

## Effects of SH-blocking Compounds on the Energy Metabolism in Isolated Rat Hepatocytes

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**ABSTRACT.** To investigate the importance of SH-groups in the energy metabolism of liver cells, isolated rat hepatocytes were exposed to various SH-blocking compounds. After 1.0 hr exposure, the cells were analyzed for the content of glycogen, lactate, pyruvate, ATP and the rate of oxygen consumption.

Without affecting the cell viability, PCMB, PCMBS, mersalyl, NEM, DTP and DSF were found to decrease glycogen levels, whereas the disulphide reagent CDPS did not affect this endogenous energy reserve. Lactate and pyruvate levels were decreased by the organic mercury compounds, whereas NEM, DTP and DSF stimulated the formation of lactate, without affecting the levels of pyruvate.

In both situations the oxygen consumption was slightly decreased. The FCCP uncoupled oxygen consumption was not affected. Up to the point of loss of cell viability, as measured by trypan blue exclusion and LDH-leakage, the liver cells maintained their ATP levels, during exposure to the various SH-reagents.

In conclusion, the results with organic mercury compounds suggest a reaction of these agents with SH-groups in the outer membrane of cells, having an inhibiting effect on the glucose uptake. The most prominent effect of DTP, DSF and NEM was an increased lactate formation, implying an intracellular effect, most probably in the TCA-cycle.

Hepatocytes in short-term and primary cultures are now being widely used as hepatic model systems for toxicity assessment. Hepatocytes in primary culture are a stable, reproducible experimental system, available for studies of prolonged duration. Hepatocytes in short term cultures are a convenient experimental model for studying the acute toxicity of chemicals (Guillouzo, 1986). Both *in vitro* systems are well-defined experimental models, used for studying the precise toxicity mechanism of chemicals, being an essential base for toxicological risk evaluation (Seinen, 1993). The purpose of the present study is to study the role of sulphhydryl groups in hepatocyte physiology, using freshly isolated rat hepatocytes as a biological test-system and SH-blocking compounds as biochemical tools.

The organic mercury compounds PCMB (p-chloromercuribenzoate), PCMBS (p-chloromercuribenzenesulphonic acid), mersalyl, the disulphide reagents DTP (dithiodipyridine), CPDS (carboxydipyridine disulphide), DSF (tetraethylthiuram disulphide, disulfiram) and the SH-alkylating agent NEM (N-ethylmaleimide) were used as model compounds. The interference of these SH-blocking compounds with glycogen reserves, lactate, pyruvate and ATP levels, cell respiration and cell viability is described.

### MATERIALS AND METHODS

**Chemicals.** p-Chloromercuribenzoate (PCMB), p-chloromercuribenzenesulphonic acid (PCMBS), o-[(3-hydroxymercuri-2-methoxypropyl)carbamoyl]phenoxyacetic acid (mersalyl), N-ethylmaleimide (NEM), 2,2'-dithiodipyridine (DTP), 6,6'-carboxydipyridine disulphide (CPDS), tetraethylthiuram disulphide (disulfiram) and carbonylcyanide p-trifluorophenylhydrazine (FCCP) were from Sigma (St Louis, USA). Hanks balanced salt solution and Waymouth MB752/1 culture medium were from Gibco Europe (Hoofddorp, The Netherlands). Collagenase (isolated from *Clostridium histolyticum*; EC 3.4.24.8), Glucoquant<sup>®</sup> test reagent and amylo-1-4,1-6 glucosidase (isolated from *Aspergillus niger* EC 3.2.1.1) were obtained from Boehringer (Mannheim Germany).

**Isolation of hepatocytes.** Male Wistar rats (body wt. 175–250 g), bred in our laboratory, were used in these studies. The animals had free access to water and food (Standard Laboratory Food, Hope Farms, Woerden, The Netherlands). The animals were anaesthetized by i.p. injection of pentobarbital (Nembutal<sup>®</sup>) at a dose of 120 mg/kg body wt. between 8:30 and 9:00 am. Hepatocytes were isolated using a two step perfusion technique, essentially as described by Seglen (1976). The cells were washed twice and resuspended in Waymouth MB752/1 culture medium. The cell viability of freshly isolated hepatocytes was 90–96% as judged by trypan blue exclu-

sion. The average cell yield was ca.  $6 \times 10^8$  cells/liver.

**Incubation of hepatocytes.** Isolated rat hepatocytes, were suspended in bicarbonate-buffered Waymouth MB752/1 culture medium at a density of  $4 \times 10^6$  cells/ml in a final volume of 3.5 ml. The cells were incubated in 25 ml Erlenmeyer flasks in a waterbath, shaken at a rate of 75 movements/min., under aeration with 95% oxygen and 5% CO<sub>2</sub>. After a 15 minute period of pre-incubation, SH-reagents were added and cells were exposed for 60 min. Stocks solutions of PCMB, PCMBS, mersalyl and NEM in distilled water and of DTP, CPDS, DSF in DMSO were freshly prepared daily. The final DMSO concentration was 1.0%.

**Analytical methods.** The glycogen content of cells was estimated according to Krack *et al.* (1980) using the Boehringer Gluco-quant<sup>R</sup> method (Mannheim, Germany). Glycogen levels were expressed as mg/10<sup>6</sup> cells, or percentages of control values. Lactate dehydrogenase (LDH, EC 1.1.1.27) lactate, pyruvate, ATP were estimated using standard procedures, as described by Bergmeyer *et al.* (1965). LDH-leakage from the cells during incubation was expressed as percentage of the total LDH activity in the cultures. Lactate and pyruvate levels were expressed as  $\mu\text{mol}/10^6$  cells. ATP content, was expressed as nmol/10<sup>6</sup> cells. After 60 min. exposure of cells to SH-agents, the cellular consumption of oxygen was estimated polarographically, in a closed thermostated chamber (37%) using Clark-electrodes. Subsequently, the un-coupling of cell respiration was realized with carbonylcyanide (p-trifluorophenylhydrazine (FCCP; 1.0  $\mu\text{M}$ ). the oxygen consumption was expressed as nmol/10<sup>6</sup> cells/min.

**Statistical analysis.** Results were expressed as means  $\pm$  SD. Student's t-test was used to calculate the significance of differences between values of treated and control suspensions.

## RESULTS

Isolated rat hepatocytes were used for studying the role of SH-groups in the energy metabolism of liver cells. Effects of various SH-reagents on glycogen, lac-

tate, pyruvate and ATP levels, cell respiration and cell viability were analyzed.

**Cell viability.** Exposure of isolated rat hepatocytes to PCMB, PCMBS, mersalyl, NEM, and DTP up to 250  $\mu\text{M}$  did not result in any significant loss of cell viability as concluded from the trypan-blue exclusion test and absence of additional LDH leakage from the cells (Table I). Cell-exposure to 1.0 mM of these compounds resulted in considerable loss of cell viability. Organic mercury compounds at a concentration of 1.0 mM inhibited the LDH-enzyme, measured in cell lysates (data not shown). Thus the leakage of LDH could not be used as a viability test for cells exposed to organic mercury compounds. The trypan blue exclusion test of cell viability, however, proved to be a useful alternative. Cell-exposure to 1.0 mM CPDS and DSF did not result in any loss of cell viability.

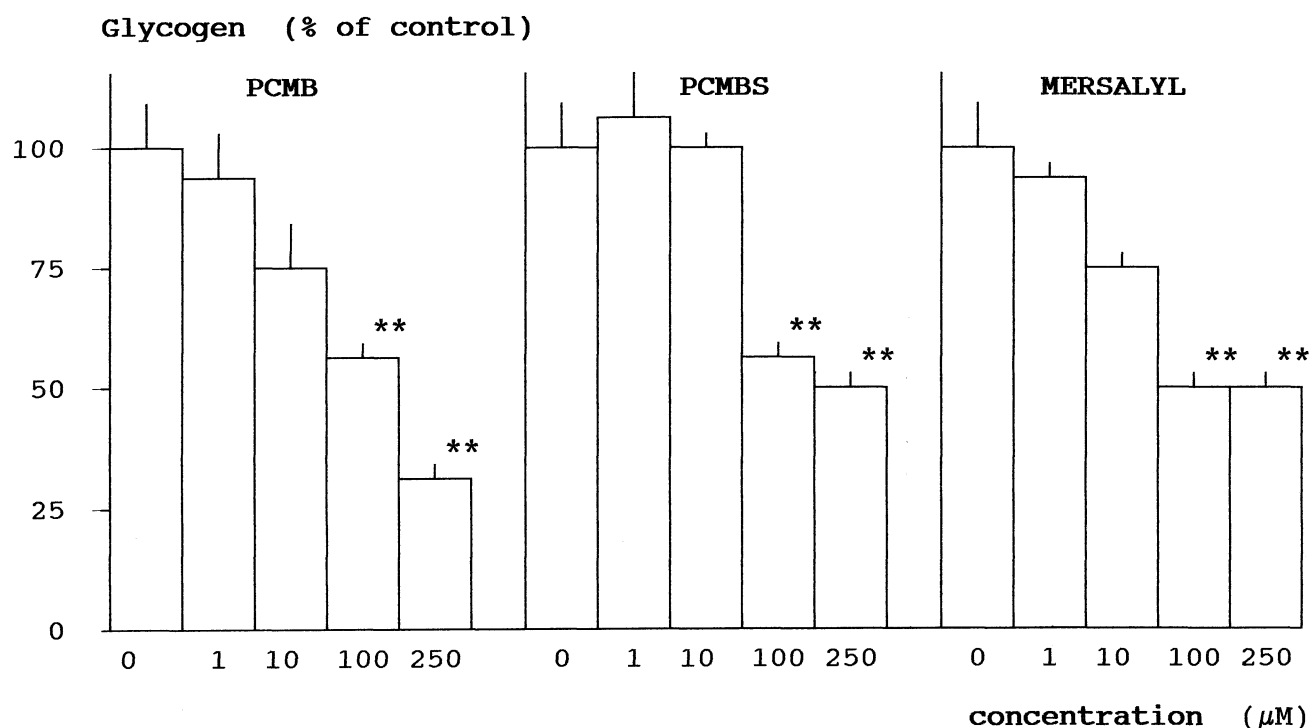
**Glycogen levels.** In control suspensions of isolated rat hepatocytes, a net glycogen deposition of 0.027 mg glycogen/10<sup>6</sup> cells/hr was observed during 1.0 hr incubation (Guillouzo, 1986; Boot, 1989). Exposure to 1–250  $\mu\text{M}$  PCMB, PCMBS and mersalyl during 1.0 hr resulted in a concentration-dependent decrease in the glycogen content of cells (Fig. 1). PCMB was the most effective glycogen depleting organic mercury compound. Exposure of cells to 250  $\mu\text{M}$  NEM (Fig. 2) resulted in strongly reduced glycogen levels as well. Of the SH-reagents studied, N-ethylmaleimide was the most effective glycogen depleting reagent. Exposure of cells to 250  $\mu\text{M}$  DTP or 1.0 mM DSF resulted in glycogen depletion as well, whereas CPDS up to 1.0 mM did not interfere with the glycogen metabolism of rat hepatocytes (data not shown). Glycogen levels, reduced as a result of the exposure of cells to 250  $\mu\text{M}$  PCMB, PCMBS, mersalyl, NEM, DTP or DSF (1.0 mM) were lower as compared with glycogen levels in freshly isolated cells, indicating the occurrence of even a 'net glycogen degradation'.

**Lactate and pyruvate levels.** In suspensions of iso-

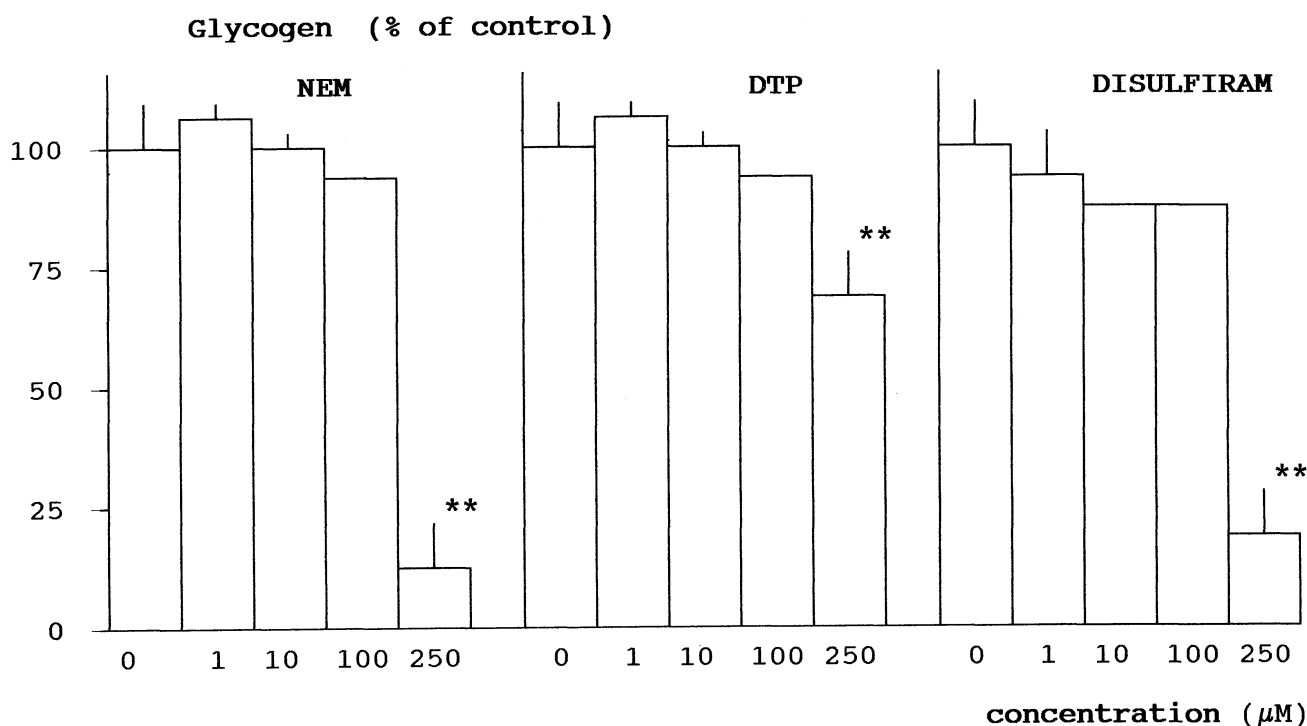
**Table I.** EFFECTS OF VARIOUS SULPHYDRYL (SH)—BLOCKING REAGENTS ON THE CELL VIABILITY IN ISOLATED RAT HEPATOCYTES.

	Leakage of lactate dehydrogenase (% of total)			
	10 $\mu\text{M}$	100 $\mu\text{M}$	250 $\mu\text{M}$	1.0 mM
PCMB	$1.8 \pm 1.2$	$2.7 \pm 2.4$	$3.3 \pm 2.7$	a)
PCMBS	$2.1 \pm 1.6$	$1.8 \pm 1.2$	$5.1 \pm 3.4$	a)
mersalyl	$3.8 \pm 1.9$	$3.8 \pm 1.5$	$3.7 \pm 2.2$	a)
NEM	$4.7 \pm 2.6$	$2.9 \pm 2.4$	$7.2 \pm 4.9$	$65.9 \pm 8.8^{**}$
DTP	$2.3 \pm 1.4$	$1.5 \pm 0.8$	$5.5 \pm 3.4$	$47.1 \pm 14.5^{**}$
CPDS	$2.1 \pm 1.6$	$2.5 \pm 1.5$	$2.2 \pm 1.1$	$3.4 \pm 2.0$
DSF	$2.0 \pm 1.6$	$2.5 \pm 1.4$	$3.0 \pm 2.0$	$6.4 \pm 3.9$

LDH-leakage during 1 hr of exposure of cells to various SH-reagents. Results are presented as means  $\pm$  SD (n = 9 incubations derived from 3 rats). Student's t-test was used, to calculate the significance of differences between control values ( $3.3 \pm 1.9$ ) and treated suspensions \*  $p < 0.05$ ; \*\*  $p < 0.01$ . a) complete loss of cell viability as shown by the trypan blue exclusion test. This could not be quantified, due to LDH-inhibition by the studied organic mercurials.



**Fig. 1.** Effects of the organic mercury compounds PCMB, PCMBS, and mersalyl on glycogen levels in isolated rat hepatocytes. Results are presented as means  $\pm$  SD ( $n=3$  rats). Student's  $t$ -test was used to calculate the significance of differences between control values and treated suspensions; \* $p<0.05$ ; \*\* $p<0.01$ .



**Fig. 2.** Effects of the alkylating reagent NEM and the disulphides DTP and DSF on glycogen levels in isolated rat hepatocytes. Incubation with CPDS (1–250  $\mu$ M) did not affect the glycogen levels (data not shown). Results are means  $\pm$  SD ( $n=3$  rats). Student's  $t$ -test was used to calculate the significance of differences between control values and treated suspensions; \* $p<0.05$ ; \*\* $p<0.01$ .

**Table II.** EFFECTS OF VARIOUS SULPHYDRYL (SH)—BLOCKING REAGENTS ON LACTATE AND PYRUVATE LEVELS IN ISOLATED RAT HEPATOCYTES.

	Lactate concentration ( $\mu\text{mol}/10^6$ cells)		
	100 $\mu\text{M}$	250 $\mu\text{M}$	1.0 mM
PCMB	$5.75 \pm 0.96$	$4.38 \pm 1.11^{**}$	b)
PCMBS	$8.30 \pm 1.99$	$5.3 \pm 1.27^{**}$	b)
mersalyl	$6.60 \pm 0.38$	$4.58 \pm 0.51^{**}$	b)
NEM	$6.31 \pm 1.32$	$9.11 \pm 1.61^{**}$	b)
DTP	$8.10 \pm 0.84$	$9.02 \pm 0.97^*$	b)
CPDS	$7.63 \pm 0.45$	n.d.	$6.90 \pm 1.45$
DSF	$9.50 \pm 2.03$	n.d.	$13.02 \pm 1.04^{**}$
	Pyruvate concentration ( $\mu\text{mol}/10^6$ cells)		
	100 $\mu\text{M}$	250 $\mu\text{M}$	1.0 mM
PCMB	$0.35 \pm 0.09^{**}$		b)
PCMBS	$0.37 \pm 0.08^{**}$		b)
mersalyl	$0.73 \pm 0.10$	$0.27 \pm 0.13^{**}$	b)
NEM	$0.67 \pm 0.07$	$0.73 \pm 0.10$	b)
DTP	$0.74 \pm 0.27$	$0.63 \pm 0.24$	b)
CPDS	$0.78 \pm 0.03$	n.d.	$0.89 \pm 0.11$
DSF	$0.76 \pm 0.09$	n.d.	$0.68 \pm 0.10$

Lactate and pyruvate levels were analyzed after 1.0 hr exposure of cells to sulphydryl (SH) reagents. In suspensions of freshly isolated hepatocytes, the lactate concentration was  $4.3 \pm 0.9 \mu\text{mol}/10^6$  cells and the pyruvate concentration  $0.34 \pm 0.18 \mu\text{mol}/10^6$  cells. After 1.0 hr of incubation, lactate control values were  $7.44 \pm 0.87$  and pyruvate levels  $0.71 \pm 0.13$ . Results are presented as means  $\pm$  SD ( $n=3$  rats). Student's *t*-test was used to calculate the significance of differences between values of control and treated suspensions \*  $p < 0.05$ ; \*\*  $p < 0.01$ . n.d.: not determined; b) loss of cell viability.

lated rat hepatocytes, the average lactate level increased from  $4.3 \pm 0.9 \mu\text{mol}/10^6$  cells to  $7.4 \pm 1.3 \mu\text{mol}/10^6$  cells during 1.0 hr of incubation. In hepatocyte suspensions, exposed to 250  $\mu\text{M}$  PCMB, PCMBS and mersalyl, the formation of lactate was considerably decreased (Table II). Increased lactate production was observed after exposure of isolated rat hepatocytes to 250  $\mu\text{M}$  N-ethylmaleimide, DTP and 1.0 mM DSF and CPDS did not interfere with the lactate production. In the cell suspensions, the average pyruvate level increased from  $0.34 \pm 0.18$

$\mu\text{mol}/10^6$  cells to  $0.71 \pm 0.05 \mu\text{mol}/10^6$  cells during 1.0 hr incubation. In hepatocyte suspensions, exposed to 250  $\mu\text{M}$  PCMB, PCMBS or mersalyl, the formation of pyruvate was strongly decreased (Table II). No significant effect of NEM, DTP, CPDS and DSF on pyruvate levels was observed.

**ATP levels.** The ATP content of isolated rat hepatocytes in control suspensions was  $33.1 \pm 9.5 \text{ nmol}/10^6$  cells. During 1.0 hr exposure of cells to 1–250  $\mu\text{M}$  of various sulphydryl reagents, the cells maintained their

**Table III.** EFFECTS OF SH REAGENTS ON THE RATE OF  $\text{O}_2$  CONSUMPTION IN SUSPENSIONS OF ISOLATED RAT HEPATOCYTES.

	Oxygen consumption (nmol oxygen/ $10^6$ cells/min)			
	10 $\mu\text{M}$	100 $\mu\text{M}$	250 $\mu\text{M}$	1.0 mM
PCMB	$13.5 \pm 3.0$	$9.5 \pm 4.0$	$9.8 \pm 1.3^{**}$	$0.7 \pm 0.7^{**}$
PCMBS	$17.4 \pm 1.4$	$16.4 \pm 1.7$	$13.2 \pm 2.3^{**}$	$5.0 \pm 2.2^{**}$
mersalyl	$17.0 \pm 3.8$	$16.0 \pm 3.1$	$15.2 \pm 3.1$	$1.1 \pm 0.7^{**}$
NEM	$17.1 \pm 1.9$	$18.0 \pm 2.3$	$10.6 \pm 4.2^{**}$	$1.1 \pm 1.1^{**}$
DTP	$15.7 \pm 1.5$	$16.0 \pm 2.3$	$15.6 \pm 1.7$	$5.5 \pm 5.5^{**}$
CPDS	$13.7 \pm 1.9$	$13.3 \pm 1.5$	n.d.	$12.4 \pm 1.9$
DSF	$14.4 \pm 2.3$	$14.5 \pm 2.6$	n.d.	$10.0 \pm 2.9^{**}$
FCCP	(1.0 $\mu\text{M}$ )	$29.3 \pm 4.3$		

Oxygen consumption was analyzed after 1.0 hr exposure of cells to various sulphydryl reagents. Results are expressed as means  $\pm$  SD ( $n=9$  incubations derived from 3 rats). Control values were  $15.8 \pm 2.5$ . Student's *t*-test was used, to calculate the significance of differences between control values and treated suspensions \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; n.d. not determined). FCCP-1.0  $\mu\text{M}$  p-trifluorophenylhydrazine) served as uncoupler of oxidative phosphorylation.

ATP levels; none of the agents caused significant differences from control values.

**Oxygen consumption.** The rate of oxygen consumption in control suspensions of isolated rat hepatocytes was  $15.8 \pm 3.1$  nmol/ $10^6$  cells/min. Up to a concentration of 10  $\mu$ M, none of the studied SH-agents interfered with the oxygen consumption (Table III). After 1.0 hr exposure of cells to 100–250  $\mu$ M PCMB, 250  $\mu$ M PCMBS and NEM or 1.0 mM DSF, decreased rates of oxygen consumption were observed. Exposure to mersalyl and DTP up to a concentration of 250  $\mu$ M and 1.0 mM CPDS did not result in any significant alterations in cellular respiration. The decrease in cell respiration caused by 1.0 mM PCMB, PCMBS, mersalyl, NEM and DTP was associated with loss of cell viability (Table I, III). Addition of the uncoupling agent carbonylcyanide p-trifluorophenylhydrazone (FCCP), as a positive control, strongly increased the cellular respiration to  $29.3 \pm 4.3$  nmol/ $10^6$  cells/min (Table III). This could be demonstrated in control and exposed suspensions.

## DISCUSSION

Isolated rat hepatocytes were cultured in Waymouth MB 752/1 culture medium. Glucose in this medium (27.8 mM) is the main exogenous energy source. ATP-levels in the cells were mainly supported by oxidative processes, as can be calculated from the rate of oxygen consumption (Table III). However, a significant non-oxygen dependent ATP-generation occurred in control suspensions of isolated rat hepatocytes, as shown by lactate formation (Table II). This simultaneous oxidative and glycolytic energy supply in isolated hepatocytes is a common phenomenon at glucose levels higher than 20 mM (Hue *et al.* 1984).

The purpose of this study was to analyse disturbances of the energy metabolism in isolated rat hepatocytes induced by various SH-reagents with distinct physico-chemical characteristics. Alterations in metabolic parameters, as a result of exposure of cells to xenobiotics are relevant as far as the effects are observed without an appreciable cell viability loss. Therefore, in addition to glycogen, lactate, pyruvate, ATP levels and cellular respiration, in each cell suspension also the leakage of LDH from the cells, as a marker for cell viability, was analyzed. The reagents PCMB, PCMBS, mersalyl, NEM and DTP showed a higher cytotoxicity as compared with CPDS and DSF (Table I). The organic mercurials PCMB, PCMBS and mersalyl provoked a concentration-dependent depletion of glycogen which is the most prevalent endogenous energy source in hepatocytes (Fig. 1). This glycogen depletion was not associated with increased lactate production. On the contrary, the lactate production was largely decreased by these

compounds (Table II). As indicated in Table I, the organic mercury compounds inhibited LDH activity, but only at a concentration of 1.0 mM. Therefore, the decreased lactate formation, observed at levels of 250  $\mu$ M of the organic mercury compounds, can not be explained by a decreased activity of LDH. Parallel to the lactate the formation of pyruvate was decreased as well by these compounds. Furthermore, the respiratory chain was not affected by these SH-reagents, as shown by unaltered FCCP-uncoupling in the presence of these chemicals. Exposure of cells to organic mercury compounds resulted in both decreased glycolytic and oxidative ATP generation, as shown by a diminished lactate formation and decreased oxygen consumption. However, the cells maintained their ATP levels up to loss of cell viability, which is for PCMB, PCMBS and NEM in accordance with studies of Morgan *et al.*. These results imply, that organic mercurials inhibit ATP-consuming physiological processes.

PCMBS and mersalyl are known as non-permeant and PCMB as relatively permeant SH-reagents (Boot, 1989). However, these distinct membrane-penetrating properties were not reflected in different effects of these compounds on the hepatocyte energy metabolism. This suggests that organic mercury compounds predominantly act on proteins located in the outer membrane of cells resulting in an inhibition the cellular uptake of glucose. This is in accordance with studies of Plagemann (1980), reporting an inhibition of cellular uptake of nucleosides, sugars and amino acids by various SH-agents. The disulphide reagents DTP and DSF induced a glycogen depletion and, in contrast to the organic mercury compounds, increased the formation of lactate (Fig. 1, Table II). DSF (disulfiram) was the most effective compound in stimulating lactate formation, demonstrated by a high L/P ratio (27.0) in exposed suspensions. Glycogen degradation and ADP phosphorylation associated with lactate production could be the result of higher energy demand of cells exposed to DTP and DSF. Hepatocytes, exposed to disulphide reagents maintained their ATP levels up to loss of cell viability.

These results suggest that DSF and DTP have the main effect on intracellular processes in the energy metabolism in hepatocytes, probably in the TCA-cycle. CPDS did not induce any significant alteration in the metabolic parameters studied. CPDS, known as non-permeant reagent, obviously reacted preferentially with peripheral rather than intracellular SH-groups. This is in agreement with *in vivo* studies by Grassetti (1983), who described CPDS as relatively non-toxic as compared with DTP and other thione-forming disulphides. NEM, as representative of SH-alkylating reagents, is known to react strongly with glutathione. Exposure of cells to 250  $\mu$ M NEM resulted in decreased glycogen, increased lactate levels, and decreased oxygen consump-

tion without affecting the respiratory chain, suggesting that NEM primarily affected the TCA-cycle. Thus, NEM showed a resemblance to disulphide compounds more than organic mercury compounds in its toxic consequences for the hepatic energy metabolism.

In conclusion organic mercury compounds, mainly reacted with SH-groups of membrane-associated proteins, probably resulting in an inhibition of the cellular uptake of glucose. NEM, a permeant SH-alkylating agent, strongly induced lactate formation, implying inhibition of the TCA-cycle. Disulphide reagents are less toxic and induced lactate formation as well. CPDS, as a non-permeant member of the disulphide reagents, did not induce any of these effects. Specific toxicity and the significance of the chemical nature of the reactive and the non-reactive part of SH-reagents, for the expression of their hepatotoxic activity, are demonstrated.

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