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Accessory Factors in Lactic Fermentation.

OTTO RAHN AND C. P. HEGARTY.

From the Laboratory of Bacteriology, New York State College of Agriculture, Cornell University, Ithaca, N. Y.

A previous paper[1] had shown that the rate of lactic-acid formation by *Streptococcus lactis* can be measured by placing the cells, obtained from a 12-hour culture by means of a centrifuge, into a buffered glucose solution and determining the acid by titration. In all experiments mentioned in the previous paper, 0.5% peptone had been added to the buffered glucose solution; this was essential for a rapid rate of fermentation. The present paper is a study of the rôle of peptone or other accessory factors.

Washing of Cells. In order to study the effect of accessory compounds, it was necessary to remove any traces of peptone or other compounds of the original medium from the cells. This was accomplished by resuspending the centrifuged cells in buffer, using an egg-beater, centrifuging again, and repeating this operation at least once. The cells from 250 cc. of culture were then suspended in 50 cc. of phosphate buffer plus 2% glucose, and the acid was titrated every half-hour. In one experiment, the cells were tested after each washing in buffered glucose solution without and with peptone. Since the number of cells after each washing was different, the rate of fermentation is measured by the fermenting capacity (abbreviated F.C.) *i. e.*, mg. acid per cell per hour, expressed in units of 10^{-10} mg. The result of washing is shown in Table I.

TABLE I.
Fermenting Capacity.

	Without peptone	With .5% peptone	Increase by Peptone %
Unwashed	1.72	6.14	256
Washed once	1.22	6.53	435
" 2 times	1.81	7.46	313
" 3 "	1.81	8.00	342
" 4 "	1.37	6.00	338

In some experiments, the effect of peptone caused an even greater increase, in others it produced a much smaller effect, but the rate of fermentation was always increased (Table II). This observation

[1] Rahn, O., Hegarty, C. P., and Deuel, R. E., *J. Bact.*, 1938, **35**, in press.

means that the rate of acid-formation is controlled not only by glucose, but also by other substances in the medium surrounding the cells. This fact helps to explain the poor growth of bacteria in synthetic media. They have not only a very limited choice of building material, but their rate of fermentation, and consequently their vital energy, is considerably lower than in the presence of peptone.

Two explanations for this additional effect of peptone present themselves. Either some part of the peptone is an accessory fermentation-factor, perhaps of the nature of a co-zymase, or the peptone contains a reducing compound which keeps out the oxygen, or establishes a redox potential more favorable for lactic-acid formation.

Previous experiments (loc. cit.) had shown an air-current to retard, and a nitrogen-current to accelerate the rate of fermentation compared with that of a suspension standing quietly in air. If peptone acted merely as oxygen-acceptor, it should have no effect in an atmosphere of nitrogen. However, experiments showed the increase in nitrogen to be larger than in an air-current.

Na_2SO_3 was used to reduce the redox potential.[2] In all experiments, with or without peptone, with amounts varying from 0.05 to 0.40% (anhydrous), fermentation was retarded by this reducing compound. Thioglycolic acid retarded slightly, and so did iron powder reduced in hydrogen. On the other hand, the oxidizing compound $K_3Fe(CN)_6$, in concentration of 0.16%, accelerated fermentation in presence of peptone, and was without influence when peptone was omitted. It does not seem probable, from these results, that the peptone compounds or other stimulating agents act by establishing a more favorable redox potential. The alternative is the assumption of an accessory fermentation-factor.

Our search for the chemical nature of the active substances in peptone was started by testing the compounds which according to Meyerhof and Lohmann, Warburg, and von Euler[3] are contained in the co-zymase system. Since we worked with living cells, it seemed sufficient to supply the simple components, nicotinic acid and adenine. Ribose as such could not be tested on account of the prohibitive price. The small amount furnished in 500γ lactoflavin per 100 cc. produced no effect. Adenine in quantities of 0.02 to 0.60% produced no effect except when in combination with several other

2 Knaysi, G., and Dutky, S. R., *J. Bact.*, 1934, **27**, 109.

3 A summary by Robison of the very extensive literature may be found in *Annual Review of Biochemistry*, 1936, **5**, 187.

compounds. Nicotinic acid (0.002%) stimulated the rate of fermentation in every experiment (Table II).

Another substance important for the production of lactic acid in muscle gave positive results with bacteria, namely magnesium ions. Concentrations of 0.05 or 0.10% $MgSO_4$ were used; 0.01% did not seem quite sufficient for maximal rates. In all later experiments, $MgSO_4$ was added to the buffer. Since the glucose buffer is free from nitrogenous substances, ammonium chloride, asparagin, alanine and cysteine were added. Only the latter gave a slight increase when 0.1% was offered. Inositol, a compound important for yeast growth, had no effect. Lactoflavin, which had been found by Orla-Jensen[4] to increase the final amount of acid in cultures of various lactic-acid bacteria, showed no change in the rate of fermentation when 500γ per 100 cc. were added.

Most of our experiments centered around the variable effect of ascorbic acid. This was always used in a concentration of 0.02%. A comparison of all experiments showed that vigorous fresh cells were not benefited by ascorbic acid, in fact, there was a decrease in some cases. When the cells were injured, by heating, by holding at 0°C. for several days, or by the use of water in place of buffer, the power of cells to produce colonies on agar was lost more rapidly than their fermenting capacity. The same has been observed before with yeast cells by Rahn and Barnes.[5] In all these cases, the cells were benefited by ascorbic acid (Table II).

In 3 experiments, the centrifuge became so overheated that the cells failed altogether to produce colonies, though they still fermented. Here, the ascorbic acid caused the greatest increase, namely, 400%, 500% and 1100%. Different kinds of injury all resulted in a need for ascorbic acid, and it seems that the greater the injury, the greater is the benefit from this compound, relatively as well as absolutely, in mg. of acid per equal quantities of cells. This may be seen from the table. It is also evident when comparing the effect of the holding-time at 0°C. in different experiments. The increase obtained from addition of ascorbic acid was

	%
with cells tested fresh	− 3
" " held ½ day at 0°	−16
" " " 1½ days " "	7
" " " 2 " " "	63
" " " 3 " " "	30

[4] Orla-Jensen, S., Otte, N. C., and Snog-Kjaer, A., *Mémoires Acad. Sciences Danemark*, 9th ser., 1936, **6**, 5.

[5] Rahn, O., and Barnes, M. N., *J. Gen. Physiol.*, 1932, **16**, 579.

TABLE II.

Increase of Rate of Lactic Acid Formation by Peptone, Nicotinic and Ascorbic Acids.

	Cell treatment	Million cells per cc.	F.C. in buffer, 10^{-10} mg.	Percentage increase by addition of .5% peptone	.002% nicotinic acid	.02% ascorbic acid	nicotinic plus ascorbic acids
I.	Washed twice	4,900	1.54	94	—	9	115
II. a	" in buffer, stored in buffer	5,000	2.52	60	—	24	—
b	" " " " as moist cells	3,800	2.52	100	—	25	—
c	" " water, " in buffer	2,000	3.15	120	—	85	—
d	" " " " " water	1,780	3.51	100	—	75	—
e	" " " " as moist cells	1,120	4.80	140	—	116	—
III. a	Cells washed twice	2,200	4.46	70	31	−21	35
b	Same cells, heated 20 min. to 42°C	1,500	4.10	130	52	0	52
c	" " " 10 " " 45°C	1,800	4.40	110	56	− 5	59
d	" " " 20 " " 45°C	1,215	4.40	120	75	20	75
IV. a	Cells washed twice, tested at once	3,700	4.22	77	15	3	67
b	Same cells, heated in centrifuge to 42°	3,250	3.88	76	22	14	63
c	" " " " " " 45°	2,080	2.37	120	46	66	135
V. a	Cells washed twice	2,600	2.42	176	58	64	—
b	Same cells heated in centrifuge to 40°C	1,400	4.28	102	30	60	—

Combinations of ascorbic and nicotinic acid are often, but not always, additive. The combined effect is rarely as high as that of peptone. It is of interest to note that in the presence of ascorbic plus nicotinic acid, adenine and cysteine increase the rate while they show little or no effect when alone. This seems to be a simple case of limiting factors.

The increased fermentation by injured cells makes it seem probable that ascorbic acid is a regular constituent of all cells, but is not needed by normal cells because they can supply their own demand. The present results do not indicate whether the injury is due to increased permeability of the membranes, to autolysis of important constituents, or to other causes. After injury, fermentation depends, at least partly, upon the ascorbic acid diffusing into the cells from the surrounding medium. Peptone and nicotinic acid do not show this increased effect with injured cells.

Summary. The formation of lactic acid by washed cells of *Streptococcus lactis* is increased by the presence of ascorbic acid or nicotinic acid. The two compounds act differently. Ascorbic acid stimulates mostly (perhaps exclusively) injured or exhausted cells while nicotinic acid affects normal and injured cells equally. The combined action of the two acids is usually, but not always additive, and approximates the effect of peptone. When both nicotinic and ascorbic acid are present, other compounds like cysteine or adenine may increase the rate of fermentation while these compounds alone have no such effect.

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Nature of the Reticulocytosis in Pernicious Anemia Following Liver Therapy.

OLIVER P. JONES. (Introduced by W. J. Atwell.)

From the Department of Anatomy, University of Buffalo, and the Hematological Laboratory, Department of Anatomy, University of Minnesota.

Since the original publications by Minot, *et al.*,[1] it has been constantly observed that, in uncomplicated cases, pernicious anemia patients during relapse exhibit a reticulocytosis in the peripheral

[1] *a.* Minot, G. R., Murphy, W. P., and Stetson, R. P., *Am. J. Med. Sci.*, 1928, **175**, 581; *b.* Minot, G. R., Cohn, E. J., Murphy, W. P., and Lawson, H. A., *ibid.*, 1928, **175**, 599.