Soc. 84, 3965.
Fischbach, F. A., and Anderegg, J. W. (1965), *J. Mol. Biol. 14*, 458.
Haggis, G. H. (1965), *J. Mol. Biol. 14*, 598.
Harrison, P. M. (1964), *in* Iron Metabolism, F. Gross, Ed., Berlin, Springer-Verlag, p 40.
Hewkin, D. J., and Griffith, W. P. (1966), *J. Chem. Soc., Sec. A*, 471.
Jacobs, I. S., and Bean, C. P. (1963), *in* Magnetism, Vol. III, Rado, G. T., and Suhl, H., Ed., New York, N. Y., Academic, p 271.
Kleinwachter, V. (1964), *Arch. Biochem. Biophys. 105*, 352.
Kurkjian, C. R., and Buchanan, D. N. E. (1964a), *Phys. Chem. Glasses 5*, 63.
Kurkjian, C. R., and Buchanan, D. N. E. (1964b), *Rev. Mod. Phys. 36*, 397.
Kurkjian, C. R., and Sigety, E. A. (1964), *Proc. VII Intern. Congr. Glass, Brussels.*
Morgan, J., and Warren, B. E. (1938), *J. Chem. Phys. 6*, 666.
Nakamoto, K. (1963), Infrared Spectra of Inorganic and Coordination Compounds, New York, N. Y., Wiley, p 161.
Neél, L. (1962), *J. Phys. Soc. Japan 17*, 676.
Pape, L., Multani, J. S., Slatman, P., and Stitt, C. (1968), *Biochemistry 7*, 606.
Schoffa, C. (1965), *Z. Naturforsch 20B*, 167.
Schugar, H., Gray, H. B., Jones, R. B., and Walling, C. (1967), *J. Am. Chem. Soc. 89*, 3712.
Spiro, T. G., Allerton, S. E., Bils, R., Renner, J., Saltman, P., and Terzis, A. (1966), *J. Am. Chem. Soc. 88*, 2721, 3147.
Stammreich, H., Bassi, D., Sala, O., and Siebert, H. (1958), *Spectrochim. Acta 13*, 192.
Van der Woude, F., and Dekker, A. J. (1966), *Phys. Status Solidi 13*, 181.
Van Loef, J. J. (1966), *Physica 32*, 2102.
Warren, B. E., Krutter, H., and Morningstar, O. (1936), *J. Am. Ceram. Soc. 19*, 202.

Reductive Alkylation of Amino Groups in Proteins*

Gary E. Means and Robert E. Feeney

ABSTRACT: When protein solutions are treated with low concentrations of simple aliphatic aldehydes or ketones and small amounts of sodium borohydride, amino groups are converted in high yield into the corresponding mono- or dialkylamino derivatives. Conditions for optimum reaction have been determined using gas–liquid partition chromatography to follow the reductive alkylation of butylamine in aqueous solution. The reaction is strongly pH dependent. At pH 9.0 and 0°, only amino groups of proteins are modified. The principal product of the reductive methylation of proteins with formaldehyde was identified as ϵ-*N*,*N*-dimethyllysine; with acetaldehyde or acetone only the corresponding monoalkylated lysines were formed. The mild conditions and low concentrations of sodium borohydride used did not result in the reductive cleavage of disulfide bonds of lysozyme, insulin, ribonuclease, turkey ovomucoid, human serum transferrin, α-chymotrypsin, or chymotrypsinogen.

Reductive methylation of ribonuclease produced an enzymatically inactive product with less than a single remaining unmodified lysine residue. Reductive alkylation of proteins occurs with minimal changes in gross physical properties as determined by studies of ultraviolet absorption spectra, sedimentation, and optical rotatory parameters.

In recent years, there has been a growing interest in the roles of specific amino acid side chains in the properties of various biologically active proteins. Toward this end, chemical modification of side chains has been widely used with generally good results. There remains a great need, however, for reagents which may be used under mild conditions and yet are specific for particular types of side chains.

Two methods for the modification of amino groups, amidination (Hunter and Ludwig, 1962) and guanidination (Schütte, 1943; Chervenka and Wilcox, 1956), partially satisfy the above needs. In addition, by main-

* From the Department of Food Science and Technology, University of California, Davis, California 95616. *Received February 19, 1968.* Supported in part by Contract 12-14-100-7715(74) from the Agricultural Research Service, U. S. Department of Agriculture, and by Grant HD-00122-5 from the National Institutes of Health. This material is taken from the thesis of G. E. M. to be submitted to the Graduate Division of the University of California, Davis, in partial fulfillment of the requirements for the Ph.D. degree in Comparative Biochemistry.

taining the positive charge of the amino groups, they do not greatly alter the charge relationships in the native protein. Either of these methods may be used to achieve low degrees of modification under relatively mild conditions, but the vigorous conditions required to achieve extensive modifications greatly limit their use with all but the most stable proteins. Most methods which modify protein amino groups under mild conditions (Fraenkel-Conrat, 1957; Haynes *et al.*, 1967) result in the loss of positive charges.

Bowman and Stroud (1950) have described a procedure for converting amino acids into corresponding *N,N*-dimethylamino acids by treatment with formaldehyde followed by catalytic hydrogenation. The reaction has been developed by Ingram (1953) into an end-group method for proteins. Fischer *et al.* (1958) have developed a method whereby labile Schiff base enzyme products may be trapped by reduction with borohydride, resulting in the alkylation of a specific lysyl residue of the enzyme. The method is generally applicable to those enzymes wherein a Schiff base is formed as a critical step in an enzymatic reaction, or where one can be induced to form by a change in pH or by addition of denaturing agents.

In the present study, simple aliphatic carbonyl compounds have been covalently bound as their respective alkyl groups to the ε-aminolysine residues of proteins *via* the use of sodium borohydride. The method is rapid and employs mild conditions. It produces extensively modified protein, with physical properties little different from those of the native proteins.

Materials and Methods

Reductive Alkylation of Proteins. Reductive methylations of proteins were generally done at 0° in 0.20 M borate buffer (pH 9.0). For each milliliter of solution containing 2.5–10.0 mg of protein, approximately 0.5 mg of sodium borohydride (Sigma Chemical Co.) was added, followed by five increments (0.5 μl/ml of reaction solution) of 37% aqueous formaldehyde solution (Baker Analyzed reagent) over a period of 30 min. Such treatment generally resulted in methylation of more than 80% of the amino groups present.[1] More extensive modification was best obtained by repetition of the same procedure. Reductive ethylation using acetaldehyde was done similarly, but resulted in less extensively modified proteins.

Reductive isopropylations[1] were done at 0° in 0.18 M borate buffer (pH 9.0) containing 10% v/v acetone. To each milliliter of solution containing 2.5–10.0 mg of protein several small portions of sodium borohydride (approximately 0.5–1 mg/ml of reaction solution) were added until the desired total (3–5-mg/ml total) had been reached. The proteins were purified by passage through Sephadex G-25 (Pharmacia Fine Chemicals).

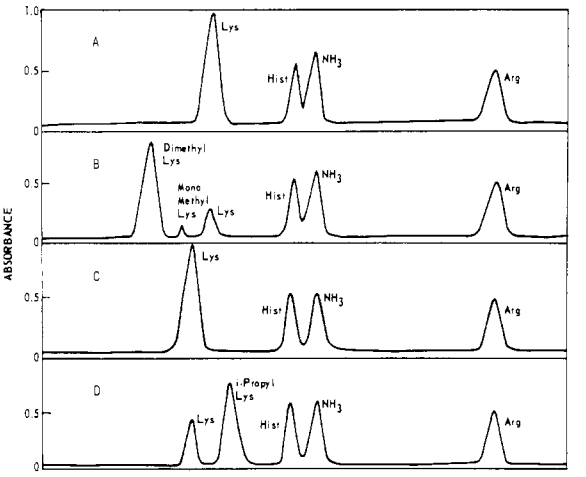


FIGURE 1: Chromatographic analysis of reductively alkylated ribonuclease after 22-hr acid hydrolysis (procedure described in the text). (A) Unmodified and (B) methylated RNase with eluent system containing 40 ml of isopropyl alcohol (see text); and (C) unmodified and (D) isopropylated RNase with eluent system containing 80 ml of isopropyl alcohol. For simplicity, all four chromatograms have been presented with their arginine peaks alined.

Reductive Alkylation of Butylamine. Reductive alkylations of butylamine were done as described in the respective figure legends. After completion of the reaction, 1 ml of 20% KOH and then 2 ml of diethyl ether were added to each sample. The mixtures were stoppered, shaken, and the ether layer was then removed and analyzed by gas–liquid partition chromatography. The gas–liquid partition chromatography analyses were done using a Varian Aerograph, Model 1520, gas–liquid partition chromatography apparatus with a 0.25 in. × 6 ft column of 150–200 mesh Porapak Q coated with 10% polyethylenimine (Wilkens Instruments, Walnut Creek, Calif.). The methylated butylamines were chromatographed at a column temperature of 135°, and the other alkylated butylamines at 165°. The chromatographic results were recorded on a 1-mV Leeds and Northrup type G recorder. Compositions of reaction mixtures were calculated assuming a direct relationship between peak areas and the weight per cent of the respective components.

Analyses of Proteins. Protein concentrations were determined both by dry weight and by optical density at absorption maxima. Bovine pancreatic ribonuclease A, type II-A, was obtained from Sigma Chemical Co. Its enzymatic activity was determined by the procedure of Kunitz (1946), using yeast ribonucleic acid (Sigma, type VI) as substrate. Turkey ovomucoid was prepared according to Feeney *et al.* (1967). Chicken lysozyme was prepared by the procedure of Alderton and Fevold (1946). Human serum transferrin (iron form) was prepared from human serum Cohn fraction IV-7 by a method similar to that of Charlwood (1963). Crystalline bovine zinc insulin was obtained from Sigma Chemical Co. Chicken ovalbumin was crystallized four times

[1] Reductively methylated proteins contain both ε-*N*-methyl- and ε-*N*,*N*-dimethyllysine residues with the latter usually predominating. Methylated proteins may therefore be referred to as either the methyl or dimethyl derivatives; we have named the methylated RNase derivatives dimethyl-RNase. Quantitation of isopropyllysine was done using the ninhydrin color factor determined for ε-*N*-methyllysine. The slight apparent increase in the total lysine may be due to this approximation.

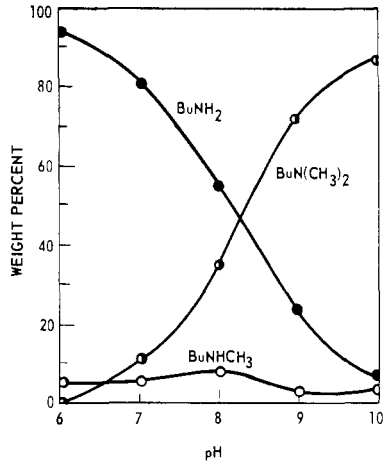


FIGURE 2: Reductive methylation of butylamine with formaldehyde. Effect of pH upon composition of reaction mixture. Reactions were carried out at 0° in 15-ml solutions of 2×10^{-2} M butylamine and 8×10^{-2} M formaldehyde at the indicated pH. Reactions were initiated by the addition of 15 mg of sodium borohydride. The pH was maintained by 0.20 M maleate at pH 6.0, by 0.15 M phosphate at pH 7.0, by 0.10 M triethanolamine at pH 8.0, by 0.20 M borate at pH 9.0, and by 0.25 M borate at pH 10.0. Analyses of the reaction mixtures were done by gas–liquid partition chromatography as described in the text.

from ammonium sulfate and then chromatographed on carboxymethylcellulose (Whatman). Three-times-crystallized, salt-free, lyophilized trypsin, chromatographed α-chymotrypsin, and salt-free, five-times-crystallized chymotrypsinogen A were obtained from Worthington Biochemical Corp. Reduced glutathione was obtained from Nutritional Biochemicals Corp.

Values for pH were determined with a Leeds and Northrup No. 7664 pH meter equipped with a L & N No. 124138 miniature glass electrode. Titration curves were obtained using a difunctional recording titrator (International Instrument Co., Canyon, Calif.) at 20° in 0.1 M KCl. Protein samples were adjusted before titration to a pH near maximum proton binding. No corrections were made for titration of solvent. Spectral titrations were done at 23° on samples in 0.1 M KCl. ϵ-*N*,*N*-Dimethyl-α-*N*-acetyllysine and *N*,*N*-dimethylalanine were prepared from α-*N*-acetyl-L-lysine (Cyclo Chemical Corp.) and L-alanine (Nutritional Biochemicals Corp.), respectively, by an adaptation of the procedure used to methylate butylamine. Both products were isolated as their hydrochloride salts.

Amino groups were determined using TNBS[2] according to the procedure of Habeeb (1966) after first diluting the reaction solution with an equal volume of 0.1 M HCl to destroy any borohydride present. Sulfhydryl groups were determined with DTNB (Ellman, 1959) after similar acidification to destroy borohydride. In all those cases where no sulfhydryl groups were detected, a minimum of 1.5 mg of protein was used in the analysis. Amino acid analyses were performed after hydrolysis of the protein in 6 M HCl *in vacuo* at 110° for 22 hr using a Technicon Autoanalyzer and a linear buffer gradient. This gradient was from 70 ml of 0.050 M citrate, 0.5% thiodiglycol, and 1% Brij 35[3] (pH 3.80) to 280 ml of a solution containing either 40 or 80 ml of isopropyl alcohol and made to volume with 0.050 M citrate, 1.0 M sodium chloride, 0.5% thiodiglycol, and 1% Brij 35 (pH 5.00). The higher concentration of isopropyl alcohol was used for chromatographing the methylated lysines and the lower concentration for the ethyl and isopropyl derivatives (Figure 1).

Determination of Borohydride. It was observed that the spectrum of protein solutions containing small amounts of sodium borohydride developed a strong absorption near 480 mμ when treated with TNBS. This same color formation was observed in the absence of protein but not in the absence of borohydride. When 0.5-ml solutions containing small amounts of sodium borohydride were added to a solution of 0.05% TNBS in 2 ml of 2% $NaHCO_3$ (pH 8.5), an orange color formed rapidly. When the reaction was stopped after 15 min by the addition of 0.5 ml of 1 M HCl, a strong absorption was observed with its maximum at 480 mμ. The intensity of this absorption was a linear function of the amount of borohydride from 0 to 0.10 mg and its amount could be calculated from the relationship $A_{480}/2.5 \times 10^6$ = moles of borohydride. The presence of low levels of amino groups did not affect these results.

N-Terminal amino acids were determined by reaction with DNFB (Levy and Li, 1955), followed by hydrolysis in 6 M HCl and separation of the DNP-amino acids by paper chromatography (Blackburn and Lowther, 1951). The DNP-amino acids were eluted from the paper with 0.5% $NaHCO_3$ and quantitated by measurement of the absorption at 360 mμ.

Absorption measurements and spectra were obtained with a Cary 15 recording spectrophotometer. Sedimentation coefficients were determined using a Beckman Model E analytical ultracentrifuge at 59,780 rpm and 20°. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter at 20° over the range 313–589 mμ in a 100-mm cell. λ_c for the one-term Drude equation was obtained from the slope of a plot of $[\alpha]_\lambda\lambda^2$ *vs.* $[\alpha]_\lambda$. The constants a_0 and b_0 were calculated from the Moffit equation, assuming λ_0 = 212 mμ. Assays for inhibitory activities of turkey ovomucoid against trypsin and α-chymotrypsin were done as recently described (Haynes *et al.*, 1967).

Results

The Effect of Reaction Conditions upon the Reductive Alkylation of Amines in Dilute Aqueous Solution. The effect of pH upon the final composition of reaction mixtures of butylamine and formaldehyde in the presence of excess sodium borohydride is shown in Figure 2. An important feature of this product profile is the low level of monoalkylamine throughout the pH range

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: TNBS, 2,4,6-trinitrobenzenesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DNFB, 2,4-dinitrofluorobenzene.

[3] Trademark of Atlas Powder Co., Wilmington, Del.

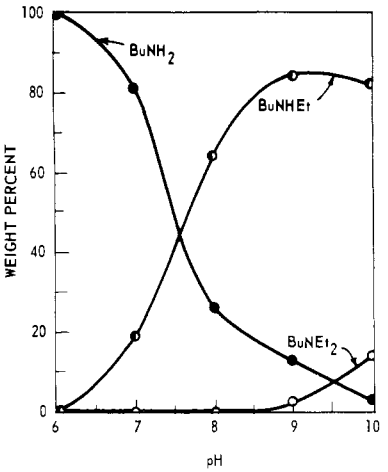
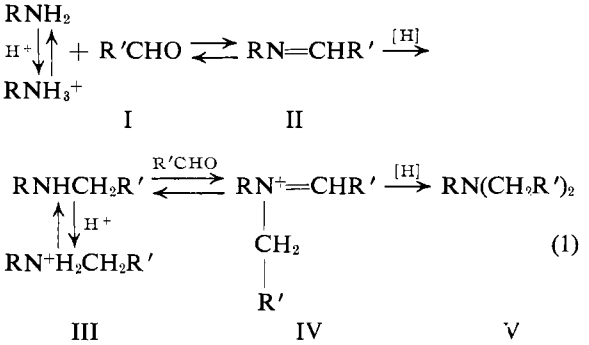


FIGURE 3: Reductive ethylation of butylamine with acetaldehyde. Effect of pH upon the composition of reaction mixtures. The reactions were performed at 0° using the same buffers described in Figure 2. Reaction solutions (12 ml) contained 2×10^{-2} M butylamine and 0.11 M acetaldehyde; reactions were initiated by addition of 15 mg of sodium borohydride. Analyses of the reaction mixtures were done by gas–liquid partition chromatography as described in the text.

studied. This is in contrast to the results obtained when butylamine was treated with either acetaldehyde (Figure 3) or acetone. With acetone, only the monoalkylated product was observed; with acetaldehyde, the diethylamine was formed only in small amounts and at high pH.

Yields of alkylated products from all carbonyl compounds studied had similar, very strong pH dependencies. These profiles are probably a reflection of the stability of sodium borohydride and of the pH dependence for formation of the respective Schiff bases (II and IV of eq 1). The reaction proceeds slowly or not at all below



(1)

pH 7, and optimum conditions are near pH 10 or above. The instability of most proteins at such a high pH, however, led to the choice of pH 9.0 for subsequent investigations.

Increasing the concentration of formaldehyde above its stoichiometric requirement (CH_2O:butylamine > 2) leads to an increased competition for the reducing agent. This results in a concomitant lowering in the yield of alkylated products as the reducing agent becomes limiting, *i.e.*, $CH_2O:NaBH_4 > 4$ (Figure 4). This competition appears to be made particularly effective by a greater displacement of the amine aldehyde–Schiff's base equilibrium in favor of the unreactive dihydroxymethyl compound VII (eq 2). At constant formaldehyde concentration, increasing the amount of borohydride above its stoichiometric requirement ($NaBH_4:CH_2O > 0.25$) has very little effect upon the yield of reaction products.

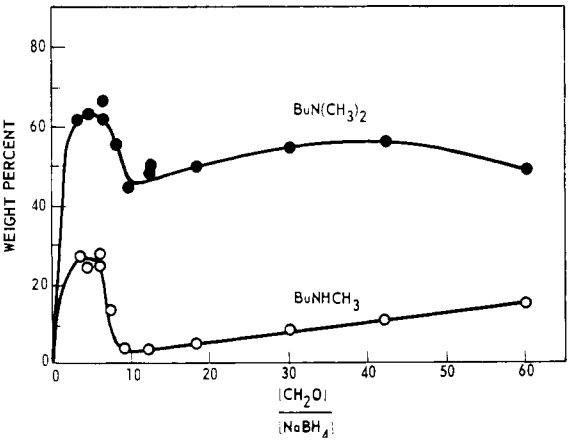
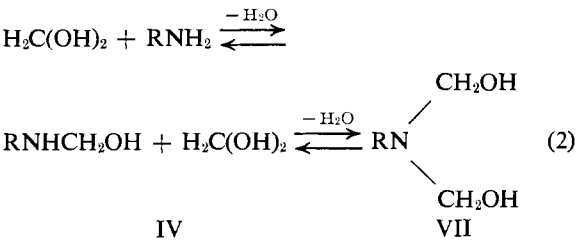


FIGURE 4: Product composition for the reductive alkylation of butylamine with formaldehyde and sodium borohydride in dilute aqueous solution. The reactions were carried out in 0.2 M borate buffer at pH 9.0 and 0°, butylamine concentrations were 2×10^{-2} M and sodium borohydride 4×10^{-2} M; formaldehyde was varied between 0.12 and 2.4 M. Analysis of the reaction mixtures by gas–liquid partition chromatography is described in the text.



(2)

Quantitation of the Reaction. Reductive alkylation of amino groups in proteins occurs with little change in directly observable properties, and affords no simple direct measure of the extent of reaction. The decrease in primary amino groups provides the simplest means for following the course of the reaction. Habeeb (1966) has shown TNBS to be an extremely useful reagent for determining amino groups in proteins. Okuyama and Satake (1960) have reported that TNBS does not react with the secondary amino group of proline, but they did not report results with other secondary amines. To investigate the possible reaction of TNBS with simple secondary amino groups, *N*-methylbutylamine was treated with TNBS using the procedure of Habeeb (1966). No reaction occurred after 2 hr at 40° as determined by comparison of the sample spectrum with that of a control. This was taken as evidence for a lack of reactivity with the secondary amino group of ε-*N*-methyllysine, which was substantiated by comparison of the TNBS analyses and amino acid analyses of several alkylated proteins.

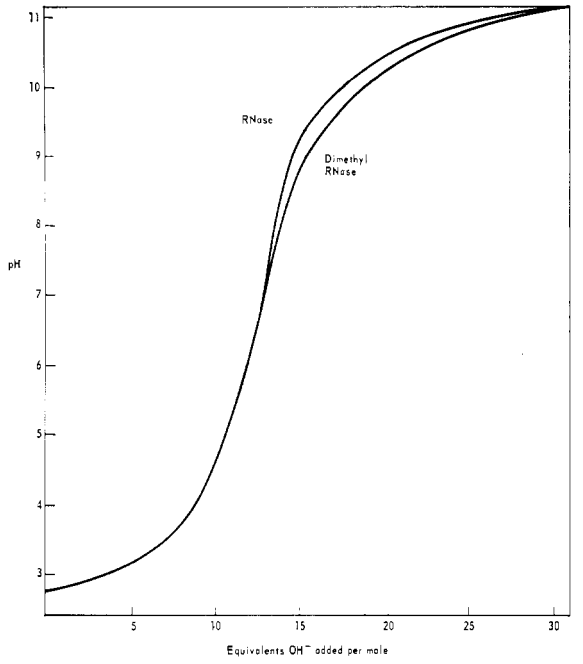


FIGURE 5: Titration of RNase and dimethyl-RNase with 0.10 M KOH done in 0.10 M KCl at 20°. No correction has been made for titration of the solvent.

Both ε-*N*-methyl- and ε-*N*,*N*-dimethyllysine were stable in 6 M HCl at 110° for 22 hr, permitting their quantitation by amino acid analysis. Because of the variety of alkylated lysines, overlapping of hydrolysate components precluded use of the normal gradient system (Technicon Autoanalyzer 21-hr run) and a modified buffer system was used (see Methods). This system, taking only 10 hr for each run, resolved into a discrete peak each of the alkaline amino acids encountered (see Figure 1). Relative ninhydrin color yields for lysine and its mono- and dialkylated derivatives were obtained using weighed samples of mono- and dimethyllysine (Table I).

Determination of the pK Values of Reductively Alkylated Amino Acids. α-*N*-Acetyl-ε-*N*,*N*-dimethyllysine, α-*N*,*N*-dimethylalanine, and the corresponding unalkylated compounds were titrated with 0.1 M KOH in 0.1 M KCl at 20° under a stream of water-saturated, CO_2-free nitrogen. The change in pH was recorded as a function of added alkali, and the p*K* values were estimated from the midpoints of the titration curves. Results are shown in Table II. As indicated, the dimethylamines are slightly weaker bases than their unmethylated parent compounds with the difference amounting to a decrease in p*K* of 0.4–0.6 pH unit. This change in p*K* is seen as a slight shift in the alkaline portion of the titration curve of methylated ribonuclease relative to that of the native protein (Figure 5).

TABLE I: Relative Molar Ninhydrin Color Yields of Methylated Lysine Derivatives.[a]

Compound	Ninhydrin Color
Lysine	1.00
ε-*N*-Methyllysine	0.881
ε-*N*,*N*-Dimethyllysine	0.822

[a] Values are relative to lysine as determined using the ninhydrin analysis system of Moore and Stein (1948).

TABLE II: Values for p*K* of Methylamines.

Compound	p*K*
α-*N*-Acetylysine	10.8
α-*N*-Acetyl-ε-*N*,*N*-dimethyllysine	10.4
Alanine	9.8
α-*N*,*N*-Dimethylalanine	9.2

Effect of Borohydride and Formaldehyde upon the Enzymatic Activity of Ribonuclease A. The reduction of ribonuclease by sodium borohydride has been studied by Moore *et al.* (1958). While they showed the enzyme to be relatively resistant to such borohydride reduction, as measured by the liberation of sulfhydryl groups, it was necessary to test the stability of the enzymic activity under the conditions used for reductive alkylation. Such treatment resulted in no measurable change in the activity of ribonuclease A during the time interval observed (Figure 6).

To investigate the reported inactivation of ribonuclease by formaldehyde (Zittle, 1948), solutions of the enzyme similar to those used in the alkylation procedure were treated with small amounts of formalin. There was an immediate rapid loss of enzymatic activity, followed by a slower second-stage loss (Figure 6). This initial inactivation was proportional to the amount of formaldehyde added, and was reversed upon dilution and short incubation. The half-life of this inactivation was similar to that for the formation of corresponding hydroxymethyl derivatives from formaldehyde and either butylamine or bovine serum albumin (G. E. Means and R. E. Feeney, unpublished data). This reaction was accompanied by a small decrease in absorbance between 245 and 260 mμ, but there was no change at either 277 or 230 mμ, characteristic wavelengths, respectively, for reaction with tyrosine and histidine (Martin and Marini, 1967). This initial inactivation apparently resulted from the reaction of formaldehyde with the catalytically vital amino group of lysine number 41 (Klee and Richards, 1957; Hirs, 1962). The second stage of inactivation was not reversed by dilution and appears to be related to those reactions of formaldehyde investigated by Fraenkel-Conrat and Olcott (1948).

The Reductive Methylation of Ribonuclease. A solution of bovine ribonuclease A (5 mg/ml) at 0° in 0.2 M borate buffer (pH 9.0) was adjusted to a concentration of 0.5 mg/ml of sodium borohydride and 0.5-μl/ml increments of formalin (12.1 M formaldehyde) were

TABLE III: Properties of Reductively Alkylated Ribonuclease.

	Lysine	Monoalkyl-lysine	Dialkyl-lysine	b_0[a]	a_0[a]	λ_c[b]	s_{20}[c] (S)
Dimethyl	0.7	0.3	8.4	−150	−350	242	1.8
Isopropyl	2.5	7.8	0	−209	−190	274	1.9
Native	10	0	0	−133	−465	236	1.8

[a] Calculated from the equation of Moffit and Yang (1956). [b] Calculated from the simple Drude equation (Yang and Doty, 1957); determined in 0.02 M acetate (pH 5.0). [c] Determined in 0.02 M acetate (pH 5.0).

added at 5-min intervals. There was an immediate loss of enzymatic activity, which continued with increasing additions of formaldehyde and was paralleled by the loss of TNBS-reactive amino groups (Figure 7). As shown, the rate of enzymatic inactivation exceeded the over-all rate of amino group modification and in this respect is similar to the results with dinitrophenylation (Hirs, 1962). The product, after the addition of 2.5 μl of formalin/ml of reaction solution, had less than 1% of its initial enzymatic activity and only 12%, or 1.3 of its 11 amino groups, as measured by TNBS. Quantitative amino acid analysis of the hydrolyzed protein detected only 0.71 mole of unreacted lysine/mole of protein. The remainder having been converted into ε-*N*-methyllysine (0.29 mole) and ε-*N*,*N*-dimethyllysine (8.4 moles). Recovery of 9.4 of the 10 original lysine residues indicated modification of the N-terminal α-amino group, a lysine, to be incomplete.[4] Amino acid analysis failed to detect unusual products other than those already mentioned and no loss of any amino acid other than lysine was found as compared with the native enzyme. No sulfhydryl groups were produced during the reaction as determined by the DTNB procedure of Ellman (1959). Amino acid analysis of a sample taken after addition of only 1 μl of formaldehyde/ml of the reaction solution showed much of the lysine to be present as monomethyllysine. At this stage the protein contained 6.2 lysine, 2.2 monomethyllysine, and 1.6 dimethyllysine residues.

Properties of the extensively modified enzyme are given in Table III. It was homogeneous on starch gel electrophoresis at pH 8.7. It was also homogeneous in the ultracentrifuge having the sedimentation coefficient of the native enzyme within experimental error. The methylated protein was slightly less levorotatory than the native enzyme but the values of b_0 calculated from the Moffit equation were similar.

Treatment of Ribonuclease with Borohydride Plus Acetone. When ribonuclease dissolved in 0.18 M borate buffer (pH 9.0) containing 10% (v/v) acetone was treated with small amounts of sodium borohydride at 0°, there was a decrease in enzymatic activity similar to that found during reductive methylation (Figure 8). Determination of amino groups with TNBS indicated a decrease in their number, which was confirmed by amino acid analysis. The most extensively modified product lost 73% of its initial enzymatic activity and 60% of its 11 amino groups as measured by TNBS. Amino acid analysis showed the presence of 2.5 of its original 10 lysine residues, and 7.8 moles of ε-*N*-isopropyllysine.[1] In contrast to the results of reductive methylation, no dialkylated lysine was found and the N-terminal α-amino group appeared to be completely unmodified. The extensively isopropylated enzyme was homogeneous at pH 8.7 on starch gel electrophoresis but had an apparent isoelectric point higher than either the native or dimethylated proteins. It was homogeneous in the ultracentrifuge with a sedimentation coefficient similar to that of the unmodified enzyme. The isopropyl enzyme was slightly less levorotatory than either the unmodified or methylated enzyme and b_0 was slightly more negative (Table III).

Specificity of the Reaction. Bowman and Stroud

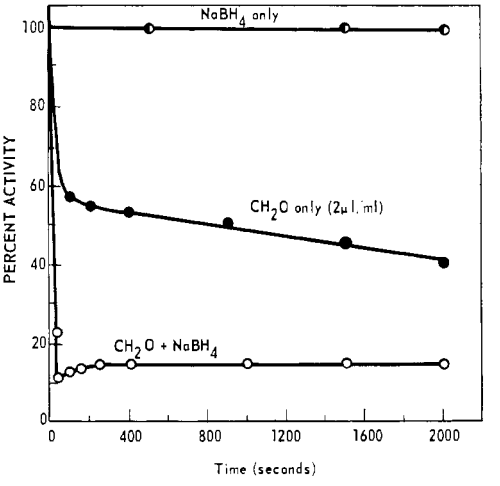


FIGURE 6: The effect of sodium borohydride and formaldehyde on ribonuclease. Solutions of bovine ribonuclease A (2.5 mg/ml) in 0.2 M borate buffer (pH 9.0) at 0° were treated with 0.5 mg/ml of sodium borohydride (◐), 2.0 μl/ml of formalin (●), and with 0.5 mg of sodium borohydride plus 2 μl/ml of formalin (○). The enzymatic activities were followed using the procedure of Kunitz (1946).

[4] Alkylation of the α-amino group of an N-terminal amino acid would render it unreactive with ninhydrin and therefore it would not be detected by amino acid analysis. The decrease of only 0.6 mole in the total lysine therefore indicates only partial modification of this N-terminal α-amino group.

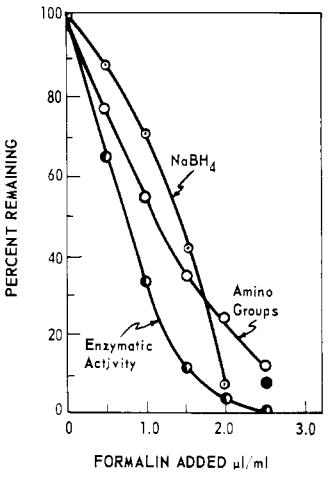


FIGURE 7: Reductive methylation of ribonuclease (procedure described in the text). Aliquots of the reaction mixture were removed after each addition of formaldehyde and analyzed for amino groups with TNBS (○); the sum of mono- and dimethyllysine by amino acid analysis (●); sodium borohydride using TNBS (⊙) and enzymatic activity (◐) according to Kunitz (1946).

(1950) and Ingram (1953) studied the reaction of formaldehyde with amino acids during catalytic hydrogenation and observed the formation of α-dimethyl derivatives from most of the common amino acids. Only a monomethylated derivative was formed from the secondary amino group of proline. Our results indicated, however, that the N-terminal residue of ribonuclease, an α-amino group of a lysine residue, was relatively resistant to alkylation. Insulin, with one ε-amino group and two N-terminal α-amino groups, was chosen for closer study. Evans and Saroff (1957) have shown the N-terminal glycine residue of zinc insulin to be relatively more susceptible to guanidination than the N-terminal phenylalanine residue. Hunter and Ludwig (1962) observed the same difference in reactivity upon amidination. When zinc insulin (5 mg/ml) in the presence of sodium borohydride (0.75 mg/ml) was treated with increments of formalin, its amino group content measured by TNBS decreased slowly, leveling off near 50% of the original value. Quantitative determination of N terminals with DNFB showed 77% of its ε-amino group had been methylated, while its two α-amino groups, those of glycine and phenylalanine, were only 63 and 20% alkylated, respectively. When zinc insulin was alkylated with acetone or acetaldehyde, similar results were obtained but with a slight decrease in alkylated ε-amino groups relative to α-amino groups (Table IV). The difference in reactivity between ε-amino groups and α-amino groups is therefore not great. The rather low extent of modification of the ε-amino groups of some proteins as compared with that observed with others under similar conditions (see Tables IV and V) suggests that the variation in reactivity of ε-amino groups may be almost as great as the differences between particular ε- and α-amino groups.

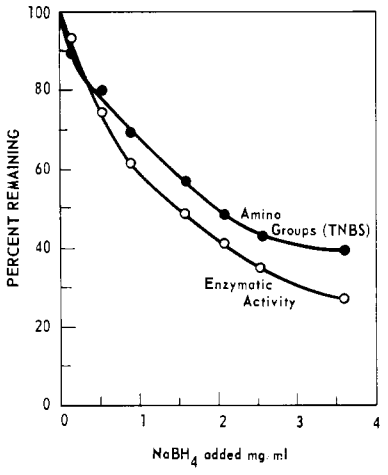


FIGURE 8: Reduction of ribonuclease in the presence of acetone (procedure described in the text). Analysis for amino groups was done with TNBS after first acidifying with an equal volume of 0.1 M HCl to remove the remaining borohydride. Enzymatic activity was determined by the procedure of Kunitz (1946).

TABLE IV: Reductive Alkylation Specificity in Insulin.[a]

Treatment	% Modified		
	ε-*N*-Lysine[b]	N-Terminal Glycine[c]	N-Terminal Phenylalanine[c]
Methylation	77	63	20
Ethylation	67	74	47
Isopropylation	56	49	22

[a] Bovine zinc insulin (Sigma). [b] Determined by amino acid analysis. [c] Determined by DNP quantitative end-group analysis.

When reduced glutathione (0.1 mg/ml) was treated with borohydride plus formaldehyde under the same conditions used for the proteins studied, more than 60% of its TNBS reactive amino groups disappeared without any detectable loss of sulfhydryl groups as determined using DTNB (Ellman, 1959). A similar result was obtained when the sulfhydryl-containing protein, chicken ovalbumin, was so treated.

The extents of modification obtained upon reductive alkylation of several proteins are shown in Table V. A portion of each protein was treated with DTNB by a procedure similar to that of Ellman (1959) immediately after its modification to determine the presence of any free sulfhydryl groups. No sulfhydryl groups were detected in any of these proteins. With turkey ovomucoid, the extent of methylation by formaldehyde was approximately similar to the extent of modification reported for high acetylation with acetic anhydride (Stevens and Feeney, 1963). The enzyme inhibitory activities of the alkylated turkey ovomucoid were determined because modification of its amino groups has been found to cause loss of inhibitory activity against trypsin

TABLE V: Reductive Alkylation of Selected Proteins.

Protein	Distribution of Products: Dialkyllysine (%)	Monoalkyllysine (%)	Lysine (%)
Chymotrypsin			
Methyl	98	1	<1
Chymotrypsinogen			
Methyl	98	1	<1
Human serum transferrin (iron form)			
Methyl	78	6	16
Isopropyl	0	29	71
Lysozyme			
Methyl	98	<1	<1
Cyclopentyl	0	32	68
Turkey ovomucoid			
Methyl	60	14	26
Isopropyl	0	22	78

but not against α-chymotrypsin (Stevens and Feeney, 1963; Haynes *et al.*, 1967). The reductively alkylated turkey ovomucoid was found to have <10% activity against trypsin and full activity against α-chymotrypsin.

Spectrophotometric titrations of methylated ribonuclease were done to determine the extent of its departure from the "native state," as measured by changes in the spectrophotometric behavior of its six tyrosyl residues at alkaline pH. Both the native and methylated protein gave a clear isosbestic point during initial stages of the titration. This isosbestic point at 278.5 mμ for native enzyme was maintained up to pH 12.2. Titration below this point resulted from the reversible ionization of three of the protein's six tyrosyl residues (Tanford and Hauenstein, 1956). Results with the methylated enzyme were qualitatively similar, but deviated from the clear isosbestic point (278 mμ) above pH 11.8 (Figure 9). The difference between the native and dimethyl protein measured from the displacement of the spectrophotometric titration curves above pH 12, at 295 mμ, was 0.4–0.5 pH unit. This displacement is matched by a similar displacement of the simple acid–base titration curves (Figure 5) and these two, taken together, suggest that the upper limit above which the "buried" tyrosyls are titrated occurs at a point of common net charge. This observed difference in behavior of the modified protein correlates with the determined difference of 0.4 pH unit between the p*K* values of α-*N*-acetyllysine and its ε-*N*,*N*-dimethyl derivative (Table II).

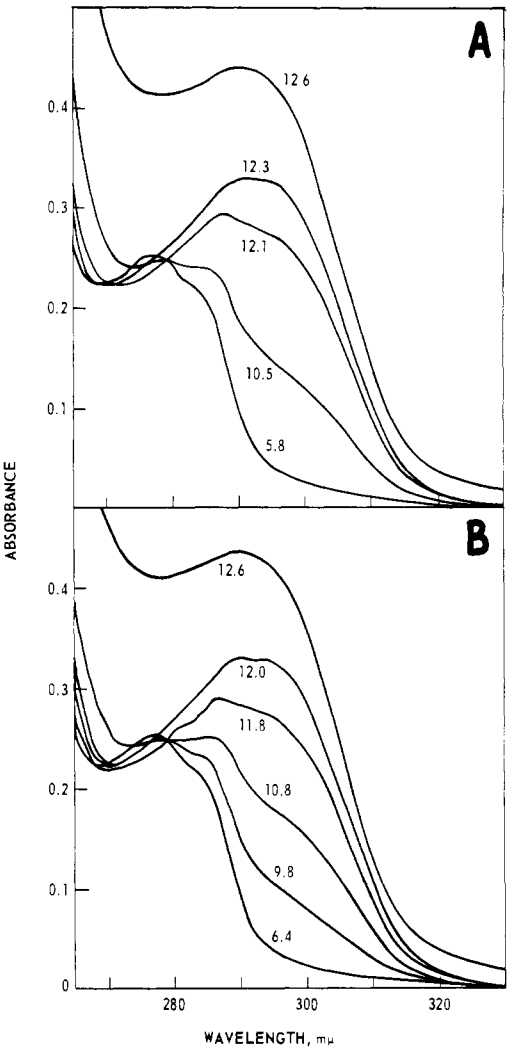


FIGURE 9: Spectrophotometric titrations of RNase and reductively methylated RNase showing the loss of the 278-mμ isosbestic points above (A) pH 12.2 for the native and (B) pH 11.8 for the methylated enzyme.

Discussion

The importance of amino groups in stabilizing the native structure of proteins by virtue of their positive charges and as functional parts of catalytic and allosteric sites has made them a prime objective of chemical modification. A large number of reagents with varied characteristics have been used in such studies. Introduction of differently charged substituents in place of positively charged amino groups has been used to study the effect of such charges upon physical and biological properties and to differentiate between simple charge effects and the absolute essentiality of amino groups. Guanidination or amidination of amino groups does not change their net charge and both methods have been widely used to determine the essential nature of lysyl residues in the maintenance of biological activity. While both methods maintain the gross charge of the native protein, the loci of the individual positive charges are displaced by almost 1.5 Å. The potential effects of many such small displacements is difficult to ascertain and may account, in part, for the decreased solubility of many proteins which have been so modified (Hughes *et al.*, 1949; Chervenka and Wilcox, 1956). The failure of trypsin to cleave at such residues is also explicable

in such terms (Shields *et al.*, 1959; Hunter and Ludwig, 1962). Reductive alkylation not only maintains the total charge of the protein, but also maintains the spacial distribution of charges. The size of added substituents is easily controlled during reductive alkylation by the choice of carbonyl compound, with a lower limit of two carbon atoms either as two methyl or as a single ethyl substituent.

Conditions for reductive alkylation are extremely mild and the reaction is easily done without special reagents or equipment. The brief exposure to mildly reducing conditions during alkylation is such as to not greatly affect the stability of disulfide bonds which require more extensive exposure for reductive cleavage (Moore *et al.*, 1958). Exposure to low concentrations of carbonyl compounds is, in general, not harmful to proteins. However, to circumvent the potentially deleterious effects of formaldehyde, the most damaging carbonyl compound employed, its concentration should be kept as low as possible. For this reason, reductive methylation is best done under conditions wherein borohydride is in excess of the formaldehyde. Such precautions are not necessary with most other carbonyl compounds. Reductive alkylation occurs most rapidly near pH 10 (Figure 2), but, depending upon the alkali stability of the protein being modified, a lower pH may be employed. At pH 9.0 the reaction proceeds rapidly and in general leads to extensive alkylation.

The mechanism for reductive alkylation presumably involves the formation of an intermediate Schiff base, the reduction of which yields the corresponding alkyllysine. The high yields of alkylated products which have been obtained suggest the Schiff base intermediate to be reduced at a rate many times faster than the carbonyl compound itself. While the extent of formation of Schiff base from simple aliphatic carbonyl and amino compounds in aqueous solution is small, their rate of formation and decomposition may be very high (Cohn and Urey, 1938). That formaldehyde gives predominantly dialkylated product is a reflection of its great reactivity and of the greater reactivity of the intermediate methylamine as compared with other secondary alkylamines (Kallen and Jencks, 1966).

Reductive alkylation appears highly specific for amino groups. Careful examination of acid hydrolysates by amino acid analysis has failed to demonstrate the loss of any amino acids except those which are N-terminal and lysine. No derivatives other than those from these amino acids have been observed.

The inactivation of ribonuclease by reductive alkylation is similar to that produced by guanidination (Klee and Richards, 1957) and dinitrophenylation (Hirs, 1962) but is surprising in light of the work of Marfey *et al.* (1965). The rapid loss of enzymatic activity upon reductive methylation is evidence that addition of even a single methyl group to the catalytically vital lysine number 41 (Hirs, 1962) so perturbs the catalytic site as to inactivate the enzyme. Marfey *et al.* (1965) have shown, however, that cross-linking lysine number 41 to lysine number 7 with a bulky dinitrophenyl group results in the loss of only 85% of the catalytic activity against cytidine 2′,3′-cyclic monophosphate. This difference in activity between the cross-linked and methylated enzyme may partly be due to the use of different substrates, a possibility which is presently under study.

At low levels of reductive alkylation the amount of ε-*N*-methyllysine exceeds the amount of ε-*N*,*N*-dimethyllysine. At intermediate levels, the ratios of mono- to dimethyllysine are similar to the natural distribution of these residues in certain histone fractions (Paik and Kim, 1967). Reductive alkylation should greatly facilitate the laboratory synthesis of similar materials containing known sequences, radioactive labels or unnatural alkyl groups.

Gorecki and Shalitin (1967) have reported that trypsin does not cleave ε-*N*,*N*-dimethyllysine ester or amide bonds. Trypsin digestion of reductively methylated peptides or proteins should result, therefore, in cleavage at arginine residues only. The positively charged lysine residues would be retained but would not be susceptible to trypsin cleavage. Trypsin hydrolysates of the methylated and unmethylated materials would therefore differ mainly in the number of peptide bonds which had been split and comparisons of peptide maps of such alkylated and unalkylated materials should facilitate the identification and ordering of the peptide sequences present.

The extreme ease with which amino groups are alkylated by such procedures is generally unrecognized. The common usage of acetone to decompose excess borohydride in protein solutions can be expected to lead to the incorporation of large numbers of isopropyl residues, adding unnecessary and apparently often unrecognized difficulties to subsequent usages of these proteins.

The single most important feature of reductive alkylation is its unique ability under mild conditions to extensively, yet specifically, modify protein amino groups, while retaining the same three-dimensional distribution of charged groups of the native protein. The availability of materials and the convenience in using this method should aid greatly in determining the role and mechanism of action of amino groups in proteins.

Acknowledgment

The supply of proteins by David T. Osuga and Stanley K. Komatsu and the technical assistance and advice of Mrs. Caroline Ouchida, Mrs. Judy Miller, Frank C. Greene, and Woo Hoe Liu, all of this laboratory, are gratefully acknowledged.

References

Alderton, G., and Fevold, H. L. (1946), *J. Biol. Chem. 164.*

Blackburn, S., and Lowther, A. G. (1951), *Biochem. J. 48*, 126.

Bowman, R. E., and Stroud, H. H. (1950), *J. Chem. Soc., 1342.*

Charlwood, P. A. (1963), *Biochem. J. 88*, 394.

Chervenka, C. H., and Wilcox, P. E. (1956), *J. Biol. Chem. 222*, 621.

Cohn, M., and Urey, H. C. (1938), *J. Am. Chem. Soc. 60*, 679.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Evans, R. L., and Saroff, H. A. (1957), *J. Biol. Chem. 228*, 295.
Feeney, R. E., Osuga, D. T., and Maeda, H. (1967), *Arch. Biochem. Biophys. 119*, 124.
Fischer, E. H., Kent, A. B., Snyder, E. R., and Krebs, E. G. (1958), *J. Am. Chem. Soc. 80*, 2906.
Fraenkel-Conrat, H. (1957), *Methods Enzymol. 4*, 247.
Fraenkel-Conrat, H., and Olcott, H. S. (1948), *J. Biol. Chem. 174*, 827.
Gorecki, M., and Shalitin, Y. (1967), *Biochem. Biophys. Res. Commun. 29*, 189.
Habeeb, A. F. S. A. (1966), *Anal. Biochem. 14*, 328.
Haynes, R., Osuga, D. T., and Feeney, R. E. (1967), *Biochemistry 6*, 541.
Hirs, C. H. W. (1962), *Brookhaven Symp. Biol. 15*, 154.
Hughes, W. L., Saroff, H. A., and Carney, A. L. (1949), *J. Am. Chem. Soc. 71*, 2476.
Hunter, M. J., and Ludwig, M. L. (1962), *J. Am. Chem. Soc. 84*, 3491.
Ingram, V. M. (1953), *J. Biol. Chem. 202*, 193.
Kallen, R. G., and Jencks, W. P. (1966), *J. Biol. Chem.* 241, 5864.
Klee, W. A., and Richards, F. M. (1957), *J. Biol. Chem. 229*, 489.
Kunitz, M. (1946), *J. Biol. Chem. 164*, 563.
Levy, A. L., and Li, C. H. (1955), *J. Biol. Chem. 213*, 487.
Marfey, P. S., Nowak, H., Uziel, M., and Yphantis, D. A. (1965), *J. Biol. Chem. 240*, 3264.
Martin, C. J., and Marini, M. A. (1967), *J. Biol. Chem. 242*, 5736.
Moffit, W., and Yang, J. T. (1956), *Proc. Natl. Acad. Sci. U. S. 42*, 596.
Moore, S., Cole, R. D., Gundlach, H. G., and Stein, W. H. (1958), *4th Intern. Congr. Biochem.*, 52.
Moore, S., and Stein, W. H. (1948), *J. Biol. Chem. 176*, 367.
Okuyama, T., and Satake, K. (1960), *J. Biochem. (Tokyo) 47*, 454.
Paik, W. K., and Kim, S. (1967), *Biochem. Biophys. Res. Commun. 27*, 479.
Schütte, E. (1943), *Z. Physiol. Chem. 279*, 59.
Shields, G. S., Hill, R. L., and Smith, E. L. (1959), *J. Biol. Chem. 234*, 1747.
Stevens, F. C., and Feeney, R. E. (1963), *Biochemistry 2*, 1346.
Tanford, C., and Hauenstein, J. D. (1956), *J. Am. Chem. Soc. 78*, 5287.
Yang, J. T., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 761.
Zittle, C. A. (1948), *J. Franklin Inst. 246*, 266.

The Determination of Association Constants for a Two-Solute System. I. Colligative Methods*

Robert F. Steiner

ABSTRACT: A general method is outlined for obtaining association constants for a system of two different associating species, which can associate with themselves or with each other in any proportion. The theory applies to colligative methods, which yield number-average molecular weights.

The problem of determining the consecutive equilibrium constants for an associating system from molecular weight data is a recurrent one in biochemistry. While proteins furnish many examples of associating systems (Reithel, 1963), there are also numerous cases of small molecules or ions which associate in solution (Ts'o *et al.*, 1963).

Several experimental approaches are available for obtaining molecular weights for systems of this kind. Of these, osmotic pressure and the other colligative methods yield number-average molecular weights, while light-scattering and sedimentation equilibrium yield weight-average quantities. The colligative methods other than osmotic pressure, such as freezing point depression and vapor pressure lowering, are primarily applicable to small molecules of molecular weight less than 10^3, while the other techniques apply to biopolymers.

Several procedures are available for computing the association constants of a self-associating system, in which a single component forms dimers, trimers, etc. For an association process of the type

$$A + A \rightleftarrows A_2;\ A_2 + A \rightleftarrows A_3;\ \ldots\ A_{i-1} + A \rightleftarrows A_i \quad (1)$$

* From the Laboratory of Physical Biochemistry, Naval Medical Research Institute, Bethesda, Maryland. Research Task MR005.06-0005. *Received February 9, 1968.* The opinions in this paper are those of the author and do not necessarily reflect the views of the Navy Department or the naval service at large.