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PHENYLALANINE METABOLISM AND FOLIC ACID ANTAGONISTS *

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Amethopterin and related folic antagonists inhibit the reduction of folic acid to tetrahydro folic acid (2). The clinical effects of these drugs are thought to be related in turn to the role of tetrahydro folic acid in single-carbon transfer reactions during nucleic acid synthesis (3). Recently, pteridines such as folic acid have been implicated in another area of metabolism, the conversion of phenylalanine to tyrosine. In this conversion the pteridine is thought to act as a cofactor which is reduced in a separate enzymatic reaction, then takes part in the actual hydroxylation of phenylalanine. The reaction sequence as it is currently deduced from studies *in vitro* is presented in Figure 1. Enzymatic activities corresponding to phenylalanine hydroxylase ("rat enzyme") and the cofactor-reducing enzyme ("sheep enzyme"), as well as the cofactor itself, have been observed in normal human liver (4).

It has been shown that folic acid antagonists such as amethopterin inhibit tyrosine synthesis in a partially purified enzyme system, and *in vivo* in the rat (5). The site of inhibition is believed to be the enzymatic reduction of the pteridine cofactor. The work reported here is an investigation of the effects of amethopterin on phenylalanine metabolism in man, and the possible relevance of this effect to the clinical properties of folic antagonists. Experiments were also performed on rodents to explore the possible interrelationship of folic acid metabolism, phenylalanine metabolism, and cancer chemotherapy.

METHODS

Patients receiving amethopterin (Methotrexate) for the treatment of histologically-proven metastatic choriocarcinoma or leukemia were studied before, during and after therapy. Amethopterin was given in either of two dosage schedules: patients with carcinoma received 20 to 25 mg intramuscularly daily for 5 days in repeated courses (6); patients with leukemia received 5 to 10 mg orally daily, as continuous therapy. Several patients were studied who received 6-mercaptopurine orally in daily doses of 100 to 200 mg and several who received vincaleukoblastine, 5 to 10 mg intravenously, each day for 3 days (7). Six adults were studied who did not have known malignant disease and did not receive chemotherapy.

Phenylalanine metabolism was measured by an oral phenylalanine tolerance test. The DL or L form of the amino acid was given after an overnight fast in a dose of 0.1 g per kg body weight mixed in orange-ice. Blood was drawn at 0, 2, 4, and 6 hours after the phenylalanine was given. The serum was analyzed for phenylalanine by the method of La Du and Michael (8), and several determinations confirmed by the method of Kaufman and Levenberg (5). Tyrosine was measured by the method of Udenfriend and Cooper (9).

Recoveries of added phenylalanine and tyrosine were quantitative from sera of patients before, during and after therapy. The decay constants for serum phenylalanine were calculated for the 2- to 4-hour, and 4- to 6-hour

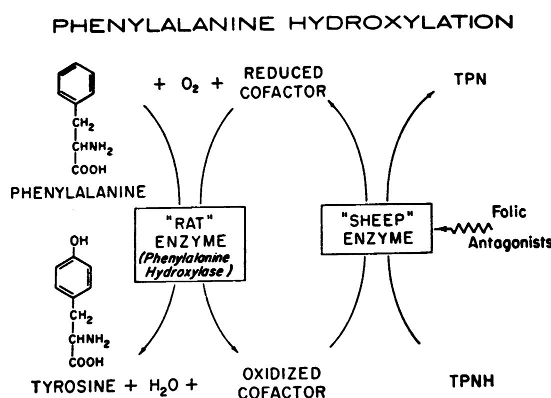

FIG. 1. SCHEMATIC REPRESENTATION OF THE ENZYME SYSTEM FOR HYDROXYLATION OF PHENYLALANINE IN MAMMALIAN LIVER AND THE LOCUS OF INHIBITION BY FOLIC ANTAGONISTS.

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* A preliminary report of the clinical findings described here has been presented at the Forty-Fourth Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., April 13, 1960 (1).

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intervals, from the equation

$$k = -\frac{2.303}{2}\log_{10}\frac{10(C_2 - C_4)}{C_2}, \quad \text{or} \quad \frac{10(C_4 - C_6)}{C_4}$$

where C_2, C_4, and C_6 represent the increment of serum phenylalanine above the fasting level at 2, 4, and 6 hours, respectively. The greater of the two values for k was included in Table I. These calculations assume that serum phenylalanine fell in accordance with a first order rate equation after an oral dose. The assumption is supported by the agreement of the k values for each time interval after the second hour; i.e., the logarithms of the phenylalanine values at 2, 4, and 6 hours fell on a straight line when plotted against time. The other parameters of phenylalanine tolerance were the maximal serum phenylalanine achieved during the test and the increment in plasma tyrosine during the test. Each patient provided her own control before or after therapy. Several tests were performed in which oral L-tyrosine was used in place of phenylalanine, and the data were treated in the same way.

In some studies urine was collected in 6-hour samples on the days of the tolerance tests and assayed for phenylpyruvic acid by the method of Følling and Sydnes (10).

The therapeutic response to the drugs was assessed by hormonal and radiological criteria in the cases of choriocarcinoma, as described by Hertz and co-workers (6), and by hematological criteria in the cases of leukemia. Drug toxicity was assessed clinically. The parameters for toxicity included mucous membrane ulceration, skin eruptions, and bone marrow suppression as reflected in peripheral blood counts.

Several tissues were assayed for the presence of the enzyme, phenylalanine hydroxylase, by using the two independent methods described by Kaufman (11). The tissue samples were homogenized in either 0.01 M acetic acid, or in 0.01 M potassium phosphate buffer, pH 7.0. Simultaneous assays were performed on rat liver as a day-to-day check of the assay procedure.

Folic deficiency in Sprague-Dawley rats was induced by feeding weanling animals either a commercial folic-deficient diet with 2 per cent succinylsulfathiazole (Sulfasuxidine, Nutritional Biochemicals) for 7 weeks, or a high-fat, folic-deficient diet for 16 weeks. The latter diet, developed by Silverman and Pitney, prevents the weight loss usually seen in folic deficiency in rats (12). Controls were given the former diet plus 20 mg of folic acid per kg of diet. Folic deficiency in the first group of animals was documented grossly by failure to gain weight, and by low total white blood cell counts. In the group fed the high-fat diet, deficiency was documented by the elevated urinary excretion of formiminoglutamic acid, as measured by a microbiologic assay. The livers were assayed for folic acid plus precursors with the digestion procedure of Silverman and Gardiner (13), and a microbiological assay employing *Pediococcus cerevisiae*. The same livers were assayed for the cofactor of phenylalanine hydroxylase by the method of Kaufman (14). Assays were performed in the linear range of the cofactor determination.

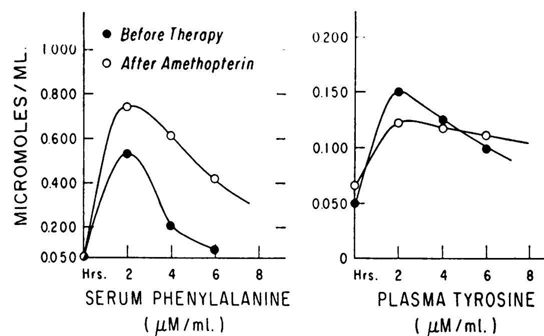

FIG. 2. A REPRESENTATIVE PAIR OF ORAL PHENYLALANINE TOLERANCE TESTS PLOTTED ON LINEAR COORDINATES (PATIENT 1, TABLE I).

A pilot study was performed to test whether changes in the phenylalanine or tyrosine content of the diet affected the response to amethopterin of transplantable mouse leukemia. The diets contained either 1 per cent L-phenylalanine and 1 per cent tyrosine (normal), 3.5 per cent L-phenylalanine and 1 per cent L-tyrosine (high-phenylalanine), or 1 per cent L-phenylalanine and no tyrosine (low tyrosine). The diets were formulated according to Franklin, Stokstad, Belt and Jukes (15), except that the casein hydrolysate was treated to remove aromatic amino acids [1] and supplemented with tryptophan and methionine. Survival times were recorded, and a dose-response curve to amethopterin was determined for each diet after inoculation of the mice with strain L-1210 transplantable leukemia. The general procedure of Goldin, Venditti, Humphreys and Mantel was employed (16).

RESULTS

Phenylalanine tolerance tests. Table I presents the results of the clinical study and includes the toxic and therapeutic effects of amethopterin, and the parameters of phenylalanine tolerance. In some patients repeated courses of therapy and repeated phenylalanine tolerance tests were performed. Every patient demonstrated impaired phenylalanine tolerance after amethopterin. This was manifest by a higher maximal serum phenylalanine level, a slower decay from this maximum (smaller k), and a smaller increment in blood tyrosine. A typical study is presented graphically in Figure 2, and phenylalanine values for several other studies are plotted on semilogarithmic coordinates in Figure 3.

The shortest time interval between the first injection of amethopterin and the performance of a tolerance test was 12 hours, (Patients 5, 6), and

[1] Supplied as compound MJ 309B by Mead, Johnson and Co., Evansville, Ind.

TABLE I

Phenylalanine tolerance tests in human subjects

		Therapy			Tolerance tests				Clinical effects			
Patient	Diagnosis	Drug*	Total drug before test	Interval, last dose and test	Dose and isomer of phenyl.	Max. serum phenyl.†	Decay constant (k) for serum phenyl.	Max. plasma tyrosine†	Fall, gonado-tropin excret.	Fall, peripheral WBC count	Toxicity to oral mucosa and skin (0 to +2)	Eventual remission p̄ therapy
			mg	*days*	*g*	*µmoles/ml*	*hrs*$^{-1}$	*µmoles/ml*	%	%		
1	Chorio-carc.		0		5.7 L	0.477	0.986	0.101				
		A	125	1.5	5.7 L	0.706	0.642	0.057	0	64	+1	Yes
2	Chorio-adenoma	V	0		5.4 L	0.398	1.070					
			25	0.5	5.4 L	0.438	0.950		59	82	0	
		A	125	3.5	5.4 L	0.891	0.437		32	0	+1	Yes
3	Chorio-carc.		0		4.8 L	0.375	0.891	0.123				
		A	125	0.5	4.8 L	0.675	0.659	0.052	99	46	+1	
		A	375	36.5	4.8 L	0.410	0.976			54	0	Yes
4	Chorio-carc.		0		5.9 DL	0.139	1.060	0.099				
		A	125	0.5	5.9 DL	0.403	0.326	0.050	99	68	+1	
		A	500	102.5	5.9 DL	0.285	0.805	0.082	72	47	0	Yes
5	Chorio-carc.		0‡		5.3 DL	0.245	0.805	0.060				
		A	25	0.5	5.3 DL	0.473	0.614	0.027	99	43	+1	Yes
6	Chorio-carc.		0		6.6 DL	0.324	0.990	0.072				
		A	25	0.5	6.6 DL	0.382	0.104	0.042	99	52	+2	Yes
7	Chorio-carc.		0		4.1 DL	0.073	0.984	0.071				
		A	125	0.5	4.1 DL	0.255	0.904	0.057	99	81	+2	Yes
8	Chorio-carc.		0		6.1 DL	0.152	0.941	0.062				
		A	100	0.5	6.1 DL	0.391	0.283	0.039	0	66	+2	Yes
9	Chorio-carc.		(300)	(120)	4.7 DL	0.453	0.995	0.047				
		A	360	2.5	4.7 DL	0.323	0.758	0.026	86	41	+1	Yes
10	Lympho. leuk.	6-M-P	75/day	0.5	3.4 L	0.398	0.737		0	Marked	0	No
		A	2.5/day × 14	0.5	3.4 L	0.740	0.533		0	0	0	Temporary
11	Chorio-carc.		0		6.4 DL	0.160	0.896	0.055				
		A	240	0.5	6.4 DL	0.413	0.606	0.027	66	0	0	
		A	490	0.5	6.4 DL	0.363	0.622	0.031	30	44	+2	No
12	Chorio-carc.		0		4.4 DL	0.121	0.875	0.068				
		A	250	9.5	4.4 DL	0.453	0.538	0.084	99	33	+2	No
13	Chorio-carc.	A	1,125	1.5	4.9 DL	0.371	0.594		0	0	0	No
		V	20	18.5	4.9 DL	0.207	0.884		72	70	0	No
14	Chorio-carc.	A	550	4.5	4.6 DL	0.277	0.139		0	59	+2	No
		V	63	16.5	4.6 DL	0.192	0.775		0	88	0	No
15	Acute myelo. leuk.		0		8.6 L	0.524	0.894					
		6-M-P	130–250/day × 15	0.5	8.6 L	0.532	0.845			Marked		No
16	Acute lympho. leuk.				6.7 L	0.223	0.995					
		6-M-P	200/day × 12	0.5	6.7 L	0.239	0.746			0		No
17	"Stein-Leventhal"	None			6.2 L	0.258	1.147					

* Drug: A = amethopterin; V = vincaleukoblastine.
† Total serum level minus fasting serum level, except in Patient 25.
‡ 700 Mg amethopterin had been administered over a period of several weeks, 78 days before listed studies were begun.
§ Derived from data published by Hsia and Paine (17).

TABLE I—(*Continued*)

Patient	Diagnosis	Therapy			Tolerance tests				Clinical effects			
		Drug*	Total drug before test	Interval, last dose and test	Dose and isomer of phenyl.	Max. serum phenyl.†	Decay constant (k) for serum phenyl.	Max. plasma tyrosine†	Fall, gonado-tropin excret.	Fall, peripheral WBC count	Toxicity to oral mucosa and skin (0 to +2)	Eventual remission on therapy
			mg	*days*	*g*	*μmoles/ml*	*hrs*$^{-1}$	*μmoles/ml*	%	%		
18	Normal	None			6.0 L	0.200	0.754					
19	Normal	None			6.7 L	0.512	0.977					
20	Hypo-gonadism	None			5.3 L	0.340	0.888					
21	"Stein-Leventhal"	None			7.8 L	0.695	0.892					
22	Obesity	None			9.0 L	0.574	1.027					
23§	Normals (mean)	None			0.1 g/kg L	0.493	0.834					
24§	Hetero-zygote phenylpy-ruvics	None			0.1 g/kg L	0.927	0.535					
25	Phenylpy-ruvic oligo-phrenia	None			0.05 g/kg L	2.633†	9.747–10	0.005				

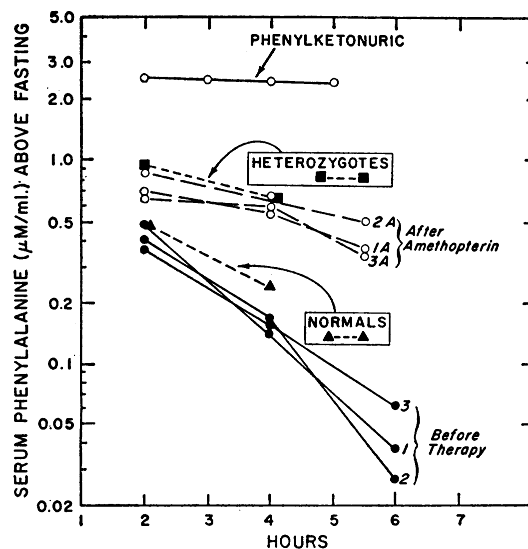

FIG. 3. ORAL PHENYLALANINE TOLERANCE TESTS, PLOTTED ON SEMILOGARITHMIC COORDINATES. The curves labeled 1, 2, 3, and 1A, 2A, 3A are from the corresponding patients in Table I. The suffix "A" indicates tests performed after amethopterin. The curves labeled "heterozygotes" and "normals" are from the data of Hsia and Paine (17). "Heterozygotes" are subjects whose children had phenylpyruvic oligophrenia. The curve labeled "phenylketonuric" is a phenylalanine test performed during the current study on a subject with known phenylpyruvic oligophrenia. All values are corrected for the fasting phenylalanine level except those for the phenylketonuric subject.

a marked change in phenylalanine tolerance was evident at that time. The changes in phenylalanine tolerance were observed 2 weeks after the last injection of amethopterin.

The control tests (before therapy) in patients with cancer did not differ from those in patients without cancer. The fasting levels of phenylalanine and tyrosine did not differ in these groups, nor did they change after therapy. Also included in the table and in Figure 3 are the mean values reported by Hsia and Paine for similar tests in normal subjects and presumed heterozygotes for the gene producing phenylpyruvic oligophrenia (17). One test in the current study was done on a patient known to have phenylpyruvic oligophrenia.

Oral tyrosine tolerance tests were performed in three patients before and after therapy. Amethopterin did not alter the absorption or metabolism of tyrosine as measured by the maximal

plasma level and the rate of decay of plasma tyrosine.

Routine liver function tests (serum bilirubin, alkaline phosphatase, and glutamic-oxalacetic transaminase levels) failed to show consistent changes after amethopterin therapy.

Patients who received 6-mercaptopurine or vincaleukoblastine demonstrated toxic side effects to the drugs which resembled those that followed amethopterin, but none of these patients showed impaired phenylalanine tolerance (Patients 2, 10, 13–16).

Within Table I the patients are arranged roughly in order of decreasing tumor response to therapy. No correlation was found between this response and the degree of impairment of phenylalanine tolerance. Furthermore, no correlation was observed between toxicity from amethopterin and phenylalanine tolerance.

The urine of Patients 1, 3–6, 8, 9, 11 and 12 was tested for phenylpyruvic acid before and after amethopterin. In those patients who received the DL-phenylalanine mixture, there was detectable phenylpyruvic acid in the urine, but the excretion did not rise after therapy. In the patients who received L-phenylalanine, no phenylpyruvic acid was detected before or after therapy.

Assay of phenylalanine hydroxylase in various tissues. Phenylalanine hydroxylase assays were performed on the following numbers of separate tissue specimens: 1 human choriocarcinoma (2 strains) grown as heterologous transplants in the cheek pouch of the hamster (18), human choriocarcinoma excised at surgery; 1 human term placenta; 1 human placenta aborted after 18 weeks' gestation; 3 rat placenta approximately 3 days from term; 4 rat mammary gland during pregnancy; 2 rat mammary gland during lactation; 20 immature rat uterus after 6 days' stimulation by exogenous estrogen; 3 rat small intestine; 1 rabbit marrow; 1 rabbit skin. These tissues were chosen for assay because of their known sensitivity to the effects of folic antagonists. There was no phenylalanine hydroxylase activity detected in any of the specimens tested. Thus, liver is the only tissue in which significant phenylalanine hydroxylase activity has been detected.

Effect of folic acid-deficient diet on cofactor content of liver. Table II presents the findings of an experiment to determine the effect of folic

TABLE II

Effects of folic-deficient diets on the content of cofactor for phenylalanine hydroxylase in rat liver

Rat	On diet	Weight	Peripheral WBC count	FIGLU* excretion	Folic acid content of liver†	Cofactor content of liver‡
	days	*g*		*μmoles/day*	*μg/g wet liver*	*U/g wet liver*
Standard diet + folic acid						
1	51	201	4,700		10.0	0.67
2	51	188			7.5	0.73
3	51	198			9.5	0.89
4	51	211	5,200		8.6	0.81
5	51	188			10.0	0.71
6	51	175	4,300		8.0	0.84
Standard diet, deficient						
7	51	183	2,400		0.48	0.36
8	51	124	3,100		0.47	0.50
9	51	165	1,500		0.50	0.57
10	51	123	2,200		0.30	0.55
11	51	116	2,600		0.49	0.49
High-fat diet, deficient						
12	120	180		133	0.56	0.77
13	120	170		113	0.46	0.80
14	120	180		113	0.49	0.69

* Formiminoglutamic acid; normal urinary excretion for the rat is less than 5 μmoles/day.
† Measured as citrovorum factor after autolysis to convert "prefolic" and folic acids to citrovorum factor (13).
‡ One unit is the amount of cofactor activity leading to the formation of 1 μmole tyrosine in a standard assay containing phenylalanine, phenylalanine hydroxylase, and "sheep enzyme" (14).

acid deficiency on the level of cofactor for phenylalanine hydroxylase in rat liver. There was a 36 per cent reduction in the mean level of cofactor activity in only those rats which demonstrated inanition. The rats fed a high-fat diet to prevent inanition failed to show any reduction of cofactor content.

The pilot study using mouse leukemia failed to yield evidence for an effect of various dietary phenylalanine: tyrosine ratios on prolongation of survival time by amethopterin.

DISCUSSION

The complete phenylalanine hydroxylase system requires two enzymes (named for their convenient animal sources), TPNH, oxygen, phenylalanine, and a cofactor (Figure 1). The exact structure of the cofactor is not known, but it has many of the chemical characteristics of a pteridine. Several synthetic tetrahydro pteridines including tetrahydro folic acid can substitute for the natural cofactor *in vitro*.

The second enzyme ("sheep liver enzyme"), catalyzes the reduction of the cofactor by TPNH (4). In the presence of reduced cofactor and oxygen, phenylalanine is converted to tyrosine in a reaction catalyzed by phenylalanine hydroxylase ("rat liver enzyme"). Folic antagonists are thought to block the enzymatic reduction of cofactor in a manner analogous to their inhibition of the activation of folic acid coenzymes by folic acid reductase (2). The experiments described here suggest that the scheme of phenylalanine hydroxylation derived from *in vitro* investigation applies as well to the human.

The inhibition of phenylalanine metabolism by amethopterin therapy is incomplete. There is, possibly, nonenzymatic reduction of the cofactor, unaffected by amethopterin, which permits phenylalanine metabolism to proceed at a reduced rate after inhibition of sheep liver enzyme. The observed inhibition of phenylalanine metabolism is of the same order of magnitude as the impaired phenylalanine metabolism found by Hsia and Paine in heterozygotes for phenylpyruvic oligophrenia (17) but far less than that seen in the homozygote. It should be emphasized that whereas amethopterin blocks sheep liver enzyme, patients with phenylpyruvic oligophrenia lack the hydroxylating enzyme itself (rat liver enzyme). The "sheep" enzyme and cofactor are present in normal amounts in the liver of patients with this disease (4).

There is little resemblance in the toxic state following folic antagonists to the clinical picture of phenylpyruvic oligophrenia. No evidence was obtained for characteristic mental or dermatologic changes. In addition, the patients with drug-induced impairment of phenylalanine metabolism did not excrete phenylpyruvic acid in the urine. This is consistent with the finding of Armstrong and Low, who failed to detect phenylpyruvic acid in the urine unless the serum phenylalanine level exceeded 0.91 μmoles per ml (19).

The results of our study, with regard to the speed of onset and duration of changes in phenylalanine tolerance after amethopterin, are in accordance with those found by Condit for folic reductase activity (20). He measured the excretion of citrovorum factor after a test dose of folic acid and found an effect within 3 days of an injection of amethopterin. The effect was evident for 2 to 10 weeks after the last dose of amethopterin, depending on the dosage schedule. Charache, Condit and Humphreys (21) found persistence of amethopterin, detected by direct assay of human liver, 3 months after the last dose of amethopterin.

Three experiments were performed to assess the importance of the "phenylalanine effect" of amethopterin in chemotherapy, and all three were negative. First, there was no correlation between the degree of impaired phenylalanine tolerance and the clinical response to treatment. Second, there was no effect from feeding additional phenylalanine or omitting dietary tyrosine on amethopterin therapy in mouse leukemia. These dietary alterations were designed to accentuate the probable metabolic consequences of inhibition of phenylalanine hydroxylation. Third, there was no evidence for the presence of phenylalanine hydroxylase in tumor tissue or in normal tissues (other than liver) which are known to be affected by folic antagonists.

The folic-deficient diet failed to reduce the hepatic level of cofactor for phenylalanine hydroxylase except when inanition was also induced. The folic acid level was consistently reduced. This

may indicate that the cofactor is not derived from folic acid, or that the cofactor is preferentially retained in the liver under conditions of folic deficiency, or both.

Hydroxylation is the first step in the sequence of reactions that convert phenylalanine to CO_2 via tyrosine and homogentisic acid. The reaction may be considered, therefore, part of a degradative pathway for excessive phenylalanine, as well as a part of several synthetic pathways which include tyrosine. Folic antagonists also inhibit the degradation of the amino acid histidine by blocking the metabolism of formiminoglutamic acid (FIGLU) (*22*). The excretion of this intermediate has been used as a test of folic deficiency or folic antagonism. There is no evidence that FIGLU excretion correlates with the therapeutic effects of amethopterin. Thus, three biochemical tests are available for the assessment of folic antagonism in mammals: the failure to excrete normal amounts of citrovorum factor after a test dose of folic acid, the excretion of abnormally large amounts of formiminoglutamic acid after the feeding of histidine, and the impairment of phenylalanine metabolism during an oral tolerance test.

SUMMARY

1. Amethopterin therapy interferes with phenylalanine metabolism in man, as measured by the oral phenyalanine tolerance test.

2. No evidence could be obtained for a relationship between impaired phenylalanine metabolism and the therapeutic or toxic effects of amethopterin in man or in the mouse.

3. Pronounced folic deficiency in the rat is sometimes accompanied by a slight reduction in the level of the cofactor for phenylalanine hydroxylase.

4. These findings have been discussed with reference to the proposed scheme of phenylalanine metabolism and the enzymatic effects of amethopterin in man.

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