low (*4*), there is an apparent conflict between the requirement of the enzyme for magnesium and the lack of effect of concentrations of fluoride which are incompatible with the maintenance of appreciable levels of magnesium ion. In this communication it is shown that the order of mixing is of the utmost importance in determining the effect of fluoride in this system. These results place definite limits on the use of fluoride in assays for hexokinase activity, and their implications require consideration in every case in which fluoride is used with ATP-requiring enzyme systems.

The assay system utilized the fact that hydrogen ion is produced as the hexokinase reaction proceeds (*1*). In each case, cresol red (*5*), glucose, hexokinase, magnesium chloride, and sodium fluoride (replaced by chloride in control samples) were mixed and preincubated for the specified times. The reaction was started by the addition of a solution of ATP in triethanolamine buffer, and the absorption at 5710 A was measured at intervals using a Beckman spectrophotometer. Final concentrations were 1m*M* glucose, 1m*M* ATP, 1m*M* Mg^{++}, 0.1*M* F^- (or Cl^-), 2m*M* triethanolamine (*p*H 8.4), 4μg/ml of cresol red, and 10μg/ml of yeast hexokinase (Sigma "practical") in a total volume of 3 ml. Temperature was controlled at 25°C. It is to be noted that under these conditions the enzyme is saturated with glucose and is sensitive to (Mg^{++}).

Results of a typical experiment are shown in Fig. 1. When the mixture contained fluoride, the time of preincubation markedly affected the measured rate of the reaction. However, the degree of inhibition did not increase during the measurement. This is emphasized in Fig. 1 by plotting the negative logarithm of the concentration of ATP against time, which shows the close adherence to first-order kinetics.

It was further observed that, although the relatively small inhibition obtained after 1 minute of pre-incubation with fluoride and the almost complete loss of activity observed after a 30-minute exposure were quite reproducible, the degree of inhibition obtained after 15-minute preincubation periods was extremely erratic. The following values of the rate constant were obtained in a series of replicate determinations: 1 minute of preincubation with fluoride, 0.032, 0.032, and 0.034; 15 minutes, 0.030, 0.023, 0.0023, 0.034, and 0.0069; 30 minutes: 0.0046 and 0.0051. In other experiments it was found that addition of sodium fluoride 1 minute *after* the other components had been mixed had no measurable effect on the rate of the reaction. This confirms previous reports (*1*).

All these observations can be explained on the basis of the assumption that the rate of removal of magnesium ion from solution by fluoride ion is a relatively slow process. If it is assumed that this occurs as follows:

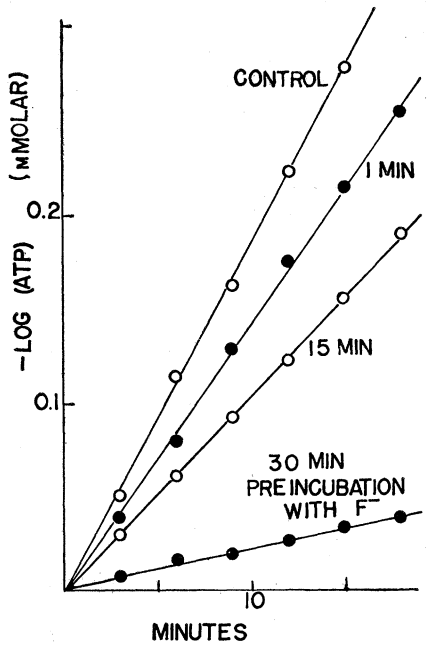

Fig. 1. Inhibition of yeast hexokinase by fluoride. The reaction was started by addition of ATP after preincubation of the other constituents for the times indicated on the curves. Chloride was substituted for fluoride in the control. Final concentrations are listed in the text.

$$Mg^{++} + F^- \rightleftarrows MgF^+ \quad (1)$$

$$MgF^+ + F^- \rightleftarrows MgF_{2(aqueous)} \quad (2)$$

$$MgF_{2(aqueous)} \rightleftarrows MgF_{2(solid)} \quad (3)$$

it seems probable that reaction 3 represents the rate-determining step in the removal of magnesium ion. The separation of a solid phase is known to depend on many factors and is frequently slow. If reactions 1 and 2 are more rapid than reaction 3, the rate of precipitation of MgF_2 would depend, among other things, on the concentration of magnesium ion. The addition of ATP to such a mixture would affect this process by reducing the concentration of free magnesium ion because of the formation of a magnesium-ATP complex as follows:

$$Mg^{++} + ATP^{-4} \rightleftarrows MgATP^{--} \quad (4)$$

This reaction occurs rapidly, and since the formation constant is of the order of 2×10^4 (*6*), the concentration of free magnesium ion in the systems studied is markedly reduced by the addition of ATP. Consequently the rate of reaction 3 is reduced to such an extent that no further effect of fluoride is detected over the period of observation.

This formulation accounts for the fact that no inhibition is observed when ATP is mixed with magnesium ion prior to the addition of fluoride, as well as the observations that fluoride mixed with magnesium ion prior to the addition of ATP exerts an inhibitory effect that is related to the time of preincubation, but does not increase after ATP is added.

The scatter in the measurements made after 15 minutes of pretreatment with fluoride is probably due to variations in the rate of reaction 3, inasmuch as the separation of a solid depends on the chance of finding or forming centers suitable for nucleation, and wide fluctuations in this variable are to be expected when mixtures of this type are made. The relatively constant inhibition found after 1 minute of preincubation may correspond to the extent of reactions 1 and 2 alone, while the considerable inhibition found after 30 minutes probably reflects a close approach to equilibrium precipitation of magnesium fluoride.

We conclude that fluoride ion can inhibit yeast hexokinase by removal of magnesium ion. This inhibition requires a finite time, and it can be minimized by attention to the order of mixing of reagents. Since it is probable that the observed effects are due to variations in the rate of precipitation of magnesium fluoride, we suggest that this variable should be evaluated in the interpretation of any enzyme assay in which fluoride is used (*7*).

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7. This investigation was supported by research grant H-1342 C3 from the National Heart Institute, National Institutes of Health, U.S. Public Health Service.

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Autoinhibition of Bacterial Endospore Germination

In the last few years, a considerable number of published works on the formation and germination of bacterial endospores has appeared. Many of these studies have been concerned with the physiological aspects of the processes. Details of the more significant investigations on this subject have been recently

reviewed by one of us (*1*), and during the preparation of this review, it was noted that many chemical inducers of germination, although effective in varying degrees, were reported as failing to produce germination of all spores in the population. Subsequent work in this laboratory has shown that, in at least one instance, the failure to obtain germination of all spores is due to an autoinhibitory effect in which a substance (or substances) excreted by germinating spores inhibits the germination of the remainder of the population (*2*).

Thus far, studies have been limited to the autoinhibitory substance produced by *Bacillus globigii*. The organism is grown on "G" agar (*3*) either with or without casein hydrolyzate at 30°C until complete sporulation occurs (usually 4 to 5 days). The spores are then harvested, washed according to the procedure of Stewart and Halvorson (*4*), and stored in the freezer. When the spores (0.2 mg/ml) are suspended in 0.067*M* phosphate buffer (*p*H 7.0), the addition of L-alanine and glucose in the ranges of concentration recommended by Church *et al.* (*3*) for complete germination fails to produce this effect when measured by the turbidimetric method of Hachisuka *et al.* (*5*) or by standard techniques involving loss of heat resistance and uptake of methylene blue (*1*). In almost all instances, 60- to 85-percent germination of the total population is obtained. Significantly lower and higher levels of inducers do not alter the basic pattern of germination, which is illustrated in Fig. 1 (curves *A* and *B*). Further additions of inducers after the germination rate has leveled off produce a very slight acceleration in the rate of germination, but the degree of increase is too small to attribute the over-all effect to a simple depletion of inducers.

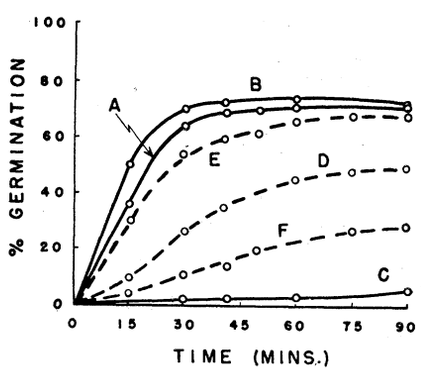

Fig. 1. Antagonism by L-alanine of the autoinhibitory effect on endospore germination. Clean, heat-shocked *B. globigii* spores (1.0 mg) were suspended in either 0.067*M* phosphate buffer (*A*, *B*, and *C*) or "supernatant" (*D*, *E*, and *F*) to which were added 10μ*M* glucose and either 10μ*M* L-alanine (*A* and *D*) or 50μ*M* L-alanine (*B* and *E*); *C* and *F* contained no inducers. "Supernatant" was obtained by centrifuging spore mixtures previously incubated with 50μ*M* L-alanine and 10μ*M* glucose for 1 hour.

Table 1. Resumption of endospore germination after replacement of inhibitory supernatant by 1.0 mg of spores in 5.0 ml of fresh phosphate buffer with 50μ*M* L-alanine and 40μ*M* glucose as inducers (except for control). After an initial induction period of 60 minutes, cells in **C and D** were centrifuged and resuspended in fresh phosphate buffer. Then additions were made as indicated, and all tubes were incubated another 60 minutes.

Tube	Additions		Percentage germination		Total
	First induction	Second induction	First induction	Second induction	
A	H_2O	None	1.4		1.4
B	L-alanine and glucose	None	81	1.0	82
C	L-alanine and glucose	L-alanine and glucose	81	13	94
D	L-alanine and glucose	H_2O	81	2.0	83

The presence of an autoinhibitory substance in induced systems in which the rate of germination has leveled off can be demonstrated in two ways. The supernatant obtained after separation of the cells in such systems is inhibitory for the germination of fresh spores, and this inhibition is antagonized by further additions of graded amounts of inducers. Curves *D* and *E* of Fig. 1 illustrate this. It should be noted that significant amounts of L-alanine may be carried over in the supernatant from the first germination, for Harrell and Halvorson (*6*) have shown that the amount of L-alanine taken up by spores during the germinative process is extremely minute. Probably this accounts for the degree of germination obtained with the supernatant in the absence of inducers (curve *F*). Preliminary kinetic studies have revealed that the autoinhibitory substance inhibits L-alanine induction of germination in a competitive manner.

The second demonstration of autoinhibition can be shown by the replacement of the supernatant after the initial rate of germination has leveled off. Addition of fresh buffer and inducers to spore populations partially germinated in this way results in an increase in the rate of germination, although 100-percent germination is still not ultimately attained. Table 1 illustrates this point.

The induction of germination with L-alanine is antagonized by the D-form of the acid, confirming in part the findings of Church *et al.* (*3*). However, the autoinhibitory substance does not appear to be alanine racemase, although low levels ($Q_n = 2.0$ μ*M* alanine racemized per milligram of cells, per hour) of the enzyme are found in the spores. This is in contrast to the data of Church *et al.* (*3*) in which no racemase was found in the spores of *B. globigii*. Evidence of the failure of racemase to contribute to the autoinhibitory effect has been shown by the inability of D-amino acid oxidase (hog-kidney cortex) to protect the spores against the autoinhibitory substance in the presence of L-alanine and glucose.

The excretion of intracellular substances during germination of bacterial endospores has been studied by other workers (*7*). Among the substances identified thus far are amino acids, peptides, a hexosamine-peptide combination, dipicolinic acid, and calcium. In the present investigation, calcium ion and dipicolinic acid, alone and in combination, have been examined for possible identity with the autoinhibitory agent. Although dipicolinic acid inhibits germination and calcium protects against such inhibition, the autoinhibitory effect is not exclusively due to the former since 100-percent germination is not achieved when calcium ion alone is added to tubes containing the inducers, L-alanine, and glucose.

A number of other substances, some of which are structurally related to alanine, have been found to antagonize L-alanine induction of germination. Included among these are lactate, pyruvate, and DL-serine, the last being the most effective of the group. Eighteen other amino acids show erratic properties in this respect, but inhibition by L-tyrosine is demonstrable in most instances. The inhibition by serine of germination induced by L-alanine is competitive in nature. Efforts to detect serine or other amino acids in the supernatant liquid by paper-chromatographic methods have been unsuccessful.

The autoinhibitory agent is heat-labile to some degree: approximately 50 percent of the activity is lost on autoclaving at 15 lb of pressure (and 121°C) for 20 minutes, and 20 percent on heating at 100°C for 10 minutes. Thus, it would appear that the autoinhibitory effect is not due exclusively to an excreted, heat-stable, free amino acid, although the possibility of a partial contribution by the latter is not ruled out.

Identification of the autoinhibitory substance and the specific metabolic reactions against which it is active may provide a tool for determining more selectively the sequence of biochemical reactions resulting in germination. Effort is being devoted at present to determine more completely the properties of the autoinhibitory substance, including its effects on specific metabolic processes.

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18 June 1956

Comparison of Suggestibility during "Light Sleep" and Hypnosis

Some recent experiments (*1*) indicate that "hypnosis" may involve a state of "light sleep." Attempting to determine directly the relationship between these "states," I recently completed a study (*2*) of 22 subjects who volunteered "to be given psychological tests at night while they were asleep." I approached the subjects—in the middle of the night while they were sleeping in their own rooms—and whispered to them: "Clasp your hands together."

After a subject clasped his hands together—all subjects did so within 10 seconds—I repeated over and over for 1 minute: "Your hands are hard . . . solid . . . completely interlocked . . . it is impossible to unclasp those hands. Try it." In this way I gave seven standard tests of suggestibility, including the following: "you cannot unclasp your hands," "you cannot open your clenched fist," "your fingers are rising," "your hand is dead and dull and numb and cannot feel anything at all," "you are becoming very thirsty and will wake up in exactly 5 minutes and drink lots of water," and "you cannot remember anything that I said . . . you cannot remember anything at all."

When tested in this way, three of the subjects "woke up" and seven either moved or opened their eyes for a moment and later stated that they were "drowsy" during the experiment. However, the remaining 12 subjects seemed to be in a stage of "light sleep," since they did not move, did not open their eyes, responded reluctantly to requests like "clasp your hands together," continued breathing slowly and easily, and later stated that they either had been in "some stage of sleep" or had complete amnesia for the experiment.

These 12 subjects, who seemed to be "lightly sleeping," responded to the seven tests of suggestibility as if they were in some stage of "hypnosis" as measured by the Davis and Husband scale of hypnotic depth (*3*). Some responded as if they were in the second stage of "hypnosis"—for instance, they were completely unable to unclasp their hands. The majority of the "lightly sleeping" subjects responded as if they were in at least the third stage of "hypnosis," since they had complete amnesia for the experiment or followed the "postsleep" suggestion to wake up in 5 minutes and drink water. (No tests were given that could have differentiated the fourth, or "deepest," stage of "hypnosis.")

In a second experiment, the same seven tests of suggestibility were given in the same way immediately after a standard hypnotic-induction procedure. In a third (control) experiment, the same tests were given in the same way to the subjects when they were normally awake.

As Table 1 indicates, there were no significant differences between the subject's responses on the seven tests of suggestibility when the subjects were "lightly sleeping" and after they had been subjected to hypnotic induction. From Table 1, it can also be seen that the subjects were more suggestible on all seven tests during the first experiment—when they were "lightly sleeping"—than they were during the control experiment when they were normally awake, and that they were significantly more suggestible on five of the seven tests.

The correlations between the subject's scores on the seven tests during the first and second experiment—when "lightly sleeping" and after hypnotic induction—were in all cases above + 0.61 and in nine out of the 12 cases above + 0.92.

I concluded from this study that the subjects of this experiment were as suggestible when they were "lightly sleeping"—that is, when they appeared to be asleep and later stated that they had been in "some stage of sleep"—as they were after hypnotic induction.

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Table 1. Mean scores on the seven tests of suggestibility when the subjects were "lightly sleeping," after hypnotic induction, and when the subjects were normally awake.

Seven tests of suggestibility	Experiment			Critical ratio of difference	
	"Sleep"	Hypnosis	Control	"Sleep" and hypnosis*	"Sleep" and control
Hand-clasp test	44.4	37	7.3	0.75	5.3†
Finger-rigidity test	40.8	35	8.5	0.54	3.7†
Finger-levitation test	4.2	2.8	1.4	0.36	1.0
Anesthesia test	1.7	1.8	0.08	0.2	4.8†
Thirst test	9.0	4.7	1.25	1.8	4.5†
Five-minute-waking test	3.3	3.0	2.0	0.2	0.9
Amnesia test	3.3	2.5	0.0	0.55	5.7†

* Not significant at the 0.05 level. † Significant at the 0.001 level.

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10 July 1956

Strontium Content of Human Bones

The refinements of analytic techniques for trace elements, together with a growing interest in the distribution of trace elements in human tissues, has resulted in a continued addition of information on these elements to the biochemical literature. The present paper (*1*) reports strontium analyses on 277 human bones from a world-wide sampling.

Hodges *et al.* (*2*), investigating the strontium content of human bones by an emission-spectrographic technique, found for a limited sampling an average of 220 ppm for bone ash. Tipton (*3*) has found a lower value for bones (120 ppm), also using an emission-spectrographic technique.

The present paper reports results indicating that there are marked regional differences. It is possible that the aforementioned discrepancy may be explained on this basis. It is also possible that regional effects may be confounded with systematic errors in the analytic data. The investigation reported here is an attempt to