Des-lysyl Glutamyl and Des-lysyl Pyroglutamyl Ribonucleases. I. Isolation and Characterization*

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ABSTRACT: Two structural homologs of bovine pancreatic ribonuclease A have been isolated from a crystallized preparation by countercurrent distribution. Chemical and physical studies indicate their structures to be des-lysyl glutamyl and des-lysyl pyroglutamyl ribonucleases. Also their properties suggest that the separation by countercurrent distribution depends on the relative ease with which they undergo reversible conformational changes in the two phases of the solvent system.

Previous studies from this laboratory have shown that solvent systems composed of low molecular weight alcohols, ammonium sulfate, and water are useful for countercurrent distribution of several globular proteins, such as insulins, ribonuclease, lysozyme, and plasma albumins (Harfenist and Craig, 1952; King and Craig, 1958; King *et al.*, 1960). However, little is known concerning the molecular parameters which influence the separations obtained. In order to shed some light on the basis of these separations, we have undertaken a detailed study of the physicochemical properties of two anomalous forms of bovine pancreatic ribonucleases. These results are to be presented in this paper.

The two anomalous ribonucleases were isolated from an unusual crystallized preparation by countercurrent distribution in a system of ethanol, ammonium sulfate, and water. Both enzymes lack the amino-terminal lysyl residue normally present in ribonuclease A, and instead they have glutamic acid, the second residue in ribonuclease A, at their amino termini (Anfinsen *et al.*, 1954; Hirs *et al.*, 1960). In one of them the glutamyl residue has cyclized in the form of a pyroglutamic acid. Consideration of the physicochemical properties of the des-lysyl ribonucleases suggests that the separation of these three homologous proteins is related to their tendencies to undergo reversible conformational changes in the two phases of the solvent system.

Experimental

Countercurrent distribution of crystalline bovine pancreatic ribonuclease (Armour lot 381-062) was carried out in a system containing, by weight, 16.5% $(NH_4)_2SO_4$, 57.6% H_2O, and 25.9% C_2H_5OH (King and Craig, 1958). A distribution train of 1000 tubes with 2-ml capacity was used. The protein was recovered from the distribution fractions as described previously. To ensure the complete removal of salts, samples were desalted on mixed bed ion-exchange columns (Dintzis, 1952). *Chromatographic analyses* of the recovered proteins were carried out on IRC-50 columns (Hirs *et al.*, 1953). *Amino acid analyses* were performed according to the published procedures (Moore and Stein, 1963; Spackman *et al.*, 1958).

Quantitative determination of the amino terminal residues was carried out with the native and the performic acid–oxidized samples (Hirs, 1956). The oxidized sample (1–2 μmoles) in 2 ml of 0.2 M $NaHCO_3$-Na_2CO_3 buffer (*p*H 9.3) was allowed to react with 4 ml of 0.007 M 1-fluoro-2,4-dinitrobenzene in ethanol at 40° for 6 hours. After acidification and ether washing, the dinitrophenyl protein was hydrolyzed in 2.5 ml of constant-boiling HCl at 110° for 10 hours in a sealed and evacuated tube. The hydrolysate, after concentration to dryness, was used directly for countercurrent distribution in a system of $CHCl_3$–HOAc–0.1 N HCl (Hausmann *et al.*, 1955). An apparatus of 2-ml capacity of each phase was used. After 250 transfers, the separated dinitrophenyl amino acids were estimated by triangular integration of the calculated curves, using molar extinctions obtained with authentic samples in the solvent system used.

Weight-average molecular weights of the samples were determined by the short column equilibrium method (Yphantis, 1964) in a buffer of 0.10 M NaCl, 0.004 M KH_2PO_4, and 0.035 M K_2HPO_4 (*p*H 7.70). *Ultraviolet spectra and rotatory dispersions* were measured with 0.1 and 1.0% protein solutions in solvents of 0.1 M KCl and deionized water, respectively. A Cary Model 14PM spectrophotometer and a Rudolph spectrophotometric polarimeter were used. Samples for these determinations were corrected for their moisture contents.

The electrophoretic properties of the fractions were compared by zone electrophoresis on cellulose acetate paper (Oxoid Division of OXO Ltd., London), using a moist chamber technique (Kohn, 1957). Runs were made in the following buffers: 0.1 M sodium acetate (*p*H 5.3), 0.05 M sodium phosphate (*p*H 6.5), and 1.0 M formic acid containing 0.1 M NaCl (*p*H 1.75). To avoid excessive heating of the paper during a 9–10 hour run, a voltage

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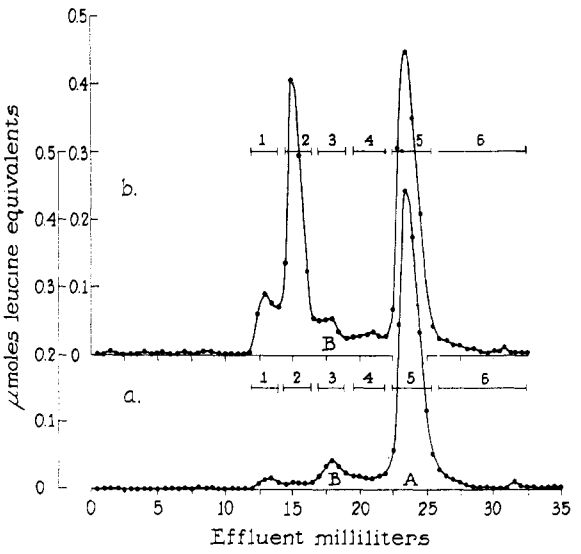

FIGURE 1: Chromatograms of commercial ribonuclease samples on a 30- × 0.9-cm column of IRC-50, in 0.2 M sodium phosphate buffer, *p*H 6.47. The ordinate indicates ninhydrin color (Hirs *et al.*, 1953). (a) Armour lot 381-059, 3.89 mg; (b) Armour lot 381-062, 5.81 mg.

gradient of 3–5v/cm was used. The proteins (*ca.* 20 μg) were located by staining the wet strips with 0.2% Ponceau S in 3% trichloroacetic acid.

Results

The commercial crystalline sample of ribonuclease (Armour lot 381-062) was observed earlier by Tanford and Hauenstein (1956; Tanford, 1962) to be different from the often studied lot (Armour lot 381-059). The 381-062 sample was found to be rich in chromatographic components less retarded than ribonuclease A, but the 381-059 sample contains little, if any of such components. These differences are shown in the comparative chromatograms (Figure 1). The chromatograms also show that the anomalous peak in the 381-062 sample is distinct from the naturally occurring B component (Martin and Porter, 1951; Hirs *et al.*, 1953). The B component has been shown recently to be a carbohydrate-containing derivative of ribonuclease A (Plummer and Hirs, 1963, 1964). In Table I are listed the compositions of these two samples as determined by chromatography.

Fractionation of the 381-062 sample by countercurrent distribution was performed in a solvent system of ethanol, water, and ammonium sulfate (King and Craig, 1958). After 1850 transfers three distinct bands with partition ratios (K) of 0.8, 1.0, and 1.5 were present (Figure 2a). The band with a K value of 1.5 was already eluted from the distribution train and was superimposed on a high base line. The high base line was due to a slight turbidity of the solution and represented very little protein. After removal of the unknown material in tubes 500–600, the train was set for recycling. At 3200 transfers (Figure 2b), the bands with K values of 0.8 and 1.0 were completely resolved. The fraction of the total sample represented by each of the three major bands is given in Table I. These figures are averages of four different experiments with sample sizes ranging from 0.3 to 2.0 g. The actual isolated yields were slightly lower due to unavoidable losses.

The cuts from the distribution pattern were analyzed by chromatography on Amberlite IRC-50, and the results are shown on the right-hand side of Figure 2. The band with a K value of 0.8 is ribonuclease A, in agreement with our earlier study. The bands with K values of 1.0 and 1.5 both emerged at the same effluent volume as that of the anomalous peak in Figure 1. In an experiment not shown, a single symmetrical peak was observed when an artificial mixture of these two components was chromatographed.

The data given in Table I show that the band with a

TABLE I: Relative Amounts of Different Components in Two Commercial Ribonuclease Samples.[a]

Chromatography			Countercurrent Distribution	
Effluent Zone (Figure 1)	Per Cent of Total (381-062)	Per Cent of Total (381-059)	Band (Figure 2)	Per Cent of Total (381-062)
1	8.9	2.4	K = 0.8	46 ± 2
2	30.5	2.3	K = 1.0	20 ± 0.5
3	6.4	7.8	K = 1.5	13 ± 2
4	5.2	5.7		
5	46.0	74.0		
6	3.0	7.0		
Total	100.0	99.2		79

[a] Calculated on the assumption that all components have identical ninhydrin color yields and ultraviolet absorbancies.

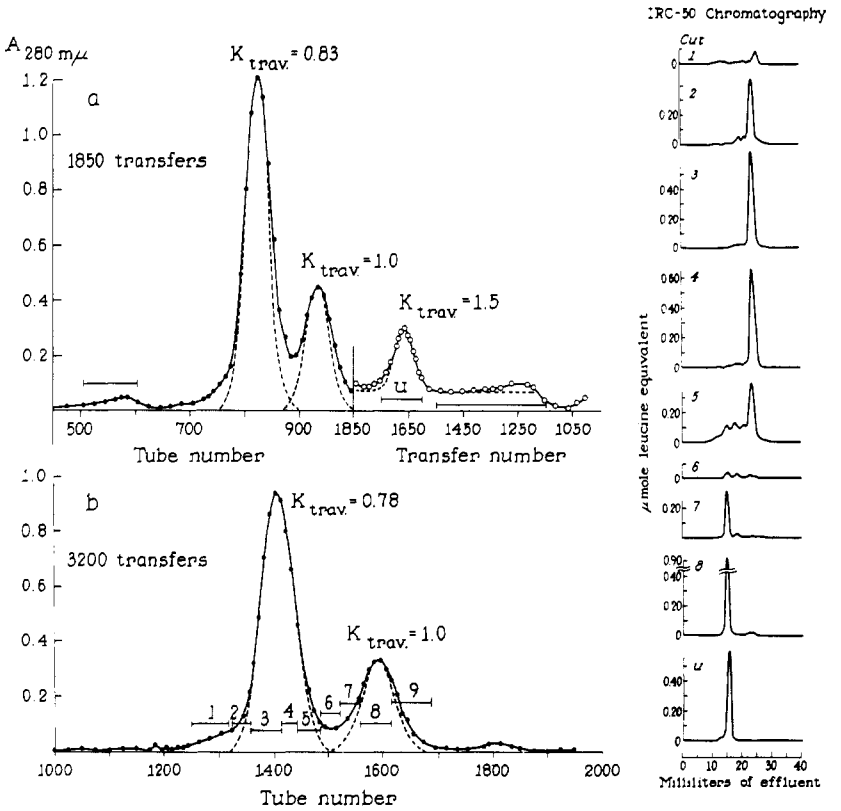

FIGURE 2: Pattern obtained by countercurrent distribution of a 952-mg sample of ribonuclease from Armour lot 381-062 in a two-phase system containing ammonium sulfate, ethanol, and water. The ordinate indicates absorbancy at 280 mμ. Solid circles indicate fractions within the distribution train and the open circles refer to effluent fractions. The dashed lines are the theoretical curves calculated for ideal solutes traveling with the partition coefficients indicated. (a) Pattern after 1850 transfers; (b) pattern after recycling of the train to 3200 transfers. At the right are the effluent ninhydrin profiles obtained upon chromatography of samples (3–5 mg) from the various distribution cuts on a 30- × 0.9-cm column of IRC-50.

K value of 0.8 accounts completely for the ribonuclease A in the sample, a figure of 46% from countercurrent distribution or chromatography. The anomalous peak in the 381-062 sample represents 31% of the total ninhydrin color and accounts almost quantitatively for the bands with *K* values of 1.0 and 1.5 which are seen to comprise, collectively, about 33% of the sample. This excellent quantitative agreement between the countercurrent distribution and chromatography data establishes unequivocally that the components with *K* values of 1.0 and 1.5 are contained within the anomalous peak in the original sample and that they are not transformation products formed during countercurrent distribution.

The amino acid analyses recorded in Table II indicate that the ribonucleases with *K* values of 1.0 and 1.5 both contain one less residue of lysine than does ribonuclease A. No other differences are apparent, and the composition of all three ribonucleases are otherwise in good agreement with the integral values reported for ribonuclease A (Hirs *et al.*, 1956, 1960).

Spots corresponding to bis-DNP-lysine and DNP-glutamic acid were identified in paper chromatograms (Levy, 1955) of ether extracts of the hydrolysates of DNP derivatives of ribonuclease A and the $K = 1.0$ ribonuclease, respectively. No α-DNP amino acids were identified for the K = 1.5 ribonuclease. For quantitative estimation, the hydrolysates of the DNP-proteins were fractionated directly by countercurrent distribution (Figure 3). Integration of the appropriate peaks yielded the values in Table III. The identities of the materials in the peaks were cross checked by paper chromatography.

In Table IV are collected some of the physical properties of these three ribonucleases. The three ribonucleases have apparently identical molecular weights, but the values reported are all 10 to 14% higher than the formula weight of ribonuclease A. This is due to aggregation, for the same sample of ribonuclease A examined five months earlier had yielded a value of 13,700 ± 3% (Yphantis, 1960). The ultraviolet spectral data in Table IV indicate that the three ribonucleases have the same number of hydrogen-bonded tyrosyl residues (Shugar, 1952; Harrington and Schellman, 1956; Her-

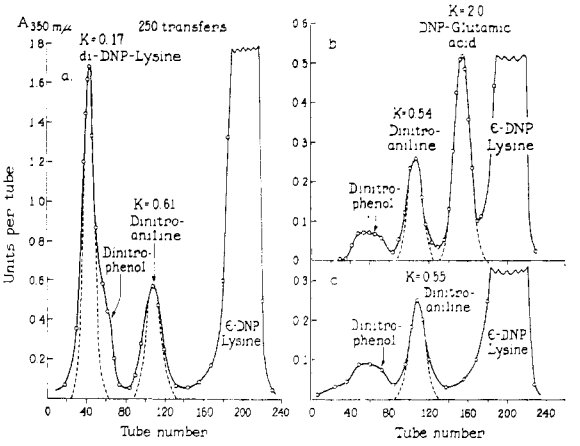

FIGURE 3: Identification of DNP-amino acids in acid hydrolysates of the derivatives obtained by reaction of performic acid–oxidized ribonuclease samples with fluorodinitrobenzene. The hydrolysates were fractionated by countercurrent distribution for 250 transfers in a system containing chloroform, acetic acid, and 0.1 N HCl. The ordinate indicates the total absorbancy units per tube. The dashed lines are the theoretical curves calculated for ideal solute traveling with the partition coefficients indicated. (a) K = 0.80 ribonuclease (ribonuclease A); (b) K = 1.0 ribonuclease; (c) K = 1.5 ribonuclease.

TABLE II: Amino Acid Composition of Ribonuclease Fractions.

Amino Acid	Ribonuclease A[a] (integral residues)	Number of Residues[b,c] (24 hour hydrolysis) K = 0.78	K = 1.0	K = 1.5
Lysine	10	*10.5*	*9.14*	*9.29*
Histidine	4	3.82	3.52	3.65
Ammonia	17	16.7	16.7	16.7
Arginine	4	3.85	3.88	3.64
Aspartic acid	15	14.9	15.0	15.0
Threonine	10	10.2	10.2	10.2
Serine	15	14.8	14.6	14.6
Glutamic acid	12	11.3	11.3	11.8
Proline	4	3.82	4.16	4.11
Glycine	3	3.10	3.03	3.18
Alanine	12	12.0	12.0	12.0
Half-cystine	8	7.73	8.20	7.71
Valine	9	7.29	7.90	7.90
Methionine	4	3.98	3.84	3.69
Isoleucine	3	1.83	1.70	1.80
Leucine	2	2.11	2.02	2.15
Tyrosine	6	5.78	5.78	5.72
Phenylalanine	3	2.97	3.07	2.95

[a] According to Hirs *et al.* (1956). [b] Expressed relative to alanine, assuming a value of 12.0 for that residue. Corrections for hydrolytic loss have been applied as follows: threonine, half-cystine, and tyrosine, 5%; serine, 10%. [c] Higher values for isoleucine and valine were obtained with 72-hour hydrolysates.

mans and Scheraga, 1961), as all have comparable absorption maxima at 277.5 mμ. The spectra of the three ribonucleases are almost superimposable with the exception that the K = 1.0 component has a significantly lower value of E_{min} which is also slightly displaced toward shorter wavelengths. The optical rotatory constants in Table IV also indicate that the three proteins have similar conformations in water. The specific rotations reported here are in good agreement with those obtained by Harrington and Schellman (1956).

Electrophoresis on cellulose acetate paper revealed that the K = 1.0 and K = 1.5 ribonucleases have equal migratory properties at *p*H 5.3 and 6.5 but that both are slower migrating than ribonuclease A. At *p*H 1.75, the K = 1.5 ribonuclease is slower migrating than ribonuclease A, and the K = 1.0 component has a mobility intermediate of those of the other two. Only a single elongated zone was observed on electrophoresis of an artificial mixture of these three components. These electrophoretic differences demonstate that the basicity of the three proteins decreases in approximately equal increments from the most basic, ribonuclease A, to the least basic, K = 1.5 ribonuclease.

The results of amino acid analyses, amino-terminal end-group determinations, and physicochemical measurements are mutually compatible with the hypothesis that the K = 1.0 and K = 1.5 ribonucleases are des-lysyl forms of ribonuclease A. As the amino terminal sequence of ribonuclease A is known to be Lys-Glu-Thr-Ala-Ala-Ala----(Anfinsen *et al.*, 1954; Hirs *et al.*, 1960), the isolation of DNP-glutamic acid from the K = 1.0 ribonuclease indicates that this molecule has somehow suffered a deletion of the lysine residue normally present at the amino terminus. Therefore, the K = 1.0 ribonuclease will be designated des-lysyl glutamyl ribonuclease.

The absence of an end group in the K = 1.5 ribonuclease can be explained most readily by the hypothesis that this molecule is a des-lysyl ribonuclease in which the amino-terminal glutamyl residue has undergone cyclization to form a pyroglutamyl residue. This hypothesis is in agreement with the observed electrophoretic differences of the two des-lysyl ribonucleases, *viz.*, the separation of the K = 1.0 and K = 1.5 ribonucleases is effective at *p*H 1.75 but not at *p*H 5.3 or 6.5. Hence, the K = 1.5 component will be called deslysyl pyroglutamyl ribonuclease. The proposed structures are shown later to be correct by degradative studies. These studies as well as the enzymatic properties and conformational stabilities of the des-lysyl ribonucleases are given in the accompanying papers (Eaker *et al.*, 1965a,b).

Discussion

An obvious explanation for the separation of the three

TABLE III: Estimation of DNP-End Groups of Ribonuclease Fractions.

DNP Derivative	μmoles Hydrolyzed	μmole Recovered[a] Bis-DNP-Lys	μmole Recovered[a] DNP-Glu	Yield[b] (cor.)
Native $K = 0.8$	0.624	0.40		0.67
Oxidized $K = 0.8$	1.70	0.90		0.54
Native $K = 1.0$	0.85	0.04	0.442	0.73
Oxidized $K = 1.0$	1.05	0.05	0.675	0.90
Native $K = 1.5$	0.76	0.04	0	
Oxidized $K = 1.5$	0.92	0.05	0	
DNP-glutamic acid	0.97		0.69	
Bis-DNP-lysine	0.483	0.475		

[a] The extinction coefficients of authentic samples of DNP-glutamic acid and bis-DNP-lysine were determined in the lower phase of the countercurrent distribution system to be 16,200 and 32,200, respectively. [b] Yields were corrected for decomposition. The correction factors were established by a control experiment in which DNP-glutamic acid and bis-DNP-lysine were exposed to the same hydrolytic conditions as the DNP-proteins.

TABLE IV: Physical Properties of Ribonuclease Fractions.

Fraction	Molecular Weight[a] (× 10^3)	Rotatory Dispersion[b] λ_c (mμ)	Rotatory Dispersion[b] $[\alpha]_D^{24}$	Ultraviolet Spectral Data[c] λ_{max} (mμ)	λ_{min} (mμ)	A_{max}/A_{min}	$A_{280m\mu}^{1cm}$ (1%)
$K = 0.8$	15.6 ± 0.5	233 ± 2	−72.2	277.5	255	2.03	6.98
$K = 1.0$	15.5 ± 0.5	233 ± 2	−72.8	277.5	253	2.50	6.84
$K = 1.5$	14.8 ± 0.5	233 ± 2	−71.3	277.5	255	2.05	6.96

[a] Determined after storage of lyophilized samples for 5 months. See text. [b] Determined with desalted samples in deionized water. [c] Measured with desalted samples in 0.1 M KCl.

homologous ribonucleases by countercurrent distribution might be their differences in polarity. At *p*H 5.5 of the solvent system, the glutamyl enzyme ($K = 1.0$) has one less positive charge than ribonuclease A ($K = 0.8$), and the pyroglutamyl enzyme ($K = 1.5$) has two less positive charges as well as one less negative charge. Thus the increased tendencies of the des-lysyl enzymes to partition into the organic solvent rich upper phase rather than into the water-rich lower phase would appear to be correlated with their decreasing polar characters. However, several observations indicate that this could not be the decisive factor.

For example, if the polar character of the solute were of importance, one would expect the partition coefficient to vary with its degree of ionization. The partition coefficient of ribonuclease A in this solvent system was 0.78 at *p*H 3.4, 0.80 at *p*H 5.5, and did not deviate significantly until *p*H 7.2 where it had increased to 1.5 (King and Craig, 1958). The latter increase might be explained as a consequence of change in polarity because of the discharge of some of the imidazolium ions, but the constancy between *p*H 3.4 and 5.5 is not interpretable in this manner because this is a region where polarity is changing due to the ionization of carboxyl groups.

An alternative explanation for the differences in partition coefficients of these three homologous proteins is that the protein can take up a unique conformation in each solvent phase, and that the three homologs differ in their ability to undergo this reversible transition. Experiments to be reported in the accompanying paper (Eaker *et al.*, 1965b) are consistent with the thesis that the des-lysyl ribonucleases differ from ribonuclease A in their conformational stabilities although the three homologs have closely similar conformations in water. The differences are shown by the increased susceptibilities of the des-lysyl ribonucleases to trypsin digestion in contrast to the resistance of ribonuclease A. The conformational similarities are indicated by the similar optical rotatory dispersion data and ultraviolet spectra. These data therefore suggest that the des-lysyl ribonucleases can undergo conformational changes more readily than ribonuclease A.

Evidence on the reversible conformational transitions of ribonuclease molecules in water–organic solvent

mixtures came from the studies of Weber and Tanford (1959). These authors found that the conformation of ribonuclease undergoes at least two transitions as the composition of a mixture of chloroethanol–water is changed. The first and the second transitions begin at about 10 and 30% chloroethanol concentrations, respectively. These reversible transitions are believed to be related to changes in the hydrophobic and the electrostatic interactions of the molecule in different solvent environments (Singer, 1962). Since the upper and the lower phases of the solvent system used for countercurrent distribution contains, respectively, 34% C_2H_5OH, 56% H_2O, 10% $(NH_4)_2SO_4$, and 16% C_2H_5OH, 60% H_2O, 24% $(NH_4)_2SO_4$ (King and Craig, 1958), it might then be expected that similar reversible conformational changes occur on partitioning of ribonuclease in these two phases.

The anion-binding effect of ribonuclease probably also plays a role in the present separation, as the *p*H dependence of the partition coefficient of ribonuclease A follows closely its known sulfate binding capacity which is 1.4 and 2.3 moles of ions bound at *p*H 6.8 and 4.5, respectively (Saroff and Carroll, 1962). The increased partition coefficients of the des-lysyl ribonucleases follow this same trend because they are found to have decreased affinities for phosphate ions and presumably they will have decreased affinities for sulfate ions too. Indications of their decreased affinities for phosphate ions came from the trypsin digestion experiments to be reported in the accompanying paper.

Further evidence on the reversible conformational changes of ribonuclease came from the finding that an isoionic preparation of ribonuclease A has a partition coefficient of 1.0 in this solvent system (Craig *et al.*, 1963). This sample of ribonuclease has a conformation different from other preparations of the same protein, as it has a slower rate of dialysis. The isoionic sample contains less than 0.5 mole of bound phosphate while the other samples studied usually contain more than 1 mole of bound sulfate or phosphate ions. The conformation of the isoionic ribonuclease was found to be metastable in the solvent system, as it gradually reverts to the normal form with a partition coefficient of 0.80 during countercurrent distribution.

When the above body of information is considered together, it is almost certain that the partition of ribonuclease molecules is associated with the process of reversible conformational changes of the solute in the two phases of the system, and the partition coefficient of ribonuclease is an over-all measure of its electrostatic interactions and of at least three interrelated equilibria, the binding affinity of the solute for sulfate ions and the tendencies of the ion-free and sulfate-bound ribonucleases to undergo the reversible transitions. The effective separation of the three homologous ribonucleases is a result of the combination of these factors.

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