Biochemical Studies on Fermentation (I)

AUTHOR(S):

Tanaka, Shozo

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Biochemical Studies on Fermentation I

By **Shozo Tanaka**

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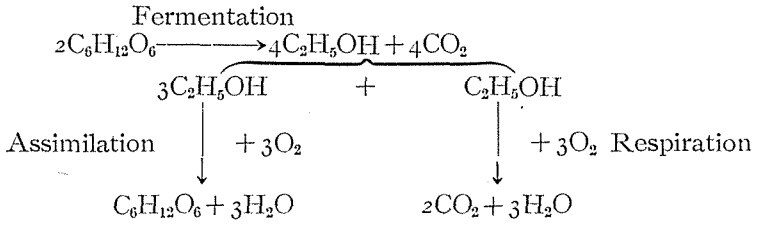
In order to release energy necessary for the growth and maintenance of life, two processes of sugar break-down are carried out by microorganisms; one is fermentation and the other is respiration. L. Pasteur[1] was the first to discover the interdependance between these processes. Fungi, especially Aspergillus and Mucor, propagate only by a respiratory process in the air, though in the absence of oxygen they ferment sugars into ethyl alcohol and carbon dioxide with the formation of spores. From these observations Pasteur came to the conclusion that fermentation is the process of intramolecular decomposition of the sugar molecules caused by the lack of oxygen. Afterward, research[2] on the effects of oxygen upon the growing and fermenting activities of yeast was made by the same investigator to discover not only the growth and the fermenting power promoted in the air, but also the cells, especially young ones, scarcely proliferating to any extent in an anaerobic condition.

Since Pasteur's discovery, the interrelation between the process of fermentation and respiration has been one of the most attractive puzzles in the field of biochemistry, and numerous investigations have been carried out on various phases of the subject, such as the effects of oxygen upon metabolic power of yeast, the distinction between the substrates subjected to these two processes and the relation between the systems of respiratory and fermenting enzymes. For seventy years heated discussions have been carried on between those[3] who recognised the stimulating effect of oxygen on the fermenting power and those[4] who denied it. At the end of the 19th century, Pflüger[5] and Wortmann[6] both expressed the opinion that ethyl acohol might be the sole substrate subjected to respiration under the assumption that fermentation is the antecedent process of respiration. This opinion was criticized by E. Godlewski,[7] J. Reinke,[8] J. Grüss,[9] H. Euler and P. Lindner[10] and M. Effront.[11] But since the discovery[12] of acetaldehyde-, ethyl alcohol- and trioses-oxidizing and- assimilating powers of yeast, Pflüger's opinion has again predominated. F. Brown

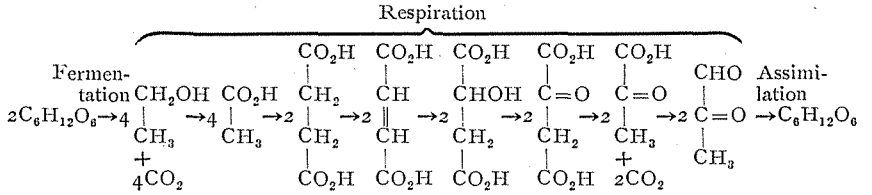
and A. Balls[13] and H. Lundin[14] strongly asserted that the yeast cells, utilizing energy released by the oxidation of a part of the ethyl alcohol produced by fermentation, assimilate another part of the alcohol as their own constituents, such as carbohydrates and proteins. O. Fürth and F. Lieben[15] differed from them to the extent of attributing the substrates of respiration and assimilation to pyruvic acid, the antecedent substance of ethyl alcohol formation.

O. Meyerhof[16] found in his investigations that the fermenting power of all the species of yeast is always suppressed in the presence of oxygen; that the ratio between the falls in this activity and the respiratory power shows usually 1.0–3.0; and that if ethyl alcohol has completely burnt up into water and carbon-dioxide in the air, the respiration quotient ought to show 0.66, but that in fact it shows only about 0.35. Therefore he came to hold the opinion that when one molecule of hexose is fermented by yeast and two molecules of ethyl alcohol thus produced is respired, six molecules of alcohol are resynthesized into hexose with energy set free by respiration, thus,



and he concluded that the yeast cell respires and assimilates the fermenting products only and the fermenting power is not affected directly by the presence of oxygen.

On the basis of Meyerhof's idea, K. Bernhauer[17] offered the following presumption on chemismus of the metabolic changes of the sugar molecules in the yeast cells:



In the field of enzyme chemistry, since the discovery of yeast zymase[18] several enzymes concerned with respiration and fermentation have appeared one after another; —Dehydrogenase,[19] yellow ferment,[20]

cytochrome[21] and so on. Nowadays the processes of respiration and fermentation are assumed to be carried out successively by the catalysis of the chain of the preceding enzymes, and the respiratory system may differ from the fermenting one only in the linkage of cytochrome-oxidase.[22] However, though a maked difference[23] is observed in the absorption spectrum shown by cytochrome between distillery yeasts which respire very faintly and bakery or wild yeasts which by nature respire strongly, repetition of the aerated culture of the former makes it gradually indistinct.[24] It shows that the respiratory enzyme-system of yeast may be composed of inconstant components and may vary under different cultural conditions.

From the biological view-point the fluctuation of respiratory and fermenting powers of yeast was examined in regard to age[25] and species,[26] and some species such as brewery top-yeast show scarcely any change in their fermenting power with varying amounts of oxygen present, but others such as wild yeast or bakery yeast result in a loss of fermenting power in the air, although under anaerobic conditions alcohol is produced by them to much the same degree as by brewery yeast. Moreover, with extension of the culure time a gradual increase in respiratory power and conversely a decrease in fermenting activity of the brewery yeast were observed by Meyerhof[26]; but these changes were denied by K. Trantwein and J. Wassermann.[27] The explanation of these contradictions was made clear by the prudent investigations of F. Windisch[28] on the metabolic change of yeast cells. He found that though fermenting power of the brewery top-yeast is promoted slowly by the repetition of an anaerobic culture, the budding power decreases conversely until at the eighth generation of culture there is no sign of proliferation; and also that as had previously been observed by F. Hayduck[29] the cells cultured under anaerobic conditions are always much larger than those grown aerobically; and that they exceed the latter in both fermenting and respiratory power; and especially that the matured yeasts almost lose proliferating power and respire most actively in the air.

So it seems probable that the contradictions between various results of investigators may be ascribed to ignorance of differences in the kinds of yeast employed and in the cultural conditions.

This led the writer to an interest in studying the distinction between the respiratory and fermenting systems of the yeast cells.

In the present experiments, quantitative investigations on the

amounts of the metabolic products formed and of the absorption of inorganic nutrients[30] were performed during the full course of the yeast culture. Thus the correlation of inorganic substances with yeast metabolism was studied, and also the relation between fermenting and respiratory processes is here discussed from the biochemical viewpoint.

Saccharomyces formosensis Nakazawa, the distillery yeast employed for alcohol production from cane molasses in Formosa, was inoculated into 30 c.c. of the Koji-extract and after cultivation for 48 hours at 30°C., the fermented liquid was withdrawn and the settled yeast was introduced into 500 c.c. of Henneberg's nutrient liquid which consisted of the following components and showed pH 6.4.

Sucrose	75 gr.
$NH_4H_2PO_4$	1 〃
KH_2PO_4	1 〃
$MgSO_4$	0.5 〃
Na_2CO_3	0.5 〃
H_2O	500 c.c.

The culture of the yeast was carried on at 30°C and every 24 hours the number of the yeast cells grown and the amounts of residual sugar, alcohol and lactic acid produced, the carbon dioxide released and the inorganic nutrients such as ammonia, phosphate, magnesium, potassium and sodium were estimated.

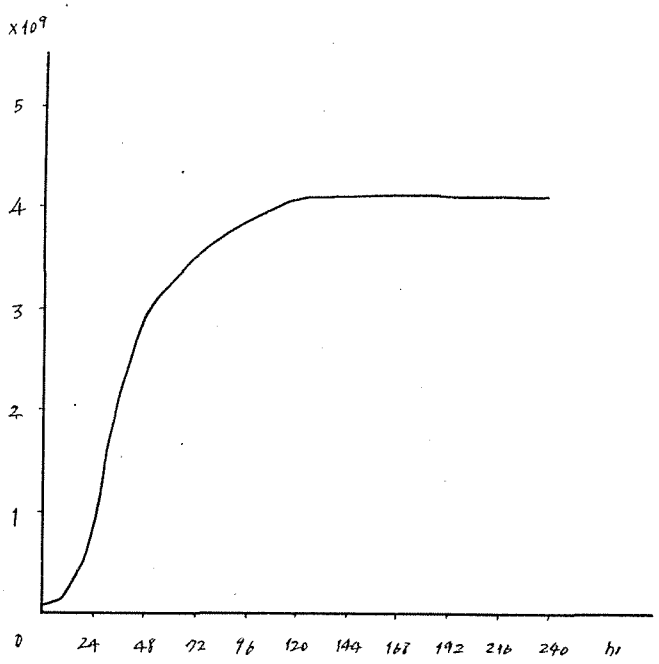
(1) The growth of the yeast

The results obtained on the growth of the yeast cells in the medium are shown in Fig. 1. For the first 15 hours there was no apparent increase in the number of the cells, but then sudden vigorous multiplication follows and the cells kept budding at an uniform rate for about 50 successive hours. Subsequently the rate of multiplication gradually descended until budding ceased at the 120th hour.

In general the proliferation of bacteria, yeasts and molds has become known with a sigmoid character of the growth curve as illustrated below and the characteristic intervals manifested on the growth curves have been distinguished as follows[31]:

a) Lag phase: initial period of slow growth, b) Logarithmic growth phase: during this phase the rate of the growth remains constant, c) Phase of negative growth acceleration: during this interval the

Fig. 1.



The yeast growth. (cell number per 1 c.c.)

microorganisms continue to increase in numbers though less rapidly than during the foregoing phase.

From the biometrical standpoint, A. Slator,[32] T. Carlson,[33] H. v. Euler and B. Palm[34] and others have derived empirically the equation of the yeast growth as that of the following monomolecular reaction;

$$\frac{dx}{dt}=K(A-x), \qquad \text{.....(I)}$$

in which K is the growth constant, A is the maximum number of cells and x is the number of cells present after time t.

As may be seen in Table I, the growth constants calculated from the writer's results according to equation (I) show indefinite values, especially during the intervals of lag phase and the phase of negative growth acceleration.

A marked fall in the value of the growth constant during the phase of negative growth acceleration had been observed by previous investigators and was attributed to the hindrance caused by the accumulation of metabolic products such as alcohol, but the irregularity at the lag phase had not yet been discussed.

Table I

time (hr)	x (10^3 cells in 100 c.c.)	K (calculated by (I))	K' (calculated by (II))
0	832		0.042
1	1040	0.109	0.041
3	1100	0.038	0.042
5	1254	0.026	0.043
7	1798	0.027	0.040
9.5	3140	0.033	0.036
25.5	8374	0.032	0.040
32	10040	0.030	0.038
48	29734	0.051	0.054
55	33320	0.047	0.043
71	34998	0.038	0.025
98	37540	0.029	0.019
120	41000	0.025	

T. Robertson and his collaborators[35] recognized that not only microorganisms but also various plants and animals show an analogous tendency to the phenomena of the monomolecular autocatakinesis[36] in regard to their growth and that the actual rate of multiplication of microorganisms is jointly proportional to two factors: the number of the cells present and the available amount of the nutrient. As the latter may be expressed by the difference between the maximum number of the cells (A) and the numbers present after time t (x), the equation may be written as follows;

$$\frac{dx}{dt}=K'x(A-x), \qquad \text{.....(II)}$$

The results of the calculation of the growth constant K' from the writer's data according to equation (II) were shown in Table I, and yet disagreement at the initial and final phases of the growth is recognized.

In equation (II), only two variable factors determining the rates of the growth were assumed. But as a matter of fact there are numerous other variable factors, some of which may be more significant.

From the writer's results, it may by presumed that this non-conformity between the theoretical and the experimental values may be referred to the differences of chemical changes brought about in the yeast cells at different stages of growth.

From another view-point, microscopical observations were made

on the morphological changes of the cells during the whole course of the growth. During the interval of the logarithmic growth phase when multiplication of the cells took place most actively, the mother cells and their daughters kept budding individually, but in connection with each other and at the end of this phase sudden separation of the binding of the cells and at the same time a noticeable decrease in their budding power were observed and finally the appearance of the sausage-shaped cells and of the giant vacuole in most of them were noticed at the 120th hour.

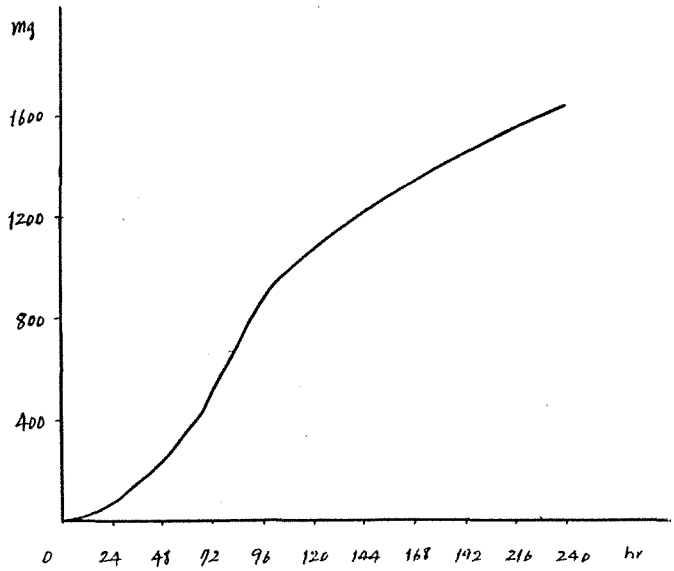
These facts lead to the assumption of variation in the physiological functions of yeasts with their ages and the course of the yeast life-cycle was differentiated into the following four stages :— (1) the latent period of the yeast, the interval from the inoculation of the yeasts to the first flexion point of the growth curve, when cells multiply at a very slow rate but the growth constants calculated by the equations show always larger than the average values, (2) the growing period of the yeast, the interval identical with the logarithmic growth phase, during which cells continue to bud in connection with each other, (3) the mature period of the yeast, the interval from the second flexion point of the growth curve to the end of the budding when mutual binding of the cells is dissolved and the absorption of inorganic nutrients decreases as shown below, until (4) the period of senility when the appearance of giant vacuoles in cells begins.

(2) The metabolic products of the yeast

Corresponding to the discrepancy in the growth constants and the morphological changes during the culture of the yeast, the accumulation of catabolic and anabolic products such as alcohol, lactic acid, carbon dioxide released by respiration, total sugar consumed, and assimilated carbohydrates, was investigated and the results obtained are shown in Figs. 2 to 6.

In the total amount of sugar consumed, a sudden increase was seen at the first flexion point of the growth curve, nevertheless no sign of rate change was noticed at the second flexion point. In other words, in the growing period of the yeast, sugar was decomposed by the reaction intimately related to the proliferation of cells, but in the mature age of the yeast other reactions might gain vigour. Accordingly, the amounts of sugar consumed by fermentation, respiration and assimilation respectively were examined.

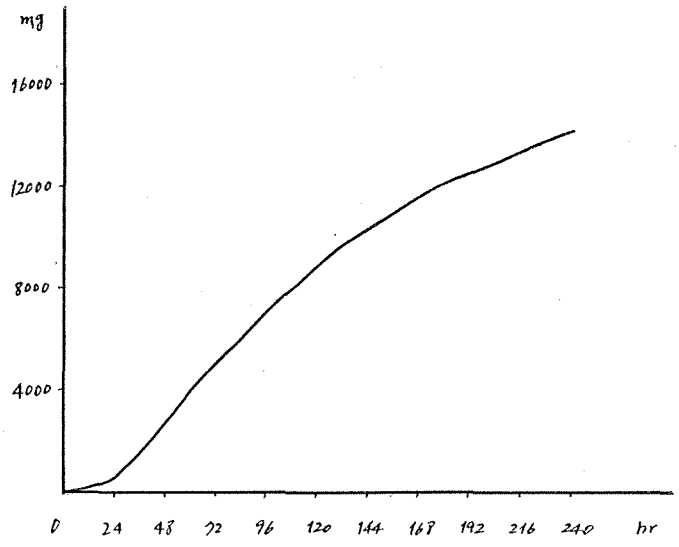
Fig. 2.



Total Sugar break-down (mg. per 100 c.c.)

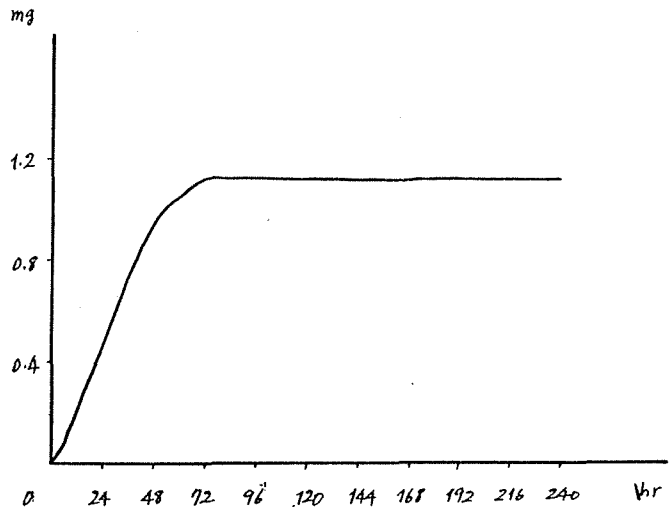
Though the rate of respiration is somewhat analogous to that of the growth, the change in the amount of sugar assimilated is quite

Fig. 3.



Sugar respired (mg. per 100 c.c)

Fig. 4.

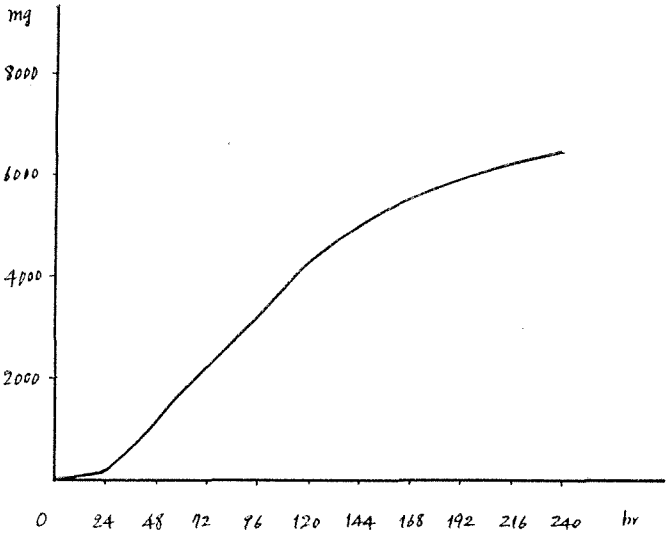


Sugar assimilated (mg. per 100 c.c.)

different and the amount of sugar fermented varies with close resemblance to that of total sugar consumed, showing that the major parts of sugar are decomposed by fermentation.

It may be assumed that respiration and assimilation correlate with the yeast growth, but that fermentation is in faint relation to it.

Fig. 5.

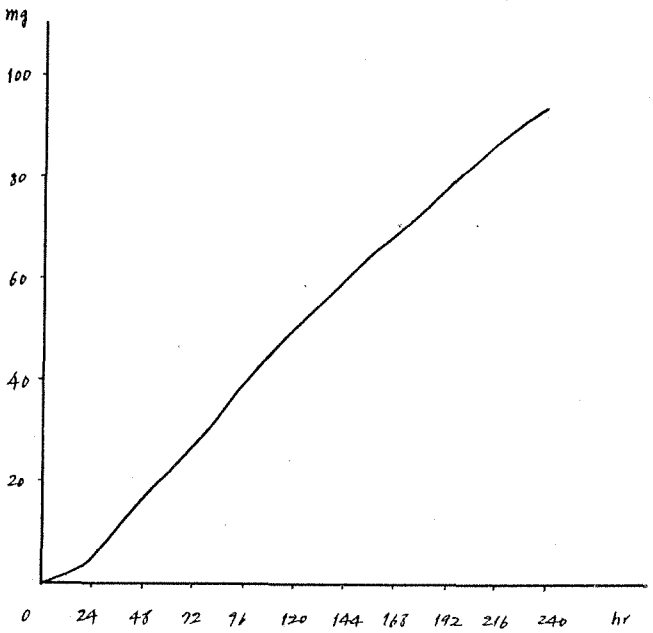


Ethyl alcohol produced (mg. per 100 c.c.)

In spite of the inclusion of lactic acid in almost all of the fermentation products, the formation of lactic acid in the process of alcoholic fermentation has been, hitherto, ascribed to the accidental dismutation[37] between pyruvic acid and glycerol phosphoric acid ester, and the former acid ought normally by the action of carboxylase to decompose into acetaldehyde and carbon dioxide.

As may be seen in Figs. 5 and 6, however, the rate of the formation of lactic acid closely resembles that of ethyl alcohol and at several different periods the ratio between the actual amount of lactic acid and of alcohol estimated is always about 0.012~0.015, as shown in Table II.

Fig. 6.



Lactic acid produced (mg. per 100 c.c.)

Table II

mg per 100 c.c. of the nutrient liquid.

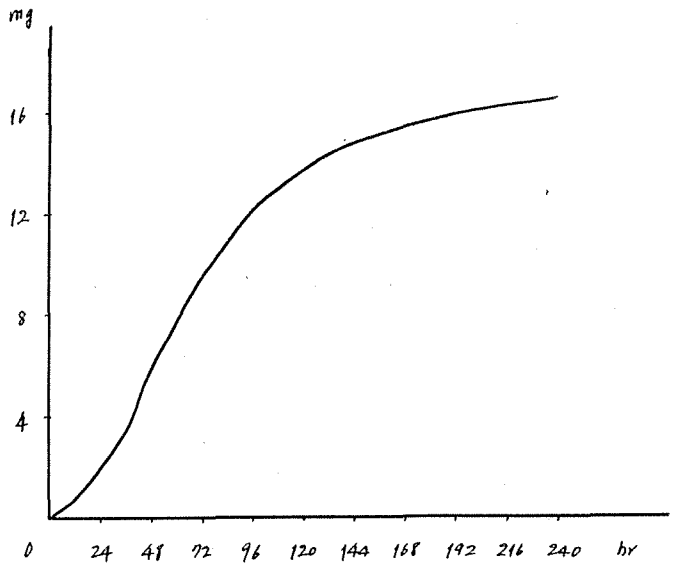
culture time (hr)	24	48	72	96	120	168	216
lactic acid	5.4	17.2	22.7	40.1	49.2	67.4	87.7
ethyl alcohol	140	1220	2240	3520	4130	5490	6040
$\frac{\text{lactic acid}}{\text{ethyl alcohol}} \times 100$	3.9	1.4	1.5	1.2	1.2	1.2	1.4

Though the amouts of lactic acid are no more than one percent of those of ethyl alcohol, its formation may be ascribed to permanent chemical changes connected with the process of alcoholic fermentation.

(3) The absorption of inorganic nutrients

The rate of the absorption of inorganic nutrients during the yeast growth is shown in Figs. 7, 8, 9 and 10. The absorption curves of ammonia, phosphate and magnesium indicated no sign of flexion in the growing phase of the yeast, but still sudden cease of the absorption occurred at the maturity of the cells.

Fig. 7.



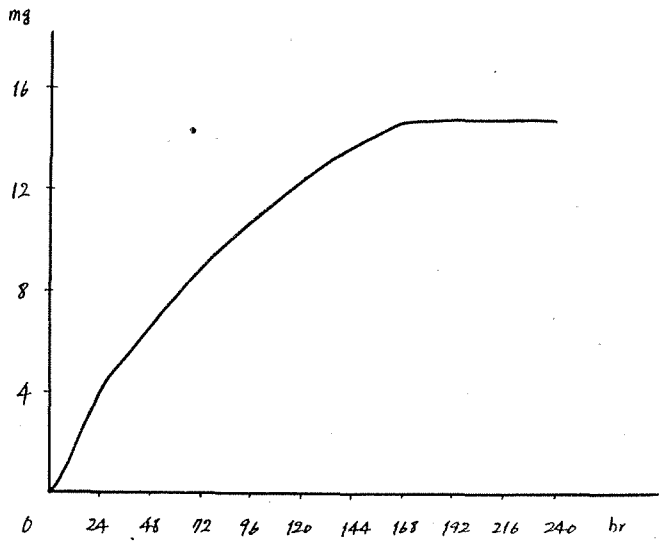
Nitrogen absorbed (mg. per 100 c.c.)

On the contrary, the rate of the absorption of potassium, showing a marked change at the end of the latent period, continued until the cells became decrepit and consequently the type of the curve is similar to those of ethyl alcohol and lactic acid formation.

These results lead to the presumption that functions of ammonia, phosphate and magnesium may be indispensable for the yeast growth and also for the processes of respiration and assimilation, while potassium may be important to the cells for fermentation.

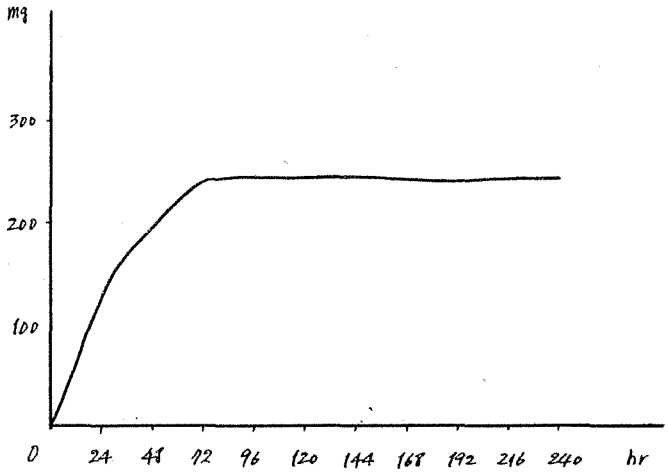
At any period, sodium was found to be absorbed scarcely if at all by the yeast cells.

Fig. 8.



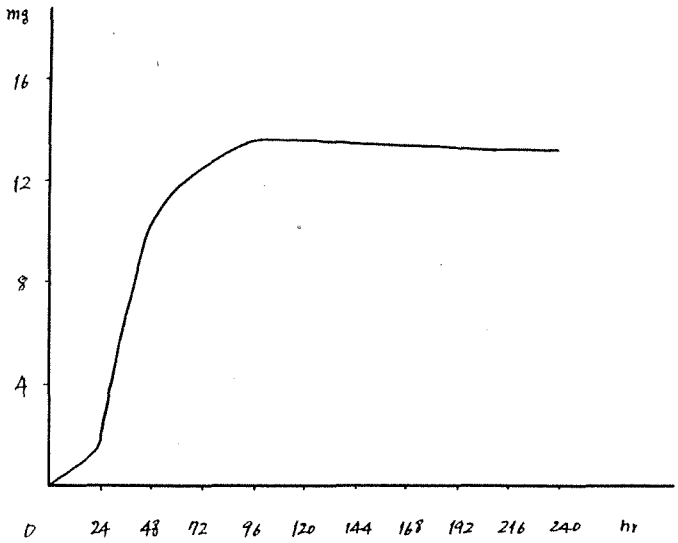
Phosphate absorbed (mg. per 100 c.c.)

Fig. 9.



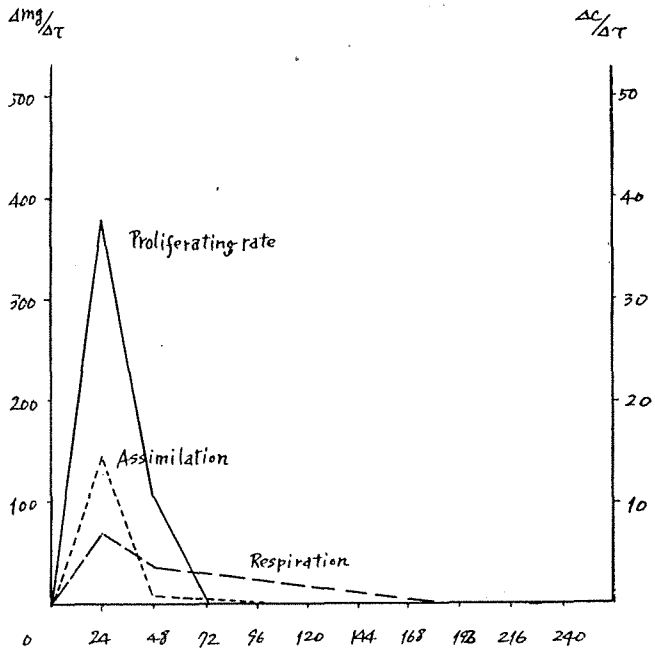
Magnesium absorbed (mg. per 100 c.c.)

Fig. 10.



Potassium absorbed (mg. per 100 c.c.)

Fig. 11.



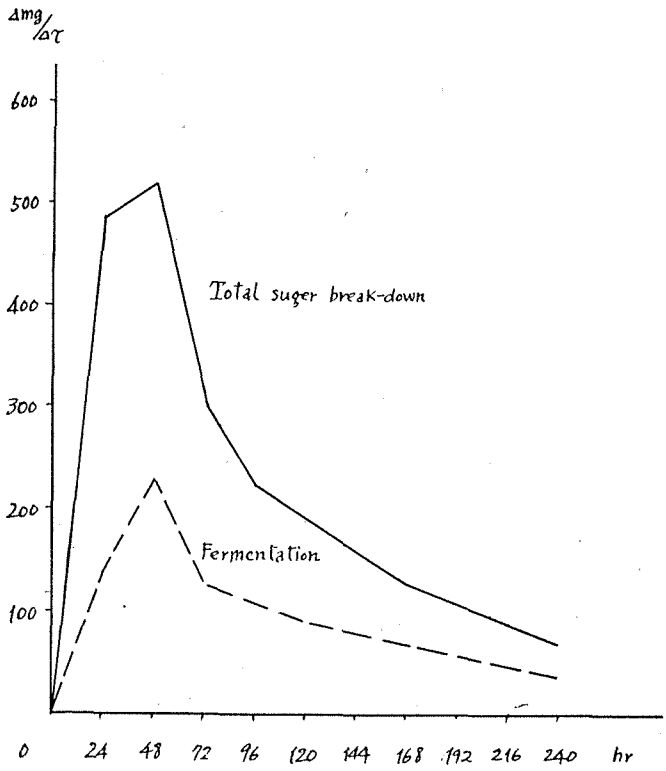
Metabolic powers (I) (mg. per 10^{10} cells per hr.)

(4) The changes of metabolic power of the yeast cells during the culture of yeast

In order to get a clear interpretation of the changes of metabolic powers of each yeast cell at its different ages, the amount per miligram of the changes brought about by unit number of the cells —10,000,000,000 cells— per hour was calculated from the above results and these results are shown in Figs. 11, 12 and 13.

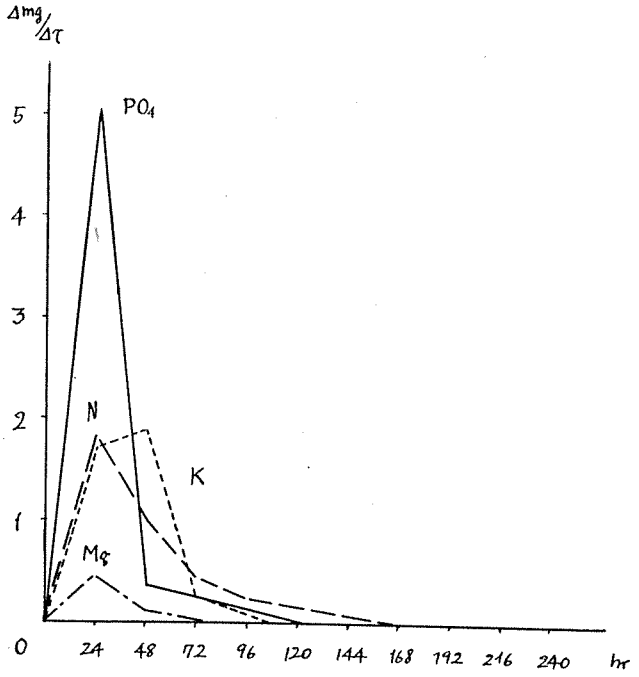
The curves for the changes in respiratory and assimilating powers and also the nutrient absoption such as ammonia, phosphate and magnesium in unit time run parallel with that of the proliferating rate, showing the maximum point at the beginning of the growing period; while, on the contrary, a marked difference is observed in the rate of fermenting power which reaches its maximum at the mature age of yeast, in coincidence with the absorption of potassium. These results indicate

Fig. 12.



Metabolic powers (II) (mg. per 10^{10} cells per hr.)

Fig. 13.



Metabolic powers (III) (mg. per 10^{10} cells per hr.)

ever-changing metabolic alteration of the yeast cells carried on during the yeast growth.

The writer comes to the conclusion that at the growing period the yeast cells, absorbing ammonia, phosphate and magnesium may grow actively with the aid of energy set free by the respiratory process and, when they reach to the mature age, gradually lose the activity of these metabolic changes and commence vigorous fermentation with the absorption of potassium.

The comparison between the metabolic power of the cells at growing and mature ages are shown in Table III. The opposite relation between respiratory and fermenting powers and the marked difference between inorganic constituents accumulated in the cells are observed. Consequently the relation between these metabolic changes can hardly be discussed without considering the quantitative differences in inorganic constituents.

Table III

Metabolic powers (mg per 10^{10} cells per hr.)		
	Growing yeast	Mature yeast
Proliferating power	38	11
Sugar break-down	485	519
Fermentation	266	450
Respiration	66	37
Assimilation	144	8
Absorbing powers of inorganic nutrients		
Ammonia	1.82	1.00
Phosphate	5.04	0.36
Magnesium	0.46	0.11
Potassium	1.71	1.89
Inorganic constituents accumulated (mg per 10^{10} cells)		
	Growing yeast	Mature yeast
Ammonia	22.5	21.9
Phosphate	62.1	22.9
Magnesium	5.6	3.2
Potassium	21.0	34.2

(5) The difference of the substrates subjected to sugar break-down

It has been commonly accepted[38] though on inadequate evidence, that carbohydrates, such as disaccharides and polysaccharides, may be fermented by yeast only when they are hydrolysed into zymohexoses by action of hydratases. According to the investigations described above, yeast might be assumed to respire and assimilate only ethyl alcohol or its antecedent substances produced in the fermenting process, and if so non-fermentable sugars ought to be hardly respired or assimilated directly. Against these assumption numerous demonstrations[39] on the assimilation of non-fermentable sugar have been reported.

The results of these investigations may suggest the probable difference between the substrates of respiratory and fermenting processes.

From this point of view, the structure of sugars contained in the nutrient liquid was examined by estimating the rate of the formation of reducing powers, and the changes in pH value of the nutrient liquid

was determined at different periods of the culture, and the results obtained are given in Table IV.

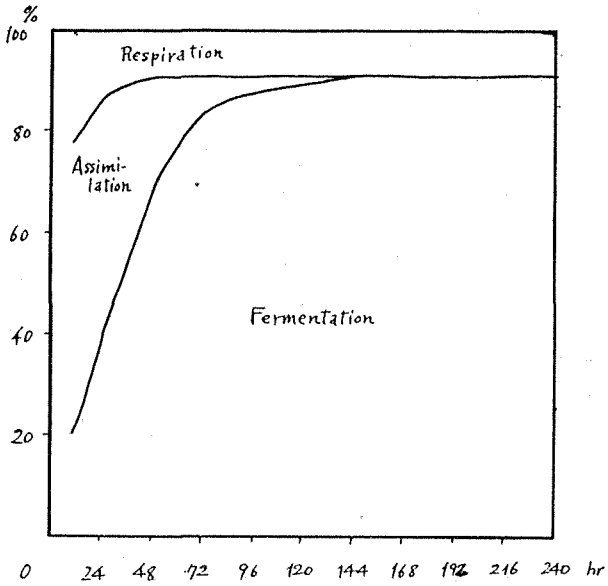
Table IV

Culture time (hr)	0	6	15	24	48	72
Percentage of reducing sugars against the total residual sugars	0	0	5.3	54.5	93.2	100
pH value of the liquid	6.4	6.4	6.2	6.0	5.0	4.2

As may be seen in the table, reducing sugars, which are never recognized until the beginning of the growing period, gradully increase thereafter and at the maturity the total residual sugars were inverted into reducing sugars. Since hydrolysis of cane sugar in the nutrient liquid, saturated with carbon dioxide or acidified to pH 5.4~5.6 with the addition of lactic acid, scarcely occurred even after keeping them at 30°C. for a long time, the formation of reducing power in the case of the inoculation of the yeast cells ought to be ascribed to action of yeast invertase which shows the optimum pH. at 4.5.[10]

Accordingly, during the interval from the beginning of cultivation

Fig. 14.



The change in the distribution of sugar consumed.

to the earlier stage of the growing period, sugars might be decomposed by yeast in the form of the sucrose molecule.

The rate of sugar distributed among respiration, fermentation, and assimilation, changes from time to time, as shown in Fig. 14, and till the rapid multiplication of cells begins, the major part of the sugar is consumed by means of respiratory and assimilating processes. Consequently the substrates sujected to these chemical changes may be supposed to be the sucrose molecule, but at the ripening age when fermentation took place very vigorously, the total residual sugars had already inverted into reducing sugars.

In other words, there may be a difference between the substrates of sugar break-down in the growing and in the mature state of the yeast.

Conclusion

Biochemical studies on the variation of physiological functions of Saccharomyces formosensis inoculated into Henneberg's nutrient liquid containing 15% of sucrose, were precisely performed and at two flexion points in the growth curve marked change in the metabolic processes was observed. At the beginning of the growing period, yeasts respire and assimilate cane sugar actively with the absorption of ammonia, phosphate and magnesium, while in their mature state they absorb potassium and ferment the inverted sugars very vigorously.

Were it assumed that metabolic changes taking place during the full course of yeast culture might appear as the result of the chemical reactions in the reaction-system composed of various constiuents which the cells contain, this system would mutate successively with the participation of the absorbed nutrients and of the metabolic products, as well as with the effects of inconstancy in the compositon of the nutrient liquid, and different reactions represented as respiratory and fermenting processes etc., would be brought about one by one.

As to inorganic constituents which activate the respiratory enzymes and the substrates of sugar break-down directly or indirectly by dissociating the water molecules which show intimate relation with the oxidative and hydrating reactions in the yeast cells, there are marked differences between young and matured yeasts, and also between the structure of the sugar molecules in the nutrient liquid at each time. From these results the conclusion may be drawn that the reaction-system in which the respiratory process is brought about actively, may

differ completely from that represented by the fermenting process.

Experimental part

1. Preparation of the nutrient liquid.

75 gr. of cane sugar was dissolved in 400 c.c. of distilled water and sterilized by steam for half an hour. To the sterilized solution 100 c.c. of inorganic nutrient liquid containing five times the amount of the salts to the above indicated composition was added. After filtering off the precipitate of ammonium magnesium phosphate, the filtrate was employed as the nutrient liquid.

2. Measurement of the yeast growth.

Measurements of increase in the numbers of yeast cells in the culture medium were carried out by means of the haemacytometer under direct microscopic observation. The results are given in Table V. The rate of the proliferation per hour was calculated from these data.

Table V

The numbers of cells ($\times 10^8$) per 100 c.c. of the nutrient liquid

Time / No.	0	1	3	5	7	9.5	25.5	32
I	1000	1123	1266	1854	2438	3856	8468	9520
II	885	1104	1179	1669	1900	3232	8300	10014
III	664	858	965	1058	1200	2658	8280	10520
IV	779	975	990	1435	1654	2814	8448	10066
average	832	1040	1100	1254	1798	3140	8374	10040
Time / No.	48	55	71	98	120	144	168	192
I	29184	31800	36396	37240	39492	39920	40030	40000
II	29380	33918	34444	38326	41038	40872	41442	40994
III	30284	32722	33600	37804	40673	40022	39978	43213
IV	30088	34840	35552	36754	42797	38744	39656	42348
average	29734	33320	34998	37540	41000	—	—	—

The proliferating rate

culture time	0	24	48	72	96	120	168	216
number of cells ($\times 10^8$)	830	8370	29730	35000	37540	41000	41000	41000
Δc (growth)		754	2136	527	254	346	0	0
$\Delta c/\Delta\tau \times 100$		37.9	10.6	0.8	0.3	—	—	—

3. Measurement of the amount of sugar break-down.

In the sterilized box 20 c.c. of the nutrient liquid was drawn out by pipette and after being heated with 2% hydrochloric acid on a water-bath for 2 hours, the total sugar content was estimated by determining the reducing power of Fehling's solution and calculated as *d*-glucose. The decrease in the reducing power of the solution after every 24 hours was designated as the sugar consumed during that interval, and also the rate of sugar break-down per hour per unit cells (10^{10}) was calculated, as shown in Table VI.

Table VI

mg. of sugar break-down per 100 c.c. of the liquid

Time / No.	0	24	48	72	96	120	168	216
I	0	400	2400	4280	6160	8000	11000	15300
II	0	540	2820	5400	7900	10380	14710	15500
III	0	360	2320	4500	6500	8300	11000	12780
IV	0	640	3340	5900	7880	9780	12320	14130
average	0	500	2830	5200	7190	9040	11660	13450
Δmg (break-down)		500	2330	2370	1990	1850	2620	1790
Δmg/Δτ per 10^{10} cells		484.5	519.2	304.8	225.3	188.0	133.1	91.0

4. Measurement of the amount of ethyl alcohol produced.

20 c.c. of the nutrient liquid after being neutralized with dilute alkaline solution and made up to 40 c.c., was distilled gently. Of 25 c.c. of the distillate, the ethyl alcohol content was estimated by determining the specific gravity of the solution. The results of the calculation of the amount of ethyl alcohol produced and the rate of alcoholic fermentation per hour per unit cell are shown in Table VII.

Table VII.

mg. of ethyl alcohol produced per 100 c.c. of the liquid

Time / No.	0	24	48	92	96	120	168	216
I	0	60	590	1800	2530	3260	4680	6100
II	0	60	800	2410	3300	4310	6100	7080
III	0	140	920	1860	2900	3750	5050	5820
IV	0	140	1520	2620	3600	4510	5930	6260

average of III & IV	0	140	1220	2240	3250	4130	5490	6040
Δmg (Formation)		140	1080	1020	1010	880	1360	550
Δmg/Δτ per 10^{10} cells		135.7	230.6	131.6	116.9	89.4	69.4	28.0

5. Measurement of the amount of lactic acid produced.

Determination of lactic acid was carried out by the method of determination of lactic acid in blood.[41] For the purpose of avoiding confusion with other metabolic products, 20 c.c. of the liquid was acidified with 30% phosphoric acid and was extracted with ether for 10 hours by means of Sudo and Kumagawa's extractor. The solution separated thoroughly from ether and volatile metabolic products by distillation was evaporated to dryness on water-bath with 20 c.c. of water and 0.2 gr. of zinc carbonate and again extracted with hot water. The content of lactic acid in the extract was determined by oxidizing it into acetaldehyde with potassium permanganate solution in Tanaka and Endo's[42] apparatus. The results are shown in Table VIII.

Table VIII

mg. of lactic acid per 100 c.c. of the liquid.

Time / No.	0	24	48	72	96	120	168	216
I	0	5.75	21.45	27.90	46.00	60.95	78.55	107.30
II	0	5.47	13.80	25.65	39.80	53.00	69.60	96.25
III	0	5.05	16.30	29.40	34.55	33.68	54.00	65.75
average	0	5.42	17.18	27.65	40.12	49.23	67.38	89.73
Δmg (Formation)		5.42	11.76	10.47	12.47	9.11	18.15	12.35
Δmg/Δτ per 10^{10} cells		5.25	2.62	1.35	1.44	0.91	0.92	0.63

6. Measurement of the amount of respiration

The differences between the total amounts of carbon dioxide released and those corresponding to the amounts of ethyl alcohol produced were designated as the gas set free by respiratory process and so the sugar respired was calculated from these data with the assumption of its burning completely into water and carbon dioxide. The results are shown in Table IX.

Table IX

mg. of carbon dioxide released per 100 c.c. of the liquid.

Time / No.	0	24	48	72	96	120	168	216
I	0	50	750	2300	3140	4100	6600	8570

II	0	50	750	2430	3780	5230	7550	8680
III	0	230	1340	2720	4200	5270	7040	8170
IV	0	230	1670	3220	4650	5770	7350	8170
average of III & IV	0	230	1510	2970	4430	5420	7190	8170
CO_2 corresponding to fermentation	0	130	1170	2200	3130	3860	5340	5790
CO_2 corresponding to respiration	0	100	340	770	1300	1560	1850	2380
Sugar respired (mg.)	0	68.2	231.8	525.0	886.4	1063.7	1254.7	1616.1
Δmg (Break-down)		68.2	163.6	293.2	361.4	177.3	191.0	361.4
Δmg/Δτ per 10^{10} cells		66.1	36.5	37.7	41.8	18.0	9.7	18.4

7. Measurement of the amount of sugar assimilated.

The difference between the amounts of the total sugar consumed and those fermented and respired were designated as those of sugar assimilated by yeast. The results of calculation are shown in Table X.

Table X

mg. of sugar per 100 c.c. of the nutrient liquid.

Time	0	24	48	72	96	120	168	216
Total sugar consumed	0	500	2830	5200	7190	9040	11660	13450
Sugar converted into alcohol	0	274	2387	4388	6359	8080	10741	11817
Sugar converted into lactic acid	0	9	28	45	69	93	127	175
Sugar respired	0	68	232	525	886	1064	1255	1616
Sugar assimilated	0	149	183	247	—	—	—	—
Δmg (Assimilation)		149	34	64	—	—	—	—
Δmg/Δτ per 10^{10} cells		144.4	7.6	8.2	—	—	—	—

8. Measurement of the amount of inorganic nutrients absorbed.

Ammonia; 20 c.c. of the nutrient liquid was mixed with 5 gr. of magnesia and made up to 200 c.c. with water. The mixture was distilled to 100 c.c. and the content of ammonia in the distillate was estimated by titration. The results are shown in Table XI.

Table XI

mg. of nitrogen absorbed per 100 c.c. of the liquid.

Time / No.	0	24	48	72	96	120	168	216
I	0	0.84	5.70	9.70	11.50	15.20	14.66	15.90
II	0	2.36	5.80	10.06	11.70	13.16	16.26	16.76
III	0	1.80	7.76	11.00	14.46	15.90	15.90	16.00
IV	0	2.50	6.36	9.00	11.16	12.90	15.46	16.00
average	0	1.88	6.36	9.90	12.20	13.78	15.58	16.16
Δmg (Absorption)		1.88	4.48	3.52	2.30	1.58	1.80	0.58
$\Delta mg/\Delta\tau$ per 10^{10} cells		1.82	1.00	0.45	0.23	0.16	0.09	0.03

Phosphate; in 10 c.c. of the nutrient liquid filtered off the suspending yeast cells, the content of phosphate was determined by Willey's method[43] with the following results.

Table XII

mg. of phosphate absorbed per 100 c.c. of the liquid.

Time / No.	0	24	48	72	96	120	168	216
I	0	4.7	8.1	12.0	15.0	16.0	20.3	20.3
II	0	3.9	6.1	7.8	10.4	10.6	11.9	11.9
III	0	6.0	6.3	8.0	8.0	8.0	8.0	8.0
IV	0	6.4	6.6	8.2	10.6	10.6	10.6	10.6
average	0	5.2	6.8	9.0	11.0	11.3	14.7	14.7
Δmg (Absorption)		5.2	1.6	2.2	2.0	0.3	3.4	0
$\Delta mg/\Delta\tau$ per 10^{10} cells		5.04	0.36	0.28	0.23	0.03	0.17	0

Magnesium; to 20 c.c. of the nutrient liquid filtered off the suspending cells, 6 N. ammonia water was added drop by drop until the precipitation of magnesium ammonium phosphate ceased.[41] This precipitate, after being washed with 1 N. ammonia water several times, was dissolved in 6 N. nitric acid and the phosphate content was estimated by the above method. The amount of magnesium absorbed was determined indirectly with the following results.

Table XIII

mg. of magnesium absorbed per 100 c.c. of the liquid.

Time / No.	0	24	48	72	96	120	168	216
I	0	0.49	0.70	1.08	1.08	1.08	1.08	1.08

II	0	0.60	0.63	1.08	1.08	1.08	1.08	1.08
III	0	0.45	1.43	1.43	1.42	1.43	1.43	1.41
IV	0	0.32	1.02	1.04	1.08	1.06	1.08	1.08
average	0	0.47	0.95	1.16	1.16	1.16	1.16	1.16
Δmg (Absorption)		0.47	0.48	0.21	—	—	—	—
$\Delta mg/\Delta\tau$ per 10^{10} cells		0.46	0.11	0.03	—	—	—	—

Potassium; in order to expel the ammonia contained, 10 c.c. of the nutrient liquid was evaporated on a water-bath with the addition of 5 c.c. of N/10 potassium-free sodium hydroxide solution. The residue was dissolved in 4 c.c. of water and the potassium content was estimated by the Jendrassik method.[45] The results are shown in Table XIV.

Table XIV

mg. of potassium absorbed per 100 c.c. of the liquid.

Time / No.	0	24	48	72	96	120	168	216
I	0	1.93	13.25	13.85	13.85	13.85	13.85	13.85
II	0	0.60	9.45	12.15	14.85	14.85	14.85	14.85
III	0	2.28	11.00	14.10	15.31	15.30	15.31	15.31
IV	0	2.23	7.30	9.20	9.23	9.23	9.23	9.23
average	0	1.76	10.25	12.32	13.31	13.31	13.31	13.31
Δmg (Absorption)		1.76	8.49	2.07	0.99	0	—	—
$\Delta mg/\Delta\tau$ per 10^{10} cells		1.71	1.89	0.27	0.11	0	—	—

Sodium; for the purpose of the removal of phosphate, 5 c.c. of sodium-free magnesium mixture was added to 5 c.c. of the nutrient liquid, and with the addition of ammonia water, phosphate was precipitated as magnesium ammonium phosphate. The filtrate from this precipitate was evaporated nearly to dryness and sodium was precipitated as $3(CH_3CO_2)\,UO_2.\,(CH_3CO_2)_2Mg.\,CH_3CO_2Na.\,9H_2O$ by E. Eineck and J. Harus' method[46] and weighed. As seen from the results shown in Table XV, no sign of the absorption of sodium was recognized at any time during the full course of the culture.

Table XV

mg. of sodium remaining per 100 c.c. of the liquid.

Time / No.	0	24	48	72	96	120	168	216
I	39.4	39.4	39.9	39.6	39.6	39.5	39.5	39.6
II	40.5	40.5	41.0	40.5	40.5	41.0	40.5	40.7

9. The formation of the reducing sugars in the nutrient liquid.

The amount of the reducing sugars formed in the nutrient liquid was determined in the ordinary way, and the percentage of this amount to that of the total residual sugar at the same period was calculated with the following results.

Table XVI

No. \ Time		0	6	15	24	48	72
I	Reducing sugar formed (mg)	97	97	641	4880	9940	9180
	Total residual sugar (mg)	13325	13225	12400	11925	11325	9175
	%	0.8	0.7	5.2	40.9	87.9	100
II	Reducing sugar formed (mg)	62	62	495	8140	10640	9360
	Total residual sugar (mg)	12875	12725	12177	11950	10800	8875
	%	0.8	0.8	5.4	68.1	98.5	100

Reducing sugars formed without inoculation of yeast (mg)

	No. \ Time	0	24	84
The liquid saturated with CO_2 (pH 5.6)	I	56	167	163
	II	64	183	183
The liquid acidified with lactic acid (pH 5.4)	I	113	182	182
	II	116	218	218

10. The change in the distribution of the sugar consumed among various processes of sugar break-down.

During the full course of the yeast culture, the changes in the distribution of the total sugar consumed among fermentation, respiration and assimilation as indicated in the above results were measured, and are indicated in Table XVII.

Table XVII

Time	0	15	24	48	72	96	120	168	216
Total sugar consumed (mg)	0	204	500	2830	5200	7190	9040	11660	13450
Sugar fermented into ethyl alcohol (mg)	0	62	200	1924	4383	6359	8080	10741	11817

ditto %	0	30.5	40.0	68.0	84.0	88.4	89.4	92.1	88.9
Sugar fermented into lactic acid (mg)	0	—	9	28	45	69	93	127	175
%	0	—	1.8	1.0	0.9	1.0	1.0	1.1	1.3
Sugar respired (mg)	0	38	68	232	525	886	1064	1255	1616
%	0	18.5	13.6	8.2	10.1	12.3	11.3	10.8	12.0
Sugar assimilated (mg)	0	114	149	183	247	—	—	—	—
%	0	51.0	29.8	6.5	4.7	—	—	—	—

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Laboratory of Biochemistry
Kyoto Imperial University

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