Journal of Chromatography, 108 (1975) 216–218

CHROM. 8196

Note

Improved bioautographic procedure for the detection of thiamine pyrophosphate or its pyrimidine precursors

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(Received December 2nd, 1974)

Bioautography combines the separatory abilities of paper chromatography with the sensitivity of microbiological assay. It depends on the separation of a mixture of growth factors via paper chromatography followed by their detection as areas which support growth of appropriate auxotrophs. Bioautography was originally developed by Winstein and Eigen[1]. Carlson and Brown[2] then used a modification to detect and identify hydroxyethyl thiamine as a product formed from thiamine pyrophosphate and either pyruvate or acetaldehyde. Subsequently, Akagi and Kuamaoka[3] identified thiamine and its phosphates using bioautography. Newell and Tucker[4] also used it to estimate the rate of phosphorylation of thiamine.

Bioautography is conventionally limited because the identification of a test compound is accomplished by the use of a suitable auxotroph. The growth requirements for the auxotroph are present in the bioautographic plate except for an essential growth factor which is chromatographed. Isolation of suitable auxotrophs, however, is time consuming and there is the risk of genetic instability.

A prototroph could be used as the indicator organism if the compound to be assayed prevented end-product inhibition of *de novo* biosynthesis. Such a procedure is advantageous since the appropriate auxotroph would not have to be isolated to use the bioautographic procedure. For example, it has been demonstrated that B_1 pyrimidine (4-amino-5-hydroxymethyl-2-methylpyrimidine), thiamine, or their phosphorylated derivatives prevented end-product inhibition of adenine and adenosine in the purine biosynthetic pathway[5–8]. The prevention of adenine inhibition made it feasible to use normal bacterial prototrophs, rather than thiamine-requiring auxotrophs, as indicator organisms. Growth would ensue when the assayed compound was eluted off the paper chromatogram onto the medium before incubation.

EXPERIMENTAL

Aliquots (2–5 ng) of thiamine, its phosphorylated derivatives, or B_1 pyrimidine were applied to descending paper chromatograms (Whatman No. 1 chromatography paper). The solvent system used for paper chromatography was *n*-propanol–isoamyl

alcohol–water–isobutyric acid–ammonium hydroxide (28%) (7.2:2.5:7.5:12.0:0.2) by volume. The air-dried chromatogram was placed on the bioautography plate for 15 min to allow elution of the test compounds. The bioautography plate consisted of a 2% agar basal layer composed of minimal medium[9], 0.15 m*M* adenine, 0.6 mg/ml of *p*-iodonitrophenyltetrazolium chloride, and a 1-ml inoculum of *Escherichia coli* B. The inoculum was prepared by growing *E. coli* B on minimal medium for 18 h at 37° in a water-bath shaker. The cells were harvested and washed two times with sterile, distilled water and re-suspended to a turbidity of 90 Klett units using a Klett–Summerson colorimeter at 540 nm. After incubation of the bioautogram at 37° for approximately 6 h, a red spot of reduced tetrazolium salts appeared wherever a growth factor that prevented adenine inhibition was present. The rest of the plate remained colorless. The R_F values of these compounds could then be determined and compared with unknowns for identification.

The optimal concentrations used were 2–5 ng of the test compounds. Reduction of the tetrazolium salt would be barely perceptable with less than 2 ng while the chromatogram would become overloaded with greater than 5 ng. Inoculum of *Salmonella typhimurium* Thi-1 was prepared similar to *E. coli* B plus 5 ng/ml of B_1 pyrimidine.

RESULTS AND DISCUSSION

Table I compares the R_F values obtained using *E. coli* B as the prototroph and Thi-1 as the B_1 pyrimidine requiring auxotroph. The R_F values were virtually identical when either an auxotroph or prototroph was used.

TABLE I

BIOAUTOGRAPHY OF THIAMINE AND RELATED COMPOUNDS USING *E. coli* B AND *S. typhimurium* Thi-1

Compound	R_F *value*	
	E. coli B	*Thi-1*
Thiamine pyrophosphate	0.14	0.13
Thiamine monophosphate	0.50	0.50
Thiamine	0.75	0.77
B_1 pyrimidine	0.72	0.73
W-11 supernatant fluid	0.75	—*

* Indicates no growth.

E. coli strain W-11 is a *pur* E mutant of K-12 with a genetic block after the biosynthesis of 5-aminoimidazole ribonucleotide (AIR), the last common intermediate between purine and B_1 pyrimidine biosynthesis. W-11 was grown on minimal medium[9] plus 0.15 m*M* hypoxanthine. A compound was accumulated in the medium that prevented adenine inhibition of *E. coli* B. After incubation for 18 h at 37° in a water-bath shaker, the medium was filtered and the resulting supernatant fluid chromatographed. Only one spot of growth was observed by bioautography (Table I). The R_F value (0.75) was not similar to any of the tested compounds except thiamine. The compound in the supernatant fluid was not thiamine, however, because it did not

support the growth of Thi-1, as does thiamine. Therefore, this isolated compound does not lie in the biosynthetic pathway between B_1 pyrimidine and thiamine pyrophosphate[10,11], though it must be closely related because it could prevent adenine inhibition.

The use of non-fastidious prototrophs as indicator organisms in bioautography makes this procedure more feasible. It eliminates the need for the isolation or procurement of the appropriate auxotrophs.

ACKNOWLEDGEMENT

This research was supported by a grant from the Brown Hazen Foundation.

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