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An Analysis of the Circular Dichroism of the Lysozyme–α-Lactalbumin Group of Proteins†

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ABSTRACT: Circular dichroism data are presented for eight lysozymes and α-lactalbumins. All have multiple Cotton effects in the near-ultraviolet region, associated with the absorption bands of the aromatic amino acids. All but the avian lysozomes have predominantly negative optical activity in this region. Hen and duck egg lysozymes are dominated by positive rotational strength, which is interpreted as arising from tryptophan residues. Calculations of on- and off-resonance interactions between the two adjacent active-site tryptophan residues show that these can give rise to sufficient rotational strength to explain the observed difference between these proteins and their homologs, which lack the two adjacent tryptophans. The apparently unrelated goose egg lysozyme, which has a low content of aromatic amino acids, shows only very small optical activity in the region of the aromatic absorption band, and marked differences in the peptide region. Differences between the egg lysozymes and the other proteins in the series in this region can be rationalized in terms of aromatic contributions. Similarities in the high-field proton magnetic resonance spectra of lysozymes, due to ring-current shifted aliphatic proton resonances, and similar effects in the same region of the inhibitor, *N*-acetylglucosamine, are described.

The interpretation of the optical activity of globular proteins in their native states is complicated by the presence of chromophores other than the peptide group, which frequently generate considerable, rotational strengths, and in some cases (Green and Melamed, 1966; Yang *et al.*, 1968) can even dominate the optical activity of the molecule as a whole. Little is known with any certainty about the nature of the perturbations that are responsible for such effects, and it is also difficult to relate the Cotton effects associated with the near-ultraviolet absorption bands of the aromatic residues to those at shorter wavelengths, where there is extensive overlap with peptide Cotton effects. We have approached this problem by considering the circular dichroism of a set of proteins, believed to possess essentially identical conformations, but differing considerably in amino acid composition and sequence. This is the lysozyme–α-lactalbumin group (Brew *et al.*, 1968; Browne *et al.*, 1969; Brew, 1970). In the light of comparisons between these molecules we consider, on the one hand, the effect of extrinsic chromophores on the interpretation of the circular dichroism of the peptide absorption bands, and also some possible explanations for the enhancement of optical activity observed in these chromophores.

Materials and Methods

Proteins. Hen egg lysozyme was the three-times-recrystallized product of Sigma Chemical Co. Duck egg and human lysozymes were gifts from Dr. C. C. F. Blake, and goose egg lysozyme from Drs. S. Kammerman and R. E. Canfield. Human and guinea pig α-lactalbumins were prepared as described elsewhere (Findlay and Brew, 1972; Brew and Campbell, 1967). Bovine α-lactalbumin was prepared following the procedure of Aschaffenburg and Drewry (1957), with the additional final step of gel filtration on Sephadex G-100 (4 × 140 cm) equilibriated with 50 mM ammonium bicarbonate. Camel α-lactalbumin was purified by a procedure essentially similar to that described by Kessler and Brew (1970) for pig α-lactalbumin. Acid-precipitated camel whey was dialyzed and lyophilized. The α-lactalbumin peak from

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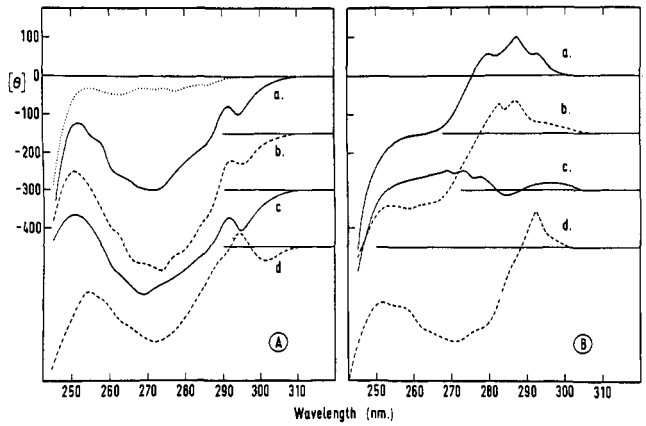

FIGURE 1: Circular dichroism in the region of the aromatic absorption bands of α-lactalbumins (A) and lysozymes (B). The α-lactalbumins are: (a) bovine (the dotted curve showing the same protein in 6 M guanidine hydrochloride), (b) camel, (c) guinea pig, and (d) human. The lysozymes are (a) hen egg, (b) duck egg, (c) goose egg, and (d) human urinary leukemic lysozyme. Conditions as described.

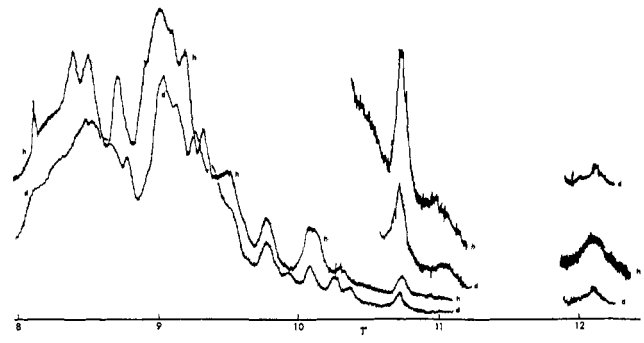

FIGURE 2: Proton magnetic resonance spectra at 220 MHz in D_2O solution at 37° of hen (h) and duck (d) egg lysozymes showing the ring-current shifted resonances in the upfield region. The higher curves were run at increased radiofrequency power.

gel filtration with Sephadex G-100 (4 × 140 cm in 50 mM ammonium bicarbonate) was lyophilized and further purified by ion-exchange chromatography on DEAE-cellulose equilibrated with 20 mM Tris-chloride (pH 7.8). The major 280-nm-absorbing peak, eluted with a gradient from 0 to 0.4 M NaCl in 20 mM Tris (pH 7.8), was dialyzed and lyophilized. All the α-lactalbumin preparations used were active in the lactose synthetase assay (Brew *et al.*, 1968) and homogeneous as judged by disc electrophoresis on polyacrylamide gels (Davis, 1965).

Circular dichroism was measured with a Jouan Mark 2 dicrograph, with a scan speed of 0.0625 or 0.125 nm per sec, and a time constant of 10 or 4 sec. The effect of finite spectral bandwidths has to be carefully considered at wavelengths above about 240 nm, and accordingly spectra were frequently run at different detector voltage settings, and at different protein concentrations and path lengths to minimize the limitations of instrumental resolution. Beer's law tests were also performed to guard against stray light artefacts. Absorbances were kept below 1.4.

Protein concentrations were determined spectrophotometrically with a Beckman DK-2A spectrophotometer. Specific absorptivities were based either on dry-weight estimations, obtained by heating samples at 120° to constant weight, or on alkaline hydrolysis, followed by ninhydrin estimation (Moore and Stein, 1954) or both. The following values were found for $E^{1\%}_{1\,cm}$; bovine lactalbumin, 20.1 (agreeing with the literature value of Kronman and Andreotti, 1964); guinea pig, 16.7; human, 19.0; camel, 19.0. For lysozymes we used: hen egg, 26.9 (Hayashi *et al.*, 1963); duck 26.6; goose, 14.8 (value provided by Dr. S. Kammerman).

Proton magnetic resonance spectra at 220 MHz were measured on two Varian instruments belonging, respectively, to I.C.I. (kindly made available by Dr. J. Becconsall) and the Science Research Council. Single scans of 1000 sec for the entire radiofrequency range were run. Protein concentrations were 10% or more. Spectra were indexed on Me_4Si in a sealed capillary.

Results

Figure 1 shows the circular dichroism in the region of aromatic side-chain absorption in a series of lysozymes and α-lactalbumins. For comparison a typical curve for a denatured protein in 6 M guanidine hydrochloride is also shown (Figure 1A, a). Some small Cotton effects from the tryptophan and tyrosine absorption bands are clearly present, but in the native protein there is an enhancement of an order of magnitude. It is clear that the egg lysozymes are very different from the other proteins in the group, which all have prominent negative Cotton effects centred at about 270 nm. There is always a positive feature at about 295 nm, which is in most cases (Figure 1) overlaid by a broad negative component. In human lactalbumin there is also a small negative Cotton effect at about 300 nm. The lowest optical activity, of the same order as that of a denatured protein, occurs in goose egg lysozyme (Figure 1B, c). Duck egg lysozyme is the only member of the group whose circular dichroism is at all close to that of hen egg lysozyme. The similarity suggests a corresponding similarity in the environments of the aromatic residues. This surmize is supported by proton magnetic resonance spectra (Figure 2), which reveal the presence of a series of similar resonances in the upfield region, arising from interactions of ring currents of the aromatic side chains with aliphatic groups.

The addition of the inhibitor, *N*-acetylglucosamine, brings about small changes in the aromatic Cotton effects of hen egg lysozyme with an enhancement maximal at about 285 nm. This agrees with earlier observations (Glazer and Simmons, 1966; Ikeda and Hamaguchi, 1969). With human urinary lysozyme a change is also observed, reflecting presumably an interaction with the aromatic residues in the active-site cleft (Blake *et al.*, 1967), but the largest effect is a diminution in the amplitude of the long-wavelength positive Cotton effect. A similar change, with additional changes in the negative Cotton effect at 270 nm, has recently been reported by Halper *et al.* (1971). The nuclear magnetic resonance (nmr) spectra of human lysozyme with and without *N*-acetylglucosamine are shown in comparison to those of hen egg lysozyme in Figure 3, and give evidence from the upfield region of an interaction with the aromatic residues of the active-site cleft.

The Cotton effects in the region of peptide absorption are shown in Figure 4, and again there is a high degree of similarity between the members of the series, despite the considerable variations in the content of chromophoric amino acids in this group of proteins. Differences do occur, notably in goose egg lysozymes, and guinea pig α-lactalbumin, which displays an apparent blue shift especially in the region of the $n-\pi^*$ peptide Cotton effect. Comparison to literature data is possible with hen egg lysozyme (Ikeda and Hamaguchi, 1969; Teichberg *et al.*, 1970) and bovine lactalbumin (Kronman,

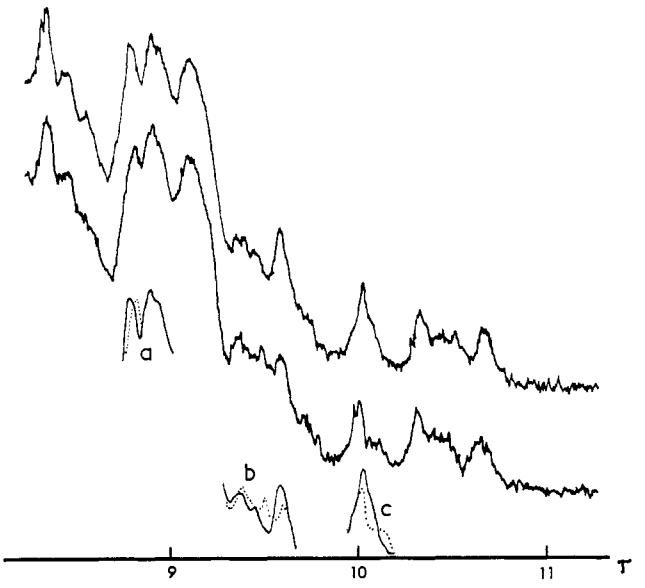

FIGURE 3: Proton magnetic resonance spectra at 220 MHz in the upfield region of human urinary leukemic lysozyme, showing the effect of introduction of *N*-acetylglucosamine (lower trace, and dotted lines (smoothed curves)), all at pD 6.9.

1968), and in both cases the agreement is good. In goose egg lysozyme the n–π^* peptide Cotton effect emerges as a peak, rather than a shoulder, and the circular dichroism is higher throughout the region.

Discussion

It is necessary to assume in all that follows that the conformations of our set of proteins are all closely similar, so that the environments of residues at given positions in the chain are directly comparable. The basis for this assumption rests on (1) the extensive sequence homologies within the group; (2) the ability of a lactalbumin sequence to fit precisely the molecular model of hen egg lysozyme (Browne *et al.*, 1969), nearly all substitutions being conservative, and external; (3) the common features of the proton magnetic resonance spectra, and their analysis in terms of conformation (Cowburn

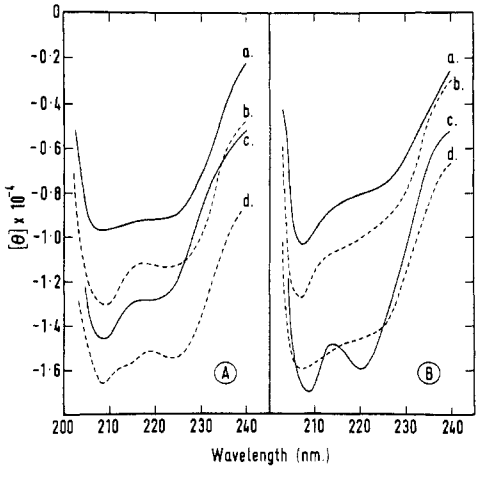

FIGURE 4: Circular dichroism in the peptide absorption region of α-lactalbumins (A) and lysozymes (B). The curves are identified according to the same key as in Figure 1. Successive curves displaced one division downward on the ordinate scale.

TABLE I: Tyrosine and Tryptophan Content of Lysozymes and α-Lactalbumins (Residues per Molecule).[a]

	Tyr	Trp
Bovine α-lactalbumin	4	4
Human α-lactalbumin	4	3
Guinea pig α-lactalbumin	3	5
Hen egg lysozyme	3	6
Duck egg lysozyme	5	6
Human leukemic lysozyme	6	5

[a] Data from complete sequences, duck egg lysozyme, from Hermann *et al.* (1971). Camel α-lactalbumin appears to be similar to, or identical with, bovine.

et al., 1970; Cowburn, 1970; unpublished observations by D. Cowburn and H. Rattle, using the guinea pig sequence); (4) the similarity of the circular dichroism spectra in the peptide region as shown above, and by Kronman (1968); and (5) the essentially similar size and shape of α-lactalbumin and lysozyme in solution as inferred from low-angle X-ray scattering (Achter and Swan, 1971; Pessen *et al.*, 1971).

Aromatic Cotton Effects. In the denatured chain, the small aromatic Cotton effects (Figure 1A, a) resemble in magnitude those of other unstructured protein chains (Yutani *et al.*, 1968; Baba *et al.*, 1969) and presumably represent the Cotton effects characteristic of the unperturbed chromophores in a peptide chain. In the native protein there is enhancement of the aromatic optical activity by one or more orders of magnitude. Apart from the egg lysozymes, the most prominent feature is a broad negative Cotton effect. A comparison with data on model compounds containing the aromatic side chains, in particular the definitive studies of Strickland *et al.* (1969), Horwitz *et al.* (1970) leaves little doubt that this Cotton effect arises largely from tyrosine residues. The number of tyrosine residues in the proteins is shown in Table I, and from the sequence data of Table II it is seen that tyrosines at positions 20 and 53 (indexed here and throughout on the hen egg lysozyme sequence) appear to be conserved, and at position 38, tyrosine is replaced only in hen egg lysozyme, where it becomes phenylalanine. It will be noted that all of these are internal residues, and if it is accepted that grossly enhanced optical activity will arise most commonly from the internal residues, which have no free rotation—a hypothesis favored by the evidence so far as it goes (Simpson and Vallee, 1966; Strickland *et al.*, 1969), as well as intuitive expectation—these must all be considered likely contributors to the 275-nm Cotton effect.

All the proteins show evidence of a positive feature at about 295 nm, which cannot be due to tyrosine (Horwitz *et al.*, 1970), and must arise from tryptophan residues. In egg white lysozymes, the feature is partly obscured, and in the lactalbumins it is superimposed on a large negative background, and so does not manifest positive ellipticity. The peak is evidently the most prominent component of the ^1L_a system of tryptophan (Strickland *et al.*, 1969). In human lactalbumin alone there is a further small negative Cotton effect at about 300 nm, which in the light of the interpretation of Strickland *et al.* (1969) of tryptophan-containing model compounds may correspond to the ^1L_a (0, 0) band. As in chymotrypsin therefore the ^1L_a system appears to dominate the tryptophan con-

tribution in the lysozyme–lactalbumin group. The conserved tryptophan residues are at positions 28, 63, and 108, the last of which is completely buried—as judged by the hen egg lysozyme model (Blake *et al.*, 1967). In hen egg lysozyme the interaction with the inhibitor, *N*-acetylglucosamine is known (Blake *et al.*, 1967) to encompass perturbation of the two adjacent active-site tryptophan residues, 62 and 63, and of tryptophan-108. This is reflected in the nmr spectra (Cohen and Jardetzky, 1968) and in the circular dichroism of hen egg lysozyme (Glazer and Simmons, 1966; Ikeda and Hamaguchi, 1969) and human urinary lysozyme (Halper *et al.*, 1971).

The presence of a strong positive contribution to the ellipticity of hen egg lysozyme with a maximum at 295 nm, from Trp-108, has been persuasively inferred by Teichberg *et al.* (1970) on the basis of the changes arising when this residue is modified by oxidation to an oxindole. The difference between the native and the oxidized hen egg lysozymes leaves negative contributions from other sources, the most prominent extrema being at 293 and 268 nm, with a molar residue ellipticity of about −160. There is the possibility that a part of the effect of oxidation of Trp-108 arises from the new oxindole chromophore, which retains its aromatic character and has a by no means negligible absorption band (Patchornik *et al.*, 1960). Trp-108 is present in all the lysozymes and lactalbumins, whose sequences are known, but the environmental features leading to any positive effect at 295 nm of the kind postulated by Teichberg *et al.*, are evidently unique to the hen egg species (Figure 1). In hen egg lysozyme there is close contact between Trp-108 and Ile-58, Ile-95, Val-99, and Met-105, and the last three of these are substituted in human lysozyme, so that the rotational constraints on Trp-108 could well be different. One can probably rule out in the case of the Cotton effect from this residue a perturbation due to a close-lying charge, or interaction with another chromophore. Whereas Teichberg *et al.* infer that the perturbation due to binding of inhibitor involves solely Trp-108, our results on human lysozyme suggest that, in this case at any rate, the perturbation may involve mainly Trp-64 (63 referred to hen lysozyme), Trp-62 being replaced by tyrosine. Both protein proton magnetic resonance (pmr) spectra show perturbation of ring-current shifted aliphatic side chains when *N*-acetylglucosamine is added (Figure 4). The inference of a similar geometric interaction with the inhibitor therefore seems reasonable.

Goose egg lysozyme is of special interest, in that it has a low tryptophan content (Dianoux and Jollès, 1967; Canfield and McMurray, 1967; Canfield *et al.*, 1971). As Figure 1 shows, the aromatic Cotton effects are of similar size to those of a fully denatured protein (Figure 1A, a). This suggests that there may also be no appreciable extrinsic contributions in the peptide region (Figure 4), and that this protein is therefore much the most likely to exhibit optical activity due only to the peptide backbone. However the fit of the experimental curve to a calculated curve made up of contributions from α helix, β chains, and random coil (Greenfield and Fasman, 1969) is not significantly better than that of hen egg lysozyme. The difference between these two species is most readily construed in terms of a positive aromatic contribution in hen egg lysozyme, centered at about 220 nm. This wavelength does indeed correspond to the absorption maximum of tryptophan (see, *e.g.*, Wetlaufer, 1962). Unfortunately it is doubtful whether any inferences based on the properties of goose egg lysozyme relate to those of other members of the group. Canfield *et al.* (1971) have shown that the part of this sequence

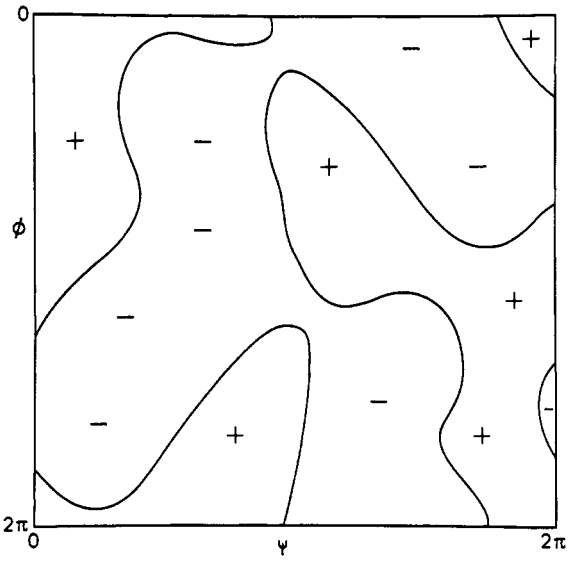

FIGURE 5: Calculated sum rotational strengths in the aromatic absorption region near 280 nm due to interaction of tryptophan residues 62 and 63 in hen egg lysozyme. On- and off-resonance terms are added, and interactions of the ^1L_a, ^1L_b, and $^1B_{a,b}$ transitions are included. The angles ϕ and ψ are angles of tilt and rotation of one chromophore relative to the other and $\phi = \psi = 0$ (defined as in text) for the conformation observed in the crystal (Blake *et al.*, 1967). The lines show the boundaries between zones of negative and positive rotational strength.

which has been determined is not all homologous with the other lysozyme sequences, and it seems likely that they are conformationally unrelated.

It is clear from the spectra of Figure 1 that the hen and duck lysozymes differ somewhat from the other proteins, even given a contribution from Trp-108 as envisaged by Teichberg *et al.* (1970). In human lysozyme the difference in the aromatic region could be accounted for by a superimposed negative circular dichroism, centered at 290–295 nm. This protein contains more tyrosine than any of the others. Tyr-45 and -122 are surface residues (in terms of hen egg lysozyme); Trp-34 is internal, and could give rise to a large contribution; Trp-111 is present in both, and is a surface residue. However the wavelength distributions of the differences between the two anomalous lysozymes and the rest of the group do not have the form of a prominent ^1L_a contribution, which is evident in other native globular proteins (Strickland *et al.*, 1969), and evidently have a different origin. The lysozymes, where data are available, differ from the lactalbumins in having two adjacent aromatic residues in the active-site cleft (Table II). Hen egg lysozyme and probably duck egg lysozyme have a tryptophan dimer (residues 62–63). We have examined whether an interaction between these adjoining chromophores could be responsible for a sufficiently large circular dichroism contribution to account for the observed curves. If the transition moment directions are known, it is possible, using the monopole formulation of Tinoco (1963) to calculate the on- and off-resonance interaction potentials between the electronic transition dipoles of a pair of chromophores (exciton and dispersion contributions), and from these the rotational strengths.

The transition moment directions for the ^1L_a, ^1L_b, and $^1B_{a,b}$ transitions were taken from the study by Yeargers (1968), and the electronic transition dipoles were estimated from the integrated absorption intensities. The orientations of the two residues were taken from coordinates of Blake *et*

TABLE II: Amino Acid Sequences of α-Lactalbumins and Lysozymes Studied.[a]

										10						
BαLA	Glu	Gln	Leu	Thr	Lys	CYS	GLU	Val	Phe	ARG	Glu	LEU	LYS			Asp
HαLA	LYS	Gln	PHE	Thr	Lys	CYS	GLU	LEU	Ser	Gln	Leu	LEU	LYS			Asp
GPαLA	LYS	Gln	Leu	Thr	Lys	CYS	Ala	LEU	Ser	His	Glu	LEU	Asn			Asp
HEWL	LYS	Val	PHE	Gly	Arg	CYS	GLU	LEU	Ala	Ala	Ala	Met	LYS	Arg	His	Gly
HLL	LYS	Val	PHE	Glu	Arg	CYS	GLU	LEU	Ala	ARG	Thr	LEU	LYS	Arg	Leu	Gly
							40									
BαLA	His	Thr	SER	GLY	TYR	Asp	THR	Glu	ALA	Ile	Val	Glu	ASN			Asn
HαLA	His	Thr	SER	GLY	TYR	Asp	THR	GLN	ALA	Ile	Val	Glu	ASN			Asp
GPαLA	His	Ile	SER	GLY	TYR	Asp	THR	GLN	ALA	Ile	Val	Lys	ASN			Ser
HEWL	Phe	Glu	SER	Asn	Phe	Asn	THR	GLN	ALA	Thr	Asn	Arg	ASN	Thr		ASP
HLL	Trp	Glu	SER	GLY	TYR	Asn	THR	Arg	ALA	Thr	Asn	Tyr	ASN	Ala	Gly	ASP
				70										80		
BαLA	Asn	Asp	Gln	Asp	PRO	His	SER	Ser	ASN	Ile	CYS	ASN	Ile	SER	CYS	Asp
HαLA	Ser	Ser	Gln	Val	PRO	Gln	SER	ARG	ASN	Ile	CYS	Asp	ILE	SER	CYS	Asp
GPαLA	Ser	Ser	Thr	THR	Val	Gln	SER	ARG	Asp	Ile	CYS	Asp	ILE	SER	CYS	Asp
HEWL	Asp	Gly	Arg	THR	PRO	Gly	SER	ARG	ASN	Leu	CYS	ASN	ILE	Pro	CYS	Ser
HLL	Asp	Gly	Lys	THR	PRO	Gly	Ala	Val	ASN	Ala	CYS	His	Leu	SER	CYS	Ser
	100										110					
BαLA	Leu		ASP	Lys	Val	GLY	ILE	ASN	Tyr	TRP	Leu	ALA	His	Lys	Ala	Leu
HαLA	Leu		ASP	Ile	Lys	GLY	ILE	ASN	Tyr	TRP	Leu	ALA	His	Lys	Ala	Leu
GPαLA	Leu		ASP	Ile	Lys	GLY	ILE	ASN	Tyr	TRP	Leu	ALA	His	Lys	Pro	Leu
HEWL	Val	Ser	ASP	Gly	Asp	GLY	Met	ASN	Ala	TRP	Val	ALA	Trp	Arg	Asn	Arg
HLL	Arg		ASP	Pro	Gln	GLY	ILE	Arg	Ala	TRP	Val	ALA	Trp	Arg	Asn	Arg

[a] Proteins are in order, bovine, human, and guinea pig α-lactalbumins, hen egg-white lysozyme, and human leukemic urinary lysozyme. (Sequences from references given in text and results of K. Brew and of J. R. C. Findlay and K. Brew, submitted for pub-

al. (1967). The rotational strengths generated by the interaction of two chromophores is given by (Tinoco, 1963)

$$R_{0;a\pm} = (\pi/2c)\left\{\mp\nu_a \mathbf{r}_{12}\cdot\boldsymbol{\mu}_{10a}\times\boldsymbol{\mu}_{20a} + \sum_{b\neq a}\frac{2\nu_a\nu_b}{h(\nu_b^2-\nu_a^2)}[V_{1a;2b}\mathbf{r}_{12}\cdot\boldsymbol{\mu}_{10a}\times\boldsymbol{\mu}_{20b} + V_{1b;2a}\mathbf{r}_{12}\cdot\boldsymbol{\mu}_{10b}\times\boldsymbol{\mu}_{20a}]\right\}$$

The first term represents the exciton contribution and the second term the dispersion contribution to the interaction. In the latter we include only the three lowest energy π–π^* transitions, since the interaction falls off rapidly as the bracket term in the denominator increases. The interaction potential $V_{1a;2a}$ for, the dimers is given by

$$V_{1a;2a} = \left[\boldsymbol{\mu}_{1a}\cdot\boldsymbol{\mu}_{2b} - \frac{3(\mathbf{r}_{12}\cdot\boldsymbol{\mu}_{1a})(\mathbf{r}_{12}\cdot\boldsymbol{\mu}_{2b})}{\mathbf{r}_{12}^2}\right]\Big/\mathbf{r}_{12}^3$$

Then the frequencies of the exciton components $\nu_{a\pm}$ are related to $V_{1a;2a}$ by $\nu_{a\pm} = \nu_{0a} \pm V_{1a;2a}$ where ν_{0a} is the frequency of the given transition in the unperturbed chromophore. The terms of the type $\boldsymbol{\mu}_{10a}$ are transition dipoles for transition a in chromophore 1, r_{12} is the separation of the centres of the dipoles, and c the velocity of light. For the geometry of the two tryptophan residues, a positive rotational strength is indeed generated, with a magnitude of some 35×10^{-40} erg cm^3 rad. Per mole of protein this is nearly an order of magnitude more than the observed value. Such a discrepancy is not surprising, for the transition moment directions are not experimental values, and the magnitude of the interaction potential is critically sensitive to their geometry. In order to emphasize this argument, we have performed the calculation of the rotational strength as a function of the relative orientations of the two chromophores. Using the procedure of Cox (1967) the tilt and twist of Trp-63 relative to Trp-62 was progressively varied. The map of rotational strength as a function of their geometric relation is shown in Figure 5, from which it is apparent that a relatively modest adjustment of the angles can lead to a large change, and even a reversal of sign in rotational strength. It should be noted that a tryptophan dimer apparently occurs only in the hen and duck egg lysozymes, and a search of the model reveals no other point of close approach between aromatic residues. We conclude from the calculation (1) that the proximity of the two active-site residues should, for reasonable transition moment directions, generate an appreciable rotational strength in the aromatic absorption region, and (2) that this can certainly be of a sufficient magnitude to explain the difference between the circular dichroism of the egg-white lysozymes, and the other proteins in the series (Figure 1). There are a number of quantitative reservations about these calculations which are mentioned below. A similar calculation, using the transition moment data of Pao *et al.* (1965) for tyrosine, shows that the Trp–Tyr pair at the active site of human urinary lysozyme could also generate a significant rotational strength in the aromatic region.

Disulfide Contributions. It is now well established that cystine residues contribute significantly to the optical activity in the region of 240–260 nm (Beychok, 1965, 1968; Imanishi and Isemura, 1969). There are two possible orientations of a disulfide bridge which may be seen as corresponding to a left- or right-handed sense, and in at least one model case the Cotton effect is inverted when a transition occurs from one to

			20									30			
LEU	Lys	GLY	TYR	Gly	GLY	Val	SER	LEU	Pro	Glu	TRP	VAL	CYS	Thr	Thr	Phe
Ile	ASP	GLY	TYR	Gly	GLY	ILE	Ala	LEU	Pro	Glu	Leu	Ile	CYS	Thr	Met	Phe
LEU	Ala	GLY	TYR	ARG	Asp	ILE	Thr	LEU	Pro	Glu	TRP	Leu	CYS	Ile	Ile	Phe
LEU	ASP	Asn	TYR	ARG	GLY	Tyr	SER	LEU	Gly	Asn	TRP	VAL	CYS	Ala	Ala	Lys
Met	ASP	GLY	TYR	ARG	GLY	ILE	SER	LEU	Ala	Asn	TRP	Met	CYS	Leu	Ala	Lsy
50										60						
Gln	SER	THR	ASP	TYR	GLY	Leu	PHE	GLN	ILE	ASN	Asn	Lys	Ile	TRP	CYS	Lys
Gln	SER	THR	Glu	TYR	GLY	Leu	PHE	GLN	ILE	Ser	Asn	Lys	Leu	TRP	CYS	Lys
Asn	His	Lys	Glu	TYR	GLY	Leu	PHE	GLN	ILE	ASN	Asn	Lys	Asp	Phe	CYS	Glu
Gly	SER	THR	ASP	TYR	GLY	Ile	Leu	GLN	ILE	ASN	Ser	Arg	Trp	TRP	CYS	Asn
Arg	SER	THR	ASP	TYR	GLY	Ile	PHE	GLN	ILE	ASN	Ser	Arg	Tyr	TRP	CYS	Asn
							90									
Lys	Phe	LEU	Asn	Asn	ASP	Leu	THR	Asn	Asn	Ile	Met	CYS	Val	LYS	LYS	ILE
Lys	Phe	LEU	Asn	ASP	ASN	ILE	THR	Asn	Asn	Ile	Met	CYS	ALA	LYS	LYS	ILE
Lys	LEU	LEU	Asn	ASP	ASN	Leu	THR	Asn	Asn	Ile	Met	CYS	Val	LYS	LYS	ILE
Ala	LEU	LEU	Ser	Ser	ASP	ILE	THR	Ala	Ser	Val	Asn	CYS	ALA	LYS	LYS	ILE
Ala	LEU	LEU	Gln	ASP	ASN	ILE	Ala	Asp	Ala	Val	Ala	CYS	ALA	LYS	Arg	Val
				120										130		
CYS	Ser	GLU	Lys	Leu	Asp	GLN	TRP	Leu			Cys	Glu	Lys	LEU		
CYS	Thr	GLU	Lys	Leu	Glu	GLN	TRP	Leu			Cys	Glu	Lys	LEU		
CYS	Ser	Asp	Lys	Leu	Glu	GLN	TRP	TYR			Cys	Glu	Ala	Gln		
CYS	Lys	Gly	Thr	Asp	Val	GLN	Ala	Trp	Ile	Arg	Gly	Cys	Arg	LEU		
CYS	Gln	Asn	Arg	Asp	Val	Arg	Gln	TYR	Val	Gln	Gly	Cys	Gly	Val		

lications to *Eur. J. Biochem.*) Capitals indicate a residue identical in at least one lysozyme and one α-lactalbumin.

the other (Claeson, 1968; Ludescher and Schwyzer, 1971). It is not unlikely however that in proteins the nature of the cystine contribution will be perturbed by the orientation of the peptide residues. With the three permitted ranges of the backbone angles of rotation (Brant *et al.*, 1967), the two disulfide orientations would be capable in principle of producing 18 types of conformation. Applying the data for cystine of Imanishi and Isemura (1969) to hen egg lysozyme, which contains two disulfide bridges of either sense (Blake *et al.*, 1967), the net contribution to the ellipticity at 260 nm is −2300°, which is similar to the observed value. There is an appreciable calculated positive contribution (600°) at 300 nm, with a crossover at 280 nm. The precipitous change of the hen egg lysozyme curves between 280 and 260 nm may therefore be attributable to the disulfide bonds, but this would carry the corollary that in the other proteins the cystines are at least partly in different states of chirality (*cf.* Browne *et al.*, 1967). With one left- and three right-handed cystines the contribution is negative down to below 240 nm (molar ellipticity of −1300, −3000, −3400, and −380°, respectively, at 300, 280, 260, and 240 nm). This situation would appear best to fit the observed data.

Peptide Cotton Effects. Figure 4 shows that in the peptide Cotton effect region all the proteins in our group are broadly similar, though the egg lysozymes deviate somewhat, showing an apparent blue shift in the n–π* region, and goose egg lysozyme is distinctly different from the rest. Judged by the model compounds (Imanishi and Isemura, 1969) the disulfide contribution should not be sufficiently great here to make any important differences. We are therefore inclined to attribute the differences to a large aromatic contribution in hen and duck egg lysozyme alone. In goose egg lysozyme the aromatic Cotton effects are almost absent, and it seems likely that the circular dichroism in the peptide region is essentially unperturbed by side chain contributions. As has been noted, however (Canfield *et al.*, 1971), this protein appears to be unrelated to the other lysozymes which we have examined.

We have attempted to assess possible aromatic contributions in the region of peptide circular dichroism from the tryptophan–tryptophan interaction. A map such as that of Figure 5 was constructed for the $B_{a,b}$ transition (near 225 nm). In the region of the map corresponding to the observed geometry the exciton term in the interaction is small, and the dispersion somewhat large, though still relatively insignificant, and negative. We are however reluctant to draw any quantitative conclusions from this result, partly because of the possibility that the assumed directions of the transition moments are appreciably in error, because of the oversimplified treatment of the $^1L_{a,b}$ contributions, and also because of the possibility, important in this region of the spectrum, of interactions between the $^1B_{a,b}$ transition and transitions in nearby peptide groups. Such effects could be of critical importance. These strictures, in particular the first two, apply equally to the calculations for the aromatic region.

It is also of course possible that the presence of a charge near an aromatic ring could result in a gross enhancement of its optical activity, and the relative effects of ionization changes at the α-carbon on the optical activity of aromatic amino acids have been reported by Rosenberg (1966). From this work it is clear that the effect in the $^1B_{a,b}$ transition can be very substantial. Residues conceivably implicated might be Tyr-23, which is unique to the hen and duck lysozymes.

We note finally that the uniqueness of the circular dichroism of the latter proteins in the peptide region indicates that these

would be poor choices for attempts to fit the curve with known standards. It is qualitatively apparent, in terms of the standard curves compiled by Greenfield and Fasman (1969), that the other proteins of the group would give a better fit in this region to a mixture of the curves for α-helix random coil and β structure, but that it would still be by no means good.

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