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ABSENCE OF TOXIC AND CARCINOGENIC EFFECTS AFTER ADMINISTRATION OF HIGH DOSES OF CHROMIC OXIDE PIGMENT IN SUBACUTE AND LONG-TERM FEEDING EXPERIMENTS IN RATS

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Abstract—The pigment chromic oxide (Cr_2O_3), which as chromium oxide green (C-green 9) or pigment green 17 is used as a cosmetics colouring, was tested in BD rats for subacute toxicity (90-day test) and for long-term toxicity and carcinogenicity (2-yr study). Administration of 2 or 5% in the feed for 90 days produced no signs of toxic effect and no detectable differences from untreated controls. Fertility of the animals was normal during treatment, and the young showed no malformations. Feeding of 1, 2 or 5% Cr_2O_3 in the feed for 2 yr was well tolerated. The animals were observed throughout life and there was no reduction in the average life expectancy of the experimental animals. Even with the very high oral doses of Cr_2O_3 given, no carcinogenic action was detected.

INTRODUCTION

The inorganic pigment, chromic oxide (chromium-(III) oxide; dichromium trioxide; Cr_2O_3) is used industrially, and as chromium oxide green (C-green 9) or pigment green 17 it is also incorporated into eye make-up, particularly as eye-shadow. In the classification in the Third Communication of the Deutsche Forschungsgemeinschaft, Farbstoff-Kommission (1959), the dye is placed in the C group of colourings, since it is applied on or near mucous membranes. According to the results of toxicological studies, C-green 9 is rated in this document as "provisionally permissible" in category Ca, data being available only on acute toxicity and local tolerance following application to the skin and eye.

A Russian paper (Dvizkov & Fedorova, 1967), described the formation of lung and pleural tumours after parenteral administration of an ill-defined chromium oxide to rats. The known carcinogenic actions of various chromium compounds in man and experimental animals have recently been summarized (International Agency for Research on Cancer, 1973). Since further investigation of the long-term toxicity of the insoluble pigment appeared to be necessary, chromium oxide green was fed to rats in the diet in a 90-day test and in a long-term feeding study lasting 2 yr.

EXPERIMENTAL

Materials. The substance used in the studies was generously made available by Dr. H. Eich, Besigheim, who also provided the following information:

Chromium oxide green (Schultz no. 1451; Colour Index no. 77288; pigment green 17) was obtained by reduction of chromate at about 600°C. The product, which was washed until all

soluble components, particularly chromate, had disappeared, was a fine green powder of pure Cr_2O_3 , insoluble in water, alkali and mineral acids. The product was in a non-hydrated form. Precise analysis of the preparation used in the study gave a maximum content of 3 ppm Cr^{3+} with one of three investigators. The preparation was free from chromate (detection limits of the method of determination) 1 $\mu\text{g CrO}_4^{2-}$). With respect to heavy-metal impurities (arsenic, lead, mercury, antimony, soluble barium, copper and zinc), the product complied with the specifications of the Deutsche Forschungsgemeinschaft, Farbstoff-Kommission (1959).

Animals and diets. The studies were conducted on inbred BD rats of both sexes (Druckrey, Dannenberg, Dischler & Steinhoff, 1963), about 100 days old and weighing on average about 200 g at the start of the tests. They were housed in groups of four in Makrolon cages and both test animals and controls were given tap-water *ad lib*. Untreated controls were fed Altromin[®], while the test pigment was administered in bread, which was made twice weekly by incorporating 1, 2 or 5% Cr_2O_3 into a dough made from 2835 g flour, 150 g milk powder, 150 g mixed salts (NaCl , CaHPO_4 and Cu, Zn, Co, Mn and K), 150 g cooking oil, 150 g cod-liver oil, 300 g malt extract, 600 g sugar, 80 g yeast and 900 ml water and subsequently baked. Uneaten bread was weighed in order to determine the amount consumed. There were only small losses from crumbs.

Experimental design and conduct

90-Day test. The test pigment was given to two groups on 5 days/wk, one group of 14 males and five females receiving 2% in the feed and a second of five males and ten females being given 5%. At weekends, the test animals received the control diet with a vegetable supplement. The untreated control group consisted of six males and six females. Uptake

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of food was determined weekly and body weight fortnightly. The blood picture was determined before the beginning of the experiment and monthly during feeding. Random samples were taken during the course of the study and from all animals in the last week of the experiment for urinary determinations of protein, sugar, bilirubin, blood and sediment. During the last 30 days of treatment, males and females from the same group were paired to test fertility, and the number of young in each litter was recorded.

At the end of the feeding period, the animals were fasted for 24 hr. Blood samples were taken from the retrobulbar plexus for determinations of blood sugar, serum protein and serum bilirubin, and from the tail vein for counts of erythrocytes and total and specific types of leucocytes and for haemoglobin estimations. Blood sugar was determined enzymatically, serum protein by biuret and serum bilirubin with diazotized sulphanic acid. The animals were killed with ether and autopsied, the liver, spleen and kidney being weighed in all animals and the brain and ovaries in random samples. Samples of these organs and of lung, heart, pancreas, stomach, small intestine and urinary bladder were fixed in 4% buffered formalin and sections were embedded in paraffin wax and stained with haematoxylin and eosin.

Carcinogenicity (2-yr feeding) study. Groups of 60 male and female rats were fed 1, 2 or 5% Cr₂O₃ baked in bread on 5 days/wk for 2 yr (600 feeding days), the control diet with a vegetable supplement being given each weekend. A further group of 60 male and female rats served as untreated controls. Intake of the test pigment was calculated weekly and the animals were weighed monthly. At the end of the feeding period, surviving animals were maintained on the control diet until they died or became moribund, death being induced with ether in the latter case. At autopsy, all the important organs, including the brain and nervous system, were fixed in 4% formalin solution and studied histologically.

RESULTS

90-Day test

The treatment was well tolerated at both dose levels. One male rat fed the 5% Cr₂O₃ diet died 70 days after the start of the experiment from acute

pneumonia. Food intake was normal throughout the study, averaging 20 g/day for males and 15 g/day for females. The total amount of Cr₂O₃ consumed during the whole of the experiment in which 2% was fed was 75 g/kg body weight (25 g/animal) for males, and 72 g/kg for females (18 g/animal). In the experiment in which 5% was fed, 180 g/kg was consumed by males, and 160 g/kg by females. No significant differences in body weight were evident between the two experimental groups and the controls (Fig. 1).

The faeces of the treated animals showed an intense green coloration throughout the study, indicating significant excretion of the administered pigment. In a subsidiary experiment in which 5 g Cr₂O₃/kg was given in a single dose to four rats, excretion was evident from the green coloration of the faeces about 16 hr after administration and ended after about 4 days. A total of 5.95 g Cr₂O₃ was given to these animals and some 4.9-5.0 g Cr₂O₃ was excreted unchanged in the faeces*.

Haemoglobin determinations and counts of erythrocytes and total and differential leucocytes in blood samples from the Cr₂O₃-treated groups showed no significant deviations from the corresponding control values (Table 1) and there were no adverse findings in determinations of serum protein, serum bilirubin and blood sugar (Table 2). These estimations were carried out at the end of the Cr₂O₃ feeding and shortly before the death of the animals. Urine analyses carried out during the experiment and at termination showed no significant differences between the treated and control animals.

Altogether nine females were paired with males from the same dosage group 60 days after the start of feeding. All the females became pregnant in due course, the gestation period was normal (23 days) and the young showed no malformations or other adverse effects. Litter sizes were in the normal range, averaging eight pups. Some of the progeny were retained for lifetime observation and so far (600 days) no tumours have been detected. It was thus shown that no toxic or teratogenic effects were associated with Cr₂O₃ treatment throughout the gestation period and fertility was not adversely affected.

All the experimental animals except those from the fertility study, which were still suckling, were killed within 1 wk of the cessation of Cr₂O₃ feeding. All the major organs were investigated at autopsy. With the exception of the weights of the spleen and liver, which showed some reduction in the treated animals

* These determinations were carried out by Dr. H. Eich.

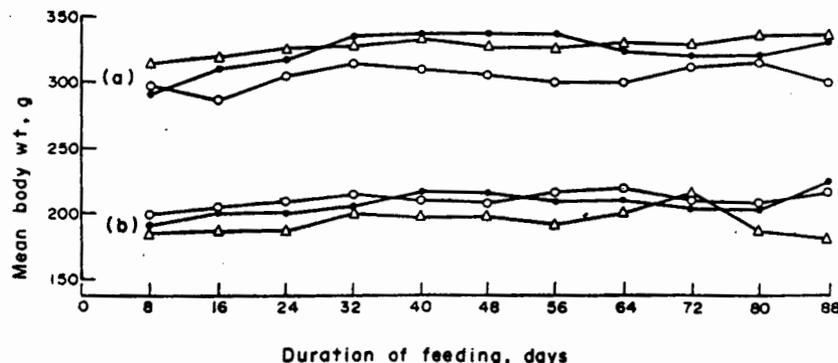


Fig. 1. Body-weight curves for (a) male rats and (b) female rats fed Cr₂O₃ at dietary levels of 0 (control; ●), 2 (Δ) or 5% (○) on 5 days/wk for a 90-day period.

Table 1. Haematological findings in BD rats fed diets containing 0, 2 or 5% Cr₂O₃ for 90 days

Sex and dietary level (%)	No. of rats	Haemoglobin (g/100 ml)	Erythrocytes (10 ⁶ /mm ³)	Total (10 ³ /mm ³)	Leucocytes			
					Differential (%)			
					N	L	M	E
Males								
0	6	16.0 ± 0.80	6.2 ± 0.76	12.3 ± 2.30	27.2 ± 1.8	70.3 ± 2.1	1.1 ± 0.3	1.4 ± 0.70
2	14	15.9 ± 0.77	6.0 ± 0.64	14.7 ± 1.80	25.7 ± 1.3	71.4 ± 0.8	1.0 ± 0.2	1.9 ± 0.65
5	4	15.1 ± 0.60	5.7 ± 0.59	10.8 ± 1.10	23.6 ± 2.0	72.2 ± 0.7	1.8 ± 0.4	2.4 ± 0.76
Females								
0	6	14.8 ± 0.50	5.9 ± 0.60	11.7 ± 2.00	25.5 ± 1.1	68.4 ± 2.0	3.1 ± 0.2	3.0 ± 0.88
2	5	13.9 ± 0.66	5.5 ± 0.63	14.4 ± 1.30	30.2 ± 1.4	67.1 ± 1.8	1.1 ± 0.3	1.6 ± 0.70
5	10	15.1 ± 0.55	5.9 ± 0.80	11.9 ± 1.60	27.0 ± 1.6	70.4 ± 2.2	0.9 ± 0.44	1.5 ± 0.80

N = Neutrophils L = Lymphocytes M = Monocytes E = Eosinophils

Values are means for the numbers of animals stated ± SEM. Those recorded for all three groups initially and at the end of months 1 and 2 of the study were closely similar to those reproduced in the table.

(Table 3), there were no macroscopic or histological changes. Compared with sections of organs from control animals, no changes could be detected that could be attributed to the Cr₂O₃ treatment.

Carcinogenicity study

Feeding of Cr₂O₃ at all three dose levels was well tolerated. As in the 90-day test, the body-weight curves for the treated animals did not differ from those for the controls. The 60 control rats reached an average life expectancy of 890 ± 45 days. Two mammary fibroadenomas, one mammary carcinoma and two hypophyseal adenomas were observed in this group. Of the 60 rats given 1% Cr₂O₃, the first died from a lung infection after 130 days and the last 1315 days from the start of the experiment. The average life expectancy was 870 ± 30 days. The total dose of Cr₂O₃ consumed was about 120 g/rat, or about 360 g/kg body weight, in 600 days of feeding. Only three mammary fibroadenomas were detected in this group. The remaining animals showed no macroscopic or microscopic signs of benign or malignant growths. The first death in the group given 2% Cr₂O₃ occurred after 127 days and the last 1230 days from the start of feeding. The average survival period was 880 ± 30 days. The total dose of Cr₂O₃ consumed in 600 days of feeding was 240 g/rat, or 720 g/kg body weight. One mammary fibroadenoma was the only tumour observed. In the group given 5% Cr₂O₃, the first rat died after 113 days and the last after 1295 days. The

average survival was 860 ± 40 days. The total dose of Cr₂O₃ consumed in 600 days of feeding was about 600 g/animal or 1800 g/kg body weight. Three mammary fibroadenomas and one hypophyseal adenoma were found, but no other benign or malignant tumours appeared.

DISCUSSION

This investigation of the pigment Cr₂O₃ (C-green 9) showed no toxic effects in BD rats given high oral doses over 90 days or 2 yr. This result is the more convincing because the doses used (up to 5% of the feed) were substantially higher than the 1% in feed recommended by national (Deutsche Forschungsgemeinschaft) and international (WHO) authorities.

In the 90-day test, there were no statistically significant differences from the values found in control animals for the haematological determinations (total and differential leucocytes, erythrocytes and haemoglobin) or for the biochemical measurements of serum protein and blood sugar and of blood, sugar, protein, bilirubin and sediment in the urine. The slightly reduced total bilirubin content in serum was within the normal limits for the strain of rat used.

The only striking observation in the subacute study was the dose-dependent reduction in the organ weights of the liver and spleen. No differences were noticed in the other organs. However, no pathological changes, either macroscopic or histological, were

Table 2. Blood analyses in BD rats fed diets containing 0, 2 or 5% Cr₂O₃ for 90 days

Sex and dietary level (%)	No. of rats	Sugar (mg/100 ml blood)	Protein (g/100 ml serum)	Total bilirubin (mg/100 ml serum)
Male/female				
0	10	74.1 ± 4.8	6.4 ± 0.30	0.57 ± 0.28
Male				
2	13	72.9 ± 5.1	6.2 ± 0.26	0.19 ± 0.11
5	4	75.5 ± 4.8	5.7 ± 0.32	0.23 ± 0.15
Female				
2	4	77.2 ± 4.4	6.2 ± 0.30	0.36 ± 0.20
5	7	73.4 ± 5.0	6.2 ± 0.28	0.34 ± 0.11

Values are means for the stated numbers of rats ± SEM.

Table 3. *Weights of organs from BD rats fed diets containing 0, 2 or 5% Cr₂O₃ for 90 days*

Sex and dietary level (%)	No. of rats	Organ weights (g)		
		Liver	Spleen	Kidneys
Males				
0	6	8.9 ± 0.7	0.82 ± 0.08	1.6 ± 0.10
2	14	8.8 ± 0.6	0.61 ± 0.15	2.2 ± 0.10
5	4	7.7 ± 0.5	0.52 ± 0.12	2.1 ± 0.08
Females				
0	6	7.3 ± 0.5	0.55 ± 0.10	1.3 ± 0.08
2	5	6.7 ± 0.7	0.48 ± 0.16	1.8 ± 0.09
5	10	5.1 ± 0.6	0.43 ± 0.15	1.2 ± 0.08

Values are means for the stated numbers of rats ± SEM.

found in the liver or spleen or any other organ studied. The reduced weights of the liver and spleen could therefore not be evaluated as a serious toxic effect without further investigation.

This interpretation is supported by the result of the long-term feeding study. Concentrations of 1, 2 and 5% Cr₂O₃ in the feed over a period of 2 yr (600 treatment days) with a total consumption between 360 and 1800 g Cr₂O₃/kg body weight were tolerated without signs of chronic toxicity. The body-weight curves of the treated animals showed no differences from those of the untreated controls and the median survival times in all three dosage groups were comparable with that of the controls. No macroscopic or histological post-mortem findings could be causally related to the Cr₂O₃ treatment.

The lack of any carcinogenic action of Cr₂O₃ is of particular importance. The type and frequency of the tumours appearing in the experimental and control animals were comparable; mammary fibroadenomas and the hypophyseal adenomas are 'spontaneous' tumours characteristic of strains of BD rats. Orally administered Cr₂O₃ has no carcinogenic action in rats even when excessive doses are given.

In contrast, Dvizkov & Fedorova (1967) described the development of malignant tumours (mainly at the site of application) after intratracheal, intrapleural and ip administration of single doses of 10–50 mg Cr₂O₃/rat. These divergent results can easily be explained by the different route of administration, since our own studies have shown that most of an orally administered dose of Cr₂O₃ is excreted unchanged in the faeces. It is known that various chromium compounds are very poorly absorbed from the gastro-intestinal tract of experimental animals (Akatsuda & Fairhall, 1934; Aronson & Rogerson, 1972). Similar observations have been made in man (Schroeder, Balassa & Tipton, 1962). On the other hand, parenterally administered chromium (either in ionized or insoluble form) is easily transported and distributed, and can be accumulated in the lungs and kidneys (Baetjer, Damron & Budacz, 1959; Grogan, 1957; Kovalchuk, 1966; Visek, Whitney, Kuhn & Comar, 1953). The occupationally conditioned susceptibility to lung cancer in workers in the chromium-processing industry may be attributed almost entirely to inhalation of chromium-containing dusts, as has been established by epidemiological studies from Germany (Gross & Kölsch, 1943; Letterer, Neidhardt & Klett, 1944), the USA (Baetjer, 1950; Brinton, Frasier

& Koven, 1952; Machle & Gregorius, 1948; Mancuso & Hueper, 1951) and Great Britain (Bidstrup & Case, 1956).

A possible defect in our studies is that the oral route is an inappropriate mode of application for a cosmetic product which is applied to the skin, but this may be refuted on the grounds of the poor resorptive capacity of the skin. It is recognized that substances shown to be non-toxic when administered orally are generally non-toxic when applied to the skin. Far more extensive absorptive surfaces are available in the gastro-intestinal tract, and experience such as that reported by Gloxhuber (1970) has shown that the skin absorbs less readily than the mucous membranes of the gastro-intestinal tract.

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