Eur J Cancer Clin Oncol, Vol. 19, No. 1, pp. 91–99, 1983.
Printed in Great Britain.

0277-5379/83/010091-08$03.00/0

The Effect of Divalent Cations on Cloudman Melanoma Cells*

JAN BOROVANSKÝ† and PATRICK A. RILEY

Department of Biochemical Pathology, University College School of Medicine, London WC1E 6JJ, U.K.

Abstract—*The effect of* Ca^{2+}, Cd^{2+}, Cu^{2+}, Mg^{2+} *and* Zn^{2+} *as acetates* (10^{-3}–10^{-5}M) *and of 2% DMSO on the proliferation and differentiation of clone M3 of the Cloudman S91 mouse melanoma was studied and compared with the behaviour of GPK (keratocyte) and MRC5 (fibroblast) cell lines. Whereas neither calcium nor magnesium ions influenced the proliferation of the cells as measured by* [^{3}H]-*thymidine incorporation, absorbance at* 280 nm *of* NaOH *cell digests and cell counts, cadmium, zinc and copper ions selectively inhibited the melanoma line.* Cd^{2+} (10^{-5}M) *and* Zn^{2+} (10^{-4}M) *were selectively cytotoxic to melanoma cells in contrast to keratocytes and fibroblasts. No direct effect of the cations on melanogenesis, as estimated from the ratio of absorbance at* 350 nm *and* 280 nm *and by tyrosinase assays, was demonstrated. DMSO stimulated melanogenesis in melanoma cells but inhibited their growth. Experiments with ouabain indicate that active transport is involved in the uptake of zinc by melanoma cells.*

INTRODUCTION

A CHARACTERISTIC of pigmented (melanin containing) tissues is their high content of some heavy metals [1], in particular zinc [2–8]. Whether the metal accumulation has any functional significance has so far received little attention, and consideration has usually been limited to the cation exchange properties of melanins [1, 9, 10] and to the presence of copper in the marker enzyme of pigment cells, tyrosinase [11]. Recently it has been observed that Zn^{2+} ions have a cytotoxic effect on B-16 mouse melanoma *in vitro* [12].

The purpose of the investigations presented in this paper was to study the effects of a range of divalent cations (Ca^{2+}, Cd^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}) on the proliferation and differentiation of the Cloudman S91 mouse melanoma cell line in comparison with the behaviour of cell lines which do not produce melanin, such as keratocytes and fibroblasts.

Accepted 4 August 1982.

*This study was performed under the Academic Links Scheme of the British Council.

†Present address: Department of Biochemistry, Faculty of General Medicine, Charles University, Unemocnice 5, 128 53 Prague 2, Czechoslovakia.

MATERIALS AND METHODS

Cell cultures

Experiments were carried out on monolayer cultures of clone M3 of the Cloudman S91 mouse melanoma (Flow Labs) grown in William's medium E (Flow Labs) with 2 mM glutamine supplemented with 10% foetal bovine serum and with penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were seeded at a density of 5×10^4 cells/well in 1 ml of the growth medium into Multiwell™ tissue culture plates (Falcon) with a growth area of 2.1 cm^2/well. Two days after plating, aqueous solutions of Ca^{2+}, Cd^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+} in the form of their acetates were added in aliquots of 0.1 ml/well to obtain the required final cation concentration (10^{-3}–10^{-5}M). An equal volume of water was added to control cultures. Two days later the cultures were labelled with methyl-[^{3}H]-thymidine ([^{3}H]-TdR) (sp. act. 5 Ci/mmol; Radiochemical Centre, Amersham, U.K.) at a final concentration of 5 μCi/well for 3 hr unless specified otherwise. Experiments were terminated by washing the [^{3}H]-TdR-labelled cultures with PBS and 5% trichloracetic acid to remove unincorporated label. The fixed cells were dried under a hair drier and then digested for 18 hr with 1M NaOH.

For comparison the same experiments were performed with guinea-pig keratocyte (GPK) and human lung fibroblast (MRC5) cell lines. The

keratocytes were inoculated at a seeding density of 3–5 × 10^4/well and MRC5 cells were seeded at density of 1 × 10^5/well. Both lines were cultured in 20 mM HEPES-buffered Minimum Essential Medium (Eagle) supplemented with 10% foetal bovine serum and penicillin (100 U/ml) and streptomycin (100 μg/ml). All 3 types of cells were grown at 37°C in a 95% air–5% CO_2 humidified incubator.

Mycoplasma contamination of the cell cultures was excluded using autoradiographic localisation of incorporated thymidine [13].

Cell proliferation

Cell proliferation was assessed in three ways: (1) the radioactivity of the acid-insoluble material was measured by dissolving 100 μl of the NaOH-digested cells in 5 ml BBOT scintillation mixture and counted in an Intertechnique ABAC SL40 liquid scintillation spectrometer; (2) estimates of total cell mass were made by measurements on standardised aliquots of optical absorbance at 280 nm as this reflects the quantity of protein in the sample; (3) in some cases the pigment cells were harvested with 10% trypsin and counted in a Coulter counter (Model A). There was a good agreement among the three techniques employed.

Melanogenesis

The effect of the metal ions on melanogenesis was studied: (1) by means of the spectral absorbance ratio ($A_{350\,nm}/A_{280\,nm}$). The colorimetric determination of melanin was performed at 350 nm to obtain a higher sensitivity than can be attained at 400 nm [14]; (2) measurement of tyrosinase activity based on the determination of ^3H_2O released into the growth medium after 24 hr treatment with L-(3,5-[^{3}H]-tyrosine (sp. act. 50 Ci/mmol; Radiochemical Centre, Amersham, U.K.) (2 μCi/well) was performed as described by Leising and Schachtschabel [15].

For comparative purposes dimethyl sulfoxide (DMSO), which has been shown to induce differentiation of cells, including melanocytes [16, 17], was added to 48-hr-old cultures (20 μl/well) to give a final concentration of 2% in the medium.

Zinc uptake

In order to ascertain whether active transport is involved in the zinc uptake by melanoma cells, ouabain octahydrate (Sigma) in aqueous solution was added to 51-hr-old cultures with and without zinc, as shown in Table 6. The cultures were tested for growth and processed as described above.

Statistical processing

The obtained data were analysed statistically using a Student's *t*-test programme on a CBM Microcomputer (Commodore). *P*-values are shown as percentages. The calculations are based on at least 4 parallel experiments. Except for fibroblasts, all the measurements were repeated at least twice, always with analogous results.

RESULTS

At 10^{-5}M concentration none of the metals tested, with the exception of Cd^{2+}, influenced melanoma cell proliferation (Table 1).

At 10^{-4}M concentration not only Cd^{2+} but also Cu^{2+} and particularly Zn^{2+} ions were cytotoxic, whereas Ca^{2+} and Mg^{2+} ions produced no alterations (Table 2). Phase-contrast microscopic examination of the cultures showed that in the presence of zinc ions the melanoma cells lose their characteristic dendritic shape and round-up, becoming detached from the culture surface (Fig. 1a,b).

In the presence of 10^{-3}M concentration of both Zn^{2+} and Cu^{2+} no melanocytes survived. Calcium and magnesium ions at the same concentration did not alter the rate of cell proliferation (Table 3).

The results of analogous experiments with fibroblast MRC5 and keratocyte GPK cell lines are shown in Tables 4 and 5 respectively. Fibroblast growth was inhibited only by 10^{-4}M Cd^{2+} ions, whilst 10^{-5}M Cd, 10^{-4}M Cu^{2+} and 10^{-5}M Zn^{2+} were without any effect. At higher concentration (10^{-4}M) zinc appeared to stimulate the uptake of thymidine.

In the case of keratocytes, apart from 10^{-4}M Cd^{2+}, none of the ions decreased their proliferation rate, but on the contrary some of them (10^{-4}M Zn^{2+}, 10^{-4}M Cu^{2+}) increased the thymidine uptake.

When comparing the behaviour of the Cloudman S91 melanoma cell line on the one hand with that of GPK (keratocyte) and MRC5 (fibroblast) cell lines on the other, it becomes apparent that cadmium, zinc and, to a certain degree, also copper ions selectively inhibited the melanoma line. Cd^{2+} (10^{-5}M) and Zn^{2+} (10^{-4}M), which were cytotoxic to melanoma cells, stimulated the growth of keratocytes and did not alter the growth of fibroblasts.

The sensitivity of melanoma cell line towards Zn^{2+} is particularly interesting in the light of the high concentration of zinc in pigmented tissues [2–8].

The addition of ouabain mitigated the cytotoxic effect of Zn^{2+} (10^{-4}M) on melanoma cells in relation to the ouabain concentration (see Table 6). The protective effect of ouabain was more pronounced in terms of the optical density

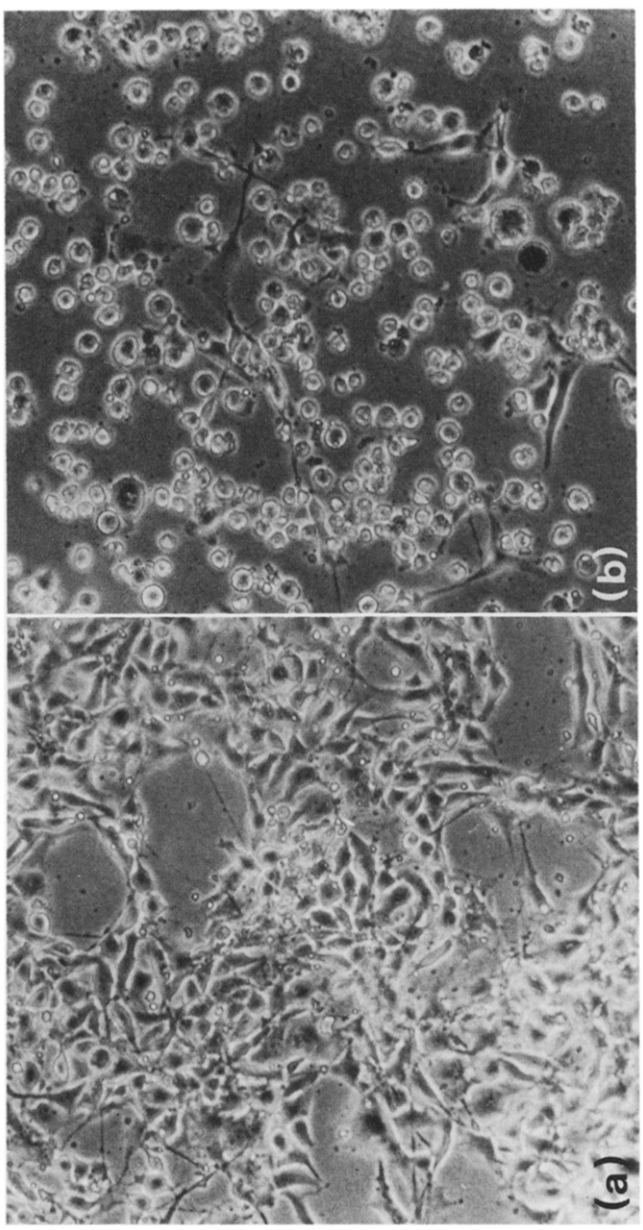

Fig. 1. Phase-contrast microscopic appearance of the Cloudman S91 melanoma cell line (×65). A = control cell culture; B = cell culture grown in the presence of 10^{-4} M Zn^{2+}.

Table 1. *The effect of divalent cations* (10^{-5}M) *on the proliferation of the Cloudman S91 melanoma cell line*

10^{-5}M:	[^{3}H]-TdR uptake (mean counts/min ± S.D.) Treated cells	Control cells	P(%)	$A_{280\ nm}$ Treated cells	Control cells	P(%)
Zn	31,204± 803	31,165±2470	n.s.	0.504±0.047	0.530±0.029	n.s.
Cu	31,420±2603	34,420±1816	n.s.	0.581±0.067	0.618±0.059	n.s.
Cd	16±8	34,420±1816	2.4×10^{-3}	0.003±0.001	0.618±0.059	5.9×10^{-3}
Ca	28,703±5640	31,165±2470	n.s.	0.511±0.078	0.530±0.029	n.s.
Mg	44,422±2418	41,231±3269	n.s.	0.903±0.038	0.880±0.059	n.s.

n.s. = not significant ($P > 5\%$). Cell cultures were grown and processed as described in Materials and Methods. The dried cells were dissolved in 0.5 ml 1M NaOH/well.

Table 2. *The effect of divalent cations* (10^{-4}M) *on the proliferation of the Cloudman S91 melanoma cell line*

10^{-4}M:	[^{3}H]-TdR uptake (mean counts/min ± S.D.)	P(%)	$A_{280\ nm}$ (mean ± S.D.)	P(%)	Cell counts*
Zn	1094± 359	2.15×10^{-3}	0.043±0.011	1.3×10^{-3}	9.8–10.5
Cu	29,636±4403	4.33×10^{-2}	0.551±0.077	2.2×10^{-1}	59.3–92.1
Cd	19±14	8.08×10^{-3}	0.005±0.002	1.2×10^{-3}	1.0–1.4
Ca	55,414±1828	n.s.	0.811±0.012	n.s.	102.7
Mg	52,229±5898	n.s.	0.797±0.021	n.s.	129.0
cc	53,913±2496	—	0.798±0.017	—	100.0

cc = control cultures; n.s. = not significant ($P > 5\%$). Cell cultures were grown and processed as described in Material and Methods. Labelling period = 3¾ hr; the dried cells were dissolved in 0.5 ml 1M NaOH/well.
*Cell number is expressed as % cell counts of control cultures.

Table 3. *The effect of divalent cations* (10^{-3}M) *on the proliferation of the Cloudman S91 melanoma cell line*

10^{-4}M:	[^{3}H]-TdR uptake (mean counts/min ± S.D.)	P(%)	$A_{280\ nm}$ (mean ± S.D.)	P(%)
Zn	39±38	2.23×10^{-3}	0.000	
Cu	26±19	2.23×10^{-3}	0.000	
Ca	54,732±2149	n.s.	0.810±0.034	n.s.
Mg	56,477±2502	n.s.	0.805±0.030	n.s.
cc	56,994±2816	—	0.814±0.041	—

cc = control cultures; n.s. = not significant ($P > 5\%$). Cell cultures were grown and processed as described in Material and Methods. Labelling period = 4 hr; the dried cells were dissolved in 0.5 ml 1M NaOH/well.

Table 4. *The effect of divalent cations on the proliferation of the MRC5 fibroblast cell line*

Cation	[^{3}H]-TdR uptake (mean counts/min ± S.D.)	P(%)	$A_{280\ nm}$*
Zn 10^{-4}M	1157±38	n.s.	0.50
Zn 10^{-5}M	904±197	n.s.	0.48
Cd 10^{-4}M	346±131	8.9×10^{-2}	0.09
Cd 10^{-5}M	1096±153	n.s.	0.44
Cu 10^{-4}M	1042±118	n.s.	0.46
control	1006±105	—	0.51

n.s. = not significant ($P > 5\%$). Cell cultures were grown and processed as described in Material and Methods. Labelling period = 5 hr; the dried cells were dissolved in 0.3 ml 1M NaOH/well.
*Value of 4 pooled samples.

Table 5. The effect of divalent cations on the proliferation of the GPK (keratocyte) cell line

Cation		[^{3}H]-TdR uptake (mean counts/min±S.D.) Treated cells	Control cells	P(%)	$A_{280\ nm}$* tc	cc
Zn	10^{-4}M	83,942±7361	44,587±5055	5.9×10^{-2}	1.18	1.08
Zn	10^{-5}M	68,833±2866	66,313±7653	n.s.	1.22	1.21
Zn+Cd	10^{-4}M	142±92	44,587±5055	8.3×10^{-3}	0.05	1.08
Cd	10^{-4}M	31±31	44,457±5055	8.3×10^{-3}	0.03	1.08
Cd	10^{-5}M	63,308±2902	44,587±5055	1.9×10^{-1}	1.24	1.08
Cu	10^{-4}M	65,618±5264	44,587±5055	3.0×10^{-1}	1.30	1.08
Mg	10^{-4}M	41,980±11,339	44,908±4730	n.s.	1.64	1.46
Ca	10^{-4}M	21,024±2734	20,685±2100	n.s.	1.72	1.72

n.s. = not significant ($P > 5\%$); tc = treated cells; cc = control cells. Cell cultures were grown and processed as described in Material and Methods. The dried cells were dissolved in 0.4 ml 1M NaOH/well.
*Value of 4 pooled samples.

Table 6. Effect of ouabain on zinc-induced toxicity in Cloudman S91 melanoma cells

Experimental conditions	[^{3}H]-TdR uptake (mean counts/min ± S.D.)	P(%)*	P(%)†	$A_{280\ nm}$ (mean ± S.D.)	P(%)*	P(%)†
Control	47,022±811	aaaa		0.680±0.030	aaaa	
10^{-4}M Zn^{2+}	2166±263	0.001	bbbb	0.082±0.010	0.008	bbbb
10^{-5}M ouabain	44,254±840	0.68		0.655±0.010	20.10	
10^{-4}M ouabain	37,249±690	0.08		0.633±0.008	4.38	
10^{-4}M Zn^{2+} 10^{-5}M ouabain	2800±562		12.10	0.106±0.006		1.56
10^{-4}M Zn^{2+} 10^{-4}M ouabain	3504±910		4.42	0.151±0.016		0.14

Significance estimates are related to *controls and to †cultures with 10^{-4}M Zn^{2+}. Cell cultures were seeded at density of 6×10^4 cells/well and grown and processed as described in Material and Methods.

of cell digests than in terms of [^{3}H]-TdR uptake, probably because ouabain also inhibits other energy-requiring transport processes such as [^{3}H]-TdR uptake (cf. Table 6). The reduction of zinc cytotoxicity by ouabain indicates that active transport is involved in the uptake of zinc by the melanoma cells.

The ratios of spectral absorbance at 350 nm and 280 nm, reflecting the amount of melanin in cell digests, are summarized in Table 7. Calcium,

Table 7. Spectral absorbance ration ($A_{350\ nm}/A_{280\ nm}$) of digests of melanoma cells exposed to metal ions and DMSO

Additive	Concentration	Mean	S.D. $\times 10^{-3}$	P(%)
Control	—	0.1295	9.7	—
Zn	10^{-4}M	0	0	
Cu	10^{-4}M	0.1340	5.6	n.s.
Mg	10^{-4}M	0.1268	2.8	n.s.
Ca	10^{-4}M	0.1276	4.0	n.s.
Cd	10^{-4}M	0	0	
Control	—	0.099	4.6	—
Zn	10^{-5}M	0.110	7.0	n.s.
DMSO	2%	0.131	11.2	0.47

n.s. = not significant ($P > 5\%$). Cell cultures were grown and processed as described in Material and Methods. The dried cells were dissolved in 0.5 ml 1M NaOH/well.

cupric, magnesium (10^{-4}M) and zinc ions (10^{-5}M) had no direct effect on melanogenesis, whereas after treatment with Zn^{2+} or Cd^{2+} (both 10^{-4}M) zero values were obtained due to zinc and cadmium toxicity. Tyrosinase activity measurements also did not reveal any significant changes after treatment with Ca^{2+}, Cd^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+} (10^{-4}M). The tyrosinase activity in the cultures was low, in the range 1200–1800 counts/min/well.

The addition of DMSO to the culture medium increased the melanin content in the Cloudman melanoma line (Table 7).

DISCUSSION

The present study demonstrates that not only cadmium but also copper and particularly zinc ions are selectively toxic to cells of the Cloudman S91 melanoma line. Cd^{2+} (10^{-5}M) and Zn^{2+} (10^{-4}M) were cytotoxic to melanoma cells but not to keratocytes and fibroblasts.

Relatively high resistance of fibroblasts to Zn^{2+} has been confirmed by the results of Priestley and Brown [18], who in cell cultures of human skin fibroblasts receiving 1.7×10^{-4}M $ZnSO_4$ observed only a 25% reduction in the proliferation rate.

As zinc ions have been reported to be cytotoxic also to B-16 mouse melanoma cell line [12], it

seems that susceptibility to Zn^{2+} may be a general feature of melanoma cells. These findings are remarkable considering the high content of zinc in all melanin-containing tissues [2–8] in which at the subcellular level zinc is concentrated in melanosomes [5–8, 19, 20].

The presence of zinc and other metals in pigmented tissues has been ascribed to cation exchange properties of melanin [1, 9, 10] and copper is, of course, also present in tyrosinase. The only known chemical role of zinc specific for pigment cells is its catalytic effect on the oxidoreduction rearrangement of dopachrome to 5,6-dihydroxyindole [21] during melanogenesis.

The usual mechanisms suggested for selective melanocytotoxicity are associated with the process of melanogenesis, the intermediates of which are known to be cytotoxic in two main ways: (a) the quinone products of tyrosine oxidation have highly reactive-SH-binding properties which may inhibit enzymes with important cellular functions, such as the DNA-polymerase of the melanocytes [22]; (b) free radical products such as semiquinones may initiate lipid peroxidation in melanocytes and thus destroy them [23].

However, no direct effect of Zn^{2+} and Cu^{2+} on melanogenesis was observed in the present study (Table 7), which is consistent with the inability of Zn^{2+} to activate mammalian tyrosinase as described by Lerner [11]. Moreover, zinc exerts a protective effect in maintaining the integrity of both cellular and organelle membranes through decreased lipid peroxidation [24, 25].

Nevertheless, electron spin resonance experiments have revealed that dipositive metal ions bind to *o*-semiquinone radical centres within melanin polymers to yield chelate complexes. This binding is often accompanied by large increases in total radical concentration [26]. An increase in the spin concentration in tissue after incubation with Zn^{2+} has been observed [26].

Zinc has an ubiquitous distribution within cells and is involved in various processes, and therefore the recognition and investigation of Zn-related critical events is not easy [27]. The exact mechanism of the zinc melanocytotoxicity remains to be elucidated. Weser *et al.* [28] have observed inhibitory effects of 10^{-4}M zinc on the growth of Ehrlich ascites tumour cells due to the inhibition of DNA-polymerase, but in general it has more often been zinc deficiency rather than excess which has exhibited an inhibitory effect on tumours (reviewed by van Rij and Pories [27]).

Cadmium proved to be specifically melanocytotoxic in amounts an order of magnitude smaller than zinc (Table 1). As cadmium is known to interfere with zinc in biochemical reactions [29, 30], its influence on pigment cells might be in relation to the effects of zinc.

On addition of ouabain to the Cloudman S91 cell line the cytotoxicity of Zn^{2+} was attenuated (Table 6), which suggests that active transport is involved in the zinc uptake by melanoma cells. The transport mechanism might be responsible for a higher concentration of zinc against copper in melanin-containing structures [5–7], as melanin itself has a higher affinity for copper [31]. Both ^{59}Fe and ^{67}Ga appear to enter the melanoma cells using transferrin as a carrier molecule [32]. This could be the case with zinc, too, since zinc–transferrin complex has been reported to have a special role in zinc metabolism [25, 33].

DMSO has been known to induce differentiation of various kinds of cells, including human melanoma [16] and B-16 mouse melanoma [17]. DMSO stimulated melanogenesis in Cloudman melanoma cells (Table 7) but inhibited their proliferation, as evidenced by the reduced incorporation of [^{3}H]-TdR label, which was 57,068 ± 2759 counts/min in control cells compared with 11,573 ± 3468 counts/min in DMSO-treated cells. The doubling time increased from 22.9 hr (Controls) to 36.6 hr (DMSO-treated cells). The stimulation of melanogenesis accompanied by a reduction of cell proliferation is in agreement with the results of Huberman *et al.* [16] and contrary to the findings of Iwata and Inui [17].

The observed effect of DMSO on cell differentiation (cf. Table 7) also suggests that the method used would be sensitive enough to detect changes in melanin content after divalent cation treatment if there were any.

REFERENCES

1. BRUENGER FW, STOVER BJ, ATHERTON DR. The incorporation of various ions *in vivo* and *in vitro* produced melanin. *Radiat Res* 1967, **32**, 1–12.
2. BOWNESS JM, MORTON RA. Distribution of copper and zinc in the eyes of fresh-water fishes and frogs. Occurrence of metals in melanin fractions from eye tissues. *Biochem J* 1952, **51**, 530–535.
3. BOWNESS JM, MORTON RA, SHAKIR MH, STUBS AL. Distribution of copper and zinc in mammalian eyes. Occurrence of metals in melanin fractions from eye tissues. *Biochem J* 1952, **51**, 521–530.

4. KURUS E. Über den histochemischen Nachweis von Zink als Spurenelement im Auge des Menschen. *Klin Monatsbl Augenheilkd* 1959, **134**, 338–350.
5. HORČIČKO J, BOROVANSKÝ J, DUCHOŇ J. Verteilung von Zink und Kupfer im menschlichen Kopfhaar verschiedener Farbtöne. *Dermatol Monatschr* 1973, **159**, 206–209.
6. HORČIČKO J, BOROVANSKÝ J, DUCHOŇ J, PROCHÁZKOVÁ B. Distribution of zinc and copper in pigmented tissues. *Hoppe-Seylers Z Physiol Chem* 1973, **354**, 203–204.
7. PROCHÁZKOVÁ B. Trace Elements in Melanoma. (In Czech.) Dissertation. Prague, Charles University, 1978.
8. BOROVANSKÝ J, HEARN PR, BLEEHEN SS, RUSSELL RGG. Distribution of ^{65}Zn in mice with melanomas and in the subcellular fractions of melanomas. *Neoplasma* 1980, **27**, 247–252.
9. WHITE LP. Melanin: a naturally occurring cation exchange material. *Nature (Lond)* 1958, **182**, 1427–1428.
10. LARSSON B, TJÄLVE H. Studies on the melanin affinity of metal ions. *Acta Physiol Scand* 1978, **104**, 479–484.
11. LERNER AB. Effect of ions on melanin formation. *J Invest Dermatol* 1952, **18**, 47–52.
12. BOROVANSKÝ J, BLEEHEN SS. Effect of Zn^{2+} on proliferation and differentiation of B-16 melanoma cells *in vitro*. In: WOHLRAB W, ed. *Malignes Melanom: Experimentelle Aspekte*. Martin Luther Universität Halle-Wittenberg Wissenschaftl. Beiträge, 1979, 52–54.
13. PEREZ AG, KIM JH, GELBARD AS, DJORDJEVIC B. Altered incorporation of nucleic acid precursors by mycoplasma-infected mammalian cells in culture. *Exp Cell Res* 1972, **70**, 301–310.
14. WHITTAKER JR. Changes in melanogenesis during the dedifferentiation of chick retinal pigment cells in cell culture. *Dev Biol* 1963, **8**, 99–127.
15. LEISING HB, SCHACHTSCHABEL DO. Enhanced melanization of Harding–Passey mouse melanoma cells following treatment with exogenous melanosomes in monolayer culture. *Z Naturforsch* 1979, **34c**, 124–130.
16. HUBERMAN E, HECKMAN C, LANGENBACH R. Stimulation of differentiated functions in human melanoma cells by tumor-promoting agents and dimethyl sulfoxide. *Cancer Res* 1979, **39**, 2618–2624.
17. IWATA K, INUI N. Stimulation of melanogenesis in melanoma cells growing in serum free medium by fetal bovine serum and dimethylsulfoxide. *Proc Japan Acad Ser B* 1980, **56**, 562–567.
18. PRIESTLEY CC, BROWN JC. Acute toxicity of zinc pyrithione to human skin cells *in vitro*. *Acta Derm Venereol (Stockh)* 1980, **60**, 145–148.
19. STEIN WD. Chemical composition of the melanin granule and its relation to the mitochondrion. *Nature (Lond)* 1955, **175**, 256–257.
20. SEIJI M, FITZPATRICK TB, SIMPSON RT, BIRBECK MSC. Chemical composition and terminology of specialized organelles (melanosomes and melanin granules) in mammalian melanocytes. *Nature (Lond)* 1963, **197**, 1082–1084.
21. HARLEY-MASON J, BU'LOCK JD. Synthesis of 5,6-dihydroxyindole derivatives: an oxidoreduction rearrangement catalysed by zinc ions. *Nature (Lond)* 1950, **166**, 1036–1037.
22. WICK MM. An experimental approach to the chemotherapy of melanoma. *J Invest Dermatol* 1980, **74**, 63–65.
23. RILEY PA. Mechanism of pigment cell toxicity produced by hydroxyanisole. *J Pathol* 1970, **101**, 163–169.
24. CHVAPIL M. New aspects in the biological role of zinc: a stabilizer of macromolecules and biological membranes. *Life Sci* 1973, **13**, 1041–1049.
25. CHVAPIL M. Effect of zinc on cells and biomembranes. *Med Clin North Am* 1976, **60**, 799–812.
26. FELIX CC, HYDE JS, SARNA T, SEALY RC. Interactions of melanin with metal ions.Electron spin resonance evidence for chelate complexes of metal ions with free radicals. *J Am Chem Soc* 1978, **100**, 3922–3926.
27. VAN RIJ AM, PORIES WJ. Zinc and tumor growth. In: SIEGEL H, ed. *Carcinogenicity and Metal Ions*. New York, Marcel Dekker, 1980, Vol. 10, 207–252.
28. WESER U, SEEBER S, WARNECKE P. Zur Wirkung von Zn^{2+} auf die Nukleinsäurebiosynthese von Aszites-Tumorzellen. *Experientia* 1969, **25**, 489–490.
29. COTZIAS GC, BORG DC, SELLECK B. Specificity of zinc pathways in the rabbit: zinc–cadmium exchange. *Am J Physiol* 1961, **201**, 63–66.

30. PRASAD AS. *Trace Elements and Iron in Human Metabolism*. New York, Plenum, 1978, 349–362.
31. POTTS AM, AU PC. The affinity of melanin for inorganic ions. *Exp Eye Res* 1976, **22**, 487–491.
32. TERNER UK, WONG H, NOUJAIM AA, LENTLE BC, HILL JR. ^{67}Ga and ^{59}Fe uptake in human melanoma cells. *Int J Nucl Med Biol* 1979, **6**, 23–28.
33. PHILLIPS JL, AZARI P. Zinc transferrin. Enhancement of nucleic acid synthesis in phytohemagglutinin-stimulated human lymphocytes. *Cell Immunol* 1974, **10**, 31–37.