OPTICAL ROTATION AND HELICAL POLYPEPTIDE CHAIN CONFIGURATION IN COLLAGEN AND GELATIN*

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INTRODUCTION

Polarimetry has been used to detect the occurrence of configurational changes accompanying denaturation in various proteins (Jirgensons, 1950, 1951, 1952; Kauzmann *et al.*, 1953; Yang and Foster, 1954; Linderstrøm-Lang and Schellman, 1954). However, because of the present incomplete knowledge of protein structure, no concrete proposals have yet been made connecting observed rotational values and details of polypeptide chain configuration. The collagen-gelatin system is particularly suitable for such a correlation, since progress has been made in the interpretation of both the collagen wide-angle x-ray diagram (Bear, 1952; Pauling and Corey, 1953; Cohen and Bear, 1953) and the optical rotivity of gelatin (Robinson, 1953).

Gelatin exhibits a well known and striking mutarotation. The reversible, temperature-dependent, sol-to-gel transformation is accompanied by a change in the specific rotation $[\alpha]_D$ from about $-100°$ to near $-300°$ (Trunkel, 1910; Smith, 1919). An apparent relationship between increase of aggregation and of optical rotation has led some investigators to attribute the mutarotation solely to the effect of molecular interactions (Kraemer and Fanselow, 1925). Katz (1932), however, by wide-angle x-ray diffraction studies, proved the existence of two different molecular forms in the sol and gel states, showing that the configuration of the molecules in the gel state is similar to that in native collagen. He explained the mutarotation by this fact. More recently, Robinson has presented evidence from infrared absorption and optical rotation of gelatin films (Robinson and Bott, 1951; Robinson, 1953) and has interpreted the mutarotation as due, for the most part, to the gain and loss of a "collagen fold" in the polypeptide chains of the gelatin molecules.

A quantitative comparison between the optical rotation of native collagen and a

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gelatin derived therefrom has not previously been reported and is of significance for the mutarotation phenomenon in gelatin, as well as for more general aspects of the problem of optical rotation and protein structure. Thaureaux (1945) examined the rotation of acid extracts of collagen and found that the high rotation values of the extracts dropped sharply when gelatin was produced by heating. Since then purer preparations have become available because of developments initiated by the work of Orekhovich, Tustanovskii, Orekhovich, and Plotnikova (1948) on the isolation and identification of citrate-soluble fractions of collagen ("procollagen"). Similar fractions have been studied physicochemically and electron optically by other investigators (Bresler, Finogenov, and Frenkel, 1950; Gross, Highberger, and Schmitt, 1954; Randall *et al.*, 1953). Gallop (1955) has characterized, by physicochemical means, a citrate extract from the tunic of carp swim bladder, as well as monodisperse gelatin ("parent gelatin") prepared therefrom. Preparations obtained by the method of Gallop have been used in the present investigation.

Experimental Methods

For procedures employed in the isolation and "recrystallization" of the citrate-extracted ichthyocol and its conversion to parent gelatin see Gallop (1955). Concentrations were determined by a colorimetric method, involving the biuret reaction, similar to that described by Gornall, Bardawill, and David (1949) and also used by Gallop. Unless otherwise stated, solutions were prepared with 0.15 M citrate at pH 3.7, and the term ichthyocol always refers to the citrate-extracted materials.

Two polarimetric methods were employed:—

1. Preliminary measurements were made with a Schmidt and Haensch polarimeter, which could be read to 0.05° and was used with an unfiltered sodium lamp. Temperature regulation was maintained by means of water from a thermoregulated bath circulated through metal-jacketed 2 dm. polarimeter tubes. Temperature was controlled to about ±0.5°C.

2. Rotatory dispersion measurements were made using a Rudolph high precision polarimeter which could be read to 0.002°. Under the conditions of experimentation readings were reproducible within about 0.01°. Metal-jacketed 4 dm. polarimeter tubes were used, and water from the thermoregulated bath was circulated through three tubes in series. The maximum temperature differential between the water bath and the tubes was about 1°C.

In all polarimetric work at least ten observations were averaged for each recorded reading. Four wave lengths of visible light were used for study of dispersion. Sources and filters are presented in Table I.

TABLE I

Sources and Filters for Dispersion Studies

Wave length, λ	Source	Filter
mμ		
436	G.E., H-4 Hg arc	Corning Glass Nos. 3389 and 5113
546	G.E., H-4 Hg arc	Baird interference filter No. V-1-250, Corning Glass No. 3-69, and didymium No. 1–63
578	G.E., H-4 Hg arc	Corning Glass Nos. 3480 and 502
589	G.E., Na-1 lamp	Yellow filter supplied with Rudolph polarimeter

Concentrations of ichthyocol from 0.02 to 0.46 per cent were examined at three different temperatures. The protein solutions were stored in the refrigerator at about 4°C., then transferred to the polarimeter tubes and equilibrated for 12 hours with the water bath at 10°C. Readings were then taken as representative of the native state. The solutions were kept overnight in the tubes at this temperature. On the following day the temperature of the bath was raised to 42°C. during the course of 5 hours and conversion to parent gelatin occurred. The tubes were allowed to equilibrate at this temperature for 1 hour, after which readings were taken. After 30 hours with the bath at 1°C., to allow complete mutarotation, readings were again made.

EXPERIMENTAL RESULTS

The Conversion of Ichthyocol to Parent Gelatin

Polarimetric method 1 was used. Fig. 1 shows rotation as a function of temperature at various times during a heating and recooling cycle. The optical rotation of unheated ichthyocol in citrate buffer was initially $[\alpha]_D^{10} = -350° \pm 30$. Upon heating the solutions to above 30°C., the rotation fell to values of $[\alpha]_D^{35} = -110° \pm 20$. The reaction time varied near 30°C., depending upon factors which were not analyzed further, but at 40°C. the reaction proceeded very rapidly, with specific rotation falling from −350° to −110° within about 30 minutes.

When the gelatin solutions were cooled, the rotation gradually rose and values of $[\alpha]_D$ up to about −290° were obtained, depending upon temperature, protein concentration, and length of cooling.

Once the value $[\alpha]_D = -110°$ has been reached by the system, the mutarotation phenomenon is reproducible. Upon cooling to a certain temperature the rotation rises, and when the solutions are reheated the rotation falls until, at a temperature greater than 30°C., the limiting value of −110° is again obtained.

Solutions made from citrate extracts of calf hide showed a slightly different behavior, requiring somewhat higher temperatures (increase of *ca.* 4°C.) to obtain comparable changes in rotation, but the values for the specific rotations (within about 15 per cent) and the mutarotation phenomenon were like those for the ichthyocol preparations.

Correlation with Viscosity Studies

Preliminary experiments were carried out with Dr. Paul M. Gallop to determine the time relationships, in ichthyocol and calf hide solutions, between the changes of specific rotation and of intrinsic viscosity during the conversion to gelatin. As Gallop has already reported (1955), in several cases the rotation decreased more rapidly than did the intrinsic viscosity, indicating the separability of these two phenomena. The kinetic relationships in this system are worthy of further study.

Rotatory Dispersion Measurements

Rotatory dispersion measurements were carried out using method 2 to analyze further the temperature effect on the specific rotation of the solu-

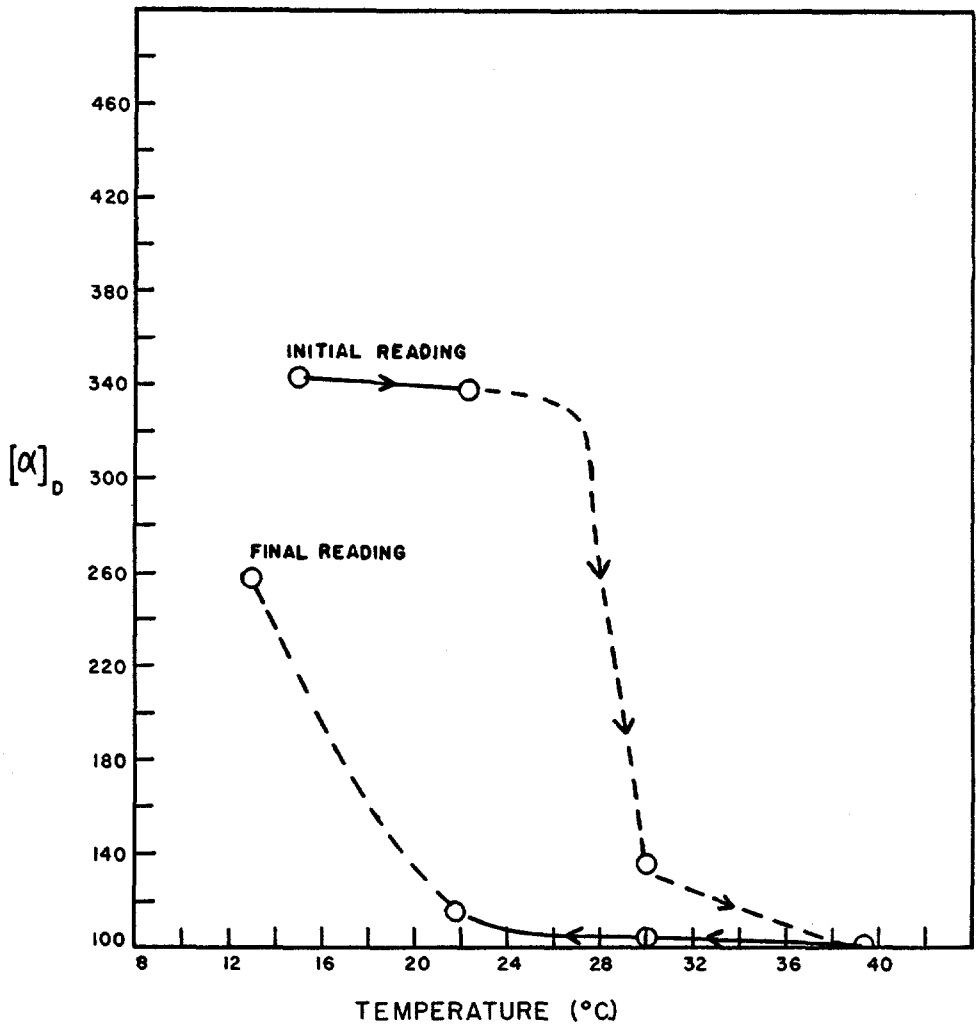

FIG. 1. Specific optical rotation, $[\alpha]_D$, of ichthyocol in pH 3.7 citrate buffer as a function of temperature. Concentration of ichthyocol 0.363 per cent. Time proceeds along the arrows, and dotted lines indicate where results were variable and not analyzed further in these experiments (see text).

tions of ichthyocol and parent gelatin. The results of these experiments are presented in Figs. 2 to 4, which show the readings at $T = 11°$, 41°, and 2°C., respectively. In order to include all of the data most clearly the figures show bilinear plots of $1/[\alpha]_\lambda^T$ *vs.* $(\lambda^2 \times 10^3 + 100\ c)$, in which c is the protein concentration in grams per 100 ml.

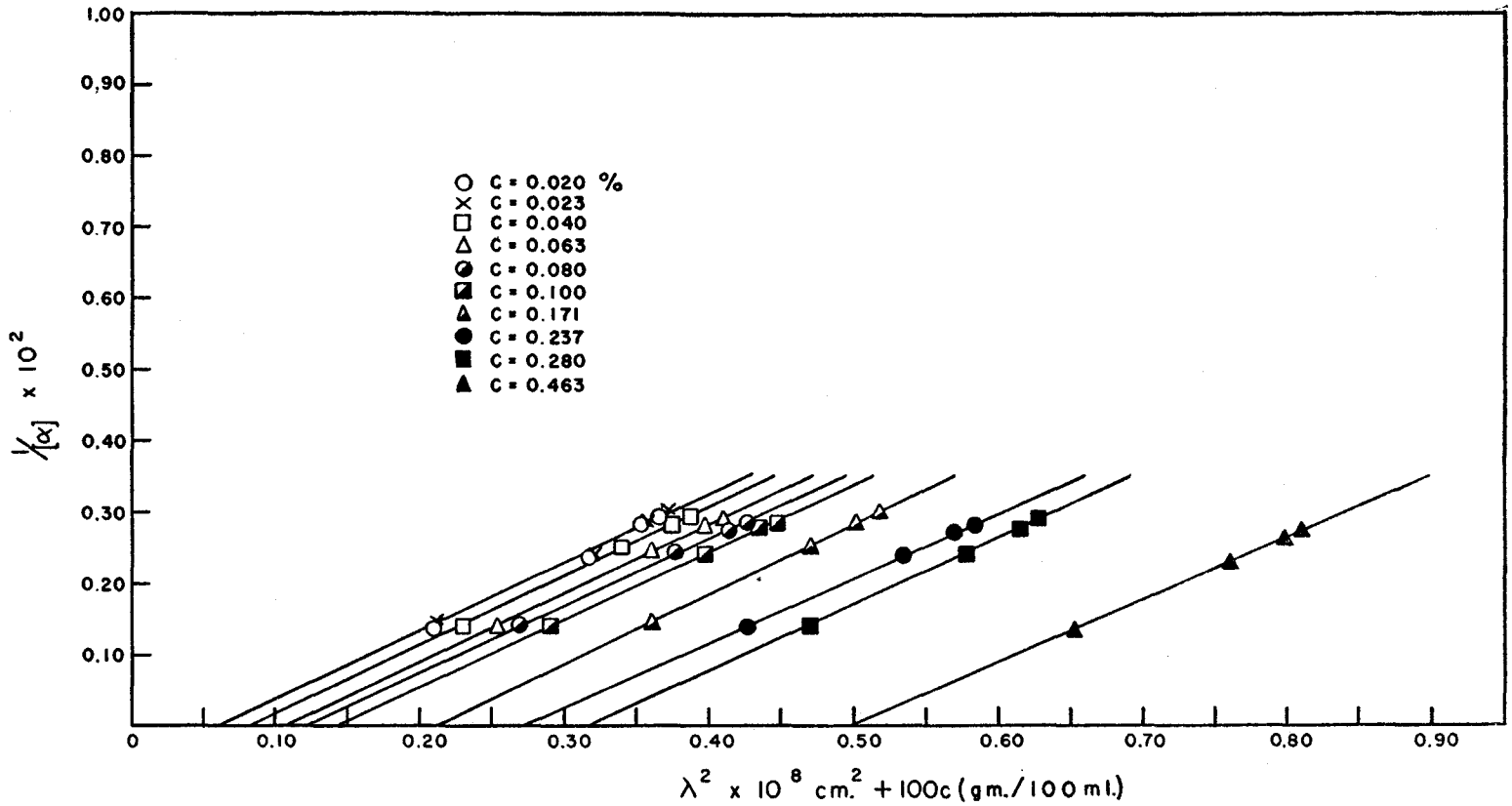

FIG. 2. Rotatory dispersion of ichthyocol in pH 3.7 citrate buffer at 11°C.

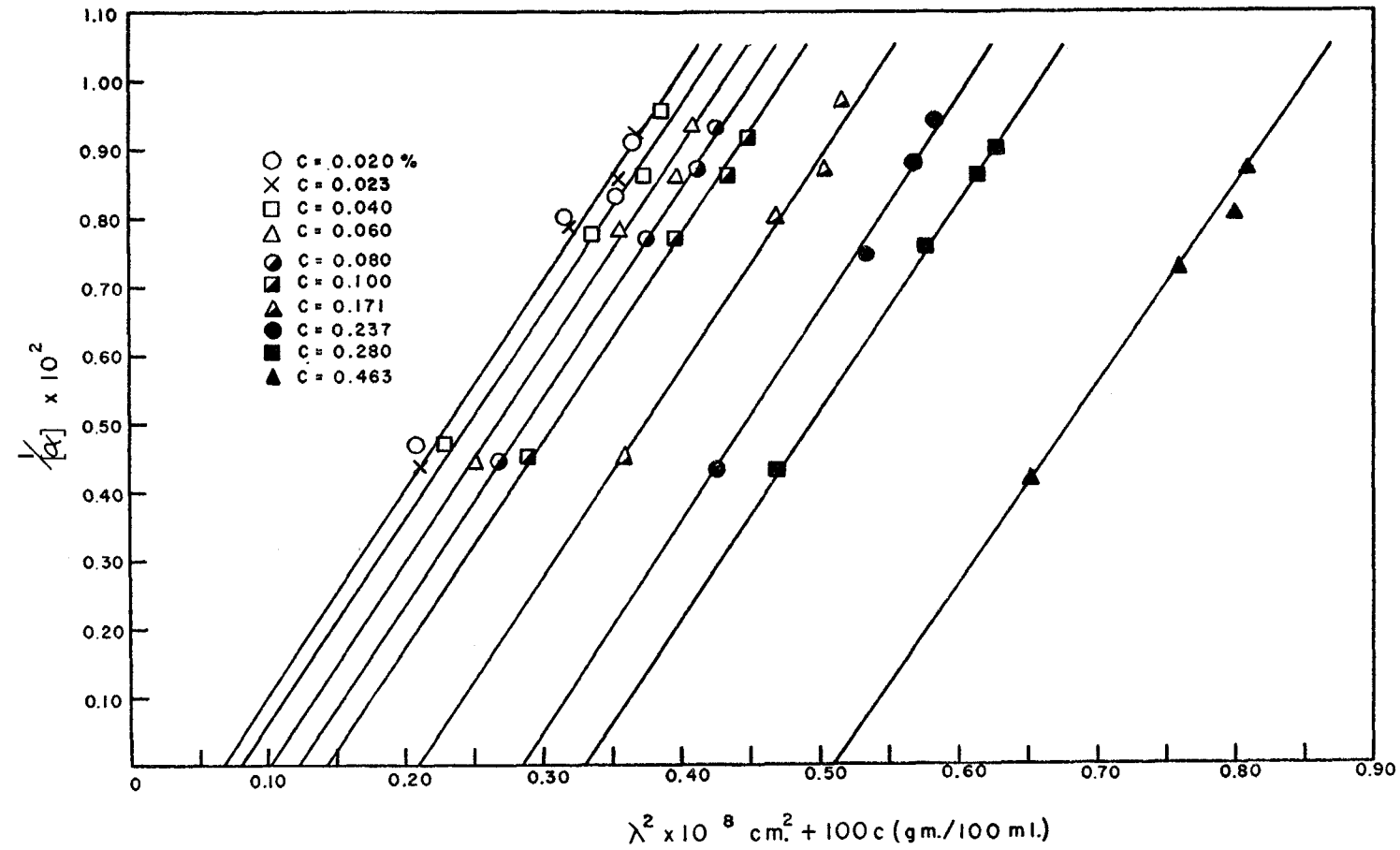

FIG. 3. Rotatory dispersion of ichthyocol parent gelatin in pH 3.7 citrate buffer at 41°C.

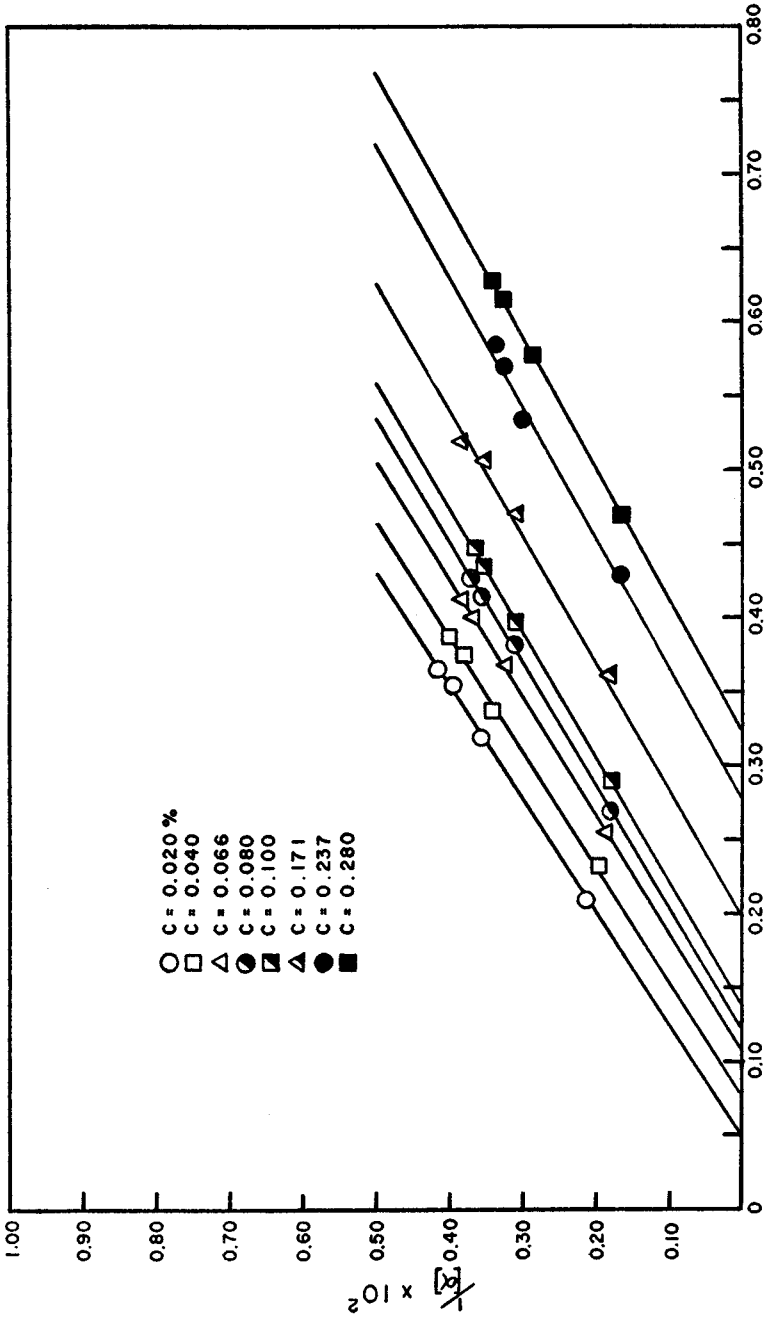

FIG. 4. Rotatory dispersion of ichthyocol parent gelatin in pH 3.7 citrate buffer at 2°C.

The results at each of the three temperatures indicate a linear relationship between $1/[\alpha]$ and λ^2, so that a single-term Drude equation,

$$[\alpha]_\lambda = \frac{k}{\lambda^2 - \lambda_0^2}$$

is applicable, in which k and λ_0 are parameters characteristic of the state of the system, which is in turn determined largely by the temperature employed. The parameter λ_0^2 is $0.042 \pm 0.006 \times 10^{-8}$ cm.^{2} and remains the same within experimental error for all cases. On the other hand, the rotation constants, k, for the medium concentration range are:—

at $T = 11°C.$, $k = 106 \times 10^{-8}$; at $T = 41°C.$, $k = 34 \times 10^{-8}$; and at $T = 2°C.$, $k = 84 \times 10^{-8}$.

Only the parent gelatin at 2°C. shows a significant concentration dependence. The experimental error is at least 10 per cent for the lower concentration so that one cannot derive the detailed shape of the concentration dependence curve from these data. The order of magnitude involved in the concentration dependence is in agreement with the data of Robinson (1951) and of Ferry and Eldridge (1949), who used higher concentrations (their lowest was 0.1 per cent) and other kinds of gelatin.

Dependence on Ionic Strength

A solution of ichthyocol which had been dialyzed exhaustively against 0.05 per cent HAc (pH about 3.5) yielded a rotatory dispersion curve similar to that obtained in citrate buffer of higher ionic strength (0.15 M citrate). This was true of parent gelatin also at 40°C. in solutions with the two different ionic strengths. However, when the parent gelatin was cooled the specific rotation observed in 0.05 per cent HAc (−210°) did not reach values as high as those characteristic of cool parent gelatin in citrate buffer (*ca.* −280°), although the dispersion constant λ_0^2 was the same, within experimental error, for the two cases.

That the above result is an effect of ionic strength is shown by the rotation of cold parent gelatin dissolved in 0.05 per cent HAc containing 0.1 M NaCl. The specific rotation of this solution was similar to that of parent gelatin in the citrate buffer. It should be noted also that parent gelatin in 0.05 per cent HAc does not visibly gel at concentrations at which gelling occurs in higher ionic strength solutions.

Observations with Films

A few observations carried out on parent gelatin films, prepared by evaporation on optical flats, are in agreement with Robinson's experience (Robinson and Bott, 1951; Robinson, 1953). In the present instances very high specific rotation (*ca.* −620°) was observed for films formed by evaporation in the cold, but the films produced by evaporation at higher temperatures gave too low rotation to be measured accurately by the polarimetric method used.

DISCUSSION

The dispersion studies presented above indicate that the optical rotation of the several states of ichthyocol and its parent gelatin can be expressed by a single-term Drude equation. The parameter λ_0 (corresponding to a far ultraviolet absorption of unknown molecular significance) remains the same within experimental error (2050 ± 150 A) for all states. These results are in agreement with the extensive work on the rotatory dispersion of gelatin

by Carpenter and Lovelace (1935), who report the same λ_0 (2200 A) for both the hot and cold states of gelatin.

Linderstrøm-Lang and Schellman (1954) have recently observed that λ_0 changes for some globular proteins from about 2500 A in the native condition to around 2200 A after denaturation. The information cited above indicates that collagens and gelatins, in both native (or configurationally ordered) and denatured conditions, exhibit relatively constant and low λ_0, although further study will be needed to establish this accurately.

In any event, it would appear that the parameter k is more sensitive than λ_0 to changes of state. Values of specific rotation referred to the Na D line, which has wave length far from λ_0, essentially demonstrate the alterations in k. It then becomes possible to use $[\alpha]_D$ directly, as is done below, to consider the major aspects of the variations in optical rotivity.

So little is known about the theoretical significance of optical rotivity in complex systems that for interpretations one must at present rely on experimental information regarding the molecular configurations associated with the observed rotations. Gallop (1955) has shown by physicochemical studies that the ichthyocol solutions examined in this study contain long, thin, protofibrillar particles, which Boedtker and Doty (1955) have found are of relatively uniform length (*ca.* 2900 A) and weight (*ca.* 300,000). All such studies show that the particles have mass per unit of contour length equal to 88 to 120 avograms[1] per A, which corresponds to about one amino acid residue per angstrom unit of length. Essentially the same result is calculated from x-ray and density considerations for protofibrils in native fibrils (Bear, 1952). Fibrils can be reconstituted from the solutions to yield the collagen wide-angle x-ray diagram and the 640 A macro period. From these facts it is clear that the particles retain native properties throughout dispersion and reprecipitation. The high specific rotation of the acid solutions ($[\alpha]_D = -350°$) accordingly is associated with individually dispersed particles whose polypeptide chain configurations have largely retained their native structure.

Recent progress in the analysis of the wide-angle x-ray diagram indicates that at least part of the polypeptide chains in native collagen have a helical configuration (Pauling and Corey, 1953; Cohen and Bear, 1953). A helical configuration might be expected to contribute to the optical rotivity of the protein in two ways: (1) by contributions due to the helical dissymmetry[2] alone, and (2) by affecting the field about each asymmetric carbon atom. In the second case an appreciable net effect on rotation might result from the

[1] The term "avogram" has been approved by the Committee on Nomenclature of Physical Chemistry of the American Chemical Society as "a quantity of matter which is one gram divided by Avogadro's number."

[2] A dissymmetric structure need not be devoid of symmetry (asymmetric) but is not superposable upon its mirror image (see Partington, 1953).

fact that a helix would be expected to yield the same sign for the induced change in each elementary, asymmetric component. Both types of effect would result from *intra*protofibrillar helical structure, and they would be operative even in the absence of *inter*protofibrillar interactions. It seems possible, therefore, that it is specifically the helical configuration of the protofibrillar particles which is responsible for the high rotation found in collagen.

Gallop has demonstrated (1955) that, in the conversion to parent gelatin, the native protofibrillar particles are depolymerized and become transformed into monodisperse molecules of weight 70,000. The low effective length of the gelatin molecules (200 A) and the amorphous character of the wide-angle x-ray diagram of hot-evaporated gelatin films indicate that a randomization of the native polypeptide chain configuration of the molecules has also occurred. The change in specific rotation from $-350°$ to $-110°$ would accordingly be interpreted as due for the most part to this randomization of the chain configuration, after which the rotation is essentially the sum of the independent rotations of the L-amino acid residues present. In agreement with this view is the fact that other denatured proteins show specific rotations near $-100°$ (Cohen, 1955).

When gelatin is cooled, the rotation rises and the collagen wide-angle diagram is yielded by the gels and by evaporated films. The helical configuration characteristic of the native particles is thus partially regained, along with a return of correspondingly high optical rotation (*ca.* $-300°$). However, the solutions, at the concentrations and temperatures examined, do not regain the full rotation of $-350°$, indicating that not all of the helical configuration of native collagen is restored.

Other facts are consistent with this interpretation. For example, the experiment with ionic strength reported above is compatible with the view that the "native" collagen in solution is well stabilized intramolecularly, but that once gelatinization has occurred charge effects at low ionic strength interfere with the tendency of the "randomized" gelatin molecules to assume the more ordered configuration, thus inhibiting the regain of rotation. Various alkali halides and urea cause a large decrease in levorotation of gelatin gel and a smaller change of rotation of the sol (Carpenter, 1927; Carpenter and Lovelace 1935, 1938) as is to be expected since these and similar reagents affect intramolecular linkages, particularly hydrogen bonds, which stabilize the collagen configuration. Gustavson (1949) has emphasized the importance of the breaking of these bonds in thermal contraction, which these reagents facilitate.

Although interparticle interactions do not seem to be influential in determining the high rotation of the acid solutions of ichthyocol, in the concentration range examined, these factors may not be negligible in all cases. In the cold-evaporated gelatin films, for example, condensation results in increasing the specific rotation greatly, even to levels above the level experienced in the ichthyocol solutions. Note, however, that close approximation of molecular chains does not *per se* enhance rotation, as is shown by the low rotations observed with hot-evaporated films. In the cold gelatin preparations, both films and solutions, with which increase of rotation with concentration is most apparent, one can demonstrate that helically wound chains are present. It is, therefore, possible that the effects of chain association are either (*a*) to improve the proportion or perfection of the existing regular coiling, or (*b*) through directed interaction between adjacent helices to increase the rotivity of all contributing elements in

the same sense. In either event, the initial specific helical organization would be fundamental, and chain association or interaction would play a secondary role. Further studies on concentrated systems of collagen and gelatin are needed to assess more fully the relative importance of these factors.

Collagen is unique among proteins in its high negative optical rotation, as well as in the high proportion of proline and hydroxyproline residues (more than 20 per cent). Study of models of polypeptide chain structure shows that these imino residues are not equally well incorporated into left- and right-handed helices. Their prevalence in collagen may thus provide a stereochemical reason for the presence of helices of one sense of twist. Mixtures of helices of opposite twists would cause cancelling effects (as may be the case in native globular proteins; Cohen, 1955), reducing the resultant rotation to low levels.

The pyrrole ring of the imino acids involves the α-carbon atom. Strains in this ring could have a large effect on the optical activity. However, if one were to attribute the high rotations found in collagen to the rotivity of proline and hydroxyproline, a value for the specific rotation of $-350°$ for the protein would correspond to about $-1200°$ for the specific rotation of the imino residues. Although this seems unlikely, further knowledge of rotation data on proline and hydroxyproline is required before this possibility can be dismissed.

SUMMARY

The optical rotation phenomena exhibited by a citrate-extracted fraction of ichthyocol (from carp swim bladder), as well as by the parent gelatin derived therefrom, have been studied. Dispersion data for all cases follow a single-term Drude equation, but the variations with state are adequately expressed by simple reference to changes in $[\alpha]_D$ as follows:—

1. The native collagen fraction, dispersed in 0.15 M citrate buffer at pH 3.7 in the cold (11°C.), yields a high negative specific rotation, $[\alpha]_D$, near $-350°$.

2. During equilibration at 40°C., which causes conversion to a monodisperse parent gelatin, the rotation drops to about $-110°$.

3. Gelation at 2°C. results in a partial regain of rotation to around $-290°$. This mutarotation is reversible, depending on temperature.

4. In the range 0.02 to 0.28 per cent the native ichthyocol and the warm gelatin solutions show little concentration dependence, but with the cold gelatin solutions the specific rotation increases with concentration. Gelatin films formed by cold evaporation yield high specific rotation (*ca.* $-620°$), but those formed by hot evaporation retain low optical activity.

5. Since this same collagen-gelatin system has been investigated physicochemically, it is possible to relate molecular changes to the observed variations in optical rotation. Conclusions are similar to those of Robinson (1953), who studied other gelatins: high negative rotation is believed related to a native collagen polypeptide configuration, herein specified as helical (from

x-ray diffraction considerations) and destroyed by heating. The possible roles of intermolecular interactions and of prevalent pyrrolidine constituents in influencing the helical configuration and optical activity are discussed.

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BIBLIOGRAPHY

Bear, R. S., *Advances Protein Chem.*, 1952, **7**, 69.
Boedtker, H., and Doty, P., *J. Am. Chem. Soc.*, 1955, **77**, 248.
Bresler, S. E., Finogenov, P. A., and Frenkel, S. Y., *Doklady Akad. Nauk S.S.S.R.*, 1950, **72**, 555.
Carpenter, D. C., *J. Physic. Chem.*, 1927, **31**, 1873.
Carpenter, D. C., and Lovelace, F. E., *J. Am. Chem. Soc.*, 1935, **57**, 2337, 2342.
Carpenter, D. C., and Lovelace, F. E., *J. Am. Chem. Soc.*, 1938, **60**, 2289.
Cohen, C., *Nature*, 1955, **175**, 129.
Cohen, C., and Bear, R. S., *J. Am. Chem. Soc.*, 1953, **75**, 2783.
Ferry, J. D., and Eldridge, J. E., *J. Physic. and Colloid Chem.*, 1949, **53**, 184.
Gallop, P. M., *Arch. Biochem. and Biophysics*, 1955, **54**, 486, 501.
Gornall, G. A., Bardawill, C. J., and David, M. M., *Canad. J. Research, Sect. B*, 1949, **27**, 791.
Gross, J., Highberger, J. H., and Schmitt, F. O., *Proc. Nat. Acad. Sc.*, 1954, **40**, 679.
Gustavson, K. H., *Advances Protein Chem.*, 1949, **5**, 354.
Jirgensons, B., *J. Polymer Sc.*, 1950, **5**, 179.
Jirgensons, B., *J. Polymer Sc.*, 1951, **6**, 477.
Jirgensons, B., *Arch. Biochem. and Biophysics*, 1952, **39**, 261; **41**, 333.
Katz, J. R., *Rec. trav. chim. Pays-bas*, 1932, **51**, 835.
Kauzmann, W., *et al.*, *J. Am. Chem. Soc.*, 1953, **75**, 5139, 5152, 5154, 5157, 5167.
Kraemer, E. O., and Fanselow, J. R., *J. Physic. Chem.*, 1925, **29**, 1169.
Linderstrøm-Lang, K., and Schellman, J. A., *Biochim. et Biophysic. Acta*, 1954, **15**, 156.
Orekhovich, V. N., Tustanovskii, A. A., Orekhovich, K. D., and Plotnikova, N. E., *Biokhimiya*, 1948, **13**, 55; *Doklady Akad. Nauk S.S.S.R.*, 1948, **60**, 837.
Partington, J. R., An Advanced Treatise on Physical Chemistry, London, Longmans, Green and Company, 1953, **4**, 296.
Pauling, L., and Corey, R. B., *Proc. Roy. Soc. London, Series B*, 1953, **141**, 21.
Randall, J. T., Brown, G. L., Jackson, S. F., Kelly, F. C., North, A. C. T., Seeds, W. E., and Wilkinson, G. R., *in* Nature and Structure of Collagen, (J. T. Randall, editor), New York, Academic Press Inc., 1953, 213.
Robinson, C. R., *in* Nature and Structure of Collagen, (J. T. Randall, editor), New York, Academic Press Inc., 1953, 96.
Robinson, C. R., and Bott, M. J., *Nature*, 1951, **168**, 325.
Smith, C. R., *J. Am. Chem. Soc.*, 1919, **41**, 135.
Thaureaux, J., *Bull. Soc. chim. biol.*, 1945, **27**, 327.
Trunkel, H., *Biochem. Z.*, 1910, **26**, 493.
Yang, J. T., and Foster, J. F., *J. Am. Chem. Soc.*, 1954, **76**, 1588.