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Comparative inhibition of plant dihydrofolate reductases by folate analogues

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Fisiologia. — *Comparative inhibition of plant dihydrofolate reductases by folate analogues.* Nota di PAOLO CROSTI e RENATO BIANCHETTI, presentata (*) dal Socio S. TONZIG.

RIASSUNTO. — L'efficacia di alcuni tra i più conosciuti inibitori delle diidrofolico-riduttasi dei batteri, di vari organismi patogeni e di animali è stata misurata sull'attività diidrofolico riduttasi di *Euglena gracilis, Neurospora crassa, Pisum sativum* e *Zea mais.*

Aminopterina e ametopterina, analoghi del folato appartenenti alla classe dei derivati delle 2–4–diaminopteridine, si sono mostrati, con l'eccezione per l'enzima di *Euglena gracilis,* inibitori molto efficaci sulle diidrofolico-riduttasi saggiate, con un 50 % di inibizione attorno alla concentrazione di 10^{-9} M. Questo grado di inibizione è del tutto paragonabile a quello mostrato per gli enzimi di altre fonti.

Tra gli analoghi del folato appartenenti alle 2–4–diaminopirimidine si è usato il trimetoprim, inibitore conosciuto per la grande diversità di azione sulle diidrofolato–riduttasi di diverse fonti. La concentrazione richiesta per avere il 50 % di inibizione va da 4×10^{-2} per l'enzima di *Euglena* a 4×10^{-7} per l'enzima di pisello. I dati suggeriscono che i derivati 2–4–diaminopirimidinici possono essere utili per evidenziare nelle piante differenze specifiche di risposta agli antifolici.

INTRODUCTION

The major metabolic pathways of folic acid and its reduced derivatives have been substantially established in a variety of prokaryotic and eukaryotic organisms, plants included [1]. The reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THFA) is generally considered the most important reaction for a normal functioning of the THFA cycle. The enzyme catalyzing this reaction, dihydrofolic reductase, has been extensively studied in a very wide range of organisms, but not in plants. Studies with selective inhibitors showed rather striking differences between enzymes from different, sometimes phylogenetically rather closely related, sources [2]. The logical consequence of these studies was the attempt to design or to modify chemical molecules for obtaining species specific inhibitors [3]. These compounds, referred to as folate analogues, are now extensively used in chemotherapy and, in addition, are useful tools for the elucidation of the cellular processes strictly related to folate metabolism.

In this work the responses of the dihydrofolate reductases from different groups of plants to the more common folate analogues have been evaluated. A comparison with dihydrofolate reductases from bacterial and mammalian sources is also reported.

(*) Nella seduta del 13 gennaio 1979.

METHODS

Dihydrofolic acid was synthetized from folic acid according to the method of Futterman [4]. Homogenates were obtained by extraction (1 g fresh weight: 2 ml buffer) of seedlings of maize, pea or actively growing *Euglena gracilis* or *Neurospora crassa*. The extraction buffer was: 10^{-1} M Tris, pH 7.4, 2×10^{-3} M, Mg acetate, 10^{-3} M mercaptoethanol, 10^{-4} M EDTA.

After centrifugation at 100,000×g for two hours, the *fraction* 30–60 % *of* $(NH_4)_2SO_4$ *saturation* of the supernatants was collected and dialysed against the extraction buffer. These enzyme preparations are substantially stable when stored at – 20 °C. Dihydrofolate reductases were assayed according to the method of Osborn and Huennekens [5].

RESULTS AND DISCUSSION

The inhibitory effect of folate analogues on plant dihydrofolate reductases was studied.

The term analogue is normally used in its broadest sense; that is, it denotes those compounds which have a chemical structure sufficiently similar to that of folic acid to exhibit inhibitory properties which stem from this structural similarity. The term therefore includes 2, 4-diamino-pteridines as well as 2,4-diamino pyrimidines. These two classes of inhibitors are considered separately. Many derivatives of 2,4-diamino-pteridine are powerful inhibitors of dihydrofolate reductase, the best known being aminopterin (4-amino-deoxyfolate) and amethopterin (4-amino-10-methyl-deoxyfolate). Both these inhibitors are bound so tightly to dihydrofolate reductase from most sources

TABLE I

The inhibitory effect of aminopterin and amethopterin on plant dihydrofolate reductases.

	Aminopterin (I_{50})	Amethopterin (I_{50})
	M	M
Zea mais	5×10^{-9}	2×10^{-9}
Pisum sativum	4×10^{-9}	10^{-9}+
Neurospora crassa	10^{-8}	1.7×10^{-9}
Euglena gracilis	3×10^{-7}	4×10^{-8}

+ stoichiometric

that its behaviour at least approximates that of a stoichiometric inhibitor. The effect of aminopterin and amethopterin on plant dihydrofolate reductase is reported in Table I. Pea enzyme and amethopterin is the only system in which a competitive stoichiometric inhibition can be noticed.

However, with the exception of the *Euglena gracilis* enzyme, the inhibitor concentration for 50 % inhibition (I_{50}), is very low for the enzymes tested, and sufficiently close to that of other sources (10^{-9} average value) [6]. Trimethoprim, (2,4-diamino-5-(3′,4′,5′-trimethoxibenzil pyrimidine) is the most interesting inhibitor of the 2–4-diamino-pyrimidine derivatives. The concentration of trimethoprim required for a 50 % inhibition of the mammalian enzymes is greater by a factor of 16,000 than that required for a similar inhibition of the bacterial enzymes. The effect of trimethoprim on plant dihydrofolate reductase is reported in Table II. The I_{50} ranges from 4×10^{-7} M

TABLE II

The inhibitory effect of trimethoprim on plant dihydrofolate reductases and comparison with enzymes from other sources.

	Trimethoprim (I_{50})
	M
Zea mais	6×10^{-5}
Pisum sativum	4×10^{-7}
Neurospora crassa	7×10^{-4}
Euglena gracilis	4×10^{-2}
Mammals	3×10^{-4} +
Bacteria	8×10^{-9} +

+ average values.

for the pea enzyme to 4×10^{-2} M for the *Euglena* enzyme. This range practically covers the strong difference in effectiveness shown by this inhibitor for bacterial and mammalian enzymes. It is also to be noted that the maize enzyme requires a concentration of inhibitor almost a hundred times greater than that required for the pea enzyme. Thus, trimethoprim has a smaller effect than aminopterin and amethopterin but shows a broader discrimination of the tested reductases. On this basis it seems possible to suggest that the 2-4-diamino-pyrimidines are useful tools for evidencing selected responses of plants to the antifolates.

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