Nitric oxide (NO) has a diverse array of physiologic effects which may be either beneficial or detrimental, depending on the anatomic site and rate of NO synthesis. Accordingly, regulation of NO synthesis rates, particularly by the inducible nitric oxide synthase (iNOS), is critical in determining whether the net effect of NO will be good or ill. Rates of NO production might inhibit hepatic conversion of phenylalanine to tyrosine was modified by changes in NO synthase activity, and conversely whether NO syntheses was limited by the rate of phenylalanine conversion to tyrosine and perfused. NO production was decreased only slightly, when flux through PAH was maximized in isolated perfused livers, and in isolated hepatocytes only when BH4 synthesis was inhibited.

Increases in NO synthesis did not reduce tyrosine formation from phenylalanine. Phenylalanine markedly increased biopterin synthesis, whereas arginine had no effect. Thus, basal BH4 synthesis appears to be adequate to support iNOS activity, whereas BH4 synthesis is increased to support PAH activity.

Tetrahydrobiopterin (BH4) is an important cofactor for two hepatic enzymes, inducible nitric oxide synthase (iNOS) and phenylalanine hydroxylase (PAH), and competition for BH4 between the two enzymes might limit hepatic iNOS or PAH activity. To test this hypothesis, we determined whether conversion of phenylalanine to tyrosine was modified by changes in NO synthase activity, and conversely whether NO synthesis was limited by the rate of phenylalanine conversion to tyrosine in rat hepatocytes and perfused livers. NO production was decreased only slightly, when flux through PAH was maximized in isolated perfused livers, and in isolated hepatocytes only when BH4 synthesis was inhibited.

Increases in NO synthesis did not reduce tyrosine formation from phenylalanine. Phenylalanine markedly increased biopterin synthesis, whereas arginine had no effect. Thus, basal BH4 synthesis appears to be adequate to support iNOS activity, whereas BH4 synthesis is increased to support PAH activity.
in cultured cells. The present study is unique in simultaneously studying NO and tyrosine synthesis in both intact liver and isolated hepatocytes.

EXPERIMENTAL PROCEDURES

Animals—Male Sprague-Dawley rats (200–240 g) were fasted for 24 h prior to the experiment, with free access to water, and anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg intraperitoneally). "NOS Induction—To induce iNOS, C. parvum (28 mg/kg intravenously; Wellcome Biotechnology, London, UK) was injected 5 days before liver perfusion. We have previously shown that this treatment results in high levels of nitrite (NO₂⁻) in rat liver perfusion (19), and isolated hepatocytes (14).

Liver perfusion—Livers were perfused in situ with a Krebs-Henseleit-bicarbonate (KHB) buffer (118 mM NaCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 4.7 mM KCl, 26 mM NaHCO₃, 2.5 mM CaCl₂) as described in detail previously (19). All components (from Sigma) were chosen to avoid any contamination with nitrate. The perfusate was oxygenated with a mixture of 95% O₂, 5% CO₂, and the pH was maintained at 7.40.

Arginine and phenylalanine were added to the perfusate as described under "Results." After the surgical procedure, the livers were allowed to recover over 30 min using a nonrecirculation KHB perfusion. Because metabolite measurements are limited by low concentrations in a single pass model, the liver was perfused in a recirculation system during the experimental periods to allow accumulation of the end products of NO (NO₂⁻ + NO₃⁻), tyrosine and total biopterin in the perfusate. The total volume of recirculation was 105 ml (40 ml for reservoir and 65 ml for system and tubing). Three recirculation periods of 20 min each, designated throughout as E₁, E₂, and E₃, were performed. Each recirculation period was preceded by a 5-min rinse period during which the livers were perfused with the same buffer and chemicals as during the following experimental period. The rinse periods allowed the elimination of any toxic metabolites that may have accumulated during the previous period.

Livers (n = 5) were perfused with KHB during the first recirculation period (E₁), KHB + 0.5 mM phenylalanine during the second period (E₂), and KHB + 5 mM phenylalanine in the third period (E₃). In a second group (n = 5), to optimize NO₂⁻ + NO₃⁻ release, 0.1 mM arginine was added to the previous perfusion conditions. Finally, to determine the effect of NO inhibition on tyrosine release, four additional livers were perfused with 5 mM N⁴⁰-monomethyl-L-arginine (L-NMMA) in similar conditions (KHB, KHB + 0.5 mM phenylalanine, KHB + 5 mM phenylalanine).

The viability of perfused livers was assessed by oxygen consumption, perfusate lactate, ampereometry, and lactate dehydrogenase release at the end of each 20-min recirculation period as described previously (19). At the end of each experiment, the livers were weighed and then freeze-dried over 48 h. The wet/dry weight ratios were calculated to determine any swelling induced by the perfusion or by the C. parvum administration. All metabolic results were normalized to the dry weight of the liver.

Hepatocyte Isolation and Cell Cultures—Rat hepatocytes were isolated using a modification of the in situ collagenase B (Boehringer Mannheim) perfusion technique of Seglen (20). Hepatocytes were separated from nonparenchymal cells by differential centrifugation at 50 x g, four times. Hepatocyte purity assessed by microscopy was greater than 98%, and viability consistently exceeded 95% by trypan blue exclusion. Hepatocytes were isolated from three separate rats, 5 days after a C. parvum injection (28 mg/kg intravenously).

Hepatocytes from C. parvum-stimulated rats were plated onto six-well gelatin-coated tissue dishes, 1.5 x 10⁶ cells/well in 2 ml or onto 10-cm Petri dishes at 4.5 x 10⁶ cells/plate in 5 ml of culture medium. Culture medium consisted of Williams’ Medium E (Life Technologies, Inc.) with 0.5 mM L-arginine, 1 µM insulin, 15 mM HEPES, 1-glutamine, penicillin, streptomycin, and 10% low endotoxin dialyzed calf serum (Hyclone, Logan, UT). After a 2-h incubation at 37°C in 95% air and 5% CO₂ to allow attachment, the medium was discarded, and the cells were washed twice with phosphate-buffered saline (Life Technologies, Inc.). Then the cells were incubated for 18 or 24 h in an amino acid-free incubation medium (Earle’s balanced salt solution; Life Technologies, Inc.) with 1 µM insulin, 15 mM HEPES, penicillin, streptomycin, and 2% low endotoxin dialyzed calf serum (Life Technologies, Inc.). In this incubation medium, arginine (0 or 5 mM) and phenylalanine (0, 0.05, 0.1, or 0.5 mM) were added as well as 2,4-diamino-6-hydroxypteridine (DAHP), an inhibitor of GTP-cyclohydrolase I (0 or 10 mM) to some cells. After incubation, culture medium was collected for nitrite (NO₂⁻) and nitrate (NO₃⁻), tyrosine, and biopterin content. Then cells were lysed by freeze thawing for determination of intracellular biopterin, BH₄, and protein content.

Assay—To determine the NO release in the cell supernatant or in the perfusate, NO₂⁻ + NO₃⁻ were measured by a procedure based on the Griess reaction, as described elsewhere (21, 22). Tyrosine release and total biopterin were measured with a high performance liquid chromatography. Oxidized and reduced forms of biopterin were analyzed by the differential oxidation method of Fukushima and Nixon (23). The concentration of protein was determined by the method of Smith et al. (24) using the bicinchoninic acid protein assay reagent (Pierce). Statistical Analysis—The results are expressed as mean ± S.E. In each group, data were analyzed using one-way analysis of variance with repeated measurements. When the results were significant, the mean values were compared by the Fisher test. Comparisons between groups were analyzed using one-way analysis of variance or Student’s t test. Significance was established at a p value <0.05.

RESULTS

C. parvum injection was used to induce hepatic iNOS expression since we have previously characterized the in vivo induction of iNOS in rat liver during chronic inflammation triggered by C. parvum. A high level of iNOS induction is achieved in the intact liver by 5 days postinjection (19), and hepatocytes have been shown to be the major site of NO production in this model (14, 15).

NO₂⁻ + NO₃⁻, Tyrosine, and Biopterin Release in Perfused Livers

In the absence of phenylalanine, tyrosine release (Fig. 1, upper) was low and unaffected by addition of arginine or L-NMMA to the perfusate. Phenylalanine (0.5 or 5 mM) increased tyrosine release, in KHB, KHB + 0.1 mM L-arginine, and KHB + 5 mM L-NMMA perfusions. Tyrosine release was not affected by the addition of arginine, whereas L-NMMA perfusion, which completely blocked NO₂⁻ + NO₃⁻ release, increased tyrosine release at 5 mM phenylalanine concentration compared to KHB perfusion.

Consistent with our previous observations (19), in the absence of added arginine, perfused livers released high levels of NO₂⁻ + NO₃⁻ (Fig. 1, middle). This release was increased by the addition of 0.1 mM arginine, whereas L-NMMA, as already mentioned, completely blocked this release. Addition of phenylalanine (0.5 or 5 mM) slightly decreased NO₂⁻ + NO₃⁻ release in the three conditions (KHB, KHB + 0.1 mM arginine, and KHB + L-NMMA). Both concentrations of phenylalanine induced a marked rise in biopterin release (Fig. 1, lower), whereas neither arginine or L-NMMA had an effect on the release of biopterin from the perfused livers.

When phenylalanine (0.5 and 5 mM) was added to the KHB perfusate, hepatic oxygen consumption significantly increased from 5.8 ± 0.5 to 7.5 ± 0.5 µmol/min/dry weight g, probably as a result of the catabolism of the carbon skeleton of phenylalanine. Addition of arginine or L-NMMA had no effect on the hepatic oxygen consumption. Liver injury increased slightly during the KHB perfusion since lactate dehydrogenase release increased from 17.8 ± 2.7 to 87.2 ± 9.0 milliunits/min/dry weight g from E₁ to E₃. However, this injury was not associated with a decline in the biosynthetic capacity of the livers, since tyrosine and biopterin release were similar during the early (E₁) and late (E₃) perfusion periods. L-NMMA perfusion significantly worsened the hepatic injury compared to the KHB perfusion (257.2 ± 51.7 versus 87.2 ± 9.0 milliunits/min/dry weight g, during E₃ perfusion).

NO₂⁻ + NO₃⁻, Tyrosine, and Biopterin Release in Hepatocytes

In the Absence of Phenylalanine—In order to allow more specific manipulation of the two pathways, the relationships between iNOS and PAH activities and BH₄ availability were also studied in hepatocytes isolated from rats previously in-
Cultured hepatocytes, in amino acid-free incubation medium, released small amount of tyrosine (42.0 ± 7.6 nmol/1.5 × 10⁶ cells/18 h) and moderate levels of \( \text{NO}_2^- + \text{NO}_3^- \) (164.0 ± 4.0 nmol/1.5 × 10⁶ cells/18 h) in the supernatant. Addition of 0.5 mM arginine caused a 2.7-fold increase in \( \text{NO}_2^- + \text{NO}_3^- \) release but had no effect on tyrosine release. NO synthesis in the hepatocytes exhibited some dependence on the \( \text{de novo} \) biosynthesis of BH4, since \( \text{NO}_2^- + \text{NO}_3^- \) release decreased by 16.5% when DAHP was added in the medium. DAHP did not influence the low basal levels of tyrosine synthesis seen in the absence of phenylalanine. Thus, iNOS in the hepatocytes is slightly dependent on \( \text{de novo} \) BH4 biosynthesis but any increase in BH4 requirement for iNOS activity with arginine addition had no consequence on PAH activity.

**In the Presence of Phenylalanine**—In the absence of arginine (Fig. 2, upper), increasing phenylalanine concentrations in the medium increased tyrosine release. In these conditions, the high requirement of BH4 for PAH has only a slight impact on \( \text{NO}_2^- + \text{NO}_3^- \) release at 0.5 mM phenylalanine concentration.

When 0.5 mM arginine was added in the medium (Fig. 2, middle), the addition of phenylalanine had no effect on \( \text{NO}_2^- + \text{NO}_3^- \). When \( \text{de novo} \) BH4 biosynthesis was inhibited by DAHP (Fig. 2, lower), the release of tyrosine associated with phenylalanine addition was dramatically reduced. In the presence of DAHP, the addition of phenylalanine, which only slightly increased tyrosine release, resulted in a concentration-dependent decrease in \( \text{NO}_2^- + \text{NO}_3^- \). This indicates that iNOS activity is likely to be limited only when BH4 levels are dramatically lowered by the inhibition of BH4 synthesis plus increased flux through PAH.

We then tested the capacity of exogenous BH4 to overcome the effects of DAHP and phenylalanine. As shown in Table I, the addition of 100 \( \mu \text{M} \) BH4 increased basal \( \text{NO}_2^- + \text{NO}_3^- \) release and also overcame the inhibitory effects of DAHP and phenylalanine on \( \text{NO}_2^- + \text{NO}_3^- \) release. BH4 also overcame the inhibitory effect of DAHP on tyrosine release in the presence of phenylalanine but did not increase basal levels of tyrosine release in the presence of phenylalanine. As shown in Table II, the addition of phenylalanine increased total biotin release, although intracellular levels were not affected. Also shown is the suppression of intracellular biotin and BH4 levels in DAHP-treated cells and even further reductions when phenyl-
competition for tetrahydrobiopterin in the liver

**Table I**

| Substrates² | NO₂⁻ + NO₃⁻ in supernatant | Tyrosine in supernatant |
|-------------|-----------------------------|-------------------------|
|             | nmol/4.5 × 10⁶ cells/24 h   |                         |
| None        | 2373 ± 90                   | 134 ± 23                |
| Phenylalanine (0.5 mM) | 2493 ± 303                 | 678 ± 156⁶              |
| DAHP (10 mM) | 2080 ± 313                  | 126 ± 5                 |
| Phenylalanine (0.5 mM) + DAHP (10 mM) | 1069 ± 228⁸ | 150 ± 6⁶                |
| BH₄ (100 μM) | 326 ± 32³                   | 143 ± 8                 |
| Phenylalanine (0.5 mM) + BH₄ (100 μM) | 3653 ± 34³⁴ | 465 ± 63                |
| Phenylalanine (0.5 mM) + BH₄ (100 μM) + DAHP (10 mM) | 3536 ± 54⁵⁵ | 709 ± 26                |

² Hepatocytes from *C. parvum*-treated rats were harvested and incubated (4.5 × 10⁶ cells/plate) in the incubation medium (5 ml) containing no amino acids (except 0.5 mM arginine) and substrates as indicated. Results represent mean ± standard error of the mean of two separate cultures of hepatocytes from three separate animals.

⁶ p < 0.05 versus none.

³ p < 0.05 versus phenylalanine.

**Table II**

| Substrates² | Cellular BH₄ | Total biopterin in cells | Total biopterin in supernatant |
|-------------|--------------|--------------------------|-------------------------------|
|             | pmol/mg protein |                       |                               |
| None        | 19.2 ± 1.4  | 31.8 ± 4.3              | 4.9 ± 0.9                     |
| Phenylalanine (0.5 mM) | 17.0 ± 0.7   | 29.1 ± 2.9              | 20.6 ± 2.4                   |
| DAHP (10 mM) | 1.8 ± 0.1⁴  | 5.1 ± 1.0³              | 0.6 ± 0.3²                   |
| Phenylalanine (0.5 mM) + DAHP (10 mM) | 1.0 ± 0.4⁶  | 3.8 ± 0.6⁶              | ND                            |

² Hepatocytes from *C. parvum*-treated rats were harvested and incubated (4.5 × 10⁶ cells/plate) in the incubation medium (5 ml) containing no amino acids (except 0.5 mM arginine) and substrates as indicated. Results represent mean ± standard error of the mean of two separate cultures of hepatocytes from three separate animals.

⁴ p < 0.05 versus none.

⁵ p < 0.05 versus similar substrates without DAHP.

⁶ Not determined.

Alanine is combined with DAHP, which is consistent with the effects of DAHP on NO₂⁻ + NO₃⁻ and tyrosine release.

**Discussion**

BH₄ is an essential cofactor for several enzymes, including aromatic acid hydroxylases and all three NO synthases (13). When enzymes requiring BH₄ coexist in a cell, it is not known how BH₄ is regulated or whether competition for BH₄ will take place, limiting enzyme activity. It is unlikely that BH₄ would be limiting in sites, such as the brain or adrenal glands, where a low output or constitutive NO synthase coexists with aromatic acid hydroxylases, due to the relatively low activity of the constitutive NO synthase enzymes. In this regard, hepatocytes are unique, containing high PAH activity in the presence of activating concentrations of phenylalanine (25) and expressing high levels of the high output or inducible NOS during inflammatory conditions (14). Using both isolated perfused livers and cultured hepatocytes from rats prestimulated to express high levels of iNOS, we studied whether the availability of BH₄ could limit NO or tyrosine synthesis. We found little evidence that competition for BH₄ limited the activity of either PAH or iNOS. This finding was true even if flux through PAH or iNOS were maximized by the addition of excess substrate. Under conditions in which de novo BH₄ production was limited by DAHP, PAH was more susceptible to inhibition than iNOS. Furthermore, only when de novo BH₄ synthesis was inhibited did increased flux through PAH limit NO synthesis. Our results also indicate that, whereas basal BH₄ production was adequate to maintain near-maximal NO synthesis, PAH activity was maintained by increasing biopterin synthesis.

Several reasons may explain that increased flux through PAH has little effect on NO synthesis. First, even though the utilization of BH₄ by PAH is relatively high (1 mol of BH₄ per mol of tyrosine produced) (11), phenylalanine stimulates an increase in biopterin synthesis to meet the BH₄ requirement of PAH (17, 18). This production is likely to take place through the interaction of phenylalanine with the recently described feedback regulator protein for GTP cyclohydrolase I, resulting in increased activity of this rate-limiting enzyme for biopterin biosynthesis (18). Second, the *K₅ₐ* for BH₄ for the NOS is 0.02 to 0.3 μM (4) and minor changes in BH₄ availability would not be expected to have a major effect on NO synthesis. Furthermore, there is no evidence for stoichiometric BH₄ turnover by NO. In fact, Giovanelli *et al.* (4) demonstrated that the NOS produced 18 mol of NO per mol of BH₄. Studies on murine (6) and human (7) iNOS indicate that BH₄ functions to maintain the enzyme in an active configuration. Similar arguments are likely to explain the minimal effect of increased flux through iNOS on the conversion of phenylalanine to tyrosine by PAH. Here, the low utilization of BH₄ by iNOS would be expected to have little impact in the face of increased BH₄ production, resulting from the interaction of phenylalanine and GTP cyclohydrolase I (18). Furthermore, as BH₄ is utilized, any negative feedback exerted by BH₄ on GTP cyclohydrolase I would be removed. This may explain our finding that BH₄ and biopterin content within the cells remain constant. We did observe a slight increase in tyrosine production when NOS activity was blocked with L-NMMA. Whether this is due to increased BH₄ availability is unclear. Since NO can interfere with a number of enzyme systems, such as mitochondrial respiration (26), nonspecific effects on cellular metabolism must also be considered.

Our *in vitro* experiments using excess exogenous BH₄ also suggest important differences in the utilization and actions of this cofactor by the two enzymes. Added BH₄ increased NO₂⁻ + NO₃⁻ release both in the presence and absence of phenylalanine, whereas the addition of Phenylalanine alone, which stimulates BH₄ synthesis, did not. The observation that only extracellular release of biopterin and not intracellular levels of BH₄ increased with phenylalanine addition suggests that the higher quantities of BH₄ produced with phenylalanine addition are rapidly oxidized by PAH and released into medium. Thus, this BH₄ may not be available to iNOS. It is also interesting to note that excess BH₄ suppressed tyrosine release. The reason for this is not readily apparent, but high levels of BH₄ have been
shown to block phenylalanine-induced activation of PAH in a preparation of purified enzyme (28). Our cell culture experiments also show that up to 40% of the bipterin in the cells is not BH4. If it were BH2, it might be expected that the BH2 could inhibit NOS activity (29); however, because BH2 binds to NOS with an affinity 10-fold lower than BH4, it is unlikely to effectively compete for binding to NOS in the presence of higher levels of BH4. Furthermore, much of the measured total bipterin may be in the form of bipterin which does not bind to NOS. Any BH2 in the cultures should be converted to BH4 by dihydrofolate reductase. The high levels of oxidized bipterin in the medium by the cells in culture.

When one considers the major differences in the functions of iNOS and PAH, as well as the differences in the functions of BH4 in each enzyme, it is not surprising that BH4 availability to support the activity of each enzyme is also differentially regulated. PAH is a constitutively expressed enzyme which must respond to rapid changes in ambient phenylalanine concentrations. PAH also has a higher $K_m$ for BH4 than iNOS and utilizes BH4 at a much faster rate. To support the high demand for BH4 in this metabolic pathway, increases in phenylalanine lead to a rapid increase in BH4 availability for the PAH enzyme. Whereas PAH is part of a constitutive metabolic pathway, iNOS, which functions to form NO from arginine, is expressed in hepatocytes only under inflammatory conditions and is a biosynthetic pathway. We have found that arginine for this reaction is most likely derived from exogenous sources, although an endogenous source not associated with the urea cycle also exists (19). As the $K_m$ of iNOS for BH4 is low, ambient intracellular BH4 levels, which appear to remain constant, are adequate to support NOS activity. However, our finding that exogenous BH4 increases NO synthesis in hepatocytes raises the possibility that intracellular levels of BH4 may not support maximal NO synthesis, at least in cultured cells. We recently showed that exogenous BH4 increased NO-dependent killing of Plasmodium falciparum in human hepatocytes (27). Whether these effects are only an in vitro phenomenon and whether exogenous BH4 will be a useful therapeutic approach to modify NO synthesis remains to be determined. Thus, we conclude that the rate of flux through either iNOS or PAH is unlikely to limit the activity of the other enzyme.

Acknowledgment—We are grateful to Sidney M. Morris, Jr., Ph.D., for helpful suggestions and for reviewing the manuscript.

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