Isotope Effects in the Binding of NADH to Equine Liver Alcohol Dehydrogenase†

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ABSTRACT: The isotope effect upon the binding constant of NADH to equine liver alcohol dehydrogenase is determined with a method in which the isotopic ratio is measured concurrently in the free and the bound form of the coenzyme, by use of a propellent-pressurized ultrafiltration apparatus for separation of the two. The value for K_H/K_D for the binding constants was 1.00 ± 0.02 at pH 10.3 and 25 °C.

In order to explain the rate acceleration observed in catalysis by some enzymes, the introduction of strain into the substrate has been hypothesized to occur during its binding to the enzyme. This is called "Strain or Distortion Theory" (Jencks, 1969a). Evidence in favor of this hypothesis with certain enzymes comes from the observations that transition-state analogues have a greater affinity for the enzyme than do the corresponding substrates. Examples of transition-state analogues have been reviewed by Lienhard (1973) and by Wolfenden (1972). Also, evidence from x-ray crystallographic studies as well as binding studies with lysozyme indicates that the *N*-acetylmuramic acid is distorted in the binding reaction (Rupley et al., 1967; Van Eikeren and Chipman, 1972).

If any bonds were strained upon binding of a substrate to the active site of an enzyme, the vibrational frequencies of that bond should change. Therefore, an isotope effect would be expected on the binding of a substrate in which an isotope were substituted for an atom subtending one of the strained bonds, and the measurement of the isotope effect on the binding constant should provide an estimation of the extent of substrate strain in the enzyme-substrate complex.

Since the substrate in the enzyme-substrate complex is unlikely to be so strained that its conformation is identical with that of the unbound transition state for the reaction, the expected isotope effect on the binding constant may be small, i.e., smaller than the calculated primary isotope effect in a bond-breaking reaction (See Discussion). Therefore, rather precise methods might be necessary for the measurement of an isotope effect on a binding constant.

Isotope effects on the dissociation constants of NADH have been calculated from steady-state kinetic data for rabbit muscle lactate dehydrogenase by the use of rate equations whose derivation depended upon the predominance of an ordered, sequential mechanism (Thompson et al., 1964). Since Silverstein and Boyer (1964) demonstrated that such a mechanism does predominate under similar reaction conditions, the hypothesis of strain in the enzyme-NADH complex is supported by the evidence. Such an hypothesis was advanced by Everse and Kaplan (1973). Although isotope effects on the Michaelis constant for NADH (NADD)[1] have been reported for horse liver alcohol dehydrogenase (Mahler et al., 1962), the K_m does not exclusively reflect the binding reaction since this constant may also be affected by the rate constants from other reaction steps. Bush et al. (1971) calculated the isotope effect for the binding of NADH to LADH from measurements of the respective binding constants of NADH and NADD by two different methods, fluorometric titration and gel-exclusion chromatography with spectrophotometric measurement of coenzyme.

Because of the importance of an isotope effect on the binding constant with regard to strain theory and because of the requirement for considerable precision in the measurement of such an isotope effect, we have reinvestigated the binding of NADH (NADD) to LADH with a method developed in this laboratory. This method enables us to measure directly the ratio of the binding constants in a mixture of the ligands and it is generally applicable to a variety of enzymes.

Materials and Methods

NAD was purchased from P-L Biochemicals and used without further purification.

Adenine[2,8-^{3}H]NAD (3.28×10^3 Ci/mol) was purchased from New England Nuclear and [U-^{14}C]NAD (2.74×10^2 Ci/mol) from Amersham/Searle. Both showed radiochemical purity greater than 95% by TLC in this laboratory (cellulose, developed with isobutyric acid–water–ammonia (66:44:1), made 10^{-4} M in EDTA). Each radioactive nucleotide was taken to dryness in vacuo, diluted to 0.5 ml, and stored at −10 °C. These solutions were used in subsequent experiments and syntheses.

Horse liver alcohol dehydrogenase (once crystallized and lyophilized) was purchased from Sigma Biochemicals and used without further purification. It was found to have the reported specific activity. Fully deuterated ethanol was purchased from Amersham/Searle and nonisotopic ethanol was purchased from U.S. Industrial Chemical Co. The scintillation cocktail was "ScintiVerse" from Fisher Scientific Co. All other chemicals were of reagent grade.

Preparation of [^{3}H]NADH. Approximately 150 000 cpm of [^{3}H]NAD was placed in 10 ml of 0.04 M ammonia. To this was added 1.0 ml of water which contained 1 mg of LADH and 0.037 mg of NAD. Absolute ethanol (20 μl) was added and the mixture was left 10 min at 25 °C. The reaction mixture was then lyophilized. In order to measure the extent of the reaction and the purity of the product, a sample of the lyophilized reaction mixture was subjected to electrophoresis on cellulose acetate under conditions known to separate NAD and NADH (20 min, 250 V, 0.05 M piperazine phosphate, pH 8.8). The

† From the Department of Biochemistry, University of Alabama in Birmingham, University Station, Birmingham, Alabama, 35294. *Received November 14, 1975.* The results are taken from the thesis of E. deJuan submitted to U.A.B. in partial fulfillment of the requirements for the Master of Science degree. This investigation was supported by a grant from the University of Alabama Faculty Research Grant Committee.

[1] The abbreviation NADD is nicotinamide adenine dinucleotide that was reduced with ethanol in the presence of equine liver alcohol dehydrogenase, and LADH in equine liver alcohol dehydrogenase (EC 1.1.1.1).

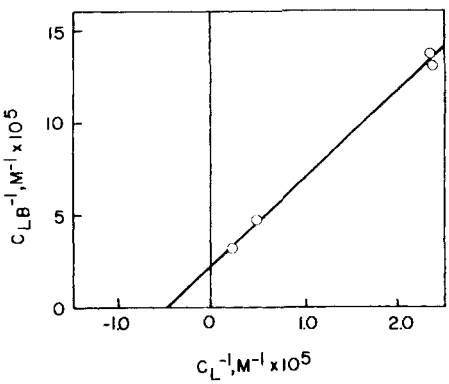

FIGURE 1: Determination of the dissociation constant of LADH for NADH by ultrafiltration. For each experimental determination, the ultrafiltration cell contained: ammonia (4.0×10^{-3} M), LADH (0.2 mg/ml), and [^{3}H]NADH (1.25×10^5 cpm) in a total volume of 1.0 ml (pH 10.3). The total concentration of NADH was adjusted by the addition of unlabeled reduced coenzyme. The difference between the radioactivity associated with the membrane in an experiment performed in the absence of enzyme and that associated with the membrane in each of the experiments performed in the presence of enzyme was divided by the specific activity of NADH in that particular experiment in order to determine the concentration of bound NADH (C_{LB}). The concentration of free coenzyme (C_L) was determined from the difference between the total coenzyme and the bound coenzyme. A double-reciprocal plot was made of the two quantities and a least-squares straight line was drawn through the points according to the equation, $1/C_{LB} = 1/C_L(K_d/nC_E^0) + 1/nC_E^0$, ($C_E^0$ is the total enzyme concentration). Values for the dissociation constant (K_d) and the number of sites per molecule of enzyme (n) were calculated from the equation for the line.

spot corresponding to NADH contained 85% of the applied radioactivity.

In preparation for a binding experiment, the lyophilized material was diluted to 10 ml in 0.004 M ammonia. As a result the concentration of LADH was 2.4×10^{-6} M of sites, and that of [^{3}H]NADH was 4.8×10^{-6} M.

Preparation of [U-^{14}C]NADD. The procedure was the same as that for [^{3}H]NADH except that [U-^{14}C]NAD and deuterated ethanol were used instead of [^{3}H]NAD and alcohol, respectively.

Measurement of Binding Isotope Effects. Five milliliters of the solution of [^{3}H]NADH plus enzyme was added to 5 ml of the solution of [^{14}C]NADD plus enzyme, and the mixture (pH 10.3, 25 °C) was placed in a ultrafiltration cell (Amicon 10PA) such that a small air space remained above the solution after complete assembly of the cell. After the application of pressure (70 psi), the flow rate ranged from 0.2 to 1.2 ml/min. The first 1.0 ml of filtrate was collected and its radioactivity was counted in 10 ml of scintillation cocktail to give a direct measurement of the isotopic ratio in the free coenzymes, [^{3}H]NADH/[^{14}C]NADD. At the termination of the filtration, the gas container was removed. The cell was inverted and swirled to coalesce any droplets on the membrane. The membrane was placed into a scintillation vial which also contained 1.0 ml of water and the vial was agitated on a vortex mixer for 10 min in order to remove protein adhering to the membrane. After removal of the membrane from the scintillation vial, 10 ml of liquid scintillation cocktail was added and the radioactivity in the vial was counted as described below. These latter counts provide a direct measurement of the ratio of the radioactive isotopes in the bound coenzymes. The total radioactivity associated with the membrane from experiments performed in the presence of enzyme was tenfold that from experiments performed in the absence of enzymes (see Discussion).

Counting Procedure. All counting was done with a Nuclear Chicago, Mark I scintillation counter. Sufficient counts were collected for each sample to give an error of less that 1% with 99.9% confidence. Radioactive standards contained 1.0 ml of water, 10.0 ml of scintillation cocktail, and either [^{3}H]NAD or [^{14}C]NAD. The channel limits were set so that channel C has 0.36% efficiency for the tritium standard. When the radioactivity of the carbon-14 standard was counted, channel A detected 25.92% of the counts detected by channel C.

Calculations. The isotope effect on the binding constant (K_I) is defined in eq 1, where K_{bH} and K_{bD} are the binding constants for the substrates that contained hydrogen or deuterium, respectively.

$$K_I = k_{bH}/k_{bD} \tag{1}$$

Substitution of the appropriate equilibrium equations for K_{bH} and K_{bD}, and rearrangement gives eq 2.

$$K_I = \frac{([^3\text{H}]\text{NADH})/([^{14}\text{C}]\text{NADD})\ \text{bound}}{([^3\text{H}]\text{NADH})/([^{14}\text{C}]\text{NADD})\ \text{free}} \tag{2}$$

If the counting efficiency for both radioactive isotopes in both channels is assumed to be the same in samples of the bound, the free, and the [^{14}C]NAD standard, and if the efficiency of tritium in channel C is assumed to be negligible (efficiency = 0.0036), the equations relating the radioactivity in each of two isotopes to the counts in the two channels of the scintillation counter may be substituted into eq 2 to give eq 3

$$K_I = (R_b r - 1)/(R_f r - 1) \tag{3}$$

where R_b is the ratio of the counts in channel A to those in channel C for the sample of the bound coenzyme, R_f is the same ratio for the free coenzyme, and r is ratio of the counts in channel C to those in channel A for the [^{14}C]NAD standard. The isotope effect, K_I, was calculated by the use of eq 3 and reported under Results. When [^{3}H]NADD and [^{14}C]NADH were the ligands, the ratio in eq 3 was inverted so the K_I has the same meaning as before.

Results

In order to demonstrate binding under the conditions of the ratio experiments, the binding constant for NADH to LADH was determined with the ultrafiltration apparatus by the method of Paulis (1969). The conditions were those of the ratio experiment except only one isotope, [^{3}H]NADH, was used. A plot of the reciprocal of the concentration of bound coenzyme vs. the reciprocal of the concentration of the free coenzyme is analogous to a double-reciprocal plot of initial velocity vs. substrate concentration and the binding constant as well as the concentration of total occupiable sites can be determined from the slope and intercept (Figure 1). Both the number of sites (1.83) and the dissociation constant (2.0×10^{-5} M) determined from Figure 1 agree with the values for these constants determined by other methods at the same pH, 2.0 and 2.3×10^{-5}, respectively (Shigehiko et al., 1967). Therefore, the binding of NADH to LADH under the ultrafiltration conditions described here is quantitatively similar to that determined by other methods and it as well as the binding found in the ratio experiments is likely to be mechanistically the same as that detected by other methods.

Table I contains the results of a representative ratio experiment and its control. Coenzyme binding is demonstrated by comparison of the radioactivity associated with the membrane in the presence of enzyme with that in the absence of enzyme. Upon electrophoresis of the initial incubation medium on cellulose acetate 84.3% of the radioactivity migrated with

TABLE I: Representative Experiments to Determine the Isotope Effect upon the Binding of [^{3}H]NADD and [^{14}C]NADH to LADH.

Incubation Medium	Radioactivity in Filtrate: Channel A (cpm)	Radioactivity in Filtrate: Channel C (cpm)	Radioactivity in Filtrate: Ratio A/C	Radioactivity on Membrane: Channel A (cpm)	Radioactivity on Membrane: Channel C (cpm)	Radioactivity on Membrane: Ratio A/C	K_I
Complete	11 834	4976	2.378	18 181	8006	2.278	1.049
Minus enzyme	12 987	5966	2.177	1 519	679	2.237	0.970

TABLE II: Isotope Effects on the Binding Constant of NADH to Alcohol Dehydrogenase.

Group No.	No. of Expts	Ligands	Enzyme	$K_I \pm$ SD
Group 1	10	NADH[a]	Lyophilized	1.00 ± 0.02
	3	NADH[b]	Lyophilized	1.02 ± 0.03
Group 2	1	NADH[a]	Fresh	1.00
	2	NADH[b]	Fresh	1.03
Group 3	1	NAD	Fresh	0.96
	1	NAD	Boiled	0.97
	1	NAD	None	1.03
	1	NAD	None	1.01[c]
	1	NAD	None	1.04[d]
Group 4	1	NADH[a]	None	1.02
	1	NADH[a]	Boiled	1.00
	1	NADH[b]	None	1.00

[a] [^{3}H]NADH and [^{14}C]NADD. [b] [^{3}H]NADD and [^{14}C]NADH. [c] The membrane was Amincon, UM10. [d] The membrane was Amincon, UM50.

NADH and upon electrophoresis of the filtrate, 83.0% of the radioactivity migrated with NADH. Therefore, there was no significant destruction of NADH during the ultrafiltration.

The isotope effect (K_I) upon the binding of NADH to LADH is very close to 1.00 from the results in Table I as is the value for K_I from the results of the control experiment. Table II contains the results of numerous such ratio experiments and control experiments. Group 1 and group 2 in the latter table contain the results from experiments with reduced coenzymes. The experiments reported in group 1 were done with relyophilized enzyme as described in Methods, whereas those in group 2 were done with coenzyme from which the enzyme was removed by ultrafiltration and to which fresh enzyme was added. The latter experiments were done to assure that active enzyme was present. Each group contains results from additional experiments in which the radioactive labels were switched with respect to the deuterium in order to control for any anomalous effects associated with the radioactive nucleotides. The mean of the value for K_I from the experiments in both group 1 and group 2 showed no isotope effect (K = 1.00). Groups 3 and 4 are from a set of control experiments. Group 3 contains the results from experiments in which the oxidized coenzyme was used in the presence of fresh enzyme and boiled enzyme as well as in the absence of enzyme. They confirm that there was no experimental difference associated with NAD, which might have compensated for an isotope effect on the binding constant. Also different membranes (Amicon UM10, XM50) were used in experiments to expose any effect due to the membrane. None was detected. Group 4 contains the results from a set of control experiments with reduced coenzyme in the presence of boiled enzyme as well as in the absence of enzyme. Again the possibility of anomalous, differential binding was excluded.

In order to determine whether pressure (70 psi) and possible denaturation affected the activity of LADH irreversibily during an ultrafiltration experiment, the amount of enzyme in the ultrafiltration cell was assayed before and after a filtration experiment (Dalziel, 1957). The fact that 94% of the original activity was recovered from the filtration chamber at the end of a run demonstrates that the enzyme was not irreversibly inactivated during the experiment.

The results of the experiments reported in Table I demonstrate that there was no detectable isotope effect on the binding of NADH to equine liver alcohol dehydrogenase, at pH 10.3, 25 °C, when the C-4 position of the nicotinamide ring is substituted by deuterium in the "A" position.

Discussion

The Method. The sensitivity of the determinations described here are ultimately dependent upon the discrimination of different radioactive isotopes by the scintillation counter, which depends upon the difference in the counting efficiency of the two isotopes in the two channels. Since the data from which K_I is calculated are ratios, most sources of error cancel out, i.e., volumetric error, etc. The major source of error remaining is counting error, which was 1% in these experiments. This counting error produces an error in K_I, calculated from eq 3, of 6% at a 99.9% confidence limit. If the values for K_I (Table I) are assumed to have a normal distribution, the calculated error is 8% at a 99.9% confidence limit. Therefore, the ultrafiltration method for determination of the binding ratios of two ligands is sufficiently precise that a difference of 0.08 in the K_I would have a greater than 99.9% chance of being significant.

In the use of this method it is advantageous to have the radioactivity associated with the membrane in experiments performed in the presence of high enzyme compared with that from experiments performed in the absence of enzyme. The realization of this advantage would be promoted by the use of a high concentration of enzyme and the use of sufficient substrate concentration to assure that enough of the coenzyme is bound. However, in the use with enzymes and substrates for which the binding constant is small, the radioactivity associated with the membrane in the presence of enzyme may be corrected for the radioactivity associated with the absence of enzyme by the use of eq 4 in place of eq 3

$$K_I = \frac{r\left(\frac{R_m - \alpha R_s}{1 - \alpha}\right) - 1}{rR_f - 1} \qquad (4)$$

where R_m is the ratio of counts in channel A to those in channel C associated with the membrane from experiments performed in the presence of enzyme, and α is the ratio of counts in channel C associated with the membrane from an experiment performed in the presence of enzyme to those in channel C from an identical experiment in the absence of enzyme. If the counting error is 1%, the volumetric error on α is 5% and the

value of α is 0.4, the error in K_I is 9% at a 99.9% confidence level. Therefore, the method can still give quite precise results.

The method may be used with a low enzyme concentration, because nearly all of the protein may be collected on the membrane (Paulis, 1969). Although enzyme concentration increases during an experiment, this increase should not affect the value of K_I.

The method could result in invalid values for K_I if one of the ligands were partially excluded or preferentially bound by the membrane. Although such discrimination is not seen with NADH, it has been observed with steroids as ligands (Ryan and Hanna, 1971). Therefore it is necessary to ascertain that preferential binding or partial exclusion of the ligand does not occur. In cases in which it does occur, membranes of different chemical composition and pore size are available and might be less discriminatory (Amicon XM50, UM Series, etc.).

Since the ultrafiltration method is relatively quick (less that 10 min), it may be used under conditions in which the active constituents (protein and ligands) are labile. Also the simplicity of manipulations and data acquisition makes this method very attractive.

Binding Isotope Effects. Binding isotope effects come about from the difference in the vibrational status of the bond subtended by the isotopically substituted atom in the bound substrate and the free substrate. These isotope effects cannot be categorized exclusively as primary or secondary isotope effects since the bond that is to be broken is probably not completely broken upon binding to the enzyme. Therefore, any observed isotope effect would probably be smaller that a "normal" primary isotope effect (K_I = 6–10 for deuterium; Jencks, 1969b). Within these limits the size of a binding isotope effect will depend upon two factors: (1) the degree of bond breaking; and (2) the relative concentration of nonproductive complexes. (1) The more the bond is broken (or strained) in the binding step, the more the isotope effect should approximate a "normal" primary isotope effect. (2) Those complexes that would be productive are the only ones that are likely to exhibit a binding isotope effect. The nonproductive complexes, which would be particularly apt to occur in experiments with loosely binding substrates, may obscure an isotope effect on the productive complex. Careful attention should be given to the possibility of nonproductive complexes in similar experiments particularly when: (1) there is no detectable isotope effect on the binding constant and (2) when the ligands bind loosely (i.e., K_d greater than 10^{-4}). In these cases there is a increased chance for the binding isotope effect on the productive complex to be obscured.

Results

The isotope effect on the binding constant of NADH to LADH as determined by the method described here was 1.00 ± 0.02. These results contrast with the results reported by Bush et al. in which an "inverse" isotope effect (K_H/K_D = 0.58 ± 0.10) was calculated from results of independent determinations of the dissociation constants for NADH and NADD by both gel exclusion chromatography and flurometric titration. If the latter ratio were expressed as the ratio of binding constants, the value would be the reciprocal of the above (K_H/K_D = 1.72). In recognition of the significant scatter in their data, the authors pointed out that their methods were "extremely sensitive to a number of external influences", and that the gel filtration method "required extreme instrumental sensitivity". Their experiments were done at pH 7 in phosphate buffer and the gel filtration experiments lasted about 45 min each. Since NADH tends to be destroyed and racemized under these conditions (Miles et al., 1968; Fawcett et al., 1961), an isotope effect in this decomposition might be reflected in the isotope effect on the binding constant. Alternatively the magnitude of isotope effects in their experiments and ours might correspond more closely, if they were both done at the same pH. Further experiments are necessary to test this possibility.

The fact that a binding isotope effect was not detected in the experiments reported here indicates that strain involving the "pro-*R*" hydrogen in the C-4 position of the nicotinamide ring does not play an important role in the binding of the reduced coenzyme to liver alcohol dehydrogenase prior to the binding of the second substrate (i.e., the aldehyde). Further experiments will be necessary to determine whether strain is more important under other conditions of pH and temperature.

The absence of an isotope effect in the binding (or dissociation) of NADH is consistent with the presence of a kinetic isotope effect in the oxidation of ethanol (Bush et al., 1973) and with the evidence that the dissociation of NADH is rate limiting for the overall reaction (Shore and Gutfreund, 1970). Northrup (1975) showed that an isotope effect in the overall reaction, particularly a small one such as that observed in the oxidation of ethanol, may originate from a reaction step that is faster than the rate-determining step. Therefore, the isotope effect in the enzymatic oxidation of ethanol might originate in the interconversion of the internal complexes, whereas the rate-determining step is the dissociation of NADH from the enzyme.

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Use of Dimethyl Suberimidate and Novel Periodate-Cleavable Bis(imido esters) to Study the Quaternary Structure of the Pyruvate Dehydrogenase Multienzyme Complex of *Escherichia coli*†

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ABSTRACT: Two new symmetrical bis(imido esters), *N,N'*-bis(2-carboximidoethyl)tartarimide dimethyl ester dihydrochloride and *N,N'*-bis(2-carboximidomethyl)tartarimide dimethyl ester dihydrochloride, have been synthesized. Tests with the tetrameric enzyme, fructose diphosphate aldolase, show that these reagents closely resemble dimethyl suberimidate in their ability to cross-link protein subunits. However, identification of the cross-linked species, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, is greatly facilitated since the cross-links can be broken by a simple treatment with sodium periodate. The periodate cleavage step can be introduced between the two dimensions of a diagonal gel electrophoretic separation, the contributors to a cross-linked species then moving off the diagonal formed by uncross-linked proteins and reverting to the positions in the gel that correspond with their regenerated monomeric form. When the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* was treated with dimethyl suberimidate or *N,N'*-bis(2-carboximidoethyl)tartarimide dimethyl ester dihydrochloride, cross-links rapidly formed between the subunits of the transacetylase and lipoamide dehydrogenase components. On the other hand, cross-links failed to form between the subunits of the decarboxylase component themselves, or between the decarboxylase and the other two types of subunit in the complex. Cross-linking experiments with the isolated lipoamide dehydrogenase were compatible with the accepted dimeric structure of this enzyme in free solution, whereas the isolated pyruvate decarboxylase component also failed to cross-link when treated with dimethyl suberimidate in free solution. The cross-linking experiments with the intact multienzyme complex provide evidence for the existence of the lipoamide dehydrogenase dimer in the assembled enzyme and show the need to interpret such experiments with care since, from other evidence, the pyruvate decarboxylase component is known to be bound to the transacetylase "core" of the complex.

The pyruvate dehydrogenase multienzyme complex of *Escherichia coli* contains three different types of polypeptide chain, responsible for the three component enzyme activities; these are: E1, pyruvate decarboxylase; E2, lipoyl transacetylase; and E3, lipoamide dehydrogenase. The component enzymes can be taken apart and reassembled in vitro to form the original structure which is somewhat larger than a ribosome (for reviews, see Reed and Oliver (1968) and Reed (1974)). The intact enzyme and the lipoyl transacetylase "core" probably have octahedral symmetry (Reed, 1974), but the subunit structure is still a matter of some controversy (Eley et al., 1972; Vogel et al., 1972; Perham, 1975; Bates et al., 1975). The number of polypeptide chains in the complex has been reported to be 48 (Vogel et al., 1972) or 60 (Eley et al., 1972).

Cross-linking reactions have proved helpful in analyzing the geometrical arrangements of protein subunits in a wide variety of situations, such as oligomeric enzymes (Cohlberg et al., 1972), membranes (Ji and Ji, 1974), ribosomes (Bickle et al., 1972), and histones (Kornberg and Thomas, 1974). Reagents based on bis(imido esters), e.g. dimethyl suberimidate (Davies and Stark, 1970), have proved particularly useful since they react specifically with protein amino groups and yield stable products. The reaction causes no change of charge at the modified residue, and the tertiary and quaternary structures of the proteins are therefore likely to remain unaffected (Perham, 1973).

The principal method of analyzing the products of cross-linking reactions has been sodium dodecyl sulfate gel electrophoresis in which the various species are characterized by measuring their molecular weights. However, in studies of complex systems, ambiguities in identifying the protein chains contributing to a cross-linked species must inevitably arise. To help overcome this difficulty, the use of bis(imido esters) containing a disulfide bond has been described (Sun et al., 1974; Wang and Richards, 1974). The disulfide bond can readily be cleaved after an initial electrophoretic separation of cross-linked species; this breaks the cross-links and permits the regenerated monomers to be characterized in a second dimension of gel electrophoresis. The strategy is therefore akin to that of diagonal paper electrophoresis of peptides (Hartley, 1970; Perham, 1969).

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[1] Abbreviations used are: CETD, *N,N'*-bis(2-carboximidoethyl)tartarimide dimethyl ester dihydrochloride; CMTD, *N,N'*-bis(2-carboximidomethyl)tartarimide dimethyl ester dihydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.