# Electron Microscopic and Biochemical Studies of Pyruvate Dehydrogenase Complex of Escherichia coli

Abstract. *Examination with the electron microscope of the pyruvate dehydrogenase complex and its component enzymes from* Escherichia coli *indicates that the complex has a polyhedral structure with a diameter of about 300 to 350 angstroms and a height of 200 to 250 angstroms. The reconstituted complex closely resembles the native complex in appearance. A tentative model of this multienzyme complex is proposed on the basis of the correlated biochemical and electron-microscopic data.*

The pyruvate dehydrogenase complex (PDC) of *Escherichia coli* is a highly organized multienzyme system (molecular weight of approximately 4.8 million) that catalyzes a multistage oxidative decarboxylation of pyruvate (*1, 2*) consisting successively of decarboxylation, reductive acetylation (acetyl generation), acetyl transfer, and electron transfer. Because of the wealth of available biochemical data on this complex, a unique opportunity is provided to correlate functional properties, as revealed by biochemical analysis, with ultrastructure, as revealed by electron microscopy. The concept of the structural organization of this pyruvate dehydrogenase complex which emerged from biochemical studies is that of an organized mosaic of enzymes in which each of the component enzymes is uniquely located to permit efficient implementation of the consecutive reactions. This concept has been confirmed and extended by the correlative electron-microscope studies reported in the present communication.

The pyruvate dehydrogenase complex is composed of three enzymes: (i) pyruvate decarboxylase (molecular weight 183,000), (ii) lipoic reductase-transacetylase (LRT), and (iii) a flavoprotein, dihydrolipoic dehydrogenase (molecular weight 112,000). There are about 16, 64, and 8 molecules of each, respectively, per molecule of complex. Lipoic reductase-transacetylase is an aggregate of a subunit whose molecular weight is about 27,000 (*2, 3*), the total molecular weight being approximately 1.6 million.

The methods for resolving pyruvate dehydrogenase complex into its component enzymes and for reconstituting the complex from the isolated enzymes have been described (*2*). Briefly, selective dissociation of the flavoprotein from the pyruvate dehydrogenase complex ($s_{20,w}$ about 60*S*) is accomplished by fractionation in the presence of 4*M* urea on calcium phosphate gel suspended on cellulose. This procedure yields free flavoprotein ($s_{20,w}$ = 6.3*S*) and a complex of decarboxylase and LRT ($s_{20,w}$ about 50*S*). The latter complex is separated into the decarboxylase ($s_{20,w}$ = 9.2*S*) and LRT ($s_{20,w}$ about 27*S*) by fractionation on gel-cellulose at *p*H 9.5. The pyruvate dehydrogenase complex is reconstituted by mixing the isolated decarboxylase, the LRT aggregate, and the flavoprotein in 0.05*M* potassium phosphate buffer (*p*H 7.0) in a ratio of 3:2:1 by weight, respectively. Examination of this mixture in an ultracentrifuge (Spinco model E) reveals the virtual absence of peaks corresponding to the individual components. Instead, a major, faster-moving peak ($s_{20,w}$ = 52.4*S*) is observed with which the yellow color of the flavoprotein is associated. This major component is isolated as a yellow pellet by centrifugation for 2½ hours at 173,000*g*. The composition and enzymatic activities of the yellow pellet are very similar to those of native pyruvate dehydrogenase complex (*2*).

For electron microscopy, the specimens were negatively stained and embedded by the microdroplet cross-spraying technique (*4*). By means of a special multiple-spraying device provided with suitably arranged separate capillaries for specimens and reagents, we obtained controlled brief interaction of microdroplets of the specimen with microdroplets of 1 to 2 percent potassium phosphotungstate at *p*H 7.2 to 7.4. The cross-sprayed microdroplets (about 0.1 to 10 $\mu$ diameter) collide and interact very rapidly shortly before impinging on the specimen grid. As a result of the small size and relatively high speed of the cross-sprayed microdroplets, there is a favorable combination of rapid cooling of the specimen and limited drying to attain a unique degree of preservation of labile structures (*4, 5*). Positive staining with 1 or 2 percent uranyl acetate was also accomplished by this technique. All reagents were filtered through 100-Å Millipore filters. Operations were carried out at 0° to 5°C. Specimens were examined in the native state shortly after preparation, or after they had been frozen and thawed under conditions that prevented significant loss in activity. They were examined generally at a final concentration of 0.5 mg of protein per milliliter in 0.02 to 0.05*M* potassium phos-

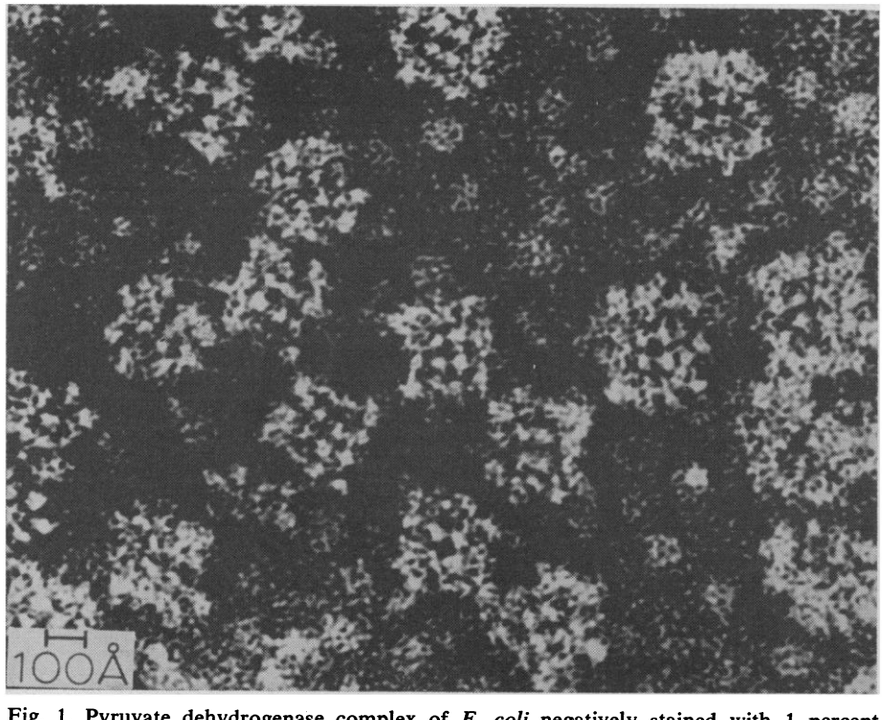


Fig. 1. Pyruvate dehydrogenase complex of *E. coli* negatively stained with 1 percent phosphotungstate (*p*H 7.4) by microdroplet cross-spraying technique (× 520,000).

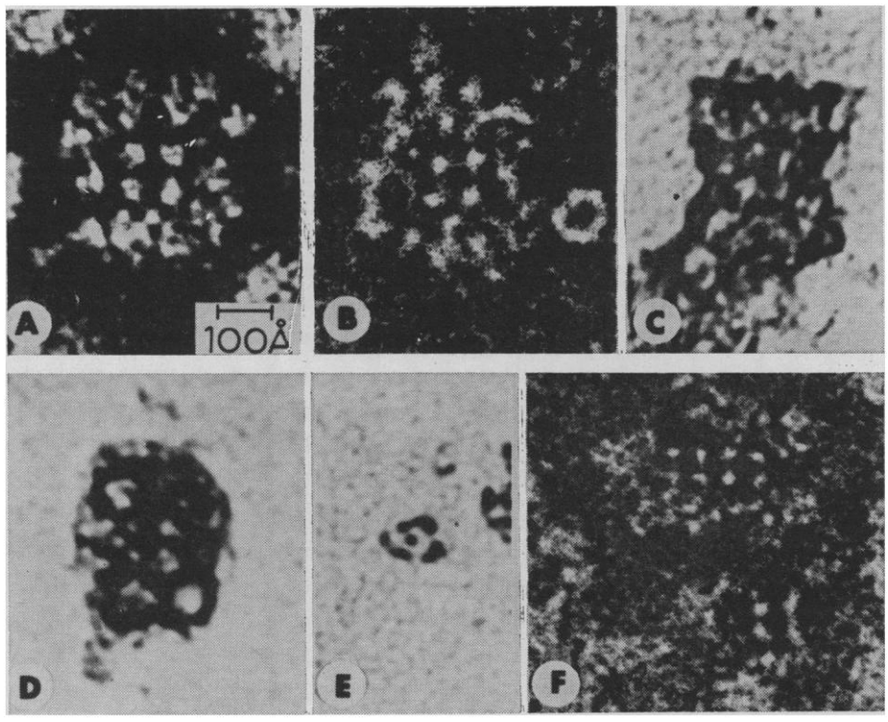


Fig. 2. Electron micrographs of *E. coli* pyruvate dehydrogenase complex (PDC): (*A*) Native PDC; (*B*) reconstituted PDC and Ferritin negatively stained with phosphotungstate; (*C* and *D*) native PDC positively stained with uranyl acetate; (*E*) isolated pyruvate decarboxylase stained with uranyl acetate; (*F*) isolated LRT aggregate negatively stained (× 700,000).

phate buffer. Other buffers, such as ammonium acetate, were also used with similar results.

Extensive control experiments were carried out with standard preparation techniques. The specimens were mounted on very thin Formvar films, or supported on special fenestrated substrates (*4*). For electron microscopy we used a Siemens Elmiskop I with multiple objective apertures and a pointed filament of single-crystal tungsten (*4, 6*) that provided microbeam illumination of high coherence. The micrographs were recorded at electron optical magnifications of 20,000 to 80,000 on Ilford high-resolution plates. Ferritin molecules of uniform size (118 to 120 Å in diameter) prepared by density-gradient ultracentrifugation were used as an internal calibration reference. Irradiation damage and specimen contamination were substantially reduced with improved specimen cooling devices and low-intensity electron optics (*4, 5*).

A typical field of particles of pyruvate dehydrogenase complex negatively stained with phosphotungstate is shown in Fig. 1. The particles have the appearance of polyhedrons of diameter about 300 to 400 Å. There are four well-defined subunits arranged as a tetrad with a side of 130 to 150 Å in the central portion of each polyhedron. Surrounding this central tetrad is an orderly array of subunits, 60 to 90 Å in diameter. The appearance of some of the projections of negatively and positively stained preparations suggests a double layer of these peripheral subunits. The relatively large variation in the diameter of the particles, that is, 300 to 400 Å, as seen in negative contrast appears to be due mainly to swelling caused by phosphotungstate. Ultracentrifuge studies indicate that phosphotungstate is adsorbed by the particles, and that some separation of subunits occurs. The electron micrographs indicate a tendency for the peripheral subunits to dissociate from the complex in the presence of phosphotungstate. Heavy staining of the particles by uranyl acetate as indicated by the electron micrographs also would result in larger than actual dimensions. In view of these complications, the minimum dimensions observed, 300 by 200 Å, are believed to represent a reasonable estimate of the actual dimensions of the PDC particle.

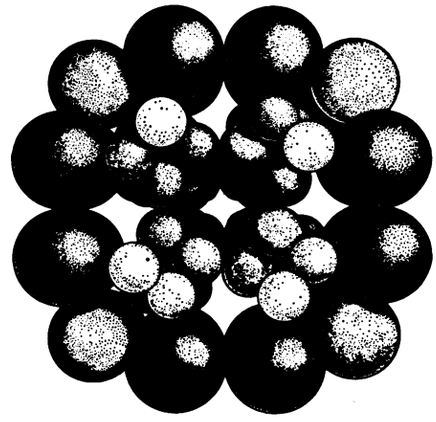

Fig. 3. Tentative model of *E. coli* pyruvate dehydrogenase complex.

The pyruvate dehydrogenase complex reconstituted from the isolated decarboxylase, LRT aggregate, and flavoprotein is shown in negative contrast in Fig. 2*B*. The central tetrad of the complex stands out clearly, and the peripheral subunits are also well-defined. Comparison with Figs. 1 and 2*A* reveals the virtual identity of appearance of the reconstituted and native complexes. This supports the biochemical data indicating that the sedimentation characteristics, composition, and enzymatic activities of the reconstituted and native complex are very similar and points up the uniqueness of the structural organization.

Figures 2*C* and 2*D* show different projections of the pyruvate dehydrogenase complex positively stained with 2 percent uranyl acetate. The appearance of the central LRT tetrad is not as distinct in these micrographs as it is in the negatively stained preparations. However, the individual peripheral subunits are more clearly outlined. Many of the peripheral subunits have the appearance of oval rings; their longer diameter is 70 to 90 Å. Figure 2*D* represents a side view of the pyruvate dehydrogenase complex. A double layer of peripheral subunits is clearly demonstrated in this micrograph. The width of the complex in this projection is 200 to 250 Å.

The appearance of the isolated pyruvate decarboxylase positively stained with uranyl acetate is shown in Fig. 2*E*. This projection of the molecule looks like an oval ring of diameter 70 to 90 Å with a dark spot of diameter about 15 Å in the center. The former dimension is in reasonable agreement with a spherical diameter of 75 Å calculated from the molecular weight of pyruvate decarboxylase. The appearance and dimensions of pyruvate decarboxylase as seen in positive contrast closely resem-

ble those of some of the repeating subunits along the periphery of the complex (Figs. 2*C* and 2*D*).

Electron micrographs of the isolated LRT negatively stained with phosphotungstate show arrays of tetrads (Fig. 2*F*). These tetrads have the same average dimension (130 to 150 Å) as, and closely resemble, the central tetrad of the native complex (Figs. 1 and 2). The dense core of the tetrads may be partly attributed to penetration of the phosphotungstate.

On the basis of the biochemical and electron-microscopic data, a tentative model of the pyruvate dehydrogenase complex has been constructed (Fig. 3). The component enzymes of the complex are represented as spheres, the diameters of which were calculated from the molecular weights of the enzymes and an assumed partial specific volume of 0.73 ml/g. These diameters would be 75, 40, and 64 Å for the decarboxylase, LRT subunit, and flavoprotein, respectively. The model consists of 16 molecules of pyruvate decarboxylase (large spheres) and 8 molecules of flavoprotein (medium-size spheres) arranged in two rings one above the other. These two rings surround the lipoic reductase-transacetylase aggregate which comprises 64 subunits (small spheres) arranged into four stacks. We cannot as yet specify the sequence of pyruvate decarboxylase and dihydrolipoic dehydrogenase molecules in the two rings.

The volume of PDC calculated from electron microscope measurements (minimal dimensions 300 by 200 Å), on the assumption that the shape of the particle may be approximated as either an oblate ellipsoid or a right circular cylinder, is about $9.5 \times 10^{-18}$ cm$^3$. The anhydrous volume of the complex calculated from hydrodynamic data (*1*) is about $5.8 \times 10^{-18}$ cm$^3$. The difference (approximately 64 percent) between the two volumes may be attributed to the open structure of the complex which is indicated by the electron micrographs and represented schematically by the model. This interpretation adequately explains the high frictional ratio ($f/f_0$) of the complex, which has been calculated to be 1.6.

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## Fluoride: Its Effects on Two Parameters of Bone Growth in Organ Culture

Abstract. *Bones of the forepaws of young rats were subjected to varying concentrations of fluoride ions in organ culture. The formation of DNA and protein synthesis were evaluated by measurements of the uptake of tritiated thymidine and $C^{14}$-labeled proline. Fluoride concentrations as high as 10 to 20 parts per million had no demonstrable effect in vitro on these basic parameters of skeletal growth.*

Interest in the effects of fluoride ions on growth was originally aroused by the observation that deficient growth accompanied skeletal and dental lesions in sheep and cattle exposed to very high concentrations of fluoride (*1*). Further studies were initiated when it was recognized that small amounts of fluoride were of value in preventing human dental caries and it was consequently necessary to evaluate the potential hazards of its use. It has been shown that fluoride concentrations in the drinking water must be maintained constantly above 100 parts per million in order to inhibit growth in domestic and experimental animals (*2*). The decrease in stature and weight seen in animals exposed to large amounts of fluoride for long periods is accompanied by mottling of dental enamel and by signs of early skeletal fluorosis, but no other indications of pathologic changes are usually seen (*3*). Extensive studies of humans exposed to natural water supplies having considerably more than the currently recommended fluoride concentration of 1 ppm have revealed no evidence of effects on growth (*4*).

Data from a study of multiplication of HeLa cells in vitro were recently interpreted as indicating inhibition of growth by fluoride (*5*). In such systems of isolated cells, growth is defined solely by rate of cell division, a more limited definition than that employed in studies of intact animals or of tissues in organ culture. In view of these results, we thought that the concentration of fluoride necessary to cause depression of protein synthesis as well as cell division should be determined in organ culture. Organ culture of rapidly growing bone was chosen for such an investigation because of the obvious relation between skeletal tissues and statural growth, and because in the intact animal fluoride is accumulated by the skeleton.

For each of the three experiments in this series, eight littermate 5-day-old Sprague-Dawley rats were used. Endochondral ossification of the bones of the rat forepaw begins shortly before birth. At 5 days of age the phalanges and metacarpals each consist of a single ossifying area, with cartilaginous ends and an epiphyseal plate at which rapid cartilage proliferation is occurring. Their appearance is that of a miniature long bone.

The first, second, and third proximal phalanges and the second metacarpal from each forepaw were dissected free from soft tissue, explanted into a plasma-thrombin clot on a small Millipore filter, and then placed in tubes for culture at 37°C. The tubes were rotated slowly in a roller drum. Standard 199 medium, supplemented with 10-percent horse serum and with a penicillin-streptomycin mixture, was employed. Tritiated-thymidine, 0.5 $\mu$c/ml, was added as a tracer for DNA synthesis; $C^{14}$-labeled proline, 0.2 $\mu$c/ml, was used in the same medium to monitor synthesis of collagen (structural protein) (*6*). Analysis of the standard medium showed that the fluoride concentration was 0.05 to 0.10 ppm (*7*). Sodium fluoride was added to adjust the concentration of fluoride ions to desired levels between one and 500 ppm. The medium was changed every 48 hours.