GUMMIFERIN, AN INHIBITOR OF THE ADENINE-NUCLEOTIDE TRANSLOCATION. STUDY OF ITS BINDING PROPERTIES TO MITOCHONDRIA

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1. Introduction

Atractyloside is known to be a competitive [1, 2] and specific inhibitor of the adenine-nucleotide translocation [2–8] across the inner [9–11] mitochondrial membrane. In the course of extracting atractyloside from the thistle *Atractylis gummifera*, another glycoside having solubility properties similar to those of atractyloside was isolated [12]. Until its structure and its possible relationship to atractyloside could be definitively established we proposed to name it *gummiferin* [12].

In this report gummiferin is shown to be as potent and specific an inhibitor of the ADP-translocation as atractyloside. But whereas the inhibition of the ADP-translocation by atractyloside is reversed by high ADP concentrations, the gummiferin-dependent inhibition is not. The binding properties of (^{35}S)-gummiferin to rat liver mitochondria were compared to those of (^{35}S)-atractyloside taken as reference [11]. The fractional saturation of mitochondria with gummiferin was found to parallel the fractional inhibitory effect of gummiferin on the ADP translocation; both the inhibition curve and the saturation curve are sigmoidal. The strong cooperative interactions observed during (^{35}S)-gummiferin binding – up to saturation – are increased by added ADP and decreased by atractyloside, but neither ADP nor atractyloside alter the number of

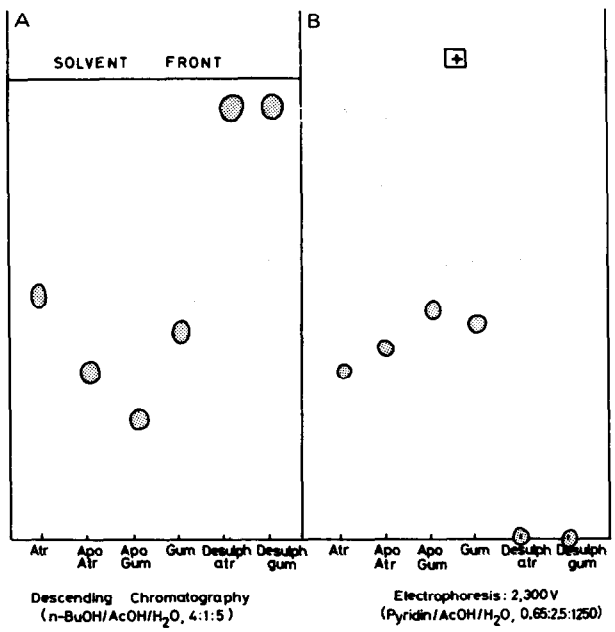

Fig. 1. Chromatographic and electrophoretic separation of atractyloside (Atr), apo-atractyloside (Apo-Atr), gummiferin (Gum), apo-gummiferin (Apo-gum), desulphated atractyloside and desulphated gummiferin. The spots give a pink color with vanillin [13] (spray: 0.5 g of vanillin in 50% phosphoric acid). (A) Descending chromatography on Whatman no. 3 paper in *n*-butanol/acetic acid/water, 4:1:5, by vol., 18 hr. Mean of the R_F values: atractyloside: 0.72, gummiferin: 0.60, apo-atractyloside: 0.45, apo-gummiferin: 0.35. (B) Electrophoresis on Whatman no. 3 paper for 1 hr in pyridin/acetic acid/water, 0.65:2.5:1250, by vol., 66 V/cm, in a Pherograph apparatus.

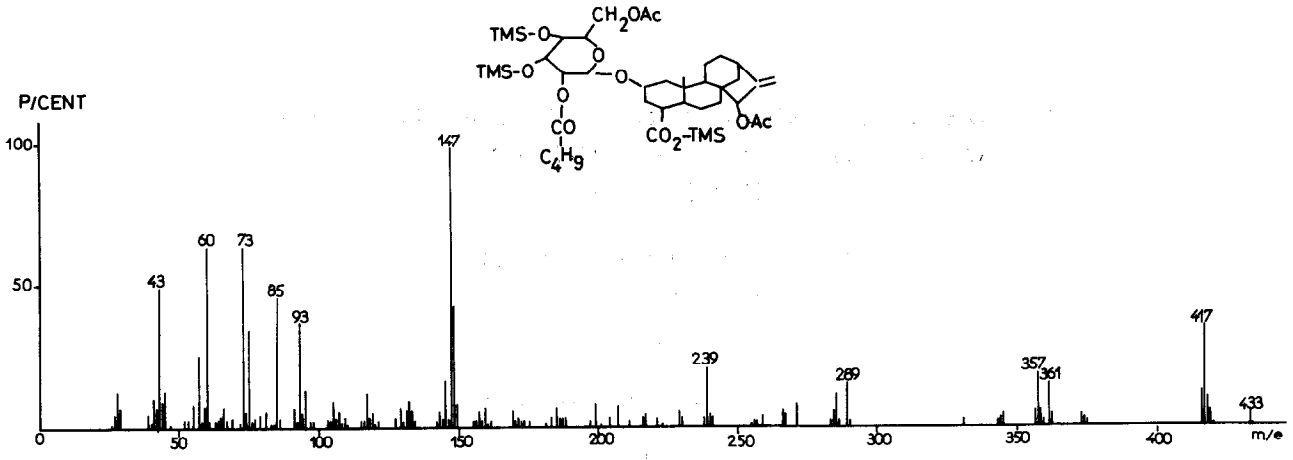

Fig. 2. (A) Mass spectrometry spectrum of silylated and acetylated atractyloside.

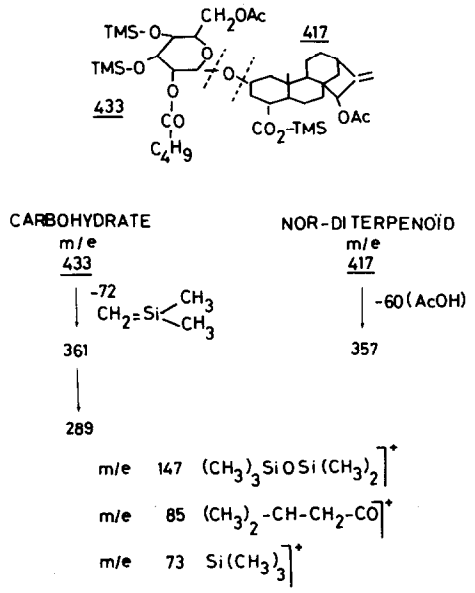

Fig. 2. (B) Fragmentation of silylated and acetylated atractyloside observed in mass spectrometry. The free hydroxyl groups of atractyloside were acetylated by acetic anhydride in pyridin prior to displacing the two sulphate groups by a silylating mixture (pyridine/hexamethyldisilazane trimethylchlorosilane). AEI (MS-9) Mass Spectrograph.

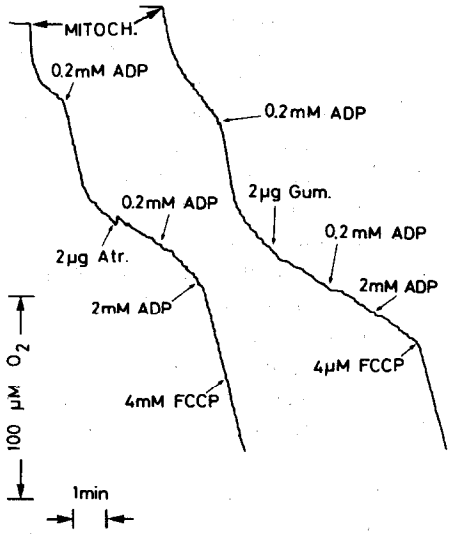

Fig. 3. Effect of atractyloside (Atr) and gummiferin (Gum) on the ADP-stimulated respiration of mitochondria. The reaction medium contained 110 mM KCl, 16 mM phosphate buffer (ph 7.4), 6 mM $MgCl_2$, 10 mM glutamate and 4 mg of mitochondrial protein; other additions: ADP, atractyloside, gummiferin, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) as indicated on the traces. Final volume 2 ml. Temperature 25°.

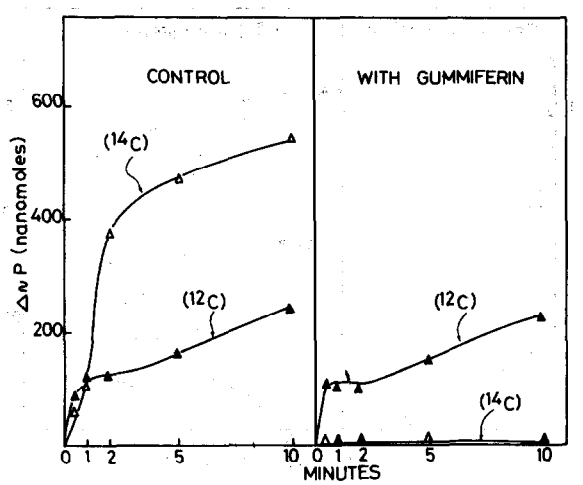

Fig. 4. Inhibition, by gummiferin, of the phosphorylation of added (^{14}C)-ADP as contrasted with the absence of effect on the phosphorylation of internal adenine-nucleotides. Mitochondria (17 mg of protein) were incubated at 2° in 110 mM KCl, 10 mM phosphate, 0.1 mM EDTA and 0.5 mM (^{14}C)-ADP. Gummiferin where present was 5 μM, pH was 7.4 and the total volume 4.0 ml. The incubation was ended by addition of perchloric acid to a final concentration of 2%. After neutralization of the extracts, the amounts of AMP, ADP and ATP were determined according to Adam [16]. The increase of phosphate bonds in adenine nucleotides (~P) was determined as the increase of the sum: ADP + 2 ATP. Labelled adenine nucleotides arising from (^{14}C)-ADP added to mitochondria were separated by paper chromatography [17] and located under UV light. Their respective areas were cut out for determination of their radioactivity by means of a thin window gas flow counter.

gummiferin binding sites. On the contrary, bongkrekic acid produces a net decrease of the number of gummiferin binding sites without interfering significantly with the co-operative interactions.

2. Results

2.1. *Isolation and characterization of gummiferin*

Gummiferin which is recovered together with atractyloside from extracts [12] of the rhizomes of the thistle *Atractylis gummifera* can be separated from atractyloside by paper chromatography or electrophoresis [12] (fig. 1). Gummiferin contains sulfur in its molecule and this allowed the isolation of (^{35}S)-gummiferin from plants that had been grown in the presence of (^{35}S)-sulfate. (^{35}S)-gummiferin recovered from electrophoregrams by elution was estimated by determination of the 50% inhibition of either the ADP-stimulated respiration or the ADP translocation (see below) after having calibrated the systems with purified gummiferin. The specific radioactivity of (^{35}S)-gummiferin (and (^{35}S)-atractyloside) used in this work ranged from 0.3 to 1 × 10^6 dpm/μmole.

Glucose is the only sugar released by total hydrolysis of gummiferin. To further analyze the structure of gummiferin, two types of derivatives were prepared from gummiferin and atractyloside: (1) the apo-derivatives (nomenclature proposed by Piozzi et al. [14] obtained by removal of isovaleric acid by barium hydroxide in dry methanol); (2) the desulphated derivatives obtained by removal of the sulphate groups [15]. Fig. 1 shows the chromatographic and electrophoretic behaviour of those compounds: as for gummiferin with regards to atractyloside, apo-gummiferin is slightly more polar and more negatively charged at pH 4.5 than apo-atractyloside; on the contrary, in these conditions, the migrations of the de-sulphated derivatives of atractyloside and of gummiferin are similar. The mass spectrum obtained from gummiferin is the same as that of atractyloside (fig. 2) [15], a result which indicates that the carbon skeleton of the two compounds is the same.

2.2. *Gummiferin as an inhibitor of the ADP translocation in mitochondria*

As shown in fig. 3, both gummiferin and atractyloside inhibit the mitochondrial respiration stimulated by low concentrations of ADP, but whereas the inhibition by atractyloside of the ADP-stimulated respiration is reversed by high ADP concentrations (2 mM) the gummiferin-dependent inhibition is not. The effects of both compounds on the ADP-stimulated respiration can be taken, in first approximation, as indicative of their interference with the ADP translocation.

Fig. 4 points to the specificity of action of gummiferin which, at concentrations which totally inhibit the oxidative phosphorylation of extra-mitochondrial (^{14}C)-ADP, does not alter the oxidative phosphorylation of the intra-mitochondrial (^{12}C)-ADP. This result indicates that gummiferin prevents the

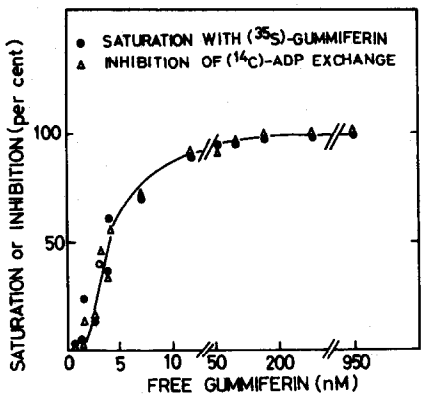

Fig. 5. Relationship between the saturation of the gummiferin binding sites and the inhibition of the ADP translocation by gummiferin. (1) Adenine-nucleotide translocation: Rat liver mitochondria were pre-loaded with (^{14}C)-adenine nucleotides as follows: mitochondria (20 mg/ml of 0.27 M sucrose) were incubated with 30 μM (^{14}C)-ADP for 15 min at 4° then sedimented by centrifugation, washed twice and resuspended in 0.27 M sucrose. The labelled mitochondria were then incubated for 45 min at 2° with different concentrations of gummiferin (up to 11.5 μM) in series of tubes (6.6 mg mitochondrial protein per tube) containing 10 ml of 110 mM KCl, 6 mM $MgCl_2$ and 10 mM Tris-sulfate, pH 7.2. The adenine-nucleotide translocation assay was then performed as follows: 2 μ, of a 0.5 ml aliquot. The rate of translocachondrial suspension and the incubation was ended after a period of 18–20 sec by filtration through millipore filter HAWP, 0.45 μ, of a 0.5 ml aliquot. The rate of translocation in the absence of gummiferin was 6.1 nmole of (^{14}C)-ADP/min/mg protein. (2) (^{35}S)-gummiferin binding: The conditions of incubation were similar to those described for assessing the adenine-nucleotide translocation. Rat liver mitochondria (6.6 mg) were incubated for 45 min at 2° with different concentrations of (^{35}S)-gummiferin (ranging from zero to 11.5 μM) in series of tubes containing 10 ml of 110 mM KCl, 6 mM $MgCl_2$, 10 mM Tris-sulfate, pH 7.2, and 200 μM ADP. The incubation was ended by centrifugation. The mitochondrial pellets were dissolved in 1 ml of formamide at 180° and their radioactivity was determined by liquid scintillation.

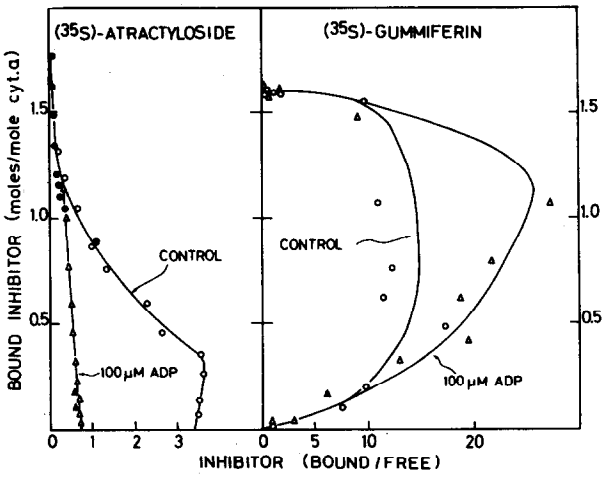

Fig. 6. Scatchard plot of the binding of (^{35}S)-atractyloside and (^{35}S)-gummiferin to rat liver mitochondria. Same conditions of incubation as in fig. 5 (2); 8.5 mg of mitochondrial protein per tube. (^{35}S)-atractyloside concentrations: up to 0.80 μM (○), from 0.13 μM (●); (^{35}S)-gummiferin concentrations: up to 0.96 μM. Since, upon incubation and centrifugation of the mitochondrial suspensions, there is a substantial loss of matrix protein not recovered in the pellets (25 to 30%), bound (^{35}S)-atractyloside and (^{35}S)-gummiferin are correlated to cytochrome *a*. The cytochrome *a* content of the rat liver mitochondrial preparations used was found to be 0.14 ± 0.02 nmole of cytochrome *a*/mg protein.

translocation of external ADP into mitochondria without interfering with the coupling mechanism,

2.3. *Binding properties of gummiferin to mitochondria*

The inhibition of the rate of the ADP translocation caused by increasing concentrations of gummiferin has been compared to the fractional saturation of mitochondria with (^{35}S)-gummiferin. As shown in fig. 5, the saturation curve and the inhibition curve are sigmoidal and strictly superimposable. The fact that the fractional inhibitory effect of gummiferin, on the rate of the ADP translocation, is equal to the fractional saturation of its binding sites confirms that, in the range of the above mentioned concentrations, gummiferin acts selectively on the adenine nucleotide transport in mitochondria.

(^{35}S)-gummiferin binding has been compared to (^{35}S)-atractyloside binding under equilibrium conditions, using mitochondrial preparations from different sources [18]. In this note, results obtained with rat liver mitochondria are presented (fig. 6). The *atractyloside binding curve* consists of two typical regions. The first region which corresponds to small concentrations of added (^{35}S)-atractyloside is characterized by a steep slope which may reflect either a

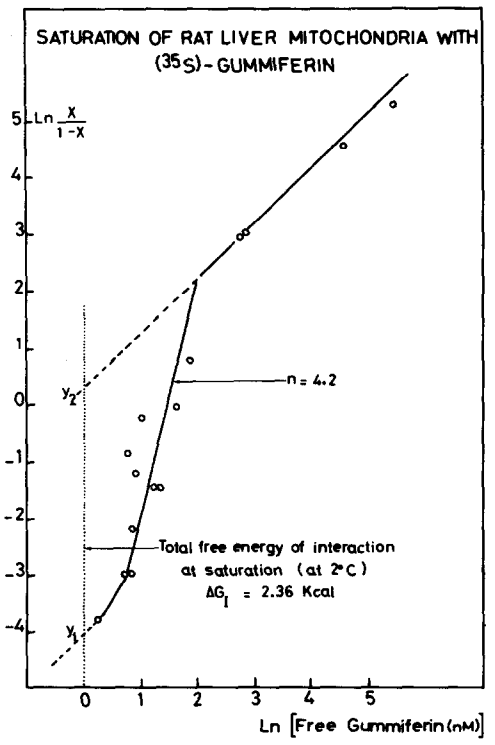

Fig. 7. Hill plot for saturation of rat liver mitochondria with (^{35}S)-gummiferin. The curve is asymptotic to lines of slope 1 at both ends. The total free energy of interaction between zero and full saturation is calculated from the distance y_1-y_2 (intercepts of the asymptotes with the Y axis) multiplied by RT [20].

low affinity binding due to a titration, by atractyloside [11], of the mitochondrial bound ADP and ATP or a positive co-operativity. The second region exhibits a marked curvature which may be interpreted as the result either of negative interactions or of the presence of more than one class of binding sites. Taking the latter alternative restricted to two classes of independent sites, the decomposition of the atractyloside binding curve into 2 slopes of low and high affinity allowed us to calculate a K_d value of about 15 nM for the high affinity sites.

Whereas the saturation of the atractyloside binding sites by (^{35}S)-atractyloside is not yet reached at concentrations of inhibitor as high as 27 μM, *total saturation of mitochondria with (^{35}S)-gummiferin* is attained at gummiferin concentrations less than 1 μM when using 5 to 8 mg of mitochondrial protein. Assuming similar molecular weights for atractyloside and gummiferin, a value of 1.6 mole of bound gummiferin at saturation per mole of cytochrome *a* was found in the experiment reported in fig. 6. Values obtained in five experiments varied from 1.3 to 1.7 mole of bound gummiferin at saturation per mole of cytochrome *a*, values which are in the same range as those reported by Weidemann et al. [19] for the number of ADP binding sites on the ADP carrier.

The binding of (^{35}S)-gummiferin to mitochondria is also characterized by *strong positive co-operative interactions.* Co-operativity indexes higher than 2 were usually found. As an example, from the Hill plot for saturation of rat liver mitochondria with (^{35}S)-gummiferin given in fig. 7, a co-operativity index of 4.2 was determined and a total free energy of interaction of 2.4 kcal per mole of sites at saturation was calculated [20], using Wyman's convention of making ΔG_I positive when the interaction is a stabilizing one, i.e. when $n>1$. This stabilizing energy is to be compared to the total free energy (10.3 kcal/mole, calculated from a K_d value of 6 nM and at 2°) involved in the binding of one mole of gummiferin to rat liver mitochondria (cf. fig. 9B).

As evidenced by experiments to be reported elsewhere, gummiferin selectively binds to the inner mitochondrial membrane as atractyloside does [10]. It is therefore implicit that the gummiferin binding properties, as assessed here with whole mitochondria, concern essentially the inner mitochondrial membrane.

2.4. *Effect of ADP*

The binding affinity of mitochondria for atractyloside is strikingly decreased by added ADP (fig. 6) a result correlative with the fact that ADP in excess reverses the inhibitory effect of atractyloside on the ADP translocation. On the contrary, ADP increases the positive co-operativity for gummiferin binding to mitochondria (fig. 6). Previous experiments carried out with inner mitochondrial membrane vesicles [11, 21] which are virtually depleted of phosphotransferases, indicated that the effect of ADP on atractyloside binding is not shared by other nucleoside diphosphates such as CDP, UDP,

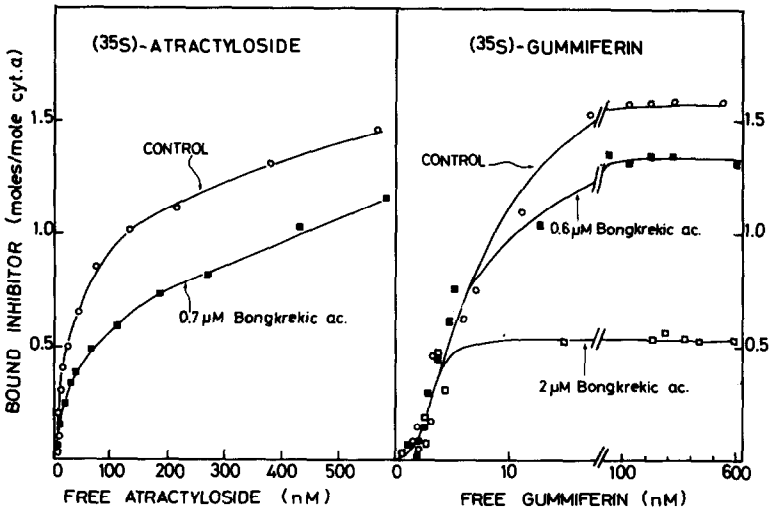

Fig. 8. Effect of bongkrekic acid on atractyloside binding to rat liver mitochondria. Mitochondria (8 mg) were preincubated for 1 min at 20° with bongkrekic acid at the indicated concentrations in series of tubes containing 10 ml of 110 mM KCl, 6 mM $MgCl_2$, 10 mM Tris-sulfate, pH 7.2. The tubes were then immediately chilled at 2° and the incubation was started by the addition of (^{35}S)-atractyloside or (^{35}S)-gummiferin. Other conditions as in fig. 6.

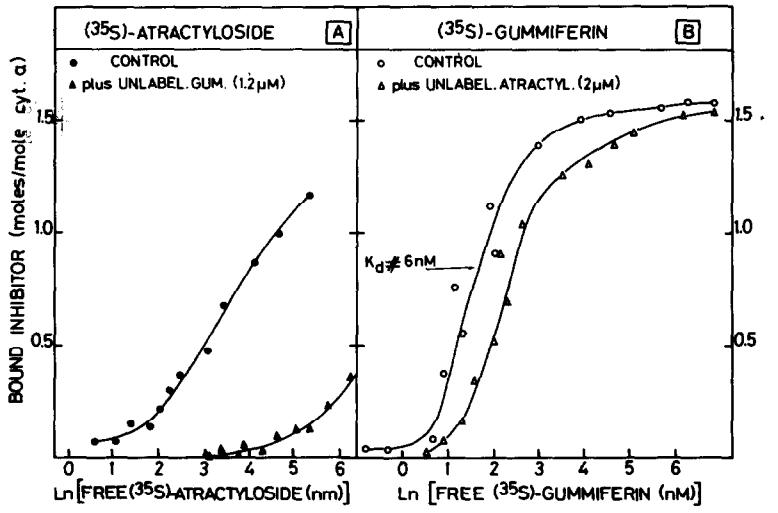

Fig. 9. Effect of gummiferin on the binding of (^{35}S)-atractyloside and, reciprocally, of atractyloside on (^{35}S)-gummiferin binding. Labelled and unlabelled inhibitors were incubated together with mitochondria (8 mg protein/tube). Experimental conditions as in fig. 6.

and GDP; the same observation holds for the effect of ADP on gummiferin binding [18].

2.5. *Effect of bongkrekic acid*

Bongkrekik acid which has been reported first by Henserson and Lardy [22] to inhibit the ADP translocation, does not significantly modify the

binding affinity of mitochondria for atractyloside and gummiferin but unambiguously depresses the total number of atractyloside or gummiferin binding sites (fig. 8).

2.6. *Effect of atractyloside on (^{35}S)-gummiferin binding and of gummiferin on (^{35}S)-atractyloside binding*

Unlabelled atractyloside added in large excess together with (^{35}S)-gummiferin to mitochondria significantly diminishes the degree of positive cooperativity and the affinity for gummiferin but does not interfere with the total amount of bound (^{35}S)-gummiferin at saturation (fig. 9B). Conversely, unlabelled gummiferin displaces bound (^{35}S)-atractyloside, but with a much higher efficiency than atractyloside does in the case of bound (^{35}S)-gummiferin (fig. 9A). The apparent competitivity between atractyloside and gummiferin for binding to mitochondria as illustrated in fig. 9 may be contrasted with the non competitive nature of the inhibition, by bongkrekic acid, of the binding of atractyloside and gummiferin (fig. 8).

3. Discussion

In spite of their similar structures, as revealed by mass spectrometry, gummiferin and atractyloside may be discriminated by their inhibitory effects on the adenine nucleotide translocation and their binding properties to mitochondria. The inhibition of the ADP translocation by gummiferin is not reversed by high concentrations of ADP, a property which is paralleled by the fact that ADP does not displace bound (^{35}S)-gummiferin. In contrast, the inhibition of the ADP translocation by atractyloside is of an apparent competitive type and the affinity of mitochondrial membranes for (^{35}S)-atractyloside is decreased by added ADP.

That gummiferin binds to the ADP carrier (or to a membrane area in the close neighbourhood) is shown by the the following observations: (1) the degree of saturation of the gummiferin binding sites strictly parallels the degree of inhibition, by gummiferin, of the ADP translocation; (2) the amount of gummiferin bound at saturation to mitochondria roughly equals the number of ADP binding sites on the ADP carrier [19]; (3) bound gummiferin is displaced by atractyloside and bongkrekic acid, two other inhibitors of the ADP translocation postulated to bind to the ADP carrier.

It is recognized that oligomeric allosteric enzymes [23] are characterized by homotropic and/or heterotropic interactions and that the co-operativity index relative to ligand binding is indicative of a minimal number of sub-units in each oligomer. The results presented here favor the concept of an oligomeric structure of the ADP carrier which could correspond to clusters of translocase units scattered in the inner mitochondrial membrane. Along this line it may be speculated that the ADP carrier assumes two limit interconvertible configurations, one corresponding to an active form (able to bind and to carry ADP) and the other one to an inactive form. The "active" form of the carrier would be induced by ADP acting both as a substrate for and as an activator of its own carrier. On the other hand, gummiferin would induce a transition from the active to the inactive configuration and would stabilize it irreversibly. In short, ADP and gummiferin may be considered as antagonistic ligands able to displace equilibria between two configurations of the ADP carrier.

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