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THE ISOLATION AND PROPERTIES OF A β-ALANINE PERMEASELESS MUTANT OF *PSEUDOMONAS FLUORESCENS*

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SUMMARY

A mutant of *Pseudomonas fluorescens* has been isolated which is markedly deficient in the active transport of L-alanine, L-proline, and β-alanine.

This mutant, which is also deficient in β-alanine transaminase, is capable of catalyzing facilitated diffusion and exchange transport of β-alanine at low temperatures but can maintain little or no uptake of this amino acid against a concentration gradient.

In the transport mutant, competitive stimulation of β-alanine by L-proline can be observed, whereas in the parent strain, L-proline inhibits β-alanine uptake.

The rate of efflux of β-alanine from preformed pools is 4-fold greater in the transport mutant than in the parent strain.

It thus appears that the functional block in this transport mutant resides not in the membrane carrier but in the ability of the cell to couple energy to the membrane carrier.

INTRODUCTION

Studies on the effect of respiratory inhibitors on the concentrative transport of galactose[1], α-methylglucosides[2] and lactose[3] in *Escherichia coli* and yeast have led investigators to believe that at least two components are involved in membrane systems which mediate active transport in microorganisms. One of these components is a stereo-selective membrane binding site or carrier. The binding site, when it is not coupled to an energy supply, is capable only of mediating facilitated diffusion; it cannot otherwise accumulate the solute against a concentration gradient. The second component couples energy to the carrier. The mechanism by which asymmetry in the flux of solute is achieved is still unknown.

In this paper we report on the isolation and properties of a mutant of *Pseudomonas fluorescens*, Strain 67/4MTR, which is impaired in the transport of some neutral amino acids. This mutant can catalyze facilitated diffusion and exchange of β-alanine but cannot form or maintain large intracellular pools of the free amino acid. In the transport mutant the rate of efflux of β-alanine from preformed pools is

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four times greater than in the parent strain 67 which is a catabolic mutant lacking β-alanine transaminase[4].

METHODS AND MATERIALS

Strains

The wild-type strain is *Pseudomonas fluorescens* ATCC 11250, used by HAYAISHI *et al.*[5] for the purification of β-alanine transaminase.

Strain 67 is a β-alanine transaminaseless mutant selected from ATCC 11250; the properties of this strain have been described elsewhere[4]. Strain 67 cannot degrade β-alanine to malonate semialdehyde. The ^{14}C pool derived from incubation of strain 67 cells with β-[1-^{14}C]alanine is chromatographically homogeneous, completely exchangeable with unlabeled β-alanine and is entirely removed from the cell in the presence of NaCN.

Strain 67/4MTR is a mutant selected for resistance to 4-methyl-DL-tryptophan using strain 67 as the parent strain. Strain 4MTR is a second mutant resistant to 4-methyl-DL-tryptophan which was selected using the wild type as parent strain.

Growth

Strain 67 was grown on a minimal medium containing glucose, 5 g/l; $(NH_4)_2SO_4$, 1.5 g/l; K_2HPO_4, 1.5 g/l; KH_2PO_4, 0.5 g/l; and $MgSO_4$, 0.2 g/l. Strains 67/4MTR and 4MTR were grown on minimal media *plus* 4-methyl-DL-tryptophan (20 μg/ml). 4-Methyl-DL-tryptophan was purchased from Mann Research Laboratory.

Transport studies

Log phase cells were harvested from the minimal medium by centrifugation and washed twice with Buffer A (glucose, 5 g/l; K_2HPO_4, 1.5 g/l; KH_2PO_4, 0.5 g/l and chloramphenicol, 200 mg/l). The washed cells were resuspended to a density of 0.5 mg dry wt./ml of incubation mixture. Cells were preincubated in phosphate buffer for 20 min at 30° prior to addition of isotopes, carriers or inhibitors.

Influx was stopped by withdrawing 0.1-ml aliquots from the incubation mixture and rapidly separating cells from medium on a Millipore filter connected to a suction flask. Filters were washed three times with 1-ml portions of Buffer A at room temperature and then glued to aluminium planchets for counting of ^{14}C isotope.

$^{14}CO_2$ evolution was monitored by placing the incubation mixture in a Warburg flask with 0.2 ml of 20% KOH in the center well. 10-μl aliquots of KOH were withdrawn periodically, placed in glass planchets and heated to dryness.

For short-term uptake studies β-[^3H]alanine was used and the incubation volume scaled down to 0.2 ml. Uptake was stopped by diluting the incubation mixture with 10 volumes of buffer and the diluted mixture was filtered and washed. Isotope retained on the dried filter was determined by scintillation counting.

Efflux measurements were performed by preloading cells at 0° with isotope until a steady-state concentration of substrate was achieved. Aliquots (0.1 ml) of pre-loaded cell suspension were centrifuged twice and placed on the filter connected to the vacuum flask. Amino acid-free buffer (1.0 ml at 25°) was added after the vacuum was interrupted with a spring clip. After specified intervals the efflux reaction was stopped by releasing the clip.

Materials

Unlabeled amino acids were obtained from Nutritional Biochemicals. Aminooxyaceticacid hemi-hydrochloride was purchased from Eastman Chemicals. Isotopes were obtained from New England Nuclear Corporation and their radio-chemical purity confirmed by paper chromatography in a butanol–acetic acid–water mixture (12:3:5, by vol.).

Quantitation of isotopes

Gas flow counting was performed with a Nuclear–Chicago Model D-47 detector and scalar operating at 18% efficiency. Appropriate corrections were made for background, self absorption, backscatter, and adsorption of isotope by Millipore filters.

Scintillation counting was performed with a Nuclear–Chicago Unilux II scintillation counter operating at 76% efficiency. The scintillation fluid contained 2,5-D-phenyloxazole (4 g/l) and 1,4-bis (2-(5-phenyloxazolyl))-benzene (0.1 g/l) both purchased from Packard Instrument Company, in toluene.

RESULTS

Specificity of the transport defect

Strain 67 is capable of transporting low concentrations of β-alanine against concentration gradients of greater than 100 (ref. 6). Large distribution ratios are apparent very early in the time course of β-alanine uptake (Fig. 1). A much lower

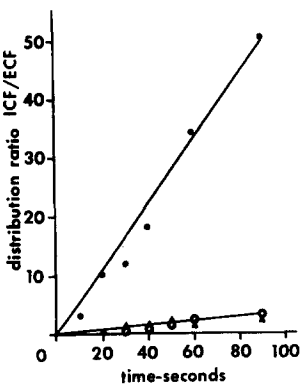


Fig. 1. Distribution ratios of β-alanine in strains 67 and 67/4MTR. Log cultures were centrifuged, washed, and resuspended to a density of 0.5 mg/ml buffer. Cells were preincubated for 20 min at 30° and 10 μC of β-[^{3}H]alanine were added along with carrier β-alanine (final concn. $2 \cdot 10^{-5}$ M). Uptake was stopped by rapid addition of 10 vol. of buffer at room temperature. Diluted cells were filtered, washed, and then dried for scintillation counting, ●, strain 67; ○, strain 67/4MTR grown in minimal medium; ▲, strain 67/4MTR grown in minimal medium *plus* 4-methyl-DL-tryptophan (20 μg/ml). ICF/ECF ratio = intracellular/extracellular fluid ratio.

ability to transport β-alanine is found in mutant 67/4MTR when this strain is grown either on minimal medium or on minimal medium *plus* 4-methyl-DL-tryptophan.

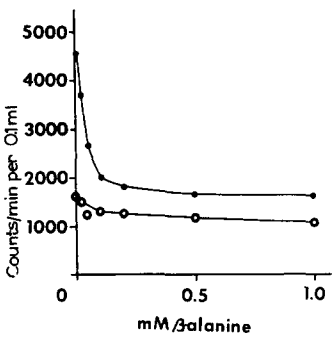
A comparison of the initial rates of uptake (performed at 1 min) for a number of amino acid substrates in strain 67 and 67/4MTR indicates that the transport defect in strain 67/4MTR is selective for only a few amino acids (Table I). L-Alanine, L-proline and β-alanine uptake is depressed in 67/4MTR; these amino acids share a common

TABLE I

SPECIFICITY OF THE TRANSPORT MUTATION

Amino acid	*Initial concn. in medium (M)*	*Counts/min per 0.1-ml cells**	
		Strain 67	*Strain 67/4MTR*
β-Alanine	$2 \cdot 10^{-5}$	950	125**
L-Proline	$2 \cdot 10^{-5}$	3900	1400**
L-Alanine	$2 \cdot 10^{-5}$	4100	2500**
L-Glutamate	$2 \cdot 10^{-5}$	1950	1750
L-Phenylalanine	$2 \cdot 10^{-5}$	2150	2050
L-Tryptophan	10^{-9} (carrier free)	80	125

* Measurements were performed at 1 min.
** Significantly lower than control ($P < 0.01$).



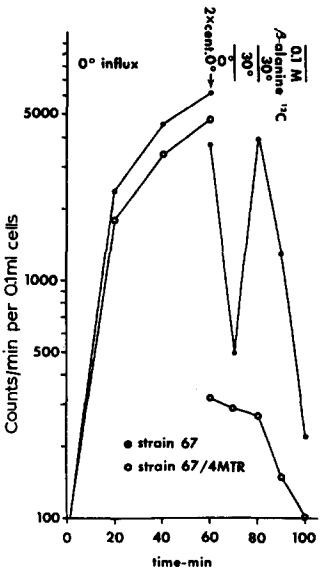


Fig. 2. L-Proline transport in strains 67 and 67/4MTR. Uniformly ^{14}C-labeled L-proline uptake was measured at 30° in the presence of β-alanine. The concentration of proline was $2 \cdot 10^{-5}$ M. 1-min uptakes were used as initial velocities. Uptake reaction was stopped by transferring 0.1-ml aliquots of incubation mixture onto Millipore filters. ●, strain 67; ○, strain 67/4MTR.

Fig. 3. Low temperature uptake of β-alanine. β-[1-^{14}C]Alanine ($1.8 \cdot 10^{-2}$ M) was added to the incubation mixture at 0°. After 1 h of uptake, incubation mixtures were centrifuged at 0° and washed with glucose–phosphate buffer. The cells were resuspended in buffer at 0° and incubated for another 10 min. The temperature of the incubation was then abruptly changed to 30°. Since the centrifugation step under these conditions removed more extracellular substrate from the incubation medium of 67/4MTR than from that of strain 67, a direct comparison of subsequent β-alanine influx at 30° could not be made. Extracellular concentrations were made equal by running parallel experiments with strain 67/4MTR until 70 min, when $2 \cdot 10^{-5}$ M β-[1-^{14}C]alanine was added to the medium. No uptake of ^{14}C occurred in this strain under these conditions. After 10 min of incubation at 30°, unlabeled β-alanine (10^{-1} M) was added to the incubation mixtures and the tubes were incubated for 20 min at 30°. ●, strain 67; ○, strain 67/4MTR.

system in strain 67 (ref. 6). At external concentration of $2 \cdot 10^{-5}$ M this transport system in strain 67 accounts for 2/3 of L-proline transport (v_{max} = 21 μmoles/min per ml intracellular fluid), 1/3 of L-alanine transport (v_{max} = 143 μmoles/min per ml intracellular fluid) and all of β-alanine transport (v_{max} = 1.5 μmoles/min per ml intracellular fluid). L-Tryptophan did not enter cells from strain 67 or 67/4MTR perhaps because in this species, the tryptophan permease may be reduced as is the case in *Pseudomonas acidovorans*[7].

The initial velocity of L-proline transport is reduced by 65% in strain 67/4MTR compared to strain 67 (Fig. 2). Moreover the sensitivity of L-proline transport to inhibition by β-alanine is negligible compared to strain 67.

Mechanism of the transport defect

In order to study various expressions of the transport deficient phenotype the uptake reaction was performed under conditions where the energy expenditure would be minimal. These conditions were achieved by allowing β-alanine influx to proceed at 0° at an external substrate concentration of $1.8 \cdot 10^{-2}$ M. The same rates of uptake were seen in strains 67 and 67/4MTR under these conditions (Fig. 3); after 1 h the distribution ratio was the same (0.35) in both strains. After 1 h the extracellular substrate was removed by centrifugation and the cells were placed in buffer containing no external amino acids. Strain 67/4MTR subsequently lost 90% of its internal pool while strain 67 lost only 30%. Further incubation at 0° for 10 min in the absence of external substrate resulted in efflux of most of the remaining internal pool in strain 67 but with little additional loss occurring in strain 67/4MTR. When the temperature of the medium was then elevated to 30° for 10 min the β-alanine pool reformed in strain 67 but not in strain 67/4MTR. The ^{14}C pools were then depleted in both strains by exchange with external β-[^{12}C]alanine at 30° in both strains. Strain 67/4MTR thus retained the ability to catalyze passive uptake of β-alanine at low temperature, and to exchange the internal pool with external β-alanine. On the other hand the ability to form and maintain pools of β-alanine against a concentration gradient is absent in this strain.

Strain 67 and 67/4MTR were preloaded with β-[1-^{14}C]alanine ($1.8 \cdot 10^{-2}$ M) at 0° for 40 min. Tracer amounts of β-[^{3}H]alanine were then added to the medium. The initial rate of ^{3}H influx, representing exchange diffusion is then more rapid than the uptake of ^{14}C which represents net accumulation (Fig. 4). The internal ^{3}H pool declines subsequently until a second phase of ^{3}H uptake appears at a rate similar to the slow accumulation of ^{14}C. In this experiment the ^{14}C distribution ratio did not exceed 0.45.

The anticipated interaction of L-proline with β-alanine transport is altered in strain 67/4MTR. The 2-min uptake and the steady-state accumulation (30 min) of β-alanine ($1.8 \cdot 10^{-2}$ M) is normally inhibited by L-proline (10^{-2} M) in strain 67 (Fig. 5). Under the same conditions, L-proline enhances the steady-state accumulation of β-alanine in mutant 67/4MTR by at least 3-fold (Fig. 5). This competitive stimulatory[8,9] effect of L-proline on β-alanine transport in strain 67/4MTR can be explained if L-proline enters the cell by a second system which excludes β-alanine and exchanges with β-alanine on a primary system shared by both amino acids. In strain 67, two active transport systems for L-proline have been described, only one of which is shared with β-alanine[6]. If strain 67/4MTR has lost the ability to accumulate L-proline and

β-alanine against a gradient by a shared system but has retained the ability to concentrate L-proline on a second system, then L-proline can enter and exchange with β-alanine. The data in Fig. 5 support this interpretation and imply that the site for β-alanine uptake has not been affected by the mutation.

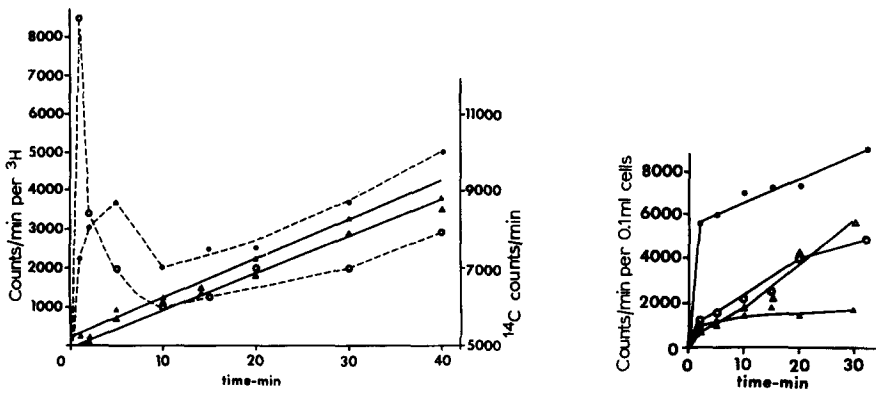


Fig. 4. Uptake and exchange of β-alanine in strains 67 and 67/4MTR. Cells were preincubated with β-[1-^{14}C]alanine ($1.8 \cdot 10^{-2}$ M) for 40 min at 0°. After 40 min a trace of β-[^{3}H]alanine was added and ^{14}C and ^{3}H uptake were monitored by scintillation methods. ●, ^{3}H strain 67; ○, ^{3}H strain 67/4MTR; ▲, ^{14}C strain 67; △, ^{14}C strain 67/4MTR.

Fig. 5. Effect of L-proline on β-alanine uptake in strains 67 and 67/4MTR. β-[1-^{14}C]Alanine ($1.8 \cdot 10^{-2}$ M) was incubated with cells in the absence and in the presence of L-proline (10^{-2} M) at 30°. ●, strain 67, β-[1-^{14}C]alanine; ▲, strain 67/4MTR, β-[1-^{14}C]alanine;, ○, strain 67, β-[1-^{14}C]-alanine *plus* L-proline; △, strain 67/4MTR, β-[1-^{14}C]alanine *plus* L-proline.

β-Alanine metabolism in mutant 4MTR

When wild-type *Pseudomonas fluorescens* is grown on β-alanine as the sole source of nitrogen the specific activity of β-alanine transaminase is 4-fold higher than the constitutive levels found when the organism is grown on $(NH_4)_2SO_4$ (ref. 4). Incubation of β-alanine-grown wild-type cells with β-[1-^{14}C]alanine results in the rapid evolution of $^{14}CO_2$. On the other hand strain 67 retains β-alanine in a metabolically inert pool because it is a blocked catabolic mutant deficient in β-alanine transaminase[4].

Preincubation of the wild-type cells with aminooxyacetic acid simulates the metabolic block of mutant strain 67 (Fig. 6A). A soluble pool of β-alanine is achieved but little $^{14}CO_2$ is produced. Preincubation of strain 4MTR (permease negative, transaminase positive) with aminooxyacetic acid does not result in accumulation of free β-alanine (Fig. 6B). β-Alanine can nonetheless be catabolized by 4-methyl-DL-tryptophan resistant cells, since $^{14}CO_2$ is produced from β-[1-^{14}C]alanine in the absence of aminooxyacetic acid.

The entry of β-alanine into 4-methyl-DL-tryptophan resistant cells is apparently mediated since L-proline inhibits the rate of $^{14}CO_2$ evolution from β-[1-^{14}C]alanine in intact 4MTR cells. This effect is not a reflection of metabolic inhibition since L-proline does not affect the catabolism of β-alanine when the membrane of wild-type cells is disrupted by toluene treatment.

Effux of β-alanine in mutant 67/4MTR

The rate of efflux of β-[1-^{14}C]alanine was measured in strains 67 and 67/4MTR preloaded with $1.8 \cdot 10^{-2}$ M β-alanine to the same internal concentration at 0° with substrate. Efflux at 25° is more complete in the permeaseless mutant (Fig. 7) suggesting that the primary transport defect in this strain is an inability to retain substrate after uptake. A defect in the "barrier function" of the membrane is implied.

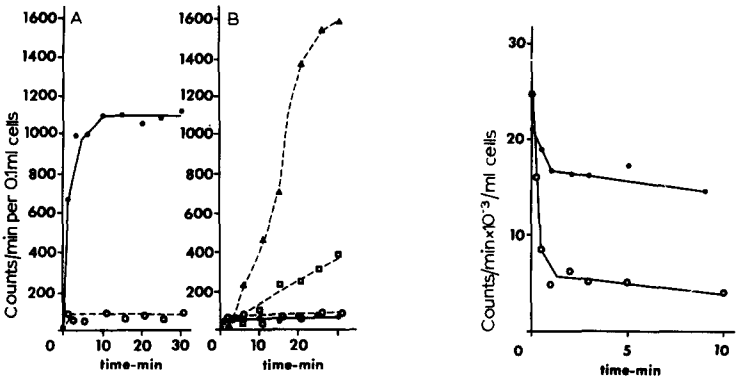


Fig. 6. β-Alanine metabolism in wild-type (A) and 4MTR cells (B). Solid lines indicate ^{14}C in soluble pool; interrupted lines indicate formation of $^{14}CO_2$. Incubations performed with β-[1-^{14}C]-alanine ($2 \cdot 10^{-5}$ M) at 30° in Warburg flasks containing 0.2 ml 20% KOH in center well. Filled and open circles, 30-min preincubation with aminooxyacetic acid (5 mM); △, no aminooxyacetic acid; □, in presence of L-proline (10^{-2} M).

Fig. 7. Efflux of β-alanine from preloaded cells. Cells were preloaded by incubation with $1.8 \cdot 10^{-2}$ M β-[1-^{14}C]alanine for 1 h at 0°. Efflux was measured by withdrawing an aliquot from the preloaded incubation mixture, centrifuging twice, and placing cells on a filter connected to a vacuum flask with a spring clip on the vacuum tubing. One ml of amino acid-free buffer at 25° was then added and efflux allowed until the reaction was stopped when the vacuum was applied by releasing the clip at specified intervals. The blank consisted of an identical incubation mixture with boiled cells. ●, strain 67; ○, strain 67/4MTR.

DISCUSSION

A large number of transport mutants in biological species have been described[10]. One example of the detailed analysis required to define mutant transport phenotypes in microorganisms is found in the description by LUBIN *et al.*[11] of a proline transport mutant in *E. coli*, strain W6. This mutant can catalyze neither concentrative uptake of L-proline nor carry out low temperature homoexchange[12]. Moreover the residual uptake of L-proline in W6 exhibits no saturation of transport velocity with respect to substrate concentration[13]. These findings are consistent with a mutation which effectively deletes the proline binding site. The type of transport mutant exemplified by the W6 mutant of *E. coli* must be distinguished from another class of transport mutants, of which *E. coli* strain ML 308-22 (ref. 14) and the present strains of *Pseudomonas fluorescens*, designated 4MTR and 67/4MTR, are apparently the first recognized examples. In the latter phenotypes, the transport carriers retain their binding properties for substrate; however in both cases the carrier is unable to achieve or maintain an internal pool despite its ability to bind and transfer substrate.

The transport mutants described here and elsewhere[14] suggest that gene product other than membrane 'binding' or 'transport' proteins[15,16] are involved in the

transport of a single substrate against a concentration gradient. This hypothesis receives indirect support from the characteristics of certain transport mutants. The observations by BOOS[17] and PARDEE *et al.*[18], that a number of transport-deficient bacterial mutants do yield soluble binding proteins when subjected to osmotic shock, suggests that a functionally active transport system requires the participation of another gene product in addition to the binding protein.

One may ask whether the altered function in the class of transport mutants described in the present work (transport negative, binding protein positive) is concerned with the coupling of energy to the carrier. There is, at present, no molecular model which explains how an energy coupling protein would react with a carrier in a bacterial active transport system. KUNDIG *et al.*[19] have described protein components in the carbohydrate–phosphate transferase system which confer a lower K_m on the enzyme which transfers phosphate to transport substrates[20,21]. The details of this reaction may be of relevance in more conventional active transport systems.

Models for active transport in bacteria have been proposed by SCARBOROUGH *et al.*[22] and by WINKLER AND WILSON[3]. SCARBOROUGH *et al.*[22] postulate that the transport carrier maintains concentration gradients of substrate by oscillating between an inactive state in response to intracellular K^+ and an active state in response to the presence of ATP in the periplasmic space. WINKLER AND WILSON[3] on the other hand, have proposed the existence of a second component of the transport system which, when complexed with the carrier couples energy to the transport system and allows unidirectional movement of substrate. On the basis of our experiments alone it is not possible to distinguish between the two models. A genetic analysis of the lactose transport mutant ML 308-22 should at least determine whether this mutation maps in the same cistron as does the carrier deficient lactose permease mutations.

A question of importance to the mechanism of active transport is: on what side of the membrane does activation of the transport site occur? In mammalian cells asymmetry in the flux of substrate is caused by the binding of Na^+ to the site on the external face of the membrane resulting in an increase of affinity of carrier for substrate[23]. Asymmetry is maintained by pumping Na^+ out of the cell. A number of investigators have found that uncoupling of energy from transport in bacteria results in an increase of the rate constant for efflux and no change in the rate of influx[1,3]. Our results support this view. In *Saccharomyces cerevisiae* on the other hand, active transport of α-glucosides is associated with a decrease in the K_m for influx[2].

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REFERENCES

1 B. L. HORECKER, J. THOMAS AND J. MONOD, *J. Biol. Chem.*, 235 (1960) 1586.
2 H. O. HALVORSON, H. OKADA AND J. GORMAN, *The Cellular Function of Membrane Transport*, Prentice Hall, Englewood Cliffs, p. 171.
3 H. H. WINKLER AND T. H. WILSON, *J. Biol. Chem.*, 241 (1966) 2200.

4 P. HECHTMAN, C. R. SCRIVER AND R. B. MIDDLETON, *J. Bacteriol.*, in the press.
5 O. HAYAISHI, Y. NISHIZUKA, M. TATIBANA, M. TAKESHITA AND S. KUNO, *J. Biol. Chem.*, 236 (1961) 781.
6 P. HECHTMAN AND C. R. SCRIVER, *J. Bacteriol.*, in the press.
7 H. ROSENFELD AND P. FEIGELSON, *J. Bacteriol.*, 97 (1969) 705.
8 J. A. SCHAFER AND J. A. JACQUEZ, *Biochim. Biophys. Acta*, 135 (1967) 741.
9 J. A. JACQUEZ, *Biochim. Biophys. Acta*, 135 (1967) 751.
10 C. R. SCRIVER AND P. HECHTMAN, *Advances in Human Genetics*, Plenum Press, New York, 1970, p. 215.
11 M. LUBIN, D. H. KESSEL, A. BUDREAU AND J. D. GROSS, *Biochim. Biophys. Acta*, 42 (1960) 535.
12 D. KESSEL AND M. LUBIN, *Biochim. Biophys. Acta*, 57 (1962) 32.
13 H. R. KABACK AND E. R. STADTMAN, *Proc. Natl. Acad. Sci. U.S.*, 55 (1966) 920.
14 P. T. S. WONG, E. R. KASHKET AND T. H. WILSON, *Proc. Natl. Acad. Sci. U.S.*, 65 (1970) 63.
15 L. H. HEPPEL, *Science*, 156 (1967) 1451.
16 J. R. PIPERNO AND D. L. OXENDER, *J. Biol. Chem.*, 241 (1966) 5732.
17 W. BOOS, *European J. Biochem.*, 10 (1969) 66.
18 A. B. PARDEE, L. S. PRESTIDGE, M. B. WHIPPLE AND J. DREYFUSS, *J. Biol. Chem.*, 241 (1966) 3962.
19 W. KUNDIG, S. GHOSH AND S. ROSEMAN, *Proc. Natl. Acad. Sci. U.S.*, 52 (1964) 1967.
20 R. D. SIMONI, M. F. SMITH AND S. ROSEMAN, *Biochem. Biophys. Res. Commun.*, 31 (1968) 804.
21 T. E. HANSON AND R. L. ANDERSON, *Proc. Natl. Acad. Sci. U.S.*, 61 (1968) 269.
22 G. A. SCARBOROUGH, M. K. RUMLEY AND E. P. KENNEDY, *Proc. Natl. Acad. Sci. U.S.*, 60 (1968) 951.
23 R. K. CRANE, *Federation Proc.*, 24 (1965) 1000.