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STUDIES OF SCHISTOSOMICIDES ANTIMONIALS ON ISOLATED MITOCHONDRIA—I

SODIUM ANTIMONY GLUCONATE (TRIOSTIB)*

ANNIBAL P. CAMPELLO,† DOREI BRANDÃO,‡ MIROSLAU BARANSKI and DINOR O. VOSS†

Instituto de Bioquímica da Universidade Federal do Paraná and Instituto de Biologia e Pesquisas Tecnológicas (Div. Bioquímica), P.O. Box 939, Curitiba, Paraná, Brazil

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Abstract—The effects of sodium antimony gluconate (Triostib) on oxidative phosphorylation and oxidation of succinate, glutamate and α-ketoglutarate by rat liver mitochondria were studied. Oxidative phosphorylation and oxidation of NAD^+-linked substrates by liver mitochondria were depressed by $4{\cdot}14 \times 10^{-3}$ M Triostib. Oxidative phosphorylation accompanying succinate oxidation was not significantly affected. Submitochondrial particles with NADH-oxidase activity were prepared and the NADH oxidation inhibited by Triostib was restored in the presence of methylene blue. It is hypothesized that Triostib acts at the NAD^+-oxidase segment of the chain, its site of action being localized between NAD^+ and the flavoprotein.

DESPITE numerous reports showing the toxic effects of antimonials upon laboratory animals and man,[1–4] a cursory search of the literature revealed that the mechanism of action of these compounds has not been entirely elucidated. A very important aspect of the mechanism of action of antimonials was established by Braun *et al.*,[5] Gammil *et al.*,[6] and by Eagle *et al.*,[7] who demonstrated the protective effect of 2,3-dimercapto-1-propanol (BAL) in the experimental intoxication produced by antimony compounds. Medina and Bacila[8] suggested that the electron transport between succinate and cytochrome b is inhibited, possibly by the inhibition of the succinic dehydrogenase. In a previous paper,[9] it was reported that Triostib inhibited the oxidation of glutamate and α-ketoglutarate in rat liver and heart mitochondria, but not that of succinate. The present paper shows the possible action of sodium antimony gluconate (Triostib) on the properties of isolated rat liver mitochondria.

EXPERIMENTAL PROCEDURE

Preparation of mitochondria. Rat liver mitochondria were prepared according to the method described for brain mitochondria by Voss *et al.*,[10] using a mannitol–sucrose medium. This medium contained 0·21 M mannitol, 0·075 M sucrose, 0·01 M Tris and 0·2 mM EDTA. The final pH was 7·4.

Methods of assay. The polarographic determinations of respiration and oxidative phosphorylation were made with an oxygen electrode as described by Voss *et al.*[11]

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‡ Departamento de Bioquímica, Universidade de Campinas, Campinas, S.P. Brazil.

The uptake of oxygen was measured by two different techniques, namely, the polarographic method of the oxygen electrode and Warburg respirometry. The first technique expresses the rate of oxygen uptake in micromoles of O_2 per second per liter, while by Warburg respirometry the values are expressed as microliters of O_2. The oxygen uptake measured polarographically was based on the consumption of oxygen from an air-saturated solution of water. The P/O ratios were calculated as ADP/O ratios according to the method of Chance and Williams[12] for assaying oxidative phosphorylation. The ADP/O ratios were calculated from the uptake of oxygen in microatoms per liter during the active state of respiration and the molar concentration of ADP was added. The respiratory control coefficients were calculated as the ratios of respiratory rates with ADP and the control respiration after ADP was consumed. Protein was determined by the method of Lowry *et al.*[13]

Particles with NADH-oxidase activity. Submitochondrial particles with NADH-oxidase activity[14] were prepared by adopting the procedure described by Kielley and Kielley[15] for preparation of ATPase. Mitochondria from 40 g liver were suspended in 55 ml of 0·003 M phosphate buffer, pH 7·5, and treated for 2 min in the cold with a Sorvall Omni-mixer homogenizer with micro-attachment (24,000 rpm). Large particles were removed by centrifugation in a model L Spinco ultracentrifuge, no. 40 rotor, at 20,000 *g* for 10 min. The supernatant solution was recentrifuged at 105,000 *g* for 30 min, and the sediment was resuspended in 0·003 M phosphate buffer, pH 7·5. The suspension was used in the NADH-oxidase assay.

Triostib, containing 30 per cent of trivalent antimony, was obtained from Burroughs Wellcome Company, London.

RESULTS

Effect of Triostib on the normal properties of rat liver mitochondria. The effect of Triostib on rat liver mitochondria was assayed polarographically using the oxygen electrode (Table 1). Aerobic mitochondria were added with 10 μmoles of either

TABLE 1. EFFECT OF TRIOSTIB ON THE NORMAL PROPERTIES OF RAT LIVER MITOCHONDRIA*

Triostib	Substrate respiration	Respiratory rate during ADP (285 μmoles)	Respiratory rate after consumption of ADP	Respiratory control coefficient	ADP/O
		Substrate: α-ketoglutarate			
None	0·65	1·96	0·65	3·0	2·6
$1{\cdot}38 \times 10^{-3}$ M	0·62	1·57	0·62	2·5	2·7
$2{\cdot}76 \times 10^{-3}$ M	0·56	0·78	0·56	1·3	
$4{\cdot}14 \times 10^{-3}$ M	0·56	0·56	0·56	1·0	
		Substrate: glutamate			
None	0·65	1·96	0·65	3·0	3·3
$4{\cdot}14 \times 10^{-3}$ M	0·37	0·37	0·37	1·0	
		Substrate: succinate			
None	0·78	1·96	0·78	2·5	2·2
$4{\cdot}14 \times 10^{-3}$ M	0·65	1·96	0·65	3·0	2·2

* System: 2·2 ml of aerobic medium containing: mannitol, 0·25 M; Tris, 0·01 M; EDTA, 0·2 mM, phosphate buffer, 0·005 M, pH 7·4; KCl, 0·01 M (final pH was adjusted to 7·4); 10 μmoles substrate; suspension of mitochondria containing 4·08 mg protein (glutamate or α-ketoglutarate) or 2·72 mg protein (succinate). Preincubation time, 2 min; total volume, 2·4 ml. Figures mean μmoles $O_2 \cdot sec^{-1}$, $liter^{-1}$.

α-ketoglutarate, glutamate or succinate, and the rate of oxygen uptake was determined (0·65 μmole $O_2 \cdot sec^{-1}$, $liter^{-1}$). This was followed by the addition of 285 μmoles ADP, which increased the rate of oxygen uptake to 1·96 μmoles $O_2 \cdot sec^{-1}$, $liter^{-1}$. When the ADP added was consumed, the rate of oxygen uptake leveled off to 0·65 μmole $O_2 \cdot sec^{-1}$, $liter^{-1}$. The respiratory control coefficient, calculated as the ratio between the respiration during ADP and after ADP, gave a value of 3·0.

The oxygen uptake by aerobic mitochondria pre-incubated for 2 min with increasing amounts of the antimonial was progressively inhibited when α-ketoglutarate was the substrate (Table 1). There was a clear effect of Triostib on the respiratory control coefficient, which indicates that oxidative phosphorylation was increasingly inhibited after the uncoupling of the mitochondria. The same was true for glutamate; however, when succinate was used as the substrate, no effect was observed on oxidative phosphorylation.

Effect of Triostib on the respiration by rat liver mitochondria. Respiration of rat liver mitochondria was studied in classical Warburg experiments with different substrates by measuring the effects of Triostib added to respiring systems in Warburg respirometers (Table 2).

Effect of methylene blue in restoring NADH oxidation inhibited by Triostib. Since the oxidation of NAD^+-linked substrates was inhibited while that of succinate was not

TABLE 2. EFFECT OF TRIOSTIB ON THE OXYGEN UPTAKE OF RAT LIVER MITOCHONDRIA*

Substrates	μl O_2/ 30 min	% Inhibition
Control	47	
Control + Triostib	14	70·2
Succinate	298	
Succinate + Triostib	210	29·5
Glutamate	216	
Glutamate + Triostib	45	79·2
α-Ketoglutarate	164	
α-Ketoglutarate + Triostib	29	82·4

* System: to 1·45 ml medium containing: mannitol, 0·25 M; Tris, 0·01 M; EDTA, 0·2 mM; phosphate buffer, 0·005 M, pH 7·4; KCl, 0·01 M (final pH was adjusted to 7·4) was added 0·5 ml of a suspension of rat liver mitochondria, 0·2 ml of a solution containing 10 μmoles substrate, $4{\cdot}14 \times 10^{-3}$ M Triostib and 0·15 ml KOH (20%) in the center well. Total volume, 2·4 ml; temperature, 37°; equilibrium time, 10 min; 90/100 shakings/min. The protein content was equal to 16·5 mg/ml in the original suspension.

TABLE 3. EFFECT OF METHYLENE BLUE IN RESTORING NADH OXIDATION INHIBITED BY TRIOSTIB*

Substrates	Oxygen uptake (μmoles $O_2 \cdot sec^{-1}$, $liter^{-1}$)
NADH + NADH-oxidase	1·30
TR + NADH + NADH-oxidase	0·34
MB + TR + NADH + NADH-oxidase	0·87
TR + NADH + NADH-oxidase + MB	0·87

* System: phosphate buffer, 0·05 M, pH 7·5; NADH, $3{\cdot}3 \times 10^{-4}$ M; NADH-oxidase (3·37 mg protein); methylene blue (MB), $4{\cdot}1 \times 10^{-5}$ M; Triostib (TR), $3{\cdot}4 \times 10^{-3}$ M. Total volume, 2·4 ml.

affected, it was assumed that Triostib acts probably at the NADH-oxidase segment of the chain. It was felt that it was possible to locate further the site of blockade by preparing submitochondrial particles with NADH-oxidase activity and testing the effect of methylene blue in restoring NADH oxidation inhibited by Triostib (Table 3).

DISCUSSION

The studies on the normal properties of rat liver mitochondria assayed polarographically, when aerobic mitochondria were preincubated for 2 min with Triostib. showed that there was a clear effect of the antimonial on the respiratory control coefficient when glutamate or α-ketoglutarate was the substrate. When succinate was the substrate, the respiratory control coefficient was not inhibited (Table 1). These results are an indication that oxidative phosphorylation is inhibited after the uncoupling of the mitochondria.

Respiration by rat liver mitochondria in the presence of glutamate or α-ketoglutarate was highly sensitive to the action of Triostib (79·2 and 82·4 per cent inhibition respectively; Table 2). A similar effect with succinate as the substrate was obtained by Lindahl and Öberg[16] with rotenone as inhibitor. The effect of Triostib on the oxidation of succinate by liver mitochondria was quite different from that described by Medina and Bacila[8] using another antimonial (sodium antimony III; bispirocathecol-2,4-dissulfonate). These authors obtained complete inhibition of succinate oxidation.

The inhibition of NADH oxidation was reversed by the addition of methylene blue (Table 3). This indicates that the block is situated on the oxygen side of the NADH-oxidase. Since the oxidation of NAD^+-linked substrates was inhibited and since Triostib has no effect on the aerobic oxidation of succinate, it was assumed that this antimonial acts, like rotenone,[16–18] in the NADH-oxidase segment of the chain, its site of action being localized between NAD^+ and the flavoprotein.

The selective action of Triostib on NADH-oxidase activity raises the question about the relationship between this drug–enzyme interaction and the chemotherapeutic effect of the antimonial in schistosomiasis. Although the tissue concentrations after the administtation of toxic doses of Triostib are several orders of magnitude lower than those inhibiting NADH-oxidase, this can not explain the mechanism of action of the antimonials in biochemical terms. However, this in no way invalidates the conclusion that Triostib, at the concentrations used in this study, inhibits the activity of NADH-oxidase between NAD^+ and flavoprotein.

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