romycin-resistant *Staphylococcus* is being treated. Since no evidence of a physical or chemical reaction between the two drugs was demonstrated, it seems possible that with erythromycin-resistant staphylococci, erythromycin stimulates a metabolic pathway which circumvents the action of lincomycin on the individual cell.

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28 December 1964

Probiotics: Growth-Promoting Factors Produced by Microorganisms

Abstract. *Several species of protozoa, during their logarithmic phases of growth, produce substances that prolong the logarithmic phase in other species. The effect is not as striking as the inhibition of growth caused by antibiotics, but a consistent 50-percent increase in growth has been obtained with* Tetrahymena pyriformis *in response to a factor produced by* Colpidium campylum.

Certain kinds of microorganisms growing in a culture medium often produce antibiotics inhibitory to the growth of other organisms. There have been several reports, however, of an opposite effect, growth stimulation, when the culture medium has been conditioned by a previous inoculation. The name given by Robertson (*1*) in the early 1920's to the effect observed in the growth of *Enchelys* and *Colpoda* was allelocatalysis. Several other workers (*2*) failed to confirm this allelocatalytic effect, but at that time there was little appreciation of the importance of axenic cultures and the desirability of chemically defined media for such experiments.

Before the development of a chemically defined medium for the ciliate now known as *Tetrahymena pyriformis*, Kidder (*3*) demonstrated that crude peptone medium could be conditioned by *Tetrahymena* so that organisms subsequently introduced into it grew more rapidly and to a greater concentration than in control cultures. Under certain conditions, inhibitory effects were also observed. In our laboratory, growth-promoting effects were obtained with different kinds of crude media and with different species of ciliates (*4*). Substances produced by *Tetrahymena* favored the growth of *Stylonychia*. Products of *Stylonychia* increased the growth of *Paramecium*, while conversely, the products of *Paramecium* favored the growth of *Stylonychia*. In all these cases, the factor responsible for the increased growth was nondialyzable and was thermolabile. In the case of *Colpidium campylum*, however, the product was not as readily destroyed by heat and yet it had a significant growth-promoting effect on *Paramecium caudatum* (*5*). It was also found possible to grow *Colpidium* in a chemically defined medium so that the unknown products, which we designate "probiotics," could be readily separated from the known components of the culture medium (*6*). The results obtained in this laboratory with ciliates are reminiscent of some of the reports in the bacteriological literature of growth-promoting effects of certain peptides. These were first described under the designation "strepogenin" (*7*). More recent work has confirmed the fact that several different kinds of peptides have growth-promoting activity for several different species of bacteria (*8*).

To test the growth-promoting activity of the protozoan product, we used two rapidly growing species that could be cultivated in a chemically defined medium. *Colpidium campylum* was used to produce the thermostable probiotic and *Tetrahymena pyriformis* (strain W) was used as the assay organism. The medium was a modification of medium A originally developed by Kidder and Dewey (*9*) for *Tetrahymena*. This had subsequently been changed to allow growth of *Colpidium campylum* (*10*), and another improvement by Holz *et al.* (*11*) permitted the growth of *Glaucoma chattoni* A without protein in the medium. To obtain satisfactory growth of both *Colpidium* and *Tetrahymena* in the same medium, it was necessary to make additional changes in the composition of the medium by inclusion of fatty acids and a sterol (Table 1). Growth of both organisms in this medium was near the maximum reported by other investigators; growth was evaluated by

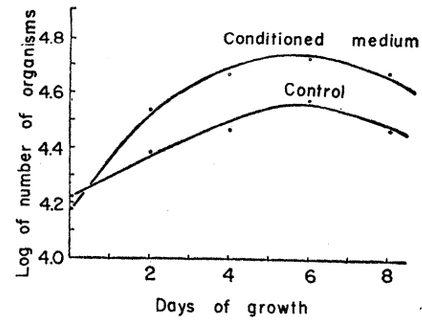

Fig. 1. Stimulation of *Tetrahymena pyriformis* by the probiotic from *Colpidium campylum*.

taking the average of four counts at each reading (*12*). The probiotic factor produced by *Colpidium* consistently prolonged the logarithmic growth phase of *Tetrahymena* by as much as 50 percent (Fig. 1), and there was a slight increase in the rate of growth in most of the experiments. Apparently, the main effect on growth regulation consisted in delaying the transition to the stationary phase and maintaining this plateau longer than in control cultures.

The probiotic effect was lost when the conditioned medium was subjected

Table 1. The chemically defined medium in which *Colpidium campylum* and *Tetrahymena pyriformis* were grown. Quantities expressed as micrograms per milliliter of final medium. Final *p*H adjusted to 7.0 with 0.1*N* NaOH. The stigmasterol and the vitamins were autoclaved separately and added aseptically to the other components which were mixed and sterilized together. The glucose was Seitz-filtered and added aseptically to the medium.

Substance	Quantity	Substance	Quantity
L-Alanine	25	Oleic acid	1.25
L-Arginine	100		
L-Aspartic acid	50	Stigmasterol	2
Glycine	25		
L-Glutamic acid	75	Calcium pantothenate	2
L-Histidine	50	Nicotinamide	4
L-Isoleucine	150	Pyridoxal HCl	4
L-Leucine	150	Riboflavin	2
L-Lysine HCl	125	Folic acid	1
L-Methionine	150	Thiamine HCl	6
L-Phenylalanine	75	Biotin	0.004
L-Proline	50	DL-6-Thioctic acid*	0.004
L-Serine	200		
L-Threonine	150		
L-Tyrosine	50	Sodium ethylenediamine tetraacetate	20
L-Tryptophan	50		
L-Valine	75		
Guanylic acid	75	$MgSo_4 \cdot 7H_2O$	40
Adenylic acid	30	$CaCl_2 \cdot 2H_2O$	20
Cytidylic acid	75	$(NH_4)_2SO_4$	10
Uridylic acid	20	$CuCl_2 \cdot 2H_2O$	2
Sodium acetate	570	$FeCl_3 \cdot 6H_2O$	0.5
		$MnCl_2 \cdot 4H_2O$	0.1
Glucose	2500	$ZnCl_2$	0.02
Linoleic acid	3.75	KH_2PO_4	570
		K_2HPO_4	570

* α-Lipoic acid.

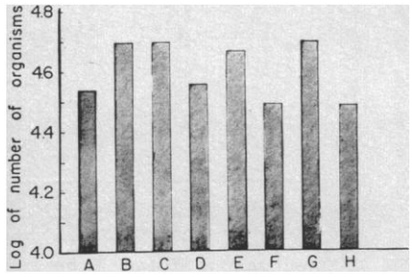

Fig. 2. Effects of different kinds of treatment on the probiotic factor produced by *Colpidium campylum*. Maximum growth of *Tetrahymena* after 6 days. *A*, Control (no factor added); *B*, conditioned medium autoclaved 20 minutes; *C*, nonfilterable factor (with membrane filter of 0.1-μ porosity); *D*, filtrate (with membrane filter of 0.1-μ porosity); *E*, factor hydrolyzed with 1*N* HCl at 100°C for 60 minutes; *F*, factor autoclaved at 20 lbs (9 kg) for 2 hours; *G*, factor separated by means of Sephadex G-25; *H*, factor hydrolyzed with 1*N* NaOH at 100°C for 30 minutes.

to prolonged autoclaving (1 hour or more) or to Seitz filtration. The active material was retained by membrane filters (Millipore and Gelman types) of fine grades with porosities of 0.1 μ, but not by filters of average porosity. Preliminary tests indicated that the separated materials contained considerable protein. Ninhydrin tests were positive after acid hydrolysis of the residue (obtained by filtration) in 1*N* HCl for 1 hour at 100°C, but this treatment did not destroy the probiotic effect. On the other hand, alkaline hydrolysis with 1*N* NaOH for 30 minutes at 100°C resulted in complete destruction of the factor. By the use of a Sephadex column (G-25) it was possible to obtain active fractions after elution with 3 to 6 ml of phosphate buffer (Fig. 2).

We could neither increase the probiotic effect more than 50 percent by concentrating the active material, nor could we dilute the original concentration of the material by more than a factor of 50. When probiotic materials produced by different species of ciliates were used together, no additive effect could be observed. Typical proteins, such as ovalbumin and casein, did not increase growth to any appreciable extent.

At present, the chief significance of probiotics is their possible mode of action in growth regulation. Since the logarithmic phase of growth in microorganisms has been significantly prolonged by the action of these products of ciliates, perhaps similar but more potent growth regulators will be found with even more striking effects. The fact that no potentiation or synergistic action of probiotics of different origin has yet been demonstrated may simply mean that these have a common mode of action. The somewhat analogous relation between the protozoan probiotic effects and the response of target cells to hormones in higher animals suggests another biological area of interest. The failure to increase the probiotic effect to the dramatic order of magnitude seen in the action of antibiotics might be explained by low responsiveness, in comparison with the better understood control mechanisms in inhibition, of the control mechanism that accelerates growth, or extends the period of growth, of microorganisms. Perhaps the current interest among bacteriologists in peptides with strepogenin activity will result in more information about stimulatory mechanisms in microorganisms generally. At least the protozoan probiotics offer an approach to the problem of growth regulation from the positive or accelerative aspect, rather than the more usual inhibitory or decelerative aspect.

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12. The numerous counts necessary for this investigation were facilitated by the use of a Coulter counter, Model A. The samples for counting were diluted 1 : 10 in a counting fluid containing 0.2 percent sodium chloride and 0.1 percent formalin.
13. We thank G. W. Kidder for the original cultures of ciliates used in this work. Supported by NSF grant GB-1121.

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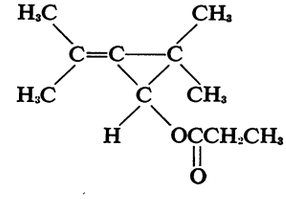
American Cockroach Sex Attractant

Abstract. *The structure (2,2-dimethyl-3-isopropylidenecyclopropyl propionate) previously assigned to the sex attractant of the American cockroach has now been shown by additional physical and chemical data and biological inactivity of the synthetic preparation to be incorrect. The structure of this attractant remains to be determined.*

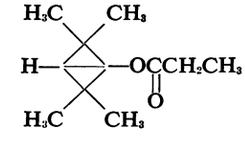
Widespread interest has been exhibited in the natural sex attractant of the American cockroach, *Periplaneta americana* (L.), whose structure we reported earlier (*1*) to be (I).



(I)

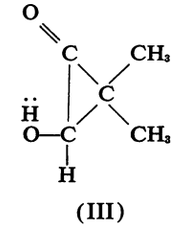
A compound having this structure has now been synthesized by Day and Whiting (*2*) and, recently, also in our laboratory by another procedure (*3*); it does not elicit a sexual response in *P. americana* males and is therefore not the sex attractant.

Day and Whiting suggest structure (II) as the correct one.



(II)

II has the same formula ($C_{11}H_{18}O_2$) as I but differs from it in the position of one of the bonds with the corresponding transfer of a hydrogen atom. We have now obtained additional evidence that reflects on the structure of the sex attractant and that of one of its oxidative degradation products, whose melting point is 55°C, for which we had suggested structure (III) (*1*).



(III)

Mass spectra (*4*), previously not available to us, have confirmed the