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Stoicheiometrical Proton and Potassium Ion Movements Accompanying the Absorption of Amino Acids by the Yeast *Saccharomyces carlsbergensis*

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1. Proton uptake into the yeast *Saccharomyces carlsbergensis*, was studied at pH 4.5–5.5 in the presence of both antimycin and 2-deoxyglucose to inhibit energy metabolism. Previous work had shown that the cells then absorbed about 20 nmol of glycine or L-phenylalanine against a considerable amino acid concentration gradient. The addition of the amino acid immediately stimulated the rate of uptake of protons two- to three-fold. About 2 extra equivalents of H^+ accompanied a given amount of the amino acids into the yeast preparations exposed to the metabolic inhibitors for 2–4 min and about 1.2 equivalents after 20 min exposure. 2. Analogous observations were made during serial additions of glycine, L-phenylalanine, L-leucine and L-lysine to preparations lacking the metabolic inhibitors and deficient in substrates needed for energy metabolism. In fresh cellular preparations the influx of glycine was then closely coupled to a stimulated flow of 2.1 equiv. of H^+ into the yeast. A similar number of K^+ ions left the cells. About 30% of the extra protons was subsequently ejected from the yeast. Deoxyglucose and antimycin together inhibited the ejection of protons. When the yeast had been fed with glucose energy metabolism was stimulated and almost as many protons as were absorbed with the amino acid were apparently ejected again. 3. Yeast preparations containing Na^+, instead of K^+, as the principal cation absorbed about 1 extra equivalent of H^+ after the addition of phenylalanine, glycine or leucine. This response was not observed in the presence of both deoxyglucose and antimycin. 4. The observations show that H^+ and, in certain circumstances, K^+ are co-substrates in the transport of the amino acids into the yeast. An analogy is drawn with the roles of Na^+ and K^+ as co-substrates in certain mammalian systems. The results lead to various models relating the physical flow of the co-substrate ions on the amino acid carrier to the transduction of chemical energy in an associated ion pump forming part of the mechanism for transporting amino acids into the yeast.

Previous work in these laboratories suggested that H^+ and K^+ might be co-substrates of the carrier systems transporting glycine and certain other neutral amino acids into the yeast cell, just as Na^+ and K^+ appear to be co-substrates in the absorption of glycine by mouse ascites tumour cells (Eddy, 1966, 1968*a*,*b*; Eddy, Backen & Watson, 1970*b*). One important piece of evidence was that yeast cells rendered deficient in ATP and similar products of energy metabolism, owing to the presence of antimycin and 2-deoxyglucose, nevertheless concentrated amino acids from the environment when there was a favourable distribution of $[H^+]$ and $[K^+]$ acting across the plasmalemma.

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According to the hypothesis that H^+ and K^+ are co-substrates of the amino acid carrier system, the absorption of the amino acid in the presence of the two metabolic inhibitors would be expected to accelerate both the influx of protons and the efflux of K^+, provided these fluxes were really responsible for driving the amino acid into the cells. The relevant mathematical equations are readily derived from those used in the mammalian case (Eddy, 1968*a*). The principal aims of the present work were first to test the above prediction about the system containing deoxyglucose and antimycin. Second, we wished to explore how in the absence of these inhibitors of energy metabolism the cellular ion pumps would respond to the presence of a given amino acid. The new findings, which have been

reported in a preliminary form (Eddy, Backen & Nowacki, 1970*a*), both strengthen the analogy between the yeast and the mammalian systems and throw new light on the mechanism of the so-called amino acid permeases of yeast.

MATERIALS AND METHODS

Preparations of the yeast (National Collection of Yeast Cultures strain no. 74) and, except where specified below, assay of the rate of amino acid transport were carried out as described by Eddy, Indge, Backen & Nowacki (1970*c*).

pH *changes*. The pH changes taking place during amino acid absorption by preparations of washed yeast cells were detected with a combination glass electrode (type GK 2321C, Radiometer A/S, Copenhagen, Denmark) containing 0.1M-KCl solution. The electrode was attached to a sensitive amplifier (Vibron C 33B-2 with 33B-2, Damper D33B; Electronic Instruments Ltd., Richmond, Surrey, U.K.) and recorder (U. R 400/M; Vitatron N.V., Dieren, The Netherlands). The yeast suspension (3–6ml) was held in a water-jacketed vessel at 30°C and stirred magnetically (Radiometer titration assembly, type TTA 31). The position of the pH electrode above the stirrer was adjusted to minimize electronic 'noise' in the recorded signal. In a typical run a full-scale deflexion (20cm) on the recorder chart corresponded to 0.3pH unit, the addition of 1μequiv. of HCl, which served to calibrate the system, producing a deflexion of 6cm at pH4.8. The acid-buffering capacity of the yeast suspension was approximately constant in a given range of 0.2units of pH from 4–5.

A larger jacketed vessel (type V 526) was used when 6–20ml of yeast suspension was to be sampled and the pH recorded. The samples (1–2ml) were filtered (filter diameter 2.5cm, pore size 0.8μm; Millipore Filter Corp., Bedford, Mass., U.S.A.) and both the yeast on the filter and the extracellular phase were collected for the assay of K^+ or ^{14}C. The yeast was washed (Eddy *et al.* 1970*c*) on the filter membrane which was then covered with water (5ml) and kept at 100°C for 10min to extract soluble cellular constituents. The K^+ was assayed in a flame photometer (Eppendorf Geratebau Netheler und Heinz G.m.b.H., Hamburg, Germany).

RESULTS

Preliminary work. The yeast was first transferred to 5mM-tris solution adjusted to a selected pH value with citric acid and contained in the apparatus described in the Materials and Methods section. When the initial pH was 4.5 the recorded pH increased rapidly (>10nequiv. of H^+ absorbed/min per mg of yeast) for 2–4min then more slowly for at least 10min. When the initial pH was near 7, on the other hand, the pH fell rapidly and then more slowly. It remained roughly constant when the initial pH was near 6. Two observations suggested that metabolism was not involved in these effects. First, similar rapid pH changes occurred at 30°C and at 0°C. Second, the addition of both 5mM-2-deoxyglucose and 0.1μg of antimycin per mg of yeast hardly affected the initial rate of change of pH at 30°C. To see whether the steady pH recorded around pH6 was the outcome of the exchange through the cell membrane of cellular and extracellular K^+ for H^+ in the opposing phase, the concentration of K^+ in the extracellular phase, which was initially at pH7, was raised to 100 mequiv./l, a value comparable with the 150mequiv./l present in the cells. The pH of the yeast suspension, which also contained both antimycin and deoxyglucose, then became steady at about pH6.0, instead of near pH6.2 as was observed when $[K^+]$ was initially about 1mequiv./l. However, if the ejection of protons by the yeast during the approach to a steady pH value was obligatorily coupled to the absorption of K^+ by the cells, the steady value would have been lowered by at least 2 pH units.

We infer from the above observations that the rapid initial pH changes at least were largely due, not to the exchange of H^+ for K^+, but to the titration of basic groups lying outside the plasma membrane. An estimate of the rate of exchange of H^+ with K^+ was inferred from other observations on the system at pH4.6–4.9 containing the two metabolic inhibitors. The rate of loss of cellular K^+ to the extracellular phase in the period from 2 to 15min was constant in each of four measurements and ranged from 2 to 4nequiv./min per mg of yeast, whereas in another six measurements the rate of absorption of H^+ in the interval from 3 to 6min was 3–5nequiv./min per mg of yeast. Hence, under these conditions, a major part of the H^+ removed from the extracellular phase apparently exchanged with K^+ that was presumably held originally inside the plasmalemma.

Dual effects of amino acids on proton absorption. These were studied principally in the concentration range (<0.2mM) where glycine absorption exhibited a K_m of about 60μM with respect to [glycine] (Eddy *et al.* 1970*a*). The amount of amino acid absorbed was usually <20nmol/mg of yeast, which is near the maximum the cells can absorb in the presence of deoxyglucose and antimycin (Eddy *et al.* 1970*b*). A similar dose of glycine caused K^+ ions to be ejected from the respiring yeast (Eddy *et al.* 1970*c*).

The addition of 0.5–1.0μmol of glycine to the system at pH4.5 containing both antimycin and deoxyglucose immediately stimulated the rate (±S.E.M.) of proton absorption from 3.4±0.5 (7) to 8.2±0.6 (7). In the representative experiment shown in Fig. 1(*b*) the faster rate was maintained for about 3min and proton influx then returned to almost its original rate. Omission of the two metabolic inhibitors altered the behaviour in one important respect. Thus 3 or 4min after the addition of the glycine, proton uptake was consistently slower than it was before the glycine was added.

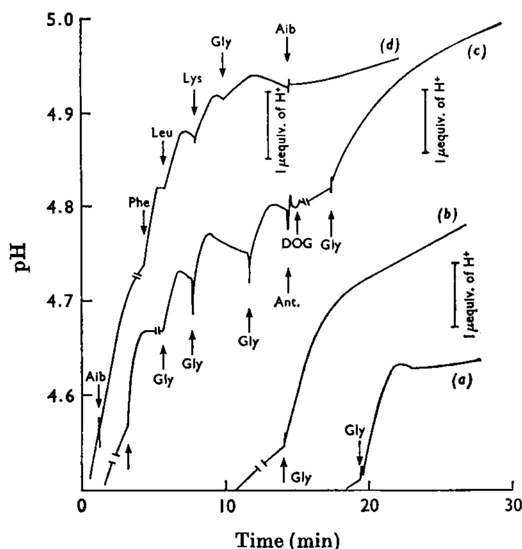


Fig. 1. Contrasting effects of a given amino acid on proton absorption in the presence and absence, respectively, of deoxyglucose and antimycin. Four experiments are compared on an arbitrary time scale. The length of the bar shows the pH change produced by the addition of 1 μmol of HCl. The yeast (10mg/ml) was initially kept with 5% (w/v) glucose at pH4.5 for 40–45min. The washed cells (50mg dry wt.) were suspended in 5mM-tris buffer (4–5ml) brought to pH4.5 with citric acid. Trace (*a*) shows the effect of adding glycine (1 μmol) at the time indicated by the arrow, which was about 4min after the yeast was put in the recording apparatus. In the separate run shown in trace (*b*), 10μg of antimycin (Ant.) and 17mM-deoxyglucose (DOG) were present throughout, and glycine (1 μmol) was added at the arrow. For trace (*c*) four successive additions of glycine (0.5μmol) were made before the inhibitors were added in the above amounts, and then a further portion (0.5μmol) of glycine was added. Trace (*d*) refers to the system without the metabolic inhibitors. The amino acids were added in the order DL-2-aminoisobutyric acid (Aib), L-phenylalanine, L-leucine, L-lysine, glycine, DL-2-aminoisobutyric acid, 0.5μmol of each being used.

An example of that behaviour is shown in Fig. 1(*a*) when the yeast in fact appeared to eject protons briefly.

All the above effects depended on the presence of the yeast and were not therefore electrode artifacts. Moreover the increase in the pH brought about by the addition of glycine was not due to the formation of ammonia, insignificant amounts of which were detected when the samples of the extracellular phase were reacted with the Nessler reagent.

Fig 1(*d*) shows that DL-2-aminoisobutyric acid, L-phenylalanine, L-leucine and L-lysine affected the proton movements in a similar fashion to glycine. Further aspects of the behaviour of glycine are illustrated in Fig. 1(*c*). Four successive additions of 0.5μmol of glycine were made and each first increased the rate of proton uptake. The pH then fell as protons left the yeast cells. A similar tendency was apparent with the other amino acids (Fig. 1*d*). Whether K^+ ions were reabsorbed when H^+ ions were expelled was not established. After 2μmol of glycine had been added (Fig. 1*c*) the two metabolic inhibitors were added. Proton ejection then ceased. The addition of a further 0.5μmol of glycine immediately increased the rate of proton uptake for about 3min, causing a net flow of protons into the yeast cells. Thus the behaviour before addition of antimycin and deoxyglucose resembled that in Fig. 1(*a*) whereas the behaviour afterwards resembled that in Fig. 1(*b*). The combined observations show clearly that whereas the ejection of protons induced by glycine was stopped by the two inhibitors of energy metabolism, the presence of these compounds actually increased the net flow of protons into the yeast.

Stoicheiometry

Proton uptake with glycine. Preliminary observations indicated that the accelerated uptake of protons ceased as the glycine content of the extracellular phase fell below 10% of the amount added. The extra H^+ absorbed after the addition of glycine, in the absence of antimycin and deoxyglucose, is compared in Fig. 2 with the amount of [^{14}C]glycine absorbed by the yeast. The extra H^+ was computed both on the assumption (1) that the endogenous rate of uptake of H^+ during the absorption of the glycine was that observed before the glycine was added, and on the assumption (2) that it equalled the slower, or even negative, rate observed after the period of accelerated proton influx. Although both methods of computation show that the uptake of glycine causes protons to flow from the extracellular phase into the yeast, the number of extra protons absorbed with each equivalent of glycine would vary from 1.6 to 2.4 dependent on the fraction of the uptake that refers to the first rather than the second assumption (Fig. 2). Because glycine in fact stimulated both proton uptake and proton ejection, in the absence of the metabolic inhibitors (Fig. 1), we prefer to use the second assumption in these circumstances. The relative positions of the two lines of Fig. 2 thus show that, once the yeast had absorbed more than about 4nmol of glycine/mg, up to 30% of the protons accompanying the further passage of glycine into the cells were subsequently ejected. Circumstances in which all the absorbed protons were apparently recycled are described below. It also seems a significant advantage for the method that the influx of H^+ estimated by using

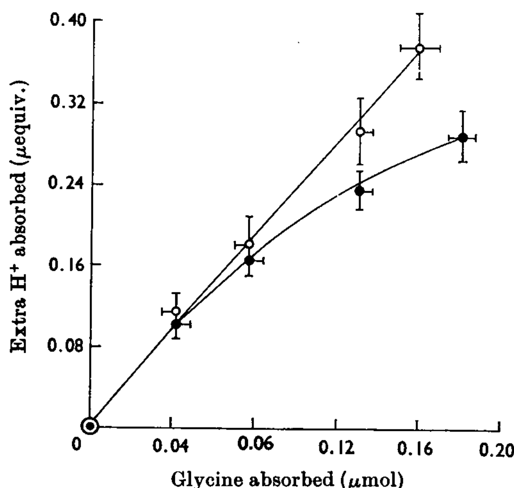


Fig. 2. Comparison of the extra number of protons entering the yeast with the amount of glycine absorbed up to that time. The yeast was kept with 5% (w/v) glucose for 40min and washed. The cells (250mg) were suspended in the electrode vessel in 5mM-tris solution (25ml) brought to pH4.5 with citric acid. Ten samples (2ml) were taken (see the Materials and Methods section) during 15min, [^{14}C]glycine (5μmol) being added after 3min. The Figure combines the results of six assays, the mean stimulated proton uptake ($\pm$S.E.M.)/ml of suspension being plotted against the mean glycine uptake ($\pm$S.E.M.)/ml, as estimated from the glycine content of the extracellular phase. Each point represents the average of four to eight observations. The extra H^+ absorbed was computed on the assumption either (1) that the endogenous rate continued at the value observed before glycine was added (●), or (2) that it changed to the larger value observed after the glycine was absorbed (○). The method is discussed in the text. The line of best fit through the results indicated by (○) corresponds to 2.14 equiv. of H^+ absorbed/glycine equivalent.

the second assumption was directly proportional to the amount of glycine absorbed (Fig. 2, upper line.)

The two methods of computation gave very similar results when the system contained the two metabolic inhibitors, because the initial endogenous rate of proton uptake was restored soon after the addition of the amino acid (Fig. 1*b*).

Variations with cellular history. When the yeast preparations were kept for up to 50min at pH4.5 and 30°C, before the addition of 0.5μmol of glycine, the subsequent net proton uptake per equivalent of glycine absorbed diminished with time (Table 1). Similar observations were made in the presence of deoxyglucose and antimycin (Table 1). The amount of glycine absorbed rapidly by the yeast then also decreased with time. For instance, after 15min exposure to the two compounds only about 50% of the added glycine, or about 10nmol/mg of yeast was absorbed during the phase of accelerated proton influx, after which glycine uptake virtually ceased. In contrast with the behaviour in the absence of glucose, keeping the yeast with glucose for up to 2h before testing it with glycine, in the presence of the two metabolic inhibitors, did not lower the number of extra H^+ ions absorbed below 2 equivalents.

Comparison of glycine and other amino acids. When the two metabolic inhibitors were absent the factor illustrated in Table 1 led to little change in the stoicheiometry during an interval of 10min during which, in the assays illustrated in Table 2, up to four additions of one or more amino acids were conveniently made. Proton uptake in series 1 of Table 2 was proportional to the size of the successive doses of glycine employed, a result in general agreement with Fig. 2. The addition of L-leucine, L-phenylalanine and L-lysine in series 2 and 3 approximately reproduced the effects observed with glycine. In series 4 the glycine concentration varied from 0.2 to 0.8mM, the glycine dose being kept near 20nmol/mg of yeast. Table 2 shows that the proton uptake per equivalent of amino acid was then roughly constant on the basis of assumption (2) above. Preliminary observations (not shown) indicated that the relative proton uptake was smaller from a 0.1mM solution of glycine. The significance of that result is uncertain however.

Efflux of K^+ *ions.* When glycine was absorbed in the absence of the metabolic inhibitors the flow of K^+ from the yeast cells increased (cf. Eddy *et al.* 1970*a*). The mean ratio, from five independent determinations, of the extra H^+ absorbed/extra K^+ displaced by the glycine was 1.03 ± 0.18, as computed by using assumption (1), and 1.20 ± 0.12, on the basis of the preferred assumption (2). We conclude that the accelerated proton influx was balanced by an equivalent efflux of K^+ ions. A similar stimulated efflux of K^+ ions, equivalent to the net proton uptake occurred in the presence of both antimycin and deoxyglucose (1 expt.).

Behaviour of 'sodium yeast'. Eddy *et al.* (1970*b*) found that the rates of glycine and phenylalanine absorption by yeast preparations containing Na^+ ions as the principal cellular cation approached the values observed with the standard 'potassium yeast'. The 'sodium yeast' absorbed the amino acids very much more slowly, however, than did the 'potassium yeast' when both antimycin and deoxyglucose were present. The effect of replacing the cellular K^+ by Na^+ is further illustrated in Fig. 3. The 'potassium yeast', serving as a control, was kept with 0.2M-potassium citrate and 100mM-glucose at pH6 for 2h before the tests with the amino acids. The aim was to simulate the conditions involved in making the 'sodium yeast'. The latter had been kept with glucose and sodium citrate for a similar period during which the initial cellular complement of K^+

Table 1. *Changes in the proton/glycine stoicheiometrical ratio in starved yeast preparations*

The yeast was manipulated as described in the legend to Fig. 1 except that the initial pH of the cell suspension in the electrode vessel was 5 in series 1 instead of 4.5 as in the other experiments. After the preliminary treatment with glucose the cells were kept in the presence (+) or absence (−) of antimycin (0.5 μg/ml of yeast) and 5mM-deoxyglucose for a period in the indicated range. Glycine (0.5 μmol) was added to the system and the extra H^+ absorbed was compared, on the basis of assumption (2) (see the text), with the amount of glycine absorbed. The number of independent determinations is given in parentheses. The yeast concentration was 17 mg/ml of suspension in series 1 and 2 and 12 mg/ml in series 3.

Series	Glycine added in the interval (min)	Deoxyglucose and antimycin	pH range	Extra H^+ absorbed/amino acid equivalent (±S.E.M.)
1	0–10	−	5.3–5.8	2.03±0.18 (10)
	10–20	−		1.42±0.12 (7)
2	0–15	−	4.5–5.0	2.05±0.06 (16)
	20–30	−		1.46±0.08 (7)
	30–50	−		1.13±0.14 (6)
3	0–4	+	4.5–5.0	1.80±0.12 (6)
	8–12	+		1.41±0.15 (3)
	14–21	+		1.22±0.07 (7)

Table 2. *Proton/amino acid stoicheiometrical ratio in various circumstances*

The assays were carried out as described in the legends to Fig. 1 and Table 1. The initial pH was 4.5. In series 1 successive portions of glycine were added to the same yeast preparations containing 110 mg dry wt. of yeast in 5 ml of buffer solution. In series 2 the indicated sequence of amino acids was added in turn to 3.0 or 3.5 ml of suspension containing 120 and 100 mg of yeast respectively. In series 3 based on three independent assays the 4 ml of suspension contained 50 mg of yeast. In series 4 the glycine concentration varied in the ranges shown, while the amount of glycine per mg of yeast was kept roughly constant by using up to 50 mg of yeast per ml of suspension.

Series		Amount of amino acid added (μmol)	Deoxyglucose and antimycin	Extra H^+ absorbed/amino acid equivalent
1	Glycine	0.25	—	2.0
		0.50	—	2.5
		0.75	—	1.9
		1.50	—	1.9
2	L-Phenylalanine	0.5	—	2.1
	L-Leucine	0.5	—	2.1
	L-Lysine	0.5	—	1.7
	L-Leucine	0.5	—	2.0
	Glycine	0.5	—	1.8
	L-Leucine	0.5	—	1.7
3	L-Phenylalanine	0.5	+	1.8±0.2 (3)
4	Glycine	0.5–5.0		
	at {	0.2 mM	—	2.05±0.10 (3)
		0.3–0.4 mM	—	2.32±0.13 (4)
		0.5–0.8 mM	—	2.16±0.14 (5)

was mostly replaced by Na^+. Fig. 3 shows that whereas the 'potassium yeast' reproduced the phenomena already illustrated in Fig. 1, no change in the rate of proton uptake occurred on the addition of glycine or of leucine to the 'sodium yeast', either in the presence of antimycin and deoxyglucose or in their absence. The 'sodium yeast' would have absorbed the added amino acid only in the latter circumstance. We infer that if the 'sodium yeast' absorbed a stoicheiometrical number of protons with the amino acids, a similar number were almost at once expelled.

After the preparation of the 'sodium yeast' had been kept for about 10 min in the apparatus used for recording the pH changes, a new phenomenon was encountered when phenylalanine, glycine or leucine was added to the system. Fig. 4 shows that each addition stimulated a net proton uptake. For

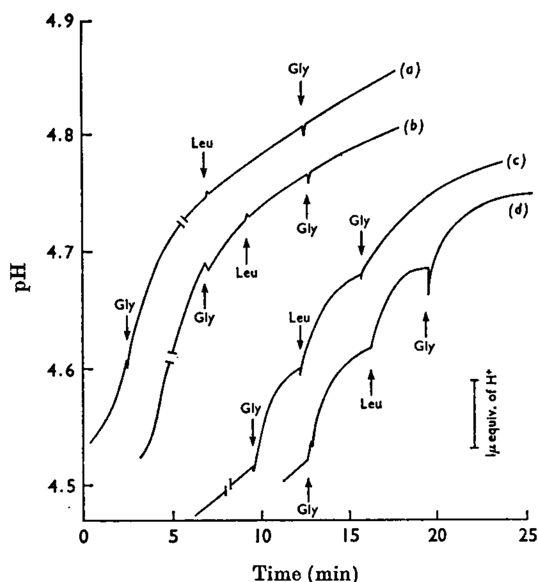


Fig. 3. The different behaviour of the 'sodium yeast' and the 'potassium yeast' in the presence of the amino acids. Traces (*b*) and (*a*) show how the pH of the suspension (4ml) of the 'sodium yeast' (50mg) changed with time in the presence and absence, respectively, of the two metabolic inhibitors (10 μg of antimycin, 5mM-deoxyglucose). Traces (*c*) and (*d*) are the corresponding traces for the 'potassium yeast'. Portions (0.5 μmol) of glycine or L-leucine were added at the times indicated by the arrows. The time at which each experiment started has been arbitrarily adjusted so as to facilitate comparison of the traces.

instance, glycine increased the rate of uptake of protons to 4–7 nequiv./min per mg of yeast, the mean increase (±S.E.M.) being 3.4±0.3 (13) nequiv. of H^+/min. An important aspect of the phenomenon, distinguishing it from the behaviour of the 'potassium yeast' illustrated in Fig. 1, was that the amino acid failed to increase the proton uptake significantly when deoxyglucose and antimycin were present (Fig. 4*d*). Further, the ratio of the extra H^+ absorbed per amino acid equivalent was about 1 (Table 3). It was therefore somewhat smaller than was observed in preparations of the 'potassium yeast' kept for 20min without the metabolic inhibitors (Table 1).

The changes in the system during the initial lag of about 10min involve the yeast itself and not simply the extracellular phase, as the following experiment shows. A sample of the 'sodium yeast' that had been kept for 10min in the recording apparatus was resuspended in fresh buffer solution at pH 4.5. The addition of glycine 1min later caused about 1 extra equivalent of protons to be absorbed.

Proton efflux during metabolism

The presence of 10mM-glucose in the standard preparations of the 'potassium yeast' resulted in a large flow of protons from the yeast and the pH fell rapidly. Fig. 5(*a*) shows that the addition of 5 μmol of glycine then caused only a small deflexion in the pH trace, as though a quantity of protons similar to that presumed to be absorbed with the glycine was ejected almost at once. This behaviour may be contrasted with that illustrated in Fig. 1(*c*), when a net outflow of protons smaller than the number initially absorbed occurred 1–2min after the addition of glycine. The factors limiting proton ejection in the latter circumstances, where energy metabolism was relatively restricted, are not fully understood. However, the deficiency both of oxygen and of fermentable substrate may have been critical. To increase the cellular carbohydrate reserves, we accordingly fed the yeast with glucose at pH 4.5 for 2h, before putting it in the electrode vessel without glucose. The yeast then ejected sufficient protons to lower the pH slowly. In the representative experiments shown in Fig. 5 the addition of 0.5 μmol of glycine caused a small uptake of protons, after which the proton flow roughly followed its original course (Fig. 5 traces *b* and *c*). The response to 2-aminoisobutyric acid was similar. The addition of L-lysine lowered rather than raised the pH (Fig. 5*c*). Next antimycin and deoxyglucose were added in turn to the system. In Fig. 5 traces (*b*) and (*c*) show that proton ejection stopped almost at once. Subsequently the addition of glycine or of lysine increased the influx of protons in the manner already illustrated in Fig. 1 traces (*b*) and (*c*). D-Arabinose, which is also absorbed by the yeast cells, failed to mimic the behaviour of the amino acids (Fig. 5*b*).

The observations in Fig. 5 strengthen the view that deoxyglucose and antimycin were potent inhibitors of the mechanism linking energy metabolism to the ejection of protons from the yeast, whereas proton influx into the 'potassium yeast' occurred even when energy metabolism had stopped.

Other yeast strains. The above behaviour was not a unique property of strain N.C.Y.C. no. 74 as two laboratory strains of *Saccharomyces cerevisiae* exhibited an accelerated influx of protons in the presence of glycine in circumstances like those illustrated in Fig. 1.

Other substrates. The addition of 0.5, 1.0, 5.0 or 10 μmol of one of the following compounds caused no significant change in the proton influx, although the subsequent addition of glycine or of phenylalanine produced effects like those shown in Fig. 1: sorbose, D-glucosamine, α-methyl D-glucoside, adenine, adenosine, 4-aminobutyric acid.

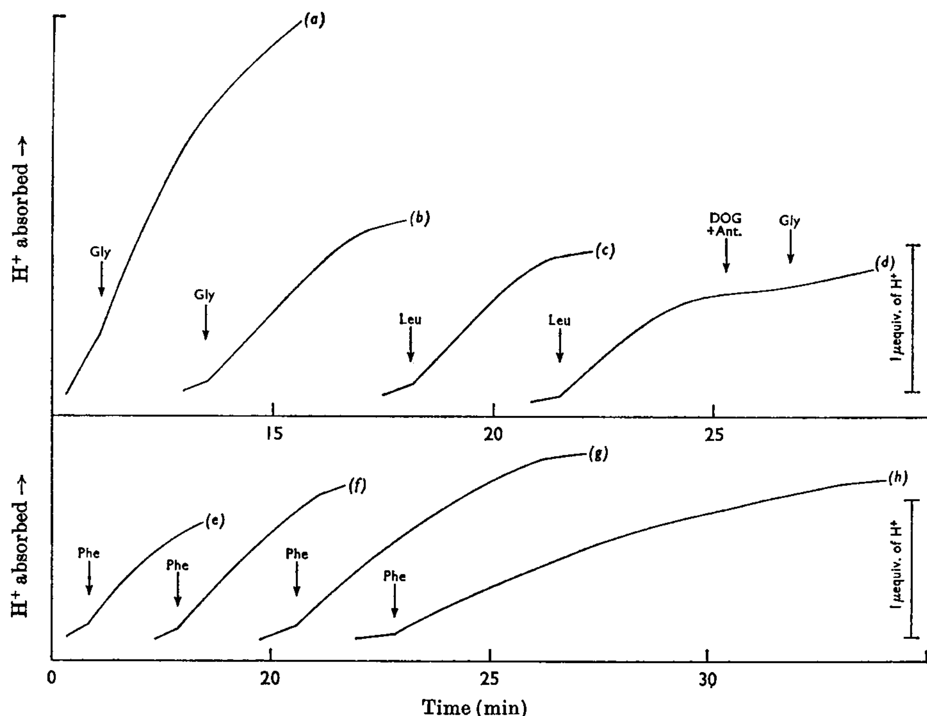


Fig. 4. Proton absorption estimated from the pH changes occurring during successive additions of phenylalanine, glycine and leucine to the aged 'sodium yeast'. A suspension of the 'sodium yeast' (50 mg) in 5 mM-tris solution (4 ml) initially at pH 4.5 was kept in the electrode system for 10 min (see the text). The addition of glycine (0.5 μmol) at the time indicated by the arrow caused only a small additional influx of H^+ in the assay illustrated in trace (*a*). A second addition caused a larger proton influx (*b*). L-Leucine (0.5 μmol) was then added for traces (*c*) and (*d*). Both antimycin (Ant.) (10 μg) and 5 mM-deoxyglycose (DOG) were added next and then glycine (0.5 μmol), which failed to cause a marked proton uptake (*d*). The time-interval between the successive additions has been shortened for convenience of representation. Incremental doses of L-phenylalanine were used in another experiment: 0.25; 0.5, 0.75 and 1 μmol, respectively, for the traces (*e*), (*f*), (*g*) and (*h*), which were obtained consecutively with the same sample of yeast. Table 3 summarizes the stoicheiometrical relations observed in these and similar assays.

DISCUSSION

Application of the co-substrate hypothesis. When energy metabolism was deficient the number of extra H^+ ions accompanying the amino acid into the yeast was a fairly reproducible quantity in a given type of yeast preparation and fell from near 2 equiv. to near 1 equiv. when the cells were starved at pH 4.5 (Tables 1 and 2). The extra H^+ exchanges with an equal number of K^+ ions from the yeast. Our earlier finding (Eddy *et al.* 1970*c*), that progressively less than 2–3 K^+ ions per glycine equivalent were ejected, as the amount of glycine already absorbed by the yeast increased, is perhaps related to the above changes in the proton stoicheiometry.

In the co-substrate model shown in Scheme 1(*a*) the amino acid enters the yeast in association with a carrier (E) that also binds up to 2 H^+ ions. The latter are carried into the cells with the amino acid (A), the observed acceleration of the proton influx being due to the relatively low rates of transfer of H^+ and A in the respective forms of EH, E2H and EA as compared with E2HA or EHA. The principles involved in the comparable mammalian case (Scheme 1*b*) are discussed by Eddy (1968*a*). By analogy with the mammalian system (Scheme 1*b*), the entry of 1 amino acid equivalent would displace up to 2 H^+ into the yeast and up to 2 K^+ ions would leave the cells. Na^+ ions inhibit glycine uptake (Eddy *et al.* 1970*c*) and may therefore be supposed to combine with E without serving the functions either of H^+, or those of K^+ that lead to the efflux of K^+ ions in the presence of glycine. Extracellular K^+ ions would lower the influx of amino acids (Eddy *et al.* 1970*b*) both by inhibiting the formation of EHA and by interfering with the efflux of cellular

Table 3. *Proton/amino acid stoicheiometrical ratios observed during the absorption of L-phenylalanine, L-leucine or glycine by 'sodium yeast'*

As explained in the legend to Fig. 4 the 'sodium yeast' (50mg) was kept in the electrode vessel for 10min and the amino acid was then added in the stated amount. Up to four such additions were made to one yeast preparation (4ml). The proton displacements computed by the two methods described in the text were similar and were averaged. The mean values obtained in a series of such assays are quoted. When deoxyglucose and antimycin were both present in the system no marked acceleration of the influx of protons occurred on the addition of glycine (three experiments) or L-phenylalanine (two experiments).

	Amount of amino acid added (μmol)	Extra H^+ absorbed/amino acid equivalent ($\pm$S.E.M.)
L-Phenylalanine	0.25	1.09 ± 0.03 (2)
	0.50	1.10 ± 0.04 (7)
	0.75	1.01 (1)
	1.00	0.96 (1)
L-Leucine	0.50	1.20 ± 0.03 (4)
Glycine	0.25	1.18 ± 0.10 (2)
	0.50	1.17 ± 0.03 (12)
	0.75	1.05 ± 0.15 (2)

K^+ ions. The failure of the proton conductor 2,4-dinitrophenol (Mitchell, 1966) to short circuit, as it were, the functioning of the amino acid carrier system seems to show that the efflux of K^+ ions plays a role in the process that is not served by the proton movements through the artificial conductor (Eddy *et al.* 1970*b*). The point is reinforced by the observation that the net efflux of K^+ ions in the absence of glycine is stimulated severalfold by dinitrophenol in these conditions (Rothstein & Bruce, 1958), as though it were then coupled to the passage of protons through the conductor.

An important feature of the model shown in Scheme 1(*a*) is that it functions without hydrolysing ATP or any similar compound. The spontaneous movements of H^+ and K^+ through the system serve to concentrate the amino acids in the yeast cells. Further work is needed, however, to reconcile Scheme 1(*a*) with (1) the magnitude of the gradients of amino acid concentration that are set up both in the presence of deoxyglucose and antimycin and in their absence (Eddy *et al.* 1970*b*); (2) the fact that the yeast absorbs only a rather small quantity of amino acid in the presence of the two metabolic inhibitors (Eddy *et al.* 1970*b*); (3) the variation in stoicheiometry, referred to above, that occurs when the yeast is starved; (4) the partial inhibition of glycine uptake that occurs in the presence of 2,4-dinitrophenol (Eddy *et al.* 1970*b*).

Behaviour of the ion pumps in energy-deficient yeast. As emphasized by Kimmich (1970), the ion movements in the system depleted of ATP might be due to an interaction with the amino acid pump in these special circumstances rather than an indication of the importance of the ions in general. Both the sodium pump of the human erythrocyte (Glynn & Lew, 1970; Lant, Priestland & Whittam, 1970) and the less well characterized proton pump in mitochondrial and photosynthetic systems (Slater, 1967), appear to catalyse reversible and vectorial processes. The spontaneous flow of the relevant cations back through the pump may in each case give rise to ATP.

Yeast cells have long been known to eject protons during energy metabolism (Conway & O'Malley, 1946; Rothstein & Enns, 1946) though little evidence is available about the mechanism. Simultaneously, either an equivalent of K^+ ions is absorbed or an equivalent of bicarbonate and succinate anions are ejected. A second, perhaps related, ion pump has also been postulated (see Rothstein, 1960), that expels Na^+ ions, in preference to K^+ ions, from the yeast cells. Unlike the proton pump the sodium pump is relatively insensitive to the presence of 2,4-dinitrophenol. Energy conservation in the yeast system might involve a high-energy intermediate (~I) shared by both the proton and amino acid pumps (Scheme 1*c*). It might normally be formed at the expense of ATP, or by a redox process (cf. Burnett, 1968), but when no metabolic energy was available, as in the yeast suspension put at pH 4.5 in the presence of deoxyglucose and antimycin, the ~I might form when protons flowed into the yeast through the proton pump in exchange for cellular K^+ ions. The amino acid pump might therefore be driven, in these circumstances, by the passage of the cations through the proton pump. Whereas the flow of cations would be stimulated owing to the removal of ~I, the cations would not necessarily be involved either in the formation of ~I from ATP or in the subsequent utilization of the intermediate by the amino acid pump (cf. Kimmich, 1970).

An essential feature of the model shown in Scheme 1(*c*) is that the relation between the amino acid flux and the direction of the proton movements would itself have a vectorial character; i.e. the reversal of the cation gradient would not lead to the amino acid being pumped out of the yeast, as happens in the mouse ascites system (Eddy, 1968*b*). We do not know whether the yeast system is vectorial in that sense. The model shown in Scheme 1(*a*) would conventionally be supposed to function equally well in either direction. It seems worthwhile remarking, nevertheless, that none of the present observations are inconsistent with the possibility that the carrier E, postulated above in connexion

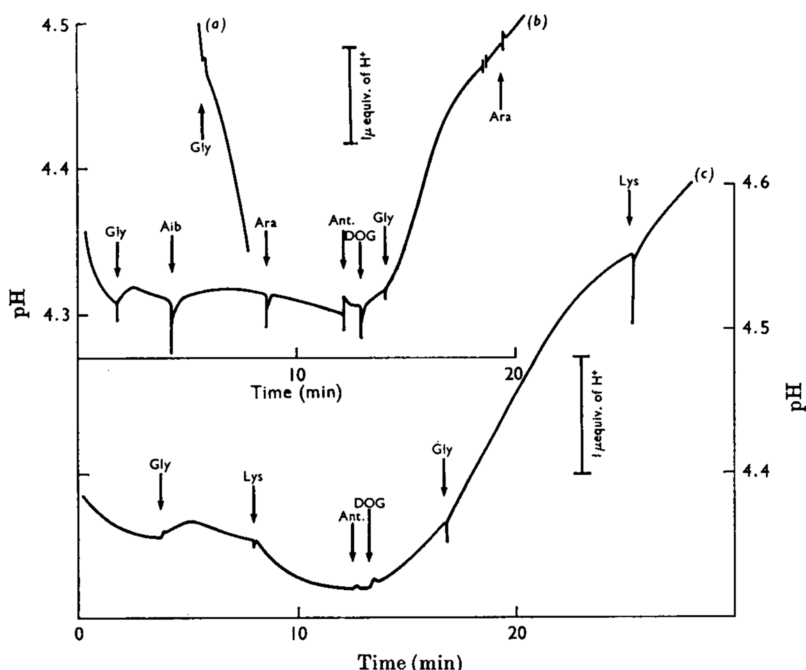
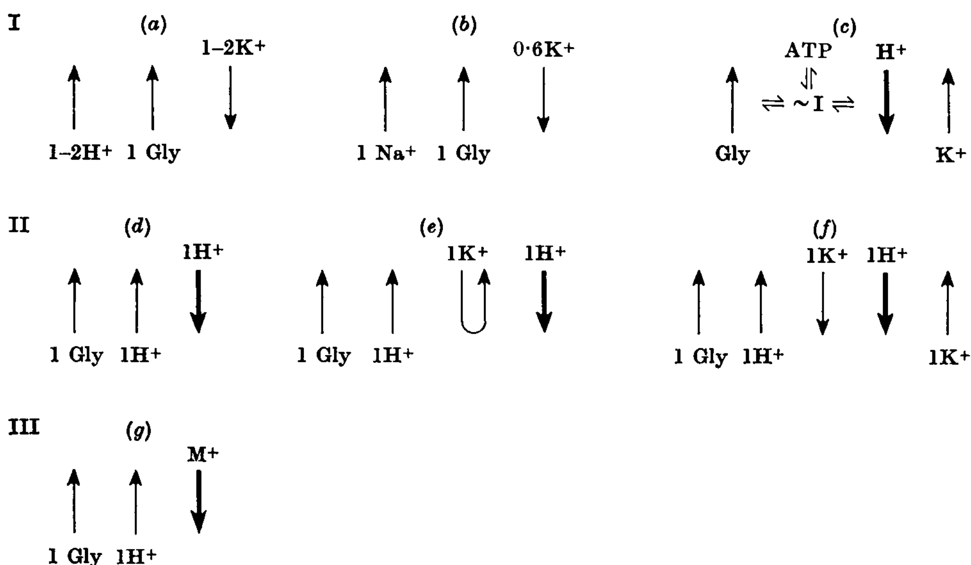


Fig. 5. Effect of stopping energy metabolism on the pH changes caused by glycine, DL-2-aminoisobutyric acid and L-lysine. For trace (*a*) the standard yeast preparation (50mg) was suspended in buffer solution (5ml) containing 10mM-glucose. Glycine (5μmol) was added at the time indicated by the arrow. For trace (*b*) the yeast (10mg/ml) was initially kept at 30°C with 5% (w/v) glucose at pH4.5 for 2h. The cells (64mg) were washed and suspended in 4ml of the 5mM-tris solution adjusted to pH4.5 with citric acid. Glycine (0.5μmol), DL-2-aminoisobutyric acid (Aib) (0.5μmol) and, as a control, D-arabinose (1μmol) were added in turn, then 25μg of antimycin (Ant.) and 7mM-deoxyglucose (DOG). Proton ejection had now stopped and further additions of glycine (0.5μmol) and D-arabinose (1μmol) were made. The experiment illustrated in (*c*) was carried out similarly and involved making successive additions of glycine (0.5μmol) and L-lysine (0.5μmol) before and after the two metabolic inhibitors.

with Scheme 1(*a*), has the properties of a 'molecular turnstile' and only catalyses the influx of the amino acid.

That issue apart, there are several more or less serious objections to the scheme depicted in Scheme 1(*c*) as opposed to Scheme 1(*a*). (1) A 5mM solution of potassium chloride that would have virtually saturated the influx of K^+ linked to the ejection of protons (Armstrong & Rothstein, 1964) only partially inhibited amino acid absorption at pH4.5 (Eddy *et al.* 1970*b*). (2) The influxes of K^+ and of glycine were not apparently in competition when glucose was present (Eddy *et al.* 1970*c*), behaviour that seems incompatible with the degree of coupling required to explain the stoicheiometry of the induced proton movements. (3) The induced influx of protons through the proton pump and their subsequent ejection, into a solution of lower pH (Fig. 1), through the very same system, seems an improbable occurrence. (4) The rate of glycine transport in the presence of glucose varied considerably with pH and, in the alkaline region, with $[K^+]$ (Eddy *et al.* 1970*c*). Thus the interactions with the cations were retained, in some degree, though apparently in a modified form, when ATP was available. (5) The presence of deoxyglucose, which serves as an efficient trap for ATP, might be expected to accelerate the breakdown of ~I in direct competition with the amino acid pump. (6) Because the 'sodium yeast' absorbed protons with the amino acids in a process that was linked to energy metabolism (Fig. 4), we infer that the protons acted as co-substrates even when metabolic energy was available to generate ~I. These various difficulties with the model depicted in Scheme 1(*c*) make it an unattractive alternative to the one in Scheme 1(*a*).

Effect of energy metabolism. The work shows very clearly that during energy metabolism the proton influx with the amino acid is neutralized in at least two ways. (1) Comparison of Fig. 1 and Fig. 5 shows that during energy metabolism the influx of protons with the amino acid was balanced by the secretion



Scheme 1. Suggested relationships between the flux of amino acid into the yeast, the cation fluxes and the ion pumps. (I) Energy metabolism deficient or stopped. (*a*) 1–2 H^+ absorbed, 1–2 K^+ ejected per glycine equivalent absorbed. The mechanism involves only physical processes, in a carrier system with distinct binding sites for H^+, or K^+, and the amino acid, respectively. (*b*) The mouse ascites system, where a bifunctional carrier has also been postulated (Eddy, 1968*a*,*b*). Model (*c*) is based on a high-energy intermediate (~I) formed, in the yeast membrane, from either ATP or, possibly, by a redox process. The same intermediate is involved both in pumping the ions and the amino acids. Further detailsare given in the text. (II) Energy metabolism functioning. The heavy arrows denote a proton pump driven by metabolic energy. Both the number of protons ejected and the number of amino acid molecules absorbed per ATP equivalent are unspecified. The pump might be structurally associated with the amino acid carrier system and otherwise resemble the proton pump described by Conway & O'Malley (1946) and Rothstein & Enns (1946). The H^+ and the amino acids are absorbed through the same system postulated in model (*a*) above. Models (*d*), (*e*) and (*f*) depict various ways, discussed in the text, of linking the physical functions of the amino acid carrier and the ion pump in which chemical energy is transduced. A ratio of 1 H^+ per amino acid equivalent is assumed simply for convenience of representation. (III) In model (*g*) a net uptake of protons with the amino acid occurs through the above sites. Charge balance is maintained in an energy-dependent process when the cation M^+, which has not been identified, is ejected through a cation pump. The latter might resemble the 'sodium pump' said to occur in yeast. The various models illustrate the events occurring during the influx of the amino acids from relatively dilute solutions (<0.2 mM). Efflux of amino acids from the yeast is very slow even in the presence of metabolic inhibitors and may involve other factors.

of almost an equivalent number of protons. The two partial processes are clearly revealed in Fig. 1 when the action of the proton pump was presumably delayed by the prevailing low rate of energy metabolism. Whether K^+ was then reabsorbed was not established. Since glycine failed to inhibit the influx of K^+ in the presence of glucose (Eddy *et al.* 1970*c*) the proton pump involved with the amino acids may be distinct from the one through which K^+ is absorbed in the absence of the amino acids. The ejection of H^+ after the uptake of H^+ with the amino acid may be simply the result of extra H^+ being absorbed, or may represent a direct modifying effect of the amino acid itself on the activity of the pump. The behaviour with L-lysine is noteworthy in this connexion in that a net outflow of protons occurred (Fig. 5*c*), as already shown in other circumstances by Eddy *et al.* (1970*c*).

Scheme 1(*d*) depicts one possible model not involving cellular K^+ and Scheme 1(*e*) another model where K^+ traverses the membrane. Because a 'sodium yeast', containing about 30 mequiv. of K^+/l of cellular water, absorbed glycine rapidly (Eddy *et al.* 1970*b*), Scheme 1(*d*) may be an adequate representation, though further work is required to clarify the roles of the cellular K^+ in the system. A model in which K^+ is ejected and a further K^+ ion reabsorbed (Scheme 1*f*) is not easily reconciled with the observation that the influx of K^+ was not increased by the addition of glycine in the presence

of glucose. It may better represent the events occurring during restricted energy metabolism as illustrated in Figs. 1(*c*) and 1(*d*).

(2) The 'sodium yeast' behaved superficially like the 'potassium yeast' fed with glucose in short term experiments (Fig. 3) and subsequently absorbed about 1 proton equivalent with the amino acid (Fig. 4). A plausible model is shown in Scheme 1(*g*) which envisages a role in the process for a cation pump, possibly like the 'sodium pump' described by Conway, Ryan & Carton (1954). The possibility that the mechanism is based on a combination of the models shown in Scheme 1(*a*) and 1(*d*) is unlikely because proton absorption stopped in the presence of the two metabolic inhibitors (Fig. 4).

Although further work is required to clarify the complex relations between these different modes of functioning of the amino acid transport system and its satellite ion pumps, the evidence presented here indicates for the first time that the movements of protons and K^+ ions with the amino acid represent a fundamental part of the mechanism.

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