Structural Studies of Ribonuclease. XVII. A Reactive Carboxyl Group in Ribonuclease*

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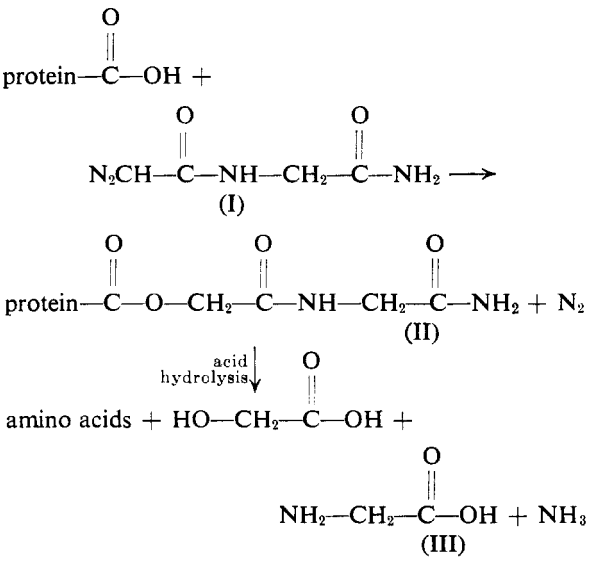
ABSTRACT: Ribonuclease A was treated with diazoacetoglycinamide under conditions designed to accomplish esterification. This reagent was chosen because acid hydrolysis of the reaction products will produce an additional glycine residue for each esterified carboxyl group.

Chromatography of the reaction products indicated that a number of derivatives were formed, the major product being a derivative which contained *four* glycine residues per mole of protein, compared to three in native ribonuclease. Subsequent tryptic and peptic digests, followed by peptide analyses, indicated that this derivative (a fully active component) was esterified at Asp-53.

In the accompanying papers (Broomfield *et al.*, 1965; Riehm *et al.*, 1965) it was reported that ribonuclease could be esterified at eight of its eleven carboxyl groups. In addition, the carboxyl groups which did not undergo esterification were identified as Asp-14, Asp-38, and Asp-83, and it was concluded that these side-chain carboxyl groups could be involved in specific tyrosine-carboxylate interactions. The partially methylated derivative appeared to have a conformation in water similar to that of native ribonuclease, even though it was prepared in an acid-alcohol medium. While we have assumed (Broomfield *et al.*, 1965; Riehm *et al.*, 1965) that the buried tyrosyl residues in the refolded partially methylated derivative are near the same carboxyl groups as they are in the native molecule, the possibility always exists that this assumption may not be valid. Therefore, it was of interest to extend our studies to protein carboxyl reactions which could be performed in an aqueous medium.

A possible means for obtaining reaction at protein carboxyl groups in an aqueous medium is the use of a water-soluble diazo compound. Indeed, esterification of protein carboxyls by diazo compounds is not a novel concept. Chibnall *et al.* (1958) employed diazomethane in an 85% ethanol solution to achieve a partial methylation of the carboxyls in β-lactoglobulin. Diazoacetamide and methyl diazoacetate were successfully utilized in an aqueous medium by Wilcox (1951) to esterify approximately twenty-five carboxyl groups in human serum albumin, and Grossberg and Pressman (1960) noted that the esterification of rabbit antibody by diazoacetamide destroyed the ability of haptens to bind to the antibody. In addition, Doscher and Wilcox (1961) have studied the reaction of chymotrypsinogen and simple carboxylic acids with diazoacetamide.

This paper reports on the reaction, in water, between diazoacetoglycinamide and ribonuclease A. The choice of diazoacetoglycinamide as the esterifying reagent was based on the observation that this compound contains a label. As is seen in the accompanying scheme, information on the extent of esterification may be obtained from an amino acid analysis of the reaction products. It is known that ribonuclease A contains three glycine residues per mole of protein. Thus, any additional quantities of glycine (III) found in the protein hydrolysate will be the result of the reaction between diazoacetoglycinamide and carboxyl groups in ribonuclease.



Employing this reagent, we have noted that the reaction yielded a number of ribonuclease derivatives, many of which differed in their glycine content. In addition we have characterized the major product of

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this reaction more fully by determining which of the carboxyls in the amino acid sequence was esterified.

Experimental

Materials. Ribonuclease (lot R23B-55), 5 times recrystallized, was purchased from Sigma Chemical Co. Ribonuclease A was obtained by chromatography of the crystalline protein on a 7.5 × 60-cm column of unsieved Amberlite IRC-50 according to the procedure of Hirs *et al.* (1953) and then deionized by passage through a 5.0 × 40-cm column of MB-1 mixed-bed resin (Rohm and Haas Co.). Salt-free trypsin and twice-recrystallized pepsin were obtained from the Worthington Biochemical Corp.

Ribonucleic acid (RNA) was obtained from Nutritional Biochemical Co. Glycylglycine methyl ester hydrochloride was purchased from Mann Research Laboratories, Inc. All other reagents were either reagent grade or the best grade available.

Diazoacetoglycine Methyl Ester. The procedure employed for the preparation of this compound differed somewhat from that described (Curtius and Darapsky, 1906) for the preparation of the corresponding ethyl ester. A solution of 9.1 g of glycylglycine methyl ester hydrochloride and 5 g of sodum nitrite in 40 ml of 2 M sodium acetate was cooled in an ice bath. Acetic acid (2 ml) was then added and the reaction mixture was allowed to stand for 2.5 hours in the ice bath. After this time, the product was extracted with 25-ml aliquots of chloroform; the extractions were continued until the chloroform layer became colorless. Petroleum ether (30–60°) was added to the combined chloroform extracts until the solution became turbid; then the mixture was allowed to stand overnight at room temperature. The precipitate was filtered and dried, *in vacuo*, over phosphorus pentoxide to yield 3.4 g (43%) of yellow product. The material was recrystallized from chloroform–petroleum ether to yield 3.1 g of yellow product melting at 127–129°.

Anal. Calcd for $C_5H_7N_3O_3$: C, 38.22; H, 4.46; N, 26.75. Found: C, 38.26; H, 4.67; N, 26.51.[1]

Diazoacetoglycinamide. This compound was prepared from diazoacetoglycine methyl ester according to the procedure of Curtius and Thompson (1906). The observed melting point of 157–159° agreed with the value of 160° reported by these workers.

Preliminary Investigation into the Reaction between Diazoacetoglycinamide and Ribonuclease A. Approximately 100 mg of ribonuclease A was dissolved in 5 ml of water and the *p*H of the solution was adjusted to 4.5 by adding 1 N $HClO_4$. The temperature of the solution was maintained at 10° by the use of a water bath and a jacketed titration vessel. To this cooled solution was added 200 mg of diazoacetoglycinamide in 3 ml of water, and the *p*H was maintained at 4.5 by the addition of 1 N $HClO_4$ with the aid of a Radiometer *p*H-stat. At various times (5, 10, 15, 24, 30, and 36 hours) additional quantities of diazoacetoglycinamide (200 mg) were added. The addition of more diazoacetoglycinamide was necessary since the reagent slowly decomposed at *p*H 4.5. This decomposition was easily followed since (a) the yellow solution gradually turned colorless and (b) nitrogen evolution ceased. At the desired times (5, 15, 30, and 48 hours), aliquots were removed and dialyzed against six changes of HCl at *p*H 3.0. Fractions of these solutions were then employed for analytical chromatography on IRC-50 columns while other fractions were prepared for amino acid analyses.

Reaction between Diazoacetoglycinamide and Ribonuclease A on a Preparative Scale. Approximately 250 mg of ribonuclease A was dissolved in 5 ml of water and the solution was adjusted to *p*H 4.5 with 1 N $HClO_4$. Again the solution temperature was maintained at 10°. To this solution was added 500 mg of diazoacetoglycinamide in 5 ml of water and the *p*H was maintained at 4.5 by the addition of 1 N $HClO_4$ with the aid of the *p*H-stat. Additional quantities of diazoacetoglycinamide (500 mg) were added at 6 and 12 hours; after 24 hours, the reaction mixture was dialyzed against six changes of HCl at *p*H 3.0. Following the dialysis step, the solution was lyophilized to yield 215 mg of protein. Chromatography of the protein (200 mg) was then performed on a 2 × 30-cm column of Amberlite IRC-50 resin. Fractions which contained the various protein components were pooled, dialyzed in a manner similar to that previously described, and then lyophilized.

Chromatography on IRC-50 Columns. Analytical chromatography was performed on 0.9 × 30-cm columns of Amberlite IRC-50 resin in conjunction with a Technicon Autoanalyzer as described by Rupley and Scheraga (1963). Columns which were 2 × 30 cm in dimensions were utilized for preparative chromatography and the effluent was collected in 4-ml fractions.

Ribonuclease Activity. Activity of the various derivatives was measured by the change in the absorbancy at 300 mμ which occurs in the enzymic depolymerization of RNA in 0.1 M sodium acetate buffer at *p*H 5.0, as described by Kunitz (1946).

Studies on Component B. Approximately 10 mg of component B, the major product of the reaction, was dialyzed in the cold for 12 hours against 0.1 M sodium borate buffer, *p*H 9.5. The solution was then dialyzed against three changes of water and a portion (containing approximately 3 mg) was chromatographed on a 0.9 × 30-cm Amberlite IRC-50 column. In another experiment, a sample of component B (15 mg) was carried through the nitrite test for esters as described by Seifter *et al.* (1960).

Performic Acid Oxidation. Oxidation of ribonuclease A and component B was performed at −10° according to Hirs (1956).

Tryptic and Peptic Digestions. Oxidized ribonuclease A and oxidized component B were digested by trypsin according to the procedure described by Hirs *et al.* (1956). Peptic digestions were carried out in *p*H 2.2, 0.2 M sodium citrate buffer at 25° according to the procedure outlined by Bailey *et al.* (1956).

Separation of Peptides on a Dowex 50-X2 Column.

[1] Analysis was performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

The procedure employed for the chromatography of the tryptic and peptic digests was similar to that employed by Hirs *et al.* (1956) and Bailey *et al.* (1956) with the modifications of Cha and Scheraga (1963) and Riehm *et al.* (1965). Approximately 20–30 mg of protein digest was employed in each chromatogram.

Amino Acid Analyses. Analyses were performed with the Technicon amino acid analyzer. All hydrolyses were carried out in 6 N HCl in sealed, evacuated ampules for 22 hours at 116°. The amino acid composition of ribonuclease and its derivatives was calculated by assuming that the hydrolysate contained fifteen residues of aspartic acid. The amino acid composition of each peptide hydrolysate was derived by assuming that an appropriate amino acid was present in the theoretical amount; the molar value obtained for this amino acid was then used to determine the number of residues for the other amino acids. The correction factors of Gundlach *et al.* (1959) and Rupley and Scheraga (1963) were employed for those amino acids which undergo decomposition during hydrolysis.

Results

Preliminary Investigations. These investigations were performed to determine to what extent the reaction between diazoacetoglycinamide and ribonuclease A had proceeded. As is seen in Table I, the concentration of glycine in the reaction product increased with each addition of reagent. If one assumes that esterification could occur at each of the eleven carboxyl groups in ribonuclease A, it is obvious that the increase in glycine content does not approach the maximum value. After seven additions of the diazo compound glycine had increased by 2.8 residues per mole of ribonuclease, indicating that approximately 25% of the carboxyl groups had been esterified.

TABLE I: Glycine Content of Ribonuclease following Treatment with Diazoacetoglycinamide (moles of glycine per mole of protein).

Additions of Diazo Compound (200 mg per addition)	Glycine content	Increase in Glycine
Native ribonuclease	3.00 (theory)	
First	3.90	0.90
Third	5.10	2.10
Fifth	5.52	2.52
Seventh	5.80	2.80

It is also noteworthy that the yields of the other amino acids in these hydrolysates approximated the theoretical values. The amino acid composition of ribonuclease following seven additions of the diazo compound (Table II, column IV) illustrates this point. In addition, amino acid analyses of the hydrolysates of the various derivatives of Table I indicated that the only ninhydrin-positive components were those which could be ascribed to the amino acids normally found in a ribonuclease hydrolysate. These observations indicate that diazoacetoglycinamide had not reacted with the ε-amino groups of lysine nor with the imidazole nitrogens of histidine residues (such reactions should not be overlooked especially if the reaction were to be carried out at higher *p*H). Any reaction with lysine and/or histidine residues would be observed in an analysis of the protein hydrolysate since (a) there would be a decrease in the lysine and/or histidine content, and (b) new amino acid derivatives would appear. These derivatives (the carboxymethyl derivatives) have been characterized by Gundlach *et al.* (1959).

Analytical chromatography of the reaction products was performed after one, three, five, and seven additions of diazoacetoglycinamide. Figure 1 illustrates the type of chromatogram obtained from such an analysis. Figure 1a, which was obtained after three treatments with the diazo compound, showed three major components. The first peak, designated peak A, appeared in a position which was identical to the elution position of ribonuclease A. Furthermore, addition of ribonuclease A to the mixture resulted in an increased area for the first peak. Peak B which was partially but not completely separated from ribonuclease A appeared to be quite symmetrical and was believed to be a single component. On the other hand, the peak which was designated C was extremely broad and unsymmetrical, thereby suggesting that it was a mixture of components. A fourth and minor peak (designated as D in Figure 1), which appeared near the completion of the chromatogram, was believed to be the ammonium ion (Rupley and Scheraga, 1963). Figure 1b, which was obtained after five treatments with diazoacetoglycinamide, showed that the concentrations of peaks B and C had increased at the expense of component A. It is also noteworthy that this chromatogram contained no components besides those observed in Figure 1a. This was also the case for the chromatogram obtained after seven additions of the diazo compound.

Reaction between Diazoacetoglycinamide and Ribonuclease A on a Preparative Scale. Chromatography of the preparative reaction mixture (200 mg) is illustrated in Figure 2. Components A and B were sufficiently separated to warrant pooling the appropriate fractions and then dialyzing these solutions against HCl at *p*H 3.0. Upon lyophilization, fraction A (tubes 49–59) produced 40 mg of protein while fraction B (tubes 66–74) yielded 35 mg. On the other hand, it was now quite obvious that peak C was a mixture and that the various components were not cleanly separated. Nevertheless, single-tube aliquots were removed at various points of C (labeled C_1, C_2, C_3, C_4, and C_5 in Figure 2) and analyzed (a) for their amino acid content and (b) for enzymatic activity. The remainder of C was pooled, dialyzed, and lyophilized to yield 65 mg of protein.

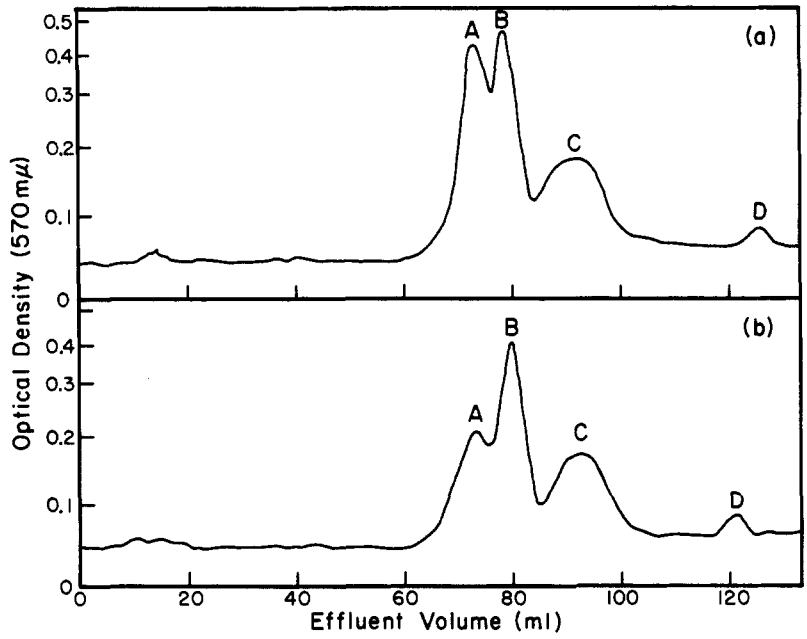

FIGURE 1: Analytical chromatography of ribonuclease following treatment with diazoacetoglycinamide. A 0.9 × 30-cm column of IRC-50 was used and the chromatogram was developed with a four-stage gradient; the first three stages contained 35 ml of 0.15 M phosphate buffer, *p*H 6.40, while the fourth stage contained 35 ml of 1 M phosphate buffer, *p*H 6.47. The ninhydrin color value of the effluent (flow rate of 20 ml/hour) was obtained with the aid of a Technicon Autoanalyzer. (a) Chromatography after three additions of the diazo compound. (b) Chromatography after five additions of the diazo compound.

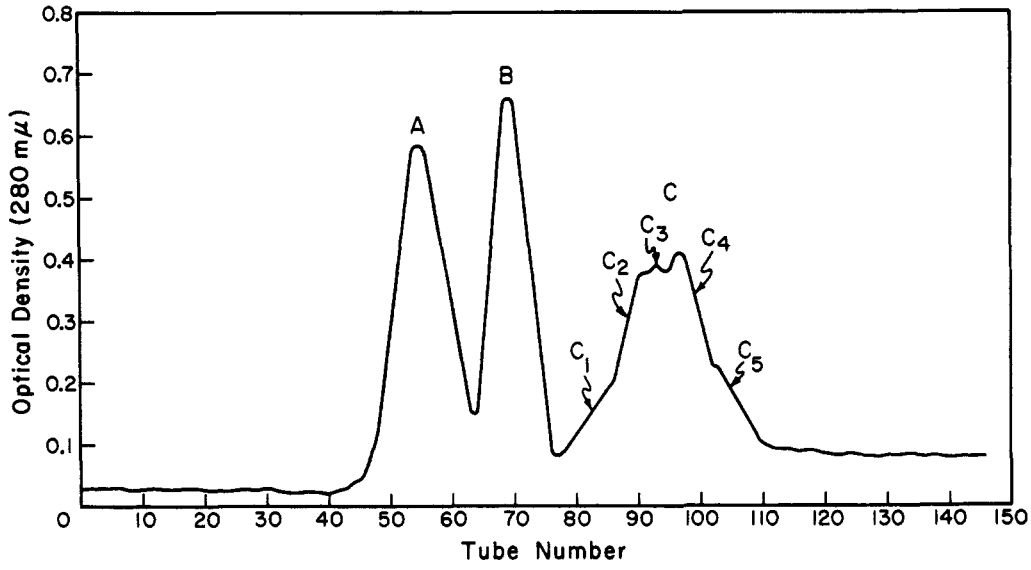

FIGURE 2: Chromatography of ribonuclease (200 mg) following three additions of diazoacetoglycinamide. A four-stage gradient was employed; the first three stages contained 150 ml of 0.15 M sodium phosphate buffer, *p*H 6.40, while the fourth stage contained 150 ml of 1 M phosphate buffer, *p*H 6.47. The effluent (flow rate of 50 ml/hour) was collected in 4-ml fractions and the optical density was read at 280 mμ.

The amino acid composition of the protein derivatives are reported in Table II. Component A contained three glycine residues per mole of protein and was assumed to be unreacted ribonuclease. Fraction B was shown to contain four glycine residues (experimentally 4.14) and thus was believed to be a component which was esterified at one carboxyl group. The glycine content of fraction C depended on the specific region

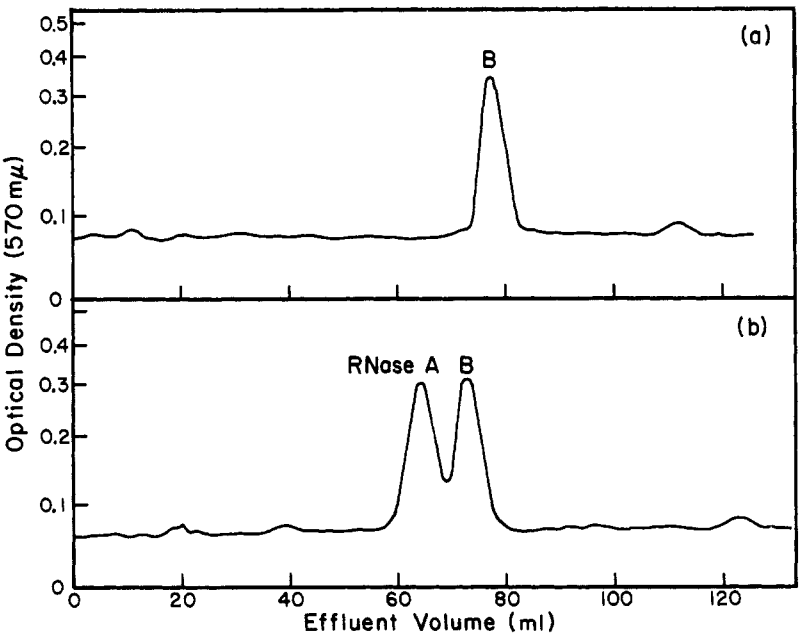

FIGURE 3: Analytical chromatography of component B. (a) Alone; (b) with added ribonuclease A. The methods for the development of these chromatograms were identical to those described for Figure 1.

TABLE II: Amino Acid Composition of the Protein Derivatives (moles of amino acid per mole of protein).

Amino acid	RNAase	RNAase Observed	Seven Additions of Diazo-Compound	A	B	C_1	C_2	C_3	C_4	C_5
Aspartic acid[a]	15	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00	15.00
Threonine	10	10.13	10.30	9.86	10.05	9.69	9.75	10.16	9.86	10.22
Serine	15	14.97	15.16	15.09	15.38	14.72	14.92	15.13	14.79	14.97
Glutamic acid	12	12.02	12.23	11.88	12.23	11.97	12.34	11.88	12.25	12.18
Proline	4	4.22	4.20	4.19	4.18	4.15	4.21	4.09	4.29	3.97
Glycine	3	3.23	5.80	3.13	4.14	4.86	4.96	5.33	5.76	6.55
Alanine	12	12.39	11.95	12.16	11.83	12.11	11.65	11.80	12.16	11.88
Half-cystine	8	7.98	8.18	8.14	8.29	7.71	8.09	7.85	8.08	7.88
Valine	9	8.49	8.52	8.62	8.78	8.57	8.68	8.85	8.63	8.71
Methionine	4	3.79	3.75	3.66	4.09	3.86	3.72	3.89	3.79	3.92
Isoleucine[b]	3	2.16	1.91	2.22	1.83	2.46	2.40	2.19	2.24	2.07
Leucine	2	2.08	1.98	1.86	1.85	1.94	1.90	1.97	2.06	1.94
Tyrosine	6	6.22	6.24	5.85	5.94	5.84	6.07	5.86	6.11	5.93
Phenylalanine	3	3.03	3.00	3.11	2.87	2.88	2.76	3.19	2.96	3.11
Lysine	10	9.76	9.70	10.18	9.92	9.83	10.17	9.83	9.93	10.11
Histidine	4	4.23	4.15	3.82	3.98	3.87	4.23	4.02	3.96	4.20
Arginine	4	4.11	4.10	4.22	4.05	3.87	4.27	4.10	4.16	4.04

[a] Assumed reference. [b] Isoleucine is known not to be completely liberated in 22-hour acid hydrolysates.

analyzed; these values ranged from 4.86 to 6.55 residues per mole of protein, thereby suggesting that fraction C was comprised, in part, of ribonuclease derivatives which were substituted at two, three, and four carboxyl groups. Nevertheless, owing to the poor resolution of the components in this fraction, the exact number and the relative concentrations of these derivatives could not be determined with accuracy. It should be pointed

out that this fraction may also have contained appreciable quantities of polymerized material. All attempts to effect an improved resolution of the components in fraction C have, up to the present time, been unsuccessful.

Activity measurements performed on the esterified ribonuclease derivatives are reported in Table III.

TABLE III: Enzymatic Activity of the Components Obtained from the Reaction between Ribonuclease and Diazoacetoglycinamide.

Derivative	Activity (%)
Ribonuclease A	100
A	102
B	98
C_1	88
C_2	78
C_3	74
C_4	57
C_5	56

All components possessed enzymatic activity toward the RNA substrate with component B being fully active. The derivatives in fraction C possessed enzymatic activities lower than that obtained for the native molecule. Therefore, it appears that esterification at one carboxyl group (as in component B) caused very little if any conformational change about the active center of the enzyme. It is also apparent that the group which is esterified in this derivative is not required for enzymatic activity. On the other hand, the decreased activity of the more-substituted derivatives indicated that these derivatives had either (a) undergone significant conformational changes, or (b) reacted, in part, at a carboxyl group(s) which participated in the catalytic function of the molecule.

The high yields and the favorable separation of component B stimulated further interest in this derivative. Figure 3a illustrates an analytical chromatogram of this derivative. The only detectable impurity appears to be a small peak which is ascribed to the ammonium ion (Rupley and Scheraga, 1963). The addition of ribonuclease A to component B resulted in the chromatogram shown in Figure 3b; thus component B (as is seen in Figure 3a) contained no detectable quantities of ribonuclease.

Component B was not stable to alkaline conditions. Treating this derivative at *p*H 9.5 for an extended period of time (12 hours), followed by analytical chromatography, indicated that component B was converted to ribonuclease A. This chromatogram showed that approximately 60% of the protein appeared in a position identical to the elution position of ribonuclease A while the remaining protein was eluted at a position identical to that of component B. Therefore, it appears that alkaline conditions produced partial saponification of the ester linkage in this derivative. Further support that component B contains an esterified carboxyl group was obtained from a positive nitrite test (Seifter *et al.*, 1960).

Tryptic Digestions. The elution patterns obtained from tryptic digests of oxidized ribonuclease A and of oxidized component B are shown in Figure 4. A visual comparison of the two patterns showed one striking difference, namely, the absence of O-T-9[2] (residues 40–61 containing Glu-49 and Asp-53) in component B. Furthermore, when all the peptides in component B were analyzed for their amino acid composition, it was noted that (a) the peak ascribed to O-T-8 showed no evidence of O-T-9 and (b) the fraction termed O-T-14 contained an additional component. This component analyzed similarly to O-T-9 with the exception that one residue of glycine was present per mole of O-T-9. The possibility that this component was O-T-13 (residues 38–62; Hirs *et al.*, 1956) was ruled out because of the complete absence of arginine in the hydrolysate (residue 39 is arginine). The amino acid analyses performed on these pertinent peptides (O-T-8, O-T-9, and O-T-14) are illustrated in Table IV.

The elution positions and the quantitative recoveries of all peptides (except O-T-9) in oxidized component B were similar to the results obtained from oxidized ribonuclease A. The methods described previously (Riehm *et al.*, 1965) for obtaining the correct analyses of O-T-2 and O-T-4 were also necessary in these investigations. These results indicated that the carboxyl groups in O-T-2 (Asp-83), O-T-4 (Asp-14), O-T-6 (Glu-86), O-T-7 (Asp-38), O-T-10 (Glu-2), and O-T-15 (Glu-9) had not undergone esterification. The state of the three carboxyls (Glu-111, Asp-121, and Val-124) could not be determined with assurance since the peptide which contains these carboxyls (O-T-16) was obtained in very low yield. It should be pointed out that O-T-16 was not observed in the oxidized ribonuclease A tryptic digest.

Peptic Digests. Figure 5 shows the elution pattern obtained from peptic digests of ribonuclease A and component B. A visual comparison of the patterns showed that O-P-3 (residues 52–55) was absent in the component B digest. In all other respects, the elution positions and the quantitative recoveries of the remaining peptides in component B compared favorably to those obtained from oxidized ribonuclease A. Therefore, the carboxyl groups in O-P-2 (Asp-121 and Val-124), O-P-5 (Glu-111), and O-P-10 (Glu-49) were believed not to have undergone esterification.

Discussion

In the preceding communications (Broomfield *et al.*, 1965; Riehm *et al.*, 1965) we reported on the prepara-

[2] The terminology employed in this communication for the identification of the peptides is that of Hirs *et al.* (1956) for the tryptic digest and of Bailey *et al.* (1956) for the peptic digest.

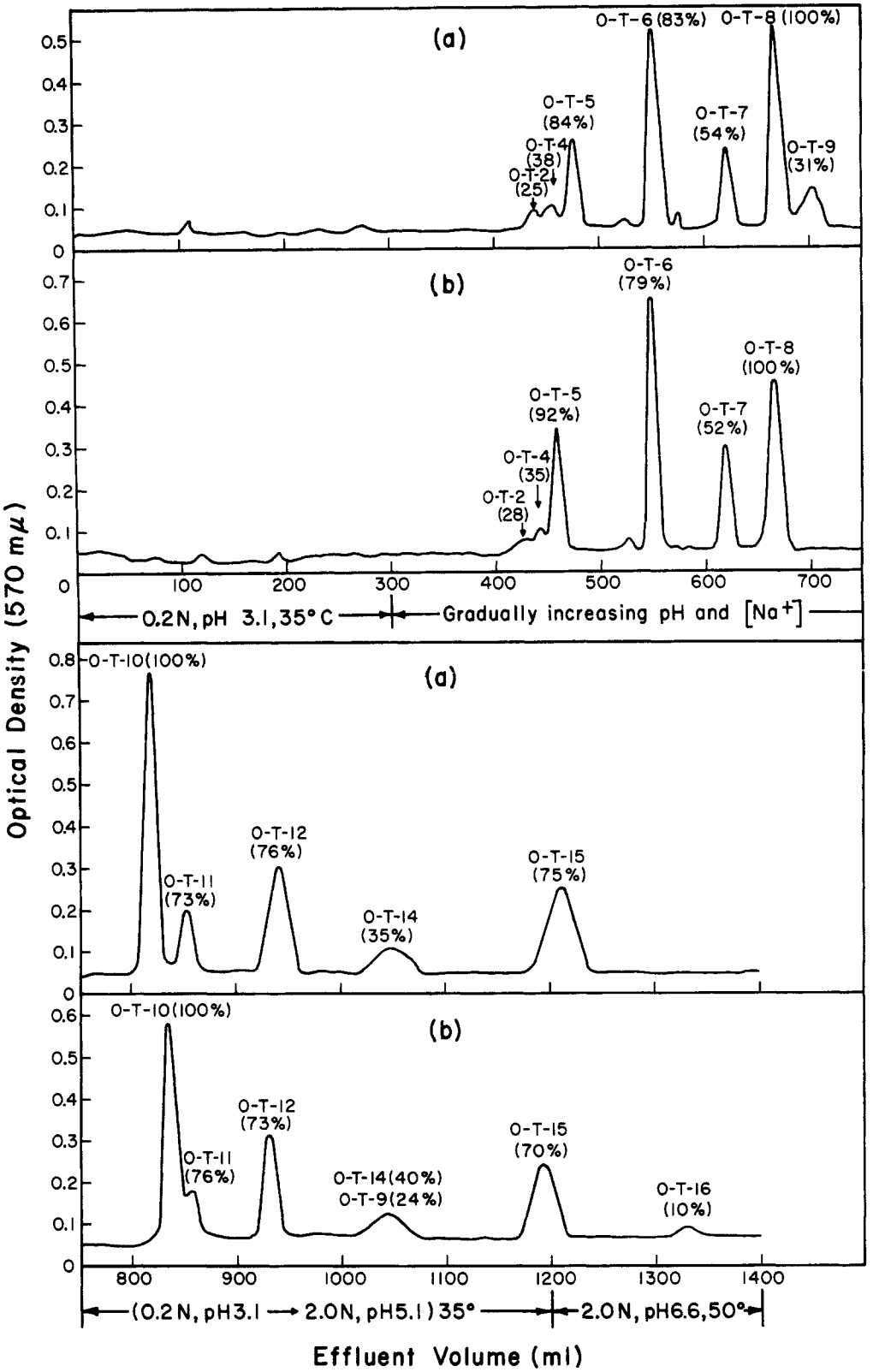

FIGURE 4: Chromatography of peptides from a 20-hour tryptic digest of oxidized protein on a 0.9 × 150-cm column of Dowex 50-X2. The effluent was collected in 2-ml fractions and the ninhydrin color values were obtained with the aid of a Technicon Autoanalyzer. The percentages in parentheses represent the yields of each peptide. The yield of peptide O-T-8 was assumed to be 100%. (a) Ribonuclease A; (b) component B.

TABLE IV: Amino Acid Compositions of the Pertinent Peptides Obtained from a Tryptic Digest of Oxidized Component B (moles of amino acid per mole of peptide).

	O-T-8 (Residues 99–104)		O-T-9 (Residues 40–61)		O-T-14 (Residues 92–98)	
Amino Acid	Theory	Observed	Theory	Observed	Theory	Observed
Cysteic acid		0.13	2	2.16	1	0.94
Aspartic acid	1	1.12	2	2.14	1	1.15
Methionine sulfone						
Threonine	2	1.72	1	1.12		
Serine			2	2.22		
Glutamic acid	1	1.08	3	2.67		
Proline			1	0.99	1	0.97
Glycine			None	1.14		
Alanine	1	1.00[a]	2	1.98	1	0.89
Valine			4	3.50		
Isoleucine						
Leucine			1	0.86		
Tyrosine					2	2.00[a]
Phenylalanine			1	1.00[a]		
Lysine	1	0.98	2	2.14	1	1.15
Histidine			1	1.24		
Arginine						

[a] Assumed reference.

tion and characterization of a methylated ribonuclease derivative. This derivative, which was prepared from a methanolic HCl medium, was shown to contain three unmethylated carboxyl groups. These carboxyl groups were identified as Asp-14, Asp-38, and Asp-83 and it was concluded that some or all of these side-chain carboxyls could be involved in tyrosine-carboxylate interactions believed to be present in the native molecule. These conclusions were based on the assumption that the methylated derivative (prepared from methanolic HCl) possessed protein-carboxylate interactions which were identical to those in the native molecule. Nevertheless, as noted previously (Riehm *et al.*, 1965), this assumption could be erroneous. Therefore, it was of interest to attempt carboxyl reactions which could be performed in an aqueous medium. Such reactions, if possible, would remove any objections to the foregoing assumption.

It was visualized that the reaction between ribonuclease A and diazoacetoglycinamide would provide a method by which the "exposed"[3] carboxyl groups of the protein could be esterified in an aqueous medium. However, it was soon noted that this reaction was quite limited. As was stated earlier, only 25% of the total carboxyls in the protein had reacted following seven additions (a 14-fold excess by weight) of the diazo compound. Therefore, this diazo compound is not a satisfactory reagent for blocking all the "exposed" carboxyl groups in ribonuclease. This observation should not be surprising in view of the experimental difficulties encountered in these examinations. In addition to reacting with carboxyl groups, the diazo compound is hydrolyzed in acidic medium; at *p*H 4.5 hydrolysis was complete in 4 hours. Hydrolysis at lower *p*H (3.0) was even more rapid, being complete in 15 minutes. Hydrolysis was minimized (most of the reagent still being present after 8 hours' reaction time) by carrying out the esterification at *p*H 6.0; however, analytical chromatography of the reaction products indicated that the reaction was not so extensive as it was at *p*H 4.5. Thus, although the *p*H selected for this study (4.5) was one at which hydrolysis of the reagent occurred to a significant extent, it was the *p*H at which the largest yield of ribonuclease derivatives was obtained.

Other factors are undoubtedly responsible for the extent to which this reaction proceeded. Local electrostatic effects could decrease the reactivity of certain carboxyls. Thus, a carboxyl group which was located in a cationic region would be expected, according to the mechanism of Doscher and Wilcox (1961), to react at a slow rate. In addition, the size of the reagent could impose restrictions. Therefore, reaction at a particular carboxyl could be hindered because of the steric effects imposed by the reagent.

Although diazoacetoglycinamide has proved inadequate in blocking all the "exposed" carboxyl groups of ribonuclease, it is interesting to note that a number

[3] The term "exposed" is defined as designating those carboxyl groups in the protein which are believed not to be involved in hydrogen bonding.

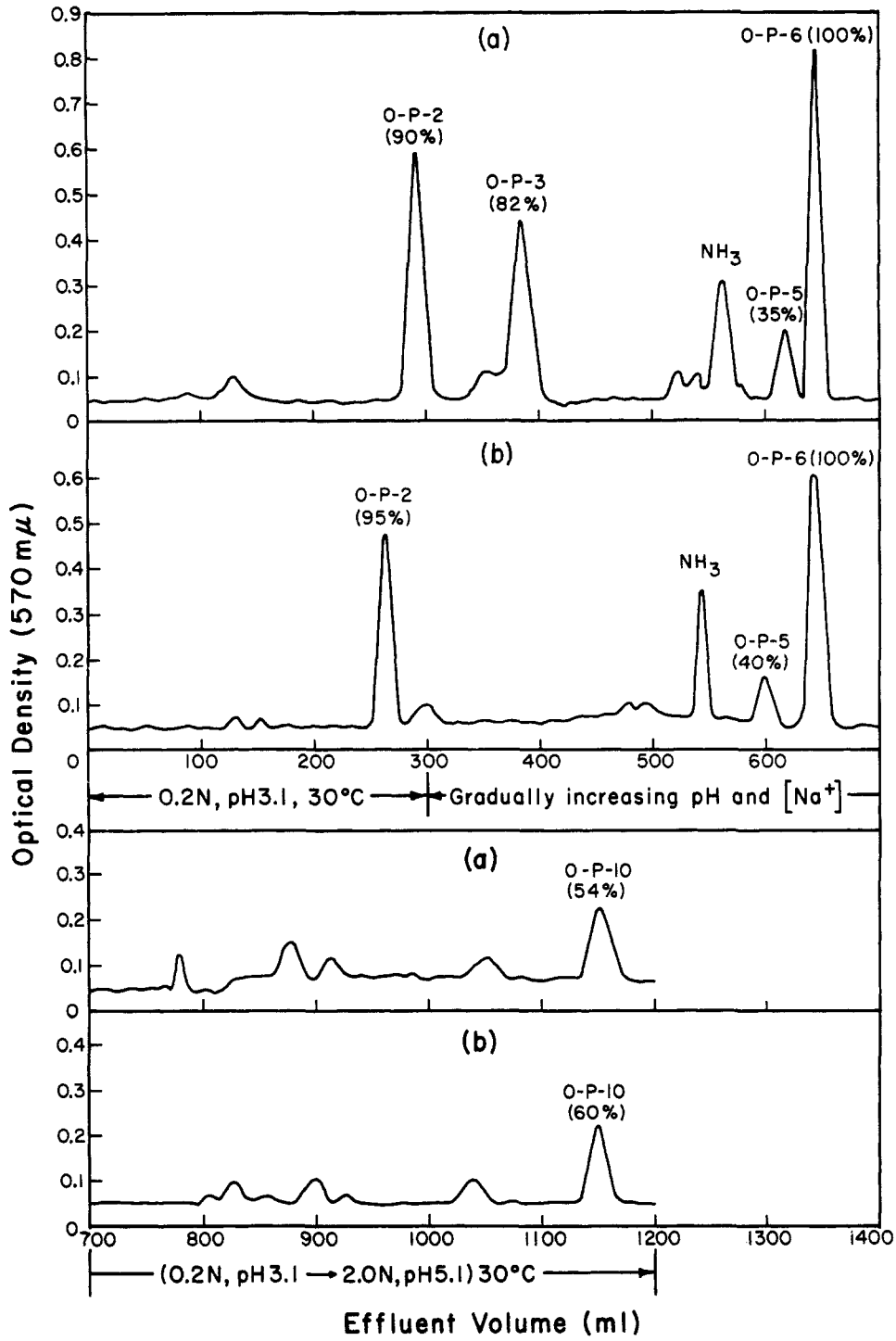

FIGURE 5: Chromatography of peptides from a 24-hour peptic digest of oxidized protein. The chromatographic conditions are the same as in Figure 4 except for the temperature and the volume of buffer change. The figures in parentheses represent the yields of each peptide. The yield of O-P-6 was assumed to be 100%. (a) Ribonuclease A; (b) component B.

of derivatives were formed. The initial product formed in this reaction was one in which a single carboxyl had been esterified. This derivative, upon amino acid analysis, was shown to contain four glycine residues (as compared to three in native ribonuclease) per mole of protein; it was fully active toward the RNA substrate, and proteolytic digestions (tryptic and peptic digests) indicated that Asp-53 had undergone reaction.

The latter conclusion is based on the following observations. Tryptic digests obtained from this oxidized component indicated that the esterified carboxyl resided in O-T-9 (residues 40–61). This point is illustrated by the complete absence in the peptide chromatogram of O-T-9 at 700 effluent ml and by the appearance of the altered peptide at a new position. The altered peptide was similar to O-T-9 in its amino acid content except for the presence of *one* glycine residue per mole of peptide. It should be noted that peptide O-T-9 possesses two carboxyl groups, namely, Glu-49 and Asp-53, and it was not possible from these observations to determine which carboxyl had undergone reaction. On the other hand, a peptic digest provided a clear distinction between these carboxyls (Bailey *et al.*, 1956). O-P-3 (residues 52–55) contains Asp-53 while O-P-10 (residues 46–51) possesses Glu-49. Subsequently, peptic digestion and chromatography of the oxidized derivative illustrated that the peptide zone at 400 effluent ml (O-P-3) was absent and that O-P-10 appeared at its normal elution position. It should be pointed out that a peptide zone corresponding to the altered O-P-3 was not observed. One possible explanation for this fact could be that esterification at Asp-53 altered the ribonuclease substrate so that peptic hydrolysis did not occur at the Glu(NH_2)-Ala (residues 55–56) peptide bond.

It is also noteworthy that component B appeared to be a single monoesterified ribonuclease, or at the most it included only small amounts of other monoesterified ribonucleases which were in quantities too small to be measured. If large amounts of other monoesterified ribonucleases were present in this component, one would expect to see appreciable quantities of O-T-9 in a tryptic digest and O-P-3 in a peptic digest. As was previously stated, this was not the case.

Although the reaction between ribonuclease and diazoacetoglycinamide yielded but a single monoesterified derivative, the amino acid analyses of fraction C indicated that derivatives which were esterified at two, three, and four carboxyls were formed. Indeed this fraction may contain even higher substituted derivatives. It is also possible and highly probable that more than one derivative would possess an identical number of esterified groups. Additional studies on the derivatives could afford information on these uncertainties.

It is interesting to speculate as to the reason why Asp-53 showed an increased reactivity toward diazoacetoglycinamide. A possible reason for this enhanced reactivity is that the side chain of Asp-53 is extremely accessible to attack by the reagent. Such an assumption implies that the side chain of Asp-53 extends into the solvent (much more than any other carboxyl) relatively free from any steric effects imposed by the remainder of the molecule. Another possibility is that Asp-53 is to be found in a region of high anionic charge. Such a region arising from the proximity of aspartate and/or glutamate side chains would be expected to enhance the reaction, according to the mechanism of Doscher and Wilcox (1961). Nevertheless, it is difficult to visualize that electrostatic effects alone would favor the production of a specific monoesterified ribonuclease. A cluster of high anionic charge would be expected to raise the *pK* of *all* carboxyls in this region and the esterification of a particular carboxyl (in this case Asp-53) should not be favored over the esterification of another carboxyl in this cluster. On the other hand, a region of high anionic charge in which one carboxyl was free from steric hindrance would alleviate this problem and would indeed be an extremely favorable situation.

It is believed that Asp-53 is not to be found in a cationic atmosphere. Such a situation would be expected to lower the *pK* of the carboxyl and thus produce an unfavorable reaction condition. Hydrogen bonding which involves Asp-53 is also excluded since this situation would again lower the *pK* of the carboxyl. The latter conclusion is in agreement with the observations of Riehm *et al.* (1965).

The observations and conclusions presented in this report indicate that diazoacetoglycinamide could not distinguish the "exposed" carboxyl groups in ribonuclease from those which are believed to be involved in protein carboxylate interactions. Nevertheless, the reaction between this reagent and ribonuclease A does indicate that this protein contains at least one extremely reactive carboxyl group (Asp-53). This observation suggests that any three-dimensional model of ribonuclease should position Asp-53 either in a region where it is quite accessible to the solvent and/or in a position of high anionic charge in which the side chain of Asp-53 is more accessible to the solvent than are the other carboxyls in this cluster.

Finally, the techniques employed in this report may find further use in investigations which are concerned with the role of the carboxyl group in other enzymatic reactions.

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Kinetic Studies of Rabbit Muscle Lactate Dehydrogenase. II. Mechanism of the Reaction*

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ABSTRACT: The rabbit muscle lactate dehydrogenase enzyme system has been investigated at *p*H 7.15 in tris(hydroxymethyl)aminomethane-chloride buffer. On the basis of information obtained from product inhibition studies, the relationship among the ϕ constants, and the Haldane relationship, it was concluded that a modification of the Theorell-Chance mechanism, involving the isomerization of at least the enzyme-oxidized coenzyme complex, is in harmony with the experimental data. Oxalate inhibition studies indicated that a primarily ordered pathway of substrate addition to the enzyme appears to be operative with the coenzyme substrates adding first. The maximum dissociation constant for the enzyme-oxidized coenzyme-oxalate complex was calculated. Oxalate at higher concentrations may interact with the free enzyme. This effect can be detected when pyruvate and reduced nicotinamide-adenine dinucleotide are substrates, but not when nicotinamide-adenine dinucleotide and L-lactate are used, because in the latter instance it is not possible to obtain measurable velocities in the presence of high concentrations of inhibitor.

Rabbit muscle lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27) has been studied kinetically (Zewe and Fromm, 1962) and fluorometrically (Fromm, 1963) in our laboratory. In a previous report (Zewe and Fromm, 1962) it was concluded, on the basis of product inhibition studies, that the reaction catalyzed by rabbit muscle lactate dehydrogenase may be represented by a simple Theorell-Chance (Theorell and Chance, 1951) mechanism in which ternary enzyme-substrate complexes are short-lived relative to the binary complexes (Alberty, 1953).

Recently Thomson and co-workers (Thomson and Darling, 1962; Thomson *et al.*, 1964) have questioned the validity of the assignment of the simple Theorell-Chance mechanism to rabbit muscle lactate dehydrogenase on the basis of their studies with deuterated NADH. They proposed that another mechanism, perhaps involving one or more ternary complexes, or one of the modifications of the Theorell-Chance mechanism proposed by Mahler *et al.* (1962), was applicable. The purpose of this report will be to examine the mechanism of this enzyme in greater detail in light of additional experimental data obtained from a study of the reaction at various *p*H values (V. Zewe and H. J. Fromm, in preparation) in the presence of reaction product, and in the presence of oxalate.

The simplest plausible reaction scheme for rabbit muscle lactate dehydrogenase appears to be a modification of the Theorell-Chance mechanism in which the enzyme-oxidized coenzyme complex undergoes isomerization. Reasons for arriving at this conclusion will be treated more fully here.

Experimental Procedure

Materials. Lactate dehydrogenase from rabbit muscle was purchased from California Corp. for Biochemical Research and was purified according to the procedure reported by Fromm (1963). Zinc L-lactate was a product of Pfanstiehl Laboratories, Inc. NAD^+ was purchased from Pabst Laboratories and was purified by column chromatography on DEAE-cellulose (Dalziel, 1963a) using Tris-chloride buffer, *p*H 7.6 (0.2 M, reservoir, 0.005 M, mixer), as the eluting buffer. The nucleotide was isolated as the barium salt. NADH was prepared

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