Biosynthesis of Biopterin: Adrenergic Cyclic Adenosine Monophosphate–Dependent Inhibition in the Pineal Gland

Abstract. *Pineal glands in organ culture synthesize and release biopterin and are able to maintain concentrations of biopterin occurring in vivo for up to 54 hours in vitro. The intracellular biopterin content is reduced 50 percent by treatment with* l-*norepinephrine or cyclic adenosine monophosphate derivatives, but not by* d-*norepinephrine. This is an indication that biopterin levels are regulated by an adrenergic cyclic adenosine monophosphate–dependent mechanism. The decline in tissue biopterin content, produced mainly by inhibition of biosynthesis, is maximal at 6 hours and is not associated with either an increase in biopterin release or a shift in the reduction state of the biopterin.*

Tetrahydrobiopterin [6-(L-*erythro*-1′, 2′-dihydroxypropyl)-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine] is a naturally occurring reducing agent; the only well-established role for this compound is as a cofactor in the enzymatic hydroxylation of aromatic amino acids by a family of pterin-dependent mixed-function monooxygenases (*1*). Recently there has been some evidence that tetrahydrobiopterin might serve another role in neuroendocrine tissue, especially in the pineal gland (*2*). In this tissue, the concentration of tetrahydrobiopterin is 10- to 300-fold higher than it is in other tissues, and the ratio of tetrahydrobiopterin content to pterin-dependent hydroxylase activity is exceedingly high (*3*).

Many aspects of pineal function are neurally regulated by an adrenergic cyclic adenosine monophosphate (cyclic (AMP)-dependent mechanism (*4*). We now report that the biosynthesis of biopterin in the pineal gland is also regulated by this type of mechanism.

Biopterin content and reduction state were analyzed by reverse-phase high-performance liquid chromatography (HPLC) with the use of fluorescence detection (*5*). Each pineal gland removed from a 4-week-old rat (100 g) contained approximately 30 pmole of biopterin (300 pmole per milligram of protein) (Table 1, experiment 1), as previously reported (*3, 4*).

To investigate the possibility of adrenergic cyclic AMP control of pineal biopterin levels, we studied pineal glands in organ culture (*6*). During the first two 24-hour incubation periods, the amount of biopterin in each gland was not decreased significantly when compared with that in uncultured fresh tissue (Table 1, experiment 1). Moreover, during each of the two 24-hour culture periods a gland releases approximately 40 pmole of biopterin into the incubation medium (*7*), an amount equivalent to the total remaining in the gland. Because biopterin may decompose in the medium, these values may be an underestimate of the total amount of biopterin that is released. After 48 hours of culture, the biopterin in the pineal gland reflects a postsynaptic location because nerve endings have degenerated by this time.

The capacity of the cultured gland to maintain in vivo levels of biopterin despite release into the medium, while suggestive, is not definitive proof of de novo biopterin biosynthesis. To investigate biopterin synthesis, we incubated pineal glands with [^{14}C]guanosine to label guanosine 5′-triphosphate (GTP), the precursor of biopterin. After initial reverse-phase HPLC analysis of tissue and medium samples, fractions containing biopterin were chromatographed again by cation-exchange HPLC (*8*) (Fig. 1F). Glands and media contained a radioactive peak that eluted with the biopterin peak detected by fluorescence. The specific activities of biopterin in the gland and media were identical. Further identification of [^{14}C]biopterin was obtained by the oxidative conversion of [^{14}C]biopterin to [^{14}C]pterin-6-carboxylic acid, as evidenced by the shift in retention time for both pterin fluorescence and radioactivity (Fig. 1G). These observations prove that pineal glands in organ culture synthesize biopterin.

Treatment of pineal glands for 6 hours with *l*-norepinephrine, cyclic AMP derivatives, or the phosphodiesterase inhibitor isobutylmethylxanthine produced approximately a 50 percent decline in tissue biopterin levels; the effect of 0.1 μ*M* *l*-norepinephrine appeared to be stereospecific (Table 1, experiment 3). The clear resolution of biopterin from other pterins and the adrenergic cyclic AMP–induced decline in biopterin content relative to the internal standard of D-*erythro*-neopterin are readily apparent in chromatographs from the HPLC analysis of biopterin in control, *l*-norepinephrine–treated, and dibutyryl cyclic AMP–treated glands (Fig. 1, A to C). These observations indicate that the concentration of biopterin in the pineal gland may be physiologically regulated by an adrenergic cyclic AMP mechanism (*9*).

The time course of the effect of *l*-norepinephrine on biopterin content was also investigated. The decline was gradual, with a significant decrease apparent as early as 3 hours ($P < .05$, Student's *t*-test). The maximum effect was seen at about 6 hours; at 12 hours a significant ($P < .05$) decrease in content was still apparent.

It appeared possible that the adrenergic cyclic AMP–induced decrease in pineal biopterin was a result of increased

Table 1. Biopterin in rat pineal glands and in organ culture medium. Each value, except those for *l*-NE and DBcAMP treatments in experiment 1, is the mean ± standard error of the biopterin content of each of three pairs of glands. Abbreviations: *l*-NE, *l*-norepinephrine; *d*-NE, *d*-norepinephrine; DBcAMP, dibutyryl N^6,O^2-adenosine 3′,5′-monophosphate; pClϕSHcAMP, 8-(parachlorophenylthio)adenosine 3′,5′-monophosphate; IBMX, isobutylmethylxanthine.

Experiment	Treatment	Biopterin (pmole/gland)	
		Gland	Medium
	In vivo		
	Control	31.3 ± 1.70	
	Organ culture		
1	24-hour control	40.7 ± 6.78	47.5 ± 0.85
	48-hour control	28.5 ± 1.82	36.5 ± 2.08
	+6-hour control	35.2 ± 3.18	
	+6-hour *l*-NE (1 μ*M*)	16.5; 16.5	
	+6-hour DBcAMP (1 m*M*)	18.7; 19.1	
2	48-hour control	32.3 ± 2.0	
	+6-hour control	32.6 ± 0.47	8.48 ± 1.27
	+6-hour *l*-NE (1 μ*M*)	23.7 ± 0.68*	
	+6-hour DBcAMP (1 m*M*)	16.5 ± 0.38*	10.2 ± 1.19
	+6-hour pClϕSHcAMP (1 m*M*)	14.0 ± 2.25*	9.75 ± 1.10
3	+6-hour control	38.6 ± 0.89	
	+6-hour *l*-NE (0.1 μ*M*)	20.4 ± 1.87*	
	+6-hour *d*-NE (0.1 μ*M*)	32.7 ± 0.76†	
	+6-hour IBMX (1 m*M*)	15.3 ± 1.65*	

*$P < .001$ when compared with 6-hour control (Student's *t*-test). †$P < .01$ when compared with 6-hour control or with 6-hour *l*-NE (Student's *t*-test).

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Table 2. Effects of *l*-norepinephrine and dibutyryl cyclic AMP on the conversion of [^{14}C]guanosine to [^{14}C]guanosine-5′-triphosphate (GTP) and [^{14}C]biopterin. Pineal glands removed from 150-g male Sprague-Dawley rats (Zivic Miller) were incubated as groups of nine or ten glands for 48 hours before treatment with medium containing [^{14}C]guanosine (40 μCi/ml) having a specific activity of 450 mCi/mmole with or without *l*-norepinephrine or dibutyryl cyclic AMP. After 6 hours, glands were removed and the specific activity of biopterin was determined (legend to Fig. 1F). The specific activity of GTP was measured on separate samples by anion exchange HPLC (Partisil SAX, Whatman) after ether extraction of a 10 percent trichloroacetic acid supernatant (10 glands per 350 μl). The solvent system was 0.5*M* KCl, 0.25*M* KH_2PO_4, *p*H 4.5, with a flow rate of 1.5 ml/min. The eluate was monitored for absorbance at 254 nm; then fractions were collected and radioactive content was determined. The number pairs represent two experiments, with each value based on the analysis of a homogenate of ten glands.

Treatment	Biopterin (pmole/gland)	[^{14}C]Biopterin		GTP (pmole/gland)	[^{14}C]GTP	
		(nCi/gland)	(nCi/nmole)		(nCi/gland)	(nCi/nmole)
Control	43.7, 44.9	0.38, 0.31	8.7, 6.9	241, 252	3.06, 2.45	12.7, 9.70
l-Norepinephrine (10 μ*M*)	34.6, 33.9	0.19, 0.21	5.5, 6.2			
Dibutyryl cyclic AMP (1 m*M*)	31.3, 29.2	0.05, 0.07	1.6, 2.4	229, 258	2.38, 2.49	10.4, 9.65

release. However, analysis of the medium from glands treated with cyclic AMP derivatives indicated that the decrease in tissue biopterin was not associated with an increase in medium biopterin (Table 1, experiment 2). Other mechanisms leading to a decrease in biopterin content include decreased synthesis or increased catabolism or a combination of both.

The possibility of an adrenergic cyclic AMP–dependent inhibition of biopterin synthesis was investigated by studying the effects of *l*-norepinephrine or dibutyryl cyclic AMP on the conversion of [^{14}C]guanosine to [^{14}C]biopterin. The specific activity of biopterin was determined by sequential reverse-phase and cation-exchange HPLC (legend to Fig. 1F). The specific activity of GTP, the biopterin precursor, in control and dibutyryl cyclic AMP–treated glands was determined by anion-exchange HPLC (legend to Table 2). In this experiment, biopterin levels and [^{14}C]biopterin content were decreased 23 and 43 percent, respectively, by *l*-norepinephrine, and 32 and 82 percent, respectively, by dibutyryl cyclic AMP (Table 2). The specific activity of biopterin synthesized by glands treated with *l*-norepinephrine or dibutyryl cyclic AMP therefore declined by 25 and 75 percent. In contrast to its effect on the specific activity of biopterin, dibutyryl cyclic AMP did not cause a significant decrease in the specific radioactivity of GTP. The specific activity of GTP was used to calculate a minimum rate of biopterin synthesis; control glands synthesize approximately 7 pmole of biopterin per hour. The entire biopterin content of the glands therefore appears to turn over within 6 hours. The apparent rates of synthesis in the presence of *l*-norepinephrine and dibutyryl cyclic AMP were 3.9 and 1.2 pmole of biopterin per hour, respectively. Because these rates of synthesis for treated glands cannot account for the levels of biopterin remaining in the glands or medium after 6 hours, biopterin degradation might also be inhibited, especially by the treatment with dibutyryl cyclic AMP. Nonetheless, the adrenergic cyclic AMP–induced decline in pineal biopterin levels appears to be primarily the result of an inhibition of biopterin biosynthesis.

To test the possibility that *l*-norepinephrine might have altered the reduction state of biopterin in the gland, we compared the oxidation products formed at *p*H 1 (Fig. 1, A and B) with those formed at *p*H 13 (Fig. 1, D and E). At *p*H 1, iodine oxidation converts 7,8-dihydro-, quinonoid dihydro-, and tetrahydrobiopterin to biopterin, whereas at *p*H 13, only 7,8-dihydrobiopterin is converted to biopterin. At *p*H 13, quinonoid dihydro-

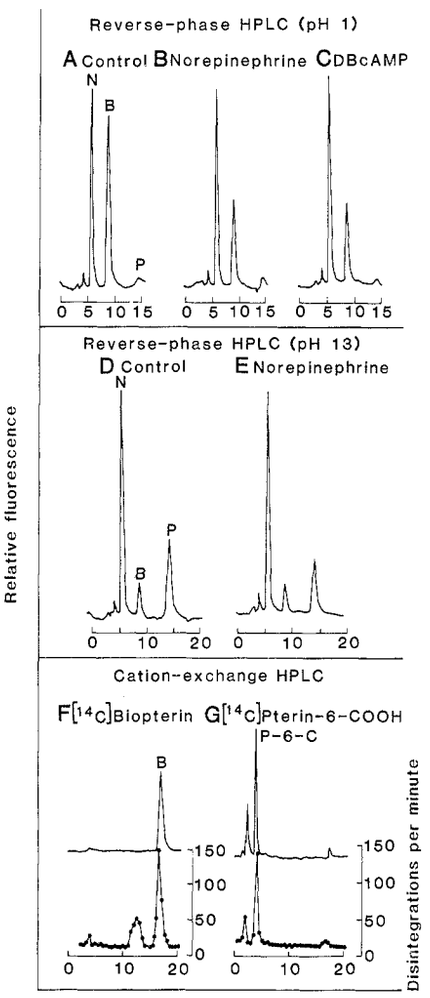

Fig. 1. Analysis by high-performance liquid chromatography of pineal biopterin content and reduction state. The method of Fukushima and Nixon was used (*5*) with an ODS-2 reverse-phase C18 column (Whatman). The solvent system was methanol and water (1 : 19), and the flow rate was 1 ml/min; chromatography was performed at room temperature (approximately 25°C). The eluate was monitored for fluorescence (excitation at 330 to 380 nm; emission at 460 to 600 nm). Biopterin content was quantified by peak area analysis. An internal standard of D-*erythro*-neopterin was used throughout to correct for biopterin recovery, which averaged 80 percent (*5*). Biopterin (*B*), D-*erythro*-neopterin (*N*), pterin (*P*). (A to C) Chromatographic recordings of preparations of pineal glands oxidized at *p*H 1 with iodine. For experimental details see the text and (*5*). (D and E) Chromatographic recordings of preparations of pineal glands oxidized at *p*H 13 with iodine. For experimental details see the text and (*5*). (F) [^{14}C]Biopterin biosynthesis by cultured pineal glands. Pineal glands were removed from 150-g male Sprague-Dawley rats (Zivic Miller) and were incubated as groups of nine glands for 24 hours in medium containing [^{14}C]guanosine (10 μCi/ml; specific activity, 450 mCi/mmole). Glands and media were separated and after reverse-phase HPLC (*5*), fractions containing biopterin fluorescence were pooled, lyophilized, and chromatographed again by cation-exchange HPLC (Partisil SCX, Whatman). The solvent system was 3 m*M* phosphoric acid, 7 percent methanol, and 1 percent acetonitrile, and the flow rate was 1.5 ml/min (*8*). The eluate was monitored for fluorescence, fractions were collected, and radioactive content was determined. The data presented are from a tissue sample. Identical results were obtained with media. Biopterin fluorescence and radioactivity eluted together. (G) Further identification of [^{14}C]biopterin. [^{14}C]Biopterin eluted from the cation exchange column was lyophilized and then oxidized under basic conditions with $KMnO_4$ to produce [^{14}C]pterin-6-carboxylic acid (*P-6-C*). Residual $KMnO_4$ was eliminated with excess methanol, and samples again chromatographed on cation-exchange HPLC. [^{14}C]Pterin-6-carboxylic acid was identified by comparison with standards of biopterin and pterin-6-carboxylic acid that had been treated with $KMnO_4$. The shift in fluorescence and radioactivity are identical to that in (F).

and tetrahydrobiopterin are converted to pterin (2-amino-4-hydroxypteridine) and nonfluorescent material. The ratio of biopterin content after oxidation at *p*H 13 to that after oxidation at *p*H 1 is therefore an indication of the relative amount of biopterin originally present in the sample as quinonoid dihydro- or tetrahydrobiopterin (*5*). Since it is unlikely that substantial amounts of quinonoid dihydrobiopterin exist in vivo, the results of this analysis indicate that more than 85 percent of the biopterin was in the tetrahydro form in both control and treated glands. The rest was either 7,8-dihydro- or oxidized biopterin.

The mechanism and physiological importance of adrenergic cyclic AMP–dependent regulation of pineal tetrahydrobiopterin biosynthesis are unknown. The possibility that a decrease in pineal content of reduced biopterin would decrease hydroxylation is not supported by the available data (*10*), which indicate that tryptophan hydroxylation within pineal glands in the presence of physiological concentrations of tryptophan is not decreased by adrenergic cyclic AMP stimulation in organ culture. Adrenergic cyclic AMP stimulation increases the activity of *N*-acetyltransferase by an induction-activation mechanism. The gradual nature of the decline in biopterin levels in response to adrenergic stimulation is similar to the gradual nature of the increase in the activity of *N*-acetyltransferase (*11*). The inhibition of biopterin biosynthesis and the resulting decline in biopterin content could be related to this increase in enzyme activity. Such a possibility is of particular interest because tetrahydrobiopterin is a reducing agent and because of indications that redox changes may play a role in the rapid neurally controlled turn-off of pineal *N*-acetyltransferase activity (*4, 12*).

Further study of the adrenergic cyclic AMP–induced inhibition of pineal biopterin biosynthesis may lead not only to an increased understanding of the regulation of biopterin synthesis, but also to the discovery of a new function of biopterin in the pineal gland and possibly a new role for the compound in other tissues.

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7. The term release is not meant to imply that a specific exocytotic secretion process exists, although such a process may eventually be found to function in this tissue. The possibility that the release of biopterin that we have detected is a result of necrosis or autolysis of the tissue is improbable because neither total biopterin or the rate of release of biopterin decreases during the 54-hour period studied. Histological examination has also indicated that there is little necrosis in glands taken from 100-g animals and incubated for 48 hours under the conditions used for organ culture.
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Carotenoids Function in Photoperiodic Induction of Diapause in a Predacious Mite

Abstract. *Predacious mites fed for two generations solely on the eggs of wild-type spider mites responded normally to short day lengths by entering diapause. However, predacious mites fed for two generations on eggs of albino spider mites, which are completely devoid of carotenoids, did not respond to short-day photoperiods. Apparently carotenoids are essential for photoperiodic induction; possibly a carotenoid or carotenoid derivative functions as the photopigment concerned in photoperiodic light reception in these mites.*

The reception of photoperiod in terrestrial arthropods presupposes the presence of a photoreceptor pigment, which is probably located in the central nervous system (*1*). Various techniques have been used in attempts to identify the pigment (or pigments) involved in photoperiodic light reception in insects and mites, such as action spectra studies, isolation and characterization of pigments from insect heads, and rearing of insects on artificial diets deficient in certain colored compounds (*2*). However, convincing evidence regarding the class of pigments involved in insect photoperiodism has not been produced. Evidence for the participation of carotenoids or derivatives of carotenoids in the photoperiodic induction of diapause has now been obtained for spider mites, by means of a genetic approach (*3*). Diapause induction appeared to be disturbed in albino spider mites, which lack all pigmentation due to a block in the uptake of carotenoids from the food plant (*4*). However, photoperiodic induction was normal in albino spider mites originating from hybrid mothers, which possess the wild pigmentation. Apparently minimal amounts of carotenoids of maternal origin suffice to restore the capability to diapause in albino mites. Using a technique based on the feeding of predacious mites with eggs of normally pigmented (wild-type) and albino spider mites, we demonstrate here that carotenoids are essential for the photoperiodic induction of diapause in these predacious mites also.

The predacious mite, *Amblyseius potentillae*, exhibits a facultative reproductive diapause, induced by short-day

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