J. Biochem. 119, 274-280 (1996)

# Uncoupling Mechanism of Glycoside Antibiotic Aculeximycin in Isolated Rat-Liver Mitochondria

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Received for publication, August 31, 1995

**Effects of basic glycoside antibiotic aculeximycin (ACM) on the oxidative phosphorylation of rat-liver mitochondria were examined. ACM was shown to be a potent uncoupler of the oxidative phosphorylation. To cause the same extent of respiration release, higher concentration of ACM was required in phosphate ($P_i$)-free medium than in $P_i$ medium. During the uncoupling caused by ACM in $P_i$ medium, large amplitude swelling and oxidation of intramitochondrial NAD(P)H occurred, indicating that ACM remarkably enhances permeability of the inner mitochondrial membrane. The $P_i$ uptake *via* $P_i/H^+$ symporter was shown to play an important, but not essential, role in the uncoupling by ACM, indicating the increase in membrane permeability is mostly due to acceleration of $P_i/H^+$ influx through $P_i/H^+$ symporter activated by ACM. ACM is the first naturally occurring antibiotic, to our knowledge, which activates $P_i/H^+$ symporter. However, since the inhibition of $P_i/H^+$ symporter by *N*-ethylmaleimide did not completely abolish the uncoupling activity of ACM, and ACM induced the uncoupling even in $P_i$-free medium, an increase in the membrane permeability for other ions, such as $Na^+$ and $K^+$, due to a different action mechanism has also to be considered. On the other hand, positively charged amine local anesthetics, like dibucaine, prevented the uncoupling activity by ACM in both $P_i$ and $P_i$-free medium. The uncoupling activity of *N*-diacetylated ACM lacking free amino groups was *ca.* 1/120th that of ACM, indicating that positively charged amino groups are important for the uncoupling activity. It is suggested that some specific interactions between positively charged amino groups of ACM and the binding site, which is probably negatively charged, are triggers that affect the permeability of the inner mitochondrial membrane. Amine local anesthetics may mask the negative charge of the binding site, thereby interfering with ACM binding.**

**Key words: antibiotic (aculeximycin), mitochondria (rat-liver), oxidative phosphorylation, uncoupling.**

Aculeximycin (ACM) is a basic glycoside antibiotic produced by *Streptosporangium albidum*. Its production and isolation were described previously (*1*) together with some of its biological and physicochemical properties. ACM (Fig. 1) is a 30-membered macrolide with a trisaccharide (aculexitriose), D-mannose, and L-vancosamine and belongs to a new class of macrolide antibiotics, which is different from the polyol macrolides produced by *Streptomyces* (*2-4*). ACM exhibited potent bactericidal activity against Gram-positive bacteria, acid-fast bacteria and trichophyton, and also considerable toxicity to mammal and fish. The precise mechanism of ACM's toxicity remains to be determined. To identify the mode of toxic action of ACM in mammal, we examined effects of ACM on the mitochondrial respiration system using rat-liver mitochondria. We found that ACM is a potent uncoupler of oxidative phosphorylation. The mechanism of the uncoupling by ACM was examined in this study.

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Abbreviations: ACM, aculeximycin; NEM, *N*-ethylmaleimide; $P_i$, inorganic phosphate; RLM, rat-liver mitochondria; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile; $TPP^+$, tetraphenylphosphonium ion.

## MATERIALS AND METHODS

*Materials*—ACM and *N*-diacetylated ACM were the same samples as those used previously (*2-4*). Rotenone, dibucaine, quinacrine, oligomycin, carboxyatractyloside, and trifluoperazine were purchased from Sigma. Cyclosporin A was a generous gift from Sandoz. Other reagents were the highest grade commercially available.

*Methods*—Mitochondria were isolated from adult male Wistar rats as reported by Myers and Slater (*5*) in a medium containing 250 mM sucrose and 2 mM Tris-HCl buffer (pH 7.4). Mitochondrial protein was assayed by the method of Lowry *et al.* (*6*) with bovine serum albumin as the standard.

The respiration of mitochondria with 10 mM sodium

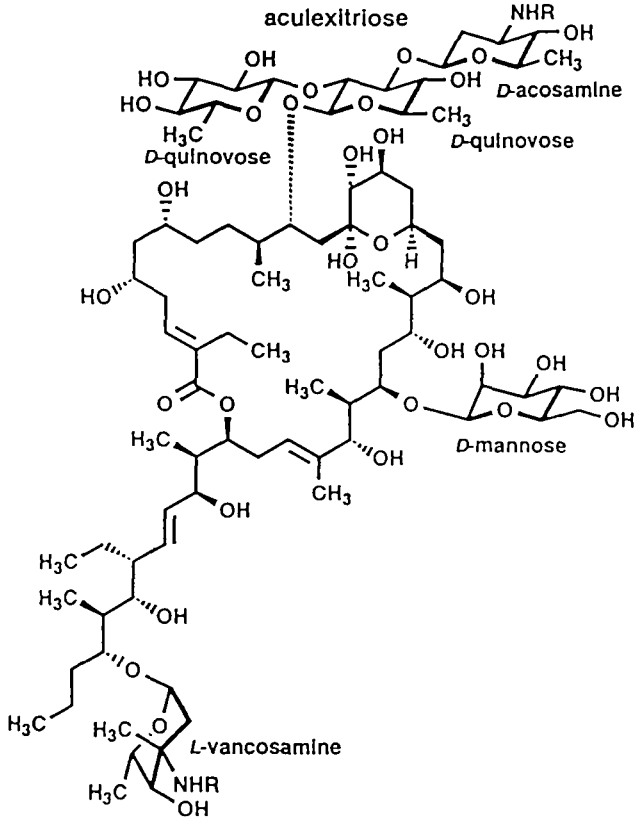


Fig. 1. **Structure of aculeximycin (R=H) and *N*-diacethylated aculeximycin (R=$COCH_3$).**

succinate (plus 2.5 $\mu$M rotenone) as a respiration substrate was monitored with a Clark-type oxygen electrode at 25°C, with the final mitochondrial protein concentration of 0.7 mg/ml. The $P_i$ incubation medium contained 200 mM sucrose, 2.5 $\mu$M rotenone, 2 mM $MgCl_2$, 1 mM EDTA, and 2.5 mM phosphate buffer (pH 7.4) in a total volume of 2.5 ml. In the $P_i$-free incubation medium, 2.5 mM Tris-HCl buffer (pH 7.4) was used in place of the phosphate buffer. In experiments on the effect of $P_i$, 10 mM Tris-HCl buffer (pH 7.4) was used. The sodium ($Na^+$) medium contained 200 mM sucrose, 10 mM sodium succinate, 2.5 $\mu$M rotenone, 2 mM $MgCl_2$, 1 mM EDTA, and 2.5 mM Tris-HCl buffer (pH 7.4). In the potassium ($K^+$) medium, 10 mM potassium succinate was used in place of sodium succinate.

The membrane potential of mitochondria was measured with a tetraphenylphosphonium ion ($TPP^+$) electrode by the method of Kamo *et al.* (7) in 15 ml of the same incubation medium as was used in the respiration experiment, except that 10 $\mu$M $TPP^+$ was included. The concentration of $TPP^+$ in the medium was calculated from calibration plots of the electrode output *vs.* the log of the $TPP^+$ concentration found in each experiment.

The oxidation-reduction state of the mitochondrial pyridine nucleotide was monitored at the wavelength pair 340-375 nm with a Shimadzu UV3000 spectrophotometer at 25°C (*8*). Mitochondrial swelling was monitored by recording of the apparent absorbance changes at 540 nm (*8*). To find when mitochondrial incubation became anaerobic, parallel experiments were done with an oxygraph. For some spectrophotometer procedures, anaerobiosis was signaled by small discontinuities in the traces, arising from changes in the redox state of cytochromes. In the figures, some optical absorbance traces from just before anaerobiosis have been omitted.

Uptake of [$^{32}$P]$P_i$ (specific radioactivity, 15.3 MBq/mmol) by mitochondria (2.0 mg/ml) during respiration with 10 mM succinate (plus 2.5 $\mu$M rotenone) as a substrate was determined at 20°C as described by Koike *et al.* (*9*), except that $P_i$ uptake was determined 30 s after the addition of a known amount of ACM and $P_i$ transport was terminated by the addition of 0.5 mM *N*-ethylmaleimide (NEM). The ATP synthesis and hydrolysis by ATPase were determined by monitoring pH changes of the medium with a Horiba F-7 pH meter according to the method of Nishimura *et al.* (*10*).

## RESULTS

The addition of ACM to state 4 mitochondria, with succinate as a substrate, accelerated respiration in $P_i$ medium (Fig. 2). The degree of stimulation of the respiration increased as the concentration of ACM increased; with about 0.3 $\mu$M, respiration release was more than sevenfold that with state 4 respiration, and this seemed to be maximum. The maximum respiration rate was almost equivalent to that obtained by a potent protonophoric uncoupler SF6847 (*11*). There was a slight lag before respiration release became constant at low concentration of the compound, but with high concentrations, respiration was rapidly stimulated. The change in the respiration rate *vs.* the ACM concentration was sigmoidal (Fig. 3, closed circles), although the effect of potent protonophore-type uncoupler is always linear (*11, 12*). The maximum respiration rate induced by ACM was constant at least up to 5 $\mu$M.

The extent of respiratory acceleration caused by a certain concentration of ACM in $P_i$ medium was larger than that in $P_i$-free medium. Respiratory titration was done in $P_i$-free medium (Fig. 3, open circles). The change in the respiration rate *vs.* the ACM concentration was also sigmoidal. The maximum respiration release was not affected by the absence of $P_i$ in the medium. The effect of $P_i$ on the ACM-induced release of state 4 respiration is shown in the inset in Fig. 3. The effect was maximum at about 2 mM $P_i$. These results show that $P_i$ in the medium was favorable, but not essential, to the respiration release by ACM. Other membrane-permeable anions such as acetate, 1-anilino-8-naphthalene, and tetraphenylborate could not replace $P_i$.

ACM dissipated the transmembrane potential, measured as uptake of $TPP^+$ (Fig. 4). The relative effects of ACM on respiration release were parallel with its effect on dissipation of transmembrane potential (data not shown). In addition, ACM released the inhibition of state 3 respiration by oligomycin, activated ATPase activity, and inhibited the synthesis of ATP in the same concentration range as that which causes respiration release (data not shown). Thus, ACM turned out to be a potent uncoupler of the oxidative phosphorylation in rat-liver mitochondria.

Effects of ACM on mitochondrial swelling and intramitochondrial $NAD(P)^+/NAD(P)H$ ratio in $P_i$ medium are shown in Fig. 5, A and B, respectively. These two mitochondrial properties are closely related to change in the inner membrane permeability (*8, 13*). Swelling occurred after the addition of ACM and the degree of swelling increased

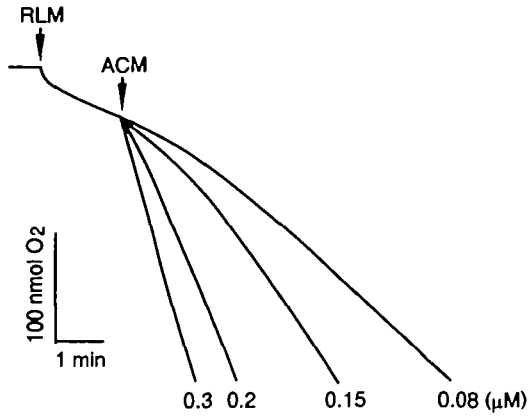


Fig. 2. **Effects of aculeximycin on state 4 respiration of mitochondria.** Mitochondria (RLM) were suspended in $P_i$ medium that contained 10 mM sodium succinate, 200 mM sucrose, 2.5 $\mu$M rotenone, 2 mM $MgCl_2$, 1 mM EDTA, and 2.5 mM phosphate buffer (pH 7.4). The final mitochondrial concentration was 0.7 mg protein/ml. Where indicated, various concentrations of aculeximycin (ACM) were added.

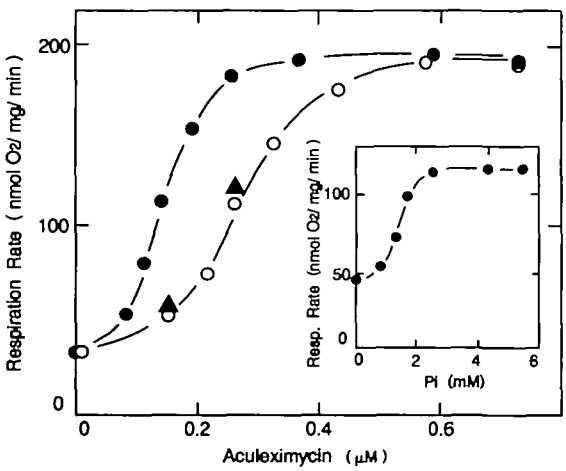


Fig. 3. **Respiratory titration curves in $P_i$ medium and $P_i$-free medium.** The closed and open circles indicate the results obtained in the $P_i$ medium (2.5 mM phosphate buffer, pH 7.4) and $P_i$-free medium (2.5 mM Tris-HCl buffer, pH 7.4), respectively. Other conditions were as described in the legend to Fig. 2. The inset shows the effect of $P_i$ on respiration release caused by 0.15 $\mu$M aculeximycin (ACM) with 10 mM Tris-HCl buffer (pH 7.4). The closed triangles indicate results observed in $P_i$ medium in the presence of 0.12 mM EDTA.

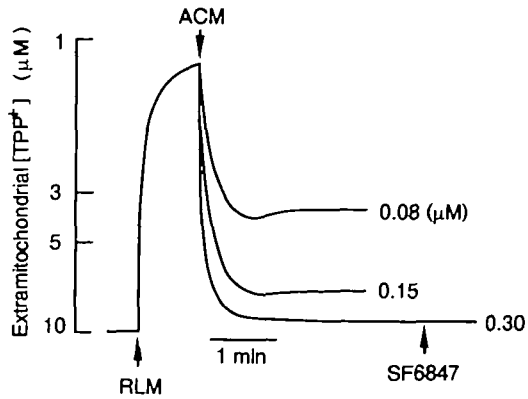


Fig. 4. **Effects of aculeximycin on the transmembrane electrical potential of mitochondria.** Changes in the transmembrane potential were monitored from changes in the extramitochondrial $TPP^+$ concentration. Mitochondria (RLM) were suspended in $P_i$ medium containing 10 $\mu$M $TPP^+$. Where indicated, various concentrations of aculeximycin (ACM) and SF6847 (60 nM) were added.

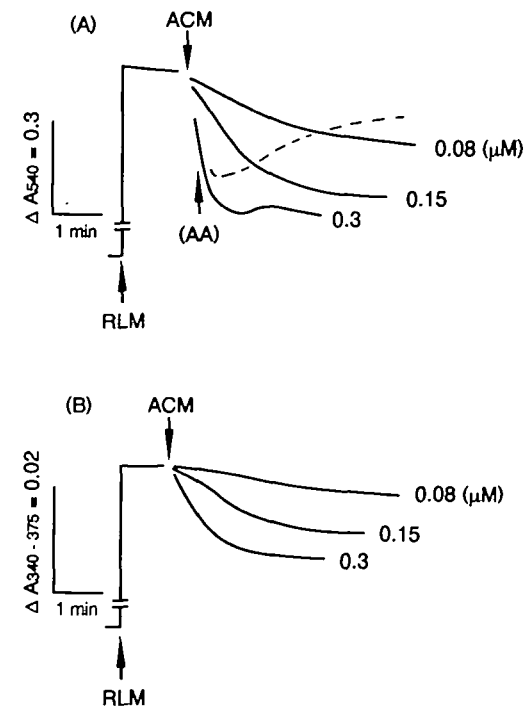


Fig. 5. **Effects of aculeximycin on the mitochondrial swelling (A) and the oxidation-reduction state of intramitochondrial pyridine nucleotide (B).** Mitochondria (RLM) were incubated in the $P_i$ medium at 0.7 mg protein/ml. Where indicated, various concentrations of aculeximycin (ACM) and antimycin A (AA, 1 $\mu$M) were added. In (B), downward deflection indicates oxidation of intramitochondrial NAD(P)H.

with an increase in the concentration of ACM. The $NAD(P)^+$/NAD(P)H ratio was also increased by the addition of ACM in a concentration-dependent way. These results indicate that ACM enhances permeability of the inner mitochondrial membrane. The relative effects of ACM on swelling were parallel with its relative effects on respiration release (Fig. 6), suggesting that the swelling (*i.e.*, an increase in the inner membrane permeability) is directly related to the uncoupling. On addition of antimycin A (1 $\mu$M) during the process of swelling, the swelling stopped and the mitochondrial volume reversed, as shown by the dotted line in Fig. 5A. When ACM was added to rotenone-treated mitochondria in the absence of succinate, the swelling was not induced (data not shown). These observations indicate that the swelling is respiration-dependent.

We examined the effects of *N*-ethylmaleimide (NEM), the commonly used inhibitor of $P_i/H^+$ symporter (*14*), on the respiration release (Fig. 7A) and swelling (Fig. 7B) induced by ACM in $P_i$ medium. When 0.12 mM NEM was

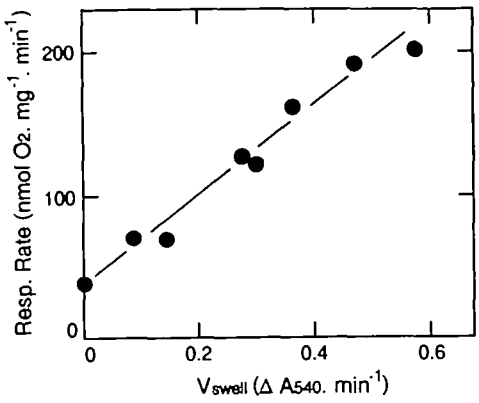


Fig. 6. **Linear relationship between the rate of swelling ($V_{swell}$) and the respiration rate induced by aculeximycin in $P_i$ medium.**

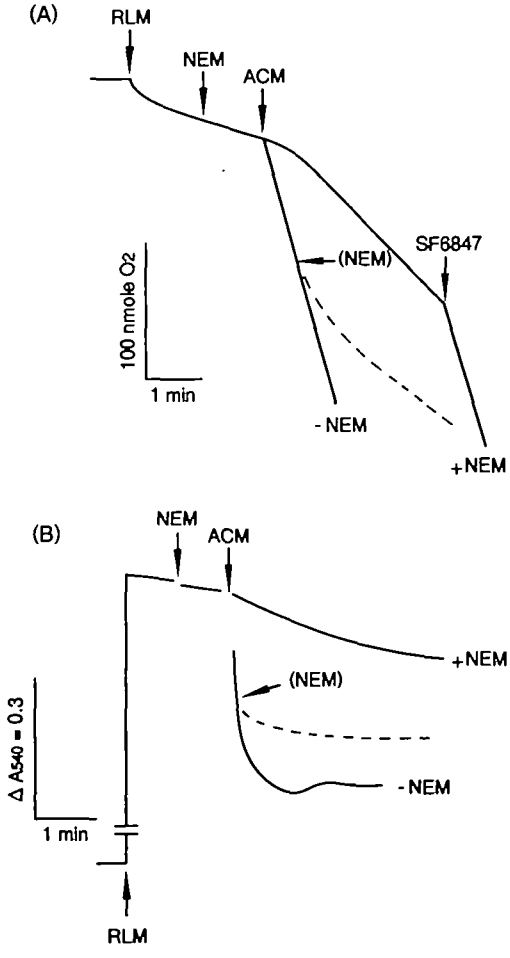


Fig. 7. **Effects of *N*-ethylmaleimide on respiration release (A) and mitochondrial swelling (B) induced by aculeximycin.** Mitochondria (RLM) were suspended in the $P_i$ medium at 0.7 mg protein/ml. Where indicated, *N*-ethylmaleimide (NEM; 0.12 mM), ACM (0.2 $\mu$M), and SF 6847 (40 nM) were added. The dotted lines show the traces when NEM (0.12 mM) was added during the uncoupling induced by ACM.

added before ACM, the respiration release and swelling were significantly, but not completely, prevented. The extents of these uncoupling properties were almost equivalent to those caused by the same concentration of ACM in $P_i$-free medium without NEM, as shown by closed triangles in Fig. 3 for the case of respiration release. The inhibition of respiration release by NEM was reversed by the addition of 40 nM SF6847, indicating that the inhibition of respiration release is not due to inhibition of the respiration chain. Addition of NEM (0.12 mM) during the uncoupling induced by ACM arrested the uncoupling as shown by dotted lines in Fig. 7, A and B. The mitochondrial volume remained constant. These results strongly suggest that the $P_i/H^+$ symport into the mitochondrial matrix is related to the uncoupling action of ACM.

To confirm this notion, we determined the effects of ACM on the uptake of [$^{32}$P]$P_i$ by mitochondria energized with 10 mM succinate. As shown in Fig. 8, the rate of $P_i$ transport into mitochondria increased with an increase in the concentration of ACM. The enhancement of $P_i$ uptake by ACM was prevented in the presence of 120 nM SF6847. Thus, the $P_i/H^+$ symport into the mitochondrial matrix appeared to participate in the uncoupling action of ACM.

The uncoupling action of ACM, however, can not be

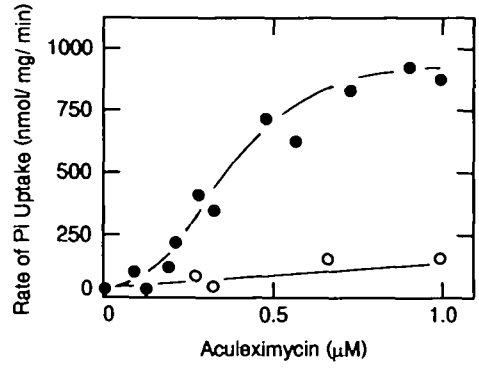


Fig. 8. **Effects of aculeximycin on the uptake of $P_i$ into mitochondria.** Uptake of [$^{32}P_i$]$P_i$ by mitochondria (2.0 mg/ml) was determined 30 s after the addition of ACM at 20°C. The experiments were carried out in the presence (open circles) or absence (closed circles) of 120 nM SF6847.

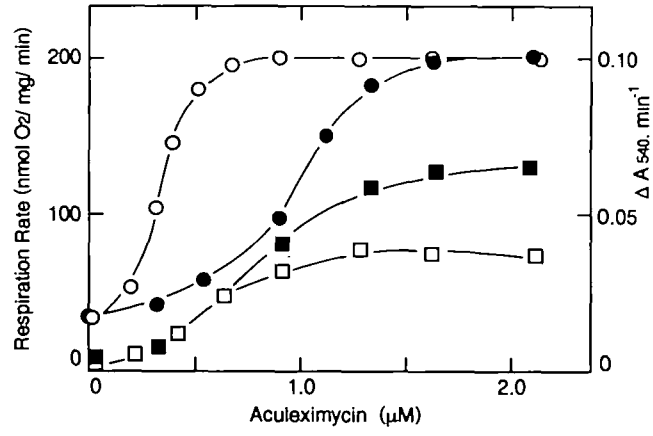


Fig. 9. **Respiratory acceleration and swelling induced by aculeximycin in $Na^+$ and $K^+$ medium.** Mitochondria were suspended in $Na^+$ (open symbols) and $K^+$ medium (closed symbols). Respiration rate (○, ●) and mitochondrial swelling (□, ■) were determined as described in "MATERIALS AND METHODS." The final mitochondrial concentration was 0.7 mg protein/ml.

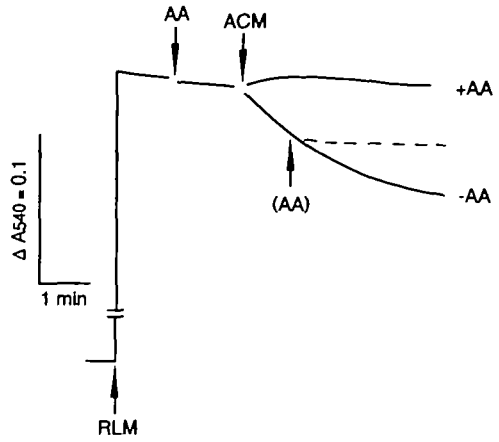


*Fig. 10.* ***Effects of aculeximycin on mitochondrial swelling in Na⁺ medium (Pi-free).*** Mitochondria (RLM) were incubated in $Na^+$ medium at 0.7 mg/ml. Where indicated, aculeximycin (ACM, 1 $\mu$M) and antimycin A (AA, 1 $\mu$M) were added. The dotted line shows the trace when antimycin A was added during the swelling.

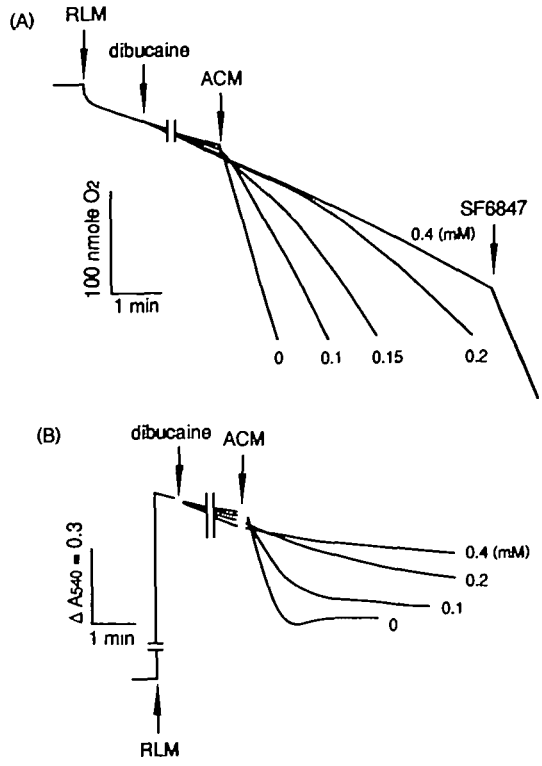


Fig. 11. **Effects of dibucaine on the uncoupling activity of aculeximycin.** (A) and (B) show respiration release and mitochondrial swelling, respectively, induced by aculeximycin (ACM) in the presence of various concentrations of dibucaine. Mitochondria (RLM) were suspended in the $P_i$ medium at 0.7 mg protein/ml. ACM (0.60 $\mu$M) was added 2 min after the addition of dibucaine. Where indicated, SF6847 (40 nM) was added.

elucidated solely by the acceleration of $P_i/H^+$ symport, since the inhibition of $P_i/H^+$ symporter by NEM did not completely abolish the uncoupling, and ACM induced the uncoupling action even in a $P_i$-free medium at higher concentrations. To identify other mechanisms, we studied the uncoupling action of ACM in the medium lacking $P_i$. The respiratory acceleration and mitochondrial swelling caused by ACM were compared in $Na^+$ and in $K^+$ medium (Fig. 9). The extent of respiration release induced by a certain concentration of ACM was greater in $Na^+$ medium than in $K^+$ medium, whereas the maximum respiration rates were identical. The concentrations of ACM required to induce the maximum respiration release were 0.6–0.8 and 1.5–1.8 $\mu$M in $Na^+$ and in $K^+$ medium, respectively. This indicates that $Na^+$ is more favorable to the uncoupling activity of ACM than $K^+$. In addition, the fact that ACM induced mitochondrial swelling indicates ion transport into mitochondrial matrix. The amplitude of maximum swelling was much less than that observed in $P_i$ medium (see Fig. 6). As was observed in $P_i$ medium, the relative effects of ACM on swelling were almost parallel with those on the respiratory release (data not shown). In the presence of antimycin A, ACM induced a slight shrinkage of mitochondria (Fig. 10). Addition of antimycin A during the process of swelling arrested the swelling as shown by the dotted line in Fig. 10, and the volume of mitochondria was maintained at the level at the time of addition of the inhibitor. These findings indicate that the swelling in $P_i$-free medium is also respiration-dependent.

The effects of the amine local anesthetic dibucaine on the uncoupling action of ACM are shown in Fig. 11. As the concentration of dibucaine with which the mitochondria were incubated increased, the extent of uncoupling as seen in the respiration release (Fig. 11A) and swelling (Fig. 11B) decreased. The state 4 respiration and the swelling were slightly accelerated in the presence of dibucaine. Treatment of mitochondria with 0.4 mM dibucaine completely prevented the uncoupling by ACM. The prevention of respiration release by dibucaine was almost completely released by the addition of 40 nM SF6847 (Fig. 11A), indicating that the prevention of respiration release is not due to inhibition of the respiration chain. Other amine local anesthetics such as trifluoperazine and quinacrine also prevented the uncoupling by ACM (data not shown). These effects of amine anesthetics were also found in $P_i$-free medium at the same concentration ranges as those in $P_i$ medium.

Treatment of ACM with 2% 1,8-diazabicyclo[5,4,0]-undecene-7 (DBU)-methanol yielded aculexitriose and an epimeric pair of the counterparts named pseudoaglycones I and II (*3, 4*). None of the products exhibited the uncoupling activity, in terms of respiration release and swelling, regardless of the presence of $P_i$. It is noteworthy that the uncoupling activity of *N*-diacetylated ACM (Fig. 1) was about 1/120th that of ACM. These facts indicate that ACM elicits the uncoupling activity in the glycosidic form, and that free amino groups of ACM, which are protonated (*i.e.*, $-NH_3^+$ form) under the experimental conditions, are essential for its activity.

## DISCUSSION

ACM uncoupled the oxidative phosphorylation in rat-liver mitochondria in both $P_i$ and $P_i$-free medium. To elicit the same extent of uncoupling activity, higher concentration of ACM was required in $P_i$-free medium than in $P_i$ medium. The uncoupling profiles of this compound differ from those of classical protonophore-type uncoupler in several ways:

in the sigmoidal relationship and $P_i$ dependence of respiration titration and in the large amplitude swelling of the mitochondria. It is, therefore, unlikely that uncoupling by ACM is due to its protonophoric action in the inner mitochondrial membrane. To begin with, ACM does not carry the physicochemical structural factors that would be required for it to function as potent protonophore (*15, 16*).

The $P_i$ uptake *via* $P_i/H^+$ symporter was shown to play an important role in the uncoupling action of ACM in $P_i$ medium, indicating that most of the uncoupling by ACM is due to acceleration of $P_i/H^+$ influx through $P_i/H^+$ symporter. The fact that the mitochondrial swelling induced by ACM is respiration-dependent supports this idea. This uncoupling mechanism seems to be similar to that of $Cd^{2+}$ (*9*) and crystal violet (*17*), for which the presence of $P_i$ in the incubation medium is essential. However, as mentioned above, the uncoupling by ACM cannot be explained by the acceleration of $P_i/H^+$ symport alone, since the inhibition of this symporter by NEM did not completely abolish the uncoupling activity, and ACM induced the uncoupling even in a $P_i$-free medium. Therefore, besides the activation of $P_i/H^+$ symporter, some other mechanism of induction of an increase in the inner membrane permeability to other ions such as $Na^+$ and $K^+$ has also to be considered, especially in the case of $P_i$-free medium. Because a higher concentration of ACM was required in $P_i$-free medium than in $P_i$ medium to elicit the same extent of uncoupling, it is likely that ACM enhances the inner membrane permeability at least by two different mechanisms in $P_i$ medium: at low concentrations, ACM activates $P_i/H^+$ symporter, and at high concentrations, it further interacts with other sites that are associated with the membrane permeability to $Na^+$ and $K^+$.

The uncoupling activity of ACM was greater in $Na^+$ medium than in $K^+$ medium in the absence of $P_i$. Several macrolide antibiotics like valinomycin induce uncoupling by working as specific ionophores. It is also possible that ACM works as an ionophore which has different specificities to $Na^+$ and $K^+$. The UV spectrum of ACM owing to a conjugated $\alpha,\beta$-unsaturated carbonyl chromophore was identical in $Na^+$ and $K^+$ media, $\lambda_{max}$ being 222 nm ($\log \varepsilon = 3.96$). In the lower dielectric media, that is, in 50% EtOH-$Na^+$ medium (v/v) and in 50% EtOH-$K^+$ medium (v/v), the UV spectrum was also identical, $\lambda_{max}$ being 225 nm ($\log \varepsilon = 3.93$). These observations suggest that ACM does not specifically encage $Na^+$ or $K^+$ into its 30-membered lactone ring. Furthermore, if ACM-mediated $Na^+$ or $K^+$ transport were to occur in $P_i$-free medium, acidification of the medium due to respiration-linked $H^+$ ejection should be observed as the case of valinomycin-mediated $K^+$ transport (*18*). However, ACM at the concentration inducing full uncoupling did not cause acidification of the medium (data not shown). It is, therefore, unlikely that ACM works as an ionophore having different specificities to $Na^+$ and $K^+$. While the uncoupling mechanism by ACM in $P_i$-free medium remains to be elucidated, we suggest that ACM affects the inner membrane permeability to these cations by acting specifically on some membrane component.

The uncoupling induced by ACM, monitored by several mitochondrial properties such as respiration release, swelling, and oxidation of intramitochondrial NAD(P)H, was prevented by treating mitochondria with amine local anesthetics regardless of the presence of $P_i$. The local anesthetics used in this study all carry a positive charge under the experimental conditions (*19, 20*). We suppose that ACM binding sites are negatively charged, which is essential to ACM binding, and amine local anesthetics mask the negative charges of the binding sites, thereby interfering with ACM binding. Considering that the uncoupling activity of *N*-diacetylated ACM is much lower than that of ACM, it is likely that the specific interactions between positively charged amino groups of ACM and negatively charged binding sites might be triggers that affect the permeability of the inner mitochondrial membrane. Then, one of the possible binding sites should be a $P_i/H^+$ symporter as discussed above. ACM is the first naturally occurring antibiotic, to our knowledge, which activates $P_i/H^+$ symporter.

A nonspecific pore model has been proposed to explain the increase in nonspecific permeability of the inner mitochondrial membrane caused by accumulated $Ca^{2+}$ and inducers such as hydroperoxide and phosphate (*21-24*). In this model, conformational change of adenine nucleotide carrier is assumed to take part in the pore formation. The participation of an adenine nucleotide carrier in the uncoupling action of ACM could be ruled out since effectors that modify the conformation of adenine nucleotide carrier, such as carboxyatractyloside and ADP or ATP (plus oligomycin) (*23*), and cyclosporin A did not affect the uncoupling activity by ACM (data not shown). Furthermore, the respiration-dependent swelling induced by ACM cannot be explained by the nonspecific pore model.

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