probably indicates structural damage to the enzyme itself. This again may represent a significant aspect of the biochemical aberration in hypertension.

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7. The StHR rats (Sprague-Dawley) had a mean blood pressure of 167.0 ± 1.7 mm-Hg, which was significantly different ($P < .01$) from their controls, which had a mean blood pressure of 111.3 ± 2.3 mm-Hg.
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Cerebral Hydroxylases: Stimulation by a New Factor

Abstract. *Cerebral tryptophan 5-hydroxylase and tyrosine hydroxylase are stimulated by a solution of lyophilized, protein-free 30,000g supernatant of brain homogenates (concentrate 1). This preparation can be further purified by passage through Sephadex G-75 and phosphocellulose columns to yield concentrate 2. Unlike concentrate 1, concentrate 2 is unstable after several days of storage even at −27°C. Neither preparation is active without the presence of a reduced pteridine cofactor. The stimulating factor appears to be thermostable, alkali-labile, dialyzable, and light-sensitive.*

Efforts to purify cerebral tryptophan 5-hydroxylase (E.C. 1.99.1.4) led us to the observation that protein-free supernatants or filtrates of cerebral homogenates contain a factor that, in nanogram amounts, stimulates hydroxylation of tyrosine or tryptophan in vitro (*1, 2*). This factor was obtained from the 30,000*g* supernatant of brain homogenates after precipitation of protein with 60 percent ethyl alcohol. Unlike the protein factor (*3*) that stimulates hepatic phenylalanine 4-hydroxylase (E.C. 1.14.3.1), this factor appears to be specific for cerebral hydroxylases of L-tryptophan and L-tyrosine. The existence of this factor has been confirmed (*4*). In view of the difficulties attendant to purification of cerebral hydroxylases (*2, 5, 6*), the knowledge of stimulatory factor, even before its ultimate characterization, will be of benefit to others.

Rats were killed by decapitation, and their brains were removed immediately in a cold room (4°C). Brains were homogenized in three volumes of 0.01*M* tris(hydroxymethyl)aminomethane (tris) acetate buffer (*p*H 7.6) containing $10^{-3}M$ 2-mercaptoethanol (or dithiothreitol), and the homogenate was centrifuged at 30,000*g* for 30 minutes at 2°C. The supernatant was cooled to −2°C, and an equal volume of cold absolute ethanol was added. The suspension was centrifuged, and the supernatant was flushed with nitrogen gas (pumped over water) for 2 minutes. It was then immersed in boiling water long enough to bring it to 70° to 80°C and diluted with an equal volume of oxygen-free boiling water. After the suspension was rapidly cooled in ice, the denatured protein was removed by centrifugation. The supernatant was concentrated under vacuum at low temperature to one-fifth of its original volume and extracted three times with an equal volume of peroxide-free ethyl ether. The aqueous solution, under nitrogen, was passed through a nitrocellulose filter [80 or 100 mμ (Millipore)]; Amicon filters inactivated the factor. The filtrate was lyophilized and the residue taken up in water. To standardize the assay conditions, the stock solution was diluted such that 50 μl diluted to 3.0 ml had an absorbance of 0.550 at 255 nm (concentrate 1). At this stage, portions of this solution stimulated partially purified cerebral tryptophan 5-hydroxylase by 40 to 50 percent. This activity remained for several weeks if the solution was sealed under nitrogen and stored at −27°C. The solution was purified further by passage through a 1 by 25 cm column of Sephadex G-25 (*7*) or G-75 equilibrated with 0.1 percent mercaptoethanol. The factor, in a manner similar to pteridines, was eluted with 0.1 percent mercaptoethanol. The fractions corresponding to pteridines, assessed by fluorescence at 450 nm, were lyophilized and applied at *p*H 7.0 on a 1 by 10 cm column of phosphocellulose (H^+) (1.76 meq/g, Schleicher and Schuell) and eluted again with 2-mercaptoethanol (*8*), yielding concentrate 2. All the above procedures must be done in the absence of light.

Tryptophan 5-hydroxylase was purified by two methods (*2, 5*), and tyrosine hydroxylase was prepared from rat brain (*6*). The activity of these enzymes was measured by separation of 5-hydroxy[^{14}C]tryptamine or [^{14}C]dopamine, respectively (*9*). Pteridine content of concentrates 1 and 2 was measured with the pteridine-dependent phenylalanine 4-hydroxylase of *Pseudomonas* (*10*). The stimulating factor

Table 1. Reactivation of tryptophan 5-hydroxylase by concentrate 1 (C1). All assays contained 3 mg of protein; tris-acetate buffer (*p*H 7.6), 50 m*M*; 2-mercaptoethanol, 1 m*M*; pargyline, 0.2 m*M*; L-tryptophan (including L-[2-^{14}C]tryptophan, 1.1×10^6 disintegrations per minute), 0.088 m*M*; and $DMPH_4$, 0.46 m*M*. Formation of 5-hydroxy[^{14}C]tryptamine was measured. Conditions of dialysis are given in the text.

Enzyme preparation	Specific activity (nmole mg^{-1} hr^{-1})
30,000*g* supernatant	0.50
30,000*g* dialyzed	.12
30,000*g* dialyzed + C1	.78
30,000*g* supernatant	.30
30,000*g* from Sephadex G-75	.16
30,000*g* from Sephadex G-75 + C1	.28

Table 2. Stimulation of tryptophan 5-hydroxylase at various stages of purification by the endogenous factor. The assay system was described in Table 1; C1, concentrate 1.

Enzyme preparation	Specific activity (nmole mg^{-1} hr^{-1})
30,000*g* supernatant	0.36
30% ethanol precipitate	.08
30% ethanol precipitate + C1	.64
$Ca_3(PO_4)_2$ gel eluate	.20
$Ca_3(PO_4)_2$ gel eluate + C1	1.70

for phenylalanine 4-hydroxylase was a preparation of sepiapterin reductase from rat liver, purified according to method A of Matsubara *et al.* (*11*). Purified lysolecithin was from egg yolk (Sigma) or from soybean (courtesy of R. L. Dryer). 5,6,7,8-Tetrahydrobiopterin (BH_4) and 2-amino-4-hydroxy-6-methyl-5,6,7,8-tetrahydropterin (6-MPH_4) were prepared in our laboratory. 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$) was purchased from Aldrich Chemical; L-[2-^{14}C]tryptophan and uniformly labeled [^{14}C]tyrosine were from Amersham/Searle.

Dialysis of a crude soluble preparation of tryptophan 5-hydroxylase was reported to stimulate enzyme activity (*5*). However, when we dialyzed fresh 30,000*g* supernatants from rat brainstem homogenates against 0.05*M* tris acetate buffer (*p*H 7.6) containing 0.01 or 0.1*M* 2-mercaptoethanol, 80 percent of the capacity to hydroxylate L-tryptophan was lost (*12*). Others confirmed this result (*4*). We reported (*1*, *2*) that the dialyzed enzyme was restored to its original activity by supplementing it with a portion of a lyophilized concentrate of the buffer containing the factor. This effect, which was not due to 2-mercaptoethanol, was the starting point of our work concerning the isolation and nature of this dialyzable factor.

Similar stimulation of enzyme protein collected from a column of Sephadex G-75 was obtained by the addition of concentrate 1 (Table 1). This material not only restored activity at various stages of purification of the enzyme, but increased its specific activity (Table 2). Concentrate 1 did not lose its stimulatory property after 5 minutes of immersion in boiling water. It is not a protein, as is evident from the character of concentrate 2, and could not be replaced in assays of cerebral tryptophan or tyrosine hydroxylases by purified sepiapterin reductase, the protein factor stimulating phenylalanine 4-hydroxylase (*3*). Lysolecithin (20 to 50 μg), which was reported to stimulate hepatic phenylalanine 4-hydroxylase (*13*), was without any effect on cerebral hydroxylases. Concentrate 1 was rapidly inactivated by prolonged exposure to light. Incubation in 0.1*N* NaOH (but not in 0.1*N* HCl) for 15 minutes at 37°C in oxygen or nitrogen led to 50 percent loss of the stimulatory effect.

The lability of the factor in light and alkali is consistent with a pterin-like structure. The pterin content of the portion of concentrate 1 that produced a three- to fivefold stimulation of tryptophan 5-hydroxylase was 0.01 to 0.02 μg (expressed as $DMPH_4$), as determined by the assay with phenylalanine 4-hydroxylase from *Pseudomonas*. The stimulatory response of the cerebral hydroxylases was linear with respect to concentrate over a range corresponding to 0.005 to 0.025 μg of pteridine. The factor is not BH_4 or its quinoid derivative, because 2.0 μg of BH_4 added to the assay system failed to result in hydroxylation of L-tryptophan in the absence of concentrate. Hydroxylation was not increased when 1 μg of BH_4 was added to system containing varying amounts of $DMPH_4$ (50 to 180 μg). Stimulation by concentrate 1 or 2 has an absolute requirement for BH_4, 6-MPH_4, or $DMPH_4$ (Table 3). This requirement is not obviated by addition of reduced nicotinamide adenine dinucleotide and dihydropteridine reductase to the assay system. Addition of borohydride or dithionate to the concentrate at *p*H 7.6 had no effect on the requirement for a pteridine cofactor.

The factor was not extractable into nonpolar solvents. Concentrates from brain did not stimulate hepatic phenylalanine 4-hydroxylase. The stimulatory effect of the cerebral concentrates appeared to be specific for brain but was not species-specific; that is, rat brain concentrate stimulated tryptophan 5-hydroxylase from hog brain, and vice versa. The factor was present in the cerebral supernatant of the different species examined. The factor is not a trace metal (the concentrates lost activity after incineration). Various compounds (0.1 to 5.0 μg) were tested in the assay system given in Table 1. Unlike concentrates 1 or 2, none of the amino acids, vitamins, pyridine nucleotides, nucleotides, nucleosides, any of their heterocyclic derivatives dithiol-containing substances, or any of the biologically important mono- and disaccharides or lipids produced stimulation of hydroxylation. Spectral and fluorescence analysis of the factor, partially purified by Sephadex G-75 chromatography of concentrate 2, revealed an excitation maximum at 365 nm with corresponding emission maxima at 430 and 510 nm at *p*H 7 to 8. The fluorescence disappeared in 0.1*N* acid or base, whereas emission of $DMPH_4$ in base is shifted from 430 to 450 nm with some quenching. The fluorescence of isobiopterin reappears at *p*H 9.5 or above. Nevertheless, the properties of the factor are indicative of a pteridine-like structure.

Table 3. Effect of the endogenous factor on partially purified tryptophan and tyrosine hydroxylases from rat brain. The assay system was that described in Table 1, except that BH_4 replaced $DMPH_4$ in some assays as indicated. Tryptophan 5-hydroxylase activity of protein was assayed after precipitation by 30 percent alcohol (*3*). Tyrosine hydroxylase activity of protein was assayed after precipitation by $(NH_4)_2SO_4$ (*8*); L-tyrosine, 0.1 m*M* (including uniformly labeled L-[^{14}C]tyrosine, 2.22×10^6 disintegrations per minute) was the substrate. L-Dopa was measured as dopamine. Abbreviations: 5-HTP, L-5-hydroxytryptophan, measured as 5-hydroxytryptamine; C2, concentrate 2; PHS, sepiapterin reductase.

Additives	Specific activity (nmole mg^{-1} hr^{-1})	
	5-HTP	Dopa
Enzyme	0.03	0.07
Enzyme + C2	.04	.07
$DMPH_4$	.06	.49
Enzyme + $DMPH_4$ + 1 μg of BH_4	.05	
Enzyme + $DMPH_4$ + C2	.45	.72
Enzyme + $DMPH_4$ + PHS	.08	
Enzyme + 20 μg of BH_4	1.10	
Enzyme + 20 μg of BH_4 + C2	1.98	

Determination as to whether this factor is an additional substance to be considered in regulation of the synthesis of cerebral catecholamines and indoleamines must await its isolation and characterization.

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Graft versus Leukemia: Quantification of Adoptive Immunotherapy in Murine Leukemia

Abstract. *Quantification of the antileukemic reactivity of transplanted immunocompetent cells from various allogeneic donors was achieved against a long-passage lymphocytic leukemia of AKR mice. Adoptive immunotherapy was the exclusive antileukemic treatment. Cells from DBA/2 donors exhibited maximal antileukemic effect, inactivating up to an estimated 10^7 leukemia cells. The cellular events were interpreted by using a theoretical cytokinetic construct.*

Immunotherapy generally is acknowledged to be most effective against what Woodruff has called "the minimal residual cancer" (*1*). Much experimental data have demonstrated that leukemia cells can be killed by adoptive immunotherapy (*2*). In the experiments reported here, we have attempted to quantify the number of leukemia cells killed (or inactivated) by a graft-versus-leukemia (GVL) reaction (*3*).

The experimental model was designed to permit an estimate of the number of leukemia cells killed, and so the measured antileukemic effect would be the exclusive result of the GVL reaction. The immunological defense system of the AKR (*H-2*^{k}) host mice was severely damaged by total body x-irradiation of 800 r, a supralethal dose (100 percent of animals dead within 15 days) in this strain. The irradiation conditions have been described (*4*). Irradiation was given the morning of day 0 as immunosuppression in order to (i) impair or eliminate host immunological participation against the leukemia and (ii) prevent rejection of the immunocompetent cells to be transplanted the following day. There was no antileukemic effect from the irradiation because it was administered before the leukemia cells were inoculated. Doses of 1, 4, 16, or 64 $\times$ 10^3 "blast" (large) cells from the spleen of an AKR mouse bearing BW5147, a long-passage lymphocytic leukemia (*5*), were given intravenously to the irradiated AKR primary hosts on the afternoon of day 0. Blast cells comprised 52 to 77 percent of cells in the spleen cell suspensions. After a delay of 24 hours, in which the leukemia cells were permitted to establish residence and resume multiplication, the mice were given separate intravenous injections of 2 $\times$ 10^7 bone marrow cells and 10^7 lymph node cells from one of four allogeneic donor strains: CBA (*H-2*^{k}), identical with the recipient at the *H-2* locus; and A (*H-2*^{a}), C57BL/6 (*H-2*^{b}), and DBA/2 (*H-2*^{d}), mismatched at this locus. The methods for preparation of the cell suspensions have been reported (*4*). The transplant on day 1 of allogeneic immunocompetent cells to the immunosuppressed, leukemic AKR mice (adoptive immunotherapy or GVL reaction) was the only therapy employed. The GVL reaction was allowed to proceed for 6 days, a period shown to be the minimal effective duration (*6*). After the 6-day GVL reaction, the primary hosts were killed and their spleens were removed. All spleen cells obtained from each primary host were injected intraperitoneally into one normal AKR secondary recipient. Secondary recipients were observed for survival. Those that died were autopsied, and all were found to have leukemia. None of the secondary recipients died after day 46, and an end point of 60 days was selected for analysis of the data.

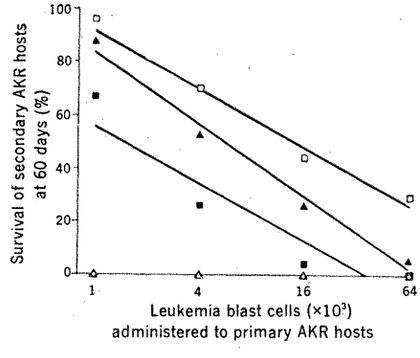

Fig. 1. Dose-response curves for survival of AKR secondary recipients at 60 days after spleen transfers from immunosuppressed AKR mice. The immunosuppressed mice had received graded doses of leukemia cells and had been treated with immunocompetent cells from allogeneic donors: □, DBA/2; ▲, C57BL/6; ■, A; △, CBA; and ○, no cells.

On the basis of three observations we elected to use the spleen as the organ for bioassay. First, Skipper *et al.* (*7*) reported that long-passage AKR leukemia invariably involved the spleen. Second, histologic examination in our laboratory of the tissues of more than 500 control AKR mice that died of BW5147 long-passage leukemia has invariably shown massive splenic involvement. Third, bioassay studies by Valeriote *et al.* (*8*) of a variety of tissues in AKR mice bearing a long-passage lymphocytic leukemia showed that the spleen contained one or more clonogenic leukemia cells after doses of cyclophosphamide that usually killed all detectable malignant cells in other organs. These results suggested that spleen cell transfer for the bioassay would afford us the greatest probability for recovery of residual leukemia cells, and that "cell cure" of the spleen might well connote "cell cure" of the entire animal.

The experimental results are presented in Table 1 and Fig. 1. The data provide evidence that immunocompetent cells from unprimed donors were capable of inactivating or killing leukemia cells and that this effect was accomplished in 6 days. Also apparent is the relative effectiveness of immunocompetent cells from the various donor strains, and the dose-response relation for each donor strain.

All secondary recipients died of leukemia after the transfer of spleen cells from control mice that were not treated with adoptive immunotherapy (Table 1). Similarly, all secondary recipients died of leukemia after spleen cell transfer from leukemic AKR mice that had been treated with immunocompetent cells from allogeneic donors that were identical at the *H-2* locus (CBA donors in Table 1). In other words, cells from CBA donors were ineffective at eliminating leukemia from AKR mice that had received as few as 10^3 BW5147 leukemia cells 1 day earlier.

Definite antileukemic activity was exhibited by immunocompetent cells