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Interactions of the Oral Microbiota
I. A System for the Defined Study of Mixed Cultures.* (27055)

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The net effect of a mixed microbial population is often greater or smaller than the simple sum of activities of the individual species growing in pure culture. It is therefore not possible to predict with certainty the influence of a single species, whose biochemical and growth characteristics have been determined solely in pure culture, on one or more different species. To the best of our knowledge there are no satisfactory techniques which allow measurement of interactions between 2 or more species of microorganisms growing in a dynamic state similar to that of nature and which at the same time permit control over basic factors of growth and population.

Until recently, attempts to assess bacterial interactions *in vitro* have fallen into 2 groups: a) growth in closed systems with selective isolation of components at varying intervals as reported by Annear(1) among others, and b) effect of metabolites produced by one species on another, the organisms being separated by a permeable membrane or similar barrier as described by Wilson and Goaz(8). A quantitative aspect was approached by Rosebury(7) with his relatively simple "drop pair" technique in which each of 2 different species diluted over a one-thousand-fold gradient are cultured as overlapping pairs on agar plates. While this technique can be evaluated mathematically, it is restricted to 2 species at a time and yields little information on products formed.

A limiting aspect of these methods (closed systems) is the lack of control over growth dynamics. Continuous culture permits controlling this factor and was suggested for early mixed populations by Rogers and Whittier(6). More recently, Zybrycki and Spaulding(9) constructed a simple continuous system for mixed cultures but cited no data on the interactions observed. A more sophisticated concept of continuous culture stems from the contribution of the chemostat by Novick and Szilard (5). The basic premise of this development assumes that multiplication of organisms in the growth cell is directly proportional to the rate at which some critical limiting nutrient is admitted to this cell. Novick(4) has also discussed the growth of pure cultures in a 2-stage arrangement in which the output of a chemostat is fed to a second growth cell. If nutrients in the second system are not limited, it becomes possible to define mathematically the steady state growth which results so that:†

$$X_2^\circ k^\circ m - F_1 + F_2/V_2 + \frac{F_1}{V_2} X_1^\circ = 0$$

In which: X_1° and X_2° respectively are the bacterial populations entering and leaving the

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† Mathematical symbols employed are those of the American Standards Assn. as cited by Gerhardt and Bartlett(3).

growth cell. k°m is maximum growth rate constant obtained from log phase of the organisms in pure culture. F_1 and F_2 are flow rates to the growth cells and V is cell volume.

Our study represents an extension of this 2-stage arrangement to permit application of previously recognized bacterial growth principles to the study of mixtures of bacteria growing under continuous and defined conditions.

Materials and Methods. To develop the desired system it seemed reasonable to experiment with a pair of organisms present in high levels in the mouth, one of which was known to furnish a metabolite utilizable by the other. The oral flora have an excellent example of this relationship in *Streptococcus salivarius* and *Veillonella alcalescens*. The former provides lactic acid from carbohydrates: the latter utilizes lactate but not the carbohydrate. Therefore, *S. salivarius* and *V. alcalescens* were grown in pure culture in individual chemostats and then combined for mixed growth at a second stage.

The chemostat cells were constructed with flat bottoms to permit the use of magnetic stirring bars. Each growth cell was equipped with additional standard taper ports to permit sampling and continuous measurement. The medium for each chemostat was metered by a peristaltic pump from an individual reservoir to an 0.0055 in. orifice at the chemostat. This size was necessary to assure constant feed rates of 8-11 ml media/hr to an 80 ml cell so that retention time in the cell was 7-10 hours. Other retention times might be used if physiologically young or physiologically aged cells were desired. Volumetric flow through each of the systems was carefully measured by calibration of the drop rate through the orifice. On the basis of experience over a number of runs, it was noted that the actual volumes deviated less than 2% from those calculated by the drop rate. The growth cell for the mixed cultures was similar in construction to the others (chemostats) but the nutrient supply to this cell was such that minimum ratio of feed rate to growth cell volume (dilution rate) exceeded the value of the maximum growth rate constant (k°m) of the faster growing species. This was a critical requirement and only in this manner could a mathematically defined maximum rate steady state be obtained in the mixed (second) system. The flow of the entire system is diagrammed in Fig. 1.

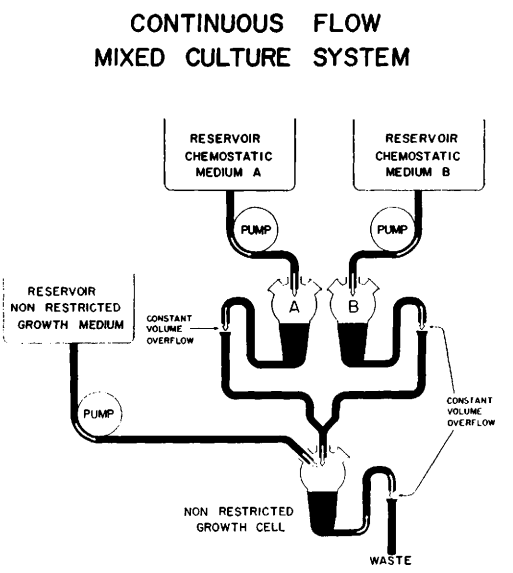

FIG. 1. Flow diagram of 2-stage mixed culture system

The basic medium, consisting of yeast extract 0.2%, tryptone 0.2%, potassium phosphate (dibasic) 0.1%, sodium bicarbonate 0.1%, sodium thioglycollate 0.02%, was adjusted to pH 7.2. The limiting substrate for *S. salivarius* was 0.01% sucrose. For *V. alcalescens* the limiting substrate was 0.01% lactate. The same basic medium which was used for mixed growth contained 0.50% sucrose and 0.50% lactate and was adjusted to pH 7.2. The pH did not go below pH 6.8 at any time during growth. Temperatures of all growth cells were controlled in thermostatically regulated glycol baths. A magnetic motor below each bath actuated a stirring bar in both the chemostat and the glycol baths to insure uniform temperature, nutrient mixture, and bacterial densities. Populations of each species were determined by viable cell counts over several intervals for replicate results. Results reported are the product of at least duplicate runs. Media highly differential for the opposite species were used for mixed growth counts. The medium routinely used for counting *S. salivarius* was 5% sucrose agar with aerobic incubation. Veillonella were cultured anaerobically under an atmosphere of 10% carbon dioxide and 90% hydrogen on Douglas agar(2). Al-

though *V. alcalescens* is an obligate anaerobe, growth of this organism in fresh media in the deep tube of the chemostat was equal to the growth rate obtained in a closed anaerobic tube. Therefore, it was not considered necessary continuously to purge the system with an inert gas.

Results. Generation times for each of the organisms growing in the second stage cell were calculated from the continuous culture growth rate equation (3):

$$FX_o - kXV = FX - V dx/d\theta$$

in which k is related to generation time (θg) by the equation $\ln 2/\theta g = k$

These times obtained for both pure and mixed cultures of *S. salivarius* and *V. alcalescens* are given in Table 1.

The generation time for each species studied was markedly influenced by the presence of growing cells of the opposite member of the pair (Table 1). For example, in the steady state (where k°_m is less than F/V) generation time of *S. salivarius* was increased from 12 to 42 minutes in the presence of roughly 5.0×10^6 cells/ml and to 48 minutes with 1.4×10^7 cells/ml of *V. alcalescens* in the system. In the reverse situation, the normal generation time of *V. alcalescens* was between 10-12 minutes, whether in a bottle (log phase) or in the continuous culture cell but was lengthened to 15-21 minutes in the presence of $1.7\text{-}2.6 \times 10^6$ cells/ml of *S. salivarius.*

When grown under non-steady state conditions (F/V less than k°_m or 0.2 in Table 1.) tremendous increases were recorded for generation times of both species. Their extension to approximately 100 minutes was remarkably constant over the last 45 hours of a 70 hour run.

Discussion. Previous studies on mixed populations have employed techniques designed to demonstrate inhibition or stimulation of one microorganism by another, growing in close association with it in tubes or bottles or on agar plates. Such procedures are limited by providing an environment that is continuously and uncontrollably changing over the time that population density undergoes a several thousand-fold increase and average age of cells changes from youth to senescence. Such patterns defy definition and do not seem representative of the indigenous microbial systems existing in the body.

TABLE I. Generation times of *S. salivarius* and *V. alcalescens* grown as pure cultures and in mixture in a 2-stage continuous culture system.

System type	Flow rate (F/V)	*S. salivarius* Generation time	*S. salivarius* Population	*V. alcalescens* Generation time	*V. alcalescens* Population
		min	cells/ml	min	cells/ml
Pure cult.	3.8	12	—	10-12	—
Mixed	3.9	42	1.8×10^6	21	4.7×10^6
"	3.8	48	2.6×10^6	15.5	14.3×10^6
"	3.9	44	1.7×10^6	17	10.5×10^6
"	0.2	101	9.35×10^7	100	14×10^6

The 2-stage continuous flow method described has obvious advantages for evaluation of interactions between 2 or more species of microorganisms because it is possible to regulate individually the ratio and levels of population for each species in the mixture by the following means: a) variation of levels of substrate furnished in the first stage chemostats, b) control of flow rate of medium to all growth cells, and c) control of flow rate of medium and number of organisms from each of the pure culture chemostats into the mixture.

Results obtained with *S. salivarius* and *V. alcalescens* demonstrate a distinct interaction between these species. The increase in generation times would not appear to be the result of depletion of critical nutrients since the medium used supported pure culture of either species at populations of at least 10^8 cells without noticeable change in generation time. This interaction is obviously subtle in nature and normally might not be detectable except by highly sensitive methodology such as we have described. Furthermore, the data can be expressed in generation time, which is a precise term with definite meaning to other investigators.

The observations reported here were obtained from non-defined reasonably complete bacteriological broth. Use of defined substrates on the one hand or use of tissue fluids on the other might well lead to different results. With proper selection of media, it should be practicable to investigate the influence of other substrates on mixed culture systems and to

note the appearance of biochemical structures not present in the pure cultures feeding into the mixture.

Summary. A continuous 2-stage system for investigations of interactions between 2 or more species of bacteria is described. The system employs 2 or more chemostatically limited stages of pure cultures feeding to a non-limited mixed continuous growth cell. This permits comparison at any point on the growth curve of numbers and products of organisms in pure culture with numbers and metabolic products resulting from interactions between the same organisms in growing in mixed culture. The systems are mathematically defined and the data can be expressed in generation time. Results obtained with a mixture of *S. salivarius* and *V. alcalescens* indicate a distinct interaction betwen the strains employed. Generation time of each of these species was noticeably increased while growing in context with the other.

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Liver Haemodynamics and Age.* (27056)

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Marked variations in the pattern of liver haemodynamics have been observed in normal rats of different weight and age, maintained under identical conditions.† When related to the metabolic processes of the liver and its capacity to react to various agents, these changes may be of considerable importance.

In the present communication, data are presented on the haemodynamic pattern of the liver of the rat in different age groups.

Technique and Materials. Three groups of white male rats of the Hebrew University strain maintained under identical conditions and fed a standard diet were used. The first group (20 animals) weighed between 80 to 150 g, the second group (100 animals) weighed between 200 and 300 g, and the third group (20 animals) weighed between 300 and 450 g. The parameters examined were hepatic blood flow per gram liver tissue and per 100 g body weight, vascular space of the liver and portal vein pressure values.

Hepatic Blood Flow Determination. Hepatic blood flow was estimated as rate of removal of radioactive colloid from the peripheral blood by the reticulo-endothelial system of the liver. Heat denaturated iodine^{131}-labeled, human serum albumin was used as described by Benacerraf *et al.*(1). The dose of radioactivity injected varied from 0.01-0.1 μc per 100 g body weight and contained 0.05 mg or less of iodinated albumin per ml of water solution. The radioactive albumin was injected into the vena dorsalis penis and samples of 0.2 ml of blood were taken from the tail at 1, 2 and 3 minute intervals. The gamma radioactivity of the blood samples was measured in an "Atomic" scintillation counter.

Liver blood flow was calculated by multi-

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