LXXI. THE INFLUENCE OF THE FATTY ACIDS AND HYDROXY-ACIDS AND THEIR SALTS ON ALCOHOLIC FERMENTATION BY LIVING YEAST.

PART II. PROPIONIC, BUTYRIC, ISO-BUTYRIC, GLYCOLLIC, LACTIC, HYDROXY-ISO-BUTYRIC, α - AND β -HYDROXYBUTYRIC ACIDS AND THEIR SODIUM SALTS.

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In his previous paper [1926], the author investigated the rate of fermentation of sugar by living yeast with buffer solutions composed of acetic acid or formic acid and their Na, K and NH_4 salts. These lowest homologues of the fatty acids exert a specific action upon the rate of fermentation and it is suggested that a hyperbolic relation exists between the concentrations of these acids in the media and the rates of fermentation. Their inhibiting effect is not the same, formic acid being 5–8 times as potent as acetic acid in diminishing the rate of fermentation.

As to the inhibiting action of fatty acids upon the rate of fermentation, Bial [1902] considered that this was due to the hydrogen ion; consequently their degree of dissociation would decide the requisite amount of the fatty acids to stop the fermentation. This conclusion was confirmed by Hägglund [1914] with acetic and butyric acids. On the other hand, Johannessohn [1912] pointed out that the inhibiting effect of the fatty acids appeared to be due to the undissociated molecule rather than to the dissociation products. With an acetic acid and Na acetate buffer solution, Euler and Heintze [1919] confirmed Johannessohn's observations. On the other hand, according to Duggan [1885], the degree of disinfecting action of fatty acids upon *B. subtilis* is inversely proportional to their molecular weights.

In the present paper, experiments were at first instituted with buffer solutions composed of higher homologues of fatty acids and their salts, in order to ascertain what influence would be exerted on the rate of fermentation, whether a hyperbolic relation would exist between the concentration of the acids and the rate of fermentation, and which acid would be more potent, the lower homologue or the higher one.

METHOD AND RESULTS.

Full details of the experimental technique were given in the previous paper [Katagiri, 1926]. The principal points are as follows: fermentation was carried out by 2 g. of pressed top yeast with 30 cc. of the various buffer solutions containing 1.5 g. of glucose after saturation with CO_2 and keeping the temperature at 25° in a water-bath. The amount of evolved CO_2 was observed at intervals of 5 minutes. For the rate of fermentation, the average number of cc. of CO_2 evolved in 5 minutes during about 1 hour's fermentation was chosen and its value was corrected for variation in the fermenting power of the yeast by a standard fermentation which was always carried out in presence of a 0.2 M acetate solution at p_H 4.7. Each p_H value was determined by the capillator method both at the beginning and at the end of the fermentation, after saturation with CO_2 .

The amount of free acid was calculated according to the amount added plus the amount that was produced from the salt in the process of saturation with CO_2 . The latter amount of the acid was calculated from the amount of CO_2 evolved by the addition of HCl of suitable strength to a mixture having the same composition as the solution used in the fermentation.

The results obtained with propionic acid, *n*-butyric acid and *iso*-butyric acid are contained in Table I and Fig. 1.

Table I.

Acid	Total concentration Propionate (M)	Ratio (salt/acid)			
		1 : 4	1 : 1	3 : 1	1 : 0
A. Propionic	0.25	0.200	0.125	0.062	0.015
	0.2	0.160	0.100	0.050	0.013
	0.1	0.080	0.050	0.025	0.010
	0.05	0.040	0.025	0.012	0.005
	0.025	0.020	0.012	0.006	0.003
B. Butyric	Butyrate (M)	Ratio (salt/acid)			
		1 : 3	1 : 1	3 : 1	1 : 0
			Concentration of free acid (M)		
	0.5	0.375	0.250	0.126	0.018
	0.2	0.150	0.100	0.050	0.010
	0.1	0.075	0.050	0.025	0.006
C. <i>iso</i> -Butyric	0.05	0.037	0.025	0.012	0.004
	0.025	0.019	0.012	0.006	0.002
	<i>iso</i> -Butyrate (M)	Ratio (salt/acid)			
		9 : 11	1 : 1	3 : 1	1 : 0
			Concentration of free acid (M)		
	0.5	0.275	0.250	0.125	0.019
	0.2	0.110	0.100	0.050	0.011
	0.1	0.055	0.050	0.025	0.006
	0.05	0.027	0.025	0.012	0.005
	0.025	0.013	0.012	0.006	0.001

The curves (Fig. 1) obtained when the rate of fermentation with the buffer solutions composed of propionic acid, *n*-butyric acid and *iso*-butyric acid with their sodium salts is plotted against the p_H , are of much the same character as was observed with formate and acetate buffer solutions in the previous paper. The p_H values did not change by more than 0.3 p_H during the fermentation and the mean values have been taken.

The concentration of the buffer solutions again appears to have a remarkable effect on the rates of fermentation, when these are compared at constant p_H , the rate of fermentation increasing rapidly as the concentration of the buffers diminishes. As in the case of acetate-acetic acid and formate-formic acid buffer solutions, however, the rate of fermentation is controlled by the amount of free acid and is almost independent of the total concentration. This is well brought out in Fig. 2. A hyperbolic relation can again be supposed to exist between the rate of fermentation and the concentration of acids in the medium.

Fig. 1.

For a direct comparison of the inhibiting effects of the various acids, the amount of each acid may be compared in two ways: one in which the rates

of fermentation are diminished to such degrees as $2/3$, $1/2$, $1/3$, $1/4$ or $1/10$ of the value observed with the least quantity of each acid, and the other in which constant rates of fermentation are observed, *e.g.* 1, 2, 5 and 8 cc. CO_2 per 5 minutes. These values are not constant, but formic acid is relatively more effective at every concentration, while the other fatty acids have nearly the same effect. As examples, the concentrations required to diminish the rate of fermentation to $1/4$ and to produce a rate of fermentation of 5 cc. per 5 minutes are given in Table II.

Fig. 2.

Table II.

Acid	Concentration of acid required to diminish the rate of fermentation to 1/4		Concentration of acid with which a rate of 5 cc. per 5 mins. was observed		Concentration of acid required completely to inhibit fermentation				Dissociation constant of the acids (25°)
	<i>M</i>	Relative	<i>M</i>	Relative	Bial [1902]		Johannessohn [1912]		
					<i>M</i>	Relative	<i>M</i>	Relative	
Formic	0.0100	1	0.007	1	0.016	1	0.0139	1	21.4 × 10 ⁻⁵
Acetic	0.0500	5.0	0.030	4.3	0.041	2.6	0.0841	6.0	1.86 × 10 ⁻⁵
Propionic	0.0675	6.7	0.040	5.7	0.066	4.1	0.1080	7.8	1.4 × 10 ⁻⁵
<i>n</i> -Butyric	0.0525	5.2	0.020	2.9	0.050	3.1	0.0926	6.6	1.48 × 10 ⁻⁵
<i>iso</i> -Butyric	0.0500	5.0	0.020	2.9	—	—	0.0926	6.6	1.5 × 10 ⁻⁵

These numerical relations of the fatty acids agree in general with the results obtained by Bial [1902], Hägglund [1914] and Johannessohn [1912], all of whom carried out the fermentation in absence of a buffer solution.

Vermast [1921] found that the disinfecting action of benzoic acid on *Bacillus coli communis* was controlled by the concentration of the undissociated acid and could be completely accounted for on the basis of the Meyer-Overton partition theory, employing the partition coefficient between water and benzene and basing the calculation on the concentration of undissociated acid.

However, the results obtained at room temperature by Kuriloff [1898] and Georgievics [1913] on the partition of the fatty acids between water and benzene, which are quoted below, show that there is no proportionality be-

tween their partition coefficients and the inhibiting action of the acids on fermentation, so that the relation found by Vermast for disinfection does not hold in the case of fermentation.

Acid	C_1/C_2	$C_1(1-\alpha)/C_2$	Author
Formic	460.5 - 340.8	455.7 - 339.5	Georgievics
Acetic	55.1 - 80.1	54 - 64	Kuriloff
<i>n</i> -Butyric	0.81 - 0.54	0.79 - 0.54	Georgievics

where C_1 and C_2 represent the concentration of acid in water and in benzene, respectively, α represents the degree of dissociation of the acid.

Thus, no reliable explanation has yet been put forward as to the effect of the fatty acids in diminishing the rate of fermentation, but it appears that the magnitude of the observed inhibiting effects of the various fatty acids is attributable, in a great measure, to their chemical nature.

HYDROXY-ACIDS.

In order to ascertain the effect of the introduction of a hydroxy-group on the inhibiting properties of the fatty acids on fermentation, experiments were made in the same manner as with fatty acids with glycollic, lactic, α -hydroxy-*n*-butyric, β -hydroxy-*n*-butyric and hydroxy-*iso*-butyric acids and their sodium salts.

The results are given in Fig. 3 and Table III, the mean values of p_H being taken as in Fig. 1.

Table III.

Acid	Total concentration Glycollate (<i>M</i>)	Ratio (salt/acid)			
		5 : 1	15 : 1	1 : 0	
A. Glycollic ($K=1.5 \times 10^{-4}$ at 25°)	0.2	0.033	0.012	0.004	
	0.1	0.017	0.006	0.002	
	0.05	0.008	0.003	0.002	
	0.025	0.004	0.001	0.0004	
B. Lactic ($K=1.38 \times 10^{-4}$ at 25°)	0.5	0.167	0.083	0.031	0.002
	0.2	0.067	0.033	0.012	0.002
	0.1	0.033	0.016	0.006	0.002
	0.05	0.016	0.008	0.003	0.0003
	0.025	0.008	0.004	0.001	0.0003
C. α -Hydroxybutyric ($K=ca. 0.45 \times 10^{-4}$ at 25°)	0.1	0.025	0.015	0.003	0.001
	0.05	0.012	0.007	0.001	0.0003
	0.025	0.006	0.004	0.0005	0.0003
D. β -Hydroxybutyric ($K=0.39 \times 10^{-4}$ at 22°)	0.1	0.067	0.033	0.019	0.004
	0.05	0.033	0.016	0.011	0.002
	0.025	0.016	0.008	0.004	0.002
E. Hydroxy- <i>iso</i> -butyric ($K=1.06 \times 10^{-4}$ at 25°)	0.2	0.067	0.033	0.012	0.003
	0.1	0.033	0.016	0.006	0.0009
	0.05	0.016	0.008	0.003	0.0007

When the rates of fermentation are plotted against the p_H (Fig. 3), the picture is totally different from that presented by the unsubstituted fatty acids (Fig. 1). Independently of the total concentration of the buffer solutions,

the values are found to be situated on regular curves which evidently approach a maximum and closely resemble those given by phosphates (see Fig. 4). The only exception is glycollic acid, which is intermediate in character between

the fatty and hydroxy-acids. These curves show that the rate of fermentation is very largely controlled by the hydrogen ion concentration, but that some other influence modifies this is clear from the fact that the curves are not identical and that the optimum p_H is not the same in each case. This points to some secondary effect due possibly to the specific nature of the acid. This is specially marked in the case of glycollic acid as will be seen from the curves in Fig. 3.

PHOSPHATE BUFFER SOLUTIONS.

In order to discuss whether the hydroxy-acids reveal any specific effect on the rate of fermentation, the author's interest was directed towards the inorganic buffer solutions containing phosphate or carbonate, which might serve as standards.

The results with Na phosphate buffer solutions are given in Table IV and Fig. 4. In this figure the p_H values measured at the end of the fermentation were used, since phosphate buffer solutions in the range of about $p_H = 3-6$ are not strongly buffered unless the most concentrated solution (0.5 M) is used.

Table IV. *Sodium phosphate buffer solutions.*

	Total concentra- tion of phosphate (M)	Concentra- tion of H_3PO_4 (M)	Concentra- tion of NaH_2PO_4 (M)	Corrected rate of fermentation cc. per 5 mins.	p_H	
					Beginning	End
Exp. 34	0.5	0.050	0.450	6.8	2.6	2.6
	0.5	0	0.500	11.6	4.2	4.0
	0.5	0	0.490	11.5	4.9	4.6
	0.5	0	0.479	11.6	5.1	4.9
	0.5	0	0.389	9.3	6.1	6.1
	0.5	0	0.137	4.4	7.0	6.9
Exp. 35	0.2	0.020	0.180	7.0	2.7	2.7
	0.2	0.005	0.195	10.8	3.7	3.4
	0.2	0	0.200	11.1	4.6	3.8
	0.2	0	0.195	11.6	5.1	4.4
	0.2	0	0.163	10.7	6.1	6.1
	0.2	0	0.090	9.4	6.8	6.8
Exp. 36	0.2	0	0.089	7.6	6.9	6.9
Exp. 36	0.1	0.010	0.090	6.9	2.7	2.7
	0.1	0.002	0.098	10.3	3.7	3.5
	0.1	0	0.100	10.1	4.4	3.7
	0.1	0	0.097	11.1	5.1	4.1
	0.1	0	0.085	11.1	6.1	5.9
Exp. 37	0.1	0	0.060	10.1	6.5	6.5
	0.1	0	0.057	9.7	6.8	6.7
Exp. 37	0.025	0.0025	0.0225	7.4	2.8	2.9
	0.025	0.0006	0.0244	9.9	3.7	3.6
	0.025	0	0.025	9.7	4.2	3.7
	0.025	0	0.024	10.0	4.5	3.7
	0.025	0	0.022	11.4	5.7	4.4
	0.025	0	0.018	11.8	6.4	6.1
	0.025	0	0.019	11.8	6.5	6.0
	0.025	0	0.015	10.5	6.6	6.6

When the fermentation rates are plotted against the p_H (Fig. 4), it is seen that the curves for different concentrations of total phosphate are not identical; both the optimum p_H and the sharpness of the maximum vary. The 0.5 M curve shows the sharpest maximum, whilst the least sharp is shown by the 0.2 M curve.

In the present paper, p_H values are determined colorimetrically; consequently, when p_H values of various concentrations of a buffer solution are compared, a certain correction for the so-called salt error must be taken into

Fig. 4.

account. The correction for salt error becomes greater as the concentration differs from about 0.1 M phosphate and the sign of the correction is different on each side of this concentration. In the case of 0.5 M or 0.2 M solution, the corrected value will be more acidic (p_H less) and in the case of 0.025 M the corrected value will be more alkaline (p_H greater). [See Büllmann and Katagiri, 1927.]

From the present uncorrected colorimetric determinations which are tabulated below it is clearly seen that the optimum p_H moves to the alkaline

side as the concentration of the phosphate buffer solution diminishes. This change would be accentuated if the p_H values were corrected for "salt error" as just explained.

Concentration of phosphate buffer solution (M)	Optimum p_H
0.5	4.45
0.2	4.8
0.1	5.0
0.025	5.25

It has not yet been decided whether Michaelis' equations [1911] for amphoteric electrolytes are applicable in the case of living yeast to the rate of fermentation and the p_H value as applied by Michaelis and Davidsohn [1911] and Davidsohn [1913] to enzyme action, but it is interesting to note that in Table V, in which K is calculated from these equations for values of fermentation rate and p_H taken from the curve in Fig. 4 for 0.5 M phosphate, the values of K obtained on each side of the maximum are approximately constant.

Table V. 0.5 M phosphate buffer solutions.

p_H	Rate of fermen- tation cc. per 5 mins.	Relative rate of fermen- tation	K_a for $\frac{[H^*]}{[H^*] + K_a} = \text{rel. rate}$	K_b for $\frac{[OH^*]}{[OH^*] + K_b} = \text{rel. rate}$
*2.6	6.8	0.59	—	0.28×10^{-11}
2.65	7.0	0.60	—	0.30×10^{-11}
2.8	8.0	0.70	—	0.27×10^{-11}
2.95	9.0	0.78	—	0.25×10^{-11}
3.15	10.0	0.86	—	0.24×10^{-11}
*4.0	11.6	1.00	—	—
Opt.				
4.45	11.6	1.00	—	—
*4.9	11.6	1.00	—	—
5.9	10.0	0.86	0.21×10^{-6}	—
*6.1	9.3	0.80	0.20×10^{-6}	—
6.35	8.0	0.70	0.19×10^{-6}	—
6.5	7.0	0.60	0.21×10^{-6}	—
6.65	6.0	0.52	0.20×10^{-6}	—
6.85	5.0	0.43	0.19×10^{-6}	—
*6.95	4.4	0.38	0.20×10^{-6}	—

* Represents the point of observation.

No simple relations have been found to exist between the rate of fermentation and the concentration of free H_3PO_4 , or of NaH_2PO_4 , or of Na_2HPO_4 .

When the rates of fermentation with the hydroxy-acids are compared with those obtained with phosphate buffer solutions, a very close resemblance is observed to exist between them; *e.g.* between the curve with lactic acid and that of 0.2 M phosphate; between those of α -hydroxy-*n*-butyric acid and 0.1 M phosphate; between those of β -hydroxy-*n*-butyric acid and 0.025 M and between those of hydroxy-*iso*-butyric acid and 0.025 M phosphate. From this resemblance it may be concluded that hydroxy-acids with the exception of glycollic acid do not exert any marked specific effect upon the rate of fermentation as compared with phosphate buffer solutions.

SODIUM BICARBONATE.

Table VI shows the results obtained with various concentrations of sodium bicarbonate buffer solutions and with the corresponding concentrations of phosphate buffer solutions. In order to make the bicarbonate buffer solutions, NaOH solutions of the corresponding concentrations were used. For each concentration of NaHCO_3 , only one p_{H} value is possible, since, under the conditions of the experiment, the solutions are saturated with CO_2 at 25° . For the phosphate buffers Na_2HPO_4 solutions were employed. Having added the requisite amount of sugar solution, they were saturated with CO_2 , yeast was added and the fermentation was carried out.

Table VI.

Sodium bicarbonate buffer solution.

Exp. 38

Concentration of bicarbonate (M)	Corrected rate of fermentation cc. per 5 mins.	p_{H} (corrected)	Relative rate of fermentation	K_c for $\frac{[\text{H}^+]}{[\text{H}^+] + K_c} = \text{rel. rate}$
0.5	4.5	7.3	0.417	0.70×10^{-7}
0.2	7.3	7.0	0.676	0.48×10^{-7}
0.1	8.9	6.8	0.824	0.34×10^{-7}
0.05	9.7	6.5	0.898	0.36×10^{-7}
0.025	10.8	6.2	1.000	—

Sodium phosphate buffer solution.

Exp. 39

Concentration of phosphate (M)	Corrected rate of fermentation cc. per 5 mins.	p_{H} (corrected)	Relative rate of fermentation	K_c for $\frac{[\text{H}^+]}{[\text{H}^+] + K_c} = \text{rel. rate}$
0.5	4.4	6.7	0.373	3.36×10^{-7}
0.2	7.6	6.6	0.644	1.38×10^{-7}
0.1	9.7	6.5	0.822	0.69×10^{-7}
0.05	10.8	6.3	0.915	0.41×10^{-7}
0.025	11.8	6.1	1.000	—

The p_{H} values in Table VI were corrected for salt error and for the barometric pressure under which the saturation of CO_2 was carried out from the values obtained electrometrically by Büllmann and Katagiri [1927] under the same experimental conditions.

In Fig. 5 (a) in which the rates of fermentation shown in Table VI are plotted against the p_{H} , it is clearly seen that the curve with bicarbonate buffer solutions is of a similar nature to that obtained with phosphate buffer solutions, but they are not perfectly identical, since in the latter curve the rate of fermentation diminishes more rapidly than in the former when the p_{H} value increases.

When the rates of fermentation are plotted against the concentration of total salts (Fig. 5 (b)), the bicarbonate and phosphate curves are again very similar, although they are not identical, but in the latter curve the rate of fermentation diminishes a little more rapidly as the concentration increases.

When Michaelis' formula for amphoteric electrolytes is applied to the

results obtained with bicarbonate and phosphate buffer solutions, the values of K_c (Table VI) vary as is to be expected in both cases with the concentration of total salt. When the values of K_c are plotted against the molar concentration of the salts (Fig. 6), a linear relation is found to exist. The inclination of the phosphate curve is greater than that of the bicarbonate curve as was to be expected from Fig. 5 (a) and (b).

Whilst the interpretation of these results is not very clear, it may be considered that with the bicarbonate buffer solution, the rate of fermentation is more exclusively controlled by the p_H value than with the phosphate buffer solution.

Fig. 5 (a)

Fig. 5 (b)

SUMMARY.

(1) Fermentation by living yeast was observed with various concentrations of propionate, butyrate, *iso*-butyrate, glycollate, lactate, α - and β -hydroxy-butyrate, hydroxy-*iso*-butyrate, phosphate and bicarbonate buffer solutions composed in each case of the acid and its sodium salt.

(2) The simple fatty acids have an inhibiting effect on the rate of fermentation, and it is suggested in each case that a hyperbolic relation exists between the rate of fermentation and the concentration of free acid.

(3) The specific effect of these fatty acids is nearly as great as that of acetic acid, but very much less than that of formic acid.

(4) The order of the specific effect of the fatty acids is not coincident with that of their partition coefficients between water and benzene or that of their molecular weights or dissociation constants. It is probably due, in a great measure, to the chemical nature of the acid itself.

Fig. 6.

(5) No specific effect of the hydroxy-acids on the rate of fermentation as compared with phosphate buffer solutions was observed, except in the case of glycollic acid which had a slight inhibiting action similar to that of the simple fatty acids.

(6) Curves showing an optimum p_H value were obtained with phosphate buffer solutions of various concentrations. As in the case of enzyme action in general, the rates of fermentation at constant concentration of buffer solutions were observed to be principally controlled by the p_H value.

(7) Not only a different optimum p_H value but also a different sharpness of optimum p_H was observed with phosphate buffer solutions of different concentration. As the concentration diminished, the optimum p_H moved to the alkaline side.

(8) The effect of buffer solutions of sodium bicarbonate and carbonic acid is very similar to that of phosphate buffers, but the rate of fermentation is more exclusively controlled by the p_H value than is the case with the phosphate buffers.

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