Isobongkrekic Acid, a New Inhibitor of Mitochondrial ADP–ATP Transport: Radioactive Labeling and Chemical and Biological Properties†

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ABSTRACT: An isomer of bongkrekic acid, designated as isobongkrekic acid, has been isolated from ethereal extracts of *Pseudomonas cocovenenans* grown on defatted coconut. Isobongkrekic acid was also obtained by alkaline treatment of bongkrekic acid. Isobongkrekic acid possesses the same ultraviolet spectrum and the same molecular weight as bongkrekic acid; it has a similar infrared spectrum but not the same nuclear magnetic resonance (NMR) spectrum. The differences in NMR data were interpreted to mean that isobongkrekic acid differs from bongkrekic acid by the configuration of the dicarboxylic end; whereas the two carboxylic groups of the dicarboxylic end have the trans configuration in bongkrekic acid, they have the cis configuration in isobongkrekic acid. Differences between bongkrekic and isobongkrekic acids are lost after catalytic hydrogenation of the molecules. Isobongkrekic acid, like bongkrekic acid, is an uncompetitive inhibitor of ADP transport in mitochondria, provided the mitochondria are preincubated in the presence of the inhibitor and a minute concentration of ADP. The inhibitory and binding efficiency of isobongkrekic acid is considerably increased below pH 7. The number of high affinity sites for [^{3}H]isobongkrekic acid is 0.13 to 0.20 nmol/mg protein in rat liver mitochondria and about 1 nmol/mg protein in rat heart mitochondria, i.e., similar to the number of high affinity sites for [^{3}H]bongkrekic acid. Isobongkrekic and bongkrekic acids compete for the same site, but the affinity of isobongkrekic acid for mitochondria is one-half to one-fourth that of bongkrekic acid.

Study of the molecular and topological properties of the mitochondrial adN[1] translocator has benefited from a large panoply of inhibitors, namely atractyloside, carboxyatractyloside, and their apo derivatives (for review, see Vignais et al., 1973; Vignais, 1976), long chain acyl-CoAs (Pande and Blanchaer, 1971; Devaux et al., 1975), and bongkrekic acid (Henderson and Lardy, 1970; Kemp et al., 1970; Scherer and Klingenberg, 1974).

This paper describes the isolation and the labeling by tritium of isobongkrekic acid, a new inhibitor of ADP transport. The binding and inhibitory properties of IsoBA are compared with those of BA. A preliminary account of this work has been given (Lauquin et al., 1975).

Experimental Procedure

Preparation of IsoBA. IsoBA was routinely prepared by alkali treatment of BA. Ten milligrams of BA in methanol was evaporated under nitrogen to dryness in a small tube; then 0.5 ml of 2 N KOH was added. The tube was sealed and incubation was carried out for 2 h at 100 °C. After acidification to pH 3 by 2 N HCl, IsoBA was extracted with peroxide-free diethyl ether and separated from BA by thin-layer chromatography using chloroform–methanol–acetic acid (94:5:1, v/v) solvent system. After location under uv light, the area corresponding to IsoBA was scraped off and IsoBA was extracted from silica by methanol. IsoBA was further purified by DEAE-cellulose chromatography, using for elution a linear gradient of NaCl in 20 mM Mops, pH 8. IsoBA was recovered at 0.25 M NaCl. After acidification at pH 2–3 by 2 N HCl, IsoBA was extracted with peroxide-free diethyl ether and concentrated under vacuum.

^{3}H-Labeling of IsoBA. Both ^{3}H-labeled isomers ([^{3}H]BA and [^{3}H]IsoBA) were obtained after treatment of BA or IsoBA by tritiated KOH. Separation and purification of the [^{3}H]BA and [^{3}H]IsoBA were carried out as described above. Tritiated KOH was prepared with tritiated water (5 mCi/ml) and potassium *tert*-butoxide (Aldrich) as described by Thomas and Biemann (1965). The specific radioactivity of IsoBA was about 1 Ci per mmol.

Methylation of BA and IsoBA. IsoBA and BA were methylated with fresh diazomethane in diethyl ether in the presence of a few drops of 2 N HCl. [^{14}C]Diazomethane was prepared from *N*-methyl[^{14}C]-*N*-nitroso-*p*-toluenesulfonamide. The ^{14}C-labeled methylated products were analyzed by thin-layer chromatography in a solvent system made of cyclohexane and ether (1:1, v/v; Figure 1). Hydrolysis of methylated BA or IsoBA by 2 N KOH for 45 min at 100 °C resulted in a mixture of BA and IsoBA.

Preparation of Hydrogenated BA and IsoBA. Catalytic hydrogenation of the NH_4 salts of BA and IsoBA was carried out at 25 °C in water with molecular hydrogen and 10% Pd/C as catalyst.

Physical Measurements. Mass spectra were taken with a GEC-AE1 MS9 apparatus. The ion source temperature was 130 °C. Infrared spectra were obtained in $CHCl_3$ with a Perkin-Elmer spectrometer. Proton magnetic resonance spectra were run on a 250-MHz Cameca spectrometer. Spectra were referenced to tetramethylsilane and expressed in parts per

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[1] Abbreviations used are: adN, adenine nucleotide; BA, bongkrekic acid; IsoBA, isobongkrekic acid; uv, ultraviolet; ir, infrared; NMR, nuclear magnetic resonance; CoA, coenzyme A; DEAE, diethylaminoethyl; Mops, 4-morpholinepropanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

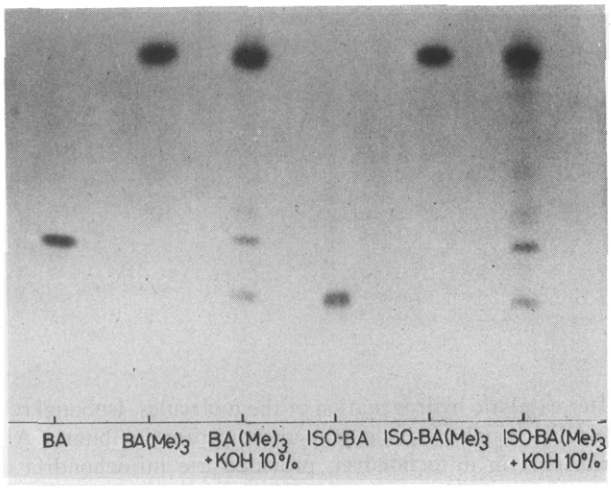

FIGURE 1: Separation of BA and IsoBA by thin-layer chromatography on silica gel GF 254 in a solvent system made of chloroform–methanol–acetic acid, 94:5:1 (v/v). Photography was under uv light.

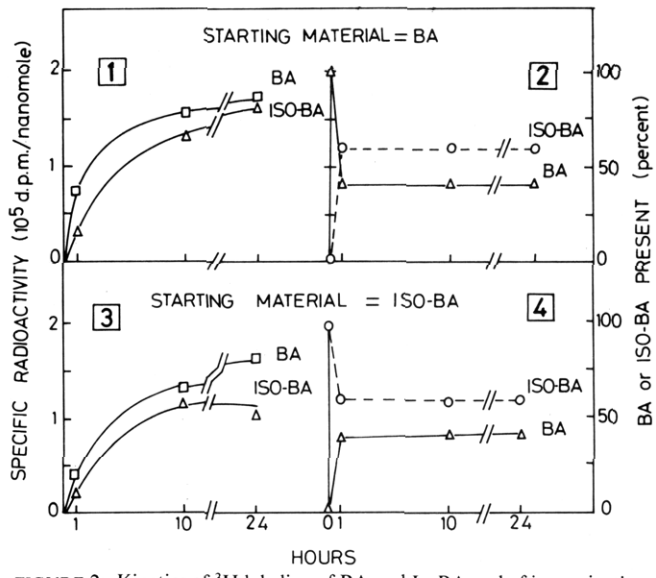

FIGURE 2: Kinetics of ^3H labeling of BA and IsoBA and of isomerization. BA or IsoBA (1.5 mg) was heated at 100 °C with 50 μl of [^3H]KOH for 1, 10, or 20 h as indicated in Experimental Procedure. After separation by thin-layer chromatography and extraction by methanol, the amount of BA and IsoBA was determined by uv absorbance and their radioactivity by liquid scintillation.

million (ppm) of the polarizing field. Titration of the carboxyl groups was performed on water–methanol–ethanol (2:1:1, v/v) solutions of BA and IsoBA, under argon, using a Radiometer pH stat; methanol and ethanol were added because BA and IsoBA are not soluble in water below pH 6.

Biological Assays. The procedure for binding of [^3H]IsoBA to mitochondria, pretreated or not by phosphate, was the same as that described for [^3H]BA in the accompanying paper (Lauquin and Vignais, 1976). Determination of the rate of ADP transport has also been described in the accompanying paper. In both cases, a standard saline medium made of 110 mM KCl, 10 mM Mes, pH 6.5, and 0.1 mM EDTA was used.

Results

IsoBA as a Natural Product. In early studies, IsoBA was detected in crude ethereal extract of *Pseudomonas cocovenenans* by thin-layer chromatography. IsoBA accounted for 5–7% of the total BA accumulated during the growth of *Ps. cocovenenans* on coconut pulp. As BA is readily isomerized into IsoBA in the presence of KOH at 100 °C (cf. Experimental Procedure), it was desirable to verify whether bicarbonate, which is used during the preparation of BA (Lijmbach et al., 1970), could be responsible for accumulation of IsoBA. No measurable accumulation of IsoBA could be detected. It can, therefore, be concluded that IsoBA is present in the fermentation products of *Ps. cocovenenans*.

Chemical Isomerization of BA into IsoBA. Isomerization of BA into IsoBA in the presence of 2 N KOH at 100 °C was a fast reaction. Equilibrium was reached in 1 h and it was slightly in favor of IsoBA (55% IsoBA, 45% BA). The reaction was fully reversible. The yield of the reaction was about 50%, starting either from BA or IsoBA (Figure 2).

Radioactive Labeling of IsoBA (or BA). As shown by Atkinson et al. (1968), the protons attached to the α-carbon atoms of the sodium or potassium salts of carboxylic acids are readily exchangeable with the deuterium of deuteriated KOH. This method was applied to ^3H labeling of BA and IsoBA, using tritiated KOH. Under these conditions, the two hydrogen atoms attached to carbon-23 in IsoBA (or BA) were exchanged with ^3H. From the specific radioactivity of [^3H]IsoBA or [^3H]BA, it could be estimated that 1.5–1.7 protons per molecule were exchanged, which is in good agreement with the theoretical value of 2. The yield of ^3H labeling was roughly 50%. As a mixture of BA and IsoBA was formed in a ratio of roughly 1:1, the yield for each isomer could be estimated to be 25% (Figure 2). Isomerization was faster than labeling. ^3H labeling was stable for at least 6 months between pH 5 and 8, i.e., at pHs used for binding experiments.

Physical and Chemical Characterization of IsoBA. IsoBA has the same uv spectrum in methanol as BA with two peaks at 237 and 267 nm. For this reason, the molecular extinction coefficients were supposed in first approximation to be identical for BA and IsoBA in methanol, namely ϵ 32 000 at 237 nm and ϵ 36 700 at 267 nm, and they were, therefore, utilized to calculate the concentration of IsoBA in solution. This assumption was validated by the following observations. (1) The specific radioactivity of [^3H]IsoBA, determined from the specific radioactivity of [^3H]KOH, was found to be identical with the specific radioactivity of [^3H]BA prepared from the same batch of [^3H]KOH. (2) Methanolic solutions of trimethylated BA and IsoBA, obtained by methylation with the same batch of [^{14}C]diazomethane, have the same specific radioactivities.

Infrared spectra of trimethylated BA and IsoBA showed at 6.3, 7.6, and 7.9 μm minor differences, which disappeared after catalytic hydrogenation. Mass spectra of trimethylated IsoBA and BA showed a similar molecular peak, corresponding to a molecular weight of 528 (Figure 3). Among the fragmentation products, the major one, found at *m/e* 183, was identical for IsoBA and BA.

NMR spectra of trimethyl-IsoBA and -BA in $CDCl_3$ showed marked differences (Figure 4A). The chemical shift of the proton resonance of BA is in excellent agreement with that reported by De Bruijn et al. (1973) and the proton assignments given by these authors were used to compare the chemical shifts of BA and IsoBA. The major differences between BA and IsoBA concerned the protons b, g and m, n of the dicarboxylic end. As these differences could be due to a cis–trans isomerization at the level of the terminal double bond of the dicarboxyl end, they were compared with those found in the cis–trans isomers of β-glutaconic acid, a compound presenting great analogy with the dicarboxyl end of BA. Interestingly the chemical shifts of protons m, n in BA and IsoBA (δ 3.32 and 3.96 ppm) differed by a value of 0.68 which was

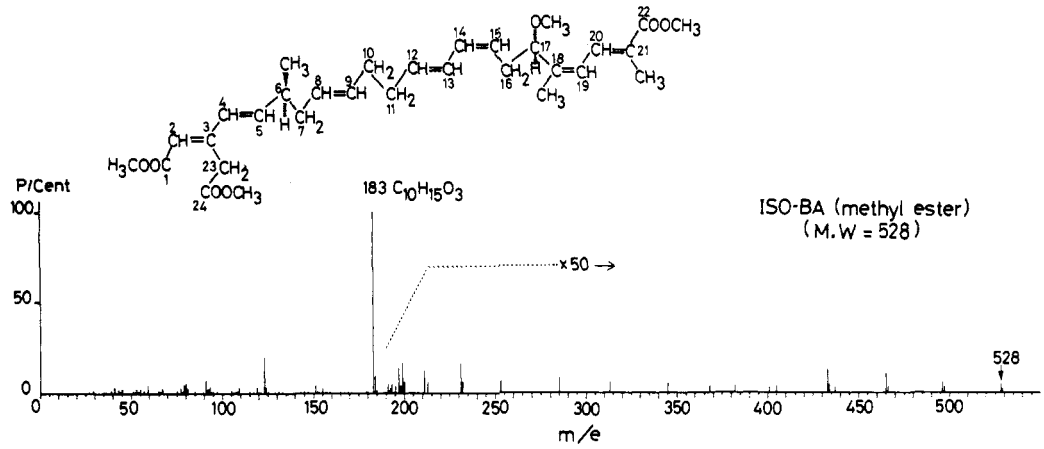

FIGURE 3: Mass spectrum of trimethyl ester of IsoBA. Conditions are as described in Experimental Procedure.

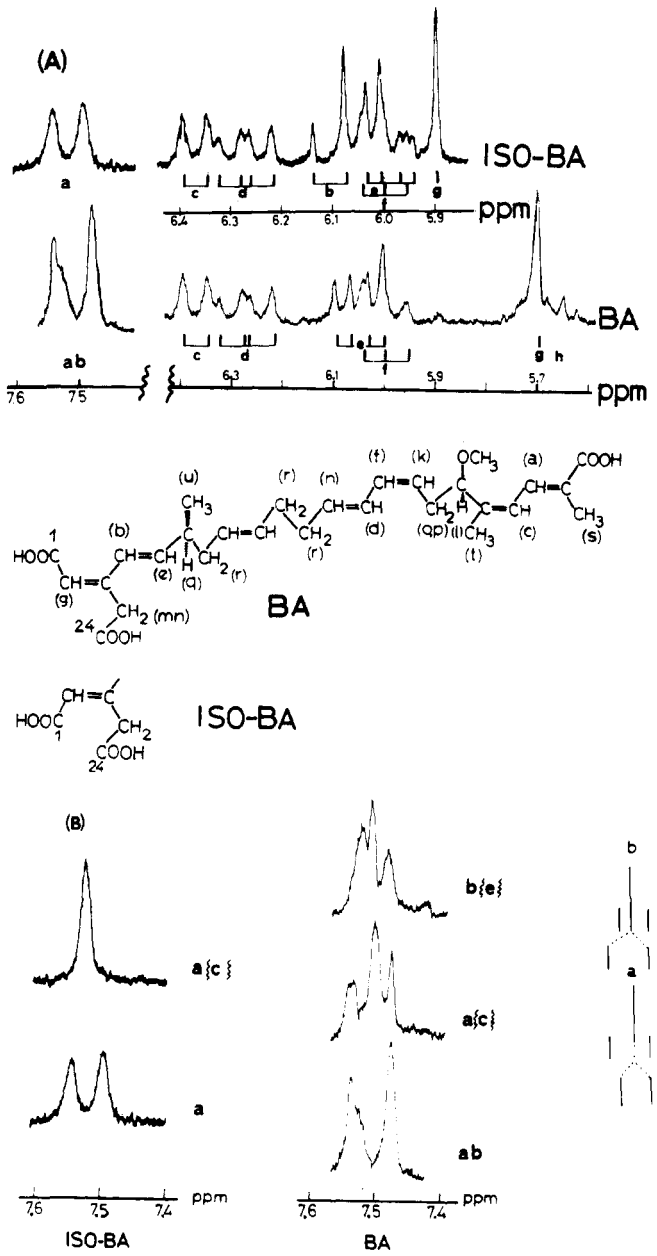

FIGURE 4: (A) The 250-MHz ^1H NMR spectra of trimethyl ester of BA and IsoBA; (B) characterization of protons a and b in decoupling experiments. Irradiation of proton c in IsoBA was at 1595 Hz, irradiation of proton c in BA at 1592 Hz, and that of proton e in BA at 1595 Hz.

Table I: Main Significant Coupling Constants in BA and IsoBA.

H-H	J_{H-H} (Hz)[a] in BA	J_{H-H} (Hz)[a] in IsoBA	Remarks
b-e	15.5	15.5	Trans coupling
b-g	~0	~0	
b-mn	0	0	
g-mn	0.5	0	Significant difference
b-q		~0	
q-u	6.7	6.7	

[a] J = coupling constant.

similar to the difference, 0.66, between the chemical shifts of equivalent protons in the trans and cis isomers of dimethyl-β-glutaconic acid (δ 3.06 and 3.72 ppm; Jackman and Wiley, 1960). Decoupling experiments were carried out to further characterize the unmasked protons a and b (Figure 4B). Protons a and b in BA were recognized at δ 7.50 and 7.52 ppm by irradiation of proton e and c, respectively. Proton a in IsoBA was found at δ 7.52 ppm by irradiation of proton c. Irradiation of proton e in IsoBA allowed us to confirm the assignment of proton b at δ 6.12 ppm in IsoBA. As shown in Table I, the main coupling constants were virtually the same for BA and IsoBA, except those concerning protons g and m, n. The long-range coupling between g and m, n was about 0.5 Hz in BA and nearly zero in IsoBA, which again indicates that BA and IsoBA differ by their dicarboxylic end. In conclusion by analogy with the cis-trans isomerization of dimethyl-β-glutaconic acid, the differences in the chemical shifts in BA and IsoBA can be explained by a cis-trans isomerization at the level of the C=C bond between C_2 and C_3 of the dicarboxyl end, the carboxyl groups being trans in BA and cis in IsoBA. Whereas BA and IsoBA could be differentiated by NMR spectroscopy and separated by thin-layer chromatography, hydrogenated BA and IsoBA behaved as a single molecular species, which is in good agreement with the conclusion that BA and IsoBA are two geometrical isomers.

Titration of carboxylic groups in IsoBA and BA was performed on water-methanol-ethanol solutions (2:1:1, v/v). The shift toward alkaline pH due to organic solvent was approximated to 1 pH unit, using the titration of succinic acid as reference. No distinct pK values could be differentiated for IsoBA or BA; the pKs of the three carboxylic groups were in the range

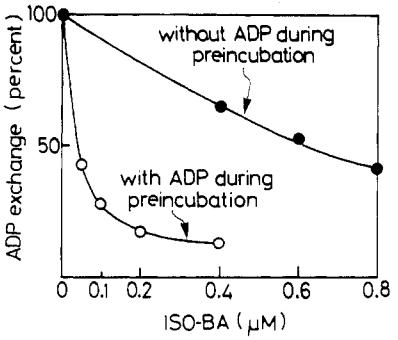

FIGURE 5: Effect of ADP in the preincubation medium on the inhibitory effect of IsoBA on [^{14}C]ADP transport. Rat heart mitochondria (1 mg protein) in 3 ml of standard saline medium, pH 6.5, were preincubated in the presence of IsoBA at the indicated concentration with or without ADP (0.5 μM) for 3 min at 20 °C and then 7 min at 0 °C. The reaction was started at 0 °C by addition of 20 μM [^{14}C]ADP. Other conditions are as described in Experimental Procedure.

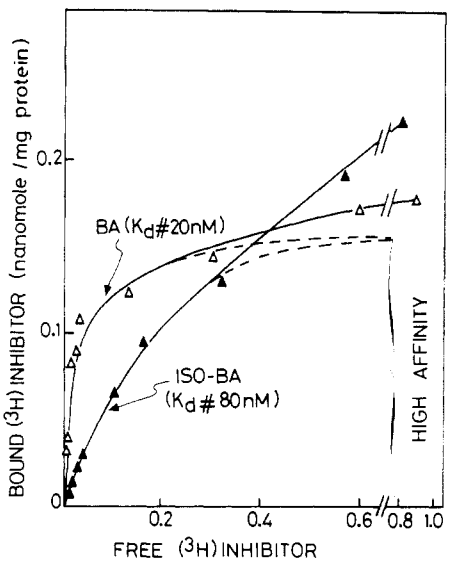

FIGURE 6: Binding of IsoBA and BA to rat liver mitochondria. Rat liver mitochondria (1 mg) pretreated by phosphate as described in Experimental Procedure were incubated with different amounts of [^{3}H]BA in 5 ml of standard medium for 3 min at 20 °C and then for 30 min at 2 °C. After centrifugation the pellets were dissolved in 1 ml of formamide at 180 °C and their radioactivity measured by liquid scintillation.

of 5.2–6.0 for IsoBA, as well as for BA (cf. Lijmbach, 1969). The end points of acid and alkaline titration, after correction for the alkaline shift, were 3.90 and 7.15 for IsoBA, and 3.90 and 6.65 for BA.

Inhibitory Properties of IsoBA. Similarly to BA, IsoBA totally inhibited the growth of yeast (*Saccharomyces cerevisiae*) at a concentration as low as 0.4 μM at pH 4.5, when the carbon source was a fermentable substrate, such as ethanol or glycerol. IsoBA inhibited respiration of *Saccharomyces cerevisiae* almost as actively as BA.

The ADP-stimulated respiration of isolated rat liver mitochondria was also inhibited by IsoBA and the inhibition was relieved by FCCP. The well-known pH dependence of the inhibitory effect of BA (Kemp et al., 1970) was also found for IsoBA. Like BA, IsoBA was a strong inhibitor of the mitochondrial ADP-stimulated respiration below pH 7.0, but its efficiency was considerably diminished above pH 7.5. Similarly to BA, the primary site of action of IsoBA on the phosphorylation of external ADP was ADP transport. When IsoBA was preincubated with rat heart mitochondria for a short period of time before the assay of ADP transport, a mixed type of inhibition was found. However, when preincubation was carried out in the presence of a minute amount of ADP (less than 0.5 μM) before the assay of ADP transport, a typical uncompetitive inhibition was observed. These features are essentially similar to those reported in the preceding paper for the inhibition of ADP transport by BA. The transition from the mixed-type inhibition to the uncompetitive type of inhibition was accompanied by a considerable enhancement of the inhibitory efficiency of IsoBA (Figure 5).

Binding Properties of [^{3}H]IsoBA. As shown in the preceding paper, the high affinity sites for [^{3}H]BA are exhibited more clearly in rat liver mitochondria or rat heart mitochondria pretreated by phosphate. The same observation holds for [^{3}H]IsoBA. The number of high affinity sites for IsoBA was between 0.13 and 0.20 nmol/mg of protein in rat liver mitochondria and about 1 nmol/mg of protein in rat heart mitochondria. However, the affinity was two to four times lower for IsoBA than for BA (Figure 6).

As for BA, IsoBA binding to heart mitochondria was found to be modified by the environmental conditions. Maximum binding capacity of [^{3}H]IsoBA to high affinity sites requires acidic pH. A clear threshold of pH, above which the binding capacity abruptly decreased, was found at pH 7.1 for IsoBA and at pH 6.7 for BA. This slight, but significant, difference may be related to the different pK values of the carboxylic groups of BA and IsoBA. The number of high affinity sites for IsoBA in rat heart mitochondria was increased by 20–30% when micromolar amounts of ADP were added to the medium or when the mitochondria were energized (see preceding paper). These effects were easily reproducible with rat heart mitochondria, but not observed with rat liver mitochondria.

Competition Between BA and IsoBA. As shown in Figures 7A and 7B, BA and IsoBA competed with each other for binding to mitochondria, which indicates that both inhibitors bind to the same site. With rat liver mitochondria, a concentration of BA as low as 0.25 μM inhibited more than 50% of the high affinity binding for [^{3}H]IsoBA, without altering the low affinity binding for IsoBA. On the other hand, IsoBA at a concentration of 0.25 μM was unable to prevent [^{3}H]BA binding; [^{3}H]BA binding was inhibited by ~80% by 27 μM IsoBA. These data are in good agreement with the finding that the affinity of mitochondria for BA is higher than for IsoBA.

Discussion

A compound structurally related to BA has been isolated among the fermentation products of *Pseudomonas cocovenenans* growing on defatted coconut. The same compound was obtained by alkaline treatment of BA. It could be easily separated from BA by silica gel thin-layer chromatography. The new compound has the same uv spectrum as BA and a similar ir spectrum; however, it differs from BA by its NMR spectrum. The differences in NMR data bear essentially on protons located at the dicarboxyl end; they are interpreted to mean that the new compound is a geometrical isomer of BA, differing from BA by the configuration of the dicarboxyl end. By analogy with NMR data for the *cis*- and *trans*-dimethyl-β-glutaconic acids, it is concluded that, whereas the carboxyl groups are trans in BA (De Bruijn et al., 1973), they are cis in IsoBA.

Referring to geometrical dicarboxylic isomers, for example, maleic acid and fumaric acids, there is a greater spread between the pK values in the cis dicarboxylic acid than in the trans isomer. The larger difference between the end points of titration of the carboxyl groups in IsoBA as compared with BA

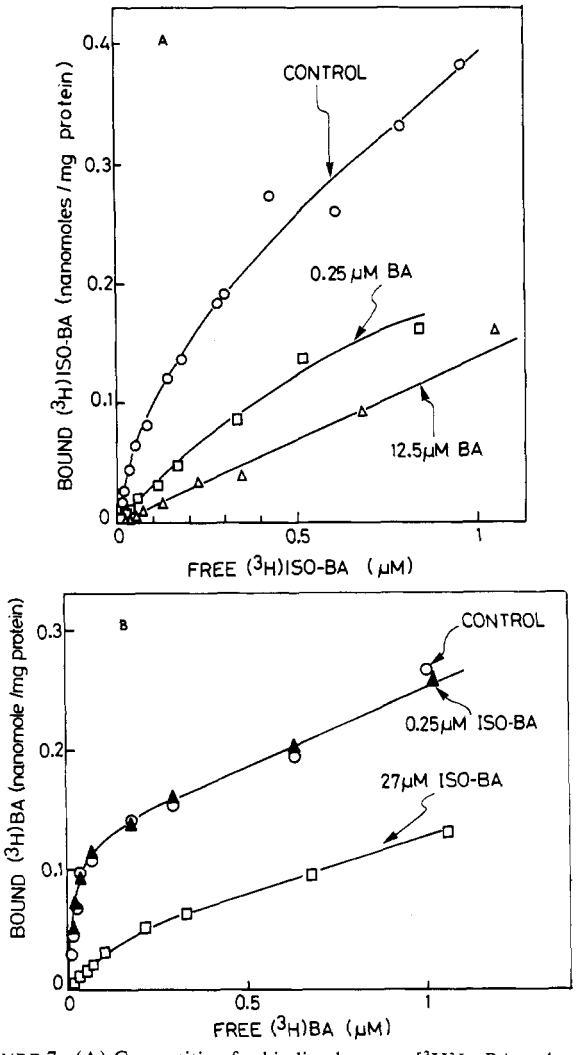

FIGURE 7: (A) Competition for binding between [^{3}H]IsoBA and unlabeled BA. Rat liver mitochondria (1 mg) were incubated for 3 min at 20 °C and 30 min at 2 °C with different amounts of [^{3}H]IsoBA and two different fixed concentrations of unlabeled IsoBA. Other experimental conditions are as in Figure 6. (B) Competition for binding between [^{3}H]BA and unlabeled IsoBA. Same conditions as in Figure 6.

is consistent with the conclusion, based on NMR data, that the terminal dicarboxyl group has the cis configuration in IsoBA and the trans configuration in BA.

IsoBA as well as BA must be protonated to inhibit ADP transport and bind to mitochondria with high affinity. It has been shown in the preceding paper that protonated BA is able to enter the inner mitochondrial membrane. It is most likely that IsoBA behaves as BA in this respect. BA and IsoBA possess similar features of binding and inhibition (same number of high affinity binding sites, same enhancing effect of ADP and of energization on the high affinity binding capacity, and same type of inhibition). They only differ by their affinity to mitochondria, the binding affinity of IsoBA being two to four times less than that of BA. This difference in affinity is obviously related to the configuration of the dicarboxyl end of the molecules. In this respect, it is interesting to recall that BA, after complete hydrogenation, becomes a much less effective inhibitor of ADP transport (Kemp et al., 1971). From these results, it appears that all portions of the BA molecule play a role in the inhibitory efficiency.

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