Hybridization of Native and Chemically Modified Enzymes. I. Development of a General Method and Its Application to the Study of the Subunit Structure of Aldolase*

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ABSTRACT: Hybridization of two variants of an oligomeric protein provides valuable information with regard to subunit structure and interactions. For most proteins, however, naturally occurring variants are not readily available; hence chemical modification of proteins was explored as a potential method for the production of a variant suitable for hybridization with the native protein. This paper considers the criteria that must be satisfied if the chemically modified protein is to be suitable as a variant. The modified protein must have a quaternary structure similar to the native protein with comparable energies of interaction between subunits. In addition, the variant must be reconstitutable after the dissociation of the oligomers into subunits. Also, if the various hybrids are to be detected readily and even separated, the chemical modification must cause a marked alteration in the physical properties (such as the electrophoretic mobility) of the protein. Finally, the modifying reagent must react specifically with the side chains of the protein in order to produce a relatively homogeneous derivative. Experimental studies on the succinylation of aldolase are presented to illustrate the various factors that must be considered in the production of a suitable variant for hybridization experiments. The experiments indicate that there is a class of about 40 lysyl residues on the surface of the enzyme which react much more rapidly with succinic anhydride than the remainder (about 60) which may be implicated in folded regions of the polypeptide chains and bonding domains between subunits. Combined ultracentrifugal and electrophoretic studies on aldolase subjected to various extents of succinylation showed that it is possible to devise a procedure which satisfied the criteria outlined above.

Fractionation experiments yielded an inactive, relatively homogeneous derivative of aldolase with quaternary structure similar to the native enzyme. Although succinylated protein had the same sedimentation coefficient as the native enzyme, its electrophoretic mobility was markedly greater due to the conversion of many positively charged lysyl residues to negatively charged succinyl-lysyl groups. Like aldolase, the modified enzyme dissociated into subunits in 4 M urea and, upon removal of the denaturant by dialysis, the intact protein was reconstituted in high yield. Dissociation and reconstitution experiments were conducted on various mixtures of the native enzyme and the variant produced by succinylation. Cellulose acetate electrophoresis and DEAE-Sephadex chromatography revealed five components in the hybrid set. The demonstration of three hybrid species constitutes strong evidence for the current model of aldolase as a tetramer composed of similar chains. The observed distribution of components produced in the hybridization experiments was in excellent agreement with that calculated on the basis of random combination of subunits. Each of the hybrid species was enzymically active; moreover the ratio of specific activities of the three hybrids relative to native aldolase indicated that each subunit in the oligomeric structure makes an independent contribution to enzyme activity. The hybridization technique and the resulting hybrids of native and succinylated aldolase serve as a valuable probe of subunits structure and interactions in oligomeric proteins.

Valuable knowledge of the subunit structure and function of proteins has been obtained from studies of the *in vitro* hybridization of two different purified forms of a single protein. With hemoglobin, in particular, hybridization experiments have been most rewarding even if, at times, they have been provoking as well as provocative (Itano and Singer, 1958; Singer and Itano, 1959; Vinograd *et al.*, 1959; Vinograd and Hutchinson, 1960; Itano and Robinson, 1960; Antonini *et al.*, 1962; Huehns *et al.*, 1962a,b; Giudotti *et al.*, 1963; Itano *et al.*, 1964; Benesch *et al.*, 1965; Guidotti, 1967). Similarly, hybridization experiments and the study of hybrids have been of considerable value in clarifying the subunit structure of lactic dehydrogenase (Markert and Møller, 1959; Markert and Appella, 1961; Markert, 1963; Kaplan, 1964; Chilson *et al.*, 1964, 1965; Di Sabato and Kaplan, 1964; Markert and Massaro, 1966). Our information of the structure of other enzymes has been enriched as well from hybridization studies (Levinthal *et al.*, 1962; Kaplan, 1963; Dawson *et al.*, 1965; Penhoet *et al.*, 1966, 1967; Kaplan, 1968; Markert, 1968; Markert and Whitt, 1968; Pietruszko *et al.*, 1969).

The detection, for example, of five variants as a consequence of hybridization of two different forms of an enzyme would constitute strong evidence indicating that the enzyme is a

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tetramer composed of identical subunits (Markert, 1963). If three isozymes are produced from two different forms of an enzyme, one has presumptive evidence that the enzyme is composed of two subunits. These conclusions are based on the assumption that each of the "mutant" forms of the enzymes employed for the hybridization experiment contains identical subunits. For such systems the number of isozymes or variants formed upon mixing the subunits is simply expressed as $i = p + 1$, where i is the number of isozymes and p represents the degree of aggregation or the number of subunits in the oligomer (Shaw, 1964). In some systems the subunits in an enzyme may not be identical even though they are functionally and structurally equivalent, *e.g.*, the various isozymes of lactic dehydrogenase (Shaw, 1964; Markert and Whitt, 1968). For these systems, too, inferences can be drawn as to the number of subunits in the protein from the general relation, $i = (n + p - 1)!/p!(n - 1)!$, where n corresponds to the total number of different subunits (Shaw, 1964; Markert and Whitt, 1968). This relationship permits valid conclusions to be drawn even from hybridization experiments with two forms of a protein which are themselves mixtures of equivalent but nonidentical subunits and the total number of dissimilar subunits exceeds the number of proteins.[1]

Although the conclusions cited above evolved from considerations of the existence of naturally occurring isozymes they are equally applicable to studies of hybridization experiments with chemically modified proteins. From the relative amounts of the different hybrids and the nature of the physical or chemical treatment required to effect hybridization, useful information can be derived about the strengths and types of interactions among the subunits in the oligomeric proteins. With some systems hybrids are formed simply by mixing the two different forms of an enzyme, whereas with other systems the production of hybrids requires the denaturation and dissociation of the protein mixture through the action of agents such as urea or guanidine hydrochloride. These hybridized proteins are effective reagents for investigating association–dissociation equilibria in proteins and for elucidating relationships among primary, secondary, tertiary, and quaternary levels of structure.

This series of papers summarizes studies on the hybridization of native and chemically modified enzymes. In this communication general aspects are considered. These include criteria which must be satisfied for the production of variants suitable for hybridization experiments and experimental findings on the succinylation of aldolase.

[1] Some systems consist of two types of subunits which are functionally and structurally distinct. These enzymes, such as tryptophan synthetase (Crawford and Yanofsky, 1958), aspartate transcarbamylase (Gerhart and Schachman, 1965), bacterial luciferase (Friedland and Hastings, 1967), and lactose synthetase (Brew *et al.*, 1968) require both types of subunits for their biological activity. The number of possible variants for such systems is described by the relation

$$i = [(n_1 + p_1 - 1)!/p_1!(n_1 - 1)!][(n_2 + p_2 - 1)!/p_2!(n_2 - 1)!]$$

where the subscripts refer to the two types of subunits which can be varied independently such as the catalytic and regulatory subunits in aspartate transcarbamylase. It should be noted that the number of variants possible for hemoglobin (Rossi-Fanelli *et al.*, 1964) is described by this equation as long as the α chains cannot be substituted for β chains (and *vice versa*) and the variations within each class are independent.

Hybridization studies with different naturally occurring FDP-aldolases (Penhoet *et al.*, 1966, 1967) have been particularly useful since they provided strong evidence that aldolase is composed of four polypeptide chains. This finding appeared in conflict with a large body of data gained earlier which supported a three-chain model for rabbit muscle aldolase. These previous experiments were carried out by a variety of techniques and were based on different approaches. Determination of the carboxyl-terminal residues of aldolase by digestion with carboxypeptidase had shown that 3 moles of tyrosine was released per mole of aldolase (Dreschler *et al.*, 1959; Kowalsky and Boyer, 1960; Rutter *et al.*, 1961; Winstead and Wold, 1964; Chan *et al.*, 1967). Similarly, three prolyl residues per molecule of aldolase were detected upon N-terminal analysis (Sine and Hass, 1967). Studies of the binding of substrates (and substrate analogs) to aldolase provided evidence successively for one, two, and at least three binding sites per molecule of enzyme (Grazi *et al.*, 1962; Horecker *et al.*, 1963; Ginsburg and Mehler, 1966; Ginsburg, 1966; Castellino and Barker, 1966; Kobashi *et al.*, 1966). Most molecular weight determinations of the native enzyme and the dissociated polypeptide chains produced by denaturing agents gave results consistent with a three-chain model (Stellwagen and Schachman, 1962; Deal *et al.*, 1963; Hass, 1964; Schachman and Edelstein, 1966; Sine and Hass, 1967). Kawahara and Tanford (1966), however, claimed from their molecular weight studies of the native enzyme and the polypeptide chains in guanidine hydrochloride that aldolase was composed of four subunits.

Subsequent to the hybridization studies, reinvestigation of the carboxyl-terminal groups of aldolase (Morse *et al.*, 1967), the N-terminal residues of aldolase (Kochman *et al.*, 1968), and the molecular weight of the dissociated enzyme (Sia and Horecker, 1968; Szuchet and Yphantis, 1968; Castellino and Barker, 1968), provided strong evidence for the four-chain model for aldolase. The discrepancies between these latter results and the earlier findings supporting the three-chain model can be traced to a variety of causes. End-group determinations and binding studies all tend to give low values for the number of subunits in a macromolecule. These misleading conclusions may stem from loss of binding sites or terminal residues by blockage or cleavage prior to experimentation, incomplete reactions, and the use of preparations of enzymes which contain inactive impurities such as denatured enzyme or other proteins. Variations in the molecular weight of the enzyme in dissociating media can be caused by difficulties in applying the required theoretical correction for possible preferential interactions in determinations of the molecular weight of macromolecules in multicomponent systems. Moreover, practical considerations such as the obvious heterogeneity of the dissociated enzyme in almost all reported experimental determinations of the molecular weight makes interpretation of the results very difficult. These discrepant findings on aldolase clearly indicate the need for additional approaches and specifically commend the use of hybridization experiments as a potential method for the elucidation of the subunit structure of proteins.

Accordingly hybridization experiments were initiated on mixtures of native and chemically modified aldolase. As shown in this paper, aldolase can be succinylated to produce a reasonably homogeneous variant. This succinylated derivative had an electrophoretic mobility markedly different

from the native enzyme. However, the sedimentation coefficient of the succinyl-aldolase preparation was virtually identical with that of the native enzyme, thus showing that quaternary structure had not been altered appreciably despite the large change in the net charge on the protein. The succinylated enzyme could be dissociated into subunits by treatment with urea. Subsequent removal of the urea led to the reconstitution of a product having properties similar to the modified intact molecules. Hybridization experiments with mixtures of succinyl-aldolase and native aldolase have been performed and upon electrophoresis five variants were detected. This result provides additional strong support for the view that aldolase is composed of four similar subunits.

Criteria for Production of Suitable Variants

Most hybridization experiments to date have involved the use of two naturally occurring variants of a single enzyme such as lactic dehydrogenase (Markert, 1963), creatine kinase (Dawson *et al.*, 1965), and aldolase (Penhoet *et al.*, 1966, 1967). Often, however, both of the required variants (or isozymes) are not available in purified form or in sufficient quantities. Under these circumstances the second variant must be produced by chemical modification of the single purified enzyme. Several criteria must be satisfied if the chemically modified species is to be suitable for hybridization with the native enzyme. First, the modified protein must possess an electrophoretic mobility (or other physical property) which is substantially different from that of the native enzyme. This alteration in properties must be sufficiently great that the two species can be readily distinguished or even separated from one another. Second, the chemical treatment must be relatively specific so as to cause uniform modification of all the enzyme molecules in the preparation and thereby produce a reasonably homogeneous derivative. Third, the quaternary structures of the modified and native enzyme must be similar with comparable interaction energies among the respective subunits; *i.e.*, the modified enzyme must not be much less (or much more) stable than the native enzyme. Fourth, the chemically altered enzyme, like the native enzyme, must be reconstitutable after dissociation into subunits. Finally, it should be noted that the modified enzyme need not be active. On the contrary, it is advantageous if the chemically altered protein molecules are enzymatically inactive; for such systems activity measurements permit the eludication of the composition of the hybrids in terms of the relative amounts of the different subunits.

Previous hybridization experiments with modified proteins have been performed with native and succinylated hemerythrin (Keresztes-Nagy *et al.*, 1965) and with native and detyrosinated aldolase produced by limited treatment of the enzyme with carboxypeptidase A (F. P. Hamburg and H. K. Schachman, unpublished data). In the former study, hemerythrin was succinylated under mild conditions in order to introduce negative charges without dissociation of the protein. Mixing the succinylated hemerythrin with the native protein produced species with a variety of intermediate electrophoretic mobilities. No distinct electrophoretic variants were detected, but this observation can be attributed to the electrophoretic heterogeneity of the succinyl-hemerythrin and to the association–dissociation behavior of the protein (Klapper *et al.*, 1966; Klapper and Klotz, 1968). In contrast, the removal of the C-terminal tyrosyl residues from aldolase by carboxypeptidase A digestion produced a reasonably homogeneous species. However, the difference in electrophoretic mobility between the native and modified enzyme was very small. As a consequence, upon dissociation and reconstitution of a mixture of the native and carboxypeptidase-treated enzyme, only a broad electrophoretic band was detected with a mobility intermediate between those for native and treated aldolase. Although this was conclusive evidence for the formation of hybrids, no resolution of intermediates was possible and thus the detection of distinct hybrids of well-defined composition was not achieved. The latter experiment illustrated the need for chemical modifications which produced a significant alteration in the net charge on the protein. Accordingly, studies were conducted on the succinylation of aldolase since this procedure is known to produce a large change in the electrophoretic mobility by the introduction of negatively charged succinyl groups onto lysyl, sulfhydryl, threonyl, and seryl residues (Maurer and Lebovitz, 1956; Habeeb *et al.*, 1958; Klotz and Keresztes-Nagy, 1963; Hass, 1964; Gounaris and Ottesen, 1965; Gounaris and Perlmann, 1967; Bethune *et al.*, 1967; Cohen, 1968). This particular modification has been investigated in an attempt to produce uniformly succinylated enzyme molecules which still possess quaternary structures analogous to those of the native enzyme. These studies on limited succinylation were necessary since extensive modification of aldolase has been found to cause its dissociation into individual, disorganized polypeptide chains (Hass, 1964).

Homogeneous succinylated enzymes are ideal for hybridization with the native enzymes since the detection of enzyme hybrids can be accomplished most readily by electrophoresis experiments. Other techniques, such as chromatography and density gradient centrifugation, can be used under certain circumstances (Zipser, 1963); but electrophoretic methods, because of their simplicity and applicability with only small amounts of material, constitute a powerful approach for the analysis of hybridization experiments. If, for example, the two enzyme forms are represented by AAAA and BBBB, then the various hybrids, AAAB, AABB, and ABBB, have electrophoretic mobilities intermediate between those for the two purified forms. Moreover, the distance of separation of the species upon electrophoresis should be constant from one to the next. Departures from this expected behavior would indicate the presence of components of different structural forms such as aggregates or the absence of some species expected from the random mixing of subunits.

Production of Homogeneous Variants by Chemical Modification

The modification of a multivalent protein with limited quantities of a specific reagent should lead to a heterogeneous population of molecules if all the groups on the protein react equally rapidly and independently. At intermediate extents of reaction, some protein molecules would be extensively modified, whereas others may have reacted to a much smaller extent.[2]

[2] For a protein containing *N*-reactive side chains, *e.g.*, lysyl residues, the distribution of groups reacting with a reagent such as succinic

When the degree of succinylation is low, the derivative may be sufficiently homogeneous, but the change in electrophoretic mobility may be too small to permit the subsequent detection (and separation) of the individual hybrids. Increasing the degree of succinylation would doubtless prove of value in permitting the resolution of the different components by electrophoresis but the increase in the heterogeneity of the modified protein would preclude its use for hybridization experiments. Although these two disadvantages are both circumvented by the use of extensive modification of the protein, this alternative also is generally unsatisfactory. Most oligomeric proteins subjected to extensive succinylation, for example, are so destabilized by the net electrostatic repulsion that dissociation into subunits occurs (Klotz and Keresztes-Nagy, 1962; Hass, 1964; Polyunovski, 1965; Spector and Katz, 1965; Poillon and Bearn, 1966; Scanu *et al.*, 1968; Jaenicke and Knof, 1968; Jaenicke *et al.*, 1968).

If, however, an oligomeric protein contains two (or more) classes of lysyl residues having different reactivities toward a given reagent, then different conclusions are reached. For example, if one class of lysyl residues can be almost completely modified and another class is essentially unmodified then a relatively homogeneous species could be obtained at the appropriate degree of chemical modification. Theoretical expressions could be given for such models but such formulations seem premature because of the inadequacy of our knowledge of the reactivity of the side chains in larger protein molecules. There is little doubt, however, that the reactivity of side chains containing amino, sulfhydryl, and phenolic groups vary substantially not only from one protein to another but also within a single protein. Thus it is not unexpected that the succinylation of carboxypeptidase A led to a modified protein which was substantially more homogeneous than that calculated from the binomial distribution for a single class of reactive groups (Bethune *et al.*, 1967). Having found a relatively sharp distribution of electrophoretic mobilities for *N*-succinyl-carboxypeptidase A and after considering the reactivity of various types of side chains, Bethune *et al.* (1967) concluded that there were different classes of reactive lysyl residues in that enzyme. Similar conclusions were reached even earlier by Fischer and Lauffer (1949) in their studies on the reaction of tobacco mosaic virus with formaldehyde.

anhydride should follow the binomial law (Baldwin *et al.*, 1951). This distribution can be expressed as

$$P_n = \frac{N!}{(N-n)!n!} f^n(1-f)^{N-n}$$

where f is the fraction of groups reacted and P_n is the probability that n groups in any molecule have reacted. The above equation shows that the distribution of modified groups in a protein after a random reaction can be calculated for any average degree of substitution. Similarly the standard deviation, σ, of the distribution of reacted side chains is readily estimated (Fischer and Lauffer, 1949) from $\sigma = [Nf(1-f)]^{1/2}$. This equation provides a direct measure of the heterogeneity of the modified protein; in 68% of the molecules the number of reacted groups will be greater than $fN - \sigma$ and less than $fN + \sigma$. The standard deviation can be used to characterize the distribution in an alternative manner since 98% of the molecules will have a number of reacted groups equal to $fN \pm 2.3\sigma$, where fN is the average number of substituted side chains. The above relations show that the products of reactions involving intermediate degrees of substitution are heterogeneous; only at very low or very high degrees of reaction are relatively homogeneous derivatives obtained.

Although as yet the variation in the reactivity of the side chains in larger proteins has not been examined in detail, there is considerable evidence for smaller protein molecules showing substantial differences in the chemical reactivity among the amino acid residues of a particular type (Cohen, 1968). Such differences have been observed for lysyl, cysteinyl, tyrosyl, histidyl, and seryl residues in proteins such as ribonuclease, lysozyme, chymotrypsin, and hemoglobin. Thus it is to be expected that chemical modification or proteins with group specific reagents would produce more homogeneous derivatives than is predicted for a random reaction.

Dissociation of Oligomeric Proteins upon Chemical Modification

Since the reaction of an oligomeric protein-like aldolase with chemical reagents (*e.g.*, hydrogen or hydroxyl ions, succinic anhydride, and sodium dodecyl sulfate) is frequently accompanied by the disruption of the tertiary and quaternary structures (Ramel *et al.*, 1961; Stellwagen and Schachman, 1962; Deal *et al.*, 1963; Hass and Lewis, 1963; Hass, 1964; Sine and Hass, 1967; Sia and Horecker, 1968), there will be at any stage of the reaction a population of modified protein molecules with some in the form of compact, intact structures and others having the conformation of disorganized polypeptide chains. Moreover, the unfolded subunits are likely to have a different reactivity toward any specific reagent. In addition, a partially reacted intact molecule may undergo a conformational change which leads to an enhanced reactivity of other side chains in the same molecule. Alternatively, the modification of several side chains of a protein may cause a diminished reactivity of other equivalent residues because of electrostatic effects. We can visualize at least two possible models for the reaction of an oligomeric protein with a reagent like succinic anhydride which causes a destabilization of the protein because of the increased net charge (Schachman, 1960; Klotz and Keresztes-Nagy, 1962). For one model (random reaction) we assume that the lysyl residues are modified at random with dissociation occurring when a critical number of residues are altered. Thus, only within a limited range of succinylation are both intact and dissociated molecules present in the reaction mixture. At the other extreme (all-or-none reaction) we assume that modification of the first lysyl residue causes a marked increase in the reactivity of the remaining lysyl residues in the same molecule along with the dissociation of the oligomeric protein into subunits. For this model the disappearance of intact molecules (or formation of subunits) is linearly dependent on the extent of succinylation.

Although these two models doubtless represent oversimplifications of the dissociation of oligomeric proteins as a consequence of chemical modification, they provide a framework for the interpretation of experimental findings on aldolase. Moreover, they indicate the type of constraints which must be considered in attempts to modify proteins chemically for the production of variants suitable for hybridization experiments. Since our present knowledge of the reactivity of side chains in oligomeric proteins is so limited and there is so little experimental data on the correlation between the extent of dissociation into subunits and the degree of chemical modification, it does not seem profitable now to consider additional possible models. Instead we

turn to experiments on the succinylation of aldolase which are analyzed in terms of the concepts outlined above.

Experimental Section

Materials. Aldolase, prepared by the method of Taylor *et al.* (1948) as modified by Kowalsky and Boyer (1960), was recrystallized two to three times to a specific activity of 12–15 μmoles of FDP cleaved per min per mg as determined by the method of Richards and Rutter (1961). Commercial aldolase having a specific activity of 9–10 μmoles of FDP of cleaved per min per mg was obtained from either Sigma Chemical Co. or C. F. Boehringer and Soehne. The protein concentration (molar) was determined spectrophotometrically on the basis of a specific absorbance coefficient of 0.91 (0.1%, 1 cm) at 280 mμ (Baronowski and Niederland, 1949) and a molecular weight of 1.6×10^5 (Kawahara and Tanford, 1966).

Samples of aldolase, succinylated to different extents, were prepared according to the procedure of Hass (1964). An aliquot of solid succinic anhydride (Eastman Organic Chemicals) was added to a 0.8% solution of aldolase in 0.05 M Tris-chloride at room temperature; the pH was maintained automatically at 8.0 by the controlled addition of 1.0 M NaOH with a Radiometer autotitrator. A sample of succinylated aldolase was removed after each aliquot of succinic anhydride had reacted as indicated by the termination of the requirement for NaOH. In some experiments various extents of succinylation were obtained by the addition of another aliquot of succinic anhydride and the procedure was repeated. The various preparations of succinylated aldolase were dialyzed at 4° against the desired buffer.

The number of moles of succinic anhydride added before removal of the sample was divided by the total number of moles of lysyl residues to give the molar ratio of succinic anhydride to lysine. According to the analysis of Velick and Ronzoni (1948) there are 104 lysyl residues/molecule of aldolase of molecular weight 1.6×10^5.

Urea from the J. T. Baker Chemical Co. was recrystallized from 70% ethanol by the method of Steinhardt (1938). All other chemicals were reagent grade.

Methods. The number of free amino groups in the protein was estimated by colorimetric analysis according to the method of Moore and Stein (1948) as modified by Fraenkel-Conrat (1957). Prior to analysis, solutions of aldolase and succinylated aldolase were dialyzed exhaustively against 0.02 M potassium phosphate (pH 6.50). Ninhydrin reagent (1 ml) was added to 0.1–0.8 mg of protein in 0.1 ml. The ninhydrin reagent was prepared by mixing 0.8 g of stannous chloride dihydrate in 500 ml, pH 5 citrate buffer (21 g of citric acid monohydrate/500 ml), with 20 g of ninhydrin in 500 ml of methyl Cellosolve. The solution was saturated with N_2 and stored in the refrigerator. The reaction mixture was heated in a boiling-water bath for 20 min. It was then rapidly mixed and cooled with 5.0 ml of 50% aqueous isopropyl alcohol solution, and the optical density was determined at 570 mμ within 40 min. Native aldolase was used for a standard curve and gave a linear relationship between the amount of protein (0.0–0.8 mg) and the absorbance at 570 mμ (0.0–1.6). All optical density values were corrected against a blank containing an appropriate volume of dialysate (A_{570} 0.03).

Sedimentation velocity experiments were performed with a Spinco Model E ultracentrifuge equipped with cylinder lens schlieren optical system having a phase plate as the schlieren diaphragm. The temperature was measured and controlled by the RTIC unit supplied by the manufacturer. Double-sector cells with a 12-mm optical path, sapphire windows, and aluminum-filled, epoxy centerpieces were used routinely. Photographic plates (Metallographic) were analyzed with the aid of a Gaertner microcomparator.

The experiments were conducted at a constant temperature (19–22°). The observed sedimentation coefficients were corrected to values for a solvent with the viscosity and density of water at 20° ($s_{20,w}$). A partial specific volume of 0.742 ml/g was used for both native aldolase (Taylor *et al.*, 1948) and succinyl-aldolase that sedimented at a rate similar to native aldolase. For dissociated succinyl-aldolase a partial specific volume of 0.704 ml/g was used (Hass, 1964). The distribution of components of different sedimentation coefficient was estimated from the area corresponding to each boundary after adequate resolution of the components had been achieved. These measured areas were corrected for radial dilution by multiplying by the square of the distance of the boundary from the axis of rotation relative to the square of the distance from the axis of rotation to the meniscus.

Zone electrophoresis experiments were performed routinely on 14.6-cm cellulose polyacetate strips (Gelman Sepraphore III or Beckman-Spinco) in a Microzone electrophoresis cell, Model R-101 (Beckman-Spinco). The buffer contained 0.002 M EDTA and 0.02 M potassium phosphate at pH 6.5. A voltage of 200–250 V (14–18 V/cm) was applied for 12–20 min. Precooling the buffer to 4° led to a slight increase in resolution of the bands over that observed at room temperature.

The protein was fixed and stained by immersion of the membrane in a solution of ponceau S in trichloroacetic acid and sulfosalicylic acid (Beckman-Spinco) for approximately 7 min. The membrane was then rinsed in 5% acetic acid. A much darker stain was obtained by immersion of the membrane already stained with ponceau S in 0.002% nigrosin (Allied Chemical Co.) in 2% acetic acid for a period of several hours or longer. The membrane, after rinsing in 5% acetic acid, was then dried at room temperature and stored.

In some experiments, the membranes were stained for enzyme activity according to the method of Penhoet *et al.* (1966). A 0.5% Noble agar solution in 0.1 M sodium arsenate (pH 7.5) containing 0.01 M NaFDP (Calbiochem), 0.001 M NAD (Sigma Chemical Co.), 0.12 mg/ml of glyceraldehyde 3-phosphate dehydrogenase (Boehringer), 0.024 mg/ml of phenazine methosulfate (Aldrich Chemical Co.), and 0.4 mg/ml of nitroblue tetrazolium chloride (Aldrich Chemical Co.) was poured into shallow Petri dishes (4–10 ml each) at 42° and allowed to solidify at 4°. After electrophoresis, the cellulose acetate strips were placed on this agar and incubated at 37° for 5–20 min to allow color development. In this system, aldolase is coupled with triosephosphate dehydrogenase so that cleavage of FDP results in formation of NADH. This in turn reduces nitroblue tetrazolium *via* mediation of phenazine methosulfate.

Electrophoretic mobilities were calculated from the distance migrated per unit time divided by the potential gradient which in turn was evaluated from the voltage applied and the length of the electrophoresis membrane. These calculated

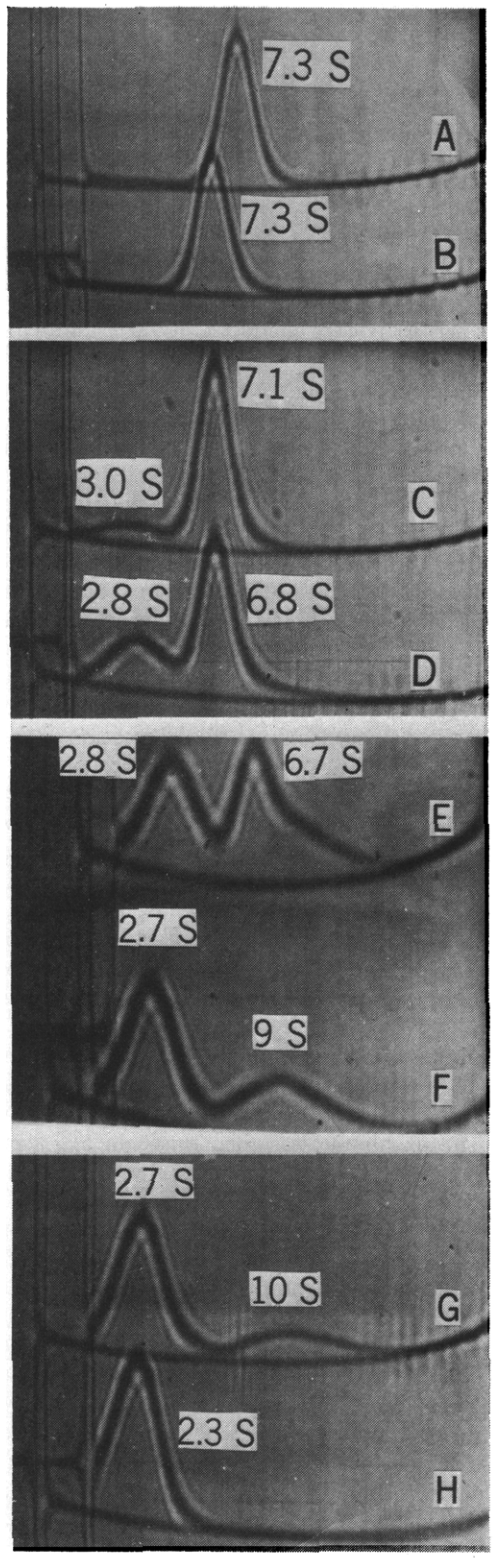


FIGURE 1: Sedimentation velocity patterns of aldolase succinylated to different extents. The reaction of different amounts of succinic anhydride with aldolase was conducted according to the procedure given in Methods. The extent of succinylation increased from sample A–H (see Table I). The ultracentrifuge experiments were conducted in a solvent containing 0.5 M NaCl, 0.002 M EDTA, 5×10^{-4} M dithiothreitol, and 0.02 M potassium phosphate (pH 6.50) at 19–22°. A double-sector cell with a negative wedge window was used in conjunction with the regular cell so that two sedimentation velocity experiments could be conducted simultaneously. Photographs were taken approximately 30 min after reaching a speed of 60,000 rpm. Movement is from left to right. The sedimentation coefficients shown in the figure were corrected to values for a solvent with the viscosity and density of water at 20°.

TABLE I: Chemical and Sedimentation Properties of Succinylated Aldolase.

Sample	Moles of Succinic Anhydride Added/ Lysyl Residue	f[a]	f_I[b]
A	0.0	0.0	1.0
B	1.3	0.24	1.0
C	2.8	0.46	0.84
D	4.3	0.60	0.57
E	5.8	0.75	0.34
F	7.6	0.87	0.12
G	11.4	0.90	0.03
H	18.0	$\geqslant 0.9$	0.0

[a] The fraction of lysyl residues succinylated (f) was estimated by ninhydrin analysis as described in Methods. [b] The fraction of intact molecules (f_I) was estimated from the sedimentation patterns in Figure 1 (or from additional patterns in the same experiments). The values for samples E–G are approximate estimates since aggregates sedimenting at rates similar to native aldolase were also present.

mobilities indicate only the relative migrations of the different components on the electropherograms as no correction was made for such effects as electroosmosis, evaporation, changes in temperature, the "effective" path length on a supporting medium, etc. (Wunderly, 1959; Kunkel and Trautman, 1959). Indeed, small differences in mobilities were observed in the present experiments from one electropherogram to another although the relative positions of the bands were not changed.

Results and Discussion

Effect of Succinylation on the Sedimentation Behavior of Aldolase. Representative sedimentation velocity patterns of aldolase samples succinylated to different extents are given in Figure 1. At low extents of modification of the lysyl residues (below about 40%) only a single sedimenting component was observed with a sedimentation coefficient (7.3 S) characteristic of the native enzyme. More extensive succinylation led to the progressive disappearance of the 7.3S component (intact modified aldolase molecules)[3] and the concomitant formation of a more slowly sedimenting species (about 2.6 S). Some aggregated material with a sedimentation coefficient of 9–10 S is observed also at the higher degrees of succinylation. It should be noted, however, that this aggregated material was not detectable in the most succinylated preparation.

[3] The slight decrease in sedimentation coefficient of the faster component (see Figure 1) upon progressive succinylation may be attributed to a concentration dependence stemming from the formation of the slower component. Support for this interpretation comes from the observation that the slower component also exhibited a decrease in sedimentation coefficient as the extent of succinylation increased. It should be noted, however, that the decrease in sedimentation coefficient of the faster component may be indicative of a slight alteration (such as swelling) of the intact molecules.

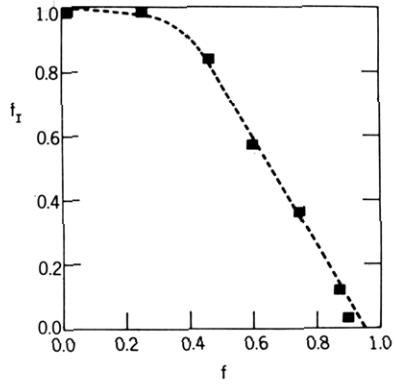


FIGURE 2: The relationship between the fraction of intact molecules (f_I) and the fraction of lysyl residues succinylated (f) in aldolase. The experimental data (■) were taken from Table I. The theoretical curve for a protein that contains 100 lysyl residues was generated for comparison. For this curve (---) it was assumed that 35 lysyl residues reacted with a first-order rate constant 50 times that of 60 of the remaining residues and that modification of the first lysyl residue in this latter group caused a marked increase in the reactivity of the remaining residues in this group along with dissociation of the protein into subunits. It was assumed further, because of the persistence of some ninhydrin reacting groups, that 5 residues did not react at all.

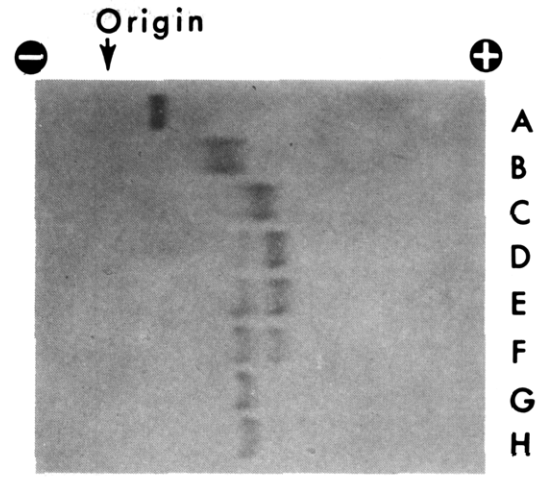


FIGURE 3: Cellulose acetate electrophoresis of aldolase succinylated to different extents (A–H from Table I). Electrophoresis was performed for 12 min at 250 V and the membrane was stained for proteins as described in Methods.

Chemical Properties of Succinylated Aldolase. Table I shows the extent of succinylation of the lysyl residues in terms of the ratio of added succinic anhydride per mole of lysyl side chains. In each experiment the reaction was carried to completion (*i.e.*, total decomposition of the added succinic anhydride). Thus only a small fraction (10–20% depending on the sample) of the succinic anhydride was consumed in the modification of the lysyl residues. Titration of the succinylated samples with *p*-hydroxymercuribenzoate showed a progressive decrease in the number of free sulfhydryl groups as the extent of succinylation was increased. The modification of other side chains in the protein was not investigated. Thus we have no evidence as to the possible formation of stable esters as a result of the reaction of the hydroxyl groups of serine and threonine with succinic anhydride (Gounaris and Perlmann, 1967). Tyrosyl residues also have been shown to react with succinic anhydride (Riordan and Vallee, 1964), but the resulting esters have been shown to be unstable and to decompose to regenerate phenolic groups. Although no quantitative data are available it seems likely that the bulk of the succinic anhydride reacted with the amino groups of the Tris buffer and with water.

Relationships between Extent of Succinylation of Aldolase and Its Sedimentation Behavior. Table I summarizes the ultracentrifuge studies in terms of the fractional amount of aldolase sedimenting as intact molecules, f_I, as a function of the extent of succinylation. These data (see also Figure 2) show that intact molecules persist even though about 40% of the lysyl residues were succinylated. At higher degrees of modification of the lysyl side chains the fraction of intact molecules decreased in approximately a linear fashion with the increase in succinylation. This behavior is consistent with that expected for a protein containing (a) a reactive class of lysyl residues on the surface of the molecule and (b) a relatively unreactive class of lysyl residues which are located in folded regions of the polypeptide chains or implicated in binding domains between subunits. According to an all-or-none model, upon succinylation of these latter lysyl residues the intact protein would dissociate with a concomitant increase in the reactivity of the remaining lysyl residues in the same class. The curve in Figure 2 was calculated for such a model with the assumption that 35 of the lysyl residues per aldolase molecule were in the class of rapidly reacting groups which act independently and at random; of the remainder, 60 residues were considered as a less reactive group, modification of which is accompanied by dissociation of the protein into subunits, and 5 residues were considered to be totally unreactive. As seen in Figure 2 the theoretical curve provides a satisfactory fit of the experimental data for the succinylation and dissociation of aldolase. Other models doubtless could account for the experimental findings equally satisfactorily; hence inferences as to the validity of an all-or-none model are unwarranted. For definitive conclusions to be drawn as to a detailed mechanism additional studies are required. Such investigations, though of interest, are not relevant to the goal of the present study aimed at devising a general method for producing a suitable variant for hybridization experiments.

Electrophoretic Properties of Succinylated Aldolase. Figure 3 shows the electrophoresis patterns of aldolase succinylated to different extents (A to H in Table I). Electrophoresis of aldolase molecules which had 25% of their lysyl residues succinylated (B) showed only one component with a mobility of about -1.1×10^{-4} cm²/V sec. This value is to be contrasted with that for the native enzyme (A) which under comparable experimental conditions had an electrophoretic mobility of -0.3×10^{-4} cm²/V sec. Thus moderate succinylation produced a derivative having the same size as the native enzyme (see Figure 1) and a mobility markedly different from the unmodified protein. In these two respects succinylation appeared to satisfy the criteria which must be considered in the design of chemical modification procedures for making variants suitable for hybridization experiments. However this particular sample was electrophoretically heterogeneous and thus not suitable as a variant.

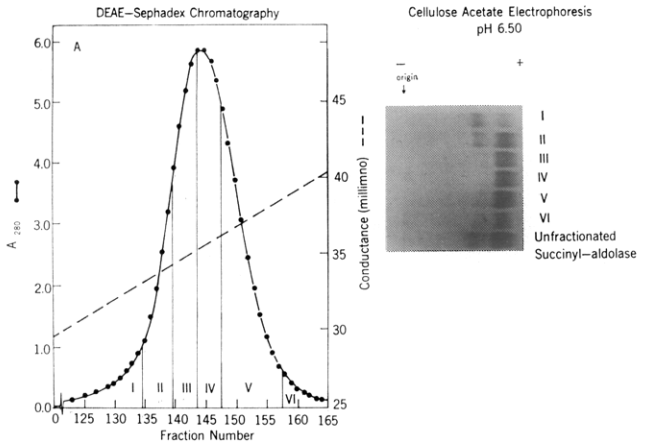


FIGURE 4: Purification of succinyl-aldolase on DEAE-Sephadex A-50. Succinylated aldolase was prepared according to the procedure given in Methods by reaction with aldolase of 2.8 moles of succinic anhydride/lysyl residue (45–50% of the lysyl residues succinylated). The sample was placed on a 3 × 34 cm column preequilibrated with 0.05 M Tris-chloride–0.001 M EDTA (pH 7.50). The fractions (5 ml) were eluted with a linear gradient of NaCl from 0 to 1.0 M over a range of 1000 ml in pH 7.50, 0.05 M Tris-chloride, 0.001 M EDTA, and 2×10^{-4} M dithiothreitol. The flow rate was 20 ml/hr per cm^2. The fractions were analyzed for absorbance at 280 mμ and conductance, and then pooled as indicated above (I–VI). Cellulose acetate electrophoresis (200 V, 15 min) of the different fractions was performed as described in Methods.

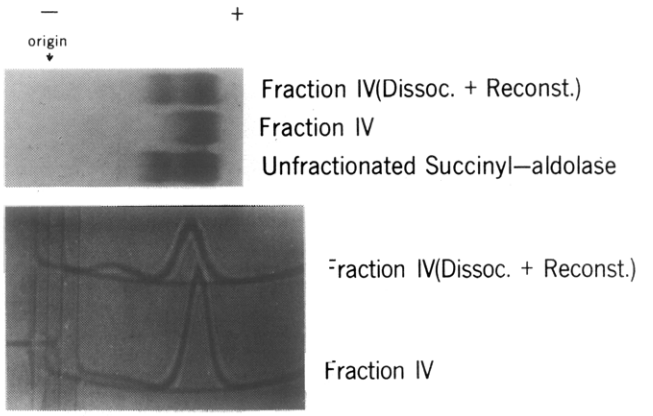


FIGURE 5: Cellulose acetate electrophoresis and sedimentation velocity patterns of succinyl-aldolase (45–50% of the lysyl residues succinylated) purified on DEAE-Sephadex (fraction IV from Figure 4) and the same sample after dissociation and reconstitution. Electrophoresis was carried out for 12 min at 300 V and the membrane (Beckman) was stained for protein as indicated in Methods. The sedimentation velocity experiments were performed in solutions containing 0.05 M Tris-chloride, 0.001 M EDTA, and 0.5 M NaCl (pH 7.50) at 21°. A double-sector cell with a wedge window was used in conjunction with the regular cell in order to permit sedimentation analysis of two samples simultaneously. The initial protein concentrations of fraction IV before and after dissociation were 0.5 and 0.4%, respectively. Sedimentation is from left to right. The photograph was taken with a phase-plate angle of 65° after 24 min at a rotor speed of 60,000 rpm.

When the extent of succinylation of the lysyl groups was increased from 25% to about 45%, two components were detected by zone electrophoresis (C). The more rapidly migrating component had an electrophoretic mobility of -1.6×10^{-4} cm^2/V sec and the zone representing that component was considerably narrower than the band observed for the 25% succinylated sample. The mobility of the slow component was -1.2×10^{-4} cm^2/V sec. In this mixture corresponding to 45% succinylation of the lysyl residues the more rapidly migrating species was the major component. At higher degrees of succinylation (D–G), once again two components were observed; but the amount of the faster component decreased and there was a concomitant increase in the amount of the more slowly migrating species. Since the ultracentrifuge experiments (Figure 1) showed that some of the modified molecules were dissociated into subunits when an average degree of succinylation of about 45% was attained, we conclude that the more rapidly migrating component in the electrophoresis experiments corresponded to intact, succinylated molecules and the slower species represented succinylated subunits.[4] Support for this conclusion came from the finding that succinylation beyond a level of 45% did not lead to any extensive sharpening of the more anodic component. Nor did it affect appreciably the mobility of that component.

Since in the samples containing two components (C–G) the faster component exhibited a relatively narrow band on electrophoresis, these samples seemed particularly suitable for hybridization experiments. Sample C, in particular, was selected since it contained only a small amount (less than 20%) of the more slowly migrating component. The removal of this material by fractionation is described in the next section.

Purification of Undissociated Succinylated Aldolase for Hybridization Experiments. Although sample C (see Table I) appeared suitable for hybridization with the native enzyme, the electrophoretic analysis of the hybrid set in the initial experiments proved complicated because of the presence of the contaminating succinylated subunits. Accordingly this succinylated aldolase preparation was subjected to chromatographic fractionation aimed at eliminating the slowly migrating, dissociated species.

As seen in Figure 4, the slow component in the succinyl-aldolase preparation was removed by chromatography on DEAE-Sephadex A-50. More than 90% of the protein applied to the column (500 mg) was recovered. The individual fractions were pooled (I–VI), the protein precipitated by dialysis against saturated ammonium sulfate, and the precipitate dissolved by dialysis against 0.02 M potassium phosphate–0.002 M EDTA (pH 6.50). Cellulose acetate electrophoresis of the different fractions (Figure 4) showed that both components were present in only the initial fractions (I and II) and that the major or fast component representing intact succinylated aldolase molecules was obtained in a relatively pure form (III–VI).

Dissociation and Reconstitution of Succinyl-aldolase. Since the hybridization studies involve dissociation and reconstitution of mixtures of the native and modified enzyme,

[4] Further evidence in support of this conclusion is presented later. When a preparation such as sample D is subjected to DEAE-Sephadex chromatography the fractions eluted first were found to have the smaller electrophoretic mobility. Though these molecules are smaller (in weight) they probably are swollen (or perhaps more anisometric) and consequently have a lower charge density as well as a smaller total net charge.

experiments were initiated on the efficiency of the reconstitution of the urea-treated succinyl-aldolase. As seen in Figure 5 the purified succinyl-aldolase after dissociation and reconstitution is contaminated by an additional component that is apparently identical in electrophoretic mobility to the slow component observed in the unfractionated succinyl-aldolase. The sedimentation velocity pattern of purified undissociated succinyl-aldolase (Figure 5) shows that only about 3% of the sample had a sedimentation coefficient less than 7 S.[5] However, after dissociation and reconstitution of the purified succinyl-aldolase approximately 20% of the sample had a sedimentation coefficient of about 3 S. No protein was lost upon dissociation and reconstitution of the succinyl-aldolase sample. A comparison of these sedimentation velocity patterns with the electrophoresis results demonstrates clearly that the slow and fast components in the electrophoresis patterns have sedimentation coefficients of about 3 and 7.5 S, respectively.

Since the slowly sedimenting component in the reconstituted material had an electrophoretic mobility intermediate between aldolase and succinyl-aldolase its presence introduced some complications in the interpretation of the electropherograms of the hybrid set of native and modified aldolase. If unfractionated succinyl-aldolase (15% slow component, 85% fast component) is dissociated and reconstituted then approximately 35–50% of the sample would exist as the slow component. Therefore prior fractionation of succinyl-aldolase is necessary to limit the amount of slow component produced upon dissociation and reconstitution of the succinyl-aldolase sample.

The results presented here were obtained by reconstitution of succinyl-aldolase by dialysis overnight against a buffer containing 0.5 M NaCl (100 volumes of buffer/volume of sample) followed by dialysis for 24 hr against two to three changes of the buffer with no added NaCl. If NaCl was not present in the reconstitution mixture the relative proportion of the slow component in the reconstituted succinyl-aldolase sample would have been higher than that shown in Figure 5. Reconstitution in solutions of high ionic strength thus appears necessary in order to obtain a good yield of the fast component in the reconstituted, succinyl-aldolase sample.

Procedure for Hybridization of Succinyl-aldolase and Aldolase. On the basis of the results presented above, the following procedure was adopted for the dissociation and reconstitution of mixtures of succinyl-aldolase and aldolase.

All operations were performed at 4°. Aldolase and succinyl-aldolase were mixed at varying molar ratios in either 0.02 M potassium phosphate–0.002 M EDTA (pH 6.50) or 0.05 M Tris-chloride–0.001 M EDTA (pH 7.50). In most cases, the initial protein concentration was 0.4%. Solid dithiothreitol and urea were added to give final concentrations of 0.1 and 4 M, respectively. The solution was stored for about 30 min before dialysis overnight against one of the above two buffers (100–200 volumes of buffer/volume of sample) containing

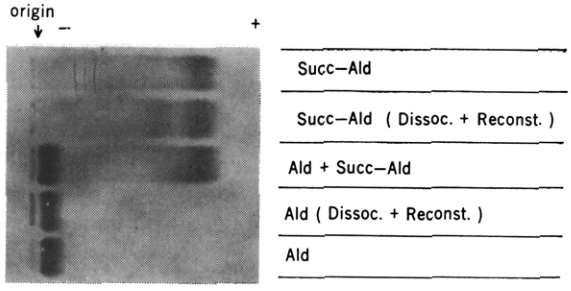


FIGURE 6: Cellulose acetate electrophoresis of the hybridization controls for succinyl-aldolase and aldolase. The mixture of succinylated and native aldolase consisted of approximately 50% of each component. Electrophoresis was performed for 12 min at 250 V and the membranes (Gelman) stained for protein as given in Methods.

0.5 M NaCl and 2×10^{-4} M dithiothreitol. The resulting mixtures were then dialyzed against two to three changes of the same buffer containing no added NaCl.

In some cases the protein was concentrated by precipitation in dialysis membranes placed in solutions of saturated ammonium sulfate (0.001 M EDTA, pH 7.50). The precipitate was collected in the clinical centrifuge and the resuspended precipitate was subsequently dissolved by exhaustive dialysis against the desired buffer.

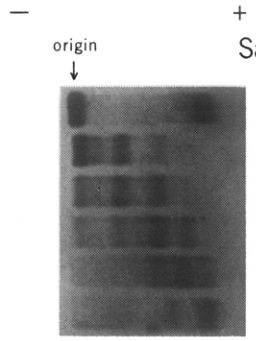
The final concentration of protein in the reconstituted mixtures was in the range of 3–15 mg/ml. No loss of protein occurred except in a few experiments where a small amount of precipitate formed upon the initial removal of urea.

Hybridization Controls. The control experiments for the hybridization of succinyl-aldolase and aldolase are given in Figure 6. No intermediate bands were detected in a mixture of succinyl-aldolase and aldolase that had not been dissociated and reconstituted. As indicated above, however, dissociation and reconstitution of succinyl-aldolase led to the formation of a small amount of an additional component representing presumably some succinyl-aldolase subunits which could not aggregate specifically to give molecules of size of the native enzyme. In contrast no additional bands were observed when native aldolase was dissociated and reconstituted. A mixture of succinyl-aldolase and aldolase, each of which had been dissociated and reconstituted separately (see Figure 8), contained only three components corresponding to native aldolase and the slow and fast components of succinyl-aldolase.

Hybridization of Succinyl-aldolase and Aldolase. Figure 7 shows patterns of the electrophoresis of different hybrid sets of aldolase and succinyl-aldolase (samples II–V). Five protein bands having different electrophoretic mobilities are clearly present. The band that migrated farthest toward the anode corresponded to succinyl-aldolase while the slowest migrating (most cathodic) band corresponded to native aldolase. Comparison of these results with those for the control leads to the conclusion that the three intermediate bands arose from mixing of subunits from succinyl-aldolase and aldolase. This type of pattern would be expected for the hybridization of two tetrameric molecules each composed of four similar chains.

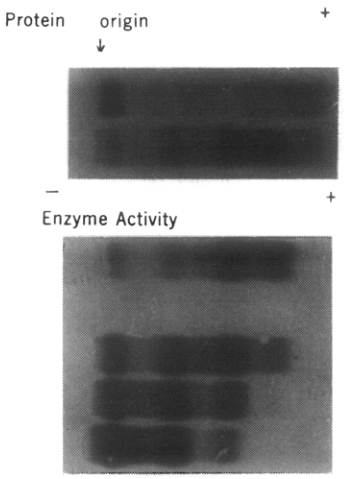
The relative proportion of each band within a hybrid set depended on the relative amounts of succinyl-aldolase and

[5] The detection of a very small amount of the 3S component in the purified succinyl-aldolase sample upon sedimentation whereas essentially no slow component was detected upon electrophoresis is probably a reflection of the difference in sensitivity of the experimental methods. In fact, a small amount of protein can be detected at the position of the slow component upon electrophoresis if larger amounts of the sample are applied to the electrophoresis membrane.



Sample	Ald	Succ—Ald
I	Control	
II	76	24
III	55	45
IV	35	65
V	17	83
VI	0	100

FIGURE 7: Cellulose acetate electrophoresis of hybrid sets of succinyl-aldolase and aldolase. Electrophoresis was performed for 12 min at 250 V and the membrane (Gelman) was stained for protein as given in Methods. Samples II–VI were prepared by dissociation and reconstitution of mixtures of aldolase and succinylated aldolase at the relative concentrations indicated. The control was a mixture of approximately 0.5 part of succinyl-aldolase and 0.5 part of aldolase that had not been dissociated and reconstituted.



Ald	Succ—Ald
Control	
17	83
17	83
0	100
35	65
55	45
76	24

FIGURE 8: Comparison of distribution of protein and enzyme activity after cellulose acetate electrophoresis of the hybrid sets of aldolase-succinylated aldolase. Electrophoresis was performed for 20 min at 200 V and the membrane (Gelman) was stained for protein and aldolase activity as indicated above. The control consisted of a mixture of aldolase and succinyl-aldolase each of which had been dissociated and reconstituted separately. The other samples were prepared by dissociation and reconstitution of mixtures of succinyl-aldolase and aldolase at the indicated percentages.

aldolase in the mixture before dissociation and reconstitution. As the percentage of succinyl-aldolase chains is increased from samples II to V, the electrophoretic pattern shifted in favor of the more anodic species. This finding is expected for random combination of the chains of succinylated and native aldolase upon hybridization.

As already shown, the dissociation and reconstitution of purified succinyl-aldolase led to the formation of a major and minor component (sample VI). Although the contaminating band does complicate the interpretation of the electrophoretic pattern of the hybrid sets, the relative intensity as well as the position of the minor or slow component between the second and third most anodic bands of the hybrid set permits it to be identified readily. Thus there is little risk that it can be identified incorrectly as one of the hybrids resulting from the combination of aldolase and succinylated aldolase chains in a single oligomer. The apparent absence of this band in the hybrid sets of samples IV and V may reflect the high background in the relevant regions of the electropherograms. However, it should be noted that in samples II and III, where the background is relatively low, this band was not detected. The absence of this band may be attributable to the ability of native aldolase subunits to combine with these chains and thereby facilitate their refolding to form members of the hybrid set.[6]

Determination of Enzyme Activity of Hybrids Resolved by Electrophoresis. Further experiments were conducted in order to detect the enzyme activity as well as the protein content of the various hybrids. Figure 8 shows the relation between the positions of the bands revealed by enzyme assay and the bands stained for protein content. It is clear that four different active components are present in the hybrid set formed from aldolase and succinylated aldolase. These species correspond in mobility to the four most cathodic protein bands in the hybrid set stained for protein (83% succinyl-aldolase and 17% aldolase). Both the minor (slow) and the major (fast) components of reconstituted succinyl-aldolase were inactive (the control showed that both protein components were present in the sample labeled 100% succinyl-aldolase). The control and the hybrid set stained for protein show clearly a band corresponding to reconstituted succinyl-aldolase that is more anodic than the four enzymatically active protein bands. This demonstration constitutes proof that the hybrid set of aldolase and succinylated aldolase consists of five members. Moreover, these results provide additional evidence that the contaminant in the reconstituted succinyl-aldolase is not a member of the hybrid set.

Chromatography of the Hybrid Set of Aldolase and Succinylated Aldolase. Since chromatography on DEAE-Sephadex has been shown to provide a successful means for separation of the members of the hybrid set of aldolase A and aldolase C (Penhoet *et al.*, 1967), similar fractionation experiments were initiated so as to resolve the hybrid set formed by aldolase and succinylated aldolase. Figure 9 gives plots of optical density (280 mμ), enzymic activity, and specific activity *vs.* fraction number for the chromatography of a hybrid set of aldolase and succinylated aldolase. The composition of the hybrid set was chosen so that all components could be detected clearly as a distinct peak of either enzyme activity or absorbance at 280 mμ. To accomplish this the hybrid set was produced from different mixtures of succinyl-aldolase and aldolase that had been dissociated and reconstituted in order that (a) all components would be present in adequate amounts, and (b) the members of the hybrid set with the lowest specific activity (*i.e.*, lowest content of active aldolase chains) would still be resolved as peaks.

From the optical density measurements of the individual fractions it is evident that five major components were present:

[6] Substantiation of this observation by more definitive experiments would be extremely significant. This result, if confirmed, would indicate that the interaction of unmodified aldolase subunits with the minor dissociated component of succinyl-aldolase induced or forced the latter species into a conformation suitable for forming a tetrameric hybrid. This possibility has a bearing on studies of interallelic complementation which occurs presumably *via* subunit interaction in oligomeric proteins.

TABLE II: Specific Activity of the Members of the Hybrid Set.

	A_4	A_3S	A_2S_2	AS_3	S_4
Experimental[a]	9.2	4.8	3.0	1.7	0.1
Calculated[b]	9.2	6.9	4.6	2.3	0.0

[a] Results obtained from Figure 9. [b] The activity of each member of the hybrid set was calculated by multiplying the specific activity of native aldolase (A_4) by the fraction of normal aldolase chains in each hybrid.

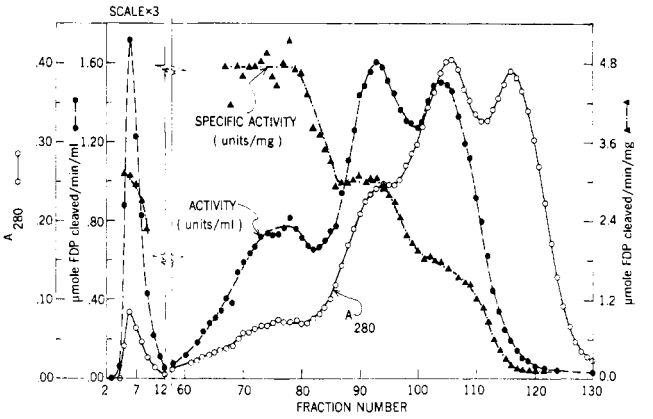


FIGURE 9: DEAE-Sephadex chromatography of an aldolase-succinylated aldolase hybrid set. The sample contained 20% (measured as protein) of a hybrid set produced from a mixture of 25% succinyl-aldolase and 75% aldolase, 36% of a hybrid set from a mixture of 65% succinyl-aldolase and 35% aldolase, and 44% of a hybrid set formed by dissociation and reconstitution of a mixture of 85% succinyl-aldolase and 15% aldolase. The sample was placed on a 1 × 50 cm column of DEAE-Sephadex A-50 preequilibrated with 0.05 M Tris-chloride–0.001 M EDTA (pH 7.50). The fractions were eluted with a linear gradient of NaCl from 0 to 1.0 M over a range of 500 ml at a flow rate of 25 ml/hr. The buffer at pH 7.50 contained 0.05 M Tris-chloride, 0.001 M EDTA, and 2 × 10^{-4} M dithiothreitol. Fractions of 2.7 ml were collected and analyzed for absorbance at 280 mμ (O—O), FDP-cleavage activity (●---●), specific activity (▲---▲), and conductance.

an initial component eluted in the dead volume and four components eluted at different sodium chloride concentrations. The first peak was eluted at a position corresponding to native aldolase (Penhoet *et al.*, 1967) and the last peak was eluted at a sodium chloride concentration corresponding to undissociated succinyl-aldolase (see Figure 4).

When the individual fractions were assayed for enzyme activity it was found, as seen in Figure 9, that activity was associated with the first four peaks and not with the fifth. This observation is to be expected for the hybridization of active aldolase chains with inactive succinyl-aldolase polypeptide chains. The preparations of succinyl-aldolase had been found to be virtually inactive with an activity less than 0.1 unit/mg as contrasted to 9–12 units/mg for native aldolase. If native aldolase and succinyl-aldolase are denoted by A_4 and S_4, respectively, then the hybrids can be denoted by A_3S, A_2S_2, and AS_3. Consequently, activity measurements on these intermediate peaks provided evidence regarding their composition and thereby furnished additional proof that they represented hybrid molecules composed of subunits of native aldolase mixed with succinyl-aldolase subunits.

When the data for enzyme activity per milliliter were combined with those for optical densities of the various fractions, the specific activities plotted in Figure 9 were obtained. The plot of specific activity *vs.* fraction number showed that a unique specific activity is associated with each peak. As indicated by the plateaus for the various hybrids, each component is relatively homogeneous with respect to activity. Because of the partial overlap of peaks three, four, and five, the plateau for peak four was not as distinct as those for the other hybrid species.

The measured specific activities of the five members of the hybrid set are compared with the calculated values (based on the specific activity of native aldolase) in Table II. As seen there, the specific activities for the hybrids containing both succinyl-aldolase and aldolase are somewhat lower than those which would be predicted if each aldolase chain contributes enzymic activity independent of the other subunits. However, the differences between observed and calculated specific activities may be no more than experimental error rather than effects on the activity of the unmodified chains due to hybridization with modified (or inactive) chains. For example, an error of only 0.020 in A_{280} at the elution peak of A_3S in Figure 9 would give an error in specific activity of 33%. An error of this magnitude could arise easily from elution of optical density due to the presence of components other than the hybrids. The general correlation of the measured and calculated specific activities of the hybrids suggests that interaction of subunits within the individual molecules has relatively little effect on the contribution of the native aldolase chains to the activity of the hybrids.

Table III presents a comparison between the measured amount of each component in Figure 9 and the theoretical distribution expected if random combination of the chains occurred in the hybridization experiments. The results show that within experimental error the chains combine in random fashion. This result suggests that either the interaction between dissimilar subunits (succinyl-aldolase and aldolase subunits) is energetically the same as between similar subunits (succinyl-aldolase–succinyl-aldolase or aldolase–aldolase interactions) or that reconstitution is kinetically controlled in that the composition of the hybrid set depends only on the initial contacts between subunits.

Discussion

Studies of the chemical modification of proteins with group-specific and group-selective reagents have produced valuable information as to the structural features responsible for the stability and function of proteins (Cohen, 1968). In the work described here, chemical modification was employed to produce a "variant" which served as a probe for investigating the subunit structure of aldolase. A variety of acylating and alkylating reagents are available for effecting changes in the electrophoretic properties of proteins since their reaction with lysyl or cysteinyl groups, for example, causes a significant alteration in the net charge on the protein. Particularly suitable for this purpose are the cyclic acid anhydrides such as succinic anhydride (Klotz, 1967), phthalic anhydride

TABLE III: Combination of the Chains of Succinyl-aldolase and Aldolase.

	Percentage of Components	
	Experimental[a]	Theoretical[b]
A_4	6	7
A_3S	12	13
A_2S_2	18	20
AS_3	32	31
S_4	32	29

[a] The percentage of components was estimated from the results given in Figure 9. [b] The theoretical distribution of members of the hybrid set was calculated assuming random combination of the aldolase and succinyl-aldolase chains. The fraction of protein with the tetrameric structure A_nS_{4-n} formed by random combination of A and S subunits is given by

$$\frac{4!x^n(1-x)^{4-n}}{(4-n)!n!}$$

where x and $1-x$ are the fraction of A and S subunits, respectively, in the solution and n is the number of A subunits in each member of the hybrid set. Since the sample chromatographed above was a mixture of three different hybrid sets (see Figure 9) the theoretical fraction of A_nS_{4-n} is

$$\frac{4!}{(4-n)!n!}\sum_{i=1}^{3} y_i x_i^n(1-x_i)^{4-n}$$

where y_i is the fraction of protein in the "i"th hybrid set, and x_i and $1-x_i$ represent the fraction of A and S subunits, respectively, in the "i" hybrid set. In the calculations it was assumed that the molar extinction coefficients of aldolase and succinyl-aldolase were the same, and that the yield of tetrameric molecules after dissociation and reconstitution was 100%.

(Epstein and Goldberger, 1964), and maleic anhydride (Butler *et al.*, 1967), and the α-halo acids such as bromoacetate and iodoacetate (Gurd, 1967). Another type of useful chemical modification involves the reaction of proteins with the *N*-carboxyanhydride of an amino acid to give a polypeptidyl derivative (Sela and Arnon, 1967; Katchalski *et al.*, 1964). This type of reaction has been used to prepare the polyglutamyl derivatives of ribonuclease and serum albumin (Wilchek *et al.*, 1966) and the polylysyl derivatives of ribonuclease, serum albumin, and γ-globulin (Sela *et al.*, 1963; Arnon *et al.*, 1965). These latter derivatives are particularly valuable as electrophoretic variants since they contain multiple positively or negatively charged polypeptidyl side chains.

As shown here, limited succinylation of aldolase had little effect on the quaternary structure of the enzyme despite the substantial change in its net charge. The modified enzyme was virtually inactive, to be sure; but no efforts had been made to prevent residues involved in the active site from reacting with succinic anhydride. Only when the less reactive lysyl residues (after the first 40%) were succinylated did the modified enzyme become so destabilized that dissociation into subunits occurred. It is as if the more reactive lysyl residues were on the "outside" of the protein molecule and were not involved in intrachain folding and intersubunit interactions. Their modification (and alteration in charge from plus to minus) also did not interfere with the process of reconstitution whereby the randomly coiled chains refold and aggregate so that the specific conformation characteristic of the native molecule is regained. In this respect succinylated aldolase is similar to chemically modified ribonuclease for which it was shown that altering the charge on some of the side chains did not prevent refolding of the randomly coiled chains to give highly organized, enzymically active molecules (Epstein and Goldberger, 1964; Epstein *et al.*, 1963).

The demonstration that the succinylated aldolase molecules could be dissociated in urea and then reconstituted in high yield satisfies the last in a series of criteria which must be met if the chemically modified molecules are to be suitable for hybridization with the native enzyme.

A large number of proteins have been modified by treatment with succinic anhydride and the results presented here for aldolase are in general accord with those found for other proteins. Extensive reactions with succinic anhydride leads to large conformational changes due presumably to the electrostatic repulsion stemming from the large increase in the net charge on the protein. With oligomeric proteins extensive succinylation frequently is accompanied by dissociation of the protein into subunits (Hass, 1964). In contrast, limited succinylation of proteins often can be effected without disruption of the tertiary or quaternary structures. Hemerythrin, for example, after limited treatment with succinic anhydride did not exhibit much dissociation into subunits; however, the succinylated protein readily formed hybrids with native hemerythrin simply by mixing the two forms of the protein (Keresztes-Nagy *et al.*, 1965). Succinylation of 55% of the lysyl residues of bovine serum albumin was accompanied by only a small change in Stokes radius, no change in the reactivity of the disulfide bonds, and little effect on its precipitability with specific antibodies (Habeeb, 1967). Reaction of 73% of the lysyl residues of carboxypeptidase A with succinic anhydride did not alter the activity of the enzyme (Bethune *et al.*, 1967). Similar results, after even more extensive succinylation, were obtained with subtilisin (Gounaris and Ottesen, 1965). Studies on α-crystallin (Spector and Katz, 1965) and high-density lipoprotein (Scanu *et al.*, 1968) have shown that 90% of the lysyl residues can be succinylated without the protein dissociating into subunits.

The hybridization of succinyl aldolase and the native enzyme to give a hybrid set of five members constitutes strong evidence that aldolase molecules are composed of four similar subunits. In this respect the results given here are identical with the earlier findings of Penhoet *et al.* (1966, 1967) who used naturally occurring aldolases A and C for hybridization experiments. Although at first glance, it appears that the oligomers are composed of identical (rather than merely similar) polypeptide chains, it should be noted that differences among the chains which do not affect the net charge on the subunits would not be detected by the electrophoretic technique. Thus the finding of five members in the hybrid set formed by native and succinylated aldolase is not, by itself

inconsistent with the proposed model of aldolase consisting of two types of polypeptide chains (Rutter *et al.*, 1961; Winstead and Wold, 1964; Edelstein and Schachman, 1966; Chan *et al.*, 1967; Morse *et al.*, 1967). The formation of five electrophoretic variants upon dissociation and reconstitution of mixtures of aldolases A and C (Penhoet *et al.*, 1966, 1967) requires that, even if the subunits within each species differ one from another, at least they must be similar in terms of their net charge. Similarly the production of a five-membered hybrid set from native and succinylated aldolase can be achieved only if each of the subunits in the native enzyme is modified to about the same extent. In considering the AABB model for aldolase (Morse *et al.*, 1967) we must recognize that the presence of two isozymes, AAAA and BBBB, in a solution in approximately equal amounts would give experimental results identical with those on which the AABB model was based. Clearly the number of subforms in each zone of the electrophoresis patterns must be determined. In this regard the recent discovery of five electrophoretic variants in "pure" aldolase A (Masters, 1967; Kochman *et al.*, 1968) is of considerable interest. This finding supports a model composed of four identical subunits, AAAA, with two different forms of the subunits present in the solution.

As seen in Figure 6, no subunit exchange occurs when the native enzyme and succinylated aldolase are mixed in neutral solutions. Apparently there is very little dissociation of the oligomeric structures under these conditions. Subunit exchange does occur, however, when the secondary, tertiary and quaternary structures are disrupted through the action of a denaturant such as urea. Whether hybrid formation can be induced without the use of reagents which cause drastic unfolding of the subunits remains to be elucidated. The two forms of aldolase hybridize in a random fashion and all of the different species appear to be stable. This may indicate that the energy of association between unlike subunits in any oligomer is essentially equal to that between identical subunits. Until detailed studies of the stability of the various hybrids are completed and possible kinetic factors are evaluated, conclusions about intersubunit interactions are not warranted. The experimental evidence indicates that the specific activity of the various hybrids is directly proportional to the number of unmodified chains within each oligomer. Apparently each subunit contributes its independent enzymic activity regardless of the composition of the other subunits in the same molecule. It is clear that a single, unmodified and potentially active aldolase chain in concert with three chemically modified and inactive chains assumes the proper conformation needed for enzyme activity. Tempting as it may be, it is nonetheless premature to conclude that the three inactive aldolase-like chains are indispensable for the stabilization of the proper conformation of the unmodified "native" polypeptide chain. Although an active monomer of aldolase has not, as yet, been isolated, it is not certain that such monomers cannot be obtained through the use of some dissociating agent. The reagents used thus far for the dissociation of aldolase, such as hydrogen or hydroxyl ions, urea, guanidine hydrochloride, and extensive succinylation, doubtless disrupted the secondary and tertiary structures as well as the quaternary structure. Only after many more experiments aimed at isolating active monomers prove negative will we be justified in concluding that monomers cannot exist to any appreciable extent in conformations which are enzymically active and that stabilization of the active conformations is dependent in part on subunit interactions in the oligomers. Such a situation would constitute an example of quaternary constraint. It seems likely that the hybridization technique illustrated here and the hybrids themselves will serve as valuable probes in investigations of the relationships among the various structural levels in multichain proteins.

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