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The Dialysis Technique in the Study of the Vitamins and Amino Acids Affecting Associations of Micro-organisms

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A dialysis cell with several compartments suitable for the study of the chemical nature of the associative, and especially symbiotic, inter-relationships among micro-organisms is described. Using this apparatus two symbiosis experiments with a five or ten compartment cell using three or seven different species of lactic acid bacteria, respectively, were made. The chemical factors affecting the close symbiosis between the different organisms were found to be certain amino acids and vitamins.

In connection with biosynthetic studies on growth factors using the symbiotic technique [1,2] a simple method was needed by which the direct contamination of different symbionts during growth could be prevented. In this paper a simple apparatus is described in which organisms are separated from each other by a dialysing membrane in a cell assembled with two or more compartments. In these experimental conditions the bacteria used can grow in associations composed of more than two species. Symbiosis experiments were made using this apparatus, in which from three to seven different species of lactic acid bacteria were able to grow together despite the fact that some growth factors essential for the symbionts were omitted from the basal medium. This has been found to be due to the fact that the growth factors produced by the bacteria in each compartment passed through the dialysing membrane to the adjoining compartment, so permitting symbiotic growth in the dialysis cell.

EXPERIMENTAL TECHNIQUE

Apparatus. The apparatus (Figs. 1 and 2) consists of two or more compartments made of Pyrex glass each furnished with a glass tube open at the top, which can be plugged with cotton wool. A detailed drawing of a dialysis cell with three compartments is shown in Fig. 1. A second and more complicated modification of the same apparatus with ten compartments is shown in Fig. 2. Each compartment has approximately 12—15 ml capacity (not including glass tube) except for the cross-shaped compartment (shown in Fig. 2) which has 50 ml capacity. A dialysis membrane (in Fig. 1 A) (Cellulose, No. 4465—A2, A. H. Thomas Co, U.S.A.) is placed between the compartments, the surfaces

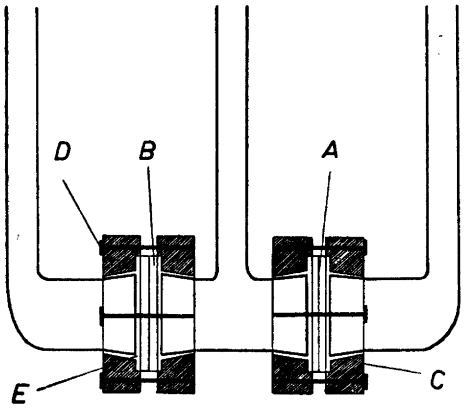

Fig. 1. Scheme showing a three-compartment dialysis cell (For description see the text).

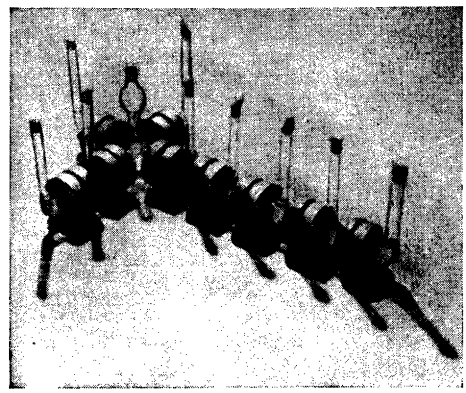

Fig. 2. Photograph showing a ten-compartment dialysis cell.

between the sections being ground smoothly, and held in place by rubber gaskets (B). The compartments are connected by aluminium hoops (C) equipped with set screws (D) and rubber packings (E). As the walls of the glass compartments and the rubber packings are slightly conical, the joints can be made water-tight by tightening the screws (D).

Cultures and Method. The organisms used were *Lactobacillus arabinosus* 17—5, *Lactobacillus casei*, *Lactobacillus fermenti*—36, *Leuconostoc mesenteroides* P—60, *Leuconostoc citrovorum* (ATCC 8081), *Streptococcus faecalis* R, *Streptococcus lactis* (ATCC 7963). The stock cultures were maintained by stab inoculation in the glucose-citrate-tryptone-yeast extract-agar as described previously [3], excluding one strain, *Str. lactis* (ATCC 7963), which was cultured in sterile skim milk. The inocula were prepared by transferring the organisms from the stab culture to 7 ml of glucose-citrate-tryptone-yeast extract medium, except for the last-mentioned strain, which was transferred to the inoculum medium as described by Anderson and Elliker[4]. After incubation for 16—18 hours at 37° C the cells were centrifuged out and washed with 0.9 % sterile saline. This process was repeated, the cells being washed 2—3 times. The cells were finally suspended in saline. One drop of barely visible suspension was used as inoculum for approximately 5 ml of the final medium.

The basal media of Henderson and Snell [5] and Anderson and Elliker [4] in slightly modified form as described previously [1,6] were used. The special omissions of some vitamins and amino acids from the basal media are noted in the text. The basal medium was diluted with an equal volume of water and placed in the dialysis cell and also in test tubes as a control (to test tubes in 5 ml portions) and autoclaved at 112° C for 5 minutes. After cooling, the dialysis cell and test tubes were inoculated aseptically and then incubated at 37° C. The growth response was followed turbidimetrically. The extent of growth was recorded as galvanometer readings of a Klett-Summerson photoelectric colorimeter with a 660 mμ filter.

RESULTS AND DISCUSSION

In the symbiosis experiments a chemically defined medium was prepared, omitting certain vitamins and amino acids which were essential for the growth of the lactic acid bacteria used. The separate compartments of the dialysis cell were then inoculated with the different strains of bacteria (and in addition each organism as a control separately into the test tube containing this incomplete medium). It should be emphasized that both inoculum cultures were washed thoroughly and that very little inoculum was used in order to avoid

Table 1. The symbiotic growth of Lb. arabinosus *17—5 (phenylalanine-requiring),* Str. faecalis *R (folic acid or folinic acid-requiring), and* Ln. mesenteroides *P—60 (phenylalanine and proline-requiring) in a five-compartment dialysis cell for 68 hours at 37° C.* (cf. *Fig. 3*). *Basal medium without phenylalanine, folic acid, folinic acid, and proline.*

Organism	Colorimeter reading		
	in dialysis cell		in test tube
	compartment		
Lb. arabinosus 17—5	2	232.0	23.0
	4	230.0	
Ln. mesenteroides P'60	1	192.0	14.0
	3	204.0	
Str. faecalis R	5	56.0	12.0
None			3.0

transference of the missing growth factors with the cells (apart from what the cells themselves contained) to the incomplete medium.

As can be seen from Table 1, *Str. faecalis* R, *Lb. arabinosus* 17—5, and *Ln. mesenteroides* P—60 could not grow alone in the test tubes, but were able to grow when inoculated into separate compartments of a cross-shaped dialysis cell despite the fact that three essential growth factors, phenylalanine, proline, and folic acid, were omitted from the basal medium (the basal medium of Henderson and Snell modified by the author was used in this experiment). Of these growth factors phenylalanine was required by *Lb. arabinosus* 17—5 and *Ln. mesenteroides* P—60, folic acid by *Str. faecalis* R, and proline by *Ln. mesenteroides* P—60. The growth of bacteria in the dialysis cell must be due to each organism releasing into the medium the growth factor required

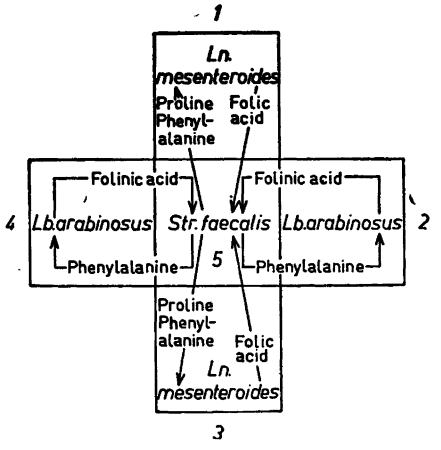

Fig. 3. The growth factors affecting the symbiotic growth of Lb. arabinosus *17—5,* Str. faecalis *R, and* Ln. mesenteroides *P—60 in a five-compartment dialysis cell.* (Cf. *the experiment described in Table 1*).

Table 2. The symbiotic growth of Lb. arabinosus *17—5 (phenylalanine-requiring),* Lb. fermenti—36 *(phenylalanine-requiring),* Lb. casei *(phenylalanine and folic acid or folinic acid-requiring),* Str. faecalis *R (folic acid or folinic acid-requiring),* Ln. mesenteroides *P—60 (phenylalanine-requiring),* Ln. citrovorum *(8081) (folinic acid-requiring), and* Str. lactis *(7963) in a ten-compartment dialysis cell for 69 hours at 37° C.* (Cf. *Fig. 4*). *Basal medium without phenylalanine, folic acid and folinic acid.*

Organism	Colorimeter reading		
	in dialysis cell		in test tube
	compartment		
Str. lactis 7963	1	39.0	48.5
	4	77.0	
Str. faecalis R	2	75.0	8.5
	8	39.0	
	10	48.0	
Lb. casei	3	96.0	9.0
Ln. citrovorum 8081	5	44.0	7.5
Lb. arabinosus 17—5	6	197.0	14.0
Lb. fermenti—36	7	78.0	4.0
Ln. mesenteroides P—60	9	44.0	7.5
None			2.0

by the strain of the adjoining compartment and to the passage of the factors through the dialysis membrane. Fig. 3 shows schematically in which compartments of the dialysis cell the different species of bacteria are situated and which are the essential growth factors produced by each organism. As can be seen, the central compartment of the dialysis cell was inoculated with *Str. faecalis* R. This organism was able to synthesize phenylalanine and proline and excrete these amino acids into the medium during growth. On the other hand, *Str. faecalis* R obtained the vitamin folic acid (pteroylglutamic acid) required by it from the adjoining compartments. This compound was produced by *Lb. arabinosus* 17—5 and *Ln. mesenteroides* P—60. On the basis of the earlier findings [1,6] it may be concluded that the folic acid produced by *Lb. arabinosus* 17—5 existed in its catalytically active form folinic acid (citrovorum factor, 5-formyl-5,6,7,8-tetrahydrofolic acid) a compound which possesses high folic acid activity for *Str. faecalis* R.

The second and more complicated symbiosis experiment is described in Table 2 and Fig. 4. This experiment was made with seven different species of lactic acid bacteria using the ten compartment dialysis cell illustrated in Fig. 2. The basal medium of Anderson and Elliker (modified by the author) was used, omitting the essential growth factors phenylalanine, folic acid, and folinic acid from the medium. Among the organisms used *Ln. citrovorum* 8081 required for growth folinic acid (citrovorum factor) and phenylalanine, *Lb. casei* folic acid (or folinic acid) and phenylalanine, *Str. faecalis* R folic acid (or folinic acid), *Lb. arabinosus* 17—5, *Lb. fermenti*—36, and *Ln. mesenteroides* P—60 phenylalanine. One strain, *Str. lactis* 7963, did not require these compounds. On the contrary, in an earlier work [6] it had already been shown that this

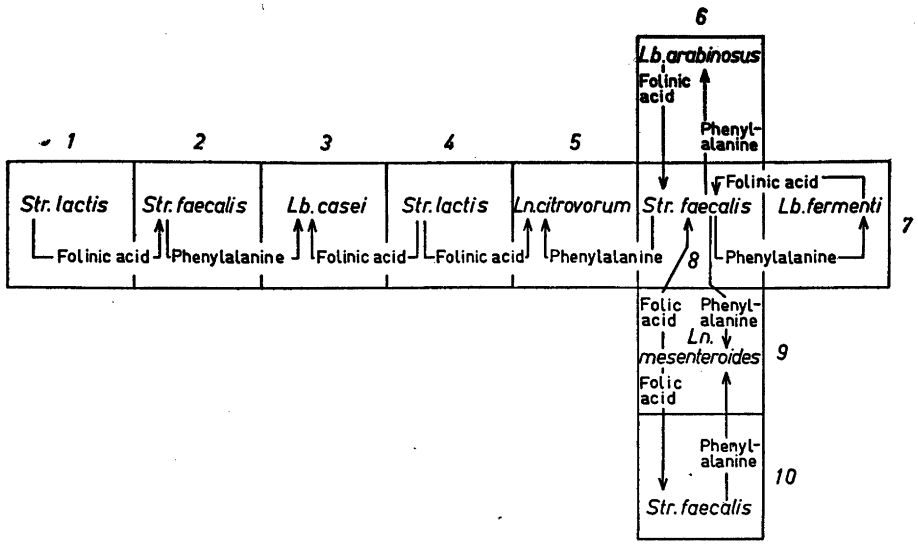

Fig. 4. The growth factors affecting the symbiotic growth of Lb. arabinosus *17—5*, Lb. fermenti *—36*, Lb. casei, Str. faecalis *R*, Str. lactis *(7963)*, Ln. mesenteroides *P—60, and* Ln. citrovorum *(8081) in a ten-compartment dialysis cell.* (Cf. *the experiment described in Table 2).*

organism was capable of producing folinic acid into the medium during growth and that phenylalanine was only a slightly stimulatory growth factor for it. As can be seen from Table 2 and Fig. 4, in the present experiment *Str. lactis* 7963 excreted folinic acid into the medium, so permitting the growth of *Ln. citrovorum* 8081, *Lb. casei*, and *Str. faecalis* R in the adjoining compartments. *Str. faecalis* R also obtained folic acid or folinic acid from the other strains, namely from *Lb. arabinosus* 17—5, *Lb. fermenti*—36, and *Ln. mesenteroides* P—60. These three last-mentioned organisms, on the other hand, benefited from *Str. faecalis* R, which was capable of producing phenylalanine into the medium.

In the symbiosis experiments described here, the growth of different species of bacteria in the dialysis cell must be due to that each organism during growth produced into the medium the growth factor (vitamin or amino acid) required by the strain of the adjoining compartment, these compounds then passing across the dialysing membrane. The results are in good agreement with those obtained in earlier symbiosis investigations [1] and are particularly interesting in view of the fact that in natural conditions micro-organisms live in mixed populations with complex inter-relationships. It seems likely that in nature also, in mixed microbial cultures, the production and excretion of growth factors such as vitamins and amino acids plays an important role in the formation of the associative relationships among micro-organisms.

In addition to symbiosis experiments with lactic acid bacteria the dialysis technique was also used in the study of the biosynthesis and the excretion of

vitamins and amino acids into the medium with the growing cells of certain other bacteria (*e. g. Escherichia coli*). In the course of this work (in progress) it has appeared that the dialysis cell described here is very useful in investigations of this kind. Evidently, the dialysis technique has also other possible applications in the investigation of the chemical nature of the associative relationships existing between micro-organisms, a field in which there are in general very few useful methods available.

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REFERENCES

1. Nurmikko, V. *Ann. Acad. Sci. Fennicae Ser. A* **II** (1954) 54.
2. Nurmikko, V. in *Biochemistry of Nitrogen*, edited by Suomalainen Tiedeakatemia, *Ann. Acad. Sci. Fennicae Ser. A* **II**, 60 (1955) 216.
3. Nurmikko, V. *Acta Chem. Scand.* **6** (1952) 1258.
4. Anderson, A. W. and Elliker, P. R. *J. Dairy Sci.* **36** (1953) 161.
5. Henderson, L. M. and Snell, E. E. *J. Biol. Chem.* **172** (1948) 15.
6. Nurmikko, V. *Suomen Kemistilehti* **B 28** (1955) 62.

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