JOURNAL OF BACTERIOLOGY, Apr. 1968, p. 1419-1424
Vol. 95, No. 4

Oxidation of D-Amino Acids by a Particulate Enzyme from *Pseudomonas aeruginosa*

VINCENT P. MARSHALL AND JOHN R. SOKATCH
Department of Microbiology, University of Oklahoma School of Medicine, Oklahoma City, Oklahoma 73104

Received for publication 20 January 1968

A particulate D-amino acid dehydrogenase has been partially purified from cell free extracts of *Pseudomonas aeruginosa* grown on DL-valine as the source of carbon and energy. A standard assay was developed which utilized 2,6-dichlorophenol-indophenol as the electron acceptor. The *p*H optimum for enzyme activity ranged from 6.0 to 8.0, depending on the amino acid assayed. The enzyme was most active with monoamino-monocarboxylic amino acids and histidine. The Michaelis constant for D-phenylalanine was found to be 1.3×10^{-3} M D-phenylalanine. Constants could not be calculated for the other amino acids oxidized because anomalous plots of V as a function of V/S were obtained. Spectra of enzyme preparations reduced with D-valine or sodium hydrosulfite exhibited adsorption bands typical of the α, β, and γ bands of cytochromes as well as bleaching in the flavin region of the spectrum. When DL-valine was added to a medium with glycerol as the energy source, D-amino acid dehydrogenase was detected after the addition of valine and was produced at a rate directly proportional to the synthesis of total protein. The enzyme was formed when D-valine, L-valine, or DL-alanine was the source of carbon and energy, but not when glucose, glycerol, or succinate was the energy source.

Early studies of the oxidation of D-amino acids by bacteria were made by Bernheim et al. (1) with whole cells of *Proteus* and by P. K. Stumpf and D. E. Green (Federation Proc. 5:I 157, 1946) with a cell-free extract prepared from *Proteus morganii*. The latter authors were the first to show that oxidation of amino acids by bacterial enzyme preparations utilized one atom of oxygen per mole of amino acid oxidized. This result is characteristic of D-amino acid oxidation by bacterial enzyme preparations and probably reflects the close association of the oxidase with the cytochrome system in these organisms. Yoneya and Adams (16), described an enzyme in *Pseudomonas striata* which catalyzed the oxidation of D-allohydroxyproline and which was induced by growth on L-hydroxyproline to Δ^1-pyrroline-4-hydroxy-2-carboxylate, which was then oxidized to α-ketoglutarate. Tsukada (13) purified two separate D-amino acid dehydrogenases from *P. fluorescens;* one was constitutive and utilized 2,6-dichlorophenol-indophenol as the electron acceptor; the other was induced by growth on D-tryptophan and utilized methylene blue as the electron acceptor.

Reports from our laboratory have shown that *P. aeruginosa* was able to grow in a medium with DL-valine as the sole carbon source. Growth in this medium resulted in ability of the organism to oxidize several D-amino acids, which could be demonstrated by the reduction of a dye when purified cell membrane preparations were incubated with D-alanine (8) or by the uptake of oxygen when sonic extracts were incubated with a number of D-amino acids (9). To evaluate the role of D-amino acid oxidation in the growth of *P. aeruginosa* on valine, we undertook the characterization of the enzyme responsible for the oxidation of D-amino acids and of the conditions which cause its induction.

MATERIALS AND METHODS

Organism and cultural conditions. The strain of *P. aeruginosa* used in these studies was the same strain used in earlier studies; it was grown in an inorganic salts medium with a single carbon source, as specified in the next paragraph, and was maintained in the same medium with 0.3% DL-valine as the carbon and energy source (8). The organism was grown in 14-liter batches of medium with aeration in a biO-Kulture fermentor produced by Fermentation Design, Inc., Fogelsville, Pa. When the culture approached stationary phase, the cells were harvested by means of the continuous-flow attachment to the Sorvall RC-2 refrigerated centrifuge. The cells were frozen and kept at —15 C.

For the studies reported in Table 4, 1 ml of a culture of *P. aeruginosa* in 0.1% glycerol medium was used to inoculate 50 ml of a medium containing 0.3% of either D-valine, L-valine, DL-alanine, glucose, suc-

cinate, or glycerol. The increase in cell population was followed by measuring the increase in turbidity, and, as the culture approached the stationary phase, the organisms were harvested by centrifugation, resuspended in 90 ml of water, centrifuged again, and finally suspended in 15 ml of phosphate buffer (0.05 M, *p*H 8.0). Cell-free extracts of the organism were prepared by sonic oscillation as outlined under *Enzyme purification.*

In the study of the kinetics of induction of D-amino acid dehydrogenase, 10 liters of medium with 0.3% glycerol as the energy source was inoculated with 1 liter of a culture grown in the same medium. The change in turbidity of the culture was followed spectrophotometrically, and 10 g of DL-valine was added in 150 ml of water after 7 hr of incubation, by which time the turbidity began to increase noticeably. Samples of 90 ml were taken at 90-min intervals, two samples being taken prior to the addition of valine. The cells were harvested, resuspended in 15 ml of 0.05 M phosphate buffer (*p*H 8.0), and broken by sonic oscillation as outlined in the following paragraph. The extract was dialyzed against the same buffer and assayed for enzymatic activity with DL-valine as the substrate as outlined under *Enzymatic methods.*

Enzyme purification. A 5-g amount of frozen cell paste was suspended in 15 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 9.0) containing 3 mg of deoxyribonuclease. The suspension was stirred at room temperature until it became fluid, and the cells were broken by sonic oscillation in a Raytheon 10-kc sonic oscillator for 15 min. The sonic extract was centrifuged for 15 min at 17,000 × *g*, and the pellet was discarded. The cell-free extract was treated with calcium phosphate gel (5), with 1.5 mg of gel solids per mg of protein. The mixture was stirred for 15 min in an ice bath, and the suspension was centrifuged at 3,000 × *g* for 10 min. The supernatant fluid from the calcium phosphate gel treatment was next treated with alumina C γ gel (15) at 2.0 mg of gel solids per mg of protein. This mixture was stirred in an ice bath for 10 min and then centrifuged for 10 min at 3,000 × *g*. The supernatant fluid from the centrifugation was brought to 45% saturation by adding 0.258 g of solid ammonium sulfate per ml, and the resulting precipitate was centrifuged for 15 min at 40,000 × *g*. The pellet was suspended in 10 ml of 0.05 M phosphate buffer (*p*H 7.5), and the enzyme was dialyzed against 1 liter of the same buffer for 1 hr in a cold room. This preparation was used throughout the studies reported here.

Enzymatic methods. Enzymatic activity was assayed by following the reduction of 2,6-dichlorophenol-indophenol at 600 mμ with a Beckman model DU spectrophotometer equipped with a Gilford model 2000 multiple absorbance recorder. The standard assay was performed with two cuvettes and contained the following components in a volume of 3.0 ml: 50 μmoles of D-valine or 100 μmoles DL-valine, 300 μmoles each of potassium phosphate and glycine (*p*H 8.0), 20 μmoles of NaCN (*p*H 8.0), 40 μg of 2,6-dichlorophenol-indophenol, enzyme. The second cuvette was a control which had all of the ingredients except amino acid. The reaction was started by the addition of enzyme, and rates of 2,6-dichlorophenol-indophenol reduction were calculated by use of a molar extinction coefficient of 21 (12). One unit of enzyme was defined as that amount which caused a reduction of 1 μmole of 2,6-dichlorophenol-indophenol per min. Specific activity was defined as units per milligram of protein. When amino acids other than valine were used in the standard assay, the phosphate-glycine buffer was adjusted to the *p*H which was optimal for the reduction of 2,6-dichlorophenol-indophenol with that amino acid.

The protocol for the standard assay was also used for the determination of the *p*H optima for the oxidation of the amino acids tested. The *p*H values were adjusted from 5.0 to 10.0 with 0.5 *p*H unit increments. A phosphate-acetate buffer was used for *p*H values below 6.0. The protocol for the studies of substrate specificity was similar to that used in the standard assay, except that 100 μmoles of amino acid were added and 300 μmoles of phosphate buffer (*p*H 7) were used in a reaction volume of 3.0 ml.

Studies of the absorption spectra of oxidized and reduced enzymes were carried out in Thunberg tubes fitted with quartz cuvettes. The reaction mixture contained 7.9 mg of enzyme, 10 μmoles of D-valine or a few crystals of sodium hydrosulfite, 0.6 ml of glycerol, and water to a volume of 3.0 ml. The cuvettes were evacuated and sealed, and valine or sodium hydrosulfite was tipped in from the side arm. Optical densities of oxidized and reduced enzyme preparations were read from 400 to 625 mμ at intervals of 5 mμ, except in the region 510 to 580 mμ where readings were made at intervals of 2 mμ. The enzyme preparations were read against a blank containing water and glycerol, and difference spectra were obtained by subtracting the readings of the oxidized enzyme from the readings of the reduced enzyme preparations.

Analytical and chemical methods. Protein concentration was determined by the method of Waddell (14). The substrates used in this study were all commercial preparations, and the amino acids were chromatographed before use by the method of Redfield (10). It was necessary to recrystallize methionine from aqueous solution before use. L-Valine used to induce formation of D-amino acid dehydrogenase was first treated with hog kidney D-amino acid oxidase (2) to remove any contaminating D-valine. L-valine was recovered by dialysis of the incubation mixture against a known volume of water, and this solution was then used to prepare the growth medium. The concentrations of D-amino acids in solutions used for kinetic studies and studies of substrate specificity were determined by oxidation with hog kidney D-amino acid oxidase (2), except in the case of D-histidine and D-allohydroxyproline; the concentrations of the latter two amino acids were estimated by a determination of the nitrogen content of the solutions (6).

RESULTS

Purification of D-amino acid dehydrogenase. The rate of dye reduction in the standard assay was directly proportional to enzyme concentration up to a rate of 5 mμmoles 2,6-dichloro-

phenol-indophenol reduced per min (Fig. 1), and the enzyme concentration in assays was adjusted so that the rate would not exceed this value. Cyanide was required in the assay; it presumably functioned by inhibiting the action of the cytochrome system which competed with the dye for electrons. This assay was more sensitive than the earlier manometric assay (9) and could be used with crude enzyme preparations with appropriate corrections for endogenous enzyme activity. When Tris buffer was used, no activity was obtained with D-valine as the substrate. This observation explained the failure to detect D-valine oxidation in an earlier study where a dye reduction assay was used to measure D-alanine oxidation by cell membranes of *P. aeruginosa* (8).

Purification of the enzyme by the procedure outlined in Materials and Methods yielded a preparation which was 10- to 13-fold purified, as determined by assay with either D-valine or D-phenylalanine (Table 1). The enzyme was exceedingly unstable (100% of the activity was lost after 40 min at 37 C); consequently, all determinations were made on the same day the enzyme was prepared. When the partially purified enzyme was centrifuged for 1 hr at 100,000 × *g* with a Spinco model L centrifuge, all of the activity was found in the pellet.

Optimal pH. The *p*H optimum for enzyme activity ranged from 6.0 with D-methionine and D-alanine to 8.0 with D-valine (Table 2). The *p*H optimum for valine oxidation in the manometric assay was close to 9.0(9).

Enzyme activity as a function of substrate concentration. Studies of the rates of 2,6-dichlorophenol-indophenol reduction as a function of substrate concentration yielded what appeared to be typical substrate saturation curves for D-valine, D-alanine, D-phenylalanine, D-serine, D-methionine, D-histidine, and D-allohydroxyproline when

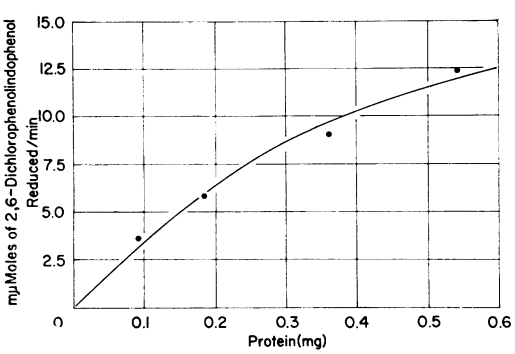

FIG. 1. *Reduction of 2,6-dichlorophenol-indophenol as a function of enzyme concentration.*

TABLE 1. *Purification of D-amino acid dehydrogenase from Pseudomonas aeruginosa*

Substance used in standard assay	Protein	Units	Specific activity
D-Valine	*mg*		*units/mg*
Sonic extract	659	2.7	0.004
Enzyme treated with calcium phosphate gel	257	2.5	0.010
Enzyme treated with alumina C_γ gel	59	1.4	0.024
Precipitate obtained with ammonium sulfate	38	1.5	0.039
D-Phenylalanine			
Sonic extract	512	2.3	0.004
Enzyme treated with calcium phosphate gel	249	2.7	0.011
Enzyme treated with alumina C_γ gel	76	2.0	0.026
Precipitate obtained with ammonium sulfate	31	1.7	0.054

TABLE 2. *Optimal pH values for oxidation of D-amino acids by the particulate enzyme of Pseudomonas aeruginosa*

Amino acid	*p*H
D-Valine	8.0
D-Histidine	7.3
D-Serine	7.0
D-Allohydroxyproline	7.0
D-Phenylalanine	6.5
D-Alanine	6.0
D-Methionine	6.0

$1/V$ was plotted as a function of $1/S$. However, when the data were plotted by the method of Hofstee (3), only those obtained with D-phenylalanine were linear. The K_m for D-phenylalanine was estimated to be 1.3×10^{-3} M; since plots of data with all other D-amino acids were curved, K_m was not calculated for these substrates. A typical result is shown in Fig. 2, where V as a function of V/S is plotted with D-valine as the substrate.

Substrate specificity. The substrate specificity of the partially purified D-amino acid dehydrogenase of *P. aeruginosa* (Table 3) was similar to that of the crude enzyme preparation of the same organism assayed manometrically (9). D-Alanine was the most rapidly oxidized of all of the substrates tested, with D-phenylalanine second and D-valine and D-norvaline next. In contrast to the crude enzyme, L-alanine was oxidized very slowly, which probably reflects removal of the alanine racemase during purification. All substrates were tested at *p*H 7.0, which represents a compromise

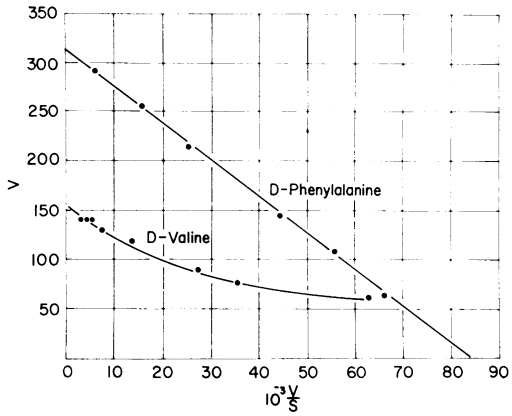

FIG. 2. *Hofstee plots of V as a function of V/S when the dye reduction assay was used to follow the oxidation of* D*-valine and* D*-phenylalanine. The dimensions of V are micromoles of 2,6-dichlorophenol-indophenol reduced per minute per liter and the dimensions of S are millimoles of* D*-valine or* D*-phenylalanine per liter.*

TABLE 3. *Substrate specificity of* D*-amino acid dehydrogenase*

Substrate	Specific activity[a]
D-Alanine	0.049
L-Alanine	0.006
D-Allohydroxyproline	0.020
D-Alloisoleucine	0.010
Glucose	0.008
D-Histidine	0.038
D-Leucine	0.024
D-Methionine	0.036
D-Norvaline	0.044
D-Phenylalanine	0.045
D-Proline	0.022
D-Serine	0.040
D-Threonine	0.030
D-Valine	0.044

[a] Activity less than 0.004 μmole per min per mg of protein was observed with the following substrates: α-aminoisobutyric acid, D- and L-arginine, D- and L-aspartic acid, D- and L-glutamic acid, glycine, L-histidine, L-hydroxyproline, L-isoleucine, L-leucine, D- and L-lysine, L-methionine L-novaline, L-phenylalanine, L-proline, L-serine, L-threonine, D- and L-tryptophane, and L-valine.

of their *p*H optima, and therefore there may ultimately be some revision in the order of activity.

Absorption spectra of D*-amino acid dehydrogenase.* The enzyme was suspended in glycerol to reduce light scattering and was reduced with D-valine or sodium hydrosulfite under anaerobic conditions in Thunberg cuvettes. Absorption maxima were observed at 412 mμ with the enzyme reduced by D-valine and at 420 mμ with the enzyme reduced with sodium hydrosulfite (Fig. 3). Maxima were also observed in the region of 525 and 555 mμ in the case of enzyme preparations reduced either with valine or hydrosulfite. Plots of difference spectra revealed peaks due to α, β, γ bands at 552, 522, 425, and 422 mμ (Fig. 4). These spectra indicate that the main cytochrome present in these preparations is cytochrome *c*, although occasionally a shoulder was observed at 560 mμ, suggesting the presence of cytochrome *b*. In studies with cell membranes from this organ-

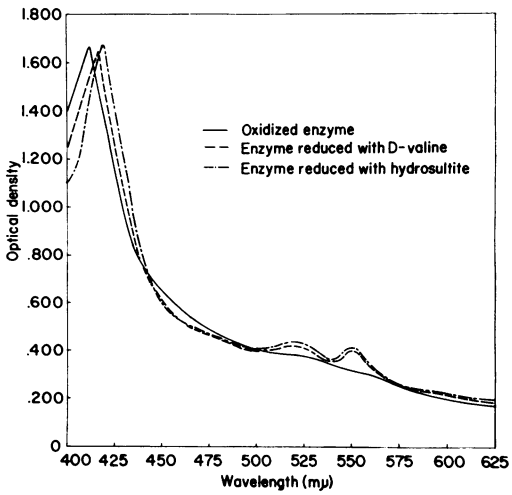

FIG. 3. *Absorption spectra of oxidized and reduced* D*-amino acid dehydrogenase preparations from Pseudomonas aeruginosa.*

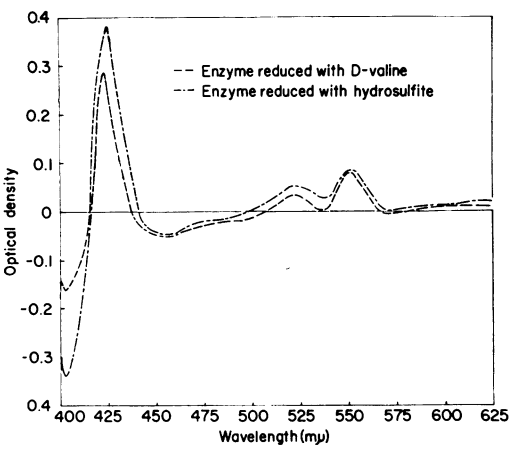

FIG. 4. *Difference spectra of* D*-amino acid dehydrogenase preparations from Pseudomonas aeruginosa reduced with* D*-valine or sodium hydrosulfite. The spectrum of the oxidized enzyme was subtracted from the spectra of the reduced enzyme preparations in order to obtain the difference spectra.*

ism, a distinct shoulder at 560 mμ was obtained with D-alanine as the reducing agent (8). Bleaching was evident in the region of 450 mμ, which may be due to the reduction of flavin (11).

Kinetics of induction of D-amino acid dehydrogenase. P. aeruginosa was inoculated into 10 liters of medium with glycerol as the energy source, and, when the culture entered the exponential phase of growth, DL-valine was added as an inducer of D-amino acid dehydrogenase. Samples of the culture were withdrawn, and sonic extracts were prepared and tested for the presence of D-amino acid dehydrogenase as outlined in Materials and Methods. There was no detectable D-amino acid dehydrogenase before the addition of the inducer, but it appeared promptly after the addition of DL-valine (Fig. 5). Enzyme activity was proportional to the protein concentration; that is, enzyme was synthesized as a constant fraction of the protein formed by the growing culture. These are the usual kinetics obtained in studies of the formation of induced enzymes (4).

Effect of the energy source on the formation of D-amino acid dehydrogenase. P. aeruginosa was grown in media with different energy sources, and enzyme extracts were tested for their ability to oxidize both isomers of valine, alanine, phenylalanine, methionine, and histidine (Table 4). Extracts prepared from cells grown on glucose, succinate, or glycerol were unable to oxidize D-amino acids. However, cells grown on either isomer of valine or in DL-alanine medium catalyzed the oxidation of the D- but not the L-amino acid substrates (Table 4). It is interesting that growth on either isomer of valine resulted in induction of the oxidase, since *P. aeruginosa* does not contain valine racemase (9).

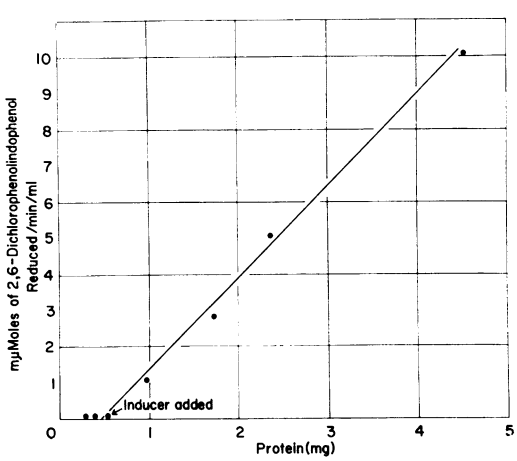

FIG. 5. *Kinetics of induction of D-amino acid dehydrogenase by DL-valine.*

TABLE 4. *Effect of the energy source on the formation of D-amino acid dehydrogenase*

Substrate	Energy source		
	L-Valine	D-Valine	DL-Alanine
D-Valine	0.005[a]	0.004	0.013
L-Valine	0	0	0
D-Alanine	0.007	0.006	0.011
L-Alanine	0.001	0.001	0.002
D-Phenylalanine	0.009	0.007	0.010
L-Phenylalanine	0	0	0
D-Methionine	0.009	0.007	0.007
L-Methionine	0	0	0
D-Histidine	0.005	0.004	0.010
L-Histidine	0	0	0

[a] Specific activity of extract; 0 = no measurable rate. D-Amino acid dehydrogenase activity was not detected in cells grown on glycerol, glucose, or succinate medium.

DISCUSSION

It has been established that the D-allohydroxyproline oxidase of *P. striata* functions in the metabolism of hydroxyproline by this organism, since the D-isomer is an obligatory intermediate in the metabolism of hydroxyproline (16). The results reported here show that the D-amino acid dehydrogenase of *P. aeruginosa* is a particulate enzyme which is induced by growth on DL-valine. Since the organism can also grow in media with D-valine as the sole carbon source and not containing valine racemase, it seems reasonable to conclude that the D-amino acid dehydrogenase of *P. aeruginosa* functions by catalyzing the oxidation of D-valine when this isomer is present in the medium. This may also be true of the D-amino acid dehydrogenases of *P. fluorescens*, although one of the two enzymes studied by Tsukada was constitutive (13). The question of why a general D-amino acid dehydrogenase should be of benefit to an organism is still unanswered, however. A number of naturally occurring D-amino acids are known, yet one of the most common, D-glutamate, is not attacked by any of the enzymes mentioned above.

There are several similarities among the enzymes from kidney (7), *P. fluorescens* (13), and *P. aeruginosa*. All of the enzymes catalyze the oxidation of monoamino-monocarboxylic D-amino acids, and none catalyze the oxidation of D-glutamate or D-aspartate. The prosthetic group of the kidney enzyme is flavin adenine dinucleotide and is easily separated from the protein, which can then be reactivated by flavin adenine dinucleotide. There is circumstantial evidence

that flavin adenine dinucleotide is also the prosthetic group of the bacterial enzymes, but there are no reports of the reactivation of an apoenzyme. Tsukada showed that the oxidized forms of both enzymes which he studied exhibited absorption bands in the region of 450 mμ, which disappeared when D-leucine was added. The absorption spectra of the particulate enzyme of *P. aeruginosa* also exhibited bleaching at 450 mμ when reduced with D-valine.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation research grant GB 4525, Public Health Service research grant AM09750 from the National Institute of Arthritis and Metabolic Diseases, and Public Health Service Research Career Development Award 2-K3-GM-18,343 made to the second author.

LITERATURE CITED

1. BERNHEIM, F., M. L. C. BERNHEIM, AND M. D. WEBSTER. 1935. Oxidation of certain amino acids by "resting" *Bacillus proteus*. J. Biol. Chem. **110**:165–172.
2. BOULANGER, P., AND R. OSTEUX. 1963. D-Amino acids, p. 367–370. *In* H. U. Bergmeyer [ed.], Methods of enzymatic analysis. Academic Press, Inc., New York.
3. HOFSTEE, B. H. J. 1952. On the evaluation of the constants Vm and Km in enzyme reactions. Science **116**:329–331.
4. JACOB, F., AND J. MONOD. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. **3**:318–356.
5. KEILIN, D., AND E. F. HARTREE. 1938. On the mechanism of the decomposition of hydrogen peroxide by catalase. Proc. Roy. Soc. (London) Ser. B. **124**:397–405.
6. LANG, C. A. 1958. Simple microdetermination of Kjeldahl nitrogen in biological materials. Anal. Chem. **30**:1692–1694.
7. MEISTER, A. M. 1965. Biochemistry of amino acids, p. 297–304. Academic Press, Inc., New York.
8. NORTON, J. E., G. S. BULMER, AND J. R. SOKATCH. 1963. The oxidation of D-alanine by cell membranes of *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **78**:136–147.
9. NORTON, J. E., AND J. R. SOKATCH. 1966. Oxidation of D- and L-valine by enzymes of *Pseudomonas aeruginosa*. J. Bacteriol. **92**:116–120.
10. REDFIELD, R. R. 1953. Two-dimensional paper chromatographic system with high resolving power for amino acids. Biochim. Biophys. Acta **10**:344–345.
11. STANIER, R. Y., I. C. GUNSALUS, AND C. F. GUNSALUS. 1953. The enzymatic conversion of mandelic acid to benzoic acid. II. Properties of the particulate fractions. J. Bacteriol. **66**:543–547.
12. STEYN-PARVE, E. P., AND H. BEINERT. 1958. On the mechanism of dehydration of fatty acyl derivatives of coenzyme A. IV. Isolation and properties of stable enzyme-substrate complexes. J. Biol. Chem. **233**:843–861.
13. TSUKADA, K. 1966. D-Amino acid dehydrogenases of *Pseudomonas fluorescens*. J. Biol. Chem. **241**:4522–4528.
14. WADDELL, W. J. 1956. A simple ultraviolet spectrophotometric method for the determination of of protein. J. Lab. Clin. Med. **48**:311–314.
15. WILLSTÄTTER, R., AND H. KRAUT. 1923. Über ein Tonerde-gel von der Formel $Al(OH)_3$. II. Mitteilung über Hydrate und Hydrogele. Chem. Ber. **56**:1117–1121.
16. YONEYA, T., AND E. ADAMS. 1961. Hydroxyproline metabolism. J. Biol. Chem. **236**:3272–3279.