The Proteins

CHEMISTRY, BIOLOGICAL ACTIVITY, AND METHODS

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VOLUME I, PART A

ACADEMIC PRESS INC., PUBLISHERS
NEW YORK, 1953

Library of Congress Catalog Card Number: 52-13366

PRINTED IN THE UNITED STATES OF AMERICA

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CONTENTS

CHAPTER 1

The Isolation of Proteins

BY JOHN FULLER TAYLOR

I. Introduction

Proteins commonly occur in complex mixtures. In some of these mixtures the number of proteins is relatively small and one variety often predominates, as does casein in milk,[1] hemoglobin in the lysate of the mammalian erythrocyte,[2] or egg albumin in the white of the avian egg.[3] In others, particularly those which compose the cells of most animal tissues and microorganisms, the number of proteins may be very large. The greater proportion of these are probably enzyme proteins, many of which are present in very small amounts.[4] In almost every kind of protein mixture there are also likely to be present lipides, carbohydrates, nucleic acids, and other organic substances, as well as many varieties of inorganic ions; any of these may be free or associated more or less closely with proteins.

The isolation of a protein from the mixture in which it occurs requires both separation from nonprotein materials (with proper respect for those which may be combined with protein), and separation from the multiplicity of other proteins which are present. The ease with which these separations can be carried out, to any desired degree of completeness, varies enormously. In practice the separations have often been far from complete, either because suitable methods have not been devised or applied, or because a partial separation has been considered satisfactory for the purpose in view.

The purposes for which protein separations are carried out differ widely. The investigation of the structure, composition, and properties

(1) T. L. McMeekin, *Advances in Protein Chem.* **5**, 201 (1950).
(2) E. Ponder, Hemolysis and Related Phenomena, Grune and Stratton, New York, 1948.
(3) H. L. Fevold, *Advances in Protein Chem.* **6**, 188 (1951).
(4) J. B. Sumner and G. F. Somers, Chemistry and Methods of Enzymes, Academic Press, New York, 1947.

of the protein molecule requires substances which are pure and homogeneous, at least within well understood and defined limits. The study of biologically active proteins requires that material with a single kind of activity be separated, if possible, without loss of that activity, from materials having other kinds of activities. For the study of biological structure and function it is important to separate substances so gently and systematically as to preserve or reveal to the utmost the relationships which existed before the separation took place. The establishment of nutritional relationships, on the other hand, may require the study of complete protein mixtures, as they occur in foods, and of purified protein fractions of known composition. The industrial uses of proteins, in plastics, fibers, adhesives, and paints, for example, demand large quantities of stable, uniform, well-characterized products, not necessarily limited to one kind of protein molecule.[5]

The information collected in this chapter is intended to serve the investigator who seeks to isolate proteins from their natural sources, for whatever purposes. Much of the material applies to the problem of isolation of pure, standard protein preparations, but much of it should also be found useful in the investigation of protein fractions and products which are actually inhomogeneous.[6–10a]

The difficulties of isolation and characterization of proteins have been far greater than those which have attended the study of most other organic compounds. Until rather recently so few proteins had been isolated in a state which could be shown to be approximately pure and homogeneous that serious doubts had been raised concerning the existence of many proteins as chemical entities.[11] With improved methods it has been recognized that many of the earlier products were mixtures,

(5) E. Sutermeister and F. L. Browne, Casein and Its Industrial Applications, Reinhold Publ. Co., New York, 1939.
(6) D. M. Greenberg, ed., Amino Acids and Proteins, C. C Thomas, Springfield, Ill., 1951.
(7) H. L. Fevold *in* Greenberg, *op. cit.*, Chap. 5, p. 256.
(8) F. Haurowitz, Chemistry and Biology of Proteins, Academic Press, New York, 1950.
(9) E. J. Cohn and J. T. Edsall, Proteins, Amino Acids and Peptides, Reinhold Publ. Co., New York, 1943.
(9a) *Ibid.*, Chap. 12.
(9b) *Ibid.*, p. 243.
(9c) *Ibid.*, p. 587.
(10) C. L. A. Schmidt, ed., The Chemistry of the Amino Acids and Proteins, C. C Thomas, Springfield, Ill., 1938.
(10a) *Ibid.*, p. 173.
(11) For a summary of the position in 1938 see R. J. Block *in* Schmidt, *op. cit.*, Chap. VII, p. 278.

some of which have since been resolved into their components. A tremendous amount of work has indeed been directed toward the complete analysis of the composition and structures of these substances. The present feeling is that more and more of the complex natural mixtures can be made to yield to sufficiently adroit fractionation procedures so as to permit their complete separation into components. The investigation of these isolated substances can be expected to yield rich rewards in further knowledge of the relation of structures to the properties, both of the pure materials and also of the parent mixtures.

The comparison of the properties of isolated materials with those of the original mixtures has often revealed evidence of the independent existence of the individual proteins in the mixture. Electrophoresis, ultracentrifugation, and electron microscopy have all provided such evidence. In many instances, however, there is also evidence that the proteins exist in nature in the form of complex associations with other substances, recognized perhaps as functional entities such as fibers, membranes, mitochondria, or other granular structures within the living cells. For many kinds of investigation, therefore, it has appeared important to dissect the living structures with care, so as to isolate these functional complexes in unmodified form. This has been referred to as their "state in nature."[12] This outlook and the related methods of investigation are of the utmost importance in furthering the knowledge of living things.

In practice, many of the methods which have been employed and which are presented below are methods which tend to break up the loose associations which are believed to be present in the state of nature. Provided, however, that the disruption of complex materials is recognized, that it does no violence to the structures of the component parts, and that, if possible, the complexes can also be studied and can be, perhaps, regenerated, the study of the parts has the great virtue of simplification. Granted that the study of the most complex systems is of the greatest ultimate value, it remains also true that we must proceed to study the simpler components first, that understanding of the complex must always be based on knowledge of the simple, and that much remains to be learned about the relatively simple, although still vastly complex, proteins which can be isolated. So far as these can be shown to behave as chemical individuals, their study permits the development of further generalizations regarding structures, properties, and particularly, biologi-

(12) E. J. Cohn, D. M. Surgenor, and M. J. Hunter, *in* J. T. Edsall, ed., Enzymes and Enzyme Systems. Their State in Nature, Harvard Univ. Press, Cambridge, Mass., 1951, p. 105.

cal activities. This leads us to distinguish the "universal and essential" from the "special, accidental, and individual."[13] This is the motivation for our endeavors to improve methods of isolation of the proteins and to encourage their further study.

II. General Principles

1. Precautions Necessary to Avoid Alterations in Protein Materials

The protein molecule is composed of one or more polypeptide chains (see Chaps. 3 and 7). The number of residues of each of the amino acids, the linear arrangement of these residues along each of the chains (which may or may not be identical), and the three-dimensional array produced by the folding or coiling of the peptide chains and their arrangement with respect to each other, all contribute to the structure of the protein molecule. The biological, chemical, and physical properties of the protein depend upon the integrity of its entire structure. These properties also may depend, often in marked degree, upon the presence within the molecule of nonprotein portions, more or less firmly attached to the polypeptide chains.

Since a slight change in the structure of the protein molecule may lead to alteration of its properties, it is important to avoid or to control changes in the molecule during the isolation procedure. Such changes can occur either (*a*) through chemical reactions which break some of the covalent bonds in the molecule, (*b*) through changes in the hydrogen bonds and saltlike linkages which support the three-dimensional structure of the molecule, or (*c*) through a change in the association with other proteins or nonprotein materials to which the protein moiety is bound. As has already been pointed out, changes of the last sort may occur without obligatory modifications of the protein molecule itself, and although they must ultimately be considered, especially in relation to cellular structure, they are not always of importance in the problem of protein isolation. The first two types of change, however, must be avoided in the isolation of intact native proteins. The significance of a particular structural change may differ from one protein to another (see Chap. 9). So also may the resistance to change differ greatly among different proteins. In the absence of specific information, however, it is generally more satisfactory to employ, whenever possible, only those procedures which are known to be least likely to produce structural alterations.

(13) J. W. Gibbs, Collected Works, Longmans, Green and Co., London and New York, 1928, Vol. II, part 2, p. 170.

a. Denaturation

This is usually considered to be any change in the structure of a protein molecule which leads to a detectable change in its properties, exclusive of changes resulting from alterations in composition or from the rupture of covalent bonds, e.g., by hydrolysis of the peptide chain itself. Such nonhydrolytic changes can be caused by a number of procedures (see Chap. 9) which usually produce profound changes in properties including loss of biological activity. In this chapter there will be considered only those conditions and procedures which must be avoided during protein isolations and investigations in order to minimize alterations in the protein. Most of these precautions have emerged from the cumulative experience of generations of chemists who have been engaged in the isolation of proteins.

The temperature should be kept as low as possible at all times. The coagulation and inactivation of protein materials at elevated temperatures are familiar to all and it must be realized that these are but extreme examples of the more general tendency of proteins to become denatured at lower temperatures, especially if other environmental conditions are also favorable. In view of the high temperature coefficient of heat denaturation, a difference of a few degrees may appear to be highly critical. It is usually important to keep the temperature low even though in the original source a particular protein may appear stable at higher temperatures. It must be remembered that a protein, prepared from a living cell, may have been undergoing constant replenishment within the cell, and that the environmental conditions in the cell are probably more favorable to the existence of the native protein than those conditions to which the investigator has exposed it.

The heat stability of proteins varies within wide limits; some proteins are known to be resistant to elevated temperatures, even to boiling.[14] This observation has been used on occasion in the purification of stable proteins by the thermal denaturation of less stable concomitants.[14,15] Such procedures, however, must be employed with discretion and with proper experimental evidence that the protein isolated has remained unaffected by the heat treatment (see sec. VIII). Low temperatures are also desirable in that they retard the growth of microorganisms as well as the action of destructive enzymes which may be present.

Extremes of pH should ordinarily be avoided. Most proteins are stable only over a rather narrow pH range and are rapidly denatured if

(14) J. H. Northrop, M. Kunitz, and R. M. Herriott, Crystalline Enzymes, Columbia University Press, New York, 1948.
(15) V. A. Najjar, *J. Biol. Chem.* **175**, 281 (1948).

their solutions are made more acidic or basic. The pH of maximum stability is likely to be rather near neutrality and is not necessarily at the isoelectric point of the protein. Plasma albumin, for example, which is isoelectric near pH 4.6, has been reported to be most stable to heat near pH 6.8.[16] Exceptions, however, are known; pepsin, for example, is most stable at a pH of about 5 and destroyed at neutral pH.[14]

Since many proteins are rapidly denatured in the presence of water-soluble organic solvents, such as ethanol or acetone, the use of these solvents for purposes of fractionation requires particular care in maintaining the temperature as low as possible.[17] On the other hand, many proteins are quite stable in glycerol solutions.

It is important in the addition of reagents, particularly of acids, bases, or organic solvents, to avoid local excesses which might bring about denaturation. Reagents should be diluted, acids and alkalies should be buffered, and the addition should take place either through a dialyzing membrane or through a narrow orifice, slowly, with adequate stirring.[18,19]

Attention should be paid to the denaturing effect of surfaces, films, and especially foams. At the surface of an aqueous solution or at an interface, proteins tend to be spread out in the form of films of denatured protein. As Bull has shown,[20] if the film of denatured protein is continually removed from the surface, denaturation continues with the formation of more film. If foam is allowed to form, the amount of denaturation can become considerable and, with dilute solutions, may constitute an appreciable proportion of the total protein. Hence it is advisable to work with concentrated solutions, to avoid the formation of large surfaces of protein solution, and particularly to avoid foaming. The use of antifoaming reagents may be of practical convenience at times but does not necessarily prevent surface denaturation.[21]

It has long been known that most proteins appear to be more stable in concentrated than in dilute solutions.[9] Methods of concentrating proteins solutions are described below (IV-1). Dilute solutions of neutral salts also increase the stability of proteins, even in dilute solutions.[9] Other special stabilizing influences are described elsewhere (IV-4).

Since the stability of different proteins, even in the same mixture,

(16) G. Scatchard, S. T. Gibson, L. M. Woodruff, A. C. Batchelder, and A. Brown, *J. Clin. Invest.* **23,** 445 (1944).

(17) R. M. Ferry, E. J. Cohn, and M. S. Newman, *J. Am. Chem. Soc.* **58,** 2370 (1936).

(18) H. Theorell, *Biochem. Z.* **268,** 46 (1934).

(19) E. J. Cohn, J. A. Luetscher, Jr., J. L. Oncley, S. H. Armstrong, Jr., and B. D. Davis, *J. Am. Chem. Soc.* **62,** 3396 (1940).

(20) H. B. Bull, *J. Biol. Chem.* **123,** 17 (1938).

(21) J. F. Taylor, unpublished observations.

can vary greatly, it is evident that every protein isolation involves a study of its own special peculiarities in order to judge the precautions which must be taken.

b. Action of Enzymes

Enzymes which hydrolyze proteins, or which attack nonprotein components of conjugated proteins, may be liberated or activated when the components of a mixture are brought into solution prior to fractionation. If these are permitted to function, the desired components of a mixture may be altered or destroyed. The importance of the prompt inactivation of the proteolytic enzymes of the pancreas for the isolation of insulin is well known.[22] Even if an enzyme is initially present in low concentrations, it may become concentrated in a particular fraction and thus cause extensive damage. An example of a non-proteolytic enzyme is the so-called prosthetic-removing (PR) enzyme of Cori and Green.[23] This enzyme alters the properties of muscle phosphorylase in such a way that the latter is difficult to separate in pure form, thus requiring the interfering enzyme to be removed at an early stage in the purification procedure.

Microorganisms are evidently undesirable in protein solutions lest their activities either modify the protein or add to the complexities of the mixture by introducing additional components. Linderstrøm-Lang has reported an example of modification of a purified protein, ovalbumin, by an enzyme of bacterial origin[24] (see sec. IV-3).

c. Complex Compounds

Complex compounds of proteins with other substances may require special precautions, depending upon the nature of the nonprotein component and upon the linkage by which the union is maintained. In dealing with nucleoproteins care must be taken to avoid splitting off the nucleic acid portion if the combined material is sought.[25] Lipoproteins have been found to be sensitive to freezing.[26] Other kinds of prosthetic groups may be altered by chemical[27] or enzymatic[23] action, leading on the one hand to alteration in the specific biological properties of the

(22) H. Jensen, The Chemistry and Physiology of Insulin, Am. Assoc. Advancement Sci., Washington, 1944.
(23) G. T. Cori and A. A. Green, *J. Biol. Chem.* **151**, 21, 31 (1943).
(24) K. Linderstrøm-Lang and M. Ottesen, *Compt. rend. trav. lab. Carlsberg. Sér. chim.* **26**, 404 (1948).
(25) J. P. Greenstein, *Advances in Protein Chem.* **1**, 209 (1944).
(26) J. L. Oncley, F. R. N. Gurd, and M. Melin, *J. Am. Chem. Soc.* **72**, 458 (1950).
(27) A. A. Green, *J. Biol. Chem.* **93**, 495 (1931); *ibid.* **95**, 47 (1932).

material, and on the other, to changes in solubility or in other properties of the conjugated protein. Changes in the state of oxidation of the prosthetic group of hemoglobin, for example, are well known as producing profound changes in solubility.[27]

d. Other Changes

Other changes in the structure or composition of proteins include changes in the state of oxidation of the sulfhydryl groups of cysteine with a resulting change in protein properties; combination with heavy metals, which may inactivate certain enzymes; or the loss of dialyzable cofactors or other components of biologically active systems. While the latter may involve merely a matter of astuteness in the testing of biological activity, such changes cannot be ignored in the course of an isolation process.

2. Choice of Material

The source from which a protein is to be isolated is often predetermined, for many reasons. If not, however, it may be worth while to explore the possibilities of selection from among different kinds of organisms, different species, or different tissues. It is desirable, in general, to choose the richest source material that can be found. Substances that are difficult to remove, such as gums or other polysaccharides, should be avoided. An interfering enzyme, such as the prosthetic-removing enzyme previously mentioned,[23] can be a serious source of difficulty; in fact, the difficulty of removing this enzyme has hitherto prevented the isolation of phosphorylase from muscle of species other than the rabbit.[28] In many instances it has been found by experience to be easier to separate or to crystallize a particular protein from one source than from another; numerous examples are found among the hemoglobins,[29] the plasma albumins,[19,30,31] the seed proteins,[32] and the enzymes.[4] If alternative methods are applied, however, the apparent advantage of a particular source may disappear.[33]

(28) G. T. Cori, private communication.

(29) A. A. Green, E. J. Cohn, and M. W. Blanchard, *J. Biol. Chem.* **109,** 631 (1935); also J. Barcroft, The Respiratory Function of the Blood. Part II. Haemoglobin. Cambridge Univ. Press, London, 1928.

(30) E. J. Cohn, T. L. McMeekin, J. L. Oncley, J. M. Newell, and W. L. Hughes, Jr., *J. Am. Chem. Soc.* **62,** 3386 (1940).

(31) T. L. McMeekin, *J. Am. Chem. Soc.* **61,** 2884 (1939); **62,** 3393 (1940).

(32) T. B. Osborne, The Vegetable Proteins, Longmans, Green and Co., London and New York, 1924.

(33) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, *J. Am. Chem. Soc.*, **69** 1753 (1947).

3. Rupture of Cells and Extraction of Proteins

In order to isolate proteins which occur within cells it is necessary first to break the cell membranes. A number of procedures have been employed, none of which is universally applicable, since the resistance of cells to disintegration varies enormously.

The erythrocyte may be readily hemolyzed by various means. This has been carried out by alternate freezing and thawing, by the addition of distilled water, or by treatment with saponin or with organic solvents such as ether or toluene.[2] The use of distilled water has been recommended for the preparation of "ghosts" or stromatin free of hemoglobin,[2] but it may dilute the hemoglobin too much when the latter is to be isolated. The use of ether contaminated with peroxides may cause the conversion of some of the hemoglobin to ferrihemoglobin. The use of toluene[34,35] has the advantage that much of the stroma material is readily removed, by centrifugation, in the form of an emulsion with the toluene.

The cells of microorganisms are generally rather difficult to disrupt. Ordinary yeast can be plasmolyzed with the aid of toluene or ethyl acetate. Yeast is often prepared for extraction by slow drying in air, which involves considerable autolysis.[36] The original method of extraction of "zymase" involved pressing in a hydraulic press after grinding with infusorial earth.[37] A variety of methods have been applied to the bacteria. Several grinding mills have been described.[38,39] Success has been reported with a method of grinding with tiny glass beads[40] instead of the more usual powdered glass or sand. Freezing and thawing, or the procedure of grinding or crushing the frozen organisms may be applied.[41] Many microorganisms are disintegrated by supersonic vibrations,[42,43] but this method must be considered likely to damage sensitive

(34) M. Heidelberger, *J. Biol. Chem.* **53,** 31 (1922).
(35) J. F. Taylor and A. B. Hastings, *J. Biol. Chem.* **131,** 649 (1939).
(36) F. Lipmann, *Compt. rend. lab. Carlsberg* **22,** 317 (1937); also R. Nilsson, *in* E. Bamann and K. Myrbäck, eds., Die Methoden der Fermentforschung, Thieme, Leipzig, 1941, p. 1284.
(37) E. Buchner and M. Hahn, Die Zymasegärung, Oldenburg, München, 1903.
(38) V. H. Booth and D. E. Green, *Biochem. J.* **32,** 855 (1938).
(39) G. Kalnitsky, M. F. Utter, and C. H. Werkman, *J. Bact.* **49,** 595 (1945).
(40) M. R. J. Salton and R. W. Horne, *Biochim. et Biophys. Acta* **7,** 19, 177 (1951).
(41) R. L. Garner and W. S. Tillett, *J. Exptl. Med.* **60,** 239 (1934).
(42) P. K. Stumpf, D. E. Green, and T. W. Smith, *J. Bact.* **51,** 487 (1945); also L. A. Chambers and E. W. Flosdorf, *Proc. Soc. Exptl. Biol. Med.* **34,** 631 (1936).
(43) E. A. Kabat and M. M. Mayer, Experimental Immunochemistry, C. C Thomas, Springfield, Ill., 1948.

proteins, especially in the presence of certain gases.[44] Similar damage must always be feared in the use of any of the more violent methods if local heating or excessive surface forces cannot be avoided. The press recently described by Hughes[45] appears to offer advantages in its speed and in its ability to rupture a variety of organisms; many yeasts as well as bacteria can be broken up fairly completely. A method of bursting bacteria by the rapid expansion of gas dissolved under pressure has been recently proposed;[46] further work may be needed to establish the resistance of proteins to surface denaturation by this procedure.

Herbert and Pinsent have given an example of the use of lysozyme to disrupt a sensitive organism, *Micrococcus lysodeikticus*, from which they prepared a crystalline catalase.[47] The use of this sort of method has thus far proved rather limited.

Many plant and animal tissues can be sufficiently broken by grinding to permit the extraction of the more soluble proteins. For mammalian muscle it is usually sufficient to grind in an ordinary meat grinder. Finer grinding can be accomplished with a "Latapie mincer," a Waring blendor or a "homogenizer."[48] Excessively fine grinding is often undesirable when soluble enzymes are sought since particulate matter is often difficult to remove and destructive enzymatic action may be enhanced. Tissues are often comminuted by crushing and grinding in the frozen state, with the use of Dry Ice.[49]

Dried materials such as seeds are usually finely ground and are often extracted with lipide solvents before extraction with aqueous solvents. Kirk and Sumner have given directions for the preparation of jack bean meal from which urease and other proteins are to be extracted.[50]

Since the drying of proteins, in some instances even from the frozen state, is likely to cause their denaturation, fresh tissue has ordinarily been employed as a source for most proteins, and especially for the more labile biologically active proteins, such as enzymes and hormones. Seed proteins have usually been prepared from the air-dried seeds,[32] and dried yeast is also often employed.[36] It is possible also to prepare dried tissue preparations either by lyophilization (see sec. IV) or as "acetone pow-

(44) L. A. Chambers and E. W. Flosdorf, *J. Biol. Chem.* **114,** 75 (1936).
(45) D. E. Hughes, *Brit. J. Exptl. Path.* **32,** 97 (1951).
(46) D. Fraser, *Nature* **167,** 33 (1951).
(47) D. Herbert and J. Pinsent, *Biochem. J.* **43,** 193 (1948).
(48) For additional details on the preparations of extracts, see W. W. Umbreit, R. H. Burris, and J. F. Stauffer, Manometric Techniques and Tissue Metabolism, Burgess, Minneapolis, 1949.
(49) A. E. Wilhelmi, J. B. Fishman, and J. A. Russell, *J. Biol. Chem.* **176,** 735 (1948).
(50) J. S. Kirk and J. B. Sumner, *Ind. Eng. Chem.* **24,** 454 (1932).

ders."[7] Finely ground, chilled tissue is rapidly mixed with several volumes of acetone previously cooled to $-10°$ or lower, and the precipitated material is rapidly filtered off and washed with more cold acetone. Many enzymes are relatively unharmed by this procedure and the dried powders can be preserved for a long time if kept cold and dry. This procedure has the advantage of removing lipide material, and the protein can be extracted as desired. Few comparative data on the yields of total protein or of individual enzymes isolated after this treatment or by extraction of the fresh tissues seem to be available.

The choice of the extracting solvent, of course, depends upon the solubility of the proteins which are to be extracted (see sec. V). It has frequently been found desirable to extract successively with different solvents, proceeding from the most selective to the most general.[9] This procedure was often employed in the extraction of the seed proteins (see Osborne[32]). It has also been employed in many other systems, and should probably always be tried. Recently Cohn *et al.*[51] have again emphasized the advantages of selective extraction. They have described a new method for the fractionation of blood plasma and other systems in which most of the protein is first precipitated and the precipitate is then successively extracted with different solvents. One great advantage of this method is that by proper choice of the extraction procedure sensitive substrates can be separated at an early stage from the enzymes by which they are attacked.

In the extraction of a whole tissue, proteins may be removed from each of the structural elements which compose the cell. Preliminary separation of the structural elements facilitates separation and identification of the components of each structure. A number of methods have been described for the separation of nuclei and of mitochondria, in relatively undamaged state, from other cellular constituents. Schneider and Hogeboom[52] have recently reviewed the methods used in this field and the results which they have yielded. Thus far the available quantities of separated cell fractions have necessarily been small. Perry[53] has isolated intact myofibrils after treatment of muscle slices with collagenase. Bacterial membranes[40] and erythrocyte "ghosts"[2] have been separated after rupture of the cells.

Preliminary treatment of a tissue, or tissue fraction, often facilitates

(51) E. J. Cohn, F. R. N. Gurd, D. M. Surgenor, B. A. Barnes, R. K. Brown, G. Derouaux, J. M. Gillespie, F. W. Kahnt, W. F. Lever, C. H. Liu, D. Mittelman, R. F. Mouton, K. Schmid, and E. Uroma, *J. Am. Chem. Soc.* **72**, 465 (1950).

(52) W. C. Schneider and G. H. Hogeboom, *Cancer Research* **11**, 1 (1951).

(53) S. V. Perry, *Biochem. J.* **48**, 257 (1951).