the curves shows that the groups responsible have a mean pK value of 8.15 and are thus probably α -amino groups. Since they behave as if they were of one species with this pK , it would be expected that half of their number would titrate between pH 8.65 and 7.65. Calculated on this basis, there are 24 present in the α -protein and 28 in the β -protein (10^5 g.). Similar values, 27 and 29 respectively, are obtained by subtracting the total H^+ bound by native elastin from the total H^+ bound by the α - and β -protein over the pH range 11.0–6.25 (the point of least slope). Thus the treatment has resulted in the production of 24–27 and 28–29 α -amino groups, in the α - and β -proteins respectively. These results are also in fair agreement with the values of 24.4 and 32.5 amino end-groups for the α - and β -proteins, obtained by the fluorodinitrobenzene technique (Partridge & Davis, 1955).

Similar analysis of the curves in the acid region shows that about 26 and 34 new acidic groups are present respectively in the α - and β -proteins. Analysis in this region is less accurate because it is not certain that all the acidic groups have been titrated at pH 2.0, which is the limit of the glass electrode. However, the comparatively good agreement between the numbers of acidic and basic groups released justifies the assumption that the former are α -carboxyl groups corresponding to the released α -amino groups.

SUMMARY

1. Titration of native elastin shows the presence (in 10^5 g. protein) of about 17 moles of acidic groups and 15–16 of basic groups. These values are in substantial agreement with those expected from the amino acid analysis.

2. The titration curves of the α - and β -proteins, derived from elastin, reveal the presence of many more acidic groups and of new groups titrating with mean pK 8.15.

3. It is shown that the new groups are α -amino in character, and that the numbers released correspond with the increase in number of acidic groups.

4. It is concluded that during the treatment of native elastin to give the α - and β -proteins, about 26 and about 31 moles of α -amino residues respectively are released in 10^5 g. of protein, with the simultaneous release of corresponding numbers of α -carboxyl residues.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

- Cohn, E. J. & Edsall, J. T. (1943). *Proteins, Amino Acids and Peptides*, p. 444. New York (U.S.A.): Reinhold Publishing Corporation.
 Partridge, S. M. & Davis, H. F. (1955). *Biochem. J.* **61**, 21.
 Partridge, S. M., Davis, H. F. & Adair, G. S. (1955). *Biochem. J.* **61**, 11.

The Effect of Redox Dyes on the Active Transport of Hydrogen, Potassium and Sodium Ions Across the Yeast Cell Membrane

By E. J. CONWAY AND R. P. KERNAN

Department of Biochemistry, University College, Dublin

(Received 27 October 1954)

Potassium ions are actively transported across the yeast cell membrane during fermentation when the external fluid contains potassium chloride. The potassium ions exchange for hydrogen ions (Conway & O'Malley, 1946; Rothstein & Enns, 1946). A similar transport of sodium ions occurs if the fermentation is carried out in the presence of 0.2M sodium citrate, without any added potassium ions (Conway & Moore, 1954). If the yeast is washed and suspended in water it slowly excretes sodium ions. These are accompanied by anions from within the cell, but if the sodium-rich yeast is suspended in 0.1M potassium chloride, the sodium is rapidly excreted in exchange for potassium ions.

The potassium and sodium ions which exchange have different carriers. Azide (0.002M) practically completely inhibits the potassium carrier but not the sodium carrier. Both are inhibited by cyanide and anoxia (Conway, Ryan & Carton, 1954). Such active transport has been interpreted as a carriage by a redox system or systems which act as the immediate energy supplier in the transport (Conway, 1951, 1953).

Apart from the immediate carriers of the ions the involvement of phosphate-bond energy at some stage in the transport of potassium ions in yeast may be inferred from the pronounced inhibitory action of 2:4-dinitrophenol (0.002M). On the other

hand, neither 2:4-dinitrophenol nor 0.002M azide has any appreciable effect on the sodium transport system in yeast.

Evidence for the redox pump mechanism for inorganic ion transport has been already advanced (Conway, 1953; Conway & Moore, 1954). Here the effects of redox dyes on the transport of potassium and sodium ions, as well as on the excretion of hydrogen ions during anaerobic fermentation, are described. They are correlated with observations of the redox potentials in the suspending fluid.

METHODS

The fermentation and electrode arrangement. The yeast suspension was contained in a corked Pyrex tube, 2.5 × 10 cm., arranged so that N₂ could be bubbled through the suspension; the cork held a glass tube containing mercury with platinum wire making contact and sealed into the bottom of the tube. The wire was fixed at the other end to a platinum foil electrode. A salt bridge of KCl in agar gel connected this fluid in the tube with saturated KCl in another vessel, which made contact with a saturated calomel electrode. The terminals were connected to a Cambridge potentiometer, with a sensitive galvanometer. The arrangement for redox potential measurement was tested, using redox mixtures of known potential, such as potassium ferricyanide/ferrocyanide and the quinhydrone half-cell at various pH values.

The yeast suspension. Fresh baker's yeast was washed twice with water and one part suspended in 20 vol. of suspending fluid. For resting yeast, the fluid contained only potassium hydrogen succinate at pH 4.5. With fermenting yeast the suspension contained 5% (w/v) of glucose in addition. The succinate was added to buffer the medium at or near to a pH of 4.5. Where the effect of the redox dyes was being examined these were incorporated in the suspending fluid in strength 10⁻⁴M, and the glucose was not added here until the oxygen was expelled. The effect of the dye was determined by comparing the results with those of a control without the dye.

Hydrogen-ion secretion. The amount of H⁺ secretion after 45 min. in the presence of a redox dye was determined by titrating to a pH of about 9.0, the supernatant fluid from centrifuged samples taken at zero time and 45 min. later. Oxygen was bubbled through the fluid before titrating. This was done to bring both the dye at zero time and after the stated period of fermentation to the same oxidized state, avoiding any possible H⁺ ion formation by the reduction of the dye itself. The result was expressed as a percentage of the amount secreted in a control mixture without the dye. The H⁺ secretion occurs partly in exchange for K⁺ and partly in association with succinate ions as succinic acid (Conway & O'Malley, 1946; Conway, 1952).

Potassium and sodium. The K and Na content was determined in the centrifuged yeast by means of the Beckman flame photometer. The centrifuged yeast, suspended in about 10 vol. of water, was boiled for about 1 min. and made up to 100 ml. with distilled water. Washing the yeast with water before making the suspension made no difference in the comparative results obtained or in the estimate of the K⁺ uptake. The result was expressed as a percentage of the amount absorbed in a control mixture without the dye.

Preparation of sodium-rich yeast. This was prepared by fermenting one part of yeast in 20 vol. of 0.2M sodium citrate (Conway & Moore, 1954) for 2.5 hr.

Determination of carbon dioxide. This was measured by the microdiffusion method (Conway, 1950b). To provide anaerobic conditions for the fermentation in the microdiffusion units, nitrogen was passed into them for some time before the glucose was added.

Determination of ethanol. The samples for ethanol assay were taken directly from the fermenting mixtures under the same conditions as for the investigations of the effect on H⁺ excretion and K⁺ uptake. These samples were centrifuged, and the ethanol content of the supernatant fluid was estimated by the microdiffusion method (Conway, 1950a). Both CO₂ and ethanol were expressed as percentages of the control.

RESULTS

Studies with normal yeast

Redox potential changes during fermentation. The redox potential of a suspension of washed resting yeast, on reaching a steady state under anaerobic conditions at pH 4.5, was in the region of -20 to -40 mv. The curves of potential with time are shown in Fig. 1. A steep fall, which may be

Fig. 1. Changes of redox potential of yeast. A, fermenting; B, fermenting in presence of Nile blue; C, fermenting in presence of *o*-chlorophenol-indophenol; D, resting. Suspending fluid 0.04M potassium hydrogen succinate at pH 4.5. A, B, and C also contain 5% of glucose; concentration of dyes 10⁻⁴M.

attributed to progressive oxygen depletion, occurs at the beginning. The potential then reaches a somewhat steady state (curve D), which may be taken as the true resting potential of the outer surface of the yeast cell. Curve A shows the effect of fermentation. After a similar rapid, but not so great, fall, the potential rises steeply to reach a steady state after about 45 min. The steady-state value was usually close to 180 mv at room temperature (18°). Kluyver & Hoogerheide (1934) found a range of 80–100 mv at pH 5.4. The pH of our

fermenting yeast without addition of redox substance was approximately 4.1 during fermentation and one could expect the observed potential to be higher than at pH 5.4.

The effect of redox dyes ($10^{-4}M$) on the redox potential during fermentation. When sufficient time was allowed for the disappearance of the oxygen during fermentation, redox dye Nile blue (E'_0 at pH 4.5 = 19 mv) showed a very marked effect. After 60 min. the potential was approximately 45 mv (curve B, Fig. 1), as compared with 185 mv in the control (curve A, Fig. 1). Other redox dyes had similar but varying effects depending on their characteristic redox potential at pH 4.5. A relatively oxidizing dye, *o*-chlorophenol-indophenol (E'_0 at pH 4.5 = 388 mv) caused, on the other hand, a marked increase in the potential (curve C, Fig. 1), the value after 60 min. being about 330 mv compared with 180 mv in the control. After about 45 min. the potential level reached a somewhat steady state in the control mixture or in the presence of oxidizing or reducing dyes.

Effect of the redox dyes on the hydrogen-ion secretion. The average production of H^+ by the control was 80 m-equiv./kg. of yeast/45 min. Zero time was regarded as that when glucose was added to the buffered suspension of yeast, following expulsion of oxygen. Thus the potential of the

mixture had approached a steady state before the first sample was taken. Fig. 2 shows the relation between the relative H^+ secretion and the E_h of the mixture containing the dyestuff. It will be seen that there is an approximate linear relation between the potential of the mixture after 45 min. and the H^+ secretion over this period.

Effect of redox dyes on the K^+ uptake during fermentation. Fig. 3 shows that the effect on the K^+ uptake is similar to that on the H^+ excretion. The average uptake of K by the control during fermentation was 26 m-equiv. of K/kg. of yeast.

Effect of inorganic redox systems on the K^+ uptake. Ceric sulphate, chromic chloride, titanous chloride and stannous chloride were used. It will be seen

Fig. 2. Effect of redox dyes on H^+ secretion by yeast fermenting in $0.04M$ potassium hydrogen succinate at pH 4.5. Glucose added after expulsion of oxygen. The dyes used were: Nile blue, $\bullet$; safranin T, $\times$; neutral red, $\otimes$; benzyl-viologen, Δ ; phenol-indo-2:6-dichlorophenol, ∇ ; 1-naphthol-2-sodium sulphonate-indophenol, $\blacktriangle$; *o*-cresol-indo-2:6-dichlorophenol, $\circ$; phenol-indo-2:6-dibromophenol, $\blacktriangledown$; *o*-chloro-indo-2:6-dichlorophenol, $\blacksquare$; *o*-chlorophenol-indophenol, $\odot$; Janus green, $+$; concentration of dyes $10^{-4}M$.

Fig. 3. Effect of redox dyes on K^+ uptake during fermentation of yeast buffered by $0.04M$ potassium hydrogen succinate. Conditions and symbols as in Fig. 2.

Fig. 4. Effect of $Ce(SO_4)_3$, $\times$; $CrCl_3$, $\blacktriangle$; $TiCl_3$, $\circ$; and $SnCl_2$, $\blacktriangledown$; ($10^{-4}M$) on K ion uptake by fermenting yeast. Conditions as in Fig. 2.

Table 1. *Production of CO₂ and ethanol during anaerobic fermentation of yeast with redox dyes in the suspending fluid*

Results expressed as percentages of the values obtained without the redox dye.

Dye	E'_0 of dye at pH 4.5 (mv)	CO ₂ production (% of control)	Ethanol production (% of control)
Phenol-indo-2:6-dibromophenol	+396	101	103
Phenol-indo-2:6-dichlorophenol	+396	101	101
<i>o</i> -Chlorophenol-indophenol	+388	101	99.5
<i>o</i> -Chlorophenol-indo-2:6-dichlorophenol	+385	100	96
<i>o</i> -Cresol-indo-2:6-dichlorophenol	+365	100.5	100
1-Naphthol-2-sodium sulphonate-indophenol	+273	98	100
Nile blue	+19	101	102
Janus green	-100	101	100
Benzyl-viologen	-109	100	100
Safranine T	-139	104	96
Neutral red	-170	99.5	100

from Fig. 4 that the trend is similar to that produced by redox dyes, a raised potential above the control causing an increased uptake of K⁺, and a reduced potential resulting in a fall in the uptake. For a given potential change the effect is smaller with the inorganic redox substances than with the organic dyes.

Effect of the redox systems used on the fermentation rates. The rate of fermentation was measured by determination of the ethanol and CO₂ production. The results in Table 1 show that there was little effect on the ethanol and CO₂ production with the various dyes (British Drug Houses Ltd.) used in 10⁻⁴M concentration.

Studies with a sodium-rich yeast

The external redox potential of sodium-yeast fermenting anaerobically. The potential levels with time of fermentation of sodium-rich yeast in sodium hydrogen succinate at pH 4.5 are illustrated in Fig. 5, curve A, which lies at a much higher potential level than that for fresh yeast in a similar medium containing potassium hydrogen succinate (Fig. 5, curve B). After 30 min. bubbling with nitrogen, when all or practically all the oxygen effect had been removed, the potential for the sodium-yeast was 220 mv and for the fresh yeast 152 mv (curve A), while after 60 min. the figures were 212 and 183 mv.

Fig. 5, curve C, shows the effect of adding KCl to the sodium-yeast. The KCl solution (3M) was added immediately on suspending the yeast, so as to give a final concentration of 0.04M in the suspending fluid. The effect is a marked reduction of the potential from the beginning when compared with that of the sodium-yeast without external KCl.

Effect of redox dyes on sodium extrusion by sodium-yeast. The same dyes, concentrations and procedure were used as in the investigations with fresh yeast. The trend of the curve (Fig. 6) is in the opposite direction to the effect produced on the fresh yeast.

Fig. 5. Redox potential changes during fermentation: A, sodium-rich yeast; B, baker's yeast; C, sodium-rich yeast in presence of 0.04M-KCl. Conditions as in Fig. 1, except that A and C were buffered by 0.04M sodium hydrogen succinate, pH 4.5.

Fig. 6. Effect of redox dyes on Na ion extrusion during fermentation of sodium-rich yeast in 5% glucose, buffered by 0.04M sodium hydrogen succinate pH 4.5. Symbols as in Fig. 2. Control (□) included.

Table 2. *Effect of redox dyes on Na⁺ extrusion by fermenting yeast*

Yeast buffered by 0.04M sodium hydrogen succinate pH 4.5 in presence of 0.1M-KCl. The redox dyes were included in the suspending mixture to form 10⁻⁴M solutions.

	Control	Neutral red included	o-Chlorophenol-indophenol included
Na ⁺ extruded in 45 min. (m-equiv./kg. of yeast)	19.5	19.5	19.6
E_h (mv)	130	120	205

When Na⁺ is being excreted in exchange for K⁺ the effect of redox dye with resulting change of potential is negligible over a wide range (Table 2). This results from the balancing of the two opposed effects on the Na⁺ carrier and on the K⁺ carrier.

DISCUSSION

The fact that the active uptake or transport of K⁺ during anaerobic fermentation can be considerably inhibited or greatly increased by very small concentrations of redox dyes, depending on the characteristic redox potential of the dyes, would appear to be strong evidence for a theory of active transport of a kind already described as the 'redox pump' (Conway, 1951, 1953). The general trend of action of inorganic redox systems is similar to the redox dyes, but the latter are more effective. This may be associated with more marked affinities of the organic dyes for the redox systems involved in the active transport.

The effect on the active secretion of H⁺ is similar in kind to the effect on the active uptake of K⁺. On the other hand, the effect on the sodium carrier goes in the opposite direction to that on the K⁺ carrier system. A redox potential increased by the redox dye decreases the active excretion of Na⁺ when these are being excreted with anions from within the cell. Conversely, potential decreased by a redox dye increased the rate of Na⁺ excretion. To account for these various facts it is concluded that the active transport of K⁺ and Na⁺ across the yeast cell membrane is directly concerned with redox systems acting as carriers which function as the immediate suppliers of the necessary energy. The detailed manner in which the redox dye system can inhibit a redox carrier system or greatly increase its action is not clear.

SUMMARY

1. The resting anaerobic potential of suspensions of baker's yeast was found to lie in the region -20 to -40 mv (E_h value at pH 4.5). Fermenta-

tion caused a marked increase in this potential, the steady-state value reaching +180 mv (at pH 4.1).

2. The steady-state potential during fermentation can be greatly altered by 10⁻⁴M redox dyes. Dyes which raise or lower the potential also raise or lower the excretion of H⁺ or the active absorption of K⁺. K absorption or H⁺ excretion are decreased to about 30 % or less of the control values when the potential is lowered by about 60 mv with the series of dyes studied. At the same time no appreciable effect is observed on the ethanol or CO₂ production.

3. Inorganic redox systems (ceric sulphate, chromic chloride, titanous chloride and stannous chloride) have a similar effect, but for a given potential change the effect is smaller with the inorganic redox system than with the redox dyes.

4. A sodium-rich yeast gives a higher potential during fermentation than normal yeast, and changes of potential produced by redox dyes, in the absence of K⁺, have the opposite effect on active Na⁺ excretion to that which they have on active K⁺ absorption.

The authors wish to thank the Irish Medical Research Council and the Rockefeller Foundation for grants.

REFERENCES

- Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, (a) p. 250; (b) p. 218. London: Crosby Lockwood.
- Conway, E. J. (1951). *Science*, **113**, 270.
- Conway, E. J. (1952). *The Biochemistry of Gastric Acid Secretion*. Illinois, U.S.A.: Thomas.
- Conway, E. J. (1953). *International Review of Cytology*, vol. II, p. 419. New York: Academy Press Inc.
- Conway, E. J. & Moore, P. T. (1954). *Biochem. J.* **57**, 523.
- Conway, E. J. & O'Malley, E. (1946). *Biochem. J.* **40**, 59.
- Conway, E. J., Ryan, H. & Carton, E. (1954). *Biochem. J.* **58**, 158.
- Kluyver, A. J. & Hoogerheide, J. C. (1934). *Biochem. Z.* **272**, 197.
- Rothstein, A. & Enns, L. H. (1946). *J. Cell. Comp. Physiol.* **28**, 231.