Mémoires de l'Académie Royale des Sciences et des Lettres de Danemark, Copenhague.
Section des Sciences, 9me série, t. VI, nº 5.

THE VITAMIN AND NITROGEN REQUIREMENTS OF THE LACTIC ACID BACTERIA

BY

S. ORLA-JENSEN
N. C. OTTE AND AGNETE SNOG-KJÆR

D. KGL. DANSKE VIDENSK. SELSK. SKRIFTER, NATURV. OG MATH. AFD., 9. RÆKKE, VI. 5.

KØBENHAVN
LEVIN & MUNKSGAARD
EJNAR MUNKSGAARD
1936

Printed in Denmark.
Bianco Lunos Bogtrykkeri A/S.

CONTENTS

The Vitamin Requirements of the Lactic Acid Bacteria.

Physiology has entered a new epoch by the discovery that life processes are very much dependent on activating and regulating catalysts. Simple metal salts in hardly detectable amounts may play a part or complex compounds called coenzymes, hormones or vitamins. These phenomena have been observed not only in the case of higher organisms, but it has also been shown that certain species of yeasts do not grow in pure synthetic media containing salts of ammonia as the only source of nitrogen unless a trace of an activator has been added, the so-called Bios. However, the amount of bios needed is so scanty, that if the medium be inoculated with a normal size inoculum, a sufficient quantity of bios will be introduced at the same time. According to the work of WILLIAMS and SANDERS[1] bios of yeast consists of three substances: inositol, vitamin B_1 and a thermostable acid of undetermined constitution, which they named "pantothenic acid". The latter constituent seems to be the most significant.

It is well known that the nutrient requirements of bacteria vary greatly from one species to another. The one extreme is represented by the autotrophic bacteria, which are satisfied with inorganic food alone, similar to green plants. Passing through species which occupy an intermediate position and are capable of utilizing nitrogen from an inorganic source, but need organic carbon compounds, the other extreme is reached in types that require both nitrogen and carbon in organic form. Some species belonging to the latter group seem to be even more particular in their food requirements than higher animals, e. g. such bacteria, as have been grown successfully on certain natural media only. The fact that a bacterium will develop in a certain natural medium only, has formerly been explained by assuming that the bacterium needed just this particular food as such. However, thinking in terms of activators one may ask whether the presence of an activator may be involved. The presence or absence of activators may possibly play an important part in the problems relating to filterable viruses and bacteriophagous activity.

We have tried to solve this problem in the case of the true lactic acid bacteria. They are apparently as fastidious in their nitrogen requirements as are the higher

[1] WILLIAMS and SANDERS, Biochem. Journ. 28 (1934) p. 1887.

animals, and they have been found to give a lively response, when small amounts of activating compounds are added. Thus in some of our earlier works it has been shown that a large number of lactic acid bacteria will not ferment carbohydrates without the presence of certain aldehyde-like activators. These are normally formed, when the carbohydrate is sterilized together with the nitrogeneous compounds[1]. In other experiments we have shown that the growth in milk of some lactic acid bacteria is increased by the addition of $^1/_{400}$ per cent autolysed yeast[2]. On the other hand, certain lactic acid bacteria grow well in pure yeast extract, but still better when milk is added[3]. This indicates that in some cases yeast extract may supply the activators not found in milk and vice-versa.

In connection with this it is of special interest to investigate why many lactic acid bacteria thrive so well on cow's milk. It may be because its nutrients as such are particularly well suited for these bacteria; or because it contains some activators; or finally, what is most likely, for both reasons. It seems natural to discuss the vitamins of milk as possible activators. The fat-soluble vitamins may be eliminated from further consideration, as the lactic acid bacteria grow just as well in skimmilk as in full milk. Of the water-soluble vitamins vitamin C may be disregarded, because the lactic acid bacteria as a whole thrive just as well in milk which has been heated considerably as they do in raw milk. The *Streptococci*, to be sure, prefer milk that has not been heated too much, but on the contrary many rodshaped bacteria are encouraged in their growth by vigorous sterilization of the milk. This is caused by the formation of aldehyde-like compounds, as mentioned above; these compounds may influence the oxidation-reduction potential in a favourable way. The conclusion must be that among the vitamins occurring in milk only the vitamins of the B group may be of significance in this connection. Vitamin B_2 is much more heat-resistant than vitamin B_1. R. Kuhn[4] has described B_2 as an orange flavin with a greenish fluorescence, giving to both whey and raw white of egg their characteristic colour. Other workers have characterized vitamin B_2 as the antipellagra vitamin. However, the flavins alone do not seem to counteract or cure pellagra; thus vitamin B_2 must contain still another component called vitamin B_6 which is alkali-fast and possibly identical with the main part of bios, namely the pantothenic acid which is not very sensitive to alkali either.

Since vitamin B_1 and lactoflavin as well as other water soluble vitamins (except B_6 or bios) in contradistinction from fat soluble ones are sensitive to alkalies, it seemed natural to try whether milk would become a poorer nutrient medium for

[1] S. Orla-Jensen, Die Abhängigkeit der Milchsäuregärung von der Art und Weise, in welcher die Sterilisierung der Nährböden ausgeführt wird, IX^e congrès international de laiterie, Copenhague 1931, Compte-rendu, 175—195.

S. Orla-Jensen, Hitherto unknown activators for the growth of lactic acid bacteria. Jour. Soc. Chem. Ind. **52** (1933), 374.

[2] S. Orla-Jensen and J. Jacobsen, Neue Untersuchungen über die baktericiden Eigenschaften der Milch, Zentralbl. für Bakteriol., II Abt., **80** (1930), 326.

[3] S. Orla-Jensen, A. D. Orla-Jensen and B. Spur, The butter aroma bacteria, Jour. Bact. **12** (1926), 333.

[4] Richard Kuhn. IX International Chem. Congress, Madrid 1934.

the lactic acid bacteria after having been heated at alkaline reaction. The procedure was as follows. The milk was brought to pH 8 and then heated 45 minutes at 100° C. The pH gradually decreased by this treatment and the milk had to be adjusted to pH 8 once again. Then it was sterilized at 115° C for 15 minutes, whereafter the temperature was brought up to 120° C only for a moment. This time, too, acid had been formed by the heat treatment, and the milk showed a pH of 6.8. It was brought to 6.5 by the addition of a 0.1 per cent cysteine hydrochloride or the corresponding quantity of hydrochloric acid, 6.5 being the pH which the milk showed after sterilization without the addition of alkali. Cysteine was added because it was presumed that especially this compound would be destroyed when heated with alkali. The milk thus treated was tested by means of various *Thermobacteria*. The figures in Table I represent parts per mille of lactic acid formed after 2 days.

Table I.

Milk		Species of bacteria				
Treated with alkali	Cysteine added	*Tbm. intestinale*[1] 314	*Tbm. lactis* 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Tbm. Jugurt*
yes	no	1.6	5.0	14.2	15.8	16.2
yes	yes	2.7	8.3	14.2	18.5	18.2
no	no	5.0	12.2	16.4	18.9	21.8

This shows that milk heated at alkaline reaction becomes a poorer nutrient medium for lactic acid bacteria, yet the effect produced varies very much from one species to another. In the case of the first two species it is considerable, and the milk cannot be regenerated by the addition of cysteine, while the action as far as *Thermobacterium helveticum* is concerned is small and can be almost completely counteracted by the addition of cysteine. Earlier experiments have taught us that the addition of cysteine to milk which was not treated with alkali gave practically no results. The treatment described here gave the milk a red-brown colour. Still more rigid treatment by means of alkali and heat changed it to a blackish brown and then it was indeed an extremely poor nutrient medium for the lactic acid bacteria. This could, however, not be taken as valid proof of the presence of alkali-sensitive activators significant for the lactic acid bacteria, because the milk had at the same time undergone profound chemical changes.

In order to remove vitamin B_1 and lactoflavin another mode of attack was consequently attempted. Shaking with Fuller's earth or with activated charcoal (norite) was tried out. The two substances adsorb both vitamin B_1 and lactoflavin. Vitamin B_1 is adsorbed predominantly at neutral reaction and may later be extracted with hydrochloric acid. Lactoflavin, on the contrary, is most successfully adsorbed at a strong acid reaction and should be extracted with pyridine. Norite charcoal was

[1] The name *Thermobacterium intestinale* is used instead of *Bacterium acidophilus* in accordance with the work of S. Orla-Jensen, Anna D. Orla-Jensen and O. Winther, Zentralblatt für Bakteriologie II Abt. 1935, Bd. 93, Heft 18—22.

preferred as Fuller's earth was not without effect on the pH of the milk but made it slightly alkaline, especially after heating. The adsorption was carried out at the normal reaction of the milk, because it would have curdled, if acid had been added.

20 g norite were used to each liter of skimmilk. In order to complete the adsorption the milk was agitated with the carbon for one hour by an electric stirrer. It was not possible to filter the norite charcoal from the milk as the particles are very fine, some of the size of ordinary bacteria. However, they could be separated off by a powerful centrifuge. For this purpose Heine's bacterial centrifuge was used with 6 containers of stainless steel, each taking 750 cc, and thus able to handle a little more than four liters at a time. After the maximum velocity had been reached, 3600 revolutions per minute, the centrifuge was kept at this speed for 15 minutes. Each lot of milk was treated twice with carbon so as to make the adsorption as complete as possible. After the second portion of charcoal was distributed in the milk, the mixture was heated to 100° C in streaming steam to remove the air layer round the minute carbon particles, thus increasing the intersurface between carbon and milk and also facilitating the centrifuging. The destruction of bacteria at the same time by this steaming was desirable since the complete procedure took considerable time. While the number of bacteria was greatly reduced by the first centrifuging, it increased rapidly again after the second one, unless the milk had been pasteurized beforehand.

Table II shows that the technic described here has actually changed the milk into an unsuitable medium for the rodshaped as well as for the coccoid lactic acid bacteria. The figures in this table and in the following indicate how much lactic acid was formed as calculated in parts per thousand, the lower row of figures giving the number of hours of incubation. The milk inoculated was always incubated at the optimal temperature for the species concerned, 37° C for the *Thermobacteria* and for *Streptococcus thermophilus* and 30° C for all the other lactic acid bacteria.

Now, what is removed from the milk by the treatment with norite charcoal? An analysis showed that no nutrient of importance was removed. The nitrogen content

Table II.

Milk	Species of bacteria													
	Tbm. lactis 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Sbm. casei* 11		*Sc. thermophilus* 7	*Sc. cremoris* 18		*Sc. cremoris* 215		*Sc. lactis* 22		*Sc. lactis* 33	
Not treated with charcoal.....	14.7	17.3	21.6	5.4	6.8	6.2	5.4	6.0	5.4	6.3	5.4	5.9	5.0	5.9
Treated with charcoal.....	1.1	2.6	0.5	0.7	0.8	1.5	0.7	0.8	0.7	0.8	0.2	0.2	0.7	0.9
Number of hours before titration	50	50	50	120	175	50	50	74	50	74	74	98	50	74

only falls 0.03 to 0.04 per cent, partly because the thorough centrifuging eliminates more of the suspended larger particles such as leucocytes, bacteria, etc. than the first centrifuging (carried out when the cream was being separated) has removed. Although the B vitamins contain about the same amount of nitrogen as do proteins, namely, about 15 per cent, their total mass in milk is so minute, that their removal cannot possibly influence the nitrogen percentage in the second decimal. As the milk is treated with charcoal at pH 6.5, it is natural to assume that it is predominantly vitamin B_1 which is adsorbed. However, experiments carried out with whey at the same reaction showed that charcoal adsorbed the larger part of lactoflavin also, the whey becoming completely decolorized. It is in fact preferable to start with clear whey instead of milk when preparing activating substances, because in that case the adsorption can be effectuated at any hydrogen ion concentration desired, and the charcoal may be separated off by simple filtration. Curiously enough the hydrogen ion concentration at which the adsorption was carried out has but a slight influence on the ability of the extracts to reactivate the milk. This might indicate that the most important constituent in the extract is neither vitamin B_1, nor lactoflavin. On the other hand, it must be emphasized that more active extracts are prepared when the charcoal is extracted with pyridine than with hydrochloric acid. This will be described later in greater detail (Tables VIa and VIb). This indicates, that if one of the vitamins here considered has an influence upon lactic acid bacteria it is probably lactoflavin rather than vitamin B_1.

The method used for preparing the extracts which can reactivate the carbon-treated milk was as follows. The charcoal used for the adsoption was washed three times with water and then stirred for one hour in equal parts of pyridine and methyl alcohol. The liquid was filtered off and the charcoal stirred for another hour using the same mixture diluted with an equal amount of water. After filtration the two yellow-greenish filtrates were united and adjusted with hydrochloric acid to pH 6. The solution was concentrated in vacuum at 40—55° C, by which procedure the pyridine and the methyl alcohol were removed at the same time. It was necessary to add a little water at intervals and to carry out the evaporation in the dark. Impurities which separated out were eliminated by filtration. The extract still had a strong odour of pyridine. The last trace of pyridine could only be removed by repeated evaporation in vacuum after addition of water, but such a treatment weakens the activating materials present. Fortunately it is unnecessary for our purpose, as the lactic acid bacteria are not inhibited in their activity before a concentration of 0.5 per cent of pyridine is reached, some species even not before 1 per cent. It is difficult to estimate how much of the activating compounds has been lost with the technic used, but if we approximate by calculating as if no loss had taken place, the treated milk used in the experiment of Table III should be reactivated by 0.75 per cent of the concentrated extract. Further experiments to illustrate this point were carried out with 0.5 and 1 per cent extract.

Table III shows that the milk treated with charcoal becomes a very much better nutrient medium for the lactic acid bacteria after the addition of the extract. 1 per cent

Table III.

Medium	Added per cent of the eluate	Treated with norite charcoal	Species of bacteria													
			Tbm. intestinale 314		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*		*Sc. cremoris* 18		*Sc. cremoris* 215		*Sc. lactis* 33	
Milk	0	yes	1.6	3.2	0.7	2.7	2.0	3.4	0.7	4.1	0.7	1.1	0.9	1.4	1.4	2.3
	0.5	„	3.2	6.8	2.9	10.1	9.7	14.0	3.8	11.3	2.5	4.7	3.4	6.3	2.9	5.2
	1	„	4.7	7.9	3.4	12.8	11.7	14.9	5.6	14.2	3.4	6.1	5.4	7.0	3.4	5.4
	0	no	5.2	9.7	7.2	15.3	11.3	18.0	12.8	21.4	4.3	7.4	6.5	7.2	4.3	6.3
Whey	0	yes	0.1	0.1	0.1	0.2	0.7	1.1	0.1	0.5	0.2	0.5	0.1	0.2	0.2	0.5
	2	„	0.5	0.5	0.3	0.7	2.5	3.8	0.9	2.9	0.9	2.0	0.9	1.4	1.1	1.6
	0	no	0.9	1.8	0.9	0.9	5.2	8.8	0.7	2.7	0.9	2.0	1.6	1.8	2.5	4.3
Number of hours before titration			48	96	24	96	24	96	24	96	24	96	48	96	24	96

is practically sufficient to reach the same amount of acid in carbon-treated milk as in the original milk as far as the *Streptococci* are concerned, but for the rods, especially for *Thermobacterium helveticum*, this amount is not sufficient to regenerate the carbon-treated milk. The data concerning whey in the latter part of the tables will be discussed in a following paper.

Using the methods described in the literature, we attempted to produce lactoflavin free from vitamin B_1, and vitamin B_1 free from lactoflavin. Preparations were made from both whey and autolyzed yeast. It was constantly found that the preparations of vitamin B_2 (containing lactoflavin) were considerably more activating for the lactic acid bacteria than those of vitamin B_1 alone. The most clear-cut experiments were obtained with preparations which were presented by the courtesy of Ferrosan Ltd. (Copenhagen) and controlled by Dr. Krieger Lassen by experiments on rats. The approximate strengths were given as follows:

> Vitamin B_1, free from vitamin B_2, one gram = 20 curative doses for rats, corresponding to about 100 γ pure vitamin. Vitamin B_2, free from vitamin B_1, one gram = 1.25 curative doses for rats, corresponding to about 12.5 γ pure vitamin.

The preparations were made from yeast. It is to be regretted that in addition they contained so much nitrogeneous food, that it cannot be left out of consideration in the series of experiments below. The preparation of vitamin B_1 was found to have 1.29 per cent nitrogen and the vitamin B_2 preparation 2.88 per cent nitrogen. Thus an addition of 4 per cent of the B_2 preparation increased the N content by 0.115 per cent. The preparations showed acid reaction and had to be neutralized before they could be mixed with the milk. The B_1 preparations could not be used in doses larger than one per cent since otherwise they coagulated the milk during sterilization. This

process was carried out by heating to 115° C for one quarter of an hour, a procedure which does not impair the activities of either B_1 or B_2. A part of each of the preparations was treated with alkali as was the milk of Table I, in order to decide whether any of the preparations contained alkali-resistent constituents, that is to say, activators related to bios. The media were titrated after 5 days.

Table IV.

Milk	Per cent preparation added	Tenth per cent N added with the preparation	γ Vitamin added per 100 cm^3 milk	Preparation treated with alkali	Species af bacteria						
					Tbm. lactis 10	*Tbm. helveticum* 12	*Sbm. casei* 11	*Sc. thermophilus* 7	*Sc. cremoris* 18	*Sc. cremoris* 215	*Sc. lactis* 22
Carbon-treated	0	0	0	no	0.2	0.6	0.3	0.2	0.5	0.2	0.2
„ „	0.5 B_1	0.07	50	„	0.8	3.2	2.5	3.8	2.9	3.0	2.3
„ „	1.0 „	0.13	100	„	1.0	5.0	3.0	4.2	4.3	3.0	2.6
„ „	1.0 „	„	?	yes	0.8	5.0	3.0	4.2	3.4	1.4	2.6
„ „	0.5 B_2	0.14	6.3	no	1.6	6.3	7.9	6.8	6.3	5.0	5.4
„ „	1.0 „	0.29	12.5	„	3.4	8.1	9.9	7.2	7.0	5.9	6.5
„ „	2.0 „	0.58	25	„	3.6	11.9	13.1	7.9	7.6	6.8	6.8
„ „	4.0 „	1.15	50	„	12.1	20.0	13.3	9.1	7.6	7.6	7.4
„ „	4.0 „	„	?	yes	7.2	12.8	11.3	9.5	8.3	8.1	8.1
Not-carbon-treated	0	0	0		13.8	21.2	9.5	6.9	7.3	6.1	7.1

0.5 per cent of B_1 preparation introduced the same amount of vitamin as did 4 per cent of B_2. The former had but a slight effect while the latter reactivated the carbon-treated milk completely. Hence everything seems to indicate that vitamin B_2 is of much more importance for the lactic acid bacteria than vitamin B_1. The favourable influence was not caused merely by the larger nitrogen content of the B_2 preparation, because 0.5 per cent of B_2 already acted better than 1 per cent of B_1, which gave both the same percentage of nitrogen. As bios is found in yeast, the possibility exists that the activating substance was neither B_1, nor B_2, but bios. That the activating power of our preparation was not destroyed by alkali treatment speaks very much for this assumption. A decrease was only noted for *Streptococcus cremoris* No. 215 as far as the B_1-preparation was concerned (this organism being very sensitive in many respects), but it must be admitted that a considerable decrease was found for many rod-shaped bacteria in the case of B_2, but strange to say all *Streptococci*, even *Streptococcus cremoris* No. 215 were rather activated by the alkali treatment. We must conclude that the slight activation produced by the B_1-preparation was caused by a substance related to bios rather than vitamin B_1, while the activating power of of the B_2-preparation in the case of the rods was not only caused by a substance related to bios but also by a not-alkali-resistent substance. The further consequence is, then, that vitamin B_1 does not play any part at all for the lactic acid bacteria; but bios

is indispensable for the whole group of these bacteria and the rod forms need an additional substance, probably lactoflavin.

But it is a question whether milk really contains bios and whether the earlier experiments will agree with this new theory. The fact that milk may be inactivated by means of charcoal does not cause any difficulty, because bios is adsorbed just as well by activated charcoal as are vitamin B_1 and vitamin B_2. Furthermore the finding that the lactic acid bacteria are not particularly sensitive to such changes in milk as are caused by the treatment with alkali (see Table I), seems to indicate that at least some of the activating constituents in milk must be fairly stable to heating with alkali.

In order to prove that the charcoal extracts from milk would encourage the growth of yeasts, this extract was tested on eight different species of yeast. None of these would grow in a pure synthetic medium when a small amount of inoculum was used. The series of yeasts used follows:

1. Brewer's yeast	Munich type.
2. — —	Carlsberg bottom yeast No. 2.
3. Wine yeast	Port.
4. — —	Chateau Margaux.
5. Distillery yeast	Rum, Cuba.
6. *Schizosaccharomyces mellacei*,	forming formic acid, important in the manufacture of rum.
7. Apiculatus yeast (lemon-shaped)	*Saccharomyces apiculatus*, Emil Chr. Hansen, non-sporulating.
8. — —	Type isolated from berries of the mountain ash.

These species were inoculated, using a very small amount of inoculum, into a 15 per cent sucrose solution, containing 0.2 per cent primary ammonium phosphate, 0.2 per cent primary potassium phosphate, and 0.1 per cent magnesium sulphate. They were also inoculated into the same solution to which moreover 0.01 per cent nitrogen had been added as autolyzed yeast or as milk bios prepared by means of activated carbon. Milk bios was used, treated as well as untreated with alkali. In the medium to which no activators had been added none of the yeasts tested showed any growth within three weeks. However, growth and fermentation were observed in the sugar solutions containing the activators described. Growth in the medium to which autolyzed yeast had been added was observed for 7 species after 2—3 days, and in the case of untreated bios after 2—5 days. The alkali-treated bios (in which vitamin B_1 is destroyed), did not produce growth before one to two days later than the untreated product. The rum yeast from Cuba behaved in a different manner as it did not produce visible growth until two weeks had passed in the case where autolyzed yeast or bios not-treated with alkali had been added, and it only started to grow after three weeks in the medium with alkali-treated bios. All in all no great differences

seem to exist between the milk bios present in our extract from the carbon and the bios of yeast found in autolyzed yeast[1].

Professor R. KUHN of Heidelberg has kindly supplied us with crystallized lactoflavin prepared from liver. Here we take the opportunity to express our gratitude to Professor Kuhn, to whom we feel very much indebted for making it possible for us to give the final proof of the significance of this compound, as well as for his work on this subject in general, which has had such a great influence on the researches described in this paper.

Experiments with Ferrosan preparations of vitamin have made us conclude that 0.5 mg of vitamin B_2 is sufficient to reactivate one liter of milk treated with charcoal. In later experiments this amount of lactoflavin was constantly used. The charcoal extract, the *eluate*, was usually added in an amount corresponding to about twice the quantity of milk from which it originated, because we lost a great deal during the preparation. It was used both with and without alkali treatment. The data obtained in a series of experiments with lactoflavin and extract from the charcoal separately and in mixture are shown in Tables Va and Vb.

The figures in these tables show that lactoflavin alone is not able to activate the lactic acid bacteria. Yet another factor is needed, namely the bios of milk, which is found in our extract. Combined with bios of milk lactoflavin has a pronounced activating action. It must be admitted that in the majority of cases one does not quite reach the amounts of acid obtained in milk not treated with coal, but in one instance almost double the amount is obtained, namely with *Streptobact. casei*. Making a reservation in the case of the latter and *Thermobact. lactis* No. 10, it may be stated that the charcoal extract as such acts as a strong activator; as far as the *Strepto-*

Table Va.

Milk	Addition	Eluate treated with alkali	Bios	Lactoflavin of the eluate	Pure lactoflavin	Species of bacteria							
						Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*	
Carbon-treated	None					0.2	0.2	0.3	0.3	0.5	0.9	0.5	0.9
„ „	Lactoflavin				+	0.3	0.6	0.3	0.5	0.6	1.1	0.5	0.9
„ „	Eluate	yes	+			5.2	7.7	0.5	1.0	1.8	4.5	2.3	4.5
„ „	„	no	+	+		7.9	9.9	0.5	1.0	5.7	11.0	3.2	9.0
„ „	Lactoflavin + Eluate	yes	+		+	9.2	14.2	1.4	5.9	5.9	13.5	5.2	9.0
„ „	„ + „	no	+	+	+	10.8	16.2	6.3	12.2	10.6	16.6	13.7	20.5
Not-carbon-treated	None					14.9	19.6	10.8	16.2	7.9	16.4	17.3	23.6
Number of hours before titration						46	118	46	118	19	118	46	118

[1] These experiments were carried out with the able assistance of Mr. C. le Dous.

Table V b.

Milk	Addition	Eluate treated with alkali	Bios	Lactoflavin of the eluale	Pure lactoflavin	Species of bacteria							
						Sbm. casei 11		*Sc. thermophilus* 7		*Sc. cremoris* 18		*Sc. lactis* 22	
Carbon-treated	None					0.2	0.2	0.1	0.3	0.3	0.5	0.3	0.5
„ „	Lactoflavin				+	0.1	0.2	0.1	0.2	0.3	0.5	0.2	0.5
„ „	Eluate	yes	+			0.1	0.2	1.6	4.5	4.1	5.9	2.3	3.4
„ „	„	no	+	+		2.3	2.5	2.3	5.2	3.4	5.4	1.6	3.4
„ „	Lactoflavin + Eluate	yes	+		+	0.9	1.1	1.8	5.9	3.6	7.0	3.6	4.7
„ „	„ + „	no	+	+	+	8.6	10.8	2.5	5.9	4.3	7.2	5.0	5.6
Not-carbon-treated	None					4.7	5.6	3.6	6.3	5.4	8.3	6.3	7.2
Number of hours before titration						118	160	23	118	19	118	46	140

cocci are concerned this factor acts almost as strongly as the mixture of extract and lactoflavin[1]. This is in agreement with our previous assumption that the *Streptococci* do not need significant quantities of lactoflavin. This fits in very well with the fact that alkali treatment of the extract, by which procedure the vitamin B_1 and lactoflavin are destroyed, influences the *Streptococci* very little, while its effect is felt in the case of *Thermobacteria* and especially of the *Streptobacteria*.

We have already mentioned that the bios of milk is adsorbed equally well at all hydrogen ion concentrations on the acid side. The extracts, however, become richer

Table VI a.

Milk	Addition	Species of bacteria							
		Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*	
Carbon-treated	None	0	0.5	0.2	0.2	0.2	0.9	0	0.8
„ „	Neutral eluate	1.4	7.9	2.3	2.3	1.0	2.5	0.7	4.7
„ „	Normal „	4.5	10.6	1.6	2.0	4.1	5.6	1.1	5.9
„ „	Acid „	5.0	11.3	0.9	1.5	5.9	9.0	1.4	8.6
„ „	Neutral „ + Lactoflavin	2.3	11.9	6.1	7.7	5.4	13.3	1.8	11.7
„ „	Normal „ + „	6.8	15.1	8.8	10.4	10.6	18.2	5.2	17.1
„ „	Acid „ + „	7.9	14.6	10.6	13.4	11.7	18.0	7.7	18.0
Not-carbon-treated	None	4.1	14.6	11.0	12.7	9.5	16.0	4.5	19.6
Number of hours before titration		18	96	66	96	18	96	18	96

[1] In this connection it must not be forgotten that our extract already contained some lactoflavin. Very probably the pure bios of milk would not activate the rod-shaped lactic acid bacteria more than does pure lactoflavin.

Table VIb.

Milk	Addition	Species of bacteria							
		Sbm. casei 11		*Sc. thermophilus* 7		*Sc. cremoris* 18		*Sc. lactis* 22	
Carbon-treated	None	0	0	0.1	0.7	0	0.6	0.2	0.2
„ „	Neutral eluate	0.6	1.4	3.2	4.3	2.9	6.3	3.8	3.8
„ „	Normal „	1.1	2.5	2.7	5.6	2.9	6.3	3.6	5.4
„ „	Acid „	1.8	2.6	2.5	6.5	2.9	5.0	3.4	3.7
„ „	Neutral „ + Lactoflavin	0.5	1.6	2.9	5.6	3.8	6.8	5.0	5.1
„ „	Normal „ + „	4.1	9.9	2.7	6.8	4.5	7.4	5.9	6.1
„ „	Acid „ + „	5.0	8.0	2.5	6.8	3.4	6.8	5.9	6.3
Not-carbon-treated	None	1.6	3.9	3.2	6.1	4.3	8.1	5.9	6.0
Number of hours before titration		66	96	21	96	18	96	66	96

in lactoflavin the higher the hydrogen ion concentration is at which the adsorption is undertaken if only the subsequent extraction be carried out with pyridine and methyl alcohol. The lactoflavin content is on the other hand low when adsorbed in neutral liquid in which case hydrochloric acid is used for the extraction. This may be plainly seen from Tables VIa and VIb. The titration was carried out after 96 hours.

The last six columns in Table VIb show that as far as the *Streptococci* are concerned the milk treated with charcoal is reactivated equally well by equivalent amounts of the three eluates. It does not make much difference whether the adsorption is carried out at pH 7 or pH 0. The results are quite different in the case of *Thermobacteria* (Table VIa), or *Streptobacteria* (Table VIb, first two columns): the more lactoflavin present in the extracts, the better the growth. Yet the amount of lactoflavin obtained even at the most acid adsorption is too small for *Thermobacterium lactis* 10 and *Streptobacterium casei* 11 to form appreciable quantities of acid. These two organisms, as is plainly shown in Tables Va and Vb, demand relatively large quantities of lactoflavin. Thus the fermentation does not become really vigorous before lactoflavin is added. Under these conditions the difference between the extracts prepared at pH 5.8 (normal eluate) and pH 0 (acid eluate) becomes very indistinct even when tested on rod-shaped bacteria. When sufficient lactoflavin is added the two extracts act equally strongly, which means that they both contain sufficient amounts of bios. The extract prepared by means of hydrochloric acid from the material adsorbed at pH 7 (neutral eluate) is too poor in bios to be fully reactivated. This is particularly pronounced in the case of *Streptobacterium casei* 11.

The evidence presented here that lactoflavin is a necessary factor for the development of the lactic acid rods is in agreement with the work of O. Warburg and W. Christian[1]. These investigators found that *Bacterium Delbrückii* (*Thermo-*

[1] O. Warburg and W. Christian, Über das gelbe Ferment und seine Wirkungen. Biochem. Zeitschr., **266** (1933), 378.

bacterium cereale, Orla-Jensen), contains a particularly large amount of lactoflavin. They further found that lactoflavin, which is the active substance of the yellow pigment, should be considered as the oxygen-carrying ferment for the lactic acid bacteria in anaerobiosis.

The experiments of these investigators and our work lend further support to each other, because of the fact that especially the more anaerobic rod-shaped lactic acid bacteria require much lactoflavin, while the less anaerobic Streptococci seem to be content with the amounts they receive with our preparations of bios.

Other workers have maintained that glutathione is a necessary factor in the lactic acid fermentation. Hence it seems natural to investigate whether lactoflavin could be replaced by glutathione. In addition we took the opportunity of trying out simultaneously whether the lactoflavin preparations with which we had so far worked and which had been prepared from liver would show exactly the same action as one obtained from milk. This latter preparation was also received through the courtesy of Professor R. Kuhn. The bios from milk which was used in these experiments had been adsorbed at pH 5.8, that is to say, the same kind of preparation and the same amount was used as for the experiments of Tables VIa and VIb. However, another lot of milk was used in the experiments the data of which are given in Tables VIIa and VIIb. The difference between the two lots is manifested in a higher acid formation for the rod-shaped lactic acid bacteria in the untreated milk, and also in the carbon-treated milk in the case where bios was the only addition. In contrast to this *Streptococcus lactis* 22 forms very little acid in the milk treated with activated carbon and

Table VIIa.

Milk	Eluate	Other Additions mg/l	Species of bacteria							
			Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*	
Not-carbon-treated			7.9	18.2	9.2	14.4	9.7	16.7	18.2	23.2
Carbon-treated	Bios	+ 1.5 Liver-Lactoflavin	6.5	12.4	6.5	11.9	9.5	15.3	16.0	20.3
„ „	„	+ 0.5 „ „	6.5	12.2	5.4	11.9	9.7	15.5	16.0	18.9
„ „	„	+ 0.5 Milk-Lactoflavin	7.0	13.5	9.2	13.3	9.9	15.5	15.8	19.8
„ „	„	+ 0.5 „ „ (treated with alkali)	5.0	10.4	5.2	8.1	7.9	10.1	8.3	13.5
„ „	„		4.3	9.9	5.2	8.1	7.4	10.6	6.1	9.7
„ „	„	+ 0.5 Glutathione	4.7	10.8	3.4	3.8	5.9	10.1	8.3	13.3
„ „	„	+ 2.0 „	4.3	11.0	2.7	4.1	6.8	9.0	5.6	13.1
„ „	„	+ 200.0 „	5.6	11.0	1.0	3.6	8.6	11.3	7.9	11.9
„ „	„	+ 2000.0 „	5.0	10.8	1.5	4.7	9.0	11.9	9.0	14.0
„ „		200.0 „	0.2	1.6	0.9	1.4	0.7	2.0	1.1	2.0
„ „		2.0 „	0.1	0.9	0.6	0.9	0.7	1.8	0.6	1.4
„ „			0	0.2	0.3	0.7	0.3	0.9	0.9	1.1
Number of hours before titration			18	90	42	90	18	90	42	90

Table VII b.

Milk	Eluate	Other Additions mg/l	Species of bacteria							
			Sbm. casei 11		*Sc. thermophilus* 7		*Sc. cremoris* 18		*Sc. lactis* 22	
Not-carbon-treated			7.0	13.5	5.6	6.1	6.8	7.9	5.6	6.3
Carbon-treated	Bios	+ 1.5 Liver-Lactoflavin	7.2	14.2	4.7	5.6	5.9	6.8	4.3	5.0
„ „	„	+ 0.5 „ „	7.4	14.2	5.0	5.6	5.9	6.8	4.3	5.2
„ „	„	+ 0.5 Milk- „	7.9	14.6	4.7	5.6	6.1	6.8	4.5	5.2
„ „	„	+ 0.5 „ „ (treated with alkali)	3.6	6.5	4.7	5.4	5.2	5.4	1.8	2.5
„ „	„		3.6	6.8	4.5	5.0	4.5	5.4	1.1	1.6
„ „	„	+ 0.5 Glutathione	3.8	7.0	5.4	6.1	5.4	5.6	2.7	2.9
„ „	„	+ 2.0 „	3.4	6.5	5.0	5.9	5.4	6.8	2.3	2.9
„ „	„	+ 200.0 „	5.0	8.8	5.6	6.1	5.4	6.5	2.9	3.8
„ „	„	+ 2000.0 „	5.4	9.0	5.0	6.1	4.5	6.3	3.2	4.7
„ „		200.0 „	1.6	3.8	1.6	2.7	0.2	0.6	0.7	1.1
„ „		2.0 „	1.4	2.9	1.4	1.8	0.2	0.7	0.2	0.7
„ „			0.9	2.3	1.1	1.8	0.1	0.7	0	0.1
Number of hours before titration			65	118	42	90	42	90	42	90

with bios added and thus appears to require lactoflavin to the same extent as the rod-shaped bacteria. This behaviour is difficult to explain, but it indicates that the *Streptococci* also need lactoflavin and that the difference in this respect so far observed is more of quantitative than of qualitative nature. If we treat with alkali the lactoflavin to be added, which has also been tried out in this connection, as low figures are generally obtained as if bios alone had been added, showing that the alkali treatment used is sufficient to destroy the lactoflavin. Yet it has not become quite inactivated as the experiment with *Thermobacterium helveticum* shows.

Tables VII a and VII b show that the amount of lactoflavin: 0.5 mg per liter, used in the previous experiments is sufficient, since we do not obtain higher concentrations of acid with three times the amount, namely 1.5 mg per liter. On the other hand, the lactoflavin of milk seems in many cases to act more favourably than the lactoflavin of liver. The difference, however, is so small, that rather than draw any conclusion as to a dissimilarity in the two flavins one should notice how well they agree.

It has already been mentioned that this experiment was undertaken in the first place to learn whether lactoflavin could be replaced by glutathione as activator for the lactic acid bacteria. The tables show distinctly that this is not the case even if large amounts of glutathione be used. Glutathione without bios has a very feeble action only, except in the case of *Streptococcus cremoris* 18, yet slightly greater than found in the case of lactoflavin. A somewhat greater effect on some of the lactic acid bacteria (*Thermobacterium bulgaricum*, *Streptobacterium casei* and *Streptococcus lactis*) was found when glutathione was used together with bios, but it did not become

significant until 200—2000 mg were reached, quantities large enough to play a part as a nitrogeneous food constituent. The most interesting finding in this connection is perhaps the discovery that glutathione may inhibit the development of certain lactic acid bacteria. This holds for *Thermobacterium lactis* No. 10 and is not an isolated fact. As will be discussed in a later paper on the nutrient requirements of the lactic acid bacteria, this bacterium is always very sensitive to small amounts of glutha-thione while it needs large amounts of lactoflavin to develop. This incident brings out still more clearly the fact that glutathione is not able to replace lactoflavin in the life processes of the lactic acid bacteria.

Table VIII.

Carbon-treated milk	Other components	Species of bacteria									
		Tbm. intestinale 314	*Tbm. lactis* 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Sbm. casei* 11	*Sc. thermophilus* 7	*Sc. cremoris* 18	*Sc. cremoris* 215	*Sc. lactis* 22	*Sc. lactis* 33
100 %	+ 0 % not-carbon-treated milk	0.7	4.3	2.5	2.3	1.4	4.3	0.5	3.2	2.3	2.7
99 -	+ 1 „ „ „ „ „	1.1	5.6	5.0	4.7	1.4	4.5	0.5	4.1	2.5	3.2
90 -	+ 10 „ „ „ „ „	3.2	11.3	9.9	14.9	2.7	5.4	0.8	5.2	4.5	4.5
75 -	+ 25 „ „ „ „ „	4.5	13.3	16.2	18.5	6.1	5.9	3.2	5.9	5.9	5.4
50 -	+ 50 „ „ „ „ „	4.5	13.3	16.9	18.5	8.6	6.8	6.3	6.1	5.9	6.3
0 -	+ 100 „ „ „ „ „	4.5	13.3	18.0	22.7	9.5	6.8	6.8	6.3	7.2	7.0
99 -	+ 1 „ autolyzed yeast	7.2	8.1	6.3	12.6	5.6	5.6	1.8	5.9	6.8	6.1
90 -	+ 10 „ „ „	7.2	7.2	14.4	15.8	8.8	6.5	7.9	7.2	6.5	7.2
99 -	+ 1 „ tomato paste	6.1	5.6	12.2	11.5	7.9	6.1	1.1	5.0	6.5	5.4
99 -	+ 1 „ malt extract	7.4	12.2	11.5	12.6	7.7	6.3	2.9	5.2	6.1	5.4
90 -	+ 10 „ „ „	16.4	16.0	16.7	16.2	12.8	5.4	7.0	7.2	8.3	6.1

In order to be able to estimate the quantity present in milk of the activators here discussed, various lactic acid bacteria were tested for acid formation in a mixture of various proportions of the same milk both in carbon-treated and in untreated condition. Thus it was found, as shown in Table VIII, that the mixtures must contain at least 50 per cent untreated milk to bring the acid production of all of our lactic acid bacteria to its full value. According to R. Kuhn's data, cow's milk does not contain more lacto-flavin than 0.5 to 1 mg per liter, the latter amount corresponding to the usual colour of whey. In our own work it was found that the rodshaped lactic acid bacteria do not reach full acid formation with less than 0.5 mg per liter. Cow's milk normally does not contain an excess of activators. Therefore if milk from individual cows is not easily brought to souring, it may be caused by a too low content of activators.

Table VIII illustrates at the same time the reactivating action of autolyzed yeast,

tomato paste, and malt extract (all rich in lactoflavin) when added to carbon-treated milk. Malt extract (EVER's) is especially activating for *Thermobacterium intestinale*, *Thermobacterium lactis*, and *Streptobacterium casei*.

Summary.

1) The true lactic acid bacteria require a thermostable alkali-fast substance. This is adsorbed by activated charcoal at all hydrogen ion concentrations within the acid range, and similarly to lactoflavin it is easily eluated by means of pyridin and methyl alcohol. This substance influences favourably the growth of yeast, and may thus be considered identical with pantothenic acid, which is the main constituent of bios. Many facts seem to indicate that this activator occurring in milk is also identical with B_6, which means that vitamin B_2 must consist of bios and lactoflavin.

2) The lactic acid bacteria require lactoflavin in varying amounts in addition to bios. The rod-shaped forms need 0.5 mg lactoflavin per liter, while the Streptococci are satisfied with a smaller amount.

3) Glutathione cannot replace lactoflavin as activator for the lactic acid bacteria.

4) It is possible to measure the content of bios and lactoflavin in various materials, adding them to carbon-treated milk and after inocculation with a suitable lactic acid bacterium titrating the acid formed. If it is desired to detect bios lactoflavin must be added to the carbon-treated milk. On the other hand, if the lactoflavin content is to be tested the treated milk has to be supplied with bios. A material which can reactivate treated milk without the addition of either bios or lactoflavin must contain both activators. The latter is the case with substances such as autolyzed yeast, tomato paste, and malt extract.

The Vitamin Requirements of various Bacteria.

In our last paper we made a close investigation of the vitamin requirements of the true lactic acid bacteria of milk only. Many lactic acid bacteria, however, thrive better in other nutrient media than milk and many bacteria belonging to the coli-aerogenes group grow even in pure synthetic media, and for this reason they may need other activators or none at all. In this work we have tested the acid forming ability of the bacteria given in Table I in milk treated and not treated with charcoal. Of these bacteria *Streptobact. plantarum*, *Betacoccus arabinosaccus* and the aerogenes bacteria are found in soured vegetable matter, while *Bacterium bifidum*, *Propionibacterium*, *Streptococcus faecium*, *Streptococcus glycerinaceus*, *Streptococcus liquefaciens*, *Betacoccus bovis*, *Microbacterium lactis* and the coli bacteria are found in faeces and manure. *Streptococcus mastitidis* is the only pathogenic *Streptococcus* thriving well in milk.

From Table I it is seen that all the true lactic acid bacteria tested here — rods as well as streptococci — and also the propionic acid bacteria and the tetracocci (that is the acid-forming grampositive micrococci) thrive better in the untreated milk than in the milk treated with charcoal and thus they need the vitamins adsorbed by the charcoal. This, on the other hand, does not seem to hold good for *Microbacterium lacticum* and the coli- and aerogenes bacteria. The results, of course, are not very clear when the bacteria form only small amounts of acid in the normal milk. *Bacterium prodigiosum* and *Bacterium fluorescens liquefaciens* as well, form more acid in the untreated than in the charcoal-treated milk, while the opposite is found in the case of the hay- and potatobacilli. From this last group of bacteria we have tried more strains than the one given in the table. This strain, however, was of special interest to us because it showed a much stronger peptonising power in the charcoal-treated than in the untreated milk. This was also the case by the fluorescent bacteria and, less clearly, by *Bacterium prodigiosum*. The explanation of this fact may be that the lactoflavin, present in the untreated milk, has a slightly inhibitive influence on the

Table I.

Species of bacteria		Milk		Number of hours before titration
		Non carbon treated	carbon treated	
Streptobacterium plantarum	23	11.5	0.7	192
„ „	30	8.3	0.2	192
Betabacterium breve	2	1.4	0	120
Bacterium bifidum	S. 5	3.2	0	192
„ „	O. 16	14.2	0.5	118
Propionibacterium technicum	16	9.7	0.1	192
Streptococcus mastitidis	5	5.4	0.3	192
„ „	9	5.9	0.2	192
„ „	12	5.4	0	192
Streptococcus faecium	1	4.3	0.6	192
„ „	8	5.4	0.5	192
„ „	11	4.7	0.1	192
„ „	19	5.0	0.1	192
Streptococcus glycerinaceus	1	4.3	0.2	192
Streptococcus liquefaciens	1	6.5	1.0	192
Betacoccus arabinosaccus	16	1.4	0.1	120
Betacoccus bovis	36	6.1	0.3	120
Tetracoccus (Pediococcus)	2	5.2	0.3	192
„ *pyogenes aureus*	13	3.8	0	192
„ „ *albus*	29	1.5	0	168
Mierobacterium lacticum	4	1.4	1.4	120
„ „	5	1.8	2.0	168
Bacterium coli	5	3.6	3.6	24
„ „	7	3.8	3.6	24
„ „	9	4.1	3.6	24
„ „	10	4.1	4.1	24
Bacterium aërogenes	2	3.6	3.4	24
„ „	3	2.5	2.7	94
„ „	4	3.8	2.3	45
Bacterium prodigiosum	7	2.9	0.7	192
„ „	8	2.5	0	192
Bacterium fluor. liquefaciens	1	1.1	0.1	192
Bacillus mesentericus	7	0.5	1.4	192

proteolysis. We have been able to show this by the gelatine method described in detail by HOLGER JØRGENSEN[1].

[1] Über die Natur der Bromatwirkung. Das Mühlenlaboratorium 1935. (Monatliche Beilage zur Wochenschrift „Die Mühle", 72. Jahrgang) S. 4. We have found it convenient to use 6 per cent gelatine instead of 4 per cent, and to try the effect of different lots of papaïn and lactoflavin during 1 hour at 70° C. instead of 40° C. Not only is 70° C. nearer to the optimum temperature for papaïn than 40° C. but at the same time a pasteurization is obtained which makes any adding of antiseptica superfluous.

The formation of pigment by many bacteria is influenced by the vitamin content of the medium. Thus the tetracocci, which are able to form more or less acid in milk, always produce more pigment in the untreated, than in the charcoal-treated milk. This is most clearly evidenced when agar slants are prepared from these two kinds of milk and inoculated as streak cultures. The red micrococci from Camembert cheese and *Tetracoccus aureus* are good examples. On the other hand, *Sarcina flava*, which turns the milk alkaline forms just the same chromo-yellow coating whether the milk contains vitamins or not. The fluorescent bacteria, *Bacterium fluorescens liquefaciens* and *Bacterium pyocyaneum*, form more pigment in untreated than in charcoal-

Table II.

	Nutrient medium	Species of bacteria			
		Tbm. lactis 10		*Sbm. casei* 11	
Non-carbon-treated milk		2.3	15.1	5.0	11.9
Carbon-treated milk	Without any addition	0.2	0.7	0.3	0.5
	With bios	0.9	6.1	1.8	3.6
	„ „ + 5 % culture of *Bact. fluor. liquefaciens*	5.4	15.3	9.5	16.2
	„ „ + 5 % „ *Bact. pyocyaneum*	8.1	15.8	10.1	17.1
	„ „ + 20 % „ „ „	11.3	15.5	11.5	18.7
Number of hours before titration		20	140	68	140

treated milk. It is interesting that the typical *Bacterium prodigiosum* behaves in the opposite way, showing the strongest colour formation in the charcoal-treated milk. In the milk culture this is seen already in the first generation; on agar slants, on the other hand, not until in the second generation. But then many strains are found to give a perfect white coating of the agar with untreated milk and a strongly red coating of the agar with charcoal-treated milk. It must be the bios of the milk rather than the lactoflavin which causes the failing of the pigment formation, because *Bact. prodigiosum* produces plently of prodigiosin on the white of an egg which is rich in lactoflavin. This seems to indicate that for *Bacterium prodigiosum* the prodigiosin plays the same part as bios. Enough of the latter substance being present it need not produce the former. It is a well known fact that *Bact. prodigiosum* after continuous transfers in laboratories easily looses the ability of forming pigment and a remedy to this will certainly be found in using substrata containing the least obtainable amount of bios.

The pigments of the fluorescent bacteria look very much like flavins, and it seemed natural therefore to try whether they belong to this group. As appears from our last paper, this question can be solved by means of the charcoal-treated milk. Such milk on addition of lactoflavin can be used for detecting bios and, on the other hand, on addition of bios for detecting lactoflavin when inoculated with

lactic acid bacteria fit for the purpose, preferably *Thermobacterium lactic* 10 and *Streptobacterium casei* 11.

In these experiments we have used one week old milk cultures of *Bacterium fluorescens liquefaciens* and *Bacterium pyocyaneum* which were sterilized by heating. The milk used for the cultures was not charcoal-treated in our first experiments (Table II) because the bacteria seemed to form more pigment here than in the treated milk. In our second experiment, the last kind of milk was also used to make sure that neither bios nor lactoflavin, originating from the milk, was transferred.

From Table II it is seen that the addition of bios and a little of a culture of a fluorescent bacterium stimulates the acid formation of the lactic acid bacteria tested. This indicates that fluorescein is a flavin. The acid formation even proceeds more quickly, to begin with, in the charcoal-treated milk with these additions, than in the untreated milk. For *Tbm. lactis* 10 the amounts of acid will, after a longer period, be alike in the two cases, while for *Sbm. casei* 11 the amount is always higher in the

Table III.

	Nutrient medium	The milk used for the culture	Species of bacteria			
			Tbm. lactis 10		*Sbm. casei* 11	
	Non-carbon-treated milk	....	7.9	15.8	5.2	11.0
Carbon-treated milk	Without any addition	....	0.2	0.3	0.1	0.2
	„ bios + 2.5 % culture of *Bact. fluor. liquefaciens*	Carbon-treated	0.8	0.9	0.9	1.7
	„ „ + „ „ „	Non-carbon-treated	1.0	1.4	0.7	1.2
	„ „ + 5 % „ „	Carbon-treated	0.8	0.8	1.0	1.8
	„ „ + „ „ „ + lactoflavin (0.5 mg/l)	„	0.8	1.1	1.1	2.7
	„ „ + 2.5 % culture of *Bact. pyocyaneum*	„	1.1	1.9	2.7	4.3
	„ „ + „ „ „	Non-carbon-treated	1.5	1.9	4.1	7.0
	„ „ + 5 % „ „	Carbon-treated	1.8	2.9	2.9	5.4
	„ „ + „ „ „ + lactoflavin (0.5 mg/l)	„	1.8	2.9	3.6	7.9
	With bios	....	2.5	5.4	1.6	3.8
	„ „ + lactoflavin (0.5 mg/l)	....	3.2	8.1	4.7	11.9
	„ „ + 2.5 % culture of *Bact. fluor. liquefaciens*	Carbon-treated	6.1	12.2	4.5	9.9
	„ „ + „ „ „	Non-carbon-treated	..	13.7	..	14.0
	„ „ + 5 % „ „	Carbon-treated	7.4	15.1	7.9	13.1
	„ „ + 2.5 % culture of *Bact. pyocyaneum*	„	8.1	15.3	6.3	12.2
	„ „ + „ „ „	Non-carbon-treated	9.0	15.5	..	14.4
	„ „ + 5 % „ „	Carbon-treated	8.6	16.0	8.1	15.3
	Number of hours before titration		24	92	67	140

milk to which fluorescein has been added. The fact is that this bacterium is not able to form its maximum amount of acid with only the small quantity of lactoflavin present in normal milk.

In the following experiment, the results of which are put together in Table III, we have also tried the cultures of the fluorescent bacteria without bios, and without bios but with lactoflavin, to find out whether these bacteria form bios as well.

From Table III it is seen that the fluorescein without bios is only able to stimulate *Tbm. lactis* 10 to a slight growth and consequently a low acid formation, and the same is the case even when lactoflavin is added. Thus the fluorescent bacteria produce hardly traceable amounts of bios. The fact is less clear in the case of *Sbm. casei* 11 because the *Streptobacteria* require less bios than the *Thermobacteria*. From the Table it is seen, moreover, that if the amount of bios, present in the normal milk, is added to the charcoal-treated milk, *Tbm. lactis* 10 on addition of only 2.5 per cent of culture of fluorescent bacteria will form more acid than with 0.5 mg/l lactoflavin, and on addition of 5 per cent of culture (especially of *Bact. pyocyaneum*) it forms just as much acid as in the original milk. Under the same conditions *Sbm. casei* 11 forms much more acid than in the original milk. The cultures of the fluorescent bacteria grown in milk not treated with charcoal always give a larger outcrop than those grown in charcoal-treated milk and, similarly, the cultures of *Bact. pyocyaneum* are always somehow stronger than those of *Bact. fluorescens liquefaciens*. *Bact. pyocyaneum*, indeed, forms, besides fluorescein, pyocyanin which, according to D. Ehrismann, is a breathing ferment[1].

Summary.

1) Not only the true lactic acid bacteria of milk but all true lactic acid bacteria, as well, need bios and lactoflavin. The propionic acid bacteria and the tetracocci also form more acid in milk not treated with charcoal, than in charcoal-treated milk. In the last substratum the pigment-producing tetracocci fail to form any colour at all. *Sarcina flava*, on the other hand, grows and forms pigment whether or not the milk is treated with coal.

2) In contradistinction from the true lactic acid bacteria the pseudo-lactic acid bacteria — the coli and aerogenes bacteria — thrive as well in the charcoal-treated as in the untreated milk, in accordance with the fact that these bacteria are able to grow in pure synthetic substrata. *Microbacterium lacticum* acts in the same manner. The hay- and potatobacteria seem to thrive even better in the charcoal-treated milk than in the untreated.

3) The fluorescent bacteria and *Bacterium prodigiosum* grow quicker in milk not treated with charcoal, than in charcoal-treated milk. The fluorescent bacteria also form more pigment in the first case while *Bacterium prodigiosum* behaves in the opposite way.

4) It is proved that fluorescein is a flavin.

5) It is found to be likely that bios is able to replace prodigiosin in the metabolism of *Bact. prodigiosum*.

[1] Pyocyanin und Bakterienatmung. Zeitschrift für Hygiene und Infektionskrankheiten 1934. **116**, 209.

Growth Factors present in Peptones.

Peptone solutions with the necessary nutrient salts are the most frequently used media for bacteria. In our work on the vitamin requirements of the lactic acid bacteria we have shown that these bacteria need milk bios and lactoflavin for their growth. Thus one may conclude that the peptone solutions, in which the lactic acid bacteria thrive, must contain at least a little of the above mentioned activators. Casein peptone was at the outset suspected of being a medium rich in activators. Prepared from the usual acid casein it proved to be an especially good medium for the lactic acid bacteria. Peptone WITTE is less suitable, and casein peptone prepared from repeatedly purified casein (according to HAMMARSTEN) is still poorer. We tried to find out whether the peptone prepared from HAMMARSTEN's casein (CH), if bios and lactoflavin were added, would equal the usual casein peptone (C). Some cysteine was also added to CH to make it comparable to C, because JONES and GERSDORFF have shown[1] that HAMMERSTEN's casein loses part of its cysteine by the repeated dissolving in alkali. Our peptone solutions were prepared so as to contain 0.5 per cent nitrogen.

It is evident from Table I that the lactic acid bacteria of milk develop much more scantily in CH than in C. The addition of cysteine had an influence only for *Streptobacterium casei* which is particularly dependent on that substance. Lactoflavin used alone has a small effect upon *Thermobacterium bulgaricum* and *Thermobacterium helveticum*. On the other hand, bios added to CH renders this medium as good if not better than C towards the lactic acid bacteria. *Thermobacterium helveticum* is an exception in as much as it grew poorly in this experiment (also in C). With the exception of *Thermobacterium bulgaricum Thermobacteria* thrive somewhat better with the additon of cysteine + bios than with bios alone. All lactic bacteria and especially *Thermobacterium bulgaricum* produce more acid in the presence of lactoflavin + bios than with bios alone. *Thermobacterium lactis* 9 and 10, and especially *Thermobacterium helveticum* produce the greatest amount of acid when all three substances are added simultaneously.

One may conclude from these experiments that the amounts of cysteine and lactoflavin present in CH are almost sufficient for the lactic acid bacteria tested. Bios

[1] Journal of biological chemistry, 1934, **104**, p. 99.

Table I.

Nutrient medium	Species of bacteria													
	Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*		*Sbm. casei* 11		*Sc. cremoris* 18		*Sc. lactis* 22	
C without any addition ...	6.8	9.2	1.6	6.5	5.2	6.8	1.8	1.8	0.2	0.3	2.5	3.4	3.2	4.1
CH „ „ „ ...	0.3	0.3	0.2	0.3	1.1	1.1	0.2	0.2	0.1	0.2	0.8	1.8	1.1	2.5
„ with cysteïne	0.3	0.3	0.1	0.2	0.5	0.5	0.3	0.3	3.2	3.8	0.8	1.8	1.0	2.0
„ „ lactoflavin	0.3	0.3	0.1	0.2	0.9	0.9	0.6	0.6	0.1	0.1	0.7	1.7	1.1	2.0
„ „ bios	8.3	9.0	3.6	5.9	8.6	9.7	0.6	0.6	3.8	5.2	3.2	4.3	3.4	3.8
„ „ cysteïne + bios	10.6	11.3	6.5	8.6	7.9	8.6	0.7	0.7	2.7	5.2	3.2	3.8	1.6	2.5
„ „ lactoflavin + bios ..	9.7	11.0	5.5	7.4	9.9	12.8	0.6	0.9	3.8	5.6	3.8	4.5	3.8	4.7
„ „ cysteïne + lactoflavin + bios	12.4	14.2	7.7	10.4	10.1	11.0	3.8	3.8	4.7	5.6	2.7	4.1	2.7	3.8
Number of hours before titration	70	119	70	145	70	119	70	119	119	145	24	119	24	119

which is removed by the process of purification is the substance lacking. When this activator is added to CH (in the same amount as that present in normal milk), the medium equals C, or even surpasses it. The amount of activators present in the casein is by no means constant, but depends on the process of manufacturing and especially on the washing. In any case casein peptone is always poorer in activators than milk, even when the nitrogen content is the same in both cases (0.5 per cent). Those lactic acid bacteria that are specific milk bacteria constantly prefer milk or peptonized milk[1] to casein peptone. The *Thermobacteria* of milk cannot be cultivated for any length of time outside milk; they die off quickly when continuously carried in peptone bouillon.

In some of our experiments we used casein peptone with double the amount of nitrogen as compared to that of milk (one per cent), because such a medium (2 C) is more satisfactory for the lactic acid bacteria than C. They do not merely produce more acid, which might be ascribed to the stronger buffer action, but frequently a greater variety of sugars is fermented in 2 C than in C[2]. This must be due to the fact that the lactic acid bacteria are more activated by 2 C than by C. There is nothing miraculous about it if we realize that 2 C must be twice as rich in activators as C prepared from the same casein. We tried to increase the acid formation in C by addition of lactoflavin and bios. In our experiments of Table II we added these activators to C in the amounts in which they are present in normal milk. We also tested the action of additional pepsin, so that C contained as much pepsin as 2 C.

Bios usually produces a favourable effect, see Table II, just as in the experiment

[1] S. Orla-Jensen, Der beste Nährboden der Milchsäurebakterien, Zentralblatt für Bakteriologie, II Abt., (1898), **4**, p. 196.

[2] S. Orla-Jensen, Anna D. Orla-Jensen and O. Winther, *Bacterium befidum* und *Thermobacterium intestinale*, Zentralblatt f. Bakteriologi, II Abt. (1935), **93**, Heft 18—22.

Table II.

Nutrient medium	Species of bacteria													
	Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*		*Sbm. casei* 11		*Sc. cremoris* 18		*Sc. lactis* 22	
C without any addition	2.9	10.6	3.6	6.8	1.4	8.3	2.0	2.9	2.3	5.4	3.2	4.3	3.8	5.2
„ with pepsin	6.3	12.4	6.1	9.5	1.6	5.6	2.5	2.9	4.5	6.5	3.2	4.3	3.8	5.0
„ „ lactoflavin	2.9	10.6	3.8	7.4	1.4	4.7	2.3	3.2	2.5	4.3	3.2	4.3	3.8	5.0
„ „ bios	5.2	10.8	8.3	11.0	5.6	11.9	2.7	4.3	6.1	7.7	3.6	4.5	3.6	4.5
„ „ lactoflavin + bios	5.9	13.1	9.2	11.0	4.7	12.2	3.2	6.3	6.8	8.6	4.1	4.7	3.8	4.5
2 C without any addition	8.6	16.2	8.6	12.8	1.4	9.0	3.4	5.9	8.3	10.6	5.9	7.0	5.9	8.1
Milk „ „ „	10.1	18.7	10.1	13.3	11.5	16.4	11.3	22.3	10.8	12.6	3.2	6.3	4.7	6.8
Number of hours before titration	25	95	52	95	25	95	25	95	113	139	25	95	25	95

with CH. Lactoflavin alone has no action, but added together with bios it improves C to such an extent that the lactic acid rods produce as much or even more acid than they do in 2 C. It is evident that the better thriving of these rods in 2 C than in C is not so much due to the additional nutrient matter and higher buffer action, as to the increased content of activators. Attention should be called to the fact that although milk contains half the nitrogen that 2 C does, it is nevertheless a better medium for the rod-shaped lactic acid bacteria, because milk possesses more activators. The *Streptococci* behave in the opposite way in all these respects. C is not at all improved for the *Streptococci* by the addition of activators. It is mainly due to the strong buffer action of 2 C that this substratum acts better than C, even better than milk, inasmuch as the *Streptococci* cannot endure a high acid concentration. Neither can they stand a high lactate ion concentration, and thus after 10 days at 30° C a higher hydrogen ion concentration was reached in C than in 2 C, while the titratable acidity was greater in 2 C than in C.

Experiments with peptone WITTE have also been carried out, like those with casein peptone; and the same results were obtained. Peptone WITTE did not improve upon the addition of lactoflavin, no matter whether lactic acid rods or *Streptococci* were tested. Upon the addition of bios, however, peptone WITTE becomes a very much better medium for the rods, whereas this addition showed no effect on the *Streptococci*. Hence one may conclude that the peptones contain enough lactoflavin even for the activation of the lactic acid rods, while their amount of bios is only sufficient for the *Streptococci*, not for the rods. The small bios content of the peptones may originate from the protein used, as already demonstrated in the case of casein peptone. The source of the relatively larger amount of lactoflavin is rather to be sought in a thing common to all peptones, namely, in enzymes used for the peptonisation. The correctness of this supposition is verified in Table II. The addition of pepsin to casein peptone improves this medium for *Thermobacterium lactis* 9

and 10, and for *Streptobacterium casei*. *Thermobacterium lactis* 9 is almost as much activated as by the addition of lactoflavin and bios.

It has been shown in our work on the vitamin requirements of the lactic acid bacteria that milk treated with norite charcoal, with bios added to compensate for the bios removed, is an excellent medium for testing the presence of lactoflavin. We used such a medium for testing the following enzyme preparations for lactoflavin content: The pepsin, which we constantly used for the preparation of casein peptone (Pepsin 1:900); a purified preparation of pepsin (Pepsin 1:10000); a pancreatin used in preparing tryptophane media for testing indol production by certain bacteria; an impure and a purified papaïn preparation; and finally some rennet solutions and rennet tablets. The proteolytic and coagulating qualities of these enzymes were destroyed by heating the aqueous solutions before use. All preparations were used in a quantity equal to one tenth of one per cent of NaCl-free dry substance. The rennet was furthermore used in the small amount usually employed in cheese making (K). The results of these experiments are presented in Table III.

Table III.

Nutrient medium		Species of bacteria									
		Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*		*Sbm. casei* 11	
Non-carbon-treated milk		9.7	18.5	10.1	14.4	10.1	16.2	9.9	22.7	3.2	5.4
Carbon-treated milk	Without any addition	0	0.5	0	0.1	0.2	0.6	0	0.5	0.2	0.7
	With Bios	4.5	9.9	0.7	0.8	2.3	7.2	1.8	7.2	1.1	2.7
	„ „ and lactoflavin 0.5 mg/l.	6.3	14.6	6.3	11.5	9.0	15.8	3.6	16.2	4.1	11.7
	„ „ „ impure pepsin (0.1 %)	8.8	13.3	8.3	11.0	7.7	12.6	7.4	16.0	6.3	11.7
	„ „ „ pure „ „	8.8	12.8	0.6	5.0	7.4	9.7	1.6	10.6	5.0	8.8
	„ „ „ pancreatin „	8.1	13.5	6.1	9.9	2.2	10.8	1.6	10.6	8.6	12.2
	„ „ „ impure papaïn „	6.5	9.3	7.0	8.6	6.8	9.7	5.0	9.9	6.1	10.4
	„ „ „ pure „ „	7.2	9.7	3.2	4.3	8.1	9.7	3.8	11.5	4.5	6.3
	„ „ „ rennet solution „	5.4	14.9	10.4	12.8	3.8	8.6	4.1	12.2	6.3	13.7
	„ „ „ „ „ K	4.7	10.1	0.9	2.3	3.6	6.1	2.0	10.6	3.2	5.0
	„ „ „ rennet tablet (0.1 %)	4.3	9.5	9.9	11.7	3.6	6.8	3.2	9.0	6.1	9.9
	„ „ „ „ „ K	3.2	10.1	0.9	2.0	3.4	5.4	2.7	11.3	1.8	3.2
Number of hours before titration		24	92	46	92	24	92	24	92	68	141

It is evident from this table that all the proteolytic enzymes tested, are relatively rich in lactoflavin, irrespectively whether they are of animal origin, as most of them are, or of vegetable origin, like papaïn. 100 mg of the enzyme usually show the same action as 0.5 mg pure lactoflavin. The action naturally becomes correspondingly weaker when smaller quantities of enzyme preparations are used. As previously stated, *Thermobacterium lactis* 10 and *Streptobacterium casei* 11 are the best suited bacteria

for testing lactoflavin, because they require such a large amount of this activator, that the negligible amount of it present in our bios preparation gives but a very slight reaction[1]. The most reliable results were therefore obtained with these two organisms. Table III shows that crude pepsin and papaïn have a stronger action than the purified products, which leads to the assumption that lactoflavin is not an important component of these enzymes, but merely an impurity. The amount of lactoflavin present in the quantity of rennet used in cheese-making is too small for the activation of lactic acid bacteria (compare bios + K with bios alone), and hence it cannot play any role in the development of the lactic acid rods so important for the cheese-ripening process. It is very strange that proteolytic enzymes contain so much lactoflavin, because this vitamin, as shown in our previous publication, has a weak inhibitive action on the proteolysis.

The lactoflavin lost by the charcoal treatment of the milk is restored, as we have seen, by adding one tenth of one per cent of any of the proteolytic enzymes tested here. This addition will at the same time give an idea of the bios content of the enzymes. Of course then bios must not be added. This has been tested in the case of pepsin and pancreatin. For the sake of comparison we tried the same amount of nitrogen also in the form of (2 per cent) autolyzed yeast. We further tested the action of these additions in the same milk which had not been treated with charcoal. The results are presented in Table IV.

Table IV.

	Milk	Species of bacteria								
		Tbm. intestinale 314	*Tbm. lactis* 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Sbm. casei* 11	*Sc. thermophilus* 7	*Sc. cremoris* 215	*Sc. lactis* 22	*Sc. lactis* 33
Carbon-treated	Without any addition	0.5	0.9	2.0	0.8	0.5	2.6	2.7	2.7	2.9
	With impure pepsin	5.9	0.9	2.3	1.7	6.2	5.2	5.3	5.0	5.0
	„ pure „	6.1	1.1	2.1	2.1	2.7	4.3	4.1	4.3	3.8
	„ pancreatin	9.9	7.7	6.3	7.9	5.4	5.4	6.3	6.3	5.9
	„ yeast autolysate	7.4	7.0	7.2	8.1	5.6	5.6	6.1	6.8	5.4
Not-carbon-treated	Without any addition	3.6	10.1	16.8	17.6	7.8	6.0	5.7	7.1	6.8
	With impure pepsin	11.4	14.0	18.0	21.6	11.1	7.1	7.5	7.5	7.7
	„ pure „	11.9	13.6	18.3	21.2	10.9	7.2	7.2	7.3	7.2
	„ pancreatin	12.6	15.8	18.2	21.4	12.6	6.8	7.4	8.3	7.7
	„ yeast autolysate	11.0	11.9	17.8	21.6	9.7	7.0	7.2	7.4	7.2
Number of hours before titration		96	48	96	48	100	48	70	100	144

[1] The vitamin requirements of the lactic acid bacteria, Tables V a and V b pp. 13 and 14. Other lactic acid rods can also be used if lactoflavin is destroyed beforehand by heating the bios with alkali.

Besides the usual lactic acid bacteria, an intestinal lactic acid organism, *Thermobacterium intestinale* (*Bacillus acidophilus*), is included in the above table. We will leave it out of consideration for the present, nor shall the untreated milk be discussed for the moment. It is well known that autolyzed yeast contains bios as well as lactoflavin, and hence our bacteria produce more acid when 2 per cent autolyzed yeast are added to the charcoal-treated milk. A similar increase in acid production is obtained when one tenth of one per cent pancreatin is added, which proves that this enzyme contains a considerable amount of bios besides lactoflavin. The pepsin preparation influences the *Thermobacteria* very slightly, but has a considerable action upon the other lactic acid bacteria. Acid production is here also more influenced by the crude than by the purified product. This is especially pronounced for *Streptobacterium casei*, whose requirements of bios seem to be smaller than of lactoflavin. Similarly to pancreatin, our pepsin preparations thus contain bios besides a relatively large amount of lactoflavin. Nevertheless the amount of bios in the pepsin preparation (when only one tenth of one per cent is added to the milk treated with charcoal) is not sufficient for the *Thermobacteria* which require considerable amounts of bios as well as of lactoflavin.

The question of the activating role of proteolytic enzymes is not solved as yet. These enzymes, especially pancreatin, contain, just as autolyzed yeast, other activators besides bios and lactoflavin, because they increase the acid production of the lactic acid bacteria, also in milk which has not been deprived of lactoflavin and bios by carbon treatment. Table IV shows that this is particularly the case with *Thermobacterium intestinale*. Here we shall not consider the problem of activation for the intestinal bacteria by means of digestive secretions, since we have treated this thoroughly elsewhere[1].

Encouraged by the results obtained with milk treated with activated charcoal, we decided to try whether the peptone solutions could be inactivated in a similar way. This seems a comparatively simple matter since charcoal is much easier to separate from such solutions than from milk, inasmuch as simple filtration can be used instead of the strong centrifuging. But besides the vitamins of the B-group several other substances, such as large molecular decomposition products of proteins, are unfortunately adsorbed by the activated charcoal. For this very reason it is used in protein chemistry for the differentiation of substances resulting from proteolytic action, just as metal salts, tannic acid, phosphotungstic acid etc., are being used. Milk with a nitrogen content slightly over 0.5 per cent loses at most 0.04 per cent after a double charcoal treatment, a considerable part of the loss being due to the separation of leucocytes, microorganisms etc. by the centrifuge. On the other hand the nitrogen content of peptone Witte drops 0.29 per cent from the original content of 0.5 per cent by such treatment. The poorer development of the lactic acid bacteria in the treated than in the untreated peptone Witte may therefore be due to the loss of nutrient substances just

[1] S. Orla-Jensen, Anna D. Orla-Jensen und O. Winther. *Bacterium bifidum* und *Thermobacterium intestinale*. Zentralblatt f. Bakteriologie, II Abt, 1935, **93**, Heft 18—22.

as much as to the loss of activators. The autolyzed yeast prepared by the method used in our laboratory did not lose as much nutrient matter by adsorption on carbon because the proteolytic action is carried much further in this medium than in peptone WITTE. Autolyzed yeast with 0.7 per cent nitrogen content, which decolorized completely after two charcoal treatments, lost only 0.15 per cent nitrogen and therefore we will consider the results obtained in an experiment with autolyzed yeast. The concentrated eluate from the charcoal contained 4.5 per cent of N, and an addition of only one per cent of this would increase the nitrogen content of the autolyzed yeast by 0.05 per cent. This error was eliminated by diluting the yeast autolysate to which eluate is added to such an extent that the same content of nitrogen was obtained in all cases.

Tables V a and V b show the results obtained with yeast autolysates containing 0.5 per cent nitrogen and 2 per cent sugar. Both alkali-treated and untreated

Table V a.

Yeast autolysate	% eluate added	Eluate treated with alkali	Species of bacteria											
			Tbm. cereale 4		*Tbm. lactis* 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*		*Tbm. Jugurt*	
Non-carbon-treated	0		9.9	14.0	2.9	8.6	3.6	4.7	0.7	3.4	8.3	9.2	10.1	11.7
Carbon-treated	0		1.6	2.5	0.9	6.1	1.8	3.4	0.2	1.4	7.7	7.9	0.9	2.7
„ „	1	0	3.8	6.1	2.5	7.7	3.6	7.4	5.6	6.5	9.2	11.0	5.6	9.0
„ „	1	+	3.2	4.5	1.4	3.8	1.1	5.2	8.1	9.2	8.3	10.1	1.8	5.9
„ „	2	0	2.7	7.7	5.0	10.4	4.3	7.9	3.2	3.2	9.5	11.3	5.0	6.5
„ „	2	+	1.6	5.0	0.9	4.3	2.3	5.0	8.6	9.0	8.6	9.7	1.1	5.9
Number of hours before titration			48	100	18	95	66	118	66	95	66	95	48	100

Table V b.

Yeast autolysate	% eluate added	Eluate treated with alkali	Species of bacteria											
			Sbm. casei 11		*Bbm. breve* 10		*Sc. cremoris* 18		*Sc. lactis* 22		*Bc. arabinosaceus* 12		*Bc. cremoris* 16	
Non-carbon-treated	0		5.0	9.9	5.0	9.2	2.3	2.5	4.7	4.7	9.7	9.7	3.2	2.9
Corbon-treated	0		1.8	5.6	1.1	5.2	2.0	3.8	3.2	4.1	9.2	8.8	0.9	0.9
„ „	1	0	4.5	7.0	3.6	7.7	3.2	3.8	3.6	3.8	10.6	10.6	2.9	2.9
„ „	1	+	3.6	5.6	1.8	7.7	3.2	4.1	2.5	2.3	13.1	10.6	1.8	1.8
„ „	2	0	3.8	6.5	3.4	7.9	2.7	3.6	3.2	3.8	11.3	9.0	4.3	3.4
„ „	2	+	3.2	5.9	2.5	8.1	3.6	5.0	1.8	3.4	9.9	8.6	0.2	0.5
Number of hours before titration			66	118	66	118	66	95	66	95	66	95	66	95

eluates were used[1]. Besides lactic acid bacteria from milk we tested several other species thriving better in yeast autolysate than in milk, namely *Thermobacterium cereale*, *Betabacteria* and *Betacocci*.

As seen from Tables V a and V b the inactivation of yeast autolysate by means of activated charcoal has a varied effect upon the bacteria tested. Inactivation was most pronounced with the mesh bacterium *Thermobacterium cereale* (Bacillus Delbruckii), and the butter aroma organism *Betacoccus cremoris*, which as a pure culture grows poorly in milk, but excellently in yeast autolysate. Inactivation was furthermore successful in the case of the two yoghurt rods, *Thermobacterium bulgaricum* and *Thermobacterium Jugurt*. Only partial inactivation was observed for the cheese ripening organism of Emmenthal cheese, *Thermobacterium helveticum*, for the two streptococci of sour milk *Streptococcus cremoris* and *Streptococcus lactis*, and almost no inactivation for the frog spawn organism, *Betacoccus arabinosaceus*. When inactivation is obtained, the addition of the eluate acts favourably to some extent. Curiously enough the addition of two per cent eluate does not always give better results than that of one per cent. The yoghurt rods and *Streptococcus cremoris* 18 produce less acid when 2 per cent eluate are added than with 1 per cent. We must conclude from this that the eluate contains inhibiting substances as well as activators.

The activation is naturally diminished by the alkali treatment of the eluate, whereby most of the lactoflavin is destroyed. When in spite of that *Thermobacterium bulgaricum* and *Streptococcus cremoris* produce more acid with alkali-treated eluate, the only explanation may be that the above-mentioned inhibiting substances also have been destroyed by the alkali treatment. It is further to be noted that the second titration gave a lower figure than the first for *Betacoccus arabinosaceus* and (only in two cases) for *Betacoccus cremoris*. This is due to the fact that the *Betacocci* later convert into gas a part of the lactic acid formed, just as does the *aerogenes* group.

Summary.

1) Peptones used for bacteriological purposes contain a sufficient amount of lactoflavin even for the normal development of the lactic acid rods. Their bios content, however, is too low for these organisms, but is sufficient for the *Streptococci*.

2) The favourable action obtained by increasing the concentration of peptone solutions is particularly due to a larger amount of activators as far as the lactic acid rods are concerned, and to the stronger buffer action in the case of the *Streptococci*.

[1] The methods for alkali treatment and for the preparation of the concentrates may be found in "The vitamin requirements of the lactic acid bacteria" p. 9. It must be stated, however, that the charcoal treatment of the autolyzed yeast was at a pH 6, and that pyridine + methyl alcohol + water were used in the preparation of the eluate. Alkali treatment (p. 7) was applied to separate bios from lactoflavin, the former being alkali-fast, the latter not.

3) The proteolytic enzymes as commercially available are very rich in lactoflavin. They also contain bios besides activators, which have a particularly favourable influence upon the intestinal lactic acid rods.

4) Lactoflavin present in peptones originates mostly from the enzymes used in the preparation of the latter, while bios may also come from the proteins, as in the case of the usual casein peptone.

5) The inactivation of peptone solutions by means of activated charcoal is not as simple as that of milk, because the charcoal adsorbs considerable amounts of peptone besides the activators. An experiment with autolyzed yeast gave results similar to those obtained with milk.

The Nitrogen Requirements of the Lactic Acid Bacteria.

It has already been stated in previous publications that the true lactic acid bacteria *Streptococci*, as well as rod forms, seem to be very particular in their nitrogen requirements[1]. Some species require milk, others mesh or sugar broth. Peptonized casein or autolyzed yeast in high concentrations are especially suitable sources of nitrogen for numerous lactic acid bacteria. The cultivation of the true lactic acid bacteria by means of amino acids, not to speak of ammonium salts as the only source of nitrogen has as yet never succeeded. This is not surprising as we have found that these bacteria require at least two activators for their development. The question arises whether the lactic acid bacteria can utilize simpler nitrogen compounds if they have at their disposal the necessary activators.

The preparation of milk bios is a tedious process and we had to economize with the lactoflavin kindly forwarded to us by Professor Kuhn. Thus we had in the preliminary experiments to use whey as a solvent for the nitrogenous compounds tested. Although whey contains the water soluble vitamins of milk, which are the necessary activators for the lactic acid bacteria, it is a very poor source of nitrogen for this group of organisms. This has been demonstrated in the latter part of Table III in our paper on the vitamin requirements of the lactic acid bacteria. Only *Thermobacterium bulgaricum* and *Streptococcus lactis* produce a mentionable amount of acid in whey. The albumin of the acid whey used was precipitated by heating at the isoelectric point. If such whey is neutralized and sterilized another precipitation takes place and the filtrate contains only 0.05 per cent nitrogen. The remaining nitrogenous substances are simple compounds like creatine, creatinine, urea, and ammonia; only 0.02 per cent nitrogen occurs as more complicated compounds which may be precipitated by the proper protein precipitants. The value of those nitrogenous compounds, especially the last mentioned, for the lactic acid bacteria is unknown. Experiments with whey can only make clear which nitrogenous substances are essential, but not which are unessential, as we do not know whether those latter substances are already present in the whey in sufficient quantity. Thus it is necessary to

[1] S. Orla Jensen. The Lactic Acid Bacteria. Det kgl. danske Videnskabernes Selskabs Skrifter 1919.

carry out supplementary experiments with pure synthetic media. Whey as well as milk contains all the mineral substances necessary for life, even traces of Cu and Zn are present. Phosphoric acid is present in considerable quantity; more in the acid whey, less in the rennet whey or in calcium chloride serum. The addition of iron not increasing the growth of the lactic acid bacteria in milk has a slight influence in whey. However, already 0.001 per cent $FeCl_3$ gives optimal results. This amount was therefore added in our experiments with whey as well as in those with synthetic media.

From Table I it appears that protein-free acid whey is a very poor medium for the lactic acid bacteria, in as much as they produce but little acid in it. The quantity of acid is calculated as one tenth of one per cent of lactic acid, as we have done elsewhere. Some more acid is produced in the rennet whey, because it contains almost double the amount of nitrogen. It seems peculiar that the lactic acid bacteria thrive so much more poorly in whey than in milk, since whey and milk contain the same soluble materials. One would not at the outset expect that the lactic acid bacteria could attack colloidal proteins, since they do not excrete proteolytic enzymes *in vivo*. Nevertheless the following experiments show that the lactic acid bacteria utilize caseinates as well as peptone WITTE. The lack of caseinates must therefore be responsible for their poor development in whey. We used a vitamin-A-free di-natrium caseinate from the British Drug House, which served us all along in our vitamin experiments. Caseinate and peptone WITTE were dissolved in whey and for the sake of comparison also in water containing lactose and nutrient salts. All media contained 0.25 per cent nitrogen, and were adjusted to a PH 6.5. Milk diluted to a similar nitrogen content was used for control. All cultures were titrated after 5 days of incubation.

This table indicates clearly that, dissolved in whey, colloidal casein is just as

Table I.

Nutrient medium	Species of bacteria									
	Tbm. intestinale 314	*Tbm. lactis* 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Sbm. casei* 11	*Sc. thermophilus* 7	*Sc. cremoris* 18	*Sc. cremoris* 215	*Sc. lactis* 22	*Sc. lactis* 33
Lactose solution + peptone "Witte".	0.3	2.1	3.4	0.3	0.6	2.6	2.6	2.7	2.5	2.7
„ „ + casein	0	0.5	0.7	0	0.5	1.1	0	0.9	1.4	1.1
Acid whey	0.2	0.2	1.6	1.1	0.2	2.0	0.9	0	1.6	0.9
„ „ + peptone "Witte"	3.7	9.4	10.0	16.0	9.6	5.2	5.6	5.0	5.2	5.6
„ „ + casein	8.6	9.5	17.3	17.8	10.4	5.9	5.4	3.8	4.5	4.7
Rennet whey	0.5	0.2	1.4	4.3	2.5	2.3	2.3	0.9	2.3	2.0
„ „ + peptone "Witte"	7.9	8.8	9.2	14.9	9.2	4.7	4.7	4.1	4.1	4.3
„ „ + casein	7.4	11.0	12.8	13.1	10.1	5.6	5.2	3.8	5.0	4.7
Milk diluted	5.4	14.0	11.9	14.6	8.1	3.8	4.5	3.8	4.3	3.8

good a source of nitrogen for the lactic acid bacteria as peptone Witte. Dissolved in sugar solution, these substances offer as poor a medium as acid whey, the reason, however, being a different one. The sugar solutions lack activators, while the whey lacks nitrogenous compounds. Albumin seems to be of no importance for the lactic acid bacteria as they thrive equally well in albumin-free whey with peptone Witte or with casein and in milk diluted to the similar nitrogen content. In certain cases even larger quantities of acid were obtained with whey media than with diluted milk. The reason is that the milk was diluted with a sugar solution, instead of with whey, and hence the amount of activators has been decreased. Table II shows that the medium used for dilution is of some importance, therefore we used whey in our further experiments[1]).

Table II.

Milk diluted to 0.2 % N with:	Species of bacteria									
	Tbm. intestinale 314	*Tbm. lactis* 9	*Tbm. lactis* 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Tbm. jugurt*	*Sbm. casei* 11	*Sc. thermophilus* 7	*Sc. cremoris* 18	*Sc. lactis* 22
Lactose solution	4.1	6.8	7.4	10.1	10.6	11.9	4.7	3.2	2.5	3.2
Acid whey	7.9	17.8	10.1	16.9	17.1	19.4	8.1	4.1	4.1	3.6
Number of hours before titration	142	116	116	116	116	116	142	142	142	142

We have already seen that milk deprived of the activators necessary for the lactic acid bacteria, but still rich in nutriment, is a suitable liquid for testing the activators in question. In a similar way protein-free whey which is a very poor source of nitrogen but rich in activators, is a very appropriate medium for testing the nitrogenous substances especially the amino acids necessary for the lactic acid bacteria. Owing to the high price of pure amino acids we had to use nutrient media which contained only 0.2 per cent nitrogen. One fourth of the nitrogen originated from the whey used. In preparing a complete mixture, we chose the proportion of amino acids similar to that occurring in casein. However, we added glycocoll, although it does not occur in casein, because it is present in lactalbumin. Ammonia was not added, a trace being already present in the whey. On the other hand, asparagine was included in addition to aspartic acid, because Virtanen[2]) found that the cereals behave quite differently with these two sources of nitrogen. While aspartic acid cannot be utilized, asparagine is just as valuable as nitrates are. The following mixture is given as an

[1] This fact may probably play a part in the nutrition of bottle-fed babies. If whey is used as a diluent instead of a saccharose solution, not only the natural vitamin content, but also the natural lactose content is retained, and the latter has a favourable action upon the intestinal flora.

[2] Report of Proceedings. Congress of pure and applied chemistry. Oslo 1933.

Table III.

g	Amino acids	Per cent N in amino acid	g N present
0.1	Glycine	18.67	0.0187
0.15	Alanine	15.73	0.0235
0.35	d-l-Valine	11.96	0.0420
0.3	l-Leucine	10.69	0.0321
0.2	d-Isoleucine	10.69	0.0214
0.2	Phenylalanine	8.50	0.0170
0.25	Tyrosine	7.74	0.0193
0.35	l-Proline	12.17	0.0427
0.2	l-Oxyproline	10.68	0.0214
0.25	l-Histidine	25.96	0.0650
0.1	l-Tryptophane	13.80	0.0138
0.3	d-Lysinedichloride	12.80	0.0384
0.25	d-Arginine	32.18	0.0805
0.1	Aspartic acid	10.53	0.0105
0.1	Asparagin	21.22	0.0212
0.6	Glutamic acid	9.50	0.0570
0.1	Cysteinechloride	8.80	0.0088
360.0	Whey	0.052	0.1872
360 cc	Nutrient medium	0.2	0.7205

example of the composition of a solution of this kind. It gives, when dissolved in whey, a nutrient medium with 0.2 per cent nitrogen which satisfies the lactic acid bacteria in every respect.

All media presented in Table IV, except the whey, contained 0.2 per cent nitrogen and were adjusted to the pH of normal milk. The sugar solutions contained, besides two per cent lactose, 0.5 per cent K_2HPO_4, 0.1 per cent $MgSO_4$, 0.1 per cent NaCl, and 0.001 per cent $FeCl_3$. A very poor medium for the lactic acid bacteria is obtained when the amino acid mixture is dissolved in the sugar solution, because of the absence of activators. On the other hand, when the amino acids are dissolved in whey, the amount of acid produced is roughly the same (or varying inside narrow limits) as that found in whey with peptone WITTE. The cultures were titrated after 5 days of incubation.

For the following experiments in which the activators of milk play the main role, only lactic acid bacteria preferring milk as a nutrient medium were used. The *Betabacteria*, *Betacocci*, and pathogenic *Streptococci* were therefore left out. *Thermobacterium intestinale* (*Bacillus acidophilus*), which as an intestinal bacterium utilizes other activators besides those found in milk was also left out.

In Table V "A" stands for the complete mixture of amino acids. We added further some diketopiperazine, a compound which according to Troensegaard[1]) is a building

[1] HOPPE-SEYLERS Zeitschrift für physologische Chemic 1930, Bd. 193, p. 171.

Table IV.

Nutrient medium	Species of bacteria									
	Tbm. acidophilum 314	*Tbm. lactis* 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Sbm. casei* 11	*Sc. thermophilus* 7	*Sc. cremoris* 18	*Sc. cremoris* 215	*Sc. lactis* 22	*Sc. lactis* 33
Lactose solut. + peptone "Witte"	0	0	0	0	0.9	3.8	0.7	2.5	1.1	0.7
Lactose solut. + mixt. of amino acids	0	0.1	0	0	0.1	0.5	0	0	0.1	0
Whey + peptone "Witte"	1.9	1.5	9.7	14.0	8.6	4.1	4.3	4.7	5.0	4.3
Whey + mixt. of amino acids	3.4	7.9	15.8	12.8	7.4	4.1	3.2	4.1	3.8	3.2
Whey	1.5	0.3	4.3	2.0	0	2.3	0.7	0.3	1.1	0.7

stone of no small importance in the structure of proteins, and for this reason possibly of physiological significance. One after the other of the amino acids was left out, the lacking nitrogen being replaced by glycocoll and alanine, as we took it for granted that none of these simple amino acids have any specific physiological influence on the lactic acid bacteria. Ammonium citrate with a small quantity of ammonium chloride and ammonium sulphate were also used for the same purpose. In this way it was possible to distinguish between the amino acids indispensable in the mixture and those which the lactic acid bacteria can do without. All the media contained 0.2 per cent nitrogen. We further took care that our solutions after sterilization had the optimal pH, namely, 6.5 for the Streptococci and 6.2 for the rods.

We tested a simpler "physiological solution" at first, using only the amino acids known to be indispensable for higher animals. It consisted of cysteine, tryptophane, phenylalanine, lysine, and histidine. Since tyrosine and phenylalanin are able to replace each other in animal nutrition, we preferred to use the latter in our first experiments, because it is more soluble. We had in addition tested whether the lactic acid bacteria behaved similarly to animals in that respect. The results are presented in Table V. As already stated, glycocoll + alanine or ammonium salts were added as fillers to reach the usual percentage of nitrogen. "B" in Table V stands for the physiologic al solution of amino acids with glycocoll and alanine, while "C" stands for the same solution with ammonium salts. The cultures were titrated after 4 days of incubation.

In order to evaluate Table V the fact must be known that the amount of acid obtained in ten tubes with identical medium, inoculated with the same lactic acid bacterium, is by no means necessarily constant. We calculate the average of such ten tubes whenever possible. Expensive amino acids forced us to curtail the amount of such media. The results were controlled, however, by repeating the experiments under varying conditions.

It is quite striking that the complete mixture of amino acids (A), is much more suited for *Thermobacterium lactis* 10 and for *Thermobacterium bulgaricum* than the

Table V.

Mixture of amino acids	Species of bacteria							
	Tbm. lactis 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Sbm. casei* 11	*Sc. thermophilus* 7	*Sc. cremoris* 18	*Sc. lactis* 22	*Sc. lactis* 33
A (complete mixt. of amino acids)	13.3	18.0	12.8	7.2	4.1	3.8	3.8	2.9
B (physiological mixt. of amino acids)	7.9	7.0	12.6	5.9	4.1	2.7	2.5	2.3
B (only half of the phenylalanine replaced by tyrosine	7.0	5.4	12.8	5.9	4.1	2.7	2.5	2.7
B in which all of the phenylalanine was replaced by tyrosine	6.5	6.3	12.8	5.6	3.8	3.2	2.7	2.7
B but histidine left out	5.9	8.3	12.6	5.0	*2.7*	*1.8*	*2.0*	*1.8*
B „ lysine „ „	3.8	5.2	11.5	5.2	3.4	2.7	2.5	2.0
B „ tryptophane „ „	7.9	5.2	12.8	6.8	3.4	2.0	2.3	2.0
B „ {histidine + tryptophane} „ „	5.6	5.0	12.6	5.4	2.9	2.3	2.3	2.0
B „ {lysine + tryptophane} „ „	5.9	5.0	12.4	5.9	3.4	2.5	2.5	2.3
B „ phenylalanine left out	5.9	5.2	11.9	5.2	2.9	2.3	2.0	2.0
B „ {phenylalanine + histidine} „ „	4.3	5.0	12.4	5.4	3.2	2.5	2.3	2.0
B „ {phenylalanine + lysine} „ „	4.1	4.7	11.9	6.8	2.9	2.5	2.3	2.3
B „ cysteine „ „	*2.3*	10.6	10.1	*1.4*	2.9	2.7	2.9	3.4
B „ {cysteine + histidine} „ „	*2.0*	9.2	10.1	*1.1*	2.3	2.0	2.0	3.2
B „ {cysteine + lysine} „ „	*1.4*	7.4	8.1	*1.6*	2.7	2.7	2.7	2.9
C (B in which glycine and alanine were replaced by ammonia)	2.5	8.8	11.5	6.3	4.3	2.0	2.7	2.5
C but tryptophane left out	2.9	5.4	11.9	6.5	3.8	2.3	2.7	2.3
C „ phenylalanine „ „	5.2	4.5	11.5	7.2	4.1	1.8	2.3	2.0
C „ cysteine „ „	*1.4*	7.9	10.1	*0.5*	3.8	2.9	3.2	3.6
C „ {tryptophane phenylalanine} + cysteine left out	*1.4*	*3.6*	*3.4*	*1.1*	3.6	3.4	2.9	3.6

physiological amino acid solution (B). These *Thermobacteria* are therefore more particular in their food requirements than the higher animals. This does not seem to be the case with other lactic acid bacteria. *Thermobacterium helveticum* and *Streptococcus thermophilus* grow equally well in A and B; and *Streptobacterium casei* and the remaining *Streptococci* produce in a few cases as much acid in C, (a mixture still simpler than B) as in A. No evident difference between the action of phenylalanine and oxyphenylalanine (tyrosine) can be inferred from Table V. Less acid is usually

produced if both are absent. The reaction given by histidine and lysine is not very clear. *Streptococci* seem to suffer when histidine is omitted and *Thermobacteria* when lysine is not included. Additional experiments verified this. Curiously enough tryptophane, so important in animal nutrition, does not seem to be required by the lactic acid bacteria. *Streptococcus cremoris* 18 and *Streptococcus lactis* 33 produce less acid in solution B when tryptophane is absent. This, however, is not confirmed by the use of solution C. As it is difficult to decide whether the whey already contains the quantity of tryptophane sufficient for the lactic acid bacteria, we shall return to this question in experiments with purely synthetic media. Cysteine shows a most pronounced effect. It is of great importance for *Streptobacterium casei* and *Thermobacterium lactis*, but to a somewhat less extent for *Thermobacterium helveticum*, which is but little influenced by changes in nitrogenous food. It seems harmful for *Thermobacterium bulgaricum*. Subsequent experiments proved, however, that cysteine is indispensable for this bacterium just as much as for other Thermobacteria. Only it soon gets too much because this amino acid strongly influences the reduction potential. In accordance herewith *Thermobacterium bulgaricum* suffers from the ommission of cysteine when tryptophane and phenylalanine are left out simultaneously. Cysteine does not seem to exercise any beneficial effect, but rather a harmful one as far as the streptococci are concerned. This was also confirmed later. Except for *Thermobacterium lactis*, the *Thermobacteria* tested grow equally well whether they have glycocoll + alanine or ammonium salts at their disposal, while *Streptobacteria* and *Streptococci* as a rule prefer the latter.

After this preliminary discussion we shall separately consider the 3 genera of lactic acid bacteria studied by us, namely, *Thermobacteria*, *Streptobacteria*, and *Streptococci*.

First we have to find out why *Thermobacterium lactis* 10 and *Thermobacterium bulgaricum* grow better in the complete mixture of amino acids than in the physiological solution. In the latter solution tryptophane was left out and one half of the phenylalanine was replaced by tyrosine. This physiological solution containing cysteine, lysine, histidine, tyrosine, and phenylalanine besides glycocoll + alanine, is presented as D in Table VI. Cultures were titrated after 6 days of incubation at 37° C.

The most important conclusion from Table VI is that the *Thermobacteria*, which are all specific milk bacteria, produced more acid in the complete, and as a rule also in the physiological solution of amino acids, than in milk diluted with whey to a 0.2 per cent nitrogen content. This proves that our *Thermobacteria* find the necessary nutrients in whey with A, and that no important substances are lacking in whey with D. The addition of tryptophane failed to produce any appreciable results in this case also. In order to decide which acids are responsible for the better growth of *Thermobacterium lactis* 10 and of *Thermobacterium bulgaricum* in A than in D the substances only present in A were tested by adding them to D. Further we have tried the effect of glutathione. The table shows that aspartic

Table VI.

Nutrient medium	Species of bacteria				
	Tbm. lactis 9	*Tbm. lactis* 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Tbm. Joghurt*
Milk diluted with whey to 0.2 % N	4.1	6.5	11.7	11.7	10.1
A (complete mixt. of amino acids)	19.6	17.3	19.4	17.1	16.7
A + glutathione	**20.7**	*11.7*	19.4	**18.9**	**20.0**
D (physiological mixt. of amino acids)	18.7	11.0	11.5	16.0	17.1
D + glutathione	18.5	*3.8*	11.9	**17.1**	**20.3**
D + tryptophane	18.9	11.5	11.7	17.1	17.8
D + leucine	18.9	10.8	12.2	16.0	18.0
D + proline	18.0	10.4	10.8	16.2	18.5
D + leucine and proline	18.9	9.2	12.4	15.8	17.6
D + arginine	18.5	13.1	**14.0**	16.7	18.9
D + aspartic acid	18.7	**15.8**	11.9	18.5	19.6
D + asparagin	18.9	**16.2**	13.1	18.7	19.6
D + leucine and asparagine	18.5	**16.2**	11.5	17.3	18.5
D + glutamic acid	**19.6**	9.7	13.1	17.1	**20.0**
D + diketopiperazine	19.1	12.4	12.4	16.4	19.1
D + ammonium salts	19.1	11.7	12.8	17.8	18.5
Whey, no addition	0.5	0.2	5.9	2.5	0.9

acid and particularly asparagine are the decisive substances for *Thermobacterium lactis* 10. On the other hand, *Thermobacterium bulgaricum* probably requires several nitrogenous substances added to D, to render it as suitable a medium as A. Of these arginine seems to act most favourably. *Thermobacterium helveticum* thrives almost equally well in D and in A, and the same holds for *Thermobacterium lactis* 9, and *Thermobacterium Jugurt*. Glutamic acid (and possibly Diketopiperazine) have a beneficial effect on *Thermobacterium lactis* 9 and on *Thermobacterium Jugurt*, and asparagine (aspartic acid) has a similar action on *Thermobacterium helveticum* and *Thermobacterium Jugurt*, besides on *Thermobacterium lactis* 10. The best, and yet the simplest possible mixture for *Thermobacteria* must contain asparagine, glutamic acid and arginine in addition to the amino acids present in D.

It has already been shown in our experiments on the vitamin requirements of the lactic acid bacteria that glutathione compared with lactoflavin has no influence upon the lactic acid fermentation. We tested it however, as it might possibly be of some importance as a building stone. Table VI shows that glutathione has a favourable effect upon the Thermobacteria, with the exception of *Thermobacterium lactis* 10, towards which it is particularly harmful when added to solution D. This is in agreement with

our previous findings[1]), and this restrained us from adding glutathione to the mixture of amino acids used above.

The *Streptobacteria* are much less particular in their nitrogen nutrition requirements than the *Thermobacteria*, which emphasizes their position as a separate genus of the rod-shaped lactic acid bacteria. Judging from Table VII — the *Streptobacteria* are satisfied with ammonium salts as long as a trace of cysteine is present. The cultures were titrated after 6 days of incubation.

Table VII.

Nutrient medium	Species of bacteria				
	Sbm. casei 11	*Sbm. casei* 34	*Sbm. plantarum* 14	*Sbm. plantarum* 21	*Sbm. plantarum* 33
Milk diluted with whey to 0.2 % N	7.2	2.9	4.5	0.7	6.3
Whey containing 0.15 % cysteine N	0	0	0	0	0
„ „ 0.015 % „ + ammonium salts	**9.7**	**8.8**	3.2	**4.1**	4.7
„ „ 0.0015 % „ + „ „	**9.7**	7.0	3.2	3.2	**6.3**
„ „ 0 „ + „ „	0.3		2.0		

Other experiments showed that *Streptobacterium casei* can produce as much acid in whey with cysteine and ammonium salts as in whey with a complete mixture of amino acids (A). *Streptobacterium plantarum* requires some extra histidine and lysine.

Finally *Streptococci*, similarly to *Streptobacteria*, are satisfied with ammonium salts when grown in whey, but in contrast to *Streptobacteria* they thrive better without cysteine. The highest acid production is not reached, however, until a little amino acid is added, and, according to Table VIII, the best results are obtained with a mixture of histidine and leucine. Cultures were titrated after 6 days of incubation.

The fact that more acid was produced in certain cases in whey with ammonium salts, histidine and leucine, than with the complete mixture A, is due to the harmful influence of cysteine present in A.

The indispensable nitrogen sources as well as the necessary growth factors have now been determined, and from these data it should be a simple matter to compose a synthetic medium suitable for the lactic acid bacteria. Nevertheless, various difficulties had still to be overcome.

[1] The Vitamin Requirement of the Lactic Acid Bacteria, Table VII a, p. 16.

Table VIII.

Nutrient medium	Species of bacteria.					
	Sc. thermophilus 7	*Sc. cremoris* 18	*Sc. lactis* 22	*Sc. fæcium* 6	*Sc. glycerinaceus* 1	*Sc. liquefaciens* 1
Milk diluted with whey to 0.2 % N	4.1	3.8	4.3	2.9	2.7	5.0
Whey + complete mixt. of amino acids	4.3	4.1	4.3	3.4	3.8	3.8
Whey + ammonium salts, histidine and leucine	**4.5**	**5.4**	**4.5**	**3.4**	**3.6**	**5.0**
+ „ „ and histidine	2.6	4.7	4.1	3.0	2.7	4.3
„ + „ „ „ leucine	2.6	5.0	4.3	3.4	3.2	4.5
„ + „ „ „ alanine	2.5	4.1	3.8	2.7	2.5	4.1
„ + „ „ „ glycine	2.5	3.4	3.4	2.5	2.3	3.8
„ + „ „ „ asparagine	2.3	3.4	3.4	2.5	2.5	3.8
„ + ammonium salts only	2.3	4.5	3.8	2.7	2.7	4.3

Salts used for the synthetic media besides the negligiable amount found in tap water, were adjusted in the following proportion: 0.5 per cent K_2HPO_4, 0.1 per cent $MgSO_4$, 0.1 per cent NaCl, 0.001 per cent $FeCl_3$, and small amounts of Cu and Zn, similar to those present in milk. In the preliminary experiments 0.05 per cent inositol and 0.1 per cent arabinose were used in addition to the usual 2 per cent glucose. Inositol has been used because it belongs to the bios complex of yeast, and arabinose because S. Orla-Jensen proved that under certain conditions pentoses can have as favourable an influence upon the lactic acid bacteria as methylglyoxal[1]). These additions proved worthless. Thiocyanates, traces of which are claimed to be present in milk according to some investigators had no influence either. Since milk and whey contain citric acid, ammonia and creatine, we supplied our media with corresponding quantities of diammoniumcitrate and creatine. The last substance, well known as the most important constituent of meat extract, has a none too pronounced, but favourable, effect upon the development of the lactic acid bacteria.

The effect of yeast nucleic acid was repeatedly tested in our experiments because the lactic acid bacteria might possibly need special nucleic substance for growth in addition to amino acids. Also the effects of proteolytic enzymes and peptone Witte were studied. Our solutions could of course, after an addition of these materials of ill defined composition, not more be considered as purely synthetic. The action of proteolytic enzymes was studied because it was proved in our work on growth factors present in peptones, that these enzymes contain thermostable compounds favouring the growth of the lactic acid bacteria, and also because whey contains a proteolytic enzyme galactase. The effect of peptone Witte was tested in order to

[1] Hitherto unknown activators for the growth of Lactic Acid Bacteria, Journal of Chemical Industry, 1933, Lll.

investigate whether the lactic acid bacteria need some of the rather complicated intermediate products of the proteins. The additions mentioned above were used in quite small amounts only, corresponding to 0.01 per cent nitrogen (when nothing else is stated). A similar amount of nitrogen originated from added bios. An extra quantity of glycocoll was added to the media not containing foreign substances, in order to bring the media to a nitrogen content of 0.22 per cent in all cases.

Experiments with whey have shown that *Thermobacteria* need the following amino acids for their optimal development: cysteine, tyrosine, phenylalanine, lysine, histidine, arginine, glutamic acid, and asparagine. As already mentioned, creatine has furthermore a favourable action. Glycocoll, alanine and ammonium citrate were used as "fillers". Small quantities of tryptophane, diketopiperazine, and glutathione were added in our first experiments, as we did not know at the time whether the reason why these compounds have no significant effect might be that they were already present in sufficient quantities in the whey. At last 0.5 mg lactoflavin per liter medium was added, and the amounts of bios occurring in milk. This mixture was called E.

Table IX.

Nitrogen source	Species of bacteria							
	Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*	
0.21 % as E + 0.01 % as glycine	2.5	5.2	0	1.9	2.0	3.3	0.6	2.4
0.21 - „ E + 0.01 - „ nucleic acid from yeast	3.8	5.6	0	2.0	2.9	3.9	0.8	2.9
0.21 - „ E + 0.01 - „ pepsin	3.6	6.3	0	3.5	3.2	4.8	1.8	4.0
0.21 - „ E + 0.01 - „ pancreatine	4.3	6.5	3.4	5.8	2.3	3.6	**3.8**	**6.8**
0.21 - „ E + 0.01 - „ peptone "Witte"	6.1	10.1	1.1	8.0	3.8	5.3	2.3	4.7
0.11 - „ E + 0.11 - „ „ „	**9.2**	**11.1**	2.9	**8.1**	4.5	5.5	3.4	4 1
0 - „ E + 0.22 - „ „ „	8.1	10.2	**3.6**	6.4	**6.1**	**6.5**	2.9	4.9
0 - „ E + 0.22 - „ „ „ without addition of bios and lactoflavin	*2.5*	*3.5*	*0.3*	*0.7*	*0.5*	*1.6*	*0*	*0.5*
Number of hours before titration	46	94	46	94	46	94	46	94

While the *Thermobacteria* produce from 10 to 20 tenths of per cent acid in whey with the optimal mixture of amino acids they formed much less acid in our experiments with synthetic media (Table IX). Growth was found although it was scanty and the rods were rather slender. Their volutin grains are, however, of normal size, thus bigger than the rods themselves. They stick out like the ribs of a starved animal and with methylene blue they are stained red instead of blue as always when the bacteria are cultivated under unfavourable conditions. The lack of one of the necessary amino acids usually giving only a small deviation, it cannot be doubted that the poor growth and the low acid formation of the *Thermobacteria* in the synthetic solutions is not caused by such a lack, but rather by the absence of some unknown

activator still present in the whey. This is also indicated by the fact that the rod-shaped lactic acid bacteria form more acid in milk than they do either in casein peptone (C) with lactoflavin and bios added, or in 2C which contains twice the nitrogen present in milk. The difference is particularly striking in the case of *Thermobacterium helveticum*, see Table II p. 27 in the paper entitled: Growth factors present in peptones. This point will be discussed later.

Table IX shows that all the compounds added and especially peptone WITTE have a favourable effect. The largest amount of acid is generally obtained with equal parts of amino acid mixture and peptone WITTE. When the activators are taken away the *Thermobacteria* form but little acid in the pure solution of peptone WITTE.

According to our experiments with whey *Streptobacteria* need only ammonium salts and a little cysteine as nitrogen source. They prefer to have some glutamic acid and creatine in addition. We further added in our first experiments tryptophane, diketopiperazine and glutathione, as we did in the case of the *Thermobacteria*. This mixture of nitrogenous compounds with bios and lactoflavin is called F in table X. The results were not much more encouraging than they were for the *Thermobacteria* although the nitrogen food was more varied than strictly necessary. Almost the same amount of acid was produced with equal parts of F and peptone WITTE as in whey with the necessary nitrogen compounds (see Table VII), and the *Streptobacteria* produced just as much in F alone as in peptone WITTE with both activators and considerably more than in peptone WITTE without activators.

Table X.

Nitrogen source	Species of bacteria	
	Sbm. casei 11	
0.21 % as F + 0.01 % as glycine	1.4	3.4
0.21 - „ F + 0.01 - „ nucleic acid from yeast	3.5	3.5
0.21 - „ F + 0.01 - „ pepsin	2.3	4.0
0.21 - „ F + 0.01 - „ pancreatine	2.0	4.3
0.21 - „ F + 0.01 - „ peptone "Witte"	2.5	5.6
0.11 - „ F + 0.11 - „ „ „	**6.3**	**7.2**
0 - „ F + 0.22 - „ „ „	0.6	3.2
0 - „ F + 0.22 - „ „ „ without addition of bios and lactoflavin	0.5	1.7
Number of hours before titration	65	113

The *Streptococci* thrive in our whey experiment with ammonium salts as the only source of nitrogen. However, a favourable effect is produced when histidine, leucine, and creatine are added; also in this case, as a precautionary measure, tryptophane, diketopiperazine, and glutathione were added in the first series of experiments. Bios was the only activator used. This mixture is designated G.

Table XI.

Nitrogen source	Species of bacteria					
	Sc. thermophilus 7		*Sc. cremoris* 18		*Sc. lactis* 22	
0.21 % as G + 0.01 % as glycine	4.3	4.6	1.4	2.5	0.9	2.0
0.21 - „ G + 0.01 - „ nucleic acid from yeast	5.2	5.7	2.0	3.6	1.8	3.4
0.21 - „ G + 0.01 - „ pepsine	4.5	5.2	3.6	4.2	2.5	3.4
0.21 - „ G + 0.01 - „ pancreatine	**5.2**	**5.6**	**4.5**	**5.6**	**5.2**	**5.6**
0.21 - „ G + 0.01 - „ peptone "Witte"	5.2	5.5	3.6	5.3	3.0	3.6
0.11 - „ G + 0.11 - „ „ „	5.0	5.5	3.6	4.5	4.7	5.0
0 - „ G + 0.22 - „ „ „	3.2	3.2	2.9	3.3	3.0	3.4
0 - „ G + 0.22 - „ „ „ no addition of bios	3.2	3.6	1.0	1.8	2.6	3.0
Number of hours before titration	46	94	46	94	46	94
Whey + ammonium salts, histidine and leucine		4.5		5.4		4.5

Table XI shows that we have reached our goal in the case of *Streptococcus thermophilus* for it forms as much acid in a pure synthetic medium as in whey with ammonium salts and amino acids. The two other *Streptococci* do not reach their optimum without the addition of a small amount of a substance of unknown composition as pancreatine or peptone Witte. Pure peptone Witte has a decidedly weaker action than if mixed with G. The omission of the activator from the peptone solution is not of the same significance for the *Streptococci* as for the lactic acid rods. Yeast nucleic acid seems to play a greater part for the *Streptococci* than for the rod-shaped lactic acid bacteria.

We have so far always added tryptophane, diketopiperazine, and glutathione to the synthetic solution together with the nitrogen nutrients absolutely necessary for the bacterium under observation. To be sure it was found in experiments with

Table XII.

The nitrogenous compounds necessary for the Thermobacteria were added in all cases	Species of bacteria							
	Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*	
M without tryptophane	13.7	20.0	5.0	17.8	11.9	13.3	14.4	18.2
M with „	15.3	21.2	4.7	18.0	12.6	13.3	16.9	17.6
S without „	5.4	7.2	0.3	6.5	1.4	2.7	0.5	2.0
S with „	1.8	8.8	0.1	5.4	0.6	4.7	0	1.6
Number of hours before titration	43	116	43	116	43	116	43	116

whey deprived of protein that lactic acid bacteria do not need tryptophane. However, it might be possible that the whey contained enough of this amino acid, so important in the life of animals. In order to gain certainty in this matter experiments with synthetic solutions were carried out, both with and without tryptophane. The solutions contained 0.22 per cent nitrogen of which the 0.02 per cent originated from tryptophane or from an equivalent amount of glycocoll. The data from these experiments can be found in Tables XII, XIII, and XIV. M, means whey, and S a synthetic medium.

Table XII shows that the acid formation of the *Thermobacteria* is usually increased a little by the addition of tryptophane to the solution of amino acid in whey, but decreased in the pure synthetic solutions. The deviations are within the experimental error and it may thus be presumed that the *Thermobacteria*, otherwise so particular in their nitrogen requirements, really do not need tryptophane.

Table XIII.

The nitrogenous compounds necessary for the Thermobacteria were added in all cases	*Streptococcus thermophilus* +									
	Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*		0	
M without tryptophane	12.8	19.6	..	..	11.0	14.0	16.9	18.2	3.8	4.1
M with „	15.8	21.4	..	18.5	12.8	16.2	17.1	17.1	3.8	4.3
S without „	7.4	11.7	6.1	9.2	5.4	5.6	5.0	7.4	4.3	5.6
S with „	4.7	5.6	5.9	7.0	5.4	5.6	5.0	5.9	5.4	5.6
Number of hours before titration ...	43	116	43	116	43	116	43	116	43	116

The conclusion drawn above is further substantiated by Table XIII. The *Thermobacteria* were here inoculated together with *Streptococcus thermophilus*. Such a mixed infection frequently occurs, e. g. *Streptococcus thermophilus* with *Thermobacterium bulgaricum* and *Thermobacterium jugurt* in Yoghurt, and with *Thermobacterium helveticum* in fresh Emmental cheese. The *Thermobacteria* have an optimum hydrogen ion concentration which is higher than that of milk, and it may be supposed that *Streptococcus thermophilus*, so to say, prepares the way for the *Thermobacteria*. When Tables XII and XIII are compared, it is not possible to confirm this supposition for the complete medium, whey with amino acids; probably because the difference is eliminated already after 43 hours. On the other hand, a favourable effect can be detected for the mixed culture in the inferior synthetic nutrient medium, where the growth of the lactic acid bacteria is slower. The acid formed after 43 hours by the mixed organisms in two cases (*Thermobacterium lactis* 10 and *Thermobacterium helveticum*) surpassed even the sum of the acidities formed by each of the bacteria. It is furthermore evident from the table that *Streptococcus thermophilus* is not influenced by the addition of tryptophane.

Similar experiments have been carried out with *Streptobacterium casei* alone

and in combination with *Streptococcus lactis* and *Streptococcus cremoris*. This mixture will occur sooner or later in all non-cooked cheeses. The data are found in Table XIV.

Table XIV.

The nitrogenous compounds necessary for the Streptobacteria were added in all cases	Bacteria:					
	Sbm. casei 11		*Sbm. casei* 11 *Sc. lactis* 22 *Sc. cremoris* 18		*Sc. lactis* 22 *Sc. cremoris* 18	
M without tryptophane	9.7	11.5	10.6	12.2	2.7	2.7
M with „	9.5	11.3	10.6	12.8	2.9	3.2
S without „	2.0	2.5	4.7	5.4	3.8	4.1
S with „	2.7	2.5	5.0	5.0	2.7	2.9
Number of hours before titration	116	139	116	139	116	139

These bacteria are still less influenced by the addition of tryptophane than the *Thermobacteria*. The largest quantity of acid is obtained when *Streptobacteria* and *Streptococci* are inoculated simultaneously. The increase in the complete nutrient medium is very small however, since the shortest period (116 hours) must be made comparatively long because the *Streptobacteria* grow so slowly.

Experiments with diketopiperazine were carried out similarly to those with tryptophane. A very feeble action could be observed for the *Streptobacteria*. Also histidine has been tried out. This amino acid which favours the growth of the *Thermobacteria* and the *Streptococci*, has no value for *Streptobacterium casei* 11. Finally the significance of glutathione in synthetic solutions was tested more closely, partly as a substitute for cysteine (used in amounts so as to give the same sulphur content) and partly as a supplement to cysteine. The latter series of experiments were extended to include some concerning the yet undiscovered activators (see the following tables).

Although we have previously shown, (in our work on the vitamin requirements of the lactic acid bacteria) that vitamin B_1 is without significance for the lactic acid bacteria, we have nevertheless considered it wise to make sure whether our synthetic media could not be improved by the addition of vitamin B_1. A little of the crystallised preparation was offered to us through the courtesy of Professor Windaus from Göttingen. It was used in the same amount as lactoflavin, 0.5 mg per liter. The data for the *Thermobacteria* and the *Streptobacteria* are found in Table XV, and for the *Streptococci* in Table XVI. Each of these bacterial groups has received its specific nitrogen compounds. As usual the rods received bios and lactoflavin in addition, and the *Streptococci* only bios. It has been investigated at the same time what happens when the rods receive glutathione instead of cysteine, and when the *Streptococci* are given lactoflavin in addition to bios.

Table XV.

Synthetic medium with bios and lactoflavin		Species of bacteria									
		Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*		*Sbm. casei* 11	
with cysteine	without B_1	0.8	5.9	1.1	2.5	0.2	1.8	1.6	2.5	0	2.0
	with „	0.9	6.5	1.2	2.9	2.3	2.5	2.0	2.5	0.6	2.5
with glutathione	without B_1	1.1	6.5	1.4	1.6	1.8	1.1	2.7	3.2	2.0	3.2
	with „	1.1	6.8	0.9	1.6	0.5	1.8	2.5	3.8	1.6	3.2
Number of hours before titration		29	144	95	144	95	144	95	144	95	144

Table XV indicates that the organisms tested grow equally poorly in the nutrient medium with cysteine and with glutathione, and irrespectively of the presence or absence of vitamin B_1. Only in the case of *Thermobacterium helveticum* and of *Streptobacterium casei* it may be to some advantage to use glutathione instead of cysteine.

Table XVI.

Synthetic medium		Species of bacteria					
		Sc. thermophilus 7		*Sc. cremoris* 18		*Sc. lactis* 22	
with bios only	without B_1	4.1	5.0	1.1	3.6	1.7	2.9
	with „	3.8	4.3	1.1	3.4	1.6	2.7
with bios and lactoflavin	without B_1	4.3	5.0	1.4	4.1	1.6	3.4
	with „	4.3	5.2	1.2	4.1	1.8	3.8
Number of hours before titration		29	144	29	144	29	144

Neither in the case of the *Streptococci* does an addition of vitamin B_1 play any role. Ou the other hand, as we have already seen, *Streptococcus cremoris* and *Streptococcus lactis* produce some more acid with bios and lactoflavin than with bios alone.

Our experiments with synthetic solutions of amino acids indicate clearly that the rod-shaped lactic acid bacteria do not reach full development if only bios and lactoflavin are added. These two activators are sufficient to give milk treated with norite charcoal its full value again for the lactic acid bacteria. Thus milk must still contain a necessary activator which differently from the two others is not adsorbed on the charcoal. As will appear from the previous discussion and from the data just presented the lacking activator cannot be vitamin B_1. It must be present in the protein-free whey, which on addition of amino acids can be made into an excellent nutrient medium for the lactic acid bacteria. An attempt has been made to isolate or to concentrate this activator. The procedure was as follows. Whey treated with acti-

vated charcoal was evaporated in vacuum to one fifth of its original volume, then placed in the refrigerator for 24 hours and filtered to get rid of as much lactose as possible. Sufficient ethyl alcohol was added to the filtrate to give a mixture containing 60 per cent alcohol. A voluminous protein-like precipitate was formed which, however, did not amount to much after having been collected on the filter. The precipitate as well as the filtrate after the alcohol had been distilled off, were tested as possible sources of activators. The filtrate did not show any action in this direction and the precipitate (P), as Tables XVII and XVIII show, had hardly as much effect as the same amount of nitrogen in the form of peptone Witte (W). Our media received a quantity of precipitate and milk bios similar to that normally present in whey. Double the amount was also used. The nitrogen contents of these admixtures were equivalent to 0.01 per cent and 0.02 per cent respectively. The significance of an addition of further 0.2 per cent glutathione was tested in the same series of experiments. The total nitrogen content was standardized with glycocoll to 0.22 per cent in all media. Of course the nitrogen compounds of specific value for the *Thermobacteria*, *Streptobacteria*, and *Streptococci* were added also.

Table XVII.

Synthetic media with bios and lactoflavin adjusted to 0.22 % N	Species of bacteria									
	Tbm. lactis 9		*Tbm. lactis* 10		*Tbm. bulgaricum*		*Tbm. helveticum*		*Sbm. casei* 11	
With glutathione, without further addition	4.5	6.8	1.6	3.2	1.2	2.0	2.0	3.6	2.0	2.3
Without „ „ „ „	4.5	6.8	2.3	4.3	1.1	4.0	2.5	3.4	2.0	2.5
„ „ with 0.01 % N as P	6.8	8.6	3.8	5.4	2.7	4.1	3.4	5.2	5.0	5.2
„ „ „ 0.02 - N - P	7.2	9.5	4.1	6.8	3.4	5.4	4.1	6.3	5.4	5.9
„ „ „ 0.01 - N - W....	7.4	13.1	7.9	10.1	8.1	10.6	6.5	8.8	6.3	7.2
„ „ „ 0.02 - N - W....	10.4	14.0	9.5	11.7	6.5	10.1	7.4	10.6	8.3	8.6
Number of hours before titration	65	116	65	116	65	116	65	116	116	142

Table XVIII.

Synthetic media with bios and lactoflavin adjusted to 0.22 % N	Species of bacteria					
	Sc. thermophilus 3		*Sc. cremoris* 18		*Sc. lactis* 22	
With glutathione, without further addition	4.7	5.2	2.5	3.4	2.5	2.9
Without „ „ „ „	3.2	4.7	2.7	3.8	2.5	3.4
„ „ with 0.01 % N as P	3.8	5.4	2.5	4.3	3.4	3.8
„ „ „ 0.02 - N - P	4.1	5.6	2.5	4.1	3.2	4.1
„ „ „ 0.01 - N - W	4.3	6.1	3.2	4.3	2.5	2.7
„ „ „ 0.02 - N - W	4.7	5.9	3.6	4.3	2.3	2.9
Number of hours before titration	22	116	41	116	65	142

These data show, as mentioned above, that it is unlikely that the precipitate (P) from whey has any other action than that of a protein. This action is less pronounced than that of the equivalent amount of peptone WITTE, except in the case of *Streptococcus lactis* 22. The data furthermore bring out the point that the media are not improved by the addition of glutathione. It must be remembered that the media for the *Thermobacteria* and for the *Streptobacteria* contain cysteine, while the media for the *Streptococci* do not contain this amino acid. We have already seen that nothing is gained (Table XV) by replacing cysteine by glutathione. Thus there is no more reason for the addition of glutathione than of tryptophane to our media.

As the activator lacking for the lactic acid rods was not to be found either in the precipitate, or in the filtrate in the last experiment, the possibility still remains that it might have been removed with the small amount of lactose crystallizing out before the addition of alcohol. Hence another lot of protein-free whey was evaporated to one fifth of its volume, this time without previous treatment with activated charcoal as the filtrate was not to be used. Five per cent of the impure lactose crystallizing out in the refrigerator were added to the synthetic solution prepared for the *Thermobacteria* as well as to the one prepared for the *Streptobacteria*. This addition was called X. All media were given bios and lactoflavin as usual. The culture tubes were titrated after 5 days in the case of the *Thermobacteria* and after 6 days in the case of the *Streptobacteria*.

Table XIX.

Nutrient medium	Species of bacteria				
	Tbm. lactis 9	*Tbm. lactis* 10	*Tbm. bulgaricum*	*Tbm. helveticum*	*Sbm. casei* 11
Synthetic medium without X.....	5.4	2.5	2.5	3.2	2.5
„ „ with X.....	17.3	11.7	7.4	13.5	6.8
Whey + amino acids.....	18.9	15.1	9.5	15.8	8.3

Table XIX shows the effect of the added impure lactose and it cannot be doubted that the activator still lacking must be present in it. By the addition of X almost as much acid is formed in the synthetic solution as in the whey with amino acids. If a larger amount of the lactose had been added we should have reached the full value. Thus we are justified in presuming that the lactic acid rods require yet another activator besides milk bios and lactoflavin.

Summary.

1) Acid whey rendered almost protein-free without the use of special protein precipitants contains all activators necessary for the development of the lactic acid bacteria. It is, however, a very poor nitrogen source and for this very reason

it is well suited for the investigation of the nitrogen compounds necessary for the lactic acid bacteria. Yet the data thus obtained must be confirmed by additional experiments with pure synthetic media.

2) The lactic acid bacteria occurring in milk can utilize colloidal caseinates as completely as peptones, although they do not excrete proteolytic enzymes. These bacteria thrive so much better in milk than in whey exclusively because of the presence of caseinates in the former. Those lactic acid bacteria that cannot utilize caseinates are not able to sour milk even though they may ferment lactose. Lactalbumin seems to be without any significance for the nutrition of the lactic acid bacteria.

3) The *Thermobacteria* as well as other lactic acid bacteria do not require tryptophane, but in other respects they have just as high requirements as to their nitrogenous food, as have the higher animals or rather still higher. Thus they need more or less cysteine, tyrosine (or phenylalanine), lysine, histidine, arginine, glutamic acid, asparagine, and creatine. An addition of glutathione may prove useful in some cases, but very harmful in others; the oxidation reduction potential probably being involved.

4) The *Streptobacteria* develop with ammonium salts and cysteine as the only sources of nitrogen. Creatine, diketopiperazine, and perhaps also glutamic acid may be useful. *Streptobacterium plantarum*, but not *Streptobacterium casei*, thrives better when histidine and lysine are added.

5) The *Streptococci* grow with ammonium salts as the only source of nitrogen, but they prefer an addition of histidine and leucine. Creatine and nucleic acid of yeast act favourably.

6) It was shown in our work on the vitamin requirements of the lactic acid bacteria that all the true lactic acid bacteria require milk bios and lactoflavin, but the rods need more lactoflavin than the *Streptococci*. This has been confirmed in the present investigation. We have further shown that the lactic acid rods require one more activator besides the two just mentioned. This activator is not adsorbed on activated charcoal, but can be obtained by evaporation of whey with the first fraction of lactose crystals.