effect on the early growth of *L. fermenti* 36. In all cases, these substances do not affect the lactic acid production after 72 hours of incubation.

In the microbiological assay of B vitamins, it is frequently found that the growth of organisms may greatly be stimulated by the natural product. We are now inclined to believe that enzyme digested starch is one of the many stimulants which may be the decisive cause of erratic results obtained in assay of B vitamins of some natural products; thus addition of it to the assay medium is recommended.

Summary. (1) Maltose or takadiastase digested starch has been found to be one of the many growth stimulants for *L. fermenti* 36 in assay of thiamine. It is also stimulatory to *L. arabinosus* 17-5 and *Strep. faecalis R.* in assays of pantothenic acid and pyridoxine. (2) Evidence has been obtained to prove that a precaution should be made on thiamine assay of starchy substances using *L. fermenti* 36 as test organism. Addition of enzyme digested starch or maltose to the assay medium is recommended.

We wish to express our appreciation to Dr. Vernon H. Cheldelin for valuable suggestions in this study.

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Chemical Inhibition of Decapsulation in the Beta Hemolytic Streptococcus. (19105)

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The following study represents an attempt to explain the loss of hyaluronic acid capsular material (HA) from beta hemolytic streptococci which occurs spontaneously in culture or in washed suspensions of young organisms. The problem has already received considerable attention(1,2,3). In the pneumococcus the disintegration of the capsule has been shown to be an enzymatic process(4) and it is possible that a similar mechanism might be involved in the streptococci. Since hyaluronidase brings about rapid decapsulation, it was necessary to consider this enzyme as a possible factor which might be operating under natural conditions. Studies by McClean(2) and Pike(5) suggest that certain capsulated strains of streptococci may produce hyaluronidase under special conditions, although it is probable that this is a result of dissociation(6). In the course of our study, extensive attempts to demonstrate an agent in cultures of various ages or in mechanically disrupted organisms which would accelerate the normal rate of capsular disintegration were unsuccessful; we were unable to duplicate the results of Morison(1) who found small amounts of such an agent released by organisms. Possibly the examination of other strains would have been successful. In these attempts the measurement of the rate of capsular loss was the same as that to be described below in connection with the demonstration of chemical inhibition of a hypothetical decapsulating enzyme which comprises the main part of this report.

Material and methods. The beta hemolytic streptococcus used in this work, strain 4, was a group C strain isolated from guinea pigs during a fatal outbreak of lymphadenitis(3). This organism was grown in a pH 7.6, 2% proteose-peptone broth containing 10% sterile horse serum, 1% dextrose, and 0.5% NaCl. Ten ml of such neutralized cultures were in-

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TABLE I. Compounds Found to be Effective in Retarding Decapsulation.

	Residual capsular polysaccharide after 2 hr at 37°C. % of total extracted by boiling		
Sodium fluoride .005 M (3 determinations)	62	76	75
Controls (distilled water)	32	34	32
Sodium azide .05 M	66		
Control	33		
Sodium arsenate 1 M	55		
Control	40		
Sodium citrate .025 M	65		
Control	34		

oculated into 90 ml of warm fresh broth and incubated at 37°C. In an actual test, cells from 4.0 ml of this broth culture were washed in distilled water and resuspended in equal amounts of the dilutions of the various chemical agents to be investigated. After 2½ hours' incubation in a 37°C waterbath, samples were centrifuged, cells washed in distilled water, and prepared for HA determination by the turbidimetric method previously described(3). Cells to be tested for residual cellular polysaccharide were resuspended in 2.0 ml of M/15 pH 7.0 phosphate buffer and boiled for 20 minutes to release capsular material. After centrifugation, 1.0 ml of the supernate was added to 5.0 ml of 0.5 M pH 4.2 acetate buffer. One ml of the dilute acidified serum reagent was then added and mixed immediately. After standing at room temperature for ½ hour, the turbidity was determined in a Klett-Duboscq photoelectric colorimeter, using various thicknesses of glass plates for comparison standards. Turbidities were converted into milligrams of hyaluronic acid by means of hyaluronic acid turbidity curves prepared with purified HA.

The following salts at the indicated concentrations were found to be ineffective in retarding decapsulation: barium chloride .00009 M to .0009 M; cadmium chloride .008 M to .08 M; lead nitrate .00005 M to .005 M; lithium chloride at .01M; manganese sulfate at .002 M; mercuric chloride at .0005 M; potassium bromide .005 M to .05 M; potassium iodide .005 M to .05 M; potassium periodate .00005 M to .005M; silver nitrate at .009 M; sodium arsenite at .05 M; sodium cyanide at .05 M; sodium oxalate at .005 M.

Table I shows the effects of fluoride, arsenate, citrate and azide ions, which caused the retention of at least 50% more cellular HA than the control cells suspended in distilled water. Since the retarding effect of fluoride on decapsulation appeared most pronounced, all further experiments were confined to this ion and a wider range of fluoride dilutions was tested. This experiment indicated that the retardation of decapsulation becomes less effective with lower concentrations of fluoride; optimal activity was obtained at a concentration of .005 M NaF. The retarding effect is not apparent in concentrations less than .00025 M NaF.

Studies were conducted to determine the duration of the fluoride ion inhibitory effect. Washed cells were resuspended in .005 M NaF and in distilled water as controls. After 2, 4, 8, 12 and 18 hours' incubation, test and control samples were examined for residual cell polysaccharide. Polysaccharide levels of the fluoride treated organisms remained high throughout the first 4 hours of incubation while the control samples showed a rapid loss. After 4 hours, however, the effect of the fluoride begins to diminish. By 8 hours all detectable amounts of polysaccharide have disappeared from the cells in the control sample. Fluoride-treated organisms tested at this same time still retained 50% of the total capsular material. At 12 hours small amounts of polysaccharide were still detected. Morison(1) reports in this connection that 0.5 to 1.0% formalin inhibits capsular disintegration up to 20 hours, an observation which we have confirmed. The optimum level is 0.25% formaldehyde assuming formalin to be 37%. It appears that the duration of this effect is considerably longer than that of fluoride.

When encapsulated streptococci are treated with hyaluronidase, the enzyme causes a rapid shedding of the capsular material. Since fluoride was seen to retard spontaneous decapsulation, experiments were conducted to determine whether fluoride would also inhibit the decapsulating activity of a streptococcal hyaluronidase. Washed cells from 4.0 ml of

TABLE II. The Effect of NaF on Streptococcal Hyaluronidase Activity.

Dilution of hyaluronidase	mg residual capsular polysaccharide after 2 hr at 37°C	
	Hyaluronidase alone	Hyaluronidase + NaF .005 M
1:2000	0	0
1:8000	0	0
1:16000	.05	.09
1:32000	.05	.10
0	.05	.10
(Total polysaccharide extracted by boiling, .13)		

a 5 hour broth culture of strain 4 were resuspended in distilled water containing various dilutions of a crude group C streptococcal hyaluronidase and incubated in a 37° water bath. At the same time, cells were also suspended in .005 M NaF containing the same dilutions of hyaluronidase used above. After 2½ hours' incubation the cells were prepared for polysaccharide determination. The results of this experiment are summarized in Table II, indicating that fluoride in no way inhibited the decapsulating activity of the group C hyaluronidase.

In view of the reported evidence that fluoride acts as an enzyme inhibitor, studies were made to determine the possible mechanism of its inhibitory activity in this instance. Some inhibitors act by combining with trace metal activators such as magnesium and calcium. If fluoride acts in this manner, it was felt that the addition of magnesium or calcium ions might block its effect. To test this possibility, varying concentrations of magnesium sulphate were added to washed cells suspended in .005 M NaF and incubated for 2½ hours. After incubation, the cells were washed and tested for residual capsular polysaccharide. It was found that the addition of magnesium ions to the test system in concentrations ranging between .05 M and .006 M resulted in an almost complete loss of the fluoride effect. With lower concentrations, the blocking action is not as pronounced. A similar experiment also indicates that calcium, like magnesium, is able to block the retarding effect of fluoride on decapsulation of strain 4. With calcium, however, greater concentrations are required to demonstrate blockage.

In addition to the inhibition of decapsulation by chemical means, the effect of temperature on the process was studied in the same manner. After exposure of young capsulated cells to various temperatures from 4°C to 98°C for 40 minutes in M/15 buffer at pH 7.0 the residual HA was determined. The results appear in Fig. 1 and indicate that the rate of loss of capsular material in relation to temperature change could be partly under the influence of an enzyme system in agreement with the conclusions drawn by Morison(1).

Discussion. Although we were unable to isolate a specific decapsulating agent from beta hemolytic streptococci, indirect evidence confirms the assumption that some enzyme is concerned. In view of the established fact that fluoride, azide and arsenate are enzyme inhibitors, it is felt that their action in delaying spontaneous decapsulation as reported here may be the result of enzyme inhibition. The failure of fluoride to affect streptococcal

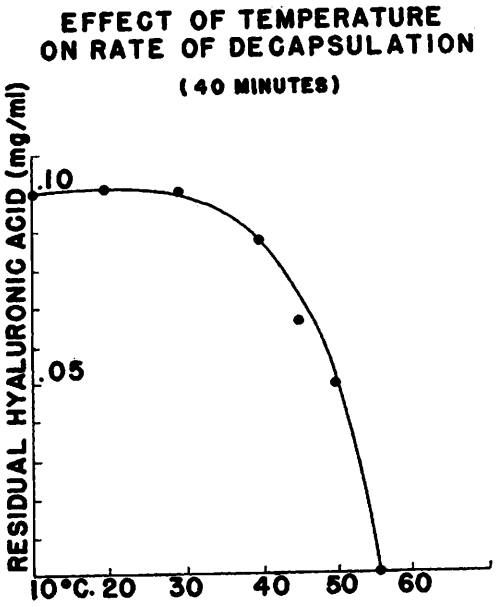

FIG. 1.

hyaluronidase, and the fact that all capsulated streptococci undergo spontaneous decapsulation even though they cannot be shown to produce hyaluronidase, would indicate that hyaluronidase is not responsible for this process.

In attempting to characterize the fluoride effect, it was found that the addition of magnesium or calcium to the test system tended to block the ability of fluoride to delay decapsulation. This may mean that fluoride acted in the system by combining with metallic ions necessary for enzyme action. However, MgF_2 and CaF_2 are highly insoluble compounds and it is felt that the observed blocking effect could also be due to the removal of available fluoride from the system.

Summary. (1) Attempts to demonstrate an agent in beta hemolytic streptococci which would accelerate the normal rate of capsular disintegration were unsuccessful. (2) Of various chemical agents tested, the following ions were ineffective in inhibiting this disintegration: Lead, cadmium, barium, manganese, lithium, silver, mercury, arsenite, iodide, bromide, periodate, cyanide and oxalate. (3) The following agents were found to retard decapsulation: Fluoride, azide, arsenate, citrate, and formaldehyde. The effect of fluoride was lost upon the addition of magnesium or calcium ions. (4) Fluoride did not inhibit the decapsulating action of streptococcal hyaluronidase. (5) The effect of temperature was found to be compatible with the assumption of a hypothetical enzyme system causing decapsulation.

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Action Mechanism of Cobra Venom, Cardiotoxin and Allied Substances on Muscle Contraction. (19106)

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The excised toad heart stops beating if perfused with a solution of cobra venom*, cardiotoxin†(1,2) or cardiac glucosides. The similarity of cobra venom to saponin was often noted by earlier workers, but later it has been shown, more closely, to resemble digitalis and digitalis-like substances(3,4). Now that a number of active principles of cobra venom have been isolated—some in a highly purified state—it was considered worthwhile to study the mode of action and to compare them with known cardiac drugs. Cardiotoxin, one of the many active constituents of cobra venom, has been isolated from it by fractional precipitation method(1). It is a protein and on weight basis it is 90-100 times more potent than crude venom. It contains less than 2% of neurotoxin and haemolysin.

Method. An isolated frog or toad heart (average weight of 250 g and 150 g respectively) was perfused with Ringer's solution. The minimum concentration of a drug required to stop the heart was taken as that amount which when perfused in the 5 cc of the test solution caused the systolic contracture. Test solutions were made up in salt mixture; the composition of which was as follows: NaCl, 6.5 g; $CaCl_2$, 0.12 g; $NaHCO_3$, 0.2 g; the total volume made up to 1000 cc. The pH was 7.2-7.4.

Results. Table I shows the concentration of each drug required for cardiac arrest. The

* Cobra venom was extracted from the poison glands of cobra (*Naja tripudians*) and dried in a vacuum desiccator over fused calcium chloride.

† Cardiotoxin brings about the stoppage of perfused toad heart or heart beat when administered intravenously into an anaesthetized cat(2).

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