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INHIBITION OF CELLULAR ACTIVITIES BY TRIETHYLLEAD

ROLE OF GLUTATHIONE AND ACCUMULATION OF TRIETHYLLEAD *IN VITRO*

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Abstract—We investigated the interaction of triethyllead with ATP-coupled cellular enzymatic activities and the role of GSH to reverse the observed inhibition of these enzymes. Triethyllead inhibited the membrane bound Na^+–K^+–ATPase from HeLa cells (IC_{50} 12 μM) and the ATP-hydrolysing activity of the mitochondrial F_0–F_1–ATPase complex (IC_{50} 17 μM). Addition of 1 mM GSH reversed both enzyme activities totally, whereas lower GSH concentrations showed a less pronounced effect. Surprisingly, in freshly isolated rat liver mitochondria the ATP-synthesizing activity was also inhibited by triethyllead (IC_{50} 16 μM), in spite of a measured high intramitochondrial GSH concentration (up to 10 mM). Further experiments in isolated submitochondrial particles revealed that ATP-synthesis and ATP-hydrolysis were inhibited by triethyllead with similar IC_{50} values, and both activities could be protected *in vitro* from the organolead compound in the presence of 1 mM GSH. Thus in all activities tested *in vitro* a high excess of GSH over triethyllead ($\geq$25-fold) is necessary to restore the inhibited enzymes. The intramitochondrial triethyllead concentration was further determined after incubation of intact mitochondria with 10 μM of the organolead compound. The organolead concentration measured was as high as 600 μM. This means that in intact mitochondria there exists only a *ca.* 16-fold excess of GSH, which has been shown to be insufficient to protect ATP-synthesizing and ATP-hydrolyzing activities of the F_0–F_1–ATPase from triethyllead *in vitro*. We concluded that in intact mitochondria the F_0–F_1–ATPase complex is inhibited by triethyllead due to its accumulation in the matrix.

Triethyllead (Et_3Pb^+) is a degradation product of the antiknocking agent tetraethyllead (Et_4Pb). Biochemical studies and experiments with cells have shown that Et_3Pb^+ is highly toxic. It was shown that this organolead compound inhibits several molecular systems [1–4]. In suspension cultures of mammalian and plant cells triethyllead showed a high membrane permeability and cytotoxicity [5]. Studying the molecular mechanism of interaction of Et_3Pb^+ with tubulin it was shown that glutathione reverses the inhibitory effect of triethyllead to this protein [6]. In order to better understand the high cytotoxicity of this organolead compound *in vivo*, we investigated its interaction with ATP-coupled cellular activities. Furthermore, we studied the capacity of glutathione to restore these enzymes affected by Et_3Pb^+, because this tripeptide is present in mammalian cells [7], shows reducing and detoxifying properties [8] and was shown to reverse the Et_3Pb^+ induced inhibition of tubulin polymerization [6]. Finally, it is discussed whether accumulation is a main toxic event in organolead poisoning.

MATERIALS AND METHODS

γ-^{32}P-ATP (sp. act. 300–600 μCi/mmol) and ^{32}P (sp. act. 20 μCi/mmol) were from Amersham Buchler (Braunschweig F.R.G.). ATP, GTP, GSH, ouabain, oligomycin were from Serva (Heidelberg F.R.G.). Et_3PbCl and MeHgCl were from Ventron (Karlsruhe F.R.G.), Et_3PbCl was further purified as described in Ref. 9. Percoll® was from Pharmacia (Uppsala Sweden).

Na^+–K^+–ATPase activity of HeLa cells (kindly provided by Dr J. Papamatheakis, Research Center of Crete, Greece) was determined in plasma membrane preparations according to Jørgensen [10]. For this, HeLa cells were homogenized in 1 mM Tris HCl pH 7.4 and the plasma membrane was partially purified by sucrose gradient centrifugation as described in Ref. 2. To determine the Na^+–K^+–ATPase activity 0.1 mg of plasma membrane preparations were incubated 2 min at 37°. In inhibition experiments, ouabain (20 μM), Et_3Pb^+ (1–100 μM) and GSH (1 mM) (final concentrations) were added. The reaction was started with 1 mM γ-^{32}P-ATP (0.1 μCi/μmol). After 10 min of incubation, the release of inorganic phosphate was measured according to the method of Martin and Doty [11], modified for $^{32}P_i$ as follows: the reaction was stopped by adding 0.3 ml of a 1:2 mixture of 8% sodium silicotungstate in 0.5 N H_2SO_4/10 mM H_3PO_4 and 0.1 ml 3.3% ammonium heptamolybdate in 4 N H_2SO_4 and vortexed for 0.5 min. To extract phosphomolybdate, 0.5 ml of a 1:1 mixture of isobutanol and benzene (v/v) were added and vortexed

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‡ Abbreviations: DTNB, 5,5′-dithiobis-nitrobenzoic acid (Ellman's reagent); Et_3Pb^+, triethyllead; $MeHg^+$, methylmercury; GSH, reduced glutathione; MOPS, 3-(*N*-Morpholino)propanesulfonic acid; HEPES, 4-(-2-hydroxyethyl)-1-piperazineethane-sulfonic acid.

Table 1. Recovery of ATP-hydrolysis activity of the F_0–F_1–ATPase complex when GSH (1 mM) was added simultaneously or after 5 min preincubation of the enzyme with 40 μM Et_3Pb^+

	ATP-hydrolysis (nmol P_i/mg protein/5 min)		
	Minute 1–5	Minute 6–10	Total
Control	446	455	901
40 μM Et_3Pb^+	15.3	11.4	26.7
40 μM Et_3Pb^+ + 1 mM GSH	433	429	862
		Addition of 1 mM GSH	
40 μM Et_3Pb^+	12.4	441	453.4

for another 5 min. Aliquots of 0.1 ml of the organic phase were counted by liquid scintillation.

Rat liver mitochondria were isolated from male Wistar rats (200–250 g) starved for 12 hr using standard procedures [12]. The preparation buffer contained 70 mM sucrose, 210 mM mannitol, 10 mM HEPES, pH 7.4. ATP-hydrolysing activity of the F_0–F_1–ATPase complex was measured as follows: 2.5 mg/ml of mitochondrial protein in buffer A (containing 50 mM Tris, 1 mM $MgSO_4$, pH 7.4) were homogenized and the ATP-hydrolysis was determined in 10 μl aliquots in the presence of Et_3Pb^+ (1–100 μM), oligomycin (20 μM) and GSH (1 mM) (final concentrations), exactly as described for the Na^+–K^+–ATPase activity.

To determine the rate of ATP-synthesis, freshly isolated mitochondria (*ca.* 0.3 mg mitochondrial protein) were incubated with 5 mM $^{32}P_i$ (0.1 μCi/μmol) and 10 mM β-hydroxybutyrate in buffer containing 250 mM sucrose, 10 mM MOPS, 2 mM $MgSO_4$, pH 7.5. In inhibition experiments triethyllead (1–100 μM) or oligomycin (20 μM) were added. After 2 min at 37°, the reaction was started with 5 mM ADP, and 5 min later, it was stopped by addition of an ammonium molybdate/silicotungstate mixture. The organic phase was discarded and aliquots of the aqueous phase were counted in a liquid scintillation counter.

Submitochondrial particles were prepared according to Hansen and Smith [13]. To rupture mitochondria three sonication intervals of 3 min were chosen. Studying ATP-synthesis and ATP-hydrolysis of submitochondrial particles the same procedures were used as for mitchondria with the exception that for ATP-synthesis measurements 1.5 mM NAD^+ was added.

Glutathione levels in freshly isolated rat liver mitochondria, isolated as described, were determined by the kinetic assay with DTNB and glutathione reductase [14]. SH-titrations of the tripeptide [15] were started by adding 2×10^{-4} M DTNB to 5×10^{-5} M GSH (final concentration) in the presence and absence of 5×10^{-5} M Et_3Pb^+ or $MeHg^+$, respectively. Reaction kinetics were followed by measuring the absorbance at 412 nm against a blank without GSH on a Perkin Elmer Lamda 15 UV/VIS spectrophotometer (Ueberlingen, F.R.G.). The molar extinction coefficient of the 3-carboxy-4-nitrothiophenolate was $E_{412} = 13.6$/mM/cm.

Intramitochondrial triethyllead accumulation was measured by the following procedure: freshly isolated rat liver mitochondria (0.2 mg) were incubated in the presence of 10 μM triethyllead at the same conditions as described for testing ATP-synthesis. The reaction mixture was layered on top of a Percoll solution of appropriate density and centrifuged (14,000 *g*, 2 min). Under these conditions no ATP-hydrolyzing activity was determined in the supernatant, indicating that mitochondria were pelleted quantitatively. The pellet was resuspended in 0.2% HNO_3, and lead concentration was determined by atomic absorption spectrometry as described previously [9].

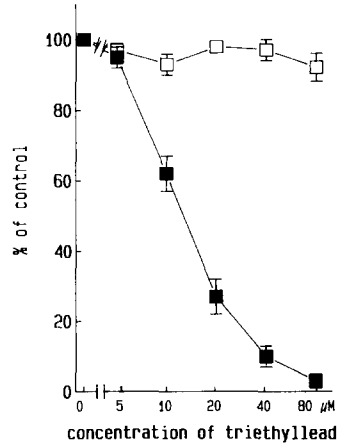

Fig. 1. Dose–response-curve of the inhibition of the Na^+–K^+–ATPase of plasma membranes of HeLa cells by triethyllead (■—■). Reversal of the inhibitory effect of Et_3Pb^+ by 1 mM GSH (□—□). Each datum point represents the mean ± SD of 4 experiments. 100% of control represents 56 ± 6.1 nmol P_i/mg protein/min.

Protein was determined according to Pelley *et al.* [16], using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Previously, it was reported that Et_3Pb^+ inhibits the membrane bound Na^+–K^+–ATPase from ascites tumor cells [2]. In order to further investigate this interaction, we tested the Na^+–K^+–ATPase from HeLa-cells in the presence of several concentrations of Et_3Pb^+ and GSH. In good agreement with the reported results [2] we found that the ouabain sensitive ATPase activity was strongly affected by the organolead compound. The dose dependent inhibition curve is shown in Fig. 1. The IC_{50} value was 12 μM Et_3Pb^+, while 50 μM of the toxin produced

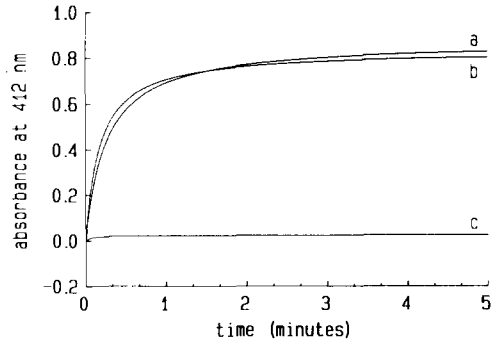

Fig. 2. Reaction kinetics of excess of DTNB (2×10^{-4} M) with 5×10^{-5} M GSH (a), 5×10^{-5} M GSH + 5×10^{-5} M Et_3Pb^+ (b), and 5×10^{-5} M GSH + 5×10^{-5} M $MeHg^+$ (c), in 1 mM Tris–HCl buffer, pH 7.4 as followed at 412 nm.

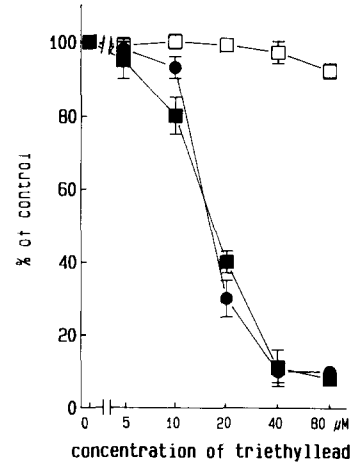

Fig. 3. Dose–response-curve of the inhibition of ATP-hydrolysis and ATP-synthesis in freshly isolated rat liver mitochondria by Et_3Pb^+. ATP-hydrolysis, measured in disrupted mitochondria (■—■), ATP synthesis determined in intact mitochondria (●—●). Reversal of the inhibitory effect of Et_3Pb^+ on the ATP-hydrolysis by 1 mM GSH (□—□). Each datum point represents the mean ± SD of 4 experiments. 100% of control represents 119 ± 8.2 nmol P_i/mg protein/min for ATP-hydrolysis and 102 ± 24.4 nmol ATP/mg protein/min for ATP-synthesis.

an almost total inhibition of the enzyme activity. In the following, we tested if GSH could protect this enzyme from the attack of Et_3Pb^+. In all cases 1 mM GSH was found to reverse the Na^+–K^+–ATPase activity (Fig. 1). In order to determine the lowest concentration necessary for this reversal, the Na^+–K^+–ATPase was incubated in the presence of 40 μM Et_3Pb^+ and varying concentrations of GSH. One millimolar cysteinic tripeptide was found to reverse the Na^+–K^+–ATPase activity totally, while lower concentrations showed a weaker protective effect. At 0.2 mM GSH, no protection could be found. We conclude that a *ca.* 25-fold excess of GSH over the added Et_3Pb^+ is necessary to protect the Na^+–K^+–ATPase activity from the organolead induced inhibition *in vitro*.

To investigate a possible interaction of GSH with Et_3Pb^+ and its capacity to reverse the inhibition of the enzymatic activity *in vitro*, we tested if GSH conjugates with Et_3Pb^+. For this, we followed the *in vitro* titration of the glutathione SH-group in the presence of equimolar organolead concentration (Fig. 2). One mole of nitrated thiophenolate anion per mole of GSH thiol group was released in the presence and absence of Et_3Pb^+ (Fig. 2a and b), showing that no difference in the SH-reactivity of glutathione can be observed. This result, indicating that there is no conjugation of GSH with Et_3Pb^+, is in line with data obtained by others [17]. On the other hand, when $MeHg^+$, another organometalic compound with similar antitubular properties as Et_3Pb^+ [18], was added to GSH, no release of nitrated thiophenolate anion was observed (Fig. 2c). In this case, a shielding effect of $MeHg^+$ on the SH-group of GSH must exist, indicating that most probably $MeHg^+$ interacts with the sulfhydryl group of glutathione.

In earlier studies it has been reported that triethyllead inhibits the mitochondrial oxidative phosphorylation [3]. We therefore investigated the influence of Et_3Pb^+ on the ATP-hydrolysis and ATP-synthesis of the mitochondrial F_0–F_1–ATPase complex. The inhibition of ATP-hydrolysis in the presence of several concentrations of Et_3Pb^+ is shown in Fig. 3. Note that the measurements of ATP-hydrolysis were performed in broken mitochondria without endogenous GSH. The IC_{50} value was 17 μM Et_3Pb^+, while the oligomycin sensitive ATP-hydrolysis was found to be 90% inhibited at 40 μM of Et_3Pb^+. Addition of 1 mM GSH restored the ATP-hydrolysis activity of this enzyme totally (Fig. 3). Lower GSH concentrations showed a much weaker protection. A concentration of 0.3 mM GSH could not protect the enzyme inhibited by 40 μM Et_3Pb^+ at all. We conclude that a *ca.* 25-fold excess of GSH is necessary to protect the ATP-hydrolysis activity of the F_0–F_1–ATPase from the Et_3Pb^+ inhibition. Similar results were also obtained by addition of glutathione to the reaction mixture when significant inhibition of the enzyme by Et_3Pb^+ had already occurred. Table 1 shows that ATP hydrolysis activity was fully recovered when 1 mM GSH was added to the enzyme preincubated for 5 min with 40 μM Et_3Pb^+.

We further investigated the influence of Et_3Pb^+ on the ATP-synthesis in freshly isolated rat liver mitochondria. Figure 3 clearly shows that ATP-synthesis is also inhibited by Et_3Pb^+. The IC_{50} value (16 μM) is in close agreement with the value of 17 μM for the inhibition of ATP hydrolysis. At 40 μM of the organolead compound given to the reaction mixture, *ca.* 90% of the ATP-synthetase activity was inhibited. However, it is known that mitochondria contain high concentrations of glutathione. In our preparations we measured in intact mitochondria GSH concentrations lying between 8 and 10 mM which are in good agreement with values determined by others [19]. As pointed out before, GSH restored the Et_3Pb^+ inhibited ATP-hydrolysis of the F_0–F_1–ATPase *in vitro*. Therefore we raised the question of how Et_3Pb^+ could interact with the ATP-synthesizing activity in freshly isolated mitochondria which contained such high GSH concentrations.

There are two possible explanations: at first, one could suppose that triethyllead interacts with the

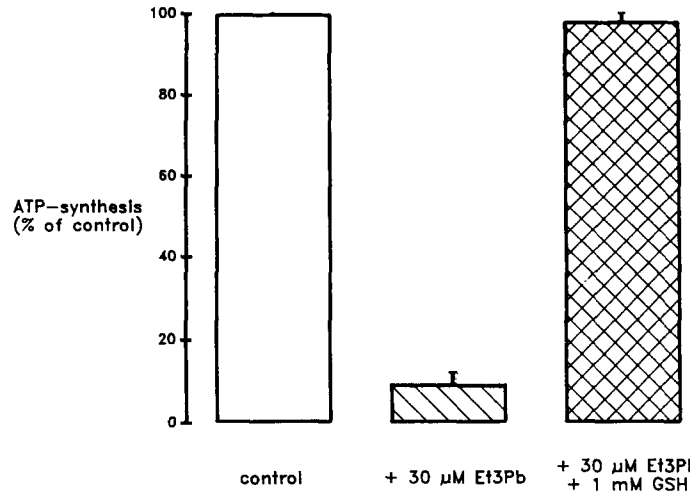

Fig. 4. Influence of ET_3Pb^+ and GSH on ATP-synthesis in submitochondrial particles. Empty bar: ATP-synthesis determined in submitochondrial particles without inhibitor. Hatched bar: ATP-synthesis inhibited by 30 μM Et_3Pb^+. Doubled hatched bar: no inhibition of ATP-synthesis by Et_3Pb^+ (30 μM) in presence of 1 mM GSH. 100% ATP-synthesis represents 27 ± 1.3 nmol ATP/mg protein/min.

enzyme complex at different sites, and that GSH can only restore the protein-organolead interaction responsible for the inhibition of the ATP-hydrolysis. Considering this hypothesis, there are several reports on the different inhibition of ATP-synthesis and ATP-hydrolysis in mitochondria explained by the proposal of the presence of two distinct catalytic sites on the F_0–F_1–ATPase complex specialized for ATP-utilization and -synthesis, respectively [20, 21]. Moreover, different influences of dithiois on organotin-inhibited F_0–F_1–ATPase activities have been reported recently [22]. In order to investigate if GSH can differentially protect the two functions of the F_0–F_1–ATPase-complex inhibited by Et_3Pb^+, we prepared submitochondrial particles and we tested ATP-synthesis and ATP-hydrolysis in the presence of Et_3Pb^+ and GSH. Both activities were found to be inhibited by this organolead compound with similar IC_{50} values, namely 18 μM for ATP-synthesis and 19 μM for ATP-hydrolysis (not shown). At 30 μM of Et_3Pb^+ given to the reaction mixture, *ca.* 90% of both activities were found inhibited (Fig. 4). However, ATP-synthesis, inhibited by 30 μM Et_3Pb^+, was fully restored by the addition of 1 mM GSH to the reaction mixture (Fig. 4). Similar results were also obtained for ATP-hydrolysis. We conclude that GSH, in a *ca.* 33-fold excess, restores *in vitro* both activities equally and that Et_3Pb^+ probably does not interact differentially with the F_0–F_1–ATPase-complex.

The second explanation is that triethyllead accumulates in mitochondria at such high concentrations, that GSH does not offer any protection. From the results obtained in this study a *ca.* 25–33-fold excess of GSH over Et_3Pb^+ was necessary to reverse totally the inhibitory effects of triethyllead on all tested ATPase activities *in vitro*. When GSH was given in less concentrations, the restoration of the inhibited activities was not complete or failed. From these results we suggested that the accumulation of Et_3Pb^+ into cells or intracellular organelles like mitochondria could play an important role for the understanding of the high toxicity of this organolead compound. In order to investigate this possibility we tested if the organolead compound could accumulate in the mitochondrial matrix. For this, freshly isolated mitochondria were incubated with 10 μM Et_3Pb^+ and the incorporated Et_3Pb^+ was determined by atomic absorption spectrometry. An intramitochondrial Et_3Pb^+ concentration as high as 600 μM ± 50 μM was measured (mean ± SD from 4 distinct experiments). This indicates that a *ca.* 60-fold accumulation of the organolead compound occurs under these conditions. Thus the measured GSH-concentration exceeded only 16-fold the Et_3Pb^+ concentration. At this GSH/Et_3Pb^+ ratio, no protection of the F_0–F_1–ATPase activity by the tripeptide could be observed *in vitro*. We conclude that the inhibition of ATP-synthesis by ET_3Pb^+ in freshly isolated rat liver mitochondria must occur by accumulation of the toxin in the matrix. At such a high Et_3Pb^+ amount the intramitochondrial GSH pool is too small to guarantee protection.

Important biological systems inhibited by Et_3Pb^+ such as tubulin, Na^+–K^+–ATPase and mitochondrial F_0–F_1–ATPase can be restored *in vitro* by GSH. Because of the large intracellular amounts of GSH the inhibition of cellular processes should not occur *in vivo*. However, in spite of the high intracellular GSH concentration, Et_3Pb^+ shows a high cytotoxicity. In this study we have demonstrated that Et_3Pb^+ inhibits ATP synthesis in freshly isolated mitochondria containing high GSH concentrations, by accumulation of the toxin in the mitochondrial matrix. Considering these results, the accumulation of triethyllead in the cell or in cellular organelles must be important for the understanding of the *in vivo* cytotoxicity of this organolead compound.

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REFERENCES

1. Zimmermann HP, Röderer G and Doenges KH, Influence of triethyllead on the *in vitro* and *in vivo* assembly and disassembly of microtubules from mammalian cells. *J Submicrosc Cytol* **16**: 203–205, 1984.
2. Haeffner EW, Zimmermann HP and Hoffmann CIK, Influence of triethyllead on the activity of enzymes of the ascites tumor cell plasma membrane and its microviscosity. *Toxicol Lett* **23**: 183–188, 1984.
3. Aldridge WN, Effects on mitochondria and other enzyme systems. In: *Biological Effects of Organolead Compounds* CRS-Press pp. 137–144, 1984.
4. Henry RA and Byington KH, Inhibition of glutathione *S*-aryltransferase from rat liver by organogermanium-lead and tin compounds. *Biochem Pharmacol* **25**: 2291–2295, 1976.
5. Stournaras C, Weber G, Zimmermann HP, Doenges KH and Faulstich H, High cytotoxicity and membrane permeability of Et_3Pb^+ in mammalian and plant cells. *Cell Biochem Funct* **2**: 213–216, 1984.
6. Faulstich H, Stournaras C, Doenges KH and Zimmermann HP, The molecular mechanism of interaction of ET_3Pb^+ with tubulin. *FEBS Lett* **174**: 128–131, 1984.
7. Boyland E and Chasseaud LF, The role of glutathione and glutathione-*S*-transferases in mercapturic acid biosynthesis. *Adv Enzymol* **32**: 173–211, 1969.
8. Sies H, Zur Biologie der Thiolgruppe: Bedeutung des Glutathions. *Naturwissenschaften* **76**: 57–64, 1989.
9. Faulstich H and Stournaras C, Potentially toxic concentrations of triethyllead in Black Forest rainwater samples. *Nature* **317**: 714–715, 1985.
10. Jørgensen PL, Isolation of (Na^+–K^+) ATPase. *Methods Enzymol* **32**: 277–290, 1974.
11. Martin JB and Doty DM, Determination of inorganic phosphate. *Anal Chem* **21**: 103–107, 1949.
12. Johnson D and Lardy H, Isolation of liver or kidney mitochondria. *Methods Enzymol* **10**: 94–96, 1967.
13. Hansen M and Smith AL, Studies on the mechanism of oxidative phosphorylation. *BBA* **81**: 214–222, 1964.
14. Tietze F, Enzymic method for quantitative determination of ng amounts of total and oxidized glutathione. *Anal Biochem* **27**: 502–522, 1969.
15. Ellman GL, Tissue sulfhydryl group. *Arch Biochem Biophys* **82**: 70–77, 1959.
16. Pelley JW, Garner CW and Little GH, A simple rapid Biuret method for the estimation of proteins in samples containing thiols. *Anal Biochem* **86**: 341–343, 1978.
17. Chand P and Clausen J, Triethyllead toxicity in relation to brain glutathione and glutathione *S*-transferase. *Toxicol Lett* **12**: 181–184, 1982.
18. Sager PR, Doherty RA and Olmsted JB, Interaction of methylmercury with microtubules in cultured cells and *in vitro*. *Exp Cell Res* **146**: 127–137, 1983.
19. Romero J, Soboll S and Sies H, Mitochondrial and cytosolic glutathione after depletion by phorone in isolated hepatocytes. *Experientia* **40**: 365–367, 1984.
20. Penefsky HS, Differential effects of adenylyl imidodiphosphate on adenosine triphosphate synthesis and the partial reactions of oxidative phosphorylation. *J Biol Chem* **11**: 3579–3585, 1984.
21. Steinmeier RC, Wang JH, Reconstitution of oxidative phosphorylation by chemically modified coupling factor F_1: Differential inhibition of reaction catalyzed by F_1-labeled with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole or 2,3-butanedione. *Biochemistry* **18**: 11–18, 1979.
22. Emanuel RL, Carver MA, Solani GC and Griffiths DE, Differential inhibition of F_0F_1–ATPase catalysed reactions in bovine-heart submitochondrial particles by organotin compounds. *BBA* **776**: 209–214, 1984.