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# ALTERATIONS IN GENE EXPRESSION AFTER CHRONIC TREATMENT OF GLIOMA CELLS IN CULTURE WITH METHYLMERCURIC CHLORIDE

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**Abstract**—The cellular and molecular mechanisms of methylmercuric chloride ($CH_3HgCl$)-induced damage to the nerve tissue were studied in a monolayer culture of glioma cells (C-6). Chronic treatment of glioma cells with low concentrations (0.05–0.1 $\mu$M) of $CH_3HgCl$ produced a significant alteration in gene expression, as evidenced by changes in the amounts and net phsophorylation profiles of specific proteins. However, the morphology and doubling time did not change.

It is well established that methylmercuric chloride ($CH_3HgCl$) causes a neurological disorder which is referred to as Minamata disease [1, 2]. $CH_3HgCl$ accumulates in the central nervous system in large amounts after ingestion, or after intravenous or intraperitoneal administration [3–6]. To investigate the cellular and molecular mechansims of $CH_3HgCl$-induced damage to nerve tissue, we have used monolayer cultures of glioma and neuroblastoma (NB) cells as an experimental model. Our previous studies have shown that: (a) glioma cells are more sensitive than NB cells to $CH_3HgCl$ [7], and (b) alteration in cyclic AMP metabolism may be one of the biochemical lesions produced by $CH_3HgCl$ in glioma and NB cells [8]. We now report that marked alterations in the amounts, and net phosphorylation profiles, of specific proteins are observed in glioma cells treated chronically with low concentrations of $CH_3HgCl$.

## METHODS

Rat glioma cells [9] (clone C-6), of passages 30–42 [10], were used in this study. The cells were grown in F12 medium containing 10% fetal calf serum, and 100 units/ml penicillin and 100 $\mu$g/ml streptomycin. Cells were maintained at 37° in a humidified atmosphere of 5% $CO_2$. Confluent cells were routinely 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 for 40 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 trypsin. Cells were centrifuged at 700 rev/min for 7 min, pellets were resuspended, and an aliquot was taken into a new flask. For chronic treatment, the cells were grown in the presence of 0.1 $\mu$M (25 ng/ml) or 0.05 $\mu$M $CH_3HgCl$ for 40 days. Cells were subcultured every 6–7 days. For the experiment, cells ($2 \times 10^6$) were plated in Lux tissue culture dishes (100 mm) and $CH_3HgCl$ (0.1 $\mu$M or 0.05 $\mu$M) was added the same day. The medium and $CH_3HgCl$ were changed 2, 3 and 5 days after plating.

The cells were harvested when confluent (6 days after plating) and were washed twice with 10 vol. of phosphate-buffered saline, pH 7.0. The subcellular fractions were prepared according to the method described previously [11]. 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 were homogenized at 5000 rev/min for 3 min (5 strokes) using a Potter–Elvehjem tissue grinder. 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 and homogenized after 30 min in a Dounce tissue grinder.

*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 fluids, free of particulate materials, constituted the cytosol fractions.

*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.

The procedures for assaying endogenous protein phosphorylation have also been described previously [12].

*Preparation of samples for phosphorylation.* Protein determinations were made on the crude nuclear, cyto-

plasmic and particulate fractions by the method of Lowry *et al.* [13]. Aliquots containing 0.6 mg of protein were transferred to 1.0 × 7.5 cm plastic tubes, diluted to 0.2 ml with the Tris–HCl buffer, and lyophilized.

*Phosphorylation reaction.* The phosphorylation reaction was carried out as outlined by Ehrlich *et al.* [14], 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°. Cyclic AMP-independent (−) phosphorylation was initiated by the addition of 0.04 ml of 18 $\mu$M [$\gamma$-$^{32}$P]ATP (specific radioactivity: 4.5 × $10^7$ dis./min nmole of ATP). The phosphorylating reagent was prepared by diluting [$\gamma$-$^{32}$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 $\mu$M [$\gamma$-$^{32}$P]ATP and 22.5 $\mu$M cAMP. The samples were incubated for an additional 10 min at 30° and the reaction was stopped by the addition of 0.6 ml of 12% sodium dodecylisulfate (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.

*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 phosphorylation reflect the net phosphorylation profile which is the result of protein kinases and phosphoprotein phosphatase activities, and does not refer to the specific enzyme.

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. The doubling time was determined on the exponential portion of the curve. Three separate determinations were made for each treatment.

## RESULTS AND DISCUSSION

The morphology and the doubling time (24–35 hr) of chronically treated glioma cells (0.05 and 0.1 $\mu$M) 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 neither cause cytotoxicity nor affect the growth rate.

There were dramatic increases and decreases in the intensities of staining of specific proteins obtained from the cytosol (Fig. 1), particulate (Fig. 2), and crude nuclear fractions (Fig. 3) of glioma cells treated chronically (40 days) with 0.1 $\mu$M (25 ng/ml) $CH_3HgCl$. A similar observation was made in cells treated with a lower concentration (0.05 $\mu$M) of $CH_3HgCl$, although the effects were relatively less (data not shown). The changes in relative amounts of proteins have been summarized in Table 1. In the particulate fraction of chronically treated cells, the protein band of mol. wt. 38,000 was not detectable, and the intensities of other protein bands were slightly increased or showed no significant changes (Fig. 2). In the cytosol fraction of chronically treated cells, the protein bands of mol. wt 85,000 and 69,000, which were not detectable in control cells, became prominent; the intensities of other bands showed an increase, a decrease or no change (Fig. 1). It was interesting to note that a protein band of mol. wt 14,000 in the core histone region was missing, and another band of mol. wt 13,200 was reduced in chronically treated cells (Fig. 3). This was not observed in acutely (5 days treatment) treated glioma cells (data not shown). We are not certain about the nature of these bands in the crude nuclear fraction; therefore, their significance cannot be evaluated at this time.

The phosphorylation levels of proteins of untreated cells were highest in the particulate (Fig. 2) and lowest in the cytosol fraction (Fig. 1). Most of the proteins in the particulate showed cyclic AMP-dependent phosphorylation, whereas in the crude nuclear fraction and cytosol, the phosphorylation of most proteins was cyclic AMP-independent. It should be pointed out that confluent cells were used in all experiments, and this, in part, may account for low phosphorylation levels in the cytosol. The levels of phosphorylation of some bands in the cytosol which were not detectable in control cells, became detectable in chronically treated cells. The phosphorylation levels of other bands appeared to increase or decrease in chronically treated glioma cells. The changes in the relative amounts of phosphorylation have been summarized in Table 2. The phosphorylation levels of the particulate fraction (mostly cyclic AMP-dependent) of several proteins markedly decreased in chronically treated cells. In the crude nuclear fraction (Fig. 3) of chronically treated cells, phosphorylation of protein bands of mol. wt. 49,000 (cyclic AMP-independent) and 37,000 (cyclic AMP-dependent), which were missing in control cells, became detectable. The phosphorylation level of another band of mol. wt 22,000 (cyclic AMP-independent), which was prominent in control cells, was markedly reduced in chronically treated cells. The phosphorylation level of a band of mol. wt 13,200 (cyclic AMP-dependent) in the core histone region was reduced in chronically treated glioma cells.

These results are of particular interest because the chronic treatment of glioma cells with as little as 12.5 ng/ml of $CH_3HgCl$ produces dramatic alterations in gene expression, as evidenced by changes in the amounts and levels of phosphorylation of specific proteins. The biological significance of these changes is unknown. However, it is interesting to note that the morphology and doubling time of glioma cells do not change after chronic treatment.

It should be emphasized that the alterations of gene expression in chronically treated glioma cells have been studied using cells in the confluent phase of growth. If similar changes are observed in chronically treated glioma cells obtained from the exponential phase of growth, one can suggest that cells need very little

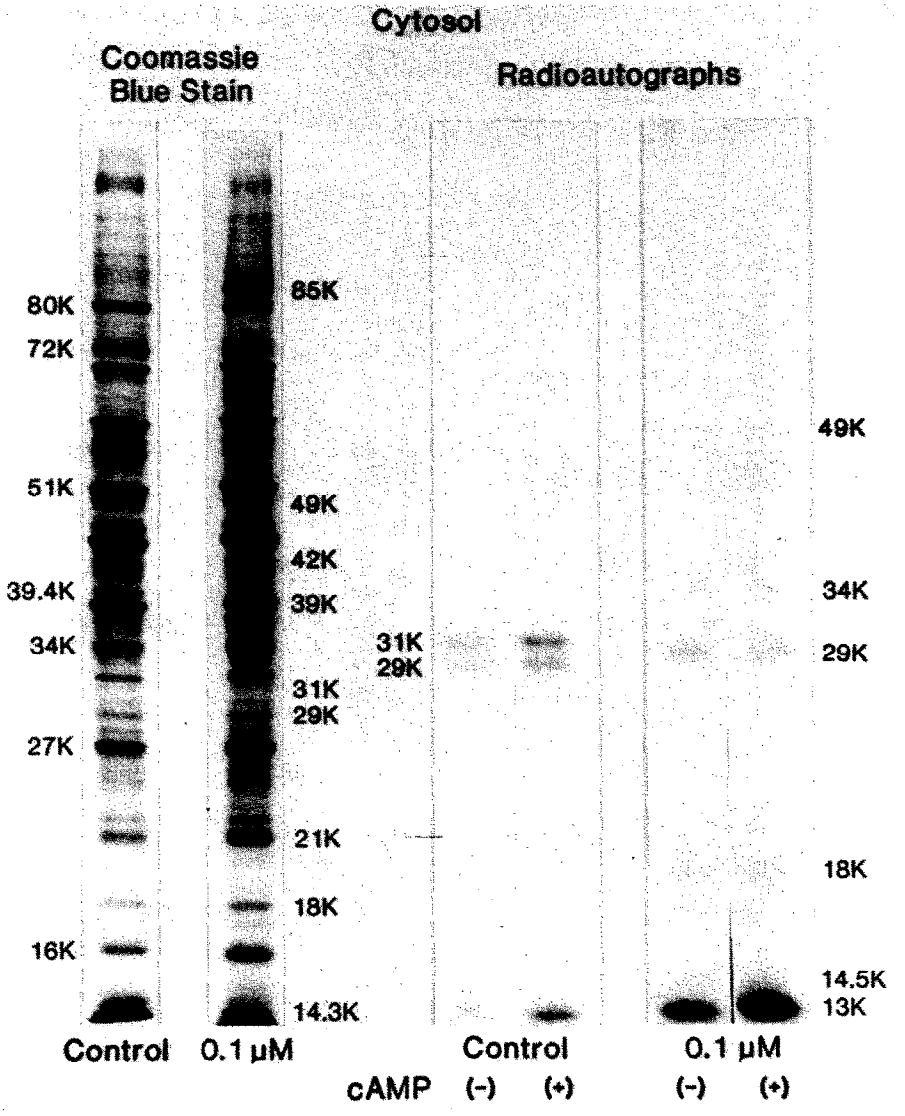


Fig. 1. Polyacrylamide cell electrophoresis of the cytosol fraction. The magnification of the gloss prints of the gels and their radio autographs (Figs. 1–3) are not identical. The approximate molecular weights of proteins are expressed as multiples of 1000 (K). The observed change in phosphorylation of a protein species represents a steady state situation and is the sum of phosphorylation and dephosphorylation reactions.

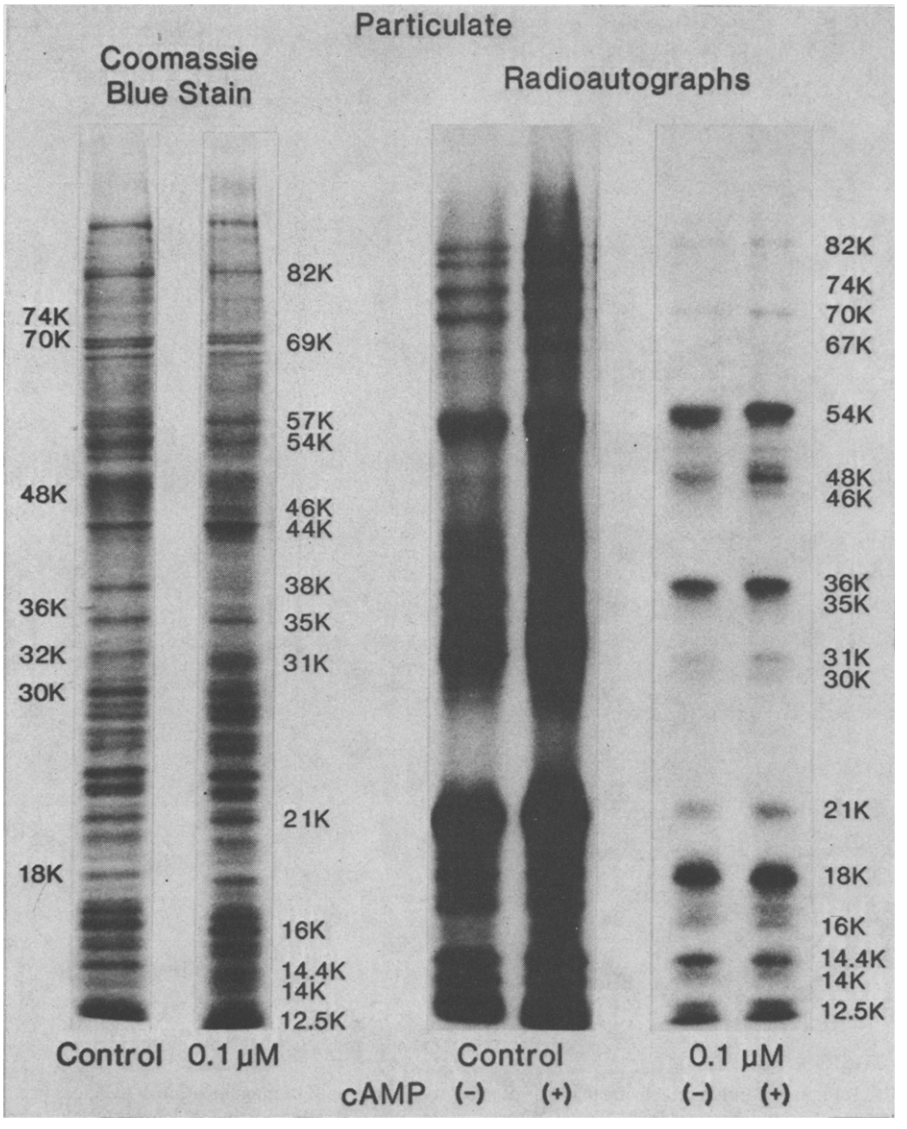


Fig. 2. Polyacrylamide cell electrophoresis of the particulate fraction. See the legend of Fig. 1 for conditions.

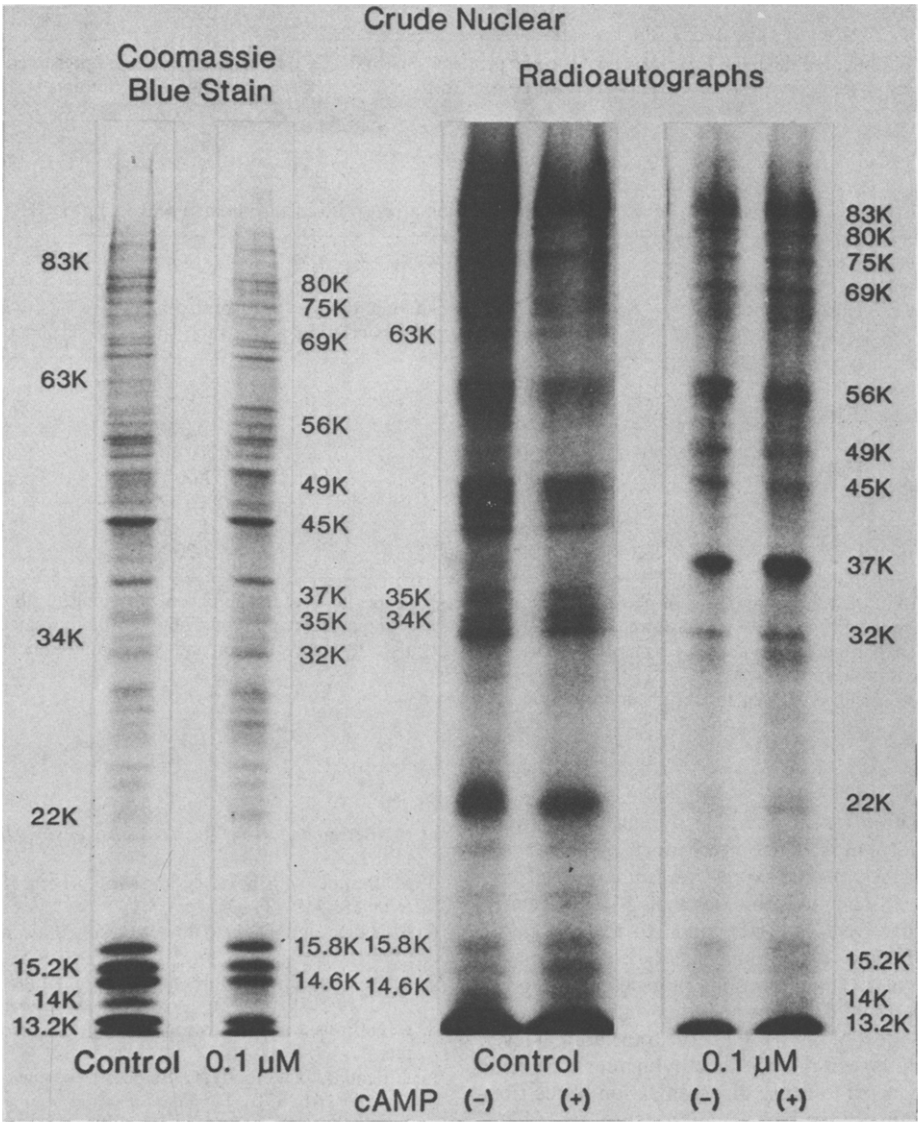


Fig. 3. Polyacrylamide cell electrophoresis of the crude nuclear fraction. See the legend of Fig. 1 for conditions.

Table 1. Alterations in relative amounts of certain protein in glioma cells chronically treated with 0.1 μM $CH_3HgCl$*

| Cytosol fraction | | Particulate fraction | | Crude nuclear fraction | |
|---|---|---|---|---|---|
| Approximate mol. wt | Relative amounts of proteins | Approximate mol. wt | Relative amounts of proteins | Approximate mol. wt | Relative amounts of proteins |
| 85,000 | + | 58,000 | – | 59,000 | + |
| 74,000 | + | 46,000 | + | 32,000 | + |
| 69,000 | + | 43,000 | + | 18,000 | – |
| 39,400 | – | 38,000 | – | 14,000 | – |

* Changes in the relative quantities of specific proteins in chronically treated glioma cells are presented as having increased (+) or decreased (–), with respect to the corresponding proteins in the untreated cells.

Table 2. Phosphorylation profile of certain proteins of glioma cells chronically treated with 0.1 μM $CH_3HgCl$*

| Cytosol fraction | | Particulate fraction | | Crude nuclear fraction | |
|---|---|---|---|---|---|
| Approximate mol. wt | Relative amounts of phohphorylation | Approximate mol. wt | Relative amounts of phosphorylation | Approximate mol. wt | Relative amounts of phosphorylation |
| 49,000 | + | 67,000–82,000† | – | 69,000 | + |
| 45,000 | + | 54,000 | – | 63,000 | – |
| 34,000 | + | 48,000† | – | 49,000 | + |
| 31,000† | – | 46,000–36,000† | – | 45,000–40,000 | – |
| 29,000 | + | 31,000–35,000† | – | 37,000† | + |
| 18,000 | + | 18,000† | – | 35,000–32,000 | – |
| | | 16,000† | – | 22,000 | – |
| | | 12,500–14,400† | – | 13,200† | – |

* Changes in the relative amounts of phosphorylation activities of specific proteins in chronically treated glioma cells are presented as having increased (+) or decreased (–), with respect to the corresponding proteins in the untreated cells. The observed change in 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.

phosphorylation for maintaining the constant growth rate and morphology. The mechanism of $CH_3HgCl$-induced changes in gene expression is unknown. However, it is pertinent to point out that 2–3 per cent of added radioactive $CH_3HgCl$ binds to the chromatin fraction [15], and this amount may be enough to alter gene expression. The alterations in gene expression in chronically treated glioma cells may also be due, in part, to the effect of $CH_3HgCl$ at the translational level. In fact, treatment of rats with methylmercuric chloride has been reported to cause disorganization of the ribosomal structures in spinal ganglion neurons [16].

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## REFERENCES

1. T. Takeuchi, in *Environmental Mercury Contamination* (Eds. R. Hartung and B. D. Dinman), p. 247. Ann Arbor Science Publishers, Ann Arbor, MI (1972).
2. H. Rustam and T. Hamdi, *Brain* **97**, 500 (1974).
3. B. Aberg, L. Ekman, R. Falk, U. Greitz, G. Persson and J. Snihs, *Archs envir. Hlth* **19**, 478 (1969).
4. M. Berlin and S. Ullberg, *Archs. envir. Hlth* **6**, 489 (1963).
5. M. Berlin, J. Fazackerley and G. Norberg, *Archs envir. Hlth* **18**, 719 (1969).
6. B. Vallee and D. D. Ulner, *A. Rev. Biochem.* **41**, 91 (1972).
7. K. N. Prasad, E. Nobles and M. Ramanujam, *Environ. Res.*, **19**, 189 (1979).
8. K. N. Prasad, E. Nobles and K. Spuhler, *Environ. Res.*, in press.
9. P. Benda, J. Lightbody, G. Sato, L. Levine and W. Sweet, *Science* **161**, 370 (1968).
10. J. Devellis, D. Inglish and F. Galey, in *Cellular Aspects of Growth and Differentiation in Nervous Tissue* (Ed. D. Pease), p. 23. University of California Press, Los Angeles (1970).
11. K. N. Prasad, P. K. Sinha, S. K. Sahu and J. L. Brown, *Cancer Res.* **36**, 2290 (1976).
12. A. Routtenberg and Y. H. Ehrlich, *Brain Res.* **92**, 415 (1975).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. Y. H. Ehrlich, E. G. Brunngraber, P. K. Sinha and K. N. Prasad, *Nature Lond.* **265**, 238 (1977).
15. M. Ramanujam, S. C. Bondy and K. N. Prasad, *Toxic. Lett.* **3**, 265 (1979).
16. N. Carmichael and J. B. Cavanagh, *Acta Neuropath.* **34**, 137 (1976).