Time-Dependent Effects of Inosine on Competence Development in *Haemophilus influenzae*

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The effect of inosine on the development of competence is dependent upon the time at which it is added to the competence medium. Competence is inhibited if inosine is added within 15 min after the development begins, but is stimulated if the addition is made at later times. Protein synthesis is stimulated by the addition of inosine, but the effect is quantitatively similar regardless of the time at which inosine is added.

Competence in the genetic transformation of *Haemophilus influenzae* develops in response to a nutritional step-down (7); transfer of cells growing logarithmically in a rich medium to a medium capable of supporting only limited growth results in the appearance of the competent state. The maximum level of competence developed in the population occurs about 2 hr after the shift to the competence medium (4). Ranhand and Herriott (5) demonstrated that the presence of inosine and lactate in the growth medium enhances the subsequent level of competence attained after transfer to competence medium.

This report shows that a stimulation of competence development in response to inosine can be demonstrated when the cells are in the competence medium, but only if added at least 10 to 15 min after the shift is made. Addition of inosine at any time prior to that is inhibitory. A stimulation of protein synthesis occurs as a result of this addition.

**MATERIALS AND METHODS**

*Media.* For growth, the synthetic medium M1 (3) was used with minor modifications. The complete medium contains both inosine and hypoxanthine, but for these experiments only one or the other was used. When grown with inosine, a level of 2,000 μg/ml was used and hypoxanthine was omitted. When grown with hypoxanthine, a level of 5 μg/ml was used and inosine was omitted. Generation times in media containing inosine are approximately 35 min, and in media containing hypoxanthine approximately 50 min. No growth occurs if both are omitted.

For competence development the MV medium (4) was used in all cases. This is a nutritionally deficient medium for *H. influenzae*, lacking in certain critical growth factors but capable of inducing the development of competence.

**Growth of cells and the development of competence.** The synthetic medium M1 was inoculated from a frozen culture of *H. influenzae* (strain Rd) to an initial density of ca. 5 X 10⁶ cells/ml. This culture was grown at 37 C to a density of 5 X 10⁹ cells/ml as determined by turbidity measurements at 650 nm in a Coleman Jr. spectrophotometer. The cells were then washed once in medium M-II (7) and suspended in a volume of medium to give a cell density of about 7 X 10⁹ cells/ml. This suspension in MV determines the zero time for competence development. After shaking at 37 C for 100–150 min, maximal levels of competence were obtained.

**Transformation.** The level of competence was determined by exposing the cells to 1 μg/ml of a purified preparation of deoxyribonucleic acid (DNA) isolated from *H. influenzae* (Rd) resistant to 2,000 μg of streptomycin per ml, by the method of Goodgal and Herriott (2). After incubation at 37 C with the DNA for 10 min, the cells were diluted in 0.37% Brain heart infusion (Difco) in 0.15 M NaCl and plated on 3.7% Brain Heart Infusion Agar (Difco). The cells were challenged 2 hr after plating, by the overlay technique, with a final concentration of streptomycin equal to 50 μg/ml. Colonies were counted after 24 to 36 hr at 37 C.

**Radioisotope Incorporation.** Incorporation of [H]-inosine (2.86 Ci/mmmole; New England Nuclear Corp., Boston, Mass.) or [14C]-phenylalanine (0.05 Ci/mmmole; Schwarz BioResearch, Inc., Orangeburg, N.Y.) was measured by pipetting 0.1 ml of the culture into 0.5 ml of cold 10% trichloroacetic acid and allowing the mixture to remain on ice for at least 5 min. The precipitate was then washed onto a 0.45-μm nitrocellulose filter (type HA; Millipore Corp.) which had been soaked in a 0.01 M solution of the unlabeled compound and washed three times with 10 ml of cold 5% trichloroacetic acid. After drying, the filters were placed into 20-ml scintillation vials (low potassium; Wheaton Glass Co., Millville, N.J.) with a toluene-based scintillation fluid, using Omnifluor (New England Nuclear Corp.) as the source of primary and
secondary fluoros, and counted in a scintillation counter. For $^1$C-labeled samples, a gas flow counter was sometimes used.

RESULTS

Effect of inosine when added at zero time or 30 min into competence development. Figure 1 shows the effect of adding 100 $\mu$g of inosine per ml, either at zero time in the MV medium or 30 min later, to cells which had been grown in M1, containing 2,000 $\mu$g of inosine per ml. There is approximately a 40-fold difference between the two in terms of transformability. There may be a slight stimulation over the control sample, to which only the inosine solvent (MV medium) had been added. In any event, the depressive effect seen at zero time is no longer evident 30 min later.

FIG. 1. Time of inosine addition and competence development. Cells were grown in M1, containing 2,000 $\mu$g of inosine per ml and were transferred to MV. They were divided into three equal portions at this time and shaken at 37 C. Inosine was added to 100 $\mu$g/ml to one sample at zero time, and to another 30 min later. The third sample served as a control and received no inosine. Competence was tested at various times.

Effect on cells grown without inosine. It was thought that the addition of inosine after 30 min in the competence medium might be able to replace the previously observed need for inosine in the growth medium (5). Figure 2 illustrates the results of an experiment comparing the competence of cells grown with inosine, cells grown without inosine, and with or without inosine addition to the competence medium. Although there is a stimulation to the cells grown with hypoxanthine when inosine is added at 45 min in the MV medium, the level of competence reached is lower than that of cells grown with inosine.

FIG. 2. Effect of inosine in the growth phase and the subsequent development of competence with or without inosine in MV. One population of cells was grown in M1, containing 2,000 $\mu$g of inosine per ml, and another population was grown in M1, containing 5 $\mu$g of hypoxanthine per ml. At the proper cell density for each culture, the cells were transferred to MV and divided into two equal portions. Forty-five min after the transfer, 100 $\mu$g of inosine per ml was added to one of them. (A) Growth in inosine, inosine addition at 45 min; (B) growth in inosine, no addition; (C) growth in hypoxanthine, inosine addition at 45 min; (D) growth in hypoxanthine, no additions.
Since the magnitude of the stimulation relative to the control is greater in these cells, however, subsequent experiments investigating this effect used cells grown without inosine.

**Optimal time of addition.** An experiment was performed to see what the effects of adding inosine would be when added at times other than zero time, 30 min, or 45 min. Figure 3 shows that the optimal time of addition was at 40 min. The addition of inosine at zero time is inhibitory to competence development, but as the time of addition is postponed, the effect becomes less inhibitory until about 15 min, when the addition becomes stimulatory.

These experiments were conducted so that the cells were transformed at 100 min in the competence medium, normally the time of peak competence, regardless of the time the inosine was added. When the experiment was repeated and the cells were transformed both at 100 min and 60 min after the addition of inosine, it was again found that the optimal time of addition was near 40 min. In all cases the peak of competence appeared near 100 min, though the peak itself was lower if the addition was not made at 40 min (Table I). The development of competence always seems to take about 100 min, and the addition of inosine can inhibit, stimulate, or have no apparent effect upon transformation, depending upon when it is added to the competence medium.

**Optimal concentration of inosine.** An inosine concentration of 100 µg/ml was arbitrarily chosen for the first few experiments, but after the optimal time of addition was established, experiments were performed to determine the effects of other concentrations. It was found that concentrations 20-fold higher or lower than 100 µg/ml do not cause stimulations dramatically different from one another. Addition of 5 µg/ml had nearly the same effect as did the addition of 2,000 µg/ml, though the cells with the higher concentration rose to a peak slightly earlier and then lost competence faster than did the cells with the lower concentrations. Further experiments established that the optimal time of addition of inosine was independent of the concentration of inosine.

**Incorporation of ³H-inosine.** It was thought that the effect of inosine might be reflected in its extent of incorporation into the cells at the different times. Incorporation of ³H-inosine into acid-precipitable form was measured after being added at different times during competence development. Figure 4 shows the results. Incorporation continued for approximately 40 min before stopping, but the final extent of incorporation was different in the three cases. The leveling off may be due to an enzymatic activity associated with

![Figure 3](http://jb.asm.org/images/Fig_3.jpg)

**Figure 3.** Optimal time of addition for inosine stimulation. Cells were grown in MI containing 5 µg of hypoxanthine per ml. They were transferred to MV in 15 equal portions. Inosine was added at a level of 100 µg/ml to individual portions at the times indicated. A control sample that received no inosine was included. All samples were tested for competence 110 min after suspension in the competence medium MV, by exposing to DNA for 30 min instead of the usual 10 min. The control sample transformed to 0.028%.

**Table 1.** Competence at 60 min after adding inosine as compared to level at usual time of peak competence

<table>
<thead>
<tr>
<th>Time of inosine addition</th>
<th>Transformation 60 min after inosine addition</th>
<th>Transformation 100 min after zero time</th>
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<tr>
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<tr>
<td>110</td>
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<td>0.017</td>
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*Cells were grown in MI containing 5 µg of hypoxanthine per ml and were transferred to MV. They were divided into six equal portions, and inosine was added to make 100 µg/ml at the times indicated. Competence was tested in each culture at two different times, once at 60 min after making the addition of inosine, and again at 100 min after the suspension in MV.*
Cells of zero, of 5 μCi per inosine per ml were added to the proper tube along with 5 μCi of 3H-inosine per ml. Samples were taken every 20 min to determine the incorporation of tritium into trichloroacetic acid-precipitable counts. Competence was tested at 120 min.

FIG. 4. Incorporation of inosine during competence development. Cells were grown in MI, containing 5 μg/ml of hypoxanthine and were transferred to MV. They were divided into three equal portions, at times of zero, 20, and 40 min after the transfer 100 μg of inosine per ml was added to the proper tube along with 5 μCi of 3H-inosine per ml. Samples were taken every 20 min to determine the incorporation of tritium into trichloroacetic acid-precipitable counts. Competence was tested at 120 min.

These effects are not related to the extent of incorporation of inosine in any obvious way. Greater quantities of inosine are incorporated when the addition is inhibitory rather than when the addition is stimulatory, which might imply that the lesser the incorporation, the greater the competence. Samples in which there is no incorporation, however, are the control samples to which the experimental samples are compared, so that this implication is apparently untrue. The stimulatory effect is rather insensitive to the extracellular concentration. The effect must therefore be a temporal one, dependent only upon the time of addition.

It is also shown that the addition of inosine at two separate times is capable of stimulating pro-

FIG. 5. Incorporation of phenylalanine during competence development. Cells were grown in MI, containing 5 μg of hypoxanthine per ml and were transferred to MV containing 0.5 μCi of 14C-phenylalanine per ml. They were divided into three equal portions, and 100 μg of inosine per ml was added to one at zero time and to another 40 min later. The third sample was a control and was not exposed to inosine. Samples were taken for incorporation of 14C into trichloroacetic acid-precipitable counts, and competence was tested at 120 min.
tein synthesis. Since the quantitative effect on protein synthesis is similar at both times, this stimulation may be irrelevant to the effects on competence that are observed. Another possibility is that the two effects are related, and there is a qualitative difference in the proteins being synthesized at the two times. The stimulation of protein synthesis at zero time may concern proteins unrelated to competence development, and the proteins made 40 min later may be involved in competence development. If this latter possibility is true, the involvement of inosine in the regulation of the cellular events leading to competence would be implicated. Experiments are in progress to test this possibility.

ACKNOWLEDGMENTS

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LITERATURE CITED