An Outer Metabolic Region of the Yeast Cell

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During fermentation with baker's yeast, free succinic acid appears rapidly in the suspending medium (Conway & Brady, 1947). With 1 part of washed centrifuged yeast to 0·6 part of 5% (w/v) glucose, forming a mixture with approximately 0·8 part of cells to 0·8 part of external fluid, about 40 m-equiv. of succinic acid per kg. cells diffuse out within 30 min., the pH of the suspending fluid falling to about 3. If the succinic acid diffused throughout the yeast cell, then under such conditions the total succinic acid plus succinate within the cells should be much higher than outside; but it is found to be about one-tenth of the external value.

When resting cells are suspended in 0·2 M-succinic acid the same relation holds. For the resting cells the ratio of the inner to outer concentrations using 0·2 M-acid is only a little increased beyond the level in fermentation, and it does not alter significantly over many hours. Such succinic acid, though not diffusing appreciably into the whole cell, yet enters some real space therein. Inulin does not enter this region, neither do peptone, used by Montgomery & White (1945) to determine the intercellular space, nor gelatin. The bivalent ions of succinic acid appear debarred, and the space can be regarded as having inner and outer membranes with different permeabilities.

The view that free succinic acid, which must be formed in the cell, is prevented from diffusing backwards by some energetic process *without a membrane barrier*, may be shown to be untenable from the rapidity of the diffusion under such circumstances and the magnitude of the required energy.

This is in no way opposed to the view that the succinate ion may be carried from within the cell outwards *across the inner membrane* of the outer region, by a process involving the expenditure of energy, combined with a special redox carrier. Evidence for such a process is being presented in a subsequent communication.

Such data related to succinic acid diffusion, and many other facts of a similar kind, are here presented as showing the existence of an outer metabolic region of the yeast cell (Conway & Downey, 1948), and for an approximate determination of its volume; also for the view that the region may be identified with the cell wall.

In relation to this outer region, and in a preliminary way, the permeability of the cell to some groups of substances of physiological importance have been examined, this study being related to that on acid production described in subsequent papers.

METHODS

Yeast suspensions. Baker's yeast was washed by suspending 0·5 kg. in 4 l. of tap water and centrifuging, the procedure being twice repeated, and the final centrifuging continued for 20 min. at about 3000–4000 rev./min. The centrifuged yeast was then suspended in an equal volume of fluid, or, very occasionally, strongly pressed to extrude most of the extracellular fluid, the resulting yeast being free from stickiness and crumbling easily to a powder. Such pressed yeast was found to contain about 0–5 g. interspace water per 100 g.

Haematocrit determinations. The following simple haematocrit was found useful. Into a thick-walled glass capillary tube of uniform bore and of suitable length for the centrifuge, the suspension was sucked by a soft rubber teat attached to one end. The column of fluid was sucked to within 3–4 cm. distance from the top of the tube. The latter was now withdrawn from contact with the solution, the column of fluid being maintained roughly at the same level, held horizontally and the rubber teat detached. Semi-molten solid paraffin was then pressed into the dry end to about 0·5–1 cm. length and the paraffin melted by holding the end of the tube, still held horizontally, over a hot plate. Cooling the end of the tube still in the same position, under the tap, the paraffin solidifies. On centrifuging up to 3000–4000 rev./min. the paraffin is not dislodged, and the space between fluid and paraffin is obliterated. The arrangement functions as a simple and accurate haematocrit. Immersion in boiling water and cleaning with ethanol and ether restores the tube for further usage.

Determination of solutes in the suspending fluid before and after mixing

Titration method. In the first group of experiments described, the suspending fluid contained only an organic acid in 0·2 M concentration. An electrometric titration of the suspending medium, reckoning the difference in titre between the two pH levels on each side of the region of maximum buffering, gave a sufficiently accurate measure of the substance. Blank titrations with water as suspending fluid were carried out, and were found to be comparatively negligible.

Gravimetric method. When washed centrifuged yeast is mixed with an equal volume of tap water, allowed to remain in contact 30 min. and centrifuged, then 5 ml. of the centrifuged fluid taken and dried, an average weight of only 0·8 mg. was found, but occasionally 2–3 mg. When mixed, say with 3% inulin, over 100 mg. are obtained. It is obvious

that such a method provides an easy and accurate procedure for measuring the inulin space and may be used with other solutes besides inulin; with peptone and gelatin for example, also with non-fermentable sugars, a suitable allowance being made in the latter case for water abstracted from the washed yeast cells on suspending in the solution. Apart from such titration and gravimetric methods, various special methods, were used, and the following may be mentioned.

Succinic acid. This was, in some instances, determined by the succinoxidase method (Weil-Malherbe, 1937; Krebs, Smyth & Evans, 1940).

Chloride. This was determined by microdiffusion methods (Conway, 1947).

Reducing sugars. These were determined by the well-known Bertrand method, comparison of reduction values before and after mixing with yeast being all that was required.

Inulin. Apart from the gravimetric method this was also determined by the method of Steinitz (1938).

Peptone. This was determined by the Kjeldahl incineration with microdiffusion procedure (Conway, 1947) for the NH_3.

Glyceric acid. This was determined in a number of experiments by the colorimetric method of Rapoport (1937).

Sodium. This was determined by a method based on the precipitate formed by zinc uranyl-acetate, as previously described for tissues (Boyle & Conway, 1941).

The pH values of mixtures, suspending fluids, etc., were determined by the Beckman glass electrode.

RESULTS

Intercellular space

A knowledge of the fluid volume surrounding the yeast cells, before they are suspended in a solution, is required for measuring the quantitative entrance of the solute into the cells. If the yeast is suspended in the solution in the proportion of 1 kg. of yeast to 1 l. of solution, the intercellular space in the yeast before suspending (S) is given as l./kg., by the relation

$$S = C_o/C_m - 1. \quad (1)$$

Here C_o is the original concentration of solute in the solution and C_m the concentration after mixing.

Inulin space. Inulin, and some other substances investigated, gave the least value for this space. For baker's yeast washed and centrifuged under the conditions described, the gravimetric method for inulin gave a mean space for twelve experiments of $0{\cdot}217 \pm 0{\cdot}005$. This gives the mean as l. water/kg. centrifuged yeast. If given per litre of centrifuged yeast it becomes $0{\cdot}23 \pm 0{\cdot}005$, since the mean specific gravity of the centrifuged yeast was found to be 1·06. The colorimetric method gave $0{\cdot}191 \pm 0{\cdot}017$ (sixteen results). The gravimetric method shows much less variability and the result therefore is taken as the intercellular space in yeast centrifuged as described.

Peptone and gelatin spaces. These were determined by the gravimetric method using dialysed 2 % (w/v) solutions. Peptone results were confirmed by total nitrogen estimations. The mean values for peptone (six results) was 0·23 (per kg. centrifuged yeast) and for gelatin (ten results), 0·21.

Theoretical interspace as that of the closest packing of spheres. At this point there arises the question of the theoretical interspace between equal and non-deformable spheres in the closest packing. If one considers first a cubical lattice connecting the centres of uniformly contacting spheres, each unit cube will have sides equal to d, the diameter of the spheres. If the number of such lattices and spheres is very large the ratio of interspace to total volume is $(d^3 - \frac{1}{6}\pi d^3)/d^3 = 0{\cdot}477$, representing the maximum interspace for uniformly contacting spheres. If the cubical lattice of the centres of the spheres are now considered deformed to parallelopipeds with 60 and 120° angles in the faces, one has the arrangement of closest packing and the interspace as a fraction of the total volume is $(d^3/\sqrt{2} - \frac{1}{6}\pi d^3)$ divided by $d^3/\sqrt{2}$ or 0·26. For centrifuged yeast the initial expectation for the interspace volume is thus 26 % of the total volume. With inulin, peptone and gelatin, we have obtained 22–24 % (by volume) under the conditions of centrifuging described. The difference may be attributed to a small degree of deformation of the yeast cells. By pressing with a simple hand press the interspace may be reduced to about 4 % or less.

From Malm's (1947) detailed account of the literature connected with such evaluations of the intercellular space, it appears that Beetlestone (1930) and Just (1940) had also arrived at the above theoretical solution of closest packing. Beetlestone, using caramel as a measure of the intercellular space, had found it to be 38 % of the cell volume when the yeast was filtered off in a Büchner funnel, and 29·5 % for yeast from the brewery press. Using haemoglobin (0·75 %) Just ascertained the intercellular space to be 26 % of the total volume.

Such determinations refer, usually, to yeast pressed out in the factory, the pressure used, from Malm's (1947) account, being of the order of 1 atm. The degree of pressure used is of the greatest consequence in such intercellular space determinations which have little significance unless such conditions are similar. Thus we have found that using a simple hand press the intercellular space as measured by inulin can be readily reduced to the level of 0–5 % of the volume, the yeast being then dry in appearance, and crumbling easily to a powder. Our own determinations are valid for the centrifuging conditions described.

Here reference may be made to Orskov's (1945) statement (based on Meyer's calculations) that the volume of n similar spheres is $4{\cdot}19n \times r^3$, the space occupied by them in the most solid packing being $6{\cdot}00n \times r^3$. This gives an interspace of 30 % and is not the correct solution. The interspace was also experimentally investigated by him using malonamide distribution and found to be 34 % of the centrifuged

yeast. This indicates that the malonamide had entered the cells, and as its further penetration is very slow it appears to have entered the outer region rapidly.

The entrance of succinic acid into yeast

When 0·2M-succinic acid is mixed with centrifuged yeast in the 1 : 1 relation the mean S value (Eqn. 1) was found to be $0{\cdot}334 \pm 0{\cdot}004$. This figure includes an increase due to the abstraction of water from the yeast cells as a result of the osmotic pressure of the succinic acid solution. With an equilibrium value, after mixing, of 0·150M-succinic acid, the amount of water leaving the cells amounts to 3·0 ml./100 g. centrifuged yeast, as shown by haematocrit observations described below, so that the interspace of the original centrifuged yeast is $0{\cdot}304 \pm 0{\cdot}004$. The mean inulin space is $0{\cdot}217 \pm 0{\cdot}005$ so that the succinic acid space in the yeast cell is $(0{\cdot}087 \pm 0{\cdot}006)/(1{\cdot}0 - 0{\cdot}217) = 0{\cdot}111 \pm 0{\cdot}008$ (l./kg.). This 'space' may be better expressed as the ratio of the total succinic acid that has entered 1 kg. of the original centrifuged yeast to the external concentration (per litre) after such entrance. Such a ratio will be referred to subsequently as the 'R value', and with mixing proportions of 1 : 1 of centrifuged yeast and allowing for water abstraction,

$$R = (C_o/C_m - 1{\cdot}22 - w)/(1 - 0{\cdot}22). \qquad (2)$$

Here 1·22 represents the inulin value and w the water abstracted from the yeast cells in 1 kg. centrifuged yeast. For substances in 0·2M concentration which do not enter the central region, or do so very slowly, w is small and experimentally determinable as 0·03. Such determination is dealt with in a later section.

Table 1. *Determination of R values for succinic acid*

(Centrifuged yeast mixed 1 : 1 with 0·2M-succinic acid, succinic entrance determined from resulting concentration in the intercellular fluid.)

Time after mixing (min.)	No. of observations	C_o/C_m* (giving S.D. of mean)	R values from Eqn. 2, in which w is 3·0
2·5	12	$1{\cdot}31 \pm 0{\cdot}004$	$0{\cdot}08 \pm 0{\cdot}005$
15	9	$1{\cdot}35 \pm 0{\cdot}005$	$0{\cdot}13 \pm 0{\cdot}006$
30	9	$1{\cdot}30 \pm 0{\cdot}007$	$0{\cdot}07 \pm 0{\cdot}009$
60	11	$1{\cdot}38 \pm 0{\cdot}012$	$0{\cdot}17 \pm 0{\cdot}015$
120	6	$1{\cdot}34 \pm 0{\cdot}007$	$0{\cdot}12 \pm 0{\cdot}009$

* C_o = the concentration before mixing, and C_m that after mixing. The total average of C_o/C_m is $1{\cdot}337 \pm 0{\cdot}004$ (S.D. of mean). The mean inulin C_o/C_m is $1{\cdot}217 \pm 0{\cdot}005$.

The total R value for succinic acid (from 2·5 to 120 min.) from Eqn. 2 ($w = 3{\cdot}1$ from Eqn. 4) is 0·114. This gives the mean ratio of the amount of succinic acid entered into 1 kg. yeast to the external concentration.

From Table 1 it will be seen that the R value for 0·2M-succinic acid does not change appreciably from 2·5 to 120 min. after mixing. Also glass electrode measurements show a very rapid entrance of succinic acid which is complete in about 1 min. or so.

R values for other organic acids

Table 2 gives R values for twenty acids, including six amino-acids, up to 60 min. contact with the cells; seven were also examined after 24 hr. contact. The R values for most of the acids in Table 2 can only be regarded as very approximate (two determinations), and many more observations would be required to arrive at the mean value accurately. The results serve the purpose of classifying such acids with respect to their entrance of the outer region, and indicate whether they enter the whole cell rapidly or at any appreciable rate over the time observed.

Table 2. *R* values for 20 organic acids in 0·2M concentration with external* pH *at or near to* 3

(The R value is the ratio of total acid that has entered 1 kg. yeast to the external resulting concentration.)

Organic acid	R value: After 15 min.	R value: After 60 min.	R value: After 1440 min.	pH of mixture after 60 min.
Succinic	0·11	0·14	0·24	2·9
Glyceric	0·15	0·15	0·11	2·8
Pyruvic	0·16	0·20	—	3·0
Malic	0·26	0·27	0·42	2·6
Fumaric	—	0·25	0·96	2·9
Lactic	—	0·19	1·44	3·2
Citric	0·11	0·10	—	2·3
Tartaric	0·20	0·22	—	—
Gluconic	—	0·24	—	2·6
Oxalic	0·06	0·08	—	3·0
Glycine	0·08	0·11	—	3·0
Alanine	—	0·15	—	2·9
Leucine	0·14	0·08	—	2·8
Aspartic	0·15	—	—	3·2
Glutamic	0·06	0·06	—	3·6
Valine	—	0·23	—	2·7
Formic	1·75	1·75	—	3·2
Acetic	1·71	1·67	1·70	3·3
Propionic	1·30	1·50	—	3·7
Butyric	1·30	1·50	—	3·6

* The R figures have been obtained from Eqn. 2, where w is 0·03 kg. water for acids with low R values. Where the acid enters throughout the cells and the R value is high w becomes 0.

For sixteen acids, including the six amino-acids, the R values lie between 0·06 and 0·27 up to 60 min. After 24 hr. there appears to have been a marked entrance of lactic acid, and of fumaric acid to a somewhat lesser degree. Appreciable entrance of malic also appears to have occurred after 24 hr.

The group of sixteen acids, showing no appreciable penetration throughout the whole yeast cell after

60 min., includes dibasic acids and those with at least one hydroxyl or amino group.

The behaviour of the ordinary monobasic fatty acids, acetic, propionic, etc., is strikingly different. Almost at once the acid penetrates throughout the cell, to reach R values about ten times greater than for the first group. The figures for such acids used in 0·2 M concentration ranged between 1·30 and 1·75. The 24 hr. figure for acetic acid was obtained and showed no difference from that after 15 min. Glass-electrode observations show a very rapid entrance, which is complete in about a minute or less.

R values as a measure of the volume of the outer region

It appears then that various organic acids enter very rapidly into some relatively small outer space of the yeast cell, into which neither inulin nor peptone enter.

While some of these organic acids, such as succinic and glyceric, do not enter the inner region at an appreciable rate; others, such as acetic acid and its group, enter the whole almost at once. The R values of these two groups may be used to determine the water for solution in the outer region and the pH of both regions.

R values for organic acids entering only the outer region may be expected to be somewhat higher than the volume of this space, if there is appreciable buffering. With high external concentrations of the acids the R values should approach the outer space volume. As the external concentration is lowered R increases and the increase can be used as a measure of the pH of the outer region, when using an acid such as glyceric which originally has no appreciable concentration therein.

The volume of the outer region could also be deduced from R values for neutral substances which enter it freely, but enter the central region not at all or very slowly. Some non-fermentable sugars are here useful, such as arabinose and galactose. Orskov (1945) has shown that arabinose enters throughout the cells at a very slow rate, and for short periods the entrance of galactose at a pH below 7·0, would also appear negligible. Adsorption effects could be largely eliminated by examining the R values for arabinose at high concentration. Relatively much water is then abstracted and may be determined as follows.

Measurement of the water abstracted from the cells on suspending in fluids of varying osmotic pressures (the w value in Eqn. 2)

The water abstracted from the cells when 1 kg. centrifuged yeast is suspended in 1 l. of fluid may be calculated from haematocrit readings as follows.

It will be assumed for simplicity of description that the yeast has been washed in tap water and the suspensions are made in the proportion of 1 kg. centrifuged yeast to 1 l. of fluid, also that the same total heights are observed in the haematocrit. Then if f be the height of the yeast cell column after centrifuging, and 1 the height after centrifuging the suspension with water, each unit volume of centrifuged yeast has decreased by $(1-f)$ due to the solute. This reduction is not altogether due to a decrease in volume of the cells but also to a proportionate reduction in the interspace volume. This latter from the inulin studies described above amounts to 0·22 l. water/kg. of centrifuged yeast. The water that has left the cells per kg. of centrifuged yeast is therefore $(1-f)-0{\cdot}22\,(1-f)$ or $0{\cdot}78\,(1-f)$, and the water that has left 1 kg. of yeast cells is $0{\cdot}78\,(1-f)/0{\cdot}78$ or $(1-f)$. Eqn. 2 may then be written

$$R=(C_o/C_m-1{\cdot}22-w)/0{\cdot}78$$
$$=[C_o/C_m-1{\cdot}22-0{\cdot}78\,(1-f)]/0{\cdot}78. \qquad (3)$$

For arabinose observed after 30 min. we have found that w is proportional to the molarity of the external solution and may be written

$$w=0{\cdot}21\,\text{M}, \qquad (4)$$

in which it is to be understood that M is the molarity observed after mixing, or suspending; and w is the

Table 3. *R values for galactose and arabinose at different concentrations, with haematocrit data*

(The galactose and arabinose determinations, respectively, were carried out by the Bertrand method and the gravimetric method as given under Methods. The numbers in brackets refer to the number of experiments.)

	Galactose			Arabinose			Haematocrit results with galactose	
Concn. of solution used, C_o (M)	Concn. outside cells after 30 min., C_m (M)	C_o/C_m	R	Concn. outside cells after 30 min., C_m (M)	C_o/C_m	R	f value	Water from cells in 1 kg. centrifuged yeast
1·0	0·695	1·44 (2)	0·10	0·690	1·45 (2)	0·11	0·820 (2)	0·141
0·5	0·367	1·36 (2)	0·08	0·362	1·38 (2)	0·11	0·911 (2)	0·070
0·2	0·150	1·33 (2)	0·11	0·150	1·33 (2)	0·10	0·961 (2)	0·031
0·133	0·102	1·31 (2)	0·10	0·100	1·325 (12)	0·11	—	—
0·1	0·076	1·31 (2)	0·10	—	—	—	0·984 (2)	0·013
0·05	0·038	1·30 (2)	0·10	—	—	—	0·990 (1)	0·008

water removed from the cells in 1 kg. of centrifuged yeast, in which there is 0·22 interspace volume.

The haematocrit data are given in Table 3.

From Orskov's (1945) results it would appear that the passage of water from the cells is rapid and may be taken as practically complete after some minutes.

It is of interest to note that the same relation for w as in Eqn. 4 may be deduced from Malm's (1947) results with sodium chloride, 6 g. yeast suspended in 10 ml. of sodium chloride solution, being shaken for 1·5 hr. at 20°, after which the weight of the dry substance of the yeast cells was determined.

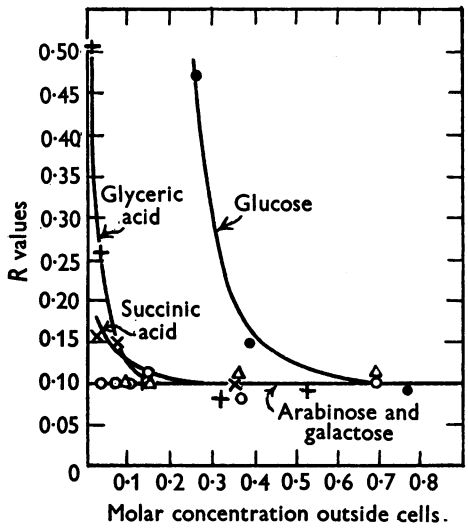

Fig. 1. The R values of arabinose (△), galactose (○), succinic (×) and glyceric (+) acids, also of glucose (●) plotted against the molar concentrations of such substances outside the cells, at the same time of observation (15–30 min.; 15 min. for glucose). At zero time 1 kg. yeast was suspended in 1 l. of a solution containing one or other of these substances. The R value of a substance is the amount that has entered 1 kg. washed cells as a ratio of the external concentration (per litre) at the time of observation. Each symbol in Fig. 1 gives the mean of two or more observations.

R values for arabinose, galactose and glucose, and for succinic and glyceric acids over a wide range of external concentration

Applying this equation for w the R values for arabinose and galactose have been determined from a range of 0·038 to 0·695 M outside the cells, and are given in Table 3. There is no appreciable effect on the R value with a 20-fold increase in the external concentration of the solute, so that surface adsorption as an explanation of such R values higher than those of inulin is untenable. In further support of such a conclusion is the fact that galactose, arabinose, and succinic acid at or beyond 0·15 M give the same or very similar R values.

In Fig. 1, R values for arabinose, galactose and glucose are plotted against the external concentrations. Arabinose and galactose give R values independent of the external concentrations, but glyceric and succinic acids show markedly increased R figures below 0·1 M and glucose below 0·4 M.

The increase in the R for glucose is obviously due to disappearance of the sugar in fermentation. The data were obtained 15 min. after mixing, which is more than sufficient for the full entrance of arabinose and galactose into the outer region.

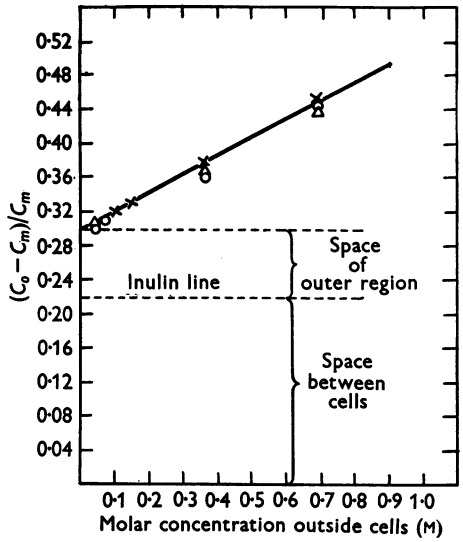

Fig. 2. Data of Table 3 plotted against the external molar concentrations at time of observation. C_o is the concentration of the substance in the original suspending fluid; C_m the concentration at the end of observation. The value of $(C_o - C_m)/C_m$ gives the 'space' occupied by the substance in excess of the original litre in which it was contained. Arabinose, ×; galactose, ●; haematocrit value for water abstracted + 0·30, △.

The increase in the R values for glyceric acid with diminishing concentration may be attributed to the increasing effectiveness of outer region buffering as the glyceric acid concentration is diminished. Succinic acid shows a similar but lesser effect. This is partly attributable to the fact that succinic acid is a weaker acid than glyceric, and partly to the presence of some succinate in the outer region. In such circumstances succinic acid may be expected to behave in the manner indicated by the curve in Fig. 1, but rising less steeply than glyceric acid at the lower concentrations.

Apart from the haematocrit readings, the water abstracted from the yeast cells may also be obtained by plotting $(C_o - C_m)/C_m$ against the external molarity. Over the range examined for arabinose, galactose and succinic acid the relation is linear as

shown in Fig. 2. The regression relation, with correlation coefficient practically 1·0, is

$$(C_o - C_m)/C_m = 0{\cdot}214\,\text{M} + 0{\cdot}30. \quad (5)$$

Here the slope gives the w value, and as M approaches zero the interspace water plus the water in the outer region is 0·30/kg. of centrifuged yeast, or $(0{\cdot}30 - 0{\cdot}22)/0{\cdot}78 = 0{\cdot}10$ water in the outer part of the cell.

If to the water abstracted from the yeast cells (per kg. centrifuged yeast), obtained from the haematocrit readings, 0·30 be added representing the water between the cells before mixing plus the water in the outer region, the values so obtained fall along the line in Fig. 2 for the arabinose, galactose and succinic acid data. In Fig. 2 the small triangles give the results so obtained from the haematocrit readings.

The R values for arabinose and galactose were also examined over the time period of 5 to 60 min. (Table 4). For arabinose no increase appears with time.

Table 4. *R values for arabinose and galactose after different times and of glucose after* 15 *min.*

(For these experiments 2% galactose and arabinose solutions were used. The brackets give the number of determinations made. With glucose a 20% solution was used, the R value being observed after 15 min.)

Time after mixing (min.)	R values		
	Arabinose	Galactose	Glucose
5	0·12 (5)	0·12 (2)	—
10	0·11 (3)	0·12 (2)	—
15	—	—	0·09 (2)
30	0·11 (3)	0·10 (2)	—
60	0·08 (2)	0·16 (2)	—

A small increase in the galactose figure may have occurred after 60 min., but its significance is not certain. Here it may be pointed out with respect to the variability of R determinations, that a change in the C_o/C_m ratio from 1·30 to 1·33 means a change in the R value from 0·09 to 0·12.

R values for ions

Potassium. Hevesey & Nielsen (1941), using labelled potassium, investigated the entrance rates into yeast cells. The entrance into resting cells was very slow, but with young fast-developing yeast cells equilibrium should be reached after about 2 hr. On the other hand, it can be shown that potassium as well as sodium and chloride ions enter the outer region or cell wall very rapidly.

Chloride. From the results to be described it appears that potassium chloride enters rapidly into the outer region of the cell. The inner membrane of this region is practically impermeable to chloride, so that over 24 hr. potassium chloride does not penetrate further to any appreciable extent.

The experiments were primarily undertaken to compare the permeability to potassium chloride of the yeast cell and muscle fibres (frog sartorius muscle used). The immersion solutions and pH value were then the same as described for muscle (Boyle & Conway, 1941). Each immersion fluid contained a constant sodium and bicarbonate concentration of 86 and 11 m-equiv./l., respectively, besides traces of calcium and phosphate, the pH being brought to 7·0 by bubbling with a gas mixture containing 3% carbon dioxide and 97% oxygen, and the washed yeast was suspended in each instance in about 50 times its volume of the suspending fluid. The potassium chloride content of the mixtures was varied from 0 to 300 m-equiv./l. The results obtained after 30 min. suspension showed no significant difference from those after 24 hr.

Table 5 shows the results obtained. It will be seen from the last column that the R value for chloride ranges over the series from 0·07 to 0·12.

It may be concluded that potassium chloride enters the outer region of the yeast cell rapidly, but the inner surface of the outer region does not allow chloride ions to pass in appreciable amounts up to 24 hr. under the given conditions.

Table 5. *Entrance of potassium chloride into yeast at various external concentrations and* pH = 7·0; *also R value for the chloride ion*

(The concentrations in brackets are interpolated. The small numbers in brackets give the number of experiments. The relative cell volume in column 6 was determined from the decrease in water content of the cells in accordance with Eqn. 4, the M value in that equation being obtained by multiplying the total c values in column 3 by 0·90 in accordance with freezing point data.)

External values m-equiv./l. Na concn. constant at 86			Concn. as m-equiv./kg. in yeast centrifuged from suspensions		Cell volume taking the washed centrifuged cell as 1·0	R values for chloride with respect to the original cell volume
K	Cl	c	K	Cl		
0	76	180	(120)	21·0	0·96	0·073
18	94	216	129 (3)	(27·0)	0·95	0·076
30	106	240	(134)	31·0 (2)	0·94	0·091
90	166	360	156 (3)	47·9 (3)	0·89	0·082
150	226	480	(185)	64·7 (1)	0·88	0·077
210	286	600	214 (3)	82·9 (3)	0·85	0·080
300	376	780	(248)	126·0 (2)	0·81	0·121

The contrast with the muscle fibre is shown in Fig. 3, in which the chloride per kg. muscle (interspace chloride deducted) is plotted against the increase of potassium chloride outside, and likewise the chloride in the yeast cell for immersion in the same solutions. The observations were taken after 24 hr. immersions and at 3–4° in the case of muscle.

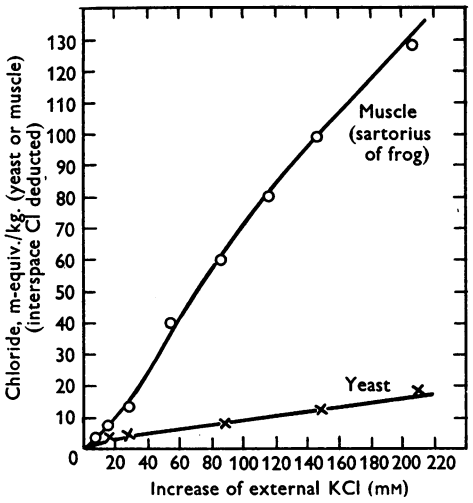

Fig. 3. The increase of chloride per kg. muscle (frog sartorius) and per kg. centrifuged yeast is plotted against the increase of KCl outside; NaCl maintained constant at 86 m-equiv./l.; pH approx. 7·0; muscle at 4°, yeast at 4° or at room temperature (no temperature effect being apparent). The chloride that would enter a space equal to the original interspace is deducted. The muscle data are taken from Boyle & Conway (1941). The yeast and muscle data give the means of several experiments.

Sodium. Sodium chloride enters as freely as potassium chloride into the outer region of the cells as shown in Table 6, and in a similar way it does not enter at an appreciable rate into the large inner region of the resting cells. On the other hand, and unlike potassium ions, sodium ions do not enter rapidly throughout the cells during fermentation in exchange for hydrogen ions.

R values for sodium and potassium chlorides using 0·1 M-solutions (1 : 1). With regard to the outer region it is of interest to know the exact amounts of sodium and potassium chlorides that enter with external values not greater than 0·1 M. If, for example, potassium ions are already held there by large fixed or non-diffusible anions, the *R* value for chloride should be lower than the arabinose and lactose value, and dependent on a Donnan relation. When sodium ions completely displace potassium ions in the outer region, on immersion in sodium chloride solution, a similar result for sodium chloride is obtained, the *R* of chloride being likewise lower than for galactose.

A number of experiments were carried out with washed centrifuged yeast suspended in 0·1 M-solutions of sodium and potassium chlorides, with and without the incorporation of 0·25 % succinic acid.

Table 6. *R values for potassium and sodium chlorides*

(The washed centrifuged yeast was suspended 1 : 1 in 0·1 M solutions. *R* values were determined from Eqns. 3 and 4.)

R values		Average of *R* values for	Time after suspending	
NaCl	KCl	NaCl and KCl	(min.)	pH
0·063 (4)	0·068 (5)	0·066±0·004	30	6·0
0·115 (5)	0·109 (5)	0·112±0·006	30	3·0
0·119 (10)	0·123 (11)	0·121±0·003	4–7	3·0

These are summarized in Table 6. It will be seen that there is no significant difference between the *R* values for sodium and potassium chlorides. There appears to be some increase in the amounts of chloride that have entered at a lower external pH. Thus at pH 6·0 the *R* value after 30 min. is 0·07, and at pH 3·0 it is 0·11. This is explicable by a decrease in non-diffusible anions in the outer region with increase of external acidity.

R values for succinate. From the following evidence it would seem that succinate ions do not enter the outer region, or do so very slowly. Washed, centrifuged yeast was pressed so that nearly all the

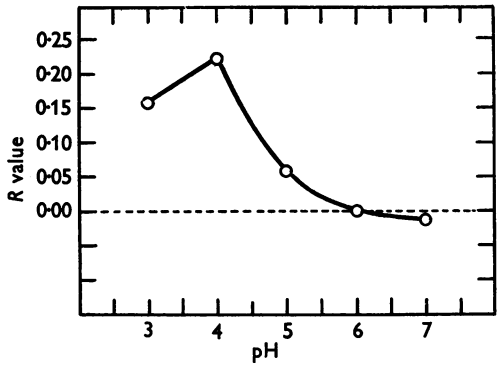

Fig. 4. *R* values for 0·02 M-succinic acid as the pH is increased by the addition of alkali. Yeast pressed to extrude the interspace fluid and suspended (1 : 1) in 0·02 M-succinic-succinate solution. Three sets of experiments summarized.

interspace fluid was extruded. Samples of this pressed yeast were then suspended in 0·02 M-succinic acid (1 : 1), and in various other solutions containing the same strength of succinic acid, but in addition various amounts of potassium hydroxide, the pH of the suspensions being determined. After 30 min. the suspensions were centrifuged, and the total succinic acid plus its ions determined by the titration method. From the results the *R* values of

the 0·02 M-succinate system (ions plus free acid) were determined at various pH values, and are plotted in Fig. 4. The points summarize the results of three sets of experiments, allowance being made for blank determinations over the pH range. It will be seen that from a pH of 6·0 onwards the R value is practically zero.

While such conditions would need to be supported by direct succinic determinations using the succinic dehydrogenase, they support the view that at least the bivalent ions of succinic acid do not readily enter the outer region of the cell.

DISCUSSION

Direct evidence for the existence of a small outer region of the yeast cell appears from the difference between the inulin and peptone space and that of the non-fermentable sugars, succinic acid and many other substances. This space with water content one-tenth of the whole cell volume is at least impermeable at its outer surface to proteins, to carbohydrate molecules as large as inulin and to divalent ions of succinic acid.

Succinic and glyceric acids and a number of organic acids, also arabinose and galactose, enter this outer region rapidly, but do not enter the central region or enter only at a very slow rate. In a third group of substances which penetrate very rapidly throughout the whole cell are included acetic, propionic and butyric acids.

Substances of the first group such as inulin or dialysed peptone are useful for determining the extracellular water. Organic acids in the second group, such as glyceric, may be used for determining the pH of the outer region, and acids of the third group for determining the pH of the yeast cell as a whole, which is very largely dependent on the inner region containing 85% of the whole water of the cell.

Concerning succinic acid the following considerations are of special interest. When baker's yeast ferments glucose (1 : 1 of 5% (w/v) solution, unbuffered) within 30 min., succinic acid appears in the outer medium to the extent of about 40 m.-equiv./l. The acid, at a pH near 3, cannot diffuse back into the main part of the cell, but does so freely into an outer region one-tenth of the whole cell volume. Succinate ions (pH = 7·0) cannot diffuse back into this region or do so very slowly.

Interpreting such facts it may be held that free succinic acid appears as such only in an outer region of the cell and then diffuses into the suspending fluid. How the free acid appears will be dealt with in a later communication. With this view the inner surface of the outer region is practically impermeable, when examined over many hours, to free succinic acid as well as to the succinate ion.

If, as an alternative explanation, it be supposed that free succinic acid can diffuse back freely into the cell, but is prevented from doing so, not by a membrane inside the outer surface of the cell, but by a continuous active extrusion, a consideration of the energy requirements makes this view untenable. Succinic acid enters the outer region very rapidly, reaching an approximate equilibrium in about a minute or less as shown by glass-electrode observations, and the energy required to extrude it with equal or similar rapidity would be far beyond the whole resting metabolism of the yeast cell. On the other hand, it would appear that the succinic system during fermentation is actively extruded from within and *across the inner membrane* of the outer region, but at a very much lesser rate than it diffuses across the outer cell boundary.

Another view to be considered is that the outer region is only apparent, and that there is a membrane surface of the cell on which the acid is adsorbed, free succinic acid not appearing as such in any part of the cell other than in relation to this membrane. Such adsorption would presumably occur as a monomolecular layer. With the average cell diameter of 11·8 μ., an Avogadro number of $6{\cdot}0 \times 10^{23}$ and a limiting value for the mean surface of the molecule of 24 $(A)^2$, it may be calculated that with concentrations of about 0·33 M for succinic acid or 0·7 M for glyceric acid the ratio of succinic or glyceric acid adsorbed on the cells to that in the external solution should be in the region of 0·01–0·02 instead of the experimental R figure of about 10 times this value.

There is also the fact that such different substances as succinic acid and glyceric acids, beyond values of 0·1 M outside the cells, or arabinose over the observed range of about 0·03 to 0·07 M, give similar R figures. The R value of a substance, or the amount that has entered the cell relative to the concentration, is thus largely independent of concentration or chemical constitution. With low concentrations of the organic acids it increases owing to buffering as may be expected.

The outer region and the cell wall. Since the volume of the outer region is scarcely affected by a wide range of arabinose or galactose concentrations, and as its outer surface must lie at the outermost rim of the cell its inner membrane must be as little distensible as the cell wall. It appears that the region is identical with the cell wall, and the water for solution therein lies between the structural units. The outer surface or membrane is impermeable to large molecules such as inulin and peptone, also to succinate ions.

SUMMARY

1. The theoretical interspace for a large number of non-deformable spheres with closest packing is 26% of the total volume. Centrifuged yeast

(3000 rev./min. for 20 min.) shows an interspace volume of 23 % with inulin as the test substance, 22 % with gelatin and 24 % with peptone, when expressed as a percentage of the total volume. The interspace fluid can be pressed out with a simple hand press, so that an amount representing only 0–5 % of the total cell volume is left.

2. If the space be measured with arabinose, galactose, or lactose, with allowance for the water abstracted from the cells, it appears as 33–34 %. The difference is due to the penetration of such substances into an outer cell region which is identified with the thick cell wall.

3. The water for solution in this outer region is 0·10–0·11 of the total cell volume. Into this part of the cell many substances penetrate readily, including sodium and potassium chloride; also succinic acid, glyceric acid, etc., but succinate ions are debarred or enter slowly.

4. The inner surface or membrane of the outer region is impermeable to chloride ions in the resting yeast. It is likewise impermeable or only very slowly permeable to succinic acid, glyceric acid and a number of other acids with hydroxyl or amino groups. On the other hand, it is very freely permeable to formic, acetic, propionic and butyric acids.

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pH Values of the Yeast Cell

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In the previous communication (Conway & Downey, 1950) it was shown that the yeast cell contained an outer metabolic region, identified as the thick cell wall, which had a content of water for free solution equivalent to about 0·10 of the whole cell volume or about 15 % of the total water of the cell.

In the resting cell the pH of this outer region appears to be very similar to that of the overall or total pH figure as determined by the different methods used here, but in the fermenting cell the overall pH increases markedly while the pH of the outer region decreases. In other words, during fermentation the inner part of the cell or cytoplasm becomes more alkaline while the cell wall itself becomes more acid. At an early stage in the study of yeast acidity (Conway & O'Malley, 1943) we found an increase of the total pH, but assigned it to changed combinations of ionizing groups. Brandt (1945) describes similar alkaline changes, but did not connect them with a corresponding production of acid.

Three methods are used here to determine the overall pH of the resting cell. In the first of these, cells of the centrifuged yeast are frozen with liquid air or oxygen and then thawed rapidly, the pH being at once determined by the glass electrode when the mixture had reached room temperature (20°).

In the second method, the fact that acetic acid very rapidly diffuses throughout the cells (Conway & Downey, 1950) was used to determine, by its distribution, the pH inside the cell. As a third method, the carbonic acid system was used in essentially the same way as acetic acid. The advantage with acetic or carbonic acid lies in the cells remaining uninjured, but in fact all three methods give the same results within the sampling error.

Brandt (1945) has given a comprehensive account of the literature relating to this subject, and has also described a method for determining the pH which consists in rapidly heating a thick suspension, by driving it through a coiled copper tube immersed in boiling water. The cells in this way are rapidly destroyed. The results obtained by Brandt's method are higher than we have obtained for resting baker's yeast by the three methods mentioned above, and we have found that heating in such a way has the