The Incorporation of Amino Acids into Protein in a Cell-Free System from Yeast*

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The factors influencing the incorporation of radioactive amino acids into proteins with cell-free preparations from yeast were studied. The complete system requires a ribosomal and supernatant fraction, an energy source, and magnesium ions. The yeast supernatant can be replaced by rat liver supernatant or *E. coli* supernatant. The incorporation is stimulated by low levels of ribosomal RNA, while higher levels inhibit. Supplemental transfer RNA depresses the incorporation, while preparations with a reconstituted terminal sequence of -pCpCpA show little effect. Ribonuclease and puromycin are potent inhibitors; deoxyribonuclease and chloramphenicol do not affect amino acid incorporation. Indirect evidence was obtained suggesting that the site of chloramphenicol sensitivity in the *E. coli* system is localized in the ribosomal fraction.

A number of cell-free preparations from microbial sources that actively synthesize protein have been described (see Novelli, 1960, and Berg, 1961, for recent reviews).

In the present paper, the characteristics of a cell-free fraction prepared from yeast which actively incorporates amino acids into protein are described. The yeast preparations show many features similar to the *E. coli* system (Lamborg and Zamecnik, 1960; Tissieres *et al.*, 1960; Matthaei and Nirenberg, 1961). However, unlike *E. coli*, the yeast is not sensitive to chloramphenicol or deoxyribonuclease.

This work extends the earlier observations of Webster (1957), who reported amino acid incorporation into yeast ribosomes, and is similar to that described recently in a preliminary report by Barnett *et al.* (1962).

MATERIALS

Crystalline ATP (disodium salt), UTP, CTP, and GTP were purchased from Pabst Laboratories. Crystalline ribonuclease and deoxyribonuclease were purchased from Worthington Biochemicals. Puromycin was purchased from Nutritional Biochemicals and chloramphenicol from Parke Davis and Co. Reduced glutathione, phosphoenolpyruvate (trisodium salt), and L-(U-C^{14}) leucine and L-(U-C^{14}) valine were purchased from California Corporation for Biochemical Research. L-(U-C^{14}) Phenylalanine was purchased from Nuclear Chicago Corporation. L-(U-C^{14}) Lysine was purchased from New England Nuclear Corporation. Pyruvic kinase was prepared from rabbit skeletal muscle as described by Bucher and Pfleiderer (1955). Transfer RNA was prepared from *Saccharomyces fragilis* by the method of Holley *et al.* (1961) and freed of residual amino acid by the procedure of Nathans and Lipmann (1961). The terminal sequence of -pCpCpA in transfer RNA was reconstituted by the procedure of Starr and Goldthwait (1960), employing a rabbit muscle enzyme. Ribosomal RNA was prepared directly from yeast ribosomes by the method of Nirenberg and Matthaei (1961). The stock L-amino acid mixture (Nutritional Biochemicals) was 0.002 M with respect to threonine, tyrosine, methionine, cysteine, isoleucine, glutamic acid, glutamine, aspartic acid, asparagine, histidine, proline, lysine, alanine, glycine, tryptophane, serine, arginine, valine, and phenylalanine when leucine incorporation was examined. When valine, lysine, or phenylalanine incorporation was studied, a similar mixture was employed, with only the respective radioactive amino acid omitted. All other chemicals were purchased commercially and were either chemically pure or reagent grade.

METHODS

Saccharomyces fragilis (American Type Culture 10022) was grown aerobically in Erlenmeyer flasks for approximately 11–12 hours at 37° in a medium described by McQuate *et al.* (1962).[1] The culture was then transferred to a second flask containing nine times the original amount of growth medium and incubated again at 37° for an additional 6–7 hours under aerobic conditions. At this time the optical density of the culture had reached a level of about 1.0–1.2 as measured at 490 mμ in a 1-cm cell. The cells were harvested by centrifugation for 15 minutes at 2000 rpm at 4°. The cells were then washed with 0.01 M Tris buffer (*p*H 7.2) containing 0.004 M magnesium acetate and 0.02 M KCl and frozen as a paste in 10–20 g portions for storage at −18°. Yeast extracts were prepared by grinding one part by weight of thawed cells with four parts of alumina (Special Alumina A-305, Bacteriological Grade, Aluminum Company of America) in a precooled mortar for 2–3 minutes. The white paste was extracted with cold 0.01 M Tris buffer (*p*H 7.2) containing 0.004 M magnesium acetate, 0.02 M KCl, and 0.0005 M glutathione or 0.0005 M mercaptoethanol. The mixture was then centrifuged two times for 30 minutes at 30,000 × *g* at 0°. The supernatant, containing approximately 40 mg of protein per ml, was passed through a 2.2 × 25 cm G-25 Sephadex column at 4°. The column had been previously equilibrated with the Tris buffer employed for extractions. The crude extract was used immediately for the amino acid incorporation studies. A variation of about 50% was found to occur from one batch of yeast to the next.

For some experiments, the ribosomes were separated from the supernatant by an additional centrifugation for 120 minutes at 105,000 × *g* and the ribosomal pellet was resuspended by gentle homogenization in the buffer employed for extraction. The ribosomal frac-

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[1] The contents of the growth medium per liter are as follows: 54 g glucose, 5 g Peptone, 10 g yeast extract, 9 g KH_2PO_4, 0.3 g $CaCl_2$, 0.5 g $MgSO_4$, 6 ml of 60% sodium lactate, 0.15 ml wheat germ oil, 5 ml Tween 80, 6 g amonium sulfate, and 20 mg. ergosterol.

tion was then centrifuged a second time and the pellet resuspended in the same buffer. This was necessary to reduce protein contamination from the supernatant fraction. Similarly, the supernatant fraction was recentrifuged for an additional 120 minutes at 105,000 × *g* to reduce the residual ribosomal contamination. The separation of ribosomes and supernatant often resulted in a marked loss of activity for amino acid incorporation.

E. coli supernatant and ribosomes were prepared by the method of Matthaei and Nirenberg (1961).

Protein was determined by the method of Gornall *et al.* (1949) or Lowry *et al.* (1951), with a fresh solution of crystalline bovine serum albumin used as a standard.

Reaction mixtures were incubated at 37°, and the reaction was terminated by the addition of 1.2 volumes of ice-cold 10% trichloroacetic acid. The precipitate was washed in the presence of nonradioactive substrate amino acids essentially by the method of Siekevitz (1952). The protein was then dissolved in 88% formic acid, a 1-ml aliquot was added to 15 ml of scintillant solution, and the radioactivity of the sample was counted in a Packard Tri-Carb Liquid Scintillation Counter. The scintillant solution contained 125 g naphthalene, 7.5 g of 2,5-diphenyloxazole (PPO), and 375 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) dissolved in 1 liter of dioxane (Hayes, 1960). The counting efficiency by this procedure with a standard C^{14}-benzoic acid sample was 54 %.

Each experiment reported in this paper was repeated at least once.

Results

General Requirements for Amino Acid Incorporation.—The requirements for amino acid incorporation into yeast protein are shown in Table I. The amino acid incorporation is dependent upon ATP, an ATP-generating system, and magnesium ions. The incorporation is not stimulated by an amino acid mixture or by the nucleotides GTP, UTP, and CTP. The system is inhibited by puromycin and ribonuclease, whereas chloramphenicol and deoxyribonuclease have little effect.

A time curve showing the incorporation of leucine and valine is shown in Figure 1. The incorporation at 37° levels off after about 6–8 minutes. This is similar to that observed for most mammalian preparations. The reticulocyte system (Allen and Schweet, 1962) and the *E. coli* system (Matthaei and Nirenberg, 1961) continue amino acid incorporation after a much longer period of incubation.

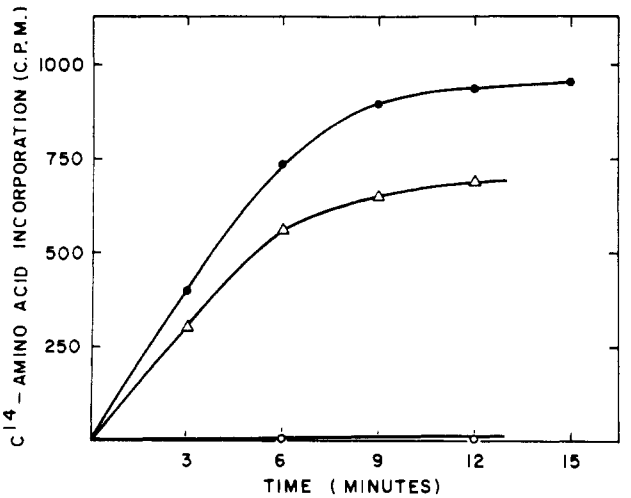

FIG. 1.—Time curve for C^{14}-leucine ●–● and C^{14}-valine △–△ incorporation into yeast protein. O–O refers to the control in the absence of ATP. See Table I for incubation conditions.

The effect of increasing concentrations of ribosomal and supernatant protein is shown in Figures 2 and 3. The incorporation of C^{14}-leucine after a 6-minute incubation is proportional to the amount of ribosomal protein added when the supernatant protein is held constant (Fig. 2). In the absence of supernatant protein little incorporation occurs, while maximal incorporation is obtained with approximately 5 mg of supernatant protein per ml (Fig. 3).

The replacement of yeast supernatant by a similar fraction from rat liver or *E. coli* is shown in Table II. In each case, the incorporation is considerably less than that found with yeast supernatant.

The *p*H optimum for C^{14}-leucine incorporation is between 7.0–7.5 (Fig. 4). In the experiments employing imidazole-Tris buffer, protein biosynthesis is depressed about 50% at *p*H 7.2 as compared to experiments with Tris buffer alone.

The effect of magnesium ion concentration on C^{14}-

TABLE I

REQUIREMENTS FOR AMINO ACID INCORPORATION INTO YEAST PROTEIN

The complete reaction mixture contained 0.008 M $Mg(OAc)_2$, 0.02 M KCl, 0.001 M ATP, 5×10^{-5} M GTP, CTP, UTP; 0.005 M PEP, 130 μg pyruvic kinase; 7.5 mg yeast supernatant protein, 2.5 mg yeast ribosomal protein; 5×10^{-5} M L-amino acid mixture, 0.100 M Tris buffer (*p*H 7.2), L-C^{14}-leucine (0.10 μmoles, containing 2.5×10^5 cpm) or L-C^{14}-phenylalanine (0.10 μmole, containing 2.5×10^5 cpm). Tubes were incubated for 6 minutes at 37° in a final volume of 1.0 ml. The puromycin, chloramphenicol, RNase, and DNase were added at zero time.

Conditions	Amino Acid Incorporation (cpm)	
	C^{14}-Leucine	C^{14}-Phenylalanine
Complete	1138	620
Zero time	80	86
−ATP, PEP, pyruvic kinase	50	60
−GTP, CTP, UTP	1028	660
−Amino acid mixture	1044	700
+0.09 μmoles Puromycin	336	310
+2 μmoles Chloramphenicol	1150	574
+10 μg RNase	74	180
+10 μg DNase	1150	530
1.5 μmoles Mg^{++} (final conc.)	330	320

TABLE II

THE EFFECT OF SUPERNATANT FRACTIONS FROM YEAST, RAT LIVER, AND *E. coli* ON AMINO ACID INCORPORATION WITH YEAST RIBOSOMES

Incubation conditions were essentially the same as those in Table I except (1) the yeast supernatant protein (3.6 mg/ml) was replaced by a similar fraction from *E. coli* or rat liver containing the same amount of protein and (2) the reaction contained 0.2 μmoles of L-C^{14}-leucine (5.0×10^5 cpm) or 0.2 μmoles of L-C^{14}-lysine (1.0×10^6 cpm).

Ribosome Fraction	Supernatant Fraction	Amino Acid Incorporation (cpm)	
		C^{14}-Leucine	C^{14}-Lysine
Yeast	Yeast	963	1987
Yeast	None	100	151
None	Yeast	34	22
Yeast	*E. coli*	356	529
None	*E. coli*	23	21
Yeast	Rat liver	200	492
None	Rat liver	2	40

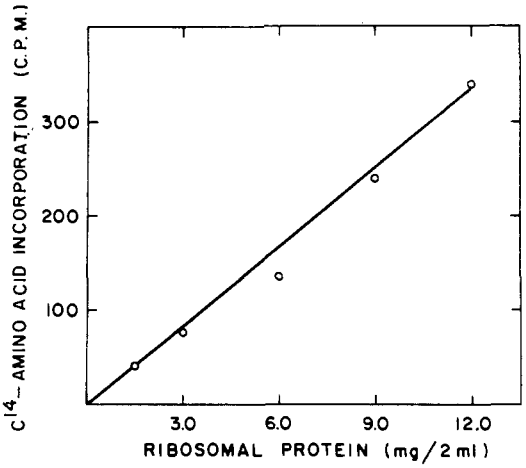

FIG. 2.—Effect of yeast ribosomal protein concentration on C^{14}-leucine incorporation into protein. Incubation conditions were the same as those in Table I except that the volume of the final incubation mixture was 2.0 ml.

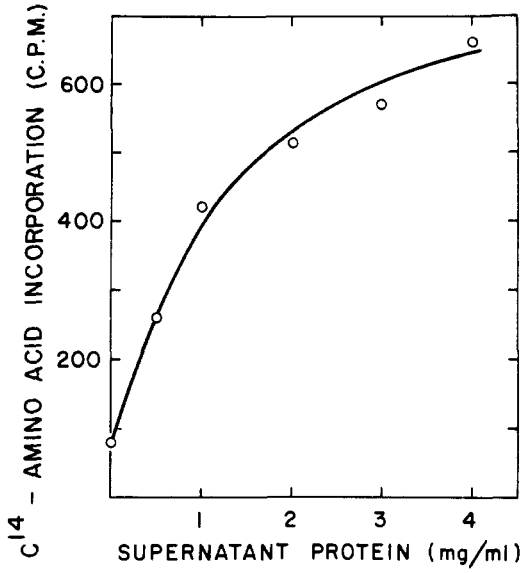

FIG. 3.—Effect of yeast supernatant protein concentration on C^{14}-leucine incorporation into protein. Incubation conditions were the same as those in Table I.

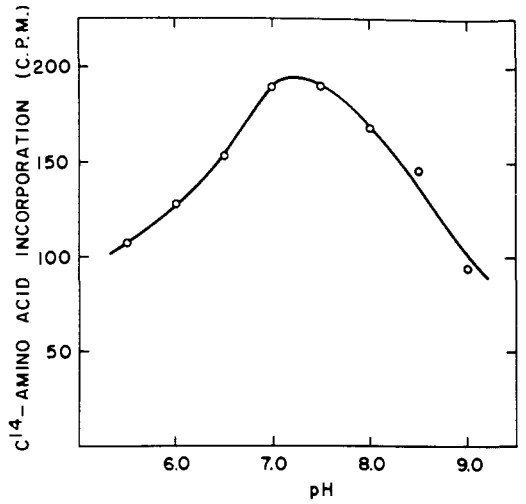

FIG. 4.—Effect of *p*H on the incorporation of C^{14}-leucine into yeast protein. Buffers were composed of 0.05 M imidazole and 0.100 M Tris titrated to the appropriate *p*H. See Table I for incubation conditions.

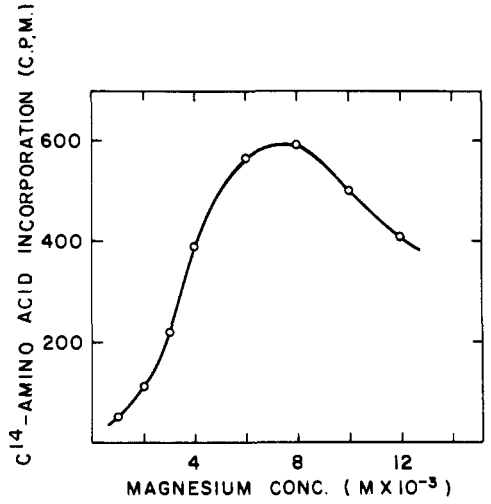

FIG. 5.—Effect of magnesium ion concentration on the incorporation of C^{14}-leucine into yeast protein. See Table I for incubation conditions.

leucine incorporation is shown in Figure 5. Under the present incubation conditions, maximal incorporation occurs at 0.008 M magnesium ion concentration.

Nature of the Final Product.—The ribosomal preparations before and after a 12-minute incubation show an ultracentrifugal pattern essentially identical to that previously reported for yeast (Chao and Schachman, 1956; Warren and Goldthwait, 1962). The distribution of the hot trichloroacetic acid–insoluble radioactivity after incorporation of C^{14}-leucine is shown in Table III. Approximately 80% of the radioactivity is associated with the ribosomal protein fraction, while the remaining 20% is found in the supernatant protein.

Effect of RNA.—Table IV shows the effect of transfer and ribosomal RNA on leucine incorporation. Transfer RNA isolated from yeast and freed of its residual amino acid inhibits incorporation, while the same preparation has little effect after reconstitution of the -pCpCpA terminal sequence. Yeast ribosomal RNA at low levels stimulates amino acid incorporation about two-fold, while larger amounts inhibit.

Localization of Chloramphenicol Sensitivity in E. coli.—Experiments described in Table I show the lack of sensitivity of the yeast system to chloramphenicol. The replacement of yeast supernatant by *E. coli* supernatant is described in Table II. To test for the site of action of this antibiotic in *E. coli*, cross experiments were carried out employing *E. coli* supernatant and yeast ribosomes and *vice versa* in the presence and absence of the drug. The results of these experiments are shown in Table V. Very little inhibition occurs in the case of *E. coli* supernatant and yeast ribosomes (tubes 5 and 6), in contrast to *E. coli* supernatant and *E. coli* ribosomes (tubes 1 and 2). Direct proof for the chloramphenicol-sensitive site was not possible by the converse experiment, since the *E. coli* supernatant could not be replaced by yeast supernatant with use of *E. coli* ribosomes (tubes 3 and 4).

DISCUSSION

The general characteristics of amino acid incorporation into protein with cell-free preparations from yeast are quite similar to those reported for *E. coli* and rat liver. The lack of amino acid stimulation and GTP requirement probably is due to contamination of the crude yeast extract. A demonstration of the ATP requirement was possible only after the extract had

TABLE III

LOCALIZATION OF C^{14}-LEUCINE IN THE SUPERNATANT AND RIBOSOMAL PROTEIN

Conditions of incubation were the same as in Table I. Reactions were stopped by chilling to 0°, and fractions were separated by centrifugation at 105,000 × *g* for 2 hours at 0°. The protein of each fraction was precipitated with trichloroacetic acid and washed, and the radioactivity was determined as described under Methods.

Incubation Time (min.)	Amino Acid Incorporation (cpm)	
	Supernatant	Ribosomes
0	18	64
7.5	121	511
15	141	569

TABLE IV

EFFECT OF RNA ON C^{14}-LEUCINE INCORPORATION INTO YEAST PROTEIN

Conditions of incubation were the same as in Table I.

Additions	Amino Acid Incorporation (cpm)
Control	400
+ 0.36 mg Transfer-RNA	298
+ 1.2 mg Transfer-RNA	250
+ 0.2 mg Reconstituted transfer-RNA[a]	390
+ 1.2 mg Reconstituted transfer-RNA[a]	385
Zero time	13
Control	265
+ 0.42 mg Ribosomal-RNA	420
+ 1.4 mg Ribosomal-RNA	630
+ 2.8 mg Ribosomal-RNA	548
Zero time	10

[a] Transfer RNA prepared with a reconstituted -pCpCpA terminal sequence.

been passed through a Sephadex column. Stimulation by an amino acid mixture or GTP is possible in *E. coli* preparations only after exhaustive dialysis of the crude preparation.

Yeast and liver preparations are active in the presence of chloramphenicol, in contrast to *E. coli* preparations. The lack of sensitivity of the yeast system to chloramphenicol made possible the cross experiments with *E. coli* supernatant and yeast ribosomes. By this procedure, it was shown that the site of the chloramphenicol sensitivity in *E. coli* is not in the supernatant fraction. This suggests that the sensitive site is probably localized in the ribosomal fraction. This is consistent with the observations of Lacks and Gros (1959) and Nathans and Lipmann (1961), who found that chloramphenicol interferes with the transfer of amino acid from amino acyl RNA to ribosomes. Other investigators (Flaks *et al.*, 1962; Speyer *et al.*, 1962), working with streptomycin-resistant and streptomycin-sensitive *E. coli* preparations, have shown that streptomycin interferes with the ribosomal fraction and not the supernatant fraction from streptomycin-sensitive cells.

The replacement of yeast supernatant by rat liver or *E. coli* supernatant differs from the cross experiments with *E. coli* and rat liver. Nathans and Lipmann (1961), employing a partially purified system from *E. coli* and rat liver, have shown a requirement for both transferring enzyme and ribosomes from the same source. Aminoacyl transfer RNA from *E. coli* was active in both systems. In the present studies with yeast ribosomes, the crude supernatants from *E. coli* or rat liver could replace the yeast supernatant, although they were not as active as the yeast supernatant itself.

TABLE V

EFFECT OF CHLORAMPHENICOL ON C^{14}-LYSINE INCORPORATION WITH YEAST AND *E. coli* SUPERNATANT AND RIBOSOMES

Incubation conditions were essentially the same as those in Table I except (1) the *E. coli* ribosomes (3.2 mg ribosomal protein/ml in Expt. 1 and 6.0 mg/ml in Expt. 2) were replaced by a similar fraction from yeast (8.0 mg ribosomal protein/ml) and the *E. coli* supernatant (4.4 mg/ml) was replaced by yeast supernatant (11 mg/ml in Expt. 1 and 3.8 mg/ml in Expt. 2); (2) the incubation time was 12 minutes; and (3) each reaction contained 0.007 μmoles of L-C^{14}-lysine (1 × 10^6 cpm). Tubes 2, 4, and 6 also contained 2 μmoles of chloramphenicol per ml. Two separate batches of yeast and *E. coli* were used in the above experiments.

Tube	Supernatant	Ribosomes	Chloramphenicol	Amino Acid Incorporation (cpm) Expt. 1	Expt. 2
1	*E. coli*	*E. coli*		5625	3905
2	*E. coli*	*E. coli*	present	196	239
3	Yeast	*E. coli*		174	170
4	Yeast	*E. coli*	present	129	74
5	*E. coli*	Yeast		4804	10,369
6	*E. coli*	Yeast	present	4460	8236
7	Yeast	None		70	44
8	*E. coli*	None		81	35
9	None	Yeast		191	1319
10	None	*E. coli*		256	180

This may be due to the enzyme complement, the transfer RNA, or both (Benzer and Weisblum, 1961). In contrast, the yeast supernatant could not replace *E. coli* supernatant with the use of *E. coli* ribosomes. Similar results have been found by Rendi and Ochoa (1961).

The experiments reported in this paper extend the earlier observations of De Kloet *et al.* (1961, 1962) and Young *et al.* (1961), who employed whole cells or protoplasts for their protein biosynthesis studies in yeast. In the present studies, the optimal magnesium ion concentration was found to be about 8 μmoles per ml, which is higher than that reported by Webster (1957), who found maximal incorporation when the magnesium ion concentration was 0.5 to 1.0 μmole per ml.

The effect of RNA on the yeast system is of particular interest. The yeast preparations show about the same stimulation of amino acid incorporation into protein by ribosomal RNA as was found by Nirenberg and Matthaei (1961) with *E. coli* preparations. Low levels of transfer RNA inhibit protein biosynthesis, whereas reconstituted transfer RNA has little effect. This inhibition might be located at (1) the activating enzyme, (2) the transfer enzyme, or (3) the ribosomal site which binds the amino acyl RNA. In each case, the transfer RNA lacking the terminal -pCpCpA sequence would be a competitive inhibitor of the transfer RNA containing the intact terminal sequence.

In studying protein biosynthesis in cell-free preparations from microbial sources, care must be taken to exclude contamination by intact cells. In the present experiments the crude extracts were centrifuged two times at 30,000 × *g* and the supernatant was carefully decanted from the pellet containing intact cells and debris. Further centrifugation of the supernatant at 105,000 × *g* yields ribosomal and supernatant fraction, neither of which are active alone for amino acid incorporation. Furthermore, a source of ATP is required in addition to the supernatant and ribosomal fraction. Thus, the amino acid incorporation observed in the

present experiments is not due to intact cell contamination.

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The Uptake of Amino Acids by Cells and Protoplasts of *S. faecalis**

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Both cells and protoplasts of *S. faecalis* contain a transport system for glycine, L-alanine, and D-alanine that requires an energy source, possesses a high temperature coefficient, is saturated at relatively low external concentrations of amino acid, and leads to high internal concentration of these amino acids in a form that is extractable as free amino acid by hot water. The affinities of the transport system for these amino acids are the same in cells and in protoplasts. Uptake of all three amino acids in protoplasts is stimulated by K^+ and inhibited by Na^+; these ions have no detectable effect in the intact cells. High concentrations of pyridoxal greatly increase accumulation of glycine and L-alanine by protoplasts; uptake of D-alanine is not changed. The effect is reasonably specific for pyridoxal, and is not observed in intact cells. 5-Deoxypyridoxal inhibits uptake in both intact cells and protoplasts. D-Cycloserine inhibits uptake of both D- and L-alanine by the transport system. No clear explanation of the differential effects of K^+ and of pyridoxal in cells and protoplasts is yet possible. In each case, the bacterial protoplast resembles mammalian cells more than it does the intact bacterial cell. Both effects may result from conformational changes in the non-rigid protoplast membrane that are not possible when this is in contact with a rigid cell wall.

Extracellular pyridoxal or pyridoxal-5-phosphate greatly increases the rate and extent of concentration of several amino acids by Ehrlich ascites tumor cells (Christensen *et al.*, 1954; Christensen, 1960). Pyridoxal phosphate also increases intestinal absorption of amino acids in animals made B_6-deficient by penicillamine treatment (Akedo *et al.*, 1960), and in isolated intestinal segments of rats previously treated with 4-deoxypyridoxine (Jacobs and Hillman, 1959).

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In contrast to these results in mammalian cells and tissues, Holden (1959a) found that vitamin B_6–deficient cells of *Lactobacillus arabinosus* absorb glutamic acid as rapidly as those grown with an excess of vitamin B_6, though not to the same extent, and Leach and Snell (1960) found that the uptake of L-alanine or of glycyl-L-alanine by cells of *Lactobacillus casei* severely deficient in vitamin B_6 was not increased by the addition of pyridoxal either to the resting cells or to growing cultures.

To determine whether the presence of a rigid cell wall in bacterial cells was responsible for these differences, and to learn more about the possible role of this structure in cell permeability, a comparative study has