THE CHEMISTRY OF PROTEIN DENATURATION

HANS NEURATH, JESSE P. GREENSTEIN, FRANK W. PUTNAM, AND JOHN O. ERICKSON

Department of Biochemistry, School of Medicine, Duke University, Durham, North Carolina, and National Cancer Institute, National Institute of Health, United States Public Health Service, Bethesda, Maryland

Received October 13, 1943

CONTENTS

I. Introduction

A. Definition

The term *denaturation* has been used rather loosely and indiscriminately to denote ill-defined changes in the properties of proteins, caused by a variety of chemical, physical, and biological agents. The observation that many unrelated processes may cause similar changes in a protein early led to the belief that any single change, such as the formation of a coagulum, suffices to characterize a "denatured" protein, and that all denaturing agents are alike in their action. Although proteins are now known to respond differently to various kinds of denaturation, the supposition of the singleness of the denaturation process has persisted.

The complexity of chemical and physical structure and the diversity of biological functions with which the proteins are endowed renders them susceptible to a variety of changes. For instance, these changes may consist of the ionization of amino, carboxyl, or phenolic groups. They may be of a more profound nature and involve the rupture of structure-determining bonds which, in turn, may lead to far-reaching intramolecular rearrangements, accompanied by the liberation of sulfhydryl or disulfide groups. Eventually changes may occur which result in a disintegration of the protein molecule as a whole. Any one of these changes may cause a decrease in protein solubility or a loss of specific biological functions; hence the term denaturation remains ambiguous unless (*a*) the nature and magnitude of the changes are defined, (*b*) criteria for their recognition are established, and (*c*) the agents which cause these changes are known. However, since the term has been implanted so firmly in the literature, little could be gained by abandoning it until it can be replaced by a more precise terminology. While denaturation processes are manifold in nature and magnitude, their mechanisms are not sufficiently understood to warrant at this time the coinage of a specific terminology for each individual process.

In the present review, the following definition is assigned to "denaturation": *Denaturation is any non-proteolytic modification of the unique structure of a native protein, giving rise to definite changes in chemical, physical, or biological properties.* This definition excludes processes which result in the hydrolysis of peptide bonds, i.e., chemical or enzymatic degradation. Such chemical reactions with protein surface groups as lead to the formation of a new chemical species (acetylation, diazotization, etc.) or to changes in the state of ionization within the pH-stability range, although not excluded by definition, will not be considered here. This limitation appears to be fully warranted, since such reactions involve primarily individual amino acid residues rather than the protein molecule as a whole. The definition is more inclusive than those previously given, in that it embraces any single deviation from the framework of the native protein.

In the past, observations of the denaturation phenomenon have been limited in scope and arbitrary in evaluation. Certain manifestations of denaturation have probably been known since the beginning of the culinary art. The coagulation of egg white upon boiling is one, the clotting of milk another. Other manifestations have since been recognized, such as a decrease in protein solubility under reference conditions, a loss in crystallizing ability, a disappearance of biological activity, or an increase in the viscosity of protein solutions (5). As new and more refined experimental tools are developed for the characterization of native protein molecules, further and more precise differences between native and denatured proteins will be found. While, in the last analysis, a full understanding of the denaturation process cannot be expected prior to a solution of the problem of the structure of proteins as a whole, a study of the conditions under which proteins undergo such changes as do occur upon denaturation is of considerable aid in the elucidation of the structure of the intact protein molecule.

The purpose of this review is twofold: (*1*) to present the widely scattered experimental data in an organized fashion and to weigh the evidence in relation to the conclusions that have been drawn; (*2*) to integrate the experimental facts with present-day concepts of protein structure. It is hoped that a realization of the incompleteness of our knowledge may serve as an aid and impetus for further research in the field.

In the following paragraphs fundamental aspects of the problem, including the qualitative chemical and biological differences between native and denatured proteins, and the nature of denaturing agents, are surveyed. In subsequent sections, the denaturation process will be considered as a truly chemical reaction with due regard to the fact that proteins are chemical entities, endowed with unique and specific biological functions.

B. QUALITATIVE DIFFERENCES BETWEEN NATIVE AND DENATURED PROTEINS

Since, fundamentally, denaturation is an intramolecular change of the protein, an exact description of the process would require a precise analysis of the dislocation and relocation of constituent atoms and groups. As such an aim is at present beyond experimental approach, recourse must be had to a description of the process in terms of measurable changes in properties as they are elicited

by the modification in structure. For this, it is imperative to establish experimental standards for quantitative measurements of the process and to select conditions which avoid the masking of events by secondary reactions of and between denaturated protein molecules, e.g., aggregation, precipitation, etc.

The types of changes discussed below have been found to accompany various kinds of denaturation.

1. Decrease in solubility

The primary act of denaturation frequently is followed by the precipitation of the denatured protein at the isoelectric point or upon the addition of smaller amounts of neutral salts than are needed to precipitate the native protein. A decrease in protein solubility has for many years been regarded as a necessary accompaniment of and a sufficient criterion for denaturation. This idea, originating from the simple observation of the coagulation of proteins upon heating, is most clearly expressed by Wu (410), as follows: "Denaturation is a change in the natural protein whereby it becomes insoluble in solvents in which it was previously soluble." This view has since gained support from the work of Northrop, Kunitz, and Herriott (*cf.* 307), who have shown that under proper conditions the loss of activity of certain proteolytic enzymes was paralleled by a proportionate decrease in the solubility of the enzyme protein.

The solubility of a protein in a given polar solvent is determined primarily by the electric charge and the polar character of the protein surface. While it is true that a decrease in solubility is frequently the most tangible manifestation of denaturation, certain other changes may occur long before the protein has lost its gross solubility properties.[1] Conversely, a denatured protein may regain the gross solubility of the native protein without fully sharing all characteristic properties of the latter. For instance, as will be discussed more fully later, tomato bushy stunt virus has been obtained in crystalline form, as soluble as the native virus protein but devoid of its specific biological activity (53). Acid-denatured hemoglobin may be made to regain the solubility and other physical properties of the native protein without being completely identical with the latter (*cf.* Part IV).

Profound changes in molecular configuration, occurring with certain kinds of denaturation (urea, guanidine hydrochloride, synthetic detergents, etc.) may readily account for a decreased solubility in polar solvents, by virtue of the exposure of non-polar groups together with a spatial redistribution of polar groups. However, the decrease in solubility accompanying milder types of denaturation for which there is no evidence for gross changes in internal structure remains inexplicable as long as the nature of these changes is not apparent.

2. Loss of biological activity

Certain manifestations of biological activity may become destroyed upon denaturation. Proteolytic enzymes become inactivated upon heating or by

[1] Slight changes in solubility may, of course, always occur. Unfortunately, comparative measurements of the *thermodynamic* solubilities of pure native and denatured proteins are unavailable.

treatment with alkali, viruses lose their power to produce disease, complement loses its ability to combine with antigen–antibody complexes, and certain hormones lose their specific regulatory functions. However, just as chemical manifestations of denaturation vary in degree from protein to protein, so do biological manifestations. For instance, in the presence of concentrated urea solutions trypsin remains active for a short period of time (16) and papain for longer periods (235), whereas tobacco mosaic virus is rapidly and completely inactivated (224). While cytochrome C (199) and crystalline ribonuclease (213) remain active after heating at 100°C., most proteolytic enzymes are rapidly inactivated.

The observation that antibodies of various kinds retain their ability to combine specifically with the homologous antigen, following denaturation by guanidine hydrochloride (136) or surface forces (348), indicates that denaturation may occur without the loss of the specific biological activity, while other observations point to the occurrence of inactivation without any other perceptible signs of denaturation (53, 54).

It may be stated, therefore, that biological inactivation, like decrease in protein solubility, is a frequent though not an invariable accompaniment of protein denaturation.

3. Loss of crystallizing ability

Many globular proteins are capable of forming crystals endowed with a regularity "down to atomic dimensions" (61). It is a matter of experience that the ability of a protein to crystallize becomes lost upon denaturation, probably as a result of gross changes in molecular structure and shape. Although upon "reversal" of denaturation (regeneration) the crystallizing ability may be regained, nevertheless the protein may still be in a denatured state (Part IV). It has been found that upon heating, protein crystals may retain their shape long after the protein has become denatured; however, after dissolution the protein can no longer be crystallized (269).

4. Increased reactivity of constituent groups

Several changes of chemical nature have been found to accompany protein denaturation. In brief, certain constituent groups such as sulfhydryl, disulfide, or phenolic groups become detectable after denaturation, while in the native protein they were either not detectable at all or else only to a small extent. The quantitative increase in the number of these groups depends on the degree of denaturation and on the nature of the protein. Available evidence indicates that the appearance of these groups is a direct result of the opening up in protein structure concurrent to denaturation. This phenomenon, fundamental to the process of denaturation, has been investigated by a diversity of quantitative methods and will be discussed in detail in Part II A.

5. Changes in molecular shape

Native proteins possess characteristic molecular dimensions, expressed in terms of size and shape. Either of these properties may be affected by the

primary denaturation process. The molar frictional ratio, a composite function of molecular shape and hydration, has been found, by various methods, to increase when certain proteins undergo denaturation. It is probable that this increase is due mainly to a change in molecular asymmetry, resulting from a transition of the molecule from a condensed to a more extended configuration. Various lines of evidence, such as viscosity and diffusion measurements, x-ray analysis, fibre formation, etc., support this view.

These changes, though sometimes too subtle to be detected by present methods, are believed in several quarters to constitute the most direct reflection of the intramolecular rearrangement occurring upon denaturation.

6. Susceptibility to enzymatic hydrolysis

The work of Bergmann and his associates on the action of proteolytic enzymes on synthetic substrates has shown that the specificity of these enzymes is directed toward either terminal or internal peptide bonds (58). Since the proteinases attack peptide bonds, it stands to reason that a denatured unfolded molecule will be more readily digested by such enzymes than a native protein. The latter requires denaturation preparatory to the act of proteolysis. This, indeed, has been confirmed in several instances. Denatured hemoglobin is digested by trypsin at a rate forty times as great as native hemoglobin (234). Urea-denatured serum albumin likewise is considerably more susceptible to tryptic hydrolysis than native serum albumin (64). The observation that peptic and tryptic fission of raw egg white is greatly enhanced upon heating of the latter points in the same direction (115). Though more experimental data are needed, it appears that an increased susceptibility to proteolysis is an accompaniment of protein denaturation.

The above list of changes in properties could be extended to include changes in absorption spectra, optical rotation, electrochemical properties, and others. These will be considered in the discussion of individual proteins, later in this review. The complex chemical and physical structure of the proteins appears to permit of an inexhaustible diversity of changes in properties, only a few of them having been investigated to date with the required precision.

C. DEGREES OF DENATURATION

Measurement of any single change is an insufficient means to characterize a denatured protein or, for that matter, to estimate the extent to which a protein has become denatured. A native protein molecule is characterized not only by its amino acid composition, i.e., the number, nature, and sequence of occurrence of the constituent amino acid residues, but also by its physical configuration, i.e., the steric arrangement of the polypeptide chains within the molecule. Fundamentally, denaturation involves a change in the physical structure of the protein rather than in its chemical composition, initiated by some, as yet ill-recognized, steps involving an "activation" of the molecule. It is obvious that there must exist various degrees of denaturation, depending on the extent to which the structure of the protein has been modified.

A completely denatured protein would be one devoid of any element of specific structure, i.e., a fully extended polypeptide chain, or at least one whose state of folding is determined in a haphazard fashion by the interaction between amino acid side chains. This state is probably realized when proteins are spread into monomolecular films (Part III D). However, not every kind of denaturation leads to such drastic changes in internal structure. Urea denaturation of serum albumin or egg albumin, for instance, gives rise to an extension of the molecule intermediate between the condensed configuration of the native proteins, and the fully expanded state characteristic for protein monolayers (Part III B).

The degree of change in structure depends on the nature of the protein and of the denaturing agents. For the purpose of discussion one may adopt a graded scale to denote the extent of denaturation, the lower limit of the scale being used to denote a native protein, and the upper limit the completely denatured protein. For instance, native hemoglobin may be thought of as existing in state A. Slight acidification, to pH 4, results in dissociation into two subunits, each of which is combined with one-half the total complement of prosthetic group (page 203). If this be denoted as state F or G, state P or Q would be reached upon further acidification, leading to a splitting off of the prosthetic complement and simultaneous denaturation of the globin moiety (page 216). Finally, state Z would correspond to a complete unfolding of the globin. Similar variations may be experienced by adding increasing quantities of urea to serum albumin, egg albumin, or serum globulin, the proteins passing from state A to increasingly denatured states F, L, P, etc. as the concentration of the denaturing agent is increased. These intermediate stages of denaturation have been recognized in several instances (160–162, 295, 296). This concept of a gradation in the extent of denaturation is incompatible with the "all-or-none" hypothesis that a given protein can exist in only one of two states, i.e., the completely native or the completely denatured.

D. DENATURING AGENTS

In keeping with the known lability of proteins, the agents capable of causing denaturation are manifold. The more important of them will be discussed in the following paragraphs.

1. Physical agents

(*a*) *Heat:* By far the most familiar denaturing agent is heat. While denaturation occurs at any temperature, the rate is greatly increased as the temperature rises. A temperature increase of 10°C. often results in a rate increase of two orders of magnitude (Part II B). At constant pH, the heat denaturation of several proteins appears to follow a first-order course, the minimum rate being at a pH intermediate between the isoelectric point of the protein and neutrality. Proteins denatured by heat, as by any other means, exhibit an enhanced susceptibility to aggregation, depending in extent on the pH, the ionic strength, and the dielectric constant of the medium. In the light of more recent kinetic data, earlier observations of a specific "coagulation temperature" characteristic

for a given protein can at best be regarded as being of qualitative significance (*vide infra*).

(*b*) *Pressure:* The limited literature on the effects of high pressures on proteins has been reviewed recently (255). Inactivation of pepsin and of rennin probably involves a series of reactions. Complete inactivation occurs at pressures of about 6000 kilos per square centimeter, and coagulation at pressures of about 10,000 kilos per square centimeter. Pressure denaturation of egg albumin results in the liberation of sulfhydryl groups (156); that of tobacco mosaic virus in inactivation and disintegration (222).

(*c*) *Freezing:* Several qualitative observations on the denaturation of proteins by freezing have been recorded and reviewed by Nord (301). The rate and extent of denaturation appear to be influenced by pH and salt concentration as well as by the temperature of freezing (143). Inactivation of tomato bushy stunt virus by freezing as by certain other means (51, 54) does not impair the serological activity or the ability of the virus to crystallize.

(*d*) *Irradiation:* Coagulation of proteins by ultraviolet light involves at least two processes, i.e., light denaturation proper, a photochemical reaction independent of temperature, followed by flocculation of the denatured protein, a reaction having a high temperature coefficient (105, 107, 108). The mechanism of the photochemical reaction is ill understood, though evidence obtained from photolysis of monolayers suggests that peptide linkages adjacent to chromophore side chains become ruptured, accompanied by a liberation of the light-absorbing side chain (88). Irradiation has been found to alter profoundly the state of aggregation of protein solutions (123, 352), to cause biological inactivation (125, 306), and to shift the pH of solutions toward the isoelectric point of the protein (62). Earlier work on the effects of ultraviolet light on proteins has been amply reviewed by Arnow (21).

Measurements of the quantum yield for the ultraviolet inactivation of urease and pepsin have shown that inactivation involves specific groups of the protein rather than changes of the molecule as a whole (217), and that enzymatic activity is destroyed before appreciable photolytic changes can be detected. Thus, the quantum yield was of the order of 10^{-2} to 10^{-3}, instead of unity, increasing with decreasing wave length, while the enzymes became inactive before a change in the molecular extinction coefficient in the absorbing region could be observed.

Cathode rays act similarly to ultraviolet light in causing biological inactivation and protein coagulation, while visible light requires the presence of a photosensitizer (104, 362, 363).

(*e*) *Sound waves:* Under the influence of intense mechanical vibrations, produced by sonic or ultrasonic waves, proteins become coagulated and enzymes inactivated (89, 90, 93). Certain specific dissolved gases appear to be essential, while others inhibit the reaction.

(*f*) *Surface forces:* When proteins are spread onto an aqueous surface, or in an interface, denaturation occurs by unfolding of the protein molecules into structures resembling fully extended polypeptide chains. The problem of surface

denaturation already has been reviewed in detail (293); its relation to the structure of denatured proteins will be considered in Part III D.

2. Chemical agents

(*a*) *Hydrogen and hydroxyl ions:* Native proteins have a characteristic pH range within which they are stable. Although great variations in the degree of ionization occur within this range, there is no evidence that they affect the intrinsic structure of the molecules. However, outside the pH-stability range denaturation occurs, usually accompanied by changes in particle size (381). The dissociation of hemoglobin or hemocyanin, the formation of edestan from edestin, and the inactivation of pepsin are representative manifestations of the denaturing action of H^+ or OH^- ions. A large excess of either acid or base eventually leads to proteolysis, particularly at elevated temperatures.

(*b*) *Organic solvents:* Addition of alcohol or acetone to aqueous protein solutions causes denaturation, and coagulation in the region of the isoelectric point (68, 367, 386, 409). This process is temperature dependent and does not seem to occur at a measurable rate at temperatures below $-15°C.$ (112). Proteins belonging to the group of prolamines, i.e., zein and gliadin, require an alcoholic medium for dissolution, no signs of denaturation becoming evident. It is probable that denaturation by organic solvents is related to the influence of the latter on the dielectric constant of the medium, though conclusive evidence concerning the underlying mechanism is lacking.

(*c*) *Organic solutes:* A number of organic compounds have been found to be excellent denaturing agents. The action of concentrated solutions of urea, guanidine salts, acetamide, and formamide has been studied systematically with a variety of chemical and physical methods (*vide infra*). Many of these organic denaturing agents increase the solubility of the denatured protein, thereby offering particularly suitable conditions for studying the process unimpaired by aggregation or flocculation.

The denaturing effects of synthetic detergents have been the subject of recent investigations. Both anionic detergents (alkyl sulfates and alkyl sulfonates, mixed alkyl-aryl sulfonates, and alkyl sulfosuccinates) (7, 247, 248, 332) and cationic detergents (alkyl-aryl-substituted ammonium halides) (209, 354) were effective, as measured by certain chemical and physical criteria. Their powerful denaturing action surpasses even that of guanidine hydrochloride when compared on the basis of equimolar concentrations (332).

3. Biological agents

(*a*) *Enzymes:* Reference has been made to the increased susceptibility to proteolytic fission following denaturation by various means, an increase resulting from the exposure of internal peptide bonds. Suggested evidence for the denaturing action of proteolytic enzymes has been adduced by Linderstrøm-Lang in a study of the temperature coefficient of the tryptic hydrolysis of native and heat-treated lactoglobulin (232), while a more conclusive proof has been furnished by Lundgren in studying the catalytic influence of papain on the

denaturation of thyroglobulin (246). The enzymatic refinement of certain immune sera and antibodies by means of proteolytic enzymes (Part III E) involves a combination of denaturation and selective proteolysis.

II. The Nature of the Reaction

A. The chemical reactivity of denatured proteins

1. Introduction

The amino acids which are the constituents of proteins may for present purposes be divided into two categories: (*a*) those which contain as polar groups only the α-amino and α-carboxyl groups, and (*b*) those which contain, in addition to these two, still a third polar group. Members of the former class include glycine, alanine, and those amino acids with hydrocarbon side chains, such as the isomeric valines and leucines. Included in the latter class are amino acids with a wide variety of polar groups attached to the side chains, i.e., the β-imidazole group in histidine, the ϵ-amino group in lysine, the δ-guanido group in arginine, the β-sulfhydryl group in cysteine, the β,β'-disulfide group in cystine, the β-phenol group in tyrosine, the γ-carboxyl group in glutamic acid, etc. Each of these polar groups in the free amino acids may be readily identified by specific reagents: e.g., the guanido group by the Sakaguchi reagent, the sulfhydryl group by nitroprusside, the disulfide group by cyanide–nitroprusside, the amino group by either electrometric titration within certain pH limits or by reaction with nitrous acid, the phenyl group by the Folin reagent, the imidazole group by diazotized sulfanilic acid, etc.

Within the protein molecule, amino acids in both categories (*a*) and (*b*) are bound each to the other by peptide linkages which involve only the α-amino and α-carboxyl groups. Only such linkages are attacked by proteolytic enzymes (159). The third polar group in each of the amino acids in category (*b*) is presumed to be free of primary linkages within the protein, for any synthetic compounds in which these groups are combined are completely resistant to the action of proteolytic enzymes (159). If these "extra" polar groups on the side chains of the acids were therefore completely free when the latter are bound within the protein, it would be expected that they would exhibit a normal reactivity to the specific agents for the groups. Synthetic peptides containing certain amino acids of category (*b*) are just as reactive toward these agents as are the free amino acids, and in some cases, as in the reaction of lysine peptides toward nitrous acid (158), the reactivity of the side-chain polar groups of the peptide-bound amino acids may be even greater than that of the same groups present in the free amino acids. The chemical reactivity of a protein may thus be expected to be a function of the number and kind of side-chain polar groups present in the constituent amino acids of category (*b*).

When, however, freshly prepared, purified, native proteins are tested *intact* for these polar groups, the proportion of the latter so noted is frequently smaller than that presumed to be present from the amino acid content of the protein as revealed by total hydrolysis. Thus, the acid- and base-combining capacities

of many proteins estimated by electrometric titration are often smaller than would be expected if all the ionizable groups presumably present contributed to the titration (111, 114). Few proteins give any reaction at all for sulfhydryl, disulfide, guanido, or phenolic groups (83, 260, 274, 394, 395), although rich in the amino acids bearing these groups, and although proteins will react with nitrous acid, thus demonstrating the presence of the ϵ-amino group of lysine, the rate of the reaction is sometimes so very low (129, 188) as to be useless for quantitative measurement. The side-chain polar groups of the pure native protein are, in general, strikingly and unexpectedly inert toward specific agents for these groups.

There are, however, two ways in which the presence of these side-chain groups may be readily revealed. The first of these, which is not of present interest, is to hydrolyze the protein completely, either with enzymes or with strong hot mineral acids, thereby splitting the protein and yielding the free constituent amino acids or peptide derivatives thereof. The second method, which is the basis of the present subject, is to treat the native protein with a variety of agents ostensibly quite mild, whereby no primary linkages could be conceivably dissolved (controls being run on synthetic peptides); the protein thus remains entirely intact, but the polar groups become readily reactive toward their specific chemical detectors. The agents which bring about this profound change within the *intact* protein are known as denaturing agents, and the increase in chemical reactivity of the protein caused thereby may be taken as a measure of the denaturing effectiveness of the agent employed. The denaturing agents are quite various and are often unrelated (page 163). In addition to the change in the chemical reactivity of the protein brought about by one or more of these agents, other alterations in molecular size and shape, solubility, and in certain cases biological specificity, may be simultaneously effected.

2. Early qualitative observations

In 1911 Arnold demonstrated that the coagulation of egg white by heating was accompanied by the appearance of chemical groupings which reacted with nitroprusside in exactly the same manner as did known mercaptan groups (20). No trace of these groups was present in the native white of the egg. Arnold believed that the positive nitroprusside reaction of the denatured protein was due to the presence of cysteine within the protein molecule and he felt that he had proved this point when, after treatment of the native protein with pepsin hydrochloride, the split products readily gave a strong nitroprusside reaction characteristic of mercaptan groups. Arnold was probably correct in his assumption, but the conduct of his enzyme experiments, particularly in the matter of controls, left something to be desired. Egg white, when treated with hydrochloric acid alone, is coagulated and becomes reactive to nitroprusside (177). The problem was further considered by Harris (177), who showed that during the course of the peptic digestion the nitroprusside-reactive groups were associated with progressively smaller degradation products of the protein. The list of denaturing agents was also increased as a result of this investigation (177), for it was

observed that treatment of solutions of egg white by alcohol, by mechanical shaking, or by ultraviolet radiation resulted in the formation of a coagulum of the protein in which nitroprusside-reactive groups made their appearance.

Not all proteins when coagulated by heat or by acid reacted with nitroprusside, e.g., the serum proteins (275). The latter proteins in the coagulated state, however, did react with nitroprusside after prior treatment with sulfite (20), tin and hydrochloric acid (20), or cyanide (275), a result which indicated the presence of disulfide groups and suggested the presence of cystine within the proteins.

The observations described above were made on coagula of the denatured proteins. That egg albumin in homogeneous media could be induced to react with nitroprusside was shown in 1930 by Hopkins (192). Many of the earlier investigators had demonstrated the unique solvent properties of amides—particularly urea—on the proteins (see 52 and 53 for collected references). Hopkins showed that when egg albumin and the serum proteins were treated in aqueous solution with concentrated urea, the former, in contrast to the latter, gave a strong nitroprusside reaction; the latter nevertheless reacted with this agent after prior treatment with cyanide. In solutions of urea, as in coagula, mercaptan appeared in egg albumin and disulfide groups in the serum proteins. Hopkins made the further interesting observation that whereas the monosubstituted alkylureas and the asymmetrically substituted dialkylureas produced qualitatively the same effect as urea, symmetrically substituted dialkylureas were completely ineffective. This point is worthy of emphasis and will be discussed below.

The qualitative observations made up to this stage were interesting, but the reactions of nitroprusside and of cyanide–nitroprusside could hardly be related to the cysteine and cystine content of the proteins. For this reason quantitative measurements of the reactive groups of the denatured proteins which could be related to the amino acid distribution of the protein were urgently required. Although many kinds of groups are presumably liberated in the denatured protein, the sulfhydryl and disulfide groups, because of their high reactivity toward specific and readily available agents, were, and have since been, the most intensively and most accurately studied.

3. Quantitative measurements of sulfhydryl and disulfide groups in coagula of denatured proteins

Mirsky and Anson (268, 274, 275) and Mirsky (265) studied coagula of denatured proteins; their methods of estimation of sulfhydryl groups included the treatment of such coagula by standardized oxidizing agents added in slight excess. The extent to which the latter agents were reduced was taken as a measure of the sulfhydryl groups. The oxidizing agents employed were cystine and ferricyanide. The determination of disulfide groups was conducted by first reducing these groups to sulfhydryl by treatment with thioglycolic acid, removing the excess and oxidized form of the latter, and then estimating the total cysteine in the hydrolyzed protein; the difference in amount between the total sulfhydryl and

the original sulfhydryl in the intact denatured protein was assumed to represent the equivalent of disulfide groups originally present in the protein. The presence of sulfhydryl groups in the coagulated proteins was checked by treating the latter with iodoacetate prior to addition of the oxidizing agents; under these conditions the protein failed to react with the latter. In the case of the muscle proteins, free sulfhydryl groups were observed in these proteins in the native state (20, 265), but after denaturation with various coagulating agents, the amount of these groups was increased (20, 396).

Coagula of serum albumin in which disulfide groups were determinable could be dissolved in dilute hydrochloric acid; after neutralization with alkali, the soluble protein possessed far fewer, if any, of these groups (275). This result was considered evidence of a "reversal" of denaturation. The possibility of a reversal of some sort is not excluded, nor is the possibility that part or all of the soluble "reversed" protein had not been completely denatured to begin with. The observation is an interesting one, but to base upon it the generalization that "denaturation is a reversible reaction" (275) is certainly hazardous. Certain specific chemical groups revealed in a coagulum of a protein are only a fraction of the number revealed in homogeneous amide or amidine solutions (see section 4 on page 170). The belief therefore upon which the concept of reversibility was based,—namely, that the coagulated protein represented the completely denatured form of the protein (275),—is at the present time hardly tenable. If there are fewer groups in the fine particles of the coagulum than in an equivalent amount of the protein dissolved in urea, then the coagulum must be composed of either (*a*) a mixture of completely native (no observable groups) and completely denatured molecules (all available groups liberated), or (*b*) molecules which are uniformly but partially denatured (fraction of available groups liberated depending upon degree of denaturation of each equally denatured molecule). If (*a*) were the case, the explanation for the presence of some soluble protein without observable groups after "reversal" is obvious; if (*b*) were the case, the statement that a protein is "either completely native or completely denatured" (275) must be untenable. In case (*b*) however, a reversal of the partially denatured protein to some other state in which the groups disappear is not unthinkable, but more evidence than a loss in groups must be presented before it can be assumed that it is possible to return the protein to its original native state. Indeed, in view of recent significant work bearing on this problem (64, 295, 296, 297, 298), the criteria for any postulated reversal of the denaturation process must involve the employment of several independent techniques.

New oxidizing agents for the titration of the sulfhydryl groups liberated in coagulated proteins were introduced later by Todrick and Walker (394) and by Kuhn and Desnuelle (211). The former used 2,6-dichlorophenolindophenol, $E_0 = + 0.22$ v., which reacts slowly with free cysteine and with denatured proteins. The latter used porphyrindin, $E_0 = + 0.57$ v., which reacts rapidly and stoichiometrically with cysteine, cysteine peptides, and denatured proteins. Neither reagent reacts with native egg albumin, but on denaturation and coagulation of the latter, an amount of either reagent is reduced to an extent corre-

sponding to 0.50–0.63 per cent of cysteine (160, 211, 394), a range of values confirmatory of the values observed earlier by Mirsky and Anson (275, 274). Table 1 includes data on the sulfhydryl and disulfide content of various coagulated proteins.

TABLE 1

Sulfhydryl and disulfide content of coagulated proteins

PROTEINS	COAGULATING AGENT	OXIDIZING AGENT	PROTEIN SULFHYDRYL AS CYSTEINE	PROTEIN DISULFIDE AS CYSTINE
			per cent	*per cent*
Egg albumin (274)		Cystine	0	
Egg albumin (274)	Heat	Cystine	0.56	
Egg albumin (275)	Shaking	Ferricyanide	0.55	
Egg albumin (275)	Surface film	Ferricyanide	0.59	
Egg albumin (394)	Heat	Dichlorophenol-indophenol	0.63	
Egg albumin (211)	Heat	Porphyrindin	0.58	
Egg albumin (160)	Heat	Porphyrindin	0.50	
Globin (276)	Acid acetone	Ferricyanide	0.38	
Serum albumin (274)	Acid acetone	Cystine	0	
Egg albumin (275)	Heat			0.57
Serum albumin (275)	Acid acetone			5.21–6.08

4. Sulfhydryl groups of proteins denatured in homogeneous media

The uncertainties inherent in heterogeneous titrations made it desirable to examine the groups of denatured proteins in homogeneous media. The discovery of Hopkins (192) that egg albumin dissolved in urea liberated sulfhydryl groups provided the starting point of a series of investigations (160–164, 166–172) of denatured proteins in solution.

The titrating oxidant first employed was the dye porphyrindin, developed by Kuhn and Desnuelle (211). Standardized solutions of the dye were added stepwise to solutions of the proteins dissolved in urea until a negative nitroprusside reaction was obtained (160). Under these conditions, the highest proportion of sulfhydryl groups revealed in egg albumin solutions was about double that observed in coagula of the protein (160). The proportion of such groups was furthermore a function of the urea concentration and was independent, within relatively wide limits, of the protein concentration. Study of the effect of compounds related to urea revealed that the guanidine halides possessed a profound effect in denaturing the protein, an effect far exceeding that of urea (160). Comparative data on the effects of urea and of guanidine hydrochloride are illustrated in table 2.

In urea solutions, up to saturation, the proportion of sulfhydryl groups liberated increases progressively with increasing urea concentration but apparently does not reach a maximum value. In guanidine hydrochloride solutions, on the other hand, the maximum proportion of groups appears to be liberated on addi-

tion of 5 *M* reagent and no further groups are revealed at higher concentrations (table 2). A further illustration of the relative effect of urea and of guanidine hydrochloride was afforded by the reaction of these substances with serum albumin (163). The failure of this protein to reveal sulfhydryl groups in concentrated urea solutions had been often demonstrated (80, 192); in guanidine hydrochloride solutions these groups are readily liberated and in terms of cysteine amount to 0.34 per cent of the protein (163).

Confirmation of the findings with denatured egg albumin in homogeneous solution was achieved shortly after by other investigators, who used not only urea and guanidine hydrochloride as denaturants but also detergents (9), and employed ferricyanide (9, 270), tetrathionate (9), mercuric benzoate (9), and iodoso-

TABLE 2

Effect of urea and of guanidine hydrochloride on egg albumin

Protein concentration, 1.09–7.7 per cent; titrating agent, porphyrindin-nitroprusside (160, 162)

UREA ADDED	PROTEIN SULFHYDRYL AS CYSTEINE LIBERATED IN UREA	GUANIDINE HYDROCHLORIDE ADDED	PROTEIN SULFHYDRYL AS CYSTEINE LIBERATED IN GUANIDINE HYDROCHLORIDE
*millimoles per 1 cc. of protein solution**	*per cent*	*millimoles per 1 cc. of protein solution**	*per cent*
0	0	0	0
4	0	2	0
7	0	3	0.18†
8	0.04	5	1.28
10	0.27	6	1.28
12	0.61	7	1.28
14	0.81	9	1.28
15	0.85	12	1.28
17	0.97	16	1.28

* Here, and elsewhere in the tables of this section, the added denaturant is described in terms of the moles or millimoles of the dry material added to 1 cc. of the protein solution.

† Precipitate.

benzoate (181) as titrating oxidants. The collected data for the maximum proportion of sulfhydryl groups liberated in egg albumin are given in table 3, and it is clear that the values obtained may be taken with some confidence as being characteristic of this protein in the denatured state.

The data collected in tables 2 and 3 strongly suggest that the maximum proportion of sulfhydryl groups, expressed as per cent cysteine, is revealed to 1.2–1.3 per cent in egg albumin treated with guanidine hydrochloride or Duponol. The reason for the somewhat lower value observed by Mirsky (270) and by Rosner (345) is not altogether clear. Although the highest proportion of groups in guanidine hydrochloride is not far from that revealed in urea, it must be borne in mind that the former is already revealed at 5 *M* concentration of guanidine, whereas at 5 *M* urea no sulfhydryl groups are at all apparent in the protein (table 2).

The general concordance of the values given in table 3 suggests that in the presence of an effective denaturing agent, most of the titrating oxidants employed are equally useful. Each has its advantages and its disadvantages and each must be carefully used within its own limitations. Porphyrindin reacts rapidly and stoichiometrically with mercaptan groups, but it requires a long and expensive synthesis, and its solutions must be freshly made and frequently standardized because of molecular instability. Such objections as have been raised against the use of this oxidant (71),—objections based to some extent upon misconceptions as to its proper and effective use,—have been recently disposed of (171). The other titrating agents, while more time-consuming in their use than porphyrindin, and like porphyrindin requiring frequent standardization of their solutions, possess the advantages of cheapness and ready availability. Since, in every case, the protein groups are estimated by the oxidant in the

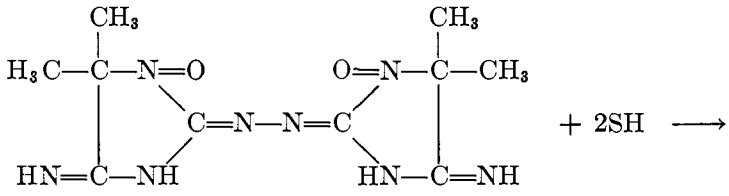
TABLE 3

Maximum proportion of sulfhydryl groups liberated in denatured egg albumin

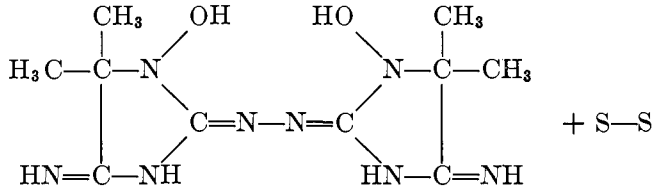
DENATURING AGENT	TITRATING AGENT	PROTEIN SULFHYDRYL LIBERATED AS CYSTEINE	REFERENCE
		per cent	
Guanidine hydrochloride	Porphyrindin	1.28	(160)
Urea	Porphyrindin	1.00	(160)
Guanidine hydrochloride	Ferricyanide, tetrathionate, or mercuric benzoate	1.24	(9)
Duponol	Ferricyanide, tetrathionate, or mercuric benzoate	1.24	(9)
Guanidine hydrochloride	*o*-Iodosobenzoate	1.29	(181)
Guanidine hydrochloride	Ferricyanide	0.96	(270)
Duponol	Ferricyanide	0.96	(270)
Urea	Ferricyanide	0.96	(270)
Urea	Iodoacetate	0.87	(345)

presence of the denaturant, the latter must be completely inert to the oxidant and must itself possess neither oxidizing nor reducing impurities (171). A method for the purification of guanidine hydrochloride has been described (171). Where the presence of oxidizing impurities in the denaturants has been suspected, the use of small amounts of cyanide has been found to be useful (9). A critical test for any sample of denaturant or oxidant is to observe the proportion of recovery of pure cysteine added to a solution of the denaturant either in water alone or in the presence of a protein which gives no sulfhydryl groups on denaturation (171).

The stoichiometry of the oxidants in each case is relatively simple. Porphyrindin and ferricyanide are readily reduced by mercaptan groups to leucoporphyrindin and ferrocyanide, respectively. Porphyrindin is changed thereby from blue to the colorless state, a change which assists in arriving at the titration end point. The nitrogen in the oxidized form is quadrivalent; this probably accounts for the instability of the molecule.



Porphyrindin (blue)



Leucoporphyrindin (colorless)

The end point with these oxidants is actually determined by that amount of the oxidant which is just sufficient to abolish the nitroprusside reaction of the denatured protein. The titer of the oxidant is ascertained by standardization against cysteine or ascorbic acid.

It is probable that *o*-iodosobenzoate could also be employed in a manner similar to porphyrindin and ferricyanide: namely, titration to the nitroprusside-negative stage of the protein. The manner in which it has been used, however, takes further advantage of the precision of iodimetry, for a known amount is generally added in slight excess to the denatured protein and the unused portion is reduced by alkali iodide in the presence of mineral acid (181); the iodine which is thereby released is titrated against standard thiosulfate. The reaction with mercaptan is as follows:

$$C_6H_4(COONa)IO + 2SH \rightarrow C_6H_4(COONa)I + S{-}S + H_2O$$

It is obvious that for these oxidative reactions, two sulfhydryl groups are necessary for the subsequent condensation to the disulfide group. It has been suggested (286) that the rigid steric configuration of the native protein does not permit such groups to be sufficiently near each other for this condensation to take place; upon denaturation, the protein assumes a more flexible configuration which permits closer contact of the sulfhydryl groups of adjacent chains and facilitates their condensation to disulfide groups. Iodoacetates, which react with only a single sulfhydryl group, may therefore substitute within native proteins.

The fact that the proportion of sulfhydryl groups liberated in urea or guanidine solutions is about double that observed in the coagulated protein is not explicable on the basis of the autoöxidation of the latter during the estimation procedures. The rate of autoöxidation of such coagula is extremely low even in the presence of appreciable amounts of iron (344).

5. The effect of urea and of guanidine derivatives

N-Methylurea and the hydrochloride of its isomer, *O*-methylisourea, had the same effect when added to solutions of egg albumin as the parent substance (160). Methylguanidine hydrochloride was as effective as the parent molecule, whereas *as*-dimethylguanidine hydrochloride possessed a definitely smaller effect. Further and more extensive substitution in the guanidine molecule, as in arginine, resulted in compounds which were completely ineffective.

When the anion of the guanidine salts was varied, several notable effects were encountered (162). Whereas the maximum proportion of sulfhydryl groups was very nearly the same in egg albumin dissolved in solutions of the guanidine halides and thiocyanate, this maximum was first revealed at 3 *M* concentration of the bromide, iodide, and thiocyanate, and only at 5 *M* in the case of the chloride (162). In sharp contrast to the profound effect of these salts of guanidine in liberating sulfhydryl groups in the protein was the complete lack of any observable effect of the sulfate, carbonate, and acetate of guanidine at concentrations as high as 6 *M* (162).

The effects of the various guanidine salts are greatly influenced by the nature of the anion. The results with guanidine sulfate, carbonate, and acetate on the one hand, and with the halides and the thiocyanate on the other, suggest that the denaturation of protein by any of the guanidine salts is a complex phenomenon in which the anion and the guanidinium cation play supplementary but quite different rôles. The sulfate and carbonate, like other salts of guanidine, are probably completely ionized, for addition of an inert alkali halide like potassium chloride to solutions of egg albumin in guanidine sulfate, carbonate, or acetate liberates the same amount of sulfhydryl groups in the protein as when it is dissolved in guanidine hydrochloride solutions alone (162). When the effect of the *N*-substituted guanidines is also taken into consideration, it would appear that it is possible to modify the effects of guanidine salts on the proteins by varying the anion on the one hand, and by extensive substitution in the guanidinium cation on the other.

The negative results with guanidine sulfate in egg albumin solutions have been interpreted on the basis of an antagonistic action between sulfate and guanidinium (85). Sulfates appear to have an inhibitory effect in general on denaturation; as an illustration it was pointed out that whereas addition of magnesium chloride to edestin liberated sulfhydryl groups in the denatured protein, addition of magnesium sulfate produced no such effect (85).

6. The effect of urea, guanidine hydrochloride, and their derivatives on various proteins

Extension of the studies of egg albumin denatured by urea, guanidine salts, and their derivatives to other proteins revealed both similarities and differences. The properties and behavior of proteins of many sorts may for convenience be assembled in the following categories: (*a*) proteins which in the native state do not give tests for sulfhydryl, but do so after treatment with concentrated guanidine hydrochloride: these include egg albumin (160), serum albumin (163),

edestin (161), excelsin (161), horse globin (161), and the tobacco mosaic virus (371); (*b*) proteins which, neither in the native state nor after treatment with concentrated guanidine hydrochloride, give tests for sulfhydryl: these include amandin (161) and insulin (167); (*c*) proteins which in the native state contain free sulfhydryl groups, the number of which is increased after treatment of the protein with concentrated guanidine hydrochloride: these include myosin (168) and urease (181); (*d*) proteins which in the native state contain free sulfhydryl groups, the number of which remains the same after treatment of the protein with concentrated guanidine hydrochloride: these include the liver nucleoproteins of several mammalian species (169).

In all of the proteins in which guanidine hydrochloride liberated sulfhydryl groups, the maximum proportion was exhibited in 5 *M* concentration of the agent, and higher concentrations of the latter produced no further effect (160–163, 168). In all of the proteins in this category, urea produced a much smaller effect.

A curious finding in regard to myosin was the fact that addition of glycine or ammonium salts to solutions of the native protein caused the abolishment of the free sulfhydryl groups (168). Subsequent addition of guanidine hydrochloride to the mixture of myosin and glycine resulted in the liberation of all the sulfhydryl groups characteristic of the intact, fully denatured protein. In the presence of glycine the sulfhydryl groups of the native myosin had simply become unavailable to specific reagents for these groups. In this sense the mixture of myosin plus glycine is an analog of egg albumin or of any of the other proteins in category (*a*) above, for all the sulfhydryl groups may be subsequently revealed in such proteins by denaturation. The amount of glycine or ammonium salts just necessary to abolish the sulfhydryl groups of the native myosin is very much smaller than the amount of guanidine hydrochloride which is necessary to liberate the full maximum of these groups (168). The free sulfhydryl groups of the native myosin may also be removed by titration with porphyrindin, but in this case subsequent addition of guanidine hydrochloride liberates sulfhydryl groups the amount of which is smaller than the total found in the originally unoxidized denatured protein by the amount of the free groups of the native protein (168). The oxidation of the native protein groups leads to their assuming a form, presumably disulfide, which is not affected by the denaturant; the disappearance of the same groups after the addition of glycine is produced by a completely different and at present unknown mechanism.

The data for the various proteins are given in table 4.

7. Relation of cysteine revealed in denatured proteins to the sulfur distribution

Comparison of the maximum proportion of sulfhydryl sulfur liberated in denatured proteins with the cystine–cysteine sulfur reveals that the former may be either equal to or less than the latter, but, with only one exception, never exceeds it. The exception occurs in the case of myosin (38), and this may be due to the fact that the value given for the cystine–cysteine sulfur is too low. The sum of the methionine sulfur and cystine–cysteine sulfur in this protein, as given,

is less than the value of the total sulfur (38). The denaturation studies on myosin yield a value for the total sulfhydryl sulfur which, when added to the methionine sulfur, gives a sum practically equal to that of the total sulfur (168).

With the exception of the titratable sulfhydryl groups, the analytical composition of the homologous liver nucleoproteins was found to be independent of the species studied (169). The myosins (38) and the brain proteins (65, 66) of several species appear to possess, like the liver nucleoproteins, an amino acid

TABLE 4

Effect of urea, guanidine hydrochloride, and their derivatives on various proteins

AGENT	PROTEIN SULFHYDRYL AS CYSTEINE												
	Concentration of agent	Egg albumin† (1,6)	Globin (161)	Serum albumin (163)	TMV‡ (371)	Edestin (161)	Excelsin (161)	Amandin (161)	Insulin (167)	Myosin (168)	Urease (181)	RLNP§ (169)
	millimoles per cc.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
	0	0	0	0	0	0	0	0	0	0.41	?	1.3
Urea	10	1.00	0.19	0	0.70	0.34	0.07	0	0	0.64		
N-Methylurea	10	1.02	0			0	0	0	0	0		
O-Methylisourea hydrochloride	10	1.05	0			0.17	0	0	0	0		
Guanidine hydrochloride	6	1.28	0.56	0.34	0.76	0.51	0.18	0	0	1.16	2.84¶	1.3
Methylguanidine hydrochloride	6	1.19	0.56			0.16	0.07	0	0	1.16		
as-Dimethylguanidine hydrochloride	6	0.75	0			0	0	0	0			
Guanidine sulfate	6*	0								0.41		
Arginine hydrochloride	4	0								0		
Glycine	0.01–1	0								0		1.3
Ammonium chloride	1.7	0								0		
Glycine + guanidine hydrochloride	1 + 6 (respectively)									1.16		

* Concentration calculated on normal basis.

† Compare table 3.

‡ Tobacco mosaic virus nucleoprotein.

§ Rabbit liver nucleoprotein.

¶ Calculated from data of Hellerman *et al.* (181).

composition which is very nearly the same for all the species studied. These observations on homologous proteins are of considerable comparative biochemical interest. It would be of further interest to see whether, like the liver nucleoproteins, such proteins could be distinguished from one species to another on the basis of their sulfhydryl-group content.

In the case of myosin, the tobacco mosaic virus, and the rabbit liver nucleoprotein (table 5), the cysteine sulfur revealed by denaturation of the proteins may be considered to account for all the non-methionine sulfur of the proteins

and to be equivalent to the cystine–cysteine sulfur observed after hydrolysis. In the case of the other proteins in table 5, where the cysteine sulfur in the denatured proteins is a fraction of the cystine–cysteine sulfur, the cause of the difference between the two values may be considered tentatively to be due to one of two factors. Either (*a*) all of the cystine–cysteine sulfur of all proteins is really cysteine sulfur, and not all of the latter is liberated in certain proteins even in concentrated guanidine solutions, or (*b*) the cystine–cysteine sulfur is actually the sum of cystine sulfur and cysteine sulfur, and the last mentioned, although completely revealed in the denatured protein, forms only a fraction of the total cystine–cysteine sulfur. Since disulfide groups (of cystine) may be observed

TABLE 5

Sulfur distribution in denatured proteins (denaturation effected in 6–12 M guanidine hydrochloride)

PROTEIN	TOTAL SULFUR	CYSTINE-CYSTEINE SULFUR IN HYDROLYSATE	METHIONINE SULFUR IN HYDROLYSATE	MAXIMUM SULFHYDRYL AS CYSTEINE SULFUR IN DENATURED PROTEINS†
	per cent	*per cent*	*per cent*	*per cent*
Egg albumin	1.62 (113)	0.48 (113)	1.12 (113)	0.34 (160)
Globin	0.49 (210)	0.27 (210)	0.20 (210)	0.15 (161)
Tobacco mosaic virus	0.2 (346)	0.2 (346)	0 (346)	0.2 (371)
Ribgrass virus strain	0.64 (204)	0.18 (204)	0.41 (204)	0.18 (204)
Serum albumin	1.89 (113)	1.53 (113)		0.09 (163)
Edestin	0.88 (113)	0.36 (113)	0.51 (113)	0.13 (161)
Excelsin	1.09 (310)	0.35* (310)		0.04 (161)
Amandin	0.43 (310)	0.22* (310)		0 (161)
Insulin	3.34 (263)	3.32 (263)	0 (263)	0 (167)
Myosin	1.10 (38)	0.21 (38)	0.73 (38)	0.31 (168)
Rabbit liver nucleoprotein	1.18 (169)	0.34 (169)	0.67 (169)	0.37 (169)
Calf liver nucleoprotein	1.14 (169)	0.37 (169)	0.67 (169)	0.19 (169)
Rat liver nucleoprotein	1.17 (169)	0.34 (169)	0.65 (169)	0.05 (169)
Rat hepatoma	1.05 (172)	0.37 (172)	0.67 (172)	0.05 (172)

* Alkali-labile sulfur, which is the same as cystine–cysteine sulfur (113, 310, 356, 417).
† In 6–10 *M* guanidine hydrochloride.

in certain intact denatured proteins (167, 274, 275), alternative (*a*) may be dismissed from consideration. Indeed, the evidence from denaturation is the most unequivocal for the presence of preformed sulfhydryl or disulfide groups, or both, in the intact protein; the ready oxidation of sulfhydryl to disulfide during the hydrolysis of any protein raises the question as to how much of the cystine found in the hydrolysate actually was preformed in the protein and how much was derived from preformed cysteine. The value of cystine–cysteine sulfur as generally given in the literature is without discrimination in this respect.

If alternative (*b*) above is correct, then the difference between the cystine–cysteine sulfur and the cysteine sulfur (third and fifth columns of table 5) should yield the cystine sulfur. The only available data relate to egg albumin and to serum albumin. The difference between the cystine–cysteine sulfur and the

cysteine sulfur of egg albumin amounts to 0.14 per cent (table 5). The disulfide groups, expressed as cystine, amount to 0.57 per cent in denatured egg albumin (275) (table 1); in terms of cystine sulfur this is 0.15 per cent. The agreement with the above difference is excellent. In the case of serum albumin, the difference between the cystine–cysteine sulfur and the cysteine sulfur amounts to 1.44 per cent (table 5). The disulfide groups, expressed as cystine, amount to 5.21–6.08 per cent in denatured serum albumin (275) (table 1); in terms of cystine sulfur this is 1.39–1.62 per cent. The fact that the overwhelming proportion of the cystine–cysteine sulfur of this protein consists of cystine, only a very small fraction belonging to cysteine, makes the observed differences subject to some error. Nevertheless, the data satisfactorily indicate that the cystine–cysteine sulfur of serum albumin may be distributed between about 94 per cent cystine sulfur and about 6 per cent cysteine sulfur.

At the present time, on the basis of the joint evidence offered by denaturation and by hydrolysis of proteins, it can be assumed that the so-called cystine–cysteine sulfur may be composed entirely of cysteine sulfur (myosin, the tobacco mosaic virus and its ribgrass variant, and the rabbit liver nucleoprotein), entirely of cystine sulfur (amandin and insulin), or of cystine sulfur plus cysteine sulfur (egg albumin, globin, serum albumin, edestin, excelsin, and the calf and rat liver nucleoproteins).

The distribution of cystine and cysteine within proteins containing the two acids may be calculated as follows: In egg albumin of molecular weight 42,000 there are six atoms of cystine–cysteine sulfur (0.48 per cent, table 5). The cysteine sulfur amounts to about two-thirds of this value; hence there are four cysteine and one cystine residues within this protein (160). In serum albumin of molecular weight 70,000 there are thirty-four atoms of cystine–cysteine sulfur (1.53 per cent, table 5). The cysteine sulfur amounts to 6 per cent of this value; hence there are two cysteine and sixteen cystine residues within this protein. In the cases of edestin and excelsin, the molecular weights obtained by physical methods differ considerably (81, 379), and therefore the minimal molecular weights given by Osborne (310) on the basis of his unexcelled analytical data have been adopted for these proteins. The value for both is 28,000 or some integral multiple thereof (310). There are three atoms of cystine–cysteine sulfur in both proteins of weight 28,000 (table 5). The cysteine sulfur in edestin is one-third of the cystine–cysteine sulfur; hence in a molecule of edestin of the weight given there is one cysteine and one cystine residue. The cysteine sulfur in excelsin is one-ninth of the cystine–cysteine sulfur; hence in a molecule of excelsin of weight $3 \times 28{,}000$ (or 84,000), there are one cysteine and four cystine residues. Similar calculations may be made for all other proteins for which the pertinent data are available.[2]

[2] *Note added January 31, 1944:* In a valuable paper, Hess and Sullivan (J. Biol. Chem. **151,** 635 (1943)) have recently determined separately (*1*) the cysteine and (*2*) the cystine content of several carefully hydrolyzed proteins, as well as (*3*) the sulfhydryl group content of the same intact unhydrolyzed proteins, (*4*) the methionine content, and (*5*) the total sulfur. For each protein, the sum of (*1*), (*2*), and (*4*), expressed as sulfur, was very nearly

8. *Tissue protein sulfhydryl*

It has been frequently observed that certain tissues such as muscle gave a positive reaction for sulfhydryl prior to any denaturation procedures (20, 265). In the case of muscle preparations, Mirsky (265) demonstrated that denaturation with trichloroacetic acid increased the number of these groups. Myosin forms nearly half the total muscle proteins, and the behavior of this individual protein on denaturation has been considered. Although the results of denaturing procedures on individual proteins yield information of a more precise character than do the results obtained with whole tissues, the latter nevertheless possess an independent interest, since they assist in the characterization of a given tissue and facilitate the comparison of such tissues among various species.

The failure of the serum proteins denatured by heat, by precipitation with acid acetone, or by treatment with urea to yield tests for the sulfhydryl groups has already been alluded to (163, 274, 275). On the other hand, when these proteins, taken either singly or as the whole serum complex, are treated with 5 *M* or more concentrated guanidine hydrochloride, they give a definite test for these groups (163). The guanidine-treated serum of the normal animals of each species gives a constant value for sulfhydryl, which may be looked upon as a characteristic of that particular species. Milk, like serum, fails to give a sulfhydryl reaction when denatured by heat or by urea, but does give this reaction when treated with guanidine hydrochloride. Under the latter condition, milk becomes nearly transparent, and the fat collects at the surface and appears like a streak of grease. The milk proteins which contribute to the sulfhydryl reaction when denatured are the albumin and globulin fractions; casein does not contribute any detectable sulfhydryl (163).

In recent years, the detection of sulfhydryl and disulfide groups in serum proteins by means of the polarograph has received considerable attention, especially by pathologists. The fundamentals of this method have been ably reviewed by Müller (281a): the applications to the study of cancerous and other pathological sera have been considered by Walker and Reimann (398). According to the general consensus, the method is not sufficiently specific to distinguish between a number of quite different pathological states.

The water-extractable proteins of mammalian liver give tests for sulfhydryl groups prior to any denaturation procedure (164). These groups may be contributed by free glutathione or by the proteins themselves. On treatment of the liver extracts with guanidine hydrochloride, the number of such groups markedly

---

the same as the value of (*5*). Category (*3*), expressed as cysteine, was found, for each protein, to be nearly the same as category (*1*), and both were in excellent agreement with the values found by other investigators for the same proteins (table 4). The observations by Hess and Sullivan are the first clear-cut indications that the sulfhydryl and the disulfide groups revealed in certain intact denatured proteins actually belong quantitatively, respectively, to the component cysteine and cystine of these proteins. The assumptions made in the above section of this review on the distribution of cysteine and cystine in proteins, based upon titration data on the intact denatured proteins, are thus completely validated by independent experimental evidence.

increases; this increase is undoubtedly due to the liberation of new sulfhydryl groups in the extracted proteins (164).

The data for the denatured sera, milk, and liver extracts for several species are given in table 6.

To a considerable proportion, the free sulfhydryl groups found in aqueous extracts of the livers of the various species, prior to denaturation, may be accounted for in terms of glutathione or some related substance (164). Following denaturation, the proteins of the rabbit tissues, whether in the serum or in the liver, reveal the presence of more sulfhydryl than do the proteins of rat tissues (164). These results are consistent with those obtained on the erythrocytes (389) and on the isolated nucleoproteins of the livers of these two species (169).

TABLE 6

Sulfhydryl groups of various tissues (groups titrated by porphyrindin-nitroprusside procedure (168) in the presence of 6–12 M guanidine hydrochloride)

TISSUE	PROTEIN SULFHYDRYL AS CYSTEINE	
	Before denaturation	After denaturation
	milligrams per milligram of protein nitrogen	*milligrams per milligram of protein nitrogen*
Rabbit serum (163)	0	0.010
Rat serum (163)	0	0.004
Guinea pig serum (163)	0	0.009
Dog serum (163)	0	0.010
Cow milk (163)	0	0.008
Rat liver (164)	0.045	0.108
Transplanted hepatoma 31 in rats (164)	0.035	0.055
Mouse liver (164)	0.046	0.128
Transplanted hepatoma 587 in mice (164)	0.042	0.120
Transplanted hepatoma 98/15 in mice (164)	0.040	0.124
Adult rabbit liver (164)	0.095	0.300
Fetal rabbit liver (17 days) (164)	0	0.016

The lack of sulfhydryl in the extractable proteins of the fetal liver prior to denaturation, and the relatively small amount found after denaturation, which is far smaller than that of the adult liver, are noteworthy but inexplicable at the present time.

9. Groups other than sulfhydryl liberated in denatured proteins

(*a*) *Disulfide:* Proteins such as egg albumin (275) and serum albumin (274, 275) in the native state give little or no reaction for disulfide groups (treatment with cyanide followed by nitroprusside). After various denaturation procedures the proteins give positive reactions for these groups (table 1). The disulfide content of denatured egg albumin expressed as cystine amounts to 0.57 per cent (275). The cystine–cysteine content of this protein estimated after complete hydrolysis is 1.78 per cent (113).

By difference, the cysteine content should be 1.21 per cent. As revealed by

denaturation of the protein in guanidine hydrochloride, the cysteine content is 1.2–1.3 per cent (table 3), in excellent agreement with the above value. In the case of serum albumin, the disulfide content of the denatured protein, expressed as cystine, amounts to 5.21–6.08 per cent (275). The cystine–cysteine content of this protein, estimated after complete hydrolysis, is 5.71 per cent (113). Both figures are quite large, and any attempt to observe a difference between them would be subject to considerable error; actually, there is a small amount of cysteine liberated as sulfhydryl—namely, 0.34 per cent—when the protein is denatured in guanidine hydrochloride (163).

These calculations demonstrate that the methods of denaturation by Mirsky and Anson (274, 275) were sufficient to liberate all the disulfide groups of the proteins but insufficient to liberate all of the sulfhydryl groups (tables 1, 2, and 3). The total cystine–cysteine content of egg albumin is readily accounted for in terms of the sum of the cystine disulfide groups liberated by heat treatment and the cysteine sulfhydryl groups liberated not by heat (which would give too low a value) but by guanidine hydrochloride. Denaturation of serum albumin by acid acetone (and by urea) readily liberates disulfide groups corresponding to nearly the whole cystine–cysteine content of the protein; sulfhydryl groups are not liberated by these agents but are liberated by guanidine hydrochloride. The various polar groups of proteins apparently differ among themselves in the ease with which they are liberated on denaturation of the protein; some groups like disulfide are readily and fully liberated by denaturation procedures insufficiently strong to liberate fully all of the sulfhydryl groups. Such differences are of considerable significance and must be taken into account in any interpretation of the protein denaturation process.

(*b*) *Phenol and indole:* These groups are derived from tyrosine and tryptophan, respectively. They have weakly reducing properties in alkaline solutions and may be tested for by oxidants such as ferricyanide or the Folin reagent (277). They may be readily determined in proteins subsequent to oxidation of the sulfhydryl groups (71, 277). The rate of reaction of oxidants with the phenol and indole groups is considerably slower and requires a higher pH than the reaction of such oxidants with sulfhydryl groups (171, 277). The chromogenic power of intact proteins toward the Folin reagent is much less than would be expected from the known content of the proteins in tyrosine and tryptophan (184, 260, 375, 395). Study of peptides and of other derivatives of tyrosine (261) revealed that these substances gave in general a considerably lower chromogenic value with the Folin reagent than did equivalent amounts of tyrosine. As far as the Folin reaction is concerned, therefore, it is not known whether the low chromogenic values of the intact protein are due to the lack of availability of the groups to the reagent (characteristic of the native state of the protein) or to the effect of substitution of the reactive amino acids within the polypeptide structure. A clearer picture is obtained when the protein is denatured, for these groups are more readily determined, i.e., there appears to be a dependence of the chromogenic power upon the completeness with which the protein is denatured (261). That not all of the phenolic groups of the native protein are available to

estimation receives support from the failure to observe all of these groups, known to be present in proteins, by electrometric titration procedures (111). The available evidence suggests that whereas some phenol and indole groups may be evident in the native protein, the number of these groups rises when the protein is denatured. There is, however, a lack of precise quantitative data on this point.[3]

(*c*) *Peptide linkages:* The proteinases like papain, trypsin, and chymotrypsin, which are active within a pH range at which few proteins are denatured (pH 5–8), are capable of splitting rapidly synthetic peptides of definite structure (57). When these enzymes act upon the complex globular proteins such as egg albumin, serum albumin, lactoglobulin, or hemoglobin, the rate of hydrolysis is relatively slow. These proteins are presumably in the native form within the pH range studied. When such proteins are denatured prior to treatment with the enzymes, the rate of reaction is much more rapid (6, 64, 234). In order to account for the relative resistance of the *native* proteins to attack by proteinases, it has been suggested by Linderstrøm-Lang (231) that the unique structure of the native protein renders the peptide bonds unavailable to the action of the enzymes. This concept puts the peptide bonds in the same category as other groups like sulfhydryl, etc., which within the native protein are also relatively unavailable to specific reagents. The initial reaction whereby the native protein is prepared for the hydrolytic action of the proteinase involves a denaturation of the protein. That the enzyme itself may induce the preliminary denaturation of the native protein substrate, prior to subsequent hydrolytic action, appears to be probable (246). Ultracentrifugal and electrophoretic experiments revealed that active native papain added to solutions of native thyroglobulin catalyzed the change of the latter into a denatured form prior to its hydrolytic fission. In substantial agreement with Linderstrøm-Lang's viewpoint (231), Lundgren states that "an initial nonenzymatic conditioning reaction occurs before the enzymatic reaction. This involves a structural change in the native protein which presumably liberates groups to serve as points of attack for the enzyme" (246). The inactivated denatured enzyme has no effect on denaturing the native protein substrate. The influence of "reversal" of denaturation on the susceptibility of the protein to enzymatic hydrolysis will be considered in Part IV.

10. Liberation of groups in relation to changes in the physical and biological properties of proteins

Since the liberation of certain reactive groups is not the only criterion of the denatured state, it is of interest to inquire about the existence of any relation

[3] *Note added February 1, 1944:* Further evidence on this particular phase of the problem is obtained from recent observations on the absorption by egg albumin and by insulin of ultraviolet light at the range of wave lengths 2800–3000 Å. (120). Within this wave-length range, the phenolic group of tyrosine absorbs strongly. When the proteins are in the native state, the absorption is minimal, but as the proteins are progressively denatured by alkali the absorption correspondingly increases until at about pH 13 the extent of absorption in solutions of insulin, but not of egg albumin, may be entirely accounted for in terms of the content of tyrosine in the former protein.

between chemical reactivity and certain physical and biological differences between native and denatured proteins.

(a) Molecular weight

Solution of the following proteins in 6.6 *M* urea produces a diminution of the molecular weight (from that in water alone): horse hemoglobin to one-half (86, 374, 415); edestin, excelsin, and amandin to one-sixth to one-eighth (81, 86); *Limulus* hemocyanin to about one-twelfth (84); tobacco mosaic virus to about one-sixtieth (371); myosin to about one-tenth (401); and myogen to one-half (154). In concentrated urea solutions, the following proteins liberate sulfhydryl groups: hemoglobin (161), edestin, excelsin (161), copper-free hemocyanin (84), tobacco mosaic virus (371), and myosin (168); amandin does not (161). The observed changes in the molecular weight occur, however, in urea concentrations which are far below that necessary for the liberation of the maximum number of sulfhydryl groups in the proteins, and which barely suffice to liberate even a few.

Solution of the following proteins in urea under the same conditions as above produces no change in the molecular weight of the proteins: egg albumin (86), serum albumin (80), gliadin (83), zein (81), and pepsin (374). In urea solution, egg albumin liberates sulfhydryl groups (160), and the serum proteins and gliadin liberate disulfide groups (83, 192).

Changes in the sedimentation or diffusion rate of the serum proteins when treated with arginine (378), clupeine (379), and relatively low concentrations of guanidine hydrochloride (294–297) have been reported. The former two reagents do not cause liberation of sulfhydryl groups in these proteins, and the last-mentioned reagent was employed at too low a concentration to liberate such groups. At the present time there does not appear to be any obvious correlation between the liberation of groups and the changes in molecular weight of the denatured proteins.

(b) Double refraction of flow

Myosin is characterized by highly asymmetric molecules which confer upon solutions of this protein the properties of streaming birefringence and structural viscosity (133). In the native state, this protein also contains free sulfhydryl groups. Various agents act upon the groups and upon the physical properties, as follows (133, 168): (*1*) High concentrations of guanidine hydrochloride, 6–12 *M*, strongly diminish the viscosity and intensity of streaming birefringence and likewise liberate the maximum proportion of sulfhydryl groups. (*2*) Low concentrations of guanidine hydrochloride, about 0.3 *M*, strongly alter the physical properties as in (*1*) but have no effect upon the liberation of sulfhydryl groups. (*3*) Relatively low concentrations of glycine and of the ammonium salts reduce the free titratable groups of the native myosin to zero; ammonium salts simultaneously diminish the double refraction of flow, but glycine leaves the latter property unaffected. Oxidation of the free sulfhydryl groups of the native protein does not affect the intensity of the birefringence. (*4*) In low concentration

salts such as potassium iodide, potassium thiocyanate, magnesium chloride, calcium chloride, and lithium chloride have no effect on the titratable groups of the native myosin but cause a rapid change in the physical properties of the latter.

Thus, substances which change the physical properties in myosin may increase the titratable sulfhydryl groups, may abolish them, or may leave them unaltered. The concentrations of reagent needed to alter the physical properties or to abolish the sulfhydryl groups are very much smaller than the amount needed to liberate the latter groups. This is analogous to the effect of urea in reducing the molecular weight of several proteins; the effective concentration of urea is considerably below that at which appreciable properties of sulfhydryl groups are liberated. Here, again, there is no obvious correlation between the liberation, disappearance, or maintenance of the sulfhydryl groups of myosin and the changes in the physical properties of this protein on denaturation.

(c) Viscosity

The globular proteins such as egg albumin (75), the serum proteins (15, 295–297, 300), and the liver nucleoproteins (169) all possess a higher viscosity in urea solutions than in water. It has been suggested that this increase in viscosity is due to an unfolding of the protein whereby the shape of the latter becomes more asymmetric (297, 300). At the concentrations of urea employed, few sulfhydryl groups if any are liberated in egg albumin and none at all in the serum or liver proteins; disulfide groups are very probably liberated in the first two.

Solutions of myosin (133) and of the tobacco mosaic virus (146) are considerably less viscous in urea solutions than in water, and at concentrations of urea well below that necessary to liberate sulfhydryl groups in the protein. The change in the physical properties of these proteins has been interpreted as a disaggregation of the latter into smaller, less asymmetric particles (133). The increase in osmotic pressure of the myosin (401) and increase in the translational diffusion constant of the virus (371) support this concept.

(d) Solubility

The coagulation of many proteins may be accompanied by the appearance of various groups (table 1). These groups may, however, also appear, and frequently in greater proportion, in proteins dissolved in solutions of urea or of guanidine hydrochloride; hence loss of solubility need not necessarily accompany the denaturation process. It is true that these agents possess a dispersive as well as a denaturing effect, and that on removal of the agent (by dialysis or dilution) part or all of the protein precipitates. In the case of myosin, the precipitate may be completely redissolved in salt solution even though the birefringence is irreversibly lost and the protein completely denatured (133). In the case of the serum proteins, removal of the denaturant causes the precipitation of a part of the total protein, part remaining in the now purely aqueous solution (295–297). Extensive physicochemical studies on this soluble fraction have revealed that certain physical properties of this protein are different from

those of the native protein (295–297). Therefore, it may be stated that whereas a coagulated protein is always a denatured protein (even if not completely), a soluble protein is not necessarily a native protein.

(e) Biological activity

Hemoglobin: At concentrations of urea and other amides which barely suffice to liberate sulfhydryl groups in the globin moiety (161), horse hemoglobin, while dissociated into half-molecules, is still able to combine with oxygen (374). It would be of interest to see whether more powerful agents such as the guanidine salts would not destroy the activity of the hemoglobin. Catalase, which possesses the same hemin group as hemoglobin, is instantaneously inactivated by 2 *M* guanidine hydrochloride (167), whereas papain is active even in 9 *M* urea (234).

Tobacco mosaic virus: The infective power of this protein is quite rapidly destroyed, with loss of its birefringent properties, at concentrations of urea and of guanidine hydrochloride which are too low to liberate appreciable amounts of sulfhydryl groups (52, 53, 371). At equivalent concentrations, guanidine hydrochloride is more effective in destroying the infectivity than is urea. The loss in infectivity is definitely unrelated to the appearance of sulfhydryl groups, for the former effect may be brought about by glycerol and by pyridine, which have no other known denaturing capacities.

Insulin: Aqueous solutions of pure amorphous insulin, concentrated by negative pressure dialysis to a concentration of about 2 per cent, do not reveal the presence of disulfide groups in the protein (cyanide–nitroprusside test). When the solutions are treated with high concentrations of urea or of guanidine hydrochloride these groups are readily observed (167). Dialysis of such solutions of the denatured proteins results in complete precipitation of the latter. Injection into rabbits of these insoluble proteins suspended in saline, or of solutions of the protein in concentrated urea, gives exactly the same biological effect *at the same rate* as does an equal amount of the original unaffected protein dissolved in water before injection (167). It has been reported that insulin denatured by spreading as a surface film is also completely active biologically (348). The process of denaturation of insulin, leaving the molecule intact but probably changing its shape and the reactivity of certain chemical groups, does not affect the biological specificity of the protein. The possibility exists, however, that the denatured protein may be converted *in vivo* either into something similar to the native form or to some other configuration which is biologically effective.

Pepsin: The activity of this enzyme is apparently unchanged in urea, as compared with aqueous solutions (374). No sulfhydryl groups are liberated; information on the possible liberation of other groups is not available.

Urease: This protein, like myosin, possesses free sulfhydryl groups when in the native state, and the proportion of these groups increases in both proteins when the latter are treated with guanidine hydrochloride (181). Oxidation of the free groups of native urease and of native myosin by porphyrindin produces neither a loss of the enzymatic activity of the former nor a decrease in the bire-

fringence of the latter. At high concentrations of guanidine which liberate sulfhydryl groups in either protein, the enzymatic activity of urease and the birefringent properties of myosin disappear. Here is a case in which a "globular" protein, urease, and a "fibrous" protein, myosin, behave similarly on denaturation. It would be of interest to see whether relatively low concentrations of guanidine hydrochloride, insufficient to liberate sulfhydryl, will destroy urease activity in the same manner as such concentrations are effective in inactivating the tobacco mosaic virus and in reducing the specific physical properties of myosin.

11. General considerations

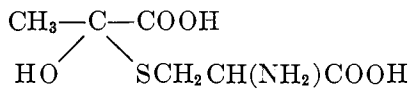
It is clear that certain chemical groups within the intact *native* protein molecule are unreactive toward specific reagents for these groups, and only become reactive, or "liberated", when the protein is *denatured.* The lack of reactivity of these groups when the protein is in the native state may be explicable on the following grounds: (*a*) that these groups within the protein exist in forms quite different from those found in the free amino acids and are only converted into the latter by the chemical transformations incident to the denaturation process; (*b*) that these groups actually exist within the protein in the same forms as those found in the free amino acids, but that the rigid steric configuration of the native form of the protein renders these groups inaccessible to their specific reagents; when the protein is denatured, and a more nearly random distribution of the group occurs, the latter are readily available to their reagents.

The weight of present evidence definitely favors alternative (*b*). Various kinds of denaturation of most if not all proteins are accompanied by an unfolding of the molecule (35, 73, 268, 278, 300), and in the course of this unfolding the chemical groups of the protein would be expected to be exposed. The further implication would be that these groups participated in the folded configuration of the native protein. The latter may be considered to be composed of bundles of polypeptide chains which are held together in the unique configuration of the native state of the protein by attractive forces of a relatively weak character. The polar groups on the side chains of the polypeptides participate in these bindings, and it is precisely these polar groups which are therefore unreactive toward specific agents when the protein is native; the denaturation process, by dissolving the loose bonds between the polar groups of adjacent polypeptide chains, leaves these groups available and reactive to the chemical agents specific for them. The nature of the loose bonds between the polar groups is at present unknown, although certain theories have been advanced (30, 95, 278).

The most completely studied of the polar groups in proteins which are liberated on denaturation of the latter is the sulfhydryl group. That this group exists as such within the native protein, according to alternative (*b*) above, is highly probable from the evidence of Anson (8). The latter observed that although native egg albumin was unreactive toward nitroprusside it would react with iodine, whereby all the sulfhydryl groups of the protein were oxidized. Further striking evidence was advanced by Anson and Stanley (19), who showed that

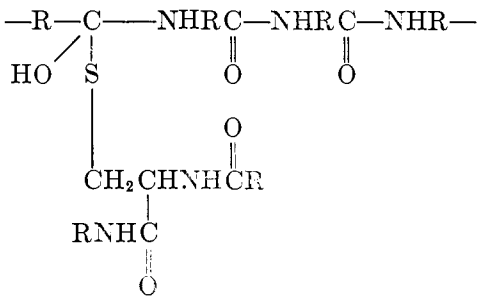
tobacco mosaic virus protein in the native state, unreactive to nitroprusside, could be treated carefully with iodine, whereby the sulfhydryl groups of the protein were oxidized without loss of infective power of the virus. What known chemical groupings involving sulfhydryl are unreactive to nitroprusside but reactive to iodine?

Cysteine may combine with pyruvic acid to form an addition compound in which the sulfhydryl group residue reacts stoichiometrically with iodine, yielding disulfide, but does not react at all with nitroprusside (355). The structure of this hypothetical compound may be represented in formula I:



I

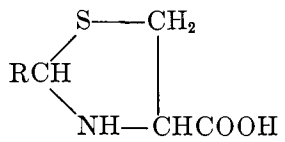
In proteins an analogous formulation may be tentatively considered, in which the sulfhydryl groups of the cysteine constituent of one polypeptide chain combine with the ketonic portion of a peptide linkage in an adjacent polypeptide chain as in formula II:



II

The critical test for the possible validity of formula II would be to observe whether the weak binding between ketone and sulfhydryl could be dissolved by solutions of guanidine salts or related compounds.

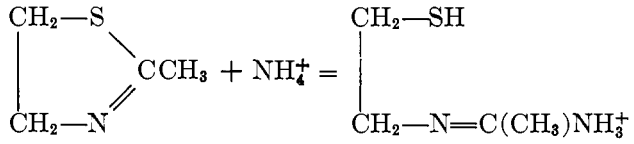
Cysteine may also condense with aldehydes, with the loss of a molecule of water, to form thiazolidines (335, 355), as in formula III:



III

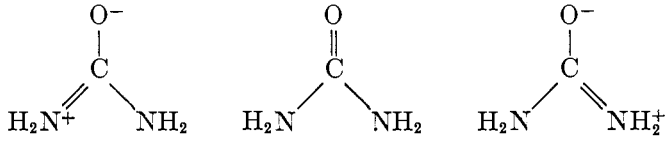
These compounds also react stoichiometrically with iodine and fail to give a nitroprusside test. The possibility that such ring structures, analogous to the oxazoline rings suggested earlier by Bergmann *et al.* (56, 59), may exist within the native protein structure was advanced by Linderstrøm-Lang. In an im-

portant paper (233), the latter demonstrated the essential lability of the thiazolidine ring toward ammonium salts, whereby the ring was opened in the presence of the latter, probably in the following manner:



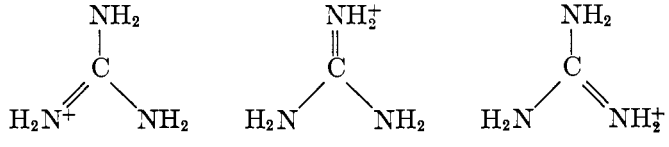
Furthermore, the ease of opening the ring was a function of the nature of the anion of the ammonium salt used and increased in the sequence sulfate, chloride, bromide. The same sequence was found in the effect of various guanidine salts in the liberation of sulfhydryl groups in egg albumin (162). The difficulty in applying these results to the problem of protein denaturation lies in the fact that the pure guanidine salts, and also urea, fail to open the thiazolidine ring. However, as Linderstrøm-Lang pointed out, only the simplest thiazolidine, the 2-methyl compound, was employed, and the further study of this effect would require the examination of more complex representatives of this class of substances.

The extent of the protein-denaturing effect which the various *N*-substituted ureas and guanidines exert has been related to the capacity of the latter to exist in several resonating structures which are in equilibrium with one another (160). Urea, for example, may be represented as resonating among the following three structures:



Replacement of hydrogen by methyl, as in *N*-methylurea, might conceivably tend to hinder the double bond from migrating to the methylated nitrogen, owing to the greater electronegativity of carbon over hydrogen. With asymmetric *N*,*N'*-dialkyl substitution this hindrance might be expected to be somewhat greater. A very much greater hindering effect would be expected for symmetrical *N*,*N'*-dialkyl substitution, for here the resonance of the double bond would be considerably restricted, and the neutral form (the center one above) would be much more important than the other two, charged, forms. The methylated ureas sometimes are weaker than urea in denaturing the proteins (table 4), and symmetrical *N*,*N'*-diethylurea possesses no denaturing effect at all (192).

The guanidinium ion resonates among the following structures:



Substitution of a methyl group in this ion produces no difference in the denaturing effect of the parent ion on certain proteins, and reduces this effect with other proteins (table 4). The *as*-dimethylguanidinium ion, in which resonance may be expected to be still more restricted, actually shows a distinctly smaller denaturing effect on the proteins than either the unsubstituted or the monosubstituted ions (table 4). An interesting series of experiments in harmony with this, as yet quite tentative, hypothesis has been described by Dickens (126). The latter observed that the Pasteur reaction in brain cortex could be inhibited by guanidine salts, and that the maximum inhibition was brought about by guanidine and methylguanidine, whereas *as*-dimethylguanidine produced little effect.

In the light of Anson's results (8) and of suggestive evidence from simpler molecules, the presence of preformed sulfhydryl groups within the native protein may be taken for granted. It is *surprising*, however, that many authors assume that the *sulfhydryl groups* which appear in the denatured protein owe their *origin* to hydrolytic fission of *disulfide linkages* (*cf.* 140), an assumption which probably had its origin in Hopkins' classic paper (192). The evidence against this assumption is considerable and may be given briefly as follows: (*a*) No synthetic peptide of cystine treated with solutions of urea or guanidine salts shows the presence of sulfhydryl; the S—S bond is completely inert to these protein denaturants (160). (*b*) Proteins such as amandin and insulin, the latter high in cystine, when denatured give no reactions for sulfhydryl groups. (*c*) All the non-methionine sulfur of myosin (168), and after denaturation of the proteins all the sulfur of the tobacco virus protein (346), can be accounted for in terms of cysteine; if the reaction were

$$\text{—S—S—} + H_2O = \text{—SH} + \text{SOH}$$

as assumed, then only half instead of all the "cystine" sulfur could be obtained in these proteins as cysteine. (*d*) If disulfide groups were converted wholly or in part to sulfhydryl by protein-denaturing agents it would be quite impossible to oxidize quantitatively the sulfhydryl groups of the denatured protein to disulfide groups in the presence of the denaturing agent; since this procedure is now commonly used, the suggestion is of course untenable.

B. THE KINETICS AND THERMODYNAMICS OF PROTEIN DENATURATION

Study of the kinetics of denaturation may give indirect evidence for protein structure as well as for the mechanism of denaturation. From the experimental determination of the order of the reaction one may hope to distinguish whether denaturation is an intramolecular or an intermolecular transformation. From the magnitude of the classical energy of activation or critical increment, or, in terms of the newer theory of absolute reaction rates, from the free energy of activation and the entropy of activation, one may estimate the number and nature of the bonds broken in the disruption of the specific native configuration. This information may then be used in the derivation of a theory of denaturation or in a test of existing theories.

However, this prospect may not be fully realized as yet because of the paucity of the data now available and because of the consistent occurrence of kinetic anomalies shortly to be described.

1. Experimental methods for following the course of the reaction

The familiar example of heat coagulation early led to the study of the effect of temperature on proteins. It was long believed that the temperature of heat coagulation, or, in the case of biologically active proteins, the temperature of heat inactivation, though somewhat influenced by ionic environment, was a physical constant characteristic of the particular protein. However, in view of later findings that heat coagulation and inactivation are measurable and not instantaneous processes, the voluminous literature on this subject may be disregarded. It will be shown later that the apparent existence of a critical temperature of denaturation arises from the high temperature coefficient characteristic of the reaction.

The discernment of the various processes involved in heat coagulation may be ascribed to Hardy (175), who first advanced the now generally accepted view that heat coagulation consists of two distinct stages. In modern terminology these are (*1*) the initial thermal denaturation,—conforming to the definition already proposed in this review; (*2*) the actual coagulation,—a colloidal phenomenon less significant for the study of the mechanism of denaturation.

Other modes of denaturation may likewise involve a subsequent step of coagulation, e.g., surface denaturation (78) and denaturation by ultraviolet irradiation (107). However, coagulation may be averted by the use of denaturants which exert a dispersive action, for example, urea and guanidine hydrochloride. Confusion has evolved from the fact that the term "coagulation", when applied to proteins, has often been used to signify both the precipitation and the preliminary denaturation.

In the measurement of the reaction kinetics of protein denaturation it is important to isolate the actual process of denaturation so that its velocity is the rate-determining step of the over-all reaction. Yet, since the appearance of a coagulum is the most tangible evidence of denaturation, the measurement of the rate of formation of insoluble protein (or conversely, the rate of disappearance of soluble protein) has been used commonly to describe the kinetics. Under conditions which facilitate rapid flocculation, that is, in the presence of a small amount of neutral salt, the initial reaction of actual *denaturation* may assume the rate-determining rôle, so that the concentration of residual protein in the supernatant solution becomes equivalent to that of unchanged protein.

This was the experimental method introduced by Chick and Martin (96–100) and commonly adopted by subsequent workers. Turbidimetric measurement of the salt-free protein denatured at its isoelectric point is an analogous procedure, in that flocculation proceeds most rapidly at the isoelectric point but differs in the empirical nature of the method of measurement of the quantity of insoluble protein (107). Certain other methods of following the course of the reaction, for example, the notation only of the apparent time of first formation of a visible precipitate (148), at best may be considered only semiquantitative.

The measurement of the change in any singular property of a protein, such as the inactivation of an enzyme or virus, affords another criterion for observing the course of reaction. The residual activity may be taken as directly proportional to the amount of unchanged protein. Where reversal of inactivation is claimed, appropriate steps may be taken to halt this process. While it is true that the thermal inactivation of a virus may proceed at a different rate from the thermal change in other properties of the same substance (223), it is unnecessary to review again here our brief that denaturation may consist of a series of concomitant changes any one of which may be used as a criterion. Furthermore, the fact that protein denaturation and enzyme inactivation possess in common a high energy of activation evinces their relationship.

2. The order of the reaction

As a result of the classical experiments of Chick and Martin (96–100) the belief that heat denaturation is a first-order reaction has become firmly implanted in the literature and has been extensively drawn upon as support for the thesis of the intramolecular character of protein denaturation. However, it is now opportune to review this question.

It will be recalled that in a first-order reaction the rate is directly proportional to the concentration of the reacting substance (149). Similarly, in a reaction of the second order the rate is proportional to the product of two concentration terms, or to the square of one, and in a zero-order reaction it is independent of the concentration. Thus, the *order* is given by the number of molecules whose concentrations determine the speed of the process. The molecularity, however, refers to the number of molecules taking part in the act leading to chemical reaction. The order and molecularity frequently prove to be identical, but, for clarity, reactions whose rate has been shown to be empirically dependent upon but one concentration term will be hereinafter designated as of the first order. Since these terms were once considered synonymous, protein denaturations following a first-order law have often been reported to be uni- or mono-molecular in nature.

With a few exceptions, protein denaturation and biological inactivation have been reported to be first-order processes. Already in 1895 Tammann (382) had found the heat destruction of emulsin, both in solution and as a dry powder, to be a first-order reaction between 60° and 75°C., the data for the temperature range 40–60°C., however, apparently deviating from this law. Colleagues of Arrhenius next reported that the heat inactivation of trypsin, pepsin, and rennet all followed a first-order law (23), as did the diminution in hemolytic activity of unpurified vibriolysin, tetanolysin, and goat serum (141).

The first rigorous kinetic studies of the heat denaturation of crystalline proteins were those of Chick and Martin (96–100), who concluded that the heat denaturation of hemoglobin and of egg albumin at constant hydrogen-ion concentration was a first-order reaction. For hemoglobin, this result has since been verified by Hartridge (178) and by Lewis (226). However, the results for egg albumin are not unambiguous, and in view of the importance subsequently attached to these experiments, the data merit review.

Protein denaturation

(*a*) *Egg albumin:* In their first paper Chick and Martin reported that "heat coagulation" of unbuffered egg albumin solutions did not conform to a simple logarithmic (first-order) law (96). After recalculation of their data, Sutherland (376) concluded that the reaction was of the second order. Chick and Martin (97) pointed out that this was true only if the time of placing the egg albumin solution into the thermostat was taken as the initial time, the period of warming up being neglected. They then showed that in a solution of constant acidity (saturated boric acid) (97) or constant alkalinity (saturated magnesium oxide) (98) the heat denaturation of egg albumin was satisfactorily a first-order reaction. However, in unbuffered solutions in the presence of concentrated salts, denaturation "proceeded in extraordinary agreement with the progress of a reaction of the second order" (97). This behavior was considered adventitious and attributed to changing hydrogen-ion concentration.[4] Subsequently, Lewis (227) found that a steady first-order constant was obtained in buffered solutions but, since the rate was much enhanced, reported only observations for unbuffered solutions for which a first-order velocity "constant" was obtained by a method of approximation.

It should be pointed out that this ambiguity with regard to the order of reaction for the heat denaturation of egg albumin is reflected in calculations of the critical increment and of the free energy and entropy of activation. For example, in the heat denaturation of egg albumin in 2 *M* ammonium sulfate (97), second-order constants (which exhibit excellent agreement and were used by Chick and Martin in the calculation of the temperature coefficient) yield a value for the energy of activation 19,000 calories greater (333) than that apparently obtained by the utilization of unsteady first-order constants (140). A similar criticism applies to the calculations of Lewis (227).

(*b*) *Hemoglobin:* Despite the ambiguity which surrounds the rate law governing the classical heat denaturation of egg albumin, it must be acknowledged that hemoglobin denaturation appears to obey a first-order law. This is true not only of heat denaturation, but holds also for the alcohol denaturation of hemoglobin (68), and apparently for the acid denaturation of the same protein (121). In contrast to the results reported for egg albumin (97), the presence of concentrated salts does not appear to change the order of reaction in the heat denaturation of hemoglobin (228).

(*c*) *Other proteins:* However, the denaturation of other proteins is not uniformly a first-order reaction. The heat denaturation of leucosin is of the first order (243), and it has been stated that the heat denaturation of insulin is likewise of the first order (148), although the velocity constant was observed to fall off for a sample of even thrice-crystallized protein. On the other hand, the well-investigated kinetics of the acid denaturation of crystalline edestin (39) may be described by either (*a*) an equation representing a reaction of high order or (*b*)

[4] The denaturation of egg albumin by sodium hydroxide at 25°C. follows a "roughly bimolecular course," the pH falling steadily during the reaction (87).

the existence of two or more simultaneous unimolecular (i.e., first-order) reactions. In fact, an empirical equation was derived which expressed the rate of formation of edestan, the product of denaturation, as a function of the rates of four independent and simultaneous exponential reactions.

Biological inactivation

A first-order law is valid for the heat inactivation of a number of enzymes, e.g., pepsin (23, 231, 304), trypsin (23, 302, 311), pancreatic lipase (251), emulsin (382), and peroxidase (418). However, in the heat inactivation of malt amylase the course of reaction is certainly not of the first-order type, and though second-order constants are more satisfactory, they likewise show deviations (244). Similarly, the heat inactivation of invertase is not of the first order, the constants falling off to one-half the initial value even in phosphate-buffered solutions (139). Furthermore, while the heat inactivation of the plant proteases papain and bromelin follows a first-order law (bromelin, however, deviating at higher temperatures), the heat inactivation of asclepain (the protease of the milkweed *Asclepias speciosa*) conforms to a second-order equation (407). The admitted presence of impurities in many of these preparations entails doubt both as to the true order of the reaction and as to the validity of calculations of activation energies by the use of unsteady rate constants. It is therefore of interest to compare the kinetics of denaturation and biological activation in the case of several well-characterized proteins.

(*a*) *Pepsin:* The heat inactivation of crystalline pepsin is of the first order (241, 304). This is also true of the inactivation of crystalline pepsin by ultraviolet radiation (306) and of crude pepsin by the beta radiation of radium (195). Inactivation by weak alkali, first reported to follow a three-halves order (258) and then to consist of an instantaneous step followed by a first-order reaction (152), has recently been shown to be completely first order for crystalline pepsin (373).

(*b*) *Trypsin:* The heat inactivation of purified trypsin is evidently of the first order (302, 311), as is inactivation of the crude product by soft x-rays (103) and by the beta radiation of radium (195). However, between pH 2.0 and pH 9.0 Kunitz and Northrop found that the irreversible inactivation of crystalline trypsin obeys a second-order equation (214). This behavior was attributed to the existence of a simultaneous reaction involving the hydrolysis of denatured trypsin by active native trypsin. On the alkaline side of pH 13.0 and on the acid side of pH 2.0, the inactivation was found to be of the first order. Fair agreement with a first-order law was observed by Pace for the heat inactivation of various purified preparations of "trypsin" and associated enzymes (311, 312, 313, 314).

(*c*) *Tobacco mosaic virus:* Sufficient data are available for a comparison of the kinetics of thermal denaturation and urea denaturation only in the case of tobacco mosaic virus nucleoprotein. The measurements are complicated by the accumulation of nucleic acid in the supernatant solution as a result of the disintegration of virus protein. In the case of the thermal denaturation (223), for

the majority of the experiments there is little choice between a first-order or second-order or even a zero-order reaction, although the first-order equation is favored by statistical analysis of the data. However, in a critical experiment the error inherent in the accumulation of nucleic acid was averted by measurement of the amount of soluble protein capable of becoming insoluble on prolonged boiling. This method of analysis strongly favored a first-order law. An analogous experiment in the case of urea denaturation gave the same result (221). As in all experimental methods based upon an analysis of the residual soluble protein, this approach involves the tacit assumption that the rate-determining step is not the flocculation or, in this instance, the disintegration of the denatured protein. As yet unexplained is the fact that the reaction velocity was observed to vary inversely with the initial virus concentration.

The denaturation of the purified virus upon exposure to high pressure (7500 kilos per square centimeter) likewise appears to be a first-order reaction, although obedience to a zero-order equation has not been rigorously excluded (222).

It has been reported that the thermal inactivation rate of unpurified tobacco mosaic virus, as well as of several other viruses, follows the course of a first-order reaction (329). Inactivation appears to proceed at a considerably faster rate than thermal denaturation and is believed to be one of the earlier reactions in a series which leads to denaturation (223).

Two modes of denaturation the kinetics of which have been inadequately studied are ultraviolet irradiation and surface denaturation. The incidence of an initial step of light denaturation preparatory to the steps of temperature-dependent denaturation and flocculation has been postulated (106–108). The initial process is reported to be of the first order for egg albumin. Surface denaturation, however, appears to be a zero-order reaction, the rate being independent of protein concentration (78, 79, 412).

Critical analysis of the abundant literature on the kinetics of protein denaturation reveals that there are few studies which satisfy the minimal criteria justifying theoretical analysis: namely, (*a*) highly purified, preferably crystalline preparations as reactants; (*b*) adequate experimental methods. It is known that the presence of impurities may affect even the order of reaction (251, 302, 407), a fact not recognized by many early investigators. The justification for the second criterion is self-evident.

When the kinetic data are considered on the basis of the number of crystalline proteins subjected to various modes of denaturation and studied by rigorous experimental methods, it must be concluded that the denaturation of proteins is not unequivocally a first-order reaction.

It may be argued that most deviations from a first-order law may satisfactorily be explained by the assumption of the existence of simultaneous reactions or of other processes whose rates are not instantaneous relative to the denaturation reaction. Inasmuch as denaturation exhibits a procession of stages, a stepwise nature of the reaction mechanism may not be gainsaid. Each one of the steps subsequent to flocculation may be of the first order or, indeed, unimolecular, but, on the other hand, the experimental methods at our disposal do not enable us to

discern the kinetics of a given step, and for a kinetic description of the process one must still rely upon the empirical dependence of the reaction velocity upon the concentration of reaction substance, i.e., the order of the reaction.

3. The molecularity of the reaction

The *a priori* acceptance of a first-order law as the factor governing the kinetics of general protein denaturation has led to the assumption of a unimolecular process of denaturation involving an intramolecular change in the intrinsic configuration of the protein molecule. The validity of this hypothesis with regard to the data derived from non-kinetic sources will be discussed in Part III. While such a mechanism does seem to be in best accord with the facts now available, the significance of water and of the concentrations of hydrogen and hydroxyl ions should not be discounted. In fact, each of these three substances has been suggested as a reactant.

It is known that a dry preparation of egg albumin is not readily susceptible to heat denaturation (41, 63, 96). Indeed, a dry protein may be exposed to temperatures exceeding 120°C. without undergoing a change in solubility (96). This observation early led to the suggestion that denaturation was a reaction taking place between protein and water (96, 97). It is notable that the kinetics of the heat denaturation of dry egg albumin do not follow a first-order law (63). Unexplainably, this denaturation resembles an autocatalytic reaction.

In addition to the rôle of water, an extraordinary catalytic influence of hydrogen and hydroxyl ions on heat denaturation and enzyme inactivation has long been known. The great majority of such processes are highly sensitive to pH, a variation in pH of one unit often being accompanied by a hundredfold change in rate. In some instances the rate is strictly proportional to some inverse or direct power of the hydrogen-ion concentration, indicating that the formation of a charged species is requisite for activation.

4. The abnormalities of protein denaturation kinetics

The most striking characteristic of the kinetics of protein reactions is the extraordinary dependence of the rate of *thermal* denaturation upon temperature. At that temperature at which coagulation or inactivation is first observed (about 50°C.), the inception is so marked that there early arose a characterization of proteins in terms of "coagulation temperatures". Raising the temperature a few additional degrees may cause the velocity to become immeasurably fast. This fact precludes rate studies over a temperature range comparable to that employed in ordinary kinetics (about 70°C.), the temperature range in the case of thermal denaturations often being limited to but 2–3°C.

The effect of temperature upon rate may be expressed approximately by the *temperature coefficient*, Q_{10}, the ratio of the velocity constant at the temperature $t + 10°$, to that at $t°$ (i.e., k'_{t+10}/k'_t). Whereas, for ordinary processes Q_{10} is about 2 to 3, that is, the velocity doubles or trebles for each 10°C. rise, temperature coefficients in excess of 600 have been reported for protein denaturations (96). This abnormality has greatly stimulated the investigation of the kinetics

of protein denaturation, for such an effect of temperature may not be explained by known processes for the redistribution of energy between molecules.

A more satisfactory method of expressing the temperature dependence of reaction velocity is given by the Arrhenius equation:

$$\frac{\mathrm{d} \ln k'}{\mathrm{d}T} = \frac{E}{RT^2} \tag{1}$$

which upon integration over the temperatures T_1 and T_2 assumes the form

$$E = \frac{RT_2T_1}{T_2 - T_1} \ln \frac{k'_2}{k'_1} \tag{2}$$

E is the *critical increment* or the *energy of activation*, k'_2 and k'_1 are the respective velocity constants,[5] and R is the molar gas constant; the dimensions of E are calories per mole. Equation 2 indicates the existence of a logarithmic relationship between the temperature coefficient and the energy of activation. For reactions conforming to the ordinary laws of chemical kinetics the value of E at 50°C. is of the order of 20,000 calories ($Q_{10} = 2.6$). For protein denaturation and enzyme inactivation, values of E ranging in magnitude up to 198,000 calories (141) have been reported (*cf.* tables 7 and 8).

On the basis of the simple collision theory of kinetic activation it is not possible to explain the observable rates of reaction without the assumption of astronomical values of the steric factor. The latter quantity is introduced to accommodate the deviation from experiment and is supposed to allow for the specific orientations of the molecules necessary for a fruitful collision. For example, if the velocity constant be given by the expression

$$k' = PZe^{-E/RT} \tag{3}$$

(in which P is the steric factor, and Z the collision number), a magnitude of P of the order of 10^{72} (216) must be assumed in the thermal denaturation of egg albumin, for which E is reported to be of the order of 140,000 calories. This is in contrast to the ordinary value of P, which usually varies from unity to 10^{-8}, though higher values have been reported (149).

5. The theory of absolute reaction rates

Such exceedingly large energies of activation as those given in tables 7 and 8 and the correspondingly large temperature coefficients have stimulated interest in the mechanism of these reactions, and the resolution of the kinetic paradox has been attempted by two different methods of approach. The school endorsing the theory of absolute reaction rates has proposed (140) that the free energy of activation, $\Delta F^{\ddagger}$, rather than E, governs the rate of the reaction. In such a formulation, the steric factor or probability factor, P, is shown to involve an entropy term, and, indeed, formally P is related to $\Delta S^{\ddagger}$, the entropy of activa-

[5] We use k' to denote the reaction velocity constant, since k, the Boltzmann constant, is introduced in the statistical definition of k'.

tion, for $P = e^{\Delta S^{\ddagger}/R}$ (216). Moreover, E, the critical increment or "experimental activation energy" obtained by substitution in the Arrhenius equation, is only approximately equal to the "heat of activation", $\Delta H^{\ddagger}$, for $\Delta H^{\ddagger} = E - RT$ (372). (At 50°C., RT equals approximately 640 calories.)

In place of the collision number, Z, a factor kT/h, having the dimensions of frequency, may be employed to represent the rate at which the activated molecules cross over the energy barrier (150). The quantity kT/h is a universal frequency dependent only on temperature, k being the Boltzmann constant and h being Planck's constant. The frequency factor PZ (equivalent to the constant A of the Arrhenius equation) is approximately equal to

$$\frac{kT}{h} e^{\Delta S^{\ddagger}/R}$$

the inequality arising from the fact that $\Delta H^{\ddagger}$ is not quite equivalent to the experimental activation energy, E.

The rate may now be expressed (140, 150) in terms of the thermodynamic functions $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta F^{\ddagger}$ (the transmission coefficient being taken as unity).

$$k' = \frac{kT}{h} e^{-\Delta F^{\ddagger}/RT} = \frac{kT}{h} e^{-\Delta H^{\ddagger}/RT} e^{\Delta S^{\ddagger}/R} \tag{4}$$

If the thermodynamic functions are known, this equation enables the calculation of the absolute rate of reaction. Conversely, the function $\Delta S^{\ddagger}$ may be calculated by substitution of the observed values of k' and $\Delta H^{\ddagger}$ (obtainable from E), $\Delta F^{\ddagger}$ may be derived by analogy to the well-known relationship $\Delta F = \Delta H - T\Delta S$.

From equation 4 it is apparent that the rate is governed not by the activation energy alone, but also by the entropy of activation. In other words, the free energy of activation is decisive in establishing the rate. For reactions characterized by abnormally high values of E, a measurable rate may still be attained if a correspondingly large entropy of activation is observed. Protein denaturation must accordingly be distinguished by large entropy changes (140), a conclusion inducing considerable speculation as to the nature of these changes, which are assumed to be intramolecular.

The importance of the application of this elegant statistical treatment to protein reactions is that a generalized theory of denaturation may be derived from analysis of the kinetic data. While the validity of the derivation of this theory may not be impugned, its application should be circumspect, for (*1*) the great majority of the data on protein denaturation do not merit quantitative interpretation, both because of the questionable purity of preparations and because of the inadequacy of experimental methods, and (*2*) the empirical values of the activation energy obtained from the Arrhenius equation may yet prove to be illusory in many cases.

An alternative method of interpretation of the abnormally large values of E is offered by Steinhardt (373) and La Mer (216). In view of the extraordinary pH dependence of reaction rate exhibited by protein denaturation (*cf. infra*), these authors suggest that part of the apparent energy of activation arises from

the heat of dissociation of the acidic equilibria involved in preparing the molecules for the kinetic step of activation. The customary method of calculating E by comparison of rates at constant pH is shown to be fallacious, inasmuch as the operation of the temperature coefficient of dissociation likewise effects a variation in the concentration of the charged species subject to the activation process. The advantage of this viewpoint is that it obviates the necessity of assuming large entropy changes of an uncertain nature. The method of appraisal has so far been restricted to pepsin (216, 373). An analogous interpretation for the acid denaturation of hemoglobin is offered in this review.

6. Application of the theory of absolute reaction rates to protein denaturation

To aid in an evaluation of the significance of the experimentally observed values of the activation energy, typical data for protein denaturation are given in table 7 and for enzyme inactivation in table 8. Since most of the existing kinetic data have already been amply reviewed (140, 372), we have selected those studies most amenable to quantitative interpretation both for reasons of purity of preparations and for explicitness of the kinetics. Without impugning the validity of the transition theory, one may well question the value of its application to impure systems. Similarly, while obedience to a first-order law is not implicit in the derivation of the theory, it does facilitate the computation of the thermodynamic functions and the comparison of rates, since the first-order constant is independent of concentration units. Moreover, by excluding reactions of indeterminate order one obviates questions as to the legitimacy of the use of unsteady constants.

In table 7 the kinetic data for the denaturation of hemoglobin are summarized. The conditions of denaturation have been taken from the original literature. In some instances, k' (given always for the lower temperature cited) is the value obtained by direct interpolation of the data for neighboring pHs. Where the rate exhibits a strong pH independence, the velocity constant read off from the graph of k' or log k' *versus* pH may differ significantly from the former value. This may lead to a value of E different in the case of pepsin, for example, by as much as 7000 calories. The values of the thermodynamic functions, $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$, and $\Delta F^{\ddagger}$ are those given by Eyring and Stearn (140).

(*a*) *Hemoglobin*: The data may be summarized as follows: (*1*) In the neutral region, the rate varies markedly with pH, attaining a minimum at pH 6.76, but E decreases only insignificantly with pH, and $\Delta S^{\ddagger}$ and $\Delta F^{\ddagger}$ are essentially constant.

(*2*) At 6.76, the pH of minimum rate, the velocity constant at first increases to a maximum, then falls off with increasing salt concentration, while E rises continuously to a value of 120,000 calories, and $\Delta S^{\ddagger}$ nearly doubles. Ammonium sulfate displaces the minimum of the pH/k' curve to the acid side. It should be noted that Lewis (228) reports that if calculation of E is made from the minima of the pH/k' curves, or from the maxima of the ammonium sulfate/k' curves, a steady value of about 79,000 calories is obtained. The former method may err in that comparison is not made at the same pH; the latter, in that it is not made

at the same salt concentration. However, in light of the view that rate comparison should be made at equal concentrations of the active species, there may be justification for this procedure.

(*3*) Even small amounts of alcohol (2 per cent and 4 per cent) effect a marked increase in the speed of denaturation. With higher concentrations the temperature of the experiment must be lowered, lest the rate become immeasurably fast. In analogy to the result observed with increasing salt concentration, the

TABLE 7

Kinetics of the denaturation of hemoglobin

SOLVENT	pH	T	k' [a]	E_{Ar}	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$ [b]
		°C.	$sec.^{-1}$	*calories per mole*	*calories per mole*	*calories per degree-mole*	*calories per mole*
Water (226)	5.7	60.5–68	4.3×10^{-4}	76,200	75,600	152.7	24,700
	6.8	60.5–68	1.05×10^{-4}	76,900	76,300	152.0	25,600
	8.0	60.5–68	3.35×10^{-4}	77,900	77,300	157.3	24,840
0.001 *M* ammonium sulfate (228)	6.76	64–70	3.41×10^{-4}	77,300	76,500	152.5	25,100
1.14 *M* ammonium sulfate	6.76	64–70	5.45×10^{-4}	87,800	87,000	184.6	24,800
2.27 *M* ammonium sulfate	6.76	64–70	9.1×10^{-5}	104,600	103,800	230.9	26,000
3.03 *M* ammonium sulfate	6.76	64–70	2.44×10^{-5}	120,600	119,800	275.8	26,900
0 per cent alcohol [c] (68, 226)	6.5	60.5–68	1.22×10^{-4}	75,750	75,050	148.5	25,550
20 per cent alcohol	6.5	40–45	3.3×10^{-5}	103,300	102,600	248.8	23,500
30 per cent alcohol	6.5	30–33	9.5×10^{-5}	120,800	120,100	319.5	23,300
Acid (121)	4.85	37–45	8.0×10^{-5}	18,500	17,760	−19.9	23,900
	4.64	25–37	1.27×10^{-4}	12,100	11,300	−38.3	22,700
	4.52	18–25	1.42×10^{-4}	11,800	11,100	−37.8	22,100
	4.08	18–25	1.1×10^{-3}	11,700	11,100	−33.7	20,900

[a] k' given for the lower temperature listed.
[b] $\Delta F^{\ddagger}$ given for the lower temperature listed.
[c] Concentration expressed as volumes per cent.

value of E rises to a magnitude of 120,000 calories, and $\Delta S^{\ddagger}$ more than doubles. However alcohol, like ammonium sulfate, shifts the minimum of the pH/k' curve to the acid side, and comparison at minimal rates instead of at constant pH would be of interest.

(*4*) The study of the denaturation of hemoglobin in the region of mild acidity (121) yields the surprising result that the energy of activation has undergone an apparent discontinuous drop to a more or less constant value of about 12,000 calories (pH 4.1–4.6). Correspondingly, $\Delta S^{\ddagger}$ has taken on a negative value. This reaction thereby assumes unusual interest, in that it is one of the rare in-

stances in protein denaturation for which the temperature coefficient is of the normal order of magnitude. It may be observed, moreover, that even in the acid region of constant E, the rate strongly depends upon pH.

(*b*) *Egg albumin:* It is of interest to note that in several respects the denaturation of egg albumin is consistent with that of hemoglobin: (*a*) At neutral reaction the rate reaches a minimum at pH 6.76, E being of the order of 130,000 calories (227). (*b*) The presence of concentrated salts depresses the rate (97); however, no initial maximum in rate is attained and E apparently drops (to 106,000 calories when calculated from second-order constants, or 88,000 if calculated from first-order "constants", for 2 M ammonium sulfate). (*c*) In the acid denaturation of egg albumin, E drops to about 97,000 calories at pH 3.4 (97) and to 48,000 at pH 2.3 (121), thereafter observing a small and rather continuous diminution to a constant value of about 36,000 over the pH range 1.1–1.7 (121). Moreover, in urea solution, the denaturation of egg albumin exhibits a negative temperature coefficient (192) corresponding to a heat of activation of about −6000 calories (140).

The meaning of the thermodynamic quantities $\Delta S^{\ddagger}$ and $\Delta F^{\ddagger}$ in terms of a theory of protein denaturation may now be examined. A cursory inspection of tables 7 and 8 will indicate that only a small variation obtains in $\Delta F^{\ddagger}$, or more precisely, in $\Delta F^{\ddagger}/T$. This is not surprising, for it is already evident from equation 4 that at constant temperature $\Delta F^{\ddagger}$ is the sole factor governing the rate of reaction. Accordingly, because of the logarithmic nature of the relationship, only a small variation in $\Delta F^{\ddagger}/T$ is admissible for the production of a conveniently measurable rate in the temperature region characteristic of protein denaturation.

Conversely, in the face of an extraordinary variation in $\Delta H^{\ddagger}$ under different conditions of denaturation, the maintenance of a rate of a given order of magnitude must be accompanied by a corresponding change in the entropy of activation. Thus, in table 7 a variation of 110,000 calories in $T\Delta S^{\ddagger}$ obtains for the denaturation of hemoglobin.

Just as the large entropy change of the over-all denaturation reaction (ΔS) is used as support for the suggested formation of random structures as a result of denaturation (278), the large *entropy of activation* ($\Delta S^{\ddagger}$) may be interpreted in terms of an "opening up" of the protein structure during the course of activation (140). While Mirsky and Pauling (278) have assumed that the molecule is held into its uniquely defined configuration by hydrogen bonds, Eyring and Stearn (140) have suggested that the specific configuration is maintained by the functioning of salt bridges as well as by bridges of the disulfide type. According to the latter theory, the energy required for thermal destruction of the cross linkages is compensated for by the large increase in entropy associated with the opening up of the specific configuration upon the breaking of the last few bridges.

In order, then, to distinguish between the degree of unfolding attributable to the *formation* of the activated complex and that associated with its *"flying apart"*, it would be desirable to learn both ΔS and $\Delta S^{\ddagger}$. We have little information as to the former, and it is now profitable to consider what confidence may be attached to the values of $\Delta S^{\ddagger}$ given by the application of the theory of absolute

reaction rates to protein denaturation. Since $\Delta S^{\ddagger}$ is obtained by substitution of k' and $\Delta H^{\ddagger}$ in the rate equation, this is tantamount to a consideration of the validity of $\Delta H^{\ddagger}$, or more precisely of E, the experimental activation energy. This evaluation is also of interest in a consideration of the Mirsky and Pauling theory of protein denaturation, for in that variant, the number of hydrogen bonds broken is obtained by dividing E by 5000 calories per mole, the bond energy observed in simple systems.

7. *Evaluation of the true activation energy*

Steinhardt (373) and La Mer (216) have already pointed out that the time-honored method of the comparison of rates at constant pH may lead to adventitious values of E. This arises from the fact that acidic equilibria are involved in the preparation of the molecule for the kinetic step of activation. Thus

$$P_0 \rightarrow P_n \qquad \text{(a)}$$

$$P_n \rightarrow P_n^{\ddagger} \qquad \text{(b)}$$

where P_0 is the initial protein molecule, P_n is the molecule after an n-fold ionization, and $P_n^{\ddagger}$ is the activated ionized complex. The operation of the temperature coefficient of dissociation in equation **a** promotes an increase in the concentration of P_n, the ionized species subject to activation. Thus, a comparison of rates at different temperatures and constant pH neglects the change in activity of the ionized molecules. This procedure leads to composite values of E; for the heat of dissociation observed in equation **a** is added to the energy of activation involved in equation **b**.

True values of E may be obtained only by rate comparison at constant concentration of the ionized species, P_n (216, 373). The separation of the true energy of activation and the total heat of dissociation is most simply accomplished by a graphical method in which log k' is plotted against pH, the slope of this line corresponding to n. To the experimental points is fitted a theoretical curve corresponding to the equation

$$\log \frac{P_n}{P_0} = n(\log K_0 - \log a_{\mathrm{H}})$$

in which K_0 is the postulated dissociation constant. In this manner the apparent dissociation constant at a given temperature may be determined (K_0 being assumed identical for the n groups involved), and ΔpH corresponding to ΔpK may be calculated. Substitution in the equation

$$\Delta H = 2.3\Delta\mathrm{pH} \cdot \frac{RT_2T_1}{T_2 - T_1} \qquad (5)$$

enables the calculation of the heat of dissociation of each group, this being multiplied n times to obtain the total ΔH. This value is then subtracted from the observed E, the remainder representing the true energy of activation.

(*a*) *Pepsin:* This method of appraisal has been applied by Steinhardt (373)

and La Mer (216) for the best-investigated case of protein denaturation, the alkaline inactivation of pepsin. Over a restricted pH range the rate was found to be inversely proportional to the fifth power of C_{H^+}. This pH dependence was ascribed to the dissociation of a proton from each of five primary amino groups of the initial molecule, leading to an increase of five in the net charge. This step corresponds to equation **a**, n being equal to 5. The quintuply ionized species was assumed to undergo the activation step in equation **b**.

Appraisal by the method already indicated yielded a value of 9040 calories per equivalent of protons dissociated, representing a total ΔH of 45,200 calories. The experimentally observed E was 63,500 calories, which upon correction for the heat of dissociation was reduced to a true value for the energy of activation of 18,300 calories.

The effect of this correction is to result in a change even in the order of magnitude of the apparent entropy of activation; at pH 5.7, for example, the corrected $\Delta S^{\ddagger}$ diminishes from 135.9 entropy units to 8.8 (*cf.* table 8). This small increase in entropy would indicate only an insignificant degree of unfolding of the activated complex. In terms of the Mirsky–Pauling theory of denaturation the correction in the value of E leads to a reduction of nine in the calculated number of hydrogen bonds broken, the corrected value of E corresponding to the destruction of but three to four hydrogen bonds.

While granting the validity of this general approach, Eyring and Stearn (140) contend that in this instance either the protein acts as a peculiar acid in the ionization process or the entropy change associated with the opening up of the protein molecule is disguised by the large entropy change of opposite sign involved in the ionization of the acid.

As Steinhardt (373) and La Mer (216) have pointed out, wherever the rate depends upon some power of the hydrogen- or hydroxyl-ion concentration, the existence of acidic equilibria may be suspected. In such cases the calculation of E by customary methods is fallacious.

Moreover, the proponents of the absolute reaction rate theory have acknowledged (140) that "some, although by no means all, of the abnormally large activation entropies of protein reactions are due to failure to include the hydrogen-ion concentration properly in calculating the specific reaction rate constants". Inasmuch as this omission has been quite general in studies of the kinetics of protein denaturation, the calculation of the entropy of activation must be formal insofar as the value of the activation energy is empirical and the standard states of the reactants remain unspecified.

8. The acid denaturation of hemoglobin

A paucity of data has precluded the extension of Steinhardt and La Mer's method of appraisal to analogous reactions. However, we have recently observed another reaction in which the pH dependence of rate may be completely interpreted in a somewhat similar manner (333). This reaction is the acid denaturation of ox oxyhemoglobin over the pH interval 4.1–4.6, well investigated by Cubin in the temperature range 18–45°C. (121). A résumé of the phenomena accompanying hemoglobin denaturation follows.

The changes undergone in the thermal denaturation of oxyhemoglobin (60.5–68°C.), a reaction characterized by an apparent E of 77,000 calories over the pH range 5.7–8.0, are not known (226). From pH 5.7–4.85 a hiatus exists over which E apparently drops to 18,500 calories. From pH 4.6–4.1 a constant value of E of 11,800 calories is observed (121). Below this acidity the kinetics have not been studied.

The molecular-kinetic data are consistent with those of reaction kinetics. As will be discussed in a following section, the stability range of hemoglobin (pH 6–8) (377) corresponds to the region of thermal denaturation. There is evidence (*cf. infra*) to indicate that acid denaturation down to pH 4.1 causes the hemoglobin molecule to split into halves along the major axis, with no consequent unfolding of the molecule. The negative value of the entropy of activation, given in table 7, is in accord with the latter conclusion. The formation of acid hematin, accompanied by the breaking of the heme–globin bond, is not observed above pH 3.9, but is complete at pH 3.1 (190). It will be shown subsequently that the intactness of this bond inhibits the denaturation of the globin moiety, the latter reaction being characteristic of acid denaturation in 0.1 N hydrochloric acid. The partially reversible reaction occurring at room temperature in the restricted pH range 4.1–4.6 accordingly may be postulated as follows:

$$H_4Gl_2 \rightleftarrows 2H_2Gl$$

H representing heme, and Gl unaltered globin.

A plot of log k' against pH for the temperatures 18°, 25°, 37°, and 45°C. leads to a slope of exactly -2 (333), indicating that the rate is directly proportional to the square of the hydrogen-ion activity. Over the portion in which the curves are parallel and linear (pH 4.1–4.6), the heat of activation is constant and equals 11,800 calories. At higher pH, the curves diverge and E increases.

A fitting of the theoretical curve,

$$\log P_2/P_0 = -2(\log K_0 - \log a_H)$$

to the experimental points enables the evaluation of pK. At 25°C. pK equals 2.70, a value of the order observed for the carboxyl group of dipeptides (189). Similarly, the value of pK for other temperatures may be determined and the apparent value of ΔpK calculated. Substitution into equation 5 yields a heat of dissociation of 5600 calories per equivalent of protons, which, multiplied by 2, represents a total heat of dissociation of 11,200 calories. The subtraction of this quantity from the experimentally observed E yields a value of only 600 calories for the true Arrhenius heat of activation. Since in this temperature range RT equals about 600 calories, the true energy of activation, $\Delta H^{\ddagger}$, is zero. This conclusion is in accord with the suggestion (140) that if a "real" $\Delta H^{\ddagger}$ for the acid denaturation of hemoglobin is calculated, its value would not be greater than zero. The fact that $\Delta H^{\ddagger}$ is zero indicates either (*1*) that the rate of the reaction subsequent to ionization is independent of temperature, or (*2*) that the acid denaturation of hemoglobin results wholly from a twofold ionization.

In order to ascertain the mechanism of the denaturation it is incumbent to inquire about the meaning of a heat of dissociation of carboxyl groups of the order

of 5600 calories. Wyman (416) has shown that the apparent heat of dissociation of the carboxyl groups of horse oxyhemoglobin in the pK range of 4 to 5 is -2000 to -3000 calories. For amino acids, the values are of the order ± 1000 calories (110), leaving some 5000 calories to be accounted for by another process. In view of the fact that hydrogen bonding has been postulated as one of the principal mechanisms operating to maintain the intact configuration of the native molecule, and that the energy required for the thermal destruction of this bond is about 5000 calories (278), it is tempting to conclude that in each ionization process one hydrogen bond has been broken. Indeed, it is stated (278) that acids act to destroy hydrogen bonds by supplying protons individually to the electronegative atoms which would otherwise share protons (i.e., the oxygen atoms of the carboxyl group). In this process the small heat of ionization of the acid and that of hydrogen-bond disruption would be observed simultaneously, leading to an apparent value for the heat of ionization of the carboxyl groups of 5600 calories. This interpretation resolves the paradox associated with the observation of an appreciable temperature coefficient for the supposed pK, despite the well-known fact that the temperature coefficient of the ionization of carboxyl groups is negligible (110).

The conclusion reached by the present analysis is that the acid denaturation of hemoglobin in the pH range 4.1–4.6 proceeds by the addition of two protons and the breaking of two hydrogen bridges. It may be observed that the same conclusion may be attained more directly in the manner of Mirsky and Pauling (278), simply by ascribing the total energy of activation to hydrogen-bond disruption occasioned by individual protons supplied by the acidic environment. A dependence of the rate upon the square of the hydrogen-ion concentration, in addition to the fact that the heat of activation is approximately equivalent to twice the postulated bond energy, might be considered ample justification for the conclusion that two hydrogen bridges were broken in the activation process.

With regard to the structure of hemoglobin, it is of interest to observe that two is the least number of bonds that might be expected to maintain a rigid configuration for two subunits, and that if the halves are postulated to have the same structure, the number of bridges should be even.

The absence of kinetic data prevents an analysis of the changes taking place in the acid denaturation of hemoglobin below pH 4.1. Since the disruption of the heme–globin bond is initiated at pH 3.9, and denaturation of the globin may occur, the observed values of E may begin to change in this region just as they do above pH 4.6 with the onset of thermal denaturation. The fact should be noted that even in fairly strong acid (0.1 N hydrochloric acid) the formation of acid hematin and the denaturation of globin require some time (128).

Since the denaturation of egg albumin at room temperature in regions of greater acidity is consistent with that of hemoglobin, it may be predicted that in this other well-investigated case of protein denaturation, a similar pH dependence of the activation energy obtains. Indeed, it has already been shown that in the alkaline denaturation of crystalline egg albumin at 25°C. (87), the rate is proportional to $(OH^-)^4$. Likewise, in the thermal denaturation of to-

bacco mosaic virus, the reaction rate varies inversely with about the third power of the hydrogen-ion concentration (223), and in the urea denaturation of the same protein the rate depends on the reciprocal of about the 1.5th power (221). These results, together with the data for the acid denaturation (dissociation) of hemoglobin, substantiate the contention of La Mer—namely, that the abnormally large values of the activation energy of protein denaturation are illusory, and that if appropriate correction of them is made, it is unnecessary to invoke large entropy changes in an explanation of the observable rates.

TABLE 8

Heat inactivation of enzymes

PROTEIN	SOLVENT	pH	T	k'	E_{Ar}	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta F^{\ddagger}$
			°C.	$sec.^{-1}$	*calories per mole*	*calories per mole*	*calories per degree-mole*	*calories per mole*
Pancreatic lipase (251)	50 per cent glycerol	6.0	40–50	1.13×10^{-4}	46,000	45,360	68.2	23,960
Trypsin (311)	24 per cent glycerol	6.51	50–60	2.83×10^{-5}	40,800	40,160	44.7	25,700
Enterokinase (312)	Water	6.50	50–60	7.37×10^{-5}	42,800	42,160	52.8	25,100
Trypsinkinase (313)	24 per cent glycerol	6.5	50–60	3.17×10^{-5}	44,900	44,260	57.6	26,200
Pancreatic proteinase (314)	Water	6.9	50–60	1.27×10^{-4}	38,500	37,860	40.6	24,700
Alkaline inactivation of pepsin (140, 373)								
Process								
$P_0 \rightarrow P_5^{\ddagger}$		6.44	15–25	1.89×10^{-5}	56,300	55,600	113.3	23,000
$P_0 \rightarrow P_5^{\ddagger}$		5.7	15–25			67,470	135.9	
$P_5 \rightarrow P_5^{\ddagger}$		5.7	15–25			22,360	8.8	
$P_0 \rightarrow P_5$		5.7	15–25			45,110	127.1	

9. Biological inactivation

Table 8 gives the kinetic and thermodynamic data for the heat inactivation of a number of well-investigated enzymes, as well as for the alkaline inactivation of pepsin. With the exception of pepsin, the preparations were non-crystalline but were purified by the methods of Willstätter and Waldschmidt-Leitz. In several cases the purity is questionable: for example, the product called "trypsin" (311) has since been acknowledged to contain two fractions, one being pancreatic proteinase, an enzyme analogous to the classical trypsinogen, and the other a carboxypolypeptidase (314). Similarly, enterokinase (studied with the presence of trypsin (312)) is included as an "enzyme" to facilitate comparison with trypsinkinase, the product obtained upon the interaction of "trypsin" with its specific activator, enterokinase (313).

The heat inactivation of the enzymes in the upper section of table 8 is characterized by about the same value of E. Indeed, the constancy of E for the

four similar proteinase preparations evokes suspicion that the same inactivation process may have been measured in each instance. It should be noted that the concentration of glycerol is without effect on the E values for purified enzymes (251, 314). However, the purity of the enzyme is unquestionably important, for McGillivray (251) found that the E of unpurified lipase dropped from the order of 100,000 calories to 35,000 calories with changing glycerol concentration.

In all these cases a marked variation of rate with pH was noted, the plot of the pH/k' curve reaching a minimum at pH 6.0 for pancreatic lipase, and at pH 6.5 for the proteinases. (The similarity in the pH of minimal rate in the case of the proteinases is another indication that the same function was being measured in all instances.) These minima are to be compared with those already given for hemoglobin and egg albumin, i.e., pH 6.76 in both instances. The existence of such minima in the region of neutrality (about 6.76 at 37°C.) has led to the suggestion that both hydrogen and hydroxyl ions are involved in the catalysis of denaturation (226). The fact that hydrogen ions are indeed involved in the dissociation of hemoglobin at pH 4.1–4.6 has already been discussed. This strong dependence of rate on pH is further evidence for the contention that the abnormal values of E may be adventitious.

Moelwyn-Hughes (281) has ably reviewed the mechanism of the inactivation of these enzymes on the basis of the collision theory of reaction kinetics.

10. Variation of the activation energy with solvent, pH, and temperature

The previous discussion has been concerned principally with the kinetics and thermodynamics of *thermal* denaturation and the explanation of the paradox associated with the presence of abnormally large activation energies. It has already been observed that the activation energy may differ greatly with the conditions of denaturation. For example, in table 7 the activation energy for the denaturation of hemoglobin is shown to vary over a whole order of magnitude, changing from 11,800 calories at pH 4.1–4.6 to 120,000 calories for the thermal denaturation in the presence of 30 per cent alcohol or 3 M ammonium sulfate. To attribute the success of each of these processes to the selfsame reaction step would be absurd. It is more profitable to attempt to break the reaction mechanism down into several successive steps, a procedure that seems admissible for the case of hemoglobin at least.

It is not feasible to list in order the result of different environmental conditions upon E; for, whereas increasing the concentration of ammonium sulfate effects a consistent increase in E for hemoglobin (228), an apparent decrease is observed for egg albumin (140). However, it appears to be a valid generalization that in acid solution the activation energy tends to a minimum value, and that in urea solution a change in sign may even be observed.

It has already been noted that in the denaturation of both egg albumin and hemoglobin, the activation energy assumes a constant minimum value at low pH. The same effect is observed in the destruction of ptyalin, for which E drops from about 123,000 calories at pH 6.8 and higher to 38,500 calories at pH 3.2 (134). Furthermore, the rate of the acid denaturation of edestin is the same at 2°C.

as at 20°C., that is, the temperature coefficient is unity and the activation energy is zero (39).

In terms of the Steinhardt–La Mer concept of the importance of ionization processes in denaturation, the effect of acidity upon E is not surprising, for in acid solutions only carboxyl groups may be dissociating, and the effect of temperature upon their dissociation is negligible (373). Correspondingly, the large temperature effect experienced in alkaline solution may in part be attributed to the ionization of amino or other basic groups whose temperature coefficient is appreciable. An alternative explanation may be found in the suggestion (140) that salt bridges may be broken either thermally or by neutralization due to a pH shift. In the latter event the heat of neutralization involved will not be observed in the measurement of the activation energy. Thus, if the breaking of salt bridges is assumed to be an integral part of the denaturation mechanism, the activation energy may decrease with pH.

Contrary to the behavior experienced for most chemical reactions and especially in contrast to that observed in the thermal denaturation of proteins, the rate of urea denaturation of egg albumin exhibits a negative temperature coefficient (192, 334), corresponding to an activation energy of about -7000 calories (140). Even more remarkable is the urea denaturation of tobacco mosaic virus (221, 371). In this reaction the rate observes a minimum at room temperature and is governed by a negative temperature coefficient below room temperature and a positive coefficient above. Other viruses exhibit similar anomalies (52). On the other hand, a positive coefficient appears to hold for the urea denaturation of fibrinogen (127) and carboxyhemoglobin (128).

The unusual temperature–rate effect observed for tobacco mosaic virus may be explained by the assumption that the native nucleoprotein is transformed into the denatured material by two (or more) simultaneous reactions, the one governed by a positive temperature coefficient, the other by a negative coefficient, the over-all effect being the resultant (221). If the two parallel processes are supposed to differ only in the number of urea molecules combining with a given virus particle, it may be predicted that the temperature coefficient would vary not only with temperature but also with urea concentration, for both these factors would be expected to govern the extent of combination.

The effect of urea upon the kinetics of protein denaturation has been ascribed to the entropy change associated with a "freezing out" of urea as the result of the strong bonding of the urea to the activated complex (140).

In concluding the discussion of the activation energy, it should be pointed out that the universally assumed temperature independence of the activation energy is not always observed in protein denaturation even over the limited temperature range available for study. A number of examples are known for which the activation energy varies markedly with temperature (e.g., ptyalin (134)) or where the plot of the ln k' *versus* $1/T$ deviates from linearity (407). Sufficient data are not available for the evaluation of the significance of this anomaly. However, an interesting analogy is to be found in the variation with temperature of the critical increment of *flocculation* for denatured horse serum. Lewis's

recalculation (230) of the data of Chick and Martin (99) shows that E decreases from 110,000 calories per mole at 44°C. to a constant value of about 23,000 calories at 76–86°C. Steinhardt has observed (373) that the fact that E becomes smaller and approaches a limiting value at higher temperatures is in accord with the effect of temperature upon the dissociation of ionizable groups, a process supposed to precede the activation step.

11. The heat of reaction

The heat of reaction, ΔH, is equal to the difference between E_1, the energy of activation of the forward reaction, and E_2, the energy of activation of the reverse reaction. E_1 may be obtained by kinetic studies, and $\Delta S_1^{\ddagger}$, the entropy of activation, may be evaluated by the Eyring method. The heat of reaction or heat of denaturation, ΔH, is measured thermochemically or by substitution in the van't Hoff equation expressing the variation in the equilibrium constant with temperature. From the well-known relations between free energy and the equilibrium constant, and between free energy and the heat of reaction, the entropy change ΔS may be obtained. If the denaturation process is reversible, from the above information one may then calculate E_2, and $\Delta S_2^{\ddagger}$ for the reverse reaction.

A knowledge of the heat change and the entropy change involved in both the activation step and the over-all reaction is fundamental for the interpretation of the mechanism of denaturation. It is therefore evident that a combination of thermochemical and kinetic data would aid greatly in the elucidation of the denaturation process. Unfortunately, both kinetic and thermochemical measurements have not yet been made upon the reversal of the denaturation process for any protein under the same conditions. The one instance where somewhat comparable data are available is the equilibrium governing the "reversible" acid denaturation of trypsin, as studied by Anson and Mirsky (17). In 0.01 N hydrochloric acid (pH 2–3) the heat of reaction calculated from the variation of the equilibrium constant with temperature is 67,600 calories per mole (17), and evaluation of the entropy change leads to a value for ΔS of 213 entropy units (372). The kinetic data of Pace (311) are for crude preparations of trypsin subjected to heat inactivation at neutral pH. The heat of activation of denaturation (*cf.* table 8) is less than 41,000 calories and the entropy of activation is but 44.7 units. If the legitimacy of the extrapolation of Pace's data to the acid region is tentatively assumed, the anomalous situation arises that the slow reverse reaction has a *negative* heat of activation of 27,000 calories per mole. However, from the above data the entropy of activation of the reverse reaction is seen to be -168.4 units per mole. Stearn (372) has suggested that it is this large loss of entropy which results in the slowing up of the reverse reaction.[6]

[6] The magnitude of the heat change, entropy change, and activation energy of denaturation of trypsin cited by Mirsky and Pauling (278) and apparently derived from the data of Northrop (305) differs considerably from the figures given above. Northrop (305) has stated that his experiments were not sufficiently accurate to permit calculation of the exact temperature coefficient. On the other hand, the extrapolation of Pace's data appears questionable. It is evident that the calculation of the number of hydrogen bonds broken in the denaturation process or of the associated entropy change still remains qualitative.

The heat of reaction of a number of other denaturation processes has been determined calorimetrically and further application of the method seems desirable. The irreversible denaturation of methemoglobin at pH 9–12 (25°C.) has been studied by an indirect calorimetric procedure, both at constant quantity of alkali in the solution and at constant pH (117). The heat of denaturation of the protein by alkali is obtained from the difference in the heats of reaction of native and denatured protein with potassium hydroxide. As a result of a pH change during denaturation the heat change at constant pH is less than that at constant quantity of alkali. At constant alkali the heat of denaturation of methemoglobin is reported to be 138 (±14) kcal. per mole, about twice that reported by Anson and Mirsky (17) for trypsin, and at constant pH is about 100 kcal. In this study, the important conclusion was reached that, contrary to statements made in the literature (5), denaturation is not an "all or none" process and the insolubility of methemoglobin at the isoelectric point is not a good measure of denaturation.

A similar study on the heats of reaction of methemoglobin and carboxyhemoglobin with sodium salicylate at pH 7.3 yielded no difference in the heats for either protein at a given salicylate concentration (336). Moreover, the heat of reaction of methemoglobin increased with salicylate concentration up to 0.8 *M*, leading to the conclusion that the reaction was not stoichiometric and that part of the heat of reaction probably arose from hydrogen-bond formation between the salicylate and the protein. This result, together with others reported in Part IV, indicates the need for a modification in the theory of the existence of a mobile equilibrium between native and denatured protein (18) insofar as this is based upon the reaction between hemoglobin and sodium salicylate.

In view of the extensive kinetic investigation of the irreversible alkaline inactivation of crystalline pepsin (373) and of the importance attached to its interpretation, the calorimetric measurement of the heat of denaturation of crystalline pepsin by alkali is significant. The surprising result was obtained (116) that the heat of denaturation fell off with pH from 85 kcal. per mole of pepsin at pH 4.3 to a negligible value at pH 6.8.[7] From pH 4.3 to 6.2, ΔH decreased slowly, but thereafter it decreased abruptly. On the other hand, the enzymatic activity remained constant to pH 6.2, thereafter falling off 70 per cent in going to pH 6.8. That ΔH and enzymatic activity are different functions of pH emphasizes the fact that the measured heat of denaturation must be regarded as the sum of changes in heat content due to several chemical processes, of which enzymatic inactivation is but one, and that these may not as yet be distinguished. This view is in accord with that already expressed in this review —namely, that denaturation consists of a series of related changes, of which biological inactivation is but one aspect.

In analogy to Steinhardt's kinetic results (373) it is interesting to observe that at pH 6.2 and 30.6°C. (the temperature of the calorimetric experiment)

[7] The heat of denaturation of crystalline *pepsinogen* calculated from the effect of temperature on the equilibrium between the native and denatured forms at pH 7.0 is 31 kcal. per mole (185).

the amount of pepsin in the ionized form is insignificant, whereas at pH 6.8 ionization is virtually complete and the energy of activation apparently undergoes a change in value. Since the kinetic experiments were made in buffered solutions at constant pH, and the calorimetric measurements were undertaken at varying initial pH in the presence of different amounts of alkali, and since the reaction has been shown to be essentially irreversible, the data are not amenable to a comparison in the manner given for trypsin.

III. Properties of Denatured Proteins

A. Molecular weight

Molecular-kinetic properties of proteins can be described in terms of size and shape. Information as to the change in these properties upon denaturation is of vital importance for the characterization of denatured proteins. Measurements of (*a*) osmotic pressure, (*b*) sedimentation and diffusion, and (*c*) diffusion and viscosity have been used to determine the molecular weight of denatured proteins. On the basis of these measurements, proteins may be divided into three groups: (*1*) those which have the same molecular weight in the denatured as in the native state; (*2*) those which are split; and (*3*) those which become aggregated upon denaturation. In the following sections the experimental data are presented and discussed according to this classification.

1. Denaturation causing no change in molecular weight

In table 9 data are given relative to the nature of the protein and of the denaturing agent, the molecular weight, and the method used for its determination. Within the limits of the resolving power of the methods, the molecular weight of the denatured protein is identical with that of the parent native material.

(*a*) *Egg albumin:* Comparative osmotic-pressure measurements yielded a value of 35,000 for the native protein in buffered solutions of pH 4.8 (36), and of 36,000 (36) and 32,000 (193) in 6.66 M[8] urea solution. Although the molecular weight of the native protein has since been found to be somewhat higher, i.e., about 45,000 (77, 381), it seems to be established that urea denaturation does not affect the molecular weight of egg albumin. While Williams and Watson (406) believed that the observed decrease in the sedimentation constant of the protein upon treatment with 6.66 M urea was due to a splitting of the molecules into halves, viscosity data indicate that the decrease in sedimentation rate is due to a change not in molecular weight but in molecular asymmetry (75). This is in accord with more recent sedimentation and diffusion measurements (347). Denaturation of egg albumin by acid alcohol or alkaline alcohol, followed by denaturation by urea, likewise yields a protein of essentially the same molecular weight as the native form (193). However, if the protein is first denatured by acids or alkali, changes in mean molecular weight occur (193).

(*b*) *Serum albumin (horse):* Osmotic-pressure measurements of Burk (80)

[8] In this and the following sections M denotes molar concentration.

TABLE 9

Proteins which undergo no change in molecular weight upon denaturation

PROTEIN	DENATURING AGENT	METHOD OF MEASUREMENT	MOLECULAR WEIGHT		REFERENCE
			Native	Denatured	
Egg albumin	6.66 *M* urea	Osmotic pressure	35,000	36,000	(36)
	6.66 *M* urea	Osmotic pressure		32,500	(193)
	6.66 *M* urea	Sedimentation, diffusion-viscosity	44,000	32,000 44,000	(75, 406) (347)
Serum albumin (horse).....	6.66 *M* urea	Osmotic pressure	74,600	73,800	(80)
	8 *M* urea	Diffusion-viscosity	71,900	77,800	(295)
	8 M guanidine hydrochloride	Diffusion-viscosity		80,600	(295)
	Heat and 6.66 *M* urea	Osmotic pressure		72,900	(80)
Serum globulin (horse).....	6.66 *M* urea	Osmotic pressure	175,000	173,000–178,000	(3, 82)
		Sedimentation-diffusion	167,000		(*cf.* 381, p. 374)
	8 *M* urea	Diffusion-viscosity	170,000	170,000	(296)
Hemoglobin and globin (sheep and dog).....	6.66 *M* urea	Osmotic pressure	65,300–66,600	63,000–69,400	(415)
Gliadin.....	6.66 *M* urea	Osmotic pressure	40,900	44,200	(83)
	1.7 *M* urethan	Osmotic pressure		42,000	(83)
Zein.....	8.33 *M* urea	Sedimentation-diffusion	40,000	40,000	(399)
Pepsin.....	1 *M* urea, 4 *M* urea, 6.5 *M* acetamide	Sedimentation	S^* = 3.0	S = 3.3	(374)
Tomato bushy stunt virus.....	Heat, freezing	Sedimentation	S = 133	S = 133	(308)
Thyroglobulin ...	Heat in salt-free solutions, urea, amino acids, high protein concentration	Sedimentation	S = 17.8 650,000	S† = 10 650,000	(245, 249)

* S = sedimentation constant.

† Interpreted as being due to change in molecular shape (245).

yielded the same molecular weight for native and for urea-denatured horse serum albumin. Combined diffusion and viscosity measurements revealed variations in apparent molecular weight following denaturation by increasing concentrations of urea (300). These have since been traced to the limitations of Kuhn's equation for the interpretation of viscosity data, as well as to an apparent retardation of the diffusion rate in concentrated urea solutions (295). Later work, on a crystalline fraction of horse serum albumin (295), indicated the molecular weight of the protein, denatured by 8 *M* urea or 8 *M* guanidine hydrochloride, to be essentially the same as that of the native form. This has been found to hold also for bovine serum albumin (331).

Although serum albumin heated at 70°C., and dissolved in 6.66 *M* urea, has the same molecular weight as the native protein (80), it appears that in this instance urea caused a depolymerization of the aggregates of heat-denatured protein (118). Sodium salicylate, in concentrations of 1 *M* and 3.5 *M*, is without effect on molecular size, as revealed by diffusion data (300).

(*c*) *Serum globulin:* Recent estimates of the molecular weight of crude preparations of native horse serum globulin vary from 150,000 (337) to 175,000 (3, 82; *cf.* also 381, p. 354 ff.). In 6.66 *M* urea, Burk (82) observed a value of 173,000 to 178,000. Combined diffusion and viscosity data also indicate no change in molecular weight of an electrophoretically monodisperse preparation of pseudoglobulin upon denaturation by concentrated urea solutions (296).

(*d*) *Hemoglobin (sheep and dog):* Unlike those of the horse, beef, and ox, the methemoglobins, oxyhemoglobins, and globins of sheep and dogs are not split by concentrated urea solutions (415).

(*e*) *Gliadin:* This protein, although not homogeneous in the ultracentrifuge (207), was found by Burk (83) to have about the same mean molecular weight in 50 to 60 per cent ethanol (40,000) as in 6.66 *M* urea solutions. Progressive aggregation occurs, however, when the buffer components of the urea solutions exceed a concentration of about 0.1 *M* phosphate. Urethan which, like urea, liberates free disulfide groups, has, in the absence of buffer components, no effect on the molecular weight of the protein.

(*f*) *Zein:* Comparative ultracentrifugal analysis in 45–49 per cent ethanol, and in 50 per cent urea solutions, failed to reveal any difference in sedimentation rate when the protein was exposed to urea for about 5 hr. ($S = 1.9$; $M = 40{,}000$). After prolonged contact with urea, fragmentation occurred (399).

(*g*) *Pepsin:* Exposure of crystalline pepsin to 1 and 4 *M* urea solutions or to 6.5 *M* acetamide for 1 to 3 hr. produces only a small increase in the sedimentation constant of the native protein (374). While diffusion measurements have not been recorded, it appears that interaction between pepsin and these amides does not involve a change in molecular weight. Enzymatic activity also was unimpaired by 3 hr. exposure to 4 *M* urea. Yet it remains to be seen whether prolonged exposure to these or higher concentrations of these amides does not exert appreciable effects on the particular properties studied.

(*h*) *Tomato bushy stunt virus:* Inactivation of this virus protein by heating or by freezing does not alter its sedimentation rate (308).

(*i*) *Thyroglobulin:* The sedimentation behavior of thyroglobulin is changed

either by raising the protein concentration of salt-free solutions or by adding urea, glycine, tyrosine, and related compounds, even in the presence of electrolytes (245, 249). The decrease in sedimentation constant, from $S = 17.8$ to $S = 10$, has been interpreted as being due to an unfolding of the molecules without any concomitant changes in size (α-protein). Although the original sedimentation constant becomes restored upon reversing the processes applied for the formation of α-protein, it is not known whether the reconstituted protein answers all the criteria of the native material. The α-protein is readily converted irreversibly into a more highly denatured form, or reversibly into fragments of lower molecular weight. Similar findings have been obtained with antitoxic diphtheria pseudoglobulin and with thymus nucleohistone. Further investigation of these processes by means of diffusion or viscosity measurements is needed before the interpretation of the ultracentrifugal measurements can be fully accepted.

2. Denaturation causing splitting of protein molecules

Molecular dissociation and denaturation

Denaturation of a number of proteins has been found to be accompanied by splitting of the molecules into subunits of well-defined kinetic properties. Whether splitting is an accompaniment of denaturation depends on the internal configuration of the protein molecule. Such proteins as the hemocyanins (72) or phycocyan (138) are capable of undergoing molecular dissociation under conditions which ordinarily are considered too mild to cause denaturation (as revealed by the liberation of sulfhydryl groups or other denaturation criteria). Edestin, excelsin, the hemoglobins and other proteins listed in table 10 are readily dissociated by typical denaturing agents, whereas serum globulin (296) and myogen (154) are so after, or concomitant to, unfolding of the molecule. Other proteins, i.e., those considered in the preceding section, resist dissociation even in the denatured state. This divergence in susceptibility to molecular fission may be related to the physical configuration of the protein molecules, in that some proteins are composed of structural subunits into which they are readily separated, whereas others consist either of a single structural unit or else of subunits which do not separate even when the protein is denatured (289).

Upon moderate variations of pH, or upon the addition of electrolytes, *Helix pomatia* hemocyanin dissociates into subunits having one-half and one-eighth of the molecular weight of the native protein (72). The dissociated molecules appear to have the same molecular shape as they have when combined in the whole molecule, as indicated by calculations of axial ratios (*vide infra*). The molecule splits lengthwise or across (289). The subunits recombine upon reversing the conditions applied for dissociation. When myogen is acted upon by urea it dissociates into halves; however, the subunits undergo further changes in that they unfold as they dissociate (154). The same is true of horse serum globulin when treated with 3 M guanidine hydrochloride (296). Obviously these two types of reactions are of different magnitude.

The molecular weight corresponds to the smallest molecular-kinetic unit which still reflects all chemical, physical, and biological properties of the protein

in bulk. If the subunits of hemocyanin or hemoglobin deviate in any single property from the whole molecules,—as they do, since they carry only one-half of the complement of prosthetic groups,—dissociation is to be considered as one type of denaturation. If a more fundamental change in properties is accepted as a criterion for denaturation, such as an intramolecular rearrangement, the dissociation of these proteins is a process different from that occurring when myogen is dissociated. Similar considerations apply for the process of recombination of the subunits. If the recombined hemoglobin or hemocyanin is identical in all respects with the original native protein, dissociation is truly reversible; otherwise it is not. However, the extent to which the original properties can be regained depends on the extent of change occurring upon dissociation: dissociation without alterations in the configuration of the subunits is more nearly reversible than dissociation with simultaneous intramolecular changes.

It was found that although the recombined molecules of *Helix pomatia* hemocyanin and of hemoglobin had the same size and shape as those of the parent native proteins, slight differences were observed in the electrophoretic mobility for hemocyanin (393) and in the shape of the oxygen dissociation curve, as well as in resistance to splitting by alkali, for hemoglobin (see below). That the method applied for dissociation may have a profound influence on the extent of recombination may be seen from the fact that with *Helix pomatia* hemocyanin recombination occurred fully when the protein was dissociated by pH changes or salts, partly when it was dissociated by ultraviolet light, and not at all when it was dissociated by ultrasonic waves (72).

An explanation for these observations may be found by considering the variations in the degree of denaturation, as exemplified in the introduction by the A–Z scale. Assume that the dissociation of hemocyanin by salts represents a change in properties corresponding in extent to a change from state A (native) to state G; that of hemoglobin by acids, where —SH groups become detectable (276), from A to L; and that of myogen by urea (154), where the subunits unfold, from A to P. All three types of dissociation are denaturation reactions in the broadest sense; however, the most apparent change in the first instance is molecular dissociation; in the latter instances, it is dissociation accompanied by further intramolecular changes.

It may be seen, therefore, that mere dissociation of protein molecules into subunits is no indication of the degree to which denaturation occurred, and that a change in molecular weight may be the result of processes of quite different orders of magnitude. An evaluation of the latter requires the application of additional standards of reference, both for the dissociation reaction and for its reversal. This will be borne out by a more detailed discussion of the dissociation of specific proteins, presented in the following paragraphs.

Experimental results

In table 10 the proteins are listed which undergo dissociation and denaturation.

TABLE 10

Proteins which undergo dissociation upon denaturation

PROTEIN	DENATURING AGENT	METHOD OF MEASUREMENT	MOLECULAR WEIGHT		REFERENCE
			Native	Denatured	
Myogen	6 *M* urea	Sedimentation-diffusion	150,000	72,000	(154)
	6.66 *M* urea	Osmotic pressure	(81,000)	(34,000)	(401)
Serum globulin (horse)	3 *M* guanidine hydrochloride	Diffusion-viscosity	170,000	96,000	(296)
Egg albumin	*N*/20 sodium hydroxide	Osmotic pressure	36,000	16,000	(193)
Edestin	6.66 Urea	Osmotic pressure	310,000	49,500	(36)
	Hydrochloric acid	Osmotic pressure		17,000	(2)
	Hydrochloric acid	Sedimentation	S^* = 12.8	S = 2.3	(39)
Excelsin	6.66 *M* urea	Osmotic pressure	214,000	35,700	(81)
Amandin	6.66 *M* urea	Osmotic pressure	206,000	30,300	(81)
Casein	6.66 *M* urea	Osmotic pressure	75,000–100,000	33,000	(36)
Limulus hemohemocyanin	6.66 *M* urea	Osmotic pressure	2,040,000	142,000	(84)
	Hydrochloric acid followed by 6.66 *M* urea	Osmotic pressure		69,000	(84)
Hemoglobin (horse, beef)	Hydrochloric acid	Sedimentation-diffusion	69,000	39,000	(155)
		Osmotic pressure		39,000	(343)
	Urea	Osmotic pressure		35,800	(193)
	Urea, formamide, acetamide	Sedimentation-diffusion	69,000	39,000	(374)
Tobacco mosaic virus	6 *M* urea	Osmotic pressure	40×10^6	40,000–100,000	(371)
	Pressure	Sedimentation		See text	(222)
Myosin	Urea	Osmotic pressure	1,000,000	100,000	(409)

*S=Sedimentation constant.

TABLE 10—*Concluded*

PROTEIN	DENATURING AGENT	METHOD OF MEASUREMENT	MOLECULAR WEIGHT		REFERENCE
			Native	Denatured	
Pepsin.....	Alkali	Sedimentation	40,000	See text	(323)
Diphtheria antitoxic globulin.....	Pepsin	Sedimentation–diffusion	184,000	98,000	(322, 391)
Insulin.....	Duponol	Sedimentation–diffusion	41,000	27,500 See text	(262)
Helix pomatia hemocyanin...	pH, salts, non-electrolytes, ultrasonics, ultraviolet light, and α-rays	Sedimentation–diffusion	8.9×10^6	4.31×10^6 1.03×10^6	(72)

(*a*) *Myogen:* Earlier osmotic-pressure measurements by Weber and Stoever (401) yielded a molecular weight of 81,000 for the native protein and of 34,000 for the protein denatured by 6.66 *M* urea. These data have been superseded by the more accurate sedimentation and diffusion measurements (154), yielding values of 150,000 and 72,000, respectively. As will be discussed in greater detail in Part III B, the denatured half-molecules are considerably more asymmetric than would be expected to result from mere dissociation.

(*b*) *Serum globulin (horse):* Whereas denaturation by 8 *M* urea is without effect on the molecular weight of horse pseudoglobulin (296), profound changes occur upon denaturation by guanidine hydrochloride. In 2 *M* solutions of this salt aggregation occurs, while in concentrations higher than 3 *M* the protein molecules split into halves as they unfold (296). Under these conditions, horse serum globulin follows the pattern of the urea denaturation of myogen.

(*c*) *Egg albumin:* Treatment of egg albumin with *N*/1 sodium hydroxide gives rise to a polydisperse material of allegedly greatly reduced antigenic activity (384). Huang and Wu (193) obtained an osmotic molecular weight of 15,900 ± 3,600 when egg albumin was exposed to *N*/20 sodium hydroxide for 24 hr., and when the precipitate, obtained after neutralization, was dissolved in 40 and 50 per cent urea. The liberation of ammonia and hydrogen sulfide, noted upon neutralization, indicates that more far-reaching, chemical changes had occurred.

(*d*) *Edestin:* Denaturation by 6.66 *M* urea reduces the molecular weight from 310,000 (36) to 49,500. At the same time four-ninths of the total sulfhydryl groups become exposed (161). Though about the same amount is set free upon denaturation by hydrochloric acid (39), splitting proceeds farther, resulting in the formation of subunits of molecular weight about 17,000 (2). The latter

reaction has been the subject of a careful study by Bailey (39), who has used several physical and chemical methods to compare the properties of the native, denatured, and reconstituted protein.

(*e*) *Excelsin:* Only a small fraction of the total sulfhydryl groups is liberated upon denaturation by 6.66 *M* urea. At the same time, the protein splits into subunits of one-sixth the molecular weight of the native material (35,700 as compared to 214,000). Dilution of the urea solutions with water results in complete precipitation of the denatured protein (81).

(*f*) *Amandin:* This protein, as previously noted, is devoid of cysteine residues. Treatment with 6.66 *M* urea lowers the molecular weight from 206,000 to 30,300 (81).

(*g*) *Casein:* This protein is polydisperse in the ultracentrifuge, with a main component having a molecular weight of 75,000–100,000 (381). Treatment with 6.66 *M* urea yields an osmotic molecular weight of 33,000 (36).

(*h*) *Limulus hemocyanin:* This copper-containing respiratory blood protein of the horseshoe crab has a molecular weight of 2,040,000 (381). Treatment with 6.6 *M* urea causes decolorization of the protein, renders it insoluble in isoelectric salt solutions, and lowers the osmotic molecular weight to 142,000 (84). The dissociated molecules contain one prosthetic group with four atoms of copper. If hemocyanin is first denatured by acids, which split off copper, and then by 6.66 *M* urea, further dissociation occurs, yielding a molecular weight of about 69,000, which is about twice the minimum molecular weight as determined by chemical analysis. *Cancer* hemocyanin (208) behaves differently in that the native and the copper-free protein are dissociated to the same extent by concentrated urea solutions. It appears, therefore, that in *Limulus* hemocyanin the copper is combined in such a way that a breakage of its linkages leads to further dissociation (84).

(*i*) *Hemoglobin and its derivatives:* We have mentioned before that the hemoglobins of the horse, beef, and ox, unlike those of the sheep and dog, are dissociated by acids and by urea and other amides. Since the literature on this subject is widely scattered, the dissociation of hemoglobin and globin will be discussed here in some detail.

(*1*) Dissociation by acids.—When horse or beef hemoglobin is treated with 0.1 *N* hydrochloric acid, it dissociates into hemin and globin (11, 190). The latter can be precipitated by acetone, dried, and redissolved in water. This acid-denatured globin is somewhat heterogeneous in the ultracentrifuge (155), with a mean sedimentation constant of about $S = 2.5$. The same value is obtained for acid-treated globin prior to the precipitation by acetone. It reveals a certain proportion of sulfhydryl groups under conditions at which the native hemoglobin reveals none (276). Rapid neutralization of acid-denatured globin yields a water-insoluble precipitate which, when dissolved in alkali, combines with heme to form hemochromogen, a pigment incapable of combining reversibly with oxygen (11). This differs from native hemoglobin in absorption spectrum. Careful neutralization of acid globin, at low temperatures, yields a fraction of a material which is soluble under conditions at which denatured

globin is not. This allegedly "native" globin (11) is now devoid of free —SH groups (276) and, although also somewhat heterogeneous, gives a sedimentation constant of $S = 2.5$ and a diffusion constant of 6.5×10^{-7}, equivalent to a molecular weight of 39,000 (155), which is in close agreement with the value derived from osmotic-pressure measurements (343).[9] It can be recombined with heme to form a substance identical with native hemoglobin in absorption spectrum (11, 190, 191) and molecular size and shape ($M = 69{,}000$ (155, 342)); however, it is somewhat less homogeneous, more susceptible to dissociation by alkali (beef (339)), and differs also in electrophoretic mobility (155) and in the shape of the oxygen dissociation curve (187). The present evidence indicates that acid treatment of hemoglobin causes not only dissociation of the molecule into halves, but also further denaturation of the dissociated subunits. What has been referred to as "native" globin is actually a regenerated product, obtained after neutralization of a solution of the denatured protein. The mere fact that it is as soluble as the native globin, is devoid of free sulfhydryl groups, and is capable of recombination with heme so as to form a substance resembling native hemoglobin is not unequivocal proof for its genuine character. Its preparation from a denatured material, together with the established differences in stability (339) and other physicochemical and biological properties already noted, suggests strongly that it is different from the globin moiety of native hemoglobin (Part IV).

(*2*) Dissociation by urea and related amides.—Treatment of horse (36) and ox hemoglobin (193) with concentrated urea solutions likewise causes dissociation into half-molecules, as determined by osmotic-pressure measurements. The effects of varying concentrations of urea, acetamide, and formamide on horse hemoglobin have been studied extensively by Steinhardt by means of sedimentation, diffusion, and other physical measurements (374). The sedimentation constant of native carbon monoxide hemoglobin was reduced from $S = 4.63$ to 3.2 by 4–7.5 M urea solutions, to 4.06 by 3 M acetamide, and to about 3.5 by 4.5–6.5 M solutions of acetamide. 4.55 M formamide reduced the sedimentation constant to $S = 3.0$, while 2 M glycine was without effect. These particular changes were complete about 1 hr. after the addition of the dissociating agents and resulted in a molecular weight of about 39,000. Unlike hydrochloric acid, these amides did not cause the formation of denatured globin, or even the splitting of the heme from the globin moiety. There was no appearance of protein sulfhydryl groups or of the hemochromogen absorption spectrum. In fact, all hemoglobin derivatives studied by Steinhardt revealed the same spectrum in water as in 4.4 M urea or 4.5 M formamide. The dissociated molecules retained their ability to combine reversibly with oxygen and carbon monoxide in the presence of the amides, though, as later work by Taylor and Hastings (383) showed, to a slightly diminished extent.

That some sort of change has occurred upon dissociation is revealed by the observation that, upon removal of the dissociating agent, the greater part of the protein failed to go in solution, whereas the soluble "regenerated" fraction was considerably less homogeneous in the ultracentrifuge than the native hemoglobin

[9] 62,300 in 65 per cent glycerol (415).

(374). Also, urea-denatured hemoglobin is digested by papain at a rate forty times as great as the native protein (234).

The action of concentrated solutions of these amides on hemoglobin is in some respects milder than that of hydrochloric acid; or, as Steinhardt points out, under the influence of the amides "hemoglobin has gone one step toward denaturation".[10] This difference in behavior may be explained by assuming that the dissociated globin undergoes further denaturation when the prosthetic group is split off, as it is by hydrochloric acid but not by urea. This may be quite similar to the difference in behavior of *Limulus* hemocyanin and *Cancer* hemocyanin toward the action of urea, already referred to in this section. These two types of dissociation of hemoglobin may be represented schematically by the following equations:

Acid dissociation:

$$H_4Gl_2 \rightarrow H_4 + 2Gl \tag{1}$$

and

$$Gl \rightarrow Gl' \tag{2}$$

where H_4 represents the heme moiety, Gl native globin, and Gl′ denatured globin.

Urea dissociation:

$$H_4Gl_2 \rightarrow 2H_2Gl \tag{3}$$

It appears that the bond between heme and globin cannot be broken (by acids) without denaturation of the latter, and that this bond inhibits the denaturation of globin by urea and other amides.

(*j*) *Tobacco mosaic virus:* The literature on this subject has been excellently reviewed by Bawden and Pirie (52). Tobacco mosaic virus, potato "X" virus, tomato bushy stunt virus, and tobacco necrosis virus are irreversibly denatured by urea, with a concomitant loss of infectivity and serological activity. The kinetics of the urea denaturation of tobacco mosaic virus has been studied extensively by Stanley and Lauffer (224, 371). The streaming double refraction and the turbidity of the virus solution decreased progressively, in like manner, with time when the virus was treated with 6 *M* urea at pH 7. This was accompanied by a proportionate decrease in the amount of protein soluble after removal of urea by dialysis. Osmotic-pressure measurements indicated that the molecular weight of the protein was between 40,000 and 100,000, as compared to 40×10^6 for the native virus. This degradation of the protein upon urea denaturation was accompanied by the splitting off of nucleic acid.

In a recent investigation it was shown (354a) that 72 hr. exposure of tobacco mosaic virus to *M*/100 glycine buffer, pH 9, at 0°C., yields partial dissociation of the virus into two components, one of which is free of nucleic acid. Both

[10] These considerations apply to the specific conditions of Steinhardt's experiments where the protein was exposed to up to 4.4 *M* urea solutions for periods of 1 to 4 hr. Drabkin (128) reports that upon prolonged contact 6 *M* urea may act on hemoglobin much in the same manner as does 0.008 *M* sodium hydroxide, 13 hr. being required for one-half completion of the conversion of horse oxyhemoglobin to hemochromogen.

components are homogeneous in sedimentation and electrophoresis, having a molecular weight of about 360,000. The nucleic acid-free fraction migrates at pH 9 with an electrophoretic mobility about 60 per cent lower than that of the unchanged virus, whereas the nucleic acid-containing component has the same mobility as the virus. Readjustment of the solutions containing either one of the biologically inactive dissociation products to pH 5 yields a material which resembles the native tobacco mosaic virus in sedimentation constant, crystal form, and electron-microscopic appearance. Also, solutions of these reformed virus proteins exhibit double refraction of flow, though the material is biologically inactive. It appears, therefore, that reassociation occurred by means of an end-to-end aggregation of about one hundred of the moderately asymmetric dissociation products, yielding a material which resembles the native protein in gross molecular properties but which is devoid of the details of internal configuration of the latter, as revealed by the lack of biological activity.

Denaturation by high pressures (222) leads at first to a disintegration into components of sedimentation constant $S = 35$, which are somewhat anisometric and do not seem to have lost an appreciable amount of nucleic acid. This inactive material is then further decomposed into insoluble protein, devoid of nucleic acid.

(*k*) *Myosin:* Weber's osmotic-pressure measurements indicate a reduction in molecular weight from about 1,000,000 to about 100,000 (400) in 6.66 M urea solution.

It has been concluded that the loss of double refraction of flow, produced also by guanidine and methylguanidine salts, arginine monohydrochloride, calcium and magnesium chlorides, potassium iodide, sodium thiocyanate, and other salts is likewise accompanied by splitting of the molecules into smaller fragments (133).

(*l*) *Pepsin:* When solutions of crystalline pepsin are brought to pH 7 or higher, the sedimentation constant decreases although the protein remains homogeneous. It is not certain whether this change is due to molecular dissociation or to a change in the degree of asymmetry or hydration. Readjustment of the pH of solutions of alkali-inactivated pepsin to a pH of 4.8 or less results in a rise in the sedimentation constant and in a decrease in the degree of homogeneity (323).

(*m*) *Diphtheria antitoxin:* Treatment of antitoxic pseudoglobulin with pepsin at pH 4.2 results in molecular dissociation, with the formation of half-molecules. Diffusion and sedimentation measurements indicate (322, 391) that cleavage occurred in a plane normal to the major axis of the molecule. The inactive pseudoglobulin contained in antitoxin preparations, purified by salting out and dialysis, is attacked by the enzyme in the same manner as the immunologically active component. However, the remaining components of antitoxic sera are digested into smaller fragments.

(*n*) *Insulin:* In the presence of 1 per cent Duponol, both native and reduced insulin have a lower molecular weight than in the absence of the detergent, a result suggestive of molecular dissociation (262). However, as pointed out by the authors, the possibility of a retardation of the sedimentation rate because of electrostatic interaction with the charged detergent micelles cannot be dismissed, and indeed deserves serious consideration.

(*o*) *Helix pomatia hemocyanin:* The work of Brohult (72) reveals that *Helix pomatia* hemocyanin having a molecular weight of 8.91×10^6 may be split reversibly into components of about one-half and one-eighth of the native molecule. Calculations of molecular shape suggest that in both instances splitting occurs in a plane parallel to the major axis of the molecules (289). The degree of dissociation depends on the pH of the solution, half-molecules appearing *in acidic* pH ranges and both half- and eighth-molecules in *alkaline* regions. Recombination results in the restoration of the original molecular-kinetic properties; however, the recombined molecules differ from the native in electrophoretic mobility (393). The dissociation by ultrasonic waves, ultraviolet light, and α-rays has already been referred to and contrasted to the dissociation by chemical agents. Dissociation by electrolytes depends on the valence of the component anion and cation and on the ionic strength. Non-electrolytes, such as glucose and fructose, glycerol, and urea, are also capable of causing dissociation.

3. Denaturation causing aggregation of protein molecules

It is a matter of experience that denatured proteins are considerably more susceptible to aggregation than native proteins (73). The degree of aggregation is dependent on external conditions, such as pH, temperature, and ionic strength of the medium, and is governed by the electrokinetic potential on the protein particles. Although more observations have been reported on the aggregation than on many other properties of denatured proteins, little is known about the actual state of dispersion of the aggregates. Since aggregation may occur under conditions for which there is no evidence that they cause denaturation,—for instance, preparatory to precipitation of native proteins by salts,—it is not a phenomenon specific for denaturation. Thus, horse serum globulin has a particle weight of about twice that of the native protein when dissolved in 1 *M* ammonium sulfate at pH 4.3 (82), or in 2 *M* guanidine hydrochloride (296), without showing any evidence of being denatured. Myogen becomes aggregated in pairs when dissolved in 1.4 *M* ammonium thiocyanate, also without the appearance of any denaturing effects (401). Examples of aggregation accompanied by denaturation are the irradiation of serum proteins by ultraviolet light, whereby the viscosity is increased and the colloid osmotic pressure is decreased (123).

Ultracentrifugal measurements have been reported for the denaturation of human serum albumin by soft x-rays. The sedimentation constant of the undenatured fraction remains constant; however, as denaturation proceeds, an increasing proportion of the protein is converted into aggregates as well as into low-molecular-weight fragments (352).

When salt-free solutions of crystalline horse serum albumin are heated to 70°C., aggregation occurs (318), varying in degree with the pH of the solution (118). The mean molecular weight of the somewhat polydisperse protein was about four times that of the native when heat-treated at pH 7.6, about nine times at pH 4.3, and about twice when heat-treated at pH 3.6. These changes are

largely irreversible; in fact, further aggregation occurs when the solutions heat-treated at pH 7.6 and 4.2 are readjusted to the isoelectric point. A preliminary announcement by Rothen indicates somewhat similar reactions of heat-denatured egg albumin (347; see also 225).

The problem of the aggregation of denatured proteins does not invite detailed consideration as long as more quantitative data on the state of dispersion, particularly in relation to other changes in properties, are lacking.

B. MOLECULAR SHAPE AND THE QUESTION OF HYDRATION

It is beyond the scope of the present review to discuss the problem of the shape of native protein molecules. Recognition of differences in shape characteristics has led to differentiation between corpuscular and fibrous proteins, the former group comprising spherical and moderately anisometric molecules, the latter, molecules of rod-shaped and fibrous configuration (29). Under the influence of certain kinds of denaturating agents, globular proteins may be converted into the fibrous state, while fibrous proteins, such as myosin and tobacco mosaic virus, assume a more nearly spherical configuration.

The methods for determining the shape of protein molecules have been reviewed elsewhere (286, 289, 309) and will be discussed here only as they have been applied to the investigation of the denaturation process. Theoretically least ambiguous are measurements of the birefringence produced by the orientation of optically isotropic, anisometric particles by hydrodynamic forces (double refraction of flow). The ratio of the major to minor axis of prolate or oblate ellipsoids may be calculated from the rotatory diffusion constant, the latter being determined by measurements of the amount of double refraction and of the angle of isocline (*cf.* 113, p. 527 ff.). For very anisometric particles, the length of the major axis may also be estimated. Fairly accurate data are available for myosin, tobacco mosaic virus, *Helix pomatia* hemocyanin, and others (*cf.* 113, p. 527 ff.). Qualitative measurements of the changes in birefringence upon denaturation have been carried out on myosin (133, 282, 284, 400; also, *cf.* 113, p. 527 ff.) and tobacco mosaic virus (256, 371). Experimental difficulties of attaining a sufficiently high velocity gradient have impeded the general application of this method to less anisometric protein molecules.

By far the most common methods for comparing the shapes of native and denatured proteins are those based on the frictional ratio, f/f_0, and viscosity measurements (286, 287, 289, 294, 309, 326). In the first of these, the molecular frictional coefficient effective in diffusion or sedimentation is compared to that expected for a spherical anhydrous molecule. The frictional ratio is a composite function of molecular asymmetry and hydration. Assuming a value for the latter, the former can be evaluated with the aid of the Perrin equation, and expressed in terms of axial ratios of prolate or oblate ellipsoids (294, 309). This method of estimation of molecular shape is attended with a certain degree of ambiguity, since (*1*) a model of ellipsoids of revolution may be an inadequate description of the shape properties of proteins, and (*2*) the degree of hydration cannot be evaluated by present-day methods.

The same limitations apply to viscosity methods where the limiting value of the intrinsic viscosity of the solutions $(\eta/\eta_0 - 1)/c$, measured under conditions which favor either complete orientation or random distribution of the solute molecules, is related to the ratio of molecular axes. Of the several equations that have been formulated, that of Simha (361) appears to be most satisfactory for proteins (294). With very anisometric molecules, the apparent viscosity varies with the rate of shear. At low rates it is appreciably higher than at high rates, since under the latter conditions the molecules become oriented, thereby requiring less energy for viscous flow. With spherical molecules, the viscosity is independent of the rate of shear (252). It has also been noted that in general with spherical molecules the relative viscosity increases linearly with solute concentration up to higher concentration ranges than it does with asymmetric molecules (262).[11]

In the past, before the shape properties of proteins had received proper consideration, there was a tendency to interpret the observed increase in viscosity of proteins upon denaturation in terms of increased hydration and aggregation (15, 173, 180, 212). As will be shown presently (page 226), this viewpoint is no longer tenable, particularly in those cases in which there is no evidence that denaturation causes aggregation of molecules. Evidence from various sources suggests that the *changes* in intrinsic viscosity or in frictional ratio (dissymetry constant) are results of changes in molecular shape.

1. Experimental results

The following proteins have been found to assume a more asymmetric shape upon denaturation by various means:

(*a*) *Serum albumin:* The specific viscosity of solutions of the crystalline protein increases with increasing urea concentration, while the diffusion constant decreases in proportion. A limiting value is approached in about 6 *M* urea solutions (300). Since the molecular weight of the protein remains unchanged (page 212), the changes in diffusion and viscosity properties have been taken as indications of alterations in shape. It is of interest to note that diffusion data reveal the solutions to be monodisperse when the urea concentration is higher than 0.5 *M*, whereas they should have been heterodisperse had the solutions contained mixtures of completely denatured and native protein molecules (270).

The increase in the degree of denaturation with increasing concentrations of the denaturing agents is paralleled by an increase in the fraction of insoluble, irreversibly denatured protein, obtained after removal of urea by dialysis under specified conditions (295). This suggests a physical correlation between the extent of distortion of intrinsic configuration of the molecules and the susceptibility of the denatured protein to irreversible changes. The fact that the water-soluble fraction (obtained after removal of urea) resembles the native protein in molecular shape (and size) shows that these globular proteins are endowed with a latent long-range elasticity, comparable in magnitude to that characteristic for certain fibrous proteins, i.e., keratin and myosin (29).

[11] Comparison of the viscosities of denatured and native proteins supports the validity of this empirical relation (118, 295, 296, 300).

In analogy with measurements of the chemical reactivity (Part II A), diffusion and sedimentation measurements have revealed a greater denaturing power of guanidine hydrochloride as compared with that of equimolar concentrations of urea. Thus, the axial ratio, calculated for a prolate ellipsoid of revolution and assuming 33 per cent hydration, was 3.3 for the native protein and 13.3 and 16.7 for serum albumin denatured by urea and by guanidine hydrochloride, respectively. Differences in the degree of denaturation were found between horse and beef serum albumin (331), and even between preparations of the latter when carried to different stages in the purification process. The effects of heat-treatment on the molecular shape of serum albumin are small as compared with those produced by urea, and conceivably may be accounted for by an end-to-end aggregation of molecules of unchanged shape (118). However, the conditions of these two sets of experiments are not strictly comparable, since in the latter instance measurements were performed in the presence of the denaturing agents, whereas in the former, they were carried out after protein solutions had been heated (at 70°C.) and cooled. No information is available on the shape changes occurring at the temperature of heating.

Ultracentrifugal experiments have furnished evidence that with one protein, i.e., thyroglobulin (245, 249), heat-treatment may produce shape changes of the type described for the urea denaturation of serum albumin.

(*b*) *Serum globulin:* The action of concentrated solutions of urea on this protein is similar to that on serum albumin (296). Denaturation by guanidine hydrochloride is a more complex process: in 2 *M* solutions the molecules become aggregated in pairs. In 3 *M* solutions of guanidine hydrochloride, the molecular assymetry is increased to the same extent as in 8 *M* urea solutions; however, the mean molecular weight is about one-half that of the native protein. Further increase of the concentration of guanidine hydrochloride, to 5.6 *M*, produces no further change in molecular shape or weight.

(*c*) *Myogen:* Sedimentation and diffusion data yield a dissymmetry constant of 1.26 for the native protein, and of 3.2 for the urea-denatured half-molecules (154). This corresponds to a change in axial ratio from 3.0 to 49.0. The action of urea on this protein is similar in nature to that of guanidine hydrochloride on serum globulin, though of considerably greater magnitude.

(*d*) *Hemoglobin:* Native hemoglobin appears to approximate in shape an oblate rather than a prolate ellipsoid of revolution (70). The dissociation products obtained by denaturation by acids have about the shape properties that would be expected to result from mere cleavage along the major molecular axis.

(*e*) *Egg albumin:* While several investigations have been recorded on the effects of denaturation on the viscosity of egg albumin solutions (15, 236, 239), most of these were performed in a limited but high concentration range in which the relative viscosity is no longer proportional to protein concentration. For this reason, and because no adequate precautions were taken to repress electro-viscous effects, the data do not lend themselves to quantitative interpretation and will not be considered here.

Quantitative viscosity measurements have been carried out by Bull (75) on solutions of crystalline egg albumin denatured (*1*) by heating at pH 8.0 for 7 min. at 100°C., followed by dilution with phosphate buffer (pH 8.0), and (*2*) by adding 1 g. of urea per cubic centimeter of protein solution and, after allowing the material to stand for 1 hr. at room temperature, diluting with phosphate buffer. The viscosity increase produced by heat denaturation was smaller than that observed after denaturation by urea. This result is in keeping with the comparative effects of these two types of denaturation on the sulfhydryl groups of egg albumin.

(*f*) *Helix pomatia hemocyanin:* The shape changes accompanying dissociation and denaturation (72) have been referred to (Part II A). Calculations of dissymmetry constants and axial ratios suggest that cleavage occurs along the major axis without any further elongation of the dissociation products (289).

Myosin and tobacco mosaic virus exemplify conditions where denaturation causes a change in the shape of anisometric molecules toward a more globular configuration. It is well to stress, however, that these changes may be the result of the dissociation of these protein molecules, occurring with both proteins in the presence of concentrated solutions of urea (pages 218, 219).

(*g*) *Myosin:* Von Muralt and Edsall (282) found that urea in concentrated solutions, and iodides and thiocyanates in dilute solutions, abolished the double refraction of flow. A variety of other agents was found by Edsall and Mehl (133) to produce similar effects, varying in extent with the nature and concentration of the substances they have studied. These include chlorides of monovalent and divalent cations, ammonium, methylammonium, and lithium salts, guanidine and methylguanidine salts, urea, and arginine hydrochloride. The double refraction of flow was also irreversibly lost upon acidification, contrary to the earlier results of Bate-Smith (44), as well as by heating. The decrease in double refraction was paralleled by a decrease in relative viscosity, indicating that the protein molecules had assumed a less asymmetric configuration.

(*h*) *Tobacco mosaic virus:* Denaturation of this virus protein by urea follows closely the pattern already described for myosin. The double refraction of flow decreases as denaturation progresses (256, 371). The relative viscosity of virus protein solutions falls off sharply, and viscosity anomalies are greatly reduced (145, 371). These findings, together with the observations of a decrease in osmotic pressure and sedimentation constant (224, 371), are indicative of changes in the direction of less anisometric shape, and disaggregation. Urea denaturation causes inactivation of the virus and an alteration of serological specificity (52). Old virus preparations, isolated by salt fractionation, reveal a tendency to aggregate to products which are more anisometric than the untreated protein. Such changes are elicited by variations of pH from pH 6.8 to pH 4.5 (256).

The effect of native and of denatured proteins on the physical properties of sodium thymus nucleate

Aqueous solutions (0.1–0.5 per cent) of sodium thymus nucleate are highly viscous and possess intense double refraction of flow (170). These properties are related to the highly asymmetric shape of the nucleate particle. The latter has been estimated to possess an axial ratio of 300 (360) and a length of 450 mμ (385). When solutions of the nucleate are treated with salts, amino acids, or proteins, the birefringence and viscosity of the nucleate diminish, and the extent of the diminution in these properties is a function of both the nature and the concentration of the added substance (170). Guanidine salts are highly effective in this respect, and the similarity of their effect upon the nucleate and upon the protein myosin (133) is indeed noteworthy. On a molar basis, the most powerful agents in reducing the viscosity and intensity of streaming birefringence of the nucleate are the *native* proteins (170). When the proteins are *denatured* prior to adding them to the nucleate, the former possess relatively little effect upon the physical properties of the nucleate. This is indeed striking, for in this phenomenon it is the native protein which is reactive and the denatured protein which is relatively inert. The effect of the protein on the properties of the nucleate through the free groups of the protein as such must be of a secondary nature; the specific configuration of the *native* protein is probably of primary importance in the effect on the nucleate. This is not wholly unexpected when it is recalled that a common method of separating nucleic acid from protein in the native nucleoprotein molecule involves the denaturation of the latter (166). There is little knowledge available concerning the nature of the interaction of native protein and nucleate. The latter in such mixtures loses its osmotic effectiveness and probably aggregates into somewhat symmetrical, large bundles (165).

It has been noted that the sedimentation constant of several native globular proteins is decreased in the presence of other native proteins (381). Since this may have been caused by changes in molecular shape rather than in molecular weight, it appears that these effects are not confined to sodium thymonucleate but are manifestations of a general denaturing action of native proteins.

2. General considerations

Values of the frictional ratio, f/f_0, greater than unity may be due to the combined effects of hydration and asymmetry. This may be expressed formally by the relation (309):

$$f/f_0 = (f/f_e)\ (f_e/f_0)$$

where the first term denotes the hydration factor and the second term the asymmetry factor, the latter being related to the ratio of molecular axes by the Perrin equation (286).

Similarly, the intrinsic viscosity, $(\eta/\eta_0 - 1)/c$, is a composite function of molecular volume and asymmetry, according to the relation:

$$\frac{\eta/\eta_0 - 1}{cV_h} = \varphi(b/a)$$

where c is the protein concentration in weight per cent, V_h the partial specific

volume of the hydrated protein, and φ a function of the axial ratio. V_h is related to the degree of hydration according to:

$$V_h = (rV_1 + V_0)$$

where V_0 is the partial specific volume of the anhydrous protein, V_1 that of the solvent, and r the number of grams of water combined with 1 g. of anhydrous protein. An increase in specific viscosity may be due to changes in r, in φ, or in both.

It is generally agreed that protein molecules are hydrated in solution as well as in the solid state (157) and that the degree of hydration may vary within certain limits with changes in environmental conditions. While there is no unambiguous method for determining the state of hydration, various lines of evidence suggest a value of the order of 30 per cent (73, 157, 292, 309). The present problem is not concerned with the actual value but with the *changes* in hydration upon denaturation.

Correlation of the following experimental facts leads to the conclusion that these changes are negligible in comparison to the changes in molecular asymmetry, i.e., *the increase in viscosity and frictional ratio is largely, if not wholly, due to changes in molecular shape:*

(*1*) If the decrease in diffusion constant and the increase in intrinsic viscosity of serum albumin upon denaturation by 8 *M* urea were to be interpreted solely by a combination between protein and urea, solvation to the extent of about 2.7 g. of urea per gram of protein would have to be assumed, equivalent to about 3200 urea molecules per protein molecule (295). This would lead to the rather unreal result that in a 1 per cent protein solution one-third of the urea molecules are bound by the protein, and in a 3 per cent solution, all of them. If solvation is assumed to remain constant, a change in axial ratio from 3.3 to 13.3 is calculated (table 10). That the latter interpretation is more nearly correct is evidenced by a comparison of the frictional ratio of egg albumin in the presence of urea (406) with the specific viscosity of urea-denatured egg albumin in aqueous buffer solutions (75). The agreement is very satisfactory, indicating that the changes in properties were not prompted by the adsorption of urea molecules but rather by the effects of the latter on the molecular shape of the protein.

(*2*) The intrinsic viscosity of serum albumin is increased by synthetic detergents (Duponol, sodium dodecylsulfate, etc. (247, 332)). A 0.035 *M* solution of sodium dodecylsulfate is about as effective as an 8 *M* urea solution. Higher concentrations of detergents are even more effective on both the acid and the alkaline side of the isoelectric point of the protein (332). Since under the conditions, not sufficient detergent molecules are present to account for the increase in intrinsic viscosity in terms of adsorption of detergent by protein, one has to assume either that water molecules are adsorbed or else that the effects are due to changes in molecular asymmetry. The initial unequal distribution of urea between the interior of the protein molecules and the surrounding medium conceivably might cause an osmotic swelling, resulting in the penetration of the protein by urea and water molecules. However, such a mechanism is unlikely

to underlie the interaction between detergent and protein, since (*1*) the detergent molecules would be too large to penetrate the protein, and (*2*) detergent is present in too low concentrations to exert appreciable osmotic effects.

(*3*) When proteins are denatured by urea or by detergents, they may be spun into fibres by forcing the solutions through narrow orifices into suitable media (35, 247, 404).

The assumption that visible fibre formation is only a result of changes in the solubility of the fibrous denatured proteins is certainly more realistic than any hypothesis which would have to postulate that hydrated spherical molecules unfold into fibrous structures upon changing the solvent composition. Moreover, there is one kind of denaturation, i.e., that by surface forces, where the conversion of native globular proteins to the fibrous denatured state has been definitely established (Part III D).

(*4*) Comparison of the x-ray diffraction patterns of native and denatured proteins, to be discussed in greater detail in Part III D, indicates the latter to yield a diffraction pattern resembling that observed with several fibrous proteins. Again, this would be unlikely to be the case if denaturation were to result merely in an increased hydration of the molecules.

(*5*) Heat-coagulated and surface-coagulated egg albumin have higher densities than the native protein when xylene is used as displacing agent (292), whereas the densities of these three forms are about the same when hydrogen is used as displacing gas (73). Although the degree of hydration is undoubtedly different in the coagulated state from what it is when the proteins are dissolved, the lower densities observed for the denatured proteins in the presence of toluene indicate that they have a denser and therefore less penetrable structure than the native protein (73).[12]

(*6*) The solubility of proteins is lower in the denatured than in the native state. This would hardly be expected if denaturation were to increase the hydration of essentially spherical molecules, whereas it can be accounted for by an increase in asymmetry accompanied by a redistribution of polar and non-polar groups.

In the light of these considerations one is led to the conclusion that, in all cases considered, the changes in viscosity and frictional coefficient are brought about by changes in molecular asymmetry. Indeed, they constitute a significant criterion for protein denaturation. In table 11 data are given for the shape changes of representative proteins, calculated with the assumption of about 33 per cent hydration, and expressed in terms of a ratio of the major to minor axis, b/a. With the exception of hemoglobin, the molecules have been assumed to approximate the shape of prolate ellipsoids.[13] The degree of change in shape

[12] Dilatometric investigations also indicate denatured proteins to be less hydrated (186).

[13] General interpretation of the shape changes in terms of flat ellipsoids has not been attempted, nor does it seem warranted at the present time. Although it has been suggested (27) that certain native proteins, such as egg albumin, consist of superposed laminae, which are separated by side chains and are liberated upon denaturation (30), there is no unequivocal evidence to sustain this hypothesis.

is seen to depend on the nature of the denaturing agent and on the susceptibility of the protein to its action.

TABLE 11

Shape changes accompanying protein denaturation

PROTEIN	DENATURING AGENT	LONG AXIS [a] / SHORT AXIS		CALCULATED FROM [c]	REFERENCE
		Native	Denatured		
Serum albumin (horse).....	1.5 *M* urea	3.3	4.1	f/f_0	(300)
	3 *M* urea		5.3	f/f_0	(300)
	6 *M* urea		13.3	f/f_0	(300)
	8 *M* urea		13.3	η	(295)
	8 *M* guanidine hydrochloride		16.7	η	(295)
Serum globulin (horse).....	5 *M* urea	5.2	9.3	η	(296)
	8 *M* urea		15.4	η	(296)
	5.6 *M* guanidine hydrochloride		15.0	η	(296)
Myogen.....	Urea	3.0	49	f/f_0	(154)
Egg albumin	Heat	3.1	5.5	η	(75)
	Urea		7.1	η	(75)
Hemoglobin.....	Acids	2.7[b]	6.4[b]	f/f_0	(155, 289)
Helix pomatia hemocyanin[d]...	pH change	2.7[e]	8.3[f]	f/f_0	(72)
			11.0[g]	f/f_0	

[a] Unless otherwise stated, values have been calculated for prolate ellipsoids of revolution, assuming 33 per cent hydration.

[b] Oblate ellipsoid of revolution.

[c] f/f_0 = calculation from the dissymmetry constant with the Perrin equation (286).
η = calculation from viscosity data with the Simha equation (361).

[d] Asymmetries calculated from f/f_0 and double refraction of flow (72).

[e] $M = 8.9 \times 10^6$.

[f] $M = 4.31 \times 10^6$.

[g] $M = 1.03 \times 10^6$.

C. ELECTROCHEMICAL PROPERTIES

1. Titration curves

Comparative measurements of titration curves have been used to determine the influence of denaturation on the amphoteric properties of native proteins. Although the results obtained are of some interest, the following considerations render their interpretation difficult: (*1*) The insolubility of denatured proteins near the isoelectric point frequently necessitates recourse to heterogeneous titrations, the latter being of doubtful significance. (*2*) While maximum acid and base combination takes place only in regions of extreme acidity (pH 1-2)

or alkalinity (above pH 12–13) (*cf.* 113, p. 444 ff.), many proteins are already denatured in these regions. Hence, comparison of these quantities with "native" proteins is impossible; moreover, in these extreme regions, titrations are least dependable. Methods for the detection of such irreversible changes during titration have been reviewed by Cannan (87).

Difficulties attending comparison of the complete titration curves of native and denatured proteins are best illustrated by hemoglobin. Titration with acids below pH 4 results in rapid denaturation, even at room temperature (67, 121), as witnessed by the fact that below this pH the titration curve cannot be reproduced by back titration with alkali (*cf.* 113, p. 444 ff.). Therefore, it is not surprising that early workers (229) reported the titration curves of allegedly native and denatured hemoglobin with acids to be identical in this region. Though other proteins may react reversibly with acid and base over a wider range (pH 1.5 to pH 11–12) (87), they are readily denatured beyond these limits (120, 121).

There is much confusion in the literature regarding even that portion of the titration curves of native and denatured proteins which lies within the pH-stability range. While some workers variously claim that the titration curve of heat-denatured egg albumin differs from that of the native (101, 182, 411, 413), others assert their identity (67, 240, 330). In part, the contradictory claims of different workers may be reconciled by correcting for the alkaline pH shift attendant on denaturation, the titration curves of native and of heat-denatured egg albumin becoming identical if calculated from a common isoelectric point (pH 4.7–4.8) (101, 240). The invalidity of this procedure will be discussed shortly.

Not only do the titration curves given by various workers for denatured egg albumin differ from one another, but also the curves for the native protein are often inconsistent with those given in recent investigations (120; also *cf.* 113, p. 444 ff.). Accordingly, these results do not merit detailed analysis. Suffice it to say that any changes in amphoteric properties that may occur on denaturation are of a small order of magnitude.

Recently, the titration curve of untreated collagen has been compared with those of collagen denatured by salts (288) and by heat (387), with the unusual result that a shift in the isoionic point was observed in opposite directions for the two modes of denaturation, a phenomenon not amenable to ready explanation.

2. Isoelectric point and electric mobility

It has been shown that in solutions of the same ionic strength the electric mobility of a protein at different hydrogen-ion concentrations is directly proportional to the number of hydrogen or hydroxyl ions bound (1). Accordingly, the electric mobility curves are superimposable on the titration curves upon proper selection of the ordinates, thus providing another means for the comparison of the amphoteric properties of native and of denatured proteins, as well as for the determination of isoelectric points. The application of the electrophoretic technique to the study of denaturation has already been reviewed (1) and will be discussed here only in brief.

(*a*) *Egg albumin:* The data of Moyer (281) indicate that the isoelectric point of surface-denatured egg albumin is pH 5.02 under the same conditions for which the dissolved native protein is isoelectric at pH 4.55 (390). The occurrence of an alkaline shift at the isoelectric point of denatured proteins is substantiated by the pH increase observed in the surface (78, 412), heat (73), and urea denaturation (413) and the ultraviolet irradiation (62) of egg albumin. In the light of these findings, comparison of the titration curves of native and of heat-denatured egg albumin, by recalculation of the data to a common isoelectric point, does not appear valid.

The electrophoretic mobility curve of surface-denatured egg albumin is displaced 0.47 pH unit to the alkaline side relative to that of the native protein, but the curves remain parallel over the limited pH range investigated (1, 281).[14] In keeping with these results, the mobility of completely heat-denatured or acid-denatured egg albumin on the alkaline side of the isoelectric point likewise is slightly less than that of the native form (237).

(*b*) *Serum albumin:* Heat denaturation of horse serum albumin brings about an alkaline shift in the electric mobility curve quite analogous to that observed in the surface denaturation of egg albumin (318). However, regeneration of urea- or detergent-denatured horse serum albumin displaces the mobility curve to the acid side above pH 5. Thus, while urea regeneration results in an increase in electric mobility above pH 5 (358), as does the regeneration of detergent-treated protein at pH 7.6 (332), heat denaturation brings about a decrease in mobility in the same pH region (318). Yet, under certain conditions, two components may be distinguished in heat-denatured horse serum albumin (118), one of lower mobility than the native (perhaps corresponding to irreversibly denatured protein) and one of greater mobility (perhaps identical with regenerated albumin). However, it is remarkable that the ratio of components may be greatly altered merely by changing the ionic strength.

On the acid side of the isoelectric point, heat denaturation of horse serum albumin results in the formation of but a single component, this migrating more rapidly than the native albumin (118, 318). At pH 3.6 urea-regenerated albumin likewise migrates somewhat faster than the native protein (358).

The results for urea regeneration of beef serum albumin are in accord with those for horse serum albumin, a small increase in mobility being experienced for the regenerated protein at pH 7.6 (331). On the other hand, at the same pH the mobility of bovine albumin regenerated by guanidine hydrochloride (299, 331) is identical with that of the native protein. However, the mobility of albumin irreversibly denatured by guanidine hydrochloride is less than that of the native or regenerated protein (299), in agreement with the results already quoted for heat-denatured horse serum albumin.

(*c*) *Other proteins:* Acid-denatured hog thyroglobulin behaves similarly to

[14] In contrast to the identical behavior usually observed for the dissolved and adsorbed proteins, the mobility curve of adsorbed egg albumin likewise undergoes a small shift to the alkaline side, the isoelectric point changing to pH 4.82. It is suggested that egg albumin may become "partially denatured" during the process of adsorption (1).

surface-denatured egg albumin and heat-denatured horse serum albumin in that the electric mobility curve remains parallel to that of the native protein but is transposed about 0.4 pH unit toward the alkaline side at the isoelectric point (179). Likewise, the electric mobillty of urea-regenerated horse serum pseudoglobulin is higher on the acid side and lower on the alkaline side of the isoelectric point (359).

The electrophoretic properties of denatured proteins are thus consistent with their titration behavior in that they indicate that only small changes in amphoteric properties occur as a result of denaturation. However, while the titration data are ambiguous, the electrophoretic data are unequivocal in indicating an alkaline shift in the acid- and base-binding properties of the denatured protein.[15]

One explanation for the shift in the isoelectric point upon denaturation may be found in the theory of Mirsky and Pauling (278), according to which denaturation results from the rupture of hydrogen bonds, the latter serving to maintain the intact native structure. Since the acid–base properties of a protein are determined primarily by the groups which are free, the liberation of paired amino and carboxyl groups in equal numbers should shift the isoelectric point towards neutrality.

Alternatively, since the strength of the acidic and basic groups in a protein molecule must depend somewhat upon the arrangement and proximity of neighboring groups, a profound alteration in molecular structure as a result of denaturation could suffice to explain a change in amphoteric properties even in the absence of the liberation of new titratable groups.

Bull (73) has discussed the small increase in acid-binding capacity observed upon denaturation in terms of both explanations: namely, (*a*) an actual increase in the number of proton-accepting groups, and (*b*) an increase in the binding strength of such groups.

3. Electrophoretic homogeneity

Although denaturation leads to an increase in molecular-kinetic heterogeneity as a result of dissociation and aggregation, the denatured proteins may yet retain the electrochemical homogeneity of the native protein. Indeed, protein mixtures may undergo an increase in electrical homogeneity upon denaturation. Egg albumin, whether denatured by heat or by acid, is electrically homogeneous at pH 6.8 and pH 10.28, but two components may be observed after prolonged dialysis at pH 12.81, the slower component apparently representing the denatured form (237). Heat-denatured serum albumin is electrophoretically homogeneous on the acid side of the isoelectric point, two components arising on the alkaline side (118).

In contrast to the splitting and aggregation often occurring upon the denaturation of proteins, it is surprising to observe that the denaturation of horse

[15] It is interesting to note that hemocyanins, dissociated into eighth-molecules outside the pH-stability range, form reassociation products at neutral pH, similar to the original molecules in molecular weight but distinguished by a 5 to 10 per cent decrease in electrophoretic mobility (393).

serum leads to an increase in electrophoretic homogeneity. Van der Scheer, Wyckoff, and Clarke (397) have found that heating of serum at 65°C. for a short time results in the progressive formation of a single denatured electrochemical component, "C," at the expense of the albumin and the globulins. This finding has been confirmed by Davis, Hollaender, and Greenstein (123), who have also observed the same phenomenon for ultraviolet irradiation. The denatured component exhibited approximately the mean mobility of the native serum. A decrease in colloid osmotic pressure accompanies the increase in electrophoretic homogeneity observed upon ultraviolet irradiation. In explanation of this phenomenon it has been suggested that ultraviolet irradiation first involves an unfolding and then a splitting and aggregation, producing molecules of similar charge distribution but of widely varying particle size (123).[16]

A marked alteration in electrophoretic pattern also occurs upon the photo-oxidation of immune rabbit and horse serum (363). Whereas the heating of individual solutions of casein or of antibody globulin produces no appreciable change in electrophoretic homogeneity, heat treatment of mixtures leads to a change in electrophoretic pattern, possibly owing to protein–protein interaction (205).

Urea-regenerated (358) and detergent-regenerated *horse* serum albumin (332), and urea-regenerated and guanidine hydrochloride-regenerated (331) *beef* serum albumin all approach the native protein in electrophoretic homogeneity. The irreversibly denatured, as well as the regenerated, form of *beef* serum albumin may be obtained in an electrochemically homogeneous state (299). Normal *horse* pseudoglobulin also gives an electrophoretic pattern nearly identical with that of the urea-regenerated protein (359).

D. STRUCTURE OF DENATURED PROTEINS: EVIDENCE FROM X-RAY AND SURFACE-FILM STUDIES

1. X-ray structure

Throughout this discussion it has been inferred that the primary effect of denaturation is an intramolecular rearrangement of the proteins. In this section we shall examine the evidence which x-ray diffraction measurements have furnished for changes in molecular structure. The pioneer work of Astbury and his associates on the structure of fibrous proteins will serve as a starting point.

The simplest diffraction pattern is revealed by silk fibroin (24–26). It can be interpreted in terms of oriented, fully extended polypeptide chains, separated by a backbone spacing of 4.6 Å. and by a side-chain spacing of about 9.5 Å., and there is little doubt that this interpretation is correct (76, 119). A similar pattern, although somewhat more diffuse, is obtained from fibres of keratin (24, 29, 37) or myosin (28, 34) by stretching them along the fibre axis; in the natural state, or rather after preliminary orientation by slight extension, these fibres yield patterns characterized by a spacing of 5.1 Å. along the fibre axis, and by

[16] Recently, it has been reported (174) that the effects of heat on the electrophoretic behavior of bovine serum may be repressed by previous saturation with glucose.

side-chain spacings of 9.8 and 27.Å., the back-bone spacing being absent altogether.

The transition of both myosin and keratin from the natural to the stretched state is, from the viewpoint of x-ray structure, a denaturation and, indeed, the least complex type of its kind. The stereochemical structure of the denatured protein is fairly well established (β configuration), while that of the native protein is open to more than one interpretation (α configuration). Astbury and Bell (32) and Huggins (194) have suggested structural schemes which satisfy bond-distance and valence-angle requirements (see also reference 55), but the final word has not yet been spoken (*cf.* 76, 291). This purely mechanical denaturation derives its apparently reversible mechanism from the strains that are imposed upon cross linkages between adjacent polypeptide chains, these cross linkages probably being established by disulfide groups of the cystine moiety (315, 365), and by ion and dipole attractions between side chains (76, 95).

A more profound type of denaturation occurs when keratin or myosin is exposed to higher temperatures or to alkali. With myosin, certain of the cross linkages are broken spontaneously in hot water or dilute alkali. In this state a diffraction pattern is observed which has been ascribed to disoriented fibres of the β configuration. With keratin, supercontraction occurs *after* the fibres are *stretched*, exposed to steam or alkali, and then allowed to contract. However, if the fibres are retained in the stretched state, new cross linkages[17] are formed and the fibres become set permanently in a fully extended configuration, yielding again a diffraction pattern of the β type. The greater susceptibility of myosin to supercontraction has been ascribed to the presence of fewer and probably more reactive cross linkages.

Astbury (30) has tabulated the amino acid composition of keratin and myosin. Though the data are somewhat incomplete and therefore tentative, they serve to show that keratin has about eight times as many cystine residues as myosin, and three times as many serine residues, both groups being influential in the formation of cross linkages, the former by virtue of covalent bonds, the latter by means of hydrogen bonds. If this is compared to the composition of silk fibroin, containing mostly glycine, alanine, and serine (44, 25, and about 14 per cent, respectively), the profound differences in reactivity and latent elasticity to which these three fibrous proteins are subject can only be ascribed to the influence of side-chain interaction.

The changes which myosin undergoes upon *dehydration* are not detectable by x-ray measurements (34). They result in aggregation of the α-form by further interaction between side chains of adjacent polypeptide chains. No sulfhydryl groups become exposed under these conditions (265–267), in contrast to the *denaturation* by heat, where the α configuration breaks down to give way to chain bundles of incompletely oriented fibres of the β configuration.

This brief survey of the intramolecular rearrangement of keratin and myosin has been given to emphasize one basic fact, i.e., that *the fibrous proteins, like the*

[17] The importance of covalent cross linkages in conferring elasticity on wool is clearly illustrated by the work of Harris and associates (147, 315).

globular proteins, are subject to denaturation by mechanical and chemical forces, and that the degree of resulting disorientation is related to the reactivity and mutual interaction of the side chains.

Side-chain interaction is more profound in the case of the *globular* proteins, if for no other reason, because the coiled configuration of the polypeptide chains affords a closer geometrical proximity of side chains. This upon denaturation conceivably may lead to either one of two opposing effects: (*1*) a rupture of cross linkages, leading to the liberation of extended, though disoriented, polypeptide chains, of the kind observed with supercontracted myosin or keratin; (*2*) the formation of new cross linkages, as in the case of the permanently "set" keratin fibres, with the net result that the molecule has passed from one rigid configuration to another, less specific one.

Remarkable progress has been made in recent years in the investigation and interpretation of x-ray diagrams of crystalline globular proteins (*cf.* reference 142). A great number of very definite reflections have been observed for hemoglobin, lactoglobulin, insulin, and other proteins when in the wet state, while upon drying many of the smaller spacings disappear.

Investigation of the denatured proteins has been less satisfactory, in regard to both the conditions of denaturation and the resolution of the diffraction patterns. Following the work of Astbury and Lomax (36), Astbury, Dickinson, and Bailey (35) investigated the denaturation of edestin, excelsin, and egg albumin by urea and heat. In a typical experiment, edestin was denatured by dissolving it in aqueous urea, and the urea solution was poured evenly over a glass slide which was then inserted in water until the protein was deposited as a thin layer. After the protein had dried, the process was repeated several times and the accumulated layers of denatured protein were peeled off, compressed between glass plates, and cut into strips. These films were extensible up to 200–250 per cent of their original length, and when released contracted to about 100 per cent of their initial length. In the unstretched state, urea-denatured edestin gave a pattern similar to that of disoriented β-keratin fibres (supercontracted) while upon stretching, orientation became more complete, the resulting diffraction diagram resembling closely that of fully extended β-keratin. It is of interest to note that in this, as well as in all other experiments that have been recorded, denaturation of these globular proteins gave rise to a sharpening of the backbone spacing of about 4.6 Å. and to the appearance of at least one outer ring corresponding to a spacing of about 3.7 Å. (31), the significance of which is not quite clear (table 12).

While the aforementioned experiments were carried out on mechanically oriented *layers* of denatured proteins, more recent work on egg albumin and other proteins, denatured by anionic detergents (314a) or heat (356a), reveals the diagonal spacing of about 3.7 Å. to appear even prior to mechanical stretching of the bulky *precipitate* of denatured protein. Stretching serves mainly to improve the orientation of the polypeptide chains, yielding a pattern similar to that observed by Astbury for layers of denatured proteins and for β-keratin, alike. A summary of the most important diffraction spacings of denatured

globular proteins is given in table 12. The agreement between the data obtained by different workers may be seen to be extremely good.

In general, one may conclude that denaturation of globular proteins results in a structure similar to that revealed by keratin when it "supercontracts," i.e., a bundle of disoriented polypeptide chains. The interesting point, however, is that this configuration has been reached in these two instances from opposite ends, i.e., from a fibrous state with keratin (or myosin) and from a more condensed, globular state with edestin, egg albumin, serum albumin, etc. When the denatured proteins become oriented, as by stretching, a structural pattern, analogous to that of fully extended β-keratin fibres is obtained. This latter has been shown most clearly by investigations of the x-ray properties of built-up monolayers of egg albumin (53).

TABLE 12

X-ray diffraction spacings of some native and denatured proteins

PROTEIN	DENATURING AGENT	SIDE-CHAIN SPACING	BACKBONE SPACING	DIAGONAL SPACING	REFERENCE
		Å.	Å.	Å.	
Egg albumin	Native	10.65	4.75		(31)
	Heat	10.2	4.75	3.67	(31)
	Heat	10.2	4.66	3.75	(314a)
	Heat	9.8	4.65	3.75	(356a)
	Alkyl benzenesulfonate	10.3	4.65	3.76	(314a)
Serum albumin	Native	9.7	4.6		(36)
	Heat	9.6	4.5	3.6	(36)
Edestin	Urea	10.0	4.5	3.7	(35)
β-Keratin		9.8	4.65	3.75	(36)

Lately, it has been reported that while fibrinogen can be made to change its pattern from one resembling α-keratin to that of the β configuration by a special method of lateral squeezing, no change in diffraction pattern occurs when fibrinogen is converted to fibrin (40). Thus, clotting does not appear to be an example of true denaturation.

Several qualitative studies on the changes in x-ray patterns of proteins upon denaturation have been reported (102, 368). However, these were not only done under ill-defined conditions of denaturation, but unfortunately on vacuum-dried preparations. Since the specific configuration of the globular proteins changes readily upon drying much in the same manner as it does upon denaturation (31), an evaluation of the meaning of these investigations is practically impossible. Spiegel-Adolf and Henny (368), in one of their investigations, claimed to have obtained evidence for the reversal of the heat coagulation of serum albumin. However, examination of the published transmission curves reveals a closer relation between native and coagulated protein, than between native and "reversibly" denatured protein.

The interpretation of x-ray data of denatured proteins in terms of a liberation of polypeptide chains receives strong support from measurements of the properties of protein monolayers. Since both types of measurements, though of quite different nature, lead to essentially the same conclusion, we shall, at this point, examine the evidence which measurements of protein films have provided:

2. Monolayers as prototypes of completely denatured proteins

A few years ago, Neurath and Bull (293) undertook a critical examination of the available data concerning the structure and properties of protein monolayers. It was concluded that under the influence of surface forces, protein molecules unfold when spread onto the surface of a water layer, or in an interface, and that the structure of these films corresponds closely to that predicted for an array of fully extended polypeptide chains of the β configuration (*cf.* 279). At low pressures, the chains were assumed to lie on their side, with the side chains parallel to the water surface, whereas upon lateral compression they were assumed to turn around until the side chains were nearly vertical. Evidence for this interpretation was derived from a comparison of mean surface area per residue and of film thickness, with the values calculated for fully extended polypeptide chains.

Further, it was concluded that in protein monolayers the polar side chains are oriented toward the aqueous phase, and the non-polar side chains toward the air phase. This, however, could only be so if along the *entire* polypeptide chains the side chains are alternately polar and non-polar, (. . . PNPNPNPNPN . . .), whereas a single deviation from this regular sequence, i.e., (. . . PNPNPPN-PNPN . . .), would result in one-half or more of the polar chains emerging from the upper surface of the film, and the corresponding fraction of the non-polar chains from the lower surface (74). There is good reason to believe that polypeptide chains will tend to assume a selective orientation in order to satisfy the respective affinities of polar and non-polar side chains for polar and non-polar media. Measurements of the adhesion tension of monolayers of egg albumin at various pressures (74) substantiate this view. Thus the films convert from a quite hydrophilic state at pressures below 0.5 dyne to a more hydrophobic state at pressures of about 1.5 dynes, owing to the forcing out of hydrophobic groups from the water surface. However, there is no need to conclude that this orientation is perfect; more often than not a hydrophobic group may remain in the water phase if the attainment of selective orientation should interfere with the steric arrangement of the chains.

This raises the question whether and to what extent a fully extended polypeptide chain is capable of internal rotation. Cockbain and Schulmann (109) suggested that separation of polar from non-polar side chains takes place by means of looping of the polypeptide chain, the mode of looping depending on the relative proportion and sequence of polar and non-polar groups. This part of their hypothesis is fully acceptable, provided that a proper rôle be accorded to the individual side chains as regards both mutual attraction (30, 76) and steric inhibition (288, 291). However, their conclusion that proteins cannot

consist of fully extended polypeptide chains of the β-keratin type, if the concept of kinking and looping is accepted, may be misleading, since by and large, and particularly from the x-ray viewpoint, it is more or less immaterial whether a long polypeptide chain is fully extended or whether it contains a few isolated folds. This is borne out by Astbury and Bell's (33) investigation of the x-ray structure and optical properties of built-up monolayers of egg albumin. To quote these authors: "The simplest description of the x-ray photographs so far obtained is that they are analogous to those of keratin that has been squeezed laterally in steam; this treatment not only brings about the α-β transformation, but also tends to orient the side chains perpendicular to the plane of flattening." By a simple mechanical device, Astbury and Bell were able to measure directly the thickness of films composed of up to 1764 monolayers, yielding a mean thickness per monolayer of 9.5 Å., in excellent agreement with surface-film measurements and with the value of the side-chain spacing of β-keratin. As is to be expected, unfolding of the protein molecule incidental to surface denaturation results in the liberation of the total complement of sulfhydryl groups of egg albumin (271).

The influence of denaturation on the spreading properties of proteins has been the subject of several investigations. The original observation that heat denaturation of serum albumin inhibited the formation of surface films at pH 4.4 (285) has been confirmed (74). The apparent lack of spreading power appears to be due to the aggregation of the heat-treated protein at this particular pH (118), since in lower pH regions spreading is not inhibited. This is true for both heat- and urea-denatured egg albumin which, at pH 2.2, exhibit the same film areas and compressibilities as the native protein (74). The two denatured proteins differed from each other and from the native material in their pH–film area relation. Ställberg (369) found also native and heat-denatured serum albumin to spread to about the same extent on a $M/150$ phosphate buffer, pH 7.0, when dispersed in an aqueous solution of 60 per cent propyl alcohol containing also 0.5 M sodium acetate. It appears, therefore, that a denatured protein unfolds under the influence of surface forces as readily as the native protein, if not more so, provided aggregation is avoided.

Langmuir (219) and Schaefer (353) reported differences between native and denatured proteins with respect to the "expansion patterns" they yield upon applying indicator oil on the protein monolayers. The meaning of these purely qualitative observations is obscure.

3. Biological activity of protein monolayers

Since surface denaturation of globular proteins results in a more or less complete liberation of fully extended polypeptide chains, it affords excellent conditions for determining the contribution of the intrinsic structure of the intact molecule to the biological activity of the protein. Unfortunately, in earlier investigations the precision requisite for obtaining truly monomolecular films has often fallen victim to the enthusiasm with which these experiments were carried out, with the result that, in many instances, what was thought to be a

protein monolayer actually was a monolayer in combination with adsorbed unspread protein. The apparent film thickness varied from anywhere between 10 and 45 Å. (discussed in reference 348). Also, the assumption that the thickness of a protein layer adsorbed on a metal slide equals that of the film on an aqueous surface often may be misleading (124).

The work of Rothen *et al.* (348) is free of such ambiguities. It was found that monolayers of metakentrin, a gonadotropic hormone of the anterior lobe of the pituitary gland, and monolayers of the oxytocic pressor hormone of the posterior lobe of the pituitary were practically inactive, in contrast to surface films of insulin which were as active as the native protein. While the specific intrinsic configuration of the molecules of the pituitary hormones is thus essential for their biological activity, this does not seem to be so with insulin, unless one assumes that surface denaturation of the latter is a reversible process. While the high cystine content of insulin may render its component polypeptide chains susceptible to reversible contraction, analogous to keratin fibres, there is no experimental evidence to sustain such a hypothesis.[18] There is, however, evidence that in the denatured state proteins may exhibit certain manifestations of biological activity. For instance, monolayers of metakentrin, although devoid of hormonal activity, react specifically with antisera to the native protein (348). Monolayers of egg albumin, of serum proteins (348, 350), and of other antigenic materials (43, 92) likewise are capable of combining specifically with their homologous antisera (Part III E). *Vice versa*, denaturation of antibodies by various means (136, 350) does not impair the specific reactivity with the homologous native antigens. It appears, therefore, that certain kinds of biological activity require the specific configuration of the native protein, whereas others reside merely in the specific amino acid structure of the polypeptide chains, regardless of the latter's mode of folding.

E. IMMUNOLOGICAL PROPERTIES OF DENATURED PROTEINS

Attempts to elucidate the relation of protein structure to immunological properties have been most fruitful in those instances in which the protein antigen was chemically modified, thereby giving rise to a new serological specificity characteristic of the chemical constituents which had been introduced (218). Although it has long been known that treatment with typical denaturing agents may also alter profoundly the immunological behavior of antigens and antisera, only recently has significant progress been made in this field, largely owing to availability of pure material and improved experimental technique. Earlier observations on the effects of heat, acids, alkali, irradiation, and sound waves already have been summarized (69, 183, 253).

1. Surface denaturation

A direct approach to the problem under consideration lies in the study of the immunological properties of protein films, for if denaturation of globular

[18] *In vivo* reversal of denaturation is not excluded by these experiments.

proteins is essentially a steric rearrangement of constituent groups in the molecule, then the complete spreading of a protein into a thin film represents an optimum degree of denaturation (see introduction). Such studies have been carried out with certain enzymes (151, 176, 220, 364) and hormones (348), as well as with a number of antigens and antibodies. In 1938 Shaffer and Dingle (359) showed that a film of egg albumin, 40 Å. thick and adsorbed on a stearate surface, could react with its antiserum. The activity of films of antibodies to types I, II, and III pneumococci, and of diphtheria toxin (33 Å.) was similarly demonstrated[19] by Porter and Pappenheimer (328). However, since it has been established that a fully spread protein monolayer rarely exceeds 8–9 Å. in film thickness (293), the above investigations leave room for doubt, for such thick films may represent merely a layer of oriented, undenatured antibody molecules. Dean *et al.* (124) have emphasized the extreme care necessary to obtain monolayers uncontaminated by dissolved protein.

By an ingenious method, Chambers *et al.* (91, 92) found the two surfaces of presumably fully expanded protein films derived from hemolytic streptococci to behave differently in agglutination reactions, indicating the indifference of serological activity to specific configuration and the possibility that activity may reside in discrete structural features. Unfortunately, the film thickness in the former communication (91) was not specified, while in the latter (92) the films (14–20 Å.) represent at best only partially unfolded molecules.

Rothen and Landsteiner (349) clearly demonstrated the ability of a monolayer of egg albumin, 8–9 Å. thick, to combine with its antiserum. This investigation was extended (348) to insulin, to oxytocic pressor hormone, and to metakentrin, the gonadotropic hormone of the anterior lobe of the pituitary gland. More recently, Rothen and Landsteiner (350) studied the serological activity of films of egg albumin, horse and human serum albumins, and serum globulins; all were found to combine specifically with their antibodies. Likewise, films of purified anti-SSS I and anti-SSS III could specifically fix the corresponding polysaccharide, as evidenced by the deposition of another layer of antibody after treating a film with antigen solution.

2. Denaturation by urea and guanidine hydrochloride

Urea, guanidine hydrochloride, and other amides in high concentrations have been shown to be effective denaturing agents, causing partial unfolding of certain protein molecules (75, 295, 296, 300). Apparent reversal of this process resulted in a molecule of size, shape (295), and electrophoretic mobility (358) approximating those of the native material, whereas the specific internal configuration was not fully regained (64, 297). An investigation of the comparative antigenic behavior of the native and the modified proteins revealed (135) that although crystalline horse serum albumin regenerated from 8 *M* urea solution had lost over 90 per cent of its ability to elicit the formation of precipitins in rabbits, it had retained its original specificity and could combine with either antisera to the same titer as the native protein.

[19] A finding not entirely in agreement with other observations (122).

Similar results were obtained with bovine serum albumin regenerated from urea or guanidine hydrochloride (254), although with this protein the more powerful denaturing action of guanidine hydrochloride was required to decrease significantly the antigenic activity of the naturally weakly antigenic, native protein. These investigations are of particular significance, not only since they were carried out with a pure protein, well defined in the native and the denatured states, but also as they demonstrate clearly the contribution of the intact internal configuration of this protein to antigenic activity.

Recently, it has been suggested that antibody globulin differs from normal globulin in the spatial configuration of the polypeptide chains rather than in chemical composition, and that denaturation of normal globulin, followed by regeneration in the presence of an antigen, should give rise to homologous antibodies (316). This hypothesis was supported by corresponding experiments in which the formation of a small amount of weak antibodies was observed (317). However, in keeping with this hypothesis, denaturation of an antibody or normal globulin, followed by regeneration in the absence of the homologous antigen, should give rise to a material essentially devoid of serological specificity; for, in the absence of specific directive forces, statistically only a small fraction of the molecules should revert to that configuration essential for activity. Such experiments were performed recently by some of the present authors, using purified antipneumococcal horse serum globulin type I and normal horse pseudoglobulin, denatured by and regenerated from 8 *M* guanidine hydrochloride (136).

It was found that the irreversibly denatured, and regenerated antibodies were strongly precipitated by the homologous antigen (SSS I), although in different optimum ratios than the native, while the native, irreversibly denatured, and regenerated normal globulins were all devoid of serological activity. These findings, to be discussed in detail elsewhere (137), suggest not only the independence of antibody activity to a specific structure, already demonstrated by surface-film studies, but also differences between normal and antibody globulin in chemical structure, i.e., the nature and sequence of amino acid residues in the polypeptide chains.

3. Miscellaneous observations

As early as 1896, Salter (351) observed a decrease in antigenicity of serum by heating. Since then, a number of reports have been published dealing with changes in the immunological properties of various antigens and antibodies upon treatment with a diversity of reagents (see 183, 218). Although there is general agreement that the altered protein antigens possess decreased antigenicity and a new specificity, and that the treated antisera react sluggishly, limited recognition of the nature of these denaturation processes renders an interpretation of these observations difficult. Nevertheless, several investigations are worth mentioning at this point.

(*a*) *Heat:* The effects of heat on the immunological properties of normal and immune sera, isolated antibodies, and a number of virus antigens have been

extensively investigated (45, 46, 47, 201–203). With all viruses studied,—i.e., tomato bushy stunt virus, tobacco mosaic virus, potato "X" virus, and tobacco necrosis virus,—infectious activity could be destroyed with little or no detectable changes in serological or physical properties. This was accomplished by mild heat treatment, irradiation with x-rays or ultraviolet light (48, 49), low concentrations of urea (52), or freezing under controlled conditions (54). Chemical agents, such as formaldehyde, nitrous acid, or hydrogen peroxide, produced similar effects (48–51, 370). More drastic treatments led to a higher degree of denaturation, as evidenced by a loss of serological activity accompanied by changes in chemical and physical properties.

Mixtures of proteins form complexes when heated to 75° to 80°C., as evidenced by salting-out (201) and electrophoretic studies (206, 397) (Part III C).

When somatic antigens are heated at 80°C. in the absence of non-specific protein and salts, they still can precipitate homologous antibodies; however, addition of non-specific proteins and salts before heating gives rise to complexes behaving like non-precipitating haptenes (45).

Injection of non-precipitating complexes of both tomato bushy stunt virus and human serum globulin into rabbits gives rise to antibodies identical in behavior with those against unheated antigens (46). Similar heat experiments on antisera and isolated antibody globulin fractions showed a difference in behavior, depending on the type of homologous antigen used (202), i.e., rod-shaped or somatic. Rabbit antisera to the former lost all serological activity upon mild heating, whereas antibodies to the latter gave rise to non-precipitating complexes when heated with non-specific albumin. These complexes were capable of combining with antigen but incapable of neutralizing infectivity, specific precipitation, or complement fixation (47). While differences were found when heated in the presence of albumin, isolated antibodies to both types of antigens were equally susceptible to heat.

(*b*) *Irradiation:* In general, irradiation of proteins by ultraviolet light, alpha rays, or visible light in the presence of a photosensitizer leads to the formation of heterogeneous solutions (pages 164 and 220). It has long been known that, following irradiation with ultraviolet light, antigens react more weakly with antisera to the untreated protein (42). More recently it has been found that ultraviolet inactivation of urease renders the enzyme non-antigenic (325), while antibodies to irradiated tobacco mosaic virus still can neutralize the infectious activity of the virus (370). Progressive photoöxidation of certain antisera causes a progressive lowering of the potency of these sera, with a simultaneous loss of tryptophan and histidine (363). Normal horse serum irradiated for several days with visible or ultraviolet light possesses a new specificity and a decreased antigenicity (183).

(*c*) *Acid and alkali:* Treatment of egg albumin with acid or alkali decreases the antigenic activity of the protein and alters its serological specificity (144, 197, 250, 405, 414). Acid treatment of pneumococcus rabbit antibodies lowers the ratio of mouse protection units to precipitable N and results in a decreased ability to fix complement, while the property of capsular swelling as well as the ability to transfer anaphylaxis passively are retained (402). The C_2' and C_4'

components of complement have been shown to be unstable in acid but stable in alkali, the opposite being true for C_1' and C_3' (324). Preparation of apparently non-antigenic beef sera by treatment with alkali has been described (22, 132).

(*d*) *Enzymatic digestion:* Treatment with papain or pepsin under special conditions produces abrupt changes in the state of dispersion of egg albumin, diphtheria antitoxin, and beef globulins (4, 319, 320, 322, 392). The antigenic qualities of diphtheria antitoxin are greatly impaired upon digestion by pepsin (327, 403). Staphylococcus toxin, although completely detoxified by controlled peptic digestion, still possesses a marked antigenic capacity (314b). Under similar treatment, solutions of anti-SSS I horse globulins become partially non-precipitable by trichloroacetic acid, while the remaining protein can still combine with antigen, although in a ratio lower than that characteristic of the untreated protein (153, 321). More recently it has been reported that digestion by pepsin or diastase renders antitoxic plasma more resistant to subsequent heat treatment, decreases the size of the antitoxic molecule, and alters its antigenic character (198).

4. General considerations

While a widespread theoretical and practical interest has stimulated numerous investigations on the immunological properties of modified proteins, in only a few instances has such a degree of precision been attained as to permit interpretation of the data in terms of protein structure. Evidence derived from denaturation of well-defined proteins by surface forces or by urea or guanidine hydrochloride, suggests that the serological activity is independent of a specific internal configuration, while the antigenic activity appears to be impaired by processes leading to profound changes in protein structure. Beyond this, little or nothing is known about those attributes of a protein which endow it with immunological activity. There is no doubt that this aspect of the problem of protein denaturation requires vigorous investigation with the precision demanded by present-day standards of immunology and protein chemistry.

IV. IS DENATURATION REVERSIBLE?

The problem of the reversibility of denaturation occupies a key position in considerations of protein denaturation and protein structure. It centers around the question whether and to what extent a denatured protein can regain the specific chemical, physical, and biological properties characteristic of the native form. Experience has shown that certain proteins, such as serum albumin, hemoglobin, trypsin, or lactoglobulin (232), are capable of reverting from the denatured insoluble state to a soluble form which resembles the parent native protein in one or more properties. Other proteins, such as egg albumin, appear to be incapable of this conversion, or at least capable to only a limited degree.[20]

[20] The observation that, following denaturation by urea or heat (75, 236, 406), a fraction of egg albumin fails to precipitate upon adjustment to the isoelectric point, suggests partial regeneration rather than incomplete denaturation of the protein.

For a given protein, the extent of "reversal" may vary with the conditions applied for denaturation, and, moreover, may depend on the procedure chosen for reversing the denaturation process. For instance, inactive denatured trypsin may be converted into a soluble active form when heat denaturation is carried out in acid, but not in alkaline, solutions (305). Rapid neutralization of acid-denatured hemoglobin yields practically no soluble protein (10), whereas more than 50 per cent may be rendered soluble upon slow neutralization (272).

The ultimate answer to the present problem lies in a precise comparative structural analysis of the native and the reversibly denatured protein, extending all the way down to the spatial arrangement of the constituent atoms and groups. As this is beyond experimental approach one has to rely on a study of measurable changes elicited on *denaturation*, with the object of ascertaining whether some or all of them can be *reversed*. *A priori*, such an analysis is not wholly conclusive, in that some irreversible changes in properties may occur which are not revealed by the particular properties open to investigation by present-day methods. On the other hand, any single irreversible change in property must constitute a sufficient criterion for the irreversibility of the denaturation process. The question can thus be answered negatively by proving the irreversibility of any single change, but not positively since it is impossible to demonstrate the identity of a multitude of properties of native and of reversibly denatured protein.

The mere fact that one or the other of the reactions accompanying denaturation can be reversed may be of considerable importance. Thus, the work of Northrop, Kunitz, and Herriott on the reversible inactivation of certain proteolytic enzymes (*vide infra*) has gone a long way toward identifying the enzyme proteins with the focus of biological activity, and has furnished valuable information on the nature of the inactivation process. Similarly, the apparently reversible changes in absorption spectrum of hemoglobin, following reversal of various kinds of denaturation to which the protein was subjected, have thrown light on the functional relation between protein carrier and prosthetic group. The nearly reversible changes in molecular asymmetry of serum proteins testify to the presence of forces which provide for the long-range elasticity of globular and fibrous proteins alike.

Just as the denaturation process has been studied by a limited number of methods, so has the reversal of denaturation. The most important of these are (*1*) solubility in salt solutions, (*2*) reactivity of chemical groups, (*3*) biological activity, (*4*) methods relating to molecular size and shapes, and (*5*) crystallinity.

1. Early observations

Michaelis and Rona (257) were probably the first to describe a reversal of protein coagulation. These authors observed that a coagulum of serum albumin, obtained by heating a solution at nearly neutral reaction and then adjusting the pH to the isoelectric point, could be redissolved in acids and remained soluble on readjustment to the isoelectric point. The protein now behaved like a "genuine protein", also in that it was again susceptible to heat coagulation. Spiegel-Adolf found that heat-coagulated serum albumin could be made to regain in part the solubility properties of the native protein by dispersing it in $N/100$

sodium hydroxide, followed by dialysis and electrodialysis (366). This "regenerated" protein resembled native serum albumin also in optical activity and serological properties. Anson and Mirsky (14) showed that if acidified solutions of serum albumin were heated and neutralized while the solutions were hot, complete precipitation of the protein ensued, while neutralization after cooling yielded about 70 per cent of soluble crystallizable protein. The same yield was obtained upon neutralization of solutions of acid acetone-precipitated serum albumin. Similar experiments were carried out by these authors with crystalline hemoglobin (10). In one (272) of a series of investigations (10–12, 272, 273) the heat-coagulated protein was dissolved in an excess of sodium hydroxide; after standing at room temperature, the solution was carefully neutralized with acid. With *oxidized ox* hemoglobin, very little soluble material could be recovered unless sodium hydrosulfite or sodium cyanide was added, whereas neither *oxidized* nor *reduced horse* hemoglobin was capable of regeneration unless cyanide was present, in which case 30 per cent of the protein became soluble. Native and regenerated hemoglobin were reported to resemble each other in crystallinity, color, and absorption spectra, and in being capable of reversible oxygenation (272). When denaturation was effected by hydrochloric acid, regeneration could be achieved by careful neutralization even without cyanide, yielding about 50 per cent of water-soluble hemoglobin (273). Similar procedures have been followed for the preparation of soluble globin from acid-coagulated globin (11, 13) with a yield of about 65 per cent for horse globin and 80 per cent for ox globin. The regenerated globins were capable of combining with heme to form compounds exhibiting the absorption spectrum of native hemoglobin, as discussed previously (page 216).

That reversal of the denaturation process may result in the reactivation of enzymes was demonstrated by the work of Northrop, Kunitz, and Herriott and of Anson and Mirsky on crystalline trypsin (17, 214, 305), chymotrypsin (215), and pepsinogen (185). Inactivation and denaturation were effected by heat or alkali, leading to a proportionate decrease in enzymatic activity and soluble protein. Following regeneration, the activity was regained in proportion to the amount of protein that became soluble. This parallelism between activity and protein solubility is significant, since later work has shown that some proteins may remain active under conditions which usually cause denaturation (235, 348), whereas others may regain their original solubility and other characteristic properties without being biologically active (53, 54).

Reversal of inactivation of insulin was observed by du Vigneaud *et al.* While heating at 100°C. in $N/10$ hydrochloric acid yielded a water-insoluble physiologically inactive precipitate, regeneration by alkali restored both biological activity and solubility in acids (130, 131). Partial reactivation has been found to occur also when insulin was inactivated by acid alcohol, yielding an easily hydrolyzable N-methyl compound (94), or when inactivated by 0.7 M ammonium hydroxide. Treatment with sodium or potassium hydroxide led to irreversible inactivation, while sodium carbonate or disodium phosphate was without effect (408).

In order to evaluate critically the experimental data pertaining to a reversal

of denaturation, we shall consider three proteins which have been most thoroughly studied with a combination of methods.

2. Serum albumin

When crystalline horse serum albumin is denatured by concentrated solutions of urea or guanidine hydrochloride, gross changes in molecular shape occur (page 222), though the molecular weight remains essentially constant (page 212). Removal of the denaturing agents by dialysis results in the separation of two fractions, one of them being insoluble in the pH region of the isoelectric point of the native protein, and the other remaining soluble (295).

At constant protein concentration, the amount of soluble "regenerated" serum albumin decreases in a sigmoidal manner with the concentration of denaturing agent originally present, reaching a lower limiting value of about 85 per cent of the total protein with concentrations of urea higher than 6 *M* and of guanidine hydrochloride higher than 4 *M*. With bovine albumin (331), the limiting yield of "regenerated" protein varies with the degree of "purity" of the preparation, being about 95 per cent for whole albumin containing 0.4 per cent of carbohydrate, in contrast to about 50 per cent for carbohydrate-free crystalline serum albumin. The extent of regeneration is thus seen to depend not only on the animal species but also on the method of preparation of the protein in question. Recent experiments (299) have shown that the quantitative distribution between insoluble "irreversibly" denatured protein and soluble regenerated protein depends also on the initial protein concentration, the yield of regenerated material increasing as the protein concentration is decreased. The parallelism between the relation of concentration of denaturing agent to increase in molecular asymmetry on the one hand, and to yield of "regenerated" protein on the other, is of significance. Since each concentration of urea, or of guanidine hydrochloride, produces a definite increase in the molecular asymmetry of each protein molecule, one may conclude that the greater the extent of change in molecular configuration, the smaller the fraction which returns to a soluble state. Since diffusion and viscosity measurements indicate that the regenerated protein resembles closely the native material in molecular size and shape properties, the regenerated protein may be associated with a more condensed configuration in contrast to the extended configuration of the asymmetric, denatured protein. It might be argued that incomplete reversal may have arisen from incomplete denaturation of the protein, the regenerated material corresponding to an undenatured fraction, or else from the presence of two fractions in the native protein, one of them being incapable of regeneration. The first of these hypotheses can be dismissed by the observed monodispersity of solutions of denatured serum albumin, as revealed by diffusion measurements, discussed in detail elsewhere (298). The second hypothesis is equally improbable, since denaturation of the regenerated fraction again gives rise to two fractions of soluble and insoluble protein, respectively, in the same distribution as was found following regeneration of the original denatured protein. The mechanism underlying this separation is not altogether clear. Comparison of the shape

properties of the denatured protein in the presence of the denaturing agent, and of irreversibly denatured serum albumin in buffer solutions, reveals the latter to be in a more condensed state than the former. Since, moreover, the relative dimensions of the denatured protein molecules are of considerably lower order of magnitude than those they would exhibit if they were fully extended polypeptide chains, it is apparent that they possess a high degree of internal cohesion which causes them to recoil when the denaturing agent is removed (297).

The suggestion has been made that incomplete regeneration was due to the interaction between denatured molecules, causing the latter to aggregate before they had an opportunity to recoil fully to the condensed configuration characteristic of the regenerated state (297). This hypothesis has received further support from the findings that the size of the fraction of regenerated protein increased upon decrease in protein concentration. However, since even in the more concentrated solutions (2 per cent) the mean interparticle distance exceeds by far the range over which common types of attractive forces are operative, the problem of the mechanism responsible for incomplete regeneration of this, as well as of other proteins that have been similarly studied (296), remains open.

The regenerated horse serum albumin resembles the native protein in molecular-kinetic properties, in crystallinity, and in serological specificity (135). However, it can be distinguished from the latter by three criteria: (*1*) It reveals a higher electrophoretic mobility on the alkaline side of the isoelectric point (358) and shows a greater amount of electrophoretic boundary spread. (*2*) It is more susceptible to tryptic fission, being hydrolyzed at a rate higher than that of the native protein and the same as that of the irreversibly denatured material (64, 331). (*3*) Although it gives rise to the same type of antibodies as the native protein, its capacity to produce antibodies is greatly impaired (135, 254). On the strength of one or all of these criteria, denaturation of serum albumin has to be considered as an irreversible process.

As discussed previously (page 222) it is not clear whether heat denaturation of serum albumin causes the same type of changes in the protein molecule as does denaturation by urea, guanidine hydrochloride, or synthetic detergents. Though at the temperature of heating the protein becomes insoluble at the isoelectric point (14, 366), the solubility is regained in part if the heated solutions are readjusted to the pH of the isoelectric point after cooling (340). Though the material is devoid of reactive disulfide groups, it can be crystallized and is serologically equivalent to the native protein (259). Its molecular-kinetic properties are different, at least under the specific conditions at which the latter were determined (118). In the absence of quantitative measurements, it is not possible to decide whether the incomplete regeneration following denaturation by heat, or by acid acetone (14), is due to the same factors previously considered for the regeneration of the urea-denatured protein.

3. Hemoglobin

The denaturation of the hemoglobins by urea, acid, heat, and sodium salicylate has been considered earlier in this review. The present discussion is concerned

primarily with the reversal of these processes and with the question of the identity of regenerated hemoglobin with the native protein. Specifically, the regeneration following denaturation by (*a*) urea, (*b*) acid, and (*c*) sodium salicylate will be considered together with the analogous reactions of the globin moiety.

(*a*) *Denaturation by urea:* Evidence previously considered (page 217) suggests that the following reaction occurs when carboxyhemoglobin (horse) is exposed to concentrated urea solutions for limited periods of time:

$$H_4Gl_2 \rightarrow 2H_2Gl \qquad (1)$$

where H_4 denotes heme and Gl denotes globin. Upon prolonged contact of the reactants denaturation is supposed to proceed further, much in the same manner as denaturation by sodium hydroxide (128), yielding a compound of oxidized heme and denatured globin; this, in turn, is split into alkaline hematin and denatured globin. However, the latter reactions occur very slowly, and considerably more slowly with carboxyhemoglobin than with oxyhemoglobin.

Reaction 1 can be reversed in part by removal of urea or similar denaturing agents (formamide and acetamide) by dialysis (374). As in the case of serum albumin, the amount of regenerated protein decreases with increasing concentrations of the denaturing agent and, in analogy with serum globulin (296), it varies also with the nature of the denaturing agent. Thus, according to Steinhardt (374), with 4 *M* urea 70 per cent of the protein becomes soluble, with 6.5 *M* urea less than 50 per cent, and with 7.5 *M* urea practically none. With formamide, the total protein can be recovered in a soluble, regenerated form.[21] The distribution is independent of the rate or other conditions of dialysis.

It is difficult to decide whether reaction 1 is reversible for that fraction that reverts to a soluble state. As has been shown in Parts II B and III A, denaturation by urea proceeds essentially along the same path as the first step of the denaturation by acids, i.e., a dissociation of the molecule into halves, initiated by the rupture of two hydrogen bonds. If bond formation is restricted to two special groupings, then recombination would likewise be restricted to these very groupings and denaturation would be a truly reversible process. However, the fact that half-molecules are formed with both types of denaturation does not necessarily indicate that they are limited to the same mechanism. Moreover, the observations that the regenerated protein shows a greater amount of boundary spread in sedimentation, and a greater tendency for aggregation, are suggestive of the existence of some differences between native and regenerated protein (374).

(*b*) *Denaturation by acid:* This reaction occurs in two stages, as discussed previously, i.e., dissociation according to reaction 1, followed by

$$H_2Gl \rightarrow H_2 + Gl' \qquad (2)$$

[21] Indicating again the inadequacy of a single criterion for estimating the extent of denaturation. Thus, any one of these amides produced the same change in molecular weight; yet the degree of regeneration is quite different.

Reaction 1 occurs between pH 6 and pH 4, while reaction 2 begins at pH 3.9 and is probably complete at pH 3.1 (190). According to a preliminary note by Drabkins, reaction 2 is a slow one (128), whereas Mirsky and Anson observed that 3 min. after exposure to dilute hydrochloric acid, all the protein was precipitated upon rapid neutralization with sodium hydroxide at 0°C. (273).

The yield of soluble regenerated hemoglobin, obtained after careful neutralization with sodium hydroxide in the presence of sodium cyanide, depends on the time of contact with cyanide and increases from 10 per cent after 1 min. to about 75 per cent after 65 hr. (273). In the absence of cyanide, about 50 per cent of the protein is obtained in soluble form. Denatured globin, prepared by acetone precipitation from a solution of acid-denatured hemoglobin, likewise yields about 70 per cent of soluble regenerated globin (13).

The question as to whether reaction 2, i.e., the conversion of native to denatured globin, is strictly reversible cannot be answered at the present time, since globin has yet to be isolated without the intermediary step of denaturation. However, if it is tentatively assumed that reaction 1 is reversible, then full identity of native and regenerated hemoglobin would prove the reversibility of reaction 2. Mirsky and Anson reported that regeneration of heat-coagulated hemoglobin yields a material which is indistinguishable from the native protein in crystal form, coagulation temperature, color, position of absorption bands, and ability to combine reversibly with oxygen and carbon monoxide (272). Holden (190, 191), in a series of investigations, demonstrated the identity between native and acid-regenerated carbon monoxide hemoglobin with respect to their characteristic absorption bands. As noted previously (page 216) the regenerated protein is devoid of free sulfhydryl groups, in contrast to the denatured protein, and exhibits the same molecular size and shape properties as the native protein (339). However, differences were observed (*1*) in susceptibility to dissociation by alkali, (*2*) in electrophoretic mobility, and (*3*) in the shape of the oxygen dissociation curve (see page 216). It is difficult to decide whether these changes, testifying to the lack of identity between native and regenerated hemoglobin, should be ascribed to the irreversible character of reaction 1 or 2. However, as the latter gives rise to more drastic changes in the protein molecule, i.e., splitting away of the heme moiety accompanied by denaturation of the globin part (as witnessed by the exposure of sulfhydryl groups, and insolubility of the protein), it is apparent that reaction 2 would be less likely to be reversible than reaction 1. We are inclined, therefore, to blame the irreversible nature of the over-all reactions mainly on the failure of denatured globin to revert to the characteristic configuration of the original protein, in analogy with the regeneration of serum albumin, although it remains to be established whether under conditions favoring reaction 1, at the exclusion of reaction 2, dissociation of hemoglobin into half-molecules can occur in a truly reversible fashion.[22]

(*c*) *Denaturation by sodium salicylate:* The reaction between hemoglobin and sodium salicylate has been cited by Anson and Mirsky (18) in support of the

[22] See, however, the lack of identity of native and recombined *Helix pomatia* hemocyanin, as revealed by electrophoresis (393).

existence of mobile equilibrium between native and denatured protein, which is progressively shifted toward the denatured side as the concentration of salicylate is increased.

According to this theory, denaturation is an all-or-none reaction in that the protein is in either a native or a completely denatured state, the concentration of the denaturing agent merely governing the quantitative distribution between these two forms. This concept, based upon limited observations, is incompatible with subsequent evidence derived from investigation of the effects of increasing concentrations of urea and other denaturing agents on proteins (160–162, 290, 295–297), according to which an increase in the concentration of a denaturing agent results in a *uniform* increase in the *degree* of denaturation. For this reason it is necessary to examine more closely the mechanism of the interaction between hemoglobin and sodium salicylate. Salicylate-denatured hemoglobin differs from the native protein in solubility, absorption spectrum, and susceptibility to tryptic digestion (18).

For measuring the postulated equilibrium between native and denatured protein, advantage was taken (18) of only one of these properties, i.e., an almost twofold increase in the absorption of green light on the part of the denatured hemoglobin, as compared to the native.[23] However, the intensity of light absorption varied with time, increasing at first, followed by a stationary level only to increase again. The mere fact that the same stationary level was established when the "equilibrium" mixture was made by starting with native or with denatured protein, is by itself not unequivocal proof for the existence of a state of equilibrium, particularly since aggregation of the denatured molecules has been found to have an influence on the degree of light absorption. Moreover, recent work has shown that colorimetric measurements alone are unreliable for determining the extent of the reaction between hemoglobin and sodium salicylate (336).

While the spectra of methemoglobin and of carboxyhemoglobin are quite different, the spectrum of methemoglobin in the presence of salicylate resembles that of carboxyhemoglobin alone, probably owing to complex formation between salicylate and the iron of methemoglobin. Though salicylate has little effect on the spectrum of carboxyhemoglobin, the heat of this reaction is about the same as that accompanying the interaction between salicylate and methemoglobin. On the other hand, while sodium benzoate changes the methemoglobin spectrum in a similar manner as does sodium salicylate, the heat of this reaction is practically zero. Therefore, not only are changes in spectrum unspecific expressions of the denaturation process, but the reaction between salicylate and hemoglobin is far from stoichiometric, since the heat of the reaction continues to increase above salicylate concentrations at which, according to Anson and Mirsky (18), the reaction has gone to completion (336).

The qualitative changes observed for the interaction between salicylate and hemoglobin have been found to be reversible. The regenerated protein is also indistinguishable from the native material in osmotic pressure (338, 341) and in

[23] Actually 1.7 fold (238).

susceptibility to splitting by alkali (339), in contrast to preparations regenerated after denaturation by heat or acid. It has been suggested that the combination between the protein and the salt is a loose though specific one, conceivably owing to the tendency of the salicylate molecule toward hydrogen-bond formation (336).

4. Trypsin

The fundamental studies of Northrop, Kunitz, and Herriott on the reversible inactivation of crystalline trypsin (214, 305), chymotrypsin (215), and pepsinogen (185) have so much in common that they may be adequately covered by a more detailed discussion of but one of these, i.e., trypsin.

(*a*) *Heat:* Crystalline trypsin may be reversibly inactivated by heat (305) in the pH range from 1 to 7, whereas in more alkaline solutions the reaction is irreversible. That at higher temperatures the enzyme is inactivated may be demonstrated by the fact that upon rapid addition of cold salt solution complete precipitation occurs, the filtrate being wholly inactive. In these, as in the following experiments, heating was confined to a short period, since prolonged exposure to high temperatures always resulted in irreversible inactivation. When salt was added after the protein solutions were allowed to cool, no precipitation occurred, leading to a complete restoration of the initial solubility and enzymatic activity. The equilibrium between active native and inactive denatured protein is rapidly established, and is governed by the temperature of heating. For instance, at 20°C. the protein is fully active and soluble, whereas upon heating at 40°C. about 50 per cent of the protein becomes precipitated by one-fourth saturation with ammonium sulfate, accompanied by a proportionate loss in activity. The same quantitative distribution is obtained by cooling solutions to 40°C. (17).

The rate of inactivation increases with increasing temperature, the reaction being nearly instantaneous at 90°C. Reactivation, however, is a comparatively slow process, requiring about 10 min. for completion when the protein in $N/20$ hydrochloric acid is rapidly heated to boiling and then poured into a mixture of $N/20$ hydrochloric acid and cracked ice.

At pH 7, the relation between inactivation and denaturation is more complex. When the protein is heated to temperatures ranging from 40° to 60°C., the amount capable of reversible inactivation and denaturation decreases in exactly the same manner as the amount which is inactivated and denatured. In this temperature range, denaturation and inactivation are irreversible processes following the general pattern of the heat inactivation of biological materials. However, above 60°C., total activity and total protein, i.e., the amounts that can be recovered following reversal of denaturation, increase with increasing temperature, approaching a value of 100 per cent above 90°C., whereas above 70°C. no residual protein or activity could be detected prior to reversal of denaturation. These observations have been interpreted by Northrop and Kunitz in terms of two competing reactions: (*1*) an equilibrium between active native and inactive denatured trypsin, which is shifted toward the right as the temperature is raised. Accordingly, the amounts of residual soluble activity

and protein decrease in proportion until all the protein is in the inactive denatured state, above 60°C. While subsequent to acidification and cooling, the enzyme tends to revert wholly to the native active state, this reaction is inhibited by another reaction, i.e., (*2*) the digestion of inactive denatured trypsin by the active native form. No reactivation occurs in the temperature range in which inactivation proceeds at a measurable rate, i.e., 40–60°C., since hydrolysis of the inactive denatured trypsin causes a progressive shift in the equilibrium of the first reaction toward the denatured form. At higher temperatures, however, the inactivation is practically instantaneous and complete, and no active trypsin remains, thereby permitting a reversal of the denaturation process proper.

This hypothesis offers an explanation for most of the observed effects, although it fails to explain why in the higher temperature range of inactivation the activity is restored to a smaller extent than the solubility. For instance, after heating at 70°C., over 80 per cent of the total protein becomes soluble in contrast to 30 per cent of the activity, the corresponding values being (by extrapolation of Northrop's data (305)) 90 and 50 per cent for 80°C. and 80 and 100 per cent for 92°C.

(*b*) *Alkali:* Exposure of solutions of active native trypsin to alkali results in rapid *irreversible* inactivation, the course of the reaction depending on the pH of the solutions (214). However, when the solutions are titrated in the cold to increasingly alkaline reactions, and then *instantly* added to cold 0.01 *M* hydrochloric acid, the inactive trypsin reverts within several hours to the native active state. This *reversibly* inactivated protein remains soluble upon the addition of *M*/1 sodium chloride, while precipitation ensues if salt is added immediately after acidification of the alkali-denatured protein. Thus, as in the case of heat inactivation, denaturation by alkali is a rapid process, while the reactivation proceeds considerably more slowly. The extent of inactivation increases with increasing pH, being complete above pH 13. Under the conditions just described, reactivation occurs in proportion to the amount of protein nitrogen that becomes soluble.

The rate of *irreversible* inactivation, occurring upon prolonged contact with alkali, depends on the pH and the temperature. For instance, while at pH 13 about 25 per cent of the enzyme may still be reactivated after 5 min. standing at 0°C., at 30°C. complete and irreversible inactivation occurs instantaneously. While upon prolonged standing the total amount of protein decreases, probably owing to slow hydrolysis, this does not occur within the period of time requisite for complete inactivation. Hence, the lack of reactivation at pH 13, as well as at pH 2, cannot be ascribed to a digestion of denatured trypsin by residual active enzyme. Within the pH range of 2.5 to 10, inactivation is also a rapid process. However, here the irreversibility of the process appears to be due to the slow digestion of the inactive denatured protein by the native active protein, analogous to the process described for the irreversible inactivation by heat.

The situation may be summed up by stating that after short contact with alkali, denatured trypsin slowly regains, on addition of acid, the activity and

solubility properties of the native enzyme, whereas longer exposure to alkali leads to permanent inactivation and denaturation.

5. General considerations

The preceding discussion has shown that under suitable conditions several denatured proteins may regain certain properties characteristic of the native state. The most tangible, though by no means most decisive, property common to native and regenerated protein is solubility in water or in salt solutions, and on the basis of this criterion the extent of denaturation varies greatly with the nature of the protein and of the denaturing agent. For instance, with horse serum albumin denatured by urea or guanidine hydrochloride 85 per cent of the protein has been recovered in a soluble regenerated state (295). Under similar conditions, 35 per cent of regenerated horse serum globulin has been obtained following denaturation by 8 *M* urea, and 15 per cent following denaturation by guanidine hydrochloride (296). With urea-denatured egg albumin less than 15 per cent of soluble protein is obtained (74), whereas urea-denatured myosin or tobacco mosaic virus protein appear to be wholly incapable of regeneration (133, 168, 381). Similar considerations apply when biological activity is used as a criterion for estimating the degree of regeneration. Thus, under special conditions trypsin, chymotrypsin, and pepsinogen may be completely reactivated, inactivation being otherwise partly or wholly irreversible. Inactivation of pepsin is always largely irreversible (303), while crystalline papain does not become inactivated for appreciable periods of time even in the presence of concentrated urea solutions (235).

In order to view the problem of reversible denaturation in the proper perspective, it is necessary to consider the nature and magnitude of the changes which a protein undergoes under the influence of a given denaturing agent, and to inquire about the reliability of specific criteria as valid indicators for the absolute identity of native and regenerated protein.

Urea denaturation of several proteins, e.g., serum albumin, serum globulin, egg albumin, and myogen, has been pictured as a transition from a specific condensed to a less specific extended configuration, caused by a partial unfolding of polypeptide chains. This interpretation appears to be fully sustained by experimental evidence. Acid denaturation of hemoglobin has been shown to be initiated by a dissociation of the molecules into halves, followed by a cleavage of the subunits into heme and denatured globin. The common feature of all these processes is a profound change in the physical structure of the protein molecule.

The first requisite for strict reversal of denaturation is a long-range elasticity of the polypeptide chains to facilitate their contraction to the condensed configuration; the second, that there be a mechanism which directs each residue and group to the same steric position which it occupied in the native protein molecule.

Evidence previously considered testifies to the contractile properties inherent in fibrous proteins (page 234) and in denatured globular proteins (page 236) alike. The question of the nature and site of the attractive forces responsible

for contraction is a matter of conjecture but is of profound importance in considerations of the second requisite for truly reversible denaturation.

On purely statistical grounds it would be extremely unlikely that each and every residue and group will find its way back to the specific relative position which it held in the native state, unless one or the other of the following hypotheses be evoked:

(*1*) The state of folding of polypeptide chains is determined primarily by attractive forces acting between constituent groups of the main chains. Hence, as long as these forces remain active, a partially unfolded polypeptide chain could revert to the original condensed configuration. This hypothesis implies that the nature and sequence of constituent amino acid side chains are relatively unimportant as structure-determining elements, and, since the main chains have the same atomic arrangement in all proteins, that proteins have a common and unique intrinsic structure. This hypothesis appears to be discredited by the noted influence of side-chain composition on the structure and contractility of protein fibres (see Part III D) and by the observed variations of globular proteins in susceptibility to denaturation and regeneration.

(*2*) Side-chain interaction being accepted as a predominant factor in molding polypeptide chains to the condensed configuration specific for individual proteins (30, 76), strict reversal of denaturation would be conceivable if, for some reason, an unfolded chain is forced back into the unique configuration of the native protein. This singleness of structure could be due either to the predominance of specific attractive forces or else to the steric requirements of the side chains, permitting of only one single state of folding.

Specific attractive forces may be found in cross linkages of the disulfide type. Indeed, the position of cystine residues is fixed in a polypeptide chain and it has already been shown that disulfide bridges confer on proteins a high degree of contractility (Part III D). However, though a cursory inspection of analytical data reveals that many proteins which are capable of regeneration have a notably high cystine content, others which are poor in, or even devoid of cystine, also exhibit a specificity of structure. Hence, it is unlikely that such covalent cross linkages are exclusive structure-determining factors. Salt linkages and hydrogen bonds between side chains undoubtedly are equally important as structure-determining elements. However, the great number of side chains capable of forming bonds of that type would permit of a variety of combinations once a polypeptide chain has become unfolded by denaturation. Therefore, the concept of side-chain interaction alone may fail to account for a singleness of structure.

It has been shown that the space requirements of amino acid residues, notably of those containing aromatic or heterocyclic rings, greatly restrict the internal rotation and steric freedom of polypeptide chains (288) and that the presence of glycine residues tends to alleviate these restrictions (291). However, analytical data reveal that with most proteins amino acids of smaller cross-sectional areas predominate, thereby allowing sufficient freedom for a chain to assume various configurations (*cf.* 55). While a more precise consideration of this problem is

unwarranted at the present time, we are inclined to accept the view that genuine polypeptide chains may fold up in a number of ways and that upon refolding, the mode of refolding will be governed to an appreciable extent by merely statistical factors, thus rendering the specific configuration of the native protein one of several equally probable ones (297). Thus, the mode of refolding but not refolding itself limits the true reversal of the denaturation process.

The opening up in protein structure concurrent to denaturation is preceded by an "activation" of the native protein molecule. The nature of this activation process is not generally understood though in two instances, i.e., pepsin and hemoglobin, it has been identified with the ionization of specific groups (Part II B). While chemical reactions of that or similar types are thus precursors of changes in physical configuration, there is no reason to believe that the former may not occur without being always followed by the latter. This, obviously, will depend on whether such groups occupy key positions in the structure of the molecule.

That chemical reactions with protein surface groups may go a long way in profoundly altering characteristic chemical and biological properties of native proteins is well known. Thus, the activity of enzymes (*viz.*, trypsin, pepsin, urease) and the solubility of proteins are highly sensitive to subtle changes in the degree of ionization as is the susceptibility of proteins to denaturation. Such purely chemical reactions may very conceivably be reversible, even if they should lead to intermolecular aggregation, provided the physical structure of the protein remains intact after redispersion of the aggregates. In short, if denaturation were to be confined to reactions of and between amino acid side chains, without significantly altering the internal configuration of the protein, such processes may suffice to influence appreciably certain characteristic properties of the native protein and may be reversible, in contrast to any process which affects the state of folding of the polypeptide chains themselves.

It is premature to decide whether such speculations have sufficient reality to account for the apparent reversal of denaturation of trypsin by heat or alkali or of hemoglobin by sodium salicylate. However, it is a matter of fact that the inactivation of trypsin is a rapid process, while reactivation occurs slowly and only under the influence of acids. If inactivation were to entail a dissociation of carboxyl groups, as it does in the case of pepsin (page 201), possibly followed by aggregation, reactivation would consist in disaggregation, usually a slow process, followed by reversal of the initial step. Under the prolonged influence of heat or alkali, interaction might proceed so far as to prevent depolymerization without causing more profound and permanent intramolecular changes. The solubility behavior of heat-denatured serum albumin or hemoglobin might be explained by similar reasoning. Thus, neutralization of the hot solutions causes precipitation of the polymerized denatured molecules; when neutralization is preceded by cooling, the depolymerized protein remains soluble subsequent to neutralization. The permanent changes in the state of aggregation occurring upon prolonged heating have been evinced by molecular-kinetic analysis (118).

Mirsky and Pauling's argument that heat-inactivated trypsin is distinguished

from the native protein by a considerably higher entropy content (278) does not invalidate the present conclusions. Calorimetric studies on the inactivation of pepsin have shown that the over-all heat of the reaction is probably that of several chemical processes, one of them being the inactivation proper (116). Indeed, ΔH, and the activity of this enzyme change in a different manner with pH, the latter decreasing less rapidly than the former. Hence, by analogy, the entropy of inactivation of trypsin is probably less than that considered in Mirsky and Pauling's calculation, which already has been shown to be of doubtful significance (page 208). Moreover, as discussed elsewhere (297), for a protein molecule comprising several thousands of bonds, such a change in entropy may be readily accounted for by an infinitesimal change in vibrational motion, thus leaving the inactive enzyme in a practically equally specified state as the native. The amount of opening up in protein structure, therefore, may be negligible.

Several proteolytic enzymes appear to be remarkably resistant to usually powerful denaturing agents. Tryptic activity is measured in the presence of concentrated urea solutions (52), pepsin remains active for several hours in 4 *M* urea (374), and papain retains its activity in 9 *M* urea for as long as 24 hr. (235). Since heat denaturation frequently produces less profound changes than does denaturation by urea and similar amides, it appears that the inactivation of these enzymes by heat is associated with other and probably milder changes than those experienced by the action of the aforementioned organic reagents.

On the basis of these considerations, the biological activity of certain proteolytic enzymes seems to be dependent not only on the intact physical configuration of the protein but also on the presence of specific "active" groups. This is in contrast to other manifestations of biological activity which, as discussed previously (page 239), are rather insensitive to gross changes of chemical or physical nature.

In summary, it may be said that the more profound the changes in a protein which occur upon denaturation, the less likely is denaturation to be reversible. While in many instances the regenerated protein has regained many of the characteristic properties of the native state, the concept of denaturation as a reversible reaction does not warrant general application.

Finally, it should be stressed that these considerations apply to the reversal of the *in vitro* denaturation of proteins, while little, if anything, is known about its occurrence *in vivo*. It is apparent that at some stage in the synthesis of proteins polypeptide chains fold up in order to be molded into a specific configuration, under the controlling influence of intracellular enzymes (60). Conversely, unfolding will occur prior to the hydrolytic action of extracellular proteinases. While the synthesis and degradation of proteins are continuous dynamic processes, it is idle to speculate whether these reversible processes ever halt at the stage of denaturation. Several specific phenomena have been postulated to involve reversible denaturation. One of these, i.e., the reversible contraction of muscle, has been referred to (Part III D). Others are the effect of temperature on the reversible quenching of bacterial luminescence (196) and the reversible conversion of visual purple to visual yellow (264).

The part of this work which was carried out at Duke University was supported by grants from the Rockefeller Foundation, the Lederle Laboratories, Inc., and the Duke University Research Council.

REFERENCES

(1) ABRAMSON, H. A., MOYER, L. S., AND GORIN, M. H.: *Electrophoresis of Proteins*. Reinhold Publishing Corporation, New York (1942).
(2) ADAIR, G. S., AND ADAIR, M. E.: Biochem. J. **28,** 199 (1934).
(3) ADAIR, G. S., AND ROBINSON, M. E.: Biochem. J. **24,** 1864 (1930).
(4) ANNETTS, M.: Biochem. J. **30,** 1807 (1936).
(5) ANSON, M. L.: In *The Chemistry of the Amino Acids and Proteins* (edited by C. L. A. Schmidt), p. 405 ff. C. C. Thomas, Springfield, Illinois (1938).
(6) ANSON, M. L.: J. Gen. Physiol. **22,** 79 (1938).
(7) ANSON, M. L.: J. Gen. Physiol. **23,** 239 (1939).
(8) ANSON, M. L.: J. Gen. Physiol. **23,** 321 (1940).
(9) ANSON, M. L.: J. Gen. Physiol. **24,** 399 (1941).
(10) ANSON, M. L., AND MIRSKY, A. E.: J. Gen. Physiol. **13,** 121 (1929).
(11) ANSON, M. L., AND MIRSKY, A. E.: J. Gen. Physiol. **13,** 469 (1930).
(12) ANSON, M. L., AND MIRSKY, A. E.: J. Phys. Chem. **35,** 185 (1931).
(13) ANSON, M. L., AND MIRSKY, A. E.: J. Gen. Physiol. **14,** 605 (1931).
(14) ANSON, M. L., AND MIRSKY, A. E.: J. Gen. Physiol. **14,** 725 (1931).
(15) ANSON, M. L., AND MIRSKY, A. E.: J. Gen. Physiol. **15,** 341 (1932).
(16) ANSON, M. L., AND MIRSKY, A. E.: J. Gen. Physiol. **17,** 159 (1933).
(17) ANSON, M. L., AND MIRSKY, A. E.: J. Gen. Physiol. **17,** 393 (1934).
(18) ANSON, M. L., AND MIRSKY, A. E.: J. Gen. Physiol. **17,** 399 (1934).
(19) ANSON, M. L., AND STANLEY, W. M.: J. Gen. Physiol. **24,** 679 (1941).
(20) ARNOLD, V.: Z. physiol. Chem. **70,** 300, 314 (1911).
(21) ARNOW, L. E.: Physiol. Rev. **16,** 671 (1936).
(22) ARNOW, L. E., KAZAL, L. A., AND DEFALCO, R. J.: J. Biol. Chem. **145,** 347 (1942).
(23) ARRHENIUS, S.: *Immunochemistry*, p. 87 ff. The Macmillan Company, New York (1907).
(24) ASTBURY, W. T.: Trans. Faraday Soc. **29,** 193 (1933).
(25) ASTBURY, W. T.: Cold Spring Harbor Symposia Quant. Biol. **2,** 15 (1934).
(26) ASTBURY, W. T.: Chem. Weekblad **33,** 778 (1936).
(27) ASTBURY, W. T.: Nature **137,** 803 (1936).
(28) ASTBURY, W. T.: Compt. rend. trav. lab. Carlsberg, Sér. chim. **22,** 45 (1938).
(29) ASTBURY, W. T.: Trans. Faraday Soc. **34,** 378 (1938).
(30) ASTBURY, W. T.: *Advances in Enzymology* (edited by F. F. Nord and C. H. Werkman) **3,** 63 (1943).
(31) ASTBURY, W. T., AND BELL, F.: Tabulae Biol. (Haag) **17,** 90 (1939).
(32) ASTBURY, W. T., AND BELL, F.: Nature **147,** 696 (1941).
(33) ASTBURY, W. T., BELL, F., GORTER, E., AND VAN ORMONDT, J.: Nature **142,** 33 (1938).
(34) ASTBURY, W. T., AND DICKINSON, S.: Proc. Roy. Soc. (London) **B129,** 307 (1940).
(35) ASTBURY, W. T., DICKINSON, S., AND BAILEY, K.: Biochem. J. **29,** 2351 (1935).
(36) ASTBURY, W. T., AND LOMAX, R.: J. Chem. Soc. **1935,** 846.
(37) ASTBURY, W. T., AND WOODS, H. J.: Trans. Roy. Soc. (London) **A232,** 333 (1933).
(38) BAILEY, K.: Biochem. J. **31,** 1406 (1937).
(39) BAILEY, K.: Biochem. J. **36,** 140 (1942).
(40) BAILEY, K., ASTBURY, W. T., AND RUDELL, K. M.: Nature **151,** 716 (1943).
(41) BARKER, H. A.: J. Gen. Physiol. **17,** 21 (1933).
(42) BARONI, V., AND JONESCO-MIHAIESTI, C.: Compt. rend. soc. biol. **68,** 393 (1910).
(43) BATEMAN, J. B., CALKINS, H. E., AND CHAMBERS, L. A.: J. Immunol. **41,** 321 (1941).
(44) BATE-SMITH, E. C.: Proc. Roy. Soc. (London) **B124,** 136 (1937).

(45) BAWDEN, F. C., AND KLECZKOWSKI, A.: Brit. J. Exptl. Path. **22,** 208 (1941).
(46) BAWDEN, F. C., AND KLECZKOWSKI, A.: Brit. J. Exptl. Path. **23,** 169 (1942).
(47) BAWDEN, F. C., AND KLECZKOWSKI, A.: Brit. J. Exptl. Path. **23,** 178 (1942).
(48) BAWDEN, F. C., AND PIRIE, N. W.: Brit. J. Exptl. Path. **17,** 64 (1936).
(49) BAWDEN, F. C., AND PIRIE, N. W.: Proc. Roy. Soc. (London) **B123,** 274 (1937).
(50) BAWDEN, F. C., AND PIRIE, N. W.: Brit. J. Exptl. Path. **19,** 66 (1938).
(51) BAWDEN, F. C., AND PIRIE, N. W.: Brit. J. Exptl. Path. **19,** 251 (1938).
(52) BAWDEN, F. C., AND PIRIE, N. W.: Biochem. J. **34,** 1258 (1940).
(53) BAWDEN, F. C., AND PIRIE, N. W.: Biochem. J. **34,** 1278 (1940).
(54) BAWDEN, F. C., AND PIRIE, N. W.: Biochem. J. **37,** 70 (1943).
(55) BECKER, B.: Thesis in Chemistry, Princeton University, 1941; cited by C. L. A. Schmidt in *Addendum to the Chemistry of the Amino Acids and Proteins*, p. 1156. C. C. Thomas, Springfield, Illinois (1943).
(56) BERGMANN, M.: Naturwissenschaften **12,** 1155 (1924).
(57) BERGMANN, M.: *Advances in Enzymology* (edited by F. F. Nord and C. H. Werkman) **2,** 49 (1942).
(58) BERGMANN, M., AND FRUTON, J. S.: J. Biol. Chem. **118,** 405 (1937).
(59) BERGMANN, M., AND MIEKELEY, A.: Z. physiol. Chem. **140,** 128 (1924).
(60) BERGMANN, M., AND NIEMANN, C.: Science **86,** 187 (1937).
(61) BERNAL, J. D., FANKUCHEN, I., AND PERUTZ, M.: Nature **141,** 523 (1938).
(62) BERNHART, F. W.: J. Am. Chem. Soc. **61,** 1953 (1939).
(63) BERNHART, F. W.: J. Phys. Chem. **45,** 1382 (1941).
(64) BERNHEIM, F., NEURATH, H., AND ERICKSON, J. O.: J. Biol. Chem. **144,** 259 (1942).
(65) BLOCK, R. J.: J. Biol. Chem. **119,** 765 (1937).
(66) BLOCK, R. J.: J. Biol. Chem. **120,** 467 (1937).
(67) BOOTH, N.: Biochem. J. **24,** 158 (1930).
(68) BOOTH, N.: Biochem. J. **24,** 1699 (1930).
(69) BOYD, W. C.: *Fundamentals of Immunology.* Interscience Publishers, Inc., New York (1943).
(70) BOYES-WATSON, G., AND PERUTZ, M. F.: Nature **151,** 714 (1943).
(71) BRAND, E., AND KASSELL, B.: J. Biol. Chem. **133,** 437 (1940).
(72) BROHULT, S.: Nova Acta Regiae Soc. Sci. Upsaliensis [4] **12,** No. 4 (1940).
(73) BULL, H. B.: Cold Spring Harbor Symposia Quant. Biol. **6,** 140 (1938).
(74) BULL, H. B.: J. Biol. Chem. **125,** 585 (1938).
(75) BULL, H. B.: J. Biol. Chem. **133,** 39 (1940).
(76) BULL, H. B.: *Advances in Enzymology* (edited by F. F. Nord and C. H. Werkman) **1,** 1 (1941).
(77) BULL, H. B.: J. Biol. Chem. **137,** 143 (1941).
(78) BULL, H. B., AND NEURATH, H.: J. Biol. Chem. **118,** 163 (1937).
(79) BULL, H. B., AND NEURATH, H.: J. Biol. Chem. **125,** 113 (1938).
(80) BURK, N. F.: J. Biol. Chem. **98,** 353 (1932).
(81) BURK, N. F.: J. Biol. Chem. **120,** 63 (1937).
(82) BURK, N. F.: J. Biol. Chem. **121,** 373 (1937).
(83) BURK, N. F.: J. Biol. Chem. **124,** 49 (1938).
(84) BURK, N. F.: J. Biol. Chem. **133,** 511 (1940).
(85) BURK, N. F.: J. Phys. Chem. **47,** 104 (1943).
(86) BURK, N. F., AND GREENBERG, D. M.: J. Biol. Chem. **87,** 197 (1930).
(87) CANNAN, R. K.: Chem. Rev. **30,** 395 (1942).
(88) CARPENTER, D. C.: J. Am. Chem. Soc. **62,** 289 (1940).
(89) CHAMBERS, L. A.: Am. J. Med. Sci. **190,** 857 (1935).
(90) CHAMBERS, L. A.: J. Biol. Chem. **117,** 639 (1937).
(91) CHAMBERS, L. A.: J. Immunol. **36,** 543 (1939).
(92) CHAMBERS, L. A., BATEMAN, J. B., AND CALKINS, H. E.: J. Immunol. **40,** 483 (1941).
(93) CHAMBERS, L. A., AND FLOSDORF, E. W.: J. Biol. Chem. **114,** 75 (1936).

(94) CHARLES, A. F., AND SCOTT, O. H.: J. Biol. Chem. **92,** 289 (1931).
(95) CHIBNALL, A. C.: Proc. Roy. Soc. (London) **B131,** 136 (1942).
(96) CHICK, H., AND MARTIN, C. J.: J. Physiol. **40,** 404 (1910).
(97) CHICK, H., AND MARTIN, C. J.: J. Physiol. **43,** 1 (1911).
(98) CHICK, H., AND MARTIN, C. J.: J. Physiol. **45,** 61 (1912).
(99) CHICK, H., AND MARTIN, C. J.: J. Physiol. **45,** 261 (1912).
(100) CHICK, H., AND MARTIN, C. J.: Kolloidchem. Beihefte **5,** 49 (1913).
(101) CHOU, C., AND WU, H.: Chinese J. Physiol. **8,** 145 (1934).
(102) CLARK, G. L., AND SHENK, J. H.: Radiology **28,** 58, 144, 357 (1937).
(103) CLARK, H., AND NORTHROP, J. H.: J. Gen. Physiol. **9,** 87 (1925).
(104) CLARK, J. H.: Am. J. Physiol. **61,** 72 (1922).
(105) CLARK, J. H.: Am. J. Physiol. **73,** 649 (1925).
(106) CLARK, J. H.: Am. J. Physiol. **113,** 538 (1935).
(107) CLARK, J. H.: J. Gen. Physiol. **19,** 199 (1936).
(108) CLARK, J. H.: Am. J. Roentgenol. Radium Therapy **40,** 501 (1938).
(109) COCKBAIN, E. G., AND SCHULMAN, J. H.: Trans. Faraday Soc. **35,** 1266 (1939).
(110) COHN, E. J.: Ergeb. Physiol. **33,** 781 (1931).
(111) COHN, E. J.: Cold Spring Harbor Symposia Quant. Biol. **6,** 8 (1938).
(112) COHN, E. J.: Chem. Rev. **28,** 395 (1941).
(113) COHN, E. J., AND EDSALL, J. T.: *Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions.* Reinhold Publishing Corporation, New York (1943).
(114) COHN, E. J., GREEN, A. A., AND BLANCHARD, M. H.: J. Am. Chem. Soc. **59,** 509 (1937).
(115) COHN, E. W., AND WHITE, A.: J. Biol. Chem. **109,** 169 (1935).
(116) CONN, J. B.., GREGG, D. C., KISTIAKOWSKY, G. B., AND ROBERTS, R. M.: J. Am. Chem. Soc. **63,** 2080 (1941).
(117) CONN, J. B, KISTIAKOWSKY, G. B., AND ROBERTS, R. M.: J. Am. Chem. Soc. **62,** 1895 (1940).
(118) COOPER, G. R., AND NEURATH, H.: J. Phys. Chem. **47,** 383 (1943).
(119) COREY, R. B.: Chem. Rev. **26,** 227 (1940).
(120) CRAMMER, J. L., AND NEUBERGER, A.: Biochem. J. **37,** 302 (1943).
(121) CUBIN, H. K.: Biochem. J. **23,** 25 (1929).
(122) DANIELLI, J., DANIELLI, M., AND MARRACK, J. R.: Brit. J. Exptl. Path. **19,** 393 (1938).
(123) DAVIS, B. D., HOLLAENDER, A., AND GREENSTEIN, J. P.: J. Biol. Chem. **146,** 663 (1942).
(124) DEAN, R. B., GATTY, O., AND STENHAGEN, E.: Nature **143,** 721 (1939).
(125) DEN HOED, D., DEJONGH, S. E., AND PEEK, A. E. J.: Biochem. Z. **205,** 144 (1929).
(126) DICKENS, F.: Biochem. J. **33,** 2017 (1939).
(127) DIEBOLD, W., AND JÜHLING, L.: Biochem. Z. **296,** 389 (1938).
(128) DRABKIN, D. L.: Proc. Soc. Exptl. Biol. Med. **41,** 225 (1939).
(129) DUNN, M. S., AND SCHMIDT, C. L. A.: J. Biol. Chem. **53,** 401 (1922).
(130) DU VIGNEAUD, V.: Cold Spring Harbor Symposia Quant. Biol. **6,** 275 (1938).
(131) DU VIGNEAUD, V., GEILING, E. M. K., AND EDDY, C. A.: J. Pharmacol. **33,** 497 (1928).
(132) EATON, A. G., AND DAVIS, H. A.: Proc. Soc. Exptl. Biol. Med. **50,** 246 (1942).
(133) EDSALL, J. T., AND MEHL, J. W.: J. Biol. Chem. **133,** 409 (1940).
(134) EGE, R.: Z. physiol. Chem. **224,** 129 (1934).
(135) ERICKSON, J. O., AND NEURATH, H.: J. Exptl. Med. **78,** 1 (1943).
(136) ERICKSON, J. O., AND NEURATH, H.: Science **98,** 284 (1943).
(137) ERICKSON, J. O., AND NEURATH, H.: In preparation.
(138) ERIKSSON-QUENSEL, I. B.: Biochem. J. **32,** 585 (1938).
(139) EULER, H. VON, AND LAURIN, I.: Z. physiol. Chem. **108,** 64 (1919).
(140) EYRING, H., AND STEARN, A. E.: Chem. Rev. **24,** 253 (1939).
(141) FAMULENER, L. W., AND MADSEN, T.: Biochem. Z. **11,** 186 (1908).
(142) *Cf.* FANKUCHEN, I.: Ann. N. Y. Acad. Sci. **41,** 157 (1941).

(143) FINN, D. B.: Proc. Roy. Soc. (London) **B111,** 396 (1932).
(144) FLOSDORF, E. W., AND CHAMBERS, L. A.: J. Am. Med. Sci. **187,** 294 (1934).
(145) FRAMPTON, V. L.: J. Biol. Chem. **129,** 233 (1939).
(146) FRAMPTON, V. L.: Arch. Biochem. **1,** 83 (1942).
(147) GEIGER, W. B., KOBAYASHI, F. F., AND HARRIS, M.: J. Research Natl. Bur. Standards **29,** 381 (1942).
(148) GERLOUGH, T. D., AND BATES, R. W.: J. Pharmacol. **45,** 19 (1932).
(149) GLASSTONE, S.: *Textbook of Physical Chemistry*, p. 1024 ff. D. Van Nostrand Company, New York (1940).
(150) GLASSTONE, S., LAIDLER, K. J., AND EYRING, H.: *The Theory of Rate Processes*, p. 5 ff. McGraw-Hill Book Company, Inc., New York (1941).
(151) GORTER, E.: Trans. Faraday Soc. **33,** 1125 (1937).
(152) GOULDING, A. M., BORSOOK, H., AND WASTENEYS, H.: J. Gen. Physiol. **10,** 451 (1926).
(153) GRABAR, P.: Ann. inst. Pasteur **61,** 764 (1938).
(154) GRALÉN, N.: Biochem. J. **33,** 1342 (1939).
(155) GRALÉN, N.: Biochem. J. **33,** 1907 (1939).
(156) GRANT, E. A., DOW, R. B., AND FRANKS, W. R.: Science **94,** 616 (1941).
(157) GREENBERG, D. M.: In C. L. A. Schmidt's *Addendum to the Chemistry of the Amino Acids and Proteins*, p. 1139 ff. C. C. Thomas, Springfield, Illinois (1943).
(158) GREENSTEIN, J. P.: J. Biol. Chem. **101,** 603 (1933).
(159) GREENSTEIN, J. P.: In *The Chemistry of the Amino Acids and Proteins* (edited by C. L. A. Schmidt), p. 252 ff. C. C. Thomas, Springfield, Illinois (1938).
(160) GREENSTEIN, J. P.: J. Biol. Chem. **125,** 501 (1938).
(161) GREENSTEIN, J. P.: J. Biol. Chem. **128,** 233 (1939).
(162) GREENSTEIN, J. P.: J. Biol. Chem. **130,** 519 (1939).
(163) GREENSTEIN, J. P.: J. Biol. Chem. **136,** 795 (1940).
(164) GREENSTEIN, J. P.: J. Natl. Cancer Inst. **3,** 61 (1942).
(165) GREENSTEIN, J. P.: J. Biol. Chem. **150,** 107 (1943).
(166) GREENSTEIN, J. P.: In *Advances in Protein Chemistry* **1.** Academic Press, New York (in press).
(167) GREENSTEIN, J. P.: Unpublished observations.
(168) GREENSTEIN, J. P., AND EDSALL, J. T.: J. Biol. Chem. **133,** 397 (1940).
(169) GREENSTEIN, J. P., AND JENRETTE, W. V.: J. Natl. Cancer Inst. **1,** 91 (1940).
(170) GREENSTEIN, J. P., AND JENRETTE, W. V.: Cold Spring Harbor Symposia Quant. Biol. **9,** 236 (1941).
(171) GREENSTEIN, J. P., AND JENRETTE, W. V.: J. Biol. Chem. **142,** 175 (1942).
(172) GREENSTEIN, J. P., JENRETTE, W. V., AND WHITE, J.: J. Natl. Cancer Inst. **2,** 305 (1941).
(173) HAND, D. B.: J. Gen. Physiol. **18,** 847 (1935).
(174) HARDT, C. R., HUDDLESON, I. F., AND BALL, C. D.: Science **98,** 309 (1943).
(175) HARDY, W. B.: J. Physiol. **24,** 158 (1899).
(176) HARKINS, W. D., FOURT, L., AND FOURT, P. C.: J. Biol. Chem. **132,** 111 (1940).
(177) HARRIS, L. J.: Proc. Roy. Soc. (London) **B94,** 426 (1923).
(178) HARTRIDGE, H.: J. Physiol. **44,** 34 (1912).
(179) HEIDELBERGER, M., AND PEDERSEN, K. O.: J. Gen. Physiol. **19,** 95 (1936).
(180) HEIM, F.: Biochem. Z. **291,** 88 (1937).
(181) HELLERMAN, L., CHINARD, F. P., AND DEITZ, V. R.: J. Biol. Chem. **147,** 443 (1943).
(182) HENDRIX, B. M., AND WILSON, V.: J. Biol. Chem. **79,** 389 (1928).
(183) HENRY, J. P.: J. Exptl. Med. **76,** 451 (1942).
(184) HERRIOTT, R. M.: J. Gen. Physiol. **19,** 283 (1936).
(185) HERRIOTT, R. M.: J. Gen. Physiol. **21,** 501 (1938).
(186) HEYMANN, E.: Biochem. J. **30,** 126 (1936).
(187) HILL, R., cited by J. H. P. Jonxis: Biochem. J. **33,** 1743 (1939).
(188) HITCHCOCK, D. I.: J. Gen. Physiol. **6,** 95 (1923).

(189) HITCHCOCK, D. I.: In *The Chemistry of the Amino Acids and Proteins* (edited by C. L. A. Schmidt), p. 596 ff. C. C. Thomas, Springfield, Illinois (1938).
(190) HOLDEN, H. F.: Australian J. Exptl. Biol. Med. Sci. **14**, 291 (1936).
(191) HOLDEN, H. F.: Australian J. Exptl. Biol. Med. Sci. **15**, 43 (1937).
(192) HOPKINS, F. G.: Nature **126**, 328, 383 (1930).
(193) HUANG, T., AND WU, H.: Chinese J. Physiol. **4**, 221 (1930).
(194) HUGGINS, M.: Ann. Rev. Biochem. **11**, 27 (1942).
(195) HUSSEY, R. G., AND THOMPSON, W. R.: J. Gen Physiol. **5**, 647 (1922); **9**, 309, 315 (1926).
(196) JOHNSON, F. H., BROWN, D., AND MARSLAND, D.: Science **95**, 200 (1942).
(197) JOHNSON, L. R., AND WORMALL, A.: Biochem. J. **26**, 1202 (1932).
(198) KASS, E. H., SCHERAGO, M., AND WEAVER, R. H.: J. Immunol. **45**, 87 (1942).
(199) KEILIN, D., AND HARTREE, E. F.: Proc. Roy. Soc. (London) **B129**, 277 (1940).
(200) KEKWICK, R. A., AND CANNAN, R. K.: Biochem. J. **30**, 227 (1936).
(201) KLECZKOWSKI, A.: Brit. J. Exptl. Path. **22**, 188 (1941).
(202) KLECZKOWSKI, A.: Brit. J. Exptl. Path. **22**, 192 (1941).
(203) KLECZKOWSKI, A.: Biochem. J. **37**, 30 (1943).
(204) KNIGHT, C. A.: J. Biol. Chem. **147**, 663 (1943).
(205) KREJCI, L. E., JENNINGS, R. K., AND SMITH, L. D.: J. Immunol. **45**, 111 (1942).
(206) KREJCI, L. E., SMITH, L. D., AND DIETZ, T. J.: J. Franklin Inst. **231**, 396 (1941).
(207) KREJCI, L. E., AND SVEDBERG, T.: J. Am. Chem. Soc. **57**, 946 (1935).
(208) KUBOWITZ, F.: Biochem. Z. **292**, 221 (1937); **299**, 32 (1938).
(209) KUHN, R., BIELIG, H. G., AND DANN, O.: Ber. **73B**, 1080 (1940).
(210) KUHN, R., BIRKOFER, L., AND QUACKENBUSH, F. W.: Ber. **72B**, 407 (1939).
(211) KUHN, R., AND DESNUELLE, P.: Z. physiol. Chem. **251**, 14 (1938).
(212) KUNITZ, M.: J. Gen. Physiol. **9**, 715 (1925).
(213) KUNITZ, M.: J. Gen. Physiol. **24**, 15 (1940).
(214) KUNITZ, M., AND NORTHROP, J. H.: J. Gen. Physiol. **17**, 591 (1934).
(215) KUNITZ, M., AND NORTHROP, J. H.: J. Gen. Physiol. **18**, 433 (1935).
(216) LA MER, V. K.: Science **86**, 614 (1937).
(217) LANDEN, E. W.: J. Am. Chem. Soc. **62**, 2465 (1940).
(218) LANDSTEINER, K.: *The Specificity of Serological Reactions*. C. C. Thomas, Springfield, Illinois (1936).
(219) LANGMUIR, I.: Cold Spring Harbor Symposia Quant. Biol. **6**, 171 (1938).
(220) LANGMUIR, I., AND SCHAEFER, V.: J. Am. Chem. Soc. **60**, 1351 (1938).
(221) LAUFFER, M. A.: J. Am. Chem. Soc., **65**, 1793 (1943).
(222) LAUFFER, M. A., AND DOW, R. B.: J. Biol. Chem. **140**, 509 (1941).
(223) LAUFFER, M. A., AND PRICE, W. C.: J. Biol. Chem. **133**, 1 (1940).
(224) LAUFFER, M. A., AND STANLEY, W. M.: Arch. Biochem. **2**, 413 (1943).
(225) LEPESCHKIN, W. W.: Biochem. Z. **314**, 135 (1943).
(226) LEWIS, P. S.: Biochem. J. **20**, 965 (1926).
(227) LEWIS, P. S.: Biochem. J. **20**, 978 (1926).
(228) LEWIS, P. S.: Biochem. J. **20**, 984 (1926).
(229) LEWIS, P. S.: Biochem. J. **21**, 46 (1927).
(230) LEWIS, W. C. M.: Chem. Rev. **8**, 81 (1931).
(231) LINDERSTRØM-LANG, K.: Proc. Roy. Soc. (London) **B127**, 17 (1939); Ann. Rev. Biochem. **8**, 37 (1939).
(232) LINDERSTRØM-LANG, K., HOTCHKISS, R. D., AND JOHANSEN, G.: Nature **142**, 966 (1938).
(233) LINDERSTRØM-LANG, K., AND JACOBSEN, C. F.: Compt. rend. trav. lab. Carlsberg **23**, 289 (1940).
(234) LINEWEAVER, H., AND HOOVER, S. R.: J. Biol. Chem. **137**, 325 (1941).
(235) LINEWEAVER, H., AND SCHWIMMER, S.: Enzymologia **10**, 81 (1941).
(236) LIU, S.: Chinese J. Physiol. **7**, 107 (1933).

(237) LONGSWORTH, L. G.: Ann. N. Y. Acad. Sci. **41**, 267 (1941).
(238) LOPES, T. L.: Anais acad. brasil cienc. **14**, 241 (1942).
(239) LOUGHLIN, W. J.: Biochem. J. **26**, 1557 (1932).
(240) LOUGHLIN, W. J.: Biochem. J. **27**, 99 (1933).
(241) LOUGHLIN, W. J.: Biochem. J. **27**, 1779 (1933).
(242) LOUGHLIN, W. J., AND LEWIS, W. C. M.: Biochem. J. **26**, 476 (1932).
(243) LÜERS, H., AND LANDAUER, M.: Z. angew. Chem. **35**, 469 (1922).
(244) LÜERS, H., AND WASMUND, W.: Fermentforschung **5**, 169 (1922).
(245) LUNDGREN, H. P.: Nature **143**, 896 (1939).
(246) LUNDGREN, H. P.: J. Biol. Chem. **138**, 293 (1941).
(247) LUNDGREN, H. P.: J. Am. Chem. Soc. **63**, 2854 (1941).
(248) LUNDGREN, H. P., ELAM, D. W., AND O'CONNELL, R. A.: J. Biol. Chem. **149**, 183 (1943).
(249) LUNDGREN, H. P., AND WILLIAMS, J. W.: J. Phys. Chem. **43**, 989 (1939).
(250) MACPHERSON, C. F. C., AND HEIDELBERGER, M.: Proc. Soc. Exptl. Biol. Med. **43**, 646 (1940).
(251) MCGILLIVRAY, I. H.: Biochem. J. **24**, 891 (1930).
(252) MARK, H.: *Physical Chemistry of High Polymeric Systems.* Interscience Publishers, Inc., New York (1940).
(253) MARRACK, J. R.: *The Chemistry of Antigens and Antibodies.* His Majesty's Stationery Office, London (1938).
(254) MARTIN, D. S., ERICKSON, J. O., PUTNAM, F. W., AND NEURATH, H.: J. Gen. Physiol. **26**, 533 (1943).
(255) MATTHEWS, J. E., JR., DOW, R. B., AND ANDERSON, A. K.: J. Biol. Chem. **135**, 697 (1940).
(256) MEHL, J. W.: Cold Spring Harbor Symposia Quant. Biol. **6**, 218 (1938).
(257) MICHAELIS, L., AND RONA, P.: Biochem. Z. **27**, 38 (1910).
(258) MICHAELIS, L., AND ROTHSTEIN, M.: Biochem. Z. **105**, 60 (1920).
(259) MILLER, B. F.: J. Exptl. Med. **58**, 625 (1933).
(260) MILLER, G. L.: J. Biol. Chem. **146**, 339 (1942).
(261) MILLER, G. L.: J. Biol. Chem. **146**, 345 (1942).
(262) MILLER, G. L., AND ANDERSSON, K. J. I.: J. Biol. Chem. **144**, 475 (1942).
(263) MILLER, G. L., AND DU VIGNEAUD, V.: J. Biol. Chem. **118**, 101 (1937).
(264) MIRSKY, A. E.: Proc. Natl. Acad. Sci. (U. S.) **22**, 147 (1936).
(265) MIRSKY, A. E.: J. Gen. Physiol. **19**, 559 (1936).
(266) MIRSKY, A. E.: J. Gen. Physiol. **20**, 455 (1937).
(267) MIRSKY, A. E.: J. Gen. Physiol. **20**, 462 (1937).
(268) MIRSKY, A. E.: Cold Spring Harbor Symposia Quant. Biol. **9**, 278 (1941).
(269) MIRSKY, A. E.: Science **93**, 285 (1941).
(270) MIRSKY, A. E.: J. Gen. Physiol. **24**, 709 (1941).
(271) MIRSKY, A. E.: J. Gen. Physiol. **24**, 725 (1941).
(272) MIRSKY, A. E., AND ANSON, M. L.: J. Gen. Physiol. **13**, 133 (1929).
(273) MIRSKY, A. E., AND ANSON, M. L.: J. Gen. Physiol. **13**, 477 (1930).
(274) MIRSKY, A. E., AND ANSON, M. L.: J. Gen. Physiol. **18**, 307 (1935).
(275) MIRSKY, A. E., AND ANSON, M. L.: J. Gen. Physiol. **19**, 427 (1936).
(276) MIRSKY, A. E., AND ANSON, M. L.: J. Gen. Physiol. **19**, 439 (1936).
(277) MIRSKY, A. E., AND ANSON, M. L.: J. Gen. Physiol. **19**, 451 (1936).
(278) MIRSKY, A. E., AND PAULING, L.: Proc. Natl. Acad. Sci. (U. S.) **22**, 439 (1936).
(279) MITCHELL, J. S.: Trans. Faraday Soc. **33**, 1129 (1937).
(280) MOELWYN-HUGHES, E. A.: Ergeb. Enzymforsch. **2**, 1 (1933).
(281) MOYER, L. S.: J. Phys. Chem. **42**, 71 (1938).
(281a) MÜLLER, O. H.: Chem. Rev. **24**, 95 (1939).
(282) MURALT, A. L. VON, AND EDSALL, J. T.: J. Biol. Chem. **89**, 315, 351 (1930).
(283) NEEDHAM, J., KLEINZELLER, A., MIALL, M., DAINTY, M., NEEDHAM, D. M., AND LAWRENCE, A. S. C.: Nature **150**, 46 (1942).

(284) NEEDHAM, J., SHEN, S. C., NEEDHAM, D. M., AND LAWRENCE, A. S. C.: Nature **147,** 766 (1941).
(285) NEURATH, H.: J. Phys. Chem. **40,** 361 (1936).
(286) NEURATH, H.: Cold Spring Harbor Symposia Quant. Biol. **6,** 196 (1938).
(287) NEURATH, H.: J. Am. Chem. Soc. **61,** 1841 (1939).
(288) NEURATH, H.: J. Phys. Chem. **44,** 296 (1940).
(289) NEURATH, H.: Cold Spring Harbor Symposia Quant. Biol. **8,** 80 (1940).
(290) NEURATH, H.: Chem. Rev. **30,** 357 (1942).
(291) NEURATH, H.: J. Am. Chem. Soc., **65,** 2039 (1943).
(292) NEURATH, H., AND BULL, H. B.: J. Biol. Chem. **115,** 519 (1936).
(293) NEURATH, H., AND BULL, H. B.: Chem. Rev. **23,** 391 (1938).
(294) NEURATH, H., COOPER, G. R., AND ERICKSON, J. O.: J. Biol. Chem. **138,** 411 (1941).
(295) NEURATH, H., COOPER, G. R., AND ERICKSON, J. O.: J. Biol. Chem. **142,** 249 (1942).
(296) NEURATH, H., COOPER, G. R., AND ERICKSON, J. O.: J. Biol. Chem. **142,** 265 (1942).
(297) NEURATH, H., COOPER, G. R., AND ERICKSON, J. O.: J. Phys. Chem. **46,** 203 (1942).
(298) NEURATH, H., ERICKSON, J. O., AND COOPER, G. R.: Science **96,** 116 (1942).
(299) NEURATH, H., PUTNAM, F. W., ERICKSON, J. O., AND VOLKIN, E.: Unpublished observations.
(300) NEURATH, H., AND SAUM, A. M.: J. Biol. Chem. **128,** 347 (1939).
(301) NORD, F. F.: Naturwissenschaften **24,** 481 (1936).
(302) NORTHROP, J. H.: J. Gen. Physiol. **4,** 261 (1922).
(303) NORTHROP, J. H.: J. Gen. Physiol. **14,** 713 (1931).
(304) NORTHROP, J. H.: Ergeb. Enzymforsch. **1,** 302 (1932).
(305) NORTHROP, J. H.: J. Gen. Physiol. **16,** 323 (1932).
(306) NORTHROP, J. H.: J. Gen. Physiol. **17,** 359 (1934).
(307) NORTHROP, J. H.: *Crystalline Enzymes.* Columbia University Press, New York (1939).
(308) OGSTON, A. G.: Biochem. J. **37,** 78 (1943).
(309) ONCLEY, J. L.: Ann. N. Y. Acad. Sci. **41,** 121 (1941).
(310) OSBORNE, T. B.: *The Vegetable Proteins.* Longmans, Green and Co., New York (1909).
(311) PACE, J.: Biochem. J. **24,** 606 (1930).
(312) PACE, J.: Biochem. J. **25,** 1 (1931).
(313) PACE, J.: Biochem. J. **25,** 422 (1931).
(314) PACE, J.: Biochem. J. **25,** 1485 (1931).
(314a) PALMER, K. J., AND GALVIN, J. A.: J. Am. Chem. Soc. **65,** 2187 (1943).
(314b) PARFENTJEV, I. A., CLAPP, F. L., AND WALDSCHMIDT, A.: J. Immunol. **40,** 189 (1941).
(315) PATTERSON, W. I., GEIGER, W. B., MIZELL, L. R., AND HARRIS, M.: J. Research Natl. Bur. Standards **27,** 89 (1941).
(316) PAULING, L.: J. Am. Chem. Soc. **62,** 2643 (1940).
(317) PAULING, L., AND CAMPBELL, D. H.: J. Exptl. Med. **76,** 211 (1942).
(318) PEDERSEN, K. O.: Nature **128,** 150 (1931).
(319) PETERMANN, M. L.: J. Phys. Chem. **46,** 183 (1942).
(320) PETERMANN, M. L.: J. Biol. Chem. **144,** 607 (1942).
(321) PETERMANN, M. L., AND PAPPENHEIMER, A. M., JR.: Science **93,** 458 (1941).
(322) PETERMANN, M. L., AND PAPPENHEIMER, A. M., JR.: J. Phys. Chem. **45,** 1 (1941).
(323) PHILPOT, J. ST.: Biochem. J. **29,** 2458 (1935).
(324) PILLEMER, L., AND ECKER, E. E.: J. Immunol. **40,** 101 (1941).
(325) PILLEMER, L., ECKER, E. E., MYERS, V. C., AND MUNTWYLER, E.: J. Biol. Chem. **123,** 365 (1938).
(326) POLSON, A.: Kolloid-Z. **88,** 51 (1939).
(327) POPE, C. G.: Brit. J. Exptl. Path. **20,** 132, 201 (1939).
(328) PORTER, E. F., AND PAPPENHEIMER, A. M., JR.: J. Exptl. Med. **69,** 755 (1939).
(329) PRICE, W. C.: Arch. ges. Virusforsch. **1,** 373 (1940).
(330) PRIDEAUX, E. B. R., AND WOODS, D. E.: Proc. Roy. Soc. (London) **B110,** 353 (1932).

(331) PUTNAM, F. W., ERICKSON, J. O., VOLKIN, E., AND NEURATH, H.: J. Gen. Physiol. **26,** 513 (1943).
(332) PUTNAM, F. W., AND NEURATH, H.: J. Biol. Chem. **150,** 263 (1943).
(333) PUTNAM, F. W., AND NEURATH, H.: Unpublished observations.
(334) RAMSDEN, W.: Nature **126,** 685 (1930).
(335) RATNER, S., AND CLARKE, H. T.: J. Am. Chem. Soc. **59,** 200 (1937).
(336) ROBERTS, R. M.: J. Am. Chem. Soc. **64,** 1472 (1942).
(337) ROCHE, A., AND BRACCO, J.: Bull. soc. chim. biol. **16,** 1479 (1934).
(338) ROCHE, J.: Compt. rend. trav. lab. Carlsberg, Sér. chim. **22,** 465 (1938).
(339) ROCHE, J., AND CHOUAIECH, M.: Bull. soc. chim. biol. **22,** 263 (1940).
(340) ROCHE, J., AND CHOUAIECH, M.: Compt. rend. soc. biol. **133,** 474 (1940).
(341) ROCHE, J., AND COMBETTE, R.: Compt. rend. soc. biol. **126,** 950 (1937).
(342) ROCHE, J., AND COMBETTE, R.: Bull. soc. chim. biol. Paris **19,** 627 (1937).
(343) ROCHE, J., ROCHE, A., ADAIR, G. S., AND ADAIR, M. E.: Biochem. J. **26,** 1811 (1932).
(344) ROSENTHAL, S. M., AND VOEGTLIN, C.: U. S. Pub. Health Service, Pub. Health Rept. No. **48,** 347 (1933).
(345) ROSNER, L.: J. Biol. Chem. **132,** 657 (1940).
(346) Ross, A. F.: J. Biol. Chem. **136,** 119 (1940).
(347) ROTHEN, A.: Ann. N. Y. Acad. Sci. **43,** 229 (1942).
(348) ROTHEN, A., CHOW, B. F., GREEP, R. O., AND VAN DYKE, H. B.: Cold Spring Harbor Symposia Quant. Biol. **9,** 272 (1941).
(349) ROTHEN, A., AND LANDSTEINER, K.: Science **90,** 65 (1939).
(350) ROTHEN, A., AND LANDSTEINER, K.: J. Exptl. Med. **76,** 437 (1942).
(351) SALTER, A.: Guy's Hosp. Rept. **53,** 241 (1896).
(352) SANIGAR, E. B., KREJCI, L. E., AND KRAEMER, E. O.: Biochem. J. **33,** 1 (1939).
(353) SCHAEFER, V. J.: J. Phys. Chem. **42,** 1089 (1938).
(354) SCHMIDT, K. H.: Z. physiol. Chem. **277,** 117 (1943).
(354a) SCHRAMM, G.: Naturwissenschaften **31,** 94 (1943).
(355) SCHUBERT, M. P.: J. Biol. Chem. **114,** 341 (1936).
(356) SCHULZ, F. N.: Z. physiol. Chem. **25,** 16 (1898).
(356a) SENTI, F. R., EDDY, G. R., AND NUTTING, G. C.: J. Am. Chem. Soc. **65,** 2473 (1943).
(357) SHAFFER, M. F., AND DINGLE, J. H.: Proc. Soc. Exptl. Biol. Med. **38,** 528 (1938).
(358) SHARP, D. G., COOPER, G. R., ERICKSON, J. O., AND NEURATH, H.: J. Biol. Chem. **144,** 139 (1942).
(359) SHARP, D. G., COOPER, G. R., AND NEURATH, H.: J. Biol. Chem. **142,** 203 (1942).
(360) SIGNER, R., CASPERSSON, T., AND HAMMARSTEN, E.: Nature **141,** 122 (1938).
(361) SIMHA, R.: J. Phys. Chem. **44,** 25 (1940).
(362) SMETANA, H.: J. Biol. Chem. **124,** 667 (1938).
(363) SMETANA, H., AND SHEMIN, D.: J. Exptl. Med. **73,** 223 (1941).
(364) SOBOTKA, H., AND BLOCH, E.: J. Phys. Chem. **45,** 9 (1941).
(365) SPEAKMAN, J. B.: Nature **132,** 930 (1933); **138,** 327 (1936).
(366) SPIEGEL-ADOLF, M.: Biochem. Z. **170,** 126 (1926).
(367) SPIEGEL-ADOLF, M.: Biochem. Z. **204,** 1 (1929).
(368) SPIEGEL-ADOLF, M., AND HENNY, G. C.: J. Phys. Chem. **45,** 931 (1941); **46,** 581 (1942).
(369) STÄLLBERG, S.: Trans. Faraday Soc. **35,** 1416 (1939).
(370) STANLEY, W. M.: Science **83,** 626 (1936).
(371) STANLEY, W. M., AND LAUFFER, M. A.: Science **89,** 345 (1939).
(372) STEARN, A. S.: Ergeb. Enzymforsch. **7,** 1 (1938).
(373) STEINHARDT, J.: Kgl. Danske Videnskab. Selskab. Math.-fys. Medd. **14,** No. 11 (1937).
(374) STEINHARDT, J.: J. Biol. Chem. **123,** 543 (1938).
(375) STERN, K. G., AND WHITE, A.: J. Biol. Chem. **122,** 371 (1938).
(376) SUTHERLAND, W.: J. Physiol. **42,** vii (1911).
(377) SVEDBERG, T.: Trans. Faraday Soc. **26,** 740 (1930).

(378) SVEDBERG, T.: Chem. Rev. **20,** 81 (1937).
(379) SVEDBERG, T.: PROC. ROY. Soc. (London) **B127,** 1 (1939).
(380) SVEDBERG, T., AND BROHULT, S.: Nature **142,** 830 (1938).
(381) SVEDBERG, T., AND PEDERSEN, K. O.: *The Ultracentrifuge.* Oxford University Press, New York (1940).
(382) TAMMANN, G.: Z. physik. Chem. **18,** 426 (1895).
(383) TAYLOR, J. F., AND HASTINGS, A. B.: J. Biol. Chem. **144,** 1 (1942).
(384) TEN BROECK, C.: J. Biol. Chem. **17,** 369 (1914).
(385) TENNENT, H. G., AND VILBRANDT, C. F.: J. Am. Chem. Soc. **65,** 424 (1943).
(386) TEORELL, T.: Biochem. Z. **229,** 1 (1930).
(387) THEIS, E. R., AND JACOBY, T. F.: J. Biol. Chem. **148,** 105 (1943).
(388) THEIS, E. R., AND JACOBY, T. F.: J. Biol. Chem. **148,** 603 (1943).
(389) THOMPSON, J. W., AND VOEGTLIN, C.: J. Biol. Chem. **70,** 793 (1926).
(390) TISELIUS, A.: Nova Acta Regiae Soc. Sci. Upsaliensis [4] **7,** No. 4 (1930).
(391) TISELIUS, A., AND DAHL, O.: Arkiv. Kemi. Mineral Geol. **14B,** No. 31, 7 pp. (1941).
(392) TISELIUS, A., AND ERIKSSON-QUENSEL, I. B.: Biochem. J. **33,** 1752 (1939).
(393) TISELIUS, A., AND HORSFALL, F. L., JR.: J. Exptl. Med. **69,** 83 (1939).
(394) TODRICK, A., AND WALKER, E.: Biochem. J. **31,** 292 (1937).
(395) TRACY, A. H., AND ROSS, W. F.: J. Biol. Chem. **142,** 871 (1942).
(396) TUNNICLIFFE, H. E.: Biochem. J. **19,** 199 (1925).
(397) VAN DER SCHEER, J., WYCKOFF, R. W. G., AND CLARKE, F. L.: J. Immunol. **40,** 39 (1941).
(398) WALKER, A. C., AND REIMANN, S. P.: Am. J. Cancer **37,** 585 (1939).
(399) WATSON, C. C., ARRHENIUS, S., AND WILLIAMS, J. W.: Nature **137,** 322 (1936).
(400) WEBER, H. H.: Ergeb. Physiol. **36,** 109 (1934).
(401) WEBER, H. H., AND STÖVER, R.: Biochem. Z. **259,** 269 (1933).
(402) WEIL, A. J., MOOS, A. M., AND CLAPP, F. L.: J. Immunol. **37,** 413 (1939).
(403) WEIL, A. J., PARFENTJEV, I. A., AND BOWMAN, K. L.: J. Immunol. **35,** 399 (1938).
(404) WEIMARN, P. P. VON: Kolloid-Z. **42,** 134 (1927).
(405) WELLS, H. G.: J. Infectious Diseases **6,** 506 (1909).
(406) WILLIAMS, J. W., AND WATSON, C. C.: Nature **139,** 506 (1937).
(407) WINNICK, T., DAVIS, A. R., AND GREENBERG, D. M.: J. Gen. Physiol. **23,** 301 (1940).
(408) WITZEMANN, E. J., AND LIVSHIS, L.: J. Biol. Chem. **58,** 463 (1923).
(409) WU, H.: Chinese J. Physiol. **1,** 81 (1927).
(410) WU, H.: Chinese J. Physiol. **5,** 321 (1931).
(411) WU, H., AND CHEN, T. T.: Chinese J. Physiol. **3,** 7 (1929).
(412) WU, H., AND LING, S. M.: Chinese J. Physiol. **1,** 407 (1927).
(413) WU, H., LIU, S. C., AND CHOU, C. Y.: Chinese J. Physiol. **5,** 309 (1931).
(414) WU, H., TEN BROECK, C., AND LI, C. P.: Chinese J. Physiol. **1,** 277 (1927).
(415) WU, H., AND YANG, E. F.: Chinese J. Physiol. **6,** 51 (1932).
(416) WYMAN, J., JR.: J. Biol. Chem. **127,** 1 (1939).
(417) ZAHND, H., AND CLARKE, H. T.: J. Biol. Chem. **102,** 171 (1933).
(418) ZILVA, S. S.: Biochem. J. **8,** 656 (1914).