Biochemical Pharmacology, Vol. 29, pp. 539–552.

0006-2952/80/0215-0539 $02.00/0

EFFECT OF METHYLMERCURIC CHLORIDE ON GENE EXPRESSION IN NEUROBLASTOMA AND GLIOMA CELLS AFTER ACUTE AND CHRONIC TREATMENTS

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(*Received* 26 *May* 1979; *accepted* 7 *August* 1979)

Abstract—To understand the cellular and molecular mechanisms of methylmercuric chloride (CH_3HgCl)-induced damage to nerve tissue, monolayer cultures of glioma cells (C-6) and of neuroblastoma cells (NBP_2) were used in this study. Chronic (6–8 weeks) and acute (5 days) treatment of glioma cells with low concentrations (0.05 to 0.1 μM) of CH_3HgCl produced marked increases and decreases in the amounts and net phosphorylation profiles of specific proteins. Chronic treatment of neuroblastoma cells (0.1 and 0.2 μM) did not produce any significant alterations in the amounts of specific proteins, but it caused marked changes in the phosphorylation levels of cellular proteins. The morphology and doubling time of chronically treated glioma and neuroblastoma cells did not change. Adenosine 3′,5′-cyclic monophosphate (cyclic AMP)-stimulating agents produced morphological changes in chronically treated glioma and neuroblastoma cells similar to those produced in untreated cells.

It is known that methylmercuric chloride (CH_3HgCl) causes neurological disorders which are referred to as Minamata Disease [1, 2]. CH_3HgCl has been reported to accumulate in the central nervous system in rather large proportions after ingestion or after intravenous or intraperitoneal administration [3–6]. In order to understand the cellular and molecular mechanisms of CH_3HgCl-induced neurotoxicity, monolayer cultures of glioma and neuroblastoma (NB) cells have been used as an experimental model. We have reported that glioma cells are more sensitive to CH_3HgCl than NB cells [7], and changes in cyclic AMP metabolism may be one of the biochemical lesions of CH_3HgCl on these cells [8]. Our preliminary results [9] have shown that marked alterations in the amounts and net phosphorylation profiles of specific proteins are observed in chronically treated glioma cells with low concentrations of CH_3HgCl. We now report the following: (a) chronic CH_3HgCl treatment of NB cells does not produce changes in specific proteins, but causes marked alterations in net phosphorylation profiles of proteins; (b) acute CH_3HgCl treatment of glioma cells produces changes in gene expression which are qualitatively different from those observed following chronic treatment; and (c) the morphology, doubling time and effect of cyclic AMP-stimulating agents on morphological differentiation do not change in chronically treated glioma cells and NB cells.

METHODS

Rat glioma cells [10] (clone C-6) of passages (30–42) [11] were used. Glioma cells were grown in F12 medium containing 10% fetal calf serum. Mouse neuroblastoma cells (clone NBP_2), which contain both tyrosine hydroxylase and choline acetyltransferase [12], were also used. Cells were grown in F12 medium containing 10% agammaglobulin newborn calf serum. Both types of medium contained 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37° in a humidified atmosphere of 5% CO_2. Glioma cells were subcultured by washing the cells twice with phosphate buffered saline (PBS), pH 7.0, and incubating them in the presence of 0.25% trypsin solution in calcium-free MEM for 40 min, whereas NB cells were subcultured by incubating them in the presence of 0.25% pancreatin solution in calcium-free MEM for 10 min. After incubation, the cells were removed from the flask surface, transferred into a centrifuge tube, and mixed well. An equal volume of growth medium was added to stop the action of proteolytic enzyme. Cells were centrifuged at 700 r.p.m. for 7 min, pellets were resuspended, and an aliquot was added to a new flask. For chronic treatment, the cells were grown in the presence of CH_3HgCl for 6–8 weeks (0.05 and 0.1 μM for glioma cells; 0.1 and 0.2 μM for NB). Cells were subcultured every 4–6 days. For the experiments, cells (2×10^6 glioma; 0.25×10^6 NB) were plated in Lux tissue culture dishes (100 mm) and CH_3HgCl (0.1 and 0.05 μM for glioma; 0.1 and 0.2 μM for NB) was added the same day. The medium (20 ml) and CH_3HgCl were changed at 2, 3 and 4 days after plating. For NB cells it was necessary to change the medium and the drug twice on day 4 because of increased production of lactic acid. For acute treatment cells were similarly plated, and CH_3HgCl was added 24 hr after plating. The drug and medium were changed in the same manner as described for the chronic treatment studies.

The cells were harvested at confluency (5 days after plating) and washed twice with 10 vol. of PBS, pH 7.0 [9]. The subcellular fractions were prepared according to a method described previously [13]. The cell pellets were suspended in approximately 2 vol. of ice-cold 0.05 M Tris–HCl, 0.25 M sucrose, 3 mM $MgCl_2$, 4 mM 2-mercaptoethanol and 2 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4, and homogenized at 5000 r.p.m. for 3 min (5 strokes) using a Potter–Elvehjem tissue grinder [9]. The homogenates were centrifuged at 800 *g* for 30 min at 4° in a Sorvall RC2-B centrifuge. The supernatant fractions were recentrifuged for an additional 30 min at 800 *g*.

Crude nuclear preparations. The appropriate nuclear pellets (800 *g*) from the two centrifugations were combined, dispersed in 5 vol. (2 ml) of the above Tris–HCl buffer, and centrifuged at 800 *g* for 30 min. The nuclear pellets were washed again by resuspending in 5 vol. of the Tris–HCl buffer and recentrifuging at 800 *g* for 30 min. The nuclei were dispersed in 1.0 ml of the Tris–HCl buffer at 4° and homogenized after 30 min in a Dounce tissue grinder [9].

Cytosols. The 800 *g* supernatant fractions obtained at the initial step were centrifuged at 100,000 *g* for 1.5 hr at 4° in a Beckman L3-40 ultracentrifuge. The clear supernatant fractions, free of particulate materials, constituted the cytosol fractions [9].

Cytoplasmic particulates. The cytoplasmic particulates (100,000 *g* pellets) were washed by dispersing in 5 vol. of the Tris–HCl buffer and centrifuging at 100,000 *g* for 1.5 hr at 4°. The pellets were suspended in 0.5 ml of the Tris–HCl buffer and homogenized in a Dounce tissue grinder [9].

The procedures for assaying endogenous protein phosphorylation have also been described previously [14].

Preparation of samples for phosphorylation. Protein determinations were made on the crude nuclear, cytoplasmic, and particulate fractions by the method of Lowry *et al.* [15]. Aliquots containing 0.6 mg protein were transferred to 1.0×7.5 cm plastic tubes, diluted to 0.2 ml with the Tris–HCl buffer and lyophilized [9].

Phosphorylation reaction. The phosphorylation reaction was carried out as outlined earlier [14, 16] with some modifications. The lyophilized protein samples were dissolved in 0.14 ml of 65 mM sodium acetate, 13 mM magnesium acetate and 2 mM PMSF, pH 6.5, and incubated for 10 min at 30° [9]. Cyclic AMP-independent (—) phosphorylation was initiated by the addition of 0.04 ml of 18 μM [γ-^{32}P]ATP (specific radioactivity: 4.5×10^7 d.p.m. - nmole ATP). The phosphorylating reagent was prepared by diluting [γ-^{32}P]ATP (New England Nuclear, Boston, MA), with non-radioactive ATP (Sigma Chemical Co., St. Louis, MO). Cyclic AMP-dependent (+) phosphorylation was initiated by the addition of 0.04 ml of 18 μM [γ-^{32}P]ATP and 22.5 μM cAMP. The samples were incubated for an additional 10 min at 30° and the reaction was stopped by the addition of 0.06 ml of 12% sodium dodecylsulfate (SDS), 20 mM Tris (base), 4 mM EDTA, 8% 2-mercaptoethanol, 40% sucrose, 2 mM PMSF and 0.1% bromophenol blue, pH 8.0. The samples were heated for 3 min in a boiling water bath, cooled in ice, and frozen until needed for electrophoresis [9].

Polyacrylamide gel electrophoresis. The frozen samples for electrophoresis were thawed and 0.01-ml aliquots were applied to 7–18% linear gradient polyacrylamide SDS gels. The samples were electrophoresed using a discontinuous buffer system at 100 V for 16 hr and at 150 V for an additional 8 hr. The gels were stained with 0.1% Coomassie brilliant blue, R-250 in 10% methanol and 7% acetic acid for 1 hr at 37° and destained in the same solvent mixture overnight at 37°. The gels were dried and photographed. Autoradiographs were prepared by exposing the dried gels to DuPont Chronex 4 X-ray films for 48–72 hr. It should be pointed out that the *in vitro* assays of endogenous protein phosphorylation provide the net phosphorylation profiles which are the result of protein kinase and phosphoprotein phosphatase activities [9].

To determine the doubling time, cells (10^5) were plated in Lux tissue culture dishes (60 mm), the cell number was counted every other day for a period of 6 days, and the growth curve was constructed [9]. The doubling time was determined on the exponential portion of the curve. Three separate determinations were made for each treatment. To study the effect of cyclic AMP-stimulating agents on morphological differentiation, cells from chronically treated cultures (glioma-10^5, NB-50,000) were plated in Lux tissue culture dishes (60 mm) and CH_3HgCl was added at the same day. Prostaglandin E_1 (PGE_1) (10 μg/ml for glioma and NB cells) and 4-(3-butoxy 4-methoxybenzyl)-2-imidazolidinone (R020-1724, 200 μg/ml for NB) were added separately 24 hr after plating. Glioma cells were not treated with R020-1724, because this inhibitor of cyclic nucleotide phosphodiesterase produced minimal changes in morphology of glioma cells [8]. The drug and medium were changed 3 days after plating, and the number of morphologically differentiated cells was determined 4 days after plating. The cells with cytoplasmic processes greater than 50 μm in length were considered morphologically differentiated. A total of 300 cells was counted and the percentage of morphologically differentiated cells was determined in the various groups.

RESULTS

Changes in growth rate, morphology and effect of cyclic AMP. The morphology and the doubling time (24–35 hr for glioma; 18–20 hr for NB) of chronically treated (0.05 and 0.1 μM) glioma cells [9] and NB cells (0.1 and 0.2 μM) were similar to those of untreated cultures. We did not select any specific type of cells during chronic treatment, since the concentrations of CH_3HgCl used in this study did not cause cytotoxicity or affect the growth rate [9]. PGE_1 produced an 85 per cent morphological differentiation in glioma cells which were not treated with CH_3HgCl. A similar effect of PGE_1 was observed in chronically treated glioma cells. PGE_1 and R020-1724 caused 45 and 75 per cent morphological differentiation, respectively, in NB cultures

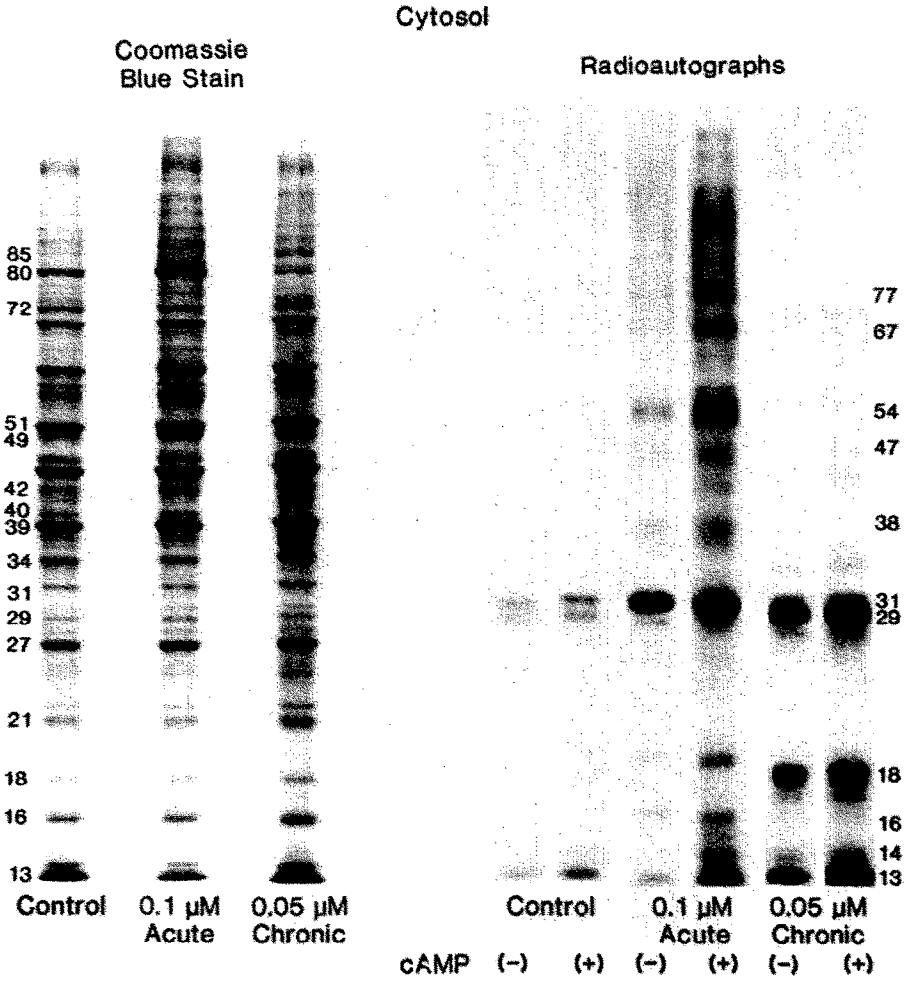

Fig. 1. Polyacrylamide gel electrophoresis of the cytosol fraction of glioma cells. The approximate molecular weights of proteins are expressed as multiples of 1000 (K). The observed change in the phosphorylation of a protein species represents a steady state situation and is the sum of phosphorylation and dephosphorylation reactions.

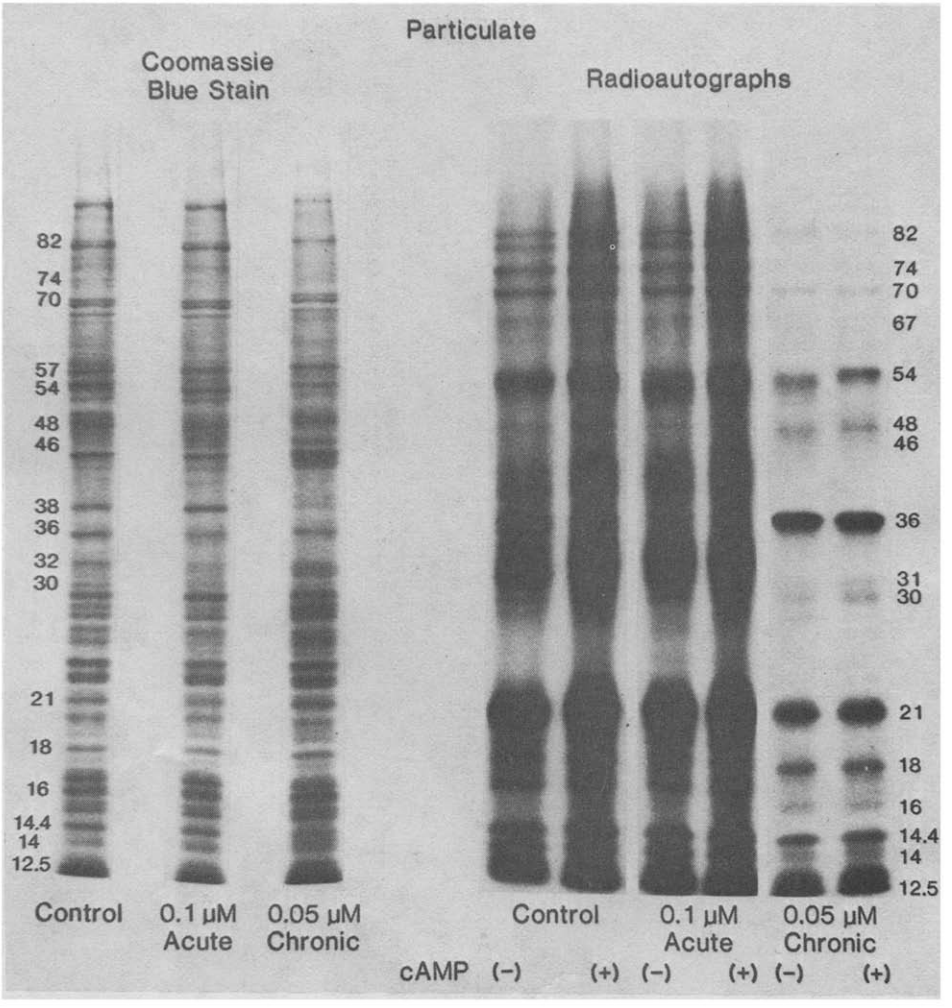

Fig. 2. Polyacrylamide gel electrophoresis of the particulate fraction of glioma cells. See the legend of Fig. 1 for additional information.

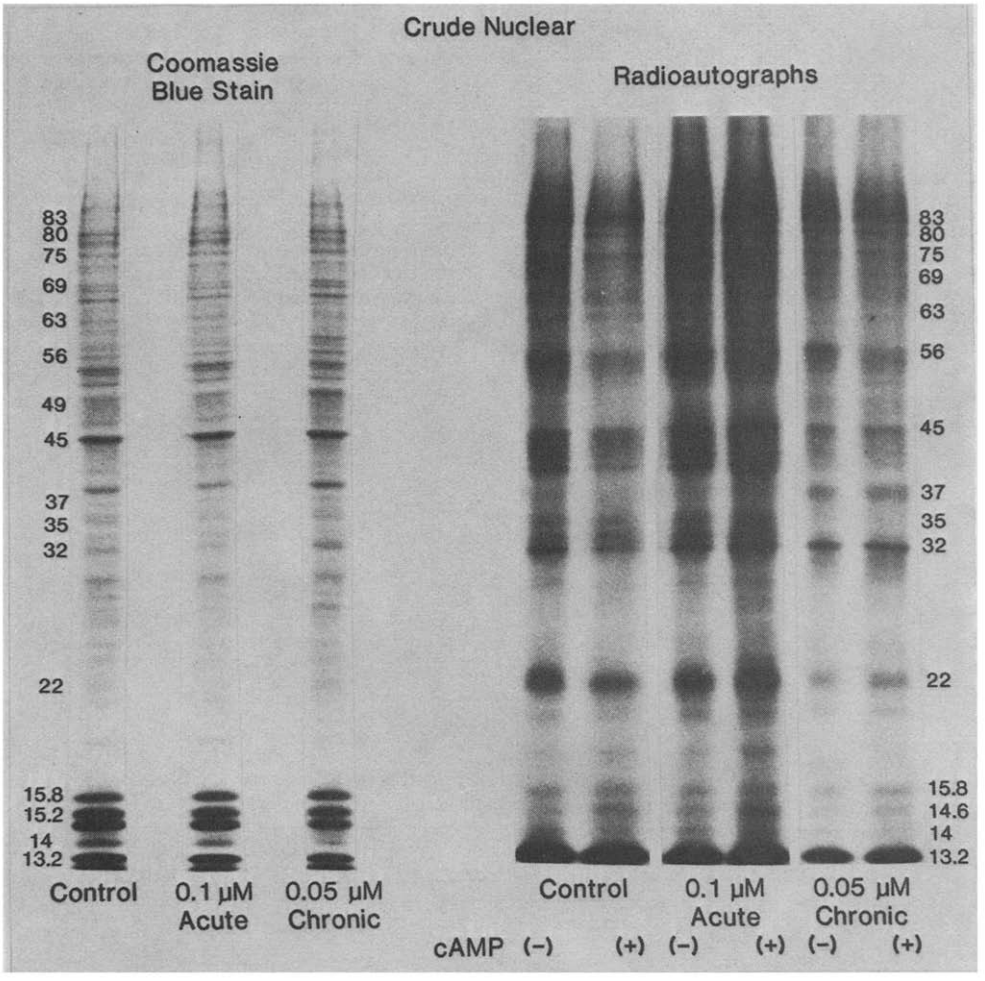

Fig. 3. Polyacrylamide gel electrophoresis of the crude nuclear fraction of glioma cells. See the legend of Fig. 1 for additional information.

Table 1. Effects of methylmercuric chloride on changes in the amounts and phosphorylation of cytosol proteins of glioma cells*

Mol. wt (×1000)	Control	Acute (0.1 μM)	Chronic (0.1 μM)	Chronic (0.05 μM)
		Coomassie blue stained protein bands		
85	Detectable	Unchanged	+	Unchanged
80	Detectable	+	−	−
49	Detectable	+	+	+
39.4	Detectable	Unchanged	−	−
		Net phosphorylation profiles		
77†	Undetectable	+	Unchanged	Unchanged
54†	Undetectable	+	Unchanged	Unchanged
47†	Undetectable	+	Unchanged	Unchanged
30†	Detectable	+	−	+
18†	Undetectable	+	Unchanged	+
16†	Undetectable	+	Unchanged	Unchanged
13†	Detectable	+	+	+

* From radioautographs, changes in the relative quantities and levels of phosphorylation of specific proteins in the acutely and chronically treated glioma cells are presented as increased (+), decreased (−) or unchanged with respect to the corresponding proteins in the untreated cells. The observed change in the level of phosphorylation of a protein species represents a steady state situation and is the sum of phosphorylation and dephosphorylation reactions.

† Refers to cyclic AMP-dependent phosphorylation.

Table 2. Effects of methylmercuric chloride on changes in the amounts and phosphorylation of proteins of particulate fraction of glioma cells*

Mol. wt (×1000)	Control	Acute (0.1 μM)	Chronic (0.1 μM)	Chronic (0.05 μM)
		Coomassie blue stained protein bands		
58	Detectable	−	−	−
46	Detectable	Unchanged	+	+
38	Detectable	Unchanged	−	−
32	Detectable	−	+	+
14	Detectable	+	+	+
12.5	Detectable	Unchanged	+	+
		Net phosphorylation profiles		
83†	Detectable	Unchanged	−	−
74†	Detectable	Unchanged	−	−
70†	Detectable	Unchanged	−	−
54†	Detectable	Unchanged	−	−
36†	Detectable	Unchanged	−	−
21†	Detectable	Unchanged	−	−
14†	Detectable	Unchanged	−	−

* From radioautographs, changes in the relative quantities and levels of phosphorylation of specific proteins in the acutely and chronically treated glioma cells are presented as increased (+), decreased (−) or unchanged with respect to the corresponding proteins in the untreated cells. The observed change in the level of phosphorylation of a protein species represents a steady state situation and is the sum of phosphorylation and dephosphorylation reactions.

† Refers to cyclic AMP-dependent phosphorylation.

Table 3. Effects of methylmercuric chloride on changes in the amounts and phosphorylation of crude nuclear proteins of glioma cells*

Mol. wt (× 1000)	Control	Acute (0.1 μM)	Chronic (0.1 μM)	Chronic (0.05 μM)
		Coomassie blue stained protein bands		
59	Detectable	Unchanged	+	+
49	Detectable	Unchanged	+	+
32	Detectable	Unchanged	+	+
22	Undetectable	Unchanged	+	+
18	Undetectable	Unchanged	−	−
14	Detectable	Unchanged	−	−
		Net phosphorylation profiles		
75	Detectable	+	Unchanged	Unchanged
56	Detectable	+	Unchanged	Unchanged
49	Undetectable	Unchanged	+	Unchanged
40–45	Detectable	+	−	−
37†	Undetectable	Unchanged	+	+
32–25	Detectable	+	−	−
22	Detectable	+	−	−
13.2†	Detectable	Unchanged	−	−

* From radioautographs, changes in the relative quantities and levels of phosphorylation of specific proteins in the acutely and chronically treated glioma cells are presented as increased (+), decreased (−) or unchanged with respect to the corresponding proteins in the untreated cells. The observed change in the level of phosphorylation of a protein species represents a steady state situation and is the sum of phosphorylation and dephosphorylation reactions.

† Refers to cyclic AMP-dependent phosphorylation.

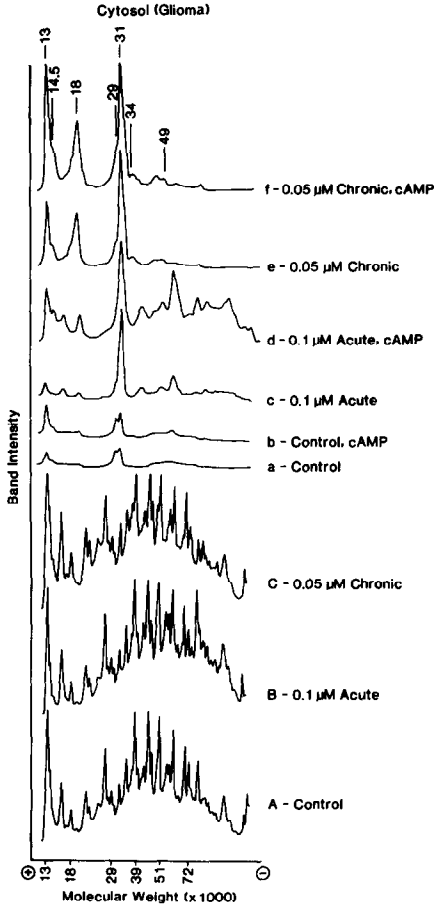

Fig. 4. Densitometeric tracings of Fig. 1. A–C represent Coomassie blue stained bands; a-f represent radioautographed bands.

which were not treated with CH_3HgCl. A similar effect of cyclic AMP-stimulating agents was observed in chronically treated NB cultures.

Changes in the relative amounts of proteins. There were dramatic increases and decreases in the intensities of specific proteins of the cytosol (Fig. 1), particulate (Fig. 2) and crude nuclear fractions (Fig. 3) of acutely (0.1 μM CH_3HgCl) and chronically (0.05 μM CH_3HgCl) treated glioma cells. The qualitative changes in the relative amounts of proteins and their phosphorylation levels have been summarized in Tables 1–3. The relative changes with intensities of specific proteins of the cytosol (Fig. 4), particulate (Fig. 5) and crude nuclear fractions of glioma cells (Fig. 6) have been also measured by densitometry. Marked alterations in the amounts of proteins were seen in glioma cells chronically treated with as little as 0.05 μM (12.5 ng/ml) CH_3HgCl. In addition, there were marked differences in the relative amounts of proteins in acutely and chronically treated glioma cells. The staining intensities of most bands (all bands of the nuclear fraction, and half of the bands of the cytosol and particulate fractions) in acutely treated glioma cells were similar to those found in the untreated glioma cells. However, the intensities of all bands presented in Tables 1–3 showed marked alterations in the chronically (0.1 μM) treated glioma cells. There were mostly quantitative differences between concentrations of 0.05 and 0.1 μM CH_3HgCl in chronically treated glioma cells. The degrees of changes were greater in cells treated with higher concentrations of CH_3HgCl.

In contrast to the chronically treated glioma cells, NB cells did not show any significant change in the amounts of proteins in the cytosol (Fig. 7), particulate (Fig. 8) and crude nuclear fractions (Fig. 9) after chronic treatment with CH_3HgCl (0.1 and

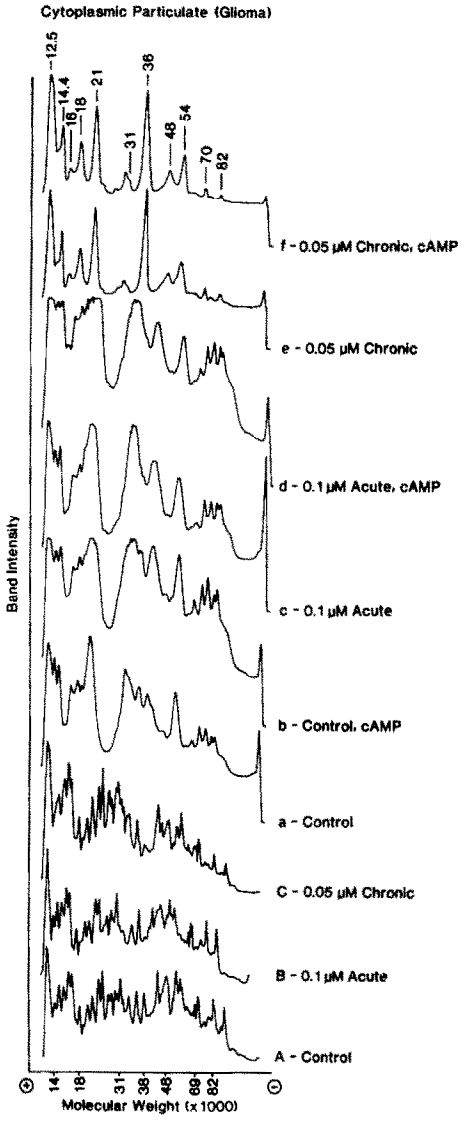

Fig. 5. Densitometeric tracings of Fig. 2. See the legend of Fig. 4 for additional information.

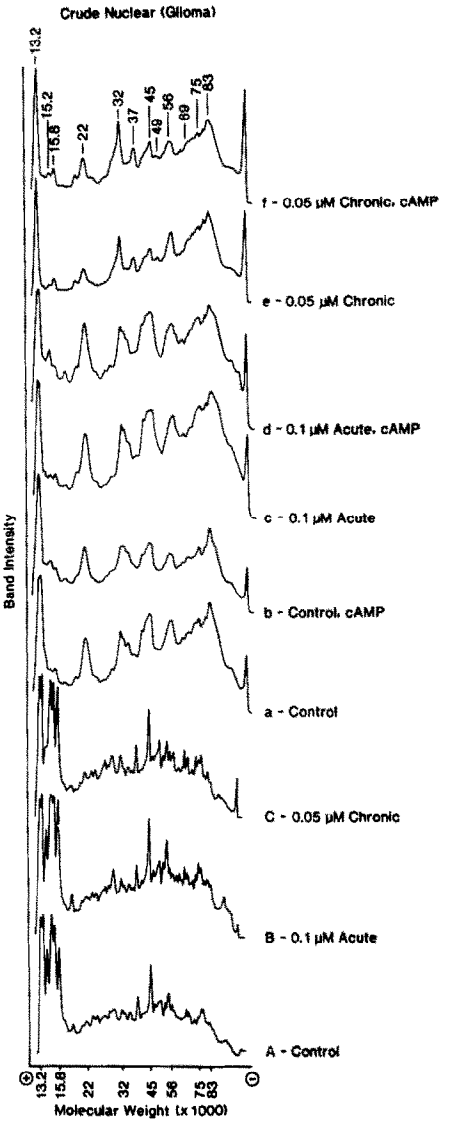

Fig. 6. Densitometeric tracings of Fig. 3. See the legend of Fig. 4 for additional information.

0.2 μM). The relative changes in the intensities of specific proteins of the cytosol (Fig. 10), particulate (Fig. 11) and crude nuclear (Fig. 12) fractions of NB cells have been measured by densitometry.

Changes in phosphorylation levels. The phosphorylation activities of proteins of untreated glioma cells and NB cells were highest in the particulate (Figs. 2 and 8) and lowest in the cytosol fractions (Figs. 1 and 7). The phosphorylation levels of most proteins in the particulate and cytosol fractions were cyclic AMP-dependent in both glioma and neuroblastoma cells (Tables 1, 3 and 4), whereas two bands in nuclear fraction of NB cells (mol. wt 67 and 79 K) or two bands in nuclear fraction of glioma cells (mol. wt 13.2 and 32 K) were cyclic AMP-dependent. It should be pointed out that confluent cells were used in these experiments, and this, in part, may account for a low phosphorylation level in the cytosol. However, the overall phosphorylation levels of cytosol proteins of NB cells were greater than those of glioma cells; the reverse was true for particulate and nuclear fractions.

Chronic treatment of glioma and NB cells produced marked alterations (increases and decreases) in net phosphorylation profiles of specific proteins. Increases and decreases in the levels of phosphorylation were observed in the cytosol fractions of NB cells (Table 4) and glioma cells (Table 1). However, the direction of the changes in the phosphorylation levels of the particulates of glioma cells was opposite to that of NB cells. For example, the phosphorylation levels of the particulate fraction decreased in chronically treated glioma cells (Table 2), whereas they were increased in similarly treated NB cells (Table 4).

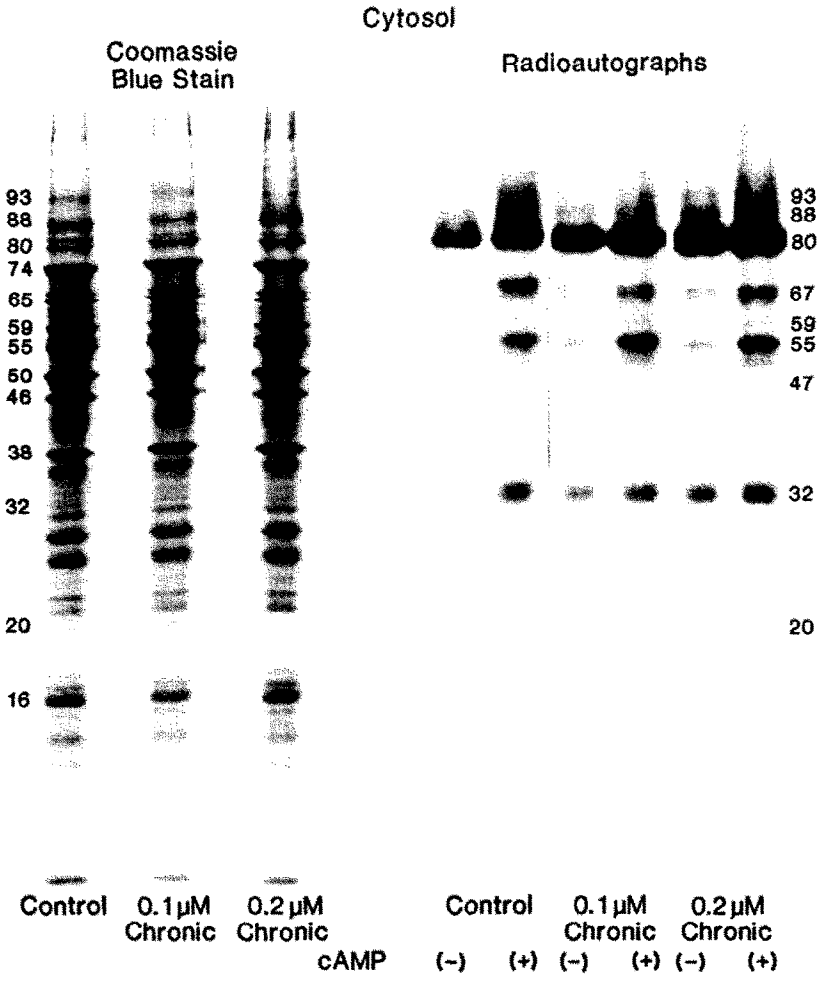

Fig. 7. Polyacrylamide gel electrophoresis of the cytosol fraction of neuroblastoma cells. See the legend of Fig. 1 for additional information.

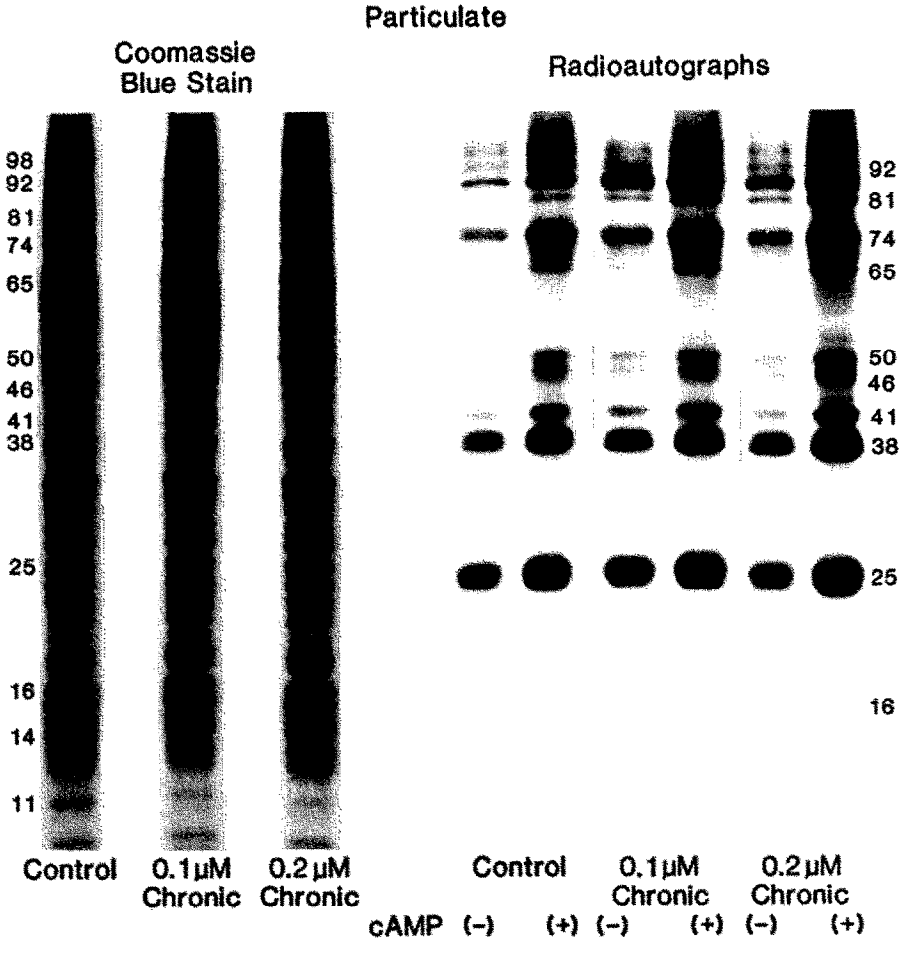

Fig. 8. Polyacrylamide gel electrophoresis of the particulate fraction of neuroblastoma cells. See the legend of Fig. 1 for additional information.

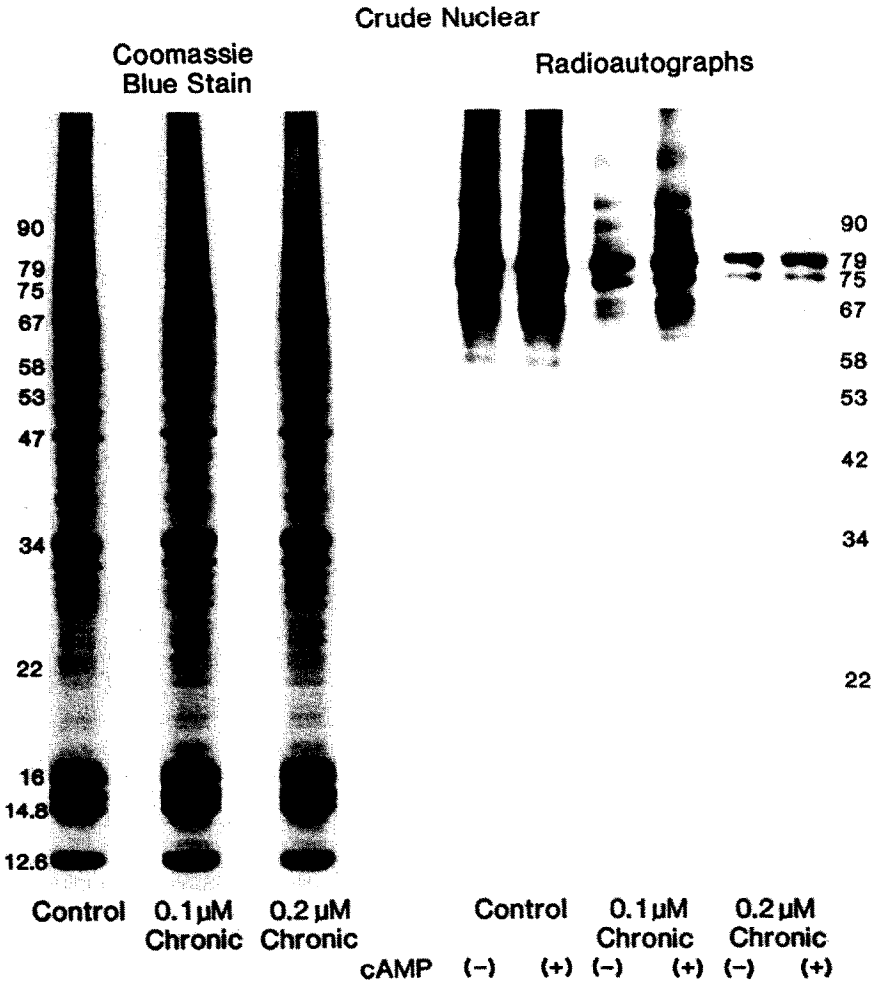

Fig. 9. Polyacrylamide gel electrophoresis of the crude nuclear fraction of neuroblastoma cells. See the legend of Fig. 1 for additional information.

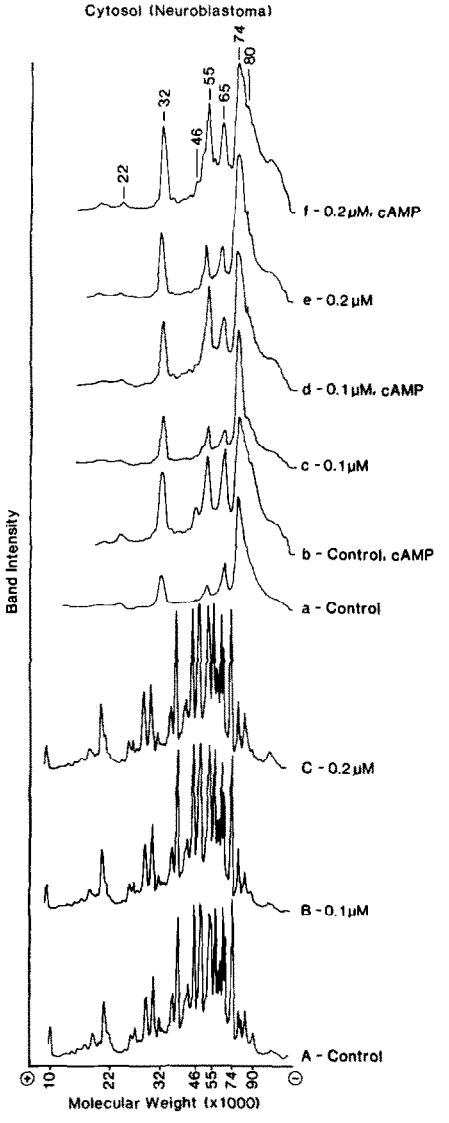

Fig. 10. Densitometeric tracings of Fig. 7. See the legend of Fig. 4 for additional information.

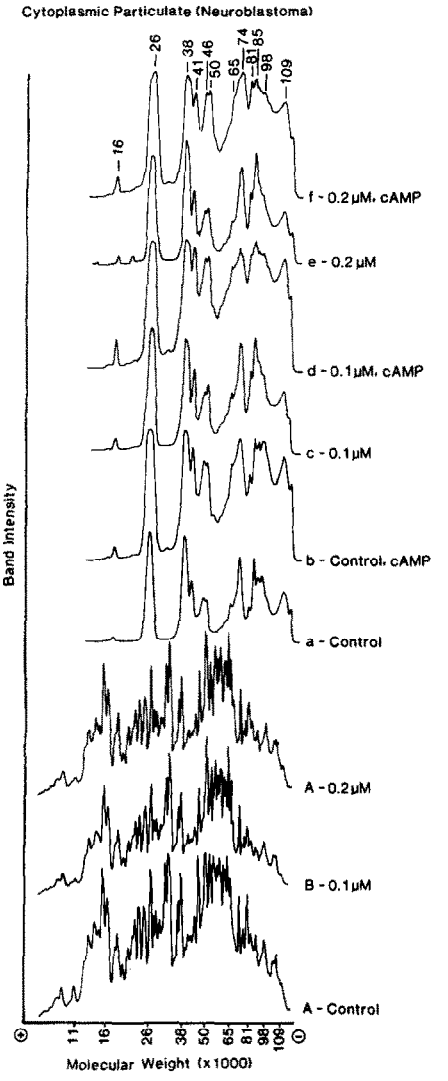

Fig. 11. Densitometeric tracings of Fig. 8. See the legend of Fig. 4 for additional information.

The direction of changes in the phosphorylation levels of crude nuclear proteins was different in both cell types. For example, the proteins of the crude nuclear fraction of chronically treated glioma cells showed increases and decreases in the levels of phosphorylation (Table 3), whereas in chronically treated NB cells, they showed only decreases in the phosphorylation levels (Table 4).

The phosphorylation levels of acutely treated glioma cells were different from those of chronically treated glioma cells. For example, the phosphorylation levels of several proteins were increased in the cytosol of acutely treated cells (Table 1), whereas the phosphorylation of these proteins in the cytosol of chronically treated glioma cells either showed no significant change or showed increases and decreases. The phosphorylation levels of several proteins remained unchanged in the acutely treated glioma cells, whereas they decreased in the chronically treated glioma cells (Table 2). The phosphorylation levels of crude nuclear proteins either remained unchanged or increased in the acutely treated glioma cells, but the phosphorylation levels of these proteins remained unchanged, increased or decreased in the chronically treated cells (Table 3). The changes in phosphorylation levels of acutely treated NB cells have not been studied.

DISCUSSION

The present study shows that the morphology, doubling time and cyclic AMP-induced morphological differentiation do not change in glioma or NB cells chronically treated with CH_3HgCl. However, the relative amounts of specific proteins change

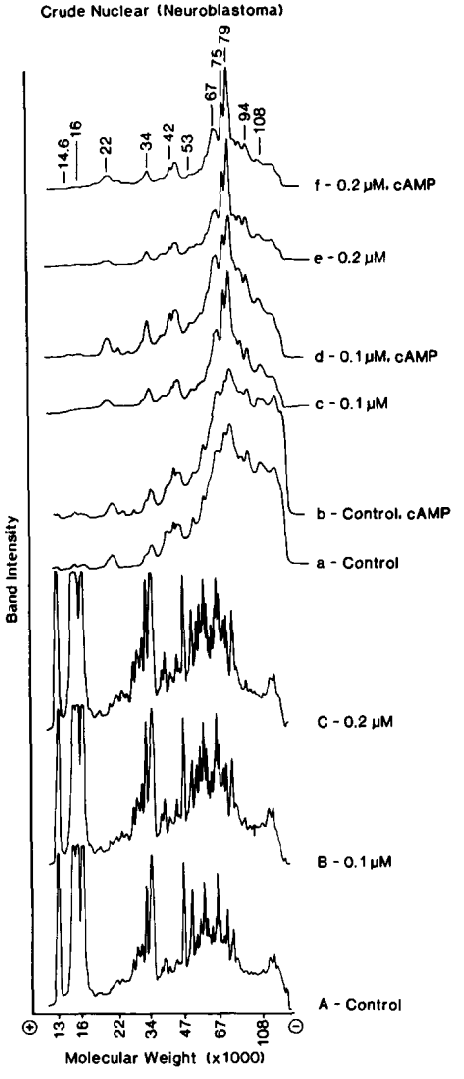

Fig. 12. Densitometeric tracings of Fig. 9. See the legend of Fig. 4 for additional information.

Table 4. Net phosphorylation profile of certain proteins of neuroblastoma cells chronically treated with CH_3HgCl*

Particulate				Crude nuclear		
Approximate mol. wt (×1000)	Relative amounts of phosphorylation (0.1 μM)	(0.2 μM)	Approximate mol. wt (×1000)	Relative amounts of phosphorylation (0.1 μM) (0.2 μM)	Approximate mol. wt (×1000)	Relative amounts of phosphorylation (0.1 μM)	(0.2 μM)
80†	+	+ +	85†	+	90, 94, 108	–	– –
67†	–	–	81†	+	67†, 79†	–	– –
55†	+	+ +	74†	+	42†	–	– –
32†	+	+ +	38†	+			
			25†	+			

* Changes in the relative amounts of phosphorylation levels of specific proteins in the chronically treated cells are presented as increased (+) or decreased (–) with respect to the corresponding proteins in the untreated cells. The observed change in the level of phosphorylation of a protein species represents a steady state situation and is the sum of phosphorylation and dephosphorylation reactions.

† Refers to cyclic AMP-dependent phosphorylation.

markedly (increase and decrease) in the chronically treated glioma cells, but not in the chronically treated NB cells. The reason for this difference is unknown. Since glioma cells are more sensitive to CH_3HgCl than are NB cells by the criterion of growth inhibition [7], it is possible that changes in the relative amounts of proteins in NB cells may be observed at concentrations higher than those used in this study. The exact reasons for the higher sensitivity of glioma cells to CH_3HgCl are unknown; however, it has been shown [13] that CH_3HgCl accumulates in the cytosol and lipoprotein fractions of glioma cells in greater proportions than in the corresponding fractions of NB cells which may, in part, account for the above phenomenon.

The levels of phosphorylation of specific proteins appear to be very sensitive to CH_3HgCl in both glioma and NB cells. However, the direction of changes in phosphorylation levels of proteins of the cytoplasmic particulate was different in these cell types. For example, the phosphorylation levels of particulate proteins decrease in chronically treated glioma, whereas they increase in chronically treated NB cells. The reasons for the high sensitivity of the phosphorylation mechanism to CH_3HgCl are unknown. The biological significance of these changes is unknown. However, it is interesting to note that the morphology, doubling time and the effect of cyclic AMP-stimulating agents on morphological differentiation remained unchanged in the chronically treated glioma cells and NB cells.

As stated earlier [9], the changes of gene expression in chronically treated cells have been studied using cells of the confluent phase of growth. If similar alterations are observed in chronically treated cells in the exponential phase of growth, it is possible that cells require very little phosphorylation activity for maintaining the growth rate, morphology and certain biological responses. The mechanism of CH_3HgCl-induced alterations in gene expression is unknown. However, it should be pointed out that 2–3 per cent of added radioactive CH_3HgCl binds to the chromatin fraction [13], and this amount may be enough to change the gene expression. The alterations in gene expression in chronically treated cells may also be due, in part, to the effect of CH_3HgCl at the translational level [9]. In fact, treatment of rats with methylmercuric chloride has been reported to cause disorganization of the ribosomal structures in spinal ganglion neurons [17].

Our results show that cyclic AMP-dependent and -independent phosphorylations of specific proteins are very sensitive to CH_3HgCl in both glioma and NB cells. Therefore, this biological system can be used to evaluate the effects of other environmental pollutants which are known to possess, or have potential to exhibit, neurotoxic effects. It is now well established that the phosphorylation of neuronal proteins is an important biological event associated with a variety of neuronal functions. If CH_3HgCl produces a similar change in the phosphorylation levels of neuronal proteins of the central nervous system *in vivo*, it is possible that changes in cyclic AMP-dependent and -independent protein phosphorylation are important biochemical lesions which could account, in part, for the expression of abnormal neurological symptoms, including alterations in behaviour.

Acknowledgements—This work was supported by NIH 1 RO1 ES NS 01576. Dr. Ramanujam is a recipient of NIH Fellowship 1F32E505137. We thank Mrs. Marianne Gaschler for her technical help.

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