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THE EFFECT OF γ,γ-*BIS*-4-ETHYLPHENYL-α,β-DIBROMOISOCROTONIC ACID ON EHRLICH ASCITES CARCINOMA AND YEAST CELLS

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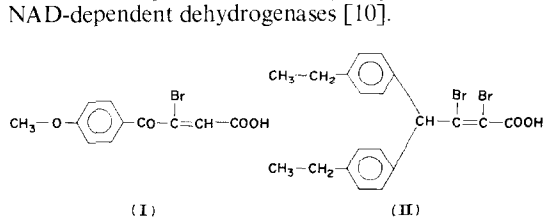
Abstract—In Ehrlich ascites carcinoma (EAC) cells γ,γ-*bis*-4-ethylphenyl-α,β-dibromoisocrotonic acid (EPBC) inhibits after short exposure the incorporation of [^{14}C]valine, [^{14}C]adenine and [^{14}C]thymidine, the rate of inhibition being directly related to the concentration of the inhibitor. This inhibition occurs even at concentrations of EPBC which stimulate the endogeneous respiration of the cells as well as the consumption of glucose and the formation of lactate. Higher concentrations fully depress the endogeneous respiration and glycolysis and cause loss of transplantability of EAC cells. The attack on glycolysis is multitarget in character. The activities of several enzymes dependent on free sulfhydryl groups, especially glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase and phosphofructokinase, are inhibited by EPBC. The changes in the total sulfhydryl group content and other findings suggest the existence of direct chemical reaction of EPBC with thiol groups. The same mechanism may explain also the effect of EPBC on glycolysis in yeast cells.

cis-β-4-Methoxybenzoyl-β-bromoacrylic acid (MBBA, I) has been used in gynecology for the therapy of carcinomas [1–4]. It inhibits the incorporation of formate into purines, and the activity of tetrahydrofolic acid formylase in extracts of pigeon heart [5], and the activity of succinate dehydrogenase (EC 1.3.99.1) in a system from rat liver [6]. MBBA decreases the endogeneous respiration of rat liver homogenates and of Yoshida ascites sarcoma cells [7]. Recently the efficient antineoplastic activity of the γ,γ-*bis*-4-ethylphenyl-α,β-dibromoisocrotonic acid (EPBC, II) has been reported [18]. This substance inhibited the growth of some experimental tumors such as adenocarcinoma, Ehrlich's ascites carcinoma, sarcoma S 37, Krebs ascites carcinoma as well as Yoshida sarcoma. The experiments *in vitro* showed that EPBC inhibited the incorporation of amino acids into proteins of tumor cells and influenced the metabolism of nucleic acids; its inhibitory effect on enzymes involved in metabolism of $C_{(1)}$-substances has been shown [9]. EPBC inhibited also the activity of succinate dehydrogenase and some NAD-dependent dehydrogenases [10].



(I) (II)

We have studied the effect of MBBA as well as of EPBC on the metabolic processes in Ehrlich's ascites carcinoma (EAC) cells *in vitro* and in yeast. The results suggested that the inhibitory effect of both substances on biosynthetic processes might be explained by a depression of the energy yielding and catabolic processes. This effect is supposed to be the result of interaction of the inhibitors with sulfhydryl groups of the enzymes involved. However, EPBC is a more potent inhibitor than MBBA. This paper deals with the effects of EPBC.

MATERIALS AND METHODS

Materials. EPBC (chromatographically pure) was a gift from Dr. M. Semonský, Research Institute of Pharmacy and Biochemistry, Praha. L-[U-^{14}C]valine (0·1 mCi per 0·094 mg), [2-^{14}C]thymidine (0·1 mCi per 0·55 mg) and [8-^{14}C]adenine sulfate (0·1 mCi per 0·307 mg) were supplied by the ÚVVVR, Praha. Chemicals and enzymes used for the determination of enzyme activities as well as for the determination of glucose and lactate were supplied by Boehringer Mannheim, except for hexokinase which was obtained from Sigma, St. Louis. 5,5'-Dithio-*bis*-(2-nitrobenzoic) acid was supplied by Fluka, Buchs. Dimethylsulfoxide (DMSO), vitamins and all other chemicals were purchased from Lachema, Brno.

Tumor cells and the yeasts. EAC cells were maintained and estimated by the method described in a previous paper [11]. The tumor cells were obtained from the peritoneal cavity of mice on the 7th or 8th day after transplanation. The cells were suspended in Krebs–Ringer phosphate buffer pH 7·4 without calcium but with added ascitic serum (2·5% v/v) and glucose (final concentration 3·0 mM). The number of the cells was adjusted to 3×10^6 per ml of the medium. Cell concentration was calibrated against the optical density of the cell suspension [11]. The endogeneous respiration of EAC cells was measured as described below. *Candida albicans* PN 10 and *Saccharomyces cerevisiae* DT XII (from Dept. Microbiol. Biochem., Slovak Polytechnical University, Bratislava) were maintained on malt agar slants and cultured in a synthetic medium containing ammonium sulfate and glucose, as the only sources of carbon and nitrogen, and mineral salts and

vitamins as described elsewhere [12]. The aerobic cultivation on a reciprocal shaker was stopped when the exponential phase of yeast growth was reached (O.D. $\frac{1\text{ cm}}{650\text{ nm}}$ = 0·2–0·3). In all experiments the EPBC was applied as a solution in DMSO, the most suitable solvent for this type of experiment [14]. The final concentration of DMSO never exceeded 1 per cent.

Respiration and glycolysis. The inhibitory effect of EPBC on the endogeneous respiration was determined on the basis of oxygen consumption in the saline–phosphate medium (154 mM NaCl, 6·2 mM KCl and 11 mM sodium phosphate buffer pH 7·4) [13]. The inhibitor dissolved in dimethylsulfoxide was mixed with 2·0 ml of this medium and 200 μl of the cell suspension in the same buffer solution were added. The concentration of the cell suspension consuming approx. 80 μmoles.min^{-1} of oxygen was chosen as standard (13·0–13·3 mg cells dry weight per 2 ml). In control experiments DMSO replaced the inhibitor solution. Rates of oxygen uptake were measured polarographically with a Clark-type oxygen electrode in a thermostatically controlled reaction vessel equipped with a stirring device [13]. Glucose and lactate concentrations were determined in the supernatant obtained after 1 ml of the EAC cells suspension was precipitated with 1 ml of 0·6 M perchloric acid (PCA) in an ice bath [12].

Enzyme assays and SH groups determination. The activities of the enzymes in crude extracts [15] were determined by the Warburg optical test in 1 cm cuvettes at room temperature. The absorbance change of nicotinamide adenine dinucleotide was determined at 340 nm, using a Specord UV VIS spectrophotometer (Zeiss, Jena). Assay conditions were selected to ensure zero-order kinetics. Assay solutions (3 ml) were as follows. For hexokinase (HK, EC 2.7.1.1): 6·65 mM glucose, 0·7 mM ATP, 0·6 mM NADP, 4 mM $MgSO_4$, 6 μg glucose-6-phosphate dehydrogenase (G-6-PD), 46 mM triethanolamine. HCl, pH 7·6. For D-glyceraldehyde-3-phosphate dehydrogenase (GAPD, EC 1.2.1.12): 4 mM 3-phosphoglycerate, 0·7 mM ATP, 0·2 mM NADH, 10 μg phosphoglyceratekinase, 0·5 mM reduced glutathione, 4 mM $MgSO_4$, 46 mM triethanolamine. HCl, pH 7·6. For phosphofructokinase (PFK, EC 2.7.1.11): 3 mM fructose-6-phosphate, 0·3 mM ATP, 3 mM cysteine, 4 mM $MgSO_4$, 40 μg aldolase, 20 μg of a mixture of glycerophosphate dehydrogenase–triosephosphate isomerase, 50 mM triethanolamine. HCl, pH 8·0. Lactate dehydrogenase (LD, EC 1.1.1.27) and G-6-PD (EC 1.1.1.49) were assayed with Biochimica Test Combination (Boehringer, Mannheim) and yeast aldolase (AD, EC 1.1.1.1) according to Ref. [16]. The concentration of thiol groups (T-SH) in sonicates was determined photometrically at 412 nm using Ellman's reagent [15]. The cells were separated by centrifugation at 1000 *g* for 10 min at 2–4°. Sonic disintegrator, VUMA UG 100 TD, (Nové Mesto and Váhom) was used to homogenize cells and the Specord UV VIS spectrophotometer (Zeiss, Jena) to measure the concentration of T-SH. Enzyme activity and T-SH content of yeast cells was determined as described in Ref. [16].

Incorporation of radioactive precursors. The incorporation of ^{14}C-precursors was measured after incubation in the same sample in which the consumption of glucose and the formation of lactate were determined. Six Erlenmeyer flasks containing the suspension of EAC cells (50 ml, 3 × 10^6 per ml) were placed in an ice bath and 500 μl DMSO with various concentrations of the inhibitor were added. From each flask three series were prepared each comprising six test-tubes with 1·0 ml of the suspension. In the first series the incorporation of adenine, in the second valine and in the third the incorporation of thymidine were studied. For these experiments 50 μl of [8-^{14}C]adenine sulfate (0·31 μCi per 1·7 μg), 50 μl of [^{14}C]valine (0·275 μCi per 4·44 μg) and 100 μl of [2-^{14}C]thymidine (1·0 μCi per 5·5 μg) were added to the respective test-tubes.

The remaining samples of EAC cell suspensions were used to measure the rate of glycolysis, the enzyme activities and the transplantability of these cells after 30 and 120 min of incubation.

The samples were incubated in a water bath at 37°. At chosen intervals they were transferred to an ice bath and 1 ml of 5% trichloroacetic acid (TCA) was added. The samples were filtered through Sympor 1 membrane filters, pore size 4 μm (Synthesia, Praha), the precipitate washed with 10 ml of cold 2·5% TCA and 10 ml water and dried at 105°. The radioactivity was measured on a methane flow counter (Frieseke and Hoepfner GmBH, FHT 200, Erlangen). The yeast cells were examined using the method described earlier [17].

Transplantability of EAC cells. The suspensions of EAC cells used to determine the incorporation of ^{14}C-precursors were incubated with various concentrations of the inhibitor for 120 min. Then 10 ml of the cell suspension was centrifuged for 5 min at 500 *g* and the pellets resuspended in 3 ml of sterile saline solution. 0·5 ml (5 × 10^6 cells) of this suspension was immediately injected intraperitoneally into each of five mice. The growth of the belly and the weight increase of the animals were measured for 30 days. In the case of death an autopsy was performed. If no tumors were found after 30 days, the cells were considered to have lost the ability of transplantation. Untreated EAC cells caused the death of animals 15 ± 2 days after transplantation.

Manometric measurements. A culture of *Candida albicans* growing aerobically on synthetic medium at 28° was used for manometric measurements. The culture was diluted with fresh medium to obtain O.D. $\frac{1\text{ cm}}{650\text{ nm}}$ = 0·075 and 3 ml of this culture was put in the main compartment of the Warburg vessel. The inhibitor dissolved in 0·1 ml DMSO was added to two vessels. In the center well of one vessel there was 0·2 ml 20% potassium hydroxide and in the second vessel the same volume of water; the incubation was performed for 10 min at 28°. The pre-incubation with the inhibitor was carried out for 15 min.

RESULTS

Effects on glycolysis and respiration. The glycolysis as well as the endogeneous respiration of EAC cells can be inhibited fully by EPBC (Figs. 1 and 2). The 50% inhibition of respiration, glucose consumption and lactate formation was obtained with concentrations of the inhibitor ranging from 230 to 265 μM (ID_{50} values). Lower concentrations stimulated these processes. At 90–100 μM EPBC, a rather high respiration and glycolysis rate exceeding that of control was observed.

Figure 3 shows the dose and time dependence of the effect of EPBC on glucose consumption and lactate

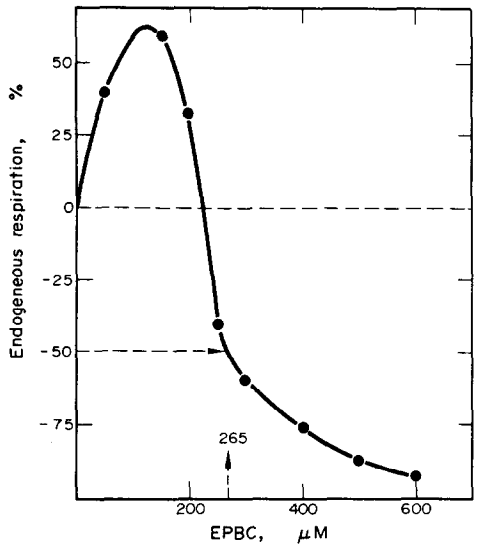


Fig. 1. Effect of EPBC on the endogeneous respiration of EAC cells. The experimental conditions were as described in the text. The oxygen uptake in the control experiment (without inhibitor) was 80 m μmoles per min. The value of ID_{50} is 265 μM.

formation in EAC cells. At doses exceeding 250 μM glycolysis was fully inhibited but, with different initial velocities dependent on inhibitor concentration. This inhibition is permanent (measured also after 5 and 10 hr).

Effects on macromolecule biosynthesis and transplantability. EPBC inhibited incorporation of [^{14}C]valine into proteins and, generally parallel but somewhat less, that of [^{14}C]adenine and [^{14}C]thymidine into nucleic acids. The curves illustrating the dependence of incorporated radioactivity on the log of concentration of EPBC are practically identical for both precursors (Fig. 4). Therefore, the ID_{50} values of EPBC (molar concentrations of EPBC required for 50% reduction of the incorporation rate) are very close for both precursors. The ratio ID_{50} adenine/ID_{50} leucine is 1·28. Such a ratio is characteristic for the known inhibitors iodoacetate and dinitrophenol as well as for other new biologically active compounds, which interfere with carbon–energy metabolism [18, 19].

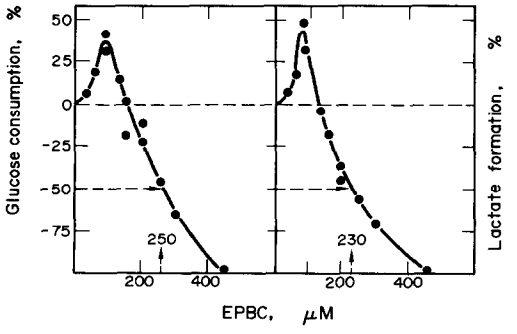


Fig. 2. Effect of EPBC on glucose consumption and lactate formation in EAC cells after 60 min incubation. The experimental conditions were as described in the text. In the control sample the glucose consumption was 1·125 μmole per 1 ml per 3 × 10^6 cells. The conversion of glucose to lactate was 87 per cent.

The kinetics of the incorporation of ^{14}C-labelled valine, adenine and thymidine into EAC cells when inhibitor in concentrations 12·5–360 μM were used are shown in Fig. 5. The complete inhibition of incorporation of all precursors was reached at the concentration 360 μM EPBC. This concentration completely depressed the glycolysis and caused loss of transplantability of cells. At a concentration of 180 μM EPBC did not cause the loss of transplantability, but the animals died in a prolonged period after transplantation (15 ± 2 days in control, 23 ± 5 days in experiment). The cells treated with the inhibitor at this concentration showed a weakly inhibited glycolysis and considerably inhibited biosynthetic processes (see Figs. 3 and 5; transplantation after 2 hours incubation of cells with inhibitor). Lower concentrations of EPBC did not alter the transplantability of cells, but exhibited significant dose–response changes of incorporation of ^{14}C-precursors (inhibition), glycolysis and respiration (stimulation; see Figs. 1, 2 and 4).

Effects on enzyme activities and thiol content. The data presented in Table 1 illustrate the dose and time dependence of EPBC inhibition of four glycolytic

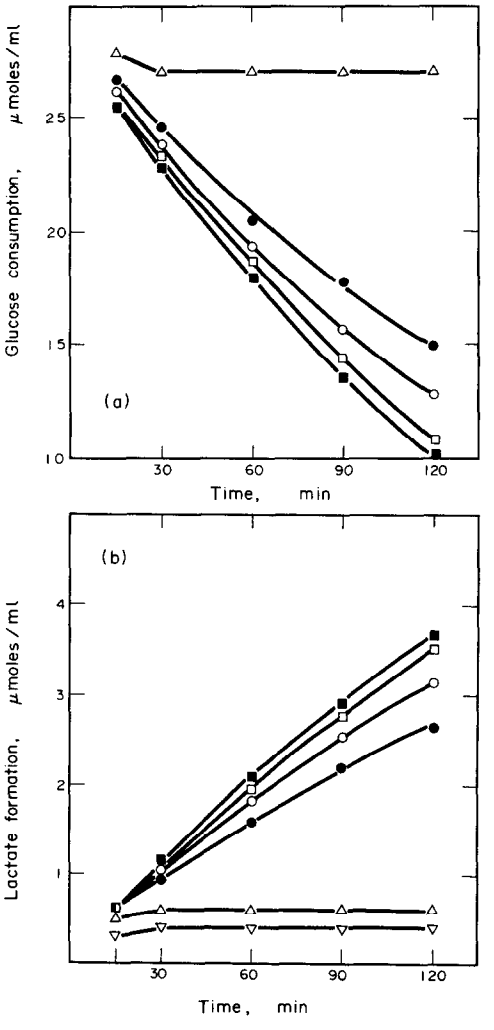


Fig. 3. Inhibition of the glycolysis of EAC cells by EPBC. Concentrations of EPBC: 720 μM (▽); 360 μM (△); 120 μM (●); 90 μM (□); 45 μM (■) and without EPBC (○).

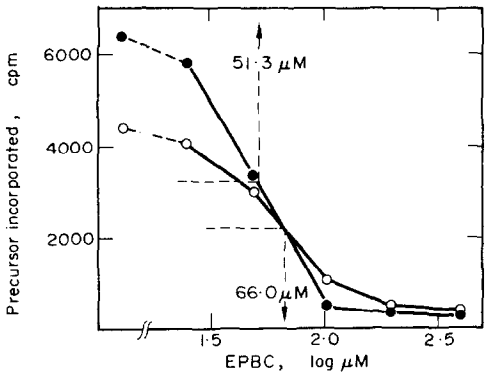


Fig. 4. Inhibition of incorporation of [^{14}C]valine and [^{14}C] adenine into EAC cells by EPBC. EAC cells suspended in the medium (see Materials and methods) were incubated for 30 min in the presence of various concentrations of inhibitor. Then [^{14}C]adenine (●) or [^{14}C]valine (○) was added and the samples incubated for another 60 min. After precipitation with TCA the radioactivity in the TCA-insoluble fraction was measured. ID_{50} value represents the inhibitor concentration (μM) giving a 50% inhibition of the incorporation.

enzymes and of G6PD in EAC cells. All these enzymes are known to be sensitive to thiol-combining agents. EPBC at a concentration of 700 μM inactivated all these enzymes and fully inhibited the glycolysis. The inhibition of PFK appears to be closely related to that of glycolysis. It seems possible therefore that the rate of glycolysis is regulated by inhibition of PFK activity.

Figure 6 illustrates changes occurring in the level of total thiols (T-SH) in homogenates prepared from suspensions of EAC cells inhibited by EPBC. Increasing amounts of EPBC reduce the concentration of T-SH in EAC cells. The initial rates and the degrees of the decrease of T-SH are directly related to the concentrations of EPBC. After 60 min the concentrations of T-SH did not change significantly. However, complete inhibition of glycolysis was reached e.g. with 360 mM EPBC after 30 min but the concentration of T-SH was 55 percent of the initial value. The equilibrium in the distribution of the inhibitor between the cells and the medium is probably reached before 30 min. We can conclude that curves given in Fig. 6 represent direct chemical reaction of the inhibitor with thiol groups of the acceptor sites of the cell components.

Effect of EPBC on yeast cells. EPBC inhibits growth of yeast and yeast-like organisms. The inhibition of the growth appears to be related to carbon dioxide production and oxygen uptake. This effect is shown in Fig. 7 where the results of experiments using *Candida albicans* are given. EPBC inhibited respiration and aerobic fermentation of glucose in growing and in resting cells without significant change in respiratory quotient (R.Q. = 2·2). In the cells treated with a high dose of EPBC (300 μM) the complete inhibition of respiration, aerobic fermentation, incorporation of ^{14}C-labelled precursors into proteins and nucleic acids, inactivation of GAPD and AD and considerable lowering of total free thiol groups was investigated.

DISCUSSION

The data presented provide some new evidence that the cytotoxic action of EPBC lies primarily in the

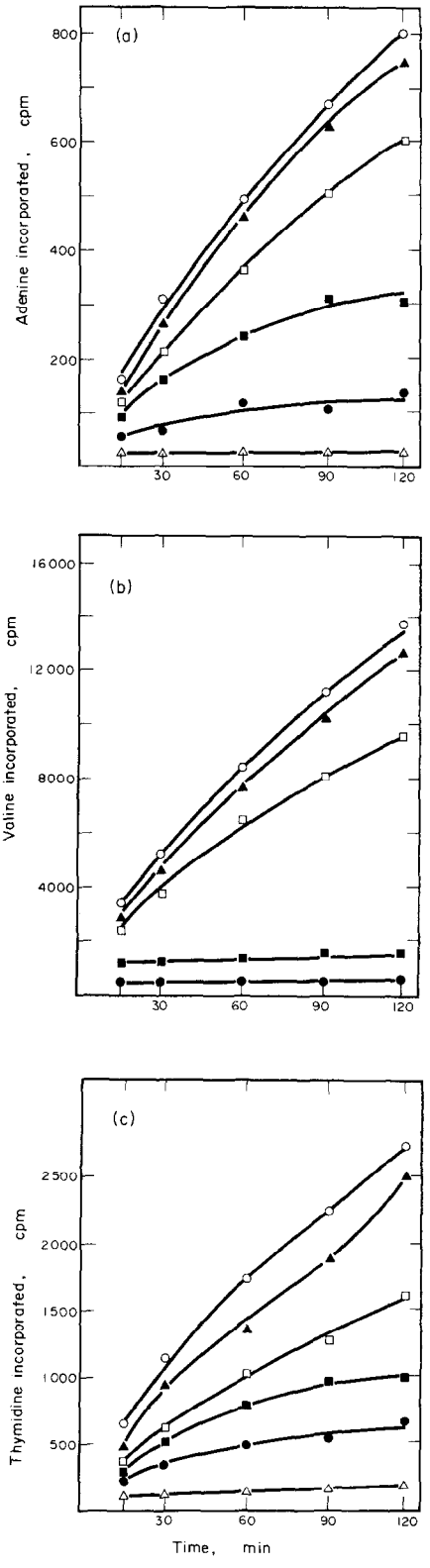


Fig. 5. Kinetics of [^{14}C]adenine, [^{14}C]valine and [^{14}C]thymidine incorporation in EAC cells influenced by various concentrations of EPBC. EPBC and radioactive precursors were added at zero time. Other experiment conditions were as described in the text. Concentrations of EPBC: 360 μM (△); 180 μM (●); 90 μM (■); 45 μM (□); 12·5 μM (▲) and without EPBC (○).

Table 1. Effect of EPBC on the enzyme activities in EAC cells

Enzymes*	Control (μmoles/min per 3 × 10^9 cells) a	b	Decrease in enzyme activity (%/EPBC per μM) 120 a	b	200 a	b	700 a	b
HK	27·5	22·0	3	10	5	27	97	–
PFK	93·0	72·0	50	81	79	98	99	99
GAPD	1160·0	770·0	40	18	79	70	99	99
LDH	2210·0	2210·0	0	23	11	40	99	–
G6PD	21·5	25·9	19	58	32	73	96	96

[a] After 30 min incubation of EAC cells with or without (control) EPBC.
[b] After 120 min incubation.
* Abbreviations: HK, hexokinase; PFK, phosphofructokinase; GAPD, D-glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase.

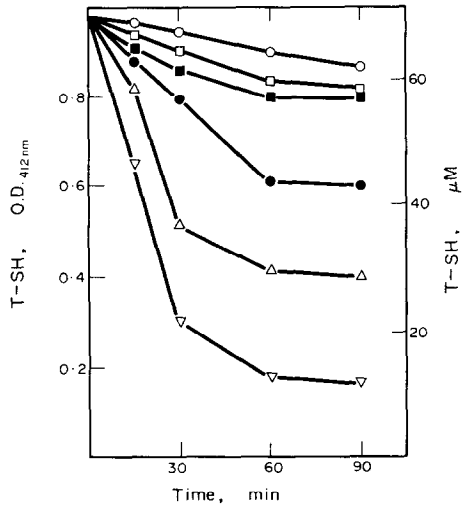


Fig. 6. Thiol content of sonicates prepared from suspensions of EAC cells inhibited by EPBC. The concentrations of EPBC are indicated in Fig. 5.

exclusion of key processes in the energy and intermediary cell metabolism by the inactivation of some enzymes, requiring SH groups for their catalytic activity. Doses of EPBC greater than 250 μM completely inhibit both the glycolysis and respiration of EAC cells and lower the concentration of T-SH. This inhibition exhibited *in vitro*, shortly before transplantation, represents very serious disorders in the metabolism of EAC cells which are reflected in the loss of transplantability.

The effect on glycolysis and respiration is multitarget in character. The inhibition of PFK and perhaps of GAPD and G6PD is responsible for the lowering of the rate and full inhibition of glycolysis. However, in cells with inhibited glycolysis the activity of other thiol enzymes e.g. HK and LD is also lowered.

EPBC in low concentrations probably primarily influences the mitochondrial functions of EAC cells. Low concentrations (< 180 μM) even, stimulated respiration and glycolysis. This selective interference with carbon–energy metabolism is sufficient to inhibit biosynthetic processes, but not to exclude the realisation of the reparation of cell mechanism *in vivo*. Interference of the mitochondria causes a partial limitation of energy demand and other important changes in metabolic processes, resulting in simultaneous inhibition of nucleic acid and protein biosynthesis. The EPBC concentration dependent drop in T-SH level is also significant.

These conclusions are supported by recent information about participation of thiols in mitochondrial function [20], in isolated rat liver mitochondria EPBC like other thiol-combining agents such as *N,N*-dimethyl-*N'*-phenyl (*N'*-fluorodichloromethylthio) sulfamide; substituted benzylisothiocyanates, 2,3-dicyano-1,4-dithiaanthraquinone and other compounds, uncouples respiration at low concentrations and inhibits respiration at high concentrations. Other thiol-combining agents uncouple and do not inhibit respiration or inhibit the respiration prior to uncoupling. There is evidence about the kind and localization of the reaction of EPBC with mitochondrial thiol groups [20].

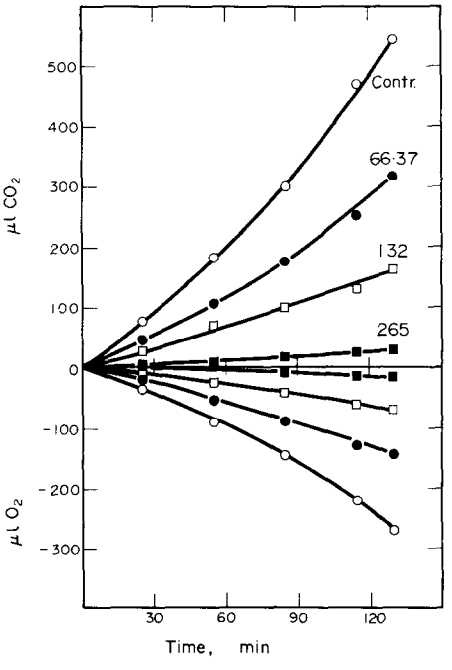


Fig. 7. Dynamics of respiration of growing *Candida albicans* cells in the presence of EPBC. Concentrations of EPBC: 265 μM (■); 132 μM (□); 66 μM (●) and without EPBC (○).

In the last few years, we have observed and described the reactions of thiols with azulene and its naturally occurring derivatives, with antibiotic A_{66} and some compounds included in the above mentioned group of new thiol-combining agents [15, 19–22]. These compounds exhibit some effects similar to those described for EPBC in EAC cells. Direct evidences about the reaction of EPBC and other thiol-combining agents with glutathione (rate constants) is included in a separate paper describing the effect of these compounds on polypeptide synthesis in cell-free system from *Escherichia coli* [23]. The chemical reaction mechanism of EPBC with SH groups is identical with the reaction mechanism generally proposed for α,β-unsaturated carbonyl grouping [24].

In yeast cells, too, EPBC inhibits carbon–energy processes due to the reaction with thiols.

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