Biochem. J. (1973) **134**, 1031–1043
Printed in Great Britain

The Absorption of Protons with Specific Amino Acids and Carbohydrates by Yeast

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(*Received* 19 *March* 1973)

1. Proton uptake in the presence of various amino acids was studied in washed yeast suspensions containing deoxyglucose and antimycin to inhibit energy metabolism. A series of mutant strains of *Saccharomyces cerevisiae* with defective amino acid permeases was used. The fast absorption of glycine, L-citrulline and L-methionine through the general amino acid permease was associated with the uptake of about 2 extra equivalents of protons per mol of amino acid absorbed, whereas the slower absorption of L-methionine, L-proline and, possibly, L-arginine through their specific permeases was associated with about 1 proton equivalent. L-Canavanine and L-lysine were also absorbed with 1–2 equivalents of protons. 2. A strain of *Saccharomyces carlsbergensis* behaved similarly with these amino acids. 3. Preparations of the latter yeast grown with maltose subsequently absorbed it with 2–3 equivalents of protons. The accelerated rate of proton uptake increased up to a maximum value with the maltose concentration (K_m = 1.6mM). The uptake of protons was also faster in the presence of α-methylglucoside and sucrose, but not in the presence of glucose, galactose or 2-deoxyglucose. All of these compounds except the last could cause acid formation. The uptake of protons induced by maltose, α-methylglucoside and sucrose was not observed when the yeast was grown with glucose, although acid was then formed both from sucrose and glucose. 4. A strain of *Saccharomyces fragilis* that both fermented and formed acid from lactose absorbed extra protons in the presence of lactose. 5. The observations show that protons were co-substrates in the systems transporting the amino acids and certain of the carbohydrates.

According to a current hypothesis the ability of various mammalian and microbial systems to concentrate certain amino acids and carbohydrates depends on the flow of the solute into the cellular phase being coupled to the flow of specific cations, such as Na^+, K^+ or H^+ ions, across the plasmalemma (Riggs *et al.*, 1958; Mitchell, 1967; Schultz & Curran, 1970; Harold, 1972). A stoicheiometric number of the appropriate cations would be absorbed as co-substrates with the amino acid or carbohydrate, the balance of charges being maintained either (1) by an induced efflux of other cations, possibly through an energy-dependent reaction, or (2) by a stimulated uptake of anions (Eddy, 1968; Eddy & Nowacki, 1971; Gibb & Eddy, 1972). Similar movements of ions might also be expected to occur in circumstances where energy metabolism was restricted so as to prevent the cellular ionic pumps from redistributing the displaced anions and cations across the plasmalemma. The absorption of Na^+ with glycine that was observed in preparations of mouse ascites-tumour cells (Eddy, 1968), as contrasted with the absence of a connexion between the uptake of L-arabinose and the uptake of Na^+ ions (Eddy, 1969), both conform to that pattern. So does the stimulated flow of Na^+ during the absorption of certain carbohydrates and amino acids both by intestinal preparations and by pigeon erythrocytes (Schultz & Curran, 1970).

The limited evidence available suggests that, at least in some micro-organisms, H^+ may serve a role like the one that Na^+ plays in the mammalian systems. Thus an accelerated uptake of H^+ occurs during the uptake of lactose by *Escherichia coli* (West, 1970; West & Mitchell, 1972), of glutamate and aspartate by *Staphylococcus aureus* (Gale & Llewellin, 1972) and during the absorption of each of four amino acids by a strain of the yeast *Saccharomyces carlsbergensis* (Eddy & Nowacki, 1971).

The study of proton movements in relation to solute transfer in various species of *Saccharomyces* has been extended in two directions in the present work. First, the proton displacements produced by maltose and α-methylglucoside, two compounds that

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certain yeast strains appear to concentrate (Harris & Thompson, 1961; Okada & Halvorson, 1964), were compared with the proton displacements produced by glucose and galactose, two compounds that appear not to be concentrated as such by these yeast strains (Kuo & Cirillo, 1970). The behaviour with lactose and sucrose was likewise examined. Secondly, certain of the genetically distinct systems transporting amino acids in the strains of *Saccharomyces cerevisiae* described by Grenson & Hennaut (1971) were each shown to function with protons as co-substrates. A preliminary account of this work was given by Seaston *et al.* (1972).

Materials and Methods

The yeasts were grown and manipulated as described by Eddy *et al.* (1970*a*). The stock cultures were maintained on nutrient agar slopes and transferred to a similar solution (10ml) from which the agar was omitted. A portion (1ml) of one of these cultures was mixed with one of the following solutions (500ml): (1) a mineral salts–nutrient medium containing 2% (w/v) glucose and 2% (w/v) $(NH_4)_2SO_4$ (Eddy *et al.*, 1970*a*); (2) a similar solution in which 3mM-L-proline replaced $(NH_4)_2SO_4$ as the principal source of N; (3) a mixture comprising yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.; 3g), malt extract (Difco; 3g), mycological peptone (Oxo Ltd., London S.E.1, U.K.; 5g), glucose (10g) and water to 1 litre; (4) a similar complex nutrient solution in which either maltose or lactose replaced glucose; (5) a similar nutrient solution from which the malt extract was omitted, glucose being the principal source of C for growth. The various cultures were agitated at 25°C for 18–22h. The yeast was harvested, washed with water and stored at 0°C for up to 3h. The yield of yeast per litre of solution was typically (*i*) 0.6g dry wt. when the nutrient lactose solutions were used; (*ii*) 2.2g when the strain 74 was grown with the complex nutrients based on (3) above; (*iii*) 0.3g when the solutions based on the mineral salts were used.

Organisms

Strain N.C.Y.C. no. 74 of *Saccharomyces carlsbergensis* and strain N.C.Y.C. no. 179 of *Saccharomyces fragilis* (*Kluyveromyces fragilis*) were obtained from the British National Collection of Yeast Cultures (Nutfield, Surrey, U.K.). Haploid cultures of strains A, B and C of *S. cerevisiae*, belonging to a series described by Grenson *et al.* (1970), were kindly supplied by Dr. M. Grenson with the following descriptions. The strain referred to in the present work as A was designated 'MG 276'. It carries the general amino acid permease and lacks the specific methionine permease associated with the gene *met-p*$_1$. Strain B was designated '2512 C'. Whereas it lacks a functional general amino acid permease, owing to the presence of the gene *gap*-1, it carries both an arginine permease and a proline permease. Strain C (*gap*-1; *met-p*$_1$) was designated '5156 d'. It lacked the general permease and the above methionine permease, and exhibited the arginine permease and the proline permease. Strains D, E and F were prepared from strain B in these laboratories by P. Earnshaw. As described by Grenson *et al.* (1966), about 10^8 cells were plated on a mineral salts–glucose–nutrient agar solution (10ml) containing $(NH_4)_2SO_4$ as the principal source of N for growth, together with 100μg of L-canavanine. About 25 yeast colonies about 4mm in diameter developed after 4 days at 25°C. Three of these substrains, chosen at random, were separately streaked on a similar growth medium and kept for 3 days at 25°C. A single colony was selected in each instance and the cells streaked again. This process was repeated altogether three times in succession. The derived strains D, E and F appear to lack the arginine permease, presumably because they carry the gene *arg-p*$_1$ (Grenson *et al.*, 1966).

Proton stoicheiometry

The proton uptake by the yeast in the presence of the carbohydrates was assayed without further treatment of the cells. The preparations that were assayed in the presence of the amino acids, however, were first put at 30°C with 5% (w/v) glucose for 20min in 20mM-Tris solution adjusted to pH4.5 with citric acid. The yeast (50mg dry wt.) was then washed with water (3 × 10ml) and used immediately for the assays.

A portion (0.5ml) of the yeast suspension (25mg or 50mg dry wt.) was mixed with an equal volume of 5mM-Tris, pH4.5. At zero time the suspension was mixed with a similar buffer solution (3 or 4ml) at 30°C in the stirred vessel of the apparatus described by Eddy & Nowacki (1971). In certain instances 10mM-iodoacetamide was added to the system at that stage. The pH of the suspension was recorded continuously. Next 25μg of antimycin and 2mM-2-deoxyglucose were quickly added. The selected amino acid (0.25–1.5μmol) or carbohydrate (1–20μmol) was usually added at 2min. (1) The uptake of L-proline, glycine and L-arginine was in various instances assayed with ^{14}C (about 1Ci/mol). (2) In other assays 2mM-[^{14}C]maltose (about 0.02Ci/mol) was employed. For (1) above the whole of the yeast suspension was poured into a centrifuge tube (15ml) at 0°C, immediately after the addition of 0.1ml of 5mM-HCl to calibrate the buffering capacity of the system. The yeast was then separated by centrifugation near 0°C for about 0.5min at 1000*g* (Eddy & Nowacki, 1971). Portions (0.1ml) of the supernatant solution were assayed for ^{14}C (Eddy *et al.*, 1970*a*). For (2) above the uptake of maltose was assayed by withdrawing portions (1ml) of the yeast suspension in a syringe and mixing them each with water (3ml) at

0°C. The yeast was separated in about 0.5min by centrifugation and suspended in the presence of 1mM-iodoacetamide (4ml) at 0°C. The cells were washed once more in a similar fashion. They were then put at 100°C with water (2ml) for 10min. The extract was assayed for ^{14}C in a scintillation spectrometer. The extracellular phase was similarly assayed. The scintillation fluid contained toluene (0.7 litre), Triton X-100 (0.3 litre), 2,5-diphenyloxazole (3g) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (100mg). Sample quenching was assessed by the channels-ratio method.

Computations. Because the buffering capacity of the yeast suspensions during pH changes of up to about 0.15 unit was roughly constant, the change in pH after the addition of the standard HCl solution served to calibrate the system in terms of the net amount of acid either produced or absorbed. The extra amount of acid absorbed with the amino acid or carbohydrate was then computed (Eddy & Nowacki, 1971) by subtracting from the total amount of acid absorbed in that interval, the amount that would have been absorbed if no solute had been added. The latter rate was assumed to be represented either by (1) the rate of proton uptake before the amino acid was added, or (2) the rate immediately after the phase of accelerated acid absorption. The two values for the extra amount of acid absorbed with the solute were nearly equal in the examples illustrated in Fig. 3 (*d* and *e*). In general their mean was determined and the results of a series of such assays averaged (Table 1). In other instances the amount of solute absorbed was determined from the distribution of ^{14}C before the solution phase was depleted of the amino acid or the carbohydrate. The relevant endogenous rate of acid absorption was then assumed to be the value exhibited just before the labelled compound was added.

Chemicals

Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were both from Packard Instruments Ltd., Wembley, Middx., U.K. The radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. They comprised [1-^{14}C]glycine and L-arginine, maltose and L-proline, each uniformly labelled with ^{14}C. Triton X-100, antimycin and 2-deoxyglucose were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; L-canavanine sulphate from Mann Research Laboratories Inc., New York 6, N.Y., U.S.A. Maltose, α-methyl-D-glucoside and lactose (bacteriologically tested) were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Results

Amino acid absorption

Cultures grown with NH_4^+. When the yeast N.C.Y.C. no. 74 (strain 74) was cultured in the mineral salts medium containing NH_4^+ as the main N source, the cells produced absorbed H^+ with various amino acids (Eddy & Nowacki, 1971). Similar preparations of the yeast strains A, B and C, however, absorbed various amino acids relatively slowly (Grenson *et al.*, 1970). We found that the addition of these compounds then had no significant effect on the pH traces observed by using the standard procedure described in the Materials and Methods section. Strain A was accordingly starved for 2h in the presence of 1% (w/v) glucose at pH4.5, a treatment that accelerated the uptake of glycine by the strain 74 (Eddy *et al.*, 1970*a*). Strain A apparently responded similarly, because the initial rate of uptake of glycine from a 0.2mM solution increased severalfold to about 13nmol/min per mg dry wt. of yeast. Definite displacements of protons then occurred in the presence of 5nmol of a given amino acid/mg dry wt. of yeast. This was shown with glycine, L-proline, L-citrulline and L-methionine, which were separately added to two preparations of the starved cells. The number of extra protons (ΔH) absorbed increased with the amount of glycine (ΔGly) absorbed (Fig. 1). A linear regression analysis of ΔH on ΔGly gave a mean value of 0.74±0.10 (S.E.M.) (14) for the stoicheiometry with a correlation coefficient of 0.899 ($P<0.001$).

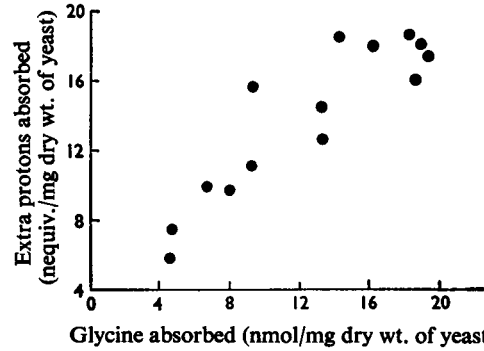

Fig. 1. *Number of extra protons absorbed in the presence of antimycin and 2mM-2-deoxyglucose as a function of the amount of glycine absorbed by preparations of strain A grown with NH_4^+ and then starved*

As explained in the Materials and Methods section, the yeast (50mg) was put at 30°C with 0.25, 0.5 or 1μmol of glycine labelled with ^{14}C. The suspension was stirred and the pH recorded continuously. After 0.5 to 4min, as appropriate, 1μequivalent of HCl was added to calibrate the system. The suspension was quickly cooled to 0°C and the yeast separated by centrifugation. The amount of ^{14}C absorbed was estimated from the amount remaining in the supernatant solution. The assays (14) were made with three preparations of the yeast.

Table 1. *Effect of genetic constitution on both the stimulated rate of proton uptake (v_H) in the presence of 0.25 μmol of one of various amino acids and the number (S) of extra protons absorbed per equivalent of amino acid*

The yeast was in each instance grown with proline as the principal N source. The washed cells (50 mg dry wt.) were put at pH 4.5 with 2 mM-2-deoxyglucose and 25 μg of antimycin in the stirred vessel of the pH meter at 30°C. Then 2 min later 0.25 μmol of the selected amino acid was added. A second addition was usually made within a further 2–3 min (see Figs. 3*d* and 3*e*). The buffering capacity of the system was calibrated by the addition of 1 μmol of HCl. The assay is described in further detail in the Materials and Methods section. The stimulated rate of proton uptake (v_H nequiv. of H^+/min per mg dry wt. of yeast) was the mean increase (±S.E.M.) in the rate after the addition of the amino acid, averaged over the indicated number of assays. The stoicheiometry (S) was similarly computed by dividing the total number of equivalents of extra protons absorbed by the number of equivalents of amino acid absorbed. Except when ^{14}C-labelled amino acid was used, the amount of amino acid absorbed was assumed to be 0.25 μmol (see the Materials and Methods section). A dash signifies that the system was not examined.

		Strain A	Strain B	Strain C	Strain D	Strain E	Strain F	Strain 74
L-Arginine	v_H	13.6 ±2.5 (5)	4.1 ±0.8 (6)	6.2 ±0.7 (4)	−0.5 ±0.1 (9)	−1.23±0.42 (3)	−1.5 ±0.6 (4)	—
	S	1.5 ±0.03 (5)	1.35±0.21 (6)	1.50±0.20 (4)	<0 (9)	<0 (3)	<0 (4)	—
L-Citrulline	v_H	9.4 ±2.1 (7)	<0.5 (3)	<0.5 (4)	—	—	—	12.8 ±0.8 (6)
	S	1.90±0.13 (7)	<0.1 (3)	<0.1 (4)	—	—	—	2.21±0.04 (6)
L-Canavanine	v_H	—	6.4 ±1.1 (4)	6.5 ±1.7 (4)	−0.7 ±0.2 (3)	—	−0.7 ±0.3 (3)	—
	S	—	1.36±0.06 (4)	1.35±0.17 (4)	<0 (3)	—	<0 (3)	—
Glycine	v_H	16.2 ±1.6 (11)	1.21±0.47 (9)	1.30±0.71 (7)	—	—	—	17.8 ±1.3 (16)
	S	1.90±0.06 (11)	0.37±0.13 (9)	0.28±0.11 (7)	—	—	—	2.03±0.07 (16)
L-Lysine	v_H	6.8 ±1.2 (8)	3.3 ±0.2 (3)	1.2 ±0.4 (4)	0.0 ±0.2 (5)	—	—	—
	S	2.31±0.26 (8)	0.80±0.14 (3)	<0.7 (4)	<0.1 (5)	—	—	—
L-Methionine	v_H	12.4 ±2.3 (10)	4.87±0.97 (11)	0.02±0.25 (12)	2.3 ±1.3 (3)	2.4 ±0.2 (3)	3.5 ±0.2 (4)	18.6 ±2.3 (4)
	S	1.72±0.11 (10)	1.03±0.18 (11)	<0.1 (12)	<1	0.75±0.0 (3)	1.05±0.13 (4)	2.10±0.04 (4)
L-Proline	v_H	2.5 ±0.2 (7)	4.1 ±0.6 (6)	3.6 ±0.2 (9)	1.3 ±0.3 (4)	4.0 ±1.5 (3)	−1.2 ±0.3 (3)	3.6 ±0.3 (33)
	S	1.30±0.21 (7)	1.16±0.09 (6) 0.90±0.04 (19)*	1.23±0.14 (9)	0.73±0.07 (4)	1.35±0.51 (3)	<0	1.23±0.07 (33)*

* Amino acid absorption assayed with ^{14}C.

There was another indication that the absorption and not the mere presence of the amino acids caused the proton displacements. In contrast with strain A, starved preparations of strain B neither absorbed [^{14}C]glycine and [^{14}C]arginine rapidly, nor exhibited proton displacements in the presence of L-arginine or any one of the above amino acids.

Cultures grown with proline

Strain 74. Table 1 shows that roughly 2 equivalents of H^+ were absorbed with a dose of 5 nmol of glycine, L-methionine or L-citrulline per mg dry wt. of yeast, when the amino acid was added as an approximately 60 μM solution (Figs. 3*d* and *e*). Fig. 2 shows that roughly 2 equivalents of K^+ left the cells during the absorption of glycine, as we had earlier found using cultures grown with NH_4^+ (Eddy & Nowacki, 1971). A stoicheiometry near 1 was observed with respect to the slower process of proline absorption from solutions containing up to 0.3 mM-proline (Table 1, Fig. 4). The correlation between the number of extra protons absorbed (ΔH) and the uptake of proline (ΔPro) was highly significant ($r = 0.654$, $P < 0.001$). A linear regression analysis of ΔH on ΔPro gave a mean value $\pm$S.E.M. of 0.86 ± 0.18 for the stoicheiometry. This is not significantly different from the value of 1.23 ± 0.06 shown in Table 1.

Strain A. This strain appeared to carry the so-called 'general amino acid permease' but it lacked the specific permease for L-methionine that exhibits a K_m with respect to methionine of about 12 μM. It rapidly absorbed various amino acids, superficially resembling strain 74 in its ability also to absorb protons rapidly in the presence of various amino acids. The effect of adding successively 0.25 μmol of each of glycine, citrulline, methionine and proline is illustrated in Fig. 3(*b*). Between about 1 and 2 extra equivalents of protons were absorbed in a series of such assays in which not more than two additions of the amino acids were made (Table 1).

Strain B. This strain lacked the general amino acid permease, but carried a number of specific amino acid permeases, including the one for L-methionine that strain A lacks. L-Citrulline is believed to be transported mainly through the general amino acid permease, so that yeast strains lacking that function grow very slowly when L-citrulline is the sole source of N (Grenson *et al.*, 1970). The observations shown in Fig. 3 are consistent with that interpretation if protons accompany the amino acids into the yeast cells. Whereas strain A produced a proton pulse with citrulline (Fig. 3*b*), strain B failed to do so but responded to the presence of three other amino acids (Fig. 3*c*). As the examples given in Fig. 3 (*d* and *e*) show, the addition of a second dose of a given amino acid caused a further uptake of protons, as though the first dose had by then been absorbed. The relative

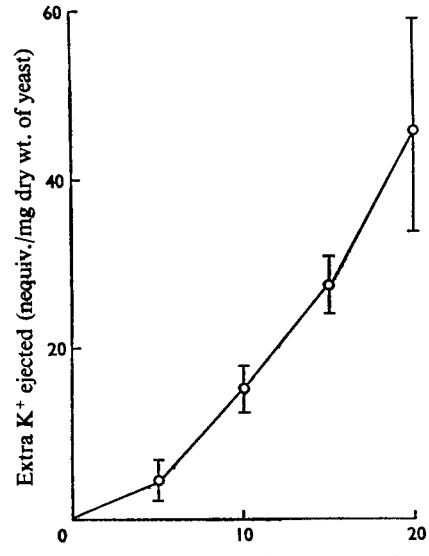

Fig. 2. *Number of extra K^+ ions leaving the yeast cells during the absorption of measured amounts of glycine by preparations of the strain* 74 *grown with proline*

The general procedure used is described in the Materials and Methods section. The amount of glycine absorbed in 4 min was varied by altering the amount of glycine added to the system in the range from 0.25 μmol to 1.0 μmol. The observations made with six preparations of yeast were combined, the mean displacement of K^+ ions $\pm$S.E.M. in four assays being illustrated as a function of the amount of glycine absorbed.

number of extra protons absorbed in a series of such assays with six amino acids are shown in Table 1, which also includes the mean values of the stimulated rate of proton uptake. The rapid absorption of protons in the presence of various amino acids seems to be a characteristic of the general amino acid permease. Presumably strain 74 carries that system. Fig. 4 illustrates the correlation that was found between the number of extra protons absorbed (ΔH) and the uptake of proline (ΔPro) by strain B in the presence of concentrations of proline in the range up to 0.3 mM. The similar observations made with strain 74 were described above. In the former case r was 0.899 for 19 pairs of observations, so that P was < 0.001. The linear regression analysis gave a mean value $\pm$S.E.M. of 0.71 ± 0.08, for the stoicheiometry, in rough agreement both with the value of 0.90 ± 0.04 given in Table 1 and with the notion that proline was absorbed with 1 equiv. of protons.

Strain C. This lacked both the general permease

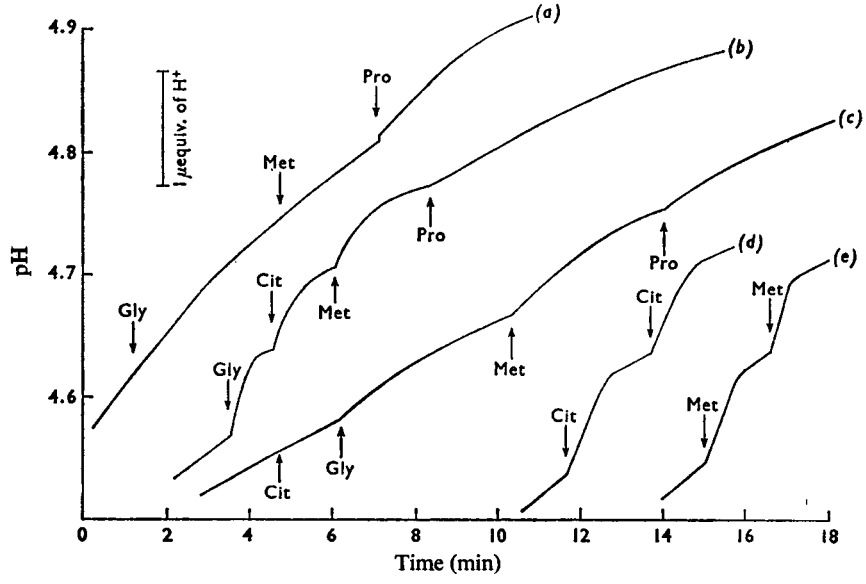

Fig. 3. *Characteristic changes in pH with time that occurred with various yeast strains in the presence of glycine,* L-*methionine,* L-*citrulline and* L-*proline*

The selected amino acid (0.25 μmol) was added at the indicated time to the stirred suspension of yeast (50 mg dry wt.) containing 2 mM-deoxyglucose and 25 μg of antimycin. Trace (*a*): glycine, methionine and proline with strain C, which lacks the general amino acid permease and one of the methionine permeases. Trace (*b*): glycine, citrulline, methionine and proline with strain A, which carries the general amino acid permease. Trace (*c*): citrulline, glycine, methionine and proline with strain B, which lacks the general permease and carries the methionine permease that strain C lacks. Trace (*d*): two additions of citrulline to strain 74. Trace (*e*): two additions of methionine to strain 74. The yeast was in each case grown with proline as the main N source.

and the specific methionine permease referred to above. The characteristic that stood out in the pH assays was the absence of an accelerated influx of protons when a 62 μM solution of L-methionine (Fig. 3*a*) was added to the system in circumstances where the responses to proline, arginine, lysine, canavanine and glycine still occurred (Table 1). During 5 min such preparations nevertheless absorbed 80–90% of the 0.25 μmol of [^{14}C]methionine that was added. The presence of a second methionine permease in this strain has been inferred (Gits & Grenson, 1967).

Canavanine-resistant strains D, E and F. These were derived from strain B by a procedure that principally selected mutants lacking the arginine permease (Grenson *et al.*, 1966). Consequently, whereas strain B absorbed in 2 min 90% of the arginine (0.25 μmol) added in the standard assay, the three derived strains absorbed 10% or less. The importance of protons in the functioning of the arginine permease may be deduced from the fact that, unlike strains B and C, strains D, E and F failed to absorb additional protons in the presence of either L-arginine (Fig. 5), or, where this compound was tested, its analogue canavanine (Table 1). There was indeed some indication that the presence of the basic amino acids retarded the uptake of protons in the latter instance. Strains D, E and F responded to L-methionine, on the other hand, like their parent strain (Fig. 5, Table 1). A similar conclusion applied to L-proline except in the case of strain F. The indicated impairment of the response to L-lysine in strain D (Table 1) may be related to the circumstance that both the arginine and lysine permeases absorb lysine (Grenson *et al.*, 1966).

Dependence on pH and [K^+]. The presence of H^+ stimulated and the presence of K^+ inhibited the uptake of various amino acids by preparations of yeast strain 74 depleted of ATP (Eddy *et al.*, 1970*b*). That strain B exhibited similar phenomena with arginine and proline was indicated, in a preliminary fashion, by the following observations made under conditions like those in which the pH changes were recorded. At pH 4.5 about 95% of the amino acid was absorbed from a 50 μM solution of proline in 2.5 min. The average rate of uptake during the interval 0.5–2.5 min was 1.3 nmol/min per mg of dried yeast, whereas the rate fell in the range 0.1–0.4 nmol/min per mg at pH 7.4 in a solution containing 100 mequiv. of K^+/l. About 40% of the amino acid was absorbed in 6 min

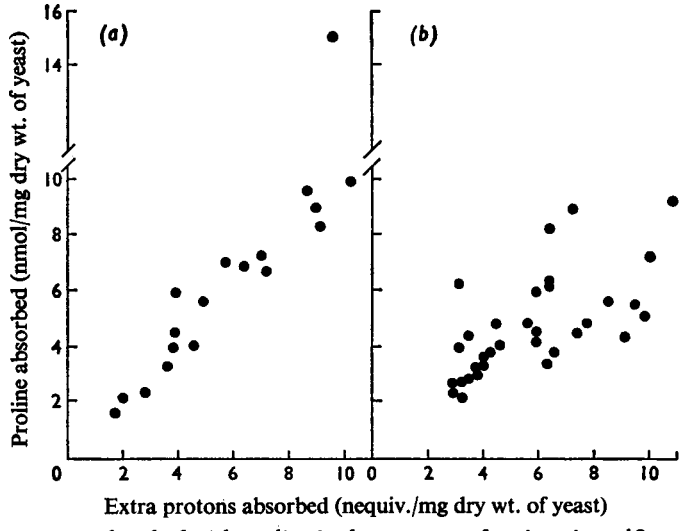

Fig. 4. *Number of extra protons absorbed with proline in the presence of antimycin and 2 mM-2-deoxyglucose by preparations of strain B and strain 74 grown with proline*

The general procedure used is described in the legend to Fig. 1 and in the Materials and Methods section. The amount of proline added to the yeast was varied between 0.2 and 1.25 μmol. The system was sampled in each case 2 min after the addition of the ^{14}C-labelled amino acid. (*a*), 19 assays made with the six preparations of strain B; (*b*), 33 assays made with ten preparations of strain 74.

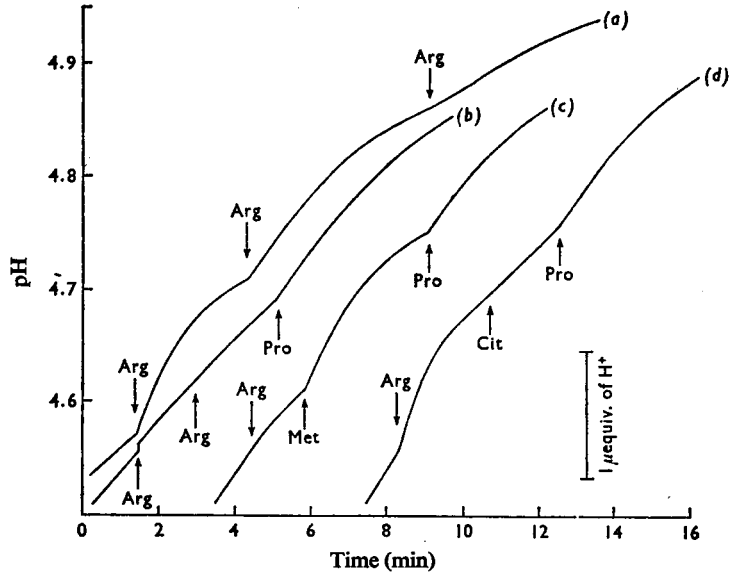

Fig. 5. *Characteristic changes in pH with time that occurred with various yeast strains in the presence of* L-*arginine,* L-*citrulline,* L-*methionine and* L-*proline*

The selected amino acid (0.25 μmol) was added at the indicated time to the stirred suspension of yeast (50 mg dry wt.) containing 2 mM-deoxyglucose and 25 μg of antimycin. Trace (*a*): successive additions of arginine to the preparations of strain B, which carried the arginine permease but not the general amino acid permease. Trace (*b*): arginine and proline with the canavanine-resistant strain D. Trace (*c*): arginine, methionine and proline with the canavanine-resistant strain E. Trace (*d*): arginine, citrulline and proline with strain C, which lacks the general amino acid permease. The yeast was in each case grown with proline as the main N source.

under the latter conditions. Similar observations were made with L-arginine. The absorption of L-methionine from a 60 μM solution both by strain B and strain C was also slower at the higher pH value. At pH 7.4, in the presence of 100 mequiv. of K^+/l, strain B absorbed about 80% of the methionine in 6 min and strain C absorbed about 40% in the same interval.

'Sodium yeast'. Preparations of strain 74 containing Na^+ ions instead of K^+ ions as the principal cation absorbed about 1 equiv. of H^+ with various amino acids (Eddy & Nowacki, 1971) such as glycine and L-methionine. Both the absorption of the amino acid and the stimulated flow of H^+ stopped in the presence of deoxyglucose and antimycin. Neither strain A nor strain B reproduced this phenomenon either with glycine or with L-methionine. The preparations of strain A containing large amounts of Na^+ ions neither (1) accumulated significant amounts of [^{14}C]glycine from a 100 μM solution, nor (2) absorbed significant net amounts of K^+ ions from a 1 mM solution of KCl at pH 4.5, unless 50 mM-glucose was present. Similar preparations of strain 74 absorbed both glycine and K^+ ions in the absence of glucose. The two yeasts thus differed in their ability to use endogenous energy metabolism to transport cations.

Absorption of specific carbohydrates by yeast strain 74

The yeast strain 74 produced acid in the presence of glucose (Eddy & Nowacki, 1971). This happened within 0.5–1.0 min of the addition of 5 μmol of the compound, which was apparently metabolized within a further 3–5 min (Fig. 6*b*). Yeast preparations adapted to utilize galactose behaved similarly with galactose (Fig. 6*d*). No acid was produced from galactose by the preparations grown with glucose (Fig. 6*e*). Other experiments (not shown) indicated that these were unable to ferment galactose.

Fig. 6(*c*) illustrates the very different behaviour exhibited when maltose was added to the preparations grown with maltose. This compound caused extra protons to be absorbed in the 0.5–1.0 min that elapsed before the pH fell. Sucrose and α-methylglucoside both behaved like maltose rather than glucose with the yeast suspensions that were grown with maltose (Fig. 6*a*). No proton uptake appeared to be

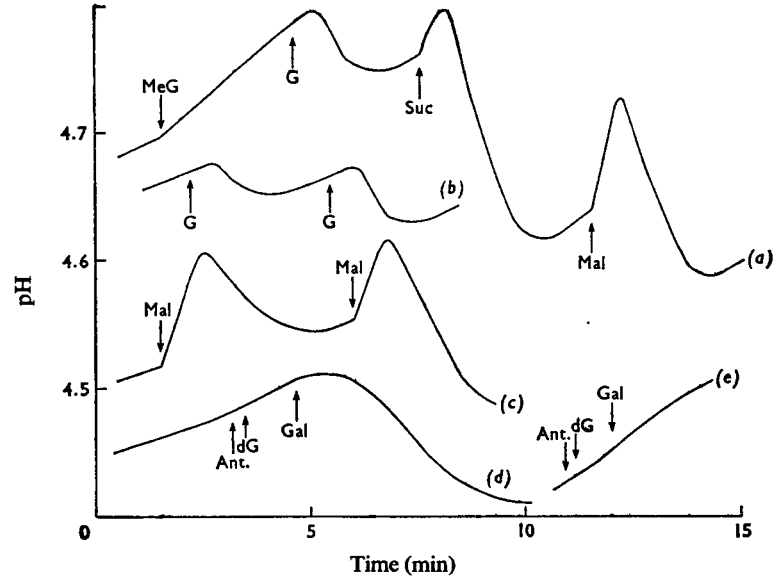

Fig. 6. *Contrasting effects of maltose, α-methylglucoside and sucrose as compared with glucose, 2-deoxyglucose and galactose on the pH changes observed with preparations of strain* 74

Trace (*a*): the yeast was grown with maltose as the principal C source. The cells (50 mg dry wt.) were put at 30°C with 2 mM-2-deoxyglucose and 25 μg of antimycin. α-Methylglucose (MeG), glucose (G), sucrose (Suc) and maltose (Mal) were added at the indicated times, 4 μmol in each case. Trace (*b*): the yeast was grown with maltose, put with deoxyglucose and antimycin and two additions of 10 μmol of glucose were made. Trace (*c*): the yeast was grown with maltose, put with deoxyglucose and antimycin and two additions of 4 μmol of maltose were made. Trace (*d*): the yeast was grown with galactose as the principal carbon source. Then 25 μg of antimycin (Ant.), 7 μmol of 2-deoxyglucose (dG) and 10 μmol of galactose (Gal) were added as shown. Trace (*e*): the yeast was grown with glucose and tested as for (*d*) above. The various traces have been moved relative to the time and pH scales. The initial pH of the suspension (4 ml) was near 4.5 in each case.

induced by 2-deoxyglucose in the experiment shown in Fig. 6(*d*) and in other similar experiments.

Effect of iodoacetamide. The metabolic inhibitors 2-deoxyglucose and antimycin together failed to inhibit acid formation either from galactose (Fig. 6*d*), maltose (Fig. 6*a*) or, in other work, from glucose. A 10mM solution of iodoacetamide virtually stopped the formation of acid from maltose (Fig. 7). The uptake of protons induced by maltose was then revealed as a separate process continuing after the time by which the pH would otherwise have started to fall. It seems reasonable therefore to assume that, after the first minute of exposure to maltose in the absence of iodoacetamide, protons were simultaneously both being absorbed with the maltose and ejected from the yeast. We made a similar inference about the absorption of glycine with H^+ (Eddy & Nowacki, 1971).

The abrupt alteration in the rate of change of pH that occurred about 1 min after the addition of maltose (Fig. 7*d*) was observed on other occasions and may mean that some acid formed even in the presence of iodoacetamide.

Adaptive phenomena. Strain 74 was grown with glucose instead of maltose as the main C source, malt extract being omitted from the growth medium. Manometric assays showed that such preparations failed to ferment either maltose or α-methylglucose at a significant rate. The influx of protons was not accelerated nor did acid accumulate after the addition of either 2mM-maltose or 4mM-α-methylglucoside to two preparations of the yeast grown in that way and tested under the conditions used for the experiments described in Fig. 6. Similar observations made with 2mM-sucrose indicated that, whereas no accelerated uptake of protons occurred, acid nevertheless formed after a delay of 0.5–1 min. The presence of 10mM-iodoacetamide stopped the pH falling. Such behaviour is consistent with a mechanism (Fuente & Sols, 1962) in which the sucrose was hydrolysed by invertase present in the yeast cell walls, the glucose and fructose produced then being absorbed by the yeast.

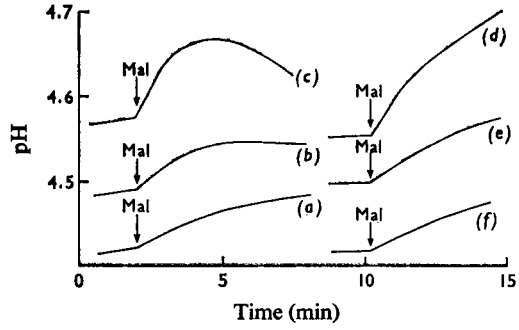

Fig. 7. *Effect of* 10mM-*iodoacetamide on acid absorption and acid production in the presence of different amounts of maltose*

Strain 74 was grown with maltose and the cells (25mg dry wt.) put at 30°C with 25μg of antimycin and 2mM-2-deoxyglucose, in the presence or absence of 10mM-iodoacetamide. Maltose (Mal) was added 2 min later as indicated. Traces (*a*), (*b*) and (*c*): no iodoacetamide, additions of 1, 2 and 16μmol of maltose respectively. Traces (*d*), (*e*) and (*f*): iodoacetamide present, additions of 16, 2 and 1μmol of maltose respectively. The traces have been moved relative to the pH and time scales for the purpose of display. The volume of the cell suspensions was 4ml in each case.

Quantitative aspects

Effect of maltose concentration. Fig. 8 shows how the initial rate of uptake of protons stimulated by maltose varied with its concentration. The observations correspond to a K_m value of 1.8mM and a $V_{max.}$ of 45 nequiv. of H^+/min per mg of dried yeast at 30°C. A second series gave 1.4mM and 36 nequiv./min per mg. Iodoacetamide lowered the rate somewhat erratically (Fig. 8).

The rate of fermentation of maltose at 30°C, in the presence of antimycin to inhibit respiration, also varied with the maltose concentration in a similar range. It corresponded to a K_m of 4.3mM and $V_{max.}$ of 250nmol of CO_2/min per mg of dried yeast in one series of assays. Values of 4.7mM and 250nmol/min per mg were observed in a second series. The latter rate is equivalent presumably to the absorption of at least about 62nmol of maltose/min per mg of dried yeast.

Effect of methylglucose concentration. The effect of α-methylglucoside concentration on the rate of proton uptake by the preparations grown with maltose corresponded to a K_m value of 1.85 ± 0.21 (3)mM and a $V_{max.}$ of 15.3 ± 4.4nmol of H^+/min per mg of dried yeast. The corresponding parameters for the fermentation of α-methylglucoside at 30°C were 13.7 ± 4.4(3)mM and 55.3 ± 8.9 (3)nmol of CO_2/min per mg of dried yeast, equivalent to about 27nmol of methylglucoside/min per mg.

Proton stoicheiometry. Fig. 9 shows that between 2 and 3 equiv. of H^+ were absorbed with each equivalent of maltose by preparations containing the three metabolic inhibitors. The maximum rate of proton absorption shown in Fig. 8 thus probably corresponded to between about 13 and 20nmol of maltose absorbed/min per mg of dried yeast. This is between about 20 and 30% of the apparent rate of absorption of maltose during fermentation if the transport is regarded as the rate-limiting step. The initial rate of absorption of maltose from a 2mM solution, in the assays on which Fig. 9 is based, varied from about 6–12nmol/min per mg of dried yeast.

Role of K^+ ions. The rate of uptake of protons

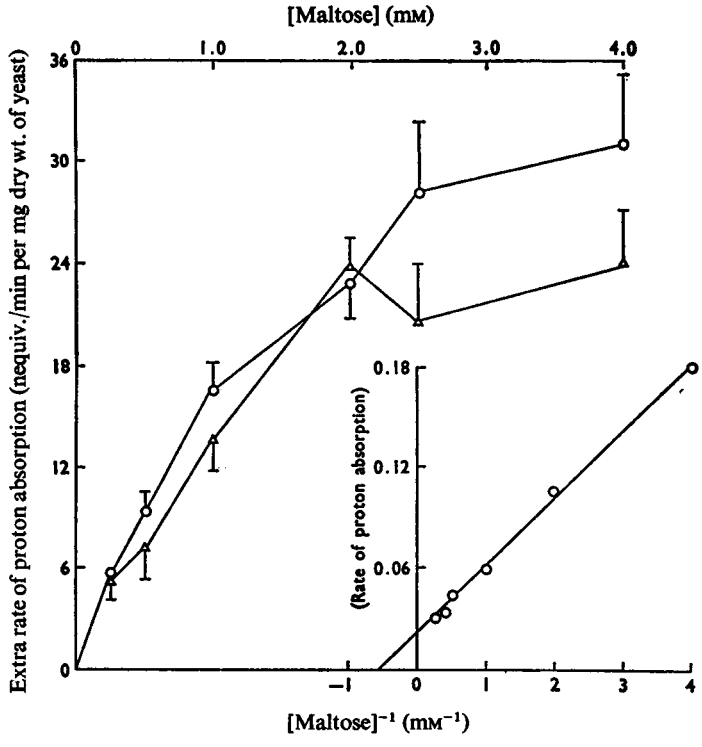

Fig. 8. *Increase in the rate of uptake of protons as a function of the maltose concentration, in the presence and absence of* 10 *mM-iodoacetamide*

Strain 74 was grown with maltose as the principal source of C. The washed yeast cells (25 mg dry wt.) were put with 25 μg of antimycin and 2 mM-2-deoxyglucose, either with or without 10 mM-iodoacetamide. After a further 2 min the indicated concentration of maltose was added. The mean increase (±S.E.M.) in the rate of proton uptake (see Fig. 7), observed in from four to eight assays with eight preparations of the yeast, was determined at six concentrations of maltose in the range up to 4 mM. ○, Iodoacetamide absent; △, iodoacetamide present. The former group of observations are represented as a double reciprocal plot in the inset to the Fig. The reciprocal rate of proton absorption is expressed as (nequiv./min per mg of protein)$^{-1}$. The K_m was about 1.8 mM and $V_{max.}$ about 45 nequiv. of H^+/min per mg dry wt. of yeast.

(±S.E.M.) induced by 1 mM-maltose was 13.4± 2.7 nequiv./30s per mg of yeast (13) in the absence of iodoacetamide. The corresponding accelerated rate of efflux of K^+, in a comparable series of assays, was determined by flame photometry to be 9.4±1.2 (4) in the same units. These observations suggest that the efflux of K^+ ions largely neutralized the initial influx of protons caused by maltose.

Yeast strain 179 *with lactose*

This strain of *Saccharomyces fragilis* utilized lactose as a main source of C for growth. When the yeast grown in that way was put at pH 4.5, under the conditions described in Fig. 6, the addition of lactose caused an immediate increase in the rate of uptake of protons of 3.7±0.4 S.E.M. (3) nmol/min per mg of dried yeast in the presence of 2.5 mM-lactose and 3.9±0.6 (3) nmol/min per mg in the presence of 5 mM-lactose. After 2–3 min the pH fell and acid accumulated at a rate of up to 5 nmol/min per mg. A similar initial response to lactose occurred when 10 mM-iodoacetamide was also present but the pH did not subsequently fall. Thus the effect of iodoacetamide on the initial increase and subsequent fall in pH, following the addition of lactose, resembled the behaviour of strain 74 with maltose. Strain 179 and strain 74 behaved similarly to one another with glucose, both producing acid after a short delay (cf. Fig. 6*b*). When strain 179 was grown with glucose instead of lactose as the principal C source, the presence of lactose did not cause either (1) an accelerated uptake of protons

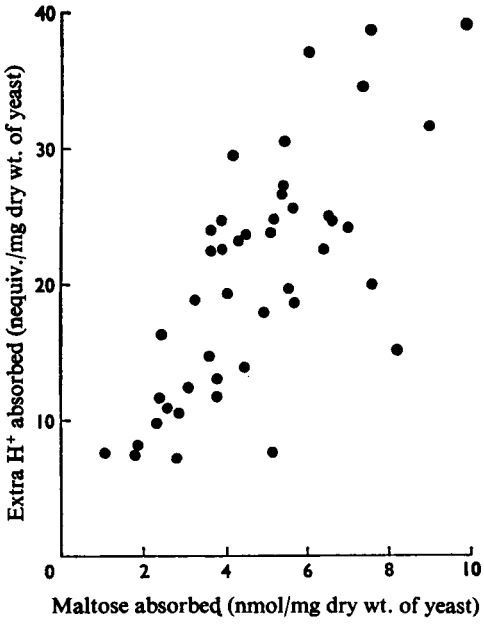

Fig. 9. *Number of extra protons absorbed by strain 74 during the uptake of [^{14}C]maltose for periods of up to 2 min*

The yeast was grown with maltose as the principal C substrate and put at 30°C with 2mM-2-deoxyglucose, 25μg of antimycin and 10mM-iodoacetamide. The pH was recorded continuously (see Fig. 7). After 2 min 2mM-[^{14}C]maltose was added. The yeast suspension was sampled about 0.5, 1.0, 1.5 or 2min later, the buffering capacity of the system having been calibrated by the addition of 1μmol of HCl. The number of extra protons absorbed in the presence of maltose was then computed and compared with the amount of ^{14}C that was extracted from the sample of yeast cells when they were put with water at 100°C for 10min (see Materials and Methods section). The results of 44 assays with four preparations of yeast are illustrated. In general the amount of ^{14}C extracted from the yeast cells increased with the length of time they had been exposed to the labelled maltose. A regression analysis indicated that 2.10±0.31 (S.E.M.) proton equivalents were absorbed with the maltose ($r = 0.726$).

under the above conditions, or, (2) the formation of acid. Acid was formed from glucose. Evidently the systems involved in the response to lactose were adaptive.

Discussion

On the basis of the present observations and those of Eddy & Nowacki (1971), the series of amino acids that the yeast strain 74 absorbs with 1–2 equivalents of protons now comprises the following: L-citrulline, glycine, L-leucine, L-lysine, L-methionine, L-phenylalanine and L-proline. The likely role of protons as co-substrates in the absorption of maltose, α-methylglucoside and sucrose by preparations of yeast strain 74 grown with maltose has been shown here for the first time. The apparent uptake of protons under these conditions might alternatively be described in terms of the efflux of hydroxyl ions, the choice of mechanism at present being arbitrary (West & Mitchell, 1972). The uptake of glucose by certain strains of yeast causes various amino acids and inorganic constituents to leak from the cellular into the extracellular phase (Lewis & Phaff, 1965; Stephanopoulos & Lewis, 1968). This phenomenon, which has been called 'shock excretion', appears to be quite distinct from the behaviour described above.

The close link between the absorption of protons and the absorption of the amino acids was evident both in the comparative behaviour of the strains A to F and in the way the previous history of the cellular preparations affected both processes. Grenson *et al.* (1970) describe the transport of various amino acids in these strains in terms of relatively specific carrier systems or permeases. While the molecular basis of these distinctions is unknown, it is convenient to use the same terminology in discussing the present observations. The latter indicate that (1) the so-called 'general amino acid permease', (2) the arginine permease, (3) the methionine permease functioning at low concentrations of methionine and (4) the proline permease probably each used protons as co-substrates.

There is no evidence to show whether either the lysine permease or the second methionine permease also function in that manner. The absorption of L-lysine nevertheless caused definite displacements of protons, which may have been associated either with the general permease or the arginine permease. It is already known that the functioning of these diverse systems may involve at least one common element that is subject to genetic variation (Grenson & Hennaut, 1971). The inability of strain F, as opposed to strain B, to absorb either arginine or proline might perhaps similarly be interpreted in terms of the modification of an element that was shared by both the proline and arginine permeases but not with the methionine permease.

Table 1 shows that the number of protons absorbed per amino acid equivalent varied both with the genetic character of the yeast and with the nature of the amino acid. Thus L-methionine was absorbed through the general permease of strain A with somewhat less than 2 proton equivalents and through the specific methionine permease of strain B with about 1 proton equivalent. Likewise, the specific proline permease exhibited a stoicheiometry not far from 1, whereas a value near

2 was found for L-citrulline with the general permease of strain A. Similar stoicheiometry was observed with the strain 74 of unknown genetic constitution. The absorption of glycine was also associated with roughly 2 protons both when the general permease of strain A was used and in the cultures of strain 74 grown with proline. On the other hand, the starved preparations of strain A grown with NH_4^+ ions absorbed glycine with, on the average, rather less than 1 proton equivalent. It is not known whether this process depended on a relatively unselective system, like the general amino acid permease of strain A, or on a system resembling the specific permeases. Inspection of Table 1 suggests that the absorption of lysine by strain B, which may have occurred through the arginine permease, was perhaps also associated with 1 proton equivalent. The behaviour of L-arginine itself was more equivocal, but is not inconsistent with these other preliminary indications that, whereas the specific permeases functioned with 1 proton equivalent, the general permease functioned with 2. Other factors that might influence the apparent stoicheiometry are (1) residual energy metabolism; (2) exchange diffusion involving the endogenous amino acid constituents; (3) variations in the endogenous rate of proton absorption with time, especially in circumstances where the amino acid was absorbed relatively slowly; (4) the existence of alternative modes of absorption without the co-substrate ion, a situation leading to non-integral stoicheiometric ratios (Schultz & Curran, 1970).

Relationship to metabolism

According to the co-substrate hypothesis, the influx of protons with these amino acids and carbohydrates might be neutralized in various ways (Eddy & Nowacki, 1971; West & Mitchell, 1972; Gibb & Eddy, 1972). As the present work shows, the efflux of K^+ ions probably served that purpose when the energy metabolism of the yeast preparations was prevented. The mechanisms involved during energy metabolism were studied in a preliminary fashion by Eddy & Nowacki (1971) but the details remain to be explored. A carbohydrate such as maltose then serves a dual role, both in accelerating the uptake of protons, and in maintaining the energy metabolism that drives the relevant ion-pumps in response to the influx of protons.

Preparations of the strain 74 grown with NH_4^+, in which energy metabolism had apparently ceased, absorbed about 20nmol of glycine/mg of yeast, less than 2% of the amount absorbed during energy metabolism (Eddy *et al.*, 1970*a*). The explanation of this behaviour is important, in relation to the problem of the validity of the co-substrate hypothesis, because it might be taken to imply that the smaller amounts of amino acid were absorbed at the expense of residual energy metabolism. Koch (1971) has recently made such a claim in connexion with the related problem of the absorption of lactose by *E. coli*. We have found (G. Watson & A. A. Eddy, unpublished work) that the amount of glycine the starved preparations of the yeast absorb, as well as the rate of absorption under standard conditions, can be lowered by allowing the yeast first to take up relatively small numbers of protons. We have also shown that the uptake of protons induced by glycine itself has a similar effect on the subsequent uptake of glycine. The factors limiting the absorption of glycine would seem therefore to depend not on residual energy metabolism but on events connected with the flow of the co-substrate ions across the plasmalemma. Further work is obviously required to define both these novel aspects of the kinetics of amino acid absorption and the factors determining the rates of uptake of the carbohydrates and amino acids when energy metabolism is initiated.

In the presence of glucose, phenylalanine is rapidly metabolized and, in such instances, it is usually assumed that the amino acid is first absorbed intact. In certain yeast strains maltose (Harris & Thompson, 1961; Robertson & Halvorson, 1957), α-methylglucoside (Okada & Halvorson, 1964) and lactose (Fuente & Sols, 1962) were apparently absorbed as such and then hydrolysed. The present work indicates that protons served as co-substrates in the absorption of these compounds, the accelerated influx of protons induced by the carbohydrate being revealed most clearly in the systems containing iodoacetamide. Sucrose also is probably absorbed directly by certain strains of yeast (Avigad, 1960; Fuente & Sols, 1962). Its utilization may, however, either alternatively or additionally, involve the initial hydrolysis of the molecule by an extracellular invertase (Sutton & Lampen, 1962). Glucose and fructose may then accumulate in the suspension medium (Fuente & Sols, 1962). Such a mechanism would seem to provide a relatively inefficient means of supplying a single cell with C for growth, unless the hydrolysis products were trapped before they diffused away. We suggest that the first mechanism, in which sucrose is absorbed intact, was involved in the absorption of protons with this carbohydrate by the preparations of strain 74 grown with maltose. The second mechanism may have been involved in the production of acid from sucrose by the preparations of yeast grown with glucose. In order to put these ideas on a firmer basis, the chemical nature of the materials accumulating in the yeasts, when they are fed with various carbohydrates, needs to be examined in relation to the adaptive history of the cultures. Van Steveninck (1970) claims that some, or all, of the glucose, 2-deoxyglucose and α-methylglucoside absorbed by the yeast strains he studied was phosphorylated in the process. It seems unlikely

that the proton displacements we have observed can be explained in that way.

According to the co-substrate hypothesis, maltose, α-methylglucoside and glucose are each absorbed by a process of facilitated diffusion. In those circumstances, where protons act as co-substrates with maltose and α-methylglucoside but not with glucose, the former pair, unlike glucose, might become concentrated in the cellular phase. The limited evidence available about a small number of strains of *S. cerevisiae* and *S. carlsbergensis* supports this notion (Harris & Thompson, 1961; Okada & Halvorson, 1964). It would be interesting to know whether the strain of *Rhodotorula gracilis* that Kotyk & Höfer (1965) found to concentrate glucose and various other monosaccharides absorbs extra protons with these compounds.

References

Avigad, G. (1960) *Biochim. Biophys. Acta* **40**, 124–134

Eddy, A. A. (1968) *Biochem. J.* **108**, 195–206

Eddy, A. A. (1969) *Biochem. J.* **115**, 505–509

Eddy, A. A. & Nowacki, J. A. (1971) *Biochem. J.* **122**, 701–711

Eddy, A. A., Indge, K. J., Backen, K. & Nowacki, J. A. (1970*a*) *Biochem. J.* **120**, 845–852

Eddy, A. A., Backen, K. & Watson, G. (1970*b*) *Biochem. J.* **120**, 853–858

Fuente, G. & Sols, A. (1962) *Biochim. Biophys. Acta* **56**, 49–62

Gale, E. F. & Llewellin, J. (1972) *Biochim. Biophys. Acta* **266**, 182–205

Gibb, L. & Eddy, A. A. (1972) *Biochem. J.* **129**, 979–981

Gits, J. J. & Grenson, M. (1967) *Biochim. Biophys. Acta* **135**, 507–516

Grenson, M. & Hennaut, C. (1971) *J. Bacteriol.* **105**, 477–482

Grenson, M., Mousset, M., Wiame, J. M. & Becket, J. (1966) *Biochim. Biophys. Acta* **127**, 325–338

Grenson, M., Hou, C. & Crabeel, M. (1970) *J. Bacteriol.* **103**, 770–777

Harold, F. M. (1972) *Bacteriol. Rev.* **36**, 172–230

Harris, G. & Thompson, C. C. (1961) *Biochim. Biophys. Acta* **52**, 176–183

Koch, A. L. (1971) *J. Mol. Biol.* **59**, 447–459

Kotyk, A. & Höfer, M. (1965) *Biochim. Biophys. Acta* **102**, 410–422

Kuo, S. & Cirillo, V. P. (1970) *J. Bacteriol.* **103**, 679–685

Lewis, M. J. & Phaff, H. J. (1965) *J. Bacteriol.* **89**, 960–966

Mitchell, P. (1967) *Advan. Enzymol.* **29**, 33–87

Okada, H. & Halvorson, H. O. (1964) *Biochim. Biophys. Acta* **82**, 547–555

Robertson, J. J. & Halvorson, H. O. (1957) *J. Bacteriol.* **73**, 186–198

Riggs, T. R., Walker, L. M. & Christensen, H. N. (1958) *J. Biol. Chem.* **233**, 1479–1484

Schultz, S. G. & Curran, P. F. (1970) *Physiol. Rev.* **50**, 637–718

Seaston, A., Inkson, C. & Eddy, A. A. (1972) *J. Gen. Microbiol.* **73**, xix

Stephanopoulos, D. & Lewis, M. J. (1968) *J. Inst. Brew. (London)* **74**, 378–383

Sutton, D. D. & Lampen, J. O. (1962) *Biochim. Biophys. Acta* **56**, 303–312

Van Steveninck, J. (1970) *Biochim. Biophys. Acta* **203**, 376–384

West, I. C. (1970) *Biochem. Biophys. Res. Commun.* **41**, 655–661

West, I. C. & Mitchell, P. (1972) *Bioenergetics* **3**, 445–462