the secondary products of oxidation (2).

Verma and Prabhakar (9) have shown that the peroxide values in oxidizing refined safflower oil are similar at low and high a_W at each storage period. The unusual low peroxide values at high a_W, therefore, can be attributed to some components like phospholipids present in the raw peanut oil (degummed peanut oil has been found to oxidize rapidly at high water activities). Reports on antioxidant and synergistic effects of phospholipids are conflicting (10,11) and the information on the role of phospholipids in oils at different a_W is scanty. These studies indicate that it would be better to trade the raw oils at high a_W in the international markets as the rate of peroxide formation is minimum at high a_W.

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Protein Conformations and Their Stability

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ABSTRACT

Our understanding of the conformations of proteins and their stability has increased substantially in recent years. A reaction of considerable interest is native (N) ⇌ denatured (D) where N is the globular, native state of the protein which is now well defined as a result of numerous structural determinations by X-ray diffraction studies, and D represents unfolded, denatured states of the protein whose structure depends on the denaturant used to promote unfolding. Through experimental studies much is known about the kinetics, thermodynamics, and mechanism of this reaction. For example, it is known that the free energy change for this reaction under physiological conditions, ΔG_D, is between 3 and 15 kcal/mol for a fairly wide range of globular proteins. Thus, the globular conformation which is absolutely essential for the biological function is only marginally stable. In addition, these ΔG_D values are remarkably sensitive to small changes in the structure of the protein. It has been shown that single amino acid substitutions can dramatically increase or decrease ΔG_D values and some substitutions surely lead to unfolding of the polypeptide chain. Most chemical alterations in the structure of a protein, e.g., cleavage of a peptide bond, or modification of an amino acid side chain, lead to decreases, often sizable, in the conformational stability. The remarkably low conformational stability of globular proteins is important, in part, because many properties of the protein, e.g., solubility and proteolytic digestibility, change substantially when the protein unfolds. Recent developments in these areas of interest to protein chemists and food scientists are illustrated and discussed.

INTRODUCTION

The important tasks in living systems are accomplished through specific interactions which depend on the globular, native conformations of proteins. (I suggest to my students that this is "the secret of life," at least at the molecular level.) The complexity and sophistication of some of these systems is awe inspiring. Thus, the interest and admiration which biochemists have for the globular conformation of proteins is understandable and is reflected in an eloquent statement by Richardson (1):

> "... high-resolution electron-density maps ... are like intricate, branched coral, intertwined but never touching. β-sheets do not show a stiff repetitious regularity but flow in graceful, twisting curves, and even the α-helix is regular more in the manner of a flower stem, whose branching nodes show the influences of environment, developmental history, and the evolution of each separate part to match its own idiosyncratic function."

The aim of the article is to review recent research on the conformational stability of globular proteins and related topics. Three recent reviews (2-4) and a book (5) have discussed conformational stability in more depth. Other reviews (6-8) and symposia (9,10) have considered protein folding from a more general point of view.

DISCUSSION

Protein Conformations

The best characterized conformation of proteins is clearly the unique, globular conformation that most proteins form spontaneously under physiological conditions in order to carry out their appointed task. This is exemplified by the title of Richardson's recent review, "The Anatomy and Taxonomy of Protein Structure" (1). Many interesting generalizations about details of the globular conformation are emerging as more and more crystal structures become available (1,6,7,11). Here we summarize some of the key features relative to conformational stability.

Figure 1 shows a globular protein model which emphasizes the difference in polarity between the interior and exterior of the molecule (see reference 11 for a more detailed

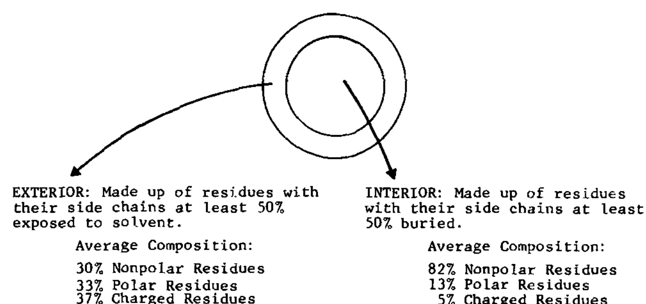

FIG. 1. A globular protein model showing the side-chain composition of the solvent accessible and solvent inaccessible portions of the protein.

analysis). It is clear why the properties of a protein change substantially when the protein unfolds. Unfolded proteins are less soluble and more hydrophobic because many more nonpolar residues are in contact with the solvent.

The interiors of globular proteins are very closely packed with surprisingly few holes and the holes that do exist are generally filled specifically with water molecules (6). Almost all of the polar groups found in the interior of globular proteins are hydrogen bonded to other polar groups (1). In α-chymotrypsin, for example, 217 of the 225 buried polar groups are hydrogen bonded (12). These hydrogen bonds are especially important in stabilizing α-helices, β-structure, and tight turns (1). Chou and Fasman (13) have shown that 88% of the residues in globular proteins are present in one of these types of structures: 38% α-helices, 20% β-structure, and 30% turns.

All of these structures can be seen in Figure 2 which illustrates another feature of globular protein structure. Note the presence of three layers of polypeptide backbone in the phosphoglycerate kinase domain. Between the layers are two hydrophobic cores. Over half of the domains of proteins have two layers and a single core, about a third have three layers and two cores, and there are a limited number of proteins with more layers (1). Rather than increase the size and number of hydrophobic cores, larger proteins generally fold to give two or more structural domains. Domains generally contain between 100 and 150 residues and are usually linked by a single polypeptide chain segment (11).

At the other end of the conformational spectrum is the random coil. When proteins are dissolved in 6 M guanidine hydrochloride (GdnHCl), with their disulfide bonds broken they approach a randomly coiled conformation (14). Even when the disulfide bonds are intact, proteins appear to unfold as completely as they can, given the conformational restraints imposed by the disulfide bonds. Higher concentrations of urea are always required to unfold proteins (15), but the resulting denatured state appears to be very similar to that found in GdnHCl solutions (16). More potent denaturants than GdnHCl are known, but little work has been done to characterize the resulting unfolded states (17).

For most proteins, thermal and acid denaturation lead to less completely unfolded products than urea and GdnHCl (2,18). This also seems to be true for salts which denature proteins such as $CaCl_2$, LiCl, and $LiClO_4$ (19). None of the partially unfolded states have been characterized in detail. Privalov (3) and Pfeil (4) point out that the thermodynamics of thermal and GdnHCl denaturation are often very similar, suggesting that the residual structure does not contribute significantly to the thermodynamics of unfolding. In contrast, Contaxis and Bigelow (20) and Aune and Tanford (21) report significant differences in free energy among some of these denatured states.

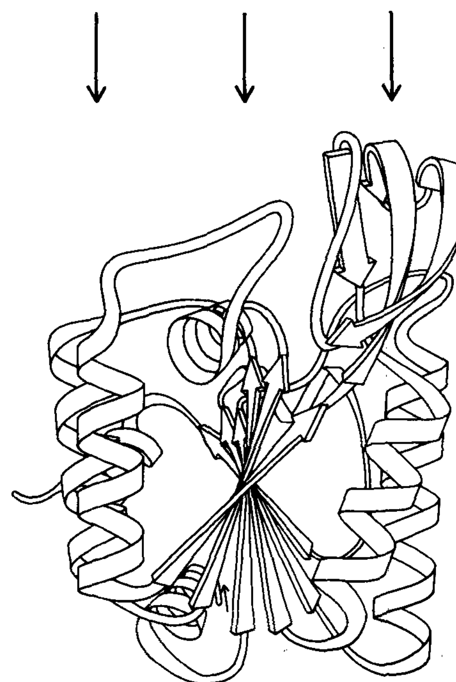

FIG. 2. Conformation of the polypeptide chain in phosphoglycerate kinase domain 2. The spiral ribbons represent α-helices and the arrows represent stands of β-sheet. The arrows above the drawing point to the backbone layers (from ref. 1).

Many detergents, sodium dodecyl sulfate (SDS) being the most popular, are potent denaturants which lead to a denatured state significantly different than a random coil (22). There has been considerable debate concerning the structure of SDS-denatured proteins (5). SDS is a valuable solvent for molecular weight studies and solubilization of proteins, but it has not been used for conformational stability studies.

McCoy and Wong (23) recently showed that the acid-, thermal- and SDS-denatured states of carbonic anhydrase B differ from each other and from the randomly coiled conformation found in 6 M GdnHCl. These denatured states can be obtained either from the native state of the protein or from the randomly coiled protein. In contrast, renaturation of the protein from the acid-, thermal- or SDS-denatured states requires first converting the protein to the randomly coiled denatured state. It seems likely that renaturation of other "irreversibly" denatured proteins might be possible by this same approach.

Very few proteins exist in an unfolded conformation under physiological conditions. One exception may be β-casein found in milk (24). However, chemical modification can decrease the stability of proteins enough so that they unfold to some extent even in the absence of a denaturant. The most successful way of accomplishing this has been to break the disulfide bonds of the protein. This can significantly decrease the conformational stability by lowering the free energy of the denatured state through an increase in the conformational entropy (25). Ribonucleases A (26) and T_1 (27), and bovine pancreatic trypsin inhibitor (6) appear to unfold almost completely when their disulfide bonds are broken. In other cases, from one to three disulfide bonds can be broken without destroying enzyme activity (11).

Experimental Determination of Conformational Stability

In 1962, just a few rough estimates of the conformational

TABLE I

Estimates of the Conformational Stability, ΔG_D, for a Variety of Proteins

Protein	Molecular weight	pH	T(C)	ΔG_D (kcal/mol)	Reference
1. Apolipoprotein A-1	28,000	9.2	37	2.4	63
2. Lac repressor (Residues 1-51)	5,700	7	25	2.6	64
3. Apolipoprotein C-II	8,800	7.4	25	2.8	65
4. α-Lactalbumin	14,200	7	37	3.8	66
5. Phycocyanin	210,000	6	25	4.4	67
6. T4 lysozyme	18,600	3	37	4.6	43
7. Troponin	72,000	7	37	4.7	68
8. G-Actin	41,700	7.5	37	5.0	69
9. Cytochrome b_5 (residues 1-90)	10,300	7	37	5.3	70
10. Phosphoglycerate kinase	46,000	7.5	25	5.3	71
11. Ig G light chain (Wes)	23,500	7	25	5.5	30
12. Ovalbumin	43,000	7	25	5.9	72
13. Penicillinase	29,000	7	25	6.0	10
14. Cytochrome c	12,400	5	37	7.8	4
15. Ribonuclease	13,700	7	37	8.0	3
16. α-Chymotrypsin	23,000	4	37	8.0	3
17. Tryptophan synthase	28,700	7	37	8.0	45
18. Pepsin	35,500	6.5	25	10.8	41
19. Trypsin inhibitor	6,500	–	25	11.0	3
20. Parvalbumin	11,400	7	25	11.0($-Ca^{2+}$)	52
				20.0($+Ca^{2+}$)	52
21. Myoglobin	17,800	10	37	12.0	3
22. Lysozyme	14,600	7	37	12.0	4
23. Trypsin	23,800	–	37	13.0	3
24. Growth hormone	21,500	8	25	14.0	73
25. Pepsinogen	39,500	6.5	25	15.7	41
26. Alkaline phosphatase	86,000	7.5	30	20 ($-Zn^{2+}$)	50
				129 ($+Zn^{2+}$)	50

stability of proteins were available (28). Since then, a variety of proteins have been investigated using several experimental techniques (see Table I). The conformational stability, which we will denote ΔG_D, is the difference in free energy between the globular conformation and unfolded states of the molecule in the absence of a denaturant. The experimental methods used for measuring ΔG_D are briefly described below.

The method of choice for measuring ΔG_D is differential scanning calorimetry. The calorimetric enthalpy change and the heat capacity change for denaturation can be determined in a single experiment with a modest amount of protein (3). As described elsewhere (4), this information can then be used to calculate ΔG_D as a function of temperature. A major advantage of this technique is that it does not require a knowledge of the mechanism of unfolding.

A more widely used approach, mainly because the equipment is more readily accessible, is to monitor unfolding by urea, GdnHCl, heat, or acid using any of several physical techniques and then analyze the resulting denaturation curve to obtain ΔG_D. The analysis is straightforward in cases where unfolding approaches a two-state mechanism (see reference 2 for details). Even for more complex mechanisms, it is sometimes possible to derive meaningful results from the data (29,30). A major problem with this approach, and to lesser extent for the calorimetric method, is extrapolating from the conditions used to promote unfolding to physiological conditions. This problem has been discussed for urea and GdnHCl denaturation (2,31,32) and for acid denaturation (33). This extrapolation can be avoided in cases where the main interest is in determining the difference in conformational stability between two similar forms of a protein, e.g., in comparing a chemically modified protein with the unmodified form of the same protein. Differences in stability of a few tenths of a kcal are readily detectable, which makes this the method of choice for analyzing these systems (34).

An interesting new method for estimating ΔG_D has been described by Hollecker and Creighton (35). Electrophoresis in 0-8 M urea gradients yields a urea denaturation curve directly visible in the gel. They show that a tangent to the denaturation curve at the midpoint can be extrapolated to 0 M urea to yield an estimate of ΔG_D.

One technique is available which yields an estimate of ΔG_D directly under physiological conditions. This method is based on measurements of the rate of exchange of peptide amide hydrogens with solvent (36). Recent developments which allow the measurement of exchange rates for identifiable, individual groups have made the technique much more useful and given a better understanding of the details of the mechanism of exchange (37,38), a topic which has stimulated considerable debate (39).

Sachs et al. (40) have described a clever approach which uses antibodies to determine the relative concentrations of native and unfolded conformations of a protein. For example, they show that a 45-residue fragment of staphylococcal nuclease has ca. .02% of the molecules folded into a native-like conformation at equilibrium.

Pfeil (4) compared results obtained by different methods for many of the proteins listed in Table I and showed that, in general, the agreement is good. This suggests that any of the techniques can be used to estimate ΔG_D when proper care is observed.

ΔG_D Values for Individual Proteins

A selection of the more reliable estimates of ΔG_D is presented in Table I. Only results for the wild type protein or for the species most frequently studied are given. No distinction is made between studies using urea and GdnHCl vs heat and acid to promote unfolding, even though the products and ΔG_D values may differ. Also, the method of extrapolation used can significantly change results from urea and

TABLE II

Denaturation Curve Midpoints May Not Reflect Conformational Stabilities Under Physiological Conditions

Protein	$(GdnHCl)_{1/2}$ (M)	$\Delta G_D^{H_2O}$ (kcal/mol)
Myoglobin	1.7	12
Lysozyme	3.1	12
	$T_{1/2}$ (C)	
Cytochrome c	80	8
Lysozyme	80	12

TABLE III

Thermal Denaturation Midpoint, $T_{1/2}$, and Conformational Stability (ΔG_D) of Wild Type and Mutant T4 Lysozymes[a]

Protein	$T_{1/2}$ (C)	ΔG_D (kcal/mole)
Wild Type	59	6.6
Glu 128 → Lys	54	4.4
Ala 146 → Thr	50	2.5
Arg 196 → His	45	2.2
Met 102 → ?	46	2.0

[a]Reference 43.

GdnHCl denaturation studies (31). Consequently, estimates from calorimetric studies are given preference when they are available.

The values given for pepsin and pepsinogen correspond to complete unfolding. Both of these molecules unfold in stages which is not surprising since they have two domain structures. The ΔG_D values for unfolding of the least stable element is 1.9 kcal/mol for pepsin and 6.2 kcal/mol for pepsinogen (41).

All of the ΔG_D values in Table I apply at a single temperature and pH. Privalov (3) and Pfeil (4) use phase diagrams to show how ΔG_D varies with pH and temperature for several of these proteins. The temperature dependence is especially interesting. The ΔG_D value for a given protein will reach a maximum between -10 and 40 C and the stability decreases at both higher and lower temperatures. There are many other interesting aspects of the thermodynamics of protein denaturation and other processes where hydrophobic bonding plays a major role (42).

It is worth noting that it is hazardous to draw conclusions about the relative conformational stabilities of proteins by comparing the midpoints of their denaturation curves. This is illustrated in Table II. Lysozyme and myoglobin have similar conformational stabilities, but a much higher concentration of GdnHCl is needed to denature lysozyme because the dependence of ΔG_D on GdnHCl concentration is much smaller. Likewise, lysozyme and cytochrome c are unfolded at about the same temperature, even though lysozyme has a substantially higher ΔG_D value.

Relationship Between Chemical Structure and Conformational Stability

Several studies have shown that small changes in the chemical structure of a protein may have substantial effects on the conformational stability or on the thermodynamics of denaturation. This can be probed using proteins which differ slightly in amino acid sequence or using specific chemical modification to change the structure. Results from a study of the thermal denaturation of T4 lysozyme mutants by Schellman et al. (43) are shown in Table III. In all cases, the mutants are less stable than the wild type protein. The mutant in which His replaces Arg at position 96 is especially interesting because the three-dimensional structures of both proteins have been determined (44). In the wild type protein, the arginine side chain is well ordered, and lies on the protein surface where the hydrophobic portion of the side chain contributes to the major hydrophobic core. When a histidine residue is substituted, a portion of the hydrophobic core will be exposed to the solvent. The histidine residue may also form a hydrogen bond to a tyrosine residue at position 88. These factors appear to cause the destabilization of the protein rather than any significant change in its tertiary structure (44).

Yutani et al. (45) have shown that ΔG_D = 8.8 kcal/mol for the α-subunit of tryptophan synthease, but the stability is increased to 13.4 kcal/mol when Glu 49 is replaced by Met and decreased to 6.3 kcal/mol when Glu 49 is replaced by Gln. Matthews et al. (46) have studied mutants of this same protein in which the Gly at position 211 is replaced by an Arg or a Glu. In this case, the midpoints of the thermal denaturation curves are about the same for the three proteins, but there are changes of ca. 16 kcal/mol in the enthalpy of denaturation. Thus, significant changes in the thermodynamics of denaturation may be occurring even in cases where small differences in the conformational stability are observed.

In some cases it may be important to the functional role of the protein to maintain the conformational stability within narrow limits. The ΔG_D values all fall between 7.2 and 8.0 kcal/mol for the cytochromes c from eight different species, some differing substantially in structure (47,48).

In most cases, cleavage of peptide bonds decreases a protein's conformational stability. For example, ribonuclease melts at a temperature 20 C lower when a single peptide bond is cleaved and both ribonuclease and staphylococcal nuclease are much less stable when a few carboxyl terminal residues are removed (2).

Chemical modification of the side chains of a protein also generally leads to a decrease in the conformational stability. Table IV shows the decreases in the conformational stability of β-lactoglobulin which result when a series of compounds are attached to the single sulfhydryl group through a disulfide bond (49). The conformational stability

TABLE IV

Differences in the Conformational Stability of Mixed Disulfide Derivatives of β-Lactoglobulin

Protein	$(Urea)_{1/2}$ (M)	Change in ΔG_D (kcal/mol)
β-1gb	4.97	
β-1gb-S-S-CH_2-CH_2-COOH	4.47	1.1
β-1gb-S-S-CH_2-CH_2-CH_3	4.23	1.6
β-1gb-S-S-CH_2-CH_2-OH	4.19	1.7
β-gb-S-S-CH_2-CH_2-NH_2	1.68	7.3

of the mercaptoethylamine derivative is decreased to such an extent that the protein is partially unfolded even in the absence of a denaturant. As another example, Hollecker and Creighton (35) have used the urea gradient electrophoresis technique to show that succinylation of the lysine amino groups in β-lactoglobulin, cytochrome c, and ribonuclease generally leads to decreases in the conformational stability. Finally, Wutrich et al. (50) have shown that seven different chemically modified forms of bovine pancreatic trypsin inhibitor are less stable to thermal denaturation than the native protein. They have also used nuclear magnetic resonance (NMR) techniques to measure the rates of proton exchange and the rates of aromatic side-chain rotation for the same series of proteins. On the basis of these results, they propose an interesting model to reconcile the results obtained when these three different techniques are used to examine protein flexibility (see below).

DISCUSSION

The most interesting finding from results such as those shown in Table 1 is that the conformational stability of globular proteins is remarkably low. Almost all ΔG_D values determined to date are between 3 and 15 kcal/mol. The only proteins with greater conformational stabilities are proteins like alkaline phosphatase (51) and parvalbumin (52) which bind metal ions to their native conformation with great affinity. (In contrast, the conformational stability of transferrin, which binds iron very tightly, is only ca. 9 kcal/mol [53]). In fact, any molecule which binds to the native conformation, but not to unfolded conformations of a protein, will enhance the conformational stability and this can be calculated if the binding constant is known (54).

In some cases, especially in the food industry, it might be desirable to be able to change the conformational stability of a protein or a group of proteins. The most effective way of doing this will probably be through changes in the intramolecular crosslinking. Breaking the disulfide bonds in a protein always leads to a sizeable decrease in the conformational stability. In contrast, if an additional crosslink is added, a protein may become much more stable. Imoto and Rupley (55) have shown that when Glu 35 is covalently linked to Trp 108 in lysozyme, the midpoint of the thermal denaturation curve is increased 20 C. This corresponds to an increase in the conformational stability of over 6 kcal/mol. These effects on the conformational stability are due mainly to changes in the conformational entropy of the denatured state. Attaching a protein to a solid support has frequently been observed to increase the protein's stability (56), probably through the same effect.

The denaturation of many globular proteins closely approaches a two-state mechanism (2). Under most conditions, only the globular native state and the unfolded denatured state are present; the concentration of partially unfolded molecules is small. This, plus the many three-dimensional structures of proteins which have been determined through X-ray diffraction studies, creates the impression that the folded conformation is rigid and static. There is now an abundance of concrete experimental evidence that globular proteins are flexible, undergoing dynamic changes in conformation (57). For example, some of the aromatic rings in the interior of the protein can rotate and some of the amide hydrogens can exchange with solvent more rapidly than would be expected just on the basis of an unfolding equilibrium. Theoretical calculations support these experimental studies (58). The emerging picture is that native proteins exist transiently in various conformations, probably not too different from the native conformation. Privalov (3) has used the term microstability to distinguish the energetics of this process from complete unfolding, macrostability. Using data from the early stages of the exchange of peptide hydrogens, he shows that microstabilities fall in the range 2.5-7.1 kcal/mol for nine proteins where data are available (59).

The remarkably low conformational stability is intriguing. It appears that the packing of nonpolar side chains and hydrogen bonding must be close to perfect in the globular conformation to reach even a marginal stability. Failure to form a single hydrogen bond in the native protein or a change in hydrophobic bonding involving only a few residues can lead to unfolding of a protein. Edsall (60) has suggested that there may be very few amino acid sequences for which a stable globular conformation is possible. At present it is not clear why the globular conformation is stable. Model compound data and theory suggest that a randomly coiled conformation should be considerably more stable than the globular conformation (61). By combining results from experimental studies of the type described here with structural information from X-ray crystallography and theoretical studies, the stage is now set for reaching a better understanding of the forces which contribute to globular protein stability. The great potential is illustrated by recent publications from Gurd's laboratory (62). Using structural and experimental information, they have refined methods for estimating the electrostatic interactions among charged groups on the surface of globular proteins. For myoglobin they suggest that electrostatic interactions make a greater contribution to the stability than nonionic forces. Thus, electrostatic interactions may make a much greater contribution to the conformational stability of globular proteins than was generally thought.

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Acetonitrile as Eluent in Silver Resin Column Chromatography

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ABSTRACT

Acetonitrile (ACN) is shown to be an effective eluent in combination with methanol (MeOH) or acetone for the rapid separation of polyunsaturated fatty acid methyl esters on a fully silver-loaded ion-exchange (XN1010) column. ACN-containing eluents thus provide on one fully siliver-loaded resin column an attractive alternative separation system to a series of partially silvered resin columns ("PARC" columns), which previously have been used for such separations. Solvent programming from 0 to 15% ACN in MeOH allows the separation of methyl oleate, linoleate, linolenate and arachidonate within 3 hr. Preparative (up to 800 mg) isocratic separations of a number of polyunsaturated fatty acid esters from natural sources (methyl linoleate from safflower esters, for example) were readily achieved.

INTRODUCTION

Macroeticular, sulfonic acid, ion-exchange resins loaded with silver ion have been useful in the chromatographic separation of unsaturated compounds, particularly geometrical isomers of unsaturated esters. The separation of geometrical isomers of methyl monoenoates was readily achieved with resins of this type using MeOH as eluent (1,2), but polyenoates were eluted only with very large volumes of MeOH or not at all. Improved resins gave practical separation of (E,E)-, (Z,E)- and (Z,Z)-nonconjugated dienoates (3). Separation of conjugated dienoates and trienoates was also possible, owing to their lower elution volumes as compared to their nonconjugated isomers (4).

The problem of elution of polyunsaturated esters with three or more nonconjugated double bonds still remained. The use of 10% 1-hexene in MeOH (5) as a step eluent was effective in eluting such esters from silver resin columns. Another approach to the problem was the development of the technique of partial silver-loading of the resin, called partial argentation resin chromatograph, or PARC (6-8). In this technique, the retention of the column for nonconjugated polyunsaturated esters was reduced by only partial loading with silver ion. The disadvantage of partial silver