Symposium on Foods:

Proteins and Their Reactions

The third in a series of symposia on foods held at Oregon State University

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Preface

The object of this book is to present for use by scientists interested in foods or proteins, or both, the excellent papers presented by 18 outstanding scientists at the *Symposium on Foods: Proteins and Their Reactions.* The book can thus serve as an authoritative up-to-date reference on *The Structure and Properties of Proteins, Protein Interactions and Degradation, Major Protein Systems and Factors Affecting Them, and The Biological Effects of Protein Interactions.*

This book should add to the value of the Symposium beyond that derived from bringing together scientists to discuss among themselves a subject of great interest and concern. It should serve as a reminder and supplement the knowledge retained or noted by each of the 275 persons who attended the Symposium. Additionally it can be of value to those who were unable to be present. In toto, we hope the publication of the book will be stimulating to those engaged in expanding our knowledge of foods, particularly the protein components.

The Symposium on Foods: Enzymes, held in 1959, *The Symposium on Foods: Oxidative Deterioration of Lipids,* held in 1961, and *The Symposium on Foods: Proteins and Their Reactions,* held in 1963, were arranged and conducted by the Department of Food Science and Technology, Oregon State University, Corvallis, Oregon. All of these have had as their primary purpose the bringing together of scientists who are engaged in research which is not necessarily related to foods and scientists who are conducting research on food systems. This affords an opportunity to form a new bridge of communication and develop a greater appreciation and usefulness of the contributions of each. Furthermore, the Symposia have been programmed to bring to light for greater research emphasis those problems believed to be given too little attention. For example, at the Symposium reported in this book, attention was drawn to the need for more knowledge of the role of foods in allergies, immunity, and the least explored aspects of nutrition.

We are deeply indebted to the authors who have so expertly covered their assigned subjects by reviewing the literature, presenting results of their own research, and later editing the transcribed recorded discussions of their respective papers which are included in this book.

Thanks are also due the members of the Department of Food Science and Technology and Mr. Roy E. Moser, Extension Food Processing Specialist, for their contributions in working out the details of the meeting and to Drs. M. W. Montgomery and G. A. Richardson in the preparation of the index of this book.

We are indeed indebted to the Division of Environmental Engineering and Food Protection, Bureau of State Services, U. S. Public Health Service, for Grant No. EF 00478-01 to cover the expenses of the Symposium and defray some publication costs. Together with the publisher's willingness to forego the usual profit, the grant permits this book to be purchased at a much reduced price which should increase the benefits of the Symposium.

January 1, 1964

H. W. SCHULTZ
A. F. ANGLEMIER

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R. David Cole

Personal Perspectives in the Practice of Protein Chemistry

INTRODUCTION

I feel very humble giving the introductory presentation in this Symposium, particularly since it is part of a series on food and my studies of proteins as food are of a knife and fork nature. In spite of being awed by the task, however, it is, as always, a pleasure to talk about proteins. When the opportunity to give this talk was presented, it was suggested that I give a philosophical talk related to basic protein chemistry, rather than give a comprehensive review. In other words, I can do just what is fun; I sincerely hope it will be profitable to you as well.

What I plan to do here is to present the background and philosophy of my own laboratory and to indicate occasionally how it bears on the subjects to be discussed at this Symposium. The talk will be personal, not out of conceit but out of the humble realization that I am not capable of expressing general philosophy but only of presenting my own. On this basis, it is hoped to express the kinds of interests and excitements which may be looked for in the future of protein chemistry and in this Symposium. While all of us here have different specialties, I trust that my interests overlap those of most people here, sufficiently for me to speak frequently for most of us and occasionally for all. I am sure that I speak for all in thanking Dr. Schultz and Dr. Anglemier and, perhaps, their unseen assistants for organizing such a promising Symposium.

As previously stated, rather than give a comprehensive review, I would like to show how the thinking in my laboratory has developed, how various key works in the literature have influenced our thinking and, finally, what we anticipate for our future in protein chemistry. I must emphasize at this point that it is our *thinking* that I shall present, and so the ideas discussed are sometimes supported by firm evidence, sometimes by preliminary results, and occasionally by nothing more than my own wild optimism. Many of the results are unpublished, but I will try to make it clear which they are. I also want to emphasize that at various times I shall be speaking for different members of my laboratory, including Dr. Michael Raftery, Dr. David Lavrin, Miss Leticia Mendiola, Miss Jean Howard, and Messrs. J. M. Kinkade, Brian Reid, William Benisek, Bryce Plapp, John Hodsdon, and T. R. Hopkins.

My interest in biochemistry was awakened by two endocrinologists, Dr. W. R. Lyons and Dr. C. H. Li, and so it ought not to be surprising that my first research was on the isolation and characterization of a

pituitary hormone, prolactin. A new simplified method of isolation for prolactin, based on countercurrent distribution (Cole and Li 1955), was developed. The material obtained using this procedure had a biological potency (35 IU/mg.) at least equal to the highest previously reported value and was quite free from all likely contaminants. It was surprising, then, to find that extensive countercurrent distribution (over 1,000 transfers) resolved this material into three components (Cole and Li 1958). Furthermore, the same three components could be resolved by extended, preparative zone electrophoresis (Cole and Li 1959). These three components all proved to have equal biological activity within the usual experimental limits of $\pm$15–20 per cent. At this point in my considerations, I was very strongly influenced by a superb review on microheterogeneity by Colvin *et al.* (1954).

I might say parenthetically that I notice in speaking to my students that I tend to divide all papers into three groups—old papers, classical papers, and recent papers. The distinction between old papers and recent ones is simply whether they were published before or after I entered biochemistry. Classical papers, then, are old papers which are useful to me. A few papers transcend these categories and become modern classics; such was the review on microheterogeneity by Colvin *et al.* (1954).

While theirs was not the first reference to microheterogeneity (Synge 1943), it was very timely and stimulating. We were faced with three very similar kinds of prolactin, and such a system seemed to fall within their definition (Colvin *et al.* 1954) of chemical microheterogeneity (the material seemed quite homogeneous biologically). Colvin *et al.* suggested that microheterogeneity might exist as such in the parent tissue, or that it might be generated during isolation by enzymatic or nonenzymatic means. Regardless of the means of formation, the differences among components of a microheterogeneous system might be due to differences in amino acid composition, amino acid sequence, physical conformation, or state of aggregation. These suggestions stimulated our thinking about the prolactin system and still form the basis for much of our thinking today. They are the subjects, then, that I would like to consider, not just as they apply to prolactin but as they affect my views on the progress and future of protein chemistry. I might add that I am looking forward to Dr. Colvin's presentation later for further stimulation.

DOES MICROHETEROGENEITY EXIST IN THE PARENT TISSUE?

This question is obviously a profound one in biology, since an affirmative answer would have many implications in considering protein biosynthesis, metabolic control systems, and evolutionary processes.

The answer to this question has often been sought by demonstrating that some systems of microheterogeneity are definitely generated during the isolation of the particular proteins in question. While there must be many examples of this, among the most striking of the early demonstrations of artificially produced microheterogeneity was the work of Dickinson (1956) (and later Chrambach and Carpenter 1960; and Sundby 1962), who considered acid catalyzed transformation (desamidation) in an attempt to explain the microheterogeneity previously demonstrated by Harfenist and Craig (1952) using countercurrent distribution, by Timasheff *et al.* (1953) using electrophoresis-convection, and by Carpenter and Hess (1956) using partition chromatography.

One other dramatic example of protein transformation during isolation may be taken from the work of Tallan and Stein (1953). These workers chromatographed lysozyme on an ion-exchange resin and found that, while isoelectric lysozyme and lysozyme chloride were stable during storage and chromatography, lysozyme carbonate formed a mixture of three enzymatically active forms upon storage and presumably in solution.

This kind of microheterogeneity formed *in vitro* is, for the most part, simply a nuisance and has probably plagued many investigators. It can frequently be controlled by keeping the temperature low, controlling metal ion or oxygen concentrations or, on the one hand, using extreme pH's to combat enzymatic transformations and, on the other hand, avoiding extreme pH's to combat chemical transformations. There is some general need in the field of protein purification for better ways to monitor and control this degradation.

As for the monitoring of samples during preparation, there are any number of techniques available. A very recent one, which might be singled out, is that of Carpenter and Hayes (1963) which involves electrophoresis on strips of cellulose acetate. This technique is unusually convenient and quick, and it possesses a high resolving power.

In our laboratory, we happened upon a rather special instance of maintaining a neutral pH while preventing enzymatic degradation. It was hoped that trypsin could be chromatographed on the ion-exchange resin IRC-50, but it was found that in the pH range suitable for chromatography, auto-digestion of the trypsin was severe. Harris (1956) had recently shown, however, that trypsin was reversibly inactivated in 8 M urea solutions. In the light of this finding, trypsin was chromatographed in buffers containing urea, and it was demonstrated that autolysis could be arrested completely so that good chromatography could be obtained (Cole and Kinkade 1961). As will become evident later, urea or similar additives may be much more generally applicable to proteins than presently thought.

The form of microheterogeneity, which is of more profound philosophical interest, is that which may exist in tissues or cells *in vivo*. Determining the state of proteins within a cell is probably as difficult to do as it is desirable. Clearly, what is desired is a quantitative balance sheet for any tissue or cell for all proteins in all their individual forms. Although this once seemed an impossible task, I have been quite impressed by progress in just this direction. Keller *et al.* (1958) have chromatographed whole pancreatic juice to give a complete analysis of the various hydrolytic enzymes of that tissue (fluid). An elegant experiment which was complementary to this work has been reported by Greene *et al.* (1963) who have chromatographed a homogenate of zymogen particles isolated from the pancreas and showed that these particles had the same pattern of proteins as the pancreatic fluid. Having a picture of the proteins excreted by the pancreas exocrine cells, and then finding the picture of the proteins as they are stored within the cells, Keller *et al.* (1963) have gone a step deeper to observe the quantitative pattern of proteins in the ribosomes. Such a series of experiments excites one with the possibility of knowing the whole history of these proteins and their interrelations *in vivo*. How interesting it would be to have such detailed, quantitative histories of several sets of proteins.

A similar excellent work was that of Dr. Feeney who chromatographed the proteins in whole egg white (Rhodes *et al.* 1958). These magnificent achievements could typify an experimental system which could advance greatly our knowledge of genetic replication, differentiation, metabolic control, growth processes and, perhaps, many other processes.

The approach used by the above workers was to chromatograph the proteins on a single column of cellulose ion-exchange resin using a very wide range of eluting conditions. The cellulose resins of Sober and Petersen (1954) are ideal for this purpose since they are so generally applicable to all proteins. Indeed, it was the work of these authors (Sober *et al.* 1956) on analyzing whole blood serum which is the first example of the kind of total analysis under discussion. The tissues for which they have been used, however, are probably simpler than most, and since the resolving power is less when the chromatography requires buffer changes than when a single buffer is used throughout the elution procedure (Moore and Stein 1956), this very general approach to the quantitative analysis for proteins may be too limited for many tissues. For this reason, one might well turn to less general, that is, more selective chromatographic systems. Actually, the most effective method would probably be a first step of the general type of chromatography to isolate groups of closely related proteins, followed by a second step using a

rigorously selective chromatography to separate closely related proteins and members of microheterogeneous systems.

In any case, it is important to get quantitative, over-all pictures of the levels of the soluble proteins of many particular tissues. For example, how do the over-all protein patterns change with biological condition in such systems as milk, seeds, etc. It would seem likely that a full understanding of such problems as morphogenesis will not be possible without such detailed quantitative data. In beginning this sort of program, in a study of the mechanism of action of prolactin in my laboratory, we are trying to determine how the protein pattern changes with the endocrine state of the mammary gland. We have shown, for example, that protein biosynthesis in the nucleus is more pronounced relative to mitochondrial and microsomal protein biosynthesis as the mammary gland is developing and preparing for lactation than during lactation when microsomal and mitochondrial protein biosynthesis predominate overwhelmingly (Lavrin and Cole 1963). Clearly the next step is to see how the total protein pattern changes with endocrine state and then to elucidate the roles of hormones in mediating such differences in protein patterns. In doing this, we would like to study the rates of synthesis of particular proteins and, for example, to compare rates of nuclear versus cytoplasmic proteins or to compare rates of particular individuals or groups of proteins in any subcellular fraction. Some progress has been made in isolating and studying the synthesis of lysine-rich histones (Cole *et al.* 1963); this has been relatively easy because of the unique nature of such proteins. Attempts to accomplish the direct, quantitative isolation of a cytoplasmic protein have been less successful. Of course, the electrophoretic systems, being used so widely in studying isozymes, might eventually be developed into a more quantitative method for analyzing complex protein mixtures (e.g., Zeldin and Ward 1963), but we have chosen to try to establish first the specificity of some direct resolving methods.

As a model, a system was developed which allowed a relatively direct isolation of insulin (Mendiola and Cole 1960). In this procedure, a whole ethanolic extract of pancreas was dried and put onto a column in which insulin was resolved from all the other proteins of the extract. The resolving power of this system was demonstrated (Cole 1960A) by the fact that it revealed the microheterogeneity of insulin (a third active component was revealed in addition to the two components described by Harfenist and Craig (1952) and was sufficiently selective to resolve rat and chicken insulins from rabbit insulin, which in turn could be resolved from ox insulin (Cole and Mendiola 1960).

In trying to develop a direct, quantitative isolation procedure for

other cytoplasmic proteins, β-glucuronidase was chosen for study since the level of its activity is very sensitive to hormonal treatment (Knobil 1952; Cole and Hopkins 1962A and B). While this enzyme has been purified (Plapp *et al.* 1963) beyond previously published levels (Bernfeld *et al.* 1953), the procedure used was by no means direct. In further attempts to develop such a direct procedure, we have been strongly influenced by the papers of Schwimmer and Balls (1949) and Brown *et al.* (1959) who have exploited enzyme-substrate complexes as specific binding forces for the isolation of enzymes. In some unpublished experiments, trypsin has been pulled out of solution with cubes of a protein gel (thiogel) and amylase from solution with spaghetti. We have even imagined a slaughterhouse in which homogenized pancreas flows along a trough into which spaghetti, protein gel, solid esters, fibers of deoxyribonucleic acid, etc. are dipped intermittently. Each adsorbent would be transferred periodically to a separate trough containing a flow of eluting buffer and leading to a faucet marked "protease," "amylase," "esterase," "DNase," etc., from which trucks could be loaded. Suffice it to say, we have considerable interest in direct specific isolation of enzymes by exploitation of enzyme substrate or enzyme inhibitor complexing. In the case of β-glucuronidase, e.g., β-aminonaphthol could be coupled to carboxymethylcellulose on the one side and to glucuronic acid on the other, to serve as a solid substrate which could be used as a chromatographic support. Indeed the cellulose resins provide an excellent matrix which could be substituted with many sorts of substrate-like organic residues, and many enzymes might thus be purified more completely, more conveniently, and in greater yield by such techniques. Moudgal and Porter (1963) have just applied a similar technique to the purification of specific antibodies, and perhaps we shall hear more of this work from Dr. Perlman (Chapter 19). In the future, we might look for widespread use of such techniques for purification of proteins.

We have departed quite a way from considerations of microheterogeneity of prolactin, but the point is that the interactions of the more recent developments in protein chemistry with our own frustrations have led our thoughts in just this way. Although it is known that at least some transformations of prolactin can occur in some isolation procedures (Squire *et al.* 1963), that is not really of prime importance. It is of considerable importance to know how the hormone exists *in vivo*, but this is what isn't known. It is at this point that our techniques are only beginning to be adequate, and it is to be anticipated that methods will soon be fully established for dealing with many problems of this general nature.

STUDIES OF AMINO ACID COMPOSITION AND SEQUENCE

The primary structure of proteins is certainly a concern common to most of us here. Primary structure was mentioned by Colvin *et al.* (1954) as a possible source of microheterogeneity in proteins, and so for the particular case of prolactin, the amino terminal residue was studied. It appears that all three components have the same sequence of at least five amino acid residues at the amino end of a single polypeptide chain (Cole *et al.* 1957), and all seem to have a carboxyl terminal half-cystine linked to the rest of the protein through a disulfide as well as a peptide bond (Li and Cummins 1958). Amino acid analysis has so far failed to reveal differences among the three forms of prolactin (Cole 1954). Most recently, the peptides of a tryptic digest of prolactin-thiosulfonate (Bailey and Cole 1959) have been mapped on an ion-exchange column (Fig. 1). Only 22 peptide peaks were found and this number is just the

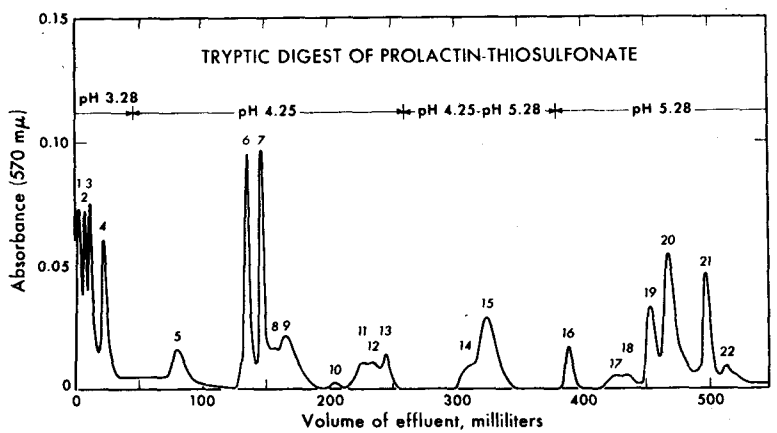

FIG. 1. CHROMATOGRAPHY OF A TRYPTIC DIGEST OF PROLACTIN-THIOSULFONATE USING THE SYSTEM DESIGNED BY SPACKMAN *et al.* (1958) FOR ANALYSIS OF BASIC AMINO ACIDS

number of lysyl and arginyl residues in prolactin. While it must be pointed out that the peaks have not been examined for purity and the recovery of ninhydrin color was only about 65 per cent, this finding is some evidence in favor of the idea that the three forms of prolactin have essentially the same primary structure. Three quite different primary structures would have been expected to give up to 70 peptides. The use of peptide maps, or "fingerprints" as they are sometimes called, was first popularized by Ingram (1958) in his most stimulating study of hemoglobin microheterogeneity. In the paper electrophoresis-chromatog-