Biochemical Pharmacology, Vol. 33, No. 15, pp. 2461–2466, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00
© 1984 Pergamon Press Ltd.

INHIBITORY ACTION OF HEXAVALENT CHROMIUM (Cr(VI)) ON THE MITOCHONDRIAL RESPIRATION AND A POSSIBLE COUPLING TO THE REDUCTION OF Cr(VI)

DAVID RYBERG* and JAN ALEXANDER†

*Institute of Occupational Health, Gydas vei 8, P.O. Box 8149 Dep. Oslo 1, Norway and †National Institute of Public Health, Dep. of Toxicology, Geitmyrsv. 75, Postuttak Oslo 1, Norway

(*Received* 13 *December* 1983; *accepted* 14 *February* 1984)

Abstract—The reduction of hexavalent chromium (Cr(VI)) in isolated liver mitochondria was studied under different redox conditions in the respiratory chain. With 25 μM Na_2CrO_4 the rates were 1.6 ± 0.7, 13.9 ± 0.6 and 12.7 ± 0.7 nmole Cr(VI) reduced 15 min/mg protein with the electron transport chain oxidized, reduced and only complex 1 reduced respectively. Electrons from succinate, bypassing complex 1, were apparently unavailable for Cr(VI)-reduction. The kinetics of chromate reduction was studied with only complex 1 in a reduced state. A rapid and a slow phase were found, probably corresponding to different electron donors in the mitochondria. Blocking the free thiols with *N*-ethylmaleimide lead to less than 10% decrease in the rapid initial Cr(VI)-reduction and to about 20% decrease during the whole incubation period (15 min). The amounts of free thiols were moderately decreased (15%) in chromate treated mitochondria during the slow reduction of Cr(VI) only. SH-groups may thus participate as reductants during the slow reduction phase. The respiration rate was inhibited about 50% by 25 μM Na_2CrO_4 when the mitochondria oxidized NAD-linked substrates. In contrast, succinate stimulated respiration was inhibited 50% by 3.6 mM Na_2CrO_4. The observed inhibition with Na_2CrO_4 in the micromolar range was therefore probably localized at complex 1 and may be coupled to the reduction of Cr(VI) at the same place. The respiration of isolated hepatocytes was also affected by Na_2CrO_4. Five micromolar chromate caused 5–10% inhibition. The inhibitory action of chromate on the mitochondrial respiration may thus constitute an important cytotoxic mechanism.

Hexavalent chromium (Cr(VI)) compounds are toxic agents capable of inducing tumors [1], mutations [2] and tissue damage [3, 4]. The biological activity of Cr(VI) is generally attributed to the oxidizing power of the chromates and to the ability of trivalent chromium (Cr(III)) to form substitution inert complexes with several different cell components. Water soluble Cr(VI)-compounds, in contrast to Cr(III)-compounds, easily penetrate the cell membrane [5]. Intracellularly they are rapidly reduced to Cr (III) [5, 6]. Various compounds and organelles may participate in the intracellular Cr(VI)-reduction. Ascorbate and sulfhydryl-compounds are assumed to be important electron donors because of their reactivity with Cr(VI) under physiological conditions [7, 8]. Similarly, the endoplasmatic reticulum may possess such an ability, since Cr(VI) is reduced when incubated with the microsomal fraction from rat liver in presence of NADPH or NADH [9]. Data on subcellular distribution of chromium in rat liver after intravenous chromate administration [10] as well as the considerable ability of isolated mitochondria to accumulate chromate [11], indicate that the mitochondria may contribute significantly to the cellular $Cr(VI)^-$ reduction. Chromate may also affect mitochondrial functions, i.e. modify mitochondrial enzymes in myocardium [12] and testicular tissue [13] in chronically dichromate poisoned animals as well as cause mitochondrial swelling in renal tubular cells after intravenous chromate administration [3]. We have therefore studied the chromate metabolism in isolated liver mitochondria and possible functional impairments caused by chromate.

MATERIALS AND METHODS

Rotenone, antimycine A, carbonyl-cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), Lubrol PX, palmitoyl-L-carnitine, bovine serum albumin (BSA) fraction V, *N*-ethylmaleimide (NEM) and ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetra-acetic acid (EGTA) were products from Sigma Chemical Company (St. Louis, U.S.A.). All other chemicals were commercially available p.a. quality. Wistar rats of 200–250 g weight from Møllegaard, Copenhagen, Denmark were used in all experiments. The animals were starved 12 hr before sacrificing by decapitation.

Preparation of mitochondria. Liver mitochondria were isolated as described by Nedergaard and Cannon [14]. The liver was homogenized in 250 mM sucrose, 20 mM K–HEPES buffer pH 7.40 and 1 mM EGTA. The 1 : 8 diluted homogenate was centrifuged at 900 *g* (max) for 12 min in about half-filled tubes (Sorvall rotor SS-34). The resulting supernatant was centrifuged at 9000 *g*(max) for 10 min. The mitochondrial pellet was resuspended in the same medium without EGTA and centrifuged at 9000 *g*(max) for 8 min. This last step was repeated once more, and the final pellet resuspended in 250 mM sucrose, 20 mM K–HEPES, pH 7.40 to a total protein concentration of 80–100 mg/ml. Mito-

chondria with respiratory control ratio lower than 5 with succinate were discarded.

Preparation of isolated hepatocytes. Parenchymal cells were prepared by the method of Seglen [15], as described previously [16]. The viability of the cells was assessed by the trypan blue exclusion test (viability better than 92% accepted).

Cr(VI)-*measurements in mitochondrial suspensions.* Cr(VI) was measured by the diphenylcarbazid (DPC) method in acid solution (pH about 2) as described by Sandell [17]. About 1 mg/ml mitochondrial protein was incubated at 37° in 150 mM KCl, 20 mM K–HEPES and 5 mM K–phosphate, pH 7.20. This medium was used in all experiments with mitochondria. Other additions are given in the figure legends. The effects and specificity of the applied inhibitors of the mitochondrial electron transport are described by Singer [18]. After 5 min preincubation, Na_2CrO_4 was added and 2.0 ml aliquots were withdrawn at different times. These samples were mixed with 0.40 ml 0.15% DPC, 0.6% Lubrol PX in 2 N H_2SO_4(w/v). Cr(VI) at zero time incubation was measured in separate experiments with all additions present other than mitochondria, which were added after the Cr-diphenylcarbazonium complex had been formed. The complex was measured after 15 min at 540 nm in a Beckham DU7 spectrophotometer with a correction program for light scattering. The complex was formed within 10 sec and was stable for at least 30 min. Standard solutions of Na_2CrO_4 were measured under identical conditions.

Cr(VI)-*measurements in hepatocyte suspensions.* The parenchymal cells were incubated at 37° in 145 mM NaCl, 4 mM KCl, 5 mM glucose, 1 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.5% BSA (w/v), 0.4 mM Na,K-phosphate and 20 mM HEPES buffer pH 7.45. This medium was used in all experiments with the hepatocytes. The experimental procedures were otherwise as described above under measurements of Cr(VI) in the mitochondrial suspensions.

Oxygen measurements. The O_2-consumpton of the mitochondria was measured with a Clark O_2-electrode in a thermostated chamber with magnetic stirring. The cellular respiration was measured in a Gilson Differential Respirometer [19]. The cell suspensions were preincubated for 10 min at 37° in the cuvettes with NaOH as CO_2-trapping agent in the central well. Na_2CrO_4 was then added. After 15 more minutes the manometer valves were closed and measurement started. Readings were done every 15th minute. The initial cellular respiration was measured in separate experiments with a Clark O_2-electrode as described above.

Other methods. Mitochondrial sulfhydryl groups were determined with Ellman's reagent as described by Sedlak and Lindsay [20], after solubilization with 0.1% Lubrol PX. Protein was analysed with the biuret method with BSA as standard.

RESULTS

Cr(VI)-*reduction in mitochondria.* The reduction of Cr(VI) in the mitochondrial suspension was very slow when the mitochondrial electron transport chain was in an oxidized state, as achieved by the combination of FCCP and no substrate added (Table 1). The few electrons released during oxidation of endogenous substrates will under such conditions easily be captured by oxygen [21]. However, some electrons may also be consumed by chromate. It is known that uncouplers affect the transport and distribution of several ions over the mitochondrial inner-membrane [22]. To eliminate a possible effect of the uncoupler FCCP on the mitochondrial uptake of chromate, this experiment was repeated with sonicated mitochondria. A similar result was obtained. Maximum Cr(VI)-reduction was seen when the whole electron transport chain was in reduced state. This was achieved by the use of glutamate as the primary electron source and KCN for inhibition of the cytochrome oxidase. The Cr(VI)-reduction was near to maximum when the mitochondria were incubated with glutamate and the electron transport chain was blocked by rotenone. During the latter incubation only the first part of the electron transport chain (complex 1) will be totally reduced [23, 24]. Neither preincubation with FCCP

Table 1. Mitochondrial reduction of Cr(VI)—the effect of redox conditions in different parts of the respiratory chain

Additions	nmole Cr(VI) reduced 15 min/mg protein
FCCP 0.5 μM	1.6 ± 0.7
Glutamate 10 mM, rotenone 4 μM	12.7 ± 0.7
3-hydroxybutyrate 10 mM, rotenone 4 μM	12.5*
Glutamate 10 mM, antimycine A 1 μg/ml	12.9 ± 0.4
Glutamate 10 mM, KCN 1 mM	13.9 ± 0.6
Succinate 10 mM, rotenone 4 μM, KCN 1 mM	7.3 ± 0.5
Malonate 10 mM, rotenone 4 μM, KCN 1 mM	6.8 ± 0.9

* Result from one experiment.

The figures are the mean with S.D. from 4 experiments.

The mitochondria (1 mg protein/ml) were preincubated for 5 min in medium (150 mM KCl, 20 mM K–HEPES and 5 mM K–phosphate, pH 7.20) with additions at 37°. 25 μM Na_2CrO_4 was added and Cr(VI) measured with diphenylcarbazid after 15 min as described in Materials and Methods. Cr(VI) at zero time incubation was analysed in separate incubations with all additions without mitochondria. Known Na_2CrO_4 solutions were used as standards.

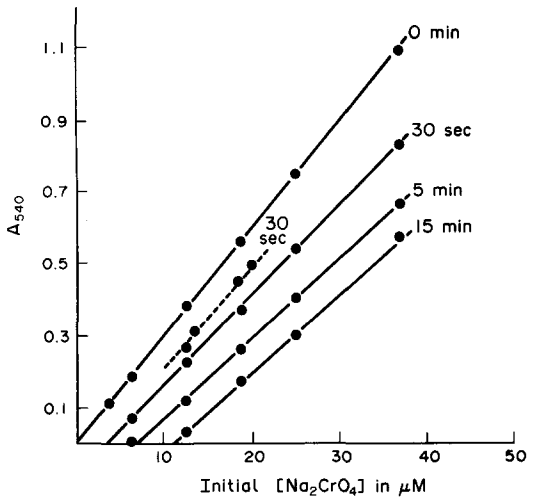

Fig. 1. Remaining Cr(VI) at different incubation times as a function of initial concentrations of Na_2CrO_4. The mitochondria (1.1 mg protein/ml) were preincubated for 5 min in medium with 10 mM glutamate and 4 μM rotenone as described in Materials and Methods. Aliquots of 2 ml were withdrawn at the indicated times, and Cr(VI) measured at 540 nm after complex formation with DPC. Cr(VI) at zero time incubation was measured in separate incubations without mitochondria. The absorbance values indicated are the mean of 2 experiments. – – –, 12.5 μM Na_2CrO_4 added 4.5 min after a prior addition of 3.5, 6.5, 12.5 and 18.0 μM. The points represents the total remaining Cr(VI) 30 sec after this second addition.

nor sonication of the mitochondria changed the observed reduction of Cr(VI). Other NAD-linked substrates, such as citrate and 3-hydroxybutyrate, could replace glutamate. Succinate as the primary electron source gave apparently about half maximum reduction rate of Cr(VI) (with rotenone added). A similar result was obtained when succinate was omitted and succinate dehydrogenase blocked by malonate (Table 1). Thus the electrons from succinate, which are delivered to complex 2, bypassing complex 1, may be unavailable for the Cr(VI)-reduction. This accords with similar results from Connett and Wetterhahn [25]. The Cr(VI)-reduction in the last experiments could be explained by the electron pressure on complex 1 due to oxidation of endogenous substrates and the efficient inhibition by rotenone.

The mitochondrial Cr(VI)-reduction, carried out keeping only complex 1 in a reduced state, can be divided in a fast and a slow phase (Fig. 1). There was a relatively large decrease in the Cr(VI) level during the first 30 sec after addition of Na_2CrO_4. The amount of Cr(VI) reduced was dependent on the initial concentration of chromate. However, the reduction rate decreased rapidly, i.e. from 11.4 to 0.29 nmole reduced/mg protein/min with 25 μM Na_2CrO_4 (mean rate the first 30 sec and the last 10 min in the incubation period respectively). A similar pattern in the Cr(VI)-reduction was found when sonicated mitochondria were used. The quantity of Cr(VI) reduced between 5 and 15 min was independent of the start concentration of chromate. The reduction rate could be raised during the slow phase if a new addition of chromate was made. However the initial decrease in the Cr(VI)-level after this second addition was considerably smaller than the first one (Fig. 1). The slow phase was not affected by a second addition of chromate (not shown). This indicates the existence of at least two electron donors with different kinetic properties, corresponding to the two phases in the Cr(VI)-reduction.

Contribution from SH-groups. Sulfhydryl-groups are assumed to be important electron donors for Cr(VI) under physiological conditions [8]. Therefore the total amount of free SH-groups in the mitochondria and the quantity of Cr(VI) reduced were measured under different incubation conditions (Table 2). No changes in the level of free thiols were observed during the first minute after chromate addition, although a major part of Cr(VI) was reduced during this period. More than 90% of the SH-groups could be blocked by NEM treatment. This lead to less than 10% decrease in the Cr(VI)-reduction during the initial 30 sec and to about 20% decrease during the whole incubation period

Table 2. Free SH-groups and Cr(VI)-reduction in mitochondria

Additions	nmole SH-groups/mg protein			nmole Cr(VI)reduced/mg protein	
	zero	1 min	15 min	30 sec	15 min
FCCP	78	80	77		—
FCCP, Na_2CrO_4	79	77	73	1.3	1.7
Glutamate, rotenone	81	79	77		—
Glutamate, rotenone, Na_2CrO_4	80	81	65	5.9	12.5
Glutamate, rotenone, NEM	6.3	6.4	4.6		—
Glutamate, rotenone, NEM, Na_2CrO_4	6.0	6.1	4.0	5.5	10.4

Typical results are shown.

The mitochondria (1 mg protein/ml) were preincubated as described in Materials and Methods in medium with all additions apart from chromate. Zero-time samples for SH-determination were withdrawn after 5 min, and 25 μM Na_2CrO_4 was added just afterwards to the current incubations. Cr(VI) was measured with DPC 30 sec and 15 min after chromate addition was described in Materials and Methods. At times indicated SH-groups were analyzed with Ellman's reagent after the method of Sedlak and Lindsay [20].

Concentration of other additions: FCCP 0.5 μM, glutamate 10 mM, rotenone 4 μM and *N*-ethylmaleimide (NEM) 0.5 mM.

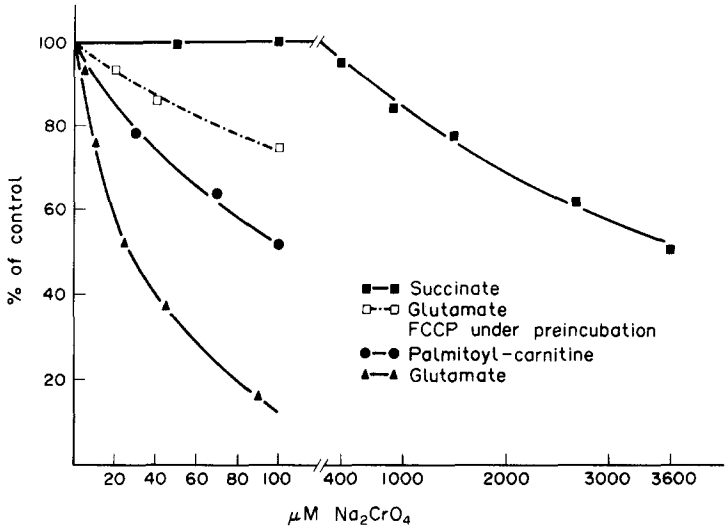

Fig. 2. Mitochondrial respiration rate at different Na_2CrO_4 concentrations as per cent of the control. Each point represents the mean of 4 experiments. The mitochondria (1 mg protein/ml) were preincubated for 5 min in the medium as described in Materials and Methods, and with additions as indicated below. Different amounts of Na_2CrO_4 were added, and after 2 more minutes the respiration was released with 0.5 μM FCCP. The O_2-consumption was measured with a Clark O_2-electrode in a 2 ml chamber at 25°C. ■——■, 10 mM succinate, 4 μM rotenone; ●——●, 15 μM palmitoyl-carnitine, 1 mM malate; ▲——▲, 10 mM glutamate, 1 mM malate; □- · - · -□, Mitochondria preincubated with 0.5 μM FCCP in medium. Two minutes after chromate addition, 10 mM glutamate and 1 mM malate were added. Mean respiratory rates of the controls were 58, 44 and 39 nmole O_2/min/mg protein for succinate, palmitoyl-carnitine and glutamate respectively.

(15 min). SH-groups are therefore probably more important as reducing equivalents for Cr(VI) during the slow reduction phase, but may also function as ligands for the Cr(III) formed.

Cr(VI) *inhibits the mitochondrial respiration.* The inhibitory effect of Na_2CrO_4 on the mitochondrial respiration was most prominent when NAD-linked substrates were oxidized (Fig. 2). Mitochondria incubated with 25 μM Na_2CrO_4 had 50% lower maximum respiration rate (with FCCP) than the control when glutamate was oxidized (Fig. 2). The inhibitory effect of chromate was similar with pyruvate or 3-hydroxybutyrate as substrates (not shown). A higher concentration of Na_2CrO_4 (110 μM) was necessary to attain 50% inhibition of palmitoyl-carnitine stimulated respiration. When the mitochondria oxidized succinate, the respiration rate was not at all affected at this concentration level, as 3.6 mM Na_2CrO_4 was necessary for 50% inhibition. The inhibitory effect was fast and similar whether the mitochondria were preincubated with Na_2CrO_4 for 1 or 3 min. A less prominent effect of chromate was seen when the mitochondria were incubated with the uncoupler FCCP and chromate *before* the respiration was started by addition of substrate (glutamate), i.e. 25% inhibition with 100 μM Na_2CrO_4. Only small quantities of Cr(VI) are reduced under such conditions (Table 1), indicating that the inhibitory effect on respiration may be coupled to the reduction of Cr(VI). As described earlier (Table 1) the reduction was probably mainly localized to the first part of the respiratory chain at low concentration of Na_2CrO_4. Addition of trivalent chromium ($CrCl_3 \leq 1$ mM) had no effect on the respiratory rate (not shown).

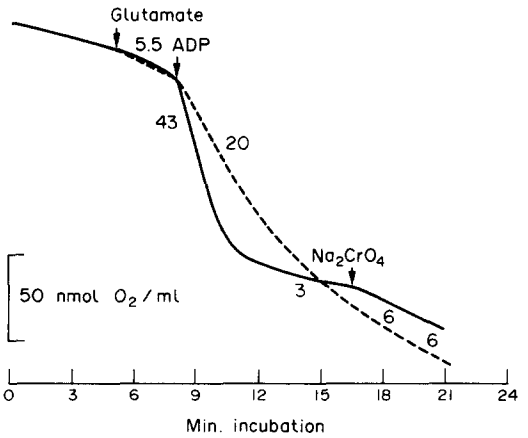

Fig. 3. The effects of Na_2CrO_4 on state 3 and 4 respiration. The mitochondria (1.1 mg protein/ml) were preincubated for 5 min in the medium with 1 mM malate. Ten millimolar glutamate was then added, and after 2 more minutes the respiration was stimulated with 600 nmol ADP. The O_2-consumption was measured in a 2 ml chamber at 25° with a Clark O_2-electrode. The figures indicated are nmole O_2 consumed/min/mg protein. Typical traces are shown. - - - Na_2CrO_4 (25 μM) was added together with 10 mM glutamate after 5 min preincubation. —— Na_2CrO_4 (25 μM) was added during state 4 respiration.

Figure 3 shows that Na_2CrO_4 also inhibited the ADP-dependent (state 3) respiration. The effect of 25 μM chromate with glutamate as substrate, was similar both on state 3 and on uncoupled (FCCP) respiration. The state 3 respiration with succinate

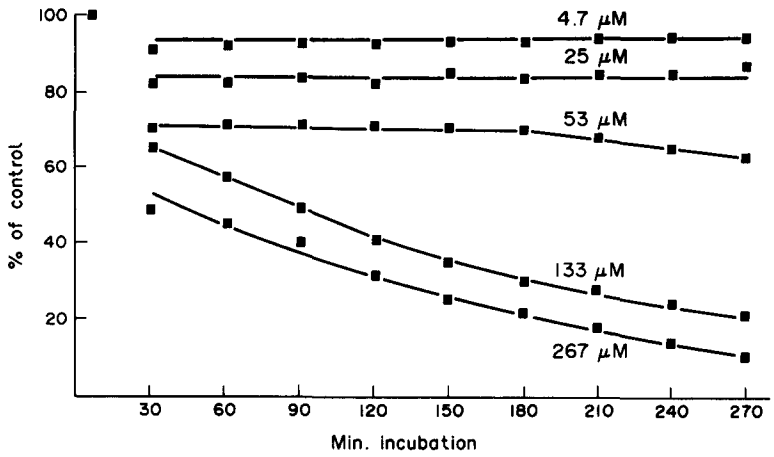

Fig. 4. The effect of Na_2CrO_4 on the respiration of isolated hepatocytes. Each point represents the mean respiratory rate during 30 min expressed as per cent of control from 3 experiments. Hepatocytes ($2 . 10^6$) were incubated in 3 ml medium and the measurement performed as described in Materials and Methods. The mean respiratory rate of control was 35 μl O_2/60 min/10^6 cells. Concentrations of chromate in micromolar are indicated.

was again not affected at this concentration range of Na_2CrO_4 (≤250 μM). The state 4 respiration was on the other hand increased (Fig. 3), an indication of lower proton-motive force probably due to membrane damage.

Cr(VI)-*effect on cellular respiration.* Na_2CrO_4 also inhibited the respiration of isolated liver parenchymal cells (Fig. 4). The effect was not immediate like that on isolated mitochondria. No change was seen in the respiration rate during the first 5 min after chromate addition, even with 500 μM Na_2CrO_4. The inhibition was however manifest 30 min after chromate addition. (The first point measured in the Gilson Respirometer.) The lowest dose with measurable effect was about 5 μM chromate, which gave 5–10% inhibition of cellular respiration. Concentrations beyond 50–100 μM Na_2CrO_4 gave an accelerated inhibition probably resulting in cell death (Fig. 4).

About 30% of Cr(VI) was reduced within 30 sec after the addition of 25 μM Na_2CrO_4 to the hepatocytes (0.67×10^6 cells/ml) (Fig. 5). This initial Cr(VI)-reduction was probably not associated directly with the mitochondria as their function seemed to be unaffected, i.e. no change in the cellular respiration rate during the first 5 min incubation with chromate. Involvement of the outer mitochondrial membrane can, however, not be excluded. Based on the inhibitory pattern of Na_2CrO_4 on respiration of isolated mitochondria and their capacity for Cr(VI)-reduction, the mitochondrial contribution in the total cellular reduction of 25 μM Na_2CrO_4 could be estimated to 15–30%. This contribution will probably vary depending on the concentration of chromate.

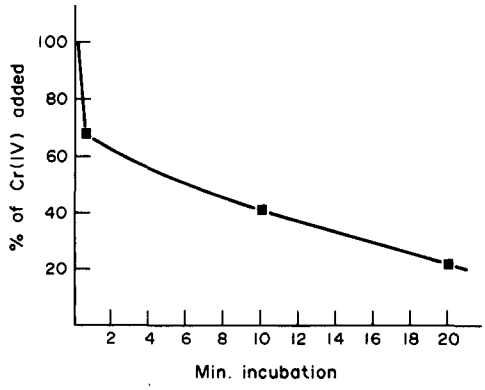

Fig. 5. Remaining Cr(VI) in the hepatocyte suspension as a function of incubation time, expressed as per cent of Cr(VI) added. The values are mean from 2 experiments. Hepatocytes (6.7×10^6) were preincubated in 10 ml medium under careful shaking. After 10 min 25 μM Na_2CrO_4 was added, and Cr(VI) analysed with DPC in 2 ml aliquotes as described in Materials and Methods.

DISCUSSION

The present results demonstrate the ability of mitochondria to reduce Cr(VI). The effects of different substrates (electron sources) and specific inhibitors of the electron transport chain, indicate that Cr(VI), in the low micromolar range, picks up electrons preferentially from NAD-linked substrates at the level of complex 1. A direct participation of NADH or NADPH in the matrix as reductants is probably negligible due to their low reactivity with chromate [26] and the small quantity of these nicotinamides in the mitochondria (NAD+NADP pool about 2.0 nmole/mg protein [27]).

The initial Cr(VI)-reduction rate is fast, but decreases fairly rapidly. The fast reduction rate is also impaired if the mitochondria are pretreated with chromate. These phenomena may be due to a reduced availability of reduction sites for Cr(VI) and a possible inhibited electron transport in the respiratory chain both caused by chromium. A slow-reducing system dominates after a few minutes and

apparently is saturated at low concentrations of chromate (less than 5 μM). The importance of this slow system increases with increasing concentrations of chromate due to a relatively lower contribution of the fast-reacting system. Sulfhydryl groups probably participate in the slow reduction of Cr(VI), but there was no correlation between the total amount of free SH-groups and the amount of Cr(VI) reduced during 15 min incubation. A complicating factor is the availability of mitochondrial SH-groups which are shown to vary under different incubation conditions [28]. The binding of *N*-ethylmaleimide to such groups in *intact* mitochondria is for example decreased by uncouplers like FCCP [29]. Reduced availability of SH-groups may thus partly explain the slow Cr(VI)-reduction in FCCP-treated mitochondria.

Na_2CrO_4 in the micromolar range caused a fast and distinct inhibition of the mitochondrial respiration, but only with NAD-linked substrates. The effect was similar both in uncoupled- and ADP-dependent respiration. This indicates that possible effects of chromate on the ATPase [30] is not involved. In separate experiments (not shown) we have not been able to identify any complex between chromium and NAD or NADH which could explain the inhibition of mitochondrial respiration. Electrons consumed in the Cr(VI)-reduction are also too few for obtaining the observed decrease in the O_2-consumption rate. We suggest that Cr(VI)-reduction at complex 1 causes an interrupting effect on the electron transport in this complex. This can be due to formation of Cr(III)-complexes with essential components or oxidative damage of such components caused by reactive intermediate oxidation states of chromium. Formation of such oxidation states under Cr(VI)-reduction are described [31], but they have received little attention in biological systems. Consequently, there is reason generally to suspect the reduction sites of Cr(VI) to be potentially vulnerable places.

The Cr(VI)-induced inhibition of respiration seen in isolated mitochondria is also present in isolated liver parenchymal cells. Mitochondria are likely contributors to the total cellular Cr(VI)-reduction, and this has also been inferred from data on subcellular distribution of chromium in rat liver [10]. A common inhibitory mechanism of mitochondrial and cellular respiration may therefore exist. However, it can not be excluded that chromium also affects other systems which are determinants for the cellular O_2-consumption.

A stongly decreased ATP level accompanied by a pronounced increase in ADP and AMP was seen in hamster fibroblasts during chromate exposure [32]. Since ATP-synthesis is mainly coupled to the mitochondrial respiration, these results indicate that mitochondria in hamster fibroblasts may also be affected by chromate. The inhibitory action of chromate on mitochondrial respiration may therefore constitute an important mechanism with potentially great consequences for the cellular functions.

Acknowledgement—We wish to express our appreciation to Anne-Lise Nordhagen and Kari Ødegaard for their expert technical assistance. This study was supported by Borregaard A/S Forskningsfond.

REFERENCES

1. T. Norseth, *Environ. Hlth Perspect.* **40**, 121 (1981).
2. A. Léonard and R. R. Lauwerys, *Mutat. Res.* **76**, 227 (1980).
3. S. K. Tandon, in *Biological and Environmental Aspects of Chromium* (Ed. S. Langård), pp. 209. Elsevier Biomedical Press, Amsterdam (1982).
4. N. B. Pedersen, in *Biological and Environmental Aspects of Chromium* (Ed. S. Langård), pp. 249. Elsevier Biomedical Press, Amsterdam (1982).
5. P. C. Rajam and A. L. Jackson, *Proc. Soc. exp. Biol. Med.* **99**, 210 (1958).
6. R. D. Barr, W. Dougherty and S. Perry, *J. Lab. clin. Invest.* **88**, 1032 (1976).
7. M. H. Samitz, D. M. Scheiner and S. A. Katz, *Archs Envir. Hlth* **17**, 44 (1968).
8. H. Sakurai, T. Tachikawa and S. Shimomura, *Analyt. Lett.* **B11**, 879 (1978).
9. J. E. Gruber and K. W. Jennette, *Biochem. biophys. Res. Commun.* **82**, 700 (1978).
10. S. Langård, *Biol. Trace Elem. Res.* **1**, 45 (1979).
11. J. Alexander, J. Aaseth and T. Norseth, *Toxicology* **24**, 115 (1982).
12. A. M. Shakhnazarov, *Archs. Path.* **35**, 67 (1973).
13. J. R. Behari, S. V. Chandra and S. K. Tandon, *Acta Biol. Med. Germ.* **37**, 463 (1978).
14. J. Nedergaard and B. Cannon, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. 55, pp. 3. Academic Press, New York (1979).
15. P. O. Seglen, *Meth. Cell Biol.* **13**, 29 (1976).
16. J. Alexander and J. Aaseth, *Biochem. Pharmac.* **31**, 685 (1982).
17. E. B. Sandell, *Colorimetric Determination of Traces of Metals*, pp. 392. Interscience New York (1959).
18. T. P. Singer, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. 55, pp. 454. Academic Press, New York (1979).
19. W. W. Umbreit, R. H. Burris and J. F. Stauffer, *Manometric Techniques*, 4th edn. Burgess Publishing (1964).
20. J. Sedlak and R. H. Lindsay, *Analyt. Biochem.* **25**, 192 (1968).
21. E. Padan and H. Rottenberg, *Eur. J. Biochem.* **40**, 431 (1973).
22. K. van Dam and C. S. Tsou, *Biochim. biophys. Acta* **162**, 301 (1968).
23. D. J. Horgan and T. P. Singer, *J. biol. Chem.* **243**, 834 (1968).
24. K. E. Öberg, *Expl Cell Ress.* **24**, 163 (1961).
25. P. H. Connett and K. E. Wetterhahn, *Structure Bonding* **53**, 93 (1983).
26. J. D. Garcia and K. W. Jennette, *J. Inorg. Biochem.* **14**, 281 (1981).
27. H. W. Heldt, N. Greif and M. Klingenberg, *J. biol. Chem.* **240**, 4659 (1965).
28. N. Sabadie-Pialoux and D. Gautheron, *Biochim. biophys. Acta* **234**, 9 (1971).
29. P. M. Vignais and P. V. Vignais, *FEBS Let.* **26**, 27 (1981).
30. M. J. Bossard and S. M. Schuster, *J. biol. Chem.* **256**, 6617 (1981).
31. J. K. Beattie and G. P. Haight Jr., *Progr. Inorg. Chem.* **17**, 93 (1972).
32. V. Bianchi, P. Debetto, A. Zantedeschi and A. G. Levis, *Toxicology* **25**, 19 (1982).