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NEW TETRAHYDROBIOPTERIN-DEPENDENT SYSTEMS*

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CONTENTS

INTRODUCTION

In 1955–1956, two lines of research with apparently nothing in common culminated in the independent discovery of biopterin. The starting point for one of these studies was the observation that maximum growth of an obscure protozoan, *Crithidia fasiculata,* in a chemically defined medium required exceptionally high concentrations of folic acid (9). In 1955, this growth-promoting substance was isolated from 4000 liters of human urine and characterized as 2-amino-4-hydroxy-6-(1,2-dihydroxypropyl) pteridine (83, 84). These workers suggested that this unconjugated pteridine be called "biopterin." All that remained to be determined about the structure of

biopterin was its optical configuration. Chemical synthesis established that the *Crithidia* factor is 2-amino-4-hydroxy-6-[1,2-dihydroxypropyl (L-*erythro*)] pteridine (85). In addition to biopterin, the structurally related unconjugated pterin neopterin (6-trihydroxypropylpterin), a derivative of the biological precursor of tetrahydrobiopterin (BH_4), can also support the growth of *Crithidia fasiculata.*

The other line of research that led to the independent discovery of biopterin originated with structural studies of the eye color pigments in *Drosophila melanogaster.* During the course of these studies, Forrest & Mitchell isolated several pteridines including a blue fluorescent compound that they characterized as biopterin (22).

These early investigations established one role for biopterin—that of pigment—and, based on the finding that it is essential for the growth of *Crithidia fasiculata,* hinted at others. A clue to what these other roles might be, at least for this organism, was provided by the observation that certain pyrimidines such as uracil and cytosine and some unsaturated fatty acids such as a mixture of oleic and linoleic acid can spare the growth requirement of *Crithidia fasiculata* for an unconjugated pteridine (52). These findings suggested that an unconjugated pteridine like biopterin might be essential for the synthesis of certain pyrimidines and unsaturated fatty acids, but this possibility was never fully realized. Thus, although preliminary evidence indicated that in *Crithidia fasiculata* the conversion of dihydroorotic acid to orotic acid involves a tetrahydropterin-dependent hydroxylation of the dihydro compound and subsequent dehydration of the hydroxylated product to orotic acid (53), this pathway appears to be unique for *Crithidia fasiculata:* In all other organisms, the conversion of dihydroorotic acid to orotic acid is catalyzed by an NAD-dependent, iron-containing flavoprotein (for literature citations, see 53). As for the possibility that BH_4 might be essential for the desaturation of long-chain fatty acids by a sequence of hydroxylation-dehydration reactions, no evidence supports such a role for this pterin.

ROLE OF BH_4 AS THE COENZYME FOR THE AROMATIC AMINO ACID HYDROXYLASES

The first metabolic role for BH_4 was established when it was shown that the enzymatic conversion of phenylalanine to tyrosine catalyzed by the multi-enzyme hepatic phenylalanine hydroxylating system is completely dependent on a new coenzyme isolated from rat liver extracts. In 1963, structural studies carried out on the naturally occurring hydroxylation coenzyme that had been isolated from rat liver proved that it is tetrahydrobiopterin, 2-amino-4-hydroxy-6-[1,2-dihydroxy(L-*erythro*)]-5,6,7,8-tetrahydropteridine, whose structure is shown in Figure 1 (40). Several synthetic unconjugated pteridines like

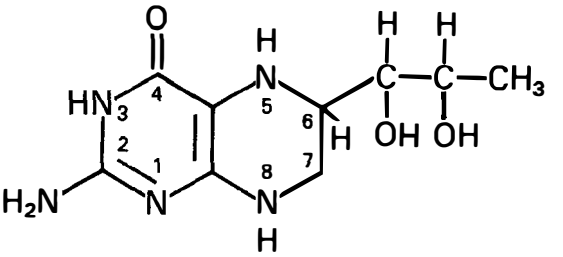

Figure 1 Structure of tetrahydrobiopterin (BH_4).

2-amino-4-hydroxy-6-methyltetrahydropteridine ($6MPH_4$) were also shown to be active in the system (38, 48).

In addition to the tetrahydropterin coenzyme ("pterin" is the trivial name for a 2-amino-4-hydroxy-pteridine), the phenylalanine hydroxylating system was shown to consist of three enzymes. The role of each component of the system is illustrated in Figure 2 with $6MPH_4$ as the coenzyme. Phenylalanine hydroxylase (PAH) catalyzes a coupled reaction in which phenylalanine is oxidized to tyrosine and the tetrahydropterin is oxidized to the corresponding 4a-hydroxytetrahydropterin; molecular oxygen, which is the source of the oxygen in the newly synthesized tyrosine, is normally reduced to water. At neutral pH, the 4a-hydroxytetrahydropterin product is unstable and breaks down rapidly to the quinonoid dihydropterin derivative; this reaction is also catalyzed by an enzyme that originally was called PAH-stimulating protein (abbreviated "PHS") (42) and later was shown to be a dehydratase (31, 43, 58). The cycle is completed by the action of the third enzyme, dihydropteridine reductase (DHPR), which catalyzes the reduction of the quinonoid dihydropterin to the tetrahydropterin. NADH is the preferred electron donor in vitro. This last reaction allows the pterin coenzyme to function catalytically in the hydroxylating system (47).

The elucidation of the roles of the individual components in the phenylalanine hydroxylating system facilitated subsequent studies showing that BH_4 and DHPR function in the same way in the tyrosine and tryptophan hydroxylating systems (reviewed in 47). Since the end products of these hydroxylase-catalyzed reactions are the tyrosine-derived neurotransmitters, dopamine and norepinephrine, and the tryptophan-derived neurotransmitter serotonin, these studies indicated that BH_4 and DHPR, are essential for normal brain development and functioning. BH_4 was also shown to be the essential

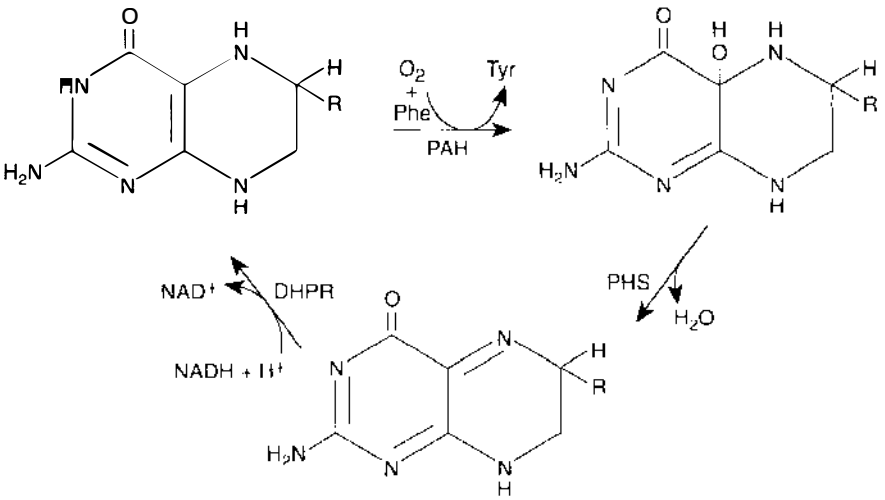

Figure 2 The enzymatic conversion of phenylalanine to tyrosine catalyzed by the multicomponent phenylalanine hydroxylase system.

coenzyme in the system that catalyzes the oxidative cleavage of glyceryl ethers (49, 103).

PHENYLKETONURIA AND ITS VARIANTS

Before discussing new roles for BH_4, it is worth noting that progress in our understanding of the biochemistry of the BH_4-dependent aromatic amino acid hydroxylating systems led to parallel progress in our understanding of aspects of phenylketonuria (PKU), a genetic disease known to be caused by a defect in the hydroxylation of phenylalanine (21, 33). The demonstration that the phenylalanine hydroxylating system consists of three essential components led to the prediction of three distinct forms of PKU, each caused by the lack of one of the essential components (41). After PAH had been identified as the missing component in the most common form of this disease, called "classical" PKU (37), researchers described several variant forms of PKU that were caused by a lack of either DHPR or BH_4; the latter condition is a consequence of a deficiency of one of the enzymes involved in the de novo synthesis of BH_4 from GTP [for review, see (45, 94)].

Note that observations made on patients who are deficient in BH_4, either because they cannot synthesize it or regenerate it (owing to a lack of DHPR), are relevant to a discussion of new roles for BH_4 because it is reasonable to expect that the BH_4 deficiency in these patients underlies most of their

pathological signs. So far, however, clinical experience with these variants, including the more-or-less successful outcome of replacement therapy with BH_4 or the products of the affected tyrosine hydroxylase- and tryptophan hydroxylase-catalyzed reactions (i.e. 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan) or a combination (reviewed in 45, 94), has not provided useful clues to new roles for BH_4. Some reasons for the failure to realize this expectation have been discussed (46); others are considered below.

The original prediction that three variant forms of PKU might exist, each caused by the lack of one of the three essential components of the PAH system, was made before the role of the dehydratase in the system had been elucidated. Because the reaction catalyzed by this enzyme occurs rapidly nonenzymatically under physiological conditions (43), it was predicted that a complete lack of the dehydratase, unlike the lack of any of the other three essential components, would probably lead to only a partial defect in phenylalanine metabolism and, hence, to only mild hyperphenylalaninemia (HPA) (44).

Recent evidence is coherent with this view. Several patients with mild HPA appear to be distinct from any that have been described previously. Their distinguishing feature is the excretion of abnormally high amounts of 7-biopterin, an isomer of biopterin with the dihydroxypropyl substituent in the 7, rather than in the 6, position of the pterin ring (6, 11, 18).

Feeding BH_4 to these patients led to the parallel increase in the excretion of both biopterin and 7-biopterin, suggesting that the 7-isomer is derived from BH_4 (12). The possibility that the patients who excrete 7-biopterin might be deficient in the dehydratase was raised by the results of in vitro experiments. These experiments demonstrated that in the absence of the dehydratase the initial pterin product of the PAH-catalyzed hydroxylation of phenylalanine, 4a-hydroxytetrahydrobiopterin, not only breaks down to its major product, quinonoid dihydrobiopterin, as shown in Figure 2, but also rearranges to give rise to small amounts of 7-biopterin, which is probably derived from the corresponding 7-quinonoid dihydrobiopterin. Furthermore, addition of the dehydratase markedly decreased the amount of 7-biopterin found (15). Qualitatively similar results were reported by Curtius et al 1990 (10). These in vitro findings suggest a possible link between excretion of 7-biopterin and a deficiency of the dehydratase, but the validity of this suggestion must await the proof that these patients actually lack the dehydratase.

The cause of the mild HPA in patients who excrete 7-biopterin has not been established. In this regard, it has been observed that 7-BH_4 is utilized inefficiently by pure rat liver PAH. Most of the 7-BH_4 is oxidized nonproductively in a reaction in which oxidation of the tetrahydropterin is largely uncoupled from hydroxylation of the amino acid (13). Additionally, in the presence of high concentrations of phenylalanine, 7-BH_4 is a potent inhibitor

of PAH (16). Either or both of these effects of 7-BH_4 on PAH could account for the impaired hydroxylation of phenylalanine and the consequent HPA seen in patients who excrete 7-biopterin.

BH_4 AND CELL PROLIFERATION

The earliest indication that BH_4 has metabolic roles in addition to that of coenzyme for the aromatic amino acid hydroxylases came from the observation, mentioned earlier, that biopterin or a structurally related unconjugated pterin like neopterin, is essential for the growth of *Crithidia fasiculata*. This dependence, together with the finding that this organism has an absolute requirement for tyrosine (9), and therefore cannot synthesize it, and is also unlikely to synthesize the tyrosine hydroxylase- and tryptophan hydroxylase-derived neurotransmitters, provided strong, albeit indirect, evidence for other roles for BH_4.

That BH_4 might also have other roles in higher organisms was indicated by the finding that the pterin is present in cells and tissues such as blood, spleen, and lung, which have negligible amounts of the aromatic amino acid hydroxylases (24).

Some insight into how biopterin may function in the physiology and development of red blood cells came from the observation that cellular levels of biopterin increase in proliferating hemopoietic cells during bone marrow transplantation in beagle dogs (118). An essential connection between biopterin synthesis and hemopoietic cell proliferation was proposed.

Also pointing in the same direction was the finding that both the content of total biopterin and the levels of GTP cyclohydrolase, the enzyme that catalyzes the first step in the *de novo* synthesis of BH_4 (See Figure 3), are strikingly higher in young rat erythrocytes, than in older erythrocytes (101). These results suggested that BH_4 levels in reticulocytes, the precursors of erythrocytes, might be high. This possibility was examined in rats treated with phenylhydrazine to stimulate the production of erythrocyte precursor cells. The biopterin concentration and the levels of GTP cyclohydrolase were found to be 17.5-fold and 23-fold higher, respectively, in hemolysates prepared from phenylhydrazine-treated rats (reticulocyte content more than 80% of total erythrocytes) than in comparable preparations from control rats (1–2% reticulocytes) (101). Very similar results were obtained with phenylhydrazine-treated mice (50).

The possibility raised by these results, i.e. that BH_4 may regulate the proliferation of erythroid cells, was examined in greater detail in murine erythroleukemia (MEL) cells as a model for erythrogenesis. In culture, these cells have a very low level of spontaneous erythroid differentiation. Treatment with certain agents, such as hexamethylene bisacetamide (HMBA), leads to

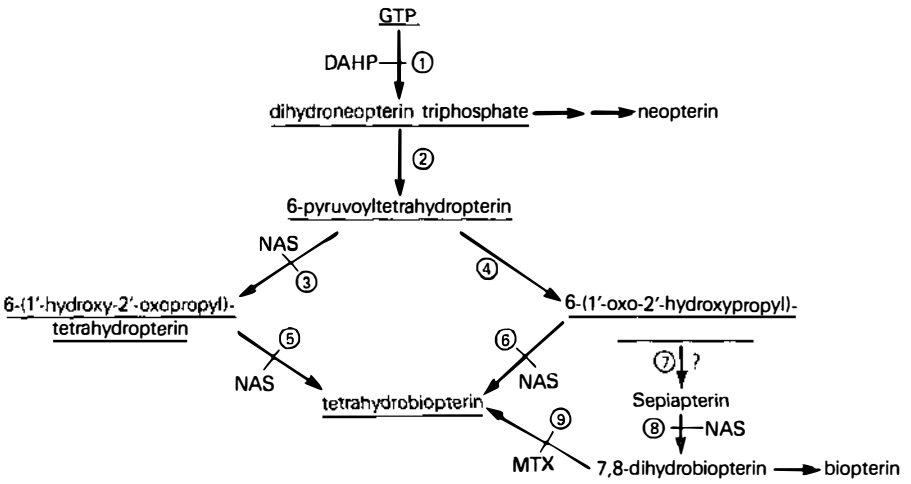

Figure 3 Proposed scheme for the biosynthesis of tetrahydrobiopterin from guanosine triphosphate (GTP).

The intermediates on the *de novo* pathway are underlined. The scheme shows two alternate routes for the conversion of 6-pyruvoyltetrahydropterin to BH_4. One involves the successive reductions of this diketo intermediate; each reduction is catalyzed by sepiapterin reductase (reactions 3 and 5). The other pathway, first demonstrated in brain tissue (68, 69), proceeds with the reduction of the 2′-oxo group of the diketo intermediate, catalyzed by an aldose reductase (reaction 4) to form the 1′-oxo-2′-hydroxy intermediate (also called 6-lactoyltetrahydropterin) and followed by reduction of the 1′oxo group, also catalyzed by sepiapterin reductase. The sepiapterin shown in the scheme is probably formed during the nonenzymatic oxidation of 6-lactoyltetrahydropterin. Conversion of sepiapterin to BH_4 through the successive actions of sepiapterin reductase and dihydrofolate reductase (reactions 8 and 9) constitutes the so-called salvage pathway for the synthesis of BH_4 (71).

The key reactions are catalyzed by the following enzymes: reaction 1, GTP cyclohydrolase; reaction 2, 6-pyruvoyltetrahydropterin synthase; reactions 3, 5, 6, 8, sepiapterin reductase; reaction 4, 6-pyruvoyltetrahydropterin-(2′-oxo)-reductase, an aldose reductase (69); reaction 9, dihydrofolate reductase. The abbreviations used are DAHP, 2,4-diamino-6-hydroxypyrimidine, an inhibitor of GTP cyclohydrolase; NAS, *N*-acetylserotonin, an inhibitor of sepiapterin reductase; MTX, methotrexate, an inhibitor of dihydrofolate reductase.

induction of differentiation culminating in terminal cell divisions and hemoglobin synthesis (62).

During a 96-hr period of exposure of MEL cells to HMBA, a progressive decrease in the initially high levels of BH_4 and GTP cyclohydrolase was accompanied by a marked increase in hemoglobin content that was detectable after 72 hr of exposure to the inducing agent (101). Cellular content of BH_4 was decreased by inhibition of its synthesis. This was accomplished by treatment of the cells with *N*-acetylserotonin (NAS), an inhibitor of sepiapterin reductase, which is the terminal enzyme in the BH_4 biosynthetic pathway (See

Figure 3). This treatment inhibited proliferation of the cells, as measured by a decrease in DNA synthesis. Although inhibition of proliferation of MEL cells is usually accompanied by differentiation, the decreased DNA synthesis and cell proliferation resulting from inhibition of BH_4 synthesis did not lead to an increase in differentiation as measured by the appearance of hemoglobin-positive cells. Inhibition of proliferation caused by treatment of the MEL cells with NAS was not due to an irreversible toxic effect of NAS on the cells, since repletion of the BH_4 content of the NAS-treated cells, by treatment with the BH_4 precursor sepiapterin, completely restored DNA synthesis (101). Indeed, increasing the BH_4 content of MEL cells that had not been treated with NAS increased DNA synthesis above control values (101).

Similar results were reported in a subclone of MEL cells (F4N); addition of either BH_4 or its precursor, sepiapterin, significantly increased DNA synthesis (51). In this subclone, in contrast to the MEL cells, only trace amounts of biopterin and GTP cyclohydrolase are present and induction (with dimethyl sulfoxide) significantly increased their levels (51).

These results indicate that BH_4 plays a role in proliferation and differentiation of murine erythroid cells. The observation that inhibition of cell proliferation by depletion of BH_4 does not culminate in hemoglobin synthesis suggests that the presence of BH_4 may enable MEL cells to enter the erythroid program that commits them to a terminal differentiation.

How BH_4 regulates the growth of these cells has not been elucidated. One possibility is based on the finding that biopterin and BH_4 inhibit the guanine: queuine tRNA transglycosylase from rabbit reticulocytes (20). This enzyme is responsible for the synthesis of the modified tRNA species in which the hypermodified base queuine is substituted for guanine. Evidence suggests that the ratio of tRNA queuine ($tRNA_Q$) to tRNA guanine ($tRNA_G$) decreases in tumor tissues during cell transformation (73, 76) and that the ratio also changes during erythroid differentiation of MEL cells. In one study, a continual decrease in $tRNA_G$ was observed during differentiation (95), whereas Lin et al (60) found an initial increase in $tRNA_G$ followed by a decrease.

Recently, the relationship between changes in cellular content of BH_4 and those of $tRNA_G$ was studied during the course of tetramethylurea-induced differentiation of MEL cells (82). Changes in levels of BH_4 and $tRNA_G$ followed similar patterns: An initial increase after the first 12 to 24 h was followed by a decrease, i.e. levels of $tRNA_G$ were high when BH_4 levels were high and these levels decreased when BH_4 levels were decreasing. Such a temporal relationship would not be expected if BH_4 was acting to inhibit the incorporation of guanine into tRNA in the MEL cells. While these results are of interest, the possibility that the BH_4 regulation of proliferation of erythroid cells is mediated by changes in the ratio of $tRNA_Q$ to $tRNA_G$ remains to be established.

These results with erythroid cells raised the possibility that BH_4 might be a general regulator of cell proliferation. Preliminary results indicate that BH_4 may also regulate proliferation of cultured neonatal rat brain astrocytes (70). On the other hand, attempts to demonstrate a similar role for BH_4 in proliferation of hamster ovary cells were negative (S. Milstien & K. Tanaka, unpublished results). Thus, it is unlikely that BH_4 is involved in the regulation of the growth of all cells.

The role of BH_4 in the proliferation of erythrocyte precursor cells is complex. Not only is there strong evidence, reviewed above, supporting the conclusion that BH_4 is necessary for the growth of these cells, but, in addition, there are indications of a reciprocal relationship, i.e. the rate of *de novo* synthesis of BH_4 is high during periods of rapid proliferation of the cells.

BH_4 AND CELL-MEDIATED IMMUNITY

Our understanding of this kind of reciprocal relationship has been advanced by studies of the relationship between pterin metabolism and activation of the immune system.

Some of the earliest evidence for such a connection came from reports that urinary levels of pterins such as neopterin (derived from the BH_4 precursor dihydroneopterin triphosphate (see Figure 3) (88, 105) and blood levels of BH_4 (54) are elevated in cancer patients. This increase was not specifically related to malignancy, but rather was part of a much broader response, involving a variety of conditions associated with activation of the immune system. Reports indicated that urinary neopterin levels are also elevated in patients suffering from viral (105) and bacterial (23) infections, including acquired immune deficiency (AIDS) (1,104), as well as from such conditions as rheumatoid arthritis and systemic lupus erythematosus (26). Thus, increased urinary excretion of neopterin is a marker for activation of the cell-mediated immune system.

These in vivo studies did not identify the cellular source of the elevated urinary neopterin. A subsequent in vitro study showed, upon stimulation with factors derived from activated T cells [activated by treatment with alloantigens or phytohemaglutinin (PHA)], that human monocytes and macrophages release neopterin into the cell culture medium and that interferon gamma (IFNγ) is the most active stimulating factor. Researchers concluded that this cytokine is the mediator of the response, because monoclonal antibodies against IFNγ were able to completely block the increased neopterin release that was induced by activated T cells (32). Macrophages and their precursors, monocytes, therefore, are probably major sources of the elevated urinary excretion of neopterin that is associated with activation of the immune system.

The mechanism for the IFNγ-induced increase in synthesis and release of

neopterin was the induction of GTP cyclohydrolase. Treatment of human macrophages with supernatants from mitrogen (PHA)-activated or alloantigen-activated T cells or with recombinant IFNγ resulted in a 10–20 fold increase in GTP cyclohydrolase activity. A comparable increase in the activity of the enzyme in the T cells, themselves, was noted after they had been activated. In T cells, the increase in GTP cyclohydrolase activity was accompanied by a modest (50–100%) increase in levels of neopterin and an increase of both biopterin and pterin (a breakdown product of BH_4) (4, 14), neither of which was detectable in unactivated T cells. By contrast, in human macrophages the elevation in neopterin induced by either activated T cell supernatants or IFNγ was not accompanied by any increase in either biopterin or pterin (92).

These results with activated T cells agree with previous reports that activation of both murine and human T lymphocytes leads to large increases in the formation and release of pteridines, including biopterin (115, 116). It was also reported that BH_4 and its biological precursors, 7,8-dihydrobiopterin and sepiapterin, are co-stimulators of concanavalin A–mediated lymphocyte proliferation, which perhaps affects the interaction of the lymphocytes with interleukin 2 (117, 119). These results suggest that in its effects on lymphocytes BH_4 is part of an autocrine signaling process in which cells respond to substances that they themselves release.

The finding that IFNγ induces in human macrophages a striking increase in neopterin, whereas biopterin remains at undetectable levels, was paradoxical because, in contrast to BH_4, no physiological function has ever been ascribed to neopterin or its derivatives.[1]

This selective effect of IFNγ on human macrophages is coherent with the observation that humans and nonhuman primates are peculiar because they have relatively large amounts of neopterin in their body fluids (19). Studies with macrophages from other species, as well as those with human cells other than macrophages, showed that the selective IFNγ-mediated increase in neopterin is unique to human macrophages. In mouse peritoneal macrophages stimulated with lipopolysaccharide and in a murine T cell line stimulated with interleukin-2, cell content of biopterin was increased twofold and four- to sevenfold, respectively, whereas neopterin was not detectable (93), i.e. this

[1]Regarding possible biological activity of this pterin, note that it is potentially active as a hydroxylase coenzyme in vivo; however, no reports have suggested that its coenzyme activity is comparable to that of BH_4. Thus, although the biosynthetic intermediate, dihydro-(D-*erythro*)-neopterin triphosphate, is not a substrate for DHFR, this enzyme does catalyze the reduction of the dephosphorylated compound, dihydroneopterin, to its tetrahydro form (74), a finding that has recently been confirmed (3). Furthermore, it has been known since 1962 that tetrahydroneopterin has high coenzyme activity with rat liver PAH (39). The maximum activity of tetrahydroneopterin, however, is only about one third that of BH_4 (77). In the case of rat brain tryptophan hydroxylase, the relative activity of tetrahydroneopterin is even less favorable—only about 5% that of BH_4 (36).

pterin pattern is just the reverse of the one seen in stimulated human macrophages.

A more detailed study of the effect of cytokines on murine macrophages, which was also extended to murine fibroblasts, showed that treatment of these cells with tumor necrosis factor (TNFα) alone increased intracellular biopterin 2-fold (macrophages) to 13-fold (fibroblasts); treatment with IFNγ alone led to modest increases, and treatment with both led to greater increases than treatment with either factor alone (109).

In human fibroblasts, in contrast to human macrophages, IFNγ increased the cell content of *both* neopterin and biopterin, with the levels of biopterin actually exceeding those of neopterin; activity of GTP cyclohydrolase increased more than 10-fold (107). In this study, unlike an earlier one (93), treatment of human macrophages with IFNγ modestly increased their biopterin content, although the amount of neopterin contained in the treated cells was about 80-times greater than that of bioperin.

The unique response to IFNγ treatment of human macrophages, leading to a selective increase in neopterin, has been traced to differences in the levels of the post-cyclase enzymes that are essential for BH_4 synthesis, particularly 6-pyruvoyltetrahydropterin synthase (Figure 3). Thus, in human macrophages, synthase activity is extremely low, and unlike GTP cyclohydrolase, is unaffected by IFNγ treatment. After this treatment, cyclohydrolase activity was about 40-times higher than that of the synthase. Sepiapterin reductase, the terminal enzyme in the BH_4 synthetic pathway, is constitutive and is in huge excess over the activities of the synthase and hydrolase (108). The ability of IFNγ to induce GTP cyclohydrolase, together with the low-constitutive levels of the synthase and its lack of response to IFNγ, account for the selective cytokine-mediated increase in neopterin in human macrophages. On the other hand, in murine macrophages as well as in nonmacrophage, cytokine-sensitive human cells that have been studied, the constitutive level of the synthase far exceeds even the elevated induced level of the hydrolase. In murine fibroblasts, for example, synthase activity is about 10 times higher than the maximally induced levels of cyclohydrolase; (109). In this enzyme pattern, where the synthase, the enzyme utilizing the neopterin derivative and converting it to BH_4, is in vast excess over the enzyme synthesizing it, the stimulation of the cyclohydrolase leads to a selective increase in biopterin rather than neopterin.

A paradoxical question was raised by the results with human macrophages: What is the physiological sense of turning on the BH_4 biosynthetic pathway only to have it almost completely short-circuited because of severely limiting amounts of the key enzyme in the pathway, 6-pyruvoyltetrahydropterin synthase? Although this question has not been answered, recent studies have uncovered possible functional connections between the immune response and increased synthesis of BH_4.

The compass for this search was provided by the observation that IFNγ plays a key role in the process. This observation focused attention on BH_4-dependent enzymes that are also induced by IFNγ and of which two are known: indoleamine-2,3-dioxygenase (IDO) and nitric oxide synthase (NOS).

Studies with Indoleamine Dioxygenase

There were several early indications that indoleamine dioxygenase, which in nonhepatic tissues catalyzes the superoxide ion-dependent, oxygenative ring cleavage of various indoleamine derivatives, may be involved in the immune response. In mammals, the enzyme catalyzes the first step in the major catabolic pathway of L-tryptophan in which the amino acid is converted to kynurenine via the formation of *N*-formyl kynurenine. The enzyme occurs in two entirely distinct forms, one in liver and one in many nonhepatic tissues. Activity of the extrahepatic enzyme in tissues such as lung and stomach was found to be markedly increased in mice under some pathological conditions e.g. after intraperitoneal injection of bacterial lipopolysaccharide, whereas the activity of the hepatic enzyme was decreased by this treatment (113). Subsequently, it was demonstrated that exposure of mouse lung slices to mouse IFN increased the activity of IDO 10- to 15-fold (114). Induction of IDO by IFNγ was also demonstrated in human peripheral blood monocytes (78).

The mechanism by which IDO mediates some of the physiological effects of IFNγ, such as its ability to fight viral and nonviral intracellular infections, was suggested by the finding that the effectiveness of this cytokine in suppressing the growth of an intracellular protozoan parasite in human fibroblasts was blocked by high levels of tryptophan in the culture medium. This observation and the finding that the IFNγ-treated fibroblasts could degrade this amino acid led to the suggestion that the increased degradation of tryptophan could limit growth of the organism either because tryptophan metabolites could be toxic to the parasite or because degradation of tryptophan could decrease the intracellular concentration of this commonly limiting amino acid (86).

Studies of the antiproliferative effects of IFNγ on a variety of human neoplastic cell lines also support the conclusion that the antiproliferative, antitumor effect of the cytokine, like its effect on growth of intracellular parasites, is due to the IFNγ-induction of IDO, with consequent starvation of the cells for tryptophan (79).

The demonstration that, in certain cells, IFNγ could induce both BH_4 synthesis and IDO appeared to make sense in light of the earlier report that BH_4 and $DMPH_4$ have cofactor activity with IDO purified from rabbit small intestine (72). The enzyme had previously been shown to require ascorbic acid and methylene blue for maximum activity. The dye was believed to be responsible for the generation of superoxide ion, which was known to

participate as a reactant in the reaction (30). The possibility that BH_4 might be the preferred coenzyme was supported by the finding that the K_m for BH_4 is lower (70 μM) (72) than the K_m for ascorbate (200 μM) (30); the values for V_{max}/K_m for the two potential coenzymes are similar (72).

Subsequent studies with IDO purified from mouse epididymis, however, made it less likely that BH_4 might function as the physiological coenzyme with this enzyme. Reduced flavin mononucleotide at 0.005 mM (with an $FMNH_2$ regenerating system) supports reaction rates that are about 40-fold higher than reaction rates in which 0.1 mM BH_4 is in the presence of its regenerating system, i.e. DHPR and NADPH (80). These in vitro data indicated that reduced flavin mononucleotide is, in all probability, the physiological coenzyme for IDO, at least for this tissue.

Despite these in vitro results, the notion that BH_4 may function in vivo as the coenzyme for IDO continues to be put forward. Recently, apparent support for this possibility was provided by the finding that both IDO and BH_4 synthesis (as measured by GTP cyclohydrolase activities and intracellular concentrations of neopterin and biopterin) are induced together by IFNγ in six human cancer cell lines, as well as in human fibroblasts and macrophages (107). As mentioned above, the level of BH_4 attained in macrophages was very low, equal to only 1–2% that of neopterin. Also suggestive of a possible functional link between IDO and pterin synthesis was the finding in macrophages that at a fixed dose of IFNγ, both IDO activity (measured by kynurenine formation) and pterin biosynthesis (measured by neopterin formation) were increased in parallel fashion by increases in L-tryptophan in the culture medium. In this study, the cofactor activity of BH_4 with IDO, originally reported with the enzyme from rabbit intestine (72), was also demonstrated with crude extracts of an IFNγ-treated human bladder carcinoma cell line. This study confirmed the observation that BH_4 showed little activity in the absence of methylene blue. The relative activities of BH_4 and ascorbate were essentially the same as in the earlier study. By contrast, the coenzyme activity of reduced flavin mononucleotide reported with IDO from mouse epididymis (80) could not be replicated with the enzyme from the human bladder carcinoma cells (107).

Despite the parallel induction by IFNγ of BH_4 biosynthesis and IDO activity in a variety of human cell lines (107) and the reported coenzyme activity of BH_4 with IDO in vitro (72, 107), the evidence does not strongly support the notion that, when the immune system is activated, BH_4 synthesis is turned on because BH_4 is the physiological coenzyme for IDO. In particular, the near-absolute dependence of the coenzyme activity of BH_4 on the presence of methylene blue, and the finding that, at least in one tissue, $FMNH_2$ is far more active than BH_4 (78), weaken support for the postulated connection.

Indeed, recent evidence has seriously undermined the IFN-IDO-BH_4

postulate. Treatment of human macrophages with IFNγ led to the expected increase in formation of kynurenine and neopterin, but treatment of the cells with 2,4-diamino-6-hydroxypyrimidine (DAHP), an inhibitor of the BH_4 biosynthetic pathway that completely prevented the IFNγ-mediated increase in neopterin, had no effect on the amount of kynurenine formed. Similar results were obtained with DAHP-treated human fibroblasts (N. Sakai, K. Saito, M. P. Heyes, et al, unpublished results)

In fibroblasts devoid of BH_4 that were obtained from patients who are deficient in 6-pyruvoyltetrahydropterin synthase, treatment with cytokines still leads to induction of IDO, as measured by kynurenine formation (N. Sakai, K. Saito, M. P. Heyes, et al, unpublished results). These results, which show that increased IDO activity can be expressed even in the absence of detectable amounts of BH_4, provide persuasive evidence against the proposal that BH_4, is the physiological cofactor for IDO.

Studies with Nitric Oxide Synthase

The other candidate enzyme that could be the link between activation of the immune system and the BH_4 biosynthetic pathway is nitric oxide synthase (NOS). This enzyme catalyzes the NADPH-dependent conversion of L-arginine to L-citrulline and nitric oxide (•NO). The enzyme is classified as an oxygenase or mixed function oxidase, since molecular oxygen, rather than water, is the source of the ureido oxygen in citrulline (56).

•NO, a highly reactive radical, has been shown to be a mediator in a bewildering array of biological processes. Not only has it been identified as the endothelium-derived relaxing factor, which is active as a smooth muscle relaxant, but it also acts as a neurotransmitter, prevents platelet aggregation, and, as part of the cell-mediated immune response, destroys or halts the growth of some tumor and bacterial cells (reviewed in 61, 63).

The synthase occurs in two distinct forms with different properties: a constitutive form, present in tissues like cerebellum, endothelial cells, and platelets, and a cytokine-inducible form, present in cells like macrophages and hepatocytes (97).

Inducible cytosolic NOS, partially purified from a murine macrophage cell line after stimulation of the cells by treatment with interferon γ and *E. coli* lipopolysaccharide, was shown to be absolutely dependent on L-arginine and on NADPH and to be partially dependent (two- to threefold stimulation) on BH_4 (57, 102), FAD, and a thiol such as glutathione (98).

The constitutive enzyme from rat cerebellum, like the inducible macrophage enzyme, also requires L-arginine and NADPH, but unlike the former enzyme, it is also dependent on exogenous Ca^{2+} and calmodulin (7). Like the macrophage enzyme, NOS from brains of various species, including rat (25, 91), porcine (67), and human (90), is stimulated by BH_4.

Both the constitutive and macrophage enzymes are homodimers, composed of identical subunits, M_r = 279,000 (7, 66, 90). The macrophage enzyme appears to be somewhat smaller (96). Both enzymes contain bound flavins [flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)] and tightly bound BH_4. The presence of bound BH_4 may account for the fact that NOS has considerable activity in the absence of added BH_4. NOS from murine macrophages contains heme-bound iron, and is sensitive to inhibition by CO (111). Although originally reported to contain nonheme iron (67), recent evidence indicates that like the macrophage enzyme, the brain enzyme is sensitive to CO (111), thereby indicating that it is also a heme-iron protein (see also 61).

It has been demonstrated that N^{ω}-hydroxyl-L-arginine is an intermediate in the conversion of L-arginine to L-citrulline and •NO (96). The overall reaction has been formulated as shown in Figure 4: 1.5 mol of NADPH is oxidized for each mole of arginine converted to •NO (measured as nitrate plus nitrite), whereas only 0.5 mol of NADPH is consumed for each mole of hydroxyarginine converted to •NO (96). Although Figure 4 depicts BH_4 as being involved only in the latter step, there is no evidence in support of this assignment.

One of the most striking differences between NOS and hepatic PAH is that the K_m for BH_4 for the NOS (0.02–0.03 μM) (25, 97) is orders of magnitude smaller than that for PAH (2 μM) (2).

The extremely low K_m of NOS for BH_4 is relevant to our understanding of the clinical picture presented by patients with variant forms of PKU caused by an inability to either regenerate or synthesize BH_4 (94). The discovery that BH_4 is a cofactor for nitric oxide synthase raised questions about why these BH_4-deficient patients do not show signs that might be expected if the BH_4 deficiency led to diminished activity of NOS with consequent impairment of

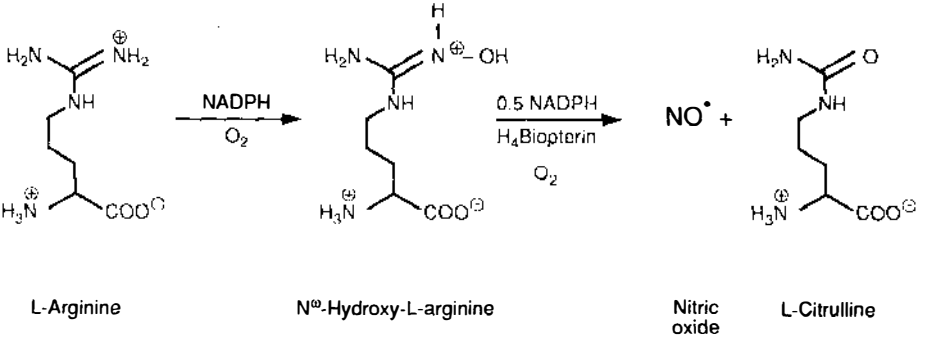

Figure 4 The reaction sequence for the enzymatic conversion of L-arginine to L-citrulline and •NO showing N^{ω} hydroxy-L-arginine as an intermediate [adapted from (99)].

the myriad physiological processes that appear to be mediated by this biofactor. To mention only one example, why do not BH_4-deficient patients exhibit signs of impaired immune function (34)? One explanation for this apparent paradox is that the deficiency of BH_4 and the consequent decrease in tissue levels of BH_4 in these patients is severe enough to impair the function of an enzyme like PAH, with a K_m for BH_4 of 2 μM, but is not severe enough to impair the function of enzyme like NOS, with a K_m for BH_4 of 0.02 μM.

Another sharp difference between NOS and PAH is that the requirement for BH_4 by the former enzyme is much more specific than it is for the latter. Thus, whereas synthetic model tetrahydropterins like $6MPH_4$ have about the same coenzyme activity for PAH as does BH_4 [i.e. the same V_{max}/K_m value (2)], $6MPH_4$ at 0.2–0.5 μM (i.e. ~ 10–20 × the K_m for BH_4) is inactive with both macrophage (57) and brain NOS (25), although it does show some activity at 50–100 μM (25, 57).

Given the large number of redox cofactors that are, or can be, bound to NOS—NADPH, FMN, FAD, heme-iron, and BH_4—the question of what role BH_4 plays in this built-in electron-transport system is of special interest. It has been postulated (57) that BH_4 functions in the NOS system in precisely the same way that it has been shown to function with the aromatic amino acid hydroxylating systems (Figure 2); the only difference is that the proposed scheme for the NOS-catalyzed reaction does not include the formation of 4a-hydroxytetrahydropterin as an intermediate in the reaction, as shown in Figure 2. If, indeed, BH_4 does function with NOS as it does with PAH (and the other aromatic amino acid hydroxylases), BH_4 must cycle between the tetrahydro and dihydro states during •NO synthesis.

There are indications, however, that BH_4 does not function with NOS in exactly the same way as it does with PAH. One difference is that both bound and exogenous BH_4 appear to function catalytically during •NO synthesis even in the absence of any obvious BH_4-regenerating system. Thus, during the first few minutes of the NOS-catalyzed reaction, even in the absence of added BH_4, about 18 times more product is formed than the amount of enzyme added. This result shows that the enzyme-bound BH_4 is functioning catalytically, and that if it is being oxidized during the reaction, it is capable of being regenerated. Alternatively, this result may indicate that BH_4 is not being oxidized to support product formation. Similar results were obtained with added BH_4; each mole of added BH_4 is capable of supporting the formation of many moles of citrulline (25).

The possibility that NOS can recycle exogenous BH_4 was examined with the use of the phenylalanine hydroxylation system as a sensitive detector of recycling. To function catalytically in that system, the quinonoid dihydropterin product must be reduced back to the tetrahydro level (see Figure 2). Under conditions where any possibility of chemical regeneration of BH_4 from its

quinonoid dihydro derivative was minimized, there was no evidence for BH_4 recycling in the presence of a complete NOS system (25). Additional evidence against recycling of BH_4 was provided by the observation that methotrexate, an inhibitor of all enzymes known to convert dihydrobiopterin to BH_4, does not inhibit BH_4-dependent NOS activity (25). Strictly speaking, these results only provide evidence against the recycling of exogenous BH_4. But unless the assumption is made that enzyme-bound and added BH_4 stimulate the reaction by different mechanisms, the data also make it unlikely that the enzyme-bound BH_4 recycles during the reaction, i.e. that the bound BH_4 is stoichiometrically oxidized (each mole of product formed is supported by the oxidation of an equivalent of BH_4) and then reduced.

Either or both of the following mechanisms have been proposed to explain these results (25). First, BH_4 may be needed to reduce NOS from an inactive to an active form, as has been demonstrated with PAH (64). In this reaction, BH_4 is oxidized but the oxidation is stoichiometric with the amount of enzyme and not with the amount of product formed. The observation that BH_4 is not an absolute requirement suggests that the purified enzyme is at least partially in the reduced, active form and that, on incubation in the absence of added BH_4, the reduced form is oxidized. Secondly, BH_4 may be an allosteric effector of NOS that is necessary to maintain the enzyme in an active form. In this regard, BH_4 has been shown to be an effector, albeit a negative one, in the activation of PAH by cyclic AMP-dependent protein kinase (87). In summary, NOS may require reduction by BH_4 to activate it, or it may require the mere presence of BH_4 to maintain it in an active form, or BH_4 may be needed to both reductively and allosterically activate the enzyme (25).

The conclusion of Giovanelli et al (25) that enzyme-bound BH_4 functions catalytically in the NOS-catalyzed reaction, as well as their proposal that one of the functions of BH_4 may be to reduce NOS to an active form, have been endorsed by other workers (27). In a further elaboration of their views, Hevel & Marletta (27) have cited results of experiments with 6-methyl-5-deaza-tetrahydropterin showing that this analogue of $6MPH_4$ has no coenzyme activity with NOS but is an inhibitor of the $6MPH_4$-stimulated NOS-catalyzed reaction. On the basis of these results, they have concluded that bound-BH_4 functions in one of the two hydroxylation steps in the NOS-catalyzed reaction in a manner similar to that observed with the amino acid hydroxylases, a conclusion that implies that oxidation of BH_4 is stoichiometric with product formation. Although it is possible that future work will prove that this is the role of BH_4, this particular argument is not persuasive. Some deazatetra-hydropterins inhibit tetrahydropterin-dependent enzymes when the pterin coenzyme does not function in a redox capacity, e.g. 5,10-dideaza-5,6,7,8-tetrahydrofolate inhibits glycinamide ribonucleotide transformylase (5). Following the same line of reasoning as that used for deazapterin and NOS, one

might conclude that the pterin coenzyme in the transformylase reaction, 10-formyltetrahydrofolate, is involved in a redox reaction with this enzyme. This conclusion, however, would be incorrect.

In contrast to the unsuccessful attempts to demonstrate a functional connection between cytokine-mediated increases in IDO activity and BH_4 levels, reviewed above, similar attempts to link NOS and BH_4 have succeeded. Evidence suggestive of such a link first came from studies of the effect of cytokine treatment on murine fibroblasts which showed that IFNγ alone, or preferably in combination with TNFα or lipopolysaccharide (LPS), induced the synthesis of NOS (measured by enhanced formation of its products, nitrite plus nitrate) and increased the intracellular content of total biopterin (110). Inhibition of the cytokine-induced increase in BH_4 synthesis by treatment of the cells with DAHP prevented almost completely the increase in BH_4 content and decreased NOS activity to 57% of the high induced levels obtained in the absence of DAHP. The effect of DAHP on NOS activity was reversed by increasing intracellular BH_4 levels by treatment of the cells with the BH_4 precursor sepiapterin. These results indicate that about half of the cytokine-induced NOS activity is dependent on the concomitant increase in BH_4 synthesis and suggest, but do not prove, that the other half may also be dependent on the BH_4 that is present prior to cytokine treatment. Evidence was also presented that DAHP could partially prevent the cytokine-induced decrease in the viability of the fibroblasts (110) and that the effect on viability was mediated by the well-established cytotoxic action of •NO (28).

A more striking dependence of NOS activity on BH_4 levels has been demonstrated in a murine macrophage cell line (RA264). Treatment of these cells with IFNγ and LPS increased BH_4 levels above the relatively high constitutive level and, in agreement with previous results (100), stimulated the synthesis of NO as measured by nitrite production (89). Incubation of the cells with DAHP in the presence or absence of cytokines resulted in 90% depletion of intracellular levels of BH_4 within 6 hr but only marginally decreased nitrite production. Depletion of total cellular BH_4 by 96% inhibited the cytokine-stimulated production of nitrite by only 52%. Nitrite formation was dramatically inhibited only after BH_4 content was depleted by more than 99% [by treatment with DAHP plus NAS, an inhibitor of sepiapterin reductase (35)]. This sharp decrease in nitrite production was largely reversed by repletion of the BH_4 content of the cells. These results indicate that BH_4 is indeed an absolute requirement for cytokine-stimulated •NO production in murine macrophages, and probably in other cells, and suggest that only a small fraction of the total intracellular pool of BH_4 in macrophages is used in the induction and expression of NOS (89).

The first indication that humans also possess a cytokine-activated NOS system came from the report that a human subject on a low nitrate diet

developed an increase in nitrate production (9-fold over basal levels) that coincided with the onset of fever and diarrhea (106). There is evidence that both the basal (59) and cytokine-mediated increased production of nitrate (29) are derived from L-arginine.

These results with humans have set the stage for what is proving to be the second unsolved mystery in this area (the first is the role of the cytokine-stimulated production of neopterin): In humans, what cell is the site of the cytokine-induced increase in NOS? To date, attempts to induce increased •NO synthesis in cultured human cells with the use of various cytokine protocols that are effective in rodent cells have been unsuccessful (8, 29, 81).

Limited success has been reported by Denis (17); upon treatment with TNFα, human monocyte-derived macrophages restricted the growth of the virulent form of *Mycobacterium avium* and enhanced the killing of the avirulent form of this organism. Granulocyte macrophage-colony stimulating factor (GM-SF) was as effective as TNFα in increasing the bacteriostatic activity of macrophages on *M. avium,* and the combination of TNFα plus GM-SF was more effective than either treatment alone. Significantly, the ability of TNFα to enhance the killing of the avirulent form of the organism was sensitive to N^G-monomethylarginine, an inhibitor of NOS, thus implicating •NO in the killing process. Also pointing to the same conclusion is the finding that the mycobacteridal activity of the macrophages correlated with nitrite production. These results appear to be peculiar to macrophages infected with *M. avium,* since nitrite production was not detectable in uninfected, cytokine-treated macrophages. Whether conditions can be found that will allow generalization of these findings to include the response of human cells to a wider variety of organisms and other invading cells remains to be seen.

BH_4 AS A NEUROTRANSMITTER-RELEASING FACTOR

An in vivo dialysis technique in which a microdialysis probe is implanted in a specific brain area, e.g. the striatum, thereby allowing perfusion of the area and the quantitative measurement of metabolites in the dialysate, has demonstrated the novel activity of (6R)-BH_4 as a dopamine-releasing agent in rat striatum (55). The addition of (6R)-BH_4 (0.25, 0.5, 1.0 mM) to the perfusion fluid increased dopamine levels in the striatal dialysates in a concentration-dependent manner. The possibility that this effect of (6R)-BH_4 was due to the stimulation by the pterin of tyrosine hydroxylase activity, leading to faster conversion of tyrosine to dopa and ultimately to more rapid formation of dopamine, is unlikely because most of the effect of BH_4 was not blocked by inhibition of tyrosine hydroxylase activity with α-methyl-*p*-tyrosine. The ability of BH_4 to stimulate the release of dopamine appears to depend on neural impulses reaching nerve terminals, since the effect was virtually

abolished by pretreatment with tetrodotoxin, a drug that inhibits neuronal activity by blocking sodium channels in neuronal tissues.

The same technique was used to show that the activity of (6R)-BH_4 as a releasing agent extends to the release of dopamine, serotonin, and glutamate from rat striatum and frontal cortex (65). The BH_4-stimulated release of glutamate was almost completely suppressed after destruction of striatal dopaminergic terminals by treatment with the neurotoxin 6-hydroxydopamine.

(6R)-BH_4 was also reported to enhance the release of acetylcholine in vivo in the rat hippocampus (75). The BH_4-induced increase in release of acetylcholine was also eliminated after inhibition of voltage-dependent Na^+ channels by tetrodotoxin, but not after depletion of catecholamines by reserpine; this would indicate that the effect of BH_4 on acetylcholine release is direct and not secondary to the effects of BH_4 on release of dopamine.

An attempt to demonstrate BH_4-mediated release of serotonin from isolated synaptosomes was unsuccessful (112). Although the reasons why this in vitro experiment failed are not known with certainty, one likely reason is that no attempt was made to inhibit re-uptake of any serotonin that might have been released. Under these conditions, therefore, a BH_4-stimulation of release would have been difficult to detect.

CONCLUDING REMARKS

One only has to think back to one of the historically important early discoveries in this field, i.e. the growth requirement of *Crithidia fasiculata* for biopterin, to be reminded that more new roles for BH_4 remain to be discovered. This conclusion follows because none of the established roles for BH_4 can readily explain why it is essential for the growth of this organism.

In addition to the likelihood that other new functions for BH_4 will be uncovered, many questions remain about how this pterin functions in the recently described BH_4-dependent systems. With respect to these systems, in particular the one responsible for the synthesis of NO, every one of the myriad functions of this biofactor represents a new physiological role for BH_4. Clearly, therefore, this field, which just a few years ago appeared to be reaching maturity, has now entered a new and exciting growth spurt.

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