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Biological Production of Acid and Alkali

2. A REDOX THEORY FOR THE PROCESS IN YEAST WITH APPLICATION TO THE PRODUCTION OF GASTRIC ACIDITY

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In a previous communication (Conway & Brady, 1950) it was shown that the H^+ ions produced in the K^+ and H^+ exchange were associated with an increase of HCO_3^- ions or alternatively a retention of succinic acid. Extreme conditions could be approached when much the greater fraction was quantitatively associated with HCO_3^- or, on the other hand, with an equivalent retention of succinate. All intermediate positions are probably possible.

In the first case, with no organic acid excretion the only fixed acidity formed outside the cell is due to the exchange of K^+ and H^+ ions. In the second, the total acidity excreted is unchanged by the addition of K^+ ions.

With the fixed acidity outside the cells arising only from the K^+ and H^+ exchange, besides no appreciable excretion of organic acid, there is also no significant increase of such metabolic ether-extractable acids present within the cell. A considerable fraction of the K^+ ions absorbed appears then to be associated with HCO_3^- ions. Yet it may be concluded that carbonic acid in the outer region of the cell cannot be a quantitatively important source of the H^+ ions exchanging for K^+ , since the free carbonic acid concentration is necessarily very low when the carbon dioxide output and pH of the region are considered.

The facts established in the present paper throw a fuller light on the mechanism involved, and lead to a redox theory of the origin of the H^+ ions excreted either in exchange for K^+ ions or as free organic acid.

METHODS

Measurement of external acidity. For present purposes it was unnecessary, apart from pH measurements, to make exact determinations of free organic acid. The total fixed

acidity was the significant quantity. This was measured by centrifuging the sample of yeast suspension, and pipetting 0.5 ml. of the supernatant into a 25 ml. Pyrex beaker, and allowing it to stand exposed to the atmosphere for about 1 hr., if the pH were no higher than about 5, to allow any free CO_2 to escape. The escape was also usually assisted by rocking, and in some cases by heating. The sample was then titrated against phenolphthalein.

Measurement of the overall pH of the yeast cells (evacuated of carbon dioxide). After centrifuging, the yeast was rapidly collected into a linen bag and pressed in a hand press, shown to be efficient in expelling the interspace water to about 0.5% of the whole. The procedure was then the same as the freezing method described in a previous paper (Conway & Downey, 1950), but prior to examination the mixture was subjected to a vacuum for a short time.

For comparative purposes another method was used in which 5 g. of the pressed yeast was introduced, a little at a time into 15 ml. boiling water, the mixture cooled and made up with water to 25 ml.

Measurement of the H^+ ions removed from the yeast cells on fermentation. This could be obtained by titrating back the frozen, thawed and evacuated yeast to the pH of the control yeast which had not fermented glucose, but a better procedure was considered to be the following. The fixed acid in a sample of the fluid outside the cells was determined as above, then the suspension was frozen as a whole, thawed, evacuated and the pH taken. From the buffering curve obtained by electrometric titration, the amount of alkali or acid necessary to bring the pH to that of the resting cells was then determined. Usually it was very small, as the external acidity was balanced by a practically equal alkalinity produced within the cells. A similar procedure was adopted in the boiling method.

Effect of inhibitors. The effect on the acid and ethanol production of a number of inhibitors was examined in different concentrations and at different pH values. The pH was adjusted by succinate, citrate or phosphate buffers.

Yeast suspensions. For the investigation of the equivalence of alkali and acid production, the washed centrifuged yeast was suspended in the proportions of 1 kg. of washed

centrifuged yeast to 0.6 l. of 5% (w/v) glucose, which in some of the experiments contained 0.1M-KCl.

For the investigation of the inhibitors, 1 kg. of centrifuged yeast was suspended in 3 l. of 0.1M-KCl, 0.3 l. of inhibitor solution added and 0.1 l. of 30% (w/v) glucose. All fermentations were conducted at room temperature which varied from about 17 to 20°. At varying times after the addition of the glucose, samples were taken, centrifuged and the supernatant fluid pipetted into clean dry tubes and stored in ice if not immediately titrated.

Ethanol determination. This was determined in 0.1 ml. samples of the suspending fluid by a microdiffusion method (modified Winnick method; Conway & Nolan, 1948).

RESULTS

The correspondence of alkali formed within the cell to acid excreted

It will be seen from Table 1, giving the results of the freezing method, that as the external solution becomes markedly acid, pH of 3.1 without potassium chloride and of 1.9 with potassium chloride, the pH within the evacuated cells becomes correspondingly alkaline. Such experiments were carried out without any external buffering.

Table 1. *Average of a series of experiments with pH determined after freezing in liquid oxygen with subsequent thawing*

(In series A the overall pH of the cells was determined by the method of first breaking the cell walls of the pressed yeast by freezing in liquid air or oxygen, and in series B after rapid destruction of the cells in boiling water as described in Methods.)

Series of expts.	Unfermented cells (pH)	pH of fermented cells		pH of fermented suspensions		pH of suspending fluid	
		Without KCl	With KCl	Without KCl	With KCl	Without KCl	With KCl
A	5.85 (9)	6.11 (5)	6.35 (5)	5.76 (8)	5.82 (8)	3.1 (5)	1.9 (5)
B	6.27 (4)	7.06 (4)	7.25 (4)	6.19 (4)	6.24 (4)	3.0 (4)	2.0 (4)

The Table shows the average of the pH of the suspensions frozen in liquid oxygen and thawed again to room temperature. With the suspensions containing added potassium chloride a correction is necessary for the potassium chloride effect on the pH as determined by the glass electrode, by comparing similar frozen suspensions with graded additions of potassium chloride in the same volume of suspending fluid. From such a study 0.11 pH is added to the pH of the potassium chloride suspensions to equalize the conditions without added potassium chloride.

It will be seen that the pH of the suspensions, despite the large amount of external acid produced, is but little different from that of the resting cells. This can only mean that nearly the same equivalent of alkali is produced *within* the cells as acid outside. If to 1 vol. of the external fluid after 30 min. fermentation were added 1 part of pressed, frozen and thawed *resting* cells then, instead of a pH of 5.8, a value at or near 5.0 could be expected and has in fact

been observed. The Table also gives the mean of four sets of experiments in which the boiling method for pH determinations was adopted. This method gives results above the true intracellular values (Conway & Downey, 1950), but is useful for comparative study. It will be seen that there is a marked increase of pH in the fermented cells, but the fermented suspensions with the cell contents released by boiling show very little change, namely, 0.08 without potassium chloride, and, 0.03 with potassium chloride.

Comparison of acid and alkali produced as m-equiv./kg. cells

This comparison was made by determining the amount of alkali required to bring the frozen thawed suspensions, or alternatively, the suspensions with the cells broken by the boiling technique to the same pH as the resting yeast similarly treated. Such values subtracted from the acid produced in the fluid outside the cells gave the alkali formed intracellularly, all values being expressed per kg. of yeast cells.

Table 2. *Acid and alkali produced, m-equiv./kg. cells*

Without KCl			With KCl		
Acid	Alkali	Difference	Acid	Alkali	Difference
38	33	- 5	40	40	0
38	26	-12	52	43	- 9
41	39	- 2	44	49	+ 5
32	33	+ 1	42	40	- 2
Oxygenated yeast					
14	12	- 2	35	38	- 3
13	4	- 9	30	30	0
15	25	+10	20	18	- 2
Mean 27	25	-2.7	38	37	-1.6

Seven sets of such experiments are available for the comparison, three with prior oxygenation (Exps. 5-7 in Table 2) and four without. It will be seen that the average figures show that with 27 m-equiv./kg. cells of acid produced, 25 m-equiv. alkali are formed, and with potassium chloride

38 m-equiv. acid are excreted and 37 m-equiv. alkali formed.

On the average, about 93 % of the acid is compensated by alkali formation or H^+ ion removal, but the difference from total compensation lies within the sampling error. Thus the total alkali required for full compensation was -2.1 ± 1.5 m-equiv., the total acid formed being 32 m-equiv./kg. cells.

The individual experiments show variations from 12 to -10 or from 5 to -9 m-equiv. alkali required to bring the mixture to the resting pH, but such could arise from some inexactitude in the pH determination or from small differences in the yeast samples, but the variation observed seems to be practically unrelated to the magnitude of the acid produced. This in turn is shown from the data in Table 3, where the acid produced over the range 13-53 m-equiv. is compared with the alkali. Thus over the range 13-23, a mean of 15.5 m-equiv. acid and 14.8 of alkali are produced, and over the range 43-53 a mean of 48.0 of acid and 46.0 of alkali.

Table 3. *Average acid and alkali produced over the total acid range*

Range (m-equiv./kg. cells)	Acid (m-equiv./kg. cells)	Alkali (m-equiv./kg. cells)
13-23	15.5	14.8
23-33	31.0	31.5
33-43	38.7	36.0
43-53	48.0	46.0

The effect of inhibitors on the acid-alkali production

The study with inhibitors has not been carried out systematically, but up to the present the results are significant for the elucidation of the main question. The relative proportions of yeast, etc., are given in Methods. Where a buffered suspending fluid was used this contained 0.1 M-succinate, citrate or phosphate.

Sodium azide. With the unbuffered mixture a practically complete inhibition (over 90 %) was obtained with inhibitor concentration outside the cells as low as 10^{-3} M. The intracellular alkalinity produced during fermentation was likewise inhibited. Table 4 shows the effect of different concentrations of azide in unbuffered solutions.

The azide effect was also examined at higher pH values, the suspending fluid being buffered. The inhibitions produced in three experiments ranged from 65 to 85 %. This seems to indicate a reduced effectiveness at the higher levels, though this would require further examination.

2,4-Dinitrophenol. A practically complete inhibition of the acid-alkali mechanism and increase of ethanol output occurs with unbuffered solutions at an inhibitor level of about 10^{-2} M. On a preliminary examination with 10^{-3} M concentration at a pH of 6.0 no appreciable inhibition was produced. From this, it was at first assumed (Conway, Brady & Carton, 1949) that dinitrophenol did not inhibit. In unbuffered solution, however, it is far less effective than azide, in the sense of requiring a much higher level for a 50 % inhibition.

Cyanide. No appreciable inhibition was produced by 0.005 M-cyanide examined over a wide range of buffered pH values in the suspending fluid.

Carbon monoxide. No appreciable inhibition was produced by carbon monoxide either by saturation with coal gas containing approximately 7 % (v/v) carbon monoxide, or by using the relatively pure gas produced from formic acid and sulphuric acid and saturating the mixture therewith before adding the glucose, the fermentation then being conducted in the dark. In the later case a 0.05 M-succinate buffer was used with pH of 6.0.

Salicylaldehyde. Using 0.008 M with the unbuffered mixture containing potassium chloride about 15 % inhibition was produced, but without

Table 4. *Inhibition of acid and increase of ethanol formation*

Exp. no.	Inhibitor (M)	Acid (m-equiv./kg. cells)	Ethanol (m-equiv./kg. cells)	Change of acid	Change of ethanol	Ratio of changes acid/ethanol
1	Azide 0.008	111 17	259 301	-94	+42	2.35
2	Azide 0.0003	92 35	264 305	-63	+41	1.39
3	Azide 0.008	135 10	245 306	-120	+61	2.05
4	Azide 0.008	93 2	249 298	-91	+49	1.86
5	Azide 0.008	72 15	232 263	-57	+31	1.84
6	Azide 0.008	94 3	260 298	-91	+38	2.39
7	Diethyldithiocarbamate 0.008	107 41	239 277	-66	+38	1.74
Mean				-83	+43	1.94 ± 0.13
						24.2

potassium chloride and at a somewhat higher pH about 40% inhibition was caused by 0.005M-salicylaldehyde.

Sodium diethyldithiocarbamate. With 0.008M concentration in the unbuffered mixture an inhibition of about 60% was produced. About the same inhibition occurred with the mixture buffered initially at 7.0 with 0.05M-succinate.

Monoiodoacetate. This produces practically complete inhibition of the acid and ethanol formation in 0.005M concentration.

on double the scale. A point of note about the acid production is that it reaches a maximum and then declines. The average uninhibited maximum is about 89 m-equiv./kg. cells. When the production is about 40–50 m-equiv. the decline in the acid production is much slower and the curve remains over 30 min. or so on a plateau. The nature of the decline in external acidity has not been investigated. In comparing the acidity produced with the increase of ethanol we have taken the maximum acidity reached.

Fig. 1. The curves summarize the course of acid and ethanol production, expressed as m-equiv. and mmol./kg. of yeast cells, in a number of experiments, in which 10 g. of washed, centrifuged yeast was suspended in 30 ml. water plus 3 ml. azide or cyanide (0.05M solutions) or plus 3 ml. water, and in addition 1 ml. 30% (w/v) glucose solution at the beginning of fermentation. The dotted curves show the mean production of ethanol and of acid in the presence of azide. The line curves show the mean production of ethanol and acid without azide. The experiments with and without azide were corresponding experiments (eight sets) done simultaneously on samples of the same yeast. Curves with azide (eight experiments): ethanol, dotted line (●); acid, dotted line (⊕). Curves without azide (eight experiments): ethanol, four experiments with cyanide (+), four experiments without cyanide (×); acid, four experiments with cyanide, and four without, averaged points (○). The averages for acid production with and without cyanide, but without azide, show no appreciable difference and are not distinguished on the graph.

Fluoroacetate. No appreciable effect on acid production resulted with fluoroacetate in 0.005M-concentration.

In conclusion, the results obtained of immediate significance are the great inhibitory power of azide and dinitrophenol on the acid-alkali mechanism, along with the fact that the ethanol production at the same time is not diminished but increased.

This inhibition not only extends to the excretion of succinic acid but also to the K^+ and H^+ exchange.

Relation of the increased ethanol production to inhibited acid

Fig. 1 shows the average curves for ethanol production without inhibitors and with cyanide and azide. The corresponding acid production is given

DISCUSSION

A redox theory of the acid-alkali formation

The facts established in the present paper lead to the conclusion that the *immediate* source of the H^+ ions outside the cell is the reduced form of a heavy metal catalyst, and that this operates cyclically (see Conway & Brady, 1948).

The manner in which it works may be briefly represented as follows. A substance transporting hydrogen derived from metabolic sources is oxidized by the catalyst, the hydrogen atoms then forming H^+ ions and electrons, which are retained. The reduced catalyst acts like an organic acid, ionizing to form hydrogen ions. This process is considered to

take place in the outer metabolic region identified as the cell wall (Conway & Downey, 1950). As only a trace of the catalyst is present, a conclusion supported by the half inhibition produced by 10^{-4} M-azide, the process must occur cyclically.

The reduced catalyst is then considered to act in the inner region of the cell, and is oxidized taking up hydrogen ions, passing hydrogen atoms to some organic acceptor.

If the activity of the catalyst is blocked by azide, whether directly or indirectly (the latter perhaps in part through the mechanism suggested by Spiegelman, Kamen & Sussman (1948) that azide inhibits the phosphorylation of adenosinediphosphoric acid (ADP) at the phosphoglyceric stage) the metabolic hydrogens are diverted off to ethanol formation, and there is an equivalent increase of ethanol produced.

It is considered, in addition, that the catalyst can move inwards carrying K^+ ions, and outwards carrying succinate ions, and possibly also bicarbonate ions. It will appear from this outline that the excretion of fixed acid will be accompanied by an equivalent alkalinity within the cell and a HCO_3^- increase. Further, if one is dealing with yeast not oxygenated prior to fermentation and containing relatively much succinate, then if potassium chloride be included in the unbuffered glucose solution the cyclical turnover of the catalyst may show only a small increase. In this case the amount of H^+ ions going to the excreted succinate to form succinic acid, as succinate, will appear retained in the cell. Thus the absorption of K^+ ions will be accompanied by a quantitative or near quantitative increase of bicarbonate ions plus retained succinic acid.

In fact, the immediate step leading to such a redox theory was the finding of conditions in which, during fermentation, no organic acid was excreted, and no appreciable extra formation of ether-extractable organic acids appeared within the cell, though the largest amount of free HCl appeared from K^+ and H^+ exchange. At the same time upwards of 80% equivalence of extra bicarbonate appeared within the cells, the mixture being bubbled with carbon dioxide at atmospheric pressure.

The HCO_3^- was originally assumed to be formed directly in decarboxylation (Conway & O'Malley, 1948). Such formation appears now to depend on conditions, which can be so varied that the decarboxylation proceeds directly through carbon dioxide, or alternatively through HCO_3^- (Conway & O'Malley, 1949). For the present interpretation the question is no longer significant, and the bicarbonate formation is interpreted as arising in the inner cell region as above discussed.

The membrane effect and active ion transport. Here a little may be said with respect to the source of succinic acid. It has been shown (Brady, 1948) that

most of such excreted succinic acid, under the conditions examined, has been already within the cells before fermentation, and necessarily then, and in by far the greater part, as succinate ions. Yet neither succinic acid nor succinate ions can at any appreciable rate go backwards into the cell if introduced outside, either when the yeast is resting or when fermenting. The conclusion may be drawn that the succinate ions pass across the membrane within outwards as a complex, in which their negative charges are balanced. Such a complex may be formed by the catalyst in the redox change across the membrane.

A somewhat similar position exists with respect to the K^+ ions. There is evidence to show that in the resting cell they can pass the membrane, but it is a relatively slow process. During fermentation they can pass much more rapidly when they exchange for H^+ ions, and here also the concept of active carriage by the same or similar catalyst may be entertained.

Application of the redox theory to formation of gastric acidity

This has already been suggested (Conway & Brady, 1948; Conway *et al.* 1949), and is dealt with in some detail elsewhere (Conway, 1949). Here it may be said that the gastric secretion of hydrochloric acid has now so many essential similarities with hydrochloric acid formation by yeast that the presence of the same underlying mechanism seems more than probable. Thus a pH as low as 1.4 can be produced by the yeast cell with free hydrochloric acid outside corresponding thereto; also with the acid excretion there is equivalent alkalization or accompanying hydrogen removal from the cell, as occurs in the parietal cells of the gastric mucosa (Hanke, 1937; Davies, 1948).

Further analogies could also be drawn and the subject is treated more fully elsewhere (Conway, 1949). A similar mechanism may also be suggested for pancreatic secretion of alkali, and acid secretion by the kidney.

SUMMARY

1. When organic acid (succinic), or free H^+ in exchange for K^+ , is excreted by fermenting yeast, H^+ ions are removed from within the cell in amount equivalent to the total titratable acid secreted.

2. This accounts for the excess HCO_3^- ions formed when potassium exchanges for hydrogen, as compared with the control without potassium chloride.

3. The acid-secreting mechanism is almost entirely inhibited by 0.005–0.001M-azide, and is about 50% inhibited by 10^{-4} M-azide.

4. At the same time ethanol production is increased by azide, and for every two equivalents of acid secreted about one extra mol. of ethanol is produced.

5. 2:4-Dinitrophenol acts like azide, but requires a much higher concentration for 50 % inhibition. Salicylaldoxime and sodium diethyl-dithiocarbamate also act like azide, but are even less effective than 2:4-dinitrophenol.

6. Monoiodoacetate (0.005 M) entirely inhibits acid and ethanol production, whereas fluoroacetate has no appreciable effect.

7. The facts of the acid secretion are well explained by a redox theory, in which it is considered that the H^+ ions are produced in an outer cell region by the ionization of the reduced form of a heavy metal

catalyst, receiving metabolic hydrogen atoms from a dehydrogenase (directly or at one or more removes) and being in turn oxidized within the cell where it receives H^+ ions and transfers hydrogen atoms to an acceptor.

8. This redox theory has an obvious application to secretion of acid by the gastric mucosa, the facts of acid secretion by yeast being now strikingly similar to acid secretion by the parietal cells. Other applications may also be entertained, such as acidification in the nephrons of the kidney or alkalinization by the pancreas.

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The Use of Radioactive Isotopes in Immunological Investigations

3. FURTHER STUDIES ON THE FATE OF INJECTED ^{32}P -CONTAINING PROTEINS

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In a previous paper (Banks, Bournsnel, Dewey, Francis, Tupper & Wormall, 1948) it was shown that the ^{32}P -containing protein antigens, lipovitellin, vitellin and artificially phosphorylated proteins, when injected intravenously into rabbits, rapidly disappear from the blood stream. With lipovitellin and vitellin, for example, only one-eighth of the injected protein could be detected in the blood 5 min. after the injection. Furthermore, in preliminary investigations on two rabbits injected with a suspension of vitellin it was found that much of the vitellin which had left the general circulation had been removed by the liver and lungs; e.g. about one-third of the injected vitellin was found in these two organs 1 min. after the injection. Vitellin is, however, a very insoluble protein, and it had to be injected as a suspension. It was considered possible that different results might be obtained with more soluble proteins, and we decided, therefore, to use the more soluble lipovitellin for a further study of this problem.

For an investigation of the immediate distribution of the injected protein it was necessary to kill the animal as soon as the injected material had become well distributed throughout the blood stream, i.e. 2-3 min. after the injection, and to do this we used the rapid coagulation technique recently introduced by Franklin & Amoroso (1948), namely the intravenous injection of Russell's viper venom, which causes clotting in 1-4 min. Subsequent immersion of the whole animal in saline containing formaldehyde fixes the protein antigen and prevents its diffusion from the blood or tissue. In the other experiments described here lipovitellin was injected intravenously, and the injected rabbits were killed, by the injection of magnesium sulphate or by stunning, after intervals ranging from 2 to 44 hr.

One object of these investigations was to study the distribution of protein antigens in the body at varying intervals after their injection into the blood stream, since information on this matter might throw light on the problem of the site of antibody