XL. NITROGEN METABOLISM IN *SACCHAROMYCES CEREVISIAE*.

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(Received November 14th, 1919.)

INTRODUCTION.

DURING the last few years, the assimilation of nitrogen by the lower organisms of the vegetable kingdom has formed the subject of an ever-increasing number of investigations, the results of which have rendered evident the great importance of this question in its bearing on the problems of both pure and applied chemistry.

Of these researches, not the least suggestive, from a purely chemical, as well as from an industrial, standpoint are those of Felix Ehrlich and his collaborators, who succeeded in establishing the fact that the "fusel oil" of the distiller is produced by the action of yeast on the amino-acids in the wort, yielding the corresponding alcohols, such amino-acids being the degradation products of the proteins originally present.

It is not necessary to trace the history of this theory, first put forward by A. Müller in 1857, and either disregarded or discredited until Ehrlich in 1903 proved that the α -amino-acids when treated with yeast in a saccharine solution give the corresponding alcohols.

The object of the work described in the present communication was the closer investigation of those factors which affect the nitrogen metabolism in yeast, not only from the point of view of the assimilation of the nitrogen detached from the amino-acid during its conversion into an alcohol, but also from the point of view of an apparent excretion of nitrogen which takes place with yeast under certain circumstances.

PART I. INVESTIGATION OF THE FACTORS WHICH INFLUENCE THE ASSIMILATION OF NITROGEN FROM AMINO-ACIDS BY YEAST.

Theoretical.

In view of the important and fundamental character of the work of Ehrlich, it was deemed advisable to repeat the final experiments of this

worker, and the results obtained with leucine confirmed Ehrlich's conclusions, namely, (1) that the amount of nitrogen lost by the amino-acid equals the amount gained by the yeast; (2) that the amount of nitrogen lost by the liquid corresponds to the amount of amyl alcohol formed; (3) that the amount of alcohol formed corresponds to about half the weight of leucine introduced initially into the solution. An extension of the same method to the desamination of asparagine gave further confirmatory results, but here, as would be expected, analysis of the resultant liquid failed to reveal the presence of any of the higher alcohols, a result quite in agreement with the theory of Ehrlich, according to whose equations the only product formed by the extraction of nitrogen from asparagine would be ethyl alcohol.

The main conditions under which these experiments were carried out may be briefly summarised as follows:

- (1) Yeast of low nitrogen content.
- (2) Non-multiplying yeast.
- (3) Large amount of sugar available for fermentation.

The first of these conditions is contingent on the statement of Ehrlich that a yeast of low nitrogen content has a greater assimilatory power for nitrogen than has one in which the percentage of protein matter is high, and a comparison of two yeasts of the same race but containing different percentages of nitrogen showed that there was a small but distinct difference between the amounts of nitrogen assimilated by the yeasts, the comparison being made with yeasts in which the difference in the nitrogen content was artificially induced by previous treatment.

The amount of yeast added to the saccharine amino-acid solution in Ehrlich's experiments precluded the possibility of cell reproduction and it consequently appeared probable that the conclusions drawn from these experiments might not be valid if the yeast were in a state of active cell multiplication during the time the nitrogen assimilation was taking place.

To elucidate this point saccharine amino-acid solutions containing the inorganic salts commonly employed as yeast nutriment (Pasteur) with the exception of the ammonium salts—the amino-acid taking their place—were seeded with a pure culture of *S. cerevisiae* in such amounts that the yeast concentration in three experiments was 1, 10, and 100 cells per unit volume respectively, and the results so obtained are of a most interesting character.

In the first place they show that an excess of yeast extracts the maximum total amount of nitrogen, that is to say, that, from equal volumes of an amino-acid solution a suspension of 100 cells/ T^1 assimilates a much greater amount of nitrogen than a suspension of 10 cells/ T . On the other hand, regarded from the point of view of a single cell, the greater the cell concentration the less the amount of nitrogen assimilated by each cell (calculated on the number of cells found in the solution at the end of the experiment).

¹ T is the symbol for the standard unit volume of 1/4000 cubic millimetre.

The following figures (mean of three typical experiments) demonstrate this:

Table I.

Rate of seeding	G. nitrogen extracted per unit cell	
	Leucine	Asparagine
100 cells/ <i>T</i>	3.37×10^{-4}	11.53×10^{-4}
10 cells/ <i>T</i>	8.87×10^{-4}	12.85×10^{-4}
1 cell/ <i>T</i>	31.95×10^{-4}	31.45×10^{-4}

The figures (see experimental section) show a noticeable lack of increase in the number of cells in those experiments seeded at the rate of 1 cell/*T*.

There are apparently three factors which might account for this, (1) lack of oxygen, (2) effect of the products of fermentation, (3) effect of some substance originally present.

Experiments proved the first factor to be of no practical importance, for aerated and non-aerated fermentations yielded similar results.

The second factor is more difficult of consideration. On the assumption—a very simple one—that the decrease in the amount of nitrogen is an indication of the amount of action which has taken place, and that consequently it is proportional to the quantity of any inhibitor formed, it follows that (taking concrete examples from the results recorded in the experimental part) in two leucine experiments (Tables III and IV) seeded with 10 (*B*) and 1 (*C*) cell per unit volume respectively, the inhibitor should be in the ratio 0.0317 to 0.0195, a concentration reached when the number of cells in these two experiments were 32 and 7 respectively. Thus it follows that each cell in *B* would be influenced by 0.0317/32 parts of inhibitor whilst each cell in *C* had to sustain the action of 0.0195/7 parts, that is 9.9 parts in *B* to 28.0 parts in *C*. Therefore if such a body is formed there is here a reason for the lack of increase in *C*, the experiment seeded with 1 cell per unit volume. It should be noted, however, that careful analysis of the fermented liquids failed to show the presence of any inhibitory body.

With regard to the third factor it must be remembered that amino-acids although providing food for yeast, exert retarding influences on the reproductive capacity of the yeast cell, and it may also be that these amino-acid preparations may contain minute traces of bodies which in the light of modern knowledge may have an inhibitory effect on the reproductive activity of the yeast. It has been shown that in the case of antiseptics, a certain concentration will stop the growth of an approximately definite number of organisms, but if the number of organisms be increased there will be a limiting value for the amount of antiseptic per cell, below which value reproduction can take place. It may be that the preparations of amino-acid used act in a similar manner, and that 100 cells/*T* gives in the above experiments a concentration of amino-acid below the limiting value, while the 1 cell/*T* is above this value and consequently reproduction is retarded.

Yet one other point of interest is attached to this series of experiments, namely, the irregularity of the final nitrogen coefficient of the yeast; but a comparison shows that the final value of this coefficient is apparently connected in some way with the reproductive activity of the yeast, and that for one set of factors the yeast has apparently a specific nitrogen coefficient. (Table VIII, p. 468.)

Experimental.

1. *Fermentation of leucine and asparagine solutions.* The preliminary experiments which were carried out during the course of these investigations were, as has already been stated, conducted along the lines given by Ehrlich himself, and consisted essentially of the fermentation of sugar solutions containing amino-acids, by yeasts of low nitrogen content. The results demonstrated clearly the truth of the conclusions which this worker himself drew. Applying the same method of experiment to the fermentation of asparagine, further confirmatory results were obtained, but in this case, as would be expected, careful analysis of the resultant liquid failed to show the presence of any higher alcohol.

2. *Effects of original nitrogen coefficient of yeast.* In order to test the statement that a yeast of low protein content extracts more nitrogen than one in which the amount of protein is high, a comparison was made between yeasts of different protein content. The seed-yeasts were prepared, in the one case by allowing the yeast to ferment without cell-multiplication in a wort rich in nitrogen, and in the other case by growing a sample of the same yeast in the wort diluted with water, the yeast being seeded at the rate of 10 cells per unit volume T . The yeasts thus prepared were finally washed and introduced at the rate of 10 cell/ T into a 10 % cane sugar solution containing 0.1 % nitrogen as asparagine, and the liquid maintained at 37°. At the end of two days the fermentation was finished, and the following results were obtained.

Table II.

Seed yeast		Total nitrogen in solution		% nitrogen assimilated
Condition	% nitrogen	Before	After	
Low	6.85	0.1000	0.0748	25.20
High	9.32	0.1000	0.0780	22.00

The distinct, although small, difference between the two numbers in the final column confirms Ehrlich's statement that the amount of nitrogen extracted is to some extent related inversely to the amount of protein originally present in the organism. At least this is so for yeasts of an *artificially* high or low nitrogen content.

3. *Effects of cell reproduction on nitrogen extraction.* The effect of active cell multiplication on nitrogen absorption was investigated by seeding a nutrient amino-acid solution with a pure culture of *S. cerevisiae*, the yeast being added to the solution as a suspension in sterile water, in such volume

that the resultant mixture contained in three series, 1, 10, and 100 cells/*T*. The composition of the nutriment itself was:

Cane sugar	10.00 %
Potassium di-hydrogen phosphate	0.20
Calcium phosphate	0.02
Magnesium sulphate	0.02
Nitrogen (as leucine or asparagine)	0.10
Water to	100.00

Tables III and IV give the results obtained in two such series.

Table III.

Source of N	Rate of seeding per unit volume	Before fermentation			After fermentation			Difference	
		<i>a</i> Total N	<i>b</i> N in yeast	<i>c</i> N in acid	<i>d</i> Total N	<i>e</i> N in yeast	<i>f</i> N in acid	<i>g</i> Increase N in yeast	<i>h</i> Decrease N in acid
Leucine } Aspar. }	100	{ 0.3256 0.3991	{ 0.2122 0.1764	{ 0.1134 0.2227	{ 0.3227 0.3976	{ 0.2444 0.2830	{ 0.0783 0.1146	{ 0.0322 0.1066	{ 0.0351 0.1081
Leucine } Aspar. }	10	{ 0.1346 0.2403	{ 0.0212 0.0176	{ 0.1134 0.2227	{ 0.1280 0.2378	{ 0.0463 0.0561	{ 0.0817 0.1817	{ 0.0251 0.0385	{ 0.0317 0.0410
Leucine } Aspar. }	1	{ 0.1155 0.2245	{ 0.0021 0.0018	{ 0.1134 0.2227	{ 0.1206 0.2254	{ 0.0267 0.0280	{ 0.0939 0.1974	{ 0.0246 0.0262	{ 0.0195 0.0253

Table IV.

Source of N	Rate of seeding per unit volume	No. of cells per <i>T</i>				N coefficient of yeast $\times 10^4$			N increase per unit cell $\times 10^4$
		<i>k</i> Before	<i>l</i> After	<i>m</i> Coefficient of multiplication	No. of new cells	<i>n</i> Before	<i>p</i> After	<i>q</i> Difference	
Leucine } Aspar. }	100	{ 97.8 97.8	{ 94.3 93.4	{ — —	{ — —	{ 21.7 18.0	{ 25.9 30.3	{ + 4.2 + 12.3	{ 3.41 11.50
Leucine } Aspar. }	10	{ 9.8 9.8	{ 32.0 33.1	{ 3.28 3.37	{ 22.2 23.3	{ 21.7 18.0	{ 14.5 17.0	{ - 7.2 - 1.0	{ 7.84 11.60
Leucine } Aspar. }	1	{ 1.0 1.0	{ 6.9 7.9	{ 6.90 7.90	{ 5.9 6.9	{ 21.7 18.0	{ 38.7 35.5	{ + 17.0 + 17.5	{ 35.65 33.16

In the above results the small increase in the number of cells in those experiments seeded at the rate of 1 cell/*T* is very noticeable, and the following experiments were conducted in order to get some idea of the state of the cells at the termination of the fermentation.

4. *Final condition of the yeast cell in experiments seeded at 1 cell/T.* A nutrient asparagine solution (of composition given above) was seeded at the rate of 1 cell/*T*, and allowed to ferment at 37° in the usual manner. At the conclusion of the fermentation, a count of the number of yeast cells was made, the yeast separated by filtration and the filtrate distilled *in vacuo* at 40°. The residue was then dissolved in 10 % sucrose solution, and the solution made up to the original volume of the asparagine solution, and finally treated with the previously separated yeast. On the completion of the second fermentation, a count of the cells was again made, with the following results:

Table V.

Fermentation	No. of cells per unit volume		Coefficient of multiplication
	First	Second	
	1.10	9.20	8.36
	9.00	31.40	3.49

This then conclusively proves that the yeast in the initial experiment (seeded at the rate of 1 cell/*T*) was not killed, but that its *reproductive activity had been retarded*.

5. *Effect of aeration on nitrogen extraction by yeast.* Fermentations were conducted as previously described (leucine and asparagine being added as the sources of nitrogen in two parallel series), except that the fermenting liquid was aerated by the passage of a stream of sterile air for a short time each day. The results are given in the following table, together with those previously shown in Table III for purposes of comparison. Experiments with asparagine gave confirmatory results.

Table VI.

Rate of seeding	Conditions		Nitrogen in solution g. per 100 cc.			No. of cells per unit volume			Coefficient of multiplication
	Aeration		Before	After	Decrease	Before	After	Increase	
100	Non-Aerated		0.1134	0.0783	0.0351	97.80	94.30	—	—
	Aerated		0.1094	0.0698	0.0396	99.00	98.00	—	—
10	Non-Aerated		0.1134	0.0817	0.0317	9.80	32.00	22.20	3.28
	Aerated		0.1094	0.0786	0.0308	9.90	33.20	23.30	3.35
1	Non-Aerated		0.1134	0.0939	0.0195	1.00	6.90	5.90	6.90
	Aerated		0.1094	0.0914	0.0180	1.00	8.40	7.40	8.40

6. *Effect of the conditions of cell reproduction on the final nitrogen coefficient of the yeast.* The following table shows that there is apparently some connection between the final nitrogen coefficient of the yeast and the conditions of cell reproduction, for the mean of the three quoted experiments seeded at 100 cells/*T* is 0.0026, at 10 cells/*T* is 0.0016, and at 1 cell/*T* is 0.0036.

Table VII.

		Nitrogen coefficient of yeast $\times 10^4$			
		Initial	Final		
Experiments			A	B	C
Amino-acid	A, B and C	100 cells/T	10 cells/T	10 cells/T	1 cell/T
Leucine	21.7	25.0	14.0	38.0	
"	18.0	24.0	16.0	35.0	
Asparagine	18.0	30.0	17.0	35.0	

PART II. CONSIDERATION OF THE THEORY OF THE MECHANISM OF THE REMOVAL OF NITROGEN FROM AMINO-ACIDS BY YEAST.

Fifteen years ago Ehrlich [1905] put forward his theory of the degradation of amino-acids by yeast, explaining the action as follows. The (NH₂) groups of the amino-acids are first split off from the parent molecule by the action of some desaminating enzyme, with the formation of the corresponding hydroxy-acid. The ammonia thus liberated is assimilated by the yeast and probably used in the construction of protein matter. At the same time the hydroxy-acid is subjected to what is presumably the ordinary zymatic action of the yeast, namely, the liberation of carbon dioxide and consequent formation of an alcohol, an action analogous to the supposed decomposition of lactic acid

under similar circumstances to carbon dioxide and ethyl alcohol. This theory states then that a hydroxy-acid is the intermediate product of the desamination.

Six years later, however, some work was published by Neubauer and Fromherz [1911] which carries Ehrlich's work a step further, in that they isolated a ketonic acid as the intermediate product of the action. They modify the theory of the action, for, in their opinion, the degradation is represented graphically as follows:

According to both these theories, the degradation of the amino-acid molecule is primarily caused by a hydrolysing enzyme, followed by a zymatic one, but Neubauer and Fromherz's explanation also requires the presence in the yeast of two others, an oxidising and a reducing enzyme.

The intermediate body, according to Ehrlich, is a hydroxy-acid, and Neubauer and Fromherz have succeeded in separating such a body, although they state it to be a reduction bye-product of the true intermediate body, the ketonic acid.

In any case the alcohol corresponding to the amino-acid, as shown in the above formulae, results: tyrosine, phenyl-amino-acetic acid, serine, and α -amino- α -methylbutyric acid, all have been shown by Ehrlich to yield the expected alcohol.

Hans Pringsheim [1906, 2] has also studied the desamination of the amino-acids more particularly in connection with the Mucors, and concludes from his results that for nitrogen to be assimilated from a complex molecule the group ($-\text{CO}-\text{CH}-\text{NH}$) must be present, and as proof of this gives the following list of bodies which he has succeeded in decomposing by moulds: glycine, alanine, leucine, tyrosine, aspartic acid, phenylglycine, allantoin, phenylalanine, and hippuric acid. He further states that a long side chain favours such a degradation.

This production of alcohols from amino-acids is, however, quite distinct from the ordinary zymatic action of the yeast, for both Ehrlich [1906] and Pringsheim [1906, 1] have proved that no amyl alcohol is formed from leucine by the action of active "acetone yeast" in the presence of sugar.

Thus the following conclusion naturally presents itself: There are two—in some ways different—actions taking place, (1) the desaminating action proper, and (2) the zymatic action. It appears probable that could these two actions be separated some light would be thrown on the extraction of nitrogen from amino-acids by yeast, and therefore it may be that by suppressing those

actions connected with the zymatic activity the presence in the yeast cell of a desaminating enzyme could be proved.

Effront [1908] apparently succeeded in doing this to a certain extent, for he found that in the decomposition of amino-acids by yeast in an alkaline medium he could demonstrate the presence of an enzyme, amidase, which under certain conditions liberates the whole of the nitrogen of amino-acids as ammonia, leaving various acids as the result of the action. For example, the whole of the amino-acid nitrogen of asparagine and part of the nitrogen of the yeast material itself are set free as ammonia in 96 hours. He also states that the enzyme can be obtained in solution.

Experiments carried out along the lines described by Effront showed two fertile sources of error, namely, bacterial growth and the presence in the liquid to be distilled of free alkali, both of which would nullify any results obtained [Effront, 1909].

The conditions of experiment are such that the yeast is dead, and a further proof that the action is quite independent of the life of the yeast was obtained by the use of dried, finely ground yeast instead of the pressed yeast before employed, the results showing that a little less than 75 % of the nitrogen was liberated as ammonia (Table IX, p. 469).

Thus the presence in the yeast cell of a desaminating enzyme, independent of the life of the yeast cell itself, seems to be proved, although it has been found impossible to extract the enzyme from the yeast in the manner described by Effront (*Experimental Section*, § 2).

Besides this desaminating ferment, amidase, however, another enzyme which causes hydrolysis analogous to the supposed hydrolysis by amidase was discovered in yeast by Shiga [1904]. This enzyme was arginase, and had previously been found in animal tissue by Dakin [1904] and its action more fully investigated by Kossel and Dakin [1904, 1, 2]. Its specific activity is the hydrolysis of arginine to ornithine and urea, although experiment proved the action to go further, the urea itself being hydrolysed.

Kossel and Dakin not only discovered this enzyme, but they also succeeded in separating it in a solid form from solution by precipitation [Kossel and Dakin, 1904, 3], and as the tissues which contain arginase act vigorously on asparagine, glutamic acid, etc., it seemed possible that this enzyme was the one at work in the experiments conducted by Effront. This possibility was more or less discountenanced by Effront's observation that amidase would not act in an acid medium, while the arginase of Kossel and Dakin is extracted by and is active in a 0.2 % acetic acid solution. Attempts at acid extraction, however, also proved abortive, and one is forced to the conclusion that the enzyme amidase is either in an insoluble state in its original form in the yeast tissue, or that it depends for its action upon a coferment which cannot be extracted by the means employed above.

If the action were a simple hydrolysis, malic acid should result from asparagine, but Effront states that the chief product of the action is propionic

acid. This being the case, the action must be a complex one and zymatic and reducing enzymes must also take part in the degradation. That yeast can, under suitable conditions, reduce the hydroxy-group of malic acid was proved by A. Fitz¹ who obtained calcium succinate from calcium malate. Reduction is also necessary in the theory of Neubauer and Fromherz.

Analysis of the liquid resulting from the action of yeast on asparagine under Effront's conditions revealed the presence of volatile fatty acids, apparently acetic and propionic, together with a non-volatile acid corresponding in molecular weight with malic which was present in an amount equal to about 60 % of the asparagine destroyed.

Presuming this removal of ammonia to be the first step in the fermentation of amino-acids, then malic acid—or ammonium malate—should be fermentable by yeast to yield ethyl alcohol alone. Experiment proved that under normal conditions of fermentation malic acid was unattacked by yeast, a result confirmed by some work of Oskar Emmerling [1899: reference to which was not found until after the work was completed] who found that while a pure culture of *B. lactis aerogenes* fermented malic acid to form succinic and formic acids with a little carbon dioxide and ethyl alcohol, pure cultures of brewery yeast had no action on malic acid, the apparent action of ordinary cultures being due to bacterial contamination.

It was thought, however, that the ammonium salt might act in an altogether different manner, for salts are often attacked while the acids themselves resist the action, *e.g.*, it has been stated that calcium malate is reduced by yeast to calcium succinate, and it seemed quite possible that the ammonium salt might serve as a source of nitrogen for the yeast cell. This was found to be the case, for, in the presence of sugar, yeast attacked ammonium malate, ethyl alcohol being the only product of the fermentation revealed by analysis of the resultant liquid. Effront states that the chief product of the action of amidase on asparagine is propionic acid, and consequently were the action of amidase the first step in the fermentation of this amino-acid then ammonium propionate should also be fermentable. This proved to be the case, although only to about half the extent of ammonium malate but quite sufficient to preclude the possibility of drawing any definite conclusions as to the rôle of malic acid in the fermentation of amino-acids.

Experimental.

1. *Desamination by yeast in alkaline solutions.* The method of conducting the desamination of amino-acids by amidase as given by Effront is as follows: 2 g. of asparagine and 10 g. of pressed yeast are mixed together with a little water, 6 cc. of *N* NaOH are then added and the mixture made up to 100 cc. with water. The ammonia formed as a result of the action of amidase on the

¹ Richter's *Organic Chemistry* (Smith), 1, 488. I cannot find reference to this work, the nearest being that of A. Fitz [1879] who found that certain schizomycetes acted upon malic acid to produce succinic and acetic acids.

asparagine is determined at intervals in a portion of the filtered liquid by distilling *in vacuo* with magnesia; controls of asparagine alone and of yeast alone are carried out concurrently.

The preliminary experiments were carried out in this manner, but as stated above it was found that abnormal results were obtained. Consequently in the following experiments toluene was in all cases added as an antiseptic and previous to distillation the sodium hydrate was neutralised by sulphuric acid¹. The following are typical results:

Table VIII.

Initial nitrogen in liquid	After 120 hours action	
	Total N	% of initial N as NH ₃
1.1200	1.1295	72
1.4500	1.1600	74

2. *Attempts to obtain amidase in alkaline solution.* In a second parallel experiment the liquid was filtered after 72 hours and the filtrate divided into two portions; to the one 5 g. of asparagine were added, whilst the other was boiled to serve as a control. The liquids were kept at 37° for three days when the free ammonia was estimated, with the result that the control liquid was found to contain the same amount as the test itself, a result directly in opposition to the statement of Effront that under such conditions the enzyme is easily extracted.

3. *Attempts to obtain amidase in acid solution.* About 100 g. of pressed yeast were ground in a mill and extracted with 100 cc. of 0.2 % acetic acid for three days at 37°. The liquid was then filtered, and the filtrate, presumably containing the enzyme, divided into two parts and treated as in the alkaline extractions. The results were quite negative.

4. *Proof that the action of amidase is independent of the life of the cell.* An amount of dried yeast, finely ground, corresponding to the amount of pressed yeast used in the previous experiments was introduced into the amino-acid solution, and the action allowed to proceed as in the cases already described. The results obtained are given in Table IX.

¹ The form of distillation apparatus used was one devised by Brown [1903, 1] a modification of the Longi apparatus as used in the Sachse-Schloesing-Longi method of ammonia estimation.

A great difficulty was at first experienced in these distillations by reason of the vigorous frothing which occurred when the pressure was reduced to about 50 mm. (at 40°) or when the temperature reached 35° if the pressure was previously reduced to 20 mm. It was found, however, that after raising the temperature to 40° and keeping the pressure at 100 mm. for about 15 to 20 minutes, the pressure could be diminished and the liquid would boil quite normally. The distillations were conducted at 40° and 12–15 mm. pressure. As a safeguard against sudden frothing carrying the liquid over into the receiver, a tap was inserted in the apparatus between the hot water jacket and the receiver so that on the slightest indication of frothing, the pressure in both the distillation flask and receiver could be immediately increased. It also served as a means of keeping the vacuum at the desired pressure in the first part of the distillation.

Table IX.

Time in hours	Total N in liquid	Total N as NH_3 in liquid	% nitrogen as NH_3
24	1.1199	0.3927	35.06
48	1.1200	0.7707	68.80
120	1.1205	0.8069	72.00

5. *Estimation of the volatile acids in resultant liquids.* The liquid, after filtration, was acidulated with sulphuric acid and distilled with additions of water as the liquid in the distilling flask became concentrated. The distillate was subsequently treated with an amount of baryta solution sufficient to neutralise the acid present, and the neutral liquid, after the separation of a certain amount of precipitate, evaporated to dryness in a weighed dish. The residue, after weighing, was treated with sulphuric acid and heated in order to remove the volatile acids. From the weight of sulphate obtained and the amount of barium salt decomposed the molecular weight of the volatile acid was calculated.

Table X.

Weight of barium salt taken	Weight of BaSO_4	% Ba in salt	Mol. weight of acid if monobasic
1.7316	1.5448	52.50	64.40
1.0844	0.9581	51.90	65.80

The percentage of Ba in barium propionate is 48.5 and in the acetate 53.8; therefore the above results appear to suggest that the volatile portion of the products of the desamination of asparagine is a mixture of acetic and propionic acids.

6. *Estimation of the non-volatile acids in resultant liquid.* With regard to the non-volatile portion of the acid products it was found a matter of considerable difficulty to obtain a reliable method of estimation. Finally the following method of procedure was decided upon. The experimental liquid was neutralised with sulphuric acid heated to boiling point and treated with an excess of precipitated calcium carbonate, and after boiling for about seven minutes filtered. The clear filtrate was concentrated and filtered to remove any calcium succinate which separated out, and then treated with about ten times its volume of 95 % alcohol. This precipitated any calcium malate, which is practically insoluble in 85 % alcohol. It was found that a convenient method of procedure was to filter through a Gooch crucible, for the salt could then be easily taken into solution again if necessary, or transferred to an evaporating basin for decomposition with sulphuric acid.

The results given below tend to show that a considerable amount of malic acid is produced in the desamination of asparagine by yeast in the conditions of experiment described.

Table XI.

Weight of Ca salt taken	Weight of CaSO_4	% Ca in salt	Mol. weight of acid
0.6166	0.4896	23.36	132.8
0.7392	0.5897	23.48	133.5

Calculated for calcium malate $C_4H_4O_5Ca$. Ca 23.25 %.

Molecular weight of malic acid = 134.

Total weight of calcium salt obtained from 2 g. asparagine = 1.3558 g.

Weight of asparagine corresponding to Ca salt obtained = 1.18 g.

Amount of asparagine decomposed (by nitrogen) = 1.623 g.

7. *Fermentation of malic acid and ammonium malate.* Investigation of the power of yeast to ferment malic acid was conducted on a solution containing 1 % malic acid and 10 % cane sugar with pure culture yeast, but the results showed that no acid was destroyed. In the case of the ammonium salt, the procedure was as follows: 1.0 g. malic acid was dissolved in water and the solution neutralised by ammonia; 20 g. of sugar and 20 g. of pressed yeast were added and the mixture made up to 250 cc. The solution was kept at 37° and a vigorous fermentation ensued which was practically over in three days. The progress of the action was followed in two ways (1) by estimation of the amount of nitrogen extracted from the liquid; (2) estimation of the decrease in the amount of malic acid present. Both series of determinations were carried out in the manner previously described, and yielded the results shown in Table XII which prove that ammonium malate is fermented by yeast.

Table XII.

Time in hours	(a) Nitrogen determinations			(b) Acid determinations		
	Nitrogen in liquid	Corresponding weight of salt	% salt fermented	Weight of Ca salt	Corresponding wt of (NH_4) salt	% salt fermented
0	0.1127	0.6762	Nil	0.7066	0.6902	Nil
24	0.0978	0.5868	13.20	0.6160	0.6017	12.82
96	0.0801	0.4806	28.90	0.5081	0.4964	28.08

8. *Products of the action of yeast on ammonium malate.* After the separation of the malic acid precipitate the filtrate was carefully evaporated to dryness at 40° *in vacuo*. This solution should contain ammonium carbonate (by the double decomposition of calcium carbonate and ammonium malate) and the calcium salt of any acid formed during the fermentation. The residue therefore of this solution was gently heated, with a result that practically the whole of it decomposed, proving it to be ammonium carbonate. The very slight amount of residue left undecomposed was quite inestimable.

Thus it appears that the only product of the fermentation of ammonium malate is ethyl alcohol.

9. *Fermentation of ammonium propionate.* The results of experiments are given in Table XIII, which show that this salt is also decomposed by yeast. The products of the action were not analysed.

Table XIII.

Time in hours	(a) Nitrogen determinations			(b) Acid determinations		
	Nitrogen in liquid	Corresponding weight of salt	% salt fermented	Weight of Ba salt	Corresponding wt of (NH_4) salt	% salt fermented
0	0.0916	0.5954	Nil	0.9350	0.6013	Nil
24	0.0875	0.5685	4.52	0.8868	0.5703	5.16
96	0.0786	0.5110	14.14	0.7980	0.5132	14.65

PART III. INVESTIGATION OF THE EFFECT OF VARIATION IN THE AMOUNTS OF AMINO-ACID AND OF SUGAR ON THE ASSIMILATION OF NITROGEN FROM AMINO-ACIDS BY YEAST.

It has already been found (Part I) that the amount of nitrogen extracted from an amino-acid by yeast varies very considerably with the change in the concentration of the yeast cells, but, so far, the experiments have always been made with an excess of amino-acid and a large amount of sugar available for fermentation.

In the first place zymatic activity is necessary for the absorption of nitrogen by yeast, the results of a series of experiments conducted by treating amino-acid solutions with yeast in the absence of sugar proving that no nitrogen was extracted. On the other hand a further series, in which the relative rates of fermentation of sugar and decomposition of amino-acids were determined, showed that, although the one is no definite function of the other, yet the activity of the zymase does exert a stimulating effect on the desamination of the amino-acids.

A rapid fermentation, however, does not mean rapid absorption of nitrogen, for experiment shows that if fermentation is extremely rapid and consequently soon over, the desaminating enzymes apparently have not sufficient time to reach a state of activity and as a result little absorption of nitrogen takes place.

As regards the effect of the second factor mentioned above, viz., variation in the amount of amino-acid, investigation shows, as would be expected, that the quantity of nitrogen extracted is not proportional to that of amino-acid originally present, although the results obtained give some evidence that the nitrogen-content of the solution does influence the nitrogen absorption.

Worthy of note are the varying amounts of nitrogen which a yeast will assimilate under conditions apparently similar. Thus it has been observed that, with a 10 % sugar solution containing 1 % nitrogen as asparagine and seeded at the rate of 100 cells/*T*, the nitrogen absorbed in 24 hours has varied from 21.7 % up to 37.2 % of the amount originally present. It has, indeed, been already shown that the nitrogen content of the seed-yeast has an influence on the absorbing capacity, but in a number of cases two samples of the same yeast, after apparently identical treatment, have absorbed appreciably different amounts of nitrogen. It seems probable that such differences depend on physiological conditions of which we are as yet ignorant.

It has been found from the large number of fermentations conducted in this investigation that on an average a normal yeast seeded at the rate of 100 cells/*T* will extract in 24 hours 0.0250 g. of nitrogen per 100 cc. of the fermenting solution, but after the lapse of 48 hours—when the extraction will generally have reached its maximum—this amount may have increased to 0.0450 g.

Experimental.

1. *Action of living yeast on amino-acids in the absence of fermentative activity.* A washed brewery yeast was seeded into a solution of asparagine containing 0.2 % of nitrogen (1.072 g. of asparagine per 100 cc.). The liquid was subsequently maintained at 37° and at intervals of 12 hours quantities of about 75 cc. were withdrawn and filtered, the nitrogen being estimated in 50 cc. of the clear filtrate. (Schleicher and Schüll's No. 572½ filter papers are found to be especially suitable for the complete separation of yeast cells from fermented solutions.) The results thus obtained are as follows:

Table XIV.

Time in hours	...	...	...	0	12	24	36	48	72
Nitrogen in liquid (g. per 100 cc.)				0.2016	0.2002	0.2022	0.2038	0.2040	0.2044

The only conclusion to which these figures lead is that without fermentation no assimilation of nitrogen takes place. No importance can be attached to the apparent slight decrease of nitrogen during the first 12 hours, although later experiments seem to show that, if the yeast is in a healthy condition, assimilation may occur to a very slight extent, this probably being due to the still incomplete subsidence of the stimulus caused by a previous fermentation.

The gradual increase observed in the nitrogen-content of the solution after the initial 12 hours is of greater importance than is at first evident; this point is considered in detail in Part IV of the present paper.

2. *Relationship between nitrogen assimilation and zymatic activity.* The relative rates of assimilation of nitrogen and of fermentation were determined by means similar to those employed in the experiment described above, except that the solution here contained 10 % of cane sugar. The progress of the fermentation was followed by estimations of the sugar content of the liquid according to Bertrand's method. The results obtained are shown in Table XV.

Table XV.

Time in hours	0	6	12	24	30	36	48	60
% sugar in solution	100.00	71.00	31.00	5.00	2.75	1.20	1.20	1.20
% nitrogen in solution	100.00	—	78.00	62.80	—	49.70	42.40	42.50

Examination of these figures reveals two important points: (1) the rate at which nitrogen was assimilated in this experiment was practically constant for the first 30 hours, but subsequently underwent rapid diminution until after 50 hours absorption of nitrogen had practically ceased: (2) although fermentation of the sugar had practically ceased after 30 hours, the assimilation of nitrogen continued for 50 hours.

3. *Effect on nitrogen assimilation of rapid fermentation.* Very rapid fermentation was induced by seeding three 10 % sugar solutions containing 1 % nitrogen as asparagine with yeast at the rate of 300 (A), 200 (B), and 100 (C) cells/T respectively. After the lapse of 12 hours visible fermentation had

ceased in *A* and had nearly ceased in *B*, whilst *C* was still fermenting vigorously. At the end of 24 hours, estimations of nitrogen in the usual way gave the following results:

Table XVI.

Experiment ...	...	<i>A</i>	<i>B</i>	<i>C</i>
Rate of seeding ...	...	300	200	100
% nitrogen assimilated		8.50	16.80	22.60

4. *Effect on nitrogen assimilation of the amount of available nitrogen present.* The liquids employed consisted of 10 % solutions of cane sugar and a saturated solution of asparagine, these being mixed in proportions to bring the nitrogen content to 2.0, 1.0, 0.4 and 0.2 % respectively. These mixed solutions were then seeded with yeast at the rate of 100 cells/*T* and allowed to ferment at 37°. The fermentations were apparently complete in 24 hours, at the end of which time the amounts of nitrogen remaining in the various solutions were estimated.

The following are the results obtained:

Table XVII.

Experiment	Total nitrogen as g. per 100 cc.		% nitrogen assimilated
	Initial	Final	
<i>A</i>	2.0000	0.9000	55.00
<i>B</i>	1.0000	0.5083	49.17
<i>C</i>	0.4000	0.2889	27.78
<i>D</i>	0.2000	0.1549	22.55

Expressed as ratio, these figures are as follows:

Total nitrogen introduced in *A* : *B* : *C* : *D*; 100 : 50 : 20 : 10.

Total nitrogen assimilated in *A* : *B* : *C* : *D*; 100 : 44.7 : 10.1 : 4.1.

It must therefore be concluded that the smaller the amount of nitrogen available for extraction from the solution, the less is the power of the yeast to extract the nitrogen.

Further elucidation of this point was attempted by means of a series of experiments in which a 10 % solution of cane sugar containing 0.03, 0.04, and 0.05 g. respectively of nitrogen as leucine per 100 cc. of solution was fermented in the manner described above. Leucine was chosen here because asparagine contains both "amino" and "amido" nitrogen, and previous experiments had shown that nitrogen is not absorbed at the same rates from these two groups. It was therefore thought that with such small amounts of nitrogen present in the solutions here involved, the use of leucine would present fewer difficulties than that of asparagine. Estimations of the amounts of nitrogen present in the solutions were made at intervals of 24 hours, with the following results:

Table XVIII.

Experiment	Initial nitrogen	Final nitrogen			
		24 hours	48 hours	72 hours	96 hours
<i>A</i>	0.0300	0.0120	0.0030	0.0037	0.0050
<i>B</i>	0.0400	0.0170	0.0060	0.0045	0.0049
<i>C</i>	0.0500	0.0230	0.0107	0.0050	0.0045

These numbers confirm that (1) the greater the amount of available nitrogen the greater the initial rate of extraction, and (2) that, in all cases but *C*, before all the nitrogen has been absorbed, an increase in the nitrogen content of the solution is to be observed. This latter is a most important point as is shown in the final part of this paper.

PART IV. THE APPARENT EXCRETION OF NITROGEN BY YEAST.

Towards the end of Part III of this communication it was pointed out that experiments seemed to indicate that under certain circumstances yeast may lose nitrogenous matter, and, for want of a better term, this may be called "nitrogen excretion."

A preliminary experiment showed that a yeast of high nitrogen content (in the case in point the yeast contained 10.3 % of nitrogen on the dry matter) would, under certain conditions, lose about 35 % of its nitrogen in fermenting a pure cane sugar solution. That this excretion did not wholly consist of bodies detrimental or useless to the yeast cell was shown by the fact that these bodies could be partly reassimilated by another yeast suggesting that the action is not a true excretion. In the following pages, however, this term has been applied to this peculiar action of the yeast.

Apparently the increase in the nitrogen content of a fermenting solution, resulting from this action, may be explained in two ways. It may be the result of (1) a "normal" excretion of nitrogen by the yeast cell, or (2) a disintegration of the cells consequent upon the commencement of auto-digestion. If the action is normal then it would be thought that a high-nitrogen yeast¹ could, cell for cell, excrete more nitrogen than a starved yeast. On the other hand, if the excretion is a pathological condition, then the starved yeast should be more prone to auto-digest and consequently should release its nitrogen constituents more rapidly. Experiment showed, however, that this was not the case, but that conversely a well-nourished, high nitrogen yeast excreted more nitrogen in fermenting a certain amount of sugar than a starved yeast. Due however to the differences in the fermentative powers of yeasts under certain conditions difficulties in comparison were found as is explained in the experimental section.

The question naturally arises, "Is this action contingent on the life of the yeast cell, or only connected with the zymatic activity?" Experiment soon proved that the action was essentially a living one, for a sample of dried yeast containing very active zymase was introduced into a sugar solution in amount to make it comparable with the living yeast experiments, and it was found that between the sixth and one hundred and twentieth hours of fermentation, the liquid only gained about one-eighth the amount of nitrogen excreted by the living yeast. Another experiment in which all the enzymes had been de-

¹ It must be emphasised that in all these experiments one race of yeast was used; reference to a high-nitrogen yeast always means a well-fed yeast, containing a large percentage of nitrogen.

stroyed gave a similar result, proving that the action is essentially a vital phenomenon.

This being the case, one would naturally expect the amount of excreted nitrogen to be proportional to the number of cells present, and experiments seeded with 10, 20, 30, etc., to 150 cells per *T*, showed that the yeast yielded to the liquid while fermenting a 10 % cane sugar solution amounts of nitrogen proportional to the number of cells present. The initial nitrogen coefficient of the yeast was 0.0019, and the average loss per unit cell was 0.00011 in two days, and 0.00014 in five days, that is to say, the yeast excreted 5.78 % of its nitrogen in 48 hours, and 7.36 % in 120 hours.

Not only, however, is this activity dependent upon the life of the yeast, but it is also dependent upon the zymatic activity. For example, fresh yeast seeded at 100 cells/*T* into pure water yielded 0.41 g. N per 100 cc. in 72 hours, which amount had not perceptibly increased after 120 hours, while a similar seeding into a 10 % cane sugar solution gave 0.71 g. in 72 hours and 1.70 g. in 120 hours. There is, therefore, some connection between nitrogen excretion and fermentative activity. Before studying this connection it was necessary to consider to what extent the time factor enters into the excretory action.

A solution of invert sugar was seeded with yeast at the rate of 75 cells/*T* and maintained at 20° during fermentation. At intervals of time the progress of the fermentation was estimated, and at the same time the amount of nitrogen in the solution was determined. From confirmatory series of experiments it was found that the rate of fermentation of the invert sugar present was constant for about 16 to 20 hours, then rapidly decreased, until at 36 hours it had ceased. On the other hand the nitrogen excretion progressed regularly for about 75 hours, after which it rapidly decreased, and after the 110th hour the action had apparently terminated.

The constant rate of excretion was approximately, in two series *A* and *B*, 0.000419 and 0.000384 g. N per hour for 75 cells/*T*. Compared with the results of a previous experiment the excretion in 24 hours per unit cell would be in these two cases 0.000134 and 0.000123 as against 0.00011 previously found. Variations to this extent have constantly been found to occur, and are probably due to the exact physiological state of the initial seed yeast.

The most important conclusion to be drawn from these two experiments is that although there is no direct relation between fermentation and excretion, the activity of the zymase stimulates the latter action, and the cessation of the metabolic changes thus stimulated does not coincide with the apparent termination of the fermentative activity. As would be expected the zymatic and excretory powers of the yeast cell are two distinct functions, but the activity of the former in some way stimulates the latter into action.

This stimulatory action of fermentation was proved very clearly as follows. Water was seeded with fresh yeast at the rate of 100 cells/*T*, and allowed to stand for 30 hours during which time two nitrogen determinations were made. At the thirtieth hour sugar was added and nitrogen determinations

were again made on the liquid at subsequent intervals of time. No sugar was added to a second experiment and the differences in the amounts of nitrogen found in the two cases were very striking. For example after 72 hours the amount of N in the sugar solution was 1.07 g. per 100 cc. and 0.41 g. in the aqueous suspension.

Consequently nitrogen excretion is, to a certain extent, dependent on the zymatic activity of the yeast, and as the expression of the latter is the amount of fermentation, it appeared desirable to investigate the effect of the variation of the amount of sugar available. Solutions containing differing amounts of cane sugar were fermented with samples of the same yeast, and nitrogen determinations made after 24, 48, 72, and 120 hours. The results obtained show the great stimulating effect on the nitrogen excretion of increase of the amount of available sugar from 1 % to 5 %—confirmatory results being obtained for all periods of time. The stimulatory effect rapidly decreases, and above 10 % sugar increase does not result in an appreciable increased rate of nitrogen excretion.

The nitrogen thus excreted may be derived from

(1) the reserve nitrogenous food material in the yeast cell, in which case the loss of nitrogen should terminate with the exhaustion of that reserve material,

(2) the nitrogenous tissue of the cell itself and, if this is the case, it is possible that the continued action may result in the death of the organism.

It is, of course, difficult to keep a yeast in a state of prolonged fermentative activity in a solution because of the accumulation of the products of fermentation which eventually destroy the zymatic activity, but a similar state was obtained by continually transferring a yeast from one sugar solution to another as soon as fermentation finished in the first case. In this way it was found that after five such fermentations the zymatic activity of the yeast was practically destroyed, although, on introducing the exhausted yeast into a wort, after 24 hours 85 % of the cells appeared to be in good condition and 55 % of them were budding. Consequently after the fifth fermentation although exhausted the cells were not dead, and rapidly regained a normal condition. During these successive fermentations the yeast lost 37.8 % of its nitrogen, its nitrogen coefficient decreasing from 0.00200 to 0.00124. This experiment demonstrates that a yeast can lose more than a third of its nitrogen, but that further loss results in the disablement of the cell, with a consequent loss of its fermentative capacity.

All the work so far described may be said to have been concerned with yeast under abnormal conditions, the abnormality consisting in the absence of nutrient matter from the sugar solution. Much work of a fruitless character was undertaken to determine whether this excretory action takes place while the assimilatory processes are also progressing, such, *e.g.* as the tracing during fermentation of solutions containing small amounts of asparagine, leucine, alanine, etc., of the amino-index, for the index of the excreted nitrogenous

matter is not that of the amino-acid. Finally it was found that the best method was precipitation of the fermented solution by phosphotungstic acid, for 35–37 % of the excreted matter is precipitated by this reagent. The method employed consisted of the fermentation of sugar solutions containing non-precipitable nitrogenous matter, with subsequent estimation at intervals of the nitrogen precipitated by the reagent.

The results conclusively proved that even when rapid assimilation of nitrogen was taking place, excreted nitrogen could also be found in the solution, although the absolute amount under different conditions was subject to wide variation, the cause of which was not determined.

Experimental.

1. *Preliminary experiment.* A 20 % cane sugar solution was seeded with yeast containing a high percentage of nitrogen, at the rate of 100/*T*, and during fermentation carried out at 10° (in order to obtain a slower action) the nitrogen in solution was determined every 24 hours. The yeast employed was a sample of brewer's yeast and from Table XIX it will be seen that during its fermentation (which, judged by the settlement of the yeast, lasted about seven days) it lost a very considerable amount of nitrogen to the liquid.

Table XIX *a*.

Time in hours	...	24	48	72	96	120	240
N in liquid as g. per 100 cc.		0.0760	0.0840	0.1520	—	0.2440	0.3360

Table XIX *b*.

	Total N in solution	Total N in yeast	% N in yeast on dry matter	% N lost by yeast
Initial	Nil	1.3520	10.30	—
Final	0.3360	1.0160	7.74	24.85

2. *Assimilation by yeast of nitrogenous matter previously excreted.* After the fifth day of fermentation, 250 cc. of the solution was extracted, filtered and vacuum distilled at 40°. In this way the residue was obtained free from alcohol and water at a temperature at which practically no change would have taken place in the constitution of the amino-acids. The residue so obtained was dissolved in 10 % sugar solution and fermented with a normal yeast in the ordinary manner. The yeast contained 8.2 % nitrogen on the dry matter; nitrogen determinations made on the solution gave the following results:

Table XX.

Time in hours	0	48	120
Total N in solution	0.0620	0.0240	0.0370

The fermentation was practically over at the end of the second day when 61.30 % of the original nitrogen had been reassimilated by the yeast, and it is interesting to note that at the end of five days a further excretion had taken

place. This suggests that about one-third of the original excretion is not assimilable by the yeast, which may be that portion precipitable by phosphotungstic acid.

3. *Comparison of the excretory powers of yeast of high and low nitrogen coefficient.* Sample *A* was a well-nourished yeast containing 9.2 % nitrogen on the dry matter, while *B* only contained 7.0 % of nitrogen. The yeast was introduced at the rate of 100 cells/*T* into 10 % sugar solution and fermentation allowed to proceed at 37° for four days, during which time determinations of the nitrogen content of the solution were made after 48 hours and at the termination of the experiment.

Table XXI.

Yeast condition	% N in yeast on dry matter	N in liquid	
		48 hours	96 hours
High nitrogen	9.2	0.3052	0.3648
Low nitrogen	7.0	0.2027	0.3500

The starved yeast was sluggish of action and did not finish fermentation until the expiration of four days, the well-fed yeast had completely finished in less than 48 hours. The figures show the stimulatory effect of fermentation on the excretion of nitrogen, an effect which is more fully studied in a later experiment.

4. *Comparison of living and air-dried yeast as regards nitrogen excretion.* A yeast which had been air dried at 37°, and afterwards ground up in a mortar, was used, the preparation having been made some 12 months before use. Sufficient was added to a 10 % sugar solution to bring the nitrogen content of the solution up to 0.1352 N per 100 cc., thus making comparison with the experiment previously described possible. The mixture was allowed to stand at 37° and a vigorous fermentation took place, showing that the zymase was still in a state of activity. After periods of 6 and 120 hours the nitrogen content of the solution was measured, the amount of nitrogen introduced into the solution mechanically being obtained from the first reading.

Table XXII.

Experiment	N in solution g. per litre	
	6 hours	120 hours
Dried yeast	0.0304	0.0568
Living yeast	0.0150	0.2440

In the case of the dried yeast it is obvious that 0.0304 g., or nearly 60 % of the total amount of nitrogen given to the solution, was introduced mechanically in a soluble form, while it is to be noted that in 120 hours the *total* amount given to the liquid is less than 25 % of that excreted by the living cell.

5. *Comparison of living yeast with one in which all the enzymes had been destroyed.* The experiment was conducted on exactly parallel lines, except that yeast previously dried at 95° was used, and gave the results set out in the following table, in which the figures for living yeast are shown for purposes of comparison.

Table XXIII.

Experiment	N in solution g. per litre	
	6 hours	120 hours
Dried at 95°	0.0535	0.0862
Living yeast	0.0150	0.2440

6. *Effect of varying the amount of yeast present.* A series of experiments was made in which 10 % sugar solution was seeded with yeast at the rate of 10, 20, 40, 80, 150, and 320 cells/*T*. On plotting the results it became apparent that as far as this series went, the excretion varied in a regular manner with the amount of yeast present. The latter points, however, were so far apart in comparison with the first three, that the experiments were repeated in an extended form, 15 fermentations being carried out, the seeding being from 10 to 150 cells per unit volume. Nitrogen determinations were made at the end of 48 and 120 hours, those in the lower seedings being made on amounts of liquid varying up to 250 cc. The results—Table XXIV—are given as grams of nitrogen per litre.

Table XXIV.

Rate of seeding ...	10	20	30	40	50	60	70	80
Nitrogen in solution after 48 hours ...	—	0.021	—	0.048	0.054	0.064	0.076	0.122
Nitrogen in solution after 120 hours ...	0.025	0.024	0.050	0.055	0.068	0.081	0.104	0.110
Rate of seeding ...	90	100	110	120	130	140	150	
Nitrogen in solution after 48 hours ...	—	—	0.116	0.132	0.136	—	0.144	
Nitrogen in solution after 120 hours ...	0.131	0.129	0.144	0.172	0.208	0.192	0.221	

The results are expressed graphically in Fig. 1, and it must be remembered on examining these figures that the vertical scale is a very large one, so that, excluding the 80 cell experiment in the second day, the greatest error occurs in the 120 cell experiment, and is equal to 0.00084 g. N per 100 cc.

The curves show without doubt that the amount of nitrogen excreted is proportional to the amount of yeast introduced into the sugar solution, other factors being constant.

The following table shows, in brief, the loss of nitrogen by the yeast.

Table XXV.

Time	Nitrogen coefficient $\times 10^4$		% N lost by yeast
	Initial	Final	
48 hours	19.0	17.9	5.78
120 hours	19.0	17.6	7.36

7. *Effect of the time factor in nitrogen excretion.* A solution of invert sugar containing 10 g. per 100 cc. was seeded with yeast at the rate of 75 cells/*T* and allowed to ferment at 20°. For the first two days the amount of sugar fermented and the nitrogen excreted were determined every two hours from

8 a.m. to 8 p.m. and afterwards with varying intervals of time for five days¹. The following are the results of two experiments:

Fig. 1.

Table XXVI.

Experiment A.

Time in hours	2	4	6	8	10	12	24	26	28	30	32	34
Sugar fermented	1.70	1.80	2.90	4.60	5.50	6.90	8.70	9.50	9.62	9.73	9.85	9.87
N excreted*	—	0.90	0.74	0.78	1.26	0.84	1.18	—	1.28	—	1.34	1.30
Time in hours	36	48	52	56	60	72	78	84	96	102	120	132
Sugar fermented	9.88	9.90	Constant.....									
N excreted*	1.56	1.66	1.70	1.64	1.94	2.14	2.12	2.12	2.02	2.20	2.22	2.26

* As g. $\times 10^2$ per 100 cc.

¹ The invert was prepared from cane sugar by taking a 40 % solution and hydrolysing in a steam steriliser for three hours with 5 cc. of strong hydrochloric acid per litre of solution. The optical rotation was then found to be constant, the acid was neutralised with sodium hydrate, and the solution diluted until it contained 10 % invert sugar. The determinations of the invert were made according to the method of Bertrand (*Manipulations de Chimie Biologique*).

Fig. 2

These figures have already been studied in the theoretical part of the paper, and it only remains to be said that graphically they demonstrate very clearly the conclusions drawn.

10. *Effect of exhausting the fermentative powers of the yeast.* A litre of 20 % cane sugar solution was seeded with yeast—nitrogen coefficient 0.0020—at the rate of 100 cells/*T*, and the fermentation allowed to proceed at 37°. At the conclusion of the fermentation—about 32 hours—the yeast was washed once

Fig. 3.

by decantation and filtered off through a Buchner funnel. The liquid, which was not quite clear, was treated with alumina cream, refiltered, and the perfectly bright filtrate acidulated with 100 cc. *N*/20 sulphuric acid (to prevent the escape of any ammonia) and concentrated to 200 cc. The nitrogen was determined in an aliquot portion of this solution.

The previously separated yeast was introduced into a further litre of 20 % sugar solution and allowed to ferment at 37°. The process of separation was carried out as described above, at the termination of the fermentation and the

whole process repeated. After the third fermentation the yeast had assumed an appearance altogether different from its original condition, for, although the cells looked perfectly healthy and approximately 5 % of them were budding, the mass was of a slimy, sticky nature, extremely difficult to filter. This appearance was much more marked after the fourth fermentation, which had been of a sluggish nature, and had taken much longer to commence, and when the yeast was introduced into the sugar solution for the fifth time the action was extremely slow. The yeast separated from this fermentation had apparently lost its zymatic power, but on being introduced into a wort (gravity 1053) it was found that after 24 hours about 85 % of the cells seemed to be in a fairly good condition and about 55 % of them were budding.

The revived yeast was again sown into wort, and after a further 24 hours only a very few poor cells were to be seen, and the majority were budding, thus demonstrating that although after the fifth fermentation the yeast was very exhausted, the cells were not dead. The following figures show the amounts of nitrogen lost during the successive fermentations:

Table XXX.

No. of fermentation	1	2	3	4	5
N excreted during fermentation	0.1160	0.1554	0.1967	0.1596	0.1293
Total amount of N excreted	0.7570				
Total N originally in the yeast	2.0006 g.				
% N lost during five fermentations	37.85				

There is apparently no explanation of the irregularity of the amounts of nitrogen excreted during different intervals, unless it be, and this is perhaps an important factor, that the aeration of the cells between the successive fermentations varied in amount and in efficiency; this might easily result from the varying states of the yeast and consequently the slightly different methods of filtration employed. However, the experiment demonstrates that during fermentation the yeast has excreted 37.8 % of its nitrogen, and not only this, but that further loss results in the disablement of the cell, with a consequent loss of its fermentative capacity.

11. *Determination of excretory matter in the presence of amino-acids.* Leucine was added in varying amounts to 20 % cane sugar solutions and fermented with yeast introduced at the rates of 50 and 100 cells/T; controls were made in which no yeast was added in one case and no sugar in another. At intervals the nitrogen was estimated as usual, and at the same time 50 cc. of the clear filtrate treated with 5 cc. phosphotungstic acid solution (10 % phosphotungstic acid in 5 % sulphuric acid). The mixture was stirred and the precipitate which formed allowed to settle, which it did very well in about 12 hours. The supernatant liquid was then decanted off and the precipitate—which in character was of a rather heavy, flocculent nature, and of an almost white or pale clay colour—mixed with about 15–20 cc. of the precipitant [Brown, 1903,

2] and filtered through an ordinary filter paper of a fairly loose texture¹; nitrogen determinations were subsequently made on the clear filtrate. Table XXXI shows the figures obtained for one such series.

Table XXXI.

Exp.	% sugar	Initial N as amino-acid	Final N after 48 hours	N pptd by phospho- tungstic acid	Change in N content of solution
<i>A</i>	0	0	0.0052	0.0020	+ 0.0052
<i>B</i>	0	0.0584	0.0600	Trace	+ 0.0016
<i>C</i>	20	0	0.0528	0.0200	+ 0.0528
<i>D</i>	20	0.0584	0.0308	0.0232	- 0.0276
<i>E</i>	20	0.0630	0.0500	0.0232	- 0.0130
<i>F</i>	20	0.1168	0.0552	0.0306	- 0.0616

These figures show that:

1. Where fermentation has not occurred, no precipitable nitrogen, or practically none, appears in the solution.
2. The greater the amount of nitrogen assimilated, the greater the amount of precipitable nitrogen found.

GENERAL CONCLUSIONS.

1. *Factors which influence the assimilation of nitrogen by yeast.*

- (a) Excess of yeast ensures the removal of the greatest *total* amount of nitrogen.
- (b) During active fermentation the greater the coefficient of multiplication, the greater the amount of nitrogen assimilated by *each* cell.
- (c) Active reproduction may result in a lowering of the nitrogen coefficient, even when a large amount of available nitrogen is present.
- (d) The final nitrogen coefficient of a yeast is independent of the initial coefficient, and tends to reach a value constant for any particular conditions of reproduction.

2. *Mechanism of the extraction of nitrogen from amino-acids by yeast.*

- (a) The amidase of Effront does not (as stated by him) produce solely a volatile acid by its action on asparagine, but also a non-volatile acid, probably malic acid.
- (b) If malic acid represents the first stage in the degradation of asparagine, it is not fermented as such, for the acid is unattacked by yeast. It may combine with NH_3 to form the ammonium salt which is completely destroyed by fermenting yeast, ethyl alcohol being the only recognised product of the action.

¹ The first runnings had always to be returned, but after a few minutes the filtrate came through quite bright. The clear liquid was warmed to about 40° and treated with ground baryta with constant stirring, in order to remove the excess of phosphotungstic and sulphuric acids. A slight excess only of the baryta was added, and the liquid allowed to settle, the clear liquid decanted off, and the remainder filtered and the precipitate washed. The excess of baryta was then removed by carbon dioxide, and the nitrogen estimated in the filtrate. It will be understood that in face of the number of processes extreme care was required to obtain concordant nitrogen readings.

(c) Propionic acid (which Effront states results from the action of amidase on asparagine) is not fermentable, and its ammonium salt is attacked with difficulty by yeast.

3. *The influence of the available amino-acids and sugar on the nitrogen assimilation by yeast.*

(a) Fermentative activity is essential to nitrogen assimilation. The two actions are not proportionate, but the former stimulates the latter, and, once induced, desamination may continue after zymatic activity ceases.

(b) Excessive zymatic activity does not ensure rapid nitrogen assimilation, in fact a quick fermentation results in only a small amount of nitrogen being extracted.

4. *Apparent excretion of nitrogen by yeast.*

(a) During fermentation yeast continually loses nitrogen to the liquid, and this action has been called "nitrogen excretion." Cases have been noted where more than 33 % of the yeast nitrogen was so lost, but further loss resulted in the disablement of the cell.

(b) Nitrogen excretion is dependent on the life of the cell, and takes place even when nitrogen is being assimilated.

(c) The bodies so excreted can be used by yeast as a source of nitrogen under suitable conditions.

(d) Increase in the amount of sugar available for fermentation increases the rate of excretion, especially between the limits 1-5 % sugar, and although zymatic activity is necessary for nitrogen excretion, the two are not proportionate, nor does the cessation of the former result in an immediate cessation of the latter.

The Author desires to acknowledge his great indebtedness to the late Professor Adrian J. Brown, F.R.S., for the kindly advice and sympathy extended to him during the course of the above research (which was concluded prior to 1914); to Mr T. H. Pope, B.Sc., for his keen interest in the work, and to Professor A. Harden, F.R.S., for his great assistance in publication.

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