*p*H were the most important vasodilating factors. He concluded with the observation that cerebral blood circulation does not seem to play a significant role in initiating the irreversibility of shock.

F. T. Moore (Harvard Medical School) summarized the sessions on the first day with a discussion of the microcirculation and its relevance to man. In his laboratories, the most useful single measurement of total body perfusion is lactate ion concentration. Lactate titer more than 10 mmole/liter is a harbinger of death; its accumulation is more sensitive to low flow state than to anoxia. From 30 to 50 percent of patients who die following shock have pulmonary insufficiency, with pulmonary embolism as a major aspect of the pathogenesis. In closing, Moore emphasized that "peripheral reflections of flow should replace other clinical indices in the monitoring of patients with shock."

E. M. Renkin (Duke University Medical Center) discussed neurogenic factors in shock, pointing out that regulator substances other than the sympathetic adrenergic vasoconstrictors, are still in the "unsolved" category. The failure of adrenergic vasoconstrictors to maintain the normal control of the microcirculation contributes to the irreversibility of shock. One avenue under investigation is the relationship between the β-receptors of the vascular bed and vasodilation. H. Viveros, in Renkin's laboratory, has been able to produce dilation after α-adrenergic blockade; the status of this mechanism as a normal physiological variable has not been clarified.

P-I Brånemark (University of Gothenburg, Sweden) discussed the microvasculature at a resolution level of 1 micron. High resolution microscopic analysis in vivo disclosed that blood cells (singly or in clumps) maintain their shape and function in shock. These findings are in contradiction with some early assumptions on cell integrity to low flow states based upon theoretical models, in vitro experiments, and low-resolution microscopy. The most important conclusion from Brånemark's experiments was that the microcirculatory system of man will return to almost normal function after several hours of reduced blood flow.

E. D. Frank (Harvard Medical School) spoke on traumatic and toxic factors in shock, emphasizing the significant role of toxic substances in the lethal aspect. He stressed the importance of monitoring a wide range of parameters while a patient is in a critical hypotensive state. For example, compensatory mechanisms such as heart rate or hematocrit do not always provide an accurate index of the vitality of the patient. Frank and co-workers were able to prevent shock in many cases as a result of direct measurement of blood volume.

The Workshop of Tracer Techniques, chaired by B. A. Burrows (Boston University School of Medicine), served the purpose of characterizing and evaluating the techniques now in use. Transport of tracers did not necessarily correlate with blood flow. Even xenon-132 clearance, once thought to approximate blood flow, is less valid at high flow rates. Rubidium can be used to measure a fraction of cardiac output if the blood is sampled within 2 minutes after injection. Permeability surface product ratios are flow dependent, and the exchange rate and the site of exchange in the capillary bed varies with the solute.

E. Selkurt (Indiana University School of Medicine) summarized the Workshop on Thermal Conductivity with the opinion that these measurements gave direct insight into metabolic derangements that occur in shock.

The Video Scanning Procedure Workshop, chaired by E. H. Bloch (Western Reserve University School of Medicine), clearly demonstrated how optical images could be enhanced by their conversion into electronic images. The latter procedures can record changes in cellular inclusions, and wavelengths which the eye cannot perceive, all in relation to time.

In E. M. Landis' (Harvard Medical School) workshop on Micro Blood Pressure Measurements, A. C. Guyton (University of Mississippi) reported that by using implanted, perforated capsules he was able to indirectly measure tissue pressure at 6 to 7 mm-Hg.

E. M. Merrill (Massachusetts Institute of Technology), reporting on the Rheology Workshop, presented data that whole blood has a higher viscosity as measured by conventional in vitro systems than in the rat-tail artery techniques. In vessels less than 80 microns in diameter, blood viscosity is irrelevant and different fluid mechanics must be applied. Furthermore, we know very little about blood rheology during low flow states. Merrill reasserted his view that the viscous property of blood is ascribable to the hematocrit and fibrinogen concentration.

B. Zweifach (University of California, San Diego) summarized the conference as follows: (i) Microvasculature is a functional but not homogeneous unit since the structure of the vessels may vary and the filtration coefficient is not constant; (ii) there is no single shock organ with a supersensitive microcirculation; (iii) the question of control (intrinsic and extrinsic) is not resolved; (iv) restoration of central pressure does not necessarily mean a concomitant return of normal transcapillary exchange and other functions of the terminal bed; (v) more attention should be given to the rheological aspects of microcirculation, and to the mechanisms resulting in peripheral shunting; and (vi) the effect of endotoxins in producing severe low flow states requires a great deal more clarification.

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Ribonuclease: Recent Advances

Pancreatic ribonuclease is one of the most intensively studied proteins in nature and probably the most completely characterized enzyme. The primary structure of this enzyme has been known for about 7 years, and it seemed possible to deduce the catalytic mechanism using the amino acid sequence, chemical modification work, and kinetic studies. Unfortunately, juxtaposition of various parts of the polypeptide chain in a catalytically active center could not be inferred from these facts; even recent attempts to do so have failed. Clearly, the three-dimensional structure of the enzyme molecule was needed for characterization of the active center and the mechanism of action. Since December 1966, the three-dimensional structure of ribonuclease A, determined by x-ray diffraction analysis, has been reported from two laboratories and that of subtilisin-treated enzyme (ribonuclease S-protein + S-peptide) reported from a third laboratory. A symposium was held at the State University of New

York at Buffalo, 31 May–1 June 1967, for purposes of comparing these three-dimensional structures and assessing the chemical properties of the enzyme and its mechanism of action in light of the new structural information.

There were three crystallographic contributions: by D. Harker and G. Kartha of Roswell Park Memorial Institute (RPMI), by H. W. Wyckoff and F. M. Richards (Yale), and by C. H. Carlisle (Birkbeck College, London). Harker and Kartha described their crystallization and x-ray diffraction analysis of ribonuclease A; described the isomorphous replacements with heavy atoms used at RPMI for phasing; and presented their model of the enzyme at a resolution of 2 angstroms. C. H. Carlisle, using the same system but a different set of heavy atom derivatives of the protein, briefly compared his lower resolution map with that of Harker *et al.* Carlisle showed several electron density diagrams which differed from those of the RPMI group. These differences were attributed to the lower resolution of the London map; Carlisle explained that these small differences should disappear when their map is at a 2-angstrom resolution. The Yale group described a unique approach to the three-dimensional structure of ribonuclease. Treatment of ribonuclease A with a protease, subtilisin, yields ribonuclease S-protein + S-peptide (the 21 amino acids from the NH_2-terminus). This is crystallized from 75 percent saturated $(NH_4)_2SO_4$ and placed in a diffractometer flow cell where small molecule and heavy atom replacements can occur, thus allowing for the needed phasing atoms and for assay of the ribonuclease S which is enzymatically active, without disturbing the crystal structure. This work has yielded a 3-angstrom resolution model.

In all three x-ray diffraction analyses, the peptide backbone appeared quite similar. A hydrophilic region was connected, by three partial chains, to a hydrophobic region, containing a cystine isolated octapeptide loop; these two regions surround a cleft for substrate. In the Yale model, the S-peptide, not covalently linked to the remaining enzyme, is slightly displaced from its position in ribonuclease A. There is very little α-helix either in ribonuclease A or S crystals. Neither the RPMI group nor the Yale group have sufficient resolution to identify all amino acids. The planar aromatic amino acids, cystine, and methionine are readily identified, but other R groups are more blurred. There are a few differences between the models of Yale and RPMI regarding the exact positions of side chains though there is no difference in identification of side chains since the primary structure is known. Higher resolution x-ray maps were suggested to solve these few discrepancies, but Richards made the point that unconstrained side chains, for example at the periphery of the molecule, would appear blurred even at a 1-angstrom resolution. One important problem that higher resolution maps might solve, however, is the nature and position of atoms in amino acid side chains which interact with pyrimidines and confer specificity to ribonuclease. The crystallographers were unable to tell what changes occurred in enzyme conformation on binding of substrate since all the x-ray work had been done with $H_2PO_4^-$, or nucleotides, or SO_4^{2-} bound to ribonuclease. The answer is important to the mechanism of ribonuclease action. Such a conformational change could provide the needed activation energy for the catalytic reaction. However, the answer may be difficult to obtain since crystallization of anion-free ribonuclease has not been achieved and such powerful approaches to isomorphous replacement as those used by the Yale group would not be directly applicable in x-ray diffractometry of substrate-free ribonuclease.

As a bridge between the crystallography and work on the mechanism of ribonuclease action, H. A. Scheraga described his current work on computing of most probable structures of macromolecules based on the assumption of minimum total energy in the molecule of interest with approximations of the contributions from different types of interaction. Results of these calculations are reflected in bond angles.

A plot of the H-C^{α}-C^1 bond angles versus H-C^{α}-N bond angles gives an energy contour map which can be used in defining the total energy of a conformation as well as the handedness of the macromolecule. Random access to the entire energy map is employed for the purpose of avoiding determination of incorrect energy minima among the many inflections. Such calculations of most probable conformation have been carried out on gramicidin-S, on oxytocin, on ribonuclease S-peptide, and the octapeptide loop of ribonuclease A. Results on S-peptide showed considerable similarity to the crystallographic models. At present, en-

zymes of known sequence, such as lysozyme, are being calculated.

If calculated conformations generated by the computer agree well with those determined by diffractometry, then the computer approach will enable relatively rapid synthesis of three-dimensional structures of macromolecules whose monomer sequences are known. However, a healthy skepticism seems warranted, as noted in discussion of this paper by G. H. Ramachandran, until Scheraga is able to solve the known structures of a number of large heteropolymers. On the other hand, if the minimal energy computations do have predictive value, a major advance will have been made in determining heteropolymer structure and in making correlations between structure and function.

The greatest number of contributions to the symposium concerned studies of ribonuclease chemistry and mechanism of action. J. Bello correlated a large body of chemical evidence with the RPMI model. Thus, the involvement of histidines-12, and -119, and lysine-41 in the active center was confirmed in the model by the close proximity of these residues to the $H_2PO_4^-$ in the substrate cleft; the participation of these residues in ribonuclease A binding and catalysis was discussed by a number of contributors. The dimerization of the enzyme by looping of two S-peptide moieties to two adjacent enzyme molecules is seen to be feasible from the model; A. M. Crestfield presented detailed analysis of his active center work based on hybrid dimers. The presence and identification of easily titratable and buried tyrosines in ribonuclease could also be explained in terms of the model. Finally, the anionic stabilization of the enzyme to urea denaturation was correlated with the model where a number of cationic sites from various parts of the polypeptide chain occur near the substrate cleft.

Another approach to the chemical study of the active center as well as the first systematic study of amino acid replacement in an enzyme was reported by F. Finn and K. Hofmann. These workers have synthesized the S-peptide and a number of S-peptide analogs and have tested enzymic activity after mixing with S-protein. The first 13 amino acids in the S-peptide (S-peptide-1-13), when mixed with S-protein in a ratio of 200 to 1, results in full enzyme activity. A ratio of 1 to 1 of S-peptide-1-14 and S-protein is fully active. Addition of amino acids 15-21, in order, to the S-peptide did not increase activity, thus indicating that these amino acids are not involved either in the binding of S-peptide to S-protein or in the catalytic activity of the complex. Methionine-13 is involved in one or both of these functions since conversion of this residue to sulfone or sulfoxide reduces enzyme activity toward RNA but not toward pyrimidine-2′,3′-cyclic phosphate substrate. All changes in histidine-12 abolish enzyme activity, thus confirming results of others. Replacement of arginine-10 with ornithine or lysine does not affect activity nor does replacement of glutamic-9 with lysine (bovine versus rat ribonuclease). Phenylalanine-8 can be replaced with tyrosine without affecting activity, but the enzyme is inactive when this residue is replaced by nonaromatic amino acids. This type of systematic study of a synthetically accessible polypeptide essential to enzyme activity is a highly informative and powerful approach in investigating enzyme mechanisms. Its general utility remains in doubt until other enzymes are found which can be converted into two reversibly dissociable pieces, each of which is essential for enzyme activity and one of which is small enough to be synthesized chemically.

G. G. Hammes described his work on perturbation analysis of ribonuclease reactions with methods involving temperature-jump and stopped-flow-temperature-jump. With very rapid perturbation and spectroscopic analysis of relaxation to a steady state, enzymic reactions as short as 1 microsecond can be analyzed and the number of intermediates in the reaction observed. Hammes has found five different relaxation times between pyrimidine-2′,3′-cyclic phosphate and the 3′-phosphomonoester, indicating at least five intermediate enzyme-substrate and enzyme-product complexes. Also, studying relaxation times as a function of *p*H, three ionizable groups at, or at least influencing, the active center were identified with *pK*'s of 5, 6, and 6.7, probably corresponding to three histidines.

From the known chemical reactivity of ribonuclease, the kinetic studies, and the enzyme model, it was possible for Hammes to deduce a plausible, if highly tentative, molecular mechanism for transesterification and -2′,3′-cyclic phosphate hydrolysis. Binding was the first step, followed by two separable enzyme conformational changes, and then

by dissociation. After binding, protonation of two enzyme bases, histidines -12, and -119, occurs in the transesterification step. A reversal of the process follows, water replacing the expelled 5′-OH with polynucleotides, thus hydrolyzing the -2′3′-cyclic phosphate in the overall general acid-base catalysis. B. R. Rabin presented a similar hypothesis, based on the same general information. His theory differed only in that one of the two histidines is protonated and the other unprotonated at the beginning with the sequential transesterification and hydrolysis involving deprotonation of the first histidine and protonation of the second, followed by a direct reversal. The other amino acid residues known to be involved in the active center are presumably involved in the binding of substrate. There seemed little to choose between these two similar alternative mechanisms except, as noted by Hammes, that the former was esthetically more pleasing and presented no difficulty of low rate constants due to particular histidines being protonated or unprotonated initially. Rabin countered with the observation that the Hammes mechanism did not involve water binding or activation which are thought, by some, to occur in ribonuclease.

A third alternative mechanism was presented by H. Witzel based on his studies of the binding and hydrolysis of a large number of base analog nucleoside-2′,3′-cyclic phosphates. The pyrimidine $-N^3-C^2=O$ appears to be required and Witzel hypothesized that the carbonyl functions as the general base regenerated by exchange with water. The major function of the enzyme in this mechanism is to stabilize transition-state pentacovalent phosphorus dianion at all stages of the reaction, with the pyrimidine ring supplying all reactive requirements. Though no evidence against this mechanism was presented, it did not appear to explain the need for all the known amino acid residues of the active center region.

E. A. Barnard showed that bromoacetate alkylation of one methionine (apparently methionine-29) occurs sharply at *p*H 6.2. Since the methionine-29 side-chain is buried in the ribonuclease A (but not S) model, this increased reactivity was suggested as being associated with a histidine ionization and conformational change. Barnard felt that the latter might be responsible for the *p*H 6 relaxation time observed by Hammes. This conformational change and the changed methionine reactivity

could be explained by movement of the S-peptide region.

There was much discussion of the mechanisms of ribonuclease A but no general consensus on which, if any, of those presented was most consistent with the model. Further work with close correlation to the three-dimensional structure will be required to resolve this problem.

Several papers were presented concerning derivatives of ribonuclease A and ribonucleases isolated from other sources. S. Beychok presented a study of the circular dichroic (CD) spectra of normal, reduced, and reoxidized ribonuclease A. The original work of Anfinsen on spontaneous recovery of enzyme activity after air reoxidation of inactive, reduced ribonuclease A appeared to represent spontaneous recovery of native conformation, and further suggested that all higher structural orders were completely dependent on the primary structure. The near-ultraviolet CD spectrum of ribonuclease A contains two bands (λ_{max} 240 mμ and 275 mμ) initially assigned to tyrosine. Further work showed the 275-mμ band to be complex, possibly including cystine disulfide bonds which may have two screw senses differentiated by CD. On reduction of the disulfides, the 275-mμ band is markedly changed. After air reoxidation, when about 90 percent of initial enzyme activity is recovered, the CD spectrum is still different from the native enzyme unless the reoxidation is carried out in the presence of 2-mercaptoethanol. This evidence for a different conformation in reoxidized ribonuclease suggests that proteins do not necessarily assume a unique lowest-energy state and, further, that a single unique conformation may not be a necessary attribute of an active enzyme. These conclusions were sharply contested by H. A. Scheraga and by R. F. Goldberger. These findings may have important theoretical implications.

E. A. Barnard, M. Gold, and E. N. Zendzian have isolated and partially purified exocrine ribonucleases from a large number of vertebrates. They have assessed the activity per unit weight of pancreas and some chemical properties of these enzymes. Surprisingly, only certain groups, for example ruminants, had much pancreatic ribonuclease. As a result of this work, a hypothesis was presented concerning the biological significance of pancreatic ribonucleases. Reactivity and substrate specificity studies of ribonucleases from the main vertebrate classes showed the

enzymes to have a number of active center similarities, thus suggesting that pancreatic enzymes are homologous.

Studies of porcine pancreatic ribonuclease were presented by C. H. W. Hirs and V. N. Reinhold. They have purified the enzyme but have found that it is molecularly inhomogeneous. These ribonucleases are glycoproteins and the inhomogeneity arises from a variable amount of polysaccharide around a constant protein core. All these enzymes contain 125 amino acids and have eight half-cystines. The protein core has other compositional similarities to bovine ribonuclease A.

K. Takahashi contrasted the primary structure and early mechanistic studies of ribonuclease T_1 with A. There is little analogy between primary structures despite highly analogous transesterification and -2′,3′-cyclic phosphate hydrolysis (of guanosine-3′-phosphodiesters). The single lysine in T_1 is not required for activity. Reaction with bromoacetate did inactivate the enzyme, but histidine was not alkylated; only esterification of glutamate-58 occurred. Thus, present evidence suggests that the two ribonucleases may have different catalytic mechanisms. It appeared that, despite the recent major advances in knowledge of the structure of ribonuclease A and the mechanistic implications of these findings, there is still a great deal of work to be done on the structure and mechanism of other ribonucleases and considerable informative work to be done on ribonuclease A as well.

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Cell Synchrony

Recent advances in the synchronization of cell division was the topic at the 2nd International Conference on Cell Synchrony, held 27–29 April 1967, in Oak Ridge, Tennessee. The program was divided into five sessions: (i) Genetic studies in cell synchrony (H. O. Halvorson, University of Wisconsin, chairman); (ii) Developmental aspects of cell synchrony (D. T. Lindsay, Uni-