BBA 66627

STUDIES ON THE DISTRIBUTION AND PROPERTIES OF THE MULTIPLE FORMS OF MAMMALIAN LIPOAMIDE DEHYDROGENASE

MAJOR L. COHN* AND I. ROSABELLE McMANUS
Department of Biochemistry, University of Pittsburgh, Pittsburgh, Pa. 15213 (U.S.A.)

(Received January 17th, 1972)

SUMMARY

Lipoamide dehydrogenase (NAD^+·NADH:lipoamide oxidoreductase, EC 1.6.4.3) was obtained from pig heart muscle by three different isolation procedures and subjected to electrofocusing and analytical acrylamide gel electrophoresis. Five or six enzymically active anodal species are resolved by these techniques. Three higher mobility species are obtained from lipoamide dehydrogenase derived from pyruvate dehydrogenase complex having isoelectric points of between pH 5.6 and 6.0, while three slower mobility species obtained from α-ketoglutarate dehydrogenase-derived lipoamide dehydrogenase have isoelectric points of between pH 6.5 and 6.8. These multiple species are invariant throughout these isolation procedures. They are identified as charge isomers with similar molecular weights and amino acid composition. Evidence is presented that these multiple species are not solely the result of alteration of lipoamide dehydrogenase by the proteolytic enzymes of pig heart.

INTRODUCTION

Lipoamide dehydrogenase (NAD^+·NADH:lipoamide oxidoreductase, EC 1.6.4.3) is the flavoenzyme catalyzing the oxidation of protein-bound dihydrolipoic acid, and associated with the mitochondrial multienzyme complexes involved in the oxidative decarboxylation of pyruvate and α-ketoglutarate. Lipoamide dehydrogenase from various sources has been shown to exist in several enzymatically active forms by starch gel electrophoresis[1,2] and polyacrylamide gel electrophoresis[3,4]. Studies by Stein and Stein[3] and Stein *et al.*[5] indicated that the multiple electrophoretic forms from pig heart differed little in their catalytic properties and were immunologically similar. It had been shown by Atkinson *et al.*[1] that a single pig heart preparation could be resolved by starch gel electrophoresis into at least six distinct forms. More recently, however, Sakurai *et al.*[6] and Wilson[7] have reported that this heterogeneity may be due to the improvident selection of isolation techniques and arises largely as a

* Present address: Department of Anesthesiology, Magee-Womens Hospital, Pittsburgh, Pa. 15213, U.S.A.

consequence of the activity of proteolytic enzymes on lipoamide dehydrogenase during its isolation.

The present work describes some of the characteristics of the multiple forms of lipoamide dehydrogenase obtained from pig heart using several isolation procedures including, in addition to the preparative method described by Massey[8,9], (1) resolution and isolation of the enzyme from purified pyruvate and α-ketoglutarate dehydrogenase complexes, and (2) isolation of the crude enzyme by a procedure designed to minimize exposure of the enzyme to proteolytic enzyme activity. The multiple forms of the pig heart enzyme have been examined by polyacrylamide gel electrophoresis, ion exchange chromatography, and isoelectric focusing. In addition, the enzyme has been isolated from pig skeletal muscle and has been shown to have substantially the same degree of microheterogeneity as was observed for the pig heart enzyme. The multiple forms show no molecular size differences and exist solely as charge isomers.

A preliminary report of some of these results has appeared[10].

EXPERIMENTAL PROCEDURES

Materials

Lipoamide (DL-6,8-thioctic acid amide), lipoic acid (DL-6,8-thioctic acid), NAD, NADH, FAD, protamine sulfate, sodium pyruvate, and thiamine pyrophosphate were obtained from the Sigma Chemical Company. Ammonium sulfate, enzyme grade, ultra-pure sucrose, urea, myoglobin, pepsin (EC 3.4.4.1), trypsin (EC 3.4.4.4), and bovine serum albumin were purchased from Schwarz–Mann Research Laboratories. Whatman DEAE-cellulose, DE 32 microgranular form, was purchased from the Burrell Corporation and cycled by the procedure described in the Whatman information brochure. Acrylamide (recrystallized for electrophoresis), methylenebisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine and β-mercaptoethanol were obtained from Eastman Organic Chemicals, and ammonium persulfate, analytical grade, was purchased from the Fisher Scientific Company. Coomassie brilliant blue R-250 was obtained from Colab Laboratories. Carrier ampholines, pH 3–10 range and pH 5–8 range, were used in the isoelectric focusing experiments and were obtained from LKB Instruments as 40% solutions. Triton X-100 was purchased from Rohm and Haas. Calcium phosphate gel–cellulose was prepared as described by Price and Greenfield[11] using Whatman CF11 fibrous cellulose powder.

Enzyme preparations

Lipoamide dehydrogenase was prepared from pig heart and skeletal muscle as described by Massey[8,9] with and without the use of heat steps. Pyruvate and α-ketoglutarate dehydrogenase complexes were isolated from pig heart mitochondrial extracts essentially as described by Hayakawa *et al.*[12] and Hirashima *et al.*[13]. Lipoamide dehydrogenase was resolved from the purified α-keto acid dehydrogenase complexes by incubating each of them at 4 °C in 6 M urea at pH 7.5 for 2.5 h, followed by chromatography of the treated complexes on calcium phosphate gel–cellulose columns[8].

Enzyme assays

Lipoamide dehydrogenase activity was determined as described by Massey[8,9]

using DL-lipoamide as substrate. The reaction was initiated by addition of lipoamide and the initial lipoamide-dependent oxidation of NADH was measured at room temperature at 340 nm. Specific activity is defined as μmoles NADH oxidized per min per mg protein.

The pyruvate dehydrogenase complex was assayed by the method of Korkes *et al.*[14]. Specific activity of the enzyme is expressed in terms of μmoles acetyl phosphate formed per h per mg enzyme.

The α-ketoglutarate dehydrogenase complex was assayed as described by Massey[15] and the reaction was initiated by the addition of α-ketoglutarate. Specific activity is expressed as μmoles NAD reduced per min per mg protein.

Other methods

Protein was determined by the method of Lowry *et al.*[16] using bovine serum albumin as the standard. Elution of proteins during column chromatography was monitored by measuring absorption at 280 nm.

Polyacrylamide disc gel electrophoresis was performed in glass tubes, 6 mm × 90 mm, essentially as described by Clarke[17]. Except where specifically noted, gels were prepared using 4% acrylamide and *N,N'*-methylenebisacrylamide in an amount to obtain 1% cross-linkage and were polymerized by addition of ammonium persulfate. Electrophoresis was performed in an analytical disc gel electrophoresis unit (Buchler polyanalyst), cooled to 3–5 °C, with 0.005 M Tris buffer, pH 8.0, in the buffer reservoirs and a current of 1.5 mA per tube for 1.5–3 h. Sample movement was to the anode. The electrophoresed gel discs were reacted with nitro-blue tetrazolium chloride, 0.3 mg/ml, and NADH, 0.6 mg/ml in 0.1 M phosphate buffer, pH 7.5, to detect diaphorase activity. In some instances, the developed gels were scanned using a Photovolt densicord, Model 542, fitted with a disc gel scanning adaptor and using a 0.1-mm aperture.

Isoelectric focusing experiments were performed with the LKB Model 8102 and followed the procedure described by Vesterberg and Svensson[18]. The enzyme samples were incorporated into the middle range of the sucrose gradient and electrofocusing was performed for approximately 60 h at 700 V with the column maintained at 4 °C using a Lauda–Brinkman K-2/R circulator. Fractions of 1.5 ml were collected at a flow rate of 18 drops per min and the pH was determined with a Corning Model 12 pH meter. Enzyme activity was determined using lipoamide as substrate and is expressed as units of activity per 1 ml. A unit is defined as an amount of enzyme which gives a change of 0.001 absorbance unit per min assayed under standard conditions at room temperature.

For recovery of proteins following electrofocusing in preparation for analysis of amino acid composition of the multiple forms, pooled fractions were dialyzed against 200 vol. of 0.01 M phosphate buffer, pH 7.5, for 96 h to remove sucrose and the major portion of the carrier ampholyte. An aliquot was reserved for analysis of protein, and the remainder was hydrolyzed under N_2 in a sealed tube in 6 M HCl at 110 °C for 48 h. Excess HCl was removed from the hydrolysate by evaporation to dryness *in vacuo* followed by repeated additions of water and evaporation to dryness. The hydrolysate was then dissolved in 2 ml water in preparation for analysis of an aliquot containing about 50 μg protein. Amino acid analysis was performed with a Beckman Model 116 amino acid analyzer using the two column system[19].

Sedimentation velocity analyses were done in a Spinco Model E ultracentrifuge equipped with Schlieren optics. All samples were run at 2 °C and the observed sedimentation coefficients were corrected to values corresponding to water at 20 °C.

RESULTS

In agreement with results reported by Stein and Stein[3] using pig heart lipoamide dehydrogenase and by Lusty[2] in studies on the enzyme isolated from beef liver, highly purified pig heart lipoamide dehydrogenase displays heterogeneity when chromatographed on DEAE-cellulose columns. A 20-mg sample of pig heart enzyme which catalyzed the oxidation of 111.2 μmoles NADH per min per mg protein using DL-lipoamide as substrate, and with a spectral ratio, $A_{280\text{ nm}}:A_{455\text{ nm}}$ of 5.4 (ref. 8) was applied to a DEAE-cellulose column, 2.2 cm × 40 cm, which had been equilibrated with 0.1 M sodium phosphate, pH 7.5. Each 4-ml fraction was assayed for protein by measuring absorption at 280 nm and for lipoamide dehydrogenase activity using DL-lipoamide as substrate. As shown in Fig. 1, two major peaks are obtained by

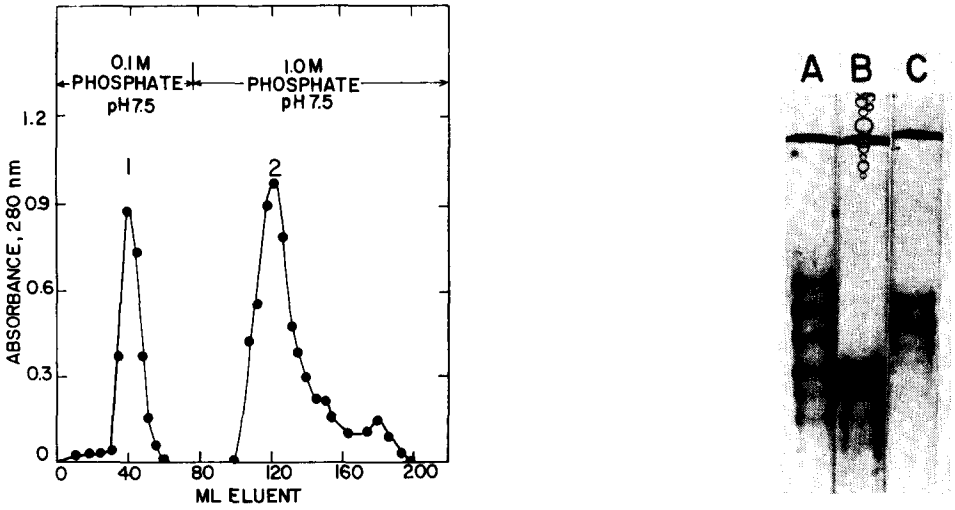


Fig. 1. DEAE-cellulose chromatography of pig heart lipoamide dehydrogenase and polyacrylamide gel electrophoresis of separate fractions. 20 mg enzyme were applied to a DEAE-cellulose column, 2.2 cm × 40 cm, equilibrated with 0.1 M sodium phosphate, pH 7.5. 4-ml fractions were collected at a flow rate of 12 ml per h. Peak 1 was eluted with 0.1 M sodium phosphate, pH 7.5; Peak 2 was eluted with 1.0 M sodium phosphate, pH 7.5. Acrylamide gel electrophoresis was performed on gels prepared as described in Experimental Procedures. Samples were electrophoresed for 3 h at 1.5 mA per tube. Tube A, 20 μg purified lipoamide dehydrogenase before chromatography; Tube B, 20-μl aliquot from concentrate of Peak 2; Tube C, 20-μl aliquot from concentrate of Peak 1. Gels were stained for diaphorase-reactive material. Movement is towards the anode.

stepwise elution with 0.1 M sodium phosphate, pH 7.5. Both of these peaks are enzymatically active. Peak 1 catalyzes the oxidation of 111 μmoles NADH per min per mg protein and has a spectral ratio, $A_{280\text{ nm}}:A_{455\text{ nm}}$ of 5.53. Peak 2 catalyzes the oxidation of 108.4 μmoles NADH per min per mg protein and has a spectral ratio, $A_{280\text{ nm}}:A_{455\text{ nm}}$ of 4.93. The highest activity fractions of Peaks 1 and 2 were dialyzed

against 0.05 M sodium phosphate, pH 7.5, and concentrated in a Diaflo ultrafiltration cell (Amicon) using a UM-10 ultrafiltration membrane. Aliquots were electrophoresed on polyacrylamide gels and the results are shown in Fig. 1. Pattern A is characteristic of the diaphorase-reactive lipoamide dehydrogenase before chromatography on DEAE-cellulose. Similarly, six species are detected when the gel is stained for protein with Coomassie blue[20]. Complete correspondence is observed between these diaphorase-active areas and lipoamide dehydrogenase activity[4]. As seen in Pattern B, Peak 2 contains species 1, 2, and 3 together with traces of a possible additional component of still higher mobility. Peak 1 contains the slower species 4, 5 and 6, as shown in Pattern C. No evidence for the presence of the complex variety of minor components observed by Stein and Stein[3] was obtained in this study.

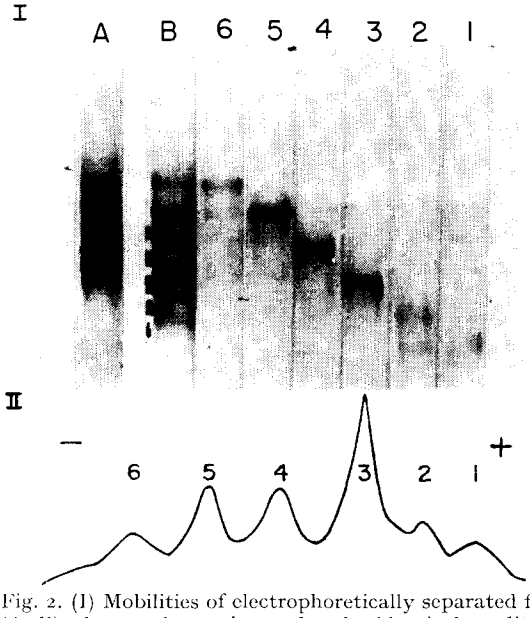


Fig. 2. (I) Mobilities of electrophoretically separated forms of pig heart lipoamide dehydrogenase (A, B) when re-electrophoresed under identical conditions (1–6). 20.5 μg enzyme were electrophoresed at 1.5 mA/tube for 3 h; the gel was then sectioned as indicated and each species was reelectrophoresed using separate gel columns. Movement is towards the anode. (II) Tracing of densitometer scan of electrophoresed lipoamide dehydrogenase (B).

As illustrated in Fig. 2-I, the enzyme species retain the same relative mobilities and discrete patterns after they are sectioned and the gel sections placed on similar polyacrylamide gel columns, followed by electrophoresis using conditions identical to those used in the original electrophoresis. Fig. 2-II shows a densitometer tracing of the diaphorase-reactive forms. The number and relative intensities of these forms in a given preparation is unaltered under a variety of conditions used for electrophoresis. Sodium phosphate buffer, customarily used at a concentration of 0.01–0.03 M to dilute the flavoenzyme, was replaced by Tris buffer by percolating the enzyme through a small Sephadex G-75 column equilibrated with 0.05 M Tris buffer (pH 7.5) – 1 mM EDTA followed by electrophoresis. The electrophoretic pattern was unaffected by this change in buffer species. Pre-electrophoresis of ammonium persulfate-containing polyacrylamide gels for 3 h at 5 mA/tube prior to electrophoresis of the enzyme,

addition of thioglycollate to the gel prior to electrophoresis[21], or use of polyacrylamide gels photopolymerized in the presence of riboflavin failed to alter the enzyme pattern, thus making it unlikely that this heterogeneity may be the result of artifacts of the electrophoretic technique.

In order to obtain larger amounts of these several species for further investigation, isoelectric focusing was examined as a preparative technique for resolution and isolation of the multiple forms of lipoamide dehydrogenase[22]. A preliminary experiment in which lipoamide dehydrogenase was electrofocused in the pH range 3–10 showed a double peak in the region between pH 5.8 and 7.2. Accordingly, in all other experiments, carrier ampholyte in the pH range of 5–8 was employed. As shown in Fig. 3, four enzymically active peak fractions are obtained when 14.7 mg lipoamide

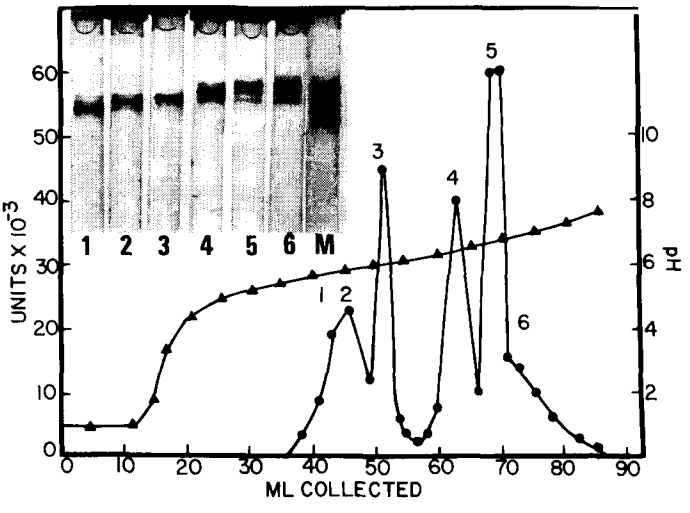


Fig. 3. Isoelectric focusing of purified pig heart lipoamide dehydrogenase. 14.7 mg purified enzyme were electrofocused in the LKB Model 8102 unit in pH 5–8 carrier ampholyte for 65 h at 700 V at 4 °C. Insert shows the patterns obtained after gel electrophoresis of 10-μl aliquots from 1.5-ml fractions collected after electrofocusing. Conditions for electrophoresis are described in Experimental Procedures. Samples were run for 1.5 h at 1.5 mA per tube. ●, lipoamide dehydrogenase activity; ▲, pH.

dehydrogenase prepared by the Massey procedure[8,9] are electrofocused in pH 5–8 carrier ampholyte for 65 h at 700 V. The activity of the enzyme was not inhibited by the presence of carrier ampholyte. The major peak fractions shown here correspond to material having isoelectric points of pH 5.6, 6.0, 6.5 and 6.8. Although not shown in this figure, close correspondence between the enzyme activity and protein concentration as measured by the absorption at 280 nm was observed. A flavoprotein absorption spectrum typical of oxidized lipoamide dehydrogenase with maxima at 358 and 455 nm, shoulders at 425 and 480 nm and a minimum at 395 nm was obtained when the material electrofocusing between pH 6.5 and 6.8 was scanned between 325 and 550 nm. The ratio of absorption at 280 and 455 nm was 5.3. Polyacrylamide disc gel electrophoresis of 10-μl aliquots from 1.5-ml fractions of the major areas shows that effective resolution of species 1, 2 and 3 has been achieved. When aliquots of each of these species were mixed and electrophoresed under the same conditions, the pattern

is the same as that observed using unfractionated material (seen in M). In order to exclude the possibility that association of carrier ampholyte with the protein might be responsible for the resolution into these several species, material eluting in the region between 60 and 80 ml, was re-electrofocused under the same conditions. The enzyme

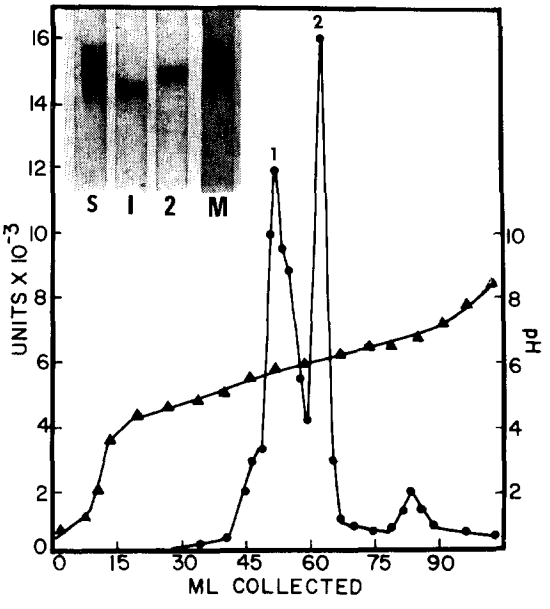


Fig. 4. Isoelectric focusing of lipoamide dehydrogenase derived from pig heart pyruvate dehydrogenase complex. 9.5 mg lipoamide dehydrogenase were electrofocused in pH 5–8 carrier ampholyte for 70 h at 700 V at 4 °C. Insert shows the patterns obtained after electrophoresis of 20-μl aliquots of Peaks 1 and 2. S indicates standard pig heart lipoamide dehydrogenase[8,9]; M indicates a mixture of material from Peaks 1 and 2. Conditions for electrophoresis are described in Experimental Procedures and samples were run for 1.5 h at 1.5 mA per tube. ●, lipoamide dehydrogenase activity; ▲, pH.

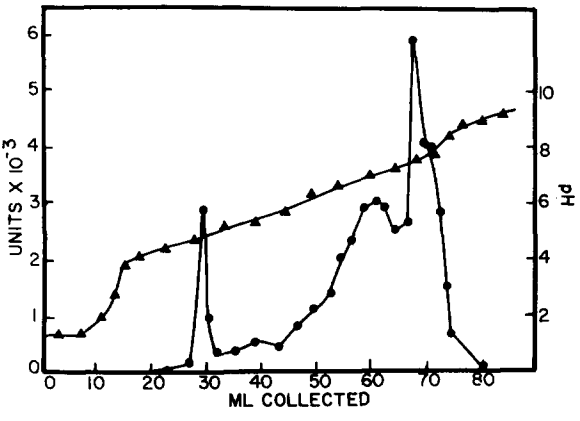


Fig. 5. Isoelectric focusing of lipoamide dehydrogenase derived from pig heart α-ketoglutarate dehydrogenase complex. 6.58 mg lipoamide dehydrogenase in 3.5 ml 0.05 M phosphate buffer (pH 7.5) were electrofocused in pH 5–8 carrier ampholyte for 70 h at 700 V at 4 °C. ●, lipoamide dehydrogenase activity; ▲, pH.

activity and position of the peak was unaltered and the gel electrophoretic pattern was unchanged.

Figs 4 and 5 show the isoelectric focusing patterns of lipoamide dehydrogenase resolved from purified pig heart pyruvate and α-ketoglutarate dehydrogenase complexes. The pyruvate dehydrogenase complex had a specific activity of 87 μmoles acetyl phosphate formed per h per mg protein as measured by the pyruvate dismutation assay[14] and the sedimentation coefficient, $s_{20,w}$, of the complex, calculated from a sedimentation velocity centrifugation carried out on 5 mg enzyme per ml 0.05 M sodium phosphate (pH 7.5) at 35 600 rev./min at 2 °C, was 63.8, in reasonable agreement with results reported by Hayakawa *et al.*[12]. The α-ketoglutarate dehydrogenase complex which was resolved to yield lipoamide dehydrogenase, catalyzed the reduction of 3.6 μmoles NAD per min per mg protein[15] and had a sedimentation coefficient, $s_{20,w}$, of 33.2, calculated from a sedimentation velocity centrifugation performed at 47 610 rev./min at 2 °C on 4 mg protein per 1 ml 0.05 M sodium phosphate, pH 7.5 in agreement with the value reported by Hirashima *et al.*[13]. As shown in Fig. 4, 9.4 mg lipoamide dehydrogenase in 2.5 ml 0.05 M phosphate buffer, pH 7.5, derived from the pyruvate dehydrogenase complex, were electrofocused in pH 5–8 ampholyte for 70 h. A sharp yellow band and one broad dim yellow band were visible and, when 1-ml fractions were collected and assayed for enzyme activity, two major enzymically active peaks were observed which correspond to species with isoelectric points of pH 5.6 and 6.0. The insert shows the gel electrophoresis patterns obtained when 20-μl aliquots of the numbered peak fractions were electrophoresed. Fraction 1 is derived from material electrofocusing at pH 5.6, and corresponds to species 1 and 2, while Fraction 2 consists of species 3 and corresponds to material having an isoelectric point of about pH 6.0. Fig. 5 shows the results of an experiment performed using lipoamide dehydrogenase derived from purified α-ketoglutarate dehydrogenase complex in the amount of 6.58 mg protein in 3.5 ml 0.05 M phosphate buffer, pH 7.5. Two major peaks are observed which occur in the higher pH region (see Fig. 3) and which correspond to the slower moving species seen in gel electrophoresis. The small enzyme active peak observed at 30 ml accounts for approximately 8.4% of total lipoamide dehydrogenase activity. Since it represented a comparatively small amount of the total activity, it was not further investigated.

Recent reports have suggested proteolytic digestion of protein during isolation as the basis for the observed electrophoretic heterogeneity of lipoamide dehydrogenase[6,7]. In an effort to evaluate the contribution of proteolytic activity to the heterogeneity of lipoamide dehydrogenase, the enzyme was isolated from a single pig heart by the method of Massey[8,9] with the complete extraction, resolution, and heating completed within 1 h after preparation of the tissue homogenate. The crude heated extract was fractionated with ammonium sulfate and the fraction precipitating between 40 and 80% was dissolved in 0.001 M phosphate (pH 7.2)–1 mM EDTA and dialyzed for 16 h against the same buffer. It was then heated at 55 °C for 5 min and centrifuged at 16 000 rev./min for 20 min. 5 ml of this supernatant containing 50 mg protein were electrofocused between pH 5–8 and the results are shown in Fig. 6. No significant alteration in the resolution of the multiple forms of the enzyme has occurred such as would be anticipated if some or all of the subspecies were attributable to proteolytic activity during the course of isolation of the enzyme.

The effect of proteolytic enzymes present in a crude pig heart extract on the iso-

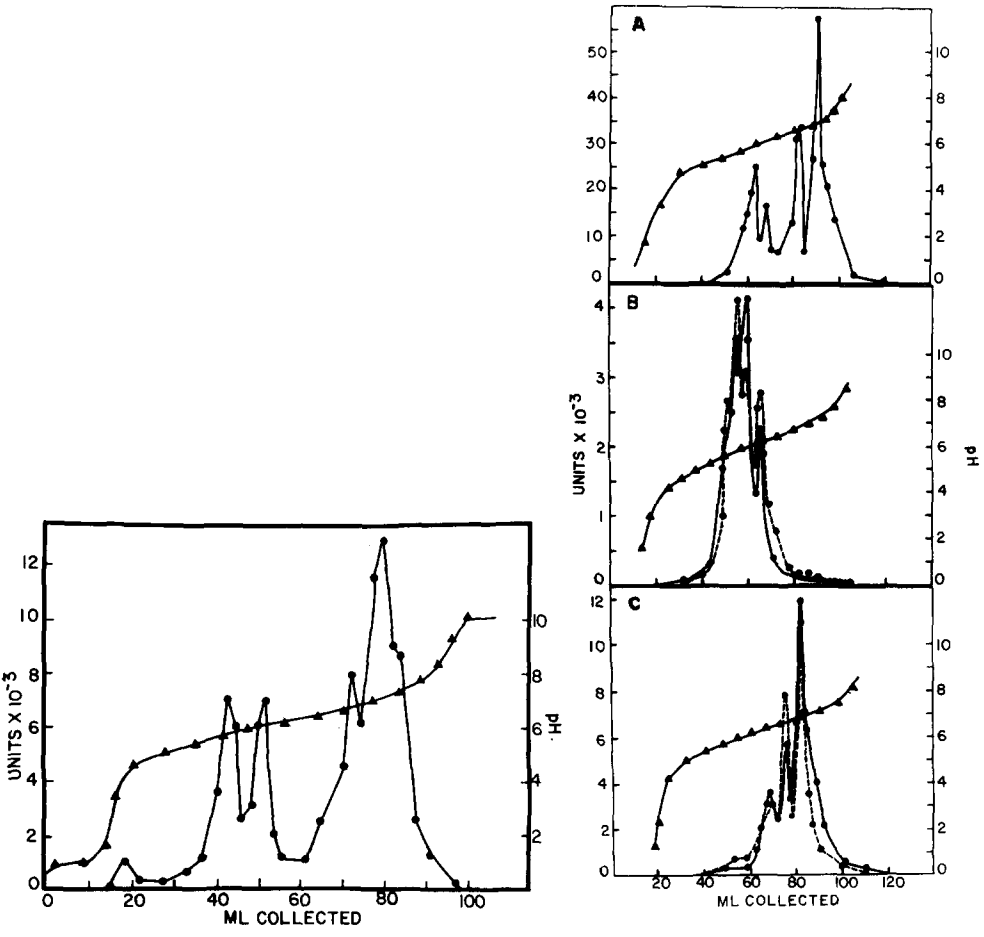


Fig. 6. Isoelectric focusing of crude pig heart lipoamide dehydrogenase. 50 mg of crude enzyme in 5 ml 0.01 M phosphate buffer (pH 7.2) were electrofocused in pH 5–8 ampholyte at 700 V for 50 h at 4 °C. See text for description of preparation.

Fig. 7. Isoelectric focusing of pig heart lipoamide dehydrogenase before and after incubation with crude pig heart extract. A, isoelectric focusing of 10.2 mg purified enzyme; B, isoelectric focusing pattern of fraction recovered in 50–78 ml region of run shown in A; ———, untreated; — — —, incubated for 18 h with crude pig heart extract; C, isoelectric focusing pattern of fraction recovered in 80–100 ml region of run shown in A; ———, untreated; — — —, incubated for for 18 h with crude pig heart extract. See text for details of preparations.

enzyme pattern of purified lipoamide dehydrogenase was examined. 10.2 mg of highly purified lipoamide dehydrogenase from pig heart were first electrofocused using pH 5–8 ampholyte at 700 V for 48 h in order to obtain resolution of the multiple forms. This pattern is shown in Fig. 7A. The fraction resolving between 50 and 78 ml in a pH range between 5.5 and 6.2 was pooled, dialyzed against 0.01 M phosphate buffer (pH 7.5) for 72 h and concentrated to a final volume of 6 ml in the Amicon Diaflo ultrafiltration cell. The material contained in the region between 80 and 100 ml was pooled and treated in an identical manner. One half of each fraction was then incubated with 3 ml fresh pig heart extract prepared by centrifuging a homogenate of 1

part pig heart to 3 parts of 0.02 M Na_2HPO_4 at 1500 × *g* for 15 min. 3 ml of 0.02 M Na_2HPO_4 were added to the remaining portions of the fractions and the samples were incubated for 18 h at 4 °C. 4 ml of each of the fractions were then re-electrofocused under conditions identical to the initial electrofocusing run. The results are shown in Figs 7B and 7C. No major alteration in the patterns can be attributed to exposure of enzyme to the crude extract, although, as seen in Fig. 7B, some increase in the pH 6.2 species is observed, a small peak has appeared at pH 5.6, and the material electrofocusing at pH 6.0 has decreased. The most notable alteration in the material having the higher isoelectric point is some increase in the pH 6.7 species. Thus, prolonged exposure to an extract which is a likely source of proteolytic enzymes fails to alter the qualitative distribution of the multiple forms of lipoamide dehydrogenase or to increase the complexity of the isoenzyme pattern.

Multiple forms of lipoamide dehydrogenase exhibiting mobilities similar to those observed for the enzyme isolated from pig heart are also obtained when the enzyme is isolated from pig skeletal muscle. Lipoamide dehydrogenase was obtained in low yield from pig skeletal muscle by the Massey procedure[8,9] with a specific activity of 44.4 μmoles NADH oxidized per min per mg protein using DL-lipoamide as substrate. The diaphorase-active pattern of this preparation after electrophoresis is shown in Fig. 8B. The pattern is qualitatively similar to that observed for an aliquot of pig heart lipoamide dehydrogenase, shown in Fig. 8A, which was run simultaneously with the skeletal muscle enzyme. Clearly, resolution of multiple forms has occurred, but species 4 and 5 are not as prominent as in the enzyme obtained from pig heart.

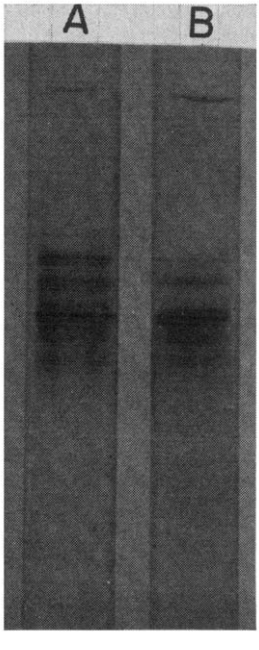
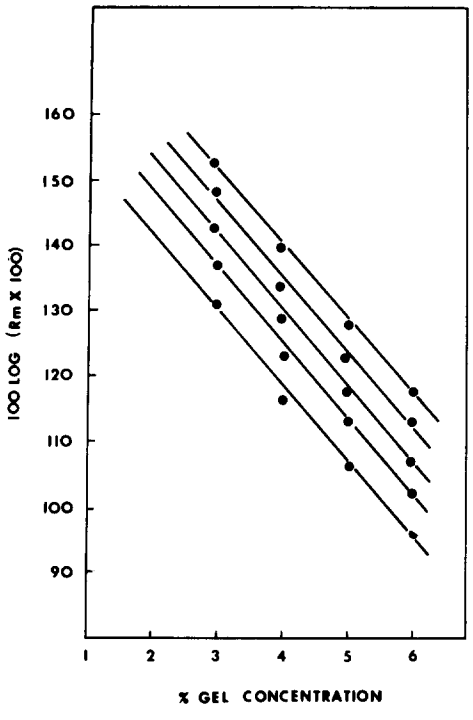


Fig. 8. Disc gel electrophoresis of pig skeletal muscle lipoamide dehydrogenase. A, pig heart lipoamide dehydrogenase, Massey preparation[8,9]; B, pig skeletal muscle lipoamide dehydrogenase. Electrophoresed in 5% polyacrylamide gel for 1.5 h at 2 mA per tube using conditions described in Experimental Procedures Movement is towards the anode.



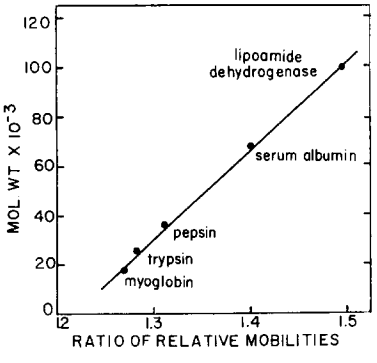


Fig. 9. The effect of different polyacrylamide gel concentrations on the electrophoretic mobility of multiple forms of pig heart lipoamide dehydrogenase. Species 1–5 are plotted here.

Fig. 10. Molecular size analysis of the multiple forms of pig heart lipoamide dehydrogenase. 25 μg of each of the standard proteins were used for polyacrylamide gel electrophoresis using 6 and 8% gels.

Millard *et al.*[23] have reported the isolation and purification of lipoamide dehydrogenase from pig brain by a quite different procedure and separated at least six enzymatically reactive bands on polyacrylamide disc gels, a result which has been confirmed in our laboratory using enzyme isolated from brain mitochondria by procedures similar to those employed for isolation of the heart and skeletal muscle enzymes.

Demonstration of the multiple forms of lipoamide dehydrogenase from various mammalian tissues provides little information on the structural basis for their separation since no efforts had been made to distinguish between heterogeneity arising from size variation and heterogeneity due to differing net charge. To decide between these possibilities, the method of Hedrick and Smith[24] for distinguishing between a size isomer family of proteins and a charge isomer group was applied to pig heart lipoamide dehydrogenase. Fig. 9 illustrates the effect of different acrylamide gel concentrations on the mobility of five lipoamide dehydrogenase isoenzymes (species 1–5) from pig heart. Purified enzyme was separated by electrophoresis on 3, 4, 5 and 6% acrylamide gel columns with the weight ratio of *N,N'*-methylenebisacrylamide/acrylamide monomer maintained at 1:30 as recommended by Hedrick and Smith[24]. The relative mobilities, expressed as 100 log ($R_m \times 100$), are plotted against percentage concentration of gel. A family of parallel curves having identical slopes are obtained, con-

sistent with heterogeneity produced by differing net charges and a constant molecular size.

Additional support for the absence of size heterogeneity as a basis for the existence of the multiple forms was afforded by an analysis of their gel electrophoretic behavior as a function of molecular weight as described by Thorun and Mehl[25] and Zwaan[26]. In this procedure, a molecular weight is obtained by calculation of the frictional ratio of a protein, expressed as the quotient of the mobilities of the protein at two gel concentrations. This ratio is independent of electrical charge, but dependent on molecular size and shape relative to the pore size of the gels. Fig. 10 shows a plot of the frictional ratios, expressed as the ratio of relative mobility in 6% gel to relative mobility in 8% gel, against molecular weights for four standard proteins, including myoglobin, trypsin, pepsin and bovine serum albumin. Imposed on this standard curve are the calculated results for the multiple forms of lipoamide dehydrogenase obtained under conditions identical to those used for the standard proteins. A molecular weight of approximately 100 000 is obtained by this procedure for each of the isoenzymes, offering further support for charge differences as a basis for the heterogeneity of the pig heart enzyme.

Table I shows the results of amino acid analyses of five fractions obtained from electrofocusing pig heart lipoamide dehydrogenase (see Fig. 3), of the enzyme before

TABLE I

AMINO ACID COMPOSITION OF LIPOAMIDE DEHYDROGENASE OBTAINED FROM PIG HEART

All samples were hydrolyzed for 48 h in 6 M HCl at 110 °C. Results represent the average of two runs and are calculated based on 49.9 moles glycine per mole FAD[27]. PDC, pyruvate dehydrogenase complex; KDC, α-ketoglutarate dehydrogenase complex.

Amino acid	*Moles amino acid per mole FAD*							
	*Total**	*PDC-derived***	*KDC-derived****	*Fraction No.*§				
				1	*2*	*3*	*4*	*5*
Lys	38.2	35.4	34.7	34.6	33.1	37.9	34.4	38.3
His	12.2	13.2	10.9	10.1	10.0	11.6	12.3	11.5
Arg	17.0	18.6	15.5	13.7	13.9	14.4	18.6	14.4
Asp	40.0	44.3	43.6	35.5	37.5	41.2	41.3	37.2
Thr	24.7	24.8	24.5	18.3	20.5	19.4	19.9	20.8
Ser	18.4	23.4	21.6	19.8	17.0	18.2	19.9	20.7
Glu	50.9	48.9	45.9	42.0	41.9	48.7	47.5	43.3
Pro	19.9	19.2	17.5	16.6	15.0	15.9	17.3	15.8
Gly	49.9	49.9	49.9	49.9	49.9	49.9	49.9	49.9
Ala	43.9	43.9	43.6	37.6	38.4	40.8	41.9	40.9
Val	43.3	37.8	35.1	33.5	37.6	35.4	37.7	37.1
Met	10.0	9.7	9.2	8.4	8.8	9.5	10.2	9.8
Ile	35.0	30.2	30.3	27.3	30.0	30.9	32.3	29.5
Leu	34.8	33.3	30.6	25.8	28.8	30.2	30.1	28.0
Tyr	8.5	10.8	8.3	7.8	7.1	8.5	9.2	10.0
Phe	16.5	15.2	14.6	11.4	13.0	12.3	12.9	13.4

* Enzyme prepared as described by Massey[8,9].

** Pyruvate dehydrogenase complex (PDC) prepared as described by Hirashima *et al.*[13].

*** α-Ketoglutarate dehydrogenase complex (KDC) prepared as described by Hayakawa *et al.*[12].

§ Fractions obtained by isoelectric focusing of lipoamide dehydrogenase. Fractions 1–5 correspond to enzymically active fractions collected from electrofocusing column and designated as shown in Fig. 3.

electrofocusing, and of the enzymes derived from the pyruvate and α-ketoglutarate dehydrogenase complexes. In general, the amino acid compositions are similar to values reported by Massey *et al.*[27] and by Sakurai *et al.*[6] for the whole enzyme except that our analyses consistently show lower arginine values than were reported by Massey *et al.*[27]. Comparison of values for the individual amino acids reveals a few instances where the differences exceed 5%. However, it must be concluded that none of these species exhibits major alterations in amino acid composition and that there are in fact remarkable similarities in their amino acid compositions. Thus, although resolution of the multiple forms depends upon charge differences, no clear evidence has been obtained for a difference in primary amino acid content as the basis for the observed charge isomers.

DISCUSSION

The most extensively used procedure for the isolation of pig heart lipoamide dehydrogenase as developed by Massey[8,9] consistently gives rise to a series of multiple forms which are distinguished by differences in anodal electrophoretic mobilities when the enzyme is subjected to starch gel or polyacrylamide gel electrophoresis. Partial resolution of these forms may also be achieved by ion exchange column chromatography. Electrofocusing of the enzyme in a pH 5–8 gradient allows better resolution, as shown in this paper and as observed recently by Wilson[28]. The forms are separable due to alteration in total net charge since the equilibrium isoelectric positions of the forms attained by isoelectric focusing correlate well with their gel electrophoretic mobilities. Further support for charge differences as the basis for their resolution is afforded by the demonstration that the forms do not differ in molecular size, at least within the limits of the methods employed for analysis. However, direct support for differences in primary structure of the forms as the basis for the occurrence of the charge isomers was not obtained in this study, since amino acid analyses of the isoenzymes recovered from the electrofocusing experiments failed to provide evidence for significant differences in amino acid composition. Similarly, comparison of tryptic digests of performic acid-treated species 3 and 5 after paper chromatography in pyridine–*n*-butanol–acetic acid–water (15:10:3:12, by vol.) followed by high voltage electrophoresis in pyridine–acetic acid–water (1:10:289, by vol.) at pH 3.6 in the second dimension failed to reveal any differences in the tryptic digest peptide pattern (unpublished observations). An analysis of the free carboxyl groups present in the multiple forms of the enzyme reported recently by Wilson[28] failed to detect any secondary modification; *i.e.* amidation of glutamate and aspartate residues, thus apparently ruling this possibility out as an explanation for the charge differences.

Examination of the lipoamide dehydrogenase components of highly purified pyruvate and α-ketoglutarate dehydrogenase complexes by the technique of isoelectric focusing provides additional evidence for the view that the several multiple forms are distributed between these two enzyme complexes. The forms having isoelectric points between pH 5.6 and 6.0 are resolved from pyruvate dehydrogenase while the forms with the higher isoelectric points are associated with α-ketoglutarate dehydrogenase. Sakurai *et al.*[6] detected two electrophoretically distinct forms of lipoamide dehydrogenase using starch gel electrophoresis which they designated as Fp-I, the main component of the α-ketoglutarate dehydrogenase complex, and Fp-II, the component

of the pyruvate dehydrogenase complex. A third species, designated Free-Fp[29,30] was shown to consist of a mixture of Fp-I and Fp-II. The faster migrating band obtained by starch gel electrophoresis at pH 7.0 corresponded to Fp-II, while the slower moving enzyme was Fp-I. These mobilities have been substantially confirmed in our laboratory using enzyme preparations which behave in our hands as multiple forms when electrophoresed on polyacrylamide gel at a more alkaline pH or when subjected to electrofocusing at pH 5–8. However, numerous criteria directed towards comparing these species, including amino acid composition, ultracentrifugal analysis, absorption spectra, molecular size determination, tryptic peptide mapping and immunochemical behavior[6,30] have failed to reveal significant substantive differences in the characteristics of these multiple forms. Some evidence in favor of conformational variation was offered by Sakurai *et al.*[6] based on circular dichroism and optical rotatory dispersion spectra of the two forms, Fp-I and Fp-II. The possible contribution of conformational differences as a basis for the heterogeneity is of considerable interest and invites further investigation.

The origin of the multiple forms remains a controversial issue. Wilson[7] has emphasized the existence of a direct relationship between the time from initial tissue preparation to enzyme extraction and the resulting heterogeneity of the extracted enzyme. Some support for this view is presented in this paper since some evidence for alteration in the relative proportions of the several species has been observed after prolonged incubation with crude pig heart extracts. However, despite serious attempts, we have failed consistently to prepare an active flavoenzyme which consists of a single species as determined by isoelectric focusing. Further, preparation of lipoamide dehydrogenase by purification of the pyruvate and α-ketoglutarate dehydrogenase complexes followed by urea resolution failed to alter significantly the observed patterns. An additional unresolved question is the precise relationship of these findings to the properties of the Fp-I and Fp-II forms obtained by Sakurai *et al.*[6]. At least a part of the discrepancies may reasonably be attributed to differences in the electrophoretic techniques used for resolution and characterization.

As pointed out by Epstein and Schlechter[31] and Markert[32], one of the possible mechanisms for the origin of isoenzymes is the structural alteration of the enzyme incurred during the isolation procedure. This mechanism is difficult to eliminate unequivocally, but we feel that it is difficult to account for all of the results reported in this paper by recourse only to this explanation. It is clear that, if proteolytic attack on a single species of lipoamide dehydrogenase has indeed given rise to the six recognizable multiple forms, the mode of attack must be highly specific and common to several tissue types, and occurs without major alteration in molecular size, amino acid composition, or absorption spectra, nor as reported by Wilson[28], in circular dichroism or fluorescence properties.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance of Miss Jackeline Peterson and Mrs Jean Lee and their appreciation to Mr Jean Paul Vergnes for the amino acid analyses.

This work was supported by Research Grants No. AM-02914 and 11147 from

the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U.S. Public Health Service.

A portion of this research is taken from a thesis submitted to the University of Pittsburgh in partial fulfillment of the requirement for the degree of Doctor of Philosophy (by M.L.C.).

REFERENCES

1 M. R. Atkinson, M. Dixon and J. M. Thornber, *Biochem. J.*, 82 (1962) 29P.
2 C. J. Lusty, *J. Biol. Chem.*, 238 (1963) 3443.
3 A. M. Stein and J. H. Stein, *Biochemistry*, 4 (1965) 491.
4 M. Cohn, L. J. Wang, W. Scouten and I. R. McManus, *Biochim. Biophys. Acta*, 159 (1958) 182.
5 A. M. Stein, B. Wolf and J. H. Stein, *Biochemistry*, 4 (1965) 1500.
6 Y. Sakurai, Y. Fukuyoshi, M. Hamada, T. Hayakawa and M. Koike, *J. Biol. Chem.*, 245 (1970) 4453.
7 J. E. Wilson, *Proc. 3rd Int. Symp. on Flavins and Flavoproteins, Durham, N. C.*, University Park Press, Baltimore, Md., 1971, p. 313.
8 V. Massey, *Biochim. Biophys. Acta*, 37 (1960) 314.
9 V. Massey, in W. A. Wood, *Methods in Enzymology*, Vol. 9, Academic Press, New York, 1966, p. 272.
10 M. L. Cohn and I. R. McManus, *Fed. Proc.*, 30 (1971) 1172.
11 V. E. Price and R. E. Greenfield, *J. Biol. Chem.*, 209 (1954) 363.
12 T. Hayakawa, M. Hirashima, S. Ide, M. Hamada, K. Okabe and M. Koike, *J. Biol. Chem.*, 241 (1966) 4694.
13 M. Hirashima, T. Hayakawa and M. Koike, *J. Biol. Chem.*, 242 (1967) 902.
14 S. Korkes, A. del Campillo and S. Ochoa, *J. Biol. Chem.*, 196 (1952) 551.
15 V. Massey, *Biochim. Biophys. Acta*, 38 (1960) 447.
16 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
17 J. T. Clarke, *Ann. N.Y. Acad. Sci.*, 121 (1964) 428.
18 O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, 20 (1966) 820.
19 D. H. Spachman, W. H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
20 A. Chrambach, R. A. Reisfield, M. Wyckoff and J. Zaccari, *Anal. Biochem.*, 20 (1967) 150.
21 J. M. Brewer, *Science*, 156 (1967) 256.
22 W. A. Susor, M. Kochman and W. J. Rutter, *Science*, 165 (1969) 1260.
23 S. A. Millard, A. Kubose and E. M. Gal, *J. Biol. Chem.*, 244 (1969) 2511.
24 J. L. Hedrick and A. J. Smith, *Arch. Biochem. Biophys.*, 126 (1968) 155.
25 W. Thorun and E. Mehl, *Biochim. Biophys. Acta*, 160 (1968) 132.
26 J. Zwaan, *Anal. Biochem.*, 21 (1967) 155.
27 V. Massey, T. Hofmann and G. Palmer, *J. Biol. Chem.*, 237 (1962) 3820.
28 J. E. Wilson, *Arch. Biochem. Biophys.*, 144 (1971) 216.
29 T. Hayakawa, Y. Sakurai, T. Aikawa, Y. Fukuyoshi and M. Koike, in K. Yagi, *Flavins and Flavoproteins*, University of Tokyo Press, Tokyo, 1968, p. 99.
30 T. Hayakawa, T. Aikawa, K. I. Otsuka and M. Koike, *J. Biochem. Tokyo*, 62 (1967) 396.
31 C. J. Epstein and A. N. Schechter, *Ann. N.Y. Acad. Sci.*, 151 (1968) 85.
32 C. L. Markert, *Ann. N.Y. Acad. Sci.*, 151 (1968) 14.