In Vivo Subunit Hybridization of Succinic Semialdehyde and 4-Aminobutanal Dehydrogenases from a *Pseudomonas* Species†

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ABSTRACT: Two minor NAD^+-specific aldehyde dehydrogenase fractions have been isolated from a *Pseudomonas* species grown on putrescine as the sole source of C and N. The chromatographic, enzymic, and electrophoretic behavior of these two fractions indicate that they are hybrids at the subunit level of two trimeric enzymes (succinic semialdehyde dehydrogenase and 4-aminobutanal dehydrogenase) previously purified from this organism. The molecular weights of these two fractions agree with the theoretical values for the hybrids. Immunodiffusion and inhibition of activity experiments conducted with antibodies to the two purified enzymes show antigenic similarities between either of the purified enzymes and the hybrids, but not between the purified enzymes themselves. Finally, electrophoresis in the presence of sodium dodecyl sulfate of the immunoprecipitate of one of the hybrids reveals the presence of subunits of both of the pure dehydrogenases. These results represent an unusual situation with no known precedent and indicate an evolutionary relationship between these enzymes.

Work in this laboratory, in part reported earlier (Padmanabhan and Tchen, 1969), suggested that a *Pseudomonas* species grown on polyamines as the sole source of C and N contains not only NAD^+-specific dehydrogenases for succinic semialdehyde, 3-aminopropanal, and 4-aminobutanal, but also hybrids of these enzymes. An NAD^+-specific succinic semialdehyde dehydrogenase [(EC 1.2.1.24) succinic semialdehyde NAD^+ oxidoreductase] (hereafter referred to as succinic semialdehyde dehydrogenase) was purified to homogeneity and its physical, chemical, and immunochemical properties were reported (Callewaert *et al.*, 1973; Rosemblatt *et al.*, 1973). Likewise, an NAD^+-specific 4-aminobutanal dehydrogenase [(EC 1.2.1.19) 4-aminobutanal NAD^+ oxidoreductase] (hereafter referred to as 4-aminobutanal dehydrogenase) was purified and its physical and chemical properties were reported (Callewaert *et al.*, 1974).

In this paper we report the physical, chemical, and immunochemical evidence supporting the identity of two minor NAD^+-specific dehydrogenases as hybrids of succinic semialdehyde and 4-aminobutanal dehydrogenases. The evolutionary significance of this is discussed.

Experimental Section

Homogeneous succinic semialdehyde and 4-aminobutanal dehydrogenases, antisera toward succinic semialdehyde dehydrogenase, general reagents, and immunochemical methods used in this paper are essentially as described in earlier papers (Callewaert *et al.*, 1973, 1974; Rosemblatt *et al.*, 1973) unless otherwise stated.

Enzymatic activity was measured at pH 8.5 in an 1.0-ml reaction mixture containing the following: 105 mM $K_2P_2O_7$, 5.0 mM SSA,[1] 4 ABA or 3 APA, 0.5 mM NAD^+, and 5.0 mM mercaptoethanol. The reaction was started by the addition of the enzyme and the formation of NADH was followed at 340 nm with a Gilford recording spectrophotometer.

The phosphate buffer used throughout was prepared by mixing 0.5 M solution of NaH_2PO_4 and K_2HPO_4 at the appropriate ratio to obtain pH 7.0 (unless otherwise stated) and then diluted to the required concentrations. All buffer solutions contained 5 mM each of ethylenediaminetetraacetic acid and mercaptoethanol and 30% (v/v) of glycerol unless otherwise stated (glycerol was required for maximum stability).

Most of the isolation procedures were performed with a 10 mM phosphate buffer which will be referred to hereafter as the standard buffer. Antisera toward 4-aminobutanal dehydrogenase were prepared in young New Zealand white rabbits. Each animal received, in the foot pads at 1-week intervals, a series of injections of 4-aminobutanal dehydrogenase emulsified in an equal volume of Freund's adjuvant. The total amount of antigen administered to each rabbit was 9.9 mg. Each animal was bled at 1-week intervals, beginning 1 week after the last injection, and the sera from these successive bleedings were pooled.

The cells were grown at 30° in a 100-l. New Brunswick fermentor in 0.2% putrescine as the sole C and N source in a salt medium described previously (Padmanabhan and Tchen, 1969).

Molecular weight determination by polyacrylamide gel electrophoresis was essentially according to the method of Hendrick and Smith (1968). Gels were stained for protein in Coomassie Blue and enzymatic activity was determined essentially as described by Davis (1964). Determination of molecular weight by gel filtration through Sephadex G-200 was according to the method of Andrews (1965) with the use of 10 mM phosphate buffer with 0.15 M NaCl and without mercaptoethanol.

Electrophoresis of the immunoprecipitate of one of the hybrids in the presence of sodium dodecyl sulfate was performed as described by Weber and Osborn (1969).

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[1] Abbreviations used are: SSA, succinic semialdehyde; 4ABA, 4-aminobutanal; 3APA, 3-aminopropanal; SSADH, succinic semialdehyde dehydrogenase; 4ABADH, 4-aminobutanal dehydrogenase; SDS, sodium dodecyl sulfate.

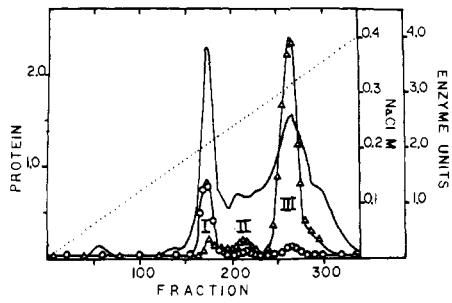

FIGURE 1: Separation of fraction B from putrescine grown cells into three fractions on DEAE-Sephadex A-50. Fraction B (700 ml) obtained from a previous DEAE-cellulose chromatography, containing 1.00 unit of 4-aminobutanal dehydrogenase/ml, 2.42 units of 3-aminopropanal dehydrogenase/ml, and 0.71 unit of succinic semialdehyde dehydrogenase/ml, was applied to a column of DEAE-Sephadex A-50 (5 × 60 cm). After a 2-l. wash, not shown, the NaCl gradient (- - -) was started; 20-ml fractions were collected and protein (—), 3-aminopropanal dehydrogenase (Δ), and succinic semialdehyde dehydrogenase (O) activities were determined and plotted. 4-Aminobutanal dehydrogenase activity (not shown) follows that of 3-aminopropanal dehydrogenase. Tubes 160–190, 200–230, and 245–278 were collected to give fractions B I, B II, and B III, respectively. Fraction B III (4-aminobutanal dehydrogenase) was further purified to homogeneity (Callewaert *et al.*, 1974). Fractions B I and B II, the suspected hybrids, were further characterized as described in the text.

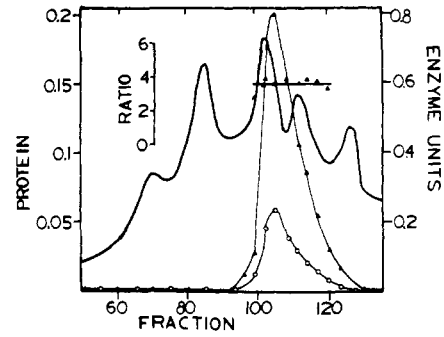

FIGURE 2: Fraction B II (20 ml), obtained from Figure 1 and concentrated on a DEAE-cellulose column, was applied to a Sephadex G-200 column (5 × 90 cm) preequilibrated with the standard buffer containing 0.15 M NaCl, and eluted with the same buffer; 15-ml fractions were collected and protein (—) and dehydrogenase activities toward 3-APA (Δ) and SSA (O) were determined. Each enzyme unit represents a change in optical density at 340 nm of one per min per ml. The ratio (▲) of the activities toward the two substrates (3APA/SSA) remained constant in all fractions with dehydrogenase activity. In a similar experiment using fraction B I, the activities for both substrates also coeluted from the same column and a similar purification was achieved.

Results

We have previously reported the isolation of pure 4-aminobutanal dehydrogenase from putrescine grown cells. During the isolation procedure, succinic semialdehyde dehydrogenase was first separated from the aminoaldehyde dehydrogenases by chromatography on cellulose. From the latter, two minor dehydrogenase fractions, with comparable activity toward succinic semialdehyde or aminoaldehydes as substracts, were isolated. These are designated as B I and B II in Figure 1. Fraction B III contains 4-aminobutanal dehydrogenase whose purification has been reported earlier (Callewaert *et al.*, 1974). It is to be noted that fractions B I and B II were eluted between pure succinic semialdehyde and 4-aminobutanal dehydrogenase and that, in contrast to these two pure dehydrogenases which showed considerable substrate specificity, fractions B I and B II showed little preference between succinic semialdehyde and 3-aminopropanal as substrate (Table I). These chromatographic and catalytic properties are consistent with the possibility that they might be hybrids of the two pure enzymes. Previously, we have shown that both succinic semialdehyde and 4-aminobutanal dehydrogenases are three subunit enzymes. If we designate these enzymes as s_3 and b_3, with s and b representing the subunits of succinic semialdehyde and 4-aminobutanal dehydrogenases, respectively, then fractions B I and B II represent hybrids with a molecular formula of s_2b and sb_2. Due to the lability of these enzymes and the fact that fractions of B I and B II have low enzymatic activities but high protein content (Figure 1), it has not been possible to purify these two fractions to any substantial degree. Preparative Sephadex G-200 column chromatography of B I and B II gave approximately five- and tenfold increase in specific activity, respectively. The ratio of activities toward succinic semialdehyde and 3-aminopropanal remained constant. These are illustrated in Figure 2. Although some purification was achieved, the resulting dehydrogenase preparations were far from pure. It was also observed that some minor degradation of the enzymes have taken place. Whereas the original B I and B II fractions each showed, upon gel electrophoresis, one sharp enzymatically active band, the pooled enzyme preparation from Figure 2 showed a broad band, probably consisting of two or three bands resulting from partial deamidation. Furthermore, although the ratio of succinic semialdehyde dehydrogenase and 3-aminopropanal dehydrogenase activities was constant for all fractions, the actual ratio had increased to 0.28 from the original ratio of 0.1. These re-

TABLE I: Properties of Dehydrogenase Fractions.

Fraction	Molecular Weight: Gel Filtration	Molecular Weight: Electrophoresis	Molecular Weight: SDS Acrylamide Electrophoresis	Precipitin Formation with Antisera to: SSADH	Precipitin Formation with Antisera to: 4ABADH	Inhibition by Antisera to: SSADH	Inhibition by Antisera to: 4ABADH	Activity Ratio (SSA/3APA)
SSADH	164,000	162,000	55,000	+	−	+	−	200
4ABADH	232,000	226,000	75,000	−	+	−	+	0.018
B I	184,000	180,000	55,000, 75,000	+	+	+	+	1.82
s_2b	185,000[a]		55,000,[a] 75,000[a]	+[a]	+[a]	+[a]	+[a]	20[a]
B II	204,000	205,000		+	+	+	+	0.1
sb_2	205,000[a]		55,000[a], 75,000[a]	+[a]	+[a]	+[a]	+[a]	5[a]

[a] Theoretical values, assuming that all subunits are fully active and that hybridization of subunits of different type does not affect the enzymatic or antigenic properties of the individual subunits.

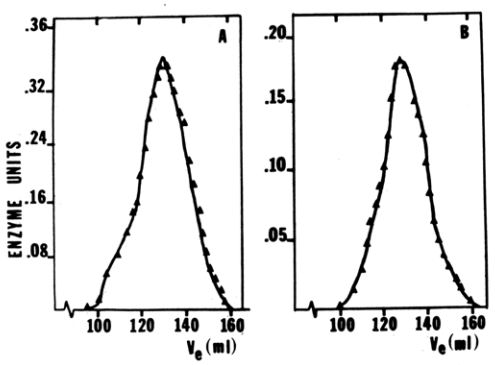

FIGURE 3: Elution profile of fractions B I and B II on Sephadex G-200. Fraction B I (2 ml) (A) or 2 ml of fraction B II (B) was applied to a Sephadex G-200 (2.0 × 45 cm) column precalibrated for molecular weight; 2-ml fractions were collected in the cold and aminoaldehyde activity (▲) was determined and plotted *vs.* elution volume.

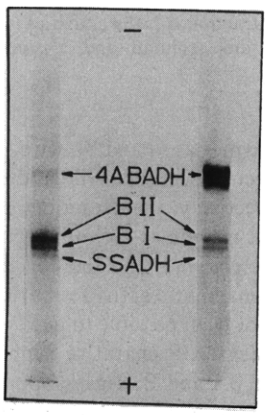

FIGURE 4: Polyacrylamide gel electrophoresis (5% acrylamide) of a mixture consisting of 4ABADH, SSADH, B I, and B II. The left gel was stained with succinic semialdehyde as substrate, the right gel with aminoaldehyde as substrate. The identity of the bands was determined by parallel electrophoresis of the separated enzyme fractions.

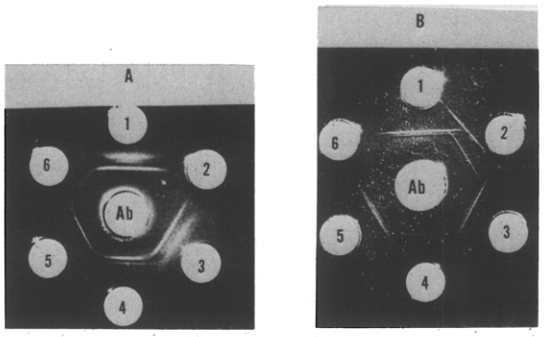

FIGURE 5: Comparative immunodiffusion studies of several enzyme fractions. Figure 5A shows an immunodiffusion plate with anti-succinic semialdehyde dehydrogenase serum (center well) and succinic semialdehyde dehydrogenase (wells 1, 3, and 5), fraction B I (well 6); fraction B II (well 4), and pure 4-aminobutanal dehydrogenase (well 2). Figure 5B shows an immunodiffusion plate with anti-4-aminobutanal dehydrogenase serum (center well) and 4-aminobutanal dehydrogenase (wells 1, 3, and 5), succinic semialdehyde dehydrogenase (well 6), fraction B I (well 4), and fraction B II (well 2). The double precipitation lines formed between purified 4-aminobutanal dehydrogenase and its antiserum was observed consistently although the enzyme appears to be pure by other criteria. The reason for this is unknown.

sults are not surprising as both parent enzymes, 4-aminobutanal dehydrogenase and to a lesser extent, succinic semialdehyde dehydrogenase, are very labile and gave multiple bands upon storage, even in 30% glycerol. The studies to be described in the following paragraphs were therefore carried out with B I and B II without further purification. The results will show that the molecular weights and immunochemical properties of B I and B II are consistent with their designation as s_2b and sb_2.

Molecular Weight Determination by Gel Filtration (Molecular Seive) Chromatography. The elution profiles of fractions B I and B II from a calibrated Sephadex G-200 column (2.5 × 45 cm) are shown in Figure 3. Both fractions B I and B II yielded a single symmetrical peak of dehydrogenase activity. The molecular weights of B I and B II were determined to be 148,000 and 204,000, respectively, as compared to 164,000 and 226,000 for pure succinic semialdehyde and 4-aminobutanal dehydrogenases, respectively (Rosemblatt *et al.*, 1973; Callewaert *et al.*, 1974).

The differences of molecular weight of these enzymes are small. When mixtures were applied to a standardized column, their elutions overlap. However, when applied individually, the peak of each enzymatic activity appeared consistently in the order of 4-aminobutanal dehydrogenase, the hybrids, and then succinic semialdehyde dehydrogenase. In a typical series of experiments, the elution peaks of these enzymes were 126, 128, 130, and 131 ml, respectively. Since these differences were too small to give confident conclusions, the molecular weight of these enzymes was also determined by gel electrophoresis.

Molecular Weight Determination by Polyacrylamide Gel Electrophoresis. Using polyacrylamide gels of six different concentrations (4–10%), the molecular weights of B I and B II were determined to be 180,000 and 205,000, respectively. In these gels, the four dehydrogenase species can be clearly separated. In Figure 4, two such gels were stained for succinic semialdehyde dehydrogenase (gel on the left) and for 4-aminobutanal dehydrogenase (gel on the right). It can be seen that the succinic semialdehyde dehydrogenase stained well with succinic semialdehyde but poorly with 3-aminopropanal as substrate. 4-Aminobutanal dehydrogenase stained well with 3-aminopropanal but poorly with succinic semialdehyde as substrate. The two suspected hybrid enzymes B I and B II stained well with either aldehydic substrate. Gel electrophoresis with B I or B II alone gave simple enzymatically active band, staining equally well with the two aldehydic substrates.

Although gel electrophoresis showed that the two dehydrogenase activities of B I (or of B II) comigrate, it was not possible to compare the relative activity of B I and B II toward these two aldehydic substrates. However, during attempts toward purification of B I and B II, it was observed that the two dehydrogenase activities remained constant as illustrated in Figure 2. There was no appreciable dissociation of either B I or B II into succinic semialdehyde dehydrogenase and 4-aminobutanal dehydrogenase.

Comparative Immunodiffusion. Using purified succinic semialdehyde dehydrogenase, 4-aminobutanal dehydrogenase, their antisera, and fractions B I and B II, it was determined that the two pure dehydrogenases do not have common antigenic sites (Figure 5A, well 2 and 5B, well 6) whereas fractions B I and B II possess common antigenic sites to both pure enzymes (Figure 5A, wells 4 and 6, and 5B, wells 2 and 4).

Inhibition of Enzyme Activity by Antibody. When antisera toward succinic semialdehyde dehydrogenase are incubated with fractions B I, B II, and purified succinic semialdehyde dehydrogenase there is a loss of enzyme activity. When the data are plotted as per cent of the remaining activity *vs.* the amount of antiserum per enzyme unit, it can be used to probe the anti-

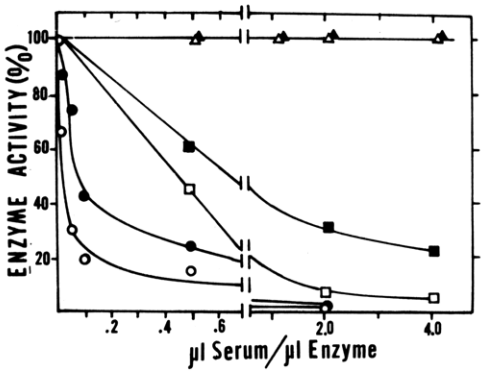

FIGURE 6: Inhibition of enzyme activity by anti-succinic semialdehyde dehydrogenase sera. Easily measurable amounts of pure succinic semialdehyde dehydrogenase and 4-aminobutanal dehydrogenase and of fractions B I and B II were incubated with various amounts of an anti-succinic semialdehyde dehydrogenase serum. After 4 hr of incubation at room temperature and then overnight at 4° the tubes were centrifuged and an aliquot of supernatant was assayed for activity using 3-aminopropanal or succinic semialdehyde as substrates. The curves represent: using succinic semialdehyde as substrate, (○) succinic semialdehyde dehydrogenase; (△) 4-aminobutanal dehydrogenase; (□) fraction B I; and (■) fraction B II; using 3-aminopropanal as substrate, (●) succinic semialdehyde dehydrogenase; (▲) 4-aminobutanal dehydrogenase. A second antiserum gave similar results. Control serum gave less than 5% inhibition of enzyme activities even at the highest ratio of serum to enzyme shown in the figure.

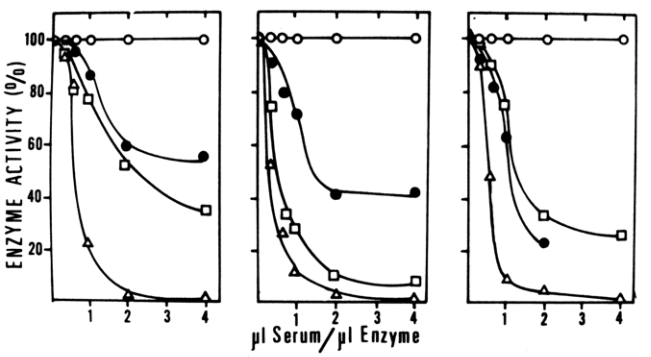

FIGURE 7: Inhibition of enzyme activity by anti-4-aminobutanal dehydrogenase sera. Easily measurable amounts of pure succinic semialdehyde dehydrogenase and 4-aminobutanal dehydrogenase and of fractions B I and B II were incubated with various amounts of anti-4-aminobutanal dehydrogenase serum. After incubation for 4 hr at room temperature and at 4° overnight the tubes were centrifuged and an aliquot was assayed for dehydrogenase activity. Left; activity toward 4-aminobutanal. The curves represent: (○) succinic semialdehyde dehydrogenase; (△) 4-aminobutanal dehydrogenase; (□) fraction B I; and (●) fraction B II. Center, activity toward 3-aminopropanal. The curves represent: (○) succinic semialdehyde dehydrogenase; (△) 4-aminobutanal dehydrogenase; (□) fraction B I; and (●) fraction B II. Right, activity toward succinic semialdehyde. The curves represent: (○) succinic semialdehyde dehydrogenase; (△) 4-aminobutanal dehydrogenase; (□) fraction B I; and (●) fraction B II. A second serum gave similar results. Control serum gave less than 5% inhibition of enzyme activities even at the highest ratio of serum to enzyme.

genic similarities of proteins. As shown in Figure 6, the amount of antiserum required to reach 50% inhibition of activity is much lower for the immunizing antigen (succinic semialdehyde dehydrogenase) than for fractions B I or B II. Purified 4-aminobutanal dehydrogenase shows no inhibition of enzyme activity when incubated with these antisera. Therefore, although succinic semialdehyde dehydrogenase is clearly the best antigen, there is some degree of antigenic similarity between fractions B I and B II and succinic semidehyde dehydrogenase while 4-aminobutanal dehydrogenase shows no antigenic similarity to succinic semialdehyde dehydrogenase.

Similarly, when antisera to 4-aminobutanal dehydrogenase are incubated with fractions B I and B II and purified 4-aminobutanal dehydrogenase, the amount of antiserum required to reach 50% inhibition of activity is much lower for the immunizing antigen (4-aminobutanal dehydrogenase) than for fractions B I or B II, while purified succinic semialdehyde dehydrogenase shows no inhibition (Figure 7). Therefore, fractions B I and B II show some degree of antigenic similarity toward both purified dehydrogenases, while the purified enzymes show no such antigenic similarity between each other.

Identification of s and b Subunits in Fraction B I. Fraction B I was precipitated with antisera to succinic semialdehyde dehydrogenase and the immunoprecipitate was thoroughly washed with 0.15 M NaCl to remove contaminating proteins. This precipitate was then dissolved and dissociated in sodium dodecyl sulfate and mercaptoethanol and subjected to electrophoresis in the presence of sodium dodecyl sulfate as described by Weber and Osborn (1969). Similarly treated crystalline rabbit immunoglobulins and other proteins of known molecular weight were run at the same time in parallel gels. The results (Figure 8) clearly show that besides the immunoglobulin bands, two additional bands are observed for the immunoprecipitate of fraction B I. Furthermore, the molecular weights calculated for these two bands correspond to the subunit molecular weights of succinic semialdehyde dehydrogenase [55,000 (Rosemblatt *et al.*, 1973)] and 4-aminobutanal dehydrogenase [75,000 (Callewaert *et al.*, 1974)]. Quantitation of the relative amount of each subunit was not possible due to incomplete dissociation of the precipitate.

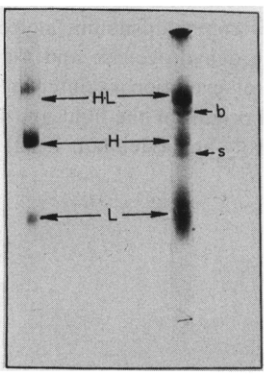

FIGURE 8: Sodium dodecyl sulfate electrophoresis of purified hybrid fraction B I. Right: results obtained on a sodium dodecyl sulfate electrophoresis of a solubilized immune precipitate formed by fraction B I and anti-succinic semialdehyde dehydrogenase. Left: results obtained in the same experiment with a crystalline rabbit immunoglobulin fraction. The letters represent: L, light chain; H, heavy chain; and H-L, heavy-light chain corresponding to one-half of the immunoglobulin molecule; s, protein band with a molecular weight corresponding to the subunit of succinic semialdehyde dehydrogenase; and b, protein band with a molecular weight corresponding to the subunit of 4-aminobutanal dehydrogenase. Results obtained in the same experiment with molecular weight standard proteins gave a linear plot for relative mobility *vs.* log molecular weight.

Discussion

The results presented herein strongly support the hypothesis of fractions B I and B II as hybrids at the subunit level of succinic semialdehyde and 4-aminobutanal dehydrogenases. The chromatographic, electrophoretic, enzymatic and immunochemical properties of these fractions are all in accord with such an hypothesis. Fraction B I has further been demon-

strated to contain subunits corresponding in size to those of the two purified enzymes.

The presence of *in vivo* hybrids of different but related enzyme subunits is admittedly an unusual situation with no known precedent, but is not surprising from theoretical considerations. Immunological studies of enzymes from different species indicate that common determinants of individual enzymes are partially retained except in cases of organisms of extremely divergent evolutionary history (Kaplan, 1965). It has also been shown that subunit hybridization requires extensive subunit structural homology (Cook and Koshland, 1969). In the course of gene mutation and recombination to allow an organism to acquire a new enzyme, the first alteration probably occurs at the active center of the enzyme to give rise to a new enzyme with different catalytic capability. This newly formed enzyme should still possess common antigenic sites and subunit interaction sites with the original enzyme. Subsequent mutation may result in further structural changes with loss of common antigenic and subunit interaction sites. Theoretically, one should find examples where the primary events of mutation and recombination have taken place while the subsequent secondary mutations have not taken place to an extent as to completely eliminate these common antigenic and/or subunit interaction sites. Indeed, common immunogenic determinants have been reported for three functionally different enzymes in *Bacillus subtilis* (Freese and Yoshida, 1965). The present work describes the presence of functionally different enzymes with common determinants for subunit hybridization. In this sense, it fills a gap in the theoretical conclusions on protein evolution.

The observed difference in subunit molecular weight of succinic semialdehyde dehydrogenase and 4-aminobutanal dehydrogenase is also of evolutionary interest. Evidence suggests common ancestral origin for the light and heavy chains of immunoglobulins (Gally and Edelman, 1972) and a similar relationship for insulin and nerve growth faction (Frazier *et al.*, 1972). The latter case, in which evidence suggests nerve growth factor arising from 1.5 copies of the ancestral gene for insulin, suggests the possibility of a similar relationship in the present study. Further investigation into the nature and extent of the evolutionary relationship of these dehydrogenases is currently in progress.

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