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Biosynthesis of Biopterin by Two Clones of Mouse Neuroblastoma

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SUMMARY

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Mouse neuroblastoma clones N-18 and N-1E were found to incorporate radioactivity from [2-^{14}C]guanosine into biopterin or its oxidation product, pterin-6-carboxylic acid. When similar incubations were carried out in the presence of [8-^{14}C]guanosine little or no incorporation of radioactivity into the pterins occurred. These results indicate that the mammalian neuroblastoma cell line can synthesize biopterin from a purine precursor. The lack of incorporation of radioactivity into the pterins from [8-^{14}C]guanosine indicates that the mammalian biosynthetic pathway may be similar to that found in bacteria.

INTRODUCTION

Tyrosine hydroxylase (EC 1.10.3.1), phenylalanine hydroxylase (EC 1.99.1.2), and tryptophan hydroxylase (EC 1.99.1.4) require reduced pterins as cofactors. Evidence has been reported that tetrahydrobiopterin may be the cofactor for all three of these mammalian enzymes (1-4). Tyrosine hydroxylase, the rate-limiting enzyme for catecholamine biosynthesis (5), is subject to several regulatory mechanisms (6). One involves end product inhibition by catecholamines (7). This inhibition is competitive with the reduced pterin cofactor (8). Thus changes in the intraneuronal concentration of tetrahydrobiopterin could influence the activity of tyrosine hydroxylase *in vivo* and, as a result, the rate of catecholamine biosynthesis. It was therefore of interest to investigate whether mammals could synthesize pterins.

Pteridine biosynthesis has been intensively studied in bacterial, insect, and amphibian systems (for review, see ref. 9). The initial step in the bacterial pathway is cleavage of the imidazole ring of guanosine triphosphate and loss of carbon 8 as formate (10, 11). This is followed by rearrangement and ring closure to form D-*erythro*-dihydroneopterin triphosphate (11) (Fig. 1). In contrast, few data are available regarding mammalian pteridine biosynthesis (12-15).

This paper presents evidence that two clones of mouse neuroblastoma cells can synthesize biopterin.

MATERIALS AND METHODS

Mouse neuroblastoma cell clones N-18 and N-1E derived from C-1300 (16) were supplied by the laboratories of Dr. M. Nirenberg, National Institutes of Health, and Dr. A. Blume, Roche Institute of Molecular Biology, respectively. Fetal calf serum and growth medium were purchased from Gibco. Male germfree rats, CD strain, 150-180 g, were purchased from Charles River Laboratories, Boston. [2-^{14}C]Guano-

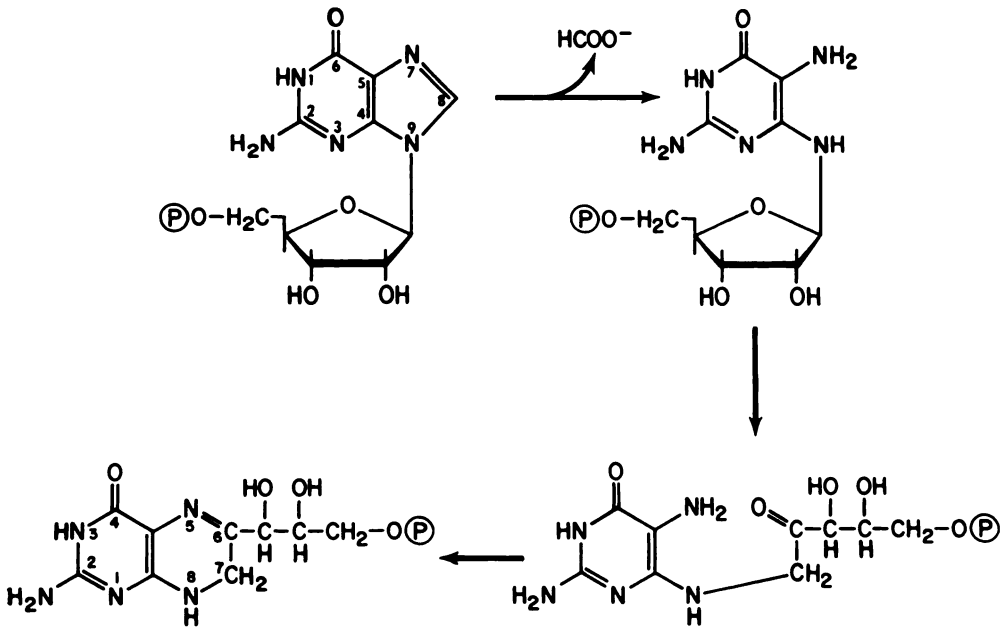

FIG. 1. *Pathway for pterin biosynthesis from guanosine triphosphate in bacteria.* Ⓟ = triphosphate.

sine (51.5 mCi/mmole), [8-^{14}C]guanosine (49 mCi/mmole), [2-^{14}C]guanine (20 mCi/mmole), and [8-^{14}C]guanine (25 mCi/mmole) were purchased from Schwarz/Mann. Tetrahydrobiopterin was obtained from Hoffmann-La Roche, Inc., and pterin-6-carboxylic acid, from Aldrich Chemical Company. Charcoal Norit A (Fisher), Ecteola cellulose (Whatman Dowex 1-X8, formate form, 200–400 mesh, Bio-Rad), and Sephadex G-25 (superfine, Pharmacia) were used.

Cell cultures. Stock cultures of neuroblastoma were maintained in Falcon flasks in a growth medium composed of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin G (10 μg/ml), and streptomycin (10 μg/ml) in an atmosphere of 10% CO_2 and 90% air at 37°. The cells were allowed to grow to about 80% of confluency in 700-ml glass prescription bottles. The medium was then decanted from two bottles and replaced with 50 ml of sterile fresh medium containing 40 μCi of either [2-^{14}C]guanosine or [8-^{14}C]guanosine. The N-18 cells were then allowed to grow for an additional 2 days, and the N-1E cells for 1 day. Following incubation the flasks were shaken vigorously to suspend the cells into the medium. A small sample was taken for determination of cell number and viability by nigrosine exclusion. All the cultures contained about 3×10^8 cells/flask. The viability was about 80% for the N-18 clone and 55% for the N-1E clone. To the cell suspension was added a 3 M solution of trichloracetic acid to a final concentration of 5%. The cells were shaken for 10 min, sonicated (Bronwill Biosonic III) for 1 min, and centrifuged at 30,000 × *g* for 10 min. The supernatant fluid was kept frozen at −17° until analyzed.

Pterin analysis. To the TCA[1] supernatant solutions, tetrahydrobiopterin carrier was added as indicated in the tables. Reduced pterins were oxidized with iodine according to Rembold *et al.* (17). After completion of the reaction, excessive iodine was reduced with ascorbic acid. The pterins were then freed from the acid and the salts of the solution by adsorption on 300 mg of charcoal. After filtration and washing with three 5-ml portions of water the compounds were desorbed from the charcoal with 15 ml of a solution of phenol-

[1] The abbreviation used is: TCA, trichloracetic acid.

saturated water–ammonia (5:1); subsequently the ammonia was evaporated and the phenol was removed by extraction with 10 ml of ether. At this stage the recovery of biopterin was routinely 30–35% of the carrier tetrahydrobiopterin. The aqueous solution was made alkaline with NaOH (pH 11–12) and then oxidized with a few drops of saturated $KMnO_4$ solution (18). This procedure results in the formation of pterin-6-carboxylic acid from biopterin (observed yield, 90%) and other 6-substituted pterins (18). Excess permanganate was reduced with several drops of methanol, as indicated by decolorization. The solution was neutralized, evaporated to a small volume, and chromatographed on a 20-ml Sephadex G-25 column (1.5 cm). The eluted pterin-6-carboxylic acid was then applied to a 4-ml Ecteola column (1.5 cm). The column was rinsed with 25 ml of water, and the pterin was then eluted with 1% formic acid. The pterin-6-carboxylic acid-containing fractions, detected by their blue fluorescence, were combined, evaporated to dryness, and subjected to paper chromatography.

The above procedure was modified in order to detect labeled biopterin. After iodine oxidation and charcoal treatment the biopterin-containing solution was chromatographed on a 10-ml Dowex 1-X8 (formate form, 200–400 mesh) column (1.5 cm) (18). The carrier biopterin-containing fractions of the water elute, detected again by their blue fluorescence, were combined, concentrated, and subjected to two or three successive paper chromatographic separations (see tables). The biopterin was oxidized with $KMnO_4$ to pterin-6-carboxylic acid. This compound was then subjected to paper chromatography.

Paper chromatography. The pterins were analyzed by as many as six consecutive paper chromatographic steps in six different systems. After each run the blue fluorescent spots were cut out and eluted from the paper with three 5-ml portions of hot water. The combined water solutions were concentrated and applied to the paper strip again for the next run. After each step the recovery of the pterins was determined spectrophotometrically by comparison of the spectra with standard solutions of biopterin or pterin-6-carboxylic acid. The radioactivity of a portion of each sample was measured in a Packard liquid scintillation spectrometer. Counting efficiency, as determined by an internal standard, was 85–87%. The solvent systems used and the corresponding R_F values for pterin-6-carboxylic acid and biopterin are listed in Table 1. In all systems the R_F values of the products of tetrahydrobiopterin oxidation were identical with standards of pterin-6-carboxylic acid or biopterin.

RESULTS

The method for detecting ^{14}C-labeled biopterin synthesized from the [^{14}C]guanosine precursor was developed in order to encompass all possible oxidation-reduction forms of this pterin that can occur in the cell. Air oxidation of tetrahydrobiopterin results in a variety of products but does not produce biopterin in a reasonable yield (17). The best oxidation procedure found for tetrahydrobiopterin appears to be iodine oxidation in acidic solution, which results in 30% conversion to biopterin (17); dihydrobiopterin will be oxidized by iodine nearly quantitatively. Therefore, in order to screen all 6-substituted pterins that can occur in the biosynthetic pathway to biopterin, the oxidation procedure used in-

TABLE 1

Paper chromatography of pterin-6-carboxylic acid and biopterin

System	Pterine-6-carboxylic acid	Biopterin
	R_F	R_F
A. 1-Butanol–acetic acid–water, 20:4:6	0.09	0.24
B. 1-Propanol–1% ammonia, 2:1	0.14	0.32
C. 4% sodium citrate	0.41	0.59
D. 3% ammonium chloride	0.53	
E. 2-Butanol–formic acid–water, 8:2:5	0.21	
F. Pyridine–ethyl acetate–water, 4:3:3	0.27	

volved iodine oxidine followed by alkaline permanganate oxidation. This procedure resulted in pterin-6-carboxylic acid as the single pterin formed from 6-substituted pterins.

Table 2 shows that the neuroblastoma clone N-18 is capable of synthesizing 6-substituted pterins from guanosine. When the growth medium contained [2-^{14}C]guanosine, radioactivity was incorporated into the pterin ring and recovered as ^{14}C-labeled pterin-6-carboxylic acid. When [8-^{14}C]guanosine was used, almost no radioactivity was incorporated into the pterin. This finding is consistent with the known biosynthetic pathway of pterins in bacteria. As shown in Table 3, similar results were obtained when pterin synthesis was investigated in clone N-1E. Again, when [2-^{14}C]guanosine was used as the precursor, the cells incorporated radioactivity into the pterin. However, with [8-^{14}C]guanosine no incorporation of radioactivity into pterin-6-carboxylic acid could be demonstrated.

TABLE 2

Pterin biosynthesis from guanosine in N-18 cell clone of mouse neuroblastoma

Cells were incubated with [2-^{14}C]- or [8-^{14}C]guanosine as described under MATERIALS AND METHODS. The total TCA supernatant volume was 120 ml. To 80-ml aliquots were added 200 μg (0.83 μmole) of tetrahydrobiopterin, and chromatography was carried out as described in the text. Over-all recovery of pterin-6-carboxylic acid after the last step (system F) was 1%.

Step	Specific radioactivity of pterin-6-carboxylic acid fractions	
	Precursor [2-^{14}C] guanosine	Precursor [8-^{14}C] guanosine
	cpm/μmole	
I_2 and $KMnO_4$ oxidation	9,315,000	9,320,000
Sephadex G-25	4,140,000	4,140,000
Ecteola	1,050,000	455,000
Successive paper chromatography		
System A	124,000	39,300
System B	124,000	6,200
System C	68,300	2,070
System D	60,000	414
System E	51,800	
System F	51,800	

In this experiment an additional control was used. To exclude the possibility of a radioactive pterin impurity in the guanosine, a control solution was prepared containing 12 μCi of [2-^{14}C]guanosine and 200 μg of tetrahydrobiopterin (0.83 μmole) in 80 ml of 5% TCA. This solution was treated in the same way as the TCA extract of clone N-1E. Following purification it was possible to dissociate the radioactivity of this control completely from pterin-6-carboxylic acid (Table 3).

Since the oxidation procedure used will convert other 6-substituted pterins to the carboxylic acid, the results of Tables 2 and 3 do not exclude the possibility that the radioactive pterin-6-carboxylic acid was formed from a 6-substituted pterin other than biopterin. In order to determine whether biopterin was in fact being synthesized, the remaining TCA extracts derived from either [2-^{14}C]guanosine or [8-^{14}C]guanosine incubations were combined, carrier amounts of nonradioactive tetrahydrobiopterin were added, and the solution was analyzed for labeled biopterin. To accomplish this the $KMnO_4$ oxidation step was omitted. Following iodine oxidation and charcoal treatment the biopterin solution was chromatographed on Dowex 1 and the entire water elute was subjected to successive paper chromatographic steps. No blue fluorescent spots other than that of biopterin could be found on the first paper chromatogram. This did not exclude the existence of other pterins in subdetectable amounts. A control sample containing 180 μg of tetrahydrobiopterin (0.75 μmole) and 7 μCi of [2-^{14}C]guanosine in 5% TCA was also subjected to the entire procedure. After three successive paper chromatographic steps the biopterin derived from the [2-^{14}C]guanosine incubation had a constant specific radioactivity (Table 4). The radioactive spot eluted from the last chromatogram was then oxidized with $KMnO_4$. The radioactivity again migrated together with pterin-6-carboxylic acid in three paper chromatographic steps and remained at a constant specific radioactivity.

Consistent with our above findings is the observation that the radioactivity of the control and of the neuroblastoma extracts incubated with [8-^{14}C]guanosine could be dissociated from both biopterin and its oxidation product, pterin-6-carboxylic acid.

It was of interest to look for biopterin synthesis in an intact animal. For this reason germfree rats were given an intraperitoneal injection with 50 μCi of [2-^{14}C] or [8-^{14}C]guanine. The procedure was carried out using sterile technique, and the animals were maintained during the course of the experiment in a sterile laminar flow environment. Either 5 or 23 hr later the animals were killed. The livers, kidneys, adrenals, and superior cervical ganglia

TABLE 3

Pterin biosynthesis from guanosine in N-1E cell clone of mouse neuroblastoma

Cells were incubated with [2-^{14}C]- or [8-^{14}C]guanosine as described under MATERIALS AND METHODS. To 85-ml aliquots were added 200 μg (0.83 μmole) of tetrahydrobiopterin, and chromatography was carried out as described in the text. A 5% TCA control solution contained 12 μCi of [2-^{14}C]guanosine and 200 μg of tetrahydrobiopterin. Over-all recovery after the last step (system C) was 1%.

Step	Specific radioactivity of pterin-6-carboxylic acid fractions		
	Precursor [2-^{14}C]guanosine	Precursor [8-^{14}C]guanosine	Control
		cpm/μmole	
I_2 and $KMNO_4$ oxidation	29,000,000	13,500,000	41,400,000
Sephadex G-25	21,470,000	8,280,000	23,800,000
Ecteola	2,480,000	662,000	869,000
Successive paper chromatography			
System E	321,000	49,700	37,300
System F	145,000	8,280	10,300
System B	82,800	3,110	1,040
System A	82,800	0	0
System C	82,800		

TABLE 4

Biopterin biosynthesis from guanosine in N-18 and N-1E cell clones of mouse neuroblastoma

Of the N-18 TCA supernatant (Table 2) 40 ml, and of the N-1E TCA supernatant (Table 3) 30 ml, were used. Aliquots from both clones, containing the same isotope, were combined, and 360 μg (1.5 μmoles) of tetrahydrobiopterin were added to each sample. A 5% TCA control solution contained 180 μg (0.75 μmole) of tetrahydrobiopterin and 7 μCi of [2-^{14}C]guanosine. The procedure was then continued as described under MATERIALS AND METHODS. Recovery of biopterin after step A was 8%, and final recovery of pterin-6-carboxylic acid was 2%.

Paper chromatographic system[a]	Specific radioactivity			
	Fractions	Precursor [2-^{14}C]guanosine	Precursor [8-^{14}C]guanosine	Control
		cpm/μmole		
C	Biopterin	711,000	877,000	2,252,000
B		52,000	28,400	213,300
A		56,900	11,900	18,960
C	Pterin-6-carboxylic acid	57,900	1,040	1,040
A		56,900	1,040	1,040
B		55,900	1,040	1,040

[a] The first three steps preceded $KMnO_4$ oxidation of biopterin; the last three followed it.

were removed, weighed, homogenized in 5% TCA, and centrifuged at 30,000 × *g* for 10 min. The supernatant fractions following addition of 200 μg of carrier tetrahydrobiopterin were analyzed for incorporation of radioactivity into biopterin or pterin-6-carboxylic acid. However, it was not possible to detect significant incorporation of radioactivity into either pterin with any of the tissues examined.

DISCUSSION

In bacteria the cleavage of the imidazole ring of GTP and the loss of carbon 8 as formate are the initial steps of pterin synthesis (10, 11). The first detectable pterin in the pathway is D-*erythro*-dihydroneopterin triphosphate (11). Our results indicate that mammalian neuroblastoma cells can also synthesize pterins from a purine. As in the bacterial system, the initial step of the pathway probably involved cleavage of the imidazole ring and loss of carbon 8. Support for this conclusion is based on the finding that cell cultures grown in the presence of [8-^{14}C]guanosine incorporated little or no radioactivity into biopterin or pterin-6-carboxylic acid, whereas appreciable incorporation resulted when [2-^{14}C]guanosine was used. From the current findings it is not possible to conclude that GTP is the immediate precursor for mammalian pterin synthesis, as is the case in bacteria. In order to investigate this point attempts will be made to demonstrate a mammalian GTP cyclohydrolase.[2] If these attempts prove successful, it may then be possible to establish the exact nature of the purine precursor.

In a preliminary communication Fukushima and Shiota (13) reported that cell cultures of neuroblastoma and Chinese hamster ovary cells produced a biopterin-like substance, based on the results of the *Crithidia* bioassay. In addition, the same authors have recently reported incorporation of [2-^{14}C]guanosine but not of [8-^{14}C]guanosine into biopterin isolated from cultures of Chinese hamster cells (15). Sugiura and Goto (14) recently reported that, following an intravenous injection of uniformly labeled GTP to mice or rats radioactive biopterin could be isolated from the urine.

Work in our laboratory has confirmed the result of Sugiura and Goto. Following the administration of [2-^{14}C]guanine to rats we also could demonstrate the urinary excretion of radioactive biopterin. In addition we have shown this to occur in germ-free animals, thus excluding any possibility of bacterial pterin synthesis. A lack of incorporation of radioactivity into biopterin was found when [8-^{14}C]guanine was administered.

In the intact rat biopterin synthesis could not be demonstrated in any of the tissues examined. This does not itself exclude synthesis in these organs. Our failure to detect pterin synthesis in liver, kidney, adrenals, and sympathetic ganglia may have been due to rapid dilution of the radioactive precursor or its metabolic products, to rapid turnover of biopterin, resulting in dilution of the newly synthesized radioactive pterin or, conversely, a low biopterin synthesis rate, or to the poor recovery of the pterin inherent in our isolation procedure.

As pointed out by Musacchio *et al.* (19), the rate of catecholamine synthesis in rat brain *in vivo* is almost two orders of magnitude less than the activity of tyrosine hydroxylase *in vitro*. Those authors suggested that at least part of this effect may be due to a suboptimal concentration of tetrahydrobiopterin *in vivo* (19). An alteration in the rate of synthesis could affect the rate of catecholamine synthesis by making more or less cofactor available to tyrosine hydroxylase. It will be of interest to determine whether mammalian pterin biosynthesis is subject to regulatory influences. That is, would biopterin systhesis increase or decrease in a compensatory manner in relation to the demand for catecholamine synthesis?

[2] The enzyme from *Lactobacillus plantarum* has been called dihydroneopterin synthetase (19).

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