5. *Streptococcus agalactiae* is strain No. 7077 of the American Type Culture Collection; *S. faecalis* 110 and *S. thermophilus* 108 are from the Department of Dairy Science, University of Illinois stock culture collection, and *S. bovis* is strain 444 obtained from H. Seeley, Cornell University, Ithaca, N.Y.
6. J. H. Roe and N. M. Papadopoulos, *J. Biol. Chem.* **210**, 703 (1954).
7. Although included in these particular experiments, cystine and $MgCl_2$ are not required for lactic dehydrogenase activity.
8. I thank Mrs. E. C. Reichelt and J. Althaus for technical assistance and W. J. Rutter and F. Wold for suggestions and criticism.

7 August 1964

Neurospora β-Galactosidase: Evidence for a Second Enzyme

Abstract. *Two enzymes which hydrolyze the artificial substrate* o-*nitrophenyl-β-*D-*galactopyranoside are produced by* Neurospora. *These enzymes precipitate at widely different concentrations of ammonium sulfate and differ with respect to induction, pH optima, sedimentation, and thermal stability.*

In addition to the β-galactosidase of *Neurospora crassa* previously described as having maximum activity at *p*H 4.5 (*1*), we have identified another β-galactosidase from this fungus, and have examined these enzymes with respect to several criteria. The *Neurospora* strain used in these studies is designated L-5, an isolate of 74A selected for its ability to grow on lactose.

Effective separation of the two β-galactosidase activities (*2*) is obtained by fractionation with ammonium sulfate (*3*). Eighty-five percent of the newly identified enzyme is contained in the protein fraction which precipitates at 20 to 40 percent saturation with ammonium sulfate in tris buffer, *p*H 8. Seventy percent of the enzyme with observed optimum activity at *p*H 3.9 precipitates in the fraction obtained at 60 to 80 per cent saturation.

Profiles of activity versus *p*H for the ammonium sulfate fractions demonstrate two distinct activity peaks (Fig. 1). The newly identified enzyme shows maximum activity at *p*H 7.5, while the other β-galactosidase shows a maximum at *p*H 3.9. Mixtures such as crude extracts (*4*) have been incubated for assay at *p*H 4.2 and at *p*H 6.8. This provides almost complete distinction between the two activities as may be seen from the *p*H profile. Ammonium sulfate fractions were used in subsequent studies reported here, and these were assayed for β-galactosidase activity at their respective *p*H optima.

Ammonium sulfate fractions were dialyzed and sedimented in a sucrose density gradient (*5*). The calculated $S_{20,w}$ values (sedimentation coefficient at 20°C in water) (*6*), based on a comparison with catalase and assuming a partial specific volume of 0.725, were 6.0 for the *p*H 3.9 enzyme and 8.6 for the *p*H 7.5 enzyme. These $S_{20,w}$ values correspond to approximate molecular weights of 96,000 and 167,000, respectively.

Thermal stability studies of the separate ammonium sulfate fractions suspended in 0.0125*M* tris-HCl buffer, *p*H 8.0, and assayed at their respective *p*H optima are summarized in Fig. 2. Samples were incubated 2.5 minutes at 50°C, chilled and centrifuged; a sample was withdrawn at that time for assay. The remainder was returned to 50°C and samples were withdrawn for assay at appropriate intervals. It is obvious that the newly identified enzyme is more stable at 50°C than its counterpart with the low *p*H optimum. The enzyme with the *p*H optimum of 3.9 showed a similar loss of activity when incubated at 50°C in citrate-phosphate buffer at *p*H 4.2.

The enzyme preparations used in these studies were extracted from "induced" cultures. The *p*H 3.9 enzyme was previously shown to be induced by xylose (*1*) which may be used as the sole carbon source. Xylose does not induce the *p*H 7.5 enzyme, and β-lactose which does induce it provides little growth as the only carbon source. Therefore, we used β-lactose plus sucrose to induce the *p*H 7.5 enzyme. In agreement with earlier studies (*1*) little induction of the *p*H 3.9 enzyme is observed in these cultures, presumably due to inhibition of induction by sucrose. We have observed that fructose plus β-lactose at *p*H 4.0 will provide reasonable growth and bring about coordinate induction of both enzymes. Induction, when it occurs, results in fivefold increases in approximate specific activity for the *p*H 7.5 enzyme and 15-fold for the *p*H 3.9 enzyme, compared to cultures grown on sucrose.

Attempts to examine the hydrolysis of β-lactose (*7*) have been hampered by interfering materials in both enzyme preparations. These materials probably consist of polysaccharides which are degraded to glucose during incubation. Preliminary results indicate that both enzymes are able to hydrolyze β-lactose in addition to the artificial substrate used for assay, but clarification of this point must await the preparation of purified enzymes.

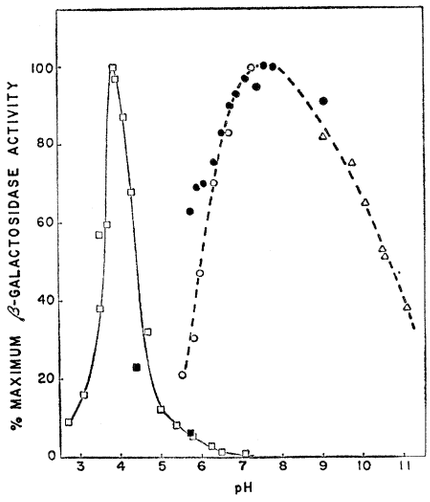

Fig. 1. Activity of β-galactosidase as a function of *p*H. Sample with optimum of *p*H 3.9 is the 60 to 80 percent ammonium sulfate fraction (□, ■). Sample with optimum of *p*H 7.5 is the 0- to 40-percent fraction (○, ●, △), heated 23 minutes at 50°C. Other symbols: □ ○, citrate-phosphate buffer; ■ ●, phosphate buffer; △, carbonate-bicarbonate buffer.

In addition to the L-5 strain used in these studies, both enzymes have been observed in wild-type 74A. The pres-

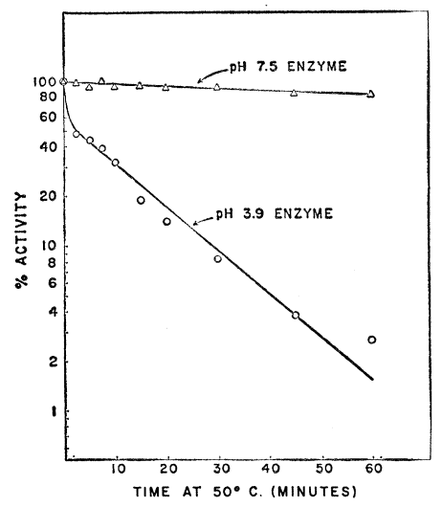

Fig. 2. β-Galactosidase stability at 50°C in 0.0125*M* tris HCl-buffer, *p*H 8.0. The *p*H 3.9 enzyme is that contained in the fraction precipitated at 60 to 80 percent saturation with ammonium sulfate; the *p*H 7.5 enzyme is that precipitated at 25 to 35 percent saturation.

ence of this second enzyme may explain why complete β-galactosidase deficient mutants of *Neurospora* have not been reported.

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References and Notes

1. O. E. Landman and D. M. Bonner, *Arch. Biochem. Biophys.* **41**, 253 (1952); O. E. Landman, *ibid.* **52**, 93 (1954).
2. Activity assayed by incubation with *o*-nitrophenyl-β-D-galactopyranoside at appropriate *p*H.
3. After brief treatment with $MnCl_2$ to a final concentration of 0.02*M* for removal of nucleic acids.
4. Ground, lyophilized mycelia were extracted into 0.0125*M* tris-HCl buffer, *p*H 8.0.
5. Spinco L-2, No. SW 39 rotor, 38,000 rev/min for 12 hours.
6 R. G. Martin and B. N. Ames, *J. Biol. Chem.* **236**, 1372 (1961).
7. By assay of glucose (Glucostat, Worthington).
8. This work was supported by USPHS grant GM-10067. One of us (W.K.B.) is a postdoctoral fellow, USPHS.

6 July 1964

Polyoma Virus: Production in Bacillus subtilis

Abstract. *Particles of complete polyoma virus are produced in competent* Bacillus subtilis *incubated with DNA isolated from purified, conventionally grown polyoma virus. The virus grown in* B. subtilis *is biologically identical to polyoma virus produced by animal cells. Quantitative parameters of the system have been established, and fluctuation tests indicate that viral replication occurs within the infected bacteria.*

Abel and Trautner (*1*) reported that vaccinia virus is formed by *Bacillus subtilis* when competent bacteria are incubated with DNA extracted from this animal virus. The virus obtained was biologically identical with conventionally grown virus used for DNA extraction. Biochemical evidence was not presented to show whether the virus replicates within the bacterium or whether the vaccinia DNA entering the bacterium merely directs the synthesis of new coat protein. Nevertheless, these important findings add further support to the concept of the universality of the genetic code. In addition, this system could be of importance for experimental virology if other animal viruses could be propagated in these bacteria, and if the synthesis of viral nucleic acids could be demonstrated to occur in this system. We now report the production of polyoma virus in *B. subtilis* and present indirect evidence for virus replication within the infected bacteria.

Competent cultures of *B. subtilis* 168-23 (a diauxotroph requiring histidine and indole) were prepared, and their competence was measured by methods previously described (*2*). Competence of the bacteria was determined by their ability to produce bacteriophage from purified phage DNA ("transfection," *3*) and to be transformed by homologous wild-type bacterial DNA. The degree of competence of the bacteria used for the polyoma experiments was such that, when they were incubated with bacteriophage SP8 DNA (1.2 μg/ml), 7.5×10^{-5} of them were infected and produced phages. The fraction of these bacteria transformed to histidine independence by exposure to DNA (1 μg/ml) from prototrophic bacteria was 2.3×10^{-3}. The total number of viable bacteria in the culture was 3.5×10^{7} per milliliter.

The "transfecting" capacity of polyoma DNA for this *B. subtilis* culture was tested essentially by the method described by Abel and Trautner (*1*). Two samples of polyoma DNA which contained 1.38×10^{12} and 1.26×10^{12} molecules per milliliter were tested. Polyoma DNA has a molecular weight of 3.5×10^{6}, is double-stranded and has a ring form (*4*). It was extracted from purified polyoma virus (*5*) by phenol (*4*), and samples were prepared by tenfold dilutions to contain from 10^{11} to 10^{2} molecules of DNA per milliliter. One-tenth milliliter of each dilution of polyoma DNA was added to 0.9 ml of the competent *B. subtilis* 168-23 culture described above and incubated at 37°C for 28 hours. The bacterial growth was removed and suspended in Eagle's medium, and the bacteria were disrupted by grinding with alumina or by grinding in a Nossal cell disintegrator. The debris was removed by centrifugation at 3000 rev/min and the supernatant was dialyzed against standard buffer at 4°C (*5*). The supernatant was tested for its plaque-forming capacity on monolayers of mouse-embryo cells (*6*) and for its focus-forming capacity on agar-suspension and monolayer cultures made with secondary fibroblast cells of rat embryo of the inbred Lewis strain susceptible to polyoma virus (*7*).

The data for the plaque-forming capacity are given in Table 1. The number of plaque-forming units (PFU) detected as a function of DNA concentration remains constant over a dilution range of five orders of magnitude; it decreases with further dilution of the polyoma virus DNA. No polyoma virus was produced with any concentration of polyoma DNA that had been incubated with deoxyribonuclease (20 μg/ml) before adding it to the competent bacteria. Both samples of polyoma DNA diluted to contain approximately 10^{6} molecules per milliliter resulted in the production of about 10^{6} PFU/ml, corresponding to about 1 polyoma PFU recovered for each molecule of DNA added to the competent bacteria. To explain these data the following possibilities were considered: (i) Most of the polyoma DNA molecules were taken up by the bacteria and simply enclosed in a protein coat, or (ii) only a certain fraction of the DNA molecules were incorporated, but those that were incorporated initiated the synthesis of more than one polyoma virus per infected bacterium.

To test these alternatives, a modified fluctuation test was performed. Competent *B. subtilis* were exposed, as in previous experiments, to polyoma DNA (10^{6} molecules/ml), evenly spread onto agar plates and incubated at 37°C for 28 hours. Twenty samples, each ap-

Table 1. The number of plaque-forming units (PFU) of polyoma virus formed by *Bacillus subtilis* as a function of polyoma DNA concentration.

Sample 1*		Sample 2*	
DNA concentration (molecules/ml)	PFU/ml	DNA concentration (molecules/ml)	PFU/ml
1.38×10^{11}	7.1×10^{5}	1.26×10^{11}	6.3×10^{5}
1.38×10^{10}	8.2×10^{5}	1.26×10^{10}	6.8×10^{5}
1.38×10^{9}	6.3×10^{5}	1.26×10^{9}	5.3×10^{5}
1.38×10^{8}	7.6×10^{5}	1.26×10^{8}	7.1×10^{5}
1.38×10^{7}	7.5×10^{5}	1.26×10^{7}	5.8×10^{5}
1.38×10^{6}	7.1×10^{5}	1.26×10^{6}	6.7×10^{5}
1.38×10^{5}	3.1×10^{4}	1.26×10^{5}	1.3×10^{4}
1.38×10^{4}	1.7×10^{2}	1.26×10^{4}	6.0×10^{2}
1.38×10^{3}	3.0×10^{1}	1.26×10^{3}	6.0×10^{1}
1.38×10^{2}	0	1.26×10^{2}	0

* All deoxyribonuclease-treated samples of polyoma DNA were inactive in this assay.