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REGULATION OF GTP CYCLOHYDROLASE I AND DIHYDROPTERIDINE REDUCTASE IN RAT PHEOCHROMOCYTOMA PC 12 CELLS

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The addition of 8-bromo cyclic AMP, forskolin, theophylline, and 3-isobutyl-1-methylxanthine to the medium of PC 12 cells resulted in an increase in GTP cyclohydrolase I activity, but had no effect on dihydropteridine reductase activity, except theophylline which caused a decrease in dihydropteridine reductase activity at 96 h. GTP cyclohydrolase I activity peaked at 24 h and returned to normal 96 h after drug treatment. Cycloheximide decreased GTP cyclohydrolase I activity at 48 and 96 h, but had little effect on dihydropteridine reductase activity. The addition of reserpine selectively increased only GTP cyclohydrolase I activity. The addition of tetrahydrobiopterin and sepiapterin, however, coordinately inhibited both GTP cyclohydrosase I and dihydropteridine reductase activities. It appears that GTP cyclohydrolase I activity in PC 12 cells is regulated by cyclic AMP stimulation and by end-product inhibition, whereas dihydropteridine reductase activity is only subject to pterin inhibition.

KEY WORDS: GTP cyclohydrolase, dihydropteridine reductase, pheochromocytoma, PC 12, cyclic AMP, tetrahydrobiopterin.

INTRODUCTION

L-*Erythro*-5,6,7,8-tetrahydrobiopterin (BH_4) is the obligatory cofactor for tyrosine hydroxylase (EC 1.14.16.2) during dopamine and norepinephrine synthesis[1,2], and for tryptophan hydroxylase (EC 1.14.16.4) during serotonin synthesis[3]. The essential role of BH_4 in the hydroxylation of aromatic L-amino acids, including the hydroxylation of L-phenylalanine by phenylalanine hydroxylase (EC 1.14.16.1), has been well established[4]. BH_4 is synthesized from GTP by an, as yet, incompletely defined pathway initiated by GTP cyclohydrolase I (EC 3.5.4.16) (GTP 7,8–8,9–dihydrolase)[5]. When BH_4 is oxidized by aromatic amino acid hydroxylases to the inactive dihydro-form, it is spontaneously regenerated by dihydropteridine reductase (EC 1.6.99.10) (NADH: 6,7-dihydropteridine oxidoreductase)[6] (Figure 1). Thus, the biosysnthesis of intracellular BH_4 is governed by two enzymes: GTP cyclohydrolase I (GTP-CH), which regulates its rate of formation, and dihydropteridine reductase (DHPR), which regulates its rate of regeneration. The normal functioning of these two enzymes maintains a catalytic pool of BH_4 sufficient to regulate the activities of aromatic amino acid hydroxylases. Both enzymes appear to be essential, however, because a defect in either can impair neurotransmitter biosynthesis[7–10].

The regulation of GTP-CH in mammals has only begun to be known. GTP-CH activity in rat adrenal medulla and cortex was induced by a pituitary-control mechan-

Abbreviations used: BH_4, L-*erythro*-5,6,7,8-tetrahydrobiopterin; GTP-CH, GTP cyclohydrolase I; DHPR, dihydropteridine reductase

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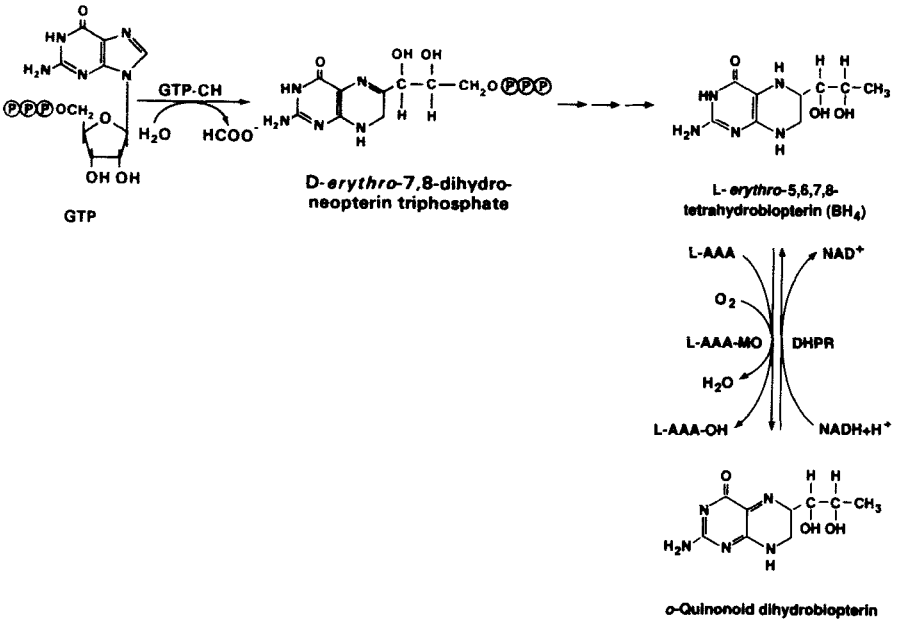

FIGURE 1 Biosynthesis, regeneration, and utilization of tetrahydrobiopterin. BH_4 is the obligatory cofactor for aromatic L-amino acid monooxygenases (L-AAA-MO). It is synthesized from GTP via intermediates of D-*erythro*-7, 8-dihydroneopterin triphosphate, 6-pyruvoyl-tetrahydropierin, and 5,6-dihydrosepiapterin. GTP cyclohydrolase I (GTP-CH) catalyzes the initial step of this biosynthetic pathway. When BH_4 is oxidized by L-AAA-MO to the inactive quinonoid dihydrobiopterin, it is spontaneously regenerated by dihydropteridine reductase (DHPR) using NADH as a cofactor. Therefore, the biosynthesis of intracellular BH_4 is governed by two enzymes: GTP-CH, which regulates its rate of formation, and DHPR, which regulates its rate of regeneration.

ism[11,12]. Using cell cultures, this hormone-dependent mechanism was further observed to be both cyclic AMP-dependent and cyclic AMP-independent[13,14]. The pituitary regulation of GTP-CH activity was also observed in the liver, spleen, bone marrow, but not the brain of rodents[15,16]. However, another pituitary peptide rather than ACTH might be responsible for the regulation of GTP-CH activity in these tissues. Moreover, brain GTP-CH activity may be independent of endocrine regulation.

Studies of GTP-CH activity in human peripheral blood mononuclear cells, on the other hand, found that phytohemagglutinin stimulated the activity of this enzyme[17,18]. Using pure macrophages and T lymphocytes in culture, Schoedon *et al.*[19] reported that γ-interferon induced GTP-CH activity in these cells, and that this increase in enzyme activity was suppressed by exogenous BH_4.

Still another aspect of the regulation of GTP-CH activity is derived from *in vitro* incubation studies. Using rat liver GTP-CH preparation, Bellahsene *et al.*[20] and Shen *et al.*[21] found that the activity of the enzyme was inhibited by pteridines. It appears, therefore, that GTP-CH activity in mammalian tissues may be subjected to hormonal regulation, end-product inhibition, lymphokine stimulation, or none of these, such as is the case in brain.

Similarly, regulatory control of DHPR activity is only partially known. *In vitro* inhibition studies found that DHPR activity was inhibited by folate analogues[22,23]. *In*

vivo studies reported that the activity of this enzyme in rat brain was not altered by nerve growth factor[24] or reserpine and 6-hydroxydopamine[25]. Purdy and Blair[26] and Shen *et al.*[27] found that catecholamines and other catechol-containing compounds were potential inhibitors of this enzyme. However, the specific activity of DHPR in mammalian tissues is far greater than that of BH_4-dependent hydroxylases[23]. The appreciable inhibition of this enzyme may not jeopardize catecholamine biosynthesis.

Rat pheochromocytoma PC 12 cells are known to contain high levels of tyrosine hydroxylase, DHPR, and GTP-CH[28-30]. This cell line was used to study first, if the activity of GTP-CH could be inhibited by pterins and activated by cyclic AMP, and second, if the activities of GTP-CH and DHPR were coordinately regulated.

MATERIALS AND METHODS

Materials

BH_4 and L-sepiapterin were purchased from Dr. B. Schirks Labs. (Jona, Switzerland). GTP, alkaline phosphatase (type VII-S from bovine intestinal mucosal), 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine, reserpine, 8-bromo cyclic AMP (Na salt), forskolin, theophylline, 3-isobutyl-1-methylxanthine, cycloheximide and RPMI 1640 medium were purchased from Sigma (St. Louis, MO). Fetal calf serum and heat-inactivated horse serum were obtained from GIBCO (Grand Island, NY).

PC 12 Culture

PC 12 Cells were grown in Falcon flasks (75 cm^2) in RPMI 1640 medium supplemented with 5% fetal calf serum and 5% heat-inactivated horse serum. The cells were maintained at 36.5°C in a humidified atmosphere of 5% CO_2 in air. Cells were plated in Falcon flasks (25 cm^2) in 10 ml medium at an initial cell density of 4×10^6 cells/flask. After incubating cells for 48 h, one-half of the medium was removed and replaced with 5 ml fresh medium containing the test compound. The cells were fed once, 48 h later, by replacing with fresh medium. The cells were harvested 0 to 96 h after drug additions by centrifugation ($86 \times g$, 5 min) and were subjected to two cycles of washing and centrifugation, with 1 ml phosphate-buffered saline each time. The cells were suspended in 0.2 ml 0.01 M sodium phosphate buffer (pH 7.7), containing 1 mM 2-mercaptoethanol, 2.6 mM EDTA, and 5% glycerol (phosphate buffer), and were frozen at −80°C. Cells were thawed at room temperature and the suspension was adjusted to contain 1% Triton X-100. The freezing-and-thawing procedure was repeated two more times, and then the cellular mixture was centrifuged to obtain the crude extract.

Enzyme and Protein Assays

Crude extracts were dialyzed against phosphate buffer with a microdialyzer (Health Products, Inc., Rockford, IL) to remove endogenous inhibitors. GTP-CH activity was assayed fluorometrically by the HPLC method of Blau and Niederwieser[31] as previously described[21]. DHPR activity was assayed spectrophotometrically by measuring the rate of disappearance of NADH according to the method of Nielsen *et al.*[32] as previously described[33]. Protein was determined by the method of Lowry *et al.*[34].

RESULTS

Time-course effects of chemicals that increase cyclic AMP levels on GTP-CH activity

Treatment of PC 12 cells with 1 mM 8-bromo cyclic AMP increased GTP-CH activity (Figure 2). Maximal increase of 1.5-fold ($p < 0.005$) was observed 24 h after the addition of the chemical. GTP-CH activity returned to the normal range 96 h after initiation of treatment. Treatment of PC 12 cells with 50 μM forskolin, an adenylate cyclase activator, also increased GTP-CH activity. The pattern and the degree of increase in GTP-CH activity were essentially the same as those observed for 8-bromo cyclic AMP. However, treatment of PC 12 cells with 0.1 mM theophylline, a cyclic AMP phosphodiesterase inhibitor, did not increase GTP-CH activity for 48 h. Elevation of GTP-CH activity ($p < 0.05$) was delayed until 96 h after the addition of theophylline. The addition of cycloheximide (5 μg/ml) greatly decreased GTP-CH activity at 48 h ($p < 0.05$) and 96 h ($p < 0.005$). However, cycloheximide at 2 μg/ml

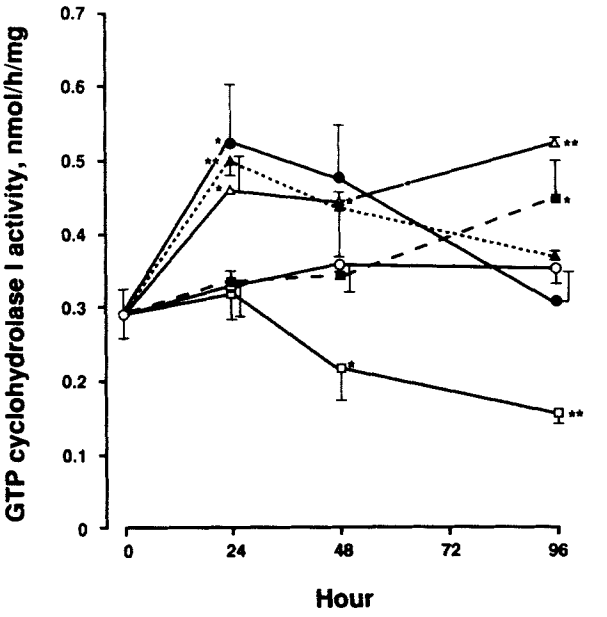

FIGURE 2 Time course effects of chemicals that increase cyclic AMP levels on GTP-CH activity. PC 12 cells were plated in Falcon flasks at a density of 4×10^5 cells/ml of RPMI 1640 medium supplemented with 5% fetal calf serum and 5% heat-inactivated horse serum. After the initial incubation of 48 h, one-half of the medium was replaced with fresh medium containing the test compound. The cells were fed once 48 h later by replacing with fresh medium, and were harvested 24, 48, and 96 h after drug treatment. Crude extracts were prepared, and endogenous inhibitors were removed by dialysis before assaying GTP-CH activity. The agents and their concentrations tested were 8-bromo cyclic AMP, 1 mM (▲. . . ..▲); forskolin, 50 μM (●—●); theophylline, 0.1 mM (■- - - -■); cycloheximide, 5 μg/ml (□—□); 8-bromo cyclic AMP (1 mM) and cycloheximide (2 μg/ml) (△—△); and controls (○—○). Each point represents mean ± SD of 3 determinations. Statistical analysis were performed by the two-tailed student t-test: **$p < 0.005$ and *$p < 0.05$, both versus controls.

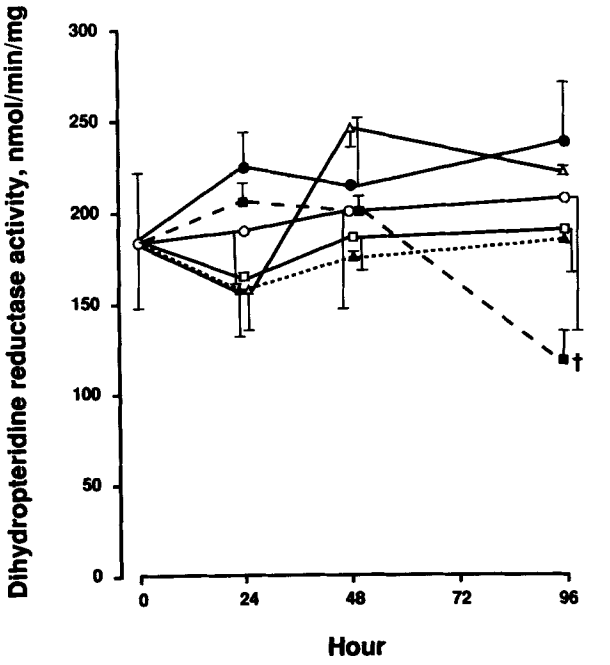

FIGURE 3 Time-course effects of chemicals that increase cyclic AMP levels on DHPR activity. See Figure 2 for cell culturing and chemicals tested.

did not block the 8-bromo cyclic AMP-mediated elevation of GTP-CH activity, which remained elevated for 4 days ($p < 0.05$).

Time-course effects of chemicals that increase cyclic AMP levels on DHPR activity

Results presented in Figure 3 show that DHPR activity in PC 12 cells were unaffected by treatment with chemicals that increase intracellular cyclic AMP levels. DHPR activity decreased, however, at 96 h after the addition of theophylline ($p < 0.1$).

TABLE 1
Activities of GTP cyclohydrolase I and dihydropteridine reductase in PC 12 cells 72 h after treatment with pterins, 3-isobutyl-1-methylxanthine, and reserpine.

Treatment	Concentration (mM)	GTP Cyclohydrolase I (% Control)	Dihydropteridine reductase (% Control)
L-Tetrahydrobiopterin	0.5	86 ± 5	80 ± 9[a]
L-Sepiapterin	0.1	87 ± 4	80 ± 15[a]
3-Isobutyl-1-methylxanthine	1	112 ± 5	101 ± 8
Reserpine	1	114 ± 4	105 ± 7

Enzyme activities in controls were GTP cyclohydrolase I, 0.29 ± 0.11 nmol/h/mg protein, and dihydropteridine reductase, 185 ± 37 nmol/min/mg protein. Values shown are means ± SD (n = 3).
[a]$p < 0.05$ versus control.

Effect of pterins, 3-isobutyl-1-methylxanthine, and reserpine on GTP-CH and DHPR activities

PC 12 cells were treated with two reduced pterins, BH_4 and sepiapterin, one cyclic AMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, and one catecholamine depleting agent, reserpine. GTP-CH and DHPR activities were measured 72 h after treatment. Results presented in Table 1 indicate that BH_4 and sepiapterin decreased the activities of both enzymes. However, only DHPR activity was significantly ($p < 0.05$) reduced by pterins. 3-Isobytyl-1-methylxanthine and reserpine slightly increased the activity of GTP-CH, but had no effect on the activity of DHPR.

DISCUSSION

Rat pheochromocytoma PC 12 cells are enriched in tyrosine hydroxylase, aromatic L-amino acid decarboxylase, dopamine β-hydroxylase, DHPR, and GTP-CH[28–30]. These cells have been used as a model system to study the regulation of tyrosine hydroxylation and catecholamine metabolism[35]. We report here that the specific activity of GTP-CH in PC 12 cells is 0.292 nmol neopterin/h/mg protein. This value is higher than those found in rat liver and brain (0.028 and 0.01 nmole neopterin/h/mg protein, respectively), and is several fold greater than those found in major mammalian tissues[36]. PC 12 cells may be useful to study the regulatory control of the activity of GTP-CH.

DHPR participates in the hydroxylation of aromatic L-amino acids by converting quinonoid dihydrobiopterin back to BH_4[6] (Figure 1). This enzyme, thus, maintains the biopterin cofactor in the active form. It has been estimated that the specific activity of DHPR in mammalian tissues is 5,000-times or more greater than that of tyrosine hydroxylase and tryptophan hydroxylases[23]. The short-term regulation of DHPR activity, therefore, might have little impact on the biosynthesis of catecholamines. Since GTP-CH is the first enzyme leading to the biosynthesis of BH_4 from GTP, this enzyme controls the supply of BH_4 for aromatic amino acid hydroxylases. Studying the regulatory control of the enzyme may have a direct consequence on the availability of BH_4.

GTP-CH in mammals has not been fully characterized. The regulation of the activity of this enzyme remains largely unknown. *In vitro* incubation studies [20,21] reveal that the end-product inhibition may play an important role on the activity of this enzyme. Using cell cultures, GTP-CH activity has been found to be stimulated by ACTH via cyclic AMP-dependent and cyclic AMP-independent mechanisms[13,14]. In this study, we found an increase in GTP-CH activity after the addition of 8-bromo cyclic AMP, suggesting that GTP-CH activity in PC 12 cells may be dependent on the intracellular level of cyclic AMP. This notion was strongly supported by the finding that the same degree of increase in GTP-CH activity was achieved by the administration of forskolin, an agent that rapidly elevates cyclic AMP levels. These findings agree with those reported by Woolf *et al.*[30]. Another support for the involvement of cyclic AMP came from the addition of 3′,5′-cyclic-nucleotide phosphodiesterase inhibitors, such as theophylline and 3-isobutyl-1-methylxanthine, which caused slight increases in GTP-CH activity.

The effect of cyclic AMP on GTP-CH activity was observed as early as 24 h after its administration. Moreover, the induction of GTP-CH activity was specific and

long-lasting. Cyclic AMP-mediated cellular activities, such as protein phosphorylation and protein synthesis, might be responsible for the increase in GTP-CH activity in PC 12 cells. The protein synthesis inhibitor, cycloheximide, decreased GTP-CH activity after 48 h ($p < 0.05$), and by 96 h the enzyme activity was still decreasing ($p < 0.005$). This result suggests that 5 μg/ml of cycloheximide was inhibitory of GTP-CH protein synthesis in PC 12 cells. However, the simultaneous addition of 2 μg/ml of cycloheximide and 1 mM 8-bromo cyclic AMP reduced by only 13% the increase in GTP-CH activity caused by 8-bromo cyclic AMP alone at 24 h. Surprisingly, GTP-CH activity at 96 h was increased 48%. This late surge of GTP-CH activity was also seen after theophylline treatment. It is not known if cycloheximide at 2 μg/ml was only partially inhibitory of GTP-CH protein synthesis. The failure of cycloheximide at 2 μg/ml to completely block the 8-bromo cyclic AMP-mediated induction of GTP-CH activity suggests that a mechanism, other than enzyme induction, may be involved in the increase in the activity of this enzyme.

The activity of DHPR was not affected by the addition of 8-bromo cyclic AMP; adenylate cyclase activators, such as forskolin; or 3′,5′-cyclic-nucleotide phosphodiesterase inhibitors, such as theophylline and 3-isobutyl-1-methylxanthine. These results would suggest that DHPR activity is not dependent on intracellular cyclic AMP levels. However, theophylline appears to decrease the activity of this enzyme at 96 h ($p < 0.1$), perhaps due to its resemblance to amino-chromes, which are known to inhibit DHPR activity[37].

Mammalian GTP-CH is known to be inhibited by reduced pterins[20,21], whereas DHPR is reported to be inhibited by folate analogues, such as aminopterin and methotrexate[22,23]. The decrease of the activities of both enzymes after sepiapterin and BH_4 treatment suggests that they share a pterin-mediated regulatory mechanism. Although DHPR appears to be more susceptible to the inhibition of pterins than GTP-CH, its physiological function may be hampered less than GTP-CH because the specific activity of DHPR is approximately 38,000 times that of GTP-CH in PC 12 cells.

Reserpine was found to increase tyrosine hydroxylase and GTP-CH activities in adrenomedullary chromaffin cells[14]. In this study, we found that it increased GTP-CH activity in PC 12 cells, but had no effect on DHPR activity. The depletion of intracellular catecholamines by reserpine might contribute to the elevation of tyrosine hydroxylase activity, but it may not cause the increase in GTP-CH activity since catecholamines are not inhibitory to GTP-CH activity (unpublished observation). The increase in GTP-CH activity might be a coordinated response to supply more BH_4 and, hence, more tyrosine hydroxylase activity. However, tyrosine hydroxylase may be activated by phosphorylation alone without the need for raising the intracellular BH_4 level[38]. The significance of the effect of reserpine of GTP-CH activity is not known.

In conclusion, this study provides evidence that cyclic AMP regulates the activity of GTP-CH, but not DHPR in PC 12 cells. The cyclic AMP-mediated stimulation of GTP-CH activity in PC 12 cells is probably similar to that found in cultured chromaffin cells[14]. The data presented here also suggests that pterins may regulate both GTP-CH and DHPR activities, probably via an end-product inhibition mechanism. Since DHPR activity is present in greater excess than GTP-CH, the regulation of GTP-CH activity may have direct consequences on the availability of BH_4 for aromatic amino acid hydroxylation.

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References

1. T. Nagatsu, M. Levitt and S. Udenfriend, *J.Biol. Chem.*, **239,** 2910 (1964)
2. A.R. Brenneman and S. Kaufman, *Biochem. Biophys. Res. Commun.*,**17,** 177 (1964).
3. D.G. Grahame-Smith, *Biochem. Biophys. Res. Commun.*, **16,** 586 (1964).
4. S. Kaufman, *J. Biol. Chem.*, **230**, 931 (1958).
5. A.W. Burg and G.M. Brown, *J. Biol. Chem.*, **243**, 2349 (1968).
6. S. Kaufman, *J. Biol. Chem.*, **239**, 332 (1964).
7. I. Smith, B.E. Clayton and O.H. Wolff, *Lancet I,* 1108 (1975).
8. S. Kaufman, N.A. Holtzman, S. Milstien, I.J. Butler and A. Krumholz, *New Engl. J. Med.*, **293,** 785 (1975).
9. A. Neiderwieser, N. Blau, M. Wang, P. Joller, M. Atares and J. Cardesa-Garcia, *Eur. J. Pediat.*, **141,** 208 (1984).
10. J.-L. Dhondt, J.-P. Farriaux, A. Boudha, C. Largilliere, J. Ringel, M. -M. Roger and R.J Leeming, *J. Pedriatr.*, **106**, 954 (1985).
11. O.H. Viveros, C.-L. Lee, M.M Abou-Donia, J.C. Nixon and C.A. Nichol, *Science,* **213,** 349 (1981).
12. M.M. Abou-Donia, D.S. Duch, C.A. Nichol and O.H. Viveros, *Endocrinol.*, **112**, 2088 (1983).
13. D.S. Duch, J.H. Woolf, M.P. Edelstein, O.H. Viveros, M.M. Abou-Donia and C.A. Nichol, *Endocrinol.*, **118**, 1897 (1986).
14. M.M. Abou-Donia, S.P. Wilson, T.P. Zimmerman, C.A. Nichol and O.H. Viveros, *J. Neurochem.*, **46,** 1190 (1986).
15. M.M. Abou-Donia, A.J. Daniels, E. Diliberto, Jr., C.A. Nichol and O.H. Viveros, *Biochemical and Clinical Aspects of Pteridines,* Vol 4. H. Wachter, H. Ch. Curtius and W. Pfleiderer (eds.), Walter de Gruyter, New York, 1985, pp. 209–220.
16. D.S. Duch, S.W. Bowers and C.A. Nichol, *Mol. Cell. Endocrinol.*, **47**, 209 (1986).
17. P.B. Rowe, E. Tripp and G.C. Craig, *Chemistry and Biology of Pteridines,* R.L. Kisliuk and G.M. Brown (eds.), Elsevier North Holland, New York, 1979, pp. 587–592.
18. N. Blau, P. Joller, M. Atares, J. Cardesa-Garcia and A. Niederwieser, *Clin. Chim. Acta,* **148,** 47 (1985).
19. G. Schoedon, J. Troppmair, G. Adolf, C. Huber and A. Niederwieser, *J. Interferon Res.*, **6**, 697 (1986).
20. Z. Bellahsene, J.-L. Dhondt and J.-P. Farriaux, *Biochem. J.*, **217,** 59 (1984).
21. R. -s. Shen, A. Alam and Y. Zhang, *Biochim. Biophys. Acta.*, **965**, 9 (1988).
22. K.G. Scrimgeour and S. Cheema, *Ann. N.Y. Acad. Sci.*, **186**, 115 (1971).
23. J.E. Craine, E.S. Hall and S. Kaufman, *J. Biol. Chem.*, **247**, 6082 (1972).
24. B. Nikodijevic, M.W. Yu and G. Guroff, *J. Neurochem.*, **28**, 851 (1977).
25. A.J. Turner, F. Ponzio and S. Algeri, *Brain Res.*, **70**, 553 (1974).
26. S.E. Purdy and J.A. Blair, *Biochem. Soc. Trans.*, **8**, 565 (1980).
27. R. -s. Shen, R.V. Smith, P.J. Davis, A. Brubaker and C.W. Abell, *J. Biol. Chm.*, **257,** 7294 (1982).
28. L.A. Greene and A.S. Tischler, *Proc. Natl. Acad. Sci. USA.*, **73**, 2424 (1976).
29. B.T. Liang, K.K. Vaccaro, B.A. Perelle and R.L. Perlman, *J. Neurochem.*, **37**, 1164 (1981).
30. J.H. Woolf, C.A. Nichol and D.S. Duch, *Chemistry and Biology of Pteridines,* B.A. Cooper and V.M. Whitehead (eds.), Walter de Gruyter, New York, 1986, pp. 283–286.
31. N. Blau and A. Niederwieser, *Anal. Biochem.*, **128**, 446 (1983).
32. K.H. Nielson, V. Simonsen and K.E. Lind, *Eur. J. Biochem.*, **9**, 497 (1969).
33. R.-s. Shen and C.W. Abell, *J. Neurosci. Res.*, **6,** 193 (1981).
34. O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
35. L.A. Greene and A.S. Tischler, *Adv. Cell. Neurobiol.*, **3**, 373 (1982).
36. D.S. Duch, S.W. Bowers, J.H. Woolf and C.A. Nichol, *Life Sci.*, **35**, 1895 (1984).
37. W.L.F. Armarego and P. Waring, *Biochem. Biophys. Res. Commun.*, **113**, 895 (1983).
38. M.A. Lazar, A.J. Lockfeld, R.J.W. Truscott and J.D. Barchas, *J. Neurochem.*, **39**, 409 (1982).