data for ATP release show ratios of k_1/k_2 that differ by a factor of 10. In addition, several of the curves of ATP release that were tested had time ratios less than the theoretical minimum of Figure 7, excluding series first order as a possible mechanism.

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Membrane D-Lactate Dehydrogenase from *Escherichia coli*. Purification and Properties†

Masamitsu Futai

ABSTRACT: D-Lactate dehydrogenase has been solubilized from the membrane of *Escherichia coli* and purified to a practically homogeneous state using conventional procedures. The enzyme had a pH optimum of 8–9 and an approximate molecular weight of 72,000 (acrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate) or 71,000 (sucrose density gradient). It contains flavine, which was found to be flavine adenine dinucleotide by the D-amino acid oxidase test and the characteristic fluorescence change as a function of pH. Fluorescence of the flavine in the purified enzyme, which is quenched at neutral pH, was increased by boiling, guanidine-HCl, or acidification. The apparent K_m for D-lactate was 6.0×10^{-4} M using an assay based on measuring the reduction of tetrazolium dye mediated by phenazine methosulfate.

One of the membrane-bound primary dehydrogenases in the respiratory chain of *Escherichia coli* (Cox *et al.*, 1970) is D-lactate dehydrogenase. Recently this enzyme has been implicated in the electron transfer reactions coupled to active transport of various amino acids and sugars into bacterial membrane vesicles (Barnes and Kaback, 1971). It is therefore of interest to characterize the enzyme and to determine its localization in the cytoplasmic membrane. In this study, D-lactate dehydrogenase has been solublized by deoxycholate from membranes of *E. coli* and purified about 400-fold to a practically homogeneous state, mainly by ammonium sulfate fractionation and DEAE-Sephadex column chromatography in the presence of Triton X-100. The purified enzyme is a flavoprotein containing FAD^+, and some of its properties are described. This enzyme has been independently purified by Kohn and Kaback (unpublished), but referred to in a review of Kaback (1972).

Materials and Methods

Growth of the Bacteria and Preparation of Membranes. *E. coli* ML 308-225 ($i^- z^- y^+ a^+$) was grown with vigorous aera-

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tion to late exponential phase in an 18-l. flask containing 15 l. of synthetic medium (Tanaka *et al.*, 1967) supplemented with 0.53% DL-lactate as carbon source. The cell pellet obtained after centrifugation was stored at −90° for a number of weeks and portions were thawed as needed. The cells were washed twice with cold 0.01 M Tris-HCl, pH 8.0, and a membrane fraction was obtained by a procedure based on lysozyme–EDTA treatment and repeated freeze–thawing as previously described (Weiner and Heppel, 1972), except that every centrifugation was done for 30 min. Almost all materials having absorption at 260 nm were recovered into the soluble fraction suggesting that the disruption of cells is complete. The membrane fraction was stable for many weeks at −90°.

Assay for D-*Lactate Dehydrogenase.* The phenazine methosulfate coupled reduction of MTT[1] was measured by a slight modification of a previously described assay (Weiner and Heppel, 1972). To 1 ml of standard assay mixture containing 0.08 M Tris-HCl, pH 8.0, 60 μg/ml of MTT, 120 μg/ml of phenazine methosulfate, and enzyme, was added 10 mM D-lactate. The increase in absorbancy at 570 nm at 23° was followed for 3 min and 1 unit of enzyme is expressed as 1 μmol of MTT reduced/min, taking ϵ as 17 mM^{-1} cm^{-1} (Kistler and Lin 1971). Between 0.012 and 0.005 unit of enzyme was used. In this condition, no consumption of oxygen was observed before virtually all MTT was reduced. For the 2,6-dichlorophenolindophenol assay, 20 μg/ml of 2,6-dichlorophenolindophenol was used instead of MTT and phenazine methosulfate.

Measurement of the Fluorescence Spectrum. The fluorescence at 530 nm excited at 450 nm was measured using a 1-cm cuvet with the Aminco Bowman spectrofluorometer and is expressed as a relative value. The measurement was made after adjusting the sample to pH 3.0, unless otherwise specified. The fluorescence of the buffer was measured and subtracted from that of the sample. For the quantitative determinations concentrations of FAD^+ that varied from 10^{-6} to 10^{-7} M were used as standards.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate. After addition of 0.2% sodium dodecyl sulfate and 1% β-mercaptoethanol the protein samples were dialyzed at 4° for 2 days against 1000 times excess of the same sodium dodecyl sulfate solution to remove detergents used in the purification procedure. In case of samples containing Triton X-100, it was dialyzed for 2 more days against the above sodium dodecyl sulfate solution containing 1 mM NaCN to avoid the growth of bacteria. Similar results were obtained when the protein samples were passed through Sephadex LH-20 using dimethylformamide–HCl buffer (Schnaitman, 1971). The electrophoresis was carried out essentially as described by Weber and Osborn (1969). Bovine serum albumin, cytochrome *c* (horse heart), and F_1 (bovine heart) were used as standards. Gels were stained for protein with Coomassie Blue.

Extraction of Flavine from the Enzyme. A solution containing 0.1 mg of enzyme protein and 6 mg of carrier albumin per ml was brought to a concentration of 10% trichloroacetic acid. The supernatant obtained after centrifugation at 10,000*g* for 10 min was extracted four times with ether and used for the measurement of fluorescence. Buffer B (0.05 M sodium phosphate buffer, pH 7.1, containing 1.0% Triton X-100) was treated as above and used as a control. Most of Triton X-100 was removed as the precipitate of trichloroacetic acid. By this procedure more than 90% of the flavine in the enzyme measured after acidification was recovered in the final solution. The carrier albumin used in this procedure had been precipitated and washed once with 10% trichloroacetic acid, followed by extensive dialysis against 0.01 M sodium phosphate buffer, pH 7.1.

Treatments Applied to Membrane Suspensions. Treatment of membranes with toluene was performed as follows: membrane suspension (20 mg of protein/ml) in 0.01 M Tris, pH 8.0, was incubated with 5% toluene for 10 min at 37°. Extraction of the membrane suspension (25 mg/ml) with cholate (0.5%) or Triton X-100 (1.0%) was done in 0.01 M Tris buffer, pH 8.0. Almost all of the D-lactate dehydrogenase appeared in the supernatant solution obtained after centrifugation at 40,000*g* for 20 min. The above supernatant was used as the extract.

Other Procedures. The D-amino acid oxidase assays were kindly carried out by Dr. S. C. Tu (Fonda and Anderson, 1967). The visible absorption spectrum was measured with an Aminco Model DU-2 spectrophotometer operated in the split beam mode.

Density gradient centrifugation was done using a gradient of 5–20% sucrose in 0.05 M sodium phosphate buffer, pH 7.1, containing 0.01% β-mercaptoethanol and 0.5% cholate. Triton X-100 in enzyme solution was replaced by cholate as follows. The purified enzyme containing Triton X-100 was absorbed to a DEAE-cellulose column equilibrated with 0.5% cholate and 0.01% β-mercaptoethanol in 0.05 M sodium phosphate buffer, pH 7.1, and eluted with 0.2 M NaCl in the same buffer. Centrifugation was at 40,000 rpm for 24 hr, using a Spinco SW-41 rotor at 4°. Enzyme (50 μg) was applied with bovine serum albumin (1 mg), ovalbumin (0.5 mg), or cytochrome *c* (1 mg). The position of the above standard was determined either by the amount of protein or absorption at 420 nm. The molecular weight was estimated by the equation of Martin and Ames (1961).

The Visking dialysis bags were washed extensively with 10 mM EDTA and distilled water. Oxygen consumption was measured by the Gilson oxygraph.

Protein was measured according to Lowry *et al.* (1951). Fractions containing detergent were assayed either after extensive dialysis against water or by making use of protein standards containing the same amount of detergent.

Materials. Chemicals were obtained as follows: DEAE-Sephadex A-50 (3.5 mequiv/g), Pharmacia Co.; DEAE-cellulose (DE52, 1 mequiv/g), Whatman Co.; D- and L-lactate and phenazine methosulfate, Calbiochem Co.; 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, Brij-58, FAD^+, FMN^+, and riboflavine, Sigma Co.; Triton X-100, Rohm and Haas Co. Cholate and deoxycholate, obtained from Aldrich Chemical Co. and Sigma, respectively, were recrystallized before use. Bovine serum albumin and cytochrome *c* were from Sigma Co. Bovine heart F_1 was a gift from Dr. E. Racker. All other compounds were of reagent grade and from commercial sources.

Results

Purification of D-*Lactate Dehydrogenase from a Membrane Fraction.* The following buffer mixtures were used: A, 0.05 M Tris-HCl (pH 7.3)–0.01% β-mercaptoethanol; B, 0.05 M sodium phosphate buffer (pH 7.1)–0.01% β-mercaptoethanol; C, 1% Triton X-100 in buffer B. All procedures were carried out at 0–4°. Unless otherwise stated, centrifugation was for 10 min at 10,000*g*.

[1] Abbreviations used are: MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; FAD, flavine adenine dinucleotide; FMN, flavine mononucleotide.

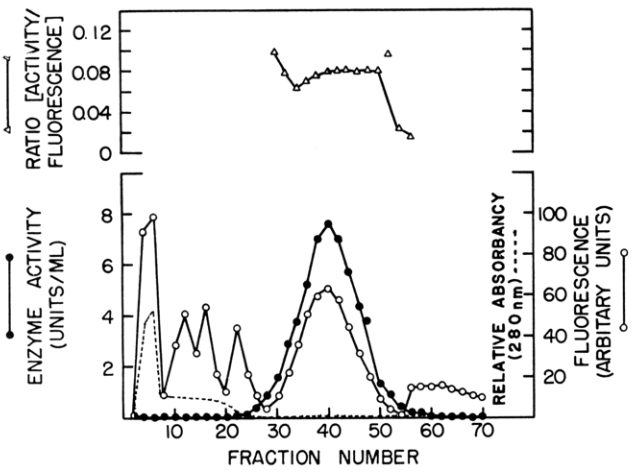

FIGURE 1: Elution patern of *E. coli* membrane D-lactate dehydrogenase from a DEAE-Sephadex column equilibrated and eluted as described under step 4 of Purification. Fractions of 3.0 ml were collected. Enzyme activity (●) was determined using the standard assay system (see Methods). Fluorescence at 530 nm (excited at 450 nm) (○) was assayed as a measure of flavine by taking 0.1 ml of each fraction and bringing the pH to 3.0. The ratio of activity to fluorescence (△) was calculated over the peak of enzyme activity. Absorbance was followed at 280 nm (- - -). See text for details.

TABLE I: Purification of D-Lactate Dehydrogenase from *Escherichia coli*.[a]

Step	Fraction	Vol (ml)	Protein (mg/ml)	Total units	Sp Act. (units/mg)
1	Membrane fraction	160	22.1	570	0.162
	Deoxycholate extract	200	8.1	750	0.461
	Ammonium sulfate	50	22.6	730	0.651
2	Chloroform and ammonium sulfate	45	11.3	815	1.84
3	Ammonium sulfate	11	7.2	420	5.25
4	DEAE-Sephadex	110	0.08	280	31.4
5	DEAE-cellulose	35	0.07	200	81.5

[a] The purification procedure is described under Results. D-Lactate dehydrogenase activity was assayed under standard conditions as described under Methods.

STEP 1. In this step, all centrifugations were at 48,000*g* for 30 min. The membrane suspension (125 ml) from 50 g of wet cells was mixed with 50 ml of 0.63% deoxycholate containing 0.63 M NaCl. After 30 min of stirring, the mixture was centrifuged. The supernatant was brought to 30% saturation with solid ammonium sulfate.[2] The pH of the solution was kept between 7.7 and 8.0 by dropwise addition of 3% ammonium hydroxide. After 30 min of stirring, the precipitate was removed by centrifugation. The supernatant fraction was brought to 55% saturation with ammonium sulfate, followed by stirring and centrifugation. This time the pellet was dissolved in 40 ml of buffer A and dialyzed against this buffer for 3 hr. A precipitate that developed during dialysis was removed by centrifugation.

[2] The percentage of saturation of ammonium sulfate using the solid salt was calculated for 25°, although experiments were carried out at 0–4°. Saturated ammonium sulfate solution was made at 0–4°.

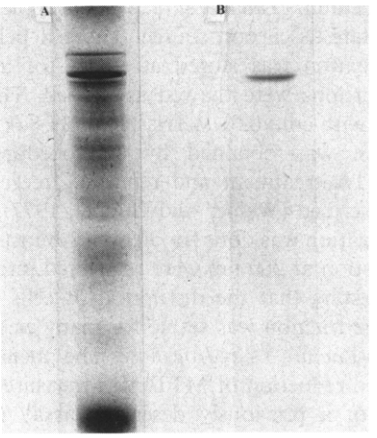

FIGURE 2: Polyacrylamide gel electrophoresis run in the presence of 0.1% sodium dodecyl sulfate. (A) Approximately 20 μg of purified enzyme after electrophoresis on 10% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate. (B) Deoxycholate extract (170 μg) (step 1) after electrophoresis as above.

STEP 2. To the dialysate from step 1, precooled chloroform was added with vigorous stirring to a final concentration of 33% and the suspension was stirred for 10 min. The turbid aqueous layer obtained after centrifugation was centrifuged at 48,000*g* for 10 min and 20% cholate was added to a final concentration of 0.5%. The solution was then made up to 50% saturation with solid $(NH_4)_2SO_4$. After stirring for 10 min, the precipitate was removed by centrifugation and dissolved in 10 ml of buffer A and dialyzed against this buffer overnight. The supernatant obtained after centrifugation at 100,000*g* for 1 hr was used for the next step.

STEP 3. To 10 ml of dialyzed material was added 1 ml of protamine sulfate (10 mg/ml of buffer A). After 10 min of stirring, the mixture was centrifuged and the supernatant solution was brought to 50% saturation by slow addition of a saturated solution of ammonium sulfate (pH 7.1). After 10 min of stirring, the precipitate was removed by centrifugation and the supernatant was brought to 66% saturation with the same reagent. This time the precipitate was collected, dissolved in 10 ml of buffer B, and dialyzed against it for 3 hr. The dialyzed solution was centrifuged at 100,000*g* for 1 hr to remove a precipitate.

STEP 4. The dialyzed solution was made 1% in Triton X-100 and applied to a column of DEAE-Sephadex previously equilibrated with 200 ml of buffer C. The column (1.8 cm diameter) was first packed with DEAE-Sephadex and glass beads (0.54 cm diameter) to a height of 16 cm and then with DEAE-Sephadex alone to a final height of 20 cm. Glass beads were used to get faster flow rate. After washing with a small amount of buffer C, a linear gradient was run with 100 ml of buffer C containing 0.05 M NaCl in the mixing vessel and 100 ml of the same buffer containing 0.15 M NaCl in the reservoir. The flow rate was 20 ml/hr and 3-ml fractions were collected. The dehydrogenase was eluted in the middle of the gradient as a single peak, coincident with one major peak of flavine fluorescence (Figure 1). The ratio of activity to fluorescence was constant over the peak. Fractions containing enzyme activity were combined and dialyzed against buffer B for 3 hr.

STEP 5. The dialyzed material was applied to a column of DEAE-cellulose (chloride form, 0.4 × 8 cm) previously equilibrated with 50 ml of buffer C. After washing the column with a small portion of buffer C, a linear gradient was run

TABLE II: Specificity of D-Lactate Dehydrogenase from *Escherichia coli*.[a]

Substrate	Rel Rate	K_m (M)
D-Lactate	100	6.0×10^{-4}
DL-α-Hydroxybutyrate	12	1.2×10^{-3}
L-Lactate	14	1.8×10^{-2}
Succinate	0	
DL-α-Glycerophosphate	0	

[a] Assays were carried out under standard conditions with the indicated compounds as substrate.

with 50 ml of buffer C in the mixing vessel and 50 ml of 0.2 M NaCl in buffer C in the reservoir. Fractions of 2 ml were collected. Enzyme activity together with flavine fluorescence were eluted as a single peak. The ratio of activity to fluorescence measured after acidification was almost constant over the peak, and the ratio was the same as obtained in DEAE-Sephadex column chromatography (Figure 1). The peak fractions were pooled and dialyzed against buffer B for 3 hr and used for the experiments reported here. In this condition Triton X-100 remained inside the dialyzing bag. The final per cent of Triton X-100 in the assay mixture was 0.005% for kinetic studies as shown below.

A summary of the results from a typical purification are given in Table I. D-Lactate dehydrogenase was purified about 400-fold from membranes with a recovery of 30%. Recovery of flavine fluorescence from the deoxycholate extract was 1.1%

Properties of the Purified Enzyme. EVIDENCE OF PURITY. Analysis of the final fraction by gel electrophoresis in 0.1% sodium dodecyl sulfate revealed only one densely stained band (Figure 2), which accounts for 95% of Coomassie Blue stain as judged from the scanning of the gel. Only this band was observed to increase in intensity with increasing specific activity of fractions in DEAE-Sephadex column chromatography. No cytochrome was detected either under oxidizing or reducing conditions. The phospholipid phosphorus detected by the method of Ames and Dubin (1960) after extracting the final fraction with chloroform–methanol (2:1, v/v) was 0.01 μg/mg of protein.

STABILITY. The final fraction could be stored at −90° in 20% ethylene glycol for at last 2 weeks. Eighty per cent of the activity was retained after storage for 5 days at 0°.

SPECIFICITY. Among the compounds tested, only D-lactate was rapidly oxidized. With this substrate normal kinetics were observed, K_m being 6×10^{-4} M for D-lactate. The K_m of phenazine methosulfate measured by oxygraph in the absence of MTT was 0.25 mM. With DL-α-hydroxybutyrate V_{max} was 10% of the value for D-lactate and K_m was 1.2×10^{-3} M (Table II). Succinate and DL-α-glycerophosphate were not attacked. A significant reduction of NAD^+ was not observed; less than 0.08% of 0.1 μmol of NAD^+ was reduced after incubation of 10 mM lactate and 0.1 unit of enzyme at pH 8 and 23° for 30 min. Cytochrome *c* could not serve as an acceptor.

OPTIMUM pH. The optimum pH for oxidation of D-lactate was between 8 and 9 (Figure 3a). This optimum pH of the pure enzyme was different from that of the enzyme activity in the crude membrane, whose pH optima were 7 and above 9 in the presence and absence of cyanide, respectively (Figure 3b). Cyanide was used to inhibit D-lactate oxidase activity of the membrane. As shown in Figure 3b, cyanide stimulated D-lactate dehydrogenase activity at neutral pH, though it inhibited the activity at alkaline pH. This inhibitory effect of cyanide at alkaline pH was also observed with pure enzyme; in the presence of 1.5 mM sodium cyanide, 2 and 50% of the activity was inhibited at pH 7.4 and 9.3, respectively.

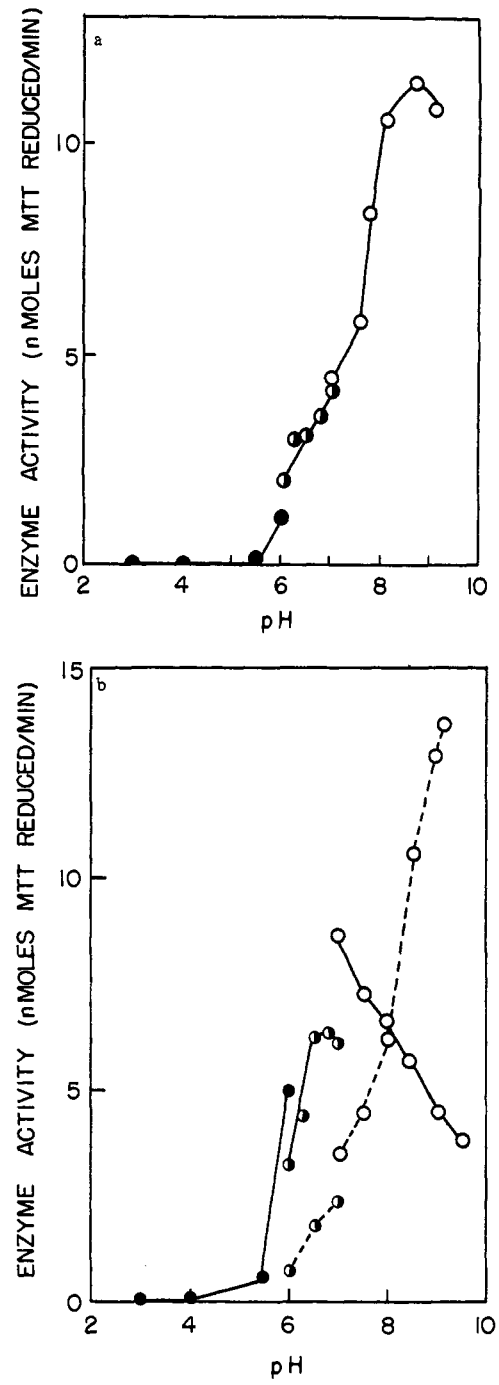

FIGURE 3: (a) pH–activity profile of pure enzyme. Standard assay conditions were used with the exception that the buffer was changed as follows: sodium acetate (●), potassium phosphate (◑), and Tris-HCl (○). At the extremes of pH studied there was less than a 0.02 pH unit change at the end of assay. (b) pH–activity profile of D-lactate dehydrogenase in crude membrane. Standard assay conditions were used except buffers were changed as in Figure 3a. Activity was measured in the presence (solid line) and absence (broken line) of 1.5 mM sodium cyanide.

DIFFERENCE IN APPARENT K_m FOR D-LACTATE DEHYDROGENASE, USING MEMBRANE FRACTIONS OR PURIFIED ENZYME. The apparent K_m of the enzyme as measured with the crude mem-

TABLE III: Apparent K_m of Pure and Membrane D-Lactate Dehydrogenase.[a]

Assay Conditions	Substrate	Addition	Apparent K_m (M)	
			Membrane Enzyme	Pure Enzyme
MTT–phenazine methosulfate, pH 8.0 (standard conditions)	D-Lactate	None	2.2×10^{-3}	6.0×10^{-4}
	D-Lactate	1.5 mM KCN	3.3×10^{-3}	1.4×10^{-3}
	DL-α-Hydroxybutyrate	None	3.9×10^{-3}	1.2×10^{-3}
MTT–phenazine methosulfate, pH 7.0	D-Lactate	None	2.5×10^{-3}	4.6×10^{-4}
Dichlorophenolindolphenol, pH 8.0	D-Lactate	None	2.2×10^{-3}	7.2×10^{-4}

[a] Assays were carried out under different conditions as indicated. See text for details.

brane fraction was 2.2×10^{-3} M under standard conditions, while the value measured with the pure enzyme was 6.0×10^{-4} M (Table II). The same K_m value of membrane enzyme was obtained either after treatment with toluene or measured anaerobically. It was of interest to determine K_m for the different fractions obtained in the course of purification. The apparent K_m became almost the same as that of the pure enzyme after extraction of membranes with deoxycholate, cholate, or Triton X-100. The extract obtained with each detergent was diluted 100-fold in the assay mixture. The final concentration of detergent had little effect on the K_m of membrane enzyme, suggesting that the change of K_m is not due to the detergent which is present in the assay mixture. The value obtained with the deoxycholate extract (4.1×10^{-4} M) was almost the same as those obtained with fractions of steps 1 and 2. The lower apparent K_m for D-lactate of the pure enzyme was also confirmed at pH 7.0, in the presence of 1.5 mM KCN and using dichlorophenolindophenol as acceptor (Table III). The K_m for DL-α-hydroxybutyrate of crude membrane was also higher than that of the purified enzyme (Table III).

EFFECT OF VARIOUS COMPOUNDS ON ENZYME ACTIVITY. None of the metal ions that were tested (Mg^{2+}, Ca^{2+}, Na^{+}, and K^{+}) had a significant effect on enzyme activity. Ions such as Zn^{2+} and Co^{2+} could not be tested because they interfered with the assay. Sulfhydryl reagents (iodoacetic acid, iodoacetamide, and *N*-ethylmaleimide) had little or no effect, confirming the results of previous workers with membrane vesicles (Barnes and Kaback, 1971). Arsenate (0.01 M) inhibited less than 10%, whereas it caused almost complete inhibition of the NAD^{+}-dependent lactate dehydrogenase of *E. coli* (Tarmy and Kaplan, 1968a). The effect of detergents was of interest since they were used in the purification. Both 0.5% deoxycholate and 0.5% cholate reduced activity by 50%, and this was reversible. Triton X-100 had very little effect, while 0.04% of sodium dodecyl sulfate caused nearly complete, irreversible denaturation. Oxamic acid (2×10^{-4} M) and 2,3-phosphoglyceric acid (2 mM) inhibited the pure enzyme by 50 and 40%, respectively.

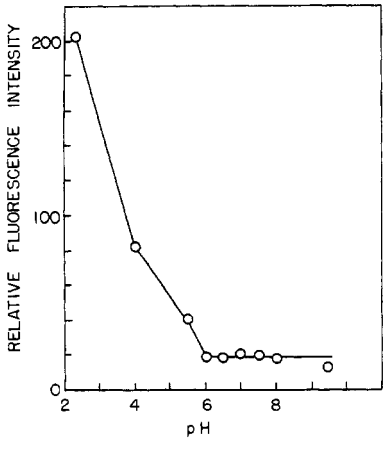

FIGURE 4: Effect of pH on the fluorescence of D-lactate dehydrogenase. Fluorescence at 530 nm (excited at 450 nm) was measured at different pH values. Buffers used were 0.1 M sodium acetate (below pH 5.5), potassium phosphate (between pH 6 and 7), and Tris-HCl (above pH 7).

ESTIMATION OF MOLECULAR WEIGHT BY GEL ELECTROPHORESIS AND SUCROSE GRADIENT CENTRIFUGATION. Sucrose gradient centrifugation in the presence of 0.5% cholate gave a mol wt of 71,000 ± 3000 for D-lactate dehydrogenase activity. This is the average of three different runs each using a different standard. This was calculated by means of an equation (Martin and Ames, 1961) in which it is assumed that partial specific volume is equal to that of standard protein. Although the method first developed did not involve the use of detergents, it could be used in the presence of cholate for a rough estimation of molecular weight. A similar value was obtained using Sephadex G-200 in the presence of 0.5% cholate and 0.1 M NaCl in 0.05 M sodium phosphate buffer, pH 7.1, although 50% of the activity was lost during the elution. The molecular weight determined by polyacrylamide electrophoresis containing 0.1% sodium dodecyl sulfate was 72,000. This last procedure determined the subunit molecular weight. Since the molecular weight estimated by sodium dodecyl sulfate gel and sucrose gradient centrifugation were the same, the enzyme is suggested to consist of a single polypeptide chain.

PROPERTIES OF THE ENZYME AS A FLAVOPROTEIN. As mentioned earlier, flavine fluorescence after acidification was eluted in the same peak as enzyme activity (Figure 1). Fluorescence was constant between pH 9 and 6, and increased as the pH was lowered. The fluorescence at pH 2.4 was 20-fold greater than that at pH 7 (Figure 4), though no enzymic activity could be detected below pH 5 (Figure 3). This quenching at a neutral pH was much greater than observed for commercial FAD^{+} or the flavine extracted from the enzyme: for them, the ratios of fluorescence at pH 2.4 compared with pH 7 were 6.3 and 6.5, respectively.

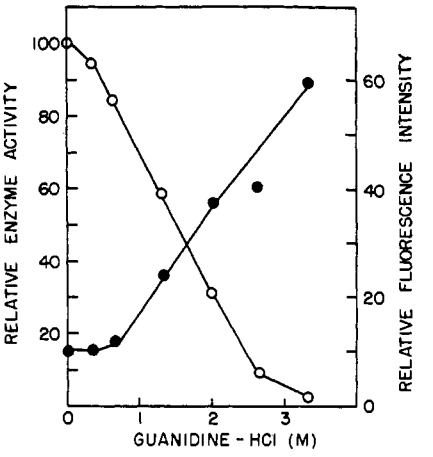

FIGURE 5: Effect of different concentrations of guanidine-HCl on the activity and fluorescence of D-lactate dehydrogenase. Purified enzyme (0.4 unit) was incubated in 1.0 ml of 0.05 M sodium phosphate buffer (pH 7.1) with different concentrations of guanidine-HCl at 23°. After 20 min, aliquots were taken and both fluoresence (●) and enzyme activity (○) were measured as described in the text.

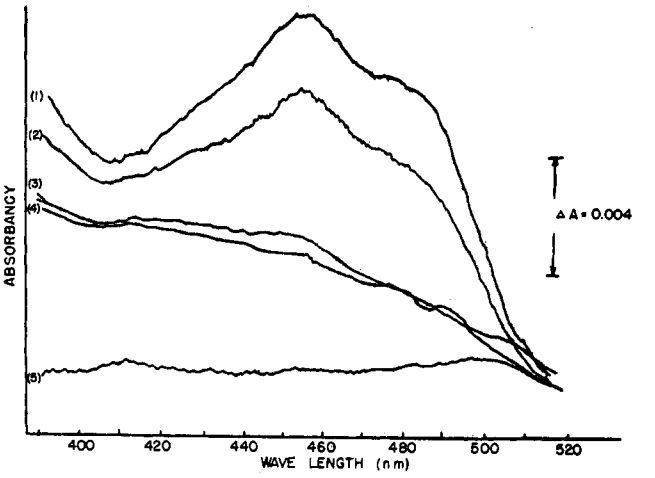

FIGURE 6: Absorption spectrum of D-lactate dehydrogenase with or without D-lactate. The absorption spectrum of purified enzyme was taken using 0.10 mg/ml of protein in 0.05 M sodium phosphate buffer, pH 7.1, containing 20% ethylene glycol and 0.01% β-mercaptoethanol in a 1-cm cuvet. The reference cuvet contained the same buffer. Spectra were taken before (1), immediately after (2), 5 min after (3), and 10 min (4) after addition of 5 mM D-lactate. The scanning speed was 1 nm/sec. The base line is curve number 5.

An increase in fluorescence was also observed by treatment with heat or guanidine·HCl. Fluorescence was increased 2.3-fold by boiling compared with 20-fold by acidification; part of the effect of acid was due to the increase of fluorescence of the liberated flavine at low pH. In virtually all conditions, increase of fluorescence and irreversible loss of enzyme activity appeared to progress in parallel fashion. This is illustrated for guanidine·HCl in Figure 5.

VISIBLE ABSORPTION SPECTRUM OF THE ENZYME. The visible absorption spectrum of the enzyme revealed a broad peak at around 455 nm and a shoulder at around 480 nm, suggesting that this enzyme is a flavoprotein (Figure 6). Addition of hydrosulfite or D-lactate (Figure 6, curves 2 and 4) resulted in the complete loss of both the peak and the shoulder. The reduced condition was maintained at room temperature for 30 min with no special precautions to obtain anaerobiosis.

DETERMINATION OF FLAVINE IN THE ENZYME. The flavine could be extracted with 10% trichloroacetic acid and removed by dialysis, so presumably it is noncovalently bound to the enzyme. It exhibited a peak of fluorescence at 529 nm when excited at 450 nm, which has been observed for FAD^+, FMN^+, and riboflavine, The pH–fluorescence curve of the flavine was the same as for FAD^+ (Figure 7); the apparent pK values as determined by the quenching of fluorescence for this material and FAD^+ were 3.8 and 3.6, respectively. The ratio of the maximum over the minimum values of the fluorescence of the flavine and FAD^+ were 15.3 and 16, respectively (Figure 7), compared with 0.7 for FMN^+.

The flavine extracted from D-lactate dehydrogenase was identified as FAD^+ by a D-amino acid oxidase test. An extract whose flavine concentration was determined to be 1.3×10^{-7} M from its fluorescence, was shown to contain 1.1×10^{-7} M FAD^+ by the D-amino acid oxidase test. From the fluorescence analysis of trichloroacetic acid extracts corresponding to a known amount of protein sample a value of 1 mol of FAD^+/72,000 mol wt was obtained (1.7, 1.2, and 0.8 for three different preparations).

Discussion

D-Lactate dehydrogenase has been purified to homogeneity from *E. coli* membranes. This enzyme differs substantially from the NADH-dependent enzyme of *E. coli* (Tarmy and Kaplan, 1968a) in location, sensitivity to arsenate and iodoacetamide, and other properties. The pyridine nucleotide linked enzyme appears to be directed toward the production of lactate (Tarmy and Kaplan, 1968b), while the enzyme of this study is a primary dehydrogenase in the respiratory chain (Cox *et al.*, 1970). The purified enzyme was inhibited by oxamate and by 2,3-phosphoglyceric acid, which are known to inhibit the D-lactate stimulated transport of membrane vesicles (Kaback, 1972). This and other properties mentioned above suggest that the present enzyme is involved in the stimulation of transport in membrane vesicles. Final proof depends on the success of reconstitution experiments, now in progress.

It may be noted that higher concentrations of deoxycholate

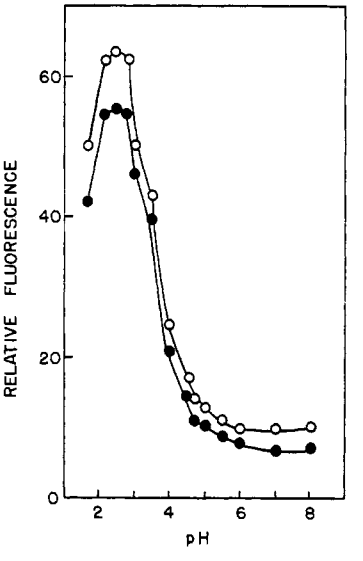

FIGURE 7: Effect of pH on the fluorescence of flavine extracted from D-lactate dehydrogenase. Fluorescence of the flavines extracted from purified enzyme with trichloroacetic acid (●) and 10^{-7} M FAD^+ (○) were measured at different pH values. Buffers used were the same as described in Figure 4 except that soduim acetate–HCl was used below pH 3. See the text for details.

and NaCl were needed to solubilize this enzyme than was the case for α-glycerophosphate dehydrogenase (Weiner and Heppel, 1972). Washing the membranes with 2 or 50 mM Tris, pH 7.4, or 4 M urea removed no activity. Total activity was increased up to 50% after solubilization, which might be explained by improved accessibility of substrate and dye to the enzyme. Most of the cytochromes having a peak of absorption at about 558 mμ in the difference spectrum (reduced *vs.* oxidized) was removed by the end of the chloroform treatment. No detergent was used in step 3, but addition of Triton X-100 was necessary in step 4. Step 4 could not be replaced by DEAE-Sephadex or DEAE-cellulose chromatography in the presence of cholate or Brij 58. It is rather surprising that DEAE-cellulose (step 5) gives a substantial further purification following DEAE-Sephadex (step 4). The entire procedure was carried out eight times with substantially similar results.

The purified enzyme appears to contain 1 mol of FAD^+/mol. The evidence for FAD^+ as a prosthetic group is based on the change of fluorescence with pH and the results of D-amino acid oxidase assays. The FAD^+ is apparently noncovalently bound since it is extractable by trichloroacetic acid. The absorption peak of the enzyme at 455 nm was completely reduced by D-lactate, but a semiquinone intermediate could not be observed. The quenching of FAD^+ in the enzyme at neutral pH was unusually high. Attempts to reversibly dissociate the flavine from the enzyme using dialysis against 1 M KBr (Massay and Curt, 1966) have been so far unsuccessful. Possibly this is due to the high degree of interaction between enzyme and FAD^+, as suggested by the quenching of flavine fluorescence.

The apparent K_m value of D-lactate dehydrogenase in the membrane was about fourfold higher than pure enzyme. A similar observation had been made on α-glycerophosphate dehydrogenase from *E. coli* (Weiner and Heppel, 1972). The K_m for α-glycerophosphate of the membrane-bound enzyme was 29-fold higher than that of the purified enzyme.

Other examples of the changes of K_m after solubilization are mitochondrial DPNH reductase (Hatefi and Stempel, 1969) and *Neurospora crassa* malate dehydrogenase (Munkres and Woodward, 1966). Treatment of the membrane with toluene did not change this value. This treatment was shown to make ferricyanide accessible to dehydrogenases inside the membrane (Weiner and Heppel, manuscript in preparation). The lower K_m value similar to that of the pure enzyme was obtained after solubilization of the activity from membranes either with deoxycholate, cholate, or Triton-X-100. This suggests that change of K_m of the enzyme is not a result of combination with detergents used in the purification procedures shown above. The change of activity of membrane enzyme after solubilization could be considered as an allotopic property of D-lactate dehydrogenase, though this might be the result of the change of accessibility of both dye and substrate to the enzyme. Greater understanding of the interaction of enzyme with detergent and phospholipid is also necessary to adequately explain this phenomenon.

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