CYTOTOXIC EFFECTS OF HEXAVALENT AND TRIVALENT CHROMIUM ON MAMMALIAN CELLS IN VITRO

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Summary.—The cytotoxic effects of hexavalent (K₂Cr₂O₇) and trivalent (CrCl₃) chromium compounds have been studied in cultured hamster fibroblasts (BHK line) and human epithelial-like cells (HEp line).

K₂Cr₂O₇ stimulates the uptake of labelled thymidine into the soluble intracellular pool (the stimulation of nucleoside uptake represents a specific effect of Cr⁶⁺) while Cr³⁺ always exerts an inhibitory action. DNA synthesis is inhibited by treatment with both chromium compounds, but especially by K₂Cr₂O₇. Moreover, the effective CrCl₃ concentrations reduce the sensitivity of DNA and RNA to hydrolysis with perchloric acid. Treatments with K₂Cr₂O₇ in balanced salt solution, where Cr⁶⁺ reduction is less marked, induce more pronounced cytotoxic effects than treatments in complete growth medium.

HEp cells turned out to be more sensitive to K₂Cr₂O₇ than BHK fibroblasts: in the former line TdR uptake is less stimulated, DNA synthesis and cell survival are more affected. Survival of BHK cells to K₂Cr₂O₇ indicates a multi-hit mechanism of cell inactivation, the extrapolation number being about 10.

On the basis of quantitative Cr determinations in the treatment solutions and in the treated cells, the cytotoxic effects of Cr are attributed to the action of Cr⁶⁺ at the plasma membrane level on the mechanisms involved in nucleoside uptake, and to the interaction of Cr³⁺ at the intracellular level with nucleophilic targets on the DNA molecule.

Mutagenic effects of environmental metals suspected of being carcinogenic have been reported (Miller and Miller, 1971; Nishioka, 1975; Sirover and Loeb, 1976) but little is known about the mechanisms of their cytogenetic action. Chromium (Cr) compounds are carcinogenic in man (Browning, 1969; Furst and Haro, 1969, IARC, 1973) and experimental animals (Hueper, 1961; IARC, 1973; Maltoni, 1974) induce cell transformation in vitro (Fradkin et al., 1975) and give rise to several cytogenetic effects in different biological systems: point mutations (Venitt and Levy, 1974; Nishioka, 1975; Bonatti, Meini and Abbondandolo, 1976), alterations of nucleic acid physico-chemical properties (Herrmann and Speck, 1954; Huff et al., 1964), chromosome aberrations (Gläss, 1956) and DNA repair synthesis (Raffetto et al., 1977). Nevertheless, the actual Cr oxidation state responsible for the above biological effects, especially for Cr interactions with genetic material, is still doubtful (Mertz, 1969; Schoental, 1975).

We have observed that Cr compounds act differentially on macromolecular syntheses and soluble-precursor uptake into the intracellular pool of mammalian cells grown in vitro (Levis and Buttignol, 1977; Levis, Buttignol and Vettorato, 1977; Levis et al., 1978), induce mitotic-cycle alterations and chromosome aberrations (Majone, 1977) and interact with nucleic acids and purified nucleotides (Tamino, 1977). In the present paper we report the cytotoxic effects of potassium dichromate
(K₂Cr₂O₇) and chromium chloride (CrCl₃), which are soluble salts of hexavalent (Cr⁶⁺) and trivalent (Cr³⁺) chromium, on cultured hamster fibroblasts (BHK line) and human epithelial-like cells (HEp line). Cr-induced alterations of thymidine (TdR) transport through the plasma membrane, of DNA synthesis, and of cell survival have been investigated.

**MATERIALS AND METHODS**

**Cells.**—Cultures of the established BHK 21 hamster fibroblast line, Clone 12, and of the HEp 2 human epithelial-like line are routinely grown in our laboratory at 37°C as monolayers, in Eagle’s minimal medium (MEM) supplemented with 10% calf serum.

**Cell survival.**—The cells are always harvested from log-phase cultures, and single-cell suspensions are made by the usual trypsinization procedures. Samples from cell suspensions, properly diluted with growth medium supplemented with 20% calf serum, are plated in 60 mm glass Petri dishes. The dishes are incubated for 8 days (BHK line) and 10 days (HEp line) at 37°C in a humidified CO₂-supplemented incubator, stained with acetic gentian violet, and scored for survivors. All colonies visible to the naked eye are counted as survivors.

**Cell treatment and labelling.**—Potassium dichromate (K₂Cr₂O₇, Mallinckrodt 6770, St Louis, Mo. 63160) and chromium chloride (CrCl₃·6H₂O, Merck, Darmstadt, Germany) are solubilized in sterile bidistilled water at 10⁻¹–10⁻⁸M concentrations immediately before use, and afterwards diluted ×100 in pre-warmed MEM or in Hanks’ balanced salt solution (HBSS) to 10⁻³–10⁻⁵M final concentrations. Experimental treatments are carried out on exponential cultures at 37°C in a climatized room; pre-warmed solutions are used in order to avoid thermic shock. After different lengths of treatment, the cultures are rinsed twice with pre-warmed HBSS and the medium containing Cr is replaced with normal growth medium. At different intervals after exposure to Cr compounds, the cultures are incubated for 1 h with tritiated nucleic acid precursors (The Radiochemical Centre, Amersham, England). Thymidine-6-H³ (³H-TdR; 2 Ci/mM) and uridine-5-H³ (³H-UR; 2–5 Ci/mM) are used at the concentration of 1 µCi/ml.

**Extraction procedures and analytical methods.**—From labelled cultures, nucleotides of the intracellular pool, RNA, DNA and proteins, are differentially extracted with perchloric acid and potassium hydroxide and measured by UV absorption as detailed elsewhere (Levis et al., 1978). Radioactivity counting of liquid samples (0.5 ml) of the different fractions is carried out by a Packard Tri-Carb 2425 scintillation counter using 10 ml of Bray’s solution. The radioactivity counts in the different fractions of a culture are normalized by dividing them by the amount of DNA in the same culture, giving values which are referred to as normalized (radio) activities. In the treated cultures, normalized activities are expressed as percentages of control values. Since Cr compounds affect the uptake of labelled nucleosides into the intracellular pool, changing their relative concentrations, the original percent values of nucleic acid normalized activities have been divided by the corresponding percent normalized activities of intracellular nucleotides. Such values therefore express the actual rates of precursor incorporation into macromolecular compounds and represent the net levels of RNA and DNA syntheses after treatment with Cr compounds (Levis et al., 1978).

**Chromium determinations.**—Determinations of oxidized, hexavalent chromium (Cr⁶⁺) and of reduced, trivalent chromium (Cr³⁺) are made by the coloured reaction complex with 1,5-diphenylcarbazide (Riedel-DeHaën AG, Hannover, Germany) after wet decomposition of the biological samples, as specified elsewhere (Levis et al., 1978). The colorimetric method is sensitive to 0.05 µg/ml of Cr in the final solution using 1 cm spectrophotometric cells, and Beer’s law is followed up to a concentration of 2 µg/ml of Cr in the final solution (or 2 parts/10⁶) as shown by the standard calibration curves.

**RESULTS**

Treatment of BHK cell cultures for 1–4 h with 10⁻⁵M–10⁻³M K₂Cr₂O₇ stimulates nucleoside uptake into the intracellular pool, inhibiting on the contrary nucleic acid synthesis (Levis et al., 1978). The effects of K₂Cr₂O₇ on TdR uptake and on DNA synthesis have been determined also in HEp cultures and compared with
Fig. 1.—$\text{K}_2\text{Cr}_2\text{O}_7$ effects on TdR uptake in BHK and HEp cultures. Normalized activities of $^3\text{H}$-TdR in the intracellular pool were determined in BHK (1) and HEp (2) cultures treated with $10^{-3}\text{M}$ (A), $10^{-4}\text{M}$ (B), $10^{-5}\text{M}$ (C) and $10^{-6}\text{M}$ (D) $\text{K}_2\text{Cr}_2\text{O}_7$ for 1 h (○—○), 2 h (○—○) and 4 h (△—△) in MEM.
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Fig. 2.—K$_2$Cr$_2$O$_7$ effects on DNA synthesis in BHK and HEp cultures. Normalized rates of DNA synthesis were determined in BHK (1) and HEp (2) cultures treated with 10$^{-3}$M (A), 10$^{-4}$M (B), 10$^{-5}$M (C) and 10$^{-6}$M (D) K$_2$Cr$_2$O$_7$ for 1 h (●—●), 2 h (○—○) and 4 h (▲—▲) in MEM.
TABLE I.—$K_2Cr_2O_7$ Effects on Survival of BHK and HEp Cultures

| $K_2Cr_2O_7$ concentration | Duratm of treatment† | Survival (% of controls)* |
|-----------------------------|----------------------|---------------------------|
|                             | (h)                  | BHK (MEM) (HBSS)          | HEp (MEM) (HBSS)          |
| $10^{-3}$M                  |                      |                           |                           |
| 1                           | 93                   | 102                      | 99                        | 104                       |
| 2                           | 96                   | 94                       | 101                       | 97                        |
| 4                           | 102                  | 98                       | 87                        | 81                        |
| $10^{-4}$M                  |                      |                           |                           |
| 1                           | 105                  | 92                       | 99                        | 86                        |
| 2                           | 93                   | 94                       | 92                        | 74                        |
| 4                           | 103                  | 87                       | 80                        | 62                        |
| $10^{-5}$M                  |                      |                           |                           |
| 1                           | 99                   | 99                       | 88                        | 72                        |
| 2                           | 97                   | 78                       | 72                        | 54                        |
| 4                           | 95                   | 70                       | 25                        | 12                        |
| $10^{-6}$M                  |                      |                           |                           |
| 1                           | 91                   | 80                       | 20                        | 2                         |
| 2                           | 61                   | 36                       | 2                         | <0.1                      |
| 4                           | 29                   | 17                       | <0.1                      | <0.1                      |
| $10^{-7}$M                  |                      |                           |                           |
| 1                           | 16                   | 7                        | <0.1                      | <0.01                     |
| 2                           | 1                    | <0.1                     | <0.01                     | <0.01                     |
| 4                           | <0.1                 | <0.1                     | <0.01                     | <0.01                     |

* Survival after $K_2Cr_2O_7$ treatment (% of control values) was determined by macroscopic colony counts in plates seeded with from $10^2$ to $10^4$ cells each.

† Treatment with $K_2Cr_2O_7$ was made in MEM or HBSS, 6 h after plating single-cell suspensions obtained by trypsinization.

those in BHK cultures. TdR uptake (Fig. 1) is stimulated less in HEp than in BHK cells by all the effective $K_2Cr_2O_7$ concentrations ($10^{-5}$M–$10^{-3}$M). Moreover, in BHK cultures the stimulation observed just at the end of exposure to dichromate (1, 2, 4 h points) increases with the length of treatment with $10^{-5}$M and $10^{-4}$M $K_2Cr_2O_7$, while it is progressively reduced with $10^{-3}$M $K_2Cr_2O_7$. In HEp cultures the latter stimulation pattern is observed even with $10^{-4}$M $K_2Cr_2O_7$.

The different sensitivity to $Cr^{6+}$ treatment of the two cell lines is confirmed by the patterns of inhibition of DNA synthesis (Fig. 2). In particular, DNA synthesis recovers after treatment with $10^{-4}$M $K_2Cr_2O_7$ only in BHK cultures, and is inhibited with $10^{-5}$M $K_2Cr_2O_7$ only in HEp cultures.

The action of dichromate is more drastic on HEp cells also on single-cell plating; HEp cell survival at a given $K_2Cr_2O_7$ concentration corresponds to that observed for BHK cells at a 10-fold higher dose (Table I). Survival is lower in both cell lines when treatment is made in HBSS.

BHK survival after 24 h treatments with different $K_2Cr_2O_7$ concentrations is shown in Fig. 3. The exponential portion
of the survival curve is preceded by a rather pronounced shoulder and the extrapolation value is \( \sim 10 \).

BHK survival is much more affected by Cr\(^{6+}\) than by Cr\(^{3+}\) (Table II): \( 10^{-2}\)M CrCl\(_3\) or \( 10^{-5}\)M K\(_2\)Cr\(_2\)O\(_7\) both reduce survival to about 0-1, after a 24 h treatment. Comparable difference of activity between Cr\(^{6+}\) and Cr\(^{3+}\) is seen when survival is measured on BHK cells chronically treated with the above Cr compounds (Table II).

Unlike K\(_2\)Cr\(_2\)O\(_7\), which is very active at \( 10^{-4}\)M, CrCl\(_3\) is ineffective at that concentration. Even \( 10^{-3}\)M CrCl\(_3\) does not affect nucleoside uptake or nucleic acid synthesis, and scarcely alters leucine uptake and protein synthesis (data not shown). Only at \( 10^{-2}\)M CrCl\(_3\), are DNA synthesis and TdR uptake drastically inhibited (Fig. 4). A stimulation of nucleoside uptake is never observed after CrCl\(_3\), while the action of K\(_2\)Cr\(_2\)O\(_7\) is always characterized by an initial period during which nucleoside uptake is stimulated. Inhibition of uptake appears only with longer exposures to dichromate (Levis et al., 1978; Bianchi et al., 1977).

On the other hand, \( 10^{-2}\)M CrCl\(_3\) affects the sensitivity of nucleic acids to hydrolysis with perchloric acid (PCA). This is proved by the altered ratio of optical densities of extracted nucleic acids, and the abnormal distributions of radioactivity in the different fractions after incubation with \(^3\)H-TdR and \(^3\)H-UR (Table III). RNA is only partially hydrolysed by PCA at \( 30^\circ\)C and is completely extracted only at \( 70^\circ\)C, thus contaminating the DNA fraction. Also a significant amount of DNA is not hydrolysed by PCA and is extracted with proteins by KOH. On the contrary, \( 10^{-2}\)M K\(_2\)Cr\(_2\)O\(_7\) does not modify the optical densities of extracted nucleic acid fractions, completely blocking nucleoside incorporation.

Determinations of Cr content in BHK
TABLE III.—Sensitivity of Nucleic Acids to the Hydrolysis with Perchloric Acid in BHK Cultures Treated with K₂Cr₂O₇ and CrCl₃

| Treatment 1 h in MEM | Labelled ³H-precursor* | O.D.† | % radioactivity‡ |
|----------------------|------------------------|-------|-----------------|
|                      | ³H-Precursor*          | RNA   | DNA            | RNA   | DNA   | Proteins |
| —                    | TdR                    | 1.95  | 0.83           | 2.35  | 0.5   | 96.0     | 3.5   |
|                      | UR                     | 1.94  | 0.81           | 2.39  | 93.0  | 6.0      | 1.0   |
| CrCl₃ 10⁻³M          | TdR                    | 1.86  | 0.80           | 2.31  | 1.0   | 94.8     | 4.2   |
|                      | UR                     | 1.87  | 0.79           | 2.36  | 91.2  | 7.6      | 1.2   |
| 10⁻²M                | TdR                    | 1.10  | 1.70           | 0.65  | 2.3   | 82.0     | 15.7  |
|                      | UR                     | 1.08  | 1.68           | 0.64  | 33.0  | 62.0     | 5.0   |
| K₂Cr₂O₇ 10⁻³M        | TdR                    | 1.86  | 0.81           | 2.29  | 0.2   | 98.0     | 1.8   |
|                      | UR                     | 1.93  | 0.82           | 2.36  | 95.0  | 4.5      | 0.5   |
| 10⁻²M                | TdR                    | 1.84  | 0.82           | 2.25  | —     | —        | —     |
|                      | UR                     | 1.77  | 0.78           | 2.27  | —     | —        | —     |

* At the end of Cr exposure, the cultures were incubated for 1 h with ³H-TdR or ³H-UR. Thereafter, soluble nucleotides, RNA, DNA and proteins were extracted with PCA and KOH as detailed in Materials and Methods.
† Determined in the fractions extracted with 10% PCA at 37°C (RNA) and 70°C (DNA) respectively.
‡ In the RNA and DNA fractions, and in the fraction extracted with 0.3M KOH (Proteins).
§ Negligible values.

TABLE IV.—Cr⁶⁺ Reduction in K₂Cr₂O₇ Solutions and Cr³⁺ Accumulation in BHK Cultures

| K₂Cr₂O₇ concentration | % Cr⁶⁺ in the solutions† | After BHK cell treatment | Cr³⁺ in the treated BHK cells‡ |
|-----------------------|--------------------------|--------------------------|-------------------------------|
|                       | At solubilation          | HBSS         | MEM         | HBSS   | MEM     | HBSS | MEM     |
| 10⁻³M                 | 100                      | 95            | 94          | 89     | 2.56    | 1.96 |
| 10⁻²M                 | 100                      | 88            | 92          | 78     | 1.06    | 0.52 |
| 10⁻¹M                 | 94                       | 47            | 82          | 38     | 0.33    | 0.18 |
| 10⁻⁰M                 | 92                       | 32            | 78          | 24     | —       | —    |

* Cr⁶⁺ and Cr³⁺ levels were determined spectrophotometrically by the diphenylcarbazide complex reaction as specified in Materials and Methods.
† % of the calibration curve levels at solubilization in bidistilled water.
‡ Determinations on about 10⁸ treated BHK cells (containing ~10⁸ μg DNA) were made in 10 ml of final solution. Cr³⁺ expressed as μg/100 μg DNA.
§ The sensitivity of the colorimetric method (see Materials and Methods) is such that it is impossible to determine <0.08 μg Cr³⁺/100 μg DNA.

cells exposed to dichromate, as well as in the solutions used for treatment, are shown in Table IV. When K₂Cr₂O₇ is brought into solution, reduction of Cr⁶⁺ occurs only in MEM, and is relatively more in less concentrated dichromate solutions. A further Cr⁶⁺ reduction takes place both in MEM and HBSS after exposure of the K₂Cr₂O₇ solutions to BHK cultures. Cr³⁺ concentration in the cells does not increase linearly with K₂Cr₂O₇ concentration in the medium, and is higher when treatment is made in HBSS. In HEp cells, which contain about twice as much DNA as BHK cells (3 × 10⁻⁵ μg vs 1.4 × 10⁻⁵ μg/ cell) the amount of Cr³⁺ bound to the cells after exposure to K₂Cr₂O₇ is also doubled (data not shown).

It should be pointed out that after treatment with 10⁻³M K₂Cr₂O₇ (~100 μg/ml of Cr⁶⁺) the amounts of Cr³⁺ in BHK cells are 20–30 times as great as those found after treatment with 10⁻⁴M CrCl₃ (~50 μg/ml of Cr³⁺) (Table V). A differ-
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Table V.—Cr\textsuperscript{3+} Accumulation in BHK Cultures after Treatment with K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} and CrCl\textsubscript{3}

| Treatment   | Cr\textsuperscript{3+} in the treated | BHK cells* Duration of treatment (h) in MEM |
|-------------|--------------------------------------|---------------------------------------------|
|             | Cr\textsuperscript{3+}               | 1   | 3   | 6   |
| K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} | 10\textsuperscript{-5}M | 0.15 | 0.30 | 0.80 |
|             | 10\textsuperscript{-4}M               | 0.65 | 1.10 | 1.80 |
|             | 10\textsuperscript{-3}M               | 1.65 | 3.25 | 4.80 |
| CrCl\textsubscript{3}     | 10\textsuperscript{-3}M               | 0.08 | 0.10 | 0.27 |

* Determined spectrophotometrically by the diphenylcarbazide complex reaction (see Materials and Methods) and expressed as \(\mu\)g of Cr\textsuperscript{3+}/100 \(\mu\)g DNA. Determinations on about 10\textsuperscript{4} treated BHK cells (containing about 10\textsuperscript{8} \(\mu\)g of DNA) were made in 10 ml of final solution.

† Owing to the sensitivity of the colorimetric method (see Materials and Methods) it is impossible to determine < 0.05 \(\mu\)g of Cr\textsuperscript{3+}/100 \(\mu\)g of DNA.

ence of about one order of magnitude in Cr\textsuperscript{3+} linked to the cells is therefore observed after exposure to equal concentrations of Cr\textsuperscript{6+} and Cr\textsuperscript{3+}, while the difference in cytotoxic activity between K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} and CrCl\textsubscript{3} is much higher.

It must be stressed that only Cr\textsuperscript{3+} can be detected in the treated cells by the present colorimetric procedure, even after treatment with 10\textsuperscript{-3}M K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}.

Discussion

The main toxic effects of chromium compounds in man are acute and chronic lesions of the skin, the nasal mucous membrane and the lungs, and are commonly referred to the oxidizing power of Cr\textsuperscript{6+} (Browning, 1969). Sensitization to Cr is related to the reduction of Cr\textsuperscript{6+} by several biological compounds and to the linkage of reduced Cr\textsuperscript{3+} in stable coordination complexes with small molecules (pyrophosphate, thiocyanate, aminoacids, nucleotides, etc.) or macromolecules (nucleic acids, proteins, etc.), particularly with proteins (Polak, Turk and Frey, 1973).

The carcinogenic action of Cr is known on the basis of epidemiological data (Browning, 1969; Furst and Haro, 1969; IARC, 1973) but a still debated question concerns the actual active agents. Most authors claim that only Cr\textsuperscript{6+} compounds (especially monochromates) are active (Browning, 1969; Furst and Haro, 1969), but Cr\textsuperscript{3+} also has been indicated as the carcinogenic agent (Grogan, 1957). As a matter of fact, some Cr\textsuperscript{6+} compounds are very powerful carcinogens in experimental animals (IARC, 1973; Maltoni, 1974), but even some Cr\textsuperscript{3+} compounds also have been shown capable of inducing tumours (Hueper, 1958, 1961; Payne, 1960; IARC, 1973; Maltoni, 1976).

As for cytogenetic effects, both Cr\textsuperscript{6+} and Cr\textsuperscript{3+} compounds can induce cell transformation in mammalian cell cultures (Fradkin et al., 1975; Raffetto et al., 1977), chromosome aberrations in cultured plant (Gläss, 1956) and animal (Raffetto et al., 1977; Majone, 1977) cells, alterations of physico-chemical properties of purified nucleic acids and nucleotides (Herrmann and Speck, 1954; Huff et al., 1964; Tamino, 1977) and infidelity of DNA replication in vitro (Sirover and Loeb, 1976). On the contrary, only Cr\textsuperscript{6+} compounds are at present known to induce DNA repair synthesis in animal cell cultures (Raffetto et al., 1977) and point mutations in yeasts (Bonatti et al., 1976) and bacteria (Venitt and Levy, 1974; Nishioka, 1975; Tamaro et al., 1976; Petrelli and De Flora, 1977). Negative results obtained with soluble Cr\textsuperscript{3+} salts in mutagenicity tests in bacteria (Venitt and Levy, 1974; Tamaro et al., 1976) and also with Cr\textsuperscript{6+} (K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}) when treatment is made in the presence of a reducing agent (Nishioka, 1975) can be related to the low ability of Cr\textsuperscript{3+} to pass through the mammalian cell’s plasma membrane (Mertz, 1969; Polak et al., 1973) and possibly also through bacterial cell membranes. Alternatively, the cytogenetic Cr action could lie in the formation of mutagenic (and carcinogenic) agents via the oxidation of cell metabolites, which is accomplished only by Cr\textsuperscript{6+} compounds (Schoental, 1975). The latter hypothesis does not account for the above mentioned
carcinogenic and cytogenetic effects of Cr\textsuperscript{3+}.

Our data on the cytotoxic effects of K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} on BHK cell cultures (Levis et al., 1978) as well as the present observations on BHK and HEp cell lines, indicate a differential action on the permeability of nucleosides through the plasma membrane, induced by treatments with concentrations from 10\textsuperscript{-5}M upwards. Namely, nucleoside uptake is at first stimulated and only secondarily inhibited, according to the length of treatment and K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} concentration (Fig. 1). A separate effect of K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} is the inhibition of macromolecular synthases, proportionate to the length of treatment and K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} concentration: DNA duplication is chiefly and immediately affected (Fig. 2), RNA and protein synthases being reduced to a smaller extent and only later (Levis et al., 1978).

On the basis of colorimetric Cr determinations (Tables IV–V) as well as of the much more sensitive atomic absorption spectrophotometry (Feldman et al., 1967), only Cr\textsuperscript{3+} is detected inside the cells even after treatment with K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}, and its level increases with the exposure period and K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} concentration, reaching a maximal value of about 5 \times 10\textsuperscript{-2} \mu g Cr\textsuperscript{3+}/\mu g of DNA (Table V; Levis et al., 1978). Cr\textsuperscript{3+} accumulation inside the cells is more rapid when cells are treated in HBSS, and the transition from the stimulation to the inhibition phase of nucleoside uptake also takes place earlier in these conditions (Bianchi et al., 1977). It has been suggested that Cr\textsuperscript{6+} interacts with specific cell-membrane components involved in nucleoside uptake (permeases), which are at first stimulated and subsequently inhibited when linked Cr\textsuperscript{3+} exceeds a critical level. Evidence that Cr interferes with nucleoside permeases is based on the specific patterns observed for the uptake of the different DNA and RNA nucleosides, as well as on the kinetics of TdR and deoxycytidine uptake in BHK cells treated with K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}: Cr acts both on the saturable, facilitated portion (i.e. on the permeases) and the linear portion (i.e. the simple diffusion) of the uptake (Bianchi et al., 1977).

The stimulation of nucleoside uptake represents a specific effect of Cr\textsuperscript{6+}. It has never been observed after treatment with Cr\textsuperscript{3+} (CrCl\textsubscript{3}), whose active concentrations always exert an inhibitory action (Fig. 4).

HEp cells turned out to be more sensitive than BHK fibroblasts to the action of dichromate, the difference between the 2 cell lines being about the same in the inhibition of DNA duplication (Fig. 2) as in the reduction of cell survival (Table I). HEp cells are more affected by K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} also in relation to TdR uptake, which is less stimulated than in BHK cells (Fig. 1). Moreover the stimulation is progressively reduced just at the end of dichromate exposure (1, 2, 4 h points) with 10\textsuperscript{-3} M K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}, both in BHK and HEp cultures (Fig. 1A) and with 10\textsuperscript{-4}M concentration only in HEp cultures (Fig. 1B), showing that in the latter cell system the transition from the stimulation to the inhibition phase of permeases is advanced.

BHK survival curve to K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} (Fig. 3) indicates a multi-hit mechanism of cell inactivation similar to that observed by treating yeast cells (Bonatti and Abbonandolo, personal communication) and corresponds to the classical survival-curve shapes, namely an initial, rather pronounced shoulder followed by a linearly logarithmic decline in the number of survivors as the dose increases. If the straight portion of the curve is extrapolated back to the axis, the intersection occurs close to 10, a form of behaviour which would be exhibited if 10 independent, Cr induced events were required for cell death. It must be stressed that no threshold dose can be inferred from survival curves to K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}, and that the extrapolation value is too low to suggest an interaction of Cr with the replication enzymes (DNA polymerases) as the more relevant inactivation mechanism. The number of such molecules in the mammalian cell greatly exceeds the hit number extrapolated on survival curves (Bollum,
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1975). Moreover, an independent, simultaneous inactivation of transcription and translation enzymatic systems would be required to explain the concomitant inhibition of RNA and protein syntheses by K₂Cr₂O₇. If on the other hand the DNA molecule is the target for the action of Cr on cell survival, it is likely that a few hits are sufficient to impair its replication capacity. The interaction between DNA and Cr could account also for a secondary inhibition of RNA and protein syntheses (Bianchi et al., 1977).

With CrCl₃ an irreversible inhibition of DNA synthesis is observed only after 2-4 h treatments with 10⁻²M concentrations (Fig. 4) whereas a comparable effect is obtained with 100 times lower K₂Cr₂O₇ concentrations (Fig. 2). The stronger cytotoxic activity of K₂Cr₂O₇ is even more evident when the reduction of BHK cell survival is measured: comparable effects are induced by K₂Cr₂O₇ concentrations 1000 times lower than CrCl₃ concentrations (Table II).

It is generally accepted that only Cr⁶⁺ penetrates through the plasma membrane, being reduced to Cr³⁺ inside the cell and firmly bound to cell components (Grogan, 1958; Mertz, 1969). Transport of Cr⁶⁺ in the form of chromate ion is very effective, its rate and therefore the final intracellular Cr concentration depending on the extracellular chromate levels (Vallee, 1969). Mechanisms for Cr³⁺ transport are presently unknown; anyhow Cr³⁺ entrance into the cells is deemed to be limited if not completely absent (Feldman et al., 1969; Mertz, 1969; Polak et al., 1973). However, significant amounts of Cr³⁺ are detected in BHK cells after treatment with 10⁻³M CrCl₃, and are about 20 times lower than those observed after treatment with the same concentration of K₂Cr₂O₇ (Table V). Therefore, the much lower effectiveness of CrCl₃ on BHK macromolecular syntheses and cell survival cannot be entirely related to a lower Cr³⁺ uptake, but seem to depend also on the stability of Cr³⁺ chelates and coordination complexes which are formed in the treatment solutions and which are characterized by a very low exchange rate with biological ligands (Mertz, 1969).

A marked difference in the cytotoxic activity of Cr⁶⁺ and Cr³⁺ compounds has been observed also in Salmonella (Petrilli and De Flora, 1977) and in mouse cells (Raffetto et al., 1977).

When BHK cells are treated with 10⁻²M CrCl₃, DNA and RNA become partially resistant to hydrolysis with PCA, an effect which is not observed with equimolar K₂Cr₂O₇ concentrations (Table III). Resistance of nucleic acids to hydrolysis with trichloroacetic acid has been described after treatment of animal tissues with Cr⁶⁺ salts, and attributed to the interaction of nucleic acids with Cr³⁺ reduced inside the cells (Herrmann and Speck, 1954). The altered extractability of nucleic acids is probably due to the stabilization of their tertiary structure by Cr³⁺, according to the modification of some physico-chemical properties of purified RNA in the presence of CrCl₃ (Huff et al., 1964).

A stabilization of the DNA double helix by Cr³⁺ is supported also by UV absorption spectra of nucleic acids purified from BHK cells treated with CrCl₃ (Tamino, 1977), by X-ray diffraction (Danchin, 1975) and NMR studies (Eisinger, Shulman and Szymbanski, 1962) of DNA-Cr³⁺ complexes. An interaction with DNA could represent also the final effect of K₂Cr₂O₇ after it is reduced to Cr³⁺ inside the cells.

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