not shown). Mitochondrial ATP content is significantly reduced after incubation with anthralin (Fig 2).

Synechococcus During dark aerobiosis and anaerobiosis the pH gradient across the thylakoid membrane is about 1.0 pH unit and between 0.2–0.5 pH units across the cytoplasmic membrane (Table I). Anthralin increases the proton gradients of dark anaerobic *Synechococcus,* resulting in an increase of 0.65 pH units across the thylakoid membrane and 0.20 pH units across the cytoplasmic membrane (Table I). In dark aerobic cells, the increase in only 0.3 pH units for the thylakoid and 0.13 pH units for the cytoplasmic membrane. Because of the very slow diffusion of KΦ into the cells it was not possible to calculate electrical potentials in *Synechococcus.* Oxygen uptake in the dark is stimulated by anthralin (Table II). This stimulation is saturated at 100 μM anthralin. Both the cyanide sensitive and insensitive oxygen uptake are stimulated and further increased by addition to saturating concentrations (100 μM) of the uncoupler CCCP. Identical respiratory rates were observed with CCCP in the absence or presence of anthralin. In the absence of intact cells no oxygen uptake was observed after addition of anthralin to the buffer. After addition of 100 μM anthralin to dark aerobic cells at ATP level decreased from 5.8 to 3.8 nmol ATP/mg protein (Fig 2).

Table I. Effects of Anthralin on Mitochondrial[a] and Cyanobacterial[b] Bioenergetic Parameters

Mitochondria	pH (internal)	ΔpH (internal-external)	ΔpH (internal-external) (mV)	Δψ (internal-external) (mV)	pmf (mV)
Control	6.78 ±0.08	−0.15 ±0.07	9 ±4	−109 ±8	−100 ±4
CCCP	6.61 ±0.06	−0.32 ±0.07	19 ±4	−9 ±4	10 ±0
Anthralin	6.96 ±0.06	0.03 ±0.01	−2 ±1	−135 ±7	−137 ±5

Cyanobacteria	pH (cytosol)	pH (thylakoid)	pH (external)	ΔpH (cytosol-thylakoid)	ΔpH (cytosol-external)
Aerobic-control	7.02 ±0.04	6.07 ±0.08	6.83 ±0.01	0.95	0.19
+Anthralin	7.15 ±0.03	5.90 ±0.04	6.83 ±0.01	1.25	0.32
Anaerobic-control	7.13 ±0.03	6.23 ±0.06	6.83 ±0.01	0.90	0.30
+Anthralin	7.33 ±0.05	5.78 ±0.02	6.83 ±0.01	1.55	0.50

[a] *Mitochondria:* pmf (proton motive force) = ΔΨ (internal-external) − 2.3 RT ΔpH (internal-external)/F. R, T, and F are the gas constant, absolute temperature and Faraday constant, respectively (2.3 RT/F = 59 mV at 25° C). The external pH was 6.93 ± 0.02, [CCCP] = 1 μM, [anthralin] = 100 μM. Mean values ± standard deviation, n = 3.

[b] *Cyanobacteria:* ΔpH (cytosol-external) is the gradient across the plasma membrane, ΔpH (cytosol-thylakoid) is the gradient across the thylakoid membrane; [anthralin] = 100 μM. Mean values ± standard deviation, n = 4. Anthralin caused a significant ($p < 0.01$) increase in the proton motive force in mitochondria and an increase ($p < 0.01$) in cyanobacterial proton gradients.

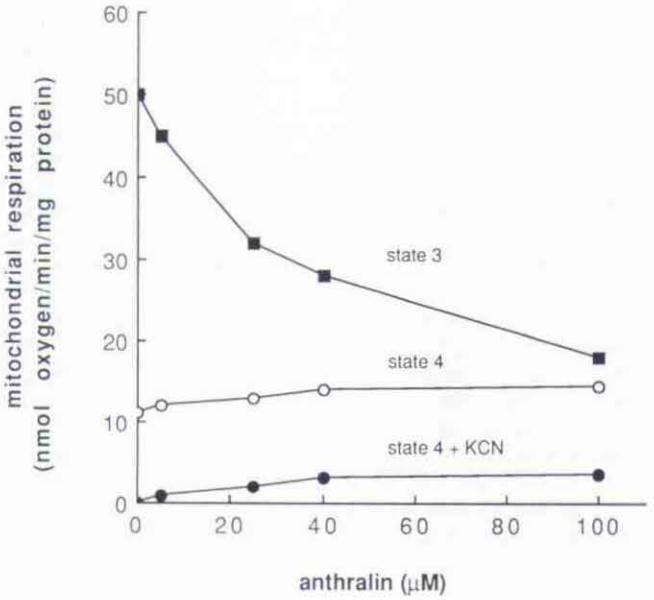

Figure 1. The effect of anthralin on mitochondrial respiration: 10 mM substrate (malate, pyruvate, α-ketoglutarate, β-hydroxybutyrate, each 2.5 mM), 1.2 mg mitochondrial protein, sucrose 250 mM, Tris-HCl 10 mM, potassium dihydrogenphosphate 10 mM, pH 7.0, 37°C. State 3 respiration was induced by addition of 600 μM ADP. Representative data are shown, n = 3 to 4.

Human Keratinocytes Incubation of human keratinocytes with 10 μM anthralin results in a significant decrease of cellular ATP content (Fig 2) and inhibition of cyanide-sensitive respiration (Table II). Oxygen uptake of keratinocytes is stimulated by the uncoupler of oxidative phosphorylation FCCP and diminished by the electron transport chain inhibitor cyanide. FCCP-stimulated oxygen uptake of keratinocytes is abolished by pretreatment of the cells with anthralin, and in cyanide-inhibited keratinocytes anthralin causes a small increase in oxygen uptake (Table II).

DISCUSSION

Mechanism of Anthralin Action in Mitochondria

Proton motive force and membrane redox components It is unequivocally shown that mitochondria are energized by anthralin, as indicated by the increase of the proton motive force. Conversely, the protonophoric uncoupler CCCP nearly abolishes the mitochondrial proton motive force (Table I). This finding is in agreement with previous studies [12] that indicate that anthralin is not an uncoupler of oxidative phosphorylation. Anthralin reduces mitochondrial ubiquinones to the corresponding ubiquinols (Table III). Ubiquinol/one redox cycles participate in mitochondrial proton translocation [32]. Low-temperature EPR (electron paramagnetic resonance) studies have also revealed significant differences between the oxidation state of redox components (iron-sulfur clusters) in anthralin-treated mitochondria as compared to control mitochondria (Fuchs, to be published), showing these centers to be in a more reduced state. The induced changes in the redox state of mitochondrial ubiquinol/ubiquinone and iron sulfur clusters might be linked to the observed increase in mitochondrial proton motive force.

Respiration Respiration during oxidative phosphorylation (respiratory state 3) and uncoupler-stimulated respiration are inhibited by anthralin (Fig 1, Table II). Because both effects are observed in the presence of NAD- and FAD-dependent substrates, the inhibition site is located between the ubiquinone pool and the terminal cytochrome oxidase. Cyanide insensitive oxygen consumption is slightly increased. No thiobarbituric acid reactive products were formed, and the lipid-soluble, chain breaking antioxidant α-tocopherol was not decreased in anthralin-treated mitochondria. This indicates the absence of lipid peroxidation. It is suggested that in mitochondria anthralin may produce reactive oxygen species by catalyzed reaction either directly with oxygen [33,34] or indirectly via reduction of mitochondrial redox components to autoxidizable products (e.g., ubisemiquinones) [35]. No significant oxygen consumption by anthralin was measured in the absence of mitochondria. Superoxide dismutase and catalase did not affect anthralin-induced stimulation of cyanide-insensitive respiration in mitochondria (data not shown). This may be due to limited access of the enzymes to hydrophobic membrane compartments in which the lipophilic anthralin is accumulated. Furthermore, anthralin is also reported to inhibit superoxide dismutase and catalase [36].

ATPase Activity Although the mitochondrial proton motive force is increased by anthralin, ATP synthesis decreases [12]. Reconstituted, oligomycin-sensitive F_0F_1-ATPase and ATPase activity in intact mitochondria is not influenced by the drug in the absence of an uncoupler [12], but in this case only a small portion of the total (latent) activity is revealed. However, uncoupler stimulated F_0F_1-ATPase activity is inhibited by anthralin, indicating an oligomycin-like effect (Fuchs, to be published).

Mechanism of Anthralin Action in *Synechococcus*

Respiration Anthralin causes an increase of oxygen uptake in *Synechococcus*. Cell-independent autoxidation of anthralin can be excluded, because stimulation of oxygen uptake is saturated at about 90 μM and no significant oxygen consumption by anthralin was

Table II. Effects of Anthralin in Uncoupler-Stimulated and Cyanide-Inhibited Oxygen Consumption in Cyanobacteria,[a] Mitochondria,[b] and Keratinocytes[c]

Membrane source	Additions	Oxygen consumption[d]
cyanobacteria	1. none	1.64 ± 0.14
	2. 100 μM anthralin	5.14 ± 0.54
	3. 100 μM anthralin 100 μM CCCP	9.46 ± 1.20
	4. 100 μM CCCP	9.50 ± 1.25
	5. 1 mM KCN	0.23 ± 0.05
	6. 1 mM KCN 100 μM anthralin	0.90 ± 0.25
	7. 1 mM KCN 100 μM anthralin 100 μM CCCP	3.39 ± 0.37
mitochondria	1. none	15.3 ± 4.2
	2. 50 μM anthralin	18.4 ± 5.3
	3. 0.5 μM CCCP	68.6 ± 12.7
	4. 50 μM anthralin 0.5 μM CCCP	13.8 ± 4.5
	5. 0.5 μM CCCP 50 μM anthralin	16.7 ± 6.3
keratinocytes	1. none	22.0 ± 2.0
	2. 10 μM anthralin	16.0 ± 3.0
	3. 2 μM FCCP	39.0 ± 6.0
	4. 10 μM anthralin 2 μM FCCP	27.0 ± 4.0
	5. 1 mM KCN	0
	6. 10 μM anthralin 1 mM KCN	2.0

[a] *Synechococcus:* 6.8 mg protein/ml, incubation time 2 min, 50 mM Hepes/Bis-tris propane buffer, pH 7.0, at 25° C, dark aerobiosis; means ± standard deviation, n = 4 to 6.

[b] *Mitochondria:* 1.2 mg protein, incubation time 2 min, sucrose 250 mM, Tris-HCl 10 mM, potassium dihydrogenphosphate 10 mM, pH 7.0 at 37° C. Metabolic state is 4: 10 mM substrate (malate, pyruvate, α-ketoglutarate, β-hydroxybutyrate, each 2.5 mM); means ± standard deviation, n = 4.

[c] *Keratinocytes:* 1.3×10^7 cells/ml Dulbecos modified Eagle medium, incubation time 45 min, 35° C; means ± standard deviation, n = 3 to 4. Inhibition of basal and uncoupler-stimulated respiration of keratinocytes by anthralin is significant, $p < 0.01$.

[d] Oxygen uptake is in natoms/min/mg protein for cyanobacteria and mitochondria, and in nmole/min/10^7 cells for keratinocytes.

Table III. Mitochondrial Ubiquinol/Ubiquinone Content and Tocopherol Concentration in the Presence of Anthralin[a]

Anthralin	Ubiquinone 9	Ubiquinol 9	Total ubiquinone 9	Ubiquinone 10	Ubiquinol 10	Total ubiquinone 10	Tocopherol
0	0.45	0.60	1.05	0.089	0.022	0.111	0.16
	±0.04	±0.08	±0.11	±0.018	±0.02	±0.033	±0.03
2.5	0.39	0.79	1.18	0.057	0.041	0.098	0.14
	±0.05	±0.18	±0.15	±0.014	±0.024	±0.025	±0.03
25	0.23	0.92	1.15	0.035	0.063	0.098	0.15
	±0.03	±0.05	±0.08	±0.008	±0.015	±0.022	±0.02
250	0.08	1.05	1.13	0.025	0.082	0.107	0.14
	±0.03	±0.05	±0.07	±0.014	±0.015	±0.23	±0.02

[a] All concentrations are nmol/mg protein. Liver mitochondria were incubated with increasing concentrations of anthralin for 5 min at 25° C in a buffer used for measuring oxygen consumption. Subsequently, mitochondrial suspensions were analyzed simultaneously for α-tocopherol and ubiquinol/one content. Values are means ± standard deviation, n = 4. Decrease in ubiquinone and increase in ubiquinol content is significant at 25 nmol anthralin/mg protein, $p < 0.01$.

measured in the absence of cyanobacteria. Anthralin-induced increase of cyanide sensitive and insensitive oxygen uptake is associated with membranes as demonstrated by its stimulation with CCCP. Cyanobacterial respiration is reduced about 80% by KCN, which represents inhibition of the terminal cytochrome aa3 [15,16]. An autoxidizable iron-sulfur cluster was suggested to be responsible for the cyanide insensitive part of oxygen uptake [15]. Because branching of both electron pathways occurs at the level of the quinone pool [37], anthralin must feed electrons into the respiratory chain before the quinone pool. Because the respiratory rates with CCCP in the absence and presence of anthralin are identical, any inhibitory effect on the respiratory chain can be excluded. The different composition of the mitochondrial and cyanobacterial electron transport chain may be responsible for the antirespiratory effect in mitochondria (in the presence of ADP) and the prorespiratory effect in cyanobacteria (in the presence of endogenous ADP).

Proton Gradients and ATP Synthesis Anthralin increases the proton gradients across the cytoplasmic and thylakoid membranes in *Synechococcus*. Thus, anthralin does not act as an uncoupler but as an electron donor for the respiratory electron transport chain. Although anthralin causes an increase in ΔpH, the ATP level decreases. We suggest that the cyanobacterial A_0A_1-ATPase is the most likely target of inhibition of ATP synthesis.

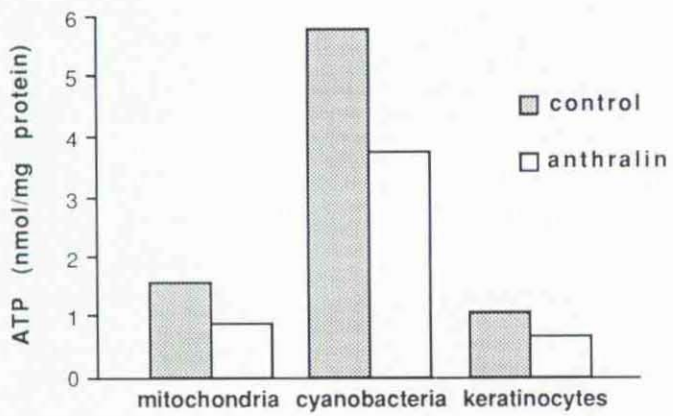

Figure 2. The effect of anthralin on ATP content of cyanobacteria, mitochondria and keratinocytes. *Synechococcus:* 6.8 mg protein/ml, 50 mM Hepes/Bis-tris propane buffer, pH 7.0, at 25°C, dark aerobiosis, anthralin 100 μM, means, n = 4. Mitochondria: 1.2 mg protein/ml incubation medium, 37°C, 10 mM substrate (malate, pyruvate, α-ketoglutarate, β-hydroxybutyrate, each 2.5 mM); anthralin 50 μM, means, n = 4. Keratinocytes: 1.3×10^7 cells/ml Dulbeco's modified Eagle's medium, incubation time 30 min, 35°C; anthralin 10 μM, means n = 3 to 4. ATP content of anthralin-treated preparations was different from that of controls, $p < 0.01$; standard deviation was less than 5%.

Anthralin and Keratinocytes We believe that the action of anthralin on keratinocyte suspensions occurs solely through its effect on mitochondria, because the data obtained from these whole cells are in agreement with the results obtained using isolated mitochondria (Figs 1 and 2, Table II) in the following ways: a) Cyanide sensitive oxygen uptake of keratinocytes is inhibited by anthralin, and consequently ATP content of keratinocytes declines; b) anthralin-induced stimulation of oxygen uptake in cyanide-inhibited keratinocytes may be due to the formation of reactive oxygen species; c) uncoupler-induced respiration is diminished by anthralin, indicating inhibition of the electron transport chain. Therefore it is suggested that the interaction of anthralin with energy-transducing membranes, such as the inner mitochondrial membrane, also occurs in keratinocytes. This is in accordance with microspectrofluorometric investigations in cultured fibroblasts, which revealed that anthralin causes striking structural alterations of fibroblast mitochondria [38].

Mechanism of Action of Anthralin: General Considerations In isolated cells anthralin is readily converted to the anthralin anion [38], which rapidly autoxidizes. The ultimate reactive species at cellular target sites is not known. Our results show that anthralin acts as an electron donor to membrane-associated redox components and inhibits ATP synthesis in three different biologic systems. ATP depletion will limit energy-dependent metabolism and thereby, e.g., may inhibit cell proliferation. Anthralin-induced changes in the cellular redox state have already been described [38]. Basically, because of the very low redox potential [$E_o' = -0.75$V] [39], anthralin is capable of reducing NAD(P), flavins, quinones, and various components of the electron transport chain. Unna already pointed out in 1916 that the antipsoriatic potency of anthranol compounds did not correlate with their reducing potential [34]. However, this conclusion was drawn from studies in non-biologic systems and did not include kinetic considerations. Krebs and Schaltegger also suggested [40] that the reducing potential of anthranol compounds does not correlate with their antipsoriatic activity. It remains to be determined which pharmacodynamic profile of antipsoriatic drugs can be attributed to their redox properties or free radical generating/modulating potential [41]. It seems plausible that such redox interactions could also occur at other membrane sites, e.g., plasma membranes such as those of keratinocytes and neutrophils. Because the redox status of plasma membranes is an important control mechanism in cell function [42], it is suggested that modulation of membrane redox status may be a common function of anthralin action.

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