

Formaldehyde
Protein

French, D. 1945

The Reactions of Formaldehyde with Amino Acids and Proteins

By DEXTER FRENCH AND JOHN T. EDSALL

Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts

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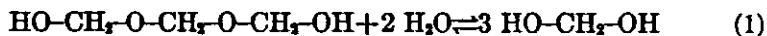
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tion of higher polymers. For accurate studies, involving determination of equilibrium and velocity constants, solutions of purified formaldehyde are, of course, decidedly preferable to those containing methanol.

Freezing-point measurements of aqueous solutions of formaldehyde (Auerbach and Barschall, 1905) indicate that at concentrations up to one molar or more, the formaldehyde is present chiefly in monomeric form. However, the ultra-violet spectra of such solutions (Bielecki and Henri, 1912) show no evidence of the characteristic absorption band near 2800 Å, which is typical of compounds containing the carbonyl group. Likewise, the Raman spectra of aqueous solutions of formaldehyde (Hibben, 1931; Krishnamurti, 1931; Kohlrausch and Köppl, 1934) show no evidence of the typical carbonyl vibration frequency near 1700 cm.⁻¹. These facts, combined with the low volatility of formaldehyde in aqueous solution, indicate that it exists chiefly as the hydrate, methylene glycol CH₂(OH)₂.² At higher concentrations, the molal freezing point depression of formaldehyde in water is considerably less than that calculated for methylene glycol, suggesting the presence of formaldehyde polymers in equilibrium with the monomeric form. Auerbach and Barschall (1905) calculated an equilibrium constant for the monomer and an assumed open chain-trimeric form, assuming the reaction to be



They found that a value of $K=0.0264$ gave a good fit to the observed data. On this basis, the concentration of monomeric formaldehyde approaches a maximum of about 11 per cent when the total concentration of formaldehyde is 20 per cent. The monomer concentration would not increase above this value with increase of total formaldehyde up to 40 per cent. It seems certain, however, that Equation 1 must be an inadequate representation of the equilibrium actually existing in formaldehyde solutions. There is every reason to expect the presence of the dimer and (in smaller concentrations) of a series of polymers larger than the trimer.

On diluting a concentrated solution of formaldehyde, a measurable amount of time is required for the depolymerization to take place. Wadano, Trogus, and Hess (1934) have studied the kinetics of the depolymerization reaction. They found the rate to vary markedly with pH, reaching a

² Confirmatory evidence may be obtained from the apparent molal volume of formaldehyde in such solutions. From the density data of Auerbach and Barschall (1905), this volume is calculated as approximately 21 cc. per mol. Employing the atomic volumes deduced by Traube (1899) (see also Cohn and Edsall, 1943, p. 157) and a co-volume of 13 cc, the value for H₂C=O should be near 34.5 cc. The calculated value for CH₂(OH)₂ is 38.0 cc. However, one mol of water, with a volume of 18 cc. is consumed in the formation of a mol of methylene glycol; hence, the expected value for the latter compound is 20 cc./mol, in very good agreement with the apparent molal volume actually observed.

minimum in the pH range 2-4 and increasing rapidly as the pH deviates from this minimum in either direction. At pH values of 7 and above, the equilibrium state is reached very quickly. There is no convincing evidence that the final equilibrium state is dependent on pH, and no such dependence would be expected if the stable forms of the monomer and polymers are uncharged molecules; see the discussion by Levy and Silberman (1937).

Formaldehyde in aqueous solution is a very weak acid, the pK value according to Wadano (1934) being 12.79, and according to Levy (1934) being 12.87. Even this very weak acidity, however, leads to the neutralization of significant amounts of alkali at pH values of 9 or even below, in moderately concentrated formaldehyde solutions—a fact which has important implications, as Levy has pointed out, for the accuracy of the formol titration.

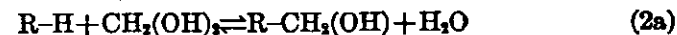
3. REACTIONS OF FORMALDEHYDE WITH FUNCTIONAL GROUPS FOUND IN AMINO ACIDS AND PEPTIDES

a. Addition and Condensation Reactions

The most frequently encountered reaction of formaldehyde is its addition to a compound containing an active hydrogen atom with the formation of a hydroxymethyl compound:



If the formaldehyde is present as methylene glycol, this may be formulated as a condensation reaction,



The hydroxyl group thus formed is usually reactive and may condense with another atomic grouping containing an active hydrogen atom to form a methylene bridge:



This condensation may take place intramolecularly with the formation of cyclic structures, intermolecularly with the formation of molecular aggregates, or with more formaldehyde molecules to form polyoxymethylene chains or bridges. In general, one may expect the formation of cyclic structures in polyfunctional amino acids if steric conditions are favorable, and there is now abundant evidence, presented later in this discussion, that such structures are actually formed.

Numerous groups found in amino acids, peptides, and proteins are capable of undergoing addition and condensation reactions with formaldehyde.

(1) *The Amino Group.* The reactions of this group with formaldehyde have certainly received more intensive study than those of any other group.

b. Reduction, Alkylation, and Other Reactions

Aldehydes in general, formaldehyde in particular, are good reducing agents and under appropriate conditions alkylating agents. Clarke, Gillespie, and Weisshaus (1933) have shown that amino acids may be methylated by boiling with formaldehyde in the presence of very concentrated formic acid. The process eventually leads to considerable deamination with the simultaneous formation of methylamines. *N*-Dimethyl glycine may be prepared in this way, but many other amino acids are rapidly deaminated.

It is possible that formaldehyde may reduce disulfide linkages and then react with the newly formed sulfhydryl groups. Convincing evidence on this point is lacking (see the discussion of keratin in Part VI).

III. METHODS APPLICABLE TO THE STUDY OF THE REACTIONS OF FORMALDEHYDE WITH AMINO ACIDS AND PROTEINS³

We shall, at this point, give only a brief summary of the methods employed for studying the reactions of formaldehyde with amino acids and proteins, since the methods can in general be more conveniently presented in connection with the discussion of the various reactions that have been studied. The isolation, crystallization, and analysis of well defined compounds has been repeatedly attempted. Particularly in the case of the polyfunctional amino acids, which can react with formaldehyde to give ring structures containing a methylene bridge, important evidence has been obtained by the actual isolation of the resulting compounds (see Part V). However, the reactions of formaldehyde with the amino group are so rapid and so readily reversible that little evidence of the nature of the equilibria existing in solution has been obtained by isolation procedures. In the study of compounds which release formaldehyde on hydrolysis, the formaldehyde can be distilled off and the distillate analyzed for formaldehyde. The difficulties involved in this procedure, and the precautions which should be taken, are further discussed below. Also, the amount of unbound and reversibly bound formaldehyde in a system may be determined by adding dimedon (dimethyldihydroresorcinol) to the mixture, and filtering off and weighing the insoluble methylene addition product. This method has been employed by Wadsworth and Pangborn (1936) in their study of the combination of various amino acids with formaldehyde. It was also employed by Nicolet and Shinn (1941) and by Martin and Synge (1941) for the isolation of the formaldehyde produced by periodate oxida-

³ A general survey of methods for the detection and analysis of formaldehyde has been given by Walker (1944), Chapters 16 and 17.

tion of serine and hydroxylysine in protein hydrolyzates.⁴ The compounds form slowly, and several days must generally be allowed for complete precipitation. Clearly, analytical methods of this sort cannot be applied to obtain information about the equilibrium state of systems in reversible equilibrium. For information about systems of the latter type, one must turn to the methods of physical chemistry.

Two methods of the latter sort have proved particularly powerful in the study of the equilibria between formaldehyde and the free amino group of amines, amino acids, peptides, and proteins:

(1) *Potentiometric Analysis*. In this method, the pH of a buffer system, made up of the acidic and basic forms of the substance under investigation, is studied as a function of the concentration of formaldehyde added. From observed changes in pH, and from the fundamental equations of acid-base equilibrium, important inferences may be drawn concerning the affinity of formaldehyde for the combining groups in the molecule being studied, and concerning the number of formaldehyde molecules which can react with such groups. The method is discussed in detail in Section IV below.

(2) *Polarimetric Analysis*. The optical rotation of optically active amino acids and their derivatives is markedly altered when formaldehyde combines with a group adjoining the center of asymmetry. If a second molecule of formaldehyde combines with the reacting group, a further change in optical rotation occurs which can, in general, be clearly distinguished from that due to the addition of one molecule of formaldehyde. Here again the detailed discussion of the application of the method is presented in Section IV.

In recent years, intensive study has been devoted to the mechanism of the action of formaldehyde as a tanning agent on collagen, casein, and other fibrous or potentially fibrous proteins. A detailed discussion of some of the principal methods employed in such studies, and the results obtained, is given in Section VI.

IV. THE REACTIONS OF FORMALDEHYDE WITH THE AMINO GROUP IN SIMPLE AMINO ACIDS

1. AMMONIA AND AMINES

Ammonia, the simplest amine, is somewhat unusual in its reaction with formaldehyde, yielding the very compact and symmetrical hexamethylenetetramine:

⁴ As Neuberger (1944) has recently pointed out, formaldehyde formed by periodate oxidation from serine may react irreversibly with histidine or other aromatic amino acids present in the hydrolyzate, the analytical determination of serine being thereby falsified. An extensive discussion of periodate oxidation methods, in the analysis of hydroxyamino acids, is given by Martin and Synge in this volume.

react with the added alkali, the optimum conditions for titration would lie at the highest obtainable F concentration. However, F is an acid of pK near 12.9 (Levy, 1934). Its presence in the solution at high concentration, therefore, has an effect which, from the point of view of the titration, is equivalent to a large increase in K_w . Thus there is an optimum region of F concentration for the formol titration, in which the pG_t values are sufficiently depressed to make the titration satisfactory, but in which the concentration of anions at the end point, due to the dissociation of F itself, is small enough to produce little error. A detailed analysis of the problem has been given by Levy (1934) who concludes that the maximum accuracy is obtained when the F concentration is 6-9 per cent at the end volume. He recommends that neutral formalin, adjusted to pH 7, should be used, that no correction for a blank should be made, and that the amino acids be titrated at as high a concentration as possible. The error of the titration is smallest when the solution at the end point contains equivalent quantities of the amino acid and the added alkali. If $F=2.3 M$, this occurs when pH is equal to $9.6 + \frac{1}{2}$ the log of the molar amino acid concentration. For a 0.1 M amino acid at the end volume, the proper end point is somewhere near pH 9.1.¹⁴ In the presence of large amounts of proline, as in gelatin hydrolyzates, the end point of the formaldehyde titration should be somewhat more alkaline than otherwise (Levy, 1934, p. 164).

The stoichiometric relations involved in the formol titration have been discussed by Van Slyke and Kirk (1933). If the titration is begun at the isoelectric point of the amino acid and carried to an end point near pH 9 in formaldehyde, the titration gives a measure of the free carboxyl groups. If the titration is started at pH 7 in water and carried to the same end point, it gives a measure of the free amino groups present. The latter method is generally the condition of choice; obviously the first method can only be applied to a single amino acid, or to a mixture of amino acids which are all of the same charge type.

Highly accurate formol titrations have been carried out by Dunn and Loshakoff (1936a) who determined pH, during the progress of the titration, on the glass electrode. Choosing the conditions of the titration to be nearly optimum according to the directions given by Levy and summarized above, they determined the electromotive force (E) as a function of the volume (V) of added alkali. The function $\Delta E/\Delta V$ passes through a sharp maximum near the stoichiometric end point, which can thus be located with very high precision. In this method, the absolute pH values are not of importance, but only their variation with V. This method is admirably suited to

¹⁴ The conditions for the titration of the dibasic amino acids require special consideration (Levy, 1935). In particular, significant errors are to be expected for histidine, if it is titrated under the same conditions as the other amino acids.

titrations of individual amino acids for determination of analytical purity; and Dunn and Loshakoff report that the error of the method for this purpose can be reduced to 0.1 per cent or less. With some modifications, methods based upon the same principle should be applicable to more complex mixtures of amino acids, but the accuracy of the titration in a mixture must necessarily be somewhat lower.

Borsook and Dubnoff (1939) described an ultramicro modification of the glass electrode formol titration of Dunn and Loshakoff. It should be noted that the conditions chosen by them for the titration deviate markedly in several respects from those recommended by Levy; presumably these conditions were chosen deliberately for special purposes, and could be suitably modified by other workers without alteration of the ultramicro technique. Sisco, Cunningham, and Kirk (1941) have described a micro titration by quantitative drop analysis, which permits the determination of 4 to 10 μ g. of amino N with an accuracy comparable to that of the macro method as ordinarily carried out. Here again (as the authors have indicated) accuracy could be improved by using a higher F concentration, with F adjusted to pH 7, and a final pH of approximately 9, following Levy's recommendation.

Martin and Synge discuss the formol titration as an analytical tool elsewhere in this volume.

V. POLYFUNCTIONAL AMINO ACIDS AND PEPTIDES

When functional groups other than the amino group are present in an amino acid, the possible complexities of the formaldehyde reaction are increased. Reactions of the types already outlined may occur at two or more points in the molecule, and any one of the initial products may react with another free functional group, with elimination of water and methylene bridge formation. However, the relative rates of the various possible reactions may be so different that a satisfactory description of the reaction mechanism, and of the products obtained, may be achieved. In the cases of cysteine and asparagine, the products obtained are well characterized, and there is considerable knowledge of the steps in the reaction. In other cases — tryptophan, tyrosine, phenylalanine, histidine — crystalline reaction products have been obtained and identified, although the mechanism of the reaction is still almost unknown. The guanidino group of arginine, and the peptide linkage, are known to react with formaldehyde, but neither the exact mechanism of the reaction, nor the exact nature of the derivatives formed, is yet known for arginine or the peptides.

1. CYSTEINE

The reaction of F with cysteine has been examined by several workers with substantially identical results. The most complete report is given by

A slow alteration in the response of arginine, in the presence of F, to the Sakaguchi test, was also observed. The usual deep carmine color was obtained with the Sakaguchi reagent immediately after mixing F and arginine; but if the reagent was not added until a half hour later, a dark green color appeared, which gradually changed to a reddish brown. This is strong evidence that the slow reaction involves the guanidino group. It should also be noted, however, that the change in optical rotation accompanying this reaction is extraordinarily great. In the presence of a large excess of F, the initial molecular rotation is near -100° , the final value greater than $+700^\circ$. Such an extremely large change would be surprising if none of the groups immediately adjoining the asymmetric carbon were involved. The total picture suggests methylene bridge formation, involving both amino and the guanidino nitrogen — compare the preceding discussion of histidine — but conclusive evidence is lacking. Wadsworth and Pangborn (1936) reported that all the F in an arginine-F mixture could be precipitated as the dimedon derivative, on standing at pH 4.4-5.0 with dimedon for three days, even when arginine and F had first been allowed to react as long as 11 days at pH 8 and 39°C . This suggests that Reaction 51 is reversible, at least with respect to F, although it has not been proved that unaltered arginine can be recovered from such systems. Further studies of the arginine-formaldehyde system should prove of great value.

9. LYSINE

Each of the two amino groups of lysine should presumably be capable of reacting, in the uncharged form, with either one or two molecules of F. Levy (1935) reported for the lysine dipolar ion the association constants $L_1^\pm = 89$, $L_2^\pm = 250$; for the anion he reported $L_1^- = 240$, $L_2^- = 310$. Frieden, Dunn, and Coryell (1943d) however, could not confirm these values by the optical rotation method, although their analysis was incomplete. They evaluated L_2^\pm/L_1^\pm as 8.72, as against 2.7 from Levy's results; and L_1^- as 35.0, much lower than Levy's figure. They concluded that the ϵ -amino group has a much stronger tendency to associate with F than the α -amino group — a result to be expected in view of the steric factors affecting the reaction (p. 299). The possibility of methylene bridge formation between the two amino groups should be considered, although there is no positive evidence for it from the available data. Titherly and Branch (1913) believed that hexahydro pyrimidine was formed from propylene diamine by a similar reaction with F. Their observations indicated a very mobile equilibrium between hexahydro pyrimidine and the open chain hydroxymethyl derivatives of propylene diamine. Further knowledge of the conditions of formation, and the stability, of such cyclic methylene diamine

derivatives may be important for understanding of the reactions of proteins with F.

VI. PROTEINS

1. INFLUENCE OF FORMALDEHYDE ON TITRATION CURVES

The formol titration of proteins does not differ in principle from that of the amino acids. The effect of F in modifying the titration curves has been studied most systematically by Kekwick and Cannan (1936) (see also Cannan, 1942, Fig. 1 and p. 402). An alkaline segment of the curve is displaced by F (1 to 8 per cent) from a pK value near 10 to one near 7. This very large displacement corresponds to what would be expected for the free ϵ -amino groups of lysine. The best procedure for titrating these groups appears to involve: (1) an initial adjustment of the aqueous protein solution to a pH of approximately 8.5; (2) addition of F, which, of course, causes a marked decrease in pH; (3) titration to a final pH of 8.5 in the F solution. The alkali consumed should be equivalent to the ϵ -amino groups. If terminal free α -amino groups are present, they should contribute very little to the titration, provided their pK values are similar to those found in peptides (pK values of 8.1 or less). If the initial adjustment of pH in water is made to 6.5 instead of 8.5, the α -amino groups are presumably included; there is then also a contribution from the imidazole groups of histidine. The ϵ -amino groups, as determined by formol titration, frequently exceed the lysine determined by analysis of the protein hydrolyzate; often this is due to inadequacies in the analytical procedure (Cannan, 1942). Lichtenstein (1940) has shown that deamination of gelatin causes virtually complete disappearance of that portion of the titration curve which is affected by F, other portions being virtually unaffected. The effect of F on the titration curves of numerous other proteins has been studied: among those investigated most systematically, we may cite egg albumin (Cannan, Kibrick, and Palmer, 1941), β -lactoglobulin (Cannan, Palmer, and Kibrick, 1942) and myosin (Dubuisson, 1941).

2. THE DETERMINATION OF BOUND FORMALDEHYDE IN PROTEINS

In the study of such processes as the tanning of collagen or casein by F, it is necessary to expose the protein to F at a given concentration and temperature, for a known time, and study the effects on the protein. At the end of the time of treatment, the sample is removed from the solution and thoroughly washed to remove F which is not firmly bound. Some of the limitations of such a procedure must be immediately apparent. Any F which is held only in a loose reversible combination will be rapidly removed by the washing procedure. This fact has generally been clearly recognized. However, F which can readily be removed in this way is not likely to play

an important part in the reactions underlying the tanning process. More important is the fact revealed by the very careful studies of Nitschmann and Hadorn (1943a) on casein, that a considerable amount of F, which still remains adherent to the solid protein even after several hours of quite thorough washing, can be removed if the washing is prolonged for a period of many days. On one sample of casein, for example, which had been soaked for 64 hours at room temperature in 38 per cent F, they were unable to demonstrate any further F in the flowing wash water after five hours of washing, even by the very delicate test of Rimini and Schryver.¹⁹ However, if the wash water were allowed to stand at this stage for 10 minutes in contact with the casein, a positive test appeared. The test did not become finally negative, in this case, until after 24 days of continuous washing. At the end of this time, analysis of the casein, by the method discussed in the following paragraph, showed an F content of 1.78 per cent. This amount of F, therefore, is very firmly held, and cannot be removed even by such prolonged washing at room temperature. In another experiment, a sample of casein which had been treated 24 hours with 38 per cent F was divided into two portions, one being continuously washed for 1½ hours and the other for 12 days. The formaldehyde content of the former was 2.59 per cent; of the latter, 1.91 per cent, so that the amount removed by the more prolonged washing was more than 35 per cent of the residual amount which remained firmly bound. It seems clear that the F which can be removed, but only by very prolonged washing at room temperature, must exist in the form of a chemical complex which does not readily dissociate. At the same time, it must be less firmly bound than the residual F which cannot be removed even by weeks of washing at room temperature. It is impossible, at present, to differentiate the chemical groups involved in these two forms of binding. This work of Nitschmann and Hadorn, however, reveals clearly the difficulties which arise in defining the concept of "bound formaldehyde" in proteins. Such a term, in order to have a definite meaning, must be very precisely defined in terms of the operations used in measuring it.

Various different techniques have been employed in the determination of bound F in proteins. Most of the linkages between F and the groups existing in the protein molecule can be broken, with the release of free formaldehyde, by prolonged hydrolysis in hot acid solution.²⁰ On thorough

¹⁹ This test is carried out by adding phenylhydrazine hydrochloride and potassium ferricyanide to the solution to be tested, and then adding a little concentrated hydrochloric acid. In the presence of formaldehyde, a strong red color appears; one part in a million of formaldehyde can be detected by this method.

²⁰ This is probably not true, however, of F which has been incorporated into tryptophan, tyrosine, phenylalanine, or histidine residues by the reactions described in Section V.

distillation, the formaldehyde passes over into the distillate, and can be determined there, for instance, by collecting it in a solution of NaHSO₃, and subsequent titration with iodine. Highberger and Retzsch (1938) determined bound formaldehyde in collagen by digesting the sample to be analyzed in 2 N H₂SO₄, and distilling over nearly all of the digestion fluid into a receiving flask containing NaHSO₃. A sufficient length of time was permitted to allow complete formation of the bisulfite addition product in the receiving flask. Iodine solution was then added in amount just sufficient to oxidize the free bisulfite, and the solution was made alkaline after the addition of a little ethanol to inhibit oxidation of sulfite by oxygen. In the alkaline solution, at pH between 9 and 10, the formaldehyde addition compound is decomposed. The liberated sulfite is then rapidly titrated with iodine and the formaldehyde content calculated from the amount of sulfite thus liberated.

Nitschmann and Hadorn (1941) confirmed the accuracy of this method of determining F in collagen digests, but showed that it gave low results for formaldehyde-treated casein, and also for vitellin and probably for certain other proteins. For casein, they discovered that accurate results could be obtained if solutions less strongly acid than those recommended by Highberger and Retzsch were employed for the digestion. Approximately 0.1 molar phosphoric acid was found to give quantitative yields. Later (Nitschmann, Hadorn, and Lauener, 1943), it was found that low results were sometimes obtained even by this technique unless the digestion fluid was twice distilled. After about 85 per cent of the original volume of liquid had been distilled, more water was added and a second distillation carried out, to a very small final volume. The second distillation leads to an added recovery, generally of the order of 1 or 2 per cent of the total amount of bound formaldehyde determined.

Theis and Jacoby (1942, 1943) and Theis (1944, 1945) have chosen not to remove free and reversibly bound F by washing, but instead press out the collagen (or other fibrous protein) several times between sheets of blotting paper at a pressure of 10,000 lbs. per sq. in. They present evidence that free water and free electrolytes are removed by this treatment, and therefore infer that free F is also removed from a protein preparation previously exposed to F solution. However, this pressure method certainly must leave in the protein considerable amounts of F which are removed even by brief washing. The values of bound F, determined by the method of Theis, are therefore not directly comparable with those determined after the protein has been thoroughly washed.

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3/8

the same reactions that appear to underlie the tanning process in collagen or casein. Nevertheless, the action of F is capable of producing very marked increase in the strength of artificial films or fibers of zein. The study of the reaction is still in a relatively early stage, and much of the work has been carried out in unpublished studies in this Department.²⁶ In attempting to interpret the nature of the groups responsible for this reaction in zein, the large number of amide groups of the dicarboxylic acids is to be noted as a distinctive feature of its composition. Furthermore, the probability that these groups are involved in the reaction is strengthened by the fact that F has little action on zein in neutral or alkaline solutions, and shows its maximum effect on treatment in fairly acid solution near pH 4. This is comparable to the conditions found most favorable by Einhorn (1905, 1908) for the reaction of amides with F to form methylol derivatives and methylene diamides (Reactions 8 and 9). It would be premature to attempt today any final interpretation of the reactions involved, but there is fairly strong evidence that methylene bridges are formed, presumably between the nitrogen of a dicarboxylic acid amide group and that of a neighboring peptide linkage or perhaps of a second amide group; this reaction is the most important underlying process which increases the mechanical strength of zein films. Ammonium salts exert a marked accelerating effect on the reaction. The general character of the findings obtained in this laboratory is fully in accord with those recently mentioned in a brief note by Wormell and Kaye (1944).²⁷

* 7. FORMATION OF TOKOIDS FROM BACTERIAL TOXINS

The action of dilute formaldehyde on solutions of soluble proteins leads to results which superficially, at least, are very different from the tanning reactions obtained with fibrous proteins. As far back as 1896, Blum observed that egg albumin, on treatment with dilute F, became resistant to heat coagulation, and a large number of similar scattered observations have been reported in the literature. These observations, however, have led to little theoretical study of the underlying mechanisms, but have led to important practical applications in the conversion of bacterial toxins to toxoids. The conditions for the most favorable course of the reaction have been determined by trial and error. Generally, the concentration of F employed is of the order of 0.12 to 0.16 per cent (0.3 to 0.4 per cent forma-

²⁶ These studies were carried out by C. C. Jensen, W. E. C. Yelland, C. R. Harmison, and Dexter French. Many other related studies on zein have been carried out at the laboratories of the Corn Products Refining Co., Argo, Illinois.

²⁷ The recent work of Fraenkel-Conrat, Cooper and Olcott (1945), discussed in the section on casein above, suggests that the terminal amide groups of asparagine and glutamine are much more reactive than the peptide groups, in F binding and methylene bridge formation. Wormell and Kaye (1945) express similar views.

lin).²⁸ The incubation is generally carried on at a temperature of 37 to 39° and over a period of several weeks. The reaction is therefore a very slow one and is essentially irreversible. Clearly, therefore, it cannot be comparable to the reaction of F with simple amino groups as in the formal titration, since the latter process is rapid and is readily reversible. No attempt will be made here to survey the earlier literature in this field. Careful and critical studies on preparations of diphtheria toxins that were still relatively impure were given by Hewitt (1930) and by Mudd and Joffe (1933), who give useful bibliographies of earlier work. The recent preparation of highly purified diphtheria toxins by Eaton (1937, 1938) and Pappenheimer (1938) has opened up the possibility of a far more precise study of the nature of the reaction. Most of the work on this problem remains for the future, but some significant observations have already been recorded by these authors. Eaton (1937) notes that much greater quantities of F are needed to convert toxin to toxoid in acid at pH 6 than in alkaline solutions near pH 9. In agreement with results of early workers, hexamethylenetetramine was found to produce unaltered toxoid from toxin at any pH value from 6 to 9. The conversion of toxin to toxoid involves no change in the ratio of protein nitrogen to the number of flocculating units (L_f) as determined by the reaction with diphtheria antitoxin. In alkaline solutions, the use of higher F concentrations than those needed to produce toxoid formation leads to marked impairment of the flocculating, combining, and immunizing properties of toxoid. The reaction is therefore very sensitive to conditions, but little is still known about the nature of the groups involved. Eaton finds that in the change from toxin to toxoid, about 30 per cent of the amino N of the toxin is slowly and irreversibly bound so that it can no longer be determined by the Van Slyke method. Pappenheimer (1938) similarly reports that 6.3 per cent of the nitrogen of toxin is free amino N, while in toxoid this has been reduced to from 2.8 to 3.3 per cent. The earlier studies of Hewitt (1930) on less highly purified toxin agree with these in showing that toxoid formation is complete when a large amount of free amino N still remains in the protein. The slow and irreversible nature of the disappearance of that amino N which is lost in the reaction suggests methylene bridge formation between amino groups and adjoining aromatic rings of tyrosine, tryptophan, or histidine residues. More exact interpretation of the nature of the reaction involves as a necessary preliminary an accurate knowledge of the amino acid composition of toxin.

²⁸ It should be noted that the statements in the literature on this point are frequently confusing. Many authors speak of F concentration when they actually mean formalin concentration. The true concentration of F is approximately 40 per cent of the concentration of formalin.

Diphtheria toxoid is much more stable to heat than is toxin and is less subject to irreversible alteration on precipitation in acid solution near pH 5 (Eaton, 1937). Toxoid precipitated by acetone at room temperature will readily redissolve and react with antitoxin as if it had been unchanged by the precipitation. Toxin, on the other hand, becomes almost completely insoluble after acetone precipitation under the same conditions, and the small residue that does redissolve no longer reacts with antitoxin as well as before.

If amino acids or peptones are present in the medium in which toxoid formation is occurring, the amount of F necessary to produce the reaction is increased, since some of the F is bound by the other substances in the medium which compete with the toxin for F. Wadsworth, Quigley, and Sickles (1937) have observed that a quantity of histidine, equivalent to the F present, almost completely inhibits toxoid formation. In view of the reaction of histidine with F already discussed in Part V, this inhibition is not surprising.

The reactions discussed in this section are drawn almost entirely from experiments with diphtheria toxins. The toxins of *B. tetani* and other bacteria presumably undergo reactions which are similar, but our knowledge of them is even more elementary than that of diphtheria toxin.

The effects of F on snake venoms are similar to those on toxins. Treatment at 38° for several days, at low F concentration, destroys or greatly reduces the toxicity of the venom, while leaving the antigenicity virtually unimpaired. The resulting products, frequently termed "anavenins," especially by the French workers in this field, are excellently adapted to the production of anti-venom on injection. For discussions and references in this field see Arthus (1930, 1931); Grasset and Zoutendyk (1933); Césari and Boquet (1939); Boquet and Vendrely (1943).

Few studies appear to have been carried out on plant toxins. Abrin (from *Abrus precatorius*) has been reported to become virtually non-toxic, while still remaining strongly and specifically antigenic, under conditions similar to those employed for the preparation of diphtheria toxoid (Ramon, 1925). On the other hand, the toxicity of ricin is not destroyed, although much diminished, by concentrations of F as high as 3.5 percent or even more, at temperatures of 38–50° C., acting over periods up to a month or more (Heymans, 1926). Moreover, ricin treated with F in this manner was found to have lost some of its immunological specificity as an antigen, in the process of reducing its toxicity.

8. TOBACCO MOSAIC VIRUS

A very thorough study of the inactivation of this virus by F was carried out by Ross and Stanley (1938). In most of their experiments they used

solutions containing approximately 2 per cent virus protein and 2 per cent F, the pH being held at 7 with phosphate buffer. They reported that a marked reactivation of virus activity could be obtained, after nearly all of the activity had been lost, if prolonged dialysis of the inactivated material at pH 3 was carried out. Reactivation under these circumstances (as measured by the infective power of the preparation for the tobacco plant) was never complete, but it was possible to increase the virus activity in many cases by a factor of 10 or more over that of the inactivated preparation before dialysis. Even preparations that were so completely inactivated that no infective power could be detected recovered an appreciable amount of virus activity on dialysis. The fully inactivated virus protein contains about 60 per cent of the amino N of the active protein. The rate of inactivation decreases with decreasing pH, being much lower at pH 6 than at 7. It was found that the amount of color obtainable when the virus protein is treated with ninhydrin or with Folin's phenol reagent was markedly reduced by the treatment with F which produced inactivation, and that definite increases in the amount of color obtained in both reactions were obtained after reactivation by dialysis. The reactions underlying the inactivation process, therefore, appeared to be at least partly reversible. Ross and Stanley inferred that the tryptophan residues in the virus protein might play an important part in the reaction. They carried out experiments with tryptophan, indole propionic acid, and glycytryptophan, showing a marked effect of F on the capacity of these substances to react with Folin's phenol reagent, whereas no similar effects were observed with tyrosine or glycytyrosine.

Kassanis and Kleczkowski (1944) were unable to find any reversal of F inactivation of virus activity, even after prolonged dialysis at pH 3; and they also found no completely regular parallelism between virus inactivation and changes in color produced with the phenol reagent. They suggest that the virus preparations employed by Ross and Stanley may have contained a virus inhibitor (other than F) which is removed by dialysis, giving apparent reactivation; or that the virus particles may have been reversibly aggregated and disaggregated under the conditions employed. Further investigation of these points is obviously desirable.

9. INFLUENZA AND OTHER VIRUSES

It has now been shown that many viruses, on treatment with F at low concentrations, lose most or all of their infectivity, while retaining full antigenicity, so that on injection the altered virus can produce a high degree of immunity to the unaltered virus. Such studies have been made with the viruses of typhus (Zinsser and Castaneda, 1931), equine encephalomyelitis (Beard, Beard and Finkelstein, 1939), malignant panleucopenia of cats

(Enders and Hammon, 1940) and recently with great success for influenza virus (Hirst, Rickard, Whitman and Horsfall, 1942; Stanley, 1945). The use of low F concentrations is very important; in the case of influenza virus, Stanley (1945) finds that the best results are obtained with concentrations of 0.01-0.10 per cent, in systems containing one to ten mg./cc. of virus material. Higher concentrations of F largely destroy antigenicity as well as virus activity, and render the material ineffective for immunization. The combination of F treatment, under carefully controlled conditions, with differential centrifugation, appears to have yielded concentrated preparations of modified influenza virus which can be used for large scale immunization of populations exposed to the disease.²⁹

The studies discussed in this section represent only a small fraction of the vast number of reports on the action of F on various proteins. However, in the present fragmentary and preliminary state of our knowledge there would be little value in multiplying instances. The use of F as a reagent for modifying specific properties of proteins is only in its infancy. Certainly, it will be used very much in the future under more definitely controlled conditions and with a gradually increasing knowledge of the nature of the underlying processes involved in the reaction. Many of the reactions discussed in this paper are given by numerous other aldehydes as well. Formaldehyde, however, owing to the very compact structure of the molecule and its high reactivity, is a particularly versatile reagent with a vast range of possible reactions.

²⁹ Dr. John F. Enders has kindly supplied us with a number of references dealing with the formal treatment of viruses, in addition to those cited in the text. For the benefit of readers interested in this field, the other references are cited here.

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