INOSINE AND LACTATE: FACTORS CRITICAL DURING GROWTH FOR DEVELOPMENT OF COMPETENCE IN HAEMOPHILUS INFLUENZAE

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Competence in bacteria is the capacity of the cells to take up extracellular DNA from the medium. The development of competence in H. influenzae takes place in two phases. In phase one the organisms are grown and in phase two competence is developed (Goodgal & Herriott, 1961). High levels of competence develop when the medium for both phases is Difco brain heart infusion broth (B-H) supplemented with hemin and nicotinamide adenine-dinucleotide (NAD). It was observed that when cells were grown in a chemically defined medium they failed to yield comparable high levels of competence even though the second phase medium was B-H (Talmadge & Herriott, 1960). This suggested that B-H contained unrecognized competence promoting activity that was essential during growth. The present report describes evidence that inosine and L(+)lactate together, have such activity.

Materials and Methods

In general the strains of H. influenzae, the growth and handling conditions, DNA preparations genetic marker and transformation techniques have been described in one or another of the earlier publications (Goodgal & Herriott, 1961; Barnhart & Herriott, 1963; and Spencer & Herriott, 1965).

Media: T_C (used for phase one): This was prepared by supplementing 100 ml quantities of 1% solution of Baltimore Biological Laboratory trypticase (after autoclaving and cooling) with (a) 17.4 ml of 10 times concentrated Earl's salts solution which

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was made up to 68 gms NaCl, 4 gms KCl, 2 gms $MgSO_4.7H_2O$, 2 gms $CaCl_2$, 1.4 gms $NaH_2PO_4.2H_2O$ and 14 gms glucose in 1000 ml H_2O, (b) 0.93 ml of 25 mgs/ml cystine or cysteine in 0.01N HCl, (c) 0.93 ml of 2.5 mgs/ml hypoxanthine in 0.01N HCl, (d) 0.93 ml of 2.5 mgs/ml uracil or uridine in 0.01N HCl or distilled water, respectively, (e) 0.53 ml of 1 mg/ml each of thiamine and calcium pantothenate in water, (f) 0.53 ml of 1 mg/ml NAD in water, (g) 2.3 ml of 1 mg/ml hemin in triethenolamine and 1 mg/ml L-histidine (free base). (a) was sterilized by autoclaving; (g) was sterilized by heating 30 minutes at 60°C and the others were sterilized by filtration through Millipore filters (pore size = 0.22-0.3μ). The above T_c medium is 2 times concentrated and was diluted with an equal volume of the solution being assayed.

B-H (used for phase two): This was the usual Difco 3.7% brain heart infusion broth sterilized by autoclaving and after cooling to room temperature it was supplemented with 10 μg/ml hemin solution (see g. above) and 2 μg/ml NAD.

Assay for Competence Promoting Factors

When cells were grown in T_c they developed low levels of competence upon transfer into B-H for phase two. However, the addition of 0.4% Difco heart infusion broth (H) to T_c for phase one led to maximal levels of competence development in B-H. Samples were assayed for competence promotion by introducing a known quantity or volume into T_c and comparing their action to one in which 0.4% H was added.

Details: Phase one: Two ml of frozen stock wild type Rd *H. influenzae* at 1-3 x 10^9/ml in 15% glycerin was washed once with, and resuspended in 2 ml 0.01M phosphate in 0.85% saline (PBS) pH 6.7; this was then diluted one to 100 in T_c containing the fraction to be tested. The cells were grown with aeration until they reached a density of about 4 x 10^8/ml as judged by turbidity at 650 mμ. at which time 5 ml was centrifuged, washed once with PBS and resuspended in 0.3 ml of PBS.

Phase two: 0.2 ml of the above concentrated cell suspension was added to 1.6 ml of B-H; this was allowed to stand without agitation for 75 min at 37°C at which time DNA carrying the streptomycin resistance marker was added to a concentration of 1 μg/ml. After standing for 15 min the cells were agitated for 30 min; samples were then diluted and plated in duplicate in the usual

manner. Transformants and viable counts were scored after 24 hours incubation at 37°C. Other controls included cells without added DNA and DNA without cells.

Evidence for Two Competence Promoting Factors in Heart Tissue Extract

When a 10% (w/v) aqueous extract (60 min at 60°C) of Difco heart tissue was passed through a Dowex 50 (H^+) column, no competence promoting activity was adsorbed; it was found in the water eluate. However, passage through a Dowex 1 (Cl^-) column followed by elution first with water then with increasing KCl to 2M revealed that neither the initial effluent nor the KCl eluate was very active alone; on mixing them, 50% of the initial activity was observed. This indicated that there were at least 2 components from heart tissue extract required during growth in order for the cells to become competent in phase two.

Nature of the Active Factors

The failure of either factor in the heart extract to adsorb to Dowex 50 suggested that they were not cationic in nature. Adsorption of one of them to Dowex 1 indicated that it was anionic, whereas the other must have been relatively neutral. Several known low molecular weight acid salts were found to have some competence-enhancing activity. Of the components of ox muscle extracts (Wood & Bender, 1957) that were not in the synthetic medium, lactate and inosine were the most interesting; the former for its abundance and the latter because its ultraviolet absorption peak at 248-250 mμ was suggestively similar to the absorption spectrum of the water eluate from the Dowex 1 column. Upon testing these substances strong activity was observed as seen in Table 1. Although this does not constitute identification of the components of H it permits the replacement of H by known substances.

Synergistic Action of Inosine and L(+)lactate

Experiments indicated that the optimal or plateau levels of inosine and L(+)lactate are in the range of 100-500μg/ml of each.

Table 1 contains the results of experiments which indicate the level of competence of cells grown in T_c containing each and both components. Phase two was performed in B-H. It is clear that while each component lifted the level of competence significantly their combined action was greater than the sum of the separate actions. This synergistic action may reflect the action

Table 1

Effect of Inosine and L(+)Lactate on Competence Development

Medium	Trans/ml x 10^{-6}	% of Standard T_c+H
T_c	0.07-0.1	0.9
T_c+H	9.7 -13.3	100
T_c + Inosine*	0.9-1.3	10
T_c + L(+) Lactate‡	1.6-3.2	30
T_c + Inosine + L(+)lac.	10.0-12.5	100
T_c + deoxyinosine + L (+) lactate	12.1	100
T_c + inosine + pyruvate	8.0-9.0	85

Cell conc. at end of 30 minute shaking period (See Methods) = 3.7×10^9/ml ± 15%

*Inosine = 100µg/ml (Calif. Biochemical Co.)

‡L(+) lactate = 500 µg/ml Na salt. (Fischer Scientific Co.) D(-) lactate (Calif. Biochemical Co.) was just as effective as L(+) lactate. The D(-) lactate contained less than 1% L(+) lactate when assayed with rabbit muscle lactic dehydrogenase. (Sigma)

of each of the components on a different essential step.

Specificity of Components

Inosine: Free purine or pyrimidine bases substituting for inosine in T_c plus lactate produced cells that became less than 20% as competent as those formed in the presence of inosine. The bases examined in 100 µg/ml concentrations were adenine, guanine, cytosine, uracil, hypoxanthine and thymine. The nucleosides cytidine, deoxycitidine, xanthosine, and thymidine and the nucleoside precursor 5-amino-4-imidazole carboxamide riboside in 150 ug/ml concentrations produced cells which were less than 30% as competent as those grown in the presence of inosine. In the presence of adenosine, guanosine, or their deoxy forms the cells were half as competent as those grown in inosine. It has been reported that in some bacterial species conversion of bases (Magasanik; Mitchell & McElroy, 1946; and Mager & Magasanik, 1960) is observed. It is of interest that deoxyinosine (Sigma Chemical Co., St. Louis, Mo.) was just as effective as inosine (Table 1) at the above concentrations and that neither hypoxanthine plus ribose nor ribose alone was very effective.

Lactate: Testing the competence promoting quality of other acid salts in the presence of inosine showed that cells grown in

the presence of β-hydroxy-butyrate, alanine or propionate were less than 30% as competent as those grown in the presence of L(+) lactate. Pyruvate, however, was 80-90% as effective as lactate. (Table 1)

Competence of Cells Grown in a Defined Medium

As noted earlier a defined medium for growth of H. influenzae was described some years ago (Talmadge & Herriott, 1960). Others (Butler, 1962; Holt, 1962; and Wolin, 1963) have made similar reports. In only one instance (Talmadge & Herriott, 1960) was competence evaluated and then the level was very low. In no instance were inosine or lactate components of the medium. A newly compounded growth medium has been developed in which cells grow readily but fail to develop competence in stage two unless inosine and lactate are included during growth. Certain irregularities in the results, however, have necessitated our withholding publication of the details until the cause of these irregularities is understood and/or removed.

DISCUSSION: It was reported recently from this laboratory (Spencer & Herriott, 1965) that H. influenzae grown in H subsequently developed high levels of competence during phase two in a defined medium (MII) notable for the absolute necessity of 4 amino acids and for the absence of several factors essential for growth. T_c+ inosine and lactate grown cells showed the same result. The amino acids in M-II that were essential for competence development were L-aspartate, L-glutamate, L-cystine and L-arginine.

In the present paper it is seen that during the growth phase of these cells 2 nutrients, inosine and L(+) lactate are needed not for growth, but for the subsequent development of competence.

These nutrients that are essential for competence development will provide convenient probes for analysis of this intriguing cellular phenomenon.

SUMMARY:

Evidence is presented to show that inosine or deoxyinosine and L(+) lactate or pyruvate are strong competence promoting factors for H. influenzae. They are not essential for growth but must be present in the growth medium for subsequent development of competence.

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