agree well with those from chromosomal (*18*) and recent anatomical studies (*19*), all of which indicate that *Alouatta* is a fairly specialized creature among living New World primates. We base this statement on the known fact that it is only in *Homo* and some pongids that we find slow-moving transferrins within the Primates (*2*). As we follow the members of this order from the prosimians to the hominoids the tendency appears to be one of acquiring increasingly slower transferrins. *Alouatta* seems to have gone further in this tendency than other known platyrrhines.

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17 August 1964

Fructose-1,6-diphosphate Requirement of Streptococcal Lactic Dehydrogenases

Abstract. *The lactic dehydrogenase of a strain of* Streptococcus bovis *specifically requires fructose-1,6-diphosphate for activity. Phosphate or fructose-1,6-diphosphate prevents inactivation of the dehydrogenase, but phosphate and other compounds cannot be substituted for the fructose-1,6-diphosphate required for activity. Lactic dehydrogenases of other species of* Streptococcus *show a similar requirement for fructose-1,6-diphosphate.*

Streptococcus bovis produces lactic acid as a major product of glucose fermentation. Examination of extracts for a pyridine nucleotide-linked lactic dehydrogenase showed occasional low rates of oxidation of reduced nicotinamide adenine dinucleotide ($NADH_2$) in the presence of pyruvate, but usually no activity was observed. After testing many possible explanations for the poor lactic dehydrogenase activity by using protective agents (thiols and chelating agents) during extract preparation, testing for nonpyridine nucleotide-linked lactate oxidation with dye acceptors, and testing other parameters without being able to explain the lack of lactic dehydrogenase activity, it was discovered that highly active lactic dehydrogenase-containing extracts could be obtained if two conditions were satisfied. These conditions were (i) the preparation of extracts in the presence of phosphate buffer and (ii) the addition of small amounts of fructose-1,6-diphosphate (F16P) to the assay system. The unusual, highly specific requirement for F16P for activity of an NAD-linked lactic dehydrogenase is described in this report, and the possible significance of the F16P requirement as a metabolic control mechanism is discussed.

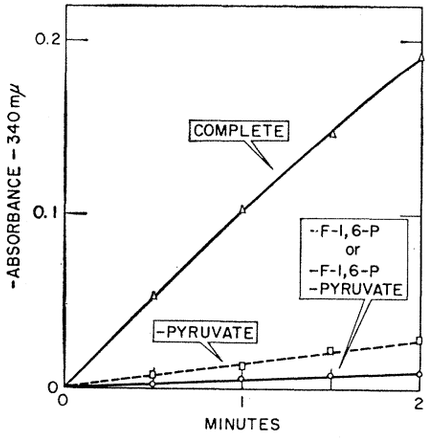

Fig. 1. Requirements for lactic dehydrogenase activity. Cuvettes (1 cm light path) contained 17m*M* tris, *p*H 7.2; 3.3m*M* neutral cysteine (*7*); 27m*M* sodium pyruvate; 20m*M* $MgCl_2$ (*7*); 0.08m*M* $NADH_2$; 1 m*M* F16P; and 10 μg of extract protein in a total volume of 3.0 ml. Extract, diluted in 0.1*M* Na_2HPO_4, *p*H 7.4, was added to start the reaction. Absorbancy changes at 340 mμ were measured with a Cary model 14 spectrophotometer.

Streptococcus bovis (*1*) was grown in 20 liters of medium as previously described (*2*). Harvested cells were washed with 0.1*M* Na_2HPO_4, *p*H 7.1, which contained 0.01 percent β-mercaptoethanol, and then suspended in the same solution. The cells were broken in a Raytheon sonic oscillator (10 kcy) under nitrogen gas and centrifuged to remove debris and residual whole cells. The supernatant solution was centrifuged at 100,000*g* for 1 hour in the model L Spinco centrifuge, and the clear supernatant solution was used in the experiments.

Figure 1 shows the absolute requirement of F16P for $NADH_2$ oxidation in the presence of pyruvate and extract. Slight $NADH_2$ oxidation was observed in the absence of pyruvate. Figure 2 shows that 10^{-4}*M* F16P was sufficient for maximum activity. No reduced nicotinamide dinucleotide phos-

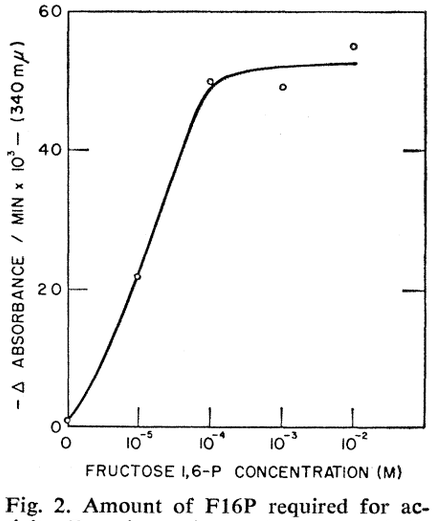

Fig. 2. Amount of F16P required for activity. Experimental procedure was as indicated for Fig. 1 except for the addition of the quantities of F16P shown in Fig. 2.

phate ($NADPH_2$) oxidation was observed in the complete assay system when $NADPH_2$ was substituted for $NADH_2$. It was necessary to maintain the enzyme in the presence of phosphate to obtain activity. Dilution of the enzyme in 0.015*M* or 0.1*M* phosphate gave maximum enzyme activity under the assay conditions indicated in Fig. 1. Dilution in 0.005*M* phosphate decreased enzyme activity approximately 40 percent, and dilution in 0.004*M* phosphate decreased activity approximately 60 percent. Sodium or potassium phosphate stabilized the enzyme, but sodium chloride did not stabilize activity. The F16P could be substituted for the stabilizing activity of phosphate, but inorganic phosphate tested at concentrations up to 0.1*M* could not be substituted for F16P in the assay system. Fructose, fructose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, ribose-5-phosphate, mannitol, sorbitol, glyceraldehyde-3-phophate, dihydroxyacetone-phosphate, glyceraldehyde, glycerol, adenosine tri-, di-, and monophosphate did not substitute for F16P in the assay system at concentrations of 0.001*M* or greater with or without $3.3 \times 10^{-3}M$ phosphate in the assay system. D-Arabitol-1,5-diphosphate (*3*), an analog of F16P, could not be substituted for F16P nor did it inhibit F16P activity when tested at a concentration of 0.001*M* in the assay system.

That the enzyme being measured is actually a lactic dehydrogenase is supported by several lines of evidence. After complete oxidation of 0.5 μmole of $NADH_2$, lactate formation was determined by the Barker-Summerson method (*4*) after removal of excess pyruvate with 2,4-dinitrophenylhydrazine, and 0.41 μmole of lactate was formed in the presence of $NADH_2$, pyruvate, and F16P. Lactate oxidation coupled to NAD reduction was difficult to measure. An alkaline *p*H was unsuitable for assay of NAD reduction because the enzyme was rapidly inactivated by exposure to *p*H greater than 7.5. Oxidation of lactate could be obtained, however, if high concentrations of lactate were used and semicarbazide was added to the reaction mixtures, especially if NAD analogs were used. Table 1 shows the rates of lactate oxidation with various NAD analogs and also demonstrates that lactate oxidation required F16P. With NAD, reduction of the nucleotide was obtained with L(+)-lactate but not with D(−)-lactate.

A limited number of strains of other *Streptococcus* species have been tested for a similar F16P requirement for lactic dehydrogenase activity. Table 2 shows that extracts of strains of *S. faecalis* (*5*), *S. thermophilus* (*5*), and *S. agalactiae* (*5*) require F16P and pyruvate for $NADH_2$ oxidation. Extracts of another strain of *S. bovis* (*5*) tested did not require F16P for $NADH_2$ oxidation in the presence of pyruvate, but a slight stimulation of lactic dehydrogenase activity was observed when F16P was present (Table 2).

The role of F16P in the lactic dehydrogenase reaction is not known. Unfortunately, the lactic dehydrogenase of *S. bovis* (*1*) has proved difficult to purify because of stability problems. It is difficult, therefore, to rule out a complex reaction requiring several enzymes in order to obtain an overall lactic dehydrogenase reaction. Measurements of F16P disappearance by the method of Roe and Papadopoulos (*6*) after the oxidation of 0.5 μmole of $NADH_2$ showed the disappearance of only 0.03 μmole of 0.22 μmole of F16P added to the reaction mixture. The small amounts of F16P required, and the high degree of specificity of the requirement, could suggest a role of F16P as an allosteric activator of the lactic dehydrogenase. The requirement of F16P for lactic dehydrogenase activity might be significant as a metabolic control mechanism.

During synthesis of metabolic intermediates of small molecular weight the organism would require pyruvate and reduced pyridine nucleotide. Glycolysis would probably proceed without accumulation of F16P in the cell and lactic dehydrogenase, which would compete with synthetic reactions, would be inactive. Pools of metabolic intermediates sufficient for synthesis of macromolecules could possibly lead to a decreased requirement for reduced pyridine nucleotide, which would accumulate and permit a pool of F16P to be built up within the cell. Activation of lactic dehydrogenase would then allow glycolysis to proceed for the purpose of providing energy for the synthesis of macromolecules from metabolic interdiates.

Table 1. Lactate oxidation with NAD and analogs. Cuvettes contained 16.7m*M* tris, *p*H 7.4; 66.7m*M* semicarbazide; 333.3m*M* sodium lactate; 0.33m*M* NAD or analog; 1m*M* F16P (where indicated); and 245 μg of extract protein to start the reaction. The total volume was 3.0 ml. Absorbance changes were measured at 340 mμ, 363 mμ, 361 mμ, 395 mμ, 358 mμ, and 356 mμ for NAD, 3-acetylpyridine-adenine dinucleotide, 3-acetylpyridine-hypoxanthine dinucleotide, thionicotinamide-adenine dinucleotide, 3-pyridinealdehyde-adenine dinucleotide, and 3-pyridinealdehyde-hypoxanthine dinucleotide, respectively.

−Δ Absorbance/min	
+F16P	−F16P
Nicotinamide-adenine dinucleotide	
0.127	0.000
3-Acetylpyridine-adenine dinucleotide	
.251	.000
3-Acetylpyridine-hypoxanthine dinucleotide	
.353	.000
Thionicotinamide-adenine dinucleotide	
.126	.000
3-Pyridinealdehyde-adenine dinucleotide	
.000	
3-Pyridinealdehyde-hypoxanthine dinucleotide	
.000	

Table 2. The F16P requirement of lactic dehydrogenases of streptococcal species. Extracts were prepared sonically from cells grown on a complex medium containing beef extract (Difco), 0.5 percent; proteose peptone (Difco), 0.5 percent; sodium thioglycolate, 0.5 percent; K_2HPO_4, 0.2 percent; NaCl, 0.5 percent; and glucose, 0.4 percent. The organisms, *S. agalactiae* and *S. faecalis*, were grown at 37°C, and *S. thermophilus* and *S. bovis* were grown at 40°C. The cells were disrupted by treatment with high frequency sound. The assay procedure was as described for Fig. 1 with the omission of cysteine and $MgCl_2$ from the reaction mixtures. Specific activity (S.A.) is defined as the number of micromoles of $NADH_2$ oxidized per minute per milligram of protein. The data of Fig. 1 were used for the specific activity calculations for *S. bovis* ATCC 15351 which are included in this table to emphasize the differences between *S. bovis* strains ATCC 15351 and 444.

Species	Reaction mixture		
	Complete (S.A.)	Minus pyruvate (S.A.)	Minus F16P (S.A.)
S. agalactiae (*5*)	5.94	0.18	0.24
S. thermophilus (*5*)	0.53	.05	.07
S. faecalis (*5*)	3.36	.00	.03
S. bovis 444 (*5*)	25.00	.00	18.00
S. bovis ATCC 15351 (*1*)	4.93	.68	0.29

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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.
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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.

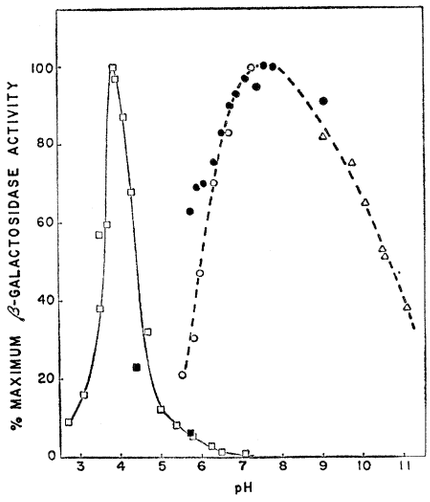

Fig. 1. Activity of β-galactosidase as a function of *p*H. Sample with optimum of *p*H 3.9 is the 60 to 80 percent ammonium sulfate fraction (□, ■). Sample with optimum of *p*H 7.5 is the 0- to 40-percent fraction (○, ●, △), heated 23 minutes at 50°C. Other symbols: □ ○, citrate-phosphate buffer; ■ ●,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

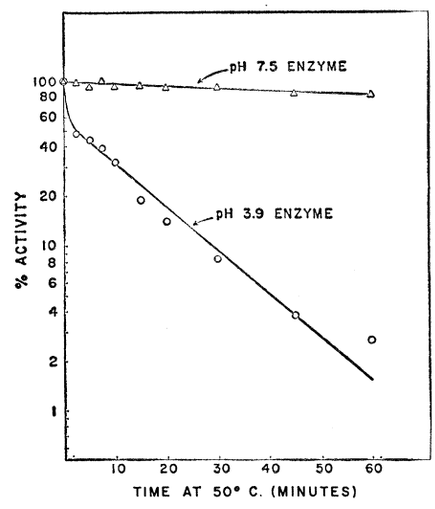

Fig. 2. β-Galactosidase stability at 50°C in 0.0125*M* tris HCl-buffer, *p*H 8.0. The *p*H 3.9 enzyme is that contained in the fraction precipitated at 60 to 80 percent saturation with ammonium sulfate; the *p*H 7.5 enzyme is that precipitated at 25 to 35 percent saturation.