Field, J., Magoun, H. W., Hall, V. E., Eds., *Am. Physiol. Soc.*, Washington, D. C., 1959, Chap. 20, p507.

5. Biedler, L. M., *Ann. Rev. Psychol.*, 1961, v12, 363.

Received January 21, 1963. P.S.E.B.M., 1963, v112.

Bifidus Factor 2 for Growth of *Lactobacillus bifidus*. (28211)

CATHARINE S. ROSE AND PAUL GYÖRGY
Department of Pediatrics, Philadelphia General Hospital, Philadelphia, Pa.

Lactobacillus bifidus, since its description by Tissier in 1899, has been studied in many laboratories. Raynaud(1) described a factor which is essential for the growth of *Lactobacillus bifidus in vitro* and which stimulates its appearance *in vivo*. This factor he calls bifidus factor 2 to distinguish it from the N-acetylglucosamine-containing substances required by *Lactobacillus bifidus* var. *pennsylvanicus*, György *et al.*(2), which he calls bifidus factor 1. Bifidus factor 2 appeared to be a peptide but differed from strepogenin or the "supplementary" factor of György and Rose(3). Through the kindness of Professor Raynaud, his strain, which has been maintained continuously since its isolation by Tissier in 1901, was made available to us so that we might compare its growth requirements with those of our strains under our laboratory conditions.

Materials and methods. The general techniques for carrying the cultures of *Lactobacillus bifidus* and for microbiological assay have been described(2,3). Our strains have been maintained for many years on a semisynthetic medium containing enzymatically hydrolyzed casein (NZCase, Sheffield). Initial trials indicated that this medium would not support growth of the "Tissier" strain of *L. bifidus* received from Professor Raynaud. In Raynaud's laboratory *L. bifidus* is maintained on Blaurock medium(1). With Blaurock medium we also obtained excellent growth of the strain. Morphologically it was typical *L. bifidus*. In carrying out microbiological assays for bifidus factor 2, two media were used: our regular NZCase medium, with modifications indicated by the requirements of the Tissier strain, and the "Garches" medium of Raynaud(1) which we will refer to as "Raynaud" medium. The latter is simpler in constitution than the NZCase medium. As a major source of nitrogen it contains a highly purified casein hydrolyzate, vitamin-free Casitone (Difco), since Raynaud found that almost all other casein preparations, including NZCase peptone, contained enough bifidus factor 2 to make them unsuitable for use in an assay medium. Several of the substances found to be active as bifidus factor 2 were analyzed for peptide and purine constitution by means of paper chromatography. Butanol-acetic acid was used as solvent system: peptides were detected by means of ninhydrin, purine bases with bromphenol blue-silver nitrate(4).

Results. Neither the NZCase medium nor the Raynaud medium alone supported growth of the Tissier strain of *L. bifidus*. With Raynaud medium a small amount of acid was produced after 48 to 72 hours incubation but it never exceeded about 2 ml of 0.1 N acid per 10 ml tube, even with a heavy inoculum. When this medium was supplemented with the crude amylase preparation from *Aspergillus oryzae* which Raynaud had found to be particularly active as bifidus factor 2 or with tryptose (Difco) there was growth in proportion to the amount of supplement added up to about 6 ml of 0.1 N acid after 48 to 72 hours incubation. NZCase peptone had much smaller activity as bifidus factor 2. NZCase medium would not support growth even when supplemented with amylase or tryptose. Additional supplementation with those components of the Raynaud medium which were not present in NZCase medium likewise failed to make it adequate for maintenance of the Tissier strain.

Among the components of the NZCase

medium not present in the Raynaud medium were adenine sulfate, guanine hydrochloride and xanthine. When this group of purine bases was omitted from the medium there was some acid production, 0.7 ml, as compared with none in the complete medium. Supplementation of the medium without purines with 50 mg of tryptose increased acid production to 4.1 ml. Addition of 50 mg of NZCase peptone beyond the amount present in the basal medium brought acid production to only 1.6 ml. Addition of the purine bases to Raynaud medium supplemented with tryptose caused marked reduction in acid production, confirming their inhibitory effect on the Tissier strain. Most of the later experiments on the relationship of the purine bases to the growth of this strain were carried out on Raynaud medium for better comparison with the work of Raynaud.

Of the 3 purine bases, adenine, guanine and xanthine, adenine was found to be the most toxic, as little as 30 μg completely overcoming the growth-promoting effect of 25 mg of tryptose. There was a competitive relationship between the two factors, the effect of a given amount of adenine being overcome by raising the level of tryptose. Guanine was about one-third, and xanthine about one-tenth as active as adenine. Adenosine and adenosine monophosphate were less toxic than adenine and 3′,5′-cyclic-AMP was almost inactive. Uracil, also a component of NZCase medium, had no inhibitory effect nor did thymine, orotic acid, uridine, thymidine, UMP, CMP, DNA or RNA. Neither did any of these substances have any effect in reversing the inhibitory effect of adenine. Magasanik(5) studied adenine inhibition in a number of microorganisms and found that its effects might be reversed by one or more of the following substances depending on the organism: succinic acid, histidine, thiamine, pantothenic acid. None of these substances had any effect in the present case.

In contrast to the other purine bases, hypoxanthine had no inhibitory effect; rather, it stimulated growth not only when added with tryptose, but when added alone to the basal Raynaud medium. Furthermore, it reversed the inhibitory effect of adenine. A typical experiment is shown in Table I. Inosinic acid could not substitute for hypoxanthine, nor could 5-amino-4-imidazolecarboxamide. Paper chromatographic analysis of the 2 active growth materials, tryptose and the amylase from *Aspergillus oryzae,* indicated that they contained hypoxanthine. However, hypoxanthine could account only in part for the growth-promoting effect of these mixtures and the importance of a peptide factor was indicated. With even high levels of hypoxanthine, production of acid did not reach the levels attainable with tryptose (Table I). NZCase peptone, very slightly active alone, added with hypoxanthine caused growth well in excess of that induced by hypoxanthine.

TABLE I. Effect of Hypoxanthine and Adenine On Growth of *L. bifidus* (Tissier).

Supplements to basal medium	Acid production (ml of 0.1 N) Adenine (μg) 0	10	25	50	100
None	2.2				
Tryptose 2.5 mg	4.7				
7.5 "	5.0	.1	0	0	0
25.0 "	6.2				
Hypoxanthine 20 μg	4.3				
50 "	4.7				
200 "	4.7				
Tryptose 7.5 mg + hypoxanthine 20 μg	7.4		.2	0	
50 "	8.1		4.7	1.1	1.0
100 "	8.1		6.8	5.9	3.8
200 "	8.4			7.7	7.1

All figures are in terms of 10 ml culture. Period of incubation 48 hr.

Skimmed human or cow's milk, both of which are active as "supplementary" factor for our strains(3), did not consistently stimulate growth of *Lactobacillus bifidus* (Tissier) when added to the Raynaud medium. Various experiments undertaken to resolve the contradictory results proved that milk did contain a growth-stimulating fraction. The inhibitory effects were in large part eliminated when the milk was deproteinized with trichloroacetic acid with subsequent removal of the trichloroacetic acid with ether. When the deproteinized preparation was dialyzed, the major part of the activity appeared in the dialyzate. An additional finding in connection with the tests on milk was the ex-

treme sensitivity of the Tissier strain of *Lactobacillus bifidus* to the "Tweens." Tween 80 (polyoxyethylene derivative of sorbitan monoleate) serves as a non-toxic source of oleic acid to certain strains of *L. bifidus* and both Tween 80 and Tween 60 (polyoxyethylene derivative of sorbitan monostearate) eliminate the toxic effect of the traces of free fatty acid which may be present in skimmed milk(6). Both of these surface-active agents proved to be very toxic to the Tissier strain. Acacia was substituted as a non-toxic agent to absorb free fatty acid.

Discussion. Raynaud(1) has discussed the various factors of peptide nature which have been reported to be essential for, or stimulatory to, the growth of *Lactobacillus bifidus*. He has indicated that his factor 2 is a specific peptide different from strepogenin. Most of the strains which we have isolated did not have an absolute requirement for such a factor. Those which did have such a requirement tended to mutate or to be overgrown by other less exacting strains. The Tissier strain has maintained its specific properties after continuous transfer for over a year.

A most interesting property of the Tissier strain is its specific requirement for hypoxanthine, and the inhibitory effects of adenine, guanine and xanthine. This characteristic was not observed by Raynaud who tested hypoxanthine among a large number of compounds which proved to inactive. It is not a chance mutation since it was observed in 3 lyophilized cultures received from Professor Raynaud, of which 2 were tested within a few days after transfer to Blaurock medium. Neither was this requirement a response which could be related to our method of preparation of the Blaurock medium. Stools of infants receiving human milk were plated on Blaurock medium. The strains of *Lactobacillus bifidus* isolated grew well in the purine-containing NZCase medium.

The growth of the Tissier organism with such preparations as peptone or the amylase from *Aspergillus oryzae,* which allow acid production far in excess of the maximum attainable with hypoxanthine, indicates that they contain an additional growth factor. NZCase peptone, which alone had almost no effect, promoted growth when it was added with hypoxanthine. It is most probable that this second factor is of the peptide nature suggested by Raynaud and previously reported to be essential for or stimulatory to the growth of many strains of *L. bifidus*(1).

Our isolation and carrying medium contains a number of components known from the work of Hassinen *et al.*(7) not to be essential. The purine bases, adenine, guanine and xanthine are among these. Their presence has precluded the possibility of isolating a strain comparable to the Tissier organism.

Inhibition of the growth of microorganisms by adenine has been reported previously. Magasanik(5) found that adenine was inhibitory to wild strains as well as to purineless mutants of *A. aerogenes, E. coli* and *S. typhimurium*. Reversal of this inhibition by histidine and thiamine indicates metabolic relationships which have not yet been elucidated. Pierce and Loring(8) observed inhibition by adenosine (but not by adenine) of the utilization of pyrimidine ribonucleotides and ribonucleosides by pyrimidineless mutants of *Neurospora*. Fries(9) reported that guanineless mutants of *Ophiostoma* were inhibited by adenine and hypoxanthine. Nelson and White(10) found that poor growth of a strain of *Streptococcus pyogene* could be overcome by omission of adenine from the medium or by addition of hypoxanthine, xanthine, guanine, or their nucleotides, or by yeast nucleic acid. Precise explanation for these effects is lacking.

Summary. Bifidus factor 2 of Raynaud(1) is required for growth of a strain of *Lactobacillus bifidus* maintained by serial transfer since its isolation by Tissier. This factor is of dual nature; it contains a) a peptide-like component and b) hypoxanthine. Growth is inhibited by adenine and, to a lesser extent, by guanine and xanthine. This inhibition is counteracted by hypoxanthine, which moreover will promote growth in the absence of adenine, guanine or xanthine.

The authors wish to express their appreciation to Prof. Marcel Raynaud, Institut Pasteur, Garches, for his kindness in furnishing lyophylized cultures of

Lactobacillus bifidus and samples of bifidus factor 2.

1. Raynaud, M., *La Semaine des Hôpitaux (Ann. de Pediat.)*, 1959, v35, 240P.
2. György, P., Norris, R. F., Rose, C. S., *Arch. Biochem. Biophys.*, 1954, v48, 193.
3. György, P., Rose, C. S., *J. Bact.*, 1955, v69, 483.
4. Smith, I., *Chromatographic Techniques*, Interscience Publishers, Inc., New York, 1958.
5. Magasanik, B., *Ann. Rev. Microbiol.*, 1957, v11, 221.
6. Tomarelli, R. M., Norris, R. F., Rose, C. S., György, P., *J. Biol. Chem.*, 1950, v187, 197.
7. Hassinen, J. B., Durbin, G. T., Tomarelli, R. M., Bernhart, F. W., *J. Bact.*, 1951, v62, 771.
8. Pierce, J. G., Loring, H. S., *J. Biol. Chem.*, 1948, v176, 1131.
9. Fries, N., *ibid.*, 1953, v200, 325.
10. Nelson, J. R., White, A. C. G., Proc. Soc. Exp. Biol. and Med., 1961, v107, 242.

Received November 26, 1962. P.S.E.B.M., 1963, v112.

Effect of Poliomyelitis Virus on Mitochondria Phospholipids Of the HeLa Cell.* (28212)

Shuang-Shine Tsao, W. E. Cornatzer and Robert G. Fischer
Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of North Dakota Medical School, Grand Forks

The uptake of radiophosphorus by HeLa cells exposed to the poliomyelitis virus was accelerated into the nucleic acid and total phospholipid fractions(1). Increased phospholipid synthesis has been observed to occur, associated with a number of other viral infections: skin of rabbits infected with Shope virus(2), chicken skin infected with fowlpox(3,4), mouse brain as a consequence of infection with Theiler's GD VII virus(5), and KB cells with adenovirus(6).

The total phospholipids of the HeLa cell after poliomyelitis virus exposure have been fractionated by chromatography in an attempt to investigate which of these fractions are involved during lipid phosphorylation (7). In the present investigation an attempt has been made to separate mitochondria of HeLa cells after poliomyelitis infection and fractionate the phospholipids by chromatography to ascertain if these lipids of this cellular fraction are involved in this process.

Materials and methods. HeLa cells were grown in growth medium in 200 ml culture flasks, in a stationary state at 36° according to the procedure of Syverton *et al.*(8). After 14 days, the growth medium was removed and cells rinsed 3 times with modified Hanks' solution(8) containing 0.1% glucose, 0.8% NaCl and 0.04% KCl. Fifteen minutes before virus inoculation, the growth medium was discarded and the HeLa cells were rinsed 3 times with 10 ml aliquots of modified Hanks' solution(8). The culture flasks to be inoculated with the virus received 8 ml of maintenance medium(8), and controls 9 ml. The maintenance medium consisted of 90 parts of maintenance solution(8) and 10 parts of chicken serum. Cultures inoculated with virus received, in addition to the maintenance medium, 1.0 ml of poliomyelitis virus† ($10^{7.42}$ $TCID_{50}$) ($TCID_{50}$ = the dilution of the virus just sufficient to kill 50% of the 5-day-old cultures in the test). Culture flasks were incubated for 4 hours. Thirty minutes before the cells were harvested 1.0 ml $NaH_2{}^{32}PO_4$ containing 80 μc was added. This time interval corresponds to the ascending part of the specific activity time curve, where synthesis of phospholipids occurs in the HeLa cell greater than breakdown(9).

At the time of harvesting the cells were scraped from the wall of 10 culture flasks with a rubber policeman and pooled and mixed in modified Hanks' solution(8), and an aliquot was removed for cell counting. The remainder of the cell suspension was centrifuged in the cold at 2000 × *g* for 3 minutes. The supernatant fluid was poured off and cells were homogenized in a Potter-

* Supported by grant from Nat. Inst. Health.

† Type I (Mahoney strain).