

THE CHEMICAL STRUCTURES OF PANCREATIC RIBONUCLEASE AND DEOXYRIBONUCLEASE

Combined text of the Nobel Lecture, December 11, 1972.

by

STANFORD M OORE AND WILLIAM H. STEIN

The Rockefeller University, New York, New York

INTRODUCTION

In introducing this summary of experiments on two enzymes, we wish to indicate that the information is representative of what biochemists are obtaining about many proteins. An understanding of the host of reactions in which proteins participate in living cells requires information on the molecular architectures of a wide variety of proteins of different origins and different functions. Such information is coming from laboratories all over the world and draws upon a rich heritage of experience from many investigators. And such knowledge is fundamental to progress in medical research; the Nobel awards this year in Chemistry (concerning ribonuclease) and in Physiology or Medicine (concerning antibodies) both concern basic researches on the chemistry and the biology of proteins.

Occasionally (1) it has been educational to write the structural formula for ribonuclease in full, in terms of its 1,876 atoms of C, H, N, O, and S. Portrayal of the complete molecule with all of the atoms of the amino groups, carboxyl groups, hydroxyl groups, guanido groups, imidazole rings, phenolic groups, indole rings, aromatic, aliphatic, and thioether side chains, sulfhydryl groups, and disulfide bonds, helps in the visualization of the almost infinite number of ways in which such groups could be arranged. This characteristic of proteins makes it possible for nature to design catalysts for such a variety of specific reactions. There is no law that says that a nucleic acid or a polysaccharide could not be an enzyme. But it is understandable that the enzymes so far isolated have turned out to be proteins; a protein is equipped to participate, sometimes through cooperation with coenzymes, in the whole lexicon of organic reactions that require catalysis in the living cell.

PURIFICATION OF RIBONUCLEASE

The first step in the study of the structure of ribonuclease was, of course, its purification. Ribonuclease was first described in 1920 by Jones (2), who showed that there was present in beef pancreas a relatively heat-stable enzyme capable of digesting yeast nucleic acid. Dubos and Thompson (3) partially purified the enzyme some eighteen years later and in 1940 Kunitz (4) described the isolation of bovine ribonuclease in crystalline form after fractionation by ammonium sulfate precipitation. In order to be as certain as possible that we were beginning the structural study with a single molecular species, we under-

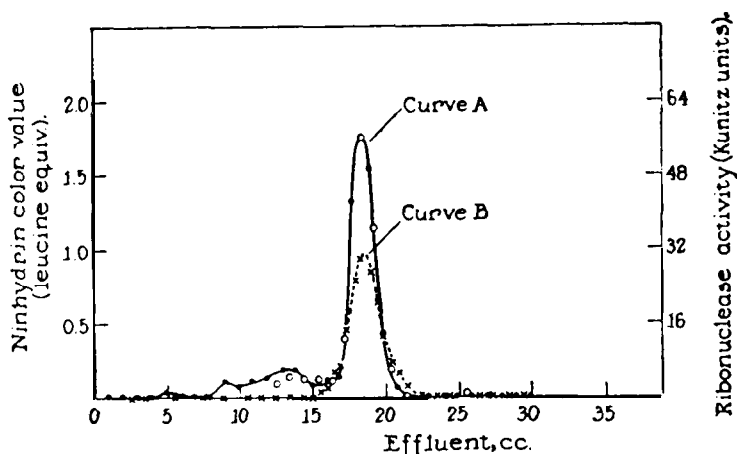


Fig. 1.

Chromatography of crystalline bovine pancreatic ribonuclease (Curve A) on the polymethacrylic acid resin Amberlite IRC-50. Elution was with 0.2 M sodium phosphate buffer at pH 6.45. Curve B was obtained upon rechromatography of material from Peak A. (From (5).)

took to apply the potential resolving power of ion exchange chromatography to ribonuclease (Fig. 1). While Werner Hirs, in our laboratory, was exploring the chromatographic purification of ribonuclease on the polymethacrylic resin Amberlite IRC-50 (5,6), Paléus and Neilands (7), in Stockholm, were studying cytochrome C on the same exchanger. These two proteins were the first molecules of their size to be thus purified. The best resolution for ribonuclease (Fig. 2) is now obtained (8) with an exchanger invented in Uppsala, a sulfoethyl cross-linked dextran, which was a development that grew from Porath and Flodin's (9) experiments on gel filtration and drew upon Sober and Peterson's (10) emphasis on the advantages of a carbohydrate matrix for the exchanger.

When pancreatic extracts were analyzed without prior fractionation, two peaks of enzymatic activity were observed by us (6) by ion exchange chromatography and by Martin and Porter (11) by partition chromatography. The major component, ribonuclease A, was selected for the first structural studies. (In later independent experiments, Plummer and Hirs (12, 13) isolated ribonuclease B in pure form from pancreatic juice and showed it to be the same as A but with the addition of a carbohydrate side chain attached to one asparagine residue.)

AMINO ACID ANALYSIS

The second step in the structural study of ribonuclease A was the determination of the empirical formula of the chromatographically homogeneous protein in terms of the constituent amino acids. Our appreciation of the importance of quantitative amino acid analysis began in the late 1930's when we had the special privilege of starting our postdoctoral studies in apprenticeship to Max

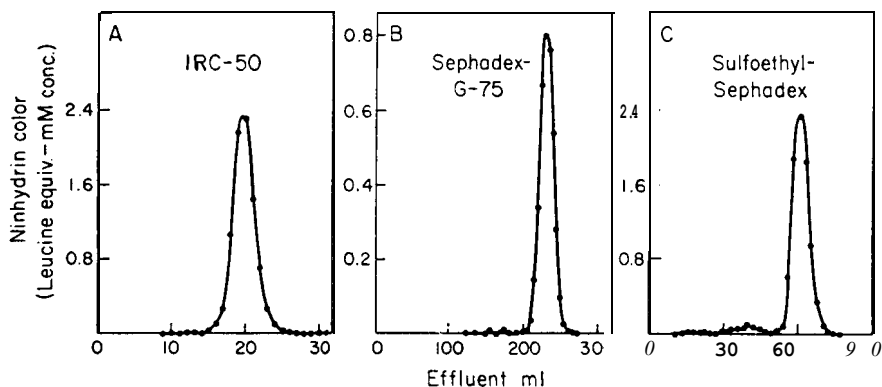


Fig. 2.

Behavior of IRC-50 purified ribonuclease on IRC-50 (A), Sephadex G-75 (B), and sulfoethyl-Sephadex (C) at pH 6.5. From (8).

Bergmann (14). In 1945 it was possible to take a new look at the subject in the light of the renaissance in chromatography stimulated by Martin and Synge in the early 1940's (15 - 17). In 1949, by combining a quantitative photometric ninhydrin method (18) with elution of amino acids from starch columns by alcohol : water eluents (19, 20) on an automatic fraction collector (21), we were able to analyze a protein hydrolysate in about two weeks by running three such chromatograms to resolve all overlaps. In the early 1950's, the process was speeded up to one week (Fig. 3) by turning to ion exchange chromatography on a sulfonated polystyrene resin (22, 23). In 1958, in cooperation with Darrel Spackman (25, 26) the process was automated (Fig. 4) to give recorded curves (Fig. 5) and the speed was increased to give an overnight run. Shorter columns and faster flow rates (27) permitted an analysis time of about 6 hours. Results from many academic and industrial laboratories have helped to make the procedures simpler and more rapid. Some recent users have adopted 2-hour systems (cf. (28)) and the ninhydrin reagent has been improved (29). In the 1970's, a number of industrially designed analyzers with increased automation have reduced the time for a complete analysis to about 1 hour and increased the sensitivity to the nanomole range. The sharing of knowledge among academic scientists and industrial designers of instruments and manufacturers of ion exchangers has played an important role in progress of biomedical research in this field.

In 1972 there are continuing developments that may make amino acid chromatography more ultramicro and more expeditious. These contributions include the introduction by Udenfriend and his colleagues of an analog of ninhydrin (30-32) that yields, at room temperature, a fluorescent product that can be detected at extremely low concentrations; there is also the continuing possibility that gas chromatography can give fully satisfactory results with amino acid derivatives.

The precision and the sensitivity of current procedures for amino acid analysis have been recently reviewed (33). The developmental research on

Amino Acid Composition of Ribonuclease A

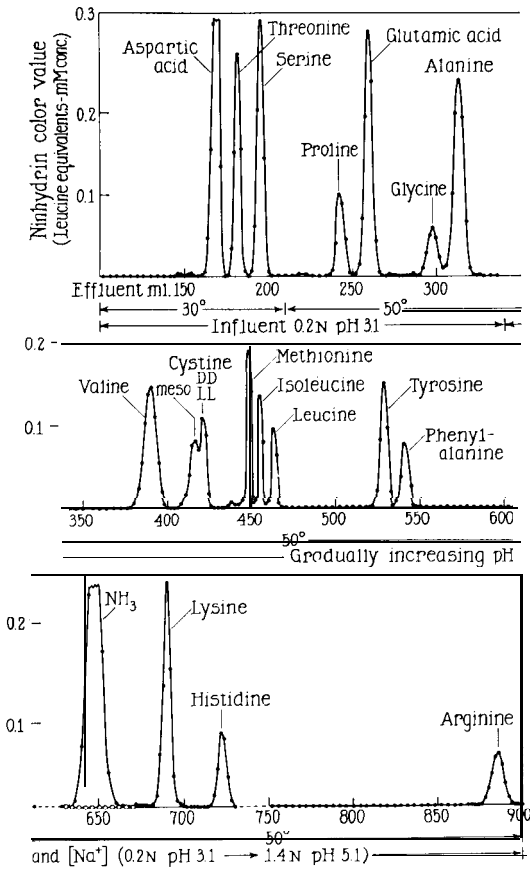


Fig. 3. The amino acids in an acid hydrolysate of ribonuclease A. The separation of the amino acids was obtained in a five-day run (23) from a 150 × 0.9 cm column of the sulfonated polystyrene resin Dowex 50-X4. From (24).

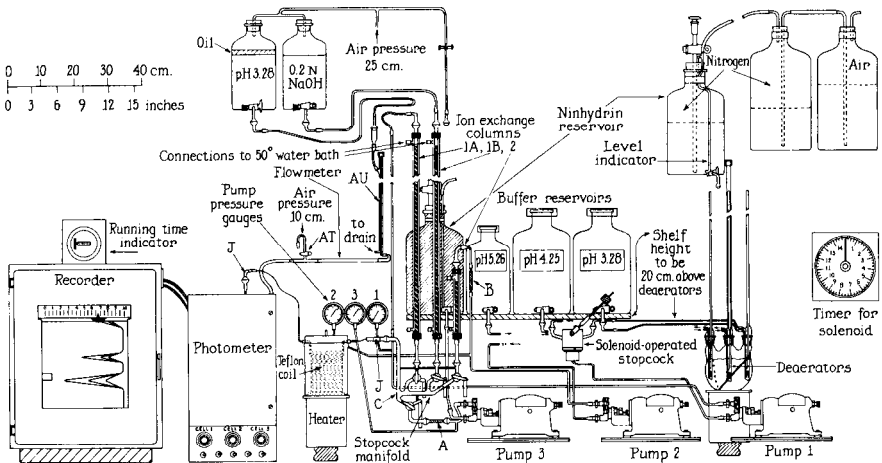


Fig. 4. Schematic diagram of automatic recording apparatus for the chromatographic analysis of mixtures of amino acids. From (26).

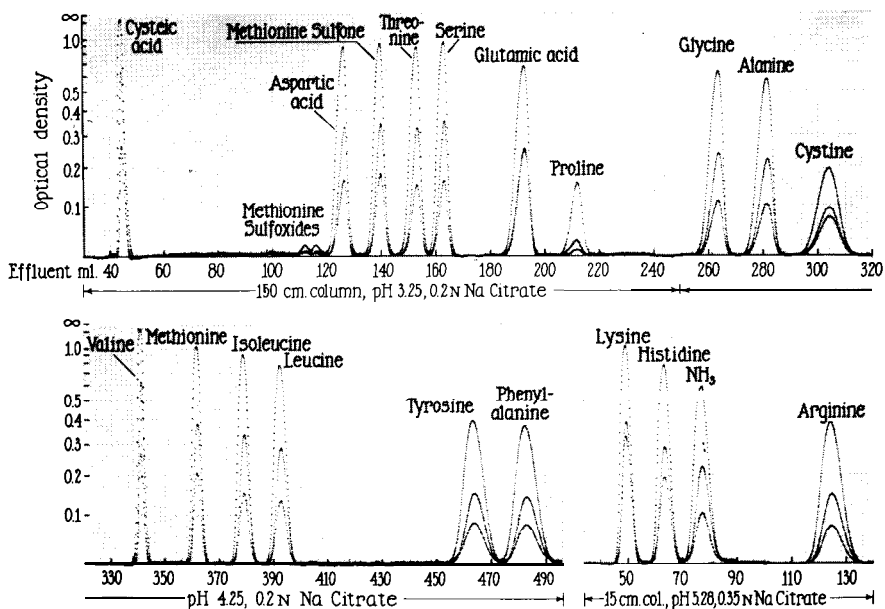


Fig. 5.

Chromatographic analysis of a mixture of amino acids automatically recorded in 22 hours by the equipment shown in Fig. 4. From (26).

quantitative amino acid analysis has also yielded procedures for the isolation of amino acids on a preparative scale (34, 35), for the determination of D- and L-amino acids (36, 37), for the analysis of hydrolysates of foods (38, 39), and for the determination of free amino acids in blood plasma (40), urine (41), mammalian tissues (42), topics that extend beyond the scope of this lecture. Specific discoveries from such studies include the findings by Harris Tallan of 3-methylhistidine (43) and tyrosine-O-sulfate (44) in human urine, acetyl-aspartic acid in brain (45), and cystathionine in human brain (46).

STRUCTURE OF RIBONUCLEASE

The empirical formula of bovine pancreatic ribonuclease (Table I), determined by the chromatographic methods applied during the structural study, turned out to be that of a molecule containing 124 amino acid residues. From the known mechanisms of protein biosynthesis, coupled with the susceptibility of the peptide bonds to enzymatic hydrolysis, all of the residues are almost certainly of the L-configuration. The calculated molecular weight is 13,683.

The experience with amino acid chromatography led us to try to develop column methods with sufficient resolving power for the separation of the peptides formed by the enzymatic hydrolysis of performic acid-oxidized ribonuclease (47), as in the chromatogram illustrated in Fig. 6 from the experiments of Werner Hirs, who was the first postdoctoral associate to join our laboratory. Fifteen young scholars began their postgraduate careers on the researches summarized in this lecture. Each citation of their contributions connotes our

TABLE I
Amino Acid Composition of Ribonuclease A (47)

Amino Acid	Number of Residues per Molecule (mol. wt. 13,683)
Aspartic acid	15
Glutamic acid	12
Glycine	3
Alanine	12
Valine	9
Leucine	2
Isoleucine	3
Serine	15
Threonine	10
Half-cystine	8
Methionine	4
Proline	4
Phenylalanine	3
Tyrosine	6
Histidine	4
Lysine	10
Arginine	4
Total number of residues	124
Amide NH,	17

recognition of the ideas, the hard work, and the enthusiasm that facilitate productive research; each of these biochemists shares the credit for the results reported on this occasion and we continue to be stimulated by their current independent accomplishments.

Werner Hirs, through gradient elution from Dowex 50-X2, obtained 100% yields of the peptides that were completely liberated by tryptic hydrolysis. The elucidation of the sequences of amino acid residues in the peptides and the crossword puzzle-like ordering of the peptides followed many of the principles established by Sanger (48) in his pioneering determination of the structure of insulin, but with the larger molecule of 124 amino acid residues quantitative methods were particularly helpful in the interpretation of the results. A key chemical method in studies of molecules of this size has been the sequential degradation method developed by Pehr Edman (49) with phenylisothiocyanate as the reagent. Instead of determining the resulting phenylthiohydantoin, we have generally used a subtractive procedure in which we utilize the amino acid analyzer to tell us which amino acid has been removed in each step.

The formula for ribonuclease (Fig. 7), largely developed by Werner Hirs (50, 51), Darrel Spackman (52) and Derek Smyth (53, 54), but drawing importantly upon the results of several key experiments by Christian B. Anfinsen and his associates (55-57), in Bethesda, is here written with the customary abbreviations. Ribonuclease was the first enzyme for which the

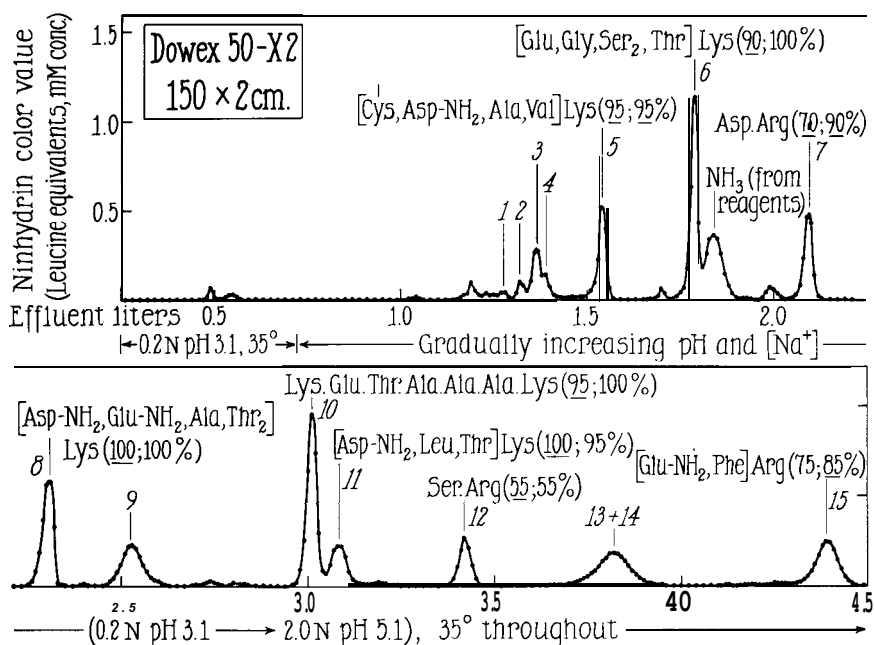


Fig. 6.

Chromatographic separation of the peptides in a tryptic hydrolysate of oxidized ribonuclease A. From (47).

sequence could be written and the determination of its structure was a logical sequel to Sanger's success with the hormone insulin.

The writing of such a two-dimensional formula is only the first step. Linderstrøm-Lang (58) referred to such a sequence as the primary structure of the protein. Catalysis is a three-dimensional operation which involves what Lang termed the secondary and tertiary structures of the chain.

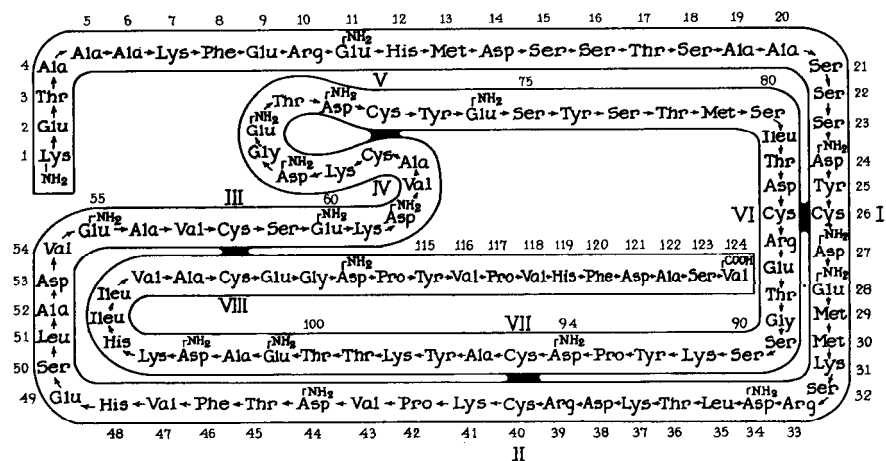


Fig. 7.

The sequence of amino acid residues in bovine pancreatic ribonuclease A. From (54), based upon (50-57).

As chemists, we had made some predictions, through derivatization experiments, about residues that were folded together to form the active center of ribonuclease. Through Gerd Gundlach's studies on the inactivation of ribonuclease by alkylation with iodoacetate (59) and Arthur Crestfield's demonstration of the reciprocal alkylation of two essential histidine residues by iodoacetate at pH 5.5 (60), we concluded that the imidazole rings of histidine-119 and histidine-12 were at the active center and were about 5 Å apart. Robert Heinrikson, in our laboratory, from experiments on the alkylation of lysine at pH 8.5 (61), and drawing upon independent dinitrophenylation experiments by Hirs *et al.* (62), further concluded that the ϵ -amino group of lysine-41 was probably 7 - 10 Å from the imidazole ring of histidine-12 and somewhat further removed from histidine-119.

But the chemical approach does not begin to provide enough data to build an adequate model of an enzyme as a whole. The great advances in X-ray crystallography pioneered by Perutz (63) and Kendrew (64) have opened a whole new chapter in this regard, with knowledge of the sequence, at least in considerable part, being a pre-requisite for the solution of the X-ray problem in the present state of the art. We were waiting with great anticipation for the results of X-ray analysis of crystals of ribonuclease which came in 1967 through the researches of Kartha, Bello, and Harker (65) on RNase A and Wyckoff and Richards and their associates (66) on RNase S. In the S-form of the enzyme (67, 68), which is fully active, the chain has been cleaved primarily between the 20th and 21st residues by controlled proteolysis with subtilisin.

Examination of the model shows the approximate positions of the imidazole rings of histidines-12 and -119 and the ϵ -amino group of lysine-41 to be compatible with the chemical predictions. The substrate for RNase (Fig. 8) is ribonucleic acid, which, from the results of experiments in the laboratories of

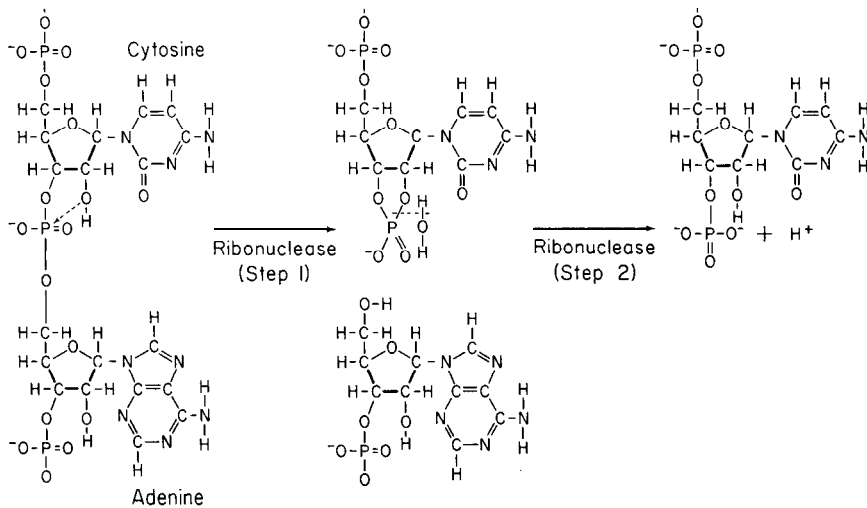


Fig. 8.

The action of ribonuclease on ribonucleic acid (reviewed in (69)).

Todd, of Cohn, and of Markham (reviewed in (69)) is cleaved at the 5'-phosphate ester bond following a pyrimidine-containing nucleotide to give, by transphosphorylation, the 2', 3'-cyclic phosphate which, in a second step, is hydrolyzed to the 3'-ester. The X-ray data show that the substrate fits in a trough on the surface of the protein with the phosphate moiety near the two imidazole rings of histidines-12 and -119 and with the pyrimidine ring tucked into a hydrophobic pocket close to the aromatic ring of phenylalanine-120

From this picture of the active site (reviewed in (70)), chemical and physical experimentation is progressing in a number of laboratories toward definition of the catalytic process in as explicit terms as possible with primary roles for one charged imidazole and one uncharged imidazole participating in the push-pull which results in transphosphorylation or hydrolysis of the phosphate ester bond.

These further experiments carry the subject into the third chapter in the history of ribonuclease, its chemical synthesis, which has grown from the many innovations in the methods for peptide synthesis in recent years. A preparation with 70 % of the activity of native ribonuclease has been synthesized through a major effort by Gutte and Merrifield (71, 72). An active RNase S-protein has been synthesized by Hirschmann, Denkwalter and associates (73). The yields in the syntheses are dependent upon a very important property of the disulfide bonds of RNase studied by White (74) and by Anfinsen and his associates (75) and reviewed particularly in terms of its special biological significance by Anfinsen (76). The reduced chain with 8 -SH groups folds to give the proper pairs of S-S bonds for the active conformation of the protein. The intramolecular forces that guide such a folding, and the similar forces that contribute to the specific aggregation of the chains of a protein with multiple subunits, such as hemoglobin, form a continuing subject of research.

The ability to synthesize RNase, or major parts thereof, opens new avenues for the identification of residues that may be essential for activity through the preparation of analogs of ribonuclease with substitutions at specific positions. Hofmann and Scoffone and their respective associates (reviewed in (70)) have done this at the amino end of the chain by synthetic variations in Richards' S-peptide. We have cooperated with Merrifield and his associates in the following recent series of experiments which illustrate the application of chemical surgery to the COOH end of the molecule and the use of synthetic replacements.

How can we examine the question of whether the proximity of the aromatic ring of phenylalanine-120 and the pyrimidine ring of the substrate leads to specific interaction between two six-membered rings in a way which is important in the binding of substrate to enzyme? Michael Lin, in our laboratory, was able to cut off, enzymatically (by pepsin according to Anfinsen (77) plus carboxypeptidase A) the last 6 residues from ribonuclease which include phenylalanine-120 and histidine-119. The resulting molecule (78) is completely inactive and does not bind substrate. Concurrently, Gutte and Merrifield had synthesized the 14-residue L-peptide from glutamic acid-111 through valine-124. When this synthetic fragment is mixed with the molecule missing residues

119 to 124, the added peptide is adsorbed and 90% of the activity of the native enzyme is regained (79). The missing histidine is thus supplied for the active center; other residues in the peptide adsorb on the protein core in such a way as to re-form the binding site and the catalytic site. This result parallels in principle the earlier experiments of Richards and Vithayathil (68) on the removal and adsorption of the S-peptide at the NH_2 end.

When leucine or isoleucine is substituted for phenylalanine at position 120 in the synthetic peptide, the combination of peptide and protein has 10% of the activity of ribonuclease and binds substrate as effectively as the native enzyme (80). In this way we conclude that the aromatic ring is not essential for binding of the pyrimidine ring or for activity. But the lower activity when leucine or isoleucine is substituted indicates that the aromatic ring of phenylalanine fits into the hydrophobic pocket more specifically than the aliphatic side chains and probably serves to orient histidine-119 more exactly in: the delicate balance with histidine-12 that gives the active site its full catalytic power.

Another way to learn what residues can be varied without loss of activity is to study the changes in pancreatic ribonucleases from different species, as has been done for the enzymes from sheep (81), rat (82), and pig (83, 84).

DEOXYRIBONUCLEASE

A further way to gain insight into what makes ribonuclease so specific for its special substrate is to look at enzymes that hydrolyze similar substrates. In the past few years we have turned our attention to pancreatic deoxyribonuclease. This enzyme, which is about twice the size of ribonuclease, hydrolyzes DNA in the presence of bivalent cations, such as Mn^{++} , to give 5'-mononucleotides and larger fragments (85). Deoxyribonuclease first attracted special attention in the classic work of Avery, MacLeod, and McCarty (86, 87), who showed that the transforming principle of the pneumococcus could be destroyed by the action of the enzyme. McCarty's (88) experiments on the purification of the enzyme from pancreas were followed by those of Kunitz (89) and of

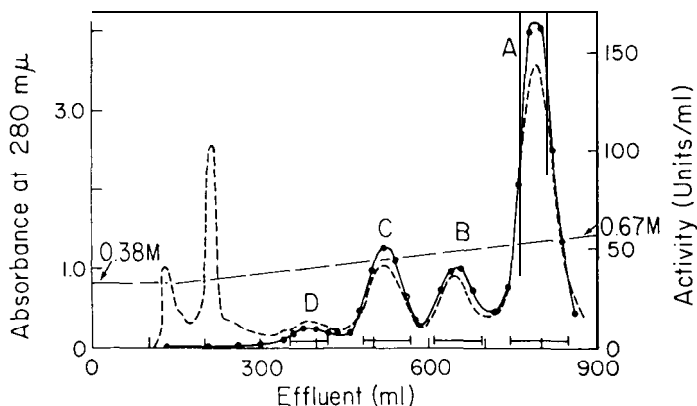


Fig. 9.

Chromatography of bovine pancreatic deoxyribonuclease, prepared by ammonium sulfate precipitation (89), on phosphocellulose at pH 4.7 with a sodium acetate buffer of increasing molarity. From (92).



Fig. 10.

sequence of amino acid residues in bovine pancreatic deoxyribonuclease A. From (93, 94).

Lindberg (90). Our studies began when Paul Price, as a graduate student, undertook the chromatographic purification of deoxyribonuclease. His initial studies showed that the enzyme, in the absence of bivalent metals, was extremely sensitive to proteolysis. Success in the purification depended upon keeping metals such as Ca^{++} present or adding diisopropyl phosphorofluoridate to inactivate the pancreatic proteases. He succeeded in resolving preparations of deoxyribonuclease into two active components on sulfoethyl-Sephadex (91) and Hans Salnikow subsequently obtained even higher resolving power (Fig. 9) with phosphocellulose (92). There are three main active components: DNase A is a glycoprotein, DNase B is a sialoglycoprotein, and DNase C is similar to A but with a proline residue substituted for one histidine. These three deoxyribonucleases were also present in the pancreatic juice from a single animal.

The determination of the chemical structure of DNase A was undertaken by

Hans Salnikow (93) and carried to completion this year by Ta-hsiu Liao (94). The working hypothesis for the structure of the molecule (Fig. 10) indicates a single chain of 257 residues with two disulfide bonds. The ordering of the tryptic and chymotryptic peptides in the reduced and carboxymethylated chain and the pairing of the half-cystine residues in the native enzyme were greatly facilitated by the cleavage of the molecule at the four methionine residues by the cyanogen bromide by the method of Gross and Witkop (95). Amino acid analyses at the nanomole level made possible sequence determinations on small amounts of peptides isolated by chromatography or paper electrophoresis.

Some of the special features of the structure can be discussed in reference to the diagram in Fig. 11. The carbohydrate side-chain, which contains 2 residues of N-acetylglucosamine and 2 to 6 residues of mannose (91, 92, 96) and which Brian Catley showed was attached via an aspartamidohexosamine linkage to a -Ser-Asn-Ala-Thr- sequence (96), is found at only one position in the chain, at residue 18. Tony Hugli studied the nitration of deoxyribonuclease (97) by tetranitromethane (98); the enzyme is inactivated by the modification of one tyrosine residue which turns out to be residue 62. Paul Price discovered that inactivation of DNase by iodoacetate in the presence of Cu^{++} and Tris buffer (99) is accompanied by carboxymethylation of one residue of histidine; from the sequences of a 3-carboxymethylhistidine-containing peptide and that of the protein, the essential imidazole ring is found to be in residue 131. DNase C (92) is the result of a mutation which causes one histidine to be replaced by a proline without any change in the activity. Hans Salnikow and Dagmar Murphy (100) have shown that this change occurs at position 118; the histidine at this position in DNase A is thus not essential for enzymatic activity.

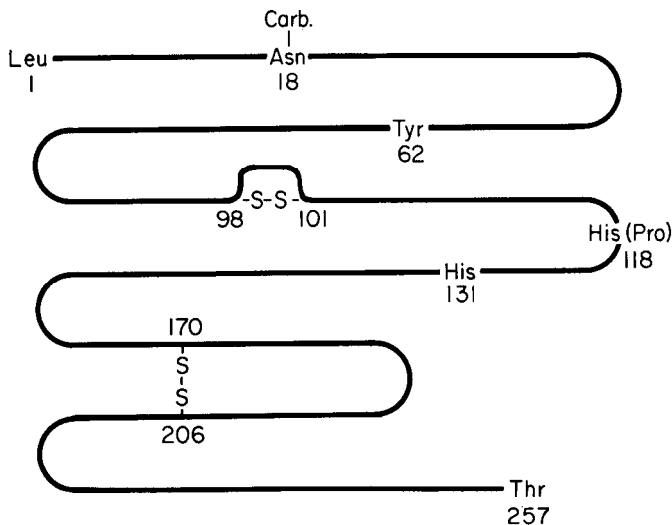


Fig. 11.

Diagram of special features of deoxyribonuclease A (94) and the substitution of Pro for His in deoxyribonuclease C (100).

The two disulfide bonds of deoxyribonuclease possess some unusual properties. Paul Price showed that even without the use of a denaturing agent both bonds are very easily reduced by mercaptans in the absence of calcium to give an inactive product. In the presence of calcium, one bond is stable and one bond is reduced (101) and the product is active. Ta-hsiu Liao has identified the non-essential disulfide bond as the one forming the small loop between residues 98 and 101 (94). When the larger loop, formed by half-cystines 170 and 206, is opened, the activity is lost.

The next step will be the correlation of the chemical evidence with the three-dimensional structure of the enzyme, if X-ray analysis of crystalline DNase A can be successfully accomplished.

CONCLUSION

In the course of studying enzymes of different functions, we have had the pleasure of cooperation with Kenji Takahashi in the identification of a carboxyl group of glutamic acid as part of the active site of ribonuclease T, (102). The essential -SH group and histidine residue of streptococcal proteinase have been studied in collaboration with Stuart Elliott and Teh-yung Liu (103, 104). The esterification of carboxyl groups at the active center of pepsin was explored with T. G. Rajagopalan (105). There is a vast amount of basic information needed on various enzymes before biochemists can explain catalytic action in full detail. Enzyme chemistry today is in a stage of development that bears some similarity to that of organic chemistry at the beginning of this century. At that time there was great activity in documenting the properties of the myriad small organic compounds conceivable by man and nature. Today, in the polypeptide field, the list of determined structures is relatively small. The enzymes that have been studied first are those that can be prepared in gram quantities, such as ribonuclease, trypsin, lysozyme, carboxypeptidase, and subtilisin. The experience in the determination of such structures is leading to ultramicrochemical methods which will extend the range of structural studies to tissue enzymes that are present in very small amounts.

From the knowledge of the structures of a large series of enzymes, underlying principles of how nature designs catalysts for given purposes will evolve. And there will be practical dividends from such research on proteins. One example of research-in-progress can illustrate this possibility. The project developed in the following way: In the course of examining the importance of the three-dimensional configuration of RNase to its activity, George Stark had occasion to dissolve the enzyme in 8 M urea at 40° (106). In one of those experiments the RNase was not active after the urea was removed by dialysis, and it turned out that traces of cyanate in the urea solution had carbamylated the ϵ -NH₂ groups of the enzyme. The chemistry of the subject carries us back to Wöhler's (107) observations on the relationship between ammonium cyanate, cyanate and urea in 1828. In 1970, Anthony Cerami and James Manning (108), two young investigators at the Rockefeller University, undertook to explore, fully on their own initiative, whether traces of cyanate in urea might have a role in the reported beneficial effect of urea on the sickling of erythro-

cytes of individuals carrying hemoglobin S. They have discovered that there is such an effect of cyanate on human erythrocytes, and that it is accompanied by carbamylation of the α -NH₂ groups of the valine residues of the α - and β -chains of hemoglobin S. The knowledge that a relatively simple chemical modification of hemoglobin S can restore nearly normal function to the deficient molecule opens the possibility that a genetic defect in man might be remedied, not by having to change the gene, but by redivatizing the protein.

Such results afford an example of the manner in which one finding leads to another in basic research and ultimately to possible benefits to man. When we consider biochemistry in 1972, it is important to realize how fragmentary is our knowledge of the molecular basis of life. Very few macromolecules can be discussed in the detail with which ribonuclease or hemoglobin can be defined. Such knowledge of structure-function relationships is basic to the rational approach to the intricate synergisms of living systems.

ACKNOWLEDGEMENTS

The researches from our laboratory on ribonuclease and deoxyribonuclease summarized in this lecture have been possible through financial backing by The Rockefeller University, The United States Public Health Service, and The National Science Foundation.

REFERENCES

1. Stein, W. H., and Moore, S. *Scientific American* 204, 81-92, 1961.
2. Jones, W., *Am. J. Physiol.* 52, 203-207, 1920.
3. Dubos, R. J., and Thompson, R. H. S., *J. Biol. Chem.* 124, 501-510, 1938.
4. Kunitz, M., *J. Gen. Physiol.* 24, 15 -32, 1940.
5. Hirs, C. H. W., Stein, W. H., and Moore, S., *J. Amer. Chem. Soc.* 73, 1893, 1951.
6. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.* 200, 493-506, 1953.
7. Paléus, S., and Neilands, J. B., *Acta Chem. Scand.* 4, 1024- 1030, 1950.
8. Crestfield, A. M., Stein, W. H., and Moore, S., *J. Biol. Chem.* 238, 618-621, 1963.
9. Porath, J., and Flodin, P., *Nature* 183, 1657-1659, 1959.
10. Sober, H. A., and Peterson, E. A., *J. Amer. Chem. Soc.* 76, 1711-1712, 1954.
11. Martin, A. J. P., and Porter, R. R., *Biochem. J.* 49, 215-218, 1951.
12. Plummer, T. H., Jr., and Hirs, C. H. W., *J. Biol. Chem.* 238, 1396-1401, 1963.
13. Plummer, T. H., Jr., and Hirs, C. H. W., *J. Biol. Chem.* 239, 2530-2538, 1964.
14. Moore, S., Stein, W. H., and Bergmann, M., *Chem. Rev.* 30, 423 -432, 1942.
15. Martin, A. J. P., and Synge, R. L. M., *Biochem. J.* 35, 1358 - 1368, 1941.
16. Martin, A. J. P., *Ann. Rev. Biochem.* 19, 517-535, 1950.
17. Synge, R. L. M., *Analyst* 71, 256-258, 1946.
18. Moore, S., and Stein, W. H., *J. Biol. Chem.* 176, 367 - 388, 1948.
19. Moore, S., and Stein, W. H., *J. Biol. Chem.* 178, 53 -77, 1949.
20. Stein, W. H., and Moore, S., *J. Biol. Chem.* 178, 79-91, 1949.
21. Stein, W. H., and Moore, S., *J. Biol. Chem.* 176, 337-365, 1948.
22. Moore, S., and Stein, W. H., *J. Biol. Chem.* 192, 663-681, 1951.
23. Moore, S., and Stein, W. H., *J. Biol. Chem.* 211, 893-906; 211, 907-913, 1954.
24. Hirs, C. H. W., Stein, W. H., and Moore, S., *J. Biol. Chem.* 211, 941-950, 1954.
25. Moore, S., Spackman, D. H., and Stein, W. H., *Anal. Chem.* 30, 1185-1 190, 1958.
26. Spackman, D. H., Stein, W. H., and Moore, S., *Anal. Chem.* 30, 1190- 1206, 1958.
27. Spackman, D. H., *Federation Proc.* 22, 244, 1963.

28. Spackman, D. H., In *Methods in Enzymology* (C. H. W. Hirs, Editor), Vol. 11, Academic Press Inc., New York, pp. 3-15, 1967.
29. Moore, S., *J. Biol. Chem.* 243, 6281-6283, 1968.
30. Samejima, K., Dairman, W., and Udenfriend, S., *Anal. Biochem.* 42, 222-236, 1971.
31. Samejima, K., Dairman, W., Stone, J., and Udenfriend, S., *Anal. Biochem.* 42, 237-247, 1971.
32. Weigele, M., De Bernardo, S. L., Teng, J. P., and Leimgruber, W., *J. Amer. Chem. Soc.* 94, 5927-5928, 1972.
33. Moore, S., In *Chemistry and Biology of Peptides* (J. Meienhofer, Editor), Ann Arbor Science Publications, Inc., Ann Arbor, Mich., pp. 629-653, 1972.
34. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.* 195, 669-683, 1952.
35. Him, C. H. W., Moore, S., and Stein, W. H., *J. Amer. Chem. Soc.* 76, 6063-6065, 1954.
36. Manning, J. M., and Moore, S., *J. Biol. Chem.* 243, 5591-5597, 1968.
37. Manning, J. M., *J. Amer. Chem. Soc.* 92, 7449-7454, 1970.
38. Schram, E., Dustin, J. P., Moore, S., and Bigwood, E. J., *Anal. Chim. Acta* 9, 149-161, 1953.
39. Dustin, J. P., Czakowska, C., Moore, S., and Bigwood, E. J., *Anal. Chim. Acta* 9, 256-262, 1953.
40. Stein, W. H., and Moore, S., *J. Biol. Chem.* 211, 915-926, 1954.
41. Stein, W. H., *J. Biol. Chem.* 201, 45-58, 1953.
42. Tallan, H. H., Moore, S., and Stein, W. H., *J. Biol. Chem.* 211, 927-939, 1954.
43. Tallan, H. H., Moore, S., and Stein, W. H., *J. Biol. Chem.* 206, 825-834, 1954.
44. Tallan, H. H., Bella, S. T., Stein, W. H., and Moore, S., *J. Biol. Chem.* 217, 703-708, 1955.
45. Tallan, H. H., Moore, S., and Stein, W. H., *J. Biol. Chem.* 219, 257-264, 1956.
46. Tallan, H. H., Moore, S., and Stein, W. H., *J. Biol. Chem.* 230, 707-716, 1958.
47. Him, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.* 219, 623-642, 1956.
48. Sanger, F., *Science* 129, 1340-1344, 1959.
49. Edman, P., *Acta Chem. Scand.* 10, 761-768, 1956.
50. Hirs, C. H. W., *J. Biol. Chem.* 235, 625-632, 1960.
51. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.* 235, 633-647, 1960.
52. Spackman, D. H., Stein, W. H., and Moore, S., *J. Biol. Chem.* 235, 648-659, 1960.
53. Smyth, D. G., Stein, W. H., and Moore, S., *J. Biol. Chem.* 237, 1845-1850, 1962.
54. Smyth, D. G., Stein, W. H., and Moore, S., *J. Biol. Chem.* 238, 227-234, 1963.
55. Anfinsen, C. B., Redfield, R. R., Choate, W. L., Page, J., and Carroll, W. R., *J. Biol. Chem.* 207, 201-210, 1954.
56. Redfield, R. R., and Anfinsen, C. B., *J. Biol. Chem.* 221, 385-404, 1956.
57. Potts, J. T., Berger, A., Cooke, J., and Anfinsen, C. B., *J. Biol. Chem.* 237, 1851-1855, 1962.
58. Linderstrom-Lang, K., *Lane Medical Lectures*, Stanford University Publications, Vol. 6, 1-115, 1952.
59. Gundlach, H. G., Stein, W. H., and Moore, S., *J. Biol. Chem.* 234, 1754-1760, 1959.
60. Crestfield, A. M., Stein, W. H., and Moore, S., *J. Biol. Chem.* 238, 2413-2420; 238, 2421-2428, 1963.
61. Heinrikson, R. L., *J. Biol. Chem.* 241, 1393-1405, 1966.
62. Him, C. H. W., Halmann, M., and Kycia, J. H., In T. W. Goodwin and I. Lindberg, (Editors), *Biological Structure and Function*, Vol. 1, Academic Press, Inc., New York, p. 41, 1961.
63. Perutz, M. F., *Science* 140, 863-869, 1963.
64. Kendrew, J. C., *Science* 139, 1259-1266, 1963.
65. Kartha, G., Bello, J., and Harker, D., *Nature* 213, 862-865, 1967.
66. Wyckoff, H. W., Hardman, K. D., Allewell, N. M., Inagami, T., Johnson, L. N., and Richards, F. M., *J. Biol. Chem.* 242, 3984-3988, 1967.
67. Richards, F. M., *C. R. Trav. Lab. Carlsberg* 29, 322-328, 329-346, 1955.

68. Richards, F. M., and Vithayathil, P. J., *J. Biol. Chem.* 234, 1459-1465, 1959.
69. Brown, D. M., and Todd, A. R., In *The Nucleic Acids: Chemistry and Biology* (E. Chargaff and J. N. Davidson, Editors), Vol. 1, Academic Press, Inc., New York, p. 409, 1955.
70. Richards, F. M., and Wyckoff, H. G., In *The Enzymes* (P. D. Boyer, Editor), Third Ed., Vol. 4, Academic Press, Inc., New York, pp. 647-806, 1971.
71. Gutte, B., and Merrifield, R. B., *J. Amer. Chem. Soc.* **91**, 501-502, 1969.
72. Gutte, B., and Merrifield, R. B., *J. Biol. Chem.* 246, 1922 - 1941, 1971.
73. Hirschmann, R., Nutt, R. F., Veber, D. F., Vitali, R. A., Varga, S. L., Jacob, T.A., Holly, F. W., and Denkwalter, R. G., *J. Amer. Chem. Soc.* 91, 507-508, 1969.
74. White, F. H., *J. Biol. Chem.* 235, 383-389, 1960.
75. Goldberger, R. F., Epstein, C. J., and Anfinsen, C. B., *J. Biol. Chem.* 328, 628-635, 1963.
76. Anfinsen, C. B., *Les Prix Nobel en 1972*, Stockholm, 1973.
77. Anfinsen, C. B., *J. Biol. Chem.* **221**, 405-412, 1956.
78. Lin, M. C., *J. Biol. Chem.* 245, 6726-6731, 1970.
79. Lin, M. C., Gutte, B., Moore, S., and Merrifield, R. B., *J. Biol. Chem.* 245, 5169-5170, 1970.
80. Lin, M. C., Gutte, B., Caldi, D. G., Moore, S., and Merrifield, R. B., *J. Biol. Chem.* **247**, 4768-4774, 1972.
81. Anfinsen, C. B., Aqvist, S. E. G., Cooke, J. P., and Jonsson, B., *J. Biol. Chem.* 234, 1118-1123, 1959.
82. Beintema, J. J., and Gruber, M., *Biochim. Biophys. Acta* 147, 612 -614, 1967.
83. Jackson, R. L., and Hirs, C. H. W., *J. Biol. Chem.* 245, 637-653, 1970.
84. Phelan, J. J., and Hirs, C. H. W., *J. Biol. Chem.* 245, 654-661, 1970.
85. Laskowski, M., Sr., In *The Enzymes* (P. D. Bayer, Editor), 3. Ed., Vol. 4, Academic Press, Inc., New York, pp. 289 - 3 11, 197 1.
86. Avery, O. T., MacLeod, C. M., and McCarty, M., *J. Exp. Med.* 79, 137 ~ 157, 1944.
87. McCarty, M., and Avery, O. T., *J. Exp. Med.* 83, 89 -96, 1946.
88. McCarty, M., *J. Gen. Physiol.* 29, 123- 139, 1946.
89. Kunitz, M., *J. Gen. Physiol.* 33, 349-362, 1950.
90. Lindberg, U., *Biochemistry* 6, 335 -342, 1967.
91. Price, P. A., Liu, T.-Y., Stein, W. H., and Moore, S., *J. Biol. Chem.* 244, 917-923, 1969.
92. Salnikow, J., Moore, S., and Stein, W. H., *J. Biol. Chem.* 245, 5685-5690, 1970.
93. Salnikow, J., Liao, T.-H., Moore, S., and Stein, W. H., *J. Biol. Chem.*, 248, 1480-1488, 1973.
94. Liao, T.-H., Salnikow, J., Moore, S., and Stein, W. H., *J. Biol. Chem.*, 248 1489-1495, 1973.
95. Gross, E., and Witkop, B., *J. Biol. Chem.* 257, 1856- 1860, 1962.
96. Catley, B. J., Moore, S., and Stein, W. H., *J. Biol. Chem.* 244, 933-936, 1969.
97. Hugli, T. E., and Stein, W. H., *J. Biol. Chem.* 246, 7191-7200, 1971.
98. Riordan, J. F., Sokolovsky, M., and Vallee, B. L., *J. Amer. Chem. Soc.* 88, 4104-4105, 1966.
99. Price, P. A., Moore, S., and Stein, W. H., *J. Biol. Chem.* 244, 924-928, 1969.
100. Salnikow, J., and Murphy, D., *J. Biol. Chem.*, 248, 1499-1501, 1973.
101. Price, P. A., Stein, W. H., and Moore, S., *J. Biol. Chem.* 244, 929-932, 1969.
102. Takahashi, K., Stein, W. H., and Moore, S., *J. Biol. Chem.* 242, 4682-4690, 1967.
103. Liu, T.-Y., Stein, W. H., Moore, S., and Elliott, S. D., *J. Biol. Chem.* 240, 1143 - 1149, 1965.
104. Liu, T.-Y., *J. Biol. Chem.* 242, 4029-4032, 1967.
105. Rajagopalan, T. G., Stein, W. H., and Moore, S., *J. Biol. Chem.* **241**, 4295-3297, 1966.
106. Stark, G. R., Stein, W. H., and Moore, S., *J. Biol. Chem.* 235, 3177-3181, 1960.
107. Wöhler, F., *Pogg. Ann.* **12**, 253-256, 1828.
108. Cerami, A., and Manning, J. M., *Proc. Nat. Acad. Sci. USA* 68, 1180 - 1183, 1971.