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The Stoicheiometry of the Absorption of Protons with Phosphate and L-Glutamate by Yeasts of the Genus *Saccharomyces*

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1. A study was made of the pH changes occurring when 0.1–4μmol of glutamate, phosphate and certain phosphate esters was added at about pH4.8 to washed cell preparations (50mg dry wt.) of strains of *Saccharomyces*. The system also contained deoxyglucose and antimycin to inhibit energy metabolism and so prevent proton ejection from the yeast. 2. A strain of *Sacc. carlsbergensis* was grown in a chemostat with a limiting supply of phosphate in order to enhance the subsequent rate of phosphate transfer into the yeast. These preparations absorbed 0.2μmol of phosphate with about 3equiv. of protons/mol of phosphate. The charge balance was maintained by the efflux of 2equiv. of K^+ from the yeast. 3. Larger amounts of phosphate were absorbed with fewer proton equivalents. 4. Arsenate and phosphate caused similar pH changes. 5. Glucose 6-phosphate, ATP and certain other phosphate esters each initiated a rise in pH, possibly because hydrolytic extracellular enzymes released phosphate that was subsequently absorbed. 6. Four strains of yeast were grown with glutamate as principal source of nitrogen. Each absorbed extra protons in the presence of L-glutamate. 7. One of them, a strain of *Sacc. cerevisiae*, absorbed 0.2μmol of glutamate with 3equiv. of protons/mol of glutamate, and in these circumstances 1–2equiv. of K^+ left the yeast cells. 8. The role of ionic gradients in the transport of these anions is discussed.

Previous work in these laboratories with *Saccharomyces* species showed that various neutral amino acids, basic amino acids and certain carbohydrates (Eddy & Nowacki, 1971; Seaston *et al.*, 1973) are absorbed with protons as co-substrates. The spontaneous inflow of protons, coupled to the efflux of an equivalent number of K^+ ions, appears to drive amino acid accumulation in yeast preparations depleted of ATP (Eddy *et al.*, 1970*b*). The subsequent ejection of protons from the yeast depends on energy metabolism (Conway & O'Malley, 1946; Rothstein & Enns, 1946; Eddy & Nowacki, 1971). A large gradient of amino acid concentration might thus form at the expense of the metabolites utilized by the proton pump (Mitchell, 1970; Harold, 1972).

The object of the present work was to show whether protons are also implicated in the permeases absorbing the anions of glutamate (Grenson *et al.*, 1970) and phosphate (Goodman & Rothstein, 1957; Borst-Pauwels, 1962) and, if so, the stoicheiometry of the process. Certain yeasts concentrate these anions extensively during energy metabolism and largely retain them when the cells are subsequently washed (Rothstein, 1963; Button *et al.*, 1973; Tang & Howard, 1973). Moreover, there is qualitative evidence that phosphate absorption raises the extracellular pH, and this has been taken to mean that $H_2PO_4^-$ is absorbed by exchanging with cellular OH^-, in a reaction linked in an unknown manner to energy metabolism (Rothstein, 1963; Suomalainen & Oura, 1971). A superficially similar process occurs in mammalian mitochondria (Scheme 1, model *a*), where each equivalent of $H_2PO_4^-$ appears to be absorbed with 1equiv. of protons, by a process of facilitated diffusion involving a specific carrier mechanism (Chappell, 1968; Mitchell & Moyle, 1969). When a steady state is reached, the ratio of the respective concentrations of $H_2PO_4^-$ inside and outside the mitochondrial matrix therefore approaches the magnitude of the H^+ ion concentration gradient between them (McGivan & Klingenberg, 1971). A similar mechanism has been proposed for the translocation of glutamate into rat liver mitochondria (Azzi *et al.*, 1967), of phosphate in yeast mitochondria (Kolarov *et al.*, 1972), of lactate in *Streptococcus faecalis* (Harold & Levin, 1974) and of both glutamate (Niven & Hamilton, 1974) and phosphate (Mitchell, 1970) in *S. aureus*. However, if anion absorption across the yeast plasmalemma is governed by the movements of protons, the power available from the above mechanism appeared to be too small to maintain either an accumulation ratio of

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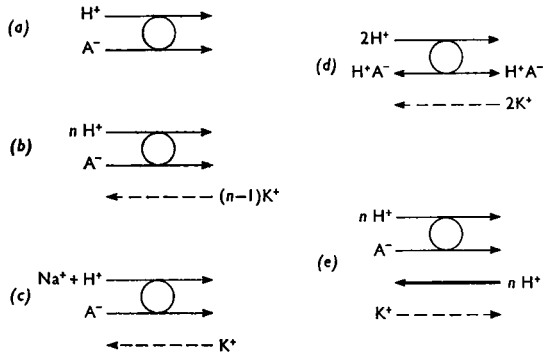

Scheme 1. *Five models relating the flow of protons, K^+ and the anions of phosphate or glutamate across the plasmalemma*

(*a*) The anion (A^-) is absorbed with one proton. (*b*) *n* protons are absorbed in an electrogenic process, causing $(n-1)K^+$ ions to leave the yeast. (*c*) Both Na^+ and H^+ act as co-substrates. (*d*) A proton-dependent anion-exchange occurs in which the net uptake of protons equals the number of K^+ ions displaced from the yeast. The former models apply when energy metabolism is restricted so as to prevent proton ejection from the yeast. (*e*) This shows how model (*b*) might function during energy metabolism, when the proton pump is working and K^+ is absorbed.

1×10^6, as Button *et al.* (1973) found for phosphate in the yeast *Rhodotorula rubra*, or a ratio of 5×10^3, as Gale & Llewellin (1972) found for glutamate in *S. aureus*. We therefore considered two other possibilities (Schemes 1*b* and 1*c*): (1) more than 1 equiv. of protons might be absorbed per anion equivalent; (2) both Na^+, or perhaps Mg^{2+}, as well as H^+ might be implicated. Thus Na^+ interacted with glutamate transport in *Escherichia coli* (Halpern *et al.*, 1973) and with melibiose transport in *Salmonella typhimurium* (Stock & Roseman, 1971), whereas Mg^{2+} was absorbed with citrate in *Bacillus subtilis* (Willecke *et al.*, 1973). Glutamate absorption by yeast can lead to the excretion of comparable amounts of 2-oxoglutarate (Lewis & Rainbow, 1963) and this was another factor to be considered.

Materials and Methods

The yeasts were grown at 25°C and manipulated as described by Eddy *et al.* (1970*a*). The same basal mineral salts–nutrient medium was used except that (1) $(NH_4)_2SO_4$ was replaced by 2.5mM-potassium glutamate as required, the glucose concentration being lowered from 2 to 1% (w/v). (2) Phosphate-limiting chemostat cultures (1 litre) were fed (about 60ml/h) with a modified basal medium containing 0.1mM-KH_2PO_4 and 10mM-KCl instead of the standard amount of KH_2PO_4. A Porton type of chemostat was used (L.H. Engineering Co. Ltd., Stoke Poges, Bucks., U.K.). The culture was kept at pH4.7 by the addition of 0.1M-KOH. It was stirred at 250rev./min and given 0.8litre of sterile air/min. The outflowing yeast was collected at 0°C. It was examined microscopically as a routine for signs of infection.

Proton absorption and K^+ efflux

Seaston *et al.* (1973) describe these assays. Typically the washed yeast (50mg dry wt.) was suspended at zero time in 4.5ml of 5mM-Tris solution adjusted to pH4.8 with citric acid. The pH of the stirred suspension was recorded continuously. Next 15μg of antimycin and 10μmol of 2-deoxyglucose were quickly added. At 2min or later the substrate (0.1–4μmol) was added. This had been adjusted to pH4.8 with either Tris or KOH. The ensuing changes in pH were used to compute the amount of acid absorbed by the yeast, the buffering capacity of the system having been calibrated by the addition of 1μmol of HCl. [U-^{14}C]Glutamate uptake and $H_2{}^{32}PO_4^-$ uptake into a given sample of the yeast taken from the above suspension were assayed by determining the respective proportions of the added ^{14}C (0.2μCi) and ^{32}P (1μCi) left in the supernatant solution when the yeast was separated. The ^{14}C was assayed in a liquid-scintillation spectrometer (Seaston *et al.*, 1973), whereas the ^{32}P was assayed in the liquid phase by using an M6 tube and the other apparatus that Eddy *et al.* (1967) used. For the assay of K^+ by flame photometry, it was sometimes convenient to filter the yeast from the above samples rather than to centrifuge them. This was done with type AA filters (0.80μm) from Millipore (U.K.) Ltd., London NW10 7SP, U.K.

Organisms

Strain N.C.Y.C. no. 74 of *Saccharomyces carlsbergensis* and strain N.C.Y.C. no. 193 of *Candida utilis* were obtained from the British National Collection of Yeast Cultures (Nutfield, Surrey, U.K.). Strain G was obtained from Dr. D. Wild, Department of Biochemistry, University of Oxford, Oxford, U.K. It is the haploid wild type (Σ 1278 b), carrying the general amino acid permease, of the series of strains A, B and C of *Saccharomyces cerevisiae* described by Seaston *et al.* (1973). Strain B, lacking a functional general amino acid permease, was also used in the present work. The unrelated strain H (code X 2180-1A) was obtained from Dr. J. Bassel, Yeast Genetic Stock Center, Donner Laboratory, University of California, Berkeley, Calif., U.S.A.

Chemicals

Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. D-

Glutamic acid, DL-2-aminoadipic acid and L-aspartic acid were from Sigma Chemical Co., St. Louis, Mo., U.S.A. L-Glutamic acid, sodium molybdate, dipotassium glucose 1-phosphate, disodium glucose 6-phosphate, $Na_4P_2O_7$ and KH_2AsO_4 were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. $Na_2ATP,3H_2O$ and 5'-AMP were products from Boehringer Corp. (London) Ltd., London W.5, U.K.

Results

Preliminary work

After growth in the mineral-salts medium containing NH_4^+ as the principal source of nitrogen, preparations of the washed cells of yeast N.C.Y.C. no. 74 were suspended at pH4.8, the subsequent changes in pH being monitored continuously for up to 5min during which time the anion to be investigated was added to the system (see the Materials and Methods section). The assays were done in either the presence or the absence of antimycin and deoxyglucose, compounds that stop energy metabolism and so prevent protons being ejected from the yeast (Eddy & Nowacki, 1971). We found that in either circumstance the addition of 1 μmol of glutamate did not significantly change the basal rate of proton absorption (less than 6nequiv./min per mg dry wt.). The addition of P_i likewise was without effect. We therefore looked for growth conditions that might increase the rate of anion absorption and so facilitate the detection of any accompanying changes in pH.

(1) Glutamate was absorbed relatively fast after aerobic growth for 19h in batch cultures in which either 2mM-proline or 2.5mM-glutamate replaced NH_4^+ as source of nitrogen. These preparations exhibited marked pH changes in the presence of glutamate (Fig. 1, traces *a*, *b* and *c*). Similar pH changes occurred with strain no. 74, with the yeast strain B lacking a functional general amino acid permease, and with strains G and H and the yeast N.C.Y.C. no. 193. The above observations are consistent with other evidence that glutamate absorption occurs by a distinct permease (Grenson *et al.*, 1970).

(2) The amount of P_i that certain yeasts absorb is enhanced (*a*) by preliminary cultivation in a medium deficient in P_i content, either in batch culture (Jeener & Brachet, 1944) or in a chemostat (Button *et al.*, 1973). (*b*) Alternatively, excess of P_i is provided during growth, after which the yeast is starved of it for several hours, in either the presence or the absence of glucose (Goodman & Rothstein, 1957). We found that preparations (50mg dry wt.) of strain no. 74, made by either procedure (*a*) or (*b*) above, would absorb at least 0.2 μmol of $H_2{}^{32}PO_4^-$ in 3min in the absence of glucose. Fig. 1 (traces *d* and *e*) shows the pH changes induced by the addition of P_i to such a preparation grown with limiting P_i in a chemostat. Yeast that was grown with excess of P_i and then starved for 16h, in the presence of 1% (w/v) glucose, behaved similarly except that the influx of protons with P_i occurred more slowly.

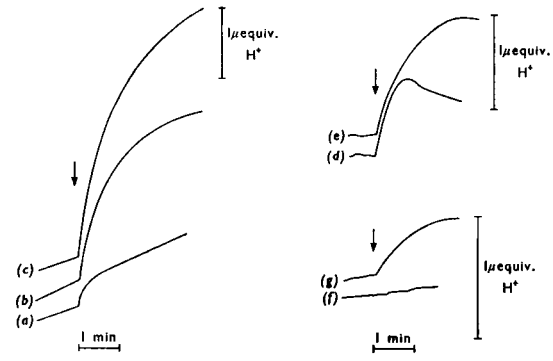

Fig. 1. *Proton absorption caused by glutamate, phosphate and glucose 6-phosphate*

For traces (*a*), (*b*) and (*c*), 0.1, 0.8 and 4 μmol of Tris glutamate, at pH4.8, was added at the indicated time to the washed cells of yeast strain G grown with 2.5mM-glutamate as principal source of nitrogen. The stirred yeast suspension (50mg dry wt.) at 30°C also contained 15 μg of antimycin, 2mM-deoxyglucose, 5mM-Tris adjusted to pH4.8 with citric acid, in a final volume of about 5ml. The pH was recorded continuously (see the Materials and Methods section). Traces (*d*) and (*e*) show the effects of adding 0.5 μmol of KH_2PO_4 to the washed cells of strain N.C.Y.C. no. 74 grown in a chemostat with P_i limiting. Deoxyglucose and antimycin were present for trace (*e*) and absent for trace (*d*). In traces (*f*) and (*g*) 0.1 μmol of glucose 6-phosphate was added to preparations of strain N.C.Y.C. no. 74 from the chemostat. The suspensions also contained 15 μg of antimycin and 2mM-deoxyglucose, with 0.5 μmol of sodium molybdate for trace (*f*).

Effect of anion dose and of addition-time on the induced rate of proton uptake

Amounts of KH_2PO_4 from 0.1 to 1.0 μmol caused roughly the same increase in the rate of proton uptake in the standard test conditions (Fig. 1). The K_m with respect to P_i was thus probably $<7\mu$M. The induced rate of uptake of protons increased systematically from about 10 to 40nequiv./min per mg of yeast, when the corresponding dilution rate used in the chemostat varied from 0.015 to 0.08 h^{-1}. A roughly proportionate increase in the rate of absorption of P_i occurred.

The above values refer to additions of P_i made about 1min after deoxyglucose and antimycin were

added to the yeast suspension. Delaying the addition of P_i resulted in a smaller response. A rate of 40nequiv./min per mg at 1min typically fell to 25nequiv./min per mg at 5min and to 5nequiv./min per mg at 10min (three experiments).

Somewhat similar observations were made with strain G and L-glutamate. When the dose was raised from 0.1 to 4.0μmol the induced proton uptake, assayed at about pH4.8, increased from roughly 22 to 67nequiv./min per mg. The reciprocal uptake rate was curvilinear with respect to [glutamate]$^{-1}$. Delaying the addition of 0.1μmol of glutamate progressively lowered the induced rate of uptake of protons that subsequently occurred, typically from 22nequiv./min per mg at 2min, through 13nequiv./min per mg at 5min, to 5nequiv./min per mg at 10min (three experiments).

Amounts of anion absorbed and apparent concentration effected

Preparations of strain no. 74 that were grown with NH_4^+ subsequently absorbed no more than about 25nmol of glycine/mg in the presence of both antimycin and deoxyglucose (Eddy *et al.*, 1970*b*). In the present work, the preparations of strain G grown with glutamate also absorbed no more than about 25nmol of glutamate/mg dry wt. in the presence of the two metabolic inhibitors. Similarly, the P_i-deficient preparations of strain no. 74 subsequently absorbed only a limited amount of P_i under these conditions. The actual limit varied with the growth history.

(1) When the dilution rate in the chemostat was increased in steps from 0.02 to 0.06h^{-1}, the amount of P_i subsequently absorbed in the standard test conditions also increased systematically from 10 to 16nmol/mg (10 assays).

(2) Preparations of the yeast that were grown with a sufficiency of P_i and then starved (see above) exhibited the even smaller capacity of about 5nmol of P_i/mg. Antimycin and deoxyglucose were omitted in other assays, so that energy metabolism could drive the proton pump. These yeast preparations absorbed at least twice as much P_i eventually as was taken up in the presence of the metabolic inhibitors. Further, some of the extra protons that entered the yeast in the presence of P_i were later ejected (Fig. 1, trace *d*). Similar behaviour occurred during the absorption of glycine, and indicates that recycling of protons across the plasmalemma probably accompanies the absorption of the solute during energy metabolism (Eddy & Nowacki, 1971).

Provided that the anion dose was within the above limits, a large proportion of the P_i or glutamate provided was absorbed into the yeast. This proportion averaged 92% in four assays in which 0.1μmol of glutamate was added to strain G in the presence of the metabolic inhibitors. It averaged 99.9% in nine assays in which 0.1μmol of P_i was added, in similar circumstances, to strain no. 74. When the ^{14}C accumulated in the yeast fed with 0.1μmol of [^{14}C]-glutamate was examined chromatographically in two solvent systems, by using the general procedures described by Eddy *et al.* (1970*b*), it behaved like the original amino acid. The maximum ratio achieved of the cellular/extracellular concentrations of glutamate was accordingly estimated to be about 70:1. The corresponding ratio for P_i was about 6×10^3,

Table 1. *Number of extra proton equivalents absorbed with different amounts of phosphate and* L-*glutamate*

Strain N.C.Y.C. no. 74 was cultured in a chemostat under conditions of phosphate deficiency. The washed yeast was suspended at pH4.8 with 15μg of antimycin and 2mM-deoxyglucose. About 1–2min later, or subsequently as specified, the indicated amount of $KH_2{}^{32}PO_4$ was added. The extra acid absorbed with the phosphate was assayed on *N* occasions (Fig. 1). The absorption of [U-^{14}C]glutamate was similarly studied in preparations of strain G grown with 2.5mM-glutamate.

	Addition (μmol)	Time (min)	*N*	Ratio: Extra H^+ absorbed / Phosphate or glutamate absorbed
Phosphate with yeast N.C.Y.C. no. 74	0.1	2	15	3.16±0.07
	0.2	2	22	3.03±0.04
		5	4	2.73±0.08
		7.5	5	2.31±0.29
		9.5	5	1.63±0.11
	0.6	2	6	2.27±0.12
	0.8	2	3	1.72±0.05
Glutamate with yeast G	0.12	2	19	2.91±0.10
		5	7	2.30±0.13
		10	4	1.23±0.08
	0.52	2	10	2.32±0.12
	2.0	2	9	1.96±0.05
	4.0	2	4	1.71±0.07

Table 2. *Uptake of protons and efflux of K^+ induced by phosphate and L-glutamate*

The procedures are outlined in Table 1 and Fig. 1. The substrate was added at 2 min. Mean values of the stoicheiometry ± S.E.M. are shown with number of assays in parentheses.

	Inducer (μmol)	Ratio $\frac{\text{Extra } H^+ \text{ absorbed}}{\text{Phosphate or glutamate absorbed}}$	Ratio $\frac{\text{Extra } K^+ \text{ excreted}}{\text{Phosphate or glutamate absorbed}}$
Phosphate with yeast N.C.Y.C. no. 74	0.2	3.03 ± 0.04 (22)	2.25 ± 0.20 (18)
	0.5	2.43 ± 0.10 (8)	2.22 ± 0.05 (8)
	1.0	1.76 ± 0.14 (7)	1.91 ± 0.20 (3)
Glutamate with strain G	0.2	2.74 ± 0.06 (8)	1.40 ± 0.25 (4)
	0.8	2.31 ± 0.09 (16)	1.76 ± 0.15 (4)

on the assumption that the P_i absorbed was dissolved as such in the cellular water.

Stoicheiometry

The number of extra proton equivalents absorbed with P_i and with glutamate were studied as a function of both the anion dose and the time at which this was added (Table 1). The early addition of 0.1 μmol of either anion displaced about 3 proton equiv. into the yeast. This is inconsistent with model (*a*) of Scheme 1 but consistent with a version of model (*b*) in which $n = 3$. Table 1 also shows that (1) larger amounts of anion were absorbed with relatively fewer protons; (2) delaying the addition of the anion had a similar consequence.

At pH 4.8 the predominant form of both glutamate and P_i would carry a single net negative charge (A^-). The proton stoicheiometry of 3 accordingly means that 2 equiv. of positive charge accompanied each equivalent of anion into the yeast. Table 2 shows that the charge balance during the absorption of 0.2 μmol of P_i was maintained by the efflux of 2 equiv. of K^+. These further observations are also consistent with model (*b*) (Scheme 1). However, the smaller proton stoicheiometry, observed when 0.5 and 1.0 μmol of P_i were used, was not accompanied by a corresponding fall in the ratio for K^+. It seemed relevant that A. Seaston, G. Carr & A. A. Eddy (unpublished work) showed that the absorption of glycine by starved preparations of yeast no. 74 appeared to involve two processes. A net uptake of protons occurred in one of these, whereas the other may have involved the extracellular glycine exchanging with intracellular amino acids. A similar notion is used in model (*d*) of Scheme 1 to account for the behaviour of the yeast preparations with the two larger doses of P_i (Table 2). It is postulated that a certain fraction of the P_i added exchanges with cellular P_i. During this process 2 equiv. each of protons and K^+ cross the plasmalemma.

Table 2 also shows the results of a preliminary investigation of the amounts of K^+ displaced during glutamate absorption. The observations are consistent with model (*b*) above but do not exclude other possibilities.

Participation of extracellular Na^+, Mg^{2+} or K^+

The above assays utilized the K^+ salt of P_i and a further quantity of K^+ leaked from the yeast, so that the extracellular concentration was at least about 0.2 mequiv./litre. The use of the Tris salt of P_i gave similar results. Likewise the presence of 50 mM-KCl, 50 mM-NaCl or 50 mM-$MgCl_2$ had no apparent effect on either the rate of uptake of protons with 0.2 μmol of KH_2PO_4, or the number of proton equivalents eventually absorbed. Similarly, when 2 mM-NaCl, 20 mM-NaCl, or 20 mM-KCl were present, the absorption of protons brought about by the addition of 0.2 μmol of Tris glutamate resembled that observed when the alkali cations were omitted. There is therefore no reason to think that model (*c*) of Scheme 1 is applicable.

Substrate analogues

In two series of assays, preparations of strain G that absorbed about 30 nequiv. of H^+/min per mg dry wt. of yeast in the presence of 1 μmol of L-glutamate responded similarly to L-aspartate, as well as to DL-2-aminoadipate. Acid absorption with 1 μmol of D-glutamate was about 70% slower than with L-glutamate.

Competition between arsenate and P_i for entry into yeast is well established (Jung & Rothstein, 1965; Button *et al.*, 1973). In keeping with that behaviour, we found in the present work with strain no. 74 that 0.1 or 0.2 μmol of KH_2AsO_4 initiated (1) roughly the same rate of proton uptake and (2) roughly the same total uptake of protons in 3 min (Table 3) as did the equivalent amounts of P_i. Table 3 shows that certain phosphate esters, namely pyrophosphate, glucose 1-phosphate, ATP and AMP, also caused a significant uptake of protons into the yeast. Glucose 6-phosphate behaved similarly (Fig. 1, trace *g*). In contrast with the

Table 3. *Extra acid absorbed after the addition of phosphate, pyrophosphate, arsenate and certain phosphate esters in the presence or absence of sodium molybdate*

Strain N.C.Y.C. no. 74 was grown in a chemostat with phosphate limitation. Portions (50mg dry wt.) of the washed cells were suspended at pH4.8 with 15μg of antimycin and 2mM-deoxyglucose. Next, 1μmol of sodium molybdate was added as required, followed by the substrate 1min later. The amount of acid displaced into the yeast during the next 3–4min is shown below (cf. Fig. 1).

Substrate	(μmol)	Sodium molybdate	Extra proton uptake (equiv.)
KH_2PO_4	0.1	−	0.31
	0.1	+	0.36
KH_2AsO_4	0.1	−	0.31
	0.2	−	0.61
	1.0	−	0.78
$Na_4P_2O_7$	1.0	−	0.70
	1.0	+	0.18
	1.0	+	0.09
followed by KH_2PO_4	0.1		0.26
Glucose 1-phosphate	0.1	−	0.26
	0.1	+	0
	1.0	−	0.77
	1.0	+	0.04
ATP	0.1	−	0.63
	0.1	+	0.03
AMP	0.1	−	0.40
	0.1	+	0.08

behaviour of P_i, the phosphate esters caused only a relatively small uptake of protons in the presence of 0.1mM-sodium molybdate. The effects of this compound were studied because it inhibits the acid phosphatases associated with the yeast cell surface (Rothstein & Meier, 1949). The proton uptake observed after the addition of these phosphate esters may therefore have been due to the absorption of P_i, released by the hydrolytic action of the extracellular acid phosphatases that are known to be carried by the yeast strain no. 74 (Tonino & Steyn-Parvé, 1963).

Discussion

It is instructive to compare the above observations on the proton stoicheiometry for phosphate and glutamate with the results of our earlier work with various neutral and basic amino acids. The so-called general amino acid permease functioned with 2 proton equiv./mol of neutral or basic amino acid (Eddy & Nowacki, 1971; Seaston *et al.*, 1973). On the other hand, both the proline permease and one of the two specific methionine permeases, variously defined by genetic analysis, each probably utilized 1 proton equiv. (Seaston *et al.*, 1973). The present work shows that phosphate and glutamate transport, each of which may involve more than one distinct carrier system with a characteristic affinity and rate of working (Tang & Howard, 1973; Borst-Pauwels & Jager, 1969), led to the absorption of a maximum of 3 proton equiv. Accordingly, in the presence of the metabolic inhibitors, a maximum of 2equiv. of positive charge were absorbed, apparently in exchange for K^+. However, during energy metabolism maintained by glucose, 1 equiv. of K^+ was absorbed with aspartate (Eddy *et al.*, 1970*a*). Extra K^+ was also absorbed with phosphate (Goodman & Rothstein, 1957). The 3equiv. of acid absorbed with the anions would be expected to be ejected through the proton pump when ATP was available (Scheme 1, model *e*). The tendency for this to happen with phosphate is demonstrated by the experiment illustrated in Fig. 1. Similar observations were made with glycine (Eddy & Nowacki, 1971).

The contrasting functions of K^+ in anion absorption, which are outlined in models (*b*) and (*e*) of Scheme 1, are consistent with other evidence (Goodman & Rothstein, 1957) that the absorption of K^+ and P_i are not necessarily linked. Morevoer, neither glycine nor aspartate caused a significant efflux of ^{42}K during energy metabolism (Eddy *et al.*, 1970*a*). As Scheme 1 shows, the efflux of K^+ caused by glutamate entering the preparations depleted of ATP (Table 2) appears to be a facultative process, as does the efflux of K^+ caused by glycine (Eddy & Nowacki, 1971).

It seems possible that the puzzling changes that occurred in the proton stoicheiometry when the anion dose and the addition time were separately varied (Tables 1 and 2) are part of a mechanism regulating the amount of anion absorbed. If (1) the value of *n* in model (*b*) of Scheme 1 varied with these experimental conditions, or (2) the proton pump was only partially inhibited (see Scheme 1, model *e*), the stoicheiometry for H^+ and that for K^+ would vary independently. This may apply to glutamate (Table 2). The assays with phosphate, however, are more consistent with a stoicheiometry of 2 for both H^+ and K^+ at the largest dose of P_i. Model (*d*) of Scheme 1 provides a tentative explanation in terms of an exchange reaction. Exchange of extracellular for intracellular phosphate is difficult to detect (Jung & Rothstein, 1965), but can occur (Button *et al.*, 1973). The possibility that endogenous glutamate or 2-oxoglutarate (Lewis & Rainbow, 1963) exchanges with some of the added glutamate, in the circumstances where the proton stoicheiometry fell, has not been ruled out.

Significance of the stoicheiometry

According to the co-substrate hypothesis, anion accumulation is driven by the spontaneous move-

ments of the associated co-substrate ions through the carrier complex. Thus the force driving the accumulation of the anion would vary both with the number of equivalents of the co-substrate ions participating and with their respective concentrations inside (suffix i) and outside (suffix o) the yeast cells. When concentrations are equated with activities, model (*a*) of Scheme 1 predicts that the ratio of the intracellular anion concentration ($[A^-]_i$) to the extracellular concentration ($[A^-]_o$) would not exceed $[H^+]_o/[H^+]_i$. In contrast, model (*b*) predicts that when $n=3$ the anion ratio would not exceed $([H^+]_o/[H^+]_i)^3\ e^{-2VF/RT}$, where V is the membrane potential and F, R and T have their usual significance. Because $[K^+]_i/[K^+]_o$ is at least 1×10^2, V would be at least about 120mV, if the distribution of K^+ was governed by the membrane potential (Scheme 1). Now $[H^+]_o/[H^+]_i$ is about 1×10^2. Hence $[A^-]_i/[A^-]_o$ would be less than 1×10^2 according to model (*a*) and less than 1×10^{10} according to model (*b*). These estimates are relevant to the work of Button *et al.* (1973), who found that a certain yeast of the genus *Rhodotorula* concentrated P_i about 1×10^5-fold at pH7 and about 1×10^6-fold at pH4. The ratio $[H_2PO_4^-]_i/[H_2PO_4^-]_o$ presumably reached roughly similar values. Thus if the same stoicheiometry of 3 proton equiv. applies to the *Rhodotorula* as to strain no. 74 of *Saccharomyces*, and if their proton gradients are of similar magnitude, the above observations imply that the efficiency of the coupling between the proton gradient and the anion movement was about 50–60%. We also conclude that model (*a*) is incompatible with the formation of such large phosphate gradients. The present work shows that the power input into the glutamate carrier is probably also relatively large. However, the maximum size of the glutamate gradient formed during energy metabolism is not known.

Recent work with *S. aureus* provides an interesting contrast with the yeast system. Gale & Llewellin (1972) showed that: (1) at pH5.5 about 0.9 proton equiv. was absorbed with glutamate and about 0.6 proton equiv. was absorbed with aspartate; (2) the amino acid accumulation ratio varied from about 10^3 to 4×10^3 during energy metabolism at pH6–8. Further, studies with ionophores led Niven & Hamilton (1974) to propose that, as in model (*a*) of Scheme 1, *S. aureus* absorbed glutamate simply in response to the trans-membrane pH gradient. However, it seems improbable that accumulation ratios of the above magnitude would be formed in that way in physiological circumstances. We suggest that at least a second equivalent of co-substrate ions may be involved, as in models (*b*) and (*c*), in which a positively charged carrier complex is formed. A similar situation may occur in the uptake of glutamate by crab nerve in which two Na^+ appear to be involved (Baker & Potashner, 1971). It also seems relevant both that Na^+ stimulates glutamate absorption in *E. coli* (Halpern *et al.*, 1973; Miner & Frank, 1974) and that Mg^{2+} is absorbed during citrate transport in *B. subtilis* (Willecke *et al.*, 1973). In each of these cases, the carrier might associate both with protons and with the inorganic cation, forming a positively charged complex with the primary substrate and thereby raising the energy input into the system.

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