utilizes the color complex formed by interaction of ferrous iron with dipicolinic acid (*6*).

The complex of ferrous iron and dipicolinic acid which is prepared from the authentic dipicolinic acid described by Black, Depp, and Corson (*7*) is unstable, but the addition of a reducing agent such as ascorbic acid yields a complex that is stable for at least 2 hours. Maximum color develops in a *p*H range of from 4.0 to 6.0. In more acidic solutions, the color is diminished, and in slightly alkaline solution, precipitation occurs. Figure 1 shows the linear relationship between the optical density at 440 mμ and concentrations over a range from 30 to 160 μg/ml.

For the colorimetric assay of dipicolinic acid in spores, 5 ml of a spore suspension of *Bacillus cereus* (ATCC 10702) (in a 15- by 125-mm test tube) containing 4 to 20 mg of spores (dry weight) is autoclaved for 15 minutes at 15 lb/in^2. The suspension is cooled, acidified with 0.1 ml of 1.0*N* acetic acid, and left at room temperature for 1 hour, during which time aggregation of the insoluble material occurs. Upon centrifugation at 1500*g* for 10 minutes, a clear supernatant is obtained. If the initial suspension contains an appreciable number of vegetative cells, it may be necessary to increase the amount of acetic acid in order to obtain a clear solution. Four milliliters of supernatant are carefully pipetted into a clean 15- by 125-mm test tube. With a pipette controller, the extract can easily be removed without disturbing the sediment. One milliliter of freshly prepared reagent, consisting of 1 percent of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 1 percent of ascorbic acid in 0.5*M* acetate buffer at *p*H 5.5, is added to the 4 ml of supernatant. The color develops immediately, and the optical density should be measured within 2 hours. The blank is a similarly treated suspension in which 1 ml of water is substituted for the color reagent. The reagent blank is negligible. The standard is prepared by adding 1 ml of the color reagent to 4.0 ml of an aqueous solution containing 100 μg of dipicolinic acid per milliliter.

Determinations of dipicolinic acid by ultraviolet absorption and by colorimetric methods gave results which were in close agreement. Free dipicolinic acid was measured in an aqueous suspension of dried spores, as described by Krishna Murty and Halvorson (*4*). An ether extract of the suspension was chromatographed on paper, the spots containing dipicolinic acid were eluted, and the ultraviolet absorption of the eluate was measured at 270 mμ, with a Beckman DU spectrophotometer. This assay showed 41.6 μg of dipicolinic acid per milligram of spores. An aliquot of the same spore suspension assayed by the colorimetric method showed 42.3 μg of dipicolinic acid per milligram of dried spores.

Although divalent iron will react similarly with other α-carboxy pyridine compounds (*6*), the agreement in results between this method and the ultraviolet absorption method, which includes isolation of the dipicolinic acid, indicates that, for spore preparations, the assay is specific. Furthermore, no other α-carboxy pyridine compounds have been reported in spores. The addition of authentic dipicolinic acid to vegetative cell preparations yielded quantitative recovery of the dipicolinic acid upon assaying by the colorimetric method. This shows that vegetative cells contain neither components that interfere with the assay nor nonspecific compounds that assay as dipicolinic acid.

Other ions, including manganese and calcium, will complex with dipicolinic acid, but in the preparations studied no effective competition of these ions with the ferrous iron has been observed. In preparations in which the concentration of these ions is excessive, the interference can be overcome by increasing the concentration of the iron in the color reagent.

Autoclaving was found to release essentially all the dipicolinic acid of the spores. By colorimetric assay, we compared the dipicolinic acid released from a preparation of dried spores by autoclaving with that released by acid digestion in 3*N* HCl for 15 minutes. The amounts were 42.3 and 43.0 μg/mg, respectively. When the washed residue of the autoclaved suspension was digested in acid, an additional 0.45 μg of dipicolinic acid was recovered.

The data for the standard curve (Fig. 1) were obtained by means of a Beckman DU spectrophotometer, but all routine colorimetric assays were made with a line-operated spectrophotometer. The local line fluctuations were such that the probable error of replicate optical density readings at times exceeds 2 percent.

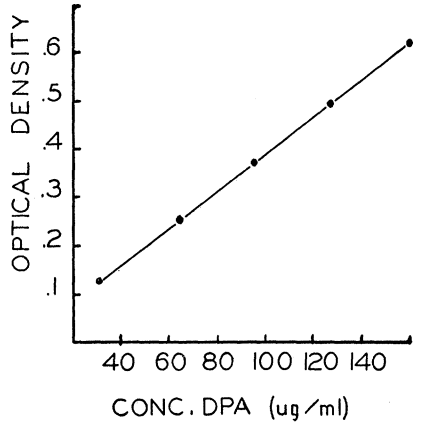

Fig. 1. The relation between optical density and dipicolinic acid concentration. Optical density was measured at 440 mμ with a Beckman DU spectrophotometer.

Under such conditions, no significance can be ascribed to the differences in results obtained with the two methods or to the trace of dipicolinic acid observed on the residues from the autoclaved preparation in the last experiment described.

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23 September 1957

Evidence for a New Growth-Promoting Acid Produced by Lactobacillus casei

Lactobacillus casei 280-16 (or 280-16A) requires either D-lactic acid or any one of a number of other D-α-hydroxy fatty acids for optimal growth (*1*, *2*). Yeast extract serves as a source of D-α-hydroxy acid for this organism, and at least part of its nutritional activity is attributable to D-lactic acid (*1*). Culture filtrates of *L. arabinosus, L. casei* (hydroxy acid-independent strain), *Leuconostoc mesenteroides, Lactobacillus brevis,* and *L. fermenti* have also been shown to provide growth promoting material assumed to be D-lactic acid (*1*). The present investigation (*3*) reveals, however, that the growth stimulant to be found in cultures of *L. casei* is not D-lactic acid, but a more lipophilic acid, possibly a higher homolog of D-lactic acid.

Lactobacillus casei 280-16A and its hydroxy acid-independent parent (American Type Culture No. 7469) were incubated at 35°C for 72 hours in a medium free of hydroxy acid. The hydroxy acid deficiency of this medium was overcome, in the case of the hydroxy acid-dependent strain, by employing a very heavy inoculum, yielding an initial population of approximately 3× 10^8 cells per milliliter of medium. The nearly normal growth which resulted under these conditions was probably due to D-α-hydroxy acids carried either in or on the washed cells. An equally dense initial population was introduced in the case of the hydroxy acid-independent strain, al-

though normal growth of this organism could have been initiated with a very much smaller inoculum.

The cultures were clarified by centrifugation, acidified by adding 2 ml of concentrated sulfuric acid to each, and steam-distilled to remove volatile acid. Titratable acid, amounting to 165 and 230 μequivalent/ml of culture, was produced by the dependent and independent strains, respectively. Acidity lost during steam distillation amounted to 140 and 138 μequivalent/ml, respectively, for the two cultures (*4*). Ammonium sulfate and sulfuric acid were added to the residual cultures to make each final solution 1*N* with respect to sulfuric acid and 30 percent with respect to ammonium sulfate. Each solution was then extracted with seven consecutive 15-ml portions of isopropyl ether-butanol mixture (2/1 by volume), and titratable acidity and growth-promoting activity were determined in a 1-ml aliquot of each extract.

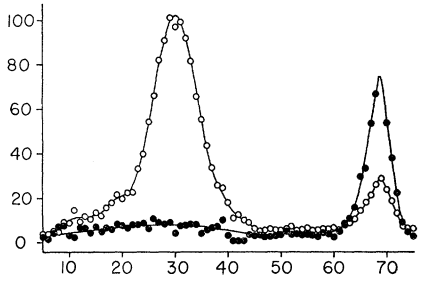

Fig. 1. Distribution of titratable acid (open circles) and growth-promoting activity (solid circles) in extracts of a *Lactobacillus casei* 7469 culture after 70 transfers in the following solvent system: upper phase isopropyl ether-butanol 2/1 by volume; lower phase 30 percent ammonium sulfate in 1*N* sulfuric acid. The values on the vertical scale represent either microequivalents of titratable acid (open circles) or units of growth-promoting activity (defined in Table 1) (solid circles) in the upper-phase solution (10 ml) of each cell. The numbers on the horizontal scale designate the consecutive cells of the counter-current extraction apparatus. The seven extracts of the *L. casei* 7469 culture (Table 1) were combined, evaporated nearly to dryness under reduced pressure, dissolved in 50 ml of upper-phase solvent, and distributed, 10 ml per cell, into cells 4, 5, and 6 at the outset of the counter-current extraction.

Table 1. Titratable acid and growth-promoting activity in extracts of cultures of *Lactobacillus casei*.

Extract No.	Strain 280–16A		Strain 7469	
	Titratable acid* (meq)	Growth-promoting activity (units)†	Titratable acid (meq)	Growth-promoting activity (units)
1‡	0.64	< 4	0.85	169
2	1.09	< 7	1.32	244
3	1.05	< 7	1.24	124
4	0.93	< 7	1.19	87
5	0.90	< 7	1.17	60
6	0.88	< 7	1.15	46
7	0.86	< 7	1.12	38

* Calculated from titrations with 0.01*N* sodium hydroxide. Phenolphthalein indicator, used with extracts 1 through 3 of the 280–16A culture, was replaced with brom thymol blue indicator for the remaining extracts of both cultures since the latter gave a sharper end point (but somewhat low titration values).

† One unit of growth-promoting activity is an amount equivalent to the growth-promoting activity 1 μmole of D-lactic acid (used as standard in the microbiological assays).

‡ During the first extraction, part of the nonaqueous solvents dissolved in the aqueous phase, and for this reason the first extract was smaller in volume and otherwise is not directly comparable to the subsequent extracts.

The results, shown in Table 1, make it fairly evident that growth-promoting activity is not associated with the major fraction of titratable acid, which appears, as would be expected, to have the correct distribution coefficient for lactic acid (0.6 in this solvent system, according to the results of a preliminary experiment with the purified compound) in either culture. That essentially none of the growth-promoting activity produced by the independent strain is associated with lactic acid is evident from the data obtained by counter-current extraction of three-fifths of the remaining combined extracts from the culture medium of this organism (Fig. 1). The solvent system employed for this extraction (carried through 70 transfers) was the same as that for the preliminary extraction. It may be seen (Fig. 1) that the distribution of the major titratable component distribution coefficient, 0.56) is close to that which could have been predicted from the distribution coefficient of lactic acid. A much smaller component (distribution coefficient, 10.6) is seen to be associated with the growth-promoting activity (Fig. 1). That this growth-promoting material was not formed artifactitiously is evident from its absence in the extracts of the dependent culture, which was treated as a control (Table 1). The growth-promoting potency of the active component appears to be about three times that of D-lactic acid when growth-promoting units (defined in Table 1) are compared with titration equivalents (Fig. 1). The high activity of the material, together with its relatively high distribution coefficient and other solubility properties, suggest that it is either a higher (by several carbons in chain length) homolog of D-lactic acid or a closely related analogous acid. Isolation of larger amounts of this material for exact identification is in process.

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3. This investigation (No. 120) was aided by grants from the National Multiple Sclerosis Society, the U.S. Public Health Service, and the University of California. We are indebted to Evelyn Brown and Audree Fowler for technical assistance.
4. The calculated acetic acid content of the acidified cultures was 146 μequivalent/ml derived from sodium acetate included in the medium. It appears, therefore, that 94 to 96 percent of the available acetic acid was removed by the steam distillation. Probably only negligible amounts of lactic acid and growth-promoting acid, if any, were removed by this process.

4 October 1957

Induction of Enzymes of the Galactose Pathway in Mutants of Saccharomyces cerevisiae

Induced biosynthesis of enzymes is dependent on the presence of a stereospecific inducer, which is not necessarily a substrate (*1*). In *Pseudomonas* it was shown that a sequential adaptation of a series of enzymes of a metabolic pathway takes place in the presence of the first (or often an intermediary) substrate of a chain of inducible enzymes (*2*). In the pathway

$$A \xrightarrow{\text{I}} B \xrightarrow{\text{II}} C \xrightarrow{\text{III}} D$$

enzyme II will arise in response to the formation of product *B*, and enzyme III will be formed when product *C* accumulates, and so forth.

In the present study (*3*, *4*) the inducible properties of the enzymes of galactose metabolism are considered in relation to the problem of sequential induction. The formation of these enzymes in response to galactose cannot be explained in terms of this pattern.

The galactose pathway (the old "galactozymase complex") which effects the complete transformation of galactose to glucose has now been completely established (*5–8*) and can be summarized as follows (*4*):

$$\text{Galactose} + \text{ATP} \xrightarrow{\text{galactokinase}} \text{Gal-1-P} + \text{ADP} \quad (1)$$

$$\text{Gal-1-P} + \text{UDPG} \underset{\text{Gal-1-P uridyltransferase}}{\rightleftharpoons} \text{UDPGal} + \text{G-1-P} \quad (2)$$

$$\text{UDPGAL} \underset{\text{(DPN)}}{\overset{\text{UDPGal-4-epimerase}}{\rightleftharpoons}} \text{UDPG} \quad (3)$$

Sum: $\text{Galactose} + \text{ATP} \rightleftarrows \text{G-1-P} + \text{ADP}$

The initial formation of the catalytical amount of uridine diphosphoglucose necessary to start reaction 2 is provided by a fourth reaction:

$$\text{UTP} + \text{G-1-P} \underset{\text{UDPG pyrophosphorylase}}{\rightleftharpoons} \text{UDPG} + \text{PP} \quad (4)$$

In the present study two haploid mu-