THE MICROHETEROGENEITY OF PROTEINS[1]

J. ROSS COLVIN, DAVID B. SMITH, AND W. H. COOK

Division of Applied Biology, National Research Laboratories, Ottawa, Canada

Received March 22, 1954

CONTENTS

I. INTRODUCTION

Pioneer work in the laboratories of Hardy, Loeb, Sørensen, and others (24, 44, 54, 96, 122) showed that the methods and concepts of the physical chemistry of solutions of small molecules were applicable to solutions of proteins. Of particular importance was the demonstration that the phase rule applied to solutions of crystalline proteins as well as to less complex substances (106, 137). Following this demonstration, interpretation of experiments on protein solutions in terms of molecularly dispersed systems, rather than as suspensions or emulsions, began to predominate. Experimental justification for this point of view accumulated rapidly in the decade following Svedberg's development of the ultracentrifuge (140), when it was shown that solutions of purified, and sometimes crystalline, proteins were often only paucidisperse mixtures, at worst. Since then, the concept of protein solutions as macromolecular systems rather than as classical polydisperse suspensions has been almost universally accepted. This point of view has been strengthened by additional new techniques, such as Tiselius' modification of electrophoresis (148, 149), which indicated that preparations which were homogeneous by one criterion were often homogeneous by several others also. As a result, in spite of the known complexity of proteins, the assumption seems to have been implicitly made by most biochemists that the careful application of present methods of purification in favorable instances would yield a collection of protein molecules which was as uniform as a collection of, say, anthracene molecules. (For examples see the recent comprehensive survey by Taylor (144).)

[1] Issued as National Research Council No. 3346.

It is customary to describe such preparations, after testing by available techniques, as pure proteins.

This attitude has prevailed in spite of a number of warnings. More than ten years ago, Pirie pointed out that the assumption that proteins can be obtained uniform was scarcely justified by the experimental evidence then available (114). In addition, he emphasized that to call a protein preparation pure is equivalent to making a null hypothesis which is not contradicted by the relevant known observations. He also indicated the many opportunities for small differences between large particles of similar construction. Synge, too, after referring to the evidence for small differences in structure between members of families of polypeptides, suggested that similar differences would be found within the more complex proteins (141). Later refinements of experimental technique, which are indicated in several recent surveys of methods (38, 94, 144), have tended to confirm this prediction. On this experimental basis Haurowitz (54) has recently drawn attention to the possibility that completely uniform preparations of proteins may not exist and that, at best, proteins must be considered to exist as families of closely related but not necessarily identical molecules.

Nonetheless, the correctness of the concept that all molecules of a very carefully prepared protein have the same unique structure and configuration is still controversial, as shown by the divergent conclusions in two recent reviews (124, 150). Pragmatically, the concept has been a useful goal in the past and therefore should not be discarded summarily, although it may require modification. Also, aside from its bearing on the problem of "purity" in these compounds, the degree to which actual protein preparations approach complete uniformity is of obvious importance for the interpretation of experiments. Unfortunately for the experimentalist, complete homogeneity of a preparation is a property which cannot be demonstrated directly (114), whereas deviations from it may be. The purpose of this review therefore is to summarize critically the recent experimental evidence which indicates microheterogeneity within or between highly fractionated protein preparations due to differences in composition, structure, or configuration of the constituent molecules. The extent to which this evidence supports or rejects the notion of complete uniformity among molecules of a carefully prepared protein will be indicated.

II. DEFINITIONS AND SCOPE OF SURVEY

For the sake of clarity, some definitions are required. The word "molecule" will be used, as by Edsall (38), to mean a kinetic unit in solution. The "structure" of a molecule (i.e., protein) will be taken, as by Wheland (162), to mean a detailed statement of the way in which each atom of the molecule is linked to each other atom. These connections will include hydrogen bonds and "salt linkages" if any. The word "configuration" will mean, following Wheland, a distinct spatial arrangement of the atoms of a molecule.

The lower limit of molecular weights of proteins will be arbitrarily set at 6000 to coincide with the possible molecular weight of the unit of insulin (49, 51). This limit is below the choice of Synge (10,000) (142), but the lower figure is in

better agreement with recent experimental work, since insulin is commonly considered to be a protein.

A protein preparation will be said to be homogeneous (or heterogeneous) as tested by a particular stated technique (57). Homogeneity as used in this report is a relative concept, defined by the given operations, and has no absolute connotation. Similarly, purity is a relative property, defined by the operations to be performed upon the preparation (41). A preparation is pure for a given purpose, if further fractionation does not change its properties for that use.

A protein preparation will be said to be microheterogeneous[2] if there is experimental evidence for one or more minor differences between individual protein molecules of the preparation, over a period which is long compared with the duration of the experiment(s). By this definition, heterogeneity contributed to a preparation by the presence of non-protein components is considered to lie outside the range of microheterogeneity. Likewise, tautomeric forms of the same molecule do not contribute to microheterogeneity. Moreover, the definition is not restricted to the properties of mass, size, or shape of the molecules. Finally, it should be noted that the heterogeneity contributed by dimers, trimers, or polymers of the unit molecular weight of the preparation, which are not in rapid equilibrium, lies outside the range of microheterogeneity.

The above definition implies an arbitrary limit beyond which differences between protein molecules are not considered to be minor. Unfortunately, lacking precise knowledge of protein structure, very few objective criteria of degrees of difference are available and discrimination between minor and major differences must be largely subjective. Consequently complete agreement with the choices exercised here cannot be expected. However, where applicable, the maximum permissible degree of deviation of a property from the average magnitude for the preparation (or from its possible alterations) was taken as approximately 10 per cent.

In addition, two aspects of the microheterogeneity of a preparation must be distinguished. One is quantitative and is determined by the size of the fraction(s) of the molecules which are different. Since the review is concerned chiefly with the presence rather than the amount of microheterogeneity, this aspect will not be emphasized. However, examples are not considered where only traces (i.e., less than 1 per cent) of a different component are present within a preparation. Usually the ratio of minor to major component(s) is much greater. The second aspect is qualitative and is concerned with how much the molecules of the various fractions differ. This problem has been referred to previously, and it will have been clear to the reader that the differences shade imperceptibly into macroheterogeneity. Moreover, even accepted minor degrees of difference may be subdivided into two categories: (*a*) those which can be related to some definite alteration in structure or configuration of the molecule; (*b*) those of which the cause is as yet unknown. As an example of the first, molecules of the unit of insulin of a single species, molecular weight 6000, have been shown to differ by a single

[2] The definition of microheterogeneity given here is a generalization from the use of the term by Synge (141) and appears to be consistent with his application.

amide group. As an example of the second type, ribonuclease has been separated unequivocally by chromatography into two forms, but as yet the difference between the two is unknown. In the present summary, no distinction is made between the microheterogeneity due to these two categories of differences.

Since complete uniformity cannot be demonstrated, whereas heterogeneity can, emphasis must necessarily be placed upon those studies which found differences within the same preparation or between similar preparations. For the present survey, results of investigations of apparently homogeneous preparations, that were not examined critically for the presence of small differences, are not relevant and have been omitted.

Likewise, species differences between corresponding proteins have been omitted, because they are not necessarily minor (11, 38, 54, 82, 150). An exception has been made in the case of insulin.

Throughout, the predominantly polypeptide chain structure of proteins will be assumed to be correct (124, 141).

With only a few exceptions, the investigations considered were published in the interval from 1946 to the end of 1953.

III. EVIDENCE OF MICROHETEROGENEITY OF PROTEINS FROM VARIOUS TECHNIQUES

A. Sedimentation and diffusion

A correspondence of the shape of the refractive index gradient curve in diffusion and sedimentation velocity experiments with the shape of normal Gaussian curves is often quoted as evidence for the homogeneity of a preparation, probably because it may be obtained conveniently as part of the determination of the molecular weight of a protein. Recognition of the limitations of this test even when recently refined experimental methods are used is now fairly general (23, 163). However, provided the deviations from theoretical are ascribed wholly to heterogeneity of the sample, these methods may be capable of detecting differences in mass or frictional coefficient between similar molecules. Williams, Baldwin, Saunders, and Squire (164) have recently developed a means of sorting out the effects of heterogeneity and diffusion on the shape of velocity sedimentation refractive index gradient curves, and have used their method to demonstrate apparently continuous measurable heterogeneity of the sedimentation coefficients among the molecules of a preparation of horse serum γ-globulin. Cann has also applied their method to an analysis of the size distribution of fractions of human γ-globulin (15). As expected, the fractions were heterogeneous, and he was able to detect a difference between the sedimentation coefficients of pseudoglobulin and euglobulin within a given preparation of γ-globulin. Moreover, within each of these subfractions there was a continuous distribution of sedimentation coefficients, with the standard deviation being about 5 per cent of the mean. No correlation was detected between electrophoretic and sedimentation heterogeneity. As a result, Cann concluded that pseudoglobulin and euglobulin are composed of molecules differing slightly in size, shape, or both, as well as charge density. The significant conclusion from both of these studies is not that heterogeneity was found in fractions which are known to be mixtures but that an ap-

proximately continuous variation was found in the hydrodynamic properties of these closely related molecules. Although the precision of the experimental method is too low to eliminate completely the possibility of discontinuous distributions in these experiments, their presence seems improbable.

Boman, in a comparison of six similarly prepared samples of bovine carbon monoxide hemoglobin, found that they varied in the relation between sedimentation and concentration (10). He concluded rather tentatively that the hemoglobin was altered by handling during preparation, but since it is not clear that experimental errors were not underestimated, the suggestion of microheterogeneity between preparations cannot be regarded as conclusive.

Putnam, Lamanna, and Sharp (120, 121) reported that a crystalline preparation of *Clostridium botulinum* type A toxin, although it gave a single peak in the electrophoretic apparatus and was serologically homogeneous, showed more boundary spreading in the ultracentrifuge than could be accounted for by diffusion alone, thus indicating the presence of small differences in molecular size in the sample. The presence of other components in similar preparations was confirmed by Wagman and Bateman (156), but no indication as to whether they were distinct impurities or members of a closely related family was given.

In theory, the equilibrium ultracentrifuge is the instrument of choice for detecting mass polydispersity among macromolecules (157), but technical difficulties have limited its usefulness so far. Kegeles (79), in a recent application, found indications of polydispersity in six-times-recrystallized ovalbumin, but the evidence was not clear-cut. Improved instrumentation would provide an opportunity for much useful work of this kind.

Recent experimental and theoretical advances in free-diffusion methods offer new hope that eventually this method may provide a sensitive tool for detecting microheterogeneity (27, 46, 47, 80, 97). Cecil and Ogston (19) found that exhaustive recrystallization of lactoglobulin did not make it homogeneous, as tested by the Gouy diffusiometer. Charlwood, in a thorough investigation of the molecular weight of normal human serum albumin by ultracentrifuge and diffusion measurements (20), noticed significant deviations in the diffusion coefficient from one preparation to another, and also the presence of more than one molecular species in a given preparation of the albumin. Because the preparations were separated electrophoretically from single samples of the serum from different patients, his results are strong evidence for variations in the diffusion coefficient of molecules of normal serum albumin within and between individuals. Furthermore, since the sedimentation coefficients of different preparations did not vary significantly, these results imply significant deviations in the molecular weight of normal human serum albumin between and within individual patients. Later, Charlwood (21) showed by the same methods that nephrotic serum albumin had a mean molecular weight higher than that of normal, while the molecular weights of the nephrotic urinary albumins were lower. Unfortunately, the experimental techniques did not permit a distinction between the possibility of an unusual distribution of normal albumin components and the presence of new components (22). No correlation between diffusion and electrophoretic hetero-

geneity was observed. Here also the significant result is not only that microheterogeneity was found in a substance which had been shown to be a mixture electrophoretically (65) but that additional small differences between molecules were found in another property.

In a single experiment using bovine plasma albumin, Akeley and Gosting detected one or more more slowly diffusing impurities by the Gouy diffusiometer (1). However, since the presence of these impurities was confirmed by velocity sedimentation experiments it is certain that all the differences are not to be considered small. This conclusion is confirmed by the difference between the estimated value of $D_{w,20}$ of this sample of bovine plasma albumin, $5.85_5 \times 10^{-7}$, and the value of $6.11_2 \times 10^{-7}$ found by Creeth (29) from an extensive investigation using the same method. This difference is as great as that which might be expected using older techniques and, considering the theoretical precision of the method, it indicates either unsuspected systematic errors or unexpectedly wide differences between samples of bovine plasma albumin.

B. Electrophoresis

The uses and limitations of the electrophoretic technique for detecting gross electrical heterogeneity or contamination in solutions of proteins are well known and have been described repeatedly (2, 57, 144). Differences of this order do not fall within the scope of this review. However, the now well-recognized electrophoretic heterogeneity of crystalline β-lactoglobulin represents an example of somewhat smaller differences between molecules which cannot differ greatly in molecular weight or shape but differ perceptibly in electrical properties and solubility (48, 93, 102, 116). Moreover, microheterogeneity exists even within the components, for isolation and crystallization of one of them gave a preparation the solubility of which increased with increasing amounts of crystals present. It would be of great interest to test this component by the reversible boundary spreading technique (see below). As yet, the reason for the small differences between and within electrophoretic components is obscure, but it does not seem to be due to techniques of preparation, dissociation, or aggregation phenomena.

Recently, methods capable of detecting very subtle differences between similar particles have been developed. Alberty (3), by extending the usefulness of an earlier equation due to Sharp, Hebb, Taylor, and Beard (133, 134), estimated quantitatively the electrical heterogeneity of protein preparations from reversible boundary spreading in electrophoresis, provided the proteins were stable and soluble at their isoelectric points, extraneous spreading effects were absent, and the mobility distribution was approximately Gaussian. By this test, all the preparations examined of bovine plasma albumin, human γ_2-globulin, chymotrypsin, chymotrypsinogen, immune lactoglobulin (pseudo), lysozyme, ovalbumin, hemocyanin, β-lactoglobulin, hyperimmune horse γ_2-globulin, normal horse γ_2-globulin, human γ_1-globulin, ribonuclease,[3] or gelatin were not homogeneous (4, 5). Of

[3] Evidence for ribonuclease was equivocal, but since the boundaries became asymmetrical during electrophoresis and symmetrical again on reversal of the current, the sample was probably heterogeneous.

these samples, the descriptions given indicate that bovine albumin, ovalbumin, ribonuclease, chymotrypsin, chymotrypsinogen, and lysozyme were highly purified crystalline materials. Once again, the significant result from these studies is not that preparations such as the globulins and gelatin which were known beforehand to be mixtures were found to be heterogeneous, but that an approximately continuous distribution of electrophoretic mobilities exists in even the best preparations examined. Although the width of the distribution varies markedly from protein to protein, these experimental observations are strong evidence for very small but definite differences between the electrical properties of the molecules in any preparation. The study showed that the method should be capable of detecting a difference in charge of three electrons between molecules of a molecular weight of the order of 160,000 at ionic strength 0.1 and that sensitivity increased as ionic strength decreased. The charge differences were tentatively assigned to variations in amino acid composition, different end-groups, different effects of neighboring atoms on ionizing groups, or different configurations of the molecule.

Studies on reversible boundary spreading in protein solutions leave no doubt about the electrophoretic microheterogeneity of the preparations examined so far and suggest several profitable avenues of research. It has been demonstrated that a Gaussian distribution of mobilities in a sample necessarily leads to a Gaussian refractive index gradient curve in electrophoresis (3). The prevalence of electrophoretic curves of this shape in boundary spreading is a temptation to invert the argument and infer that the distribution of mobilities is truly continuous. However, it is clear that this procedure is not generally valid, since the effects of diffusion superimposed upon a small number of closely related but discrete components would lead to the same form of curve within the limits of present techniques. Experiments are needed to estimate a lower limit to the number of discrete components in a reversible boundary spreading experiment which would still be consistent with a given Gaussian refractive index gradient curve. In addition, the possible contribution of a horizontal gradient in the isoelectric points of protein molecules due to a horizontal temperature gradient in the cell does not seem to have been considered. The temperature gradient between the interior of an electrophoretic cell and the wall is well known (149) and if, as seems probable, the isoelectric points of protein molecules are a function of temperature (24), there will then be a temperature-induced gradient of isoelectric points of the molecules between the interior and the wall. In contrast to the irreversible effects of convection and electroösmosis such an effect would be reversible. Some preliminary calculations indicate that the effect may contribute slightly to the heterogeneity constant, h, for proteins with isoelectric points in the range above pH 6.

Furthermore, in spite of its sensitivity, a limitation of the reversible boundary spreading method is illustrated strikingly by the observation that the heterogeneity constant for a sample of gelatin is of the same size as the values for very highly purified crystalline preparations of other proteins (5). Other restrictions on the method are caused by pH and conductivity changes across the boundary

(36). The technique has been extended to preparations having a non-Gaussian mobility distribution (14) and to preparations where there is a distribution of diffusion coefficients among the molecules (7). As yet, the method is restricted to preparations in which interactions between protein molecules may be neglected.

Electrical microheterogeneity has been detected even among proteins which cannot be studied at their isoelectric points by reversible boundary spreading, owing to insolubility or instability. The attainment of a steady state in the moving ascending boundary is a satisfactory qualitative criterion for homogeneity in such systems. Crystalline preparations of urease, trypsin, pepsin (5), and bovine serum albumin (64) have been shown not to satisfy this test. Human oxyhemoglobin, however, was homogeneous (64). Suitably prepared oxyhemoglobin should therefore provide a satisfactory provisional standard for further work on the electrical homogeneity of proteins by the steady-state method, but further experiments of this kind have not been reported. It is regrettable that no comparable experiments have been published for oxyhemoglobin using the reversible boundary spreading method.

Following earlier indications (98), Perlmann has recently given direct and conclusive evidence for analytical as well as electrical microheterogeneity among ovalbumin derivatives (111, 112, 113). She has shown that ovalbumin, as it is obtained fresh from hens' eggs or after crystallization, is a mixture of at least three forms which differ in electrophoretic properties owing to the presence of zero, one, or two phosphate groups on the molecule. As yet, no other differences in the properties of the three ovalbumins have been observed, although Perlmann has pointed out the possibility. The same worker has shown that plakalbumin (40, 107), which is derived from ovalbumin by the action of an enzyme from *Bacillus subtilis*, exists in a similar series of forms of differing phosphate content. Thus, five different stable modifications of ovalbumin are known, all of which are crystallizable and which differ from one another by one or two known small changes in structure and associated properties. It would be of the greatest interest to know if any or all of these crystalline modifications exhibit reversible boundary spreading, but such experiments have not yet been reported.

Significant differences in the chemical and physical properties of human adult and fetal hemoglobins have been known for many years. However, Pauling, Itano, Singer, and Wells first demonstrated that a significant difference could be detected between the electrophoretic mobility of adult human hemoglobin derived from erythrocytes of apparently normal individuals and that from individuals suffering from sickle-cell anemia (109). The difference is independent of buffer ions used and is not due to differences in molecular weight or molecular shape or to differences between the hemes. No differences in the basic or acidic amino acid content of the respective globins could be detected (128) or in the number of primary amide groups (35). Following this stimulus, work on abnormal forms of adult human hemoglobin has expanded rapidly and at least four distinct forms are known (68). Three of these forms are distinguished by their electrophoretic properties and the fourth by a higher solubility. The differences in charge have been tentatively assigned to differences in the configuration of the hemoglobin mole-

cules and, together with fetal hemoglobin, are further evidence for small variations among related proteins.

Yeast glyceraldehyde-3-phosphate dehydrogenase also has different electrophoretic forms, which may be separated by nucleic acid fractionation (83). All these forms appear to have about the same sedimentation constant and are not in rapid equilibrium with one another. Likewise, crystalline lactoperoxidase contains two enzymatically active forms, A and B, which differ electrophoretically and in their optical absorption properties (115). There is some suggestion that lactoperoxidase A may be convertible to lactoperoxidase B by partial oxidation of the aromatic groups in the protein. Both these recent investigations are further evidence for the existence of stable, closely related forms of the same protein from the same tissue.

Small electrophoretic differences between proteins, related or not, have been used lately for large-scale fractionation studies (16, 17, 18, 146, 147). By means of electrophoresis-convection, workers in Kirkwood's laboratory have fractionated bovine γ-globulin, horse diphtheria antitoxin pseudoglobulin, the bovine serum proteins, and insulin in quantity. Since the method depends upon differences in electrical charge density, since the insulin was reasonably purified to begin with, and particularly since no apparent limit to fractionation was found for the globulin, their success is further confirmation of small differences in the electrical properties of molecules in such preparations. Crystalline insulin was found to be heterogeneous and to contain two components, only one of which appeared to have biological activity. Both electrophoretic components were present in a sample of mixed species insulin as well as in beef insulin. They did not appear to be similar to the A and B components of Harfenist and Craig (50). The difference in charge was estimated to be about three electrons.

C. Phase distribution

The classical solubility criterion for homogeneity is one of the most sensitive available to the chemist and has been widely applied to proteins (106, 144). A résumé of its uses and limitations for the detection of distinct impurities in a substance has been given by Herriott (56), who had previously used this method with Desreux to distinguish four forms of swine pepsin (32). The test is particularly useful for the detection of small differences between two related proteins when one or both has a characteristic, accurately measurable property. The method has been carefully applied by Falconer and Taylor in the purification of pig liver esterase (42). By this test, solutions of crystalline ox liver catalase have been shown to contain two distinct forms of catalase, differing in activity (13). However, since one form may have been partially degraded erythrocyte catalase, the differences between the two are not necessarily minor. By the same method, as well as by electrophoresis, two different forms of lactic dehydrogenase from ox heart muscle have been recognized (104). Both forms have the same sedimentation constant and molecular weight but one has a higher proportion of acid groups per mole. Both investigations show that solubility methods, in properly designed experiments under favorable conditions, can detect microheterogeneity in a pro-

tein preparation. In general, however, recent investigations have suggested that classical solubility methods are better suited for the detection of small amounts of impurities of dissimilar proteins in a preparation than of appreciable quantities of very closely related proteins. Only two examples need be given. The heterogeneity of ribonuclease, which was not detected in the first careful solubility studies (86) and only indications of which were given by later experiments, has been demonstrated recently (62). A sample of insulin, which was homogeneous by classical solubility studies (43), was found by more refined methods to contain at least two major components (50). For proteins, the methods of analysis and the complexity of their structure inevitably reduce the sensitivity of liquid–solid phase distribution methods.

Some of these limitations may be avoided by phase distribution studies of another type (28). Counter-current distribution techniques are less generally applicable to proteins than to smaller molecules but have been used with great success in a favorable case recently (50). Thus, crystalline insulin from beef pancreas, which gave one peak electrophoretically (pH 1.6–3.1) and was homogeneous by solid–liquid phase distribution tests, was resolved into two or more components in a 2-butanol–1 per cent aqueous dichloroacetic acid system. All components possessed equal biological activity, and the partition ratios of the corresponding components from different species were indistinguishable. No analytical differences between the components were detected initially, but very recently Harfenist has shown that the two major components differ by a single amide group (49). This work would seem to be approaching the limit of detection of microheterogeneity in proteins, analytically at least! These results are in contrast to the unsuccessful attempt of Porter to fractionate insulin by partition chromatography on columns with the equivalent of about four hundred plates in a solvent system (118). As pointed out by Porter, insulin behaved as a single entity on several columns, suggesting that partition chromatography is a blunter tool for the demonstration of fine differences than is counter-current distribution. However, this difference may also be accounted for by Porter's use of Boot's insulin, other samples of which were shown by Harfenist and Craig to contain much less of component B than Lilly insulin. Porter's samples may therefore have been nearly homogeneous.

D. Chromatography

Although insulin has not been fractionated successfully by chromatography, some highly purified crystalline proteins have been resolved by this technique and the results are further evidence for very small differences between similar protein molecules. Martin and Porter, using the solvent system ammonium sulfate, water, and ethylene glycol on kieselguhr, separated crystalline beef pancreas ribonuclease into two enzymatically active forms (99). In later work, they were able to obtain only one form from pig pancreas, while in fetal calf pancreas the minor component was present in only trace amounts. Hirs, Stein, and Moore confirmed the presence of two components in beef pancreas ribonuclease, using carboxylic acid ion-exchange resins (62, 63). Recycling of the components gave

no further fractionation. Different preparations of crystalline salts of lysozyme have been fractionated by similar techniques into three chromatographically distinct, enzymatically active components (143). These components also underwent no further fractionation on recycling. Heterogeneity of the samples was also detected electrophoretically. The microheterogeneity of crystalline lysozyme, as reflected by its chromatographic behavior, was a function of the method of preparation and time of storage. Unlike isoelectric lysozyme and lysozyme chloride, which were stable in the solid state at room temperature, lysozyme carbonate was converted from one chromatographically distinct form to the two others under these conditions. All three forms had equal enzymatic activity. More acidic species seemed to be formed at room temperature in the solid state and (perhaps) in solution. Precise description of the small differences between the three forms and between the salts remains a fascinating challenge for future work.

Cow heart cytochrome c has been separated on carboxylic acid ion-exchange resins into three components which differ in iron content (108). Horse heart cytochrome c has been resolved similarly into two components (9). Very recently, another basic protein, chymotrypsinogen α, has been chromatographed on an ion-exchange resin (61). The best crystalline preparations were homogeneous chromatographically and no change in behavior was found after storage in the dry state at 4°C. for 5 months. Less well fractionated preparations gave two enzymatically activatable components, one of which was identified with the single component of the better preparations. The other component was thought to be an artifact produced during isolation or storage of the zymogen. There is an indication that neutral proteins like bovine plasma albumin may be fractionated on ion-exchange resins (135) (see also Boardman and Partridge (9)), but the differences probably exceed the limits chosen for microheterogeneity.

E. Immunology

Immunological techniques have been used more often for the detection of small amounts of widely different proteins in a preparation, or for differentiation between corresponding proteins from related species, than they have for the detection of small differences between similar molecules of the same preparation (11, 73, 76, 89, 90). Some qualitative suggestions of such differences have been found by this means however, although the evidence is not clear-cut or conclusive. In an early study, Kabat and Heidelberger found that crystalline horse serum albumin was homogeneous immunologically (74) in spite of the previous detection of several components as judged by solubility studies (136) and the presence of fractions differing in carbohydrate content of the protein molecules (58, 59, 60). This suggestion that immunology was insensitive to fine differences between related proteins was confirmed by two independent studies on human γ-globulins (70, 81). Several fractions of γ-globulin were immunologically homogeneous, although they were heterogeneous electrophoretically and ultracentrifugally and were known to contain a mixture of antibodies. Kabat and Murray confirmed these surprising results by showing that four different samples of human γ_2-globulin from three different laboratories were uniform immunochemically, al-

though they differed electrophoretically and ultracentrifugally (77). In contrast, Cohn, Deutsch, and Wetter were able to distinguish between four human γ-globulin fractions of different physical properties by immunological methods (25). They stressed the precautions which must be observed when using immunological techniques as criteria for homogeneity.

These conflicting results of careful studies, in some cases using the same preparations, suggest that immunochemistry is not as useful a method for detecting heterogeneity as has sometimes been thought. This conclusion is confirmed by recent studies which suggest that impurities may be overlooked because different proteins exhibit marked differences in antigenicity (159).

The immunological homogeneity of several highly purified components of egg white has been examined also. Crystalline ovalbumin which was homogeneous by the ultracentrifuge and diffusion studies but electrophoretically heterogeneous contained only traces of an impurity (26). Apparently, the immunological technique could not differentiate the two phosphorylated forms of ovalbumin. Conalbumin, which gave a single peak in electrophoresis experiments over the pH range 3.0–8.6 and in the ultracentrifuge, was heterogeneous immunologically (26). This heterogeneity was attributed to traces of protein contaminants from the egg white source. Four-times-reprecipitated ovomucoid contained at least two components immunologically, but the results did not reflect the extreme heterogeneity shown by electrophoresis (160). Crystalline lysozyme containing no contaminants detectable by the ultracentrifuge was heterogeneous immunologically, electrophoretically, and possibly by diffusion measurements (161). Evidence was observed for two distinct antigenic components of crystalline lysozyme which were present in the same ratio in egg white as in the crystalline state.

An examination of eight samples of crystalline β-lactoglobulin showed that they were heterogeneous immunologically and electrophoretically (33). However, since some of the components were certainly distinct impurities from the whey, no conclusions about the microheterogeneity of the major component are possible.

In an interesting demonstration of the effect of splitting off a hexapeptide (together with associated changes), plakalbumin was shown to be distinguishable immunologically from ovalbumin (78). Yeast glyceraldehyde-3-phosphate dehydrogenase (Yeast Protein 2) was shown to be heterogeneous by solubility studies and electrophoresis but homogeneous immunologically (84).

Summarizing, these investigations suggest that immunochemistry, although sometimes useful, is not as powerful a tool for differentiating minor differences between related proteins as it is for detecting small amounts of distinct impurities. Hence, when immunological heterogeneity is found, the differences between protein molecules of the preparation are not necessarily minor. A further difficulty with the immunological technique is that the results cannot be interpreted quantitatively or unequivocally in chemical or physical terms.

F. Amino acid composition

Variations in amino acid composition were among the earliest means of distinguishing different classes of proteins (155). Consequently, the possibility of

detecting finer differences between individual proteins by their amino acid content has been recognized for some time. Analytical methods are still too crude to carry this approach very far except in favorable cases (for instance, compare the careful, painstaking, and complete analyses of Brand (12) and of Stein and Moore (138) for β-lactoglobulin and bovine serum albumin), but it should be much more practicable in the future (49).

Meanwhile, some suggestions of microheterogeneity among the molecules of highly purified preparations of a protein have been found. On the basis of a reproducible, non-integral value per mole for tryptophan in different highly purified preparations of human serum albumin, Brand has suggested that this protein is not chemically homogeneous (12) (*cf.* Tristam (151)). Another example is the difference between serum albumin and serum mercaptalbumin (both bovine and human), which differ by the presence of a single sulfhydryl group (38, 66). The difference in the A and B components of bovine insulin due to the presence of a single extra amide group on the A component has been referred to previously.

Ovalbumin, which itself is a mixture of forms differing in phosphate content, is transformed to a distinct new protein, plakalbumin, by the action of an enzyme from *Bacillus subtilis* (40, 107). The difference in properties is due at least partially to the removal of six amino acid residues and a consequent change in composition of the protein. However, recent work by Steinberg indicates strongly that other differences between the two proteins must exist (139).

Similar transformations of the precursors of proteolytic enzymes are well known and recently have become again the subject of intense investigation. The differences between pepsinogen and pepsin fall outside the range of microheterogeneity (55, 132), but recently the splitting of a seven or eight amino acid residue from trypsinogen during the activation to trypsin has been reported (30). Some of the transformations involved in the activation of chymotrypsinogen to the various forms of chymotrypsin appear to be particularly good examples of minor differences between related proteins. Kunitz and Northrop discovered that chymotrypsin existed in three forms, α, β, and γ, which differed in crystal form, solubility, titration curves, and rate of denaturation (85, 87). The process of activation appeared to involve hydrolytic cleavage of a small number of peptide bonds. Later, Jacobsen was able to infer the existence of two additional forms of chymotrypsin, π and δ, from experiments on the kinetics of the various processes (69). These forms differed from chymotrypsinogen only by the splitting of one and two peptide bonds, respectively. Recently, Gladner and Neurath have shown these results to be consistent with the opening, at different points, of a cyclic cross-linked polypeptide chain (45). They suggested that chymotrypsinogen, of molecular weight 23,000 (129), had a ring structure with internal disulfide bridges between chains. Activation to π-chymotrypsin is accompanied by the splitting of one peptide bond to open the ring. Splitting of a second bond at a different point on the ring yields δ-chymotrypsin. Alternatively, other peptide bonds in π-chymotrypsin may be split, with the loss of a basic peptide to give α-chymotrypsin.

Differences in amino acid composition of corresponding proteins from different species are well known (150, 154). Recent work on the amino acid composition

of beef, pork, and sheep insulins, however, presents an interesting example of microheterogeneity among corresponding proteins from different species. Whereas insulins from different sources are indistinguishable immunologically (158), by crystal form (130, 131), by solubility criteria (130) (see, however, Lens and Evertzen (42)), by isoelectric point (130), or by physiological activity (92, 131), they differ perceptibly in amino acid composition (49, 52, 123). Thus, beef and pork insulins differ by one residue each of threonine, alanine, valine, and isoleucine. Sheep and beef insulins differ by one residue of serine and glycine. Clearly, the molecular weight and the sequence of amino acid residues in the polypeptide chains of insulin must vary among species.

G. Amino acid sequence

As yet, the complete amino acid sequences in the polypeptide chains are known for only one protein (125, 126) and therefore the experimental basis for generalization is very narrow. However, the determination of an amino acid sequence for the chains of beef insulin, which is consistent with all peptides found, has been used to infer that a unique sequence exists for each protein (6). The validity of this argument has been questioned and the necessarily Procrustean nature of present methods emphasized (150). Sanger's sequence for beef insulin has been partially confirmed (88), and preliminary results on lysozyme also tend to support the assumption of a unique sequence (39, 53, 72, 127, 145). The presence of a single N terminal amino acid residue in ovomucoid also tends to support the assumption (110, 152), but recent results on the serum albumins (31, 100, 153) and the γ-globulins of different species (101, 117, 119) are contradictory. Future work must decide whether different amino acid sequences within the polypeptide chains of a given protein contribute to microheterogeneity.

H. Miscellaneous

The lability of proteins is attributed to their ability to pass from a small number of specific configurations to many less well defined states (103). Because of the known complexity of proteins, reversible changes of this sort are *a priori* evidence for microheterogeneity within a preparation. Consequently, each instance of reversible denaturation (for references to examples see Neurath and Schwert (105)) is presumptive evidence for the microheterogeneity of that protein, at least under some conditions. Other recent examples of minor modifications of a protein are the following. Insulin exposed to dilute acids (e.g., 0.1 N acetic) undergoes a minor irreversible alteration which affects its solubility but not its biological activity (91). Deutsch found small differences in analytical data, activity, and conditions for crystallization among six crystalline horse erythrocyte catalase preparations; he believed these differences to be due to slight modifications of the preparative method (34). Ricin, a protein from the castor bean, apparently exists in two forms which are indistinguishable electrophoretically, ultracentrifugally, and immunologically but differ sharply in toxicity (75). If this interpretation of the experiments is correct, a relatively small difference between the physical properties of the two molecules must be responsible for or at least accompanied by a profound difference in their biological effect.

IV. CONCLUSIONS

The results of studies described in the foregoing sections have been summarized in table 1. With the possible exception of some globulins, the preparations listed were carefully prepared, highly purified products. Moreover, present methods of examination cause little, if any, degradation during characterization. Nonetheless, with two possible exceptions, all preparations were shown to be heterogeneous by one or more criteria. Only one test was applied to each of the samples of human oxyhemoglobin and chymotrypsinogen α, and although the test in each case was a searching one, there is independent evidence in the table that heterogeneity may have been missed. The data, therefore, provide a wide base for the conclusion that all protein preparations examined to date are microheterogeneous at best. In many cases, the heterogeneity may be gross.

Differences between individual molecules of preparations present a variety of forms. Differences in molecular weight (20), charge density (5), composition (113), molecular configuration (109) (the evidence for this is indirect), solubility (50), or biological activity (104) have been reported. However, more extensive studies on the same sample of a carefully purified protein by all possible methods of testing homogeneity would be rewarding. In particular, possible differences between corresponding proteins from different tissues of the same organism, and especially within preparations from the same tissue, should be borne in mind.

Since microheterogeneity is exhibited within preparations, differences between them are to be expected and have been found. The average molecular weight (20), charge density (5), and chromatographic characteristics (143) of different preparations of the same protein vary in minor but perceptible ways. This variation occurs even when the samples are prepared successively by the same worker, using the same method on similar types of starting material. The extent of differences due to random variations of the same method, as well as those due to different methods of preparation, need to be investigated more carefully.

As yet, the discrete or continuous character of the distribution function for a property of the molecules of a preparation usually cannot be decided with certainty. For some properties, such as the molecular weight or the electrokinetic aspects of a molecule, the function is probably continuous to a good approximation. For others, like primary amide content, the function can scarcely be other than discrete. In some proteins, causes of microheterogeneity are correlated (e.g., ovalbumin), but since the microheterogeneity due to any one of many properties may be superimposed upon that due to some of the remainder, a number of closely related forms of a given molecule is possible. This number of possibilities becomes very large whenever properties sensitive to small changes in configuration are included. The foregoing data indicate that many of these possibilities are actually realized.

In summary, therefore, experimental evidence makes untenable the notion that all molecules of a protein carefully prepared by present methods are identical. Irrespective of future developments, all present protein preparations must be regarded as populations of more or less closely related individuals, not as col-

TABLE 1

Summary of evidence of microheterogeneity of Proteins

Protein	Crystallinity	Sample Homogeneous by	Sample Heterogeneous by	References
Bovine plasma albumin....	+		Reversible boundary spreading	(3)
	+		Boundary steady state	(64)
Human plasma albumin....	−		Diffusion	(20)
	+		Amino acid composition	(12)
	+		Amino acid composition	(66)
Horse serum albumin.....	+	Immunology	Solubility; carbohydrate content of fractions	(58, 59, 60, 74, 137)
Human γ-globulin.....	−		Sedimentation coefficient distribution	(15)
	−	Immunology	Electrophoresis; ultracentrifuge	(81)
	−	Immunology	Electrophoresis; ultracentrifuge	(77)
	−		Immunology; electrophoresis; ultracentrifuge	(25)
Horse γ-globulin.....	−		Sedimentation coefficient distribution	(164)
β-Lactoglobulin.....	+	Ultracentrifuge	Diffusion	(19)
	+		Electrophoresis; solubility	(116)
Clostridium botulinum type A toxin.....	+	Electrophoresis; immunology	Boundary spreading in ultracentrifuge	(120, 121)
Ovalbumin.....	+		Reversible boundary spreading	(4)
	+		Electrophoresis; phosphate content	(113)
	+	Diffusion; ultracentrifuge	Electrophoresis; immunology	(26)
Ribonuclease.....	+		Reversible boundary spreading	(4)
	+		Chromatography	(62, 63, 99)
Lysozyme.....	+		Reversible boundary spreading	(4)
	+		Electrophoresis; chromatography	(143)

TABLE 1—*Concluded*

Protein	Crystallinity	Sample Homogeneous by	Sample Heterogeneous by	References
Lysozyme—*continued*	+	Ultracentrifuge	Immunology; diffusion; electrophoresis	(161)
Conalbumin	−	Electrophoresis; ultracentrifuge	Immunology	(26)
Ricin	+		Toxicity	(75)
Chymotrypsin	+		Reversible boundary spreading	(4)
Chymotrypsinogen α	+		Reversible boundary spreading	(4)
	+	Chromatography		(61)
Pepsin	+		Solubility	(5)
			Boundary steady state	(32)
Insulin	+	Solubility	Counter-current distribution	(43, 50)
Ox heart muscle lactic dehydrogenase	+	Ultracentrifuge	Solubility; electrophoresis	(104)
Lactoperoxidase	+		Electrophoresis; optical absorption	(115)
Human oxyhemoglobin	−	Boundary steady state		(64)
Human hemoglobin	−		Electrophoresis	(109)
			Solubility	(68)
Yeast glyceraldehyde-3-phosphate dehydrogenase	−	Immunology Ultracentrifuge	Solubility Electrophoresis	(84)
Cow heart cytochrome c	−		Chromatography	(108)

lections of entities with indistinguishable properties. Because of the known complexity and lability of these biological high polymers, this conclusion is not surprising (54, 142). However, the gain in simplicity afforded by the assumption that all molecules of a carefully prepared sample are equivalent, particu-

larly for those applications which are not sensitive to small differences, has diverted attention from former suggestions.

If the microheterogeneity (at least!) of all present samples of any protein is accepted, three causes can be distinguished. Part or all of the heterogeneity may arise from (*a*) distinctly different protein contaminants, (*b*) derivatives produced by the preparative procedure, or (*c*) normal variations between molecules of the protein within the cell.

These three possibilities will be considered in turn. The first, contamination by a distinctly different protein, usually called an impurity, presupposes a valid method or methods to detect it and therefore presents no problem, in principle. The microheterogeneity contributed from this source may be reduced to an acceptable level by fractionation procedures.

The second possibility, i.e., that proteins may be altered perceptibly by techniques of isolation, fractionation, storage, or examination, has been known for a long time (114). Several examples have been presented in the foregoing sections. For instance, slight differences in methods of preparation (91), obscure transformations during storage in the solid state (143), minor effects of pH or chemical reagents (61), and the ubiquitous effects of enzymes (95) have been shown to modify some or all of the molecules of protein preparations. There is a high probability therefore that some part of the microheterogeneity present in all protein preparations comes from this source. As yet no quantitative estimate of this fraction can be given for any protein.

However, admitting the effects of isolation and examination, the greater part of the observed microheterogeneity in some samples must be assigned to an inherent variation in the properties of individual molecules of the same protein (see page 706). This conclusion rests upon the experimental demonstration that the same degree of microheterogeneity observed in purified crystalline samples of some proteins is present in material which must be reasonably close to the native state. Perlmann observed that the A_1 and A_2 components of ovalbumin, containing two and one phosphorus atoms per mole, respectively, were present in diluted egg white in much the same proportions as in the crystalline ovalbumin produced by ammonium sulfate precipitation (113). Since degradation during dilution and examination by electrophoresis seems improbable, we must conclude that microheterogeneity due to the mixture of these two forms of the protein exists in "native" ovalbumin. Similarly, Wetter and Deutsch found excellent agreement between the curves of the quantitative precipitation reaction of five- and seven-times-recrystallized lysozyme and of egg white with rabbit antilysozyme γ-globulin (160). They therefore suggested that the antigenic components of lysozyme are present in the same ratio in egg white and the crystalline state. Similarly, Martin and Porter, using techniques which minimized proteolysis, found that ribonuclease from beef pancreas which had been frozen in solid carbon dioxide immediately after slaughter gave much the same distribution of components by chromatography as did crystalline material (99). They concluded that ribonuclease preëxists in two distinct forms in the gland. Their observations have been confirmed by Hirs, Moore, and Stein (62). Finally, extensive parallel im-

munochemical and toxicity assays of crude extracts of castor beans, compared with toxic crystalline ricin, have indicated that the raw bean contains two forms of the protein which are electrophoretically, ultracentrifugally, and immunologically equivalent, but that one of them is acutely toxic while the other is not (75). This conclusion was confirmed by a decrease in the proportion of toxic nitrogen to immunologically active nitrogen in the mother liquors of toxic crystalline ricin. Alternative possibilities to the existence of two forms of ricin were not consistent with the evidence, and the authors concluded that both forms must preëxist in the castor bean.

In addition, Charlwood, after isolating normal human serum albumins by electrophoresis under very mild conditions, found that a given preparation from a single patient contained more than one molecular species by diffusion measurements (20). The single, approximately Gaussian peak observed in the ultracentrifuge measurements was evidence that the differences in the hydrodynamic properties of the species were minor. All five studies therefore support the proposition that some proteins at least are microheterogeneous in the native state. It is noteworthy that the examples given above include two enzymes.

From the foregoing recent experimental evidence, the presence of perceptible minor differences between the constituent molecules of all present protein preparations is highly probable. Moreover, a substantial fraction of these minor differences may be assumed to exist in the "native" state of many, if not all, proteins. Acceptance of these conclusions raises some important questions about the correct description of present preparations and of native proteins. As stated by Pirie, components of a sample which share a common property (or properties) but which are chemically or physically distinguishable may be regarded as individuals (114). The older name is adopted for the group and each individual component given a particular name. Provided the distributions within the group are discrete, there is no objection to the logic and usefulness of this procedure. However, we have seen that there is considerable doubt about the discrete character of the distribution function for some properties of proteins. If the function is continuous, the system breaks down for this property unless supplemented by an arbitrary decision, even though molecules or groups of molecules can be distinguished physically or chemically. Particularly because of limitations in present experimental methods, it seems more correct to describe a native protein, not in terms of a finite number of definite chemical entities, but as a population of closely related individuals which may differ either discretely or continuously in a number of properties. The standard deviation of such a population varies with the property and protein considered. Fractionation may separate the population into two or more fractions which are distinguishable by some property or properties (for instance, serum albumin containing mercaptalbumin), but each of these fractions also represents a population of variable though closely related members. Thus, though fractionation may reduce the original standard deviation of a particular property in a population to zero in favorable instances, other distributions are not necessarily affected. Consequently, from this point of view a unique structure and configuration for any particular protein is improbable.

There is only a general similarity between members of a family of closely related molecules which may vary simultaneously in a large number of minor ways. The designation "same protein" refers to a population which has been shown to possess (or is inferred to have) an approximately similar range of properties.

The advantage of the viewpoint advocated above is that it gives an accurate description of any present protein preparation and of at least some "native" proteins. It does not assume a definite and perhaps unjustified individuality for components of a preparation.

The reader familiar with biological problems will recognize the similarity between the present viewpoint on proteins and that of many modern biologists on the problem of species (8, 37, 67). This similarity is not accidental, since both arise from the attempt to classify closely related complex entities the properties of which are not sharply defined. In both problems further study will permit better characterization of the populations involved, but present methods are unlikely to make separation of closely related samples easier.

V. IMPLICATIONS OF MICROHETEROGENEITY OF PROTEINS

If the idea of a normal natural microheterogeneity within a population of protein molecules is accepted, there are some general implications for future research which are worth examining. Experimental results from investigations of the structure or configuration of proteins must be interpreted always in a statistical, not a definite, sense. The most that can be said for any given result or deduction is that it represents the most probable or modal conclusion, not the only one. Eventually, an accurate description of a property of a preparation will, in general, include an estimate of the standard deviation. It is clear that the possibility of a number of minor variations on the main theme, as suggested above, complicates considerably an already extremely complex problem.

Furthermore, the idea of microheterogeneity places some limitations upon the type of mechanism whereby these large molecules may be synthesized. It has been established that in some native proteins a substantial fraction of the molecules differ detectably from the remainder. The presence of this fraction therefore excludes from consideration any mechanism of synthesis which depends very strongly upon identity between an original macromolecule and its replica (71). Confirmation of microheterogeneity in all native proteins would therefore make the applicability of such mechanisms much less probable, unless their dependence upon identity is less rigid than has been indicated. In brief, a mechanism for synthesizing similar but not necessarily identical molecules may be all that is required.

In addition, the simple concept of identical active centers within or between protein molecules in enzymic catalysis may have to be modified. If an enzyme protein varies (as some have been shown to do), then in general the effectiveness of the catalytic site must vary also, unless interaction between the points introducing microheterogeneity and the catalytic site is negligible. This follows from present notions of the effect of changes in charge density, composition, or detailed molecular configuration on the activity of enzymes. Once again, the only valid description is one in terms of an average, most probable effect. Determina-

tion of the distribution functions of enzymic activity or related properties as a function of small changes in molecular parameters must be one of the major problems for enzyme chemists of the future.

VI. SUMMARY

The recent experimental evidence for microheterogeneity in highly purified protein preparations has been reviewed. This evidence indicates that the molecules of even carefully prepared samples of a single protein from a single source may differ perceptibly in such diverse properties as molecular weight, charge density, composition, molecular shape, molecular configuration, solubility, and biological activity. The conclusion is drawn that all protein preparations prepared so far represent populations of closely related members of a family, not collections of identical molecules. Furthermore, from more limited evidence the inference is drawn that proteins in their native state are microheterogeneous. The implications of these conclusions for interpretation of experiments with this class of substances are indicated.

The authors wish to thank Drs. P. M. Laughton, P. A. Charlwood, D. R. Whitaker, and E. O. Hughes for reading and criticizing the manuscript of this paper.

VII. REFERENCES

(1) AKELEY, D. F., AND GOSTING, L. J.: J. Am. Chem. Soc. **75**, 5685 (1953).
(2) ALBERTY, R. A.: J. Chem. Educ. **25**, 426, 619 (1948).
(3) ALBERTY, R. A.: J. Am. Chem. Soc. **70**, 1675 (1948).
(4) ALBERTY, R. A., ANDERSON, E. A., AND WILLIAMS, J. W.: J. Phys. & Colloid Chem. **52**, 217 (1948).
(5) ANDERSON, E. A., AND ALBERTY, R. A.: J. Phys. & Colloid Chem. **52**, 1345 (1948).
(6) BAILEY, K., AND SANGER, F.: Ann. Rev. Biochem. **20**, 103 (1951).
(7) BALDWIN, R. L., LAUGHTON, P. M., AND ALBERTY, R. A.: J. Phys. & Colloid Chem. **55**, 111 (1951).
(8) BLAKE, S. T.: Australian J. Science **11**, 119 (1949).
(9) BOARDMAN, N. K., AND PARTRIDGE, S. M.: Nature **171**, 208 (1953).
(10) BOMAN, H. G.: Acta Chem. Scand. **5**, 1311 (1951).
(11) BOYD, W. C.: *Fundamentals of Immunology*, 2nd edition. Interscience Publishers, Inc., New York (1947).
(12) BRAND, E.: Ann. N. Y. Acad. Sci. **47**, 187 (1946).
(13) BROWN, G. L.: Biochem. J. **51**, 569 (1952).
(14) BROWN, R. A., AND CANN, J. R.: J. Phys. & Colloid Chem. **54**, 364 (1950).
(15) CANN, J. R.: J. Am. Chem. Soc. **75**, 4213 (1953).
(16) CANN, J. R., BROWN, R. A., AND KIRKWOOD, J. G.: J. Biol. Chem. **181**, 161 (1949).
(17) CANN, J. R., BROWN, R. A., AND KIRKWOOD, J. G.: J. Am. Chem. Soc. **71**, 1609 (1949).
(18) CANN, J. R., AND KIRKWOOD, J. G.: Cold Spring Harbor Symposia Quant.Biol. **14**, 9 (1949).
(19) CECIL, R., AND OGSTON, A. G.: Biochem. J. **44**, 33 (1949).
(20) CHARLWOOD, P. A.: Biochem. J. **51**, 113 (1952).
(21) CHARLWOOD, P. A.: Biochem. J. **52**, 279 (1952).
(22) CHARLWOOD, P. A.: Biochem. J. **56**, 259 (1954).
(23) CHARLWOOD, P. A.: J. Phys. Chem. **57**, 125 (1953).
(24) COHN, E. J., AND EDSALL, J. T.: *Proteins, Amino Acids and Peptides*, Chap. 20. Reinhold Publishing Corporation, New York (1943).
(25) COHN, M., DEUTSCH, H. F., AND WETTER, L. R.: J. Immunol. **64**, 381 (1950).

(26) COHN, M., WETTER, L. R., AND DEUTSCH, H. F.: J. Immunol. **61**, 283 (1949).
(27) COULSON, C. A., COX, J. T., OGSTON, A. G., AND PHILPOT, J. ST. L.: Proc. Roy. Soc. (London) **A192**, 382 (1948).
(28) CRAIG, L. C., GREGORY, J. D., AND BARRY, G. T.: Cold Spring Harbor Symposia Quant. Biol. **14**, 24 (1949).
(29) CREETH, J. M.: Biochem. J. **51**, 10 (1952).
(30) DAVIE, E. W., AND NEURATH, H.: Biochim. et Biophys. Acta **11**, 442 (1953).
(31) DESNUELLE, P., ROVERY, M., AND FABRE, C.: Compt. rend. **233**, 987 (1951).
(32) DESREUX, V., AND HERRIOTT, R. M.: Nature **144**, 287 (1939).
(33) DEUTSCH, H. F.: J. Biol. Chem. **185**, 377 (1950).
(34) DEUTSCH, H. F.: Acta Chem. Scand. **6**, 1516 (1952).
(35) DICKMAN, S. R., AND MONCRIEF, I. H.: Proc. Soc. Exptl. Biol. Med. **77**, 631 (1951).
(36) DISMUKES, E. B., AND ALBERTY, R. A.: J. Am. Chem. Soc. **76**, 191 (1954).
(37) DOBZHANSKY, T. G.: *Genetics and the Origin of Species*. Columbia University Press, New York (1937).
(38) EDSALL, J. T.: In *The Proteins*, edited by Hans Neurath and Kenneth Bailey, Vol. 1, Part B, p. 549. Academic Press, Inc., New York (1953).
(39) EDWARD, J. T., AND NIELSEN, S.: Chemistry & Industry **1953**, 197
(40) EEG-LARSEN, N., LINDERSTRØM-LANG, K., AND OTTESEN, M.: Arch. Biochem. **19**, 340 (1948).
(41) EYRING, H.: Anal. Chem. **20**, 98 (1948).
(42) FALCONER, J. S., AND TAYLOR, D. B.: Biochem. J. **40**, 835 (1946).
(43) FREDERICQ, E., AND NEURATH, H.: J. Am. Chem. Soc. **72**, 2684 (1950).
(44) FRUTON, J. S., AND SIMMONDS, S.: *General Biochemistry*. John Wiley and Sons, Inc., New York (1953).
(45) GLADNER, J. A., AND NEURATH, H.: J. Biol. Chem. **205**, 345 (1953).
(46) GOSTING, L. J., AND MORRIS, M. S.: J. Am. Chem. Soc. **71**, 1998 (1949).
(47) GOSTING, L. J., AND ONSAGER, L.: J. Am. Chem. Soc. **74**, 6066 (1952).
(48) GRÖNWALL, A.: Compt. rend. trav. lab. Carlsberg **24**, No. 8-11, 185 (1942).
(49) HARFENIST, E. J.: J. Am. Chem. Soc. **75**, 5528 (1953).
(50) HARFENIST, E. J., AND CRAIG, L. C.: J. Am. Chem. Soc. **74**, 3083 (1952).
(51) HARFENIST, E. J., AND CRAIG, L. C.: J. Am. Chem. Soc. **74**, 3087 (1952).
(52) HARFENIST, E. J., AND CRAIG, L. C.: J. Am. Chem. Soc. **74**, 4216 (1952).
(53) HARRIS, J. I.: J. Am. Chem. Soc. **74**, 2944 (1952).
(54) HAUROWITZ, F.: *Chemistry and Biology of Proteins*. Academic Press, Inc., New York (1950).
(55) HERRIOTT, R. M.: J. Gen. Physiol. **21**, 501 (1938).
(56) HERRIOTT, R. M.: Federation Proc. **7**, 479 (1948).
(57) HESS, E. L.: Science **113**, 709 (1951).
(58) HEWITT, L. F.: Biochem. J. **28**, 2080 (1934).
(59) HEWITT, L. F.: Biochem. J. **30**, 2229 (1936).
(60) HEWITT, L. F.: Biochem. J. **31**, 360 (1937).
(61) HIRS, C. H. W.: J. Biol. Chem. **205**, 93 (1953).
(62) HIRS, C. H. W., MOORE, S., AND STEIN, W. H.: J. Biol. Chem. **200**, 493 (1953).
(63) HIRS, C. H. W., STEIN, W. H., AND MOORE, S.: J. Am. Chem. Soc. **73**, 1893 (1951).
(64) HOCH, H.: Biochem. J. **46**, 199 (1950).
(65) HOCH, H., AND MORRIS, C. J. O. R.: Nature **156**, 234 (1945).
(66) HUGHES, W. L., JR.: Cold Spring Harbor Symposia Quant. Biol. **14**, 79 (1949).
(67) HUXLEY, JULIAN: *Evolution, the Modern Synthesis*. George Allen and Unwin, London (1942).
(68) ITANO, H. A.: Science **117**, 89 (1953).
(69) JACOBSEN, C. F.: Compt. rend. trav. lab. Carlsberg, Sér. chim. **25**, 325 (1947).
(70) JAGER, B. V., SMITH, E. L., NICKERSON, M., AND BROWN, D. M.: J. Biol. Chem. **176**, 1177 (1948).

(71) JEHLE, H.: J. Chem. Phys. **18**, 1150 (1950).
(72) JUTISZ, M., AND PÉNASSE, L.: Bull. soc. chim. biol. **34**, 480 (1952).
(73) KABAT, E. A.: J. Immunol. **47**, 513 (1943).
(74) KABAT, E. A., AND HEIDELBERGER, M.: J. Exptl. Med. **66**, 229 (1937).
(75) KABAT, E. A., HEIDELBERGER, M., AND BEZER, A. E.: J. Biol. Chem. **168**, 629 (1947).
(76) KABAT, E. A., AND MAYER, M. M.: *Experimental Immunochemistry*. Charles C. Thomas, Springfield, Illinois (1948).
(77) KABAT, E. A., AND MURRAY, J. P.: J. Biol. Chem. **182**, 251 (1950).
(78) KAMINSKA, M., AND GRABAR, P.: Bull. soc. chim. biol. **31**, 684 (1949).
(79) KEGELES, G.: J. Am. Chem. Soc. **69**, 1302 (1947).
(80) KEGELES, G., AND GOSTING, L. J.: J. Am. Chem. Soc. **69**, 2516 (1947).
(81) KENDALL, F. E.: J. Clin. Invest. **16**, 921 (1937).
(82) KLOTZ, I. M., BURKHARD, R. K., AND URQUHART, J. M.: J. Phys. Chem. **56**, 77 (1952).
(83) KREBS, E. G.: J. Biol. Chem. **200**, 471 (1953).
(84) KREBS, E. G., RAFTER, G. W., AND JUNGE, J. M.: J. Biol. Chem. **200**, 479 (1953).
(85) KUNITZ, M.: J. Gen. Physiol. **22**, 207 (1938).
(86) KUNITZ, M.: J. Gen. Physiol. **24**, 15 (1940).
(87) KUNITZ, M., AND NORTHROP, J. H.: J. Gen. Physiol. **18**, 433 (1934).
(88) LANDMANN, W. A., DRAKE, M. P., AND DILLAHA, J.: J. Am. Chem. Soc. **75**, 3638 (1953).
(89) LANDSTEINER, K.: *The Specificity of Serological Reactions*. Harvard University Press, Cambridge, Massachusetts (1947).
(90) LEMBERG, R., AND LEGGE, J. W.: *Hematin Compounds and Bile Pigments*. Interscience Publishers, Inc., New York (1949).
(91) LENS, J.: Biochim. et Biophys. Acta **2**, 76 (1948).
(92) LENS, J., AND EVERTZEN, A.: Biochim. et Biophys. Acta **8**, 332 (1952).
(93) LI, C. H.: J. Am. Chem. Soc. **68**, 2746 (1946).
(94) LI, C. H.: In *Amino Acids and Proteins*, edited by D. M. Greenberg. Charles C. Thomas, Springfield, Illinois (1951).
(95) LINDERSTRØM-LANG, K.: Bull. soc. chim. biol. **35**, 100 (1953).
(96) LOEB, J.: *Proteins and the Theory of Colloidal Behavior*, 2nd edition. McGraw-Hill Book Company, Inc., New York (1924).
(97) LONGSWORTH, L. G.: J. Am. Chem. Soc. **69**, 2510 (1947).
(98) LONGSWORTH, L. G., CANNAN, R. K., AND MACINNES, D. A.: J. Am. Chem. Soc. **62**, 2580 (1940).
(99) MARTIN, A. J. P., AND PORTER, R. R.: Biochem. J. **49**, 215 (1951).
(100) MCCLURE, L. E., SCHIELER, L., AND DUNN, M. S.: J. Am. Chem. Soc. **75**, 1980 (1953).
(101) MCFADDEN, M. L., AND SMITH, E. L.: J. Am. Chem. Soc. **75**, 2784 (1953).
(102) MCMEEKIN, T. L., POLIS, B. D., DELLA MONICA, E. S., AND CUSTER, J. H.: J. Am. Chem. Soc. **70**, 881 (1948).
(103) MIRSKY, A. E., AND PAULING, L.: Proc. Natl. Acad. Sci. U. S. **22**, 439 (1936).
(104) NEILANDS, J. B.: J. Biol. Chem. **199**, 373 (1952).
(105) NEURATH, HANS, AND SCHWERT, G. W.: Chem. Revs. **46**, 69 (1950).
(106) NORTHROP, J. H.: *Crystalline Enzymes*. Columbia University Press, New York (1939).
(107) OTTESEN, M., AND WOLLENBERGER, A.: Nature **170**, 801 (1952).
(108) PALEUS, S., AND NIELANDS, J. B.: Acta Chem. Scand. **4**, 1024 (1950).
(109) PAULING, L., ITANO, H. A., SINGER, S. J., AND WELLS, I. C.: Science **110**, 543 (1949).
(110) PÉNASSE, L., JUTISZ, M., FROMAGEOT, C., AND FRAENKEL-CONRAT, H.: Biochim. et Biophys. Acta **9**, 551 (1952).
(111) PERLMANN, G. E.: J. Am. Chem. Soc. **71**, 1146 (1949).
(112) PERLMANN, G. E.: J. Gen. Physiol. **35**, 711 (1952).
(113) PERLMANN, G. E.: Discussions Faraday Soc. **1953**, No. 13, 67.
(114) PIRIE, N. W.: Biol. Rev. **15**, 377 (1940).
(115) POLIS, B. D., AND SCHMUKLER, H. W.: J. Biol. Chem. **201**, 475 (1953).

(116) POLIS, B. D., SCHMUKLER, H. W., CUSTER, J. H., AND MCMEEKIN, T. L.: J. Am. Chem. Soc. **72**, 4965 (1950).
(117) PORTER, R. R.: Biochem. J. **46**, 473 (1950).
(118) PORTER, R. R.: Biochem. J. **53**, 320 (1953).
(119) PUTNAM, F. W.: J. Am. Chem. Soc. **75**, 2785 (1953).
(120) PUTNAM, F. W., LAMANNA, C., AND SHARP, D. G.: J. Biol. Chem. **165**, 735 (1946).
(121) PUTNAM, F. W., LAMANNA, C., AND SHARP, D. G.: J. Biol. Chem. **176**, 401 (1948).
(122) ROBERTSON, T. BRAILSFORD: *The Physical Chemistry of the Proteins*. Longmans, Green and Company, New York (1920).
(123) SANGER, F.: Nature **164**, 529 (1949).
(124) SANGER, F.: Advances in Protein Chem. **7**, 1 (1952).
(125) SANGER, F., AND THOMPSON, E. O. P.: Biochem. J. **53**, 353 (1953).
(126) SANGER, F., AND TUPPY, H.: Biochem. J. **49**, 463 (1951).
(127) SCHROEDER, W. A.: J. Am. Chem. Soc. **74**, 281 (1952).
(128) SCHROEDER, W. A., KAY, L. M., AND WELLS, I. C.: J. Biol. Chem. **187**, 221 (1950).
(129) SCHWERT, G. W.: J. Biol. Chem. **190**, 799 (1951).
(130) SCOTT, D. A.: J. Biol. Chem. **92**, 281 (1931).
(131) SCOTT, D. A., AND FISHER, A. M.: Trans. Roy. Soc. Canada V, **34**, 137 (1940).
(132) SEASTONE, C. V., AND HERRIOTT, R. M.: J. Gen. Physiol. **20**, 797 (1937).
(133) SHARP, D. G., HEBB, M. H., TAYLOR, A. R., AND BEARD, J. W.: J. Biol. Chem. **142**, 217 (1942).
(134) SHARP, D. G., TAYLOR, A. R., BEARD, D., AND BEARD, J. W.: J. Biol. Chem. **142**, 193 (1942).
(135) SOBER, H. A., KEGELES, G., AND GUTTER, F. J.: J. Am. Chem. Soc. **74**, 2734 (1952).
(136) SØRENSEN, S. P. L.: Compt. rend. trav. lab. Carlsberg **18**, 45 (1930).
(137) SØRENSEN, S. P. L., AND SØRENSEN, M.: Compt. rend. trav. lab. Carlsberg **19**, No. 11 (1933).
(138) STEIN, W. H., AND MOORE, S.: J. Biol. Chem. **178**, 79 (1949).
(139) STEINBERG, D.: J. Am. Chem. Soc. **75**, 4875 (1953).
(140) SVEDBERG, T., AND PEDERSEN, K. O.: *The Ultracentrifuge*. Clarendon Press, Oxford (1940).
(141) SYNGE, R. L. M.: Chem. Revs. **32**, 135 (1943).
(142) SYNGE, R. L. M.: Quart. Revs. (London) **3**, 245 (1949).
(143) TALLAN, H. H., AND STEIN, W. H.: J. Biol. Chem. **200**, 507 (1953).
(144) TAYLOR, J. F.: In *The Proteins*, edited by Hans Neurath and Kenneth Bailey, Vol. 1, Part A, p. 1. Academic Press, Inc., New York (1953).
(145) THOMPSON, A. R.: Nature **169**, 495 (1952).
(146) TIMASHEFF, S. N., BROWN, R. A., AND KIRKWOOD, J. G.: J. Am. Chem. Soc. **75**, 3121 (1953).
(147) TIMASHEFF, S. N., AND KIRKWOOD, J. G.: J. Am. Chem. Soc. **75**, 3124 (1953).
(148) TISELIUS, A.: Biochem. J. **31**, 1464 (1937).
(149) TISELIUS, A.: Trans. Faraday Soc. **33**, 524 (1937).
(150) TRISTAM, G. R.: In *The Proteins*, edited by Hans Neurath and Kenneth Bailey, Vol. 1, Part A, p. 181. Academic Press, Inc., New York (1953).
(151) TRISTAM, G. R.: Reference 150, p. 205.
(152) TURNER, R. A., AND SHMERZLER, G.: Biochim. et Biophys. Acta **11**, 586 (1953).
(153) VAN VUNAKIS, H., AND BRAND, E.: Abstracts of Papers Presented at the 119th Meeting of the American Chemical Society, Boston, Massachusetts, April, 1951, p. 28c.
(154) VELICK, S. F., AND UDENFREND, S.: J. Biol. Chem. **203**, 563, 575 (1953).
(155) VICKERY, H. B.: Ann. N. Y. Acad. Sci. **47**, 63 (1946).
(156) WAGMAN, J., AND BATEMAN, J. B.: Arch. Biochem. Biophysics **31**, 424 (1951).
(157) WALES, M.: J. Phys. & Colloid Chem. **52**, 983 (1948).
(158) WASSERMAN, P., AND MIRSKY, I. A.: Endocrinology **31**, 115 (1942).
(159) WETTER, L. R., COHN, M., AND DEUTSCH, H. F.: J. Immunol. **69**, 109 (1952).

(160) WETTER, L. R., AND DEUTSCH, H. F.: Arch. Biochem. **28**, 399 (1950).
(161) WETTER, L. R., AND DEUTSCH, H. F.: J. Biol. Chem. **192**, 237 (1951).
(162) WHELAND, G. W.: *Advanced Organic Chemistry*, 2nd edition. John Wiley and Sons, Inc., New York (1949).
(163) WILLIAMS, J. W.: Federation Proc. **7**, 474 (1948).
(164) WILLIAMS, J. W., BALDWIN, R. L., SAUNDERS, W. M., AND SQUIRE, P. G.: J. Am. Chem. Soc. **74**, 1542 (1952).