tube, and 5 ml of this denser solution was then slowly injected below the extract. A sharp and relatively stable boundary was thus formed between the colored extract and the colorless buffer-salt solution. Centrifugation was carried out in a Spinco C rotor at 42,040 rev/min for 6 hours at 7°C. The color boundary, which appears to be associated with the movement of fraction II, is blurred somewhat during the centrifugation. By means of the tube slicer, five of the tubes were sampled as follows, and the corresponding fractions were pooled: top 4 ml, middle 4 ml, and lower 1 ml plus pellet. An additional 1 ml of buffer was used to rinse the lower portion of each tube, so that the total volume of the pellet solution was 10 ml. The solutions were dialyzed against maleate buffer as indicated above, and the pellet solution was subsequently centrifuged at 25,000 *g* for 30 minutes at 7°C to remove a small amount of insoluble green material. The two remaining tubes from the separation run were used to examine the composition of the pellet itself. The supernatant fluid was simply decanted, and the pellets were dissolved in a total of 8 ml of maleate buffer. The resulting solution was then dialyzed, and the insoluble green matter was removed as described above. All solutions were analyzed for total nitrogen and examined in the ultracentrifuge.

Table 1. Ultracentrifugal and analytical data for an extract of green Burley tobacco and for various fractions obtained after separation ultracentrifugation. To facilitate comparison of the data, peak areas and nitrogen values are all expressed in terms of 4 ml of the clarified extract.

Solution	Determination		
	N (mg)	Fr. I area (cm^2)	Fr. II area (cm^2)
Clarified extract, before separation run	2.06	21.6	54.0
Fractions, after separation run:			
Top 4 ml	0.94		46.0
Middle 4 ml	0.47	4.4	8.4
Lower 1 ml plus pellet	0.60	10.6	2.6
Pellet only, after separation run	0.21	3.2	0.8

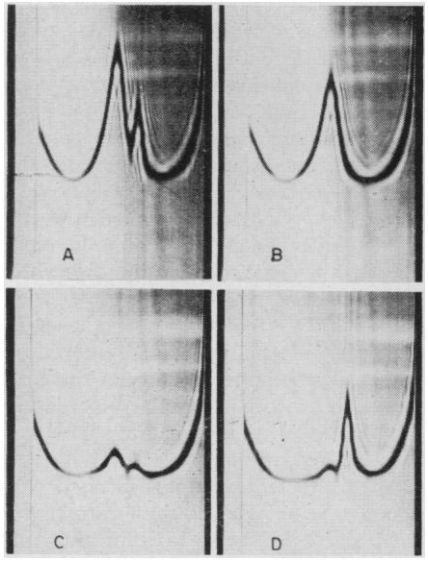

Fig. 1. Schlieren patterns of tobacco extracts to illustrate separation of protein fractions by centrifugation across a density boundary. Sedimentation is from left to right; the faster-moving component is fraction I. *A*, clarified extract before separation run, showing both fractions I and II; *B*, top 4 ml from separation run, pure fraction II; *C*, middle 4 ml from separation run, showing traces of both components; *D*, lower 1 ml of tube plus pellet (solution diluted 1:1 with maleate buffer), primarily fraction I. Patterns photographed at 35° bar angle and 8 minutes after the rotor had reached speed (50, 740 rev/min). Runs were made in a synthetic boundary cell (see *5*) at 25° ± 0.4°C.

Sedimentation patterns obtained for the clarified extract and for solutions from the separation run are shown in Fig. 1. Analytical data, including that for the pellet only, are summarized in Table 1. Peak areas, which are proportional to the concentration of the sedimenting components, and sedimentation constants were evaluated from enlarged tracings of the solution and base-line patterns. The areas were corrected for the dilution effect, but Johnston-Ogston corrections (*7*) were not made. The average sedimentation constants determined for fractions I and II were 17 and 3 Svedberg units, respectively.

Analysis of the data shows that the over-all recovery of nitrogen from the clarified extract is 97 percent. The recoveries of fractions I and II, calculated from the peak area measurements, are 70 and 105 percent, respectively. The lower apparent recovery of fraction I is believed to be associated primarily with the uncertainty entailed in estimating the precise volume of the "lower 1 ml" when the tube-slicer technique is utilized. It is also apparent that a major portion of fraction I is suspended in the solution which bathes the pellet and that the fraction I in this solution is not entirely free of fraction II. These factors may be accounted for in terms of sedimentation theory for angle-tube centrifugation and could probably be eliminated or minimized appreciably by the use of a swinging bucket rotor to provide purely horizontal centrifugation.

This relatively simple procedure, with variations, has been carried out many times. The yields of both fractions have been uniformly high, and the preparations of fraction I have been essentially colorless and have been characterized by little or no aggregation (see Fig. 1 *D*). While the present application has been confined to the proteins of green tobacco, the method should be generally applicable to the separation of other mixtures containing macromolecular constituents whose sedimentation constants vary appreciably.

DONALD F. KOENIG*
JAMES K. PALMER
CARL J. LIKES

Virginia Institute for Scientific Research, Richmond

References and Notes

1. R. W. Dorner, A. Kahn, S. G. Wildman, *J. Biol. Chem.* **229**, 945 (1957). See also earlier papers in this series.
2. J. Bonner, *Plant Biochemistry* (Academic Press, New York, 1950), pp. 260–61.
3. L. Eggman *et al.*, *J. Biol. Chem.* **205**, 969 (1953).
4. This work was sponsored and released for publication by the American Tobacco Company, Inc., Richmond, Va.
5. The separation procedure employed in this study is somewhat similar to that suggested by M. K. Brakke [*J. Am. Chem. Soc.* **73**, 1847 (1951)], in which a solution is layered over a solvent having a density gradient. The synthetic boundary cell, developed by E. G. Pickels *et al.* [*Proc. Natl. Acad. Sci. U.S.* **38**, 943 (1952)], is based on the same principle except that a solvent of uniform density is layered over the solution.
6. Millipore Filter Corp., Watertown, Mass.
7. J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.* **42**, 789 (1946).

* Present address: Thomas C. Jenkins Department of Biophysics, Johns Hopkins Medical School, Baltimore 8, Md.

10 April 1958

Identification of Growth-Promoting α-Hydroxy Fatty Acids Produced by Lactobacillus casei

It was reported in an earlier communication from this laboratory that practically all of the growth-promoting activity in extracts of *Lactobacillus casei* 7469 culture filtrates, as measured with *L. casei* 280-16A (a D-α-hydroxy fatty acid-dependent strain), was associated not with lactic acid but with a more lipophilic substance considered likely to be a higher homolog of lactic acid (*1*). A more rigorous fractionation of such extracts in the present investigation (*2*) has now revealed that they contain at least three growth-promoting acids, two of which appear to be identical with D-α-hydroxyisovaleric and D-α-hydroxyisocaproic acids, respectively.

Lactobacillus casei 7469 was incubated at 35°C for 24 to 96 hours, and the resulting cultures were clarified by passage through a Sharples supercentrifuge. Hydrochloric acid was added (180 meq/liter) to the essentially cell-free solutions, and the acidified media were subjected to continuous liquid-liquid extraction with isopropyl ether. The extracted material from each culture was fractionated by a 200-transfer countercurrent distri-

bution between isopropyl ether and 1*N* hydrochloric acid in a 100-cell Craig-Post apparatus (*3*), and the distribution of organic acids was followed by titrating the material in aliquots of the ether phase with 0.02*N* sodium hydroxide. Growth-promoting activity was determined by microbiological assay of the titrated samples with *L. casei* 280-16A.

The fraction with the highest microbiological activity was that represented by distribution peak D, Fig. 1. It had approximately twice the growth-promoting activity of D-lactic acid and appeared to consist of a single component, which was isolated both as its barium salt and as the free acid. The empirical formulae, $C_6H_{12}O_3$ for the free acid (*4*) and $(C_6H_{11}O_3)_2Ba$ for the salt (*5*), were suggested by the results of microchemical analyses (*4, 5*), and the corresponding α-hydroxy fatty acid structure, $C_4H_9 \cdot CHOH \cdot COOH$, seemed likely from the *p*H ½ value, 3.8 (*6*), and from previous correlations between structure and growth-promoting activity (*7*).

The five possible racemic (*8*) acids which fit these formulae are DL-α-hydroxyisocaproic acid, DL-α-hydroxy-*n*-caproic acid, DL-α-hydroxy-DL-β-methylvaleric acid, DL-α-hydroxy-D'L'-β-methylvaleric acid, and DL-α-hydroxy-β,β'-dimethylbutyric acid. The isolated acid was readily distinguishable from the last four of these by marked differences in their infrared absorption spectra. It was indistinguishable from DL-α-hydroxyisocaproic acid on this basis and appeared, therefore, to be identical with the latter compound (see spectra 1 and 2, Fig. 2). It was estimated from the results of semiquantitative experiments that DL-α-hydroxyisocaproic acid constituted approximately 0.2 to 0.3 percent of the total acids produced by *L. casei* 7469. Only the D-moiety of the racemic acid may be considered to be a growth-promoting product, for the results of earlier studies have indicated that L-α-hydroxyisocaproic acid probably does not support growth of the test organism (*7*).

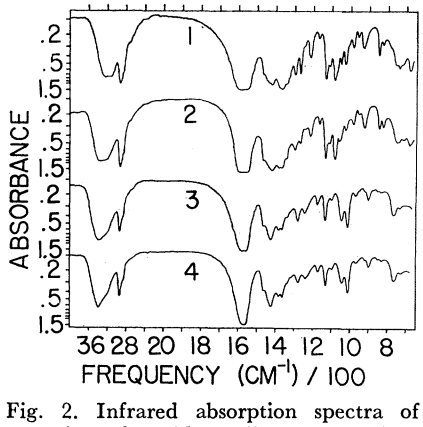

Fig. 2. Infrared absorption spectra of potassium bromide pellet preparations containing barium salts of synthetic α-hydroxyisocaproic acid (curve 1), α-hydroxyisocaproic acid from *L. casei* culture (curve 2), synthetic α-hydroxyisovaleric acid (curve 3), and α-hydroxyisovaleric acid from *L. casei* culture (curve 4).

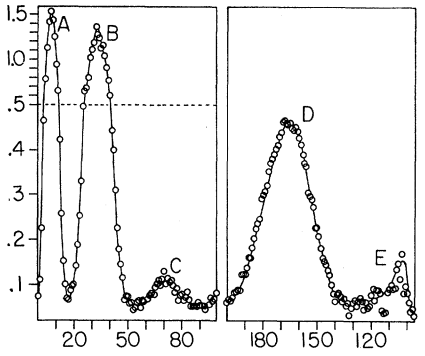

Fig. 1. Countercurrent distribution of acids from a clarified 24-hour culture of *L. casei* 7469 in a 200-transfer experiment. The solvent system consisted of isopropyl ether and 1*N* hydrochloric acid. Values on the vertical scale represent milliliters of 0.02*N* sodium hydroxide required to titrate the acids in 5-ml aliquots of the ether phase solutions (note change of scale at the 0.5-ml level). Numbers on the left horizontal scale designate the cells corresponding to the titrated samples (removed after 200 transfers). Titration values represented on the right side of the figure correspond to samples withdrawn from cell 99 after the designated number (right horizontal scale) of transfers. Distribution peaks A through E correspond to lactic acid, acetic acid (plus unidentified material), α-hydroxyisovaleric acid, α-hydroxyisocaproic acid, and an unidentified (presumably α-hydroxy fatty acid) fraction, respectively (see text).

The second best growth-promoting fraction (approximately one-third as active as D-lactic acid in this respect) was that represented by distribution peak C, Fig. 1. It appeared to consist of a single component, which was identified as α-hydroxyisovaleric acid by the same procedures as those employed with the α-hydroxyisocaproic acid fraction. The isolated α-hydroxyisovaleric acid was readily distinguishable from α-hydroxy-*n*-valeric acid by marked differences in their infrared absorption spectra, but it was indistinguishable from synthetic α-hydroxyisovaleric acid on this basis (see spectra 3 and 4, Fig. 2). Isopropyl ether solutions of this fraction (concentrates taken directly from the countercurrent extraction apparatus) were distinctly dextrorotatory, and the isolated barium salt was levorotatory ($[\alpha]_D^{25} = -4°$, 1 percent aqueous solution). The $[\alpha]_D$ of purified barium L-α-hydroxyisovalerate (1 percent aqueous solution, 25 to 27°C) is reportedly −10.1° (*9*), and on this basis the *L. casei* product was estimated to consist of approximately 70 percent L- and 30 percent D-α-hydroxyisovaleric acids. L-α-Hydroxyisovaleric acid does not appear to promote growth of the test organism (*7*), and the preponderance of this enantiomorph in the α-hydroxyisovaleric acid fraction was assumed, therefore, to account for the latter's relatively low growth-promoting activity (approximately one-third that of D-lactic acid). L- and D-α-hydroxyisovaleric acids appeared to constitute approximately 0.1 to 0.2 percent of the total acids produced by *L. casei* 7469.

The acids represented by distribution peak E, Fig. 1, did not appear to be homogeneous but had distinct growth-promoting activity (approximately one-fourth that of D-lactic acid) and yielded an average *p*H ½ value of 3.8. It seemed quite likely, therefore, that this fraction contained at least one additional D-α-hydroxy fatty acid. This fraction and those represented by distribution peaks A (lactic acid) and B (acetic acid plus unidentified components) are under further investigation (*10*).

The functions of D-α-hydroxy fatty acids in *L. casei* are far from clear. Our present working hypothesis is that they are metabolic precursors of cerebrosides since the latter from both mammalian and microbial sources are conspicuous for their content of 22–26 carbon D-α-hydroxy fatty acids (*7*). Whether the D-α-hydroxyisovaleric and D-α-hydroxyisocaproic acids produced by *L. casei* are concerned in vital metabolic processes of this nature or whether they are merely accidental by-products of amino acid metabolism (theoretically derivable from valine and leucine, respectively) appear to be important questions, which we hope may be resolved by the experimentation now in progress.

MERRILL N. CAMIEN
AUDREE V. FOWLER
MAX S. DUNN

Chemical Laboratory,
University of California, Los Angeles

References and Notes

1. M. N. Camien and M. S. Dunn, *Science* 127, 27 (1958).
2. This investigation (No. 124) 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, James Gilbert, and Claus Becker for technical assistance, to Donna M. Karasek for infrared spectrography, and to Heather King for microanalyses.
3. The analytical instrument employed had a capacity of 10 ml of each phase per cell. Preparative work was carried out with a larger apparatus (capacity 40 ml of each phase per cell), which was made available through the courtesy of Dr. John G. Pierce and the Department of Physiological Chemistry, University of California, Los Angeles.
4. Carbon: calculated 54.5 percent; found, 54.7 and 54.8 percent. Hydrogen: calculated 9.15 percent; found, 8.65 and 9.14 percent. Equivalent weight: found, 134.7 (formula weight 132.2).
5. Barium: calculated, 34.4 percent; found, 33.8 and 34.0 percent.
6. Estimated from the titration curve. This value is characteristic of simple α-hydroxy acids. The corresponding unsubstituted acids yield much higher values, and the β-hydroxy acids give values approximately halfway between those

of the unsubstituted and α-hydroxy acids. Divalonic acid (a six-carbon, growth-promoting β-hydroxy acid), for example, exhibits a *p*H ½ value of 4.3, according to Wolf *et al.* [*J. Am. Chem. Soc.* 78, 4499 (1956)].
7. M. N. Camien and M. S. Dunn, *J. Biol. Chem.* **211**, 593 (1954).
8. Neither the isolated barium salt (2 percent in water) nor the free acid (2 percent in either water or chloroform) yielded a significant optical rotation with sodium light.
9. C. G. Baker and A. Meister, *J. Am. Chem. Soc.* 73, 1336 (1951).
10. A paper describing the details of their characteristics is in preparation.

11 April 1958

Daily Rhythms in Male Harvester and Argentine Ants

The nuptial flights of various species of ants occur at characteristic times—for example, in a species of *Myrmica,* early morning; of *Lasius,* late afternoon (*1*); of *Eciton,* night (*2*). To gain a better understanding of such field observations, male and female harvester ants [*Veromessor andrei* (Mayr)] and male Argentine ants [*Iridomyrmex humilis* (Mayr)] (*3*) were studied in a constant-temperature room in alternating light and dark (*4*). Under these conditions, as is shown below, the males of both species exhibited a sharp daily activity peak, but at opposite ends of the light period.

The *Veromessor* females were obtained at a nest entrance on 26 July and kept until September with workers under room conditions (about 19° to 26°C). The males were collected at the entrance of the same nest on 13 September and kept by themselves.

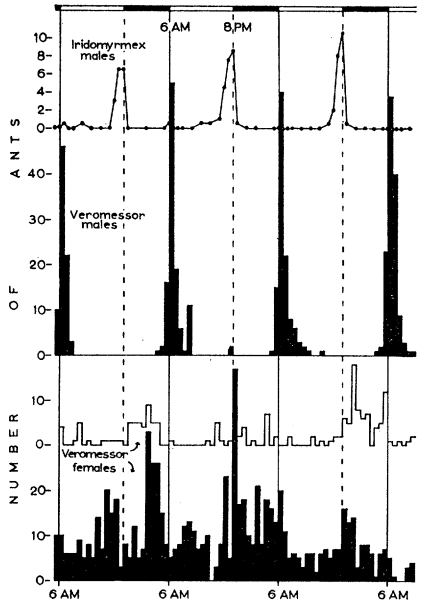

Fig. 1. Comparative activity patterns under alternating light and dark. *Iridomyrmex*: number out at time observed (mean of two counts 1 minute apart); *Veromessor*: half-time tunnel count per hour.

To obtain automatic activity samples, a circular "race track" was used. Two lots of ten males and two lots of ten females were placed in tiny chambers of clear plastic. Cotton-plugged test tubes of water and of sugar water projected down through the roof of each chamber; no other food or moisture was supplied. A transparent tube circled from one exit from the chamber back to the other, narrowing midway between to form the lining of a polystyrene tunnel. An ant passing through the tunnel, and thus between "sensitive" and "ground" screws, triggered a capacity-operated relay. In another room the total number of passes was recorded on a digital counter which was photographed each hour.

Since there were only two such relays available, a motor-driven cam device was used with each to alternate the contacts between two tunnels, so that a group of males were counted for half a minute, then females, and so forth. Thus each machine made a half-time count for each sex (*5*).

To simulate the darkness of the normal nest, the entire chamber was covered by an orange Plexiglas box (*6*). The circular "track" was left fully exposed to the light.

The counts of *Iridomyrmex* males, on the other hand, were made on a complete colony, consisting of nest queens, numerous workers and young, and about 14 males. The nest had been under laboratory conditions for over a year. Two adjacent plaster units opened onto a common board, which had legs set in DDT (*7*). The microscope-slide roof of each unit was overlapped with orange Plexiglas (*6*). Water and sugar water were continuously available in tubes suspended from a post set on the board (*7*). Solid food was provided, and the plaster was moistened, at irregular (though recorded) times; this had no observable effect on the rhythm. The males came out and wandered over the units or near them. Their wings made them easy to spot, and the total out at any given time was counted by eye.

Both species were kept on the same table in a darkroom at 25.4° ± 0.4°C. A 40-watt fluorescent light, automatically turned on at 6 A.M. and off at 8 P.M., provided an intensity of about 50 ft-ca. Two 40-watt clear ruby darkroom lights (two feet from the ants) were on continuously. Mechanical disturbance was minimized by sponge-rubber pads.

Figure 1 shows the simultaneously recorded activity patterns from 30 September to 3 October 1957, after the ants had been under the experimental conditions for several days. The replicate pattern of *Veromessor* males was similar to the one shown. Both female counts are given because of their greater variability. In fact, the activity patterns of the females are of interest, under these particular conditions, principally because of their lack of well-marked peaks, by contrast with the males.

The activity peak of *Veromessor* males occurred the first hour of each light period and was preceded consistently by a rise the last hour of the dark, with relative quiet the rest of the time (*8*). The *Iridomyrmex* males, on the other hand, were out of the nest during the last 2 hours of the light period only. The increases in activity *before* the changes in lighting suggest endogenous control of the rhythms. Further evidences for such an internal "clock" (*9*) in both species studied here were (*10*): (i) persistence of rhythm in constant darkness (that is, red light), though the *Veromessor* peak averaged 0.5 to 1 hour later each day, and (ii) a shift of phase following a single 5 P.M.–5 A.M. light period.

Veromessor mating flights occur locally early in the morning (*10*), thus suggesting the significance of the laboratory activity peaks. A species-characteristic light-phase relationship would seem valuable in synchronizing the nuptial flights of colonies of the same species. However, simple environmental response to the dawn might be insufficient, unless the ants were near enough to the surface at the right time. Endogenous control could bring them to the entry in time for the dawn. Their clocks could be "set" when the winged ants emerge on nonflight days, as do *Veromessor* (and other ants—for example, see *1, 11*). Such inherent control was postulated for worker leaf-cutting ants which were in the nest entry 1 hour before dawn, though even artificial light would not bring them up earlier (*12*). Similarly, bees trained to forage at a certain time may remain in a remote part of the hive until shortly before that time (*13*).

The value of the clocklike emergence of *Iridomyrmex* males is more difficult to explain, since mating takes place within the nest (*7, 14*). If a general male-and-female flight ever occurs, the results reported here would suggest the end of the day as the time. Perhaps the exit rhythm is a vestige of such a flight. The males sometimes fly, and they are found at artificial lights (*7, 14*). If the males normally fly before mating, a clock with the phase relationship noted could bring them out when late afternoon and night remain for encountering another nest or column of workers, before it again becomes too hot or dry for survival. In certain army ant species, where the virgin queens are wingless, some of the males, after their night flights, find their way into other colonies (*2*).

ELWOOD S. MCCLUSKEY*
Department of Biological Sciences,
Stanford University,
Stanford, California