Hormonal Stimulation of Zinc Uptake in Mammalian Cell Cultures

RODY P. COX[1]

Department of Medicine, New York University School of Medicine, New York, New York 10016

(Received March 25, 1968)

SUMMARY

Adrenal steroid hormones with glucocorticoid activity increase the uptake of Zn^{++} in certain mammalian cell cultures. Whereas in the absence of added hormones the Zn^{++} content of HeLa S_3 cells is approximately 8- to 20-fold greater than that of an equivalent volume of medium, after growth in the presence of prednisolone the cellular Zn^{++} content doubles or triples. Kinetic studies suggest that cells must grow 8–12 hr in the presence of prednisolone before an increased Zn^{++} uptake becomes apparent. Accumulation of Zn^{++} is markedly depressed at low temperatures and in the presence of sulfhydryl-blocking agents. Inhibitors of either glycolysis or oxidative metabolism have little effect on Zn^{++} uptake. However, if both glycolysis and oxidative metabolism are inhibited, Zn^{++} accumulation is decreased. $^{65}Zn^{++}$ taken up by mammalian cells is readily exchangeable and is nearly all soluble in trichloracetic acid. Hormonal effects on Zn^{++} uptake are selective in that (a) only those steroid hormones with potent glucocorticoid effects stimulate Zn^{++} uptake while the degree of effectiveness of each hormone parallels its glucocorticoid activity; (b) susceptibility to hormone effects on Zn^{++} accumulation was confined to certain established "epithelium-like" cell lines; and (c) the uptake of several other cations, for example, $^{45}Ca^{++}$ and $^{86}Rb^{++}$, was not significantly enhanced by the steroid hormone, and a number of monovalent and divalent cations did not compete with $^{65}Zn^{++}$. Certain effects of hydrocortisone on cells may be explained by an increase in the Zn^{++} content of cells.

INTRODUCTION

Regulation of cellular activities in higher animals is in part controlled by hormones. The mechanisms responsible for the effects of various hormones on cells are complex and poorly understood. Moreover, it appears that hormones may have a number of different effects, for example, stimulation of a rapidly synthesized nuclear RNA, followed by increased synthesis of certain enzymes or other proteins (1–9), translational effects on protein synthesis (10, 11), and effects on the transport of various substances into and out of cells or cell organelles (12, 13). During an investigation of the mechanisms responsible for the induction of increased alkaline phosphatase activity by hydrocortisone or its synthetic derivative prednisolone in tissue culture (10, 14, 15), it was found that adrenal glucocorticoid hormones increase the uptake of Zn^{++} by certain mammalian cell cultures.

MATERIALS AND METHODS

The methods used in this laboratory for monolayer cultures have been described (10, 14, 15). Tissue culture medium and reagents were purchased from Grand Island Biological Co., and the hormone preparations were obtained from Mann Research Laboratories. The cell lines studied and their origin are listed in Table 2. For these studies all cell lines were grown at 35° in Waymouth's medium containing 10% calf serum and antibiotics (50 units of penicillin, 50 μg of strepto-

[1] Career Scientist of the Health Research Council of the City of New York (I-251).

mycin, and 30 μg of kanamycin per milliliter). The nonradioactive Zn^{++} content of "complete" Waymouth's medium is approximately 1.2 μg/ml.[2] Radioactive isotopes with a purity greater than 99% were purchased from New England Nuclear Corporation. The specific activities of various radionuclides used were: $^{65}Zn^{++}$ preparations varied from 2.16 to 3.21 mC/mg; $^{45}Ca^{++}$, 10.3 mC/mg; and $^{86}Rb^{+}$, 8.7 mC/mg. One-tenth of a millicurie of each of the radionuclides was diluted with 2.2 ml of distilled water. Two methods were used to study the uptake of the isotopes: (a) cells were grown for several days in medium containing the radioactive isotope at a final concentration of 0.46 μC/ml; (b) cultures were exposed to pulse-labeling for 3 hr with radionuclides at a final concentration of 2.3 μC/ml. After labeling, the cells were immediately harvested by decanting the medium and washing the monolayer five times with ice-cold 0.9% saline. The last two washes had less than 50 cpm above background. The cells were lysed with 0.5% deoxycholate dissolved in 0.45% saline.

The $^{65}Zn^{++}$ content of the deoxycholate cell lysate was assayed in a well-type Packard automatic gamma detector with a thallium-activated sodium iodide crystal. Approximately 10% of the $^{65}Zn^{++}$ peak gamma emission at 1.119 m.e.v. was counted. In all experiments the uptake of the radioactive isotope was compared in replicate cultures grown in the presence and absence of hormones. The same batch of radioactive nuclide was used in an experiment, and recently prepared lots of $^{65}Zn^{++}$ were purchased every 2 weeks. The results are expressed as radioactivity per milligram of cell protein. Approximately 160,000 HeLa S_3 (Spinner line) cells contain 0.1 mg of cell protein; when these cells are grown in medium with added prednisolone, cell size is only slightly increased, about 140,000 cells containing 0.1 mg of protein.

The $^{45}Ca^{++}$ or $^{86}Rb^{+}$ content of cell lysates was assayed by counting a 0.2-ml aliquot of the deoxycholate cell lysate dissolved in 15 ml of dioxane scintillation solution (10, 16) in a Packard Tri-Carb scintillation counter. Protein determinations were carried out by the method of Lowry *et al.* (17) on an aliquot of deoxycholate cell lysate.

[2] Determinations were made by Dr. Donald Crawford, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma.

RESULTS

The specificity of steroid hormones in stimulating Zn^{++} accumulation in HeLa S_3 cells is shown in Table 1. The relative effectiveness of various steroid hormone preparations, in general, correlates with their glucocorticoid activity. At the concentrations tested, hydrocortisone and its synthetic analogue prednisolone are the most potent inducers of increased Zn^{++} uptake. Aldosterone and corticosterone also appear to facilitate slightly the uptake of Zn^{++}. These steroids have, in common, a 11β-hydroxyl group and 17β-sidechain which contains a 20-ketone and 21-hydroxyl group. Molecules with a methyl group in position 21 (11β-hydroxyprogesterone) appear to have slight and somewhat variable effects on enhancing the Zn^{++} content of cells. Cortisone, which has a carbonyl group in the 11-position, was ineffective in stimulating $^{65}Zn^{++}$ uptake. The minimal and inconstant increases in Zn^{++} accumulation observed with the other steroid hormone preparations appear to be within the range of experimental variations for untreated cultures.

The effects of adrenal glucocorticoid hormones on stimulating Zn^{++} uptake in mammalian cell cultures were selective in that only certain heteroploid epithelium-like cell lines, such as HeLa cells, Kb cells, and D-98 cells, were affected (Table 2). Two other epithelium-like established cell lines, Chang liver and Chang conjunctiva cells, showed a small increase in Zn^{++} content when grown in medium containing prednisolone. Other cell lines—for example, human skin fibroblast strains, BS-C-1 green monkey kidney cells, a minimal deviation hepatoma cell line H411E, and mouse embryo fibroblasts 3T3—showed little or no enhancement of Zn^{++} accumulation when cultured in medium with added

TABLE 1

Effects of steroids on $^{65}Zn^{++}$ uptake in HeLa S_3 cell cultures

HeLa S_3 cells were grown for 72 hr in Waymouth's medium containing 10% calf serum, 0.46 μC/ml $^{65}Zn^{++}$, and 2.5 μg/ml of each of the steroids tested. Stock solutions of the hormones were prepared by dissolving them in 0.05 ml of ethanol and adding water to make 10 ml of solution. Control cultures contained an equal volume of the alcohol–water diluent.

Steroid, 2.5 μg/ml	Number of experiments	Average $^{65}Zn^{++}$ uptake[a]	Range
No added steroid	—	1.0	—
Cholesterol	2	0.92	0.84–1.00
Pregnenolone	2	1.05	0.96–1.14
Progesterone	2	0.89	0.75–1.03
11β-Hydroxyprogesterone	4	1.25	1.10–1.37
17β-Hydroxyprogesterone	2	0.85	0.750–.95
Deoxycorticosterone	4	1.12	0.94–1.21
Aldosterone	4	1.40	1.21–1.75
Corticosterone	6	1.35	1.15–1.68
Hydrocortisone	3	2.62	2.15–3.04
Prednisolone	5	3.12	2.80–3.90
Cortisone	2	0.94	0.91–0.97
Diethylstilbestrol	3	0.87	0.74–1.03
17β-Estradiol	3	1.10	0.96–1.18
Testosterone	4	1.12	1.02–1.18

[a] $^{65}Zn^{++}$ uptake in each experiment was calculated by dividing the average counts per minute of $^{65}Zn^{++}$ per milligram of cell protein in four replicate steroid-treated cultures by the average counts per minute of $^{65}Zn^{++}$ per milligram of cell protein in four replicate cultures grown in medium without added steroids. The averages and ranges are shown.

TABLE 2

Effects of prednisolone on the $^{65}Zn^{++}$ content and alkaline phosphatase activity of various mammalian cell cultures

Cell culture[a]	Origin of cell	$^{65}Zn^{++}$ uptake		Alkaline phosphatase induction ratio[b]
		No additions	Prednisolone	
HeLa S_3 Spinner line adapted to monolayer culture	Carcinoma of human cervix	3.1 ± 0.4	8.6 ± 0.6	8.3
HeLa Ch-CL321	Carcinoma of human cervix	6.7 ± 1.0	14.2 ± 1.7	2.1
Kb cell line	Carcinoma of mouth	2.13 ± 0.3	5.65 ± 0.7	2.3
D-98	Human bone marrow	2.36 ± 0.4	7.70 ± 0.8	2.2
Chang liver cell	Fetal human liver	1.67 ± 0.6	2.44 ± 0.5	1.2
Chang conjunctival cell	Fetal human conjunctiva	2.80 ± 0.2	3.83 ± 0.6	1.5
Hepatoma H411E	Minimal deviation of hepatoma cell from rat	1.17 ± 0.1	1.36 ± 0.3	0.8
BS-C-1	African green monkey kidney	6.7 ± 1.2	7.3 ± 1.6	0.9
Human skin fibroblast	Foreskin culture, human infant	5.7 ± 0.8	5.3 ± 0.4	1.0
3T3 fibroblast	Swiss mouse embryo	2.2 ± 0.3	1.0 ± 0.2	0.8

[a] All cultures were grown in Waymouth's medium containing 0.46 μC/ml $^{65}Zn^{++}$ for 96 hr. The averages and ranges of at least four replicate cultures are expressed as counts per minute × 10^{-3} per milligram of cell protein.

[b] Alkaline phosphatase induction ratio is the ratio of specific activity of the enzyme prepared from cultures grown in medium with added prednisolone to the specific activity of replicate cultures grown in medium without added hormone.

prednisolone. Table 2 also shows the *induction ratio* for alkaline phosphatase in the various cell lines studied. All the epithelium-like lines except for the HeLa S_3 (Spinner line) had high constitutive levels of alkaline phosphatase. In those lines in which the enzyme activity increased upon growth in medium with prednisolone the Zn^{++} accumulation by the cells was also enhanced, although there was no proportionality between the extent of alkaline phosphatase induction and the increase in Zn^{++} uptake. Moreover, markedly increasing the Zn^{++} content of cells by growing them for 3–5 days in medium containing high concentrations of Zn^{++} (0.1 mM) but without added prednisolone did *not* enhance the alkaline phosphatase activity of the cells.

Prednisolone also showed specificity with respect to the type of cation affected. The uptake of $^{65}Zn^{++}$ was markedly stimulated whereas the uptake of certain other cations, such as $^{45}Ca^{++}$ and $^{86}Rb^{++}$, exhibited a variable and small increase (Table 3). Addition of increasing concentrations of nonradioactive Zn^{++} proportionately reduce the $^{65}Zn^{++}$ accumulation, thus showing that the radioactivity taken up by the cells is $^{65}Zn^{++}$ rather than a radioactive contaminant of our $^{65}Zn^{++}$ preparations. The hormonally induced stimulation of $^{65}Zn^{++}$ accumulation was observed either when HeLa cells were grown for several days in medium containing radioactive Zn^{++} and a glucocorticoid hormone or when cells grown for several days in nonradioactive medium with added prednisolone were pulse-labeled for 3 hr with $^{65}Zn^{++}$.

When prednisolone is added to growing cultures of HeLa S_3 cells, the stimulation of $^{65}Zn^{++}$ uptake is not observed until about 8–12 hr after the hormone has been added (Fig. 1). These kinetic findings indicate that the hormone does not simply act as a carrier molecule. This conclusion is also supported by the finding that HeLa S_3 cells grown for 48 hr in Waymouth's medium containing prednisolone show the same $^{65}Zn^{++}$ uptake when they are pulse-labeled for 3 hr in medium with or without added prednisolone.

Figure 2 shows the kinetics of $^{65}Zn^{++}$ uptake in replicate cultures grown for 72 hr in medium with and without added prednisolone. The rate of $^{65}Zn^{++}$ uptake is approximately 2–3 times faster in cells grown in medium containing the hormone than in replicate cultures grown without added hormone, and in both instances is relatively complete after 3 hr. The kinetics of efflux of $^{65}Zn^{++}$ from cells grown for 72 hr in radioactive medium with and without added prednisolone is shown in Fig. 3; the data are consistent with Zn^{++} in cells being readily exchangeable. Similar kinet-

TABLE 3

Comparison of the effects of prednisolone on $^{65}Zn^{++}$, $^{45}Ca^{++}$, and $^{86}Rb^{++}$ accumulation by HeLa S_3 cells grown in Waymouth's medium[a]

Cation	Cation uptake after 72-hr growth with isotope, 0.46 μC/ml[b]		Cation uptake after 3-hr pulse with isotope, 2.3 μC/ml[c]	
	Control	Prednisolone, 1.5 μg/ml	Control	Prednisolone, 1.5 μg/ml
$^{65}Zn^{++}$	3.1 ± 0.6	9.6 ± 1.4	3.8 ± 0.4	11.6 ± 0.3
$^{45}Ca^{++}$	5.09 ± 0.7	5.36 ± 0.8	15.4 ± 1.2	17.0 ± 1.0
$^{86}Rb^{++}$	23.7 ± 2.4	25.5 ± 3.1	70.0 ± 15.2	75.8 ± 8.3

[a] The averages and ranges of three replicate cultures are expressed as counts per minute × 10^{-3} per milligram of cell protein.

[b] Replicate cultures were grown for 72 hr in Waymouth's medium containing 0.46 μC/ml of radioactive nuclide.

[c] Replicate cultures were grown in Waymouth's medium with or without prednisolone (1.5 μg/ml) for 72 hr. The cultures were then pulse-labeled for 3 hr with radioactive nuclide at a final concentration of 2.3 μC/ml.

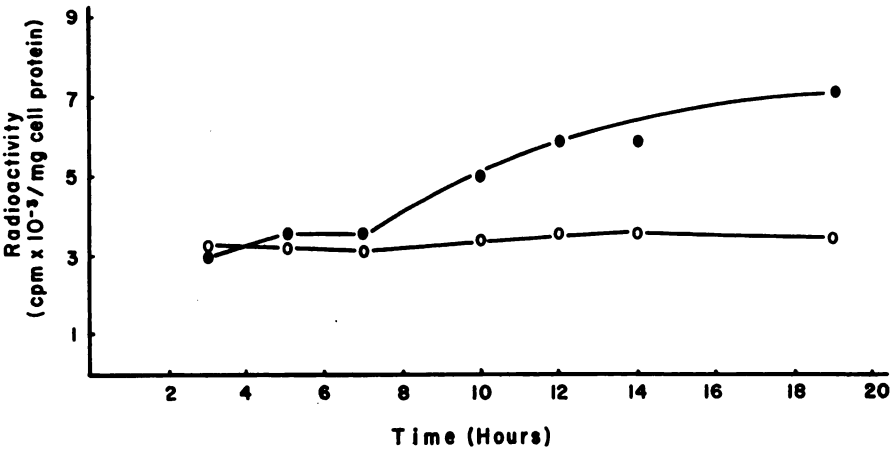

FIG. 1. *Effect of duration of growth in medium containing prednisolone on $^{65}Zn^{++}$ uptake by HeLa S_3 cells during a 3-hr pulse*

O, Control: cultures grown in medium without added hormone. ●, Prednisolone-treated: cultures grown in medium with 1.5 μg/ml of prednisolone. Prednisolone was added to prednisolone-containing cultures at time 0. At various times thereafter, 2.3 μC of $^{65}Zn^{++}$ was added per milliliter to three replicate control and three prednisolone-treated cultures. The cultures were pulse-labeled for 3 hr and then harvested as described in MATERIALS AND METHODS.

ics was observed when labeled cells were incubated in medium without added non-radioactive Zn^{++}, showing that the exchange is not dependent on increased levels of Zn^{++} in the medium. $^{65}Zn^{++}$ taken up by mammalian cultures is nearly all soluble in 10% trichloracetic acid; less than 1% of the counts remain associated with trichloracetic acid-insoluble material. Most of the $^{65}Zn^{++}$ in cell lysates can also be removed by dialysis.

The concentration of $^{65}Zn^{++}$ in HeLa S_3 cells is much higher than the $^{65}Zn^{++}$ content of a volume of medium equal to the total

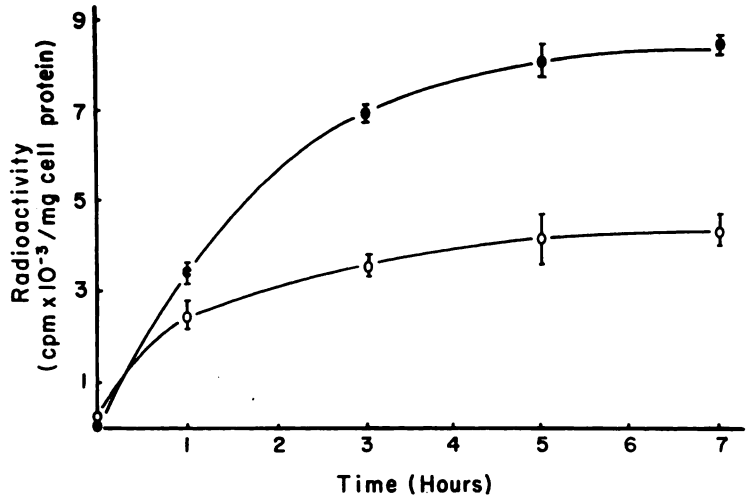

FIG. 2. *Comparison of $^{65}Zn^{++}$ uptake in HeLa S_3 cell cultures grown for 72 hr in medium with and without added prednisolone*

O, Control: cultures grown for 72 hr in medium without added hormone. ●, Prednisolone-treated: cultures grown for 72 hr in medium with 1.5 μg/ml of prednisolone. $^{65}Zn^{++}$, 2.3 μC/ml, was added to all cultures at time 0, and four replicate cultures of each were harvested at various times thereafter.

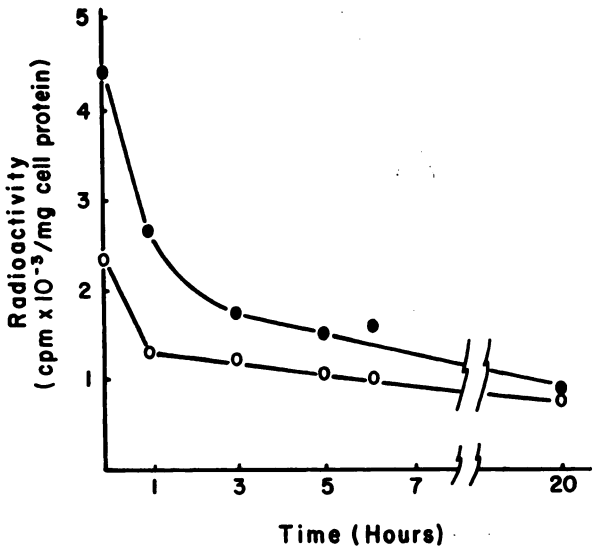

FIG. 3. *Efflux of $^{65}Zn^{++}$ from HeLa S_3 cells grown for 72 hr in medium with $^{65}Zn^{++}$, 0.46 μC (2.0 μM)*

O, Control: HeLa S_3 cells grown for 72 hr in medium without added hormone. ●, Prednisolone-treated: HeLa S_3 cells grown for 72 hr in medium with 1.5 μg/ml of prednisolone. At time 0, the radioactive medium was decanted, the cells were washed with Puck's buffer, and nonradioactive medium supplemented with 12 μM $ZnCl_2$ was added. The medium added to the "prednisolone-treated" cultures also contained 1.5 μg/ml of prednisolone. Three replicate control and three prednisolone-treated cultures were harvested at each time interval.

cell volume (distribution ratio). As seen in Table 4, the distribution ratio (cells to medium) is about 15 with respect to the cells grown in the absence of prednisolone and about 40 for cells grown in medium with added prednisolone.

The $^{65}Zn^{++}$ content of HeLa S_3 cells depends on a number of factors, for

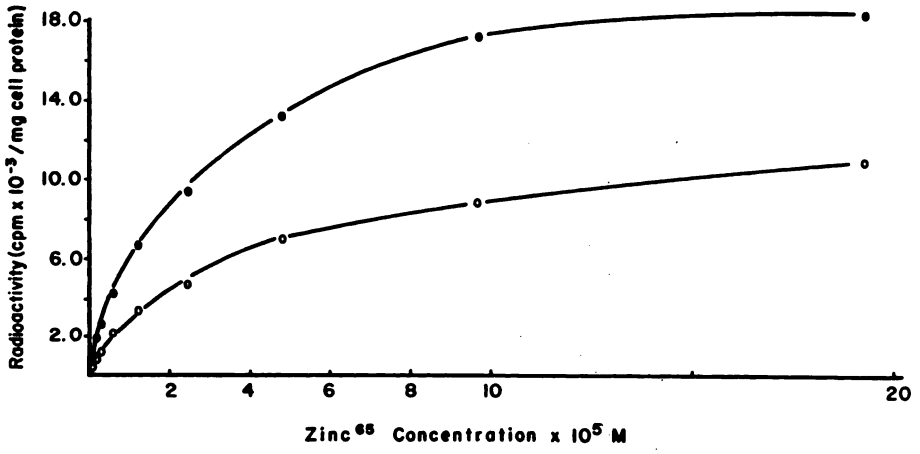

FIG. 4. *Effect of increasing concentrations of $^{65}Zn^{++}$ on the accumulation of radioactivity by HeLa S_3 cultures pregrown for 72 hr in medium with and without added prednisolone*

O, Control: cultures grown for 72 hr in medium without added hormone. ●, Prednisolone-treated: cultures grown for 72 hr in medium with 1.5 μg/ml of prednisolone. Each concentration of $^{65}Zn^{++}$ was added to three replicate control and three prednisolone-treated cultures, and the cells were harvested 3 hr later. The nonradioactive Zn^{++} content of medium was 18 μM.

TABLE 4
Distribution ratio of $^{65}Zn^{++}$ between cells and medium in HeLa S_3 cells grown in Waymouth's medium

HeLa S_3 cells grown in Waymouth's medium containing 0.46 μC $^{65}Zn^{++}$ for 72 hr	Cell volume[a] (ml)	Radioactivity in cells (cpm $\times 10^{-3}$)	Radioactivity in equal volume of medium (cpm $\times 10^{-3}$)
No addition (control)			
A	0.019	5.71	0.39
B	0.016	5.82	0.31
C	0.014	5.83	0.36
Prednisolone, 1.5 μg/ml			
A	0.014	12.92	0.24
B	0.017	12.98	0.28
C	0.019	14.61	0.30

[a] Cell volumes were measured in a Lawrence tube after centrifuging the cell suspension at 1000 rpm in a PR4 refrigerated International centrifuge for 20 min.

example, the $^{65}Zn^{++}$ concentration in the culture medium, the duration of growth in the presence of the label, and the density of the cell monolayer per volume of medium. Figure 4 shows the effect of increasing the concentration of $^{65}Zn^{++}$ in the medium on the radioactivity per milligram of cell protein in replicate cultures grown in medium with and without added prednisolone. The stimulation by the hor-

TABLE 5
Effect of increasing the number of HeLa S_3 cells per culture on the uptake of $^{65}Zn^{++}$

HeLa S_3 cell cultures[a]	Cell protein per culture (mg)	$^{65}Zn^{++}$ uptake[b]
Control	0.24 ± 0.08	5.50 ± 0.8
Prednisolone, 1.5 μg/ml	0.25 ± 0.07	10.80 ± 1.1
Control	0.48 ± 0.09	3.60 ± 0.6
Prednisolone, 1.5 μg/ml	0.51 ± 0.12	7.50 ± 0.4
Control	0.95 ± 0.10	2.40 ± 0.5
Prednisolone, 1.5 μg/ml	0.96 ± 0.07	5.10 ± 0.3
Control	1.04 ± 0.16	2.10 ± 0.7
Prednisolone, 1.5 μg/ml	1.10 ± 0.20	4.30 ± 1.1

[a] For each experiment, three replicate HeLa S_3 cultures were grown for 72 hr in 2-oz glass bottles with Waymouth's medium with or without added prednisolone (1.5 μg/ml). After 72 hr of growth, the medium was replaced with fresh medium containing 2.3 μC/ml of $^{65}Zn^{++}$; the cultures were incubated at 37° for 3 hr before harvesting.

[b] The averages and ranges of three replicate cultures are expressed as counts per minute $\times 10^{-3}$ per milligram of cell protein.

TABLE 6
Effects of temperature on the uptake of $^{65}Zn^{++}$ by HeLa S_3 cells during a 3-hr pulse label

Addition to Waymouth's medium[a]	Temperature	$^{65}Zn^{++}$ uptake[b]
No added hormone	35°	8.2 ± 1.3
Prednisolone	42°	19.1 ± 2.6
Prednisolone	40°	19.6 ± 3.1
Prednisolone	35°	16.3 ± 1.8
Prednisolone	24°	6.8 ± 1.8
Prednisolone	18°	3.4 ± 0.8
Prednisolone	7°	1.4 ± 0.6
Prednisolone	0°	0.4 ± 0.2

[a] Cultures were grown at 35° for 72 hr in Waymouth's medium containing 1.5 μg/ml of prednisolone except for the cultures designated "no added hormone," which were grown in Waymouth's medium without added hormone. The effects of temperature were studied by incubating the cultures at the indicated temperature for 30 min before adding $^{65}Zn^{++}$ to a final concentration of 2.3 μC/ml. After addition of $^{65}Zn^{++}$, cultures were maintained at the temperature shown during the 3-hr pulse.

[b] The averages and ranges of three to six replicate cultures are expressed as counts per minute $\times 10^{-3}$ per milligram of cell protein. During the course of the experiment, there were no apparent cytological changes observed in the cell monolayer.

mone of $^{65}Zn^{++}$ accumulation is approximately proportional at all $^{65}Zn^{++}$ levels tested. However, the increase in $^{65}Zn^{++}$ uptake with increasing concentration of $^{65}Zn^{++}$ in the medium reaches a plateau at high zinc concentrations, a phenomenon suggesting saturation of uptake. Table 5 shows the effect of increasing the number of cells per volume of medium on the uptake of $^{65}Zn^{++}$. As the cell population density in the culture medium increases, the $^{65}Zn^{++}$ content per milligram of cell protein decreases. However, the prednisolone-induced enhancement of $^{65}Zn^{++}$ accumulation remains approximately proportional at each population density.

Temperature profoundly influences the accumulation of $^{65}Zn^{++}$ by HeLa cell cultures. Increasing the temperature of cultures 35° to 40° or 42° during a 3-hr pulse-label with $^{65}Zn^{++}$ slightly increases the $^{65}Zn^{++}$ uptake of HeLa S_3 cells (Table 6). However, decreasing the temperature from 35° to 24° or 18° significantly decreases $^{65}Zn^{++}$ accumulation, and at temperatures of 7° or 0° there is a marked diminution in the radioactive Zn^{++} content of cells following a 3-hr pulse, as shown in Table 6. These results suggest that simple diffusion alone cannot explain the hormonal effects on Zn^{++} uptake, since cold would not be expected to have such marked effects.

Table 7 shows the effects of sulfhydryl-blocking agents on $^{65}Zn^{++}$ uptake in HeLa S_3 cells previously grown for 72 hr in Waymouth's medium containing prednisolone. The marked inhibition caused by these agents suggests that the entry or bind-

TABLE 7

Effects of sulfhydryl-blocking agents and metabolic inhibitors on the uptake of $^{65}Zn^{++}$ by HeLa S_3 cells during a 3-hr pulse

HeLa S_3 cell cultures[a]	$^{65}Zn^{++}$ uptake	
	30-min incubation with inhibitor[b]	3-hr incubation with inhibitor[b]
No additions (control)	8.2 ± 1.3	7.1 ± 0.6
Prednisolone, 1.5 μg/ml	16.3 ± 1.8	15.6 ± 1.4
Prednisolone with *N*-ethylmaleimide, 1 mM	1.16 ± 0.5	ND[c]
Prednisolone with *p*-chloromercuribenzoate, 0.1 mM	5.8 ± 1.7	ND
Prednisolone with mercurous ions, 20 μM	5.2 ± 1.3	ND
Prednisolone with iodoacetate, 0.1 mM	6.1 ± 1.9	ND
Prednisolone with a nitrogen atmosphere	17.1 ± 1.7	16.2 ± 1.8
Prednisolone with sodium cyanide, 5 mM	13.6 ± 2.6	14.8 ± 0.8
Prednisolone with potassium azide, 5 mM	12.7 ± 3.1	12.8 ± 1.4
Prednisolone with 2,4-dinitrophenol, 5 mM	13.6 ± 1.1	14.0 ± 0.6
Prednisolone with sodium fluoride, 20 mM	12.9 ± 2.3	13.2 ± 0.8
Prednisolone with potassium azide, 5 mM, and sodium fluoride, 2 mM	9.4 ± 1.7	8.7 ± 0.9
Prednisolone with ouabain, 0.1 mM	12.8 ± 0.6	11.7 ± 1.1

[a] Cultures were grown at 35° for 72 hr in Waymouth's medium containing 1.5 μg/ml of prednisolone except for the cultures designated "no additions," which were grown in Waymouth's medium without added hormone. The effects of sulfhydryl-blocking agents and metabolic inhibitors were studied by adding the reagent either 30 min or 3 hr before adding $^{65}Zn^{++}$ to a final concentration of 2.3 μC/ml. All cultures were incubated at 35° during the 3-hr pulse.

[b] The averages and ranges of six replicate cultures are expressed as counts per minute × 10^{-3} per milligram of cell protein. During the course of the experiment (30 min or 3 hr of prior treatment with inhibitors before addition of $^{65}Zn^{++}$ and 3 hr of pulse-labeling) no apparent cytological changes were observed in the cell monolayer except in cultures in which both potassium azide and sodium fluoride were present for 3 hr of incubation; in some of these cultures there was some rounding of cells and a few cells detached from the glass after a further 3 hr of radioactive labeling.

[c] ND indicates that these experiments were not done.

ing of Zn^{++} is probably mediated by interaction with sulfhydryl groups. Table 7 also shows that anaerobiosis and several inhibitors of glycolysis and oxidative metabolism when tested individually either have no effect or only slightly decrease the uptake of $^{65}Zn^{++}$. Prolonging for 3 hr the time of prior incubation with these inhibitors also did not appreciably further decrease $^{65}Zn^{++}$ uptake during a subsequent 3-hr pulse (Table 7). When sodium azide and sodium fluoride were used together so as to block both glycolysis and oxidative metabolism, there was a moderate reduction in $^{65}Zn^{++}$ accumulation after a 30-min incubation and a slightly greater effect after a 3-hr incubation with these inhibitors (Table 7). The significance of this effect is difficult to interpret since there were cytological alterations in some of the cultures after 6 hr in the presence of both inhibitors. Ouabain, an inhibitor of a membrane-bound sodium-potassium dependent ATPase, also seemed to have little effect on the $^{65}Zn^{++}$ content of cells, at least for the time course of these experiments.

TABLE 8

Effect of various cations on the uptake of $^{65}Zn^{++}$ by HeLa S_3 cells during a 3-hr pulse

HeLa S_3 cell cultures[a]	^{65}Zn uptake in the presence of various cation concentrations[b]	
	30 μM	60 μM
No additions	6.4 ± 1.3	5.4 ± 1.2
Prednisolone, 1.5 μg/ml	16.7 ± 2.0	14.3 ± 1.3
Prednisolone with manganous ions	16.5 ± 1.7	19.7 ± 2.1
Prednisolone with cobaltous ions	14.8 ± 1.3	15.5 ± 1.7
Prednisolone with cadmium ions	12.1 ± 2.4	6.5 ± 0.2
Prednisolone with cupric ions	14.7 ± 1.5	12.8 ± 1.3
Prednisolone with mercurous ions	3.0 ± 0.8	2.4 ± 0.3
Prednisolone with ferrous ions	14.8 ± 1.6	17.6 ± 0.9
Prednisolone with rubidium ions	15.1 ± 0.8	15.2 ± 2.1
Prednisolone with lithium ions	16.9 ± 1.4	12.2 ± 1.8

[a] Cultures were grown for 72 hr in Waymouth's medium containing prednisolone, 1.5 μg/ml, except for the cultures designated "no additions," which were grown in Waymouth's medium without added hormone. The effects of certain cations were determined by adding either 30 μM or 60 μM final concentration of these cations as the chloride salt to the cultures 30 min before addition of $^{65}Zn^{++}$. The total Zn^{++} content of the medium was 30 μM; this included 2.3 μC of $^{65}Zn^{++}$. After a 3-hr incubation at 37° in radioactive medium, the cells were harvested. During the course of the experiments, there were no apparent cytological changes observed in the cell monolayer.

[b] The averages and ranges of six replicate cultures are expressed as counts per minute × 10^{-3} per milligram of cell protein.

Table 8 shows that a number of divalent and monovalent cations do not appreciably affect the uptake of $^{65}Zn^{++}$ when present in the medium at the same concentration or at twice the concentration of Zn^{++}. The marked inhibition observed with mercurous ions is interpreted as sulfhydryl binding (see Table 8) rather than competition for a common binding or carrier molecule. The only cation that had an effect was cadmium, and its effects were somewhat variable unless present at twice the concentration of Zn^{++}.

DISCUSSION

The work reported indicates that certain mammalian cells in culture concentrate Zn^{++}, and this uptake is stimulated in some but not all mammalian cell lines by adrenal glucocorticoid hormones, such as hydrocortisone or its analogue prednisolone. The final concentration of $^{65}Zn^{++}$ associated with HeLa S_3 cells is about 8- to 20-fold greater than the medium concentration when cells are grown in medium without added prednisolone, and this uptake doubles or triples following growth in medium with added hormone. The stimulation of Zn^{++} uptake by hormones is selective in several ways: (a) only those steroid hormones with potent glucocorticoid effects are able to stimulate Zn^{++} uptake; (b) only certain mammalian cell lines are susceptible to the hormone-mediated stimulation; and (c) the accumulation of certain other cations is not increased by the

hormone. Kinetic studies on the rate of $^{65}Zn^{++}$ uptake in HeLa S_3 cells indicate that the prednisolone effects show an 8–12-hr delay before an increase in $^{65}Zn^{++}$ content is observed. This finding, together with evidence that HeLa cells previously grown in medium with added prednisolone take up $^{65}Zn^{++}$ at the same rate during a 3-hr pulse in the presence or absence of the hormone, indicates that the hormone does not act simply as a Zn^{++} carrier. The rate of $^{65}Zn^{++}$ uptake in cells grown for 72 hr in Waymouth's medium containing hydrocortisone or its analogue prednisolone is approximately 2 to 3 times faster than in replicate cultures grown in medium without added hormone. The $^{65}Zn^{++}$ taken up by cells is readily exchangeable and does not appear to be incorporated into macromolecules. At high $^{65}Zn^{++}$ levels, the accumulation of isotope by cells reaches a plateau.

The $^{65}Zn^{++}$ content of cells per milligram of cell protein appears to depend in part on the confluency of cultures (ratio of cells to medium). It would appear that this variation in Zn^{++} accumulation is not due to a depletion of radioative Zn^{++} from the medium. However, Stoker and Rubin recently have emphasized that the microenvironment of cells in monolayer cultures may be quite different from the composition of the bulk of the medium (18). It is also known that the metabolism of cells in confluent cultures differs from that in rapidly growing cultures (19, 20); such differences in metabolism may in part account for the effects of cell population density on Zn^{++} accumulation. Nevertheless, it is clear that the effect of prednisolone on enhancing Zn^{++} uptake is observed at all population densities studied.

Sulfhydryl groups may play an important role in mediating Zn^{++} accumulation in HeLa S_3 cells, since blocking these groups markedly reduces the $^{65}Zn^{++}$ content of the cells. Uptake also is greatly depressed by low temperatures. On the other hand, anaerobiosis and certain metabolic inhibitors of either glycolysis or oxidative metabolism appear to have little effect on Zn^{++} uptake. When glycolysis and oxidative metabolism are both blocked by using sodium fluoride and potassium azide, the uptake of $^{65}Zn^{++}$ is moderately decreased. The significance of this finding is not clear since, following 3 hr of incubation with both inhibitors and a subsequent 3-hr $^{65}Zn^{++}$ pulse, this combination of inhibitors caused a cytological change in some cultures. If energy is required for Zn^{++} accumulation, the failure of anaerobiosis or inhibitors of glycolysis or oxidative metabolism to decrease uptake markedly when used singly suggests that either glycolysis or oxidative metabolism can produce sufficient energy to sustain Zn^{++} uptake for the time course of our experiments. During the course of a pulse-labeling experiment, $^{65}Zn^{++}$ uptake also was not markedly affected by ouabain.

The mechanisms responsible for Zn^{++} uptake in mammalian cells are not known. The findings described in this study do not distinguish among the various modes of translocation of Zn^{++} from medium to cells. The marked temperature dependence of Zn^{++} accumulation observed even at temperatures of 18° and 24° and the striking inhibition of Zn^{++} uptake in the presence of sulfhydryl-blocking agents suggests that a selective temperature-sensitive Zn^{++} carrier or Zn^{++}-binding molecule containing sulfhydryl group(s) may be involved in the accumulation of this cation. A sulfhydryl-containing protein has been implicated in the transport of β-galactosides into bacterial cells (21). Prednisolone may enhance Zn^{++} uptake in certain mammalian cell cultures by increasing the level or activity of carrier molecules, or the hormone may in some way alter the cell so as to increase Zn^{++}-binding sites selectively. It is of interest that adrenal corticosteroid hormones have recently been implicated in maintaining the activity of the sodium- and potassium-activated ATPase in rat kidney (22). This effect of hormones is apparently mediated by the stimulation of ATPase synthesis (22).

The uptake or binding of Zn^{++} appears to be relatively specific, since a number of cations at approximately twice the concentration of Zn^{++} do not appreciably compete

with it for uptake. However, cadmium appears to have a significant though variable effect. It is of interest that cadmium feeding can alter the partition of $^{65}Zn^{++}$ among various animal tissues (23) and also that the teratogenic effects of cadmium can be blocked by Zn^{++} administration (24).

The increased alkaline phosphatase activity induced in certain established epithelium-like cell cultures by glucocorticoid hormones has been shown to be due to *synthesis* of an enzyme with an increased catalytic efficiency rather than to an increase in the alkaline phosphatase content of cells (10, 16). Maximal enzyme induction is not attained until the base level enzyme has been replaced by alkaline phosphatase synthesized in the presence of the hormone. The increased catalytic efficiency of the enzyme is accompanied by an alteration in some of the chemical and physical properties of the enzyme (10). A possible explanation for these observations on alkaline phosphatase induction is that the "induced" form of the enzyme has an increased number of catalytic sites. Since alkaline phosphatase is a zinc-containing metalloenzyme and a portion of the zinc ion is associated with the active site (25), it seems possible that the increased alkaline phosphatase activity of cells grown in medium with added prednisolone might be due in part to the greater availability of Zn^{++} in these cells. However, markedly increasing the Zn^{++} content of cells by growing them in medium with high Zn^{++} levels but without added prednisolone does not increase the alkaline phosphatase activity. This finding suggests that increased Zn^{++} content per se does not "induce" the alteration in enzyme activity. However, Zn^{++} deficiency in calves has been reported to decrease their serum alkaline phosphatase activity (26).

Hormones have been previously implicated in $^{65}Zn^{++}$ uptake by prostatic tissue. Uptake of $^{65}Zn^{++}$ by the dorsolateral lobe of the prostate is increased in hypophysectomized rats by testosterone (27), chorionic gonadotropin (27), and interstitial cell stimulating hormone (28). Uptake is not significantly altered in this tissue by the administration of follicle stimulating hormone, prolactin, or growth hormone alone. However, certain combinations of these hormones do augment the effects of interstitial cell stimulating hormones on $^{65}Zn^{++}$ uptake (29).

The finding that adrenal glucocorticoid hormones selectively stimulate Zn^{++} uptake in cultures of certain epithelium-like mammalian cells may be of importance in understanding certain effects of these hormones. For example, glucocorticoid hormones have been reported to "stabilize" the outer membranes of cells (30) and the membranes that surround certain cell organelles (31), causing these structures to be more resistant to cytolytic or surface-active substances. The reasons for this increased resistance to injury of cell membranes is unknown. Perhaps the stimulation by adrenal hormones of Zn^{++} accumulation in certain cells is in part responsible for this "stabilization" of membranes. The recent findings that the addition of heavy metal ions, particularly Zn^{++}, protects membranes from fragmentation during subcellular fractionation procedures provides support for this speculation (32). The concentration of Zn^{++} within cells may influence the availability of this cation for the synthesis of certain Zn^{++}-containing proteins, and this metal may also influence the rates of biochemical reactions (33).

ACKNOWLEDGMENTS

This investigation was supported by Research Grant GM 15508 from the United States Public Health Service and Research Contract U-1296 with the Health Research Council of the City of New York. I wish to acknowledge the valuable technical assistance of Dr. Adriana Ruckenstein and Mrs. Maude Levy. I thank Drs. P. Elsbach, E. Simon, D. Schacter, M. J. Griffin, and J. King for their discussion and advice.

REFERENCES

1. H. Ui and G. C. Mueller, *Proc. Natl. Acad. Sci. U. S.* **50**, 256 (1963).
2. F. T. Kenney and F. J. Kull, *Proc. Natl. Acad. Sci. U. S.* **50**, 493 (1963).
3. H. L. Segal and Y. S. Kim, *Proc. Natl. Acad. Sci. U. S.* **50**, 912 (1963).

4. T. H. Hamilton, *Proc. Natl. Acad. Sci. U. S.* **51**, 83 (1964).
5. J. E. Varner and G. R. Chandra, *Proc. Natl. Acad. Sci. U. S.* **52**, 100 (1964).
6. M. E. Dahmus and J. Bonner, *Proc. Natl. Acad. Sci. U. S.* **54**, 1370 (1965).
7. A. R. Means and T. H. Hamilton, *Proc. Natl. Acad. Sci. U. S.* **56**, 1594 (1966).
8. G. M. Tomkins, E. B. Thompson, S. Hayashi, T. Gelehrter, D. Granner and B. Peterkofsky, *Cold Spring Harbor Symp. Quant. Biol.* **31**, 349 (1966).
9. B. W. O'Malley and P. O. Kohler, *Proc. Natl. Acad. Sci. U. S.* **58**, 2359 (1967).
10. M. J. Griffin and R. P. Cox, *Proc. Natl. Acad. Sci. U. S.* **56**, 946 (1966).
11. L. Sokoloff, C. M. Francis and P. L. Campbell, *Proc. Natl. Acad. Sci. U. S.* **52**, 728 (1964).
12. G. A. Porter, R. Bogoroch and I. S. Edelman, *Proc. Natl. Acad. Sci. U. S.* **52**, 1326 (1964).
13. H. Rasmussen and E. Ogata, *Biochemistry* **5**, 733 (1966).
14. R. P. Cox and C. M. MacLeod, *J. Gen. Physiol.* **45**, 439 (1962).
15. R. P. Cox and C. M. MacLeod, *Proc. Natl. Acad. Sci. U. S.* **49**, 504 (1963).
16. M. J. Griffin and R. P. Cox, *J. Cell Biol.* **29**, 1 (1966).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
18. M. Stoker and H. Rubin, *Nature* **215**, 171 (1967).
19. E. M. Levine, Y. Becker, C. W. Boone and H. Eagle, *Proc. Natl. Acad. Sci. U. S.* **53**, 350 (1965).
20. G. J. Todaro, G. K. Lazar and H. Green, *J. Cell. Comp. Physiol.* **66**, 325 (1965).
21. C. F. Fox and E. P. Kennedy, *Proc. Natl. Acad. Sci. U. S.* **54**, 891 (1965).
22. P. L. Jørgensen, *Biochim. Biophys. Acta* **151**, 212 (1968).
23. G. C. Cotzias and P. S. Papavasilioa, *Am. J. Physiol.* **206**, 787 (1964).
24. V. H. Ferm and S. J. Carpenter, *Nature* **216**, 1123 (1967).
25. L. Engström, *Biochim. Biophys. Acta* **52**, 49 (1961).
26. W. J. Miller, W. J. Pitts, C. M. Clifton and J. D. Morton, *J. Dairy Sci.* **48**, 1329 (1965).
27. S. A. Gunn and T. C. Gould, *J. Endocrinol.* **16**, 18 (1957).
28. S. A. Gunn, T. C. Gould and W. A. D. Anderson, *Proc. Soc. Exptl. Biol. Med.* **104**, 348 (1960).
29. S. A. Gunn, T. C. Gould and W. A. D. Anderson, *J. Endocrinol.* **32**, 205 (1965).
30. G. Melynkovych, *Science* **152**, 1086 (1966).
31. G. Weissmann and J. Dingle, *Exptl. Cell Res.* **25**, 207 (1962).
32. L. Warren, M. C. Glick and M. K. Nass, *in* "The Specificity of Cell Surfaces" (B. D. Davis and L. Warren, eds.), p. 109. Prentice-Hall, Englewood Cliffs, New Jersey, 1967.
33. M. Fujioka and I. Lieberman, *J. Biol. Chem.* **239**, 1164 (1964).