BBA 1040

THE INFLUENCE OF SOME 4-AMINO ANALOGUES OF TETRAHYDROFOLATE COENZYMES ON PURINE BIOSYNTHESIS

VĚRA SLAVÍKOVÁ AND KAREL SLAVÍK

Institute of Hematology and Blood Transfusion and Laboratory for Protein Metabolism, Charles University, Prague (Czechoslovakia)

Received August 8th, 1962

SUMMARY

1. Aminopterin and other 4-amino analogues of folic acid coenzymes do not show any appreciable inhibitory effect on the biosynthesis of inosinate in pigeon liver.

2. Tetrahydroaminopterin and some of its derivatives substitute the tetrahydrofolate coenzymes in both transformylation reactions participating in purine biosynthesis.

3. The deamination of tetrahydroaminopterin and its derivatives to tetrahydrofolate compounds as a possible cause of their coenzymic activity could not be established.

INTRODUCTION

In our previous papers we described the preparation[1] and some biochemical properties[2,3] of several 4-amino analogues of the folic acid coenzymes, especially the formyl and methylene derivatives of tetrahydroaminopterin. Besides the strong inhibition of folate reductase, the moderate non-competitive inhibition of N^5,N^{10}-methylenetetrahydrofolate dehydrogenase and of formiminotransferase was observed. To establish the mechanism of the biochemical action of these antimetabolites, we studied the action of the 4-amino analogues on some enzymic reactions involving transformylation[2] and transhydroxymethylation[4] by tetrahydrofolate coenzymes.

This paper describes the influence of the tetrahydroaminopterin derivatives on the transformylation reaction participating in purine biosynthesis.

MATERIALS AND METHODS

Aminopterin (Lepetit) and folic acid (Light) were purified as their crystalline magnesium salts. The 4-amino analogues and tetrahydrofolate derivatives were prepared in our laboratory[1]. The purity of all substances used was checked by chromatography and ultraviolet absorption.

The enzyme was prepared by extracting acetone powder of pigeon liver with 10 vol. of 0.05 M Veronal buffer (pH 7.6) at 0°. After 20 min at 0° the insoluble part was removed by centrifugation and the clear supernatant was used for the experiments[5].

The purine biosynthesis was determined by the incorporation of [^{14}C]formate into inosinic acid isolated by paper chromatography.

Incubation mixture: 19.9 μmoles of ATP, 19.2 μmoles of homocysteine, 27.3 μmoles of glutamine, 19.0 μmoles of glycine, 10.0 μmoles of ribose 5-phosphate, 7.3 μmoles of barium 2-phosphoglycerate, 47.0 μmoles of $MgSO_4 \cdot 7H_2O$, 1.3 μmoles of sodium [^{14}C]formate (activity 2.0 μC), 2.0 μmoles of tetrahydrofolate, 2.0 ml of enzymic preparation and 0.05 M Veronal buffer (pH 7.6) in a total volume of 4.4 ml. The sample was incubated at 37°. Samples of 1.8 ml each were withdrawn immediately after the addition of the enzyme and after incubation for 45 min; both samples were deproteinized with 1.0 ml of 10% trichloroacetic acid. To each sample 2.0 mg of hypoxanthine was added as a carrier. Trichloroacetic acid and Veronal were removed by 3-fold extraction with 5.0-ml portions of ether. For hydrolysis of the nucleotides and destruction of the radioactive coenzymes, 0.5 ml of 2% $FeCl_3$ dissolved in 2 N HCl was added and the samples were heated at 100° for 30 min. After cooling, the solutions were dried under an infrared lamp. The residues were dissolved in 0.5 ml of water and applied to preparative chromatograms (Whatman No. 3). The chromatograms were developed with water-saturated butanol and the band of hypoxanthine was then cut out and eluted directly onto an aluminium planchette. The eluates were taken to dryness and counted by a thin end-window tube using an apparatus of Frieseke–Hoepfner.

RESULTS

During investigations on the inhibition of purine biosynthesis by some aminopterin derivatives, no inhibitory effects were observed (Table I). However, aminopterin derivatives with a hydrogenated pyrazine ring showed an appreciable activating effect. In further experiments, tetrahydroaminopterin and its derivatives were used instead of tetrahydrofolate as possible coenzymes. It was found that, to a certain extent, they are able to substitute for tetrahydrofolic acid in its coenzyme function. Fig. 1 demonstrates the activation effect of tetrahydroaminopterin compared with that of tetrahydrofolate and its diglutamyl conjugate.

TABLE I

THE EFFECT OF SOME AMINOPTERIN DERIVATIVES ON THE INCORPORATION OF [^{14}C]FORMATE INTO INOSINIC ACID IN THE PRESENCE OF TETRAHYDROFOLATE

The antimetabolite was added in a concentration equal to that of tetrahydrofolate, *e.g.* 2.0 μmoles for each sample.

Antimetabolite added	*Radioactivity in hypoxanthine isolated (counts/min)*	*Activity of the sample without antimetabolite (%)*
None	1900	100
N^{10}-Formylaminopterin	1690	88
N^{10}-Methylaminopterin	1490	78
N^{10}-Hydroxymethylaminopterin	2245	118
Aminopterin	2080	110
Tetrahydroaminopterin	2840	149
N^5-Formyltetrahydroaminopterin	3090	168.5
Trimethyloltetrahydroaminopterin	2982	157
N^{10}-Formyltetrahydroaminopterin	4579	240
N^5,N^{10}-Methylenetetrahydroaminopterin	5413	287

The activation effect of tetrahydroaminopterin is even more pronounced than the effect of the diglutamyl conjugate of tetrahydrofolic acid. Several aminopterin derivatives (Table II) showed an analogous activation effect on purine biosynthesis.

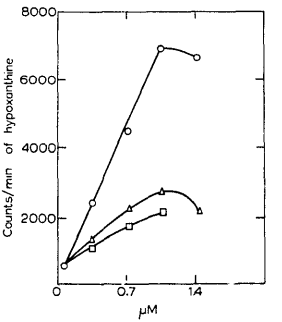

Fig. 1. Effect of coenzymes on inosinate biosynthesis. O—O, tetrahydrofolate; Δ—Δ, tetrahydroaminopterin; □—□, tetrahydroteropterin.

N^5-Formyl and N^5,N^{10}-methylene derivatives of dibromotetrahydroaminopterin showed higher coenzymic activities than the analogous non-brominated 4-amino analogues. The possible deamination of the antimetabolite to the tetrahydrofolate compound acting as the actual coenzyme could be supposed as an explanation of the coenzymic activity of tetrahydroaminopterin. Therefore, the formylating coenzymes were isolated from the incubation mixture by means of paper chromatography in 0.1 N formic acid using non-radioactive synthetic compounds as carriers. Using the tetrahydrofolate coenzyme the radioactivity could be detected in both N^{10}-formyltetrahydrofolate and N^5,N^{10}-methenyltetrahydrofolate bands. With tetrahydroaminopterin as coenzyme, radioactivity could be detected in one band only,

TABLE II

THE EFFECT OF SOME AMINOPTERIN DERIVATIVES ON THE INCORPORATION OF ^{14}C-FORMATE INTO INOSINIC ACID IN THE ABSENCE OF TETRAHYDROFOLATE

Cofactor added	*Counts/min in hypoxanthine synthesized using various concentrations of antimetabolite*		
	2 μmoles	*3 μmoles*	*4 μmoles*
Tetrahydrofolic acid	1170	—	—
Tetrahydroaminopterin	1157	1506	1917
3′,5′-Dibromotetrahydroaminopterin	490	545	676
N^5-Formyltetrahydroaminopterin	—	601	620
N^5-Formyl-3′,5′,-dibromotetrahydroaminopterin	1110	1070	1031
Trimethyloltetrahydroaminopterin	694	612	668
Dimethylol-3′,5′-dibromotetrahydroaminopterin	350	260	238
N^5,N^{10}-Methylenetetrahydroaminopterin	317	410	—
N^5,N^{10}-Methylene-3′,5′-dibromotetrahydroaminopterin	478	700	—

which by comparison with a synthetic sample was identified to be N^{10}-formyltetrahydroaminopterin. No radioactivity could be detected in the band of N^5,N^{10}-methenyltetrahydrofolate added as carrier (Table III). From these experiments it can be concluded that the deamination of tetrahydroaminopterin to tetrahydrofolate does not represent the real mechanism of the coenzymic function of tetrahydroaminopterin.

In further experiments the distribution of ^{14}C between C-2 and C-8 of the purine skeleton in the inosinate synthesized was followed using either tetrahydrofolate or tetrahydroaminopterin as the coenzyme. The radioactive hypoxanthine was oxidized by means of chlorate to alloxan containing C-2 and to urea containing C-8[8].

TABLE III

THE DISTRIBUTION OF ^{14}C ACTIVITY IN THE SUPPOSED COENZYMICALLY ACTIVE COMPOUNDS (COUNTS/MIN/μMOLE)

The composition of the incubation mixture was the same as in the usual experiments. After incubation, the samples were deproteinized with 10% trichloroacetic acid and 1.1 μmoles of anhydroleucovorin was added as a carrier. Trichloroacetic acid and Veronal were extracted by three portions of ether and the samples were evaporated to dryness in a vacuum desiccator over KOH. The chromatography was carried out on Whatman No. 3 paper with 0.1 M aqueous formic acid. The bands of the coenzyme were detected under Mineralight and then cut out. They were eluted with water directly on to planchettes for counting.

Substance isolated	*Coenzyme added*	
	Tetrahydrofolate	*Tetrahydroaminopterin*
N^5,N^{10}-Methenyltetrahydrofolate	127	5
N^{10}-Formyltetrahydrofolate	481	—
N^{10}-Formyltetrahydroaminopterin	—	365

TABLE IV

THE PERCENTAGE DISTRIBUTION OF THE ACTIVITY IN C-2 AND C-8 OF THE INOSINATE SYNTHESIZED USING EITHER TETRAHYDROFOLATE OR TETRAHYDROAMINOPTERIN AS COENZYME

Hypoxanthine was isolated from the deproteinized incubation mixture by paper chromatography, using water-saturated butanol, and was then oxidized by xanthine oxidase[7] in a total volume of 5 ml. The oxidation was carried out under vigorous stirring until dichlorophenolindophenol solution added dropwise to the sample was discoloured no more. The sample was deproteinized by heating at 100° for a brief period and centrifuged. After the acidification of the supernatant fraction with conc. HCl to pH 2–3, uric acid crystallized on standing overnight in a cold box. The uric acid crystals were isolated and their specific activity was determined. They were then used for chemical degradation: uric acid (30 mg) was suspended into 1.0 ml of 5.5 N HCl in a centrifugation tube, 30 mg of $KClO_3$ in 0.5 ml water were added, and the solution was heated for several minutes in a boiling-water bath. After cooling, the crystals of alloxan were centrifuged down and washed with 1.0 ml of water. The washing was added to the alloxan mother liquor. Alloxan was suspended in water and evaporated on the planchette for counting. To the combined mother liquors, neutralized to pH 7 with 25% ammonia, an equal volume of glacial acetic acid and 1.0 ml of a saturated methanolic solution of xanthydrol were added. After standing overnight the dixanthylurea was centrifuged down, washed 5 times with methanol, and suspended in 1.0 ml of dimethylformamide. The dimethylformamide solution was evaporated to dryness on a planchette for counting.

Coenzyme	*% incorporation of $H^{14}COONa$*	
	C-8 (urea)	*C-2 (alloxan)*
Tetrahydrofolate	50.6	49.4
Tetrahydroaminopterin	53.3	46.7

Table IV shows the distribution of the radioactivity between C-2 and C-8 of inosinate synthesized with either the tetrahydrofolate or the tetrahydroaminopterin coenzyme. The distribution of activity between both carbon atoms did not differ significantly when tetrahydrofolate, or tetrahydroaminopterin, was used as the coenzyme. Therefore, it may be supposed that in the course of purine biosynthesis tetrahydroaminopterin can participate in both glycinamide ribotide and aminoimidazolecarboxamide ribotide transformylations.

DISCUSSION

The activation effect of some 4-amino analogues with a hydrogenated pyrazine ring on the biosynthesis of purine can be most probably explained by the fact that they substitute for the natural tetrahydrofolate coenzymes in transformylation reactions. All hydrogenated 4-amino analogues show an activation effect both in the presence, and in the absence, of tetrahydrofolate, but a comparison of Tables I and II shows a difference in the degree of activation observed. This effect could be explained by several more complicated mechanisms, *e.g.* the ability of the hydrogenated 4-amino analogues to prevent the oxidation of tetrahydrofolate derivatives, or the utilization of the formyl or methylene groups of the antimetabolites themselves for the biosynthesis of purine.

Considering these findings the biochemical action of the 4-amino analogues with the hydrogenated pyrazine ring might differ, to some extent, from that of the non-hydrogenated 4-amino analogues of folic acid. It can be supposed that these substances might effect the synthesis of thymidylate either directly[9] or indirectly[10] via inhibition of folate reductase. The lack of tetrahydrofolate coenzymes for purine biosynthesis due to the inhibition of the folate reductase might be eliminated by the coenzymic action of the antimetabolites themselves.

REFERENCES

[1] K. SLAVÍK, V. SLAVÍKOVÁ AND Z. KOLMAN, *Collection Czech. Chem. Commun.*, 25 (1960) 1929.
[2] V. SLAVÍKOVÁ AND K. SLAVÍK, *Experientia*, 17 (1961) 1.
[3] S. C. HARTMAN AND J. M. BUCHANAN, *J. Biol. Chem.*, 234 (1959) 1812.
[4] G. K. HUMPHREYS AND D. M. GREENBERG, *Arch. Biochem. Biophys.*, 78 (1958) 275.
[5] D. A. GOLDTWAITH AND G. R. GREENBERG, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic Press, New York, 1955, p. 504.
[6] K. SLAVÍK, H. TOMÁŠOVÁ AND V. SLAVÍKOVÁ, *Collection Czech. Chem. Commun.*, 23 (1958) 1378.
[7] H. M. KALCKAR, *J. Biol. Chem.*, 167 (1947) 461.
[8] J. C. SONNE, J. M. BUCHANAN AND A. M. DELLUVA, *J. Biol. Chem.*, 173 (1948) 69.
[9] K. SLAVÍK AND V. SLAVÍKOVÁ, *Abstr. 5th Intern. Congr. Biochem. Moscow 1961*, p. 82.
[10] M. J. OSBORN, M. FREEMAN AND F. M. HUENNEKENS, *Proc. Soc. Exptl. Biol. Med.*, 97 (1958) 429.