Prominent Role of DT-Diaphorase as a Cellular Mechanism Reducing Chromium(VI) and Reverting Its Mutagenicity

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ABSTRACT

Rat liver postmitochondrial (S-12) fractions accounted for the bulk of the activity of whole cell homogenates in reducing chromium(VI) and accordingly in decreasing its mutagenicity. Both cytosolic (S-105) and microsomal fractions concurred to this process, which in all subcellular preparations tested was selectively induced by phenobarbital and especially by Aroclor 1254, but not by 3-methylcholanthrene. Cytosolic fractions were markedly more efficient in reducing chromium(VI) than microsomal fractions recovered from the same amount of tissue (liver or lung), although the latter preparations had a higher specific activity. The microsomal activity was exclusively NADPH dependent. A minor part of the cytosolic reduction was determined by nonenzymatic components, notably by some electron donors and chiefly by reduced glutathione, which proved to reduce chromium(VI) at physiological concentrations. However, also in cytosolic fractions, the most important contribution to chromium reduction was enzyme catalyzed, as shown by the following properties: thermolability; requirement for exogenous NADH or NADPH [supplied as such or in the form of a NADPH-generating system (S-9 mix)]; and saturation by chromium(VI). The likely involvement of DT-diaphorase in this metabolic process is supported by several findings, including its sharp pH dependence and its partial suppression by known inhibitors of this enzyme protein, such as p-chloromercuribenzoate, L-thyroxine, and dicumarol (which conversely did not counteract the metabolic deactivation of the other direct-acting mutagens 2-methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino]acridine 2HCl and epichlorohydrin). Similarly, cytosolic reduction of chromium(VI) was partially inhibited by selective metabolic depletors of both coenzymes of DT-diaphorase, i.e., NADPH and NADH. Pretreatment of rats with enzyme inducers (phenobarbital and 3-methylcholanthrene) stimulated the activity of DT-diaphorase in liver cytosolic fractions. A dramatic stimulation (35 to 40 times over untreated controls) was produced by Aroclor 1254, which also coincided the liver cytosolic activity of enzymes involved in the glucose 6-phosphate-dependent pathway of both nicotinamide adenine dinucleotide phosphate and glutathione reduction (glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and glutathione reductase). In the lung cytosol, a slight yet significant stimulation of some of these enzyme activities was determined by the daily intratracheal instillations of high doses of chromium(VI) itself for 4 weeks, a condition which has been found to enhance the pulmonary metabolism of this metal ion.

INTRODUCTION

Although chromium(VI) compounds are mutagenic and positive in a large variety of short-term test systems (14, 19), metabolic pathways and chemical reactions occurring in the organism are likely to counteract in vivo their toxic activity and carcinogenic potential. For instance, secretions in the alimentary tract of humans (saliva, gastric juice) are capable of reducing to inactive chromium(III) several mg of chromium(VI) per day (20), thus providing a readily surmountable barrier to the metal introduced by the p.o. route or swallowed following reflux from the respiratory tract. Another barrier is represented by the blood stream where chromium(VI) is selectively accumulated and reduced within erythrocytes (10, 18).

Additionally, chromium(VI) can be reduced by cytoplasmic components, which can be reproduced in vitro by combining postmitochondrial (S-9 or S-12) or other subcellular fractions with suitable cofactors. The range of efficiency varies from highly active tissues, such as the liver, to practically inactive tissues, such as striated muscle (18), which is in fact a typical target of chromium(VI) carcinogenicity in rodents given local injections (12). Subcellular preparations from both rodent and human lungs, which are the only recognized targets of chromium(VI) carcinogenicity in epidemiological studies (12), have a poor yet detectable activity in decreasing the mutagenicity of the metal (2, 5, 19, 20).

As already discussed (20, 21), this reducing process may represent a detoxification mechanism implying the occurrence of a metabolically regulated threshold in chromium pulmonary carcinogenesis. We also have evidence that this reaction is selectively inducible in the liver following systemic administrations of known enzyme stimulators and in the lung following pretreatment with not only Aroclor 1254 but also chromium(VI) itself, instilled daily i.t. at high doses for 4 weeks (21).

We have now explored in more detail the biochemical mechanisms underlying the metabolic reduction of chromium(VI). The results herein presented provide evidence that, in addition to already reported mechanisms taking place in microsomal (9) and mitochondrial preparations (24), cytosolic components do supply a quite important contribution, which is mainly attributable to NAD(P)H-dependent enzyme-catalyzed reactions. In particular, DT-diaphorase, an enzyme which has been so far demonstrated to afford detoxification of some organic compounds (25), appears...
to be involved also in chromium metabolism. Nonenzymatic components, and especially GSH, also appear to play an appreciable role in the extracellular reduction of this metal ion.

MATERIALS AND METHODS

Chemicals and Biochemicals. Sodium dichromate (Na$_2$Cr$_2$O$_7$·2H$_2$O) was purchased from Merck-Schuchardt (Munich, Federal Republic of Germany); chromic acetate [Cr(CH$_3$COO)$_3$] from BDH (Poole, England); epichlorohydrin from Carlo Erba (Milano, Italy); and ICR 191 from Polyscience, Inc. (Warrington, PA).

Various biochemical reagents, i.e., G6P, G6PD, NADP$^+$, NADPH, NADH, GSH, and GSSG, were purchased from Boehringer Mannheim GmbH (Federal Republic of Germany).

Enzyme inhibitors included dicumarol, sodium pyruvate, pCMB, and l-thyroxine. These reagents were commercial preparations provided by Sigma Chemical Co. (St. Louis, MO).

Aroclor 1254 was a kind gift from Monsanto Co. (St. Louis, MO). DPC was purchased from Carlo Erba, EDTA from Rudl Pont (Torino, Italy), and BSA from Sigma.

Treatment of Rats and Preparation of Subcellular Fractions. Experiments were carried out by testing subcellular fractions obtained from adult male Sprague-Dawley rats. The animals were either untreated or treated, 5 days before sacrifice, with a single i.p. injection (500 mg/kg) of the PCB Aroclor 1254, diluted in corn oil to a concentration of 200 mg/ml. Liver and lungs were pooled from 10 rats per group.

In some experiments, we also used the same preparations tested in another study (21), to which we refer for technical details. Six groups of Sprague-Dawley rats were treated as follows by Dr. D. Steinhoff at the Institute of Toxicology of Bayer AG (Wuppertal, Federal Republic of Germany): (a) no treatment; (b) 0.9% NaCl solution i.t., 5 times per week for 4 weeks; (c) sodium dichromate (0.25 mg/kg) i.t., 5 times per week for 4 weeks; (d) phenobarbital (60 mg/kg) i.p. for 3 consecutive days before sacrifice; (e) 3-methylcholanthrene (80 mg/kg) in a single i.p. injection 24 h before sacrifice; and (f) Aroclor 1254 (500 mg/kg) in a single i.p. injection 5 days before sacrifice.

Liver and lung cell preparations were obtained as previously described (21). Four types of preparations were assayed: (a) whole cell homogenates, obtained by homogenizing, in a Potter-Elvehjem apparatus, minced organs in a 50 mM Tris-0.25 M sucrose solution, pH 7.4 (3 ml/g of wet tissue); (b) S-12 fractions, i.e., supernatants obtained by centrifuging twice cell homogenates for 20 min at 12,000 × g; (c) S-105 or cytosolic fractions, i.e., supernatants obtained by centrifuging S-12 fractions for 1 h at 105,000 × g; and (d) microsomal fractions, i.e., the corresponding pellet, washed once and resuspended in a 50 mM Tris-0.1 mM EDTA solution, pH 7.4, supplemented with 20% glycerol (0.5 mg/g of original tissue).

All the cell preparations were divided into small aliquots and immediately stored at −80° until use. Their protein concentration was measured according to the protein-dye method of Bradford (3).

Evaluation of Chromium(VI) Reduction. For evaluating chromium(VI) reduction, varying amounts of sodium dichromate were mixed with either reducing metabolites (NADPH, NADH, GSH) or complete metabolic system. These were represented by liver or lung cell preparations, either combined with NADPH or NADH, or incorporated into S-9 mix, i.e., a NADPH-generating system composed of 8 mM MgCl$_2$, 33 mM KCl, 5 mM G6P, 4 mM NADP$^+$, and 100 mM sodium phosphate, pH 7.4. For the assays with microsomes, S-9 mix was supplemented with yeast G6PD (8 IU/ml). In some experiments, enzyme inhibitors or metabolic depleters (dicumarol, pCMB, l-thyroxine, sodium pyruvate, GSSG) or oxidizing agents (potassium permanganate) were also added. The final volume of all these mixtures (1 ml) was obtained by adding 0.2 mM phosphate-buffered saline, pH 7.4, unless otherwise specified.

After varying time intervals of mixing in a rotary shaker (10 rpm) at 37°C, the tubes were transferred into an ice-cold bath. Immediately, two 100-μl aliquots of each mixture were directly transferred into 2 cuvets. One series of cuvets (samples) was then filled with 2.5 ml of DPC reagent (40 mg of DPC in 100 ml of 9% ethanol 8% sulfuric acid in water). The second series of cuvets (corresponding blanks) was filled with 2.5 ml of the same acid-ethanol mixture, but without DPC. After 10 to 15 min at room temperature, the resulting chromium-diphenylcarbazonium complex was measured at 540 nm in a Beckman DU7 spectrophotometer. Detailed calibration curves were drawn by testing standard solutions of untreated sodium dichromate.

Mutagenicity Assays. In some experiments, we evaluated the changes in the mutagenic response elicited by sodium dichromate and, by comparison, by the other 2 direct-acting mutagens, i.e., epichlorohydrin and ICR 191. To this purpose, mixtures of mutagens, metabolic systems, and inhibitors (or suitable controls) were prepared as described in the previous section and then plated in triplicate with bacteria and top agar according to the Ames' plate incorporation test (15). Salmonella typhimurium strains were TA100 and TA102 for dichromate, TA1535 for epichlorohydrin, and TA1537 for ICR 191.

Assay of Enzyme Activities in S-105 Fractions. Enzyme activities were measured in liver and lung S-105 fractions from variously treated rats. The enzymes under scrutiny were DT-diaphorase [assayed according to Ernster et al. (8)], GSSG reductase [assayed according to Veiga Salles and Ochoa (26)], G6PD, and 6PGD [assayed according to Rudack et al. (23)]. The results were expressed as IU of enzyme activity per g of cytosolic protein.

RESULTS

Comparison of Whole Cell Homogenates, S-12, S-105, and Microsomal Fractions from Variously Treated Rats in Reducing Chromium(VI). Several assays were carried out in order to evaluate the relative ability of different cell preparations to reduce chromium(VI). No reducing effect was detectable in the presence of S-9 mix lacking subcellular fractions. A representative experiment, performed with equivalent amounts (corresponding to 25 mg of the original tissue) of liver preparations from either untreated or Aroclor-treated rats, combined with suitable cofactors (see "Materials and Methods"), is shown in the 2 graphs on the right side of Chart 1.

Some general trends, which could be consistently confirmed in several assays, are apparent: (a) irrespective of the metabolic system used, the efficiency of reduction is directly related to the initial amounts of chromium(VI); (b) all the cell preparations from Aroclor-treated rats (bottom graph) are more active than the corresponding preparations from untreated rats (upper graph); and (c) irrespective of rat treatment, S-12 fractions appear to be responsible for a large proportion of the reducing ability of whole cell homogenates and, within S-12 fractions, the major activity resides in cytosolic rather than in microsomal fractions. The relative contribution of subcellular fractions can hardly be quantitated because, as previously stated, it depends on the initial chromium(VI) concentration, as well as on other variability factors, which will be described later. To give an example, considering reduction of 50 μg of chromium(VI) by different liver fractions from Aroclor-treated rats within 1 h at 37°C (experiment shown in Chart 1), S-12 fractions were responsible for the 77% of the total reducing activity of whole cell homogenates. Under these conditions, the reducing activity of S-12 fractions was mainly recovered in cytosol (71%), the microsomal contribution being much lower (25%).
The reducing ability of microsomal fractions could be better evaluated by increasing up to 8-fold their amounts (i.e., up to amounts recovered from 200 mg of the original tissue) (data not shown), or by increasing the incubation time (see next section). Data obtained not only in colorimetric assays but also in mutagenicity test systems (Table 1) provided evidence that phenobarbital, although less efficiently than Aroclor, is also capable of stimulating the reduction of chromium(VI) and its loss of mutagenicity, while 3-methylcholanthrene did not affect the rate of this process. It is noteworthy that induction by Aroclor and phenobarbital occurred in both S-105 and microsomal components of S-12 fractions.

When the reducing efficiency was related to the protein concentration of liver subcellular fraction, rather than to the weight of the original tissue, the highest specific activity was found in microsomal preparations (Table 2). The reducing ability of liver S-12 fractions was greater than that of the corresponding lung preparations also in terms of specific activity. For instance, in the experiment shown in Table 2, the activity of lung S-12 per mg of protein was 59% as high as the one displayed by liver S-12. In the lung, the relative contribution of S-105 to the reducing ability of S-12 fractions obtained from the same amount of tissue appeared to be even more remarkable than in the liver (an example will be shown in Table 5).

Similar findings were obtained by directly supplementing liver or lung S-12 or S-105 fractions from untreated rats with NADPH or NADH instead of using the complete NADPH-generating system (i.e., S-9 mix).

Reduction of chromium(VI) was linearly related to the amounts of subcellular preparations (data not shown).

Time Dependence of Metabolic Chromium Reduction. The start of the reaction was practically immediate. In fact, even by combining at 4°C, as rapidly as possible, the various reagents, i.e., chromium(VI), the metabolic systems, and the DPC reagent, some decrease of chromium(VI) concentration could be consistently observed. The initial concentrations could be maintained only by adding the metabolic systems after the DPC-chromium(VI) complex had been already formed.

Within the first h of contact, as checked after 2.5, 5, 10, 20, 30, 45, and 60 min, reduction of the metal ion was linearly related to the incubation time with S-12 fractions (data not shown). Since 60 min on, the profiles of the curves relating reduction of chromium(VI) to the incubation time tended to flatten, and in a few h (depending on the initial amounts of the metal ion and on the efficiency of the metabolic system), they reached a plateau (Chart 2). In the experiment reported in this chart, after 1-h contact, S-105 fractions accounted for 61% and microsomal preparations for 37% of the reducing ability of S-12 fractions. The efficiency of lung S-12 fractions was 30% of that of the corresponding liver fractions.

After 24 h, the reducing ability of the same subcellular fractions, as well as of liver whole homogenates, was remarkable and again directly related to the initial amounts of the metal ion (see Table 3 for a representative experiment). Liver whole homogenates reduced almost 400 µg of chromium(VI), 93% of which was accounted for by S-12 fractions. Sixty-eight % of the activity of liver S-12 fractions was recovered in S-105 fractions and 39% in microsomal preparations. Lung S-12 reduced 35%
of the amount reduced by liver S-12 fractions.

Requirement for NADPH as a Cofactor. The 2 graphs on the left side of Chart 1 show the reducing ability of the different cell preparations, from either untreated or Aroclor-treated rats, when combined with an incomplete S-9 mix lacking NADP+. From the comparison with the 2 corresponding graphs on the right, the requirement for this cofactor appears to be almost absolute for liver microsomes, which were virtually inactive without the supply of exogenous NADP+. Conversely, whole cell homogenates, S-12, and S-105 fractions showed some reducing activity also in the absence of added NADP+, although such activity was much less pronounced than in the presence of the complete NADPH-generating system. Similar findings were obtained by testing subcellular preparations in the total absence of S-9 mix. The comparison with the corresponding graphs on the right in Chart 1 suggests that, since the 3 curves relative to homogenates, S-12, and S-105 fractions showed some reducing activity also in the absence of NADP+, the bulk of the NADPH-independent reducing activity is located in the cytosol.

Effect of Heating on Chromium-reducing Components of S-12 and S-105 Fractions. In order to check the thermal susceptibility of the components responsible for chromium reduction, some comparative assays were performed with untreated or heated (5 min at 100°C) S-12 or S-105 fractions. An example of the results obtained is shown in Chart 3. A minor part of the activity could be ascribed to thermostable components, whose reducing ability was rather constant and independent of initial chromium(VI).

On the other hand, the relative contribution of thermostable components became progressively more important upon increasing the initial chromium(VI) amounts.

Reduction of Chromium by GSH, NADH, and NADPH. Reducing metabolites were tested as possible candidates among thermostable factors responsible for chromium(VI) reduction. GSH, NADH, and NADPH, whose levels in the S-105 fractions were found to be unchanged by heating (data not shown), produced a dose-related reduction of chromium(VI), without any addition of metabolic system. On a molar basis, the reducing ability of GSH, NADH, and NADPH was of the same order of magnitude (Chart 4) and was unaffected by prior heating of their solutions. Their efficiency was further enhanced by increasing the time of contact with chromium(VI) at 37°C. For instance, 4 mM GSH reduced approximately 5 µg of chromium(VI) in 1 h, 8 µg in 2 h, 11 µg in 4 h, and 16 µg in 8 h. Control experiments, involving mixtures of GSH, NADH, or NADPH with the DPC reagent prior to addition of chromium(VI), demonstrated that these reductants do not interfere with the colorimetric assay.

The chromium(VI)-reducing ability of GSH was confirmed in the mutagenicity test system. In fact, preincubation (30 min at 37°C) of varying amounts of GSH with a fixed amount of sodium dichromate and subsequent plating with bacteria resulted in a dose-related loss of mutagenicitiy (Chart 5). Although showing a parallel trend, analytical and mutagenicity data should not be compared on a quantitative basis, because availability of GSH in the soft agar overlay and its time of reaction, prior to penetration of chromium(VI) into bacteria, remain largely undetermined.

Kinetic Analysis of Enzyme-catalyzed Chromium Reduction. The finding that the bulk of chromium reduction by cell preparations is due to thermostable, NADPH-, or NADH-requiring mechanisms, whose efficiency is directly related to the actual availability of the substrate [chromium(VI)], prompted us to examine the possibility of an enzyme-catalyzed reaction. When the initial chromium(VI) concentrations were raised over the values shown in Charts 1 and 3 and plotted versus the amounts of

Table 3

<table>
<thead>
<tr>
<th>Initial chromium(VI) (µg)</th>
<th>Amount of chromium(VI) reduced (µg)</th>
<th>Liver</th>
<th>Lung</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole homogenate</td>
<td>S-12 fraction</td>
<td>S-105 fraction</td>
</tr>
<tr>
<td>400</td>
<td>381 ± 23*</td>
<td>353 ± 41</td>
<td>240 ± 32</td>
</tr>
<tr>
<td>200</td>
<td>200 ± 0</td>
<td>200 ± 0</td>
<td>171 ± 14</td>
</tr>
<tr>
<td>100</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>98 ± 3</td>
</tr>
<tr>
<td>50</td>
<td>50 ± 0</td>
<td>50 ± 0</td>
<td>50 ± 0</td>
</tr>
<tr>
<td>25</td>
<td>25 ± 0</td>
<td>25 ± 0</td>
<td>25 ± 0</td>
</tr>
<tr>
<td>12.5</td>
<td>12.5 ± 0</td>
<td>12.5 ± 0</td>
<td>12.5 ± 0</td>
</tr>
</tbody>
</table>

*Mean ± SD.
chromium(VI) undergoing reduction by liver S-12 preparations, a
typical rectangular hyperbola was obtained (Chart 6). This indi-
cates the attainment of a steady-state saturation phenomenon,
consistent with an enzyme-catalyzed reaction mechanism.

Kinetic analysis of this cytosolic chromium-reducing system at
pH 7.4 revealed a $K_m$ for the substrate sodium dichromate of $1.5 \times 10^{-4}$ M, as estimated at a constant NADPH concentration of
1.5 mM and taking into due account the extents of chromium(VI)
reduction produced by both NADPH and the cell preparations
per se. The affinity for this chromium(VI) compound in the pres-
ence of 1.5 mM NADH was comparable, as shown by a very
similar $K_m$ value. The apparent $K_m$ values for NADPH and NADH
were approximately $3.0 \times 10^{-4}$ M and $3.5 \times 10^{-4}$ M, respectively.
The identical affinities towards both NADPH and NADH displayed
by liver preparations, in the uninduced and induced animals,
respectively, are in agreement with known mechanisms of en-
zyme induction. Thus, a quantitative variation resulting from
modified turnover of the underlying enzyme protein, rather than
expression of an alternative structural gene coding for a quali-
tatively different enzyme form, is clearly involved.

Effect of Inhibitors of DT-Diaphorase and of Depletors of
Reduced Pyridine Coenzymes. The requirement of the cyto-
solic chromium-reducing enzyme system for either NADPH or
NADH prompted us to explore the possible involvement of DT-
diaphorase in this process. Such enzyme protein is in fact known
to display reducing activity towards a number of structurally
different substrates, using NADPH or NADH in a characteristi-
cally interchangeable way (8, 11).

A number of assays provided evidence that dicumarol, the
most typical inhibitor of DT-diaphorase (8), is capable of pre-
venting the metabolic chromium reduction. In the Ames reversion
test, addition of dicumarol inhibited to a large extent the decrease
of dichromate mutagenicity produced by liver S-12 fractions,
while it did not affect at all the metabolic deactivation of other
direct-acting mutagens, i.e., epichlorohydrin and ICR 191 (Table
4).

Also, in colorimetric assays, dicumarol was successful in par-
tially counteracting the reduction of chromium(VI) afforded, to a
variable extent, by liver preparations (whole cell homogenates,
S-12, or S-105 fractions) or by lung preparations (S-12 or S-105
fractions), both from untreated and from Aroclor-treated rats
(Table 5). The same table shows, as also confirmed in a number
of additional experiments, that the reduction of chromium(VI) by
cell preparations was also inhibited, although to a lower extent
than by dicumarol, by GSSG and pyruvate. These metabolites
are expected to remove reducing equivalents through specific
enzymatic mechanisms. In particular, GSSG removes NADPH
via GSSG reductase, and pyruvate removes NADH via lactate
dehydrogenase.

Other assays were carried out by testing the effects of various
inhibitors in a low-ionic-strength buffer as the reaction medium
(0.1 M potassium phosphate buffer, pH 7.5) and by supplemen-
ting the reaction medium with BSA. Both conditions have been
reported to stabilize the DT-diaphorase activity (8). In our assays,
addition of BSA resulted in a slight enhancement of chromium(VI)
reduction (data not shown). As shown in Table 6, the cytosolic
NADPH-dependent reduction was almost totally reverted by the
strong oxidizing agent potassium permanganate. Three inhibitors
of DT-diaphorase, i.e., dicumarol, pCMB, and, to a lower yet
significant extent, L-thyroxine (8), were also effective in counter-
acting chromium reduction, although they proved to be devoid
of any oxidizing activity towards chromium, as checked by

![Chart 3. Effect of heating (5 min at 100°C) on the reducing ability of liver S-12 fractions (20% in S-9 mix) from Aroclor-treated rats, after 1 h at 37°C. Bars, SD.](chart3)

![Chart 4. Amount of chromium(VI) reduced by varying concentrations of GSH, NADPH, and NADH following incubation for 1 h at 37°C with sodium dichromate [28.6 μg, containing 10 μg of chromium(VI)]. Bars, SD.](chart4)
REDUCTION OF CHROMIUM(VI) BY DT-DIAPHORASE

Charts. Effect of varying amounts of GSH on the mutagenicity of sodium dichromate (30 μg/plate) in strain TA102 of S. typhimurium. The GSH molarities reported on the abscissa refer to final concentrations in the complete reaction medium, including top agar.

Effect of pH on the Cytosolic Chromium Reduction. Since DT-diaphorase has been reported to display optimal activity in a mildly acidic environment (11, 16), we checked the pH dependence of chromium(VI) reduction by liver S-105 fractions (Chart 7). In the presence of NADPH and without inhibitors, the reducing activity was strongly affected by the actual pH values, being approximately doubled upon lowering the pH from 7.5 to 6.5 and being still increased at 6.0 and 5.5. Again, addition of dicumarol to the system resulted in a partial inhibition of the process, which was more apparent at low pH values. In the absence of exogenous NADPH, the pH dependence of the reduction by S-105 fractions was much less pronounced and only poorly affected by dicumarol. In the absence of any metabolic system, pH variations within the range investigated (5.5 to 7.5) had no effect on chromium(VI) stability (data not shown).

Assay of Enzyme Activities (DT-Diaphorase, G6PD, 6PGD, GSSG Reductase) in Liver and Lung Cytosolic Fraction from Variousely Treated Rats. The activities of DT-diaphorase and of 3 enzymes involved in the G6P-dependent pathway of GSSG reduction, i.e., G6PD, 6PGD, and GSSG reductase, were assayed in liver and lung cytosolic fractions from rats either untreated, or treated i.t. with 0.9% NaCl solution or dichromate, or treated i.p. with enzyme inducers according to the schedule reported under "Materials and Methods." The mean results obtained (based on 3 to 5 determinations per enzyme activity) are reported in Table 7.

The i.t. treatments had no effect on any enzyme activity in liver S-105 fractions. Taking into account the statistically significant results only, the hepatic G6PD activity was slightly enhanced by phenobarbital and 3-methylcholanthrene and to a larger extent by Aroclor 1254 (about 4 times), whereas a moderate stimulation of G6PD and of GSSG reductase was afforded by Aroclor only. All 3 inducers efficiently stimulated the activity of hepatic DT-diaphorase, assayed with both NADPH and NADH as coenzymes, such stimulation being dramatic in the case of Aroclor (35 to 40 times over untreated rats).

On the other hand, Aroclor had no effect on the same enzyme activities in lung preparations. Some slight yet significant increases in activity were conversely determined by the i.t. treatment with dichromate (for G6PD, 6PGD, and DT-diaphorase) or even with NaCl (for G6PD only).

DISCUSSION

A first point deserving comment deals with the relative contribution of rat subcellular fractions in reducing chromium(VI). This problem can hardly be assessed in quantitatively precise terms, because a number of variability factors were found to markedly affect the metabolic efficiency of this process including, e.g., the cell type, pretreatments of rats, the cofactors added to subcellular preparations, the initial amounts of chromium(VI) to be

Table 4

<table>
<thead>
<tr>
<th>Compound (amount/plate)</th>
<th>Without S-12</th>
<th>With S-12</th>
<th>With S-12 + dicumarol</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA100 Water</td>
<td>169 ± 12</td>
<td>154 ± 8</td>
<td>172 ± 15</td>
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<tr>
<td>Sodium dichromate (30 μg)</td>
<td>731 ± 38</td>
<td>247 ± 19</td>
<td>610 ± 44</td>
</tr>
<tr>
<td>TA1535 Water</td>
<td>14 ± 3</td>
<td>11 ± 5</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>Epichlorohydrin (500 μg)</td>
<td>746 ± 40</td>
<td>197 ± 23</td>
<td>208 ± 11</td>
</tr>
<tr>
<td>TA1537 Dimethyl sulfoxide</td>
<td>8 ± 5</td>
<td>10 ± 2</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>ICR 191 (2 μg)</td>
<td>1428 ± 83</td>
<td>31 ± 9</td>
<td>27 ± 12</td>
</tr>
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</table>

*Mean ± SD.
REDUCTION OF CHROMIUM(VI) BY DT-DIAPHORASE

Table 5
Effect of metabolic inhibitors on the reduction of chromium(VI) by liver and lung preparations

Sodium dichromate [57.3 μg, containing 20 μg of chromium(VI)] was incubated in triplicate tubes, for 1 h at 37°C, with S-9 mix containing 20% of liver or lung cell preparations from untreated or Aroclor-treated rats, in either the absence or the presence of metabolic inhibitors. Reduction of the metal was evaluated as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Treatment of rats</th>
<th>Whole cell homogenate</th>
<th>S-12</th>
<th>S-105</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O (controls)</td>
<td>None</td>
<td>20.0 ± 0.0*</td>
<td>18.8 ± 0.8</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Aroclor</td>
<td>20.0 ± 0.0</td>
<td>20.0 ± 0.0</td>
<td>14.5 ± 0.3</td>
</tr>
<tr>
<td>Dicumarol (0.1 mw)</td>
<td>None</td>
<td>17.6 ± 0.3*b</td>
<td>11.9 ± 0.8*b</td>
<td>6.1 ± 0.4*b</td>
</tr>
<tr>
<td></td>
<td>Aroclor</td>
<td>18.4 ± 0.4*c</td>
<td>17.1 ± 0.8*c</td>
<td>10.6 ± 0.3*c</td>
</tr>
<tr>
<td>Sodium pyruvate (5 mm)</td>
<td>None</td>
<td>19.0 ± 0.3*c</td>
<td>15.3 ± 0.5*c</td>
<td>10.7 ± 0.4*c</td>
</tr>
<tr>
<td></td>
<td>Aroclor</td>
<td>20.0 ± 0.0</td>
<td>18.7 ± 0.4*c</td>
<td>13.6 ± 0.2*c</td>
</tr>
<tr>
<td>GSSG (5 mw)</td>
<td>None</td>
<td>20.0 ± 0.0</td>
<td>14.4 ± 0.7*b</td>
<td>10.9 ± 0.4*c</td>
</tr>
<tr>
<td></td>
<td>Aroclor</td>
<td>20.0 ± 0.0</td>
<td>19.5 ± 0.3*d</td>
<td>13.7 ± 0.2*c</td>
</tr>
</tbody>
</table>

* Mean ± SD.
*b Significant at P < 0.001 by Student’s t test as compared to the corresponding control.
*c Significant at P < 0.01 by Student’s t test as compared to the corresponding control.
* Significant at P < 0.05 by Student’s t test as compared to the corresponding control.

Sodium dichromate [57.3 μg, containing 20 μg of chromium(VI)] was incubated in triplicate tubes, for 1 h at 37°C, with a mixture composed of liver S-105 fractions from untreated or Aroclor-treated rats, in either the presence of metabolic inhibitors. Reduction of the metal was evaluated as described in "Materials and Methods."

Table 6
Effect of various inhibitors on the reduction of chromium(VI) by liver cytosolic preparations

Sodium dichromate [57.3 μg, containing 20 μg of chromium(VI)] was incubated in triplicate tubes, for 1 h at 37°C, with a mixture composed of liver S-105 fractions from untreated rats, 0.1 mw NADPH, various inhibitors, and BSA (7 mg/ml) in 0.1 M potassium phosphate buffer, pH 7.5. Reduction of the metal was evaluated as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mw)</th>
<th>Amount of chromium(VI) reduced (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O (controls)</td>
<td>1.2 rtiM NADPH</td>
<td>20.0 ± 0.0</td>
</tr>
<tr>
<td>Potassium permanganate</td>
<td>0.5</td>
<td>8.07 ± 0.11*</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>0.1</td>
<td>1.14 ± 0.49b</td>
</tr>
<tr>
<td>Dicumarol</td>
<td>1.0</td>
<td>4.57 ± 0.15b</td>
</tr>
<tr>
<td>pCMC</td>
<td>0.01</td>
<td>2.84 ± 0.38b</td>
</tr>
<tr>
<td>L-Thyroxine</td>
<td>0.1</td>
<td>7.74 ± 0.15c</td>
</tr>
</tbody>
</table>

* Mean ± SD.
*b Significant at P < 0.001 by Student’s t test as compared to the corresponding control.
*c Significant at P < 0.01 by Student’s t test as compared to the corresponding control.
*d Significant at P < 0.05 by Student’s t test as compared to the corresponding control.

In any case, it was evident that postmitochondrial (S-12) fractions account for the bulk but not for the totality of chromium(VI) reduction by whole homogenates, implying a partial involvement of other cellular structures and organelles as well. For instance, this phenomenon has been already documented in mitochondria, which have been estimated to supply some contribution to the total cellular reduction (24). This is consistent with the finding that chromium(VI) is easily accumulated, reduced, and then trapped as chromium(III) within mitochondria (1), where it inhibits the electron transport chain (24) and causes considerable ultrastructural damage (17).

The chromium-reducing activity of microsomal preparations was found to be almost exclusively NADPH dependent. Both in colorimetric and mutagenicity test systems, the efficiency of liver microsomes appeared to be enhanced by pretreatment of rats with phenobarbital and especially with Aroclor 1254, while 3-methylcholanthrene showed no influence on this activity. The selective induction of chromium metabolism by phenobarbital, compared to 3-methylcholanthrene, had been already reported in a previous study (9), suggesting that NADPH and NADH may supply reducing equivalents to cytochrome P-450 in rat liver microsomes, thereby operating as a reductase for chromate.

Although at equivalent protein concentration the specific activity of microsomes was superior to that of S-105 fractions, the contribution of cytosolic components to the reducing capacity of postmitochondrial fractions was markedly higher than that of microsomal preparations, when referred to an equivalent weight of tissue used for their subfractionation.

Even assuming that only a part of metabolic activities located in the cell endoplasmic reticulum can be recovered in the microsomal pellet following homogenization and differential centrifugation (7), cytosolic components appear therefore to play a prominent role in the intracellular chromium(VI) reduction. This finding prompted us to explore in more detail the cytosolic mechanisms responsible for such activity, which appeared to be selectively stimulated by the same inducers acting on microsomal metabolism.

A minor part of chromium(VI) reduction in S-105 fractions could be attributed to components which (a) were thermostable, (b) were endogenous in cell preparations, irrespective of the

Chart 7. Amount of chromium(VI) reduced after incubation for 1 h at 37°C of sodium dichromate [57.3 μg, containing 20 μg of chromium(VI)] with liver S-105 fractions from untreated rats, in the absence (A, •) or in the presence (C, O) of 1.2 mw NADPH, without (A, O) or with (C, O) 1 mw dicumarol, as related to the pH of the diluting buffer (0.2 M potassium phosphate-buffered saline). Bars, SD.

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The experimental indications supporting the role of DT-diaphorase also in chromium(VI) reduction include typical properties of this enzyme (8, 11, 16), which is mainly located in the cell cytosol. For instance, the NADPH-dependent reducing activity of the cytosol was strongly enhanced upon decreasing the pH value to 5.5 to 6.5, which conversely affected its NADPH-dependent reducing ability towards chromium(VI).

In addition to NADPH, GSH is of particular interest, since it displayed such ability at lower concentrations in liver cells (4, 27). GSH is also capable of binding chromium(VI) (22) and is therefore expected to produce complete detoxification of the metal. NADH and NADPH, although showing a saturation phenomenon, have considerably lower concentrations in liver cells (4, 27).

On the other hand, the remaining major component(s) responsible for chromium(VI) reduction in the cytosol showed opposite features, typical for enzyme-catalyzed activities, such as (a) thermostability, (b) requirement for NADH or NADPH (supplied as such or in the form of the NADPH-generating system (S-9 mix)), and (c) an efficiency which was directly related to the amount of the substrate [chromium(VI)] until reaching a plateau indicating a saturation phenomenon.

There are several indications about the likely involvement of the flavin adenine dinucleotide-containing flavoprotein DT-diaphorase, or NAD(P)H-quinone reductase, as a candidate enzyme of chromium(VI) reduction. This enzyme, firstly characterized in 1962 (8), has been shown so far to have beneficial effects in the detoxification of a variety of quinones and of certainazo dyes (8, 11, 16, 25). It catalyzes a 2-electron transfer from reduced pyridine nucleotides (NADPH and NADH) to the substrate, thereby avoiding generation of intermediate semiquinone derivatives and subsequently of highly toxic oxygen radicals, such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and their by-products (see Chart 8).

The NADPH-generating enzymes present in the cytosol. Interestingly, the DT-diaphorase activity in hepatic cytosol was strongly enhanced upon decreasing the pH value to 5.5 to 6.5, which conversely affected its NADPH-dependent activity to a very limited extent. Reduction of the metal ion by liver homogenates, S-12, and S-105 fractions and by lung S-12 and S-105 fractions, from both untreated and Aroclor-treated rats, was partially counteracted by known inhibitors of DT-diaphorase, such as dicumarol, pCMB, and L-thyroxine. The specificity of this phenomenon was checked with dicumarol, which in the Ames reversion test inhibited the decrease of chromumb(VI) mutagenicity due to liver S-12 fractions, while it did not affect the metabolic deactivation of other direct-acting mutagens, i.e., epichlorohydrin and ICR 191. A partial inhibition of the cytosolic chromium(VI)-reducing activity was also obtained with GSSG and pyruvate. Since these metabolites remove NADPH and NADH, respectively, through specific enzymatic mechanisms, they are likely to provide a metabolic switch off of DT-diaphorase activity by limiting the supply of reducing equivalents to this enzyme.

Additional data in favor of the involvement of DT-diaphorase resulted from the measurement of its activity and of the activities of NADPH-generating enzymes present in the cytosol. Interestingly, the DT-diaphorase activity in hepatic cytosol was stimulated by the known enzyme inducers phenobarbital and 3-methylcholanthrene and, especially, by Aroclor 1254. This PCB induced a striking increase in activity, which was of the same order of magnitude (35 to 40 times over the controls) as the one obtained with GSSG and pyruvate. Since these metabolites remove NADPH and NADH, respectively, through specific enzymatic mechanisms, they are likely to provide a metabolic switch off of DT-diaphorase activity by limiting the supply of reducing equivalents to this enzyme.
recently observed by using the quinone resorufin as a substrate (16). Aroclor also coinduced GSSG reductase, 6PDG, and especially 6GPD (about 4-fold) in liver cytosol, while it did not enhance any of these enzymatic activities in lung fractions. In the latter preparations, a slight yet significant stimulation of 6GPD, 6PDG, and DT-diaphorase was conversely determined by the i.t. administration of dichromate to rats (0.25 mg/kg, 5 times per week for 4 weeks).

The stimulation of these enzyme activities in the liver following systemic injection of Aroclor and in the lung following the local application of dichromate compares well with the parallel enhancement of chromium(VI) reduction which is afforded by the same cell preparations in mutagenicity test systems (21).

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All these findings support the view that DT-diaphorase, acting via a 2-electron transfer from reduced pyridine nucleotides to mitochondria. Toxolology, 24: 115-122, 1982.

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Prominent Role of DT-Diaphorase as a Cellular Mechanism Reducing Chromium(VI) and Reverting Its Mutagenicity

Silvio De Flora, Alessandro Morelli, Cristina Basso, et al.


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