Summary.—Soon after infection, partially replicated parental (PRM) DNA can be isolated in CsCl. The newly synthesized strands are associated with the parental molecule only by hydrogen bonding as they can be separated by denaturation. After sonication, this partially replicated DNA is dissociated into two classes—one parental-like, and the other having a density intermediate between parental and hybrid.

A large fraction of partially replicated DNA, when characterized in a sucrose gradient, sedimented at least twice as rapidly as unbroken DNA from T4 particles; therefore, a circular and folded tertiary structure of replicative T4 DNA is postulated.

Abbreviations: DNA, deoxyribonucleic acid; 5-BU, 5-bromodeoxyuridine; FUdR, 5-fluorodeoxyuridine; "hot," labeled with radioactive isotope; "cold," not labeled with radioactive isotope; "heavy," substituted with heavy density marker 5-BU; "light," not substituted with heavy density marker 5-BU; CM, chloramphenicol; m.o.i., multiplicity of infection; CS, citrate salt buffer; EM, electron microscope; TD, thymidine; PRM, partially replicated molecule—replicative DNA, moiety of parental DNA which, during semiconservative replication, acquires a new strand (of a new density in a particular experimental system); PNC, polynucleotide chain; CSF, citrate salt buffer with 1% formaldehyde.

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*PROTEIN-HEME INTERACTIONS IN HEME-PROTEINS: CYTOCHROME C**

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Protoporphyrin IX, when free in solution as hemin, does not rotate the plane of polarized light. Yet, when bound to its apoprotein, as in myoglobin[1] or hemoglobin,[2] its electronic transitions become optically active. The present communication considers the source of this optical activity by enumerating dominant terms in the rotational strength of the Soret transition, and by invoking the sum rule for coupled transitions which requires that other regions of the optical rotatory dispersion (ORD) curves of heme-proteins be altered as a consequence of a simple Soret Cotton effect. These considerations will be applied to ORD curves of cytochrome *c* which on superficial examination appear in contradiction with established views on the optical rotation of proteins and polypeptides. However, the incongruity is only apparent in that it assumes that cytochrome *c* follows a simple polypeptide model. Detailed analysis of the data discloses a protein-heme interaction, and

evidence will be discussed which suggests that part of the effect is due to a helix-heme interaction. Furthermore, such an interaction of heme with protein can be substantiated thermodynamically by exploiting the requirement that separate regions of the ORD curves relate in a particular way.

General Considerations in the Rotatory Dispersion of Coupled Electric Transitions.— In a general and most illuminating derivation of the rotational strength, Tinoco[3] has clarified the relationship between the polarizability,[4] one electron,[5] and many electron[6] theories of optical rotation by obtaining terms used in all three approaches. Tinoco's result, a six-term expression, provides a sound basis from which to consider special cases of particular interest. For example, when one is dealing with an electrically allowed, magnetically forbidden transition, four of the six terms are zero, leaving two terms, one of which dominates at relatively large distances. Under these conditions the major term is the electric-electric coupled oscillator term which is the basis of the Kirkwood polarizability theory.[4] Thus, if two strong electric transitions a and b are oriented such that they derive their rotational strengths from interacting, each with the other, then the rotational strength of transition a due to coupling with transition b is given by

$$R_{a,b} = \frac{-2\pi}{c} \frac{V_{a,b}\nu_a\nu_b \, (\mathbf{R}_b - \mathbf{R}_a) \cdot \boldsymbol{\mu}_b \times \boldsymbol{\mu}_a}{h(\nu_b^2 - \nu_a^2)}, \tag{1}$$

where $V_{a,b}$ is the dipole interaction potential; ν_a and ν_b are the frequencies of the transitions; $(\mathbf{R}_b - \mathbf{R}_a)$ is the distance from a to b; and $\boldsymbol{\mu}_a$ and $\boldsymbol{\mu}_b$ are the electric transition dipole moments. Similarly, one may write for the rotational strength of transition b due to coupling with transition a

$$R_{b,a} = -\frac{2\pi}{c} \frac{V_{b,a}\nu_a\nu_b \, (\mathbf{R}_a - \mathbf{R}_b) \cdot \boldsymbol{\mu}_a \times \boldsymbol{\mu}_b}{h(\nu_a^2 - \nu_b^2)}. \tag{2}$$

It is apparent that

$$V_{a,b} = V_{b,a}; \qquad (\mathbf{R}_b - \mathbf{R}_a) = -(\mathbf{R}_a - \mathbf{R}_b)$$

$$\boldsymbol{\mu}_b \times \boldsymbol{\mu}_a = -\boldsymbol{\mu}_a \times \boldsymbol{\mu}_b; \qquad (\nu_b^2 - \nu_a^2) = -(\nu_a^2 - \nu_b^2)$$

giving

$$R_{a,b} = -R_{b,a} \tag{3}$$

or

$$R_{a,b} + R_{b,a} = 0. \tag{4}$$

Equation (3) also holds for the term with steeper distance dependence. This means that such a coupling of transitions will, for example, give a positive contribution to the Cotton effect centered at wave length λ_a and a negative contribution to that at λ_b. Indeed, equation (4) is the sum rule first noted by Kuhn in treating a classical coupled oscillator model.[7] The quantum mechanical proof of a general sum rule is given by Condon.[8] This reciprocal behavior provides a specific test which may in favorable circumstances be used to obtain detailed information on the conformation of a molecular system. Thus if a chromophoric group contains an electric transition which derives part of its rotational strength from coupling with an electric transition in a spatially related group, a variable whose effect is to change

their spatial orientation must alter the respective Cotton effects in a reciprocal manner. Conversely, reciprocal behavior elicited by a variable such as temperature may be used to relate two groups spatially. The form of equation (3) and the reciprocal behavior it implies suggests the term *reciprocal relation* as a descriptive designation of this situation. In favorable cases where μ may be estimated from the absorption curves and R_A from the ORD or circular dichroism curves, it becomes possible to relate spatially two chromophoric groups in a molecule. Such considerations indicate that adenine and nicotinamide interact in a specific way in reduced β-diphosphopyridine nucleotide.[9]

Consequences of the Sum Rule (Reciprocal Relations) in the ORD of Heme-Proteins.—The heme group in myoglobin or hemoglobin derives its optical activity from dissymmetric interaction with the protein. The very large Soret transition dipole moment suggests that a considerable part of its rotational strength arises from coupling with transitions occurring in neighboring groups. Considering the electric-electric coupled oscillator contribution to the rotational strength of the Soret transition, R_S, we may write for coupling with protein transitions,

$$R_S = -\frac{2\pi}{c}\sum_p \frac{V_{s,p}\nu_s\nu_p\,(\mathbf{R}_p - \mathbf{R}_s)\cdot\underline{\mu}_p \times \underline{\mu}_s}{h(\nu_p^2 - \nu_s^2)} = \sum_p R_{s,p}, \tag{5}$$

where the summation is over all protein transitions. By the reciprocal relations noted above, we have

$$R_{s,p} = -R_{p,s}. \tag{6}$$

Therefore, even if the heme were to bind the protein in such a way as to cause no change whatever in the protein conformation, one would still expect the rotational strengths of the protein transitions to change. Thus, changes in the peak and trough in the ORD of apomyoglobin upon binding of the heme moiety[10, 11] cannot be directly interpreted as a change in helical content. Nor can the increase in peak and trough values in cytochrome *c* upon reduction in phosphate buffer[12, 13] be interpreted directly as an increase in helical content, since these may equally, in view of the reciprocal relations implicit in the sum rule, reflect a changed helix-heme orientation. However, the reappearance of the helical Cotton effects upon reduction of ferricytochrome *c* in 3 *M* guanidine hydrochloride[13] would appear to have no alternative explanation other than the regeneration of a helical region.

As the ORD extrema characteristic of helical proteins are found to change depending on the presence and state of the heme, it is of interest to consider the form which the sum rule would take for coupling of the Soret transition with transitions in helical array. It may be noted in passing that the transitions in the 260–300-mμ range of the aromatic groups in myoglobin and hemoglobin have Cotton effects of small amplitude when compared to the Soret Cotton effect and cannot themselves be coupled to produce the very large rotational strength of the Soret transition. In addition, the exciton interactions in the helical segments would resolve the electric transitions of the peptide chromophore into parallel and perpendicular components.[14] Thus, for helix-heme interactions one has the relationship for the contribution to the Soret transition's rotational strength

$$R_{S,H} = \sum (R_{s,\perp i} + R_{s,\parallel i}) = -\sum_i (R_{\perp i,s} + R_{\parallel i,s}). \tag{7}$$

Contributions to the trough at 232 mμ and the peak at about 200 mμ represent two of at least eight significant terms of differing sign in the summation. Thus, it is possible for the terms in the summation to be greater than the sum itself.

The presence of an element of symmetry in the porphyrin moiety would require that the dot product of the electric dipole and magnetic dipole moments of the Soret transition be zero, and therefore would require that the rotational strength of the Soret transition arise from coupling with dissymmetrically arranged transitions in the protein. The degeneracy of the Soret transition necessitates in this case a fourfold symmetry axis.[15] The simplicity of the Soret Cotton effect in myoglobin and hemoglobin indicates sufficient symmetry to retain the degeneracy of this transition and supports the thesis that rotatory power arises from coupling with protein transitions. The more complex Soret Cotton effects in cytochrome *c* suggest removal of the degeneracy, albeit not sufficient to be apparent in the absorption curve at room temperature. In this case a reciprocal behavior elicited by systematically varying temperature and pH may be used to assess coupling with protein transitions.

Hemoglobin presents an interesting case due to the presence of four hemes. Should a substantial dipole interaction potential exist between the Soret transition dipole moments, one would expect a splitting of the energies of the transition and an increased complexity of the Cotton effect. A simple Soret Cotton effect would suggest that this coupling is negligible. The sensitivity of this correlation as a probe to assess the nature of heme-heme interactions is being investigated.

Effect of Temperature, pH, and Guanidine HCl on Cytochrome c.—In general, the helical contents of proteins may be approximated either by using rotation at a single but sensitive wavelength or by employing the Moffitt and Yang equation[16]

$$[m'] = a_0 \frac{\lambda_0^2}{\lambda^2 - \lambda_0^2} + b_0 \left(\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2}\right)^2, \tag{8}$$

in which case the coefficient, b_0, of the second term is responsive to a helical array of identical strong electric transitions and as such is a good index of helicity. The following are three equivalent expressions from which the fraction of helix, f_H, may be obtained:

$$[m']_\lambda^{obs} = [m']_\lambda^D + f_H ([m']_\lambda^H - [m']_\lambda^D)$$

$$a_0^{obs} = a_0^D + f_H (a_0^H - a_0^D)$$

$$b_0^{obs} = b_0^D + f_H (b_0^H - b_0^D),$$

where it has been assumed that the rotatory dispersion of the protein under study may be described as a sum of helical, H, and nonhelical or disordered, D, segments[17] of a simple polypeptide or protein. The parameters a_0 and b_0 are obtained by plotting $[m']/x$ versus x, where $x = \lambda_0^2/(\lambda^2 - \lambda_0^2)$. In the 240–315-mμ range for paramyosin, a completely helical protein, the Moffitt and Yang parameters are approximately -350, -100, and 220 mμ for b_0, a_0, and λ_0, respectively.[18] These values compare very favorably with those obtained by averaging the parameters of three helical polypeptides determined over approximately the same spectral range.[19, 20]

On this basis we can now consider the effect of temperature on the ORD curve of

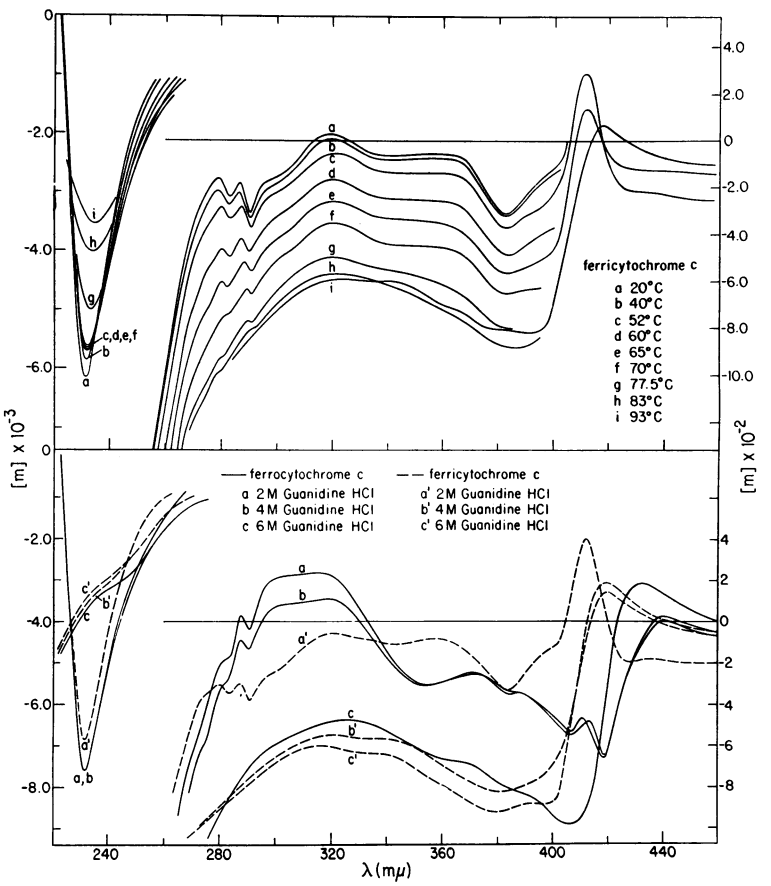

FIG. 1.—*Top:* Effect of temperature on the ORD curve of ferricytochrome *c*. *Bottom:* Effect of guanidine hydrochloride on the ORD curves of ferri (- - -) and ferro (———) cytochrome *c*.

ferricytochrome *c* (Fig. 1). It is seen that in the temperature range of 52–70°C the extremum at 232 mμ does not show any significant change, whereas the relative positive displacement and curvature diminish progressively with increasing temperature. Figure 2 shows the pH effect to exhibit qualitatively the same pattern, though with a slight enhancement of the trough. The changing background and curvature argue that the helical content is changing, whereas the constancy of the trough value argues to the contrary that there is no change in helical content. This is not simply a discrepancy due to the choice of parameters, but indicates that, whatever the choice may be, the helical content based on $[m']_{232}$ is constant while that based on $[m']_{260}$ or on a_0 and/or b_0 is decreasing with increased temperature or with approach of pH extremes.[21] However, the apparent breakdown in the determination of protein conformation by ORD is only a reflection of the inadequacy, in this case, of the simple polypeptide model.

Obviously, the gross feature which does not fit such a model is the heme with its very large Soret transition dipole. The Soret transition is implicated in a direct way in that its Cotton effect is also changing under these conditions. Yet it is not

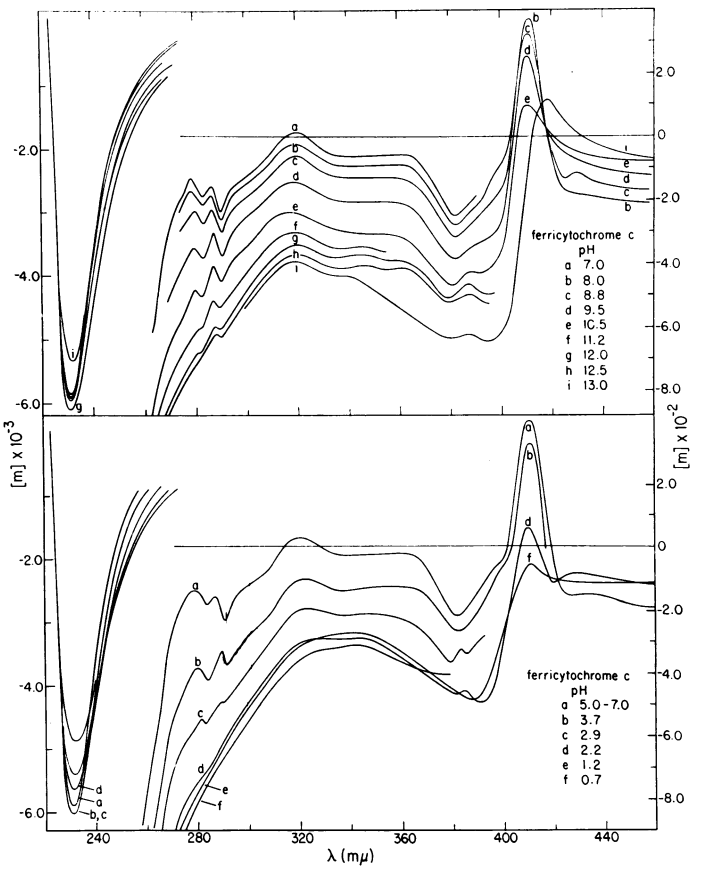

FIG. 2.—Effect of pH on the ORD curve of ferricytochrome *c*.

this Cotton effect *per se* which is responsible for the varying background rotation, as the magnitude of the changes at 280 mμ are greater than at 383 mμ, the latter being an extremum of the Soret Cotton effect. Nor is it likely that the positive displacement can be ascribed to Cotton effects of other heme transitions, as the guanidine hydrochloride denatured ferricytochrome *c* shows a strong Soret Cotton effect but at shorter wavelength exhibits little more than a characteristic curve for a denatured protein. It should also be noted that local Cotton effects such as may arise from aromatic residues in this wavelength region could not be responsible for the broad positive displacement. Accordingly, we interpret the changing background of the rotatory dispersion, conveniently measured at 280 mμ, to reflect changes in the environment of the peptide chromophore. The background rotation becomes less positive with increasing temperature, which suggests that interaction of protein transitions with the Soret transition results in a positive $\sum_p \Delta R_{p,s}$. The complex Soret Cotton effect can be approximated by a longer wavelength negative Cotton effect and a shorter wavelength positive Cotton effect, the positive extrema of which overlap. On raising the temperature, the negative Cotton effect is lost and the positive Cotton effect increases in magnitude. The

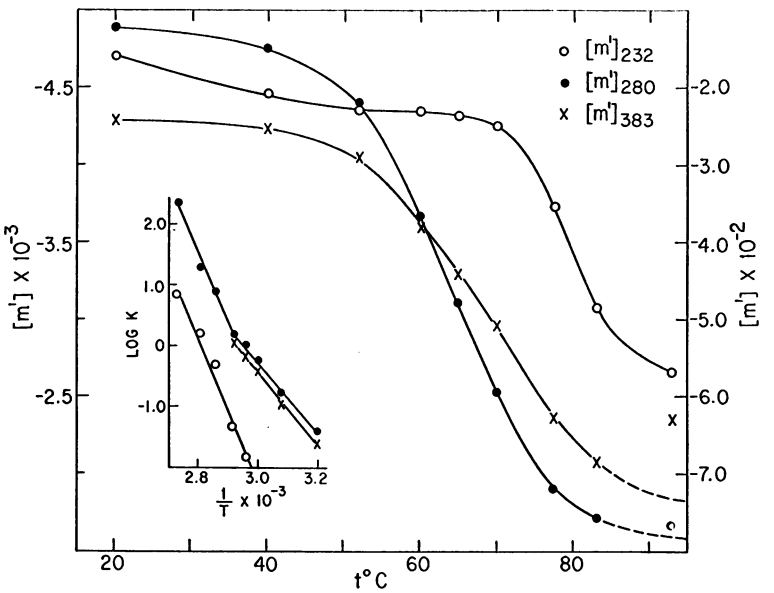

FIG. 3.—Temperature effect on the mean residue rotation at 232 mμ, an extremum characteristic of helical proteins, at 280 mμ, a wavelength exemplary of the changing background rotation, and at 383 mμ, an extremum of the complex Soret Cotton effect. Values for $[m']_{232}$ are on the left ordinate while those for $[m']_{280}$ and $[m']_{383}$ are on the right ordinate.

Insert: Plot showing that the process observed at 383 mμ has the same free energy change as that occurring at 280 mμ. It is also seen that the background rotation breaks into a steeper curve which approaches the slope of the helix-coil transition followed at 232 mμ.

$\sum_p \Delta R_{s,p}$ for the protein-heme interaction would then be negative in accord with the reciprocal relations.

A specific protein-heme interaction is clearly suggested by the above correlation. If this is so, it is necessary that the thermodynamic process exemplified by the change in $[m']_{383}$ and $[m']_{280}$ have the same free energy change, ΔF. Figure 3 is a plot showing the change in mean residue rotation at 280, 383, and 232 mμ as a function of temperature. It can be seen that $[m']_{280}$ and $[m']_{383}$ show thermal transitions at approximately the same temperature. Taking an equilibrium between the two states indicated by this transition and defining them as interacted and unfolded forms, we have

$$\text{interacted} \rightleftharpoons \text{unfolded}$$

$$K = \frac{[\text{unfolded}]}{[\text{interacted}]} = \frac{x_u}{x_i} = e^{-\Delta F/RT}$$

$$x_i = \frac{[m']_\lambda^{\text{obs}} - [m']_\lambda^u}{[m']_\lambda^i - [m']_\lambda^u} = 1 - x_u.$$

If $\log K$ is now plotted against $1/T$ as in the insert of Figure 3, it is clear that the change in background and the change in Soret Cotton effects do indeed have the same slope. ΔF for the process is approximately +30 kcal/mole. In addition, it is seen that the curve obtained from data at 280 mμ breaks into a steeper slope

which approaches that taken at 232 mμ and which represents the helix-coil transition with a ΔF of approximately +50 kcal/mole. The temperature denaturation of ferricytochrome *c* is therefore a two-step process, the first step being an unfolding of a protein segment from close interaction with the heme, and the second step being a helix-coil transition.

Additional observations may be cited in support of such a view. Ferricytochrome *c* having been denatured at 90°C may then be cooled to 20°C. The ORD curves of this sample show by the magnitude of the trough that the helix has been reformed, but the native Soret Cotton effect and the positive displacement are not regained. Even after a month, complete renaturation has not occurred. However if, on cooling, the protein is reduced with dithionite and reoxidized with potassium ferricyanide, then a more nearly native Soret Cotton effect is regenerated and the positive displacement has recurred. Thus by the ORD criteria *reduction has a renaturation effect* and may be interpreted as effecting reformation of the protein-heme interaction under these conditions. Furthermore, in the temperature range 20–70°C ferrocytochrome *c* does not show a similar gross conformational change. Thus the protein-heme interaction in ferrocytochrome *c* involves a considerably greater free energy of interaction than in ferricytochrome *c*. Also, ferrocytochrome *c* does not exhibit the pH effects seen in ferricytochrome *c*, as the reduced form has an ORD curve which is altered surprisingly little over the pH range 2.9–12.5. From this, one may conclude that *reduction appears to stabilize the protein-heme interaction.*

A parallel situation is found in guanidine hydrochloride (GHCl) (see Fig. 1). If the concentration of GHCl is increased stepwise to 4 *M*, ferricytochrome *c* is found to be completely denatured by ORD criteria. Reduction in the presence of 4 *M* GHCl results in reformation of considerable amount of helix, regeneration of the heme environment, and recurrence of the positively displaced background. This renaturation effect can be achieved essentially undiminished up to GHCl concentrations of 4.5 *M*. *Reduction therefore stabilizes a helical segment.* The reformation of helix upon reduction and the simultaneous re-establishment of a near-native heme environment suggest that the protein-heme interaction can be stated in more specific terms. Thus it may be that the protein-heme interaction is more specifically a helix-heme interaction in which a helical segment is in close association with the heme and is likely held in position by the binding of a ligand from the protein to the heme iron, the ligand being much more strongly bound in the reduced protein. The broad positive displacement supports the prospect that one is observing an enhancement of the rotational strength of many regularly oriented and identical transitions due to coupling with heme transitions, as is to be expected from a helix-heme interaction.

Summary.—It has been noted that, insofar as the Soret transition derives its rotational strength from coupling with protein transitions, the rotational strengths of the protein transitions must have been altered in a reciprocal manner governed by the sum rule. Retention of the degeneracy of the Soret transition in myoglobin and hemoglobin, as measured by the intensity of the Soret band and the simplicity of the Soret Cotton effect, indicates that the chromophore has retained its inherent symmetry and therefore supports the proposition that a substantial contribution to the rotational strength of the Soret transition in these heme-proteins arises from coupling with protein transitions.

Interpretation of the cytochrome *c* data based solely on a polypeptide or simple protein model leads to an apparent contradiction which is resolved by consideration of coupling of heme and protein transitions. The reciprocal relations required for this coupling are noted and the expected thermodynamic relations are found. Thus, evidence has been presented for the coupling of protein and heme transitions in cytochrome *c*.

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[21] A best set of Moffitt parameters, obtained from smoothed curves, indicates that b_0 is little affected by the protein-heme interaction (-112 at 52°C and -114 at 70°C), that the major change occurs in a_0 ($+37$ at 52°C and -148 at 70°C), and that a small change occurs in λ_0 (221 mμ at 52°C and 219–220 mμ at 70°C). Also on raising the pH from 7.0 to 10.8, the positive extremum at 200 mμ appears to shift 2 or 3 mμ toward shorter wavelengths.

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*STRUCTURE OF A POLYSACCHARIDE PROTEIN COMPLEX**

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The presence of complex sulfated polysaccharides in connective tissue has been recognized for approximately 100 years. Cartilage, in particular, is a rich source of such materials, where they comprise up to 30 per cent or more of the dry weight of the tissue. Chemical studies on the polysaccharide components of connective tissue carried out primarily by Meyer and co-workers have demonstrated certain