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# Differential Inhibition/Inactivation of Mitochondrial Complex I Implicates Its Alteration in Malignant Cells

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**Abstract**—Methylglyoxal strongly inhibited mitochondrial respiration of a wide variety of malignant tissues including sarcoma of mice, whereas no such significant effect was noted on mitochondrial respiration of normal tissues with the exception of cardiac cells. This inhibition by methylglyoxal was found to be at the level of mitochondrial complex I (NADH dehydrogenase) of the electron transport chain. L-Lactaldehyde, which is structurally and metabolically related to methylglyoxal, could protect against this inhibition. NADH dehydrogenase of submitochondrial particles of malignant and cardiac cells was inhibited by methylglyoxal. This enzyme of these cells was also inactivated by methylglyoxal. The possible involvement of lysine residue(s) for the activity of NADH dehydrogenase was also investigated by using lysine-specific reagents trinitrobenzenesulfonic acid (TNBS) and pyridoxal 5′ phosphate (PP). Inactivation of NADH dehydrogenase by both TNBS and PP convincingly demonstrated the involvement of lysine residue(s) for the activity of the sarcoma and cardiac enzymes, whereas both TNBS and PP failed to inactivate the enzymes of skeletal muscle and liver. Together these studies demonstrate a specific effect of methylglyoxal on mitochondrial complex I of malignant cells and importantly some distinct alteration of this complex in cancer cells.

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*Key words*: sarcoma, NADH dehydrogenase, methylglyoxal, lactaldehyde

The anticancer effect of methylglyoxal (MG) has been known for a long time. Several *in vitro* and *in vivo* studies demonstrated that it acts specifically against malignant cells (for review see [1-4]). From our laboratory we also showed that MG inhibits the respiration of both Ehrlich ascites carcinoma (EAC) cells [5, 6] and leukemic leukocytes without affecting the respiration of normal leukocytes [7]. Moreover, we showed that MG inhibits ATP production in mitochondria of EAC and human leukemic leukocytes. This causes the cells to die. We also reported that MG inhibits flow of electrons through mitochondrial complex I (EC 1.6.5.3) of EAC [6] as well as leukemic leukocytes [7] but not of normal leukocytes and a wide variety of normal cells from animals [8, 9].

In the present study we have investigated whether MG could inhibit mitochondrial complex I of diverse types of malignant cells, thus providing further evidence of a unique alteration in malignant cells of this vital enzyme responsible for ATP production. For this we performed experiments using mouse skeletal muscle and sarcoma developed by 3-methyl cholanthrene as well as mitochondria of diverse types of postoperative human tissues. To investigate the mechanism of this MG inhibition, we studied different aspects of this inhibition employing different techniques such as polarographic studies and spectrophotometric assay of NADH dehydrogenase. By using specific amino acid modifying reagents, we also investigated the possible difference(s) in the mechanism of catalytic activity of mitochondrial NADH dehydrogenase of sarcoma and other normal tissues. The results provide strong evidence that MG specifically acts against mitochondrial complex I in malignant cells, and possible alteration of this complex in malignancy is due to some difference(s) in the catalytic site.

*Abbreviations*: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EAC, Ehrlich ascites carcinoma; MG, methylglyoxal; α-OG, α-oxoglutarate; PP, pyridoxal 5′ phosphate; SMP, submitochondrial particles; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine; TNBS, trinitrobenzenesulfonic acid.

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## MATERIALS AND METHODS

**Chemicals and enzyme.** 3-Methyl cholanthrene, MG, protease VIII (product No. P5380), rotenone, α-oxoglutarate (α-OG), ADP (disodium salt), succinate, malonate, hexokinase, Dowex 50W $H^+$ resin, NADH, dithiothreitol (DTT), trinitrobenzenesulfonic acid (TNBS), pyridoxal 5′ phosphate (PP), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co. (USA). All other reagents used were of analytical grade and purchased from local manufacturers.

**Development of sarcoma in mice.** Animal experiments were carried out in accordance with the guidelines of the Institutional Ethics Committee (IEC). Appropriate precautions were taken to minimize pain or discomfort to the animals. Sarcoma tissue was developed as described previously by injecting 3-methyl cholanthrene at the dose of 10 mg/kg body weight in the hind leg of Swiss albino female mice. Malignancy was confirmed by histological examination [10]. Sarcoma tissues were excised soon after sacrificing the mice and immediately transferred to ice cold buffer.

**Human postoperative malignant and normal tissues.** For the collection of human tissue samples, informed consent was taken from the donors, and the institutional ethics committee (IEC) approved the studies with human postoperative tissues, which were colleted both from male and female donors (age group range 21 to 75 years). Tissues were collected immediately after surgery in ice-cold normal saline and experiments were conducted within half an hour of surgery. Malignant tissues were compared with the distal part of the operated tissues with no evidence of malignancy. These distal tissues were considered as normal.

**Preparation of mitochondria from different sources.** Unless mentioned otherwise all operations were carried out at 0-4°C.

*Mouse skeletal muscle and postoperative human tissues (both normal and malignant).* Skeletal muscle from hind leg was excised soon after sacrificing the mice and transferred immediately after removing fat and connective tissues to ice-cold buffer containing 0.1 M KCl, 0.05 M Tris-HCl, pH 7.4, 2 mM EGTA. The tissues were washed several times in this buffer (postoperative human tissues were washed with 0.9% normal saline). All tissues were minced finely with scissors. Tissues were suspended in 10 volumes of buffer containing 0.1 M KCl, 0.05 M Tris-HCl, pH 7.4, 2 mM EGTA, 0.5% BSA (fatty acid free), 5 mM $MgCl_2 \cdot 6\ H_2O$, 1 mM ATP and protease VIII (3 U/ml) and were incubated with stirring for 3 min in the case of mouse skeletal muscle and 5 min for human postoperative tissues. The suspension was then homogenized in an Omni GLH homogenizer for 3 × 15 sec periods with 1 min intervals. The tissue homogenate was centrifuged at 490*g* for 10 min to remove nuclei and plasma membrane fragments. The supernatant was filtered with cotton gauze and centrifuged at 10,370*g* for 10 min to collect a mitochondrial pellet. The pellet was suspended in the above-mentioned buffer but without the protease. It was then washed twice by centrifuging at 10,370*g* for 10 min. Final mitochondrial pellet was resuspended in a minimum volume of the protease-free buffer.

*Sarcoma tissue.* Mitochondria of sarcoma tissue were prepared as described previously [10]. In brief, sarcoma tissue was collected and washed in buffer containing 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, and 0.1% BSA (fatty acid free). After finely mincing the tissue, it was homogenized in six volumes of the buffer with 10 up and down strokes of a Potter–Elvehjem homogenizer and centrifuged at 1500*g* for 5 min. The supernatant was collected and centrifuged at 8000*g* for 15 min. The pellet was washed twice with same buffer by centrifuging at 8000*g* for 15 min and finally suspended in a minimum volume of the buffer.

*Mouse heart.* Mitochondria were prepared basically by the method of Smith [11]. Heart tissue was rinsed in buffer containing 0.25 M sucrose, 20 mM Tris-HCl, pH 7.8, 1 mM sodium succinate, 0.2 mM EDTA and was minced finely with scissors. The minced tissue was suspended in six volumes (w/v) of buffer and homogenized in a Potter–Elvehjem homogenizer with 10 up and down strokes. The homogenate was centrifuged at 1000*g* for 10 min. The supernatant was taken and centrifuged at 15,000*g* for 10 min. The pellet was washed with the same buffer by centrifuging at 15,000*g* for 10 min and finally suspended in a minimum volume of the buffer.

*Mouse liver.* Soon after sacrificing the mouse, the liver was excised and transferred immediately to buffer containing 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% BSA (fatty acid free). After removing blood and connective tissues, the liver was minced finely and then homogenized in six volumes (w/v) of the same buffer. The homogenate was centrifuged at 650*g* for 10 min. The supernatant was taken and centrifuged at 14,000*g* for 10 min. The pellet was suspended in the same buffer and centrifuged again in 14,000*g* for 10 min. Final pellet was resuspended in a minimum volume of the buffer.

**Preparation of submitochondrial particles (SMP) from mouse sarcoma, cardiac, skeletal muscle, and liver tissue mitochondria.** The respective mitochondrial suspension was sonicated by four pulses of 15 sec duration under ice-cold condition. The sonicated mitochondria were diluted with two volumes of buffer containing 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, and centrifuged for 15 min at 29,000*g* to sediment the unbroken mitochondria. The supernatant was then centrifuged for 30 min at 100,000*g*. The pellet was suspended again in the buffer and centrifuged for 30 min at 100,000*g*. Finally the pellet was resuspended in a minimum volume of the buffer.

**Preparation of L-lactaldehyde.** L-Lactaldehyde was prepared from L-threonine and ninhydrin according to the method of Huff and Rudney [12]. The solution of L-lactaldehyde was concentrated by lyophilization. The concentration of the L-lactaldehyde was calculated assuming a yield of 17% [12, 13].

**Measurement of mitochondrial respiration.** Mitochondrial oxygen consumption was measured with a Hansatech (GB) Oxygraph fitted with a Clark electrode. The respiratory medium in a total volume of 2 ml contained 125 mM KCl, 10 mM Tris-MOPS (pH 7.4), 1 mM $KH_2PO_4$, 1 mM $MgCl_2$, 1 mM EGTA, and the respiratory substrates were usually 10 mM α-OG or 10 mM pyruvate plus 10 mM malate or 5 mM succinate. The mitochondrial protein in the medium was 0.3-0.6 mg. The temperature of the respiratory medium was 30°C. Other additions such as ADP (0.4 mM), methylglyoxal (2.5 mM/5.0 mM), malonate (0.5 mM), etc. are mentioned in the figures. ADP was added to start phosphorylating respiration, and the respiratory control ratio for mitochondria was usually >5.

**Oxygen consumption by SMP.** The oxygen consumption by SMP was measured at 30°C in the same incubation buffer as described for mitochondrial respiration but without any respiratory substrate and ADP. The reaction was started with addition of 0.15 mM of NADH and monitored for at least 10 min. The amount of protein was 0.09-0.11 mg of the SMP preparation.

**Assay of mitochondrial NADH dehydrogenase (complex I).** The NADH dehydrogenase activity was assayed polarographically and spectrophotometrically.

*Polarographic assay.* The NADH dehydrogenase activity was assayed by monitoring the consumption of oxygen in a Hansatech Oxygraph fitted with a Clark electrode. The reaction medium was same as described for oxygen consumption by SMP at 30°C.

*Spectrophotometric assay.* The NADH dehydrogenase activity was assayed spectrophotometrically by monitoring the utilization of NADH at 340 nm with two different assay systems. Assay mixture I contained in a total volume of 1 ml, 75 mM of sodium phosphate buffer, pH 7.8, 0.25 mg cytochrome *c* and 0.15 mM of NADH. Other components where added are indicated in appropriate legends of figures and tables. The reaction was started by addition of SMP.

Assay mixture II. Since cytochrome *c* is associated with other redox components of SMP, we also measured the activity of NADH dehydrogenase using the artificial coenzyme Q analog decylubiquinone as described by Janssen et al. with some modification [14]. Assay mixture II contained, in total volume of 1 ml, 25 mM sodium phosphate buffer, pH 7.8, 0.35 mg BSA, 75 μM decylubiquinone, 1.2 μM antimycin A, and 0.15 mM NADH. Decylubiquinone and antimycin A were dissolved in dimethylsulfoxide.

**Chemical modification of NADH dehydrogenase (complex I).** For this experiment, SMP preparation from the mitochondria of sarcoma or mouse skeletal muscle or cardiac or liver tissue was treated with TNBS or PP as described below. TNBS was dissolved in water and its concentration was determined by measuring the adduct formation with sodium glycine buffer, pH 9.0, at 345 nm. The extinction coefficient of the adduct is $1.45 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$. A total volume of 0.2 ml SMP containing 0.2-0.3 mg protein was incubated in 50 mM phosphate buffer, pH 8.2, with different concentrations of TNBS or PP. In the case of PP, the tubes were protected from light. After the indicated time interval the requisite amount of aliquot was withdrawn and assayed for residual enzyme activity spectrophotometrically with two different assay methods using either cytochrome *c* or decylubiquinone as the electron acceptor. A control tube was maintained with the same amount of SMP but without any addition of TNBS or PP.

Protein was estimated with BSA as standard by the method of Lowry et al. as described by Layne [15].

**Statistical analysis.** Statistical analysis was performed using Origin 6 software. Each experiment was performed 3 to 5 times, results are expressed as mean ± SD, and Student's *t*-test for significance was performed and $p < 0.05$ was considered significant.

## RESULTS

**Effect of MG on respiration of mouse skeletal muscle and sarcoma tissue mitochondria.** As mentioned in the introduction, we reported earlier that MG inhibits the respiration of mitochondria of EAC cells [6] as well as that of leukemic leukocytes, but it had no significant effect on mitochondrial respiration of normal leukocytes [7] with the exception of cardiac cell mitochondria [8, 9]. To investigate whether this inhibitory property of MG is a unique phenomenon, we studied the effect of MG on the respiration of mitochondria prepared from mouse skeletal muscle and sarcoma tissue.

Methylglyoxal at a concentration of 2.5 mM inhibited α-OG-dependent respiration of sarcoma mitochondria by 75% (Fig. 1, solid line). This suggests that MG inhibits sarcoma mitochondrial complex I of the respiratory chain. Succinate (5 mM), a substrate for complex II, when added to the system, started respiration immediately, and this can be inhibited by malonate (0.5 mM), a known inhibitor of succinate dehydrogenase. Rotenone (5 μM), a well-known inhibitor of mitochondrial complex I, also completely inhibited this respiration (data not shown). Similar observation regarding MG-mediated inhibition was noted using pyruvate plus malate as respiratory substrate instead of α-OG (data not shown). Both α-OG and pyruvate plus malate donate electrons to complex I, whereas succinate donates electrons to complex II bypassing complex I. The restoration of respiratory activity by succinate indicates that MG has no effect on mito-

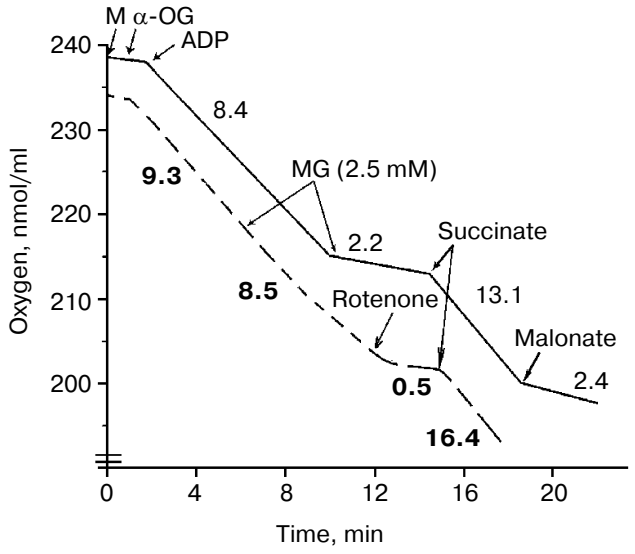


**Fig. 1.** Effect of MG on oxygen consumption by mouse sarcoma tissue (solid line) and skeletal muscle mitochondria (dashed line). The addition of different compounds is indicated by the arrows. The values on the ordinate axis represent oxygen consumption (nmol/min per mg protein). The details of the incubation medium and other conditions are described in "Materials and Methods". M, mitochondria; MG, methylglyoxal; α-OG, α-oxoglutarate.

chondrial complex II, III, and IV. Moreover, to exclude the possibility of MG inhibition on other respiratory complexes, artificial respiratory substrates such as duroquinone and N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD) were tested. Duroquinone and TMPD donate electron directly to complex III and IV, respectively. Both duroquinone and TMPD could restore the MG-inhibited sarcoma mitochondrial respiration (α-OG-dependent). This duroquinone- and TMPD-dependent respiration could be inhibited by antimycin A and sodium azide, respectively, in a very orthodox pattern (data not shown). No significant inhibition by MG was noted in similar experimental condition using mouse skeletal muscle mitochondria (Fig. 1, dashed line), but rotenone (5 μM) could completely inhibit this respiration. These experiments confirm that MG has an inhibitory effect specifically on complex I of sarcoma mitochondria.

**Effect of MG on mitochondrial respiration of normal and malignant human postoperative tissues.** Since we have noted a significant differential effect of MG between mitochondria of normal muscle and sarcoma, we investigated its effect on mitochondrial respiration of human postoperative normal and malignant tissues. Figure 2 represents typical Oxygraph data showing the effect of MG on mitochondria isolated from adenocarcinoma of stomach, normal stomach, adenocarcinoma of colon, and normal colon. It is interesting to note that, similar to its effect on mouse sarcoma, MG (5 mM) strongly inhibited the mitochondrial respiration of adenocarcinoma of both stomach and colon but not of mitochondria of normal cells. It was also observed that the inhibitory effect of MG is only at the level of complex I. Experiments with other complex specific substrates and inhibitors show that MG did not inhibit the activity of other complexes (data not shown). Table 1 also shows that methylglyoxal is inhibitory to mitochondrial respiration specifically of malignant cells.

**Protection by L-lactaldehyde.** L-Lactaldehyde, structurally similar and metabolically related to MG, is formed by NAD(P)H-dependent methylglyoxal reduc-

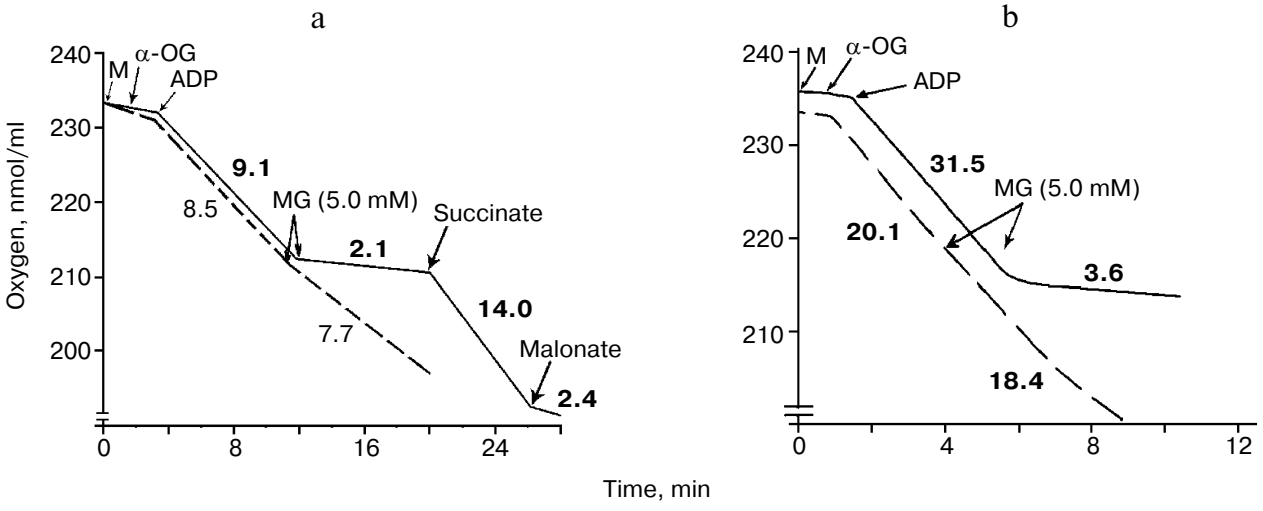


**Fig. 2.** Effect of MG on mitochondrial respiration of human postoperative tissues. a) Mitochondria of human adenocarcinoma of stomach (solid line) and normal stomach (dashed line); b) mitochondria of adenocarcinoma of colon (solid line) and normal colon (dashed line). Other details of values and abbreviations are similar to Fig. 1.

**Table 1.** Effect of MG on different malignant and normal human tissue mitochondrial respiration

| Type of tissue | Rate of oxygen consumption, nmol/min per mg protein | | Activity retained, % |
|---|---|---|---|
| | – MG | + MG | |
| Adenocarcinoma | | | |
| Colon (3) | 29.0 ± 4.0 | 7.8 ± 1.8 | 27 |
| Stomach (4) | 11.3 ± 2.8 | 0.14 ± 0.02 | 2 |
| Breast (2) | 11.6 ± 1.8 | 5.8 ± 1.1 | 50 |
| Rectum (1) | 7.2 | 3.4 | 47 |
| Liver (1) | 6.2 | 2.4 | 39 |
| Ovary (1) | 3.0 | 1.06 | 35 |
| Gall bladder (2) | 14.2 ± 0.3 | 5.06 ± 0.7 | 36 |
| Normal tissue | | | |
| Colon (4) | 26.4 ± 6.2 | 26.5 ± 4.8 | 100 |
| Stomach (3) | 8.6 ± 1.4 | 7.9 ± 0.8 | 92 |
| Rectum (1) | 8.4 | 9.6 | 114 |
| Liver (3) | 7.1 ± 1.5 | 7.4 ± 1.4 | 104 |
| Gall bladder (1) | 6.4 | 6.8 | 106 |

Note: After addition of ADP (0.4 mM) the oxygen consumption was monitored for at least 15 min at 30°C. The final concentration of MG was 5 mM. Numbers in the parentheses represent the number of samples obtained. α-OG (10 mM) was used as respiratory substrate. Other conditions of the assay are described in the "Materials and Methods". The data are means ± S.D; $p < 0.05$.

tase in several organisms [13, 16]. In our previous studies we showed that L-lactaldehyde could exert a protective effect against MG mediated respiratory inhibition to the malignant cells [5, 8]. The result shows that MG at a concentration of 5 mM inhibited the respiration of sarcoma mitochondria completely, but in presence of L-lactaldehyde (2.5 mM), the inhibition is reduced to only 10% (Fig. 3). However, rotenone could readily inhibit the respiration. A similar observation was noted in respiration of human postoperative malignant tissue mitochondria (data not shown). Other metabolites which are structurally and metabolically related to MG such as pyruvate and acetol (α-hydroxyacetone) were also tested and showed neither any inhibition on sarcoma mitochondrial respiration nor any protective effect against inhibition by methylglyoxal (data not shown). These results suggest that the presence of two vicinal keto and aldehyde group of MG are essential for inhibition of sarcoma mitochondrial respiration.

**Inhibition of NADH dehydrogenase activity by MG on sarcoma and cardiac SMPs.** The exclusive involvement of complex I in the inhibitory effect of MG has been further substantiated by experiments with SMP of different tissues. Submitochondrial particles are freely permeable to NADH, and therefore metabolite transporters have no function in these preparations. Consequently, respiration in these particles is only limited by the NADH dehydrogenase activity of complex I of the respiratory chain.

Table 2 shows that MG (2.5 mM) could readily inhibit the dehydrogenation (oxidation) of NADH by SMP of sarcoma and cardiac cellular mitochondria, but it has no inhibitory activity of succinate oxidation (data not shown) confirming that NADH dehydrogenase of complex I is involved in MG inhibition. Moreover, MG has no inhibitory effect on NADH oxidation by SMP of skeletal muscle and liver tissue.

The results clearly demonstrated that MG at the concentration of 2.5 mM inhibits the NADH dehydrogenase activity of sarcoma and cardiac SMP to the extent of approximately 75%. NADH dehydrogenase activity of skeletal muscle and liver SMP remain unaffected as measured spectrophotometrically using decylubiquinone and polarographically by monitoring oxygen consumption. Same results were obtained spectrophotometrically when NADH oxidation was estimated using cytochrome *c* as the electron accepter.

**Inactivation of sarcoma and cardiac cellular NADH dehydrogenase by MG.** While attempting experiments to understand the mode of lactaldehyde protection, we observed that MG also could strongly inactivate the enzyme from sarcoma and cardiac mitochondria. Figure 4 shows that at a concentration of 0.5 mM, MG inactivated both the sarcoma and cardiac enzymes to the extent of about 50%. This inactivation was increased to about 70-75% with the increase of MG concentration to 1 mM, whereas NADH dehydrogenase from normal muscle and

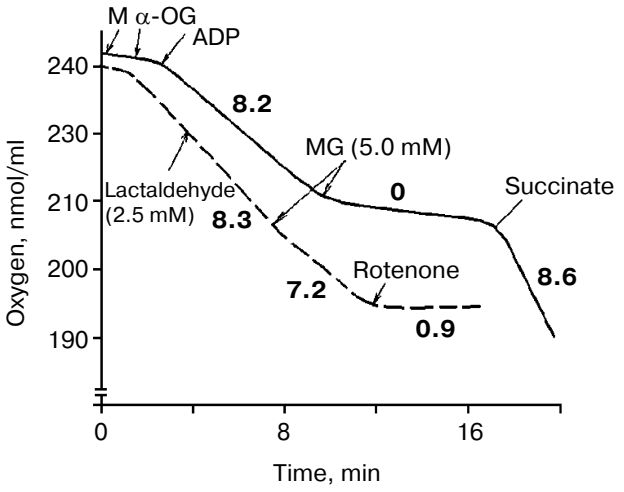


**Fig. 3.** Inhibition by MG of sarcoma tissue mitochondrial respiration in absence (solid line) and in presence (dashed line) of 2.5 mM L-lactaldehyde. Other details are described in "Materials and Methods" and in Fig. 1.

liver mitochondria retained 100% activity even at a higher MG concentration of 2 mM.

**Inactivation of sarcoma and cardiac cell NADH dehydrogenase by TNBS and PP.** It had been shown that MG inhibits mitochondrial respiration of malignant cells and does not inhibit the mitochondrial respiration of other normal cells with the exception of cardiac cell mitochondria [8]. This inhibitory effect was brought through the inhibition of complex I. It is evident from Table 2 that MG inhibits the NADH dehydrogenase of sarcoma and cardiac cells but it has no effect on the NADH dehydrogenase activity of normal muscle and liver. Methylglyoxal could also inactivate the sarcoma and cardiac enzymes. These results suggest that cardiac cell NADH dehydrogenase has some similarity with malignant cell NADH dehydrogenase in respect to MG inhibition/inactivation. These findings further prompted us to investigate whether both sarcoma and cardiac mitochondrial NADH dehydrogenase are different from this enzyme of other normal tissues in relation to the presence of critically involved amino acid residue(s) at the active site of the enzyme. The strong possibility of binding of carbonyl group(s) of MG with free amino group at the ε-position of lysine prompted us to investigate whether malignant and cardiac enzyme are similar to each other but different from other normal cell enzyme in relation to the presence of critically involved lysine residue(s) at the MG binding site. The presence of two vicinal lysine residues was reported at the nucleotide-binding domain of 51 kDa subunit of NADH dehydrogenase (complex I) [17, 18]. Therefore, we tested lysine specific reagents TNBS and PP on the activity of NADH dehydrogenase from different tissue sources.

Table 3 shows that both the sarcoma and cardiac cell enzymes were inactivated by TNBS or PP. With 100 μM TNBS the sarcoma and cardiac cell enzymes were inactivated by approximately 75 and 70%, respectively. PP at a concentration of 1.5 mM could also inactivate the sarcoma and cardiac cell enzymes to the extent of about 75 and 65%, respectively. Because TNBS or PP at the indicated concentrations failed to inactivate the normal muscle and liver enzyme this suggests that lysine residue(s) might be critically involved at the MG binding site of both sarcoma and cardiac cell enzyme but not at the binding domain of normal muscle and liver enzyme.

We also observed that NADH dehydrogenase of sarcoma, cardiac, and other normal tissue mitochondria were strongly inactivated by thiol reagent DTNB. Moreover, the inactivated enzyme could be reactivated to a significant extent by DTT (Fig. 5, columns 1 and 2), indicating the involvement of cysteine residue(s) for the catalytic activity of the enzyme from all the sources studied.

TNBS is known to react with cysteine residue at lower pH values [19]. To test whether TNBS reacted with -SH group(s), we performed reactivation experiments with thiol-containing reagent DTT. The results show that the sarcoma and cardiac cellular enzymes inactivated by TNBS could not be reactivated on incubation with DTT, suggesting that TNBS reacts with lysyl residue rather than with cysteine.

**Double inhibition studies with DTNB and TNBS.** As mentioned earlier, the sarcoma and cardiac NADH dehydrogenase were inactivated by thiol reagent DTNB,

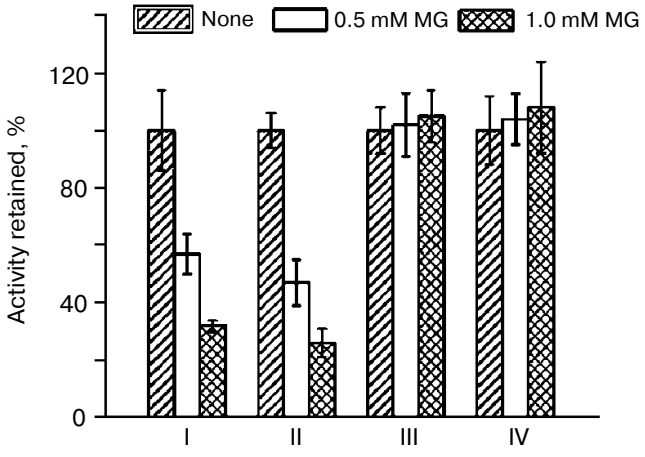


**Fig. 4.** Inactivation of NADH dehydrogenase activity of sarcoma (I), cardiac (II), skeletal muscle (III), and liver SMP (IV) by MG. SMPs from various sources containing 0.2-0.3 mg of protein were incubated in 0.2 ml of 50 mM phosphate buffer, pH 8.2, with or without MG. After 30 min of incubation the NADH dehydrogenase activity in an aliquot from each tube was measured for residual activity. The tubes incubated without MG served as the control. The activity of the control tube was taken as 100%. The assays were done spectrophotometrically using decylubiquinone or cytochrome *c* as electron accepter and polarographically by monitoring consumption of oxygen.

**Table 2.** Inhibition of NADH dehydrogenase activity and oxygen consumption of sarcoma, cardiac, skeletal muscle and liver SMP by MG

| Type of tissue | Addition | A | | B | |
|---|---|---|---|---|---|
| | | NADH dehydrogenase, μmol/min per mg protein | Activity retained, % | Oxygen consumption, nmol $O_2$/min per mg protein | Activity retained, % |
| Sarcoma | none (control) | 1.30 ± 0.2 | 100 | 206 ± 24 | 100 |
| | 2.5 mM MG | 0.36 ± 0.2 | 28 | 63 ± 12 | 30 |
| Cardiac | none (control) | 6.1 ± 1.03 | 100 | 326 ± 13 | 100 |
| | 2.5 mM MG | 1.4 ± 0.3 | 23 | 88 ± 19 | 27 |
| Skeletal muscle | none (control) | 2.0 ± 0.16 | 100 | 263 ± 27 | 100 |
| | 2.5 mM MG | 1.9 ± 0.13 | 95 | 247 ± 23 | 94 |
| Liver | none (control) | 1.9 ± 0.4 | 100 | 250 ± 13 | 100 |
| | 2.5 mM MG | 1.8 ± 0.25 | 95 | 252 ± 28 | 101 |

Note: Sarcoma, cardiac, muscle and liver SMPs were prepared from highly coupled mitochondria by sonication and NADH dehydrogenase activity was assayed by monitoring NADH oxidation both spectrophotometrically using decylubiquinone (A) and polarographically (B). The protein content of added SMP in the assay was 0.09-0.1 mg. Other conditions of the assay mixture are described in "Materials and Methods". Spectrophotometric assay using cytochrome *c* as the electron accepter showed similar results (data not shown).

and the inactivated enzymes could be reactivated by DTT. By taking advantage of inactivation–reactivation, we performed a double inhibition study by TNBS and DTNB [20] to ascertain whether TNBS specifically binds to the lysine residue(s) of sarcoma and cardiac cell enzymes. In this experiment, the enzyme was first inactivated by DTNB and then treated with TNBS. If both the reagents reacted with a thiol group, then modification with DTNB would protect the thiol against the subsequent reaction with TNBS and the inactive enzyme would be at least partially reactivated after incubation with DTT. If the inactivation by TNBS was due to modi-

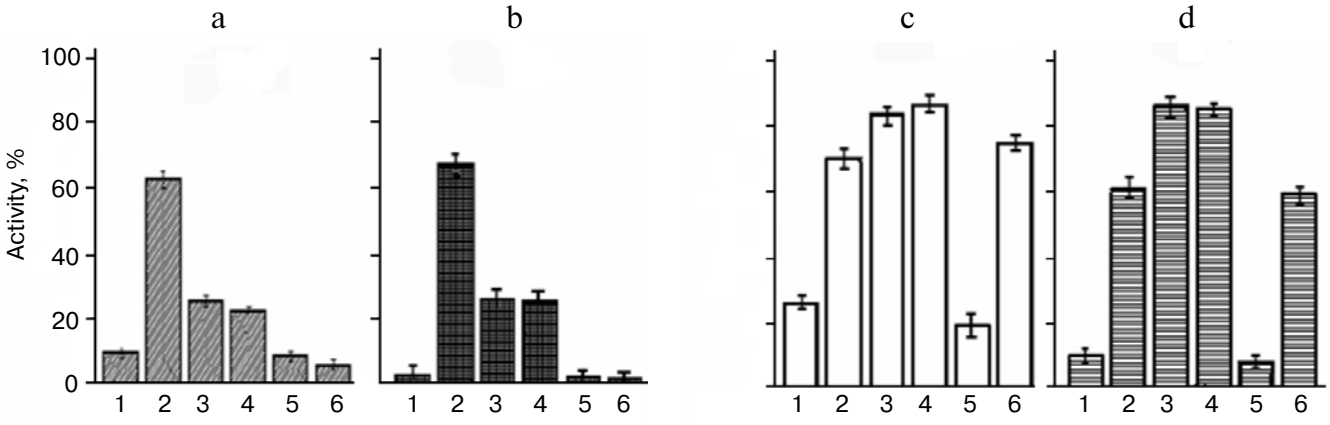


**Fig. 5.** Reversal of activity by DTT of sarcoma (a), cardiac (b), skeletal muscle (c), and liver (d) NADH dehydrogenase inactivated by DTNB and/or TNBS. SMP from various sources containing 0.2-0.3 mg protein were incubated for 30 min in a total volume of 0.2 ml containing 50 mM phosphate buffer, pH 8.2, with 50 μM DTNB (1, 2) or 100 μM TNBS (3, 4). Contents of tubes 5 and 6 were first incubated for 30 min with DTNB and then TNBS was added and incubated for another 30 min. After incubation, the residual enzyme activity was measured by taking an aliquot. The contents of the tubes represented by bars 2, 4, and 6 were then allowed to react with 10 mM DTT and incubated for 30 min, while the other tubes were kept without DTT. After incubation, the remaining enzyme activity was measured by taking an aliquot from each tube. In all cases a control tube was monitored without addition of any modifying reagent or DTT, and its activity was considered as 100%. The assay was done spectrophotometrically using decylubiquinone as electron acceptor. Similar results were obtained when cytochrome *c* was used instead of decylubiquinone.

**Table 3.** Inactivation of NADH dehydrogenase activity and oxygen consumption of sarcoma, cardiac, skeletal muscle and liver SMP by lysine specific reagents TNBS and PP

| Type of tissue | Addition | A | | B | |
|---|---|---|---|---|---|
| | | NADH dehydrogenase, residual activity | | $O_2$ consumption rate | |
| | | μmol/min per mg protein | % | nmol/min per mg protein | % |
| Sarcoma | control | 1.30 ± 0.20 | 100 | 206 ± 24 | 100 |
| | 100 μM TNBS | 0.32 ± 0.08 | 25 | 61 ± 17 | 30 |
| | 1.5 mM PP | 0.31 ± 0.03 | 24 | 70 ± 19 | 34 |
| Cardiac | control | 6.1 ± 1.03 | 100 | 326 ± 13 | 100 |
| | 100 μM TNBS | 1.8 ± 0.51 | 29 | 101 ± 6 | 31 |
| | 1.5 mM PP | 2.2 ± 0.70 | 36 | 127 ± 9 | 39 |
| Skeletal muscle | control | 2.0 ± 0.16 | 100 | 263 ± 27 | 100 |
| | 100 μM TNBS | 1.76 ± 0.40 | 88 | 248 ± 21 | 94 |
| | 1.5 mM PP | 1.9 ± 0.09 | 95 | 243 ± 21 | 92 |
| Liver | control | 1.9 ± 0.4 | 100 | 252 ± 13 | 100 |
| | 100 μM TNBS | 1.71 ± 0.10 | 90 | 234 ± 18 | 93 |
| | 1.5 mM PP | 1.8 ± 0.06 | 95 | 252 ± 12 | 100 |

Note: SMP from various sources containing 0.2-0.3 mg of protein were incubated in 0.2 ml of 50 mM phosphate buffer, pH 8.2, with or without (control) TNBS or PP. After 30 min of incubation NADH dehydrogenase activity and oxygen consumption was measured for the residual activity. Tubes without any treatment served as control and its activity was considered as 100%. The assays were performed spectrophotometrically using decylubiquinone as electron accepter (A) and polarographically (B) by measuring oxygen consumption.

fication of lysine residue(s), then the initial modification with DTNB would fail to protect the enzyme against subsequent irreversible reaction by TNBS. In this case, final incubation with DTT would be unable to reactivate the enzyme. The results of such an experiment are presented in Fig. 5.

As shown in panels (a) and (b) (columns 5 and 6), the sarcoma or cardiac enzymes were first inactivated with DTNB and then incubated with TNBS. The inactivated enzymes could not be reactivated by DTT. Moreover DTT failed to reactivate the enzyme from either source inactivated by TNBS only (columns 3 and 4). The activity of the enzyme inactivated by DTNB only could be reversed by DTT, indicating that TNBS specifically reacted with lysyl residue(s).

In contrast, skeletal muscle and liver enzyme could not be inactivated by TNBS (panels (c) and (d); columns 3), but DTNB-inactivated enzyme (columns 1) could be reactivated by DTT (columns 2), indicating that DTNB binds to the thiol group present in the catalytic domain.

Together these studies convincingly demonstrated: i) the critical involvement of lysyl residue(s) in the activity of sarcoma and cardiac enzymes indicating similarity between these two enzymes; ii) lysyl residue(s) might not be critically involved in the activity of enzymes from the other normal tissue mitochondria studied.

## DISCUSSION

The results clearly indicate that the action of MG is selective against malignant cells. This selectivity is observed in the results obtained by different experimental approaches such as polarographic study of mitochondrial respiration and spectrophotometric and polarographic assay of NADH dehydrogenase. The spectrofluorimetric studies of mitochondrial membrane potential, cytochrome *c* release, and atomic force microscopy of possible structural alteration induced by MG are further described in the following paper [21]. Studies with human

tissues also demonstrated that MG inhibits diverse types of malignant mitochondria. Moreover, mitochondrial complex I is the target site for MG, which is evidenced from the studies using several mitochondrial-complex-specific substrates, artificial electron donors/acceptors and inhibitors, and also the work with SMP. Together these studies convincingly demonstrate that this vital enzyme of cellular energy metabolism might be critically altered in malignant cells.

Although it is generally assumed that enhanced glycolysis is a hallmark of malignancy, numerous reports have indicated that oxidative phosphorylation is not decreased in malignant cells (for a review see [22, 23]). In fact, it had been shown that in the highly dedifferentiated malignant cell AS-30D hepatoma the cellular ATP was mainly provided by oxidative phosphorylation. Moreover, mitochondrial complex I was the site that exerted most of the control of oxidative phosphorylation in these cells [24]. We recall that in normal cells nearly 80% of cellular ATP is generated through this pathway.

The protection by L-lactaldehyde from MG inhibition suggests a possible mechanism of this inhibition. We assume that complex I of these malignant tissues have two vicinal binding sites for MG, one is attached through the keto and another through the aldehyde group, and that is why a stable linkage is formed. The protection from inhibition by L-lactaldehyde also supports the two-binding-site idea. Lactaldehyde has only an aldehyde group, through which it might compete with MG and not allow MG to form a stable adduct occupying the two binding sites.

It was previously shown from our laboratory with different tissue sources that MG does not inhibit mitochondrial respiration of normal cells with the exception of cardiac cell mitochondria [8, 9], suggesting that cardiac and malignant cell mitochondria have some similarities that distinguish them from those mitochondria that are not affected by MG.

Studies with lysine-specific reagents TNBS and PP provide strong evidence for the involvement of lysine residue(s) with the activity of complex I of sarcoma and cardiac cell mitochondria and also provide evidence for the difference from other normal cell mitochondrial complex I.

Because lysine has one free amino group at the ε-position that may interact with a carbonyl group, it is most likely that MG binds with two amino groups of lysine vitally needed for the catalytic activity of complex I. Considering the presence of two vicinal lysine residues at the nucleotide-binding domain of the 51 kDa subunit of complex I, this proposition is reasonable [17].

Many reports are available in the literature pertaining to structural and functional relationship of mitochondrial complex I [17, 25-29], but we emphasize here that to our knowledge all the studies involving mammalian species are of cardiac cell mitochondria [27-29]. The possible similarity between complex I of cardiac and malignant cell mitochondria on one hand and the possible difference of complex I of these two sources with that of other normal cell mitochondrial complex I had not been previously investigated and hence remained unnoticed.

In conclusion, the results presented in this paper strongly suggest that the molecular architecture of complex I of malignant, cardiac and other normal cells should be precisely determined to find the possible similarities and differences. If some vital component(s) of cardiac and malignant enzyme is/are found to be similar, then it is important to investigate how it is engendered. A successful investigation in this regard will contribute greatly to understanding of the precise biochemical difference between normal and malignant cells and also of carcinogenesis.

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