Local Protons and Uncoupling of Aerobic and Artificial $\Delta\tilde{\mu}_H$-Driven ATP Synthesis†

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ABSTRACT: Gramicidin D causes inhibition of ATP synthesis either in the absence or in the presence of depression of $\Delta\tilde{\mu}_H$, in low-salt and in high-salt media, respectively, at concentrations 2 orders of magnitude higher in the former with respect to the latter case. When the number of active redox pumps is reduced by increasing the antimycin concentration, the P/O ratio of respiring, gramicidin-treated mitochondria either is slightly increased in low-salt media or is first decreased and then constant in high-salt media. Addition of gramicidin D in low-salt media to mitochondria synthesizing ATP by means of artificially imposed $\Delta\tilde{\mu}_H$ gradients results in (a) no effect on the K^+ efflux ratio ± ADP (equivalent to the aerobic respiratory control ratio) and (b) no effect on the ATP/K^+ ratio (equivalent to the P/O ratio) except at the low gramicidin D concentrations where there is also a slight enhancement of the rate of ATP hydrolysis. During respiration-driven ATP synthesis, addition of valinomycin plus K^+ causes depression of $\Delta\tilde{\mu}_H$ with little inhibition of ATP synthesis while addition of gramicidin D causes inhibition of ATP synthesis with little depression of $\Delta\tilde{\mu}_H$. The view is discussed that the gramicidin-accessible protons which uncouple aerobic ATP synthesis in a $\Delta\tilde{\mu}_H$-independent manner are of a different class from the gramicidin-inaccessible protons which uncouple diffusion potential driven ATP synthesis in a $\Delta\tilde{\mu}_H$-dependent manner. The gramicidin-accessible protons are suggested to be pump associated and to reflect primary events in energy transduction.

In the preceding paper (Luvisetto & Azzone, 1989), we have shown that gramicidin possesses a dual mechanism of action: (a) in high-salt media, uncoupling is paralleled by increased proton conductance and depression of $\Delta\tilde{\mu}_H$,[1] effects reflecting a classical membrane proton cycling mode of action; (b) in low-salt media, uncoupling is accompanied by absence of increase of proton conductance as well as of depression of $\Delta\tilde{\mu}_H$, effects which may be explained on the basis of proton cycling at or near the pump. In the present paper, we take into consideration the effects of gramicidin on ATP synthesis whether driven by respiration or by artificially generated proton electrochemical gradients.

The dependence of the rate of ATP synthesis on the value of $\Delta\tilde{\mu}_H$ and of the inhibition of ATP synthesis upon the decline of $\Delta\tilde{\mu}_H$ are among the keystones of the chemiosmotic hypothesis. It was therefore largely a surprise when it was reported that (a) the value of $\Delta\tilde{\mu}_H$ could be depressed by ion transport considerably, i.e., almost 50 mV, without appreciable inhibition of the rate of ATP synthesis (Zoratti et al., 1982) and (b) some agents were capable of causing a large inhibition of ATP synthesis without appreciable depression of $\Delta\tilde{\mu}_H$ (Rottenberg & Hashimoto, 1986; Luvisetto et al., 1987). The lack of proportionality between ATP synthesis and $\Delta\tilde{\mu}_H$ has been considered to support the concept that delocalized protonic circuits are not the only mechanism for energy transduction between primary and secondary proton pumps (Zoratti et al., 1982; Westerhoff et al., 1984; Rottenberg & Hashimoto, 1986). It has been noted, however, that several of the effects taken to support the *decoupling* concept could also be explained by assuming that some of these agents, such as fatty acids and anesthetics, act as *slip inducers* in the proton pumps (Pietrobon et al., 1981; Luvisetto et al., 1987). Not all the observations can, however, be accounted for by either the decoupling or the slip concept. For example, the increase of the P/O ratio in chloroform-treated mitochondria during titration with antimycin cannot be explained by a decoupling mechanism while the slip concept does not explain the observation of a depression of $\Delta\tilde{\mu}_H$ not followed by a decline of the rate of ATP synthesis (Zoratti et al., 1982).

Artificial $\Delta\tilde{\mu}_H$ gradients either in natural or in artificial membranes have often been used to investigate the mechanism of ATP synthesis. Below, we have tested the effect of gramicidin D on both respiration-driven and artificial $\Delta\tilde{\mu}_H$ gradient-driven ATP synthesis. The fact that in low-salt media gramicidin D uncouples presumably by means of a pump proton cycling mechanism suggests gramicidin D as a tool to assess whether artificial $\Delta\tilde{\mu}_H$ gradient- and respiration-driven ATP syntheses are processes of a similar nature. Some reports suggest that they may not be completely equivalent. Zoratti et al. (1986a) reported that the proton fluxes through the ATPase during artificial $\Delta\tilde{\mu}_H$ gradient-driven ATP synthesis are much lower and with a different $\Delta\tilde{\mu}_H$ dependence than needed to account for aerobic phosphorylation. Rottenberg and Steiner-Mordoch (1986) showed that fatty acids do not inhibit ATP synthesis when driven by artificially imposed $\Delta\tilde{\mu}_H$

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[1] Abbreviations: J_o^{sh}, rate of respiration in static head; J_o^{max}, maximal rate of respiration; J_p, rate of ATP synthesis; J_{ATP}, rate of ATP hydrolysis; J_H^l, proton flux through leaks; $J_K(\pm ADP)$, rate of K^+ efflux in the presence and in the absence of ADP; ATP/K^+, ratio between ATP synthesized and K^+ release; $\Delta\psi$, transmembrane electrical potential gradient; $\Delta\tilde{\mu}_H$, transmembrane proton electrochemical potential gradient; n_e, H^+/e^- stoichiometry; n_p, H^+/ATP stoichiometry; P_i, inorganic phosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate.

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gradients. Below, we will report evidence indicating that in low-salt media the same concentrations of gramicidin D which uncouple aerobic phosphorylation are practically without effect on artificial $\Delta\tilde{\mu}_H$ gradient-driven ATP synthesis.

Materials and Methods

Materials. Rat liver mitochondria were prepared according to standard procedures (Massari et al., 1972), and all the experiments were performed within 4 h of preparation. The mitochondrial protein was assayed with the biuret method using serum albumin as a standard. The composition of the reaction medium is given in the legends of the figures. All the reagents were of maximal purity commercial grade. Inhibitors, valinomycin and gramicidin D, were obtained from Sigma.

Determination of $\Delta\tilde{\mu}_H$ and Rate of Respiration. The transmembrane electrical potential and the rate of respiration were estimated as essentially described in the preceding paper (Luvisetto & Azzone, 1989). The operative conditions and times of incubations with the reagents are described in the legends of the figures.

Determinations of Rates of ATP Hydrolysis and of ATP Synthesis. The rate of ATP hydrolysis, J_{ATP}, was measured spectrophotometrically on an AMINCO DW 2a dual-wavelength spectrophotometer equipped with magnetic stirring and thermostatic control, following continuously the decrease in absorbance at 340 minus 374 nm due to NADH oxidation in the presence of excess phosphoenolpyruvate, piruvate kinase, and lactate dehydrogenase. The rates of ATP hydrolysis were corrected for the contribution of extramitochondrial ATPases, which was determined in control samples in the presence of oligomycin (1 μg/mg of protein) and atractyloside (200 μM). The measurements of ATP hydrolysis in low-salt media were performed in the absence of an enzymatic ATP-regenerating system, to avoid any presence of cation, expecially K^+, and the rate of ATP hydrolysis was estimated by the rate of P_i formation determined by the colorimetric method.

To determine the rate of ATP synthesis, J_p, three 1-mL samples were withdrawn from the suspension within 1 min after addition of ADP and were quenched in $HClO_4$ (5 M). After centrifugation of the denatured protein and neutralization of an aliquot of the supernatant with triethanolamine/KOH, the ATP content of the samples was determined by standard enzymatic methods, following fluorometrically with an Eppendorf spectrofluorometer the reduction of NADP in the presence of hexokinase, glucose, and glucose-6-phosphate dehydrogenase. The rate of ATP synthesis was calculated from linear regression analysis of the ATP concentration vs time plot.

For the determination of the P/O ratio, the rates of respiration in the presence of ADP, $J_o^{st.3}$, and of ATP synthesis were measured in parallel samples under identical conditions.

Results

Effects of Gramicidin D on Oxidative Phosphorylation. Figure 1 shows the effects of gramicidin D on the rate of phosphorylation and on the respiratory rate and membrane potential in the presence of ADP, measured both in low-salt and in high-salt media. It is seen that in low-salt media, increase of the gramicidin D concentration up to 1.5 μg/mg resulted in complete abolition of ATP synthesis with little depression of $\Delta\tilde{\mu}_H$. The rate of respiration was slightly depressed at the lower, and then increased at the higher, concentrations of gramicidin D, parallel to the stimulation of the resting respiratory rate. In the right panels are reported the effects of gramicidin D in high-salt media. There was also

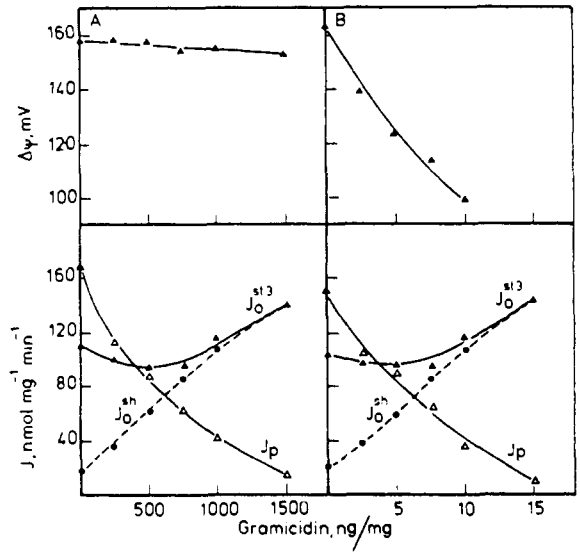

FIGURE 1: (Lower panels) Mitochondrial rate of respiration in static head, J_o^{sh} (●), rate of respiration is state 3, J_o^{st3} (▲), and rate of ATP synthesis, J_p (△) as a function of increasing concentrations of gramicidin in low-salt media (panel A) and in high-salt media (panel B). (Upper panels) Difference of electrical potential across the inner mitochondrial membrane in state 3 as a function of the same gramicidin concentrations. Standard (low-salt) medium composition was 0.2 M sucrose, 10 mM succinate (Tris salt), 30 mM Tris/MOPS, 5 mM P_i/Tris, 1 mM EDTA/Tris, and 5 μM rotenone, pH 7.4, T = 25 °C. In cation-supplemented media, 5 mM KCl was added to the standard medium. After 2 min of incubation of rat liver mitochondria (1 mg of protein/mL), gramicidin was added. After 2 min of incubation, ADP (cyclohexylamine salt, 1 mM) was added, and J_o^{st3} and J_p were measured.

inhibition of ATP synthesis but with two major differences. First, the concentrations of gramicidin D were 2 orders of magnitude lower (Luvisetto & Azzone, 1989). Second, the inhibition of ATP synthesis was accompanied by a marked depression of $\Delta\tilde{\mu}_H$. Also, in high-salt media, there was a slight inhibition of the respiration at lower, and a stimulation at higher, concentrations of gramicidin D.

Titration of the P/O Ratio with Respiratory Inhibitors. A recent paper (Pietrobon et al., 1987) has reported the pattern of the P/O ratio during a titration with respiratory inhibitors as carried out with mitochondria where uncoupling was induced by increasing either the leak or the slip. It was shown that the decrease of the number of active redox pumps was accompanied by a decrease of the P/O ratio only when uncoupling was caused by an increase of the leak but not when uncoupling was due to an increase of the slip. Figure 2 shows a similar experiment as carried out with mitochondria incubated either in low-salt or in high-salt media. It is seen that the decrease of the number of active redox pumps was accompanied by a constant P/O ratio in low-salt media, while it was accompanied first by a decrease and then by a constant P/O ratio in high-salt media. The two behaviors correspond to what has been interpreted as due to the induction first of the slip and then of the leak. It may be argued that the decline of the P/O ratio is less marked in the high-salt media than previously reported in the presence of uncouplers (Pietrobon et al., 1987). However, this may be explained by remembering that the uncoupling by gramicidin D in high-salt media is not due directly to membrane proton cycling but rather to the combination of respiration-induced cation uptake followed by H^+/K^+ exchange. The proton–cation cycling obtained by the combination of the two processes depends on the rate of respiration in that it is reduced in magnitude proportionally to the decrease of the number of active pumps; this leads to a

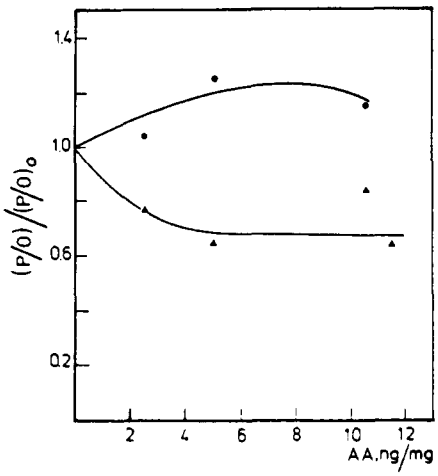

FIGURE 2: Normalized P/O ratio as a function of antimycin concentration in the presence of a constant amount of gramicidin [250 ng/mg (●)] in low-salt media and of gramicidin [5 ng/mg (▲)] in the presence of 5 mM KCl. Medium as in Figure 1. Rat liver mitochondria (1 mg/mL) were incubated for 5 min with increasing concentrations of antimycin and for 2 min with gramicidin; then ADP (1 mM) was added, and the rate of respiration and the rate of ATP synthesis were m easured in parallel samples. $(P/O)_0$ is the value of J_p/J_o in the absence of antimycin. $(P/O)_0$ was, respectively, 1.5 in low-salt media and 1.32 in high-salt media.

decrease of the extent of uncoupling proportionally to the inhibition of the rate of respiration and thus to a more limited lowering of the P/O ratio at the higher antimycin concentrations.

Uncoupling Effect of Gramicidin D on Artificial $\Delta\tilde{\mu}_H$ and Respiration-Driven ATP Syntheses. The phenomenon of the decrease of the respiratory control ratio (RCR) upon addition of uncoupling agents is known since the first studies on oxidative phosphorylation (Chance & Williams, 1958). More recently, Rossi and Azzone (1970) reported that addition of ADP to respiratory-inhibited valinomycin-treated mitochondria where K^+ was flowing down a concentration gradient resulted in a large stimulation of the rate of K^+ efflux. The acceleration of K^+ efflux was shown to be due to the artificial gradient-driven ATP synthesis. The effect may then be considered as equivalent to the ADP-induced stimulation of the respiration since in both cases it depends on the utilization of $\Delta\tilde{\mu}_H$, or of some other energy source, to form ATP. The artificial $\Delta\tilde{\mu}_H$ gradient-driven ATP synthesis lends itself, therefore, as a model to analyze the mechanism of gramicidin D uncoupling under conditions where the uncoupling is due to pump proton cycling. Figure 3 shows an experiment where the effect of gramicidin D on the respiratory control ratio is compared with that of gramicidin D on the rate of K^+ efflux in the absence and presence of ADP (*K^+ efflux control ratio*). Both effects were measured in low-salt media. It is seen that addition of gramicidin D caused a marked decline on the respiratory control ratio while it had almost no effect on the K^+ efflux control ratio. The negligible effect of gramicidin D on the K^+ efflux ratio is a consequence of the fact that neither the K^+ efflux in the absence of ADP [see also Figure 2 of Luvisetto and Azzone (1989)] nor the K^+ efflux in the presence of ADP is appreciably modified by gramicidin. The experiment thus indicates that also the mechanism by which the rate of K^+ efflux is enhanced by ADP during ATP synthesis, in respiratory-inhibited mitochondria, is not modified by the presence of gramicidin D.

Rossi and Azzone (1970) also reported that during the ADP-induced stimulation of the rate of K^+ efflux there was a large synthesis of ATP. Figure 4 shows that addition of

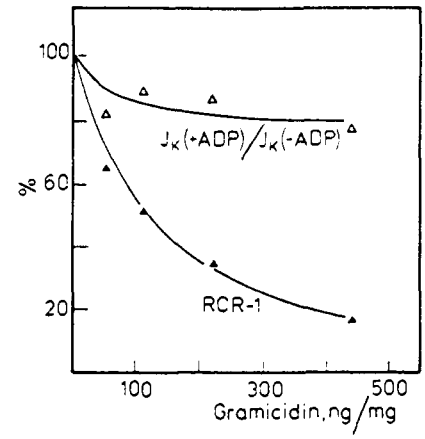

FIGURE 3: Comparison between percent of rate of K^+ efflux, in the presence and in the absence of ADP, and of respiratory control ratio as a function of increasing concentrations of gramicidin in low-salt media. RCR was determined by the ratio J_o^{st3}/J_o^{sb}. Rate of K^+ efflux was determined essentially as described in Zoratti et al. (1986b) by measuring the initial rate of K^+ efflux immediately after addition of valinomycin to antimycin-inhibited mitochondria. In the absence of gramicidin, RCR and J_K(+ADP)/J_K(−ADP) were, respectively, 5.57 and 4.98.

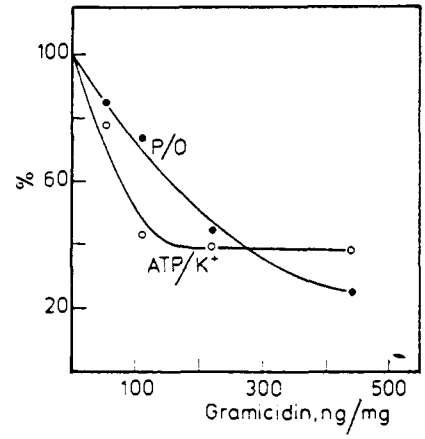

FIGURE 4: Comparison between percent of P/O and ATP/K^+ ratios as a function of increasing gramicidin concentrations in low-salt media. P/O ratio was determined as described in the legend of Figure 2. ATP/K^+ ratio was determined by measuring the ATP formation and K^+ release from valinomycin-treated, antimycin-inhibited mitochondria.

increasing concentrations of gramicidin D had a biphasic effect on the K^+-driven ATP synthesis. The results have been expressed in terms of the ATP/K^+ ratio which may be considered as equivalent to the P/O ratio of respiring mitochondria. Below 100 ng of gramicidin/mg of protein, there was an inhibition of about 50% of the number of moles of ATP synthesized per mole of K^+ released from the mitochondria. Above 100 ng/mg, there was practically no inhibition of the ATP/K^+ ratio. This biphasic effect may be compared with the effect of gramicidin D on the P/O ratio in respiring mitochondria. It is seen that the effect of gramicidin D was not biphasic and reached an extent of inhibition which was much larger than that observed on the artificial $\Delta\tilde{\mu}_H$ gradient-driven ATP synthesis. In practice, gramicidin D was able to cause complete inhibition of the respiration-driven but not of the artificial $\Delta\tilde{\mu}_H$ gradient-driven ATP synthesis.

The results of Figure 4 may be considered in contradiction with those of Figure 3 where gramicidin D had almost no effect on the K^+ efflux ratio ±ADP. The discrepancy receives an explanation from the data of Figure 5 which show the effect of gramicidin D on the rate of ATP hydrolysis as measured

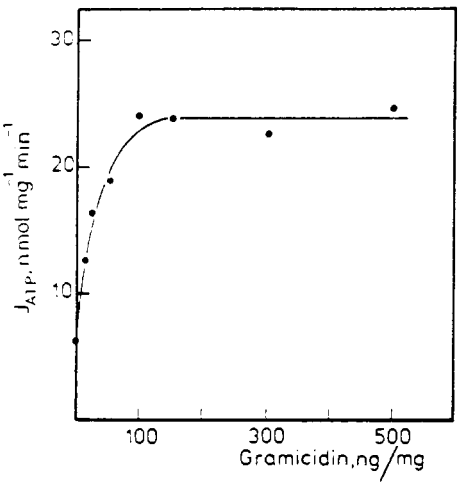

FIGURE 5: Rate of ATP hydrolysis as function of increasing concentrations of gramicidin in low-salt media. Medium composition was 0.2 M sucrose, 30 mM Tris/MOPS, 1 mM EGTA/Tris, and 2 mM $MgCl_2$. ATP-regenerating enzymatic system was not included in the medium. The rate of ATP hydrolysis was determined by measuring with colorimetric standard methods the formation of inorganic phosphate.

in low-salt media. It is seen that low gramicidin D concentrations induced an enhancement of the ATPase activity which, however, reached a maximum below 100 ng/mg. Above this gramicidin D concentration, there was no further increase of the rate of ATP hydrolysis. The rate of ATP hydrolysis shown in Figure 5 was low because the assay was carried out in the absence of an ATP-regenerating system due to the necessity of avoiding the presence of cations in the medium. The experiment of Figure 5 indicates that gramicidin D is capable of stimulating the ATPase proton pump in a manner which is not very dissimilar from that occurring with the redox proton pumps. However, while in the case of the redox proton pumps the stimulation by gramicidin D increases proportionally to the gramicidin D concentration to reach very high values, in the case of the ATPase proton pumps the stimulation by gramicidin D reaches a limit at low rates of ATP hydrolysis. The slight enhancement of the ATP hydrolysis by gramicidin may explain the decrease of the ATP/K^+ ratio caused by low gramicidin concentrations. That the decrease of the ATP/K^+ ratio may be due to slight enhancement of the ATP hydrolysis is also supported by the fact that low gramicidin concentrations inhibited more the anaerobic than the aerobic system. Clearly, the consequences of inducing a slight ATPase activity are larger the lower the rate of ATP synthesis. This is the case with the anaerobic with respect to the aerobic system.

Relationship between $\Delta\tilde{\mu}_H$ and Aerobic Phosphorylation. Figure 6 shows the relationship between the rate of aerobic ATP synthesis and $\Delta\tilde{\mu}_H$ as studied in the presence of increasing concentrations of either only gramicidin D or both gramicidin D and K^+. It is seen that at the lowest K^+ concentrations, i.e., 1 mM, the inhibition of ATP synthesis occurred in the presence of a very small depression of $\Delta\tilde{\mu}_H$, in accord with Figure 1. However, with the increase of the K^+ concentrations, there was a gradual increase of the extent of depression of $\Delta\tilde{\mu}_H$. By connecting with dashed lines the points obtained at the same gramicidin D concentrations, a network tends to become apparent with the vertical lines uniting the points at increasing gramicidin D concentrations and the horizontal lines uniting the points at increasing K^+ concentrations. This would seem to suggest that the effect of increasing the K^+ concentrations is mainly of depressing the level of $\Delta\tilde{\mu}_H$ while that of increasing the gramicidin D concentrations is mainly that of depressing the rate of ATP synthesis.

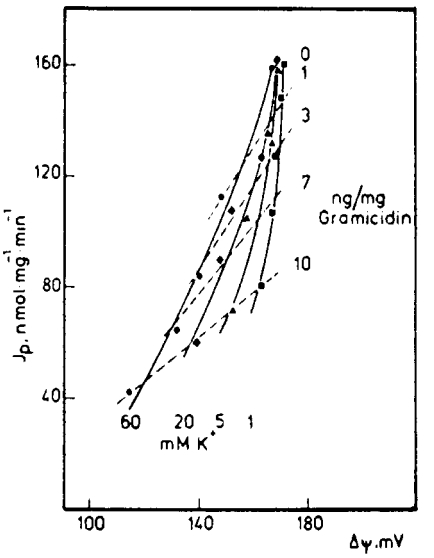

FIGURE 6: Flow–force relationships between J_p and $\Delta\psi$ as obtained in the presence of different constant amounts of gramicidin and of different constant amounts of K^+ in the high-salt media. The compositon of standard medium was the same as described in the legend of Figure 1. Different stationary states are obtained by increasing concentrations of gramicidin (0, 1, 3, 7, and 10 ng/mg) in various K^+-supplemented media (1, 5, 20, and 60 mM). The phosphorylation was initiated by addition of mitochondria (1 mg/mL) to the medium containing all the reagents. After 1 min of incubation, the rate of ATP synthesis and the transmembrane electrical potential were determined.

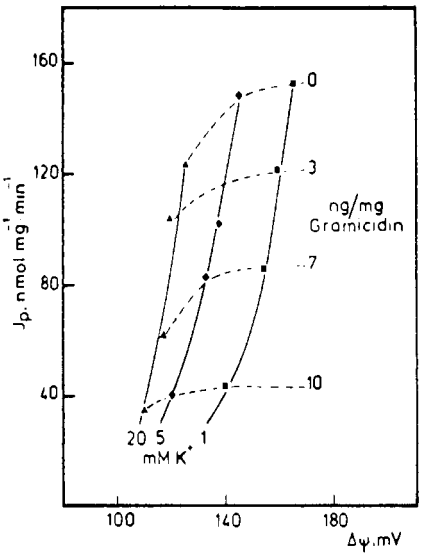

FIGURE 7: Flow–force relationships between J_p and $\Delta\psi$ in the presence of increasing concentrations of gramicidin, in different K^+-supplemented media, as obtained by valinomycin-treated mitochondria. The experimental procedure was as described in the legend of Figure 6. Valinomycin was 23 pmol/mg of protein.

In Figure 7 is reported an experiment similar to that of Figure 6 but carried out in the presence of both valinomycin and gramicidin D. The rationale of the experiment is that if depression of $\Delta\tilde{\mu}_H$ and inhibition of ATP synthesis are two independent effects, by a suitable choice of agents acting either on one or on the other parameter, it should be possible to inhibit one without the other. The result should be a network similar to that of Figure 6 but with almost horizontal lines. To realize this experiment under ideal conditions, it would be necessary to depress $\Delta\tilde{\mu}_H$ without necessarily adding ions to the incubation medium, i.e., to use a substance which causes a depression of $\Delta\tilde{\mu}_H$ independently of any ion transport. Such

a substance is not available. On the other hand, an ionophore such as valinomycin is available which causes a depression of $\Delta\tilde{\mu}_H$ proportionally to the rate of K^+ transport and leads to a K^+ distribution at electrochemical equilibrium. It is, however, possible to obtain in the presence of very low valinomycin concentrations a steady state of continuous K^+ cycling across the membrane and of constant but depressed $\Delta\tilde{\mu}_H$. The steady state is due to the fact that K^+ is extruded though the H^+/K^+ antiporter at a rate which is equal to that of K^+ influx. Under these conditions, the rate of respiratory stimulation and the extent of $\Delta\tilde{\mu}_H$ depression are porportional to the rate of K^+ cycling.

Figure 7 shows that increase of the K^+ concentration in the medium caused a depression of $\Delta\tilde{\mu}_H$ in mitochondria treated with a low valinomycin concentration. However, in accord with Zoratti et al. (1982), the depression of $\Delta\tilde{\mu}_H$ was accompanied by only a very limited inhibition of ATP synthesis and only when the depression was larger than 20–30 mV. It is important that the sum of the K^+ transport induced and ATP synthesis induced stimulations of the respiration be not higher than the maximal rate of respiration in order to allow the two processes of ion transport and of ATP synthesis to proceed in parallel. Figure 7 shows also that when the valinomycin-treated, K^+-supplemented, mitochondria were supplemented with increasing gramicidin concentrations, there was, in accord with Figure 6, a marked inhibition of ATP synthesis with a relatively little change of $\Delta\tilde{\mu}_H$.

The values of $\Delta\tilde{\mu}_H$ reported particularly in Figures 6 and 7 are presumably largely overestimated. This is due to two facts. First, no correction has been included for $TPMP^+$ binding, a correction which becomes more relevant at low values of $\Delta\tilde{\mu}_H$. Second, no correction has been introduced for the increase of matrix volume due to K^+ uptake. It may be easily calculated that such a correction would lower the value of $\Delta\tilde{\mu}_H$ considerably, particularly in the low range of values. By taking into account these two facts, the insensitivity of ATP synthesis to the $\Delta\tilde{\mu}_H$ depression becomes much larger. The result of a $\Delta\tilde{\mu}_H$ depression without inhibition of ATP synthesis and of an ATP inhibition without $\Delta\tilde{\mu}_H$ depression is a network where the vertical lines are a function of the gramicidin D concentration and the horizontal lines a function of the K^+ concentration. The experiment of Figure 7 strongly supports the concept that the rate of ATP synthesis is not a straight function of the level of $\Delta\tilde{\mu}_H$ and that the depression of $\Delta\tilde{\mu}_H$ does not necessarily lead to a proportional inhibition of ATP synthesis.

Discussion

The mechanism of action of the agents, capable of *uncoupling* the $\Delta\tilde{\mu}_H$-controlled respiratory rate in a manner which is not membrane proton cycling, has been attributed either to *decoupling* or to *intrinsic uncoupling* (Rottenberg, 1983; Rottenberg & Hashimoto, 1986; Pietrobon et al., 1981, 1987; Luvisetto et al., 1987).

An effect of gramicidin D on proton-involving reactions in chloroplasts has been repeatedly observed [cf. Theg and Junge (1983) and Opanasenko et al. (1985)] and tentatively attributed to interaction of gramicidin D with photosystem 2a. The presence of proton-buffering domains not in equilibrium with the intrathylakoid bulk phase has already been suggested [Prochaska & Dilley, 1978a,b; Theg & Homann, 1982; Theg & Junge, 1983; Laszlo et al., 1984; cf. also Westerhoff et al. (1984)]. The $\Delta\tilde{\mu}_H$-independent uncoupling and the very steep flow–force relationships (Luvisetto & Azzone, 1989) allow the operational definition of a class of gramicidin D accessible protons which are cycling at or near the proton pump. These gramicidin D accessible protons differ from the protons in the bulk aqueous phase, which reflect the membrane proton conductance and the bulk to bulk $\Delta\tilde{\mu}_H$, in that the cycling through the membrane of the gramicidin D inaccessible protons gives rise to the $\Delta\tilde{\mu}_H$-dependent uncoupling and the very smooth flow–force relationships. Accessibility to gramicidin becomes then a distinctive feature of the two types of protons.

Effect of Gramicidin on the P/O Ratio. Under the assumption of completely coupled proton pumps and from the condition of zero net proton flux (Pietrobon et al., 1987), the relationship between rates of phoshorylation and respiration can be derived:

$$J_p/J_e = n_e/n_p - J_H^i/n_pJ_e \quad (1)$$

where J_H^i is the rate of passive proton influx and n_e and n_p are the H^+/e^- and the H^+/ATP stoichiometries, respectively. Equation 1 indicates that the flux ratio J_p/J_e is approximately equal to the ratio of the mechanistic stoichiometries of the two redox and ATPase proton pumps as long as the rate of the dissipative proton flux is low with respect to the rate of proton extrusion via the redox proton pumps. The lower the dissipative proton flux, the more extended is the range in which reduction of the number of active redox proton pumps is accompanied by a nearly constant P/O ratio.

The presence of slips in both the redox and ATPase proton pumps complicates the analysis. In fact, in the presence of intrinsic uncoupling of the redox proton pumps, low antimycin concentrations inhibit mainly the uncoupled electron flow. This leads to a slight increase of the J_p/J_e ratio at low antimycin concentrations. On the other hand, in the presence of intrinsic uncoupling of the ATPase proton, the J_p/J_e ratio tends to remain constant in an extended range due to the very steep dependence of the dissipative proton flux through the ATPase on $\Delta\tilde{\mu}_H$. The very small dependence of the J_p/J_e ratio in native mitochondria upon reduction of the number of active redox proton pumps may then be explained on the basis of a low leak and a small slip in the redox pumps. The constancy of the P/O ratio in gramicidin-treated mitochondria incubated in low-salt media during the antimycin titration is in accord with the conclusion that under this condition there is no increase of passive proton influx and then of proton cycling. On the other hand, the partial decrease of the P/O ratio in gramicidin-treated mitochondria in high-salt media indicates a proton cycling by a more complex mechanism, i.e., combination of the influx of cations into the matrix via gramicidin D with the efflux of cations from the matrix, in exchange with protons, due to activation of H^+/K^+ antiporter during membrane stretching (Bernardi & Azzone, 1983). Upon increasing the antimycin concentration, the lowering of the P/O ratio is less marked with gramicidin D than with protonophoric agents because the gramicidin D mechanism depends on the extent of cation uptake; i.e., the reduction of the number of active pumps causes an inhibition of cation influx and of generation of the proton cycling circuit. Thus, in this system, the extent of proton cycling would depend partly on the concentrations of gramicidin D and univalent cations and partly on the rate of respiration. Higher antimycin concentrations, by decreasing the cation uptake, decrease also the generation of the proton–cation cycling circuit.

Aerobic Phosphorylation and Bulk $\Delta\tilde{\mu}_H$. While it is generally accepted that aerobic phosphorylation requires a minimal level of $\Delta\tilde{\mu}_H$, the quantitative relationships between the rate of ATP synthesis and $\Delta\tilde{\mu}_H$ remain unclear. For example, it has been repeatedly reported that the rate of ATP synthesis is very steeply dependent on $\Delta\tilde{\mu}_H$. Furthermore, the transition from the resting to the phosphorylating state is accompanied

by a $\Delta\tilde{\mu}_H$ depression not larger than 20 mV. Also, titrations of the respiratory rate with hexokinase or of phosphorylating mitochondria with oligomycin or atractyloside yield very steep flow–force relationships. Recently, Slater (1987) has pointed out that such a steep dependence amounts to a virtual gating effect of $\Delta\tilde{\mu}_H$ which may not be easily explained by the present formulation of the chemiosmotic hypothesis. The insensitivity of the rate of ATP synthesis to the depression of $\Delta\tilde{\mu}_H$ as reported previously (Zoratti et al., 1982; Rottenberg, 1983) and confirmed here in Figures 6 and 7 is in sharp contrast with the report of steep flow–force relationships and with the concept that the inhibition of ATP synthesis by uncouplers is uniquely dependent on the depression of bulk $\Delta\tilde{\mu}_H$. In fact, the data of Figures 6 and 7 indicate that depression of $\Delta\tilde{\mu}_H$ and inhibition of ATP synthesis can be split, in that it is possible to obtain depression of $\Delta\tilde{\mu}_H$ without inhibition of ATP synthesis or, *vice versa*, inhibition of ATP synthesis without depression of $\Delta\tilde{\mu}_H$; i.e., by a suitable combination of agents or conditions, any extent of inhibition of ATP synthesis may occur at all extents of depression of $\Delta\tilde{\mu}_H$. This suggests that the overlapping of depression of $\Delta\tilde{\mu}_H$ and inhibition of ATP synthesis may be the result of an effect of the uncoupling agents on both parameters in parallel but not in sequence. That the uncoupling process does not depend primarily on the depression of bulk $\Delta\tilde{\mu}_H$ does not mean that it is independent of proton cycling. On the contrary, the uncoupling effect of gramicidin D, a classical protonophoric agent, indicates that it is the topology of the proton cycling process and not its occurrence which needs to be revised.

Artificial $\Delta\tilde{\mu}_H$ Gradient-Driven ATP Synthesis. Addition of gramicidin D to mitochondria which are synthesizing ATP by means of artificial $\Delta\tilde{\mu}_H$ gradients results in two type of effects: (1) addition of ADP enhances the rate of K^+ efflux about 5-fold; however, the rate of K^+ efflux, whether in the presence or in the absence of ADP, remains almost unchanged after addition of gramicidin D; and (2) addition of gramicidin D results in a biphasic effect on the ATP/K^+ ratio (equivalent to the aerobic P/O ratio)—first a net depression and then a constant ATP/K^+ ratio independently of the presence of gramicidin D.

The first observation is partly equivalent to that reported in the preceding paper (Luvisetto & Azzone, 1989) where gramicidin in low-salt media was unable to stimulate the passive proton influx, because in the absence of ATP synthesis the rate of passive proton influx depends exclusively on the leak. However, in the presence of ADP, the rate of K^+ efflux is determined by the utilization of the proton electrochemical gradient to perform proton-driven ATP synthesis. Thus, the insensitivity of the ratio of K^+ efflux (±ADP) to the addition of gramicidin D indicates that also the rate of utilization of the proton gradient for ATP synthesis, capable of ATP synthesis, is not affected by gramicidin D. Note that addition of the same gramicidin D concentrations to mitochondria catalyzing an aerobic synthesis of ATP causes a marked decline of the respiratory control ratio (RCR); i.e., the energy source for ATP synthesis is uncoupled by gramicidin D when mitochondria generate ATP aerobically.

The second observation concerns the biphasicity of the effect of gramicidin on the diffusion potential driven ATP synthesis. At low gramicidin D concentrations, there is an inhibitory effect which is even higher on the artificial $\Delta\tilde{\mu}_H$-driven than on the respiration-driven ATP synthesis. We attribute this first rapid decline to two facts. First, low gramicidin concentrations cause a sharp enhancement of the rate of ATP hydrolysis which, however, reaches rapidly a limit (Figure 5). Second, the rate of ATP synthesis under aerobic conditions is more than 10 times higher than that under anaerobic conditions and thus much less sensitive to the induction of a limited rate of ATP hydrolysis. Above the gramicidin D disappears completely, and the diffusion potential driven ATP synthesis becomes insensitive to gramicidin D. If we accept that the first phase of inhibition is due to the enhancement of ATP hydrolysis, it appears that the artificial $\Delta\tilde{\mu}_H$-driven ATP synthesis *sensu strictu* is relatively insensitive to gramicidin D. It is highly significant that, in the range of high gramicidin D concentrations, the same concentrations of gramicidin D which are without effect on the ATP/K^+ ratio cause a marked depression of the P/O ratio in aerobic mitochondria. The constancy of the ATP/K^+ ratio at higher gramicidin D concentrations is then in agreement with the results of Figure 3 where gramicidin D is practically without effect on the ratio of K^+ efflux ±ADP.

Pick et al. (1987) have suggested that gramicidin, as well as palmitic acid, interferes with a direct H^+ transfer between specific electron transport and ATPase complexes, a H^+ transfer which provides an alternative coupling mechanism in parallel with bulk to bulk $\Delta\tilde{\mu}_H$. This view was considered to be directly supported by the observation that fatty acids, while inhibiting aerobic phosphorylation, do not interfere with ATP synthesis when driven by artificially imposed $\Delta\tilde{\mu}_H$ in submitochondrial particles (Rottenberg & Steiner-Mordoch, 1986). Strangely, however, fatty acids do inhibit the P_i–ATP exchange, an effect which is attributed to interference with $\Delta\tilde{\mu}_H$ generated by ATP. The question then arises as to why fatty acids interfere with the $\Delta\tilde{\mu}_H$ generated by ATP but not with the ATP generated by $\Delta\tilde{\mu}_H$. The discrepancy has been tentatively explained by assuming that the intrinsic coupling in the ATPase is asymmetric with respect to the origin of protons. The *ad hoc* assumption is therefore made that during fatty acid uncoupling the asymmetry allows the recognition as to whether the protons are provided by the ATPase or by an artificially generated $\Delta\tilde{\mu}_H$ gradient.

The insensitivity of $\Delta\tilde{\mu}_H$-driven ATP synthesis in submitochondrial particles to fatty acids is largely in accord with the insensitivity to gramicidin in low-salt media obtained in the present study. In fact, we find both that the K^+ efflux ratio (±ADP) is insensitive to gramicidin and that the process of $\Delta\tilde{\mu}_H$-driven ATP synthesis is gramicidin D insensitive above a certain gramicidin concentration. However, we also find a certain inhibition of the ATP synthesis in the lower range of the gramicidin D concentrations and consider this inhibition of importance for two reasons. First, assuming that fatty acids act like gramicidin D, it explains the inhibition caused by fatty acids on the P_i–ATP exchange (Rottenberg & Steiner-Mordoch, 1986); this becomes a consequence of the enhancement of ATP hydrolysis and is not due to an improbable asymmetry. Second, it suggests that in principle the effect of gramicidin at or near the ATPase proton pump is not basically different, although more limited in extent, from that at or near the redox proton pump.

Proton Domains and Pump-Associated Protons. In the preceding paper (Luvisetto & Azzone, 1989), we have discussed the implications of the terms *proton domain, intrinsic uncoupling, and decoupling*. The proposal that in low-salt media gramicidin D catalyzes proton cycling at or near the redox pumps explains the two major observations of the preceding paper (Luvisetto & Azzone, 1989), namely, that the uncoupling effect of gramicidin D on the aerobic processes is not accompanied by (1) depression of $\Delta\tilde{\mu}_H$ and (2) increase of the proton conductance. Since proton cycling at or near

the redox proton pump is implicated in all three different concepts, it appears that the analysis of the properties of the isolated redox and ATPase proton pumps does not allow a distinction between them [cf. Zoratti et al. (1982), Westerhoff et al. (1984), Azzone et al. (1984), Rottenberg and Hashimoto (1986), Slater et al. (1986), Rottenberg (1983), Luvisetto et al. (1987), and Pietrobon et al. (1987)].

However, the suggestion of proton cycling at or near the redox proton pumps does not explain the two major observations of the present paper, namely, that (1) the same gramicidin concentrations which cause an extensive uncoupling of respiration-driven ATP synthesis leaves instead almost unaffected the artificial $\Delta\tilde{\mu}_H$-driven ATP synthesis except for a partial inhibitory effect below 100 ng of gramicidin/mg and (2) during aerobic ATP synthesis it is possible to observe in the presence of gramicidin inhibition of ATP synthesis with little or no depression of $\Delta\tilde{\mu}_H$ and in the presence of valinomycin depression of $\Delta\tilde{\mu}_H$ with little or no inhibition of ATP synthesis. These two groups of observations indicate that during each cycle of electron transfer a specific class of protons originates at the level of the redox pump. This class is different from the class of protons released into the bulk water phase although it appears as already expression of an energy transduction step, as further indicated by two properties: first, it exerts thermodynamic control over the rate of respiration; second, it may be used for ATP synthesis. These protons, now tentatively identified as gramicidin accessible and proton pump associated, may represent not the parallel but the real primary event of energy conservation at the molecular level.

The existence of a particular class of pump-associated protons explains other observations which have not yet received an adequate explanation: (1) the increase of the $\Delta G_p/\Delta\tilde{\mu}_H$ ratio with the decrease of $\Delta\tilde{\mu}_H$ (Azzone et al., 1978; Petronilli et al., 1986; Guffanti et al., 1984); (2) the much more rapid rate and the much steeper dependence on $\Delta\tilde{\mu}_H$ of ATP synthesis during aerobic phosphorylation with respect to artificial $\Delta\tilde{\mu}_H$ gradient-driven phosphorylation (Zoratti et al., 1986a); and (3) the lack of proton release in the water phase during oxygen pulses parallel to the rise of the membrane potential in anaerobic mitochondria or bacteria (Conover & Azzone, 1980; Gould & Cramer, 1977; Gould, 1979; Kell & Hitchens, 1982; Hitchens & Kell, 1984). The last two observations are relevant in order to draw a distinction between the concepts of decoupling and of proton domains. In fact, observation 3 supports the concept of electron-transfer steps in the redox pumps accompanied by formation of electrical fields across the membrane but not by release of protons to the bulk phase. Furthermore, observation 2 suggests that the redox pump associated protons responsible for ATP synthesis during respiration possess different kinetic properties from the bulk-phase protons responsible for ATP synthesis during K^+ diffusion. This is what is predicted by the proton domain concept (Westerhoff et al., 1984; Azzone et al., 1984; Pietrobon et al., 1987). At all events, the present information does not allow a distinction as to the association of the protons either directly to the pump or indirectly to a protonophore coupled to the transmembrane redox enzymes by means of electrical fields as recently suggested by Kamp et al. (1988).

In conclusion, while the proposal of intramembranal proteic pathways for proton transfer, as suggested by the decoupling concept, remains for the moment not open to experimental test, the proton domain concept is supported by the operational definition of a particular class of protons. The elucidation of the properties of these protons may be crucial for understanding the topology of the proton domains and the molecular steps of the energy coupling machinery.

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Location and Magnetic Relaxation Properties of the Stable Tyrosine Radical in Photosystem II†

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ABSTRACT: Dipolar interactions with neighboring metal ions can cause enhanced spin-lattice relaxation of free radicals. We have applied the theory of dipolar relaxation enhancement and shown that the dependence of the enhanced relaxation on the protein structure surrounding the free radical can be used to obtain distances from the free radical to the protein surface. To test the theoretical predictions, we have examined the effect of added Dy^{3+} complexes on the microwave power saturation of free radicals in two protein complexes of known structure: myoglobin nitroxide and the reaction center from *Rhodobacter sphaeroides*. Three cases have been considered: (1) metal ions bound to a specific site, (2) metal ions bound randomly over the protein surface, and (3) metal ions distributed randomly in solution. Only case 3, which assumes no specific binding, gave good agreement between the distances obtained by using the two model systems. The effect of added Dy^{3+} complexes on the microwave power saturation of signal II_{slow} from photosystem II (PSII) was used to determine the location of the stable tyrosine radical giving rise to signal II_{slow}. Assuming that the surface of a membrane-bound protein can be approximated as planar, we have obtained distances from the tyrosine radical to the membrane surface in thylakoids, in PSII membranes, and in Tris-washed PSII membranes. The distances we have determined are in good agreement with those predicted on the basis of a structural homology between the D1 and D2 subunits of PSII and the structurally characterized L and M subunits of the reaction center from purple non-sulfur bacteria. We have also examined the temperature dependence of the microwave power at half-saturation ($P_{1/2}$) of signal II_{slow} from 4 to 200 K in dark-adapted PSII membranes. Above 70 K, the $P_{1/2}$ increases as $T^{2.5}$, which is consistent with a Raman relaxation mechanism. But between 10 and 70 K, the $P_{1/2}$ is nearly independent of temperature. Such temperature independence of the $P_{1/2}$ is highly unusual.

The EPR[1] signal II_{slow} species, D^+, was the first radical observed from PSII (Commoner et al., 1956) and is known to be associated with the electron-donor side of the photosystem. Recent studies have identified D^+ as a tyrosine cation radical (Barry & Babcock, 1987), and site-specific mutagenesis has indicated it to be Tyr-160 of the D2 polypeptide from the PSII reaction center core (Debus et al., 1988). Signal II_{slow} is called "slow" because it is stable on the order of days at 0 °C. D is oxidized in the light, via the S_2 and S_3 states of the O_2-evolving complex (Babcock & Sauer, 1973). These S states refer to the model of Kok et al. (1970) postulating five intermediate oxidation states (S states S_0-S_4) of the O_2-evolving complex which store the equivalents to oxidize H_2O to O_2. Evidence that D^+ oxidizes the S_0 state to S_1 in the dark has led to speculation that the role of D^+ is to maintain oxidizing equivalents that prevent PSII from being trapped in the dark in the possibly unstable S_0 state (Styring & Rutherford, 1987).

Signal II_{slow} has a line width of ~19 G and a g value of 2.0047 ± 0.0002 (Kohl, 1972). The signal exhibits partially resolved hyperfine structure attributed to protons (O'Malley et al., 1984). There have been a number of studies on the decay kinetics of signal II_{slow} and great interest in identifying the molecule giving rise to the signal. Nevertheless, many aspects of the magnetic properties of this free radical, including the temperature dependence of the spin relaxation rates, had not been explored until recently. In order to characterize some of the magnetic properties of D^+, we have studied the temperature dependence of the microwave power saturation of signal II_{slow} between 4 and 200 K in dark-adapted thylakoids, PSII membranes, and Tris-treated PSII membranes (which lack three extrinsic polypeptides and Mn).

Likewise, the location of D^+ in the PSII reaction center has not been established. To obtain distance information, we have used the effect of added Dy^{3+} complexes on the microwave power saturation of signal II_{slow}. Bloembergen (1949) and Abragam (1955) developed the theory for the enhanced relaxation caused by dipolar interactions with rare earth ions. Changes in relaxation and line widths have since been used

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[1] Abbreviations: $(BChl)_2^+$, bacterial chlorophyll dimer; chl, chlorophyll; D^+, tyrosine radical in photosystem II giving rise to EPR signal II_{slow}; EDTA, ethylenediaminetetraacetic acid; EPR, electron paramagnetic resonance; Fe-Q_A, iron-quinone electron acceptor of photosystem II; HEDTA, *N*-(hydroxyethyl)ethylenediaminetriacetic acid; kDa, kilodalton(s); MbNO, myoglobin nitroxide; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PSII, photosystem II; *Rb. sphaeroides*, *Rhodobacter sphaeroides*; Tris, tris(hydroxymethyl)aminomethane.

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