Biopterin derivatives in human body fluids and tissues

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SYNOPSIS Levels of biopterin derivatives in urine, serum, milk, cerebrospinal fluid, brain, and liver have been measured with the Crithidia fasciculata assay. Normal levels in serum and urine have been given and compared with those in a number of benign and malignant proliferative disorders, phenylketonuria, kidney disease, Parkinson's disease, schizophrenia, controlled epilepsy, rheumatoid arthritis, and pernicious anaemia.

The active component of Crithidia factor in serum was 7,8-dihydrobiopterin. Tissue, urine, and some serum samples contained two active materials, the principal one being 7,8-dihydrobiopterin; a minor constituent was probably tetrahydrobiopterin.

Serum biopterin levels following methotrexate administration were raised and subsequent administration of folic acid and 5-formyltetrahydrofolic acid further increased serum levels of biopterin derivatives; this was in contrast to the total absence of response to oral folates without prior methotrexate and to 5-methyltetrahydrofolic acid either with or without methotrexate being given.

Tetrahydrobiopterin is the cofactor in the enzymatic hydroxylation of phenylalanine to tyrosine (Kaufman, 1963) and the further hydroxylation of tyrosine to dopa which limits the rate of catecholamine biosynthesis (Levitt *et al*, 1965). Hosoda and Glick (1966) gave an additional role for tetrahydrobiopterin in the hydroxylation of tryptophan to 5-hydroxytryptophan. Biopterin (1) and its biologically active reduced derivatives 7,8-dihydrobiopterin (2) and 5,6,7,8-tetrahydrobiopterin (3) differ from folic acid (4) in the substituent at C6 of the pteridine ring. In the rat, biopterin has been shown to be excreted in the absence of dietary biopterin (Pabst and Rembold, 1966).

Raised levels of biopterin derivatives have been reported in the blood of patients with gout (Baker *et al*, 1961) and in gout, uraemia, and alcoholic liver disease (Baker *et al*, 1974). Biopterin derivatives have been measured in human brain, liver, and cerebrospinal fluid (Baker *et al*, 1974).

Fukushima and Shiota (1972) showed that large oral doses of folic acid did not affect urinary levels of biopterin derivatives.

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In an earlier paper (Leeming and Blair, 1974), methods for the assay and identification of biopterin derivatives in urine were given. This paper now reports a detailed survey of the levels of biopterin derivatives in normal human body fluids and tissues and in various disease states. A well-defined set of conditions under which serum biopterin levels are increased by oral administration of folates and related compounds is also described.

Material and Methods

MATERIAL

Biopterin was a gift from Roche Products Ltd. Pteric acid was a gift from Professor I. H. Rosenberg. 5-Formyltetrahydrofolic acid was a gift from Lederle Laboratories Ltd. The calcium salt of 5-methyltetrahydrofolic acid was prepared by the method of Blair and Saunders (1970). 5-Methyl-5,6-dihydrofolic acid was prepared by dissolving 400 mg of 5-methyltetrahydrofolic acid in 200 ml of distilled water containing 5 mg of copper sulphate (Blair *et al*, 1975). The mixture was stirred under a slow stream of oxygen for 60 to 100 minutes, and the reaction was timed by testing for ultraviolet absorption maxima at 248 nm and 280 nm at pH 7.0 in 0.1 M phosphate buffer. Further purification was carried out according to Gapski *et al* (1971). 5-Methyl-5,8-dihydrofolic acid was prepared by dissolving 10 mg of 5-methyl-5,6-dihydrofolic acid in 0.01 M hydrochloric acid 30 minutes before administration. Neutralization with dilute sodium hydroxide immediately preceded use. 7,8-Dihydrofolic acid was prepared according to the method of Futterman (1963). 10-Formylfolic acid was prepared by the method of Blakley (1959). Folic acid (Kodak Ltd) and methotrexate (Lederle Laboratories Ltd) were commercial products.

SUBJECTS STUDIED

The normal sera were taken from 42 males and 72 females aged 18 to 60 years. The normal urines were from 30 males and 30 females within the same age group. Patients with kidney disease and schizophrenia were inpatients; the remainder were mostly outpatients.

Cerebrospinal fluids were those submitted for routine analysis and which subsequently appeared normal. Expressed breast milk was obtained from normal females within one week of parturition. All samples were taken randomly during the day from non-fasting subjects. Liver and brain were obtained post mortem.

DRUGS

Children with leukaemia had been receiving be-

tween 5 mg and 7.5 mg methotrexate daily for five days. Psoriatics on azothioprine were taking 50 mg once a day, and psoriatics on methotrexate had had from 5 mg to 25 mg orally or parenterally from one to six days before sampling. Patients on Septrin were taking 960 mg twice daily. The schizophrenics were having multiple treatment from a wide selection of drugs. The controlled epileptics were having various doses of phenytoin and phenobarbitone. Patients with Parkinson's disease were, with one exception, taking L-dopa.

PREPARATION OF SAMPLES

Serum samples were frozen until the day of the test, diluted 1:20 with 0.2 M phosphate buffer pH 5.0, autoclaved at 115°C for 3 minutes, and centrifuged. The clear supernatant was added in 0.5 ml quantities to three tubes and then treated in a similar way to the standards. Whole blood anticoagulated with dipotassium EDTA was assayed in the same way as serum; where necessary, further 1:5 dilutions of extracts were made in buffer. Urine samples were diluted 1:1000 in buffer from which three further 1:5 dilutions were made. Assays were then carried out as for sera.

Cerebrospinal fluids (CSF) were diluted as sera but not autoclaved. Tissue was homogenized at 50 mg/ml, autoclaved, and assayed as serum. Some samples of tissue were lyophilized to compare the weights of fresh and dehydrated material. Human milk was defatted by centrifugation (Ford, 1974) and then treated as serum. Duplicate samples of milk were filtered through 8/32 inch Visking tubing and the filtrates were assayed.

CHROMATOGRAPHY

Serum, urine, and tissue extracts were chromatographed on cellulose thin layer plates to 15 cm in 0.5% sodium carbonate, 5% acetic acid, and 3% ammonium chloride. Eluates were assayed for Crithidia active materials.

FOLATES AND ANTIFOLATES

Fourteen patients with psoriasis, who had had methotrexate within the previous six days, had serum Crithidia factor levels measured. These were compared with nine psoriatics without systemic therapy, four psoriatics on azothioprine, 12 children with leukaemia who had had methotrexate from 1 to 14 days before sampling, three patients on Septrin (trimethoprim with sulphamethoxazole) and normals.

Blood samples were taken at 0, 1, 2, 3, and 6 days from a patient who had received a single 25 mg dose of methotrexate intravenously for extensive erythrodermic psoriasis, and at 0, 1, 2, 3, 4, and 24 hours

from two other psoriatics with severe lesions who had had 5 mg of methotrexate orally. Nine patients had a single 10 mg dose of methotrexate orally followed 24 hours later by different pteridines. Two of these had 5 mg of oral folic acid, two had 10 mg of 5-methyltetrahydrofolic acid orally, two had 10 mg of 5-formyltetrahydrofolic acid orally, two had 5 mg of 5-formyltetrahydrofolic acid intravenously, and one had 5 mg of biopterin orally. Blood samples were taken before the methotrexate was given, immediately before the pteridine, and at 1, 2, 3, 4, and 24 hours after the pteridine for Crithidia factor assay.

Blood samples were taken for up to 3 hours from normal subjects who had taken oral folates.

MICROBIOLOGICAL ASSAY

The culture medium for *Crithidia fasciculata* was that described by Guttman and Wallace (1964), with the addition of 1% casamino acids (Iwai *et al*, 1970). Experiments showed that casamino acids produced a considerable increase in maximum growth, and it was not necessary to pretreat with charcoal. The medium was prepared without vitamins, haemin or triethanolamine at 10 fold concentration, steamed for 20 minutes, and stored in screw-capped bottles at 4°C. The vitamins were prepared as a dry mix, with the exception of folic acid which was added from a stock kept for folic acid assays. The complete double-strength medium was adjusted to pH 7.5. The addition of buffered biological material or standard produced a final pH of 6.5. This optimum pH value was arrived at independently by Baker *et al* (1974).

The standard (in triplicate) was prepared by adding 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, and 150 µg of biopterin/tube in 0.5 ml quantities of 0.2 M phosphate buffer pH 5.0. The volume was made to 4.0 ml by adding 1.5 ml distilled water and 2.0 ml double-strength medium.

The inoculum was prepared by taking one drop of a two-day culture in maintenance medium (Leeming and Blair, 1974) and adding it to 15 ml single-strength medium, incubating for four days to exhaust endogenous growth factor, and diluting 1:100 in single-strength medium; 1 drop was added to each test. Ampicillin, 25 mg/ml, added to this inoculum assisted in preventing bacterial contamination. Incubation was at 29°C in the dark for four days. Reading was carried out on a semi-automated system (Leeming and Graham, 1973).

The assay was specific for biopterin, 7,8-dihydro-biopterin, 5,6,7,8-tetrahydrobiopterin, and L-neopterin. Folates and antifolates at the levels described in this paper did not affect the growth of *Crithidia fasciculata*.

Results

BODY FLUID AND TISSUE LEVELS

Mean serum levels of biopterin derivatives in normal subjects and patients with various diseases are given in table I. Urine levels are given in table II. Normal serum biopterin derivative levels ranged from 0.4 ng/ml to 3.6 ng/ml (mean 1.81 ± 0.06 ng/ml), and normal urine levels from 0.3 µg/ml to 6.9 µg/ml (mean 2.1 ± 0.38 µg/ml).

Biopterin derivatives in other body fluids and tissues are given in table III. CSF levels were close to serum levels whereas milk and tissues contained markedly higher concentrations. Brain varied considerably in water content, between both sites of sampling and different patients, while liver seemed fairly constant at around 75%.

Crithidia factor levels in solid tissues were very much higher than those found in serum and CSF (table III), probably resulting from intracellular biosynthesis. Similar levels in brain and liver have been reported (Baker *et al*, 1974). Levels in milk were more akin to those found in tissues than in serum, although total folate levels measured by *L. casei* were very similar to those found in serum (5.5 ng/ml vs 1.0 ng/ml). The difference between Crithidia factor in unfiltered and protein-free milk (table III) was statistically significant ($P < 0.01$). Folates are also bound to whey proteins (Ford, 1974).

LEVELS IN DISEASE

Serum values from patients with cirrhosis, Parkinson's disease, and epilepsy did not show any statistically significant variation at the 5% level by Student's *t* test from the normal group. Those with proliferative disorders were all low although carcinoma patients were not significantly lower (only 6 samples were tested). Patients with pernicious anaemia, schizophrenia, rheumatoid arthritis, and regional enteritis all had low serum values. Phenylketonuric and uraemic subjects had significantly raised results. The hospital control group of children gave a similar level to normal adults. Urine levels were low in controlled epilepsy but only at the 5% level of significance by Student's *t* test. If, however, the number of results below 1.0 µg/ml was compared with the number of normals below this figure, the value obtained by applying the χ^2 test was more significant ($P < 0.01$). Urine levels in rheumatoid arthritis and kidney dysfunction were significantly low. Schizophrenics gave values which, although raised, were not significantly elevated; the standard error reflects the variations within this group.

CHROMATOGRAPHY

Chromatography showed the material in patient

Disease	No. of Observations	Mean (ng/ml) $\pm$ Standard Error	Significance by Student's <i>t</i> test
Normal	114	1.81 $\pm$ 0.06	—
Children (hospital control) and peer group for phenylketonuria	10	1.78 $\pm$ 0.25	—
1a		Higher than normal	
Kidney dysfunction	4	8.75 $\pm$ 0.98	< 0.001
Phenylketonuria	30	4.86 $\pm$ 0.51	< 0.001
1b		Lower than normal	
Leukaemia	25	1.30 $\pm$ 0.12	< 0.001
Pernicious anaemia	4	1.0 $\pm$ 0.22	< 0.001
Psoriasis	9	1.43 $\pm$ 0.08	< 0.001
Schizophrenia	17	1.51 $\pm$ 0.07	0.001-0.01
Regional enteritis	11	0.98 $\pm$ 0.13	< 0.001
Rheumatoid arthritis	55	1.46 $\pm$ 0.11	0.01-0.001
1c		No statistically significant difference	
Carcinomatosis	6	1.42 $\pm$ 0.30	0.2-0.3
Cirrhosis	20	1.63 $\pm$ 0.10	0.1-0.2
Controlled epilepsy	18	1.93 $\pm$ 0.14	NS
Parkinson's disease	25	2.05 $\pm$ 0.17	0.2-0.3

Table I Serum levels of biopterin derivatives in disease and the significance of their variation from normal

Disease	No. of Observations	Mean (μ g/ml) $\pm$ Standard Error	Significance by Student's <i>t</i> test
Normal	60	2.1 $\pm$ 0.19	Control
Rheumatoid arthritis	17	0.75 $\pm$ 0.18	< 0.001
Controlled epilepsy	16	0.41 $\pm$ 0.35	0.05-0.1
Kidney dysfunction	4	0.077 $\pm$ 0.048	< 0.001
Schizophrenia	20	3.55 $\pm$ 1.09	0.1-0.2

Table II Urine levels of biopterin derivatives in disease and the significance of their variation from normal

White Matter			Grey Matter		Substantia Nigra	
ng/fresh g	Dry wt Fresh wt $\times$ 100		ng/fresh g	Dry wt Fresh wt $\times$ 100	ng/fresh g	Dry wt Fresh wt $\times$ 100
Brain						
1	25	—	25	—	53	—
2	20	27%	20	16%	500	11%
3	112	—	22	—	175	—
4	28	—	35	17%	175	24%
Liver						
1	41	23%				
2	100	27%				
3	163	24%				
4	144	—				

Table III Biopterin derivatives in human body fluids and tissues

No. of Estimations	Mean (ng/ml) $\pm$ Standard Error of Mean
Whole blood (10)	4.0 $\pm$ 0.18
Red blood cells (10)	7.8 $\pm$ 0.55
CSF (19)	1.9 $\pm$ 0.13
Milk (6)	131.0 $\pm$ 13.6
Milk after filtration through Visking tubing (6)	79.3 $\pm$ 6.3

with kidney disease to be the same as in normal serum (probably 7,8-dihydrobiopterin) although increased in quantity. One patient with untreated myeloma had a very raised level of Crithidia activity (70 ng/ml), and chromatography exposed a second material which co-chromatographed with

biopterin and tetrahydrobiopterin. The urine of epileptics and rheumatoid arthritics showed two peaks identical with those found in normal urine, probably tetrahydrobiopterin and 7,8-dihydrobiopterin (Leeming and Blair, 1974).

Chromatography of brain and liver produced

two materials, one of which co-chromatographed with 7,8-dihydrobiopterin and the other with 5,6,7,8-tetrahydrobiopterin and biopterin.

FOLATES AND ANTIFOLATES

Table IV details the levels in untreated psoriasis compared with treated psoriasis and other patients on antifolate drugs. Azothioprine did not have any great effect on biopterin derivatives in serum while methotrexate and Septrin caused substantial increases.

Table V shows that oral folates did not affect the serum levels of biopterin derivatives. Methotrexate orally (fig 1) or intravenously (fig 2) gave rise to increased serum biopterin derivatives which were sustained for a considerable period of time. In two of the 12 leukaemic children on methotrexate the level was measured at 11 days and 14 days and gave results of 4.8 ng/ml and 5.6 ng/ml respectively. Oral 5-methyltetrahydrofolic acid after oral methotrexate did not increase the level of biopterin derivatives in serum but oral folic acid and oral and intravenous 5-formyltetrahydrofolic acid caused a substantial rise (fig 3). Oral biopterin (fig 4) caused a dramatic rise in the serum value which continued after 4 hours.

Discussion

The levels in normal subjects varied considerably from those of Frank *et al* (1963), who gave values

Fig 1 Serum *Crithidia* factor levels in two patients following oral methotrexate.

of 27 ng/ml in serum and 48 ng/ml in whole blood but were similar in that whole blood gave approximately twice the serum value. Subsequently, Baker *et al* (1974) reported plasma and blood levels 0.9 ng/ml $\pm$ 0.2 and 1.9 ng/ml $\pm$ 0.8 in 31 subjects roughly half the values given in this paper. These

	Normals	Untreated Psoriatics	Psoriatics on Azothioprine	Psoriatics on Methotrexate	Leukaemic Children on Methotrexate	Patients on Septrin
No. of samples	114	9	4	14	12	4
Mean serum level (ng/ml) with standard error of mean	1.81 $\pm$ 0.06	1.43 $\pm$ 0.08	1.18 $\pm$ 0.23	15.24 $\pm$ 4.94	4.3 $\pm$ 0.31	7.35 $\pm$ 2.49
Range (ng/ml)	0.4-3.8	1.0-1.8	0.7-1.7	3.0-60.0	2.8-5.6	1.4-13.5

Table IV Biopterin derivative levels in treated and untreated psoriatics compared with other patients on dihydrofolate reductase inhibiting drugs

	5-Methyl-tetrahydro-folic Acid	Folic Acid	Pteric Acid	5-Formyl-tetrahydro-folic Acid	10-Formyl-folic Acid	5-Methyl-5,6-dihydrofolic Acid	5-Methyl-5,8-dihydrofolic Acid	7,8-Dihydro-folic Acid
No. of subjects	5	3	2	4	2	5	3	4
Hours								
0	2.07 $\pm$ 0.13	1.17 $\pm$ 0.17	1.63 $\pm$ 0.12	1.63 $\pm$ 0.30	0.55 $\pm$ 0.05	2.36 $\pm$ 0.62	1.20 $\pm$ 0.23	2.15 $\pm$ 0.48
½	1.88 $\pm$ 0.11	1.23 $\pm$ 0.09	1.50 $\pm$ 0.20	1.70 $\pm$ 0.24	0.53 $\pm$ 0.17	2.42 $\pm$ 0.55	1.30 $\pm$ 0.26	1.90 $\pm$ 0.26
1	1.73 $\pm$ 0.09	1.27 $\pm$ 0.20	1.50 $\pm$ 0.10	1.43 $\pm$ 0.21	0.58 $\pm$ 0.22	2.28 $\pm$ 0.53	1.33 $\pm$ 0.35	1.78 $\pm$ 0.24
1½	1.80 $\pm$ 0.09	1.30 $\pm$ 0.12	1.53 $\pm$ 0.02	1.20 $\pm$ 0.33	0.50 $\pm$ 0.30	2.24 $\pm$ 0.57	1.17 $\pm$ 0.37	1.83 $\pm$ 0.24
2	1.80 $\pm$ 0.09	1.38 $\pm$ 0.16	1.55 $\pm$ 0.05	1.25 $\pm$ 0.13	0.68 $\pm$ 0.02	1.94 $\pm$ 0.36	1.53 $\pm$ 0.32	1.78 $\pm$ 0.22
2½	—	—	—	—	—	—	—	—
3	1.85 $\pm$ 0.15	1.30 $\pm$ 0.09	1.65 $\pm$ 0.45	1.18 $\pm$ 0.28	0.80 $\pm$ 0	1.96 $\pm$ 0.36	1.30 $\pm$ 0.35	1.93 $\pm$ 0.23

Table V Biopterin derivative levels (ng/ml) in serum following oral folates in normal fasting adults

Fig 2 Serum Crithidia factor levels over six days in a patient following intravenous methotrexate.

Fig 3 Serum Crithidia factor levels when folates were given 24 hours after 10 mg methotrexate.

same workers gave values for CSF which were considerably lower than those presented here—0.4 ng/ml $\pm$ 0.21 as opposed to our 1.9 ng/ml $\pm$ 0.13. These differences could reflect different techniques; in our hands, recovery of added biopterin and tetrahydrobiopterin was very close to 100% over a range of concentrations.

Urinary excretion was found to be approximately 1.6 mg/day compared to the findings of Baker *et al* (1974)—2.1 mg/day and Fukushima and Shiota (1972)—1.4 mg/day.

Fig 4 Serum Crithidia factor levels when biopterin was given 24 hours after 10 mg methotrexate.

The levels of biopterin derivatives found in the serum and urine of patients in a number of diseased conditions (table I) could provide clues to the metabolism of these materials. The very high levels found in the serum of patients with kidney disease and the concurrently low level found in urine suggest that the kidney plays a vital role in maintaining serum levels within narrow limits. The raised levels in phenylketonuria can be correlated with amino acid levels and will be presented separately (Leeming *et al*, 1976). The disorders with abnormal cellular proliferation all showed low serum levels. The depressed levels in the serum of schizophrenics are difficult to explain. The cirrhotics did not have significantly different levels from normals in contrast to the findings of Baker *et al* (1974) who found raised levels in patients with alcoholic liver disease, which they assumed to reflect impairment of biopterin catabolism accompanied by a lesser impairment in biopterin synthesis. In Parkinson's disease the mean serum level was slightly above normal but not significantly so ($P = 0.2-0.3$). However, only one patient was certainly unmedicated and had a raised level (5.2 ng/ml). Any change at tissue level may not necessarily be reflected in serum concentrations of biopterin derivatives if the blood/brain barrier is intact. In rats, tetrahydrobiopterin has difficulty in penetrating in the opposite direction (Kettler *et al*, 1974).

That three of the four myelomatous patients had significantly lower serum levels than normal was in agreement with findings in the other malignant diseases. However, one patient had a very raised level (70 ng/ml) in plasma taken off for replacement with

protein-free substrate before maintenance on cytotoxic drugs. Protein binders for folates are currently of interest to haematologists (Waxman, 1975) and nutritionists (Ford, 1974) alike, and the possibility exists that there is a protein which binds biopterin or its derivatives, and in this patient it was stimulated to overproduction. Chromatography of serum from this patient in the three solvent systems showed a considerable quantity of the normally undetectable (in serum) component of Crithidia factor which co-chromatographed with biopterin and tetrahydrobiopterin. The other material normally found in serum and previously described as 7,8-dihydrobiopterin was also raised.

As the Crithidia assay showed a low level of growth in the absence of added biopterin, any inhibitory substance would have been detected during the chromatography. Therefore inhibition can be ruled out as a contributory factor to low levels in specimens where chromatography was employed.

The lack of response to oral folates (table V) demonstrates that in normal circumstances these compounds do not contribute to the synthesis of biopterin derivatives. Concurrent measurement of folates showed normal absorption. Folate is readily absorbed (Ratanasthien *et al*, 1974) with the exception of pteric acid (Brown *et al*, 1973; Blair *et al*, 1974). Fukushima and Shiota (1972) found that oral folic acid did not raise the level of biopterin derivatives in urine.

The raised levels of biopterin derivatives following folate antagonists (figs 1 and 2) could be caused by minor pathways to biosynthesis being fed by unmetabolized folate; or unreduced biopterin derivatives, accumulated by inhibition of reduction back to the tetrahydro forms, may be the trigger for increased synthesis of reduced forms. Biopterin, when given to a patient on methotrexate (fig 4), led to a very raised level in the serum, much above the levels following oral biopterin in unmedicated subjects (Leeming, 1975). This may have been due in part to renal dysfunction (blood urea 9.1 mmol/l), which could have accounted for the raised level (4.0 ng/ml) before methotrexate was given (Baker *et al*, 1974). Chromatography of serum from this patient 24 hours after biopterin was given showed a raised level of 7,8-dihydrobiopterin as well as a substantial amount of tetrahydrobiopterin and/or biopterin.

The rises in serum levels following oral folic acid and both oral and intravenous 5-formyltetrahydrofolic acid in patients on methotrexate (fig 3) contrasted with the failure in response to oral 5-methyltetrahydrofolic acid. The increases demonstrated between 3 and 4 hours occurred after the normal peak serum folate level following oral folates

(Ratanasthien *et al*, 1974). This may suggest that in the presence of methotrexate these compounds contribute to serum biopterin derivatives by their metabolism either providing a substrate for bio-synthesis or stimulating synthesis from another starting point.

Very little is known about the metabolism of biopterin derivatives in man except for their specific role in the enzymatic hydroxylation of phenylalanine, tyrosine, and tryptophan. The work in rats suggests an endogenous source (Pabst and Rembold, 1966) and non-passage of the blood brain barrier (Kettle *et al*, 1974), which, in the context of the variable levels in brain and liver presented here, point to widely dispersed synthesis dependent on demand. A defect in the reduction of tetrahydrobiopterin has been suggested as a cause of dietary resistant phenylketonuria (Smith *et al*, 1975). Faulty bio-synthesis could have a similar effect which would be distinctive following assay and identification of biopterin derivatives.

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