OBSERVATIONS ON THE REGULATION OF THE SYNTHESIS OF THE TRICARBOXYLIC ACID CYCLE ENZYMES IN BACILLUS SUBTILIS, MARBURG

by

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Our recent studies on several mutants of B. subtilis, Marburg incapable of synthesizing aconitase indicate that in a sporogenic strain the full operation of the tricarboxylic acid (TCA) cycle enzymes is necessary for the expression of the sporulating capacity. (Hanson, R.S. et al., 1964). Transformation experiments carried out with DNA obtained from these mutants further confirmed this conclusion. These experiments together with those obtained with B. cereus (Nakata and Halvorson, 1960; Hanson et al., 1963a,b) have directed our attention to the regulation mechanism controlling the synthesis of the TCA cycle enzymes because of the importance it may play in cellular metabolism in general and because it may also control the physiological process of sporulation.

The repression of the TCA cycle enzymes during exponential growth of B.cereus in a complex medium containing glucose has been described (Hanson et al., 1963a,b). Further experiments from their laboratory suggested that both a carbon source and a nitrogen source are required for the repression of these enzymes. The data presented here indicated that this is indeed the case in B. subtilis.

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The experiments summarized in table I show an almost complete repression of aconitase activity during logarithmic growth in complex media containing glucose, while the repression is relatively low in nutrient broth without the addition of glucose (some carbohydrates may be still present in this medium). Following glucose exhaustion (T_0), there is an increase in the specific activities of these two enzymes.

Table I : Repression of Aconitase and Isocitric dehydrogenase by glucose during exponential growth of B. subtilis, in complex medium .

Time of harvesting	Nutrient Broth		Nutrient Broth + 0.1% glucose		G Medium +)	
	Aconi-tase	Isocit. DH-ase	Aconi-tase	Isocit. DH-ase	Aconi-tase	Isocit. DH-ase
Exponential growth	152	0.163	0-43	0.190	0	0.009
T_0	---	0.161	----	0.930	33	0.090
T_1	412	0.450	900	-----	220	0.240
T_3	232	0.323	800	1.12	386	0.250

+) Because the pH changes in Nutrient Broth + glucose and in G medium, these media were neutralized by the addition of sterile KOH. Specific activities are expressed in µmoles of $NADPH_2$/mg prot./min (isocitric dehydrogenase) or in Δ OD240/mg prot./min (aconitase).

Because glutamate synthesis is one of the important functions of the TCA cycle, glutamate was tested for its effect on enzyme synthesis when added to a minimal medium containing glucose (MG medium) as a carbon source. Three cultures of B. subtilis, wild type, were grown on minimal medium with different nitrogen sources, under otherwise identical conditions. In the first, ammonia was the only nitrogen source, glutamate was added to the second and yeast extract to the third. The cultures were harvested at different times during exponential growth and the extracts were assayed for aconitase, isocitric dehydrogenase and malic dehydrogenase. The results of one such experiment are shown in table II.

Table II : Activities of the enzymes of the tricarboxylic acid cycle during exponential growth of B. subtilis, Marburg (SMY) in various media.

		Sp. activities		
		1	2	3
Medium	O.D. of culture	Aconitase	Isocit. DH-ase	Malic DH-ase
MG	0.600	680	0.82	1.21
MG + 25mM glutamate	0.600	68	0.22	0.49
MG + 0.1% Yeast extract	0.600	48	0.03	0.14

The basal medium (MG) is composed of a mineral solution supplemented with 0.5% glucose (Anagnastopoulos and Spizizen, 1960). Other additions are noted above. Cells were harvested at exponential phase and the specific activities of the various enzymes in the extracts were determined. Specific activities are expressed in µmoles of $NADH_2$ or $NADPH_2$/mg prot./min. for 2 and 3 and in ΔOD_{240}/mg prot./min. for 1. The specific activities of cells harvested at an OD of 0.600 are presented here.

It can be seen that if only glucose is present in minimal medium the specific activities of these enzymes are relatively high. However, when glutamate or yeast extract is added to the culture, the synthesis of these enzymes is repressed. The repression of the synthesis of isocitric and malic dehydrogenases is more severe when yeast extract is added to the medium than when glutamate is used, although the latter is also quite effective. Arginine can replace glutamate in the repression of the aconitase as shown in table III.

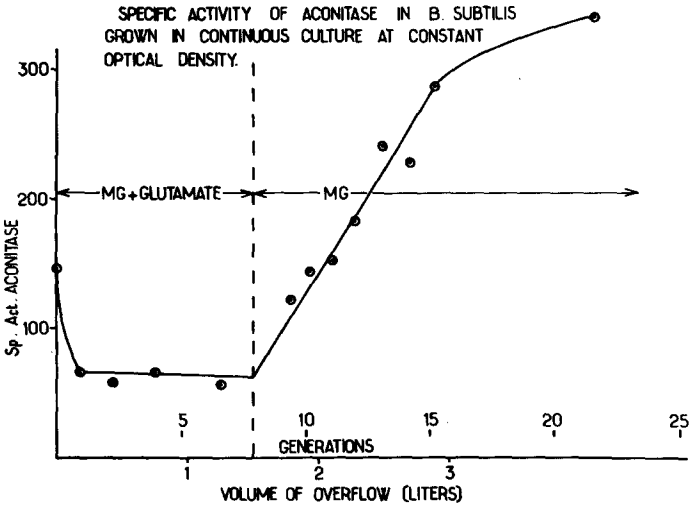
Table III : The effect of addition of various amino acids to the growth medium on aconitase synthesis.

Amino acid added to the growth medium	Conc. (mM)	Aconitase activity
None	25	723
Glutamate	25	18
Aspartate	25	736
Glutamate + Aspartate	25 , 12.5	107
Arginine	12.5	49
Lysine	12.5	403

The cells were harvested from G medium at an OD of 0.600 and the extracts assayed for the various enzyme activities. Additions to the growth medium are given above.

This is to be expected since, arginine, as shown by Wiame et al., 1961, is converted to glutamate by B. subtilis and also supports growth of glutamate requiring aconitase⁻ mutants (Ramos et al., 1962; Hanson et al., 1964). Lysine partially represses aconitase while aspartate is not effective and perhaps slightly relieves the repression of glutamate.

Figure I shows the specific activity of aconitase in B. subtilis grown in continuous culture at constant optical density. The synthesis of this enzyme increases several fold when cells growing in a medium with glucose and glutamate are allowed to continue growth in the same medium without glutamate.



The optical density of the bacterial population was maintained at approximately 0.550 by adjusting the flow rate of the medium into the growth flask. Samples of the overflow were collected in an ice bath, and the extracts of these cells were assayed for aconitase as described previously (Hanson et al., 1964).

We have also examined the effect of different carbon sources on the repression of the synthesis of aconitase in a minimal medium in the presence of glutamate. Table IV shows that glucose and glycerol, but not acetate or lactate, support repression or aconitase synthesis.

This experiment also demonstrates that glucose alone, without any external addition of glutamate, causes considerable repression of the enzyme, as compared to the derepression obtained in the presence of lactate or acetate plus glutamate.

(lactate or acetate alone do not support growth of B. subtilis) Perhaps this is due to the accumulation of a glutamate pool during exponential growth (see Aubert et al., 1963).

Table IV : Effect of different carbon sources on the repression of the synthesis of the tricarboxylic acid cycle enzymes.

Additions to minimal medium	Conc. (mM)	Aconitase	Isocit. DH-ase	Malic DH-ase
		Specific activities		
Glucose	28	890	---	3.30
Glucose + glut.	28;25	20	0.03	2.20
Glycerol + glut.	56;25	75	0.02	1.77
Lactate + glut.	56;25	1163	0.52	2.60
Acetate + glut.	84;25	2780	0.56	2.50

The mineral medium was that indicated in table II. The cells were harvested at an O.D. of 0.600 and the extracts assayed for the enzymes shown.

Since glutamate and glucose produce repression of the aconitase synthesis, one could expect the inhibition of sporulation under conditions of such repression. These conditions are similar to those created by the deficiency of aconitase synthesis in the aconitase$^-$ mutants, which, as we have shown (Hanson et al., 1964), are unable to sporulate, although they are genetically Sp$^+$. Attempts were therefore made to inhibit sporulation of B. subtilis, (try$^-$ mutant 168, Marburg) grown in the synthetic medium described by Donnellan et al., (1964). The results have shown that in the presence of an excess of glutamate (25 or 50 µmoles/ml) and glucose (50 µmoles/ml) the inhibition of sporulation reaches about 60-80% as compared to a control of the same medium containing only 5 µmoles/ml of glutamate and 50 µmoles/ml of glucose. Similar results have been obtained by Donnellan et al. (1964) using B. subtilis (ATCC 6051).

The regulation of the tricarboxylic acid cycle in whole cells probably involves other mechanisms than the one described here, such as repressible permease (Ramos et al., 1962) or feed-back inhibition. The dual requirement

for a glucose (or glycerol) catabolite and an organic nitrogen source is not understood as yet. Perhaps glutamate, in our case, spares the hypothetical corepressor postulated by Magasanik (1961) to be responsible for catabolite repression. It is also possible that glutamate itself, or a derivative of glutamate, is one of the corepressors in a system sensitive to multivalent repression (Freundlich et al., 1962) by some of its products. Derepression should also be expected to occur when the concentrations of such hypothetical corepressors could be experimentally lowered.

Regardless of the mechanism of the regulation of the TCA cycle, it is an important phenomenon because it provides the cells with a primary means of regulating the synthesis of glutamate and amino acids derived from it. It is especially important in the sporogenic bacilli because of the apparent relationship between this regulation and sporulation process.

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