has clearly defined the relation between vapor pressure and the degree of protein hydration.

A comparison of protein hydration in crystals suspended in ammonium sulfate solutions (data from Table I) with the total water content of salt-free crystals equilibrated at the same relative humidity is shown in Table III.

It may be noted that the values obtained by these methods essentially agree and also that they vary in a consistent manner with relative humidity as would be expected. Adair and Adair[7] and Adair and Robinson[26] have reported a similar relation for hemoglobin. Results for the hydration of β-lactoglobulin in sucrose as obtained by this calculation[9] are considerably less than would be expected from the vapor phase measurements of the water content of β-lactoglobulin crystals at the same relative humidity. Thus the hydration or "bound water" for β-lactoglobulin crystals in 34.7% sucrose with a relative humidity of 96.8% was found to be 0.32 g. of water per g. of protein, as compared to a value of 0.64 g. of water from vapor phase measurements. This difference between these two values may be attributed to the binding of sucrose by the protein or to the difficulty of making accurate vapor phase measurements at high humidities.

It is apparent from these results on the composition of protein crystals suspended in various media that the calculation for hydration or "bound water" varies with the method chosen for the determination.

(26) G. S. Adair and M. E. Robinson, *J. Physiol.*, **72**, 2P (1931).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

An X-Ray Diffraction Investigation of Selected Types of Insulin Fibrils

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The X-ray diffraction patterns of insulin fibrils prepared from native and chemically modified insulins are examined. Within the limits set by inherent reflection diffuseness and incomplete orientation, the patterns obtained from air-dried fibrils prepared from methylated insulin (FE), acetylated insulin (FA), diazonium coupled insulin (FD), fibrils obtained by seeding and other fibril types are identical with those obtained from native insulin (FN). The various insulins, therefore, do not form distinctly different fibrous structures but extensive alterations of monomer side chains can be accommodated within the same basic fibril structure. Small-angle meridional reflections suggest a fibril period of 48.5 Å. The largest equatorial spacings, 55 and 30.8 Å., are in close approximation to $\sqrt{3}$:1, indicating pseudo-hexagonal packing with a = 61.6 Å. However, the equatorial reflections taken together are more consistent with an orthorhombic unit cell having dimensions of 55 and 30.8 Å. normal to the fibril period. The closeness of the unit cell dimensions of insulin fibrils to that of Low's orthorhombic insulin sulfate crystals (a = 44 Å., b = 51.4 Å., c = 30.4 Å.), the similarities between the smaller spacings given by insulin fibrils and the fiber pattern given by rapidly dried insulin sulfate crystals, and the similar conditions required for fibril formation and insulin sulfate crystallization (*p*H below 3.5) suggest a general correspondence in monomeric structure and packing. An intense 4.8 Å. meridional spacing is the most characteristic reflection of insulin fibrils. It is absent in other crystalline and denatured insulins and is probably the 10th order of the 48.5 Å. fibril period. The X-ray diffraction patterns and low value of the observed positive intrinsic double refraction indicate that in insulin fibrils the polypeptide chains are in their normal folded state and predominantly parallel to the fibril axis.

Insulin and chemically modified insulins have been shown to undergo spontaneous transformations in which are produced fibrils having diameters of hundreds and lengths of thousands of ångström units.[2-4] Not only are almost all of these transformations known to be reversible, but the low temperature growth of each type of fibril in the presence of monomer of another type suggests that they are structurally compatible.[5] In order to determine whether the family of fibrils have common and/or specific structural features, the present investigation was undertaken by means of X-ray diffraction and polarization optical methods.

Experimental

Armour Zn-insulin crystals, recrystallized using a modification of the final crystallization technique of Romans, Scott and Fisher,[6] were used.

Fibrils prepared from native insulin in mineral acids are termed FN. Fibrils prepared from modified insulin are designated as follows: esterified insulin, FE; acetylated insulin, FA; and diazonium-coupled insulin, FD.

Under appropriate conditions phenol causes essentially complete reversion of FN, the product formed having all of the characteristics of native insulin. However, if the reaction time of the reversion process is extended a new set of fibrils, termed FN–FN(P), will form. A superficially similar process also occurs with FN in high concentrations of low molecular weight organic acids. These are termed FN–FN(O).[5]

A previous publication details the methods by which modified insulins were prepared.[6] Per 12,000 molecular weight unit esterification will cover the twelve available carboxyl groups (the eight free carboxyl groups of glutamic acid and four terminal carboxyl groups); acetylation will cover all of the six available amino groups (the two ε-amino groups of lysine and four terminal amino groups, but no phenyl acetates will be formed since they are labile in acidic solutions); coupling with diazonium salts will modify the eight tyrosine and four imidazolium side chains.

In view of the facts that fibrils prepared from these several types of insulin were to be examined for structural differences and that fibrils could be prepared from extensively

(1) The work reported here was submitted to the Massachusetts Institute of Technology in partial fulfillment for the degree of Doctor of Philosophy.

(2) (a) D. F. Waugh, THIS JOURNAL, **66**, 663 (1944); (b) D. F. Waugh, *ibid.*, **68**, 247 (1946).

(3) D. F. Waugh, *ibid.*, **70**, 1850 (1948).

(4) J. L. Farrant and E. H. Mercer, *Biochim. Biophys. Acta*, **8**, 355 (1952).

(5) D. F. Waugh, D. F. Wilhelmson, S. L. Commerford and M. L. Sackler, THIS JOURNAL **75**, 2592 (1953).

(6) R. G. Romans, D. A. Scott and A. M. Fisher, *Ind. Eng. Chem.*, **32**, 908 (1940).

modified insulins (esterification and acetylation), conditions were chosen to give complete modification. As reagents, methyl alcohol, acetic anhydride and benzenediazonium chloride were used, the latter in amount sufficient to give 4–8 groups per 12,000 molecular weight unit.

Preparation of FN.—The technique required to prepare standard insulin gel has been described recently. Such gels contain a negligible residue of native insulin since, in large amounts, they give no detectable biological response and their content of fibrils is completely centrifugable.[5]

Fibrils from Modified Insulins.—The technique used to prepare FN proved satisfactory for preparing FE and FD. However, fibril formation with acetylated insulin proceeded more slowly and with greater difficulty. The following method was adopted for the preparation of FA.

A 2% solution of acetylated insulin in 0.05 *N* hydrochloric acid was heated continuously at 100° until the first appearance of flow double refraction (usually 25–30 minutes). The ampule was then frozen and thawed and reheated for 2 minutes. After a second freeze-thawing it was given a final heat treatment of 5 minutes.

Phenol Fibrils (FN–FN(P)).[5]—To a solution of 0.15 ml. of melted phenol and 1.85 ml. of distilled water was added 0.5 ml. of standard 2% gel (FN) which had previously been frozen in a solid carbon dioxide–acetone mixture and thawed. The final phenol concentration was 6% by volume. Incubation at 60° for 1 hour produced a weak gel. Prior to preparing specimens for the X-ray camera, these fibrils were dialyzed in the cold for 72 hours against 0.025 *N* hydrochloric acid to remove phenol and then concentrated at 50° to yield a final protein concentration of 1.2%.

Gels Produced by Seeding.[7]—A 0.2% homogeneous suspension of FN in 0.05 *N* hydrochloric acid, prepared by diluting standard gel, was added to an equal volume of a 2% solution of insulin in 0.05 *N* hydrochloric acid. Growth was carried out at (a) room temperature for one week, (b) 59° for 35 minutes and (c) 100° for 3 minutes. Under these conditions the unseeded insulin solution will show negligible or small (c) amounts of fibril formation.

Heat Precipitate.[2]—Spherites were prepared by heating a 2% solution of insulin in 0.025 *N* sulfuric acid at 100° for 30 minutes.

Preparation of Specimens for X-Ray Diffraction Examination.—In gels of FN the fibrils are not cross linked, the physical characteristics of the suspension of fibrils being due largely to the electrostatic repulsion between fibrils. Gels produced from esterified insulin and diazonium-coupled insulin are of similar structure, while those prepared from acetylated insulin are cross linked. For example, the latter will not disperse into a suspension of individual fibrils on dilution but will give rise to small gel-like clumps; in contrast FN, FE and FD gels are readily dispersed.[5]

Insulin fibrils were examined in the form of stacks of thin films, the fibrils of which had been oriented by stroking the insulin gel. The X-ray beam passed parallel to the film surfaces of the stack. In a number of preliminary investigations in which fibril orientation had been obtained by cursory stroking by hand with a thin knife blade, it was noted that the resulting diffraction patterns were badly arced. Such could be the result of poor orientation either of fibrils or of intrafibrillar molecular chains. Since it was desirable to eliminate the former as much as possible, a number of methods for improving orientation were tried: rolling, stretching, spinning, electrical orientation, centrifugation and unidirectional stroking. The latter, although it did not eliminate the arcing, was most successful. The stroking was carried out as follows: a chrome-plated steel blade, 0.0038 cm. thick, was passed in a series of parallel strokes through a gel layer of insulin. The blade velocity was 24 cm. per sec. At the end of each return stroke the gel layer was shifted laterally 0.0015 cm. The gel was stroked completely six times with the blade passing through the gel on both the forward and return strokes. This was followed by complete stroking six times with the blade passing through the gel only on the forward stroke. The oriented gel layer was then allowed to dry before application of the next aliquot of gel. An insulin film contained six gel layers, each approximately 0.0025 cm. thick when dry.

Almost all of the specimens examined contained glycerol which helped to decrease friability and facilitate stripping from siliconed glass slides. Glycerol was introduced either by dialysis or by preparing fibrils in the presence of glycerol.

X-Ray Equipment.—Details of construction of the cameras used have been given by Rugo.[8] Both had pin-hole collimation, were of the vacuum type and were capable of resolving 100 and 200 Å. spacings. In addition, a wide-angle camera of lower angular resolution, having a variable specimen-to-film distance up to 10 cm., was used for more rapid registration of patterns. Ni-filtered Cu Kα radiation (λ 1.54 Å.) was used.

Double Refraction Curve of FN.—Detailed descriptions of the theory and practice for constructing form double refraction curves are available.[9,10] In the present study a standard polarizing microscope employing a wedge compensator was used to measure retardations. Immersion media were pure solvents or mixtures of cyclohexane, benzene, chlorobenzene, bromobenzene and iodobenzene.

Results

Comparison of Native and Regenerated Zn-insulin Crystals.—The spacings observed with crystalline powders obtained from native insulin and insulin regenerated from FN are listed in Table I. Wet crystals have more numerous reflections and are also similar. The close resemblance of comparable X-ray patterns supports the identity of the two.

TABLE I

SPACINGS OF DRY Zn-INSULIN CRYSTALS FROM NATIVE AND REGENERATED INSULIN

Intensity = rough relative intensity; s = strong; ms = moderate to strong; m = moderate; mw = moderate to weak; w = weak

Native crystals, Å.	Regenerated crystals, Å.	Intensity
27.9	28.2	s
21.6	20.3	ms
18.5	19.1	ms
15.4	15.4	m
	14.2	w
11.6	11.6	m
10.3	10.2	mw
9.2	9.4	mw
8.56	8.53	mw

Fibrous Insulin (FN).—Figure 1 is a composite drawing of more than 100 diffraction patterns obtained from air-dried FN. The corresponding spacings are listed in Table II. Section A, which includes small-angle meridional reflections, suggests a fibril period of 48.5 Å. There is some doubt as to the presence of the 4th order of this series due to the fact that this reflection is not only diffuse but overlaid with a highly arced equatorial diffraction. The choice of 48.5 Å. as the fibril period rests upon two observations: (a) a correspondence in structure between insulin sulfate crystals and insulin fibrils, as detailed below and (b) the fact that the wide-angle reflections, section B, column 3, clearly indicate a pseudo period of 4.8 Å., which could be the tenth order of a 48.5 Å. fibril period.

Section C cites the equatorial reflections. The diffuseness of many of these reflections, causing an uncertainty in measuring corresponding spacings, in conjunction with the lack of diffractions between

(7) D. F. Waugh, R. E. Thompson and R. J. Weimer, *J. Biol. Chem.*, **185**, 85 (1950).

(8) H. Rugo, Ph.D. Thesis, Mass. Inst. of Tech., 1949. See also O. E. A. Bolduan and R. S. Bear, *J. Appl. Phys.*, **20**, 983 (1949).

(9) F. O. Schmitt, "Medical Physics," O. Glasser, ed., Yearbook Publ., Inc., Chicago, Ill., 1944, p. 1586.

(10) H. S. Bennett in McClung's "Microscopical Technique," P. B. Hoeber, Inc., New York, N. Y., 1950, p. 591.

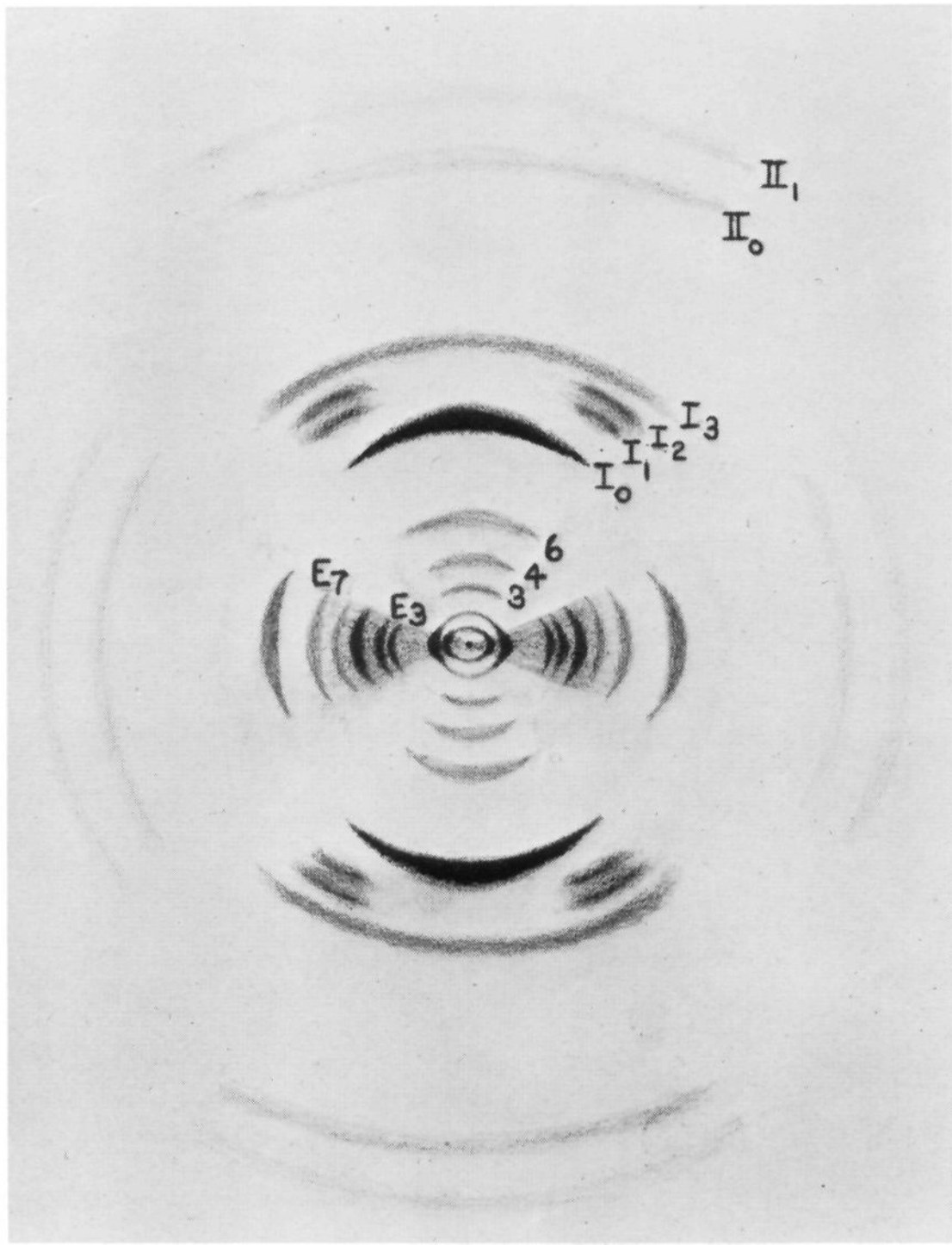

Fig. 1.—A composite drawing of the diffraction pattern of native insulin fibrils. Equatorial reflections are more diffuse than indicated; fiber axis vertical.

TABLE II

A. Small Angle Meridional Reflections of Insulin Fibrils

The column headed d contains the measured Bragg spacings which, when multiplied by the assigned k index, yield the fibril period b_0. Intensities are moderate to weak.

k	d, Å.	b_0, Å.	Intensity
3	16.1	48.3	mw
4	12.0	48.0	mw
6	8.2	49.2	mw
	Av.	48.5	

B. Wide-Angle Meridional and Near Meridional Reflections

The first column designates the wide-angle pattern notation. Roman number I refers to the first layer line and II to the second layer line. Subscript 0 designates on-axis reflections; 1, first off-axis spot on layer line; 2 second off-axis spot on layer line, etc. ζ and ξ are the Bernal reciprocal space coördinates. Intensity indicates rough relative intensities: vs = very strong; s = strong; ms = moderate to strong; m = moderate; mw = moderate to weak; w = weak; vw = very weak.

Reflection	d, Å.	ζ	ξ	Intensity
I_0	4.79	0.321	0.000	vs
I_1	4.12	.318	.176	m
I_2	3.80	.341	.218	m
I_3[a]	3.54	(.325)	.286	m
II_0	2.40	.639	.000	w
II_1[a]	2.25	(.650)	.189	vw

C. Equatorial Reflections

The column headed E contains the equatorial spacings numbered by starting from the innermost spot

E	d, Å.	Intensity	E	d, Å.	Intensity
1	55	ms	6	8.0	mw
2	30.8	s	7	6.9	m
3	14.0	ms	8	5.5	m
4	11.8	ms	9	3.08	vw
5	9.5	ms	10	2.77	vw

[a] Highly arced; ξ determined using average ζ (0.325) of I_0, I_1, I_2, II_0.

30.8 and 14.0 Å., makes it difficult to determine the packing perpendicular to the fibril axis. The largest equatorial spacings, 55 and 30.8 Å., are in close approximation to $\sqrt{3}$:1 (1.78 compared to 1.73), suggesting pseudo-hexagonal packing with a = 61.6 Å. However, the equatorial reflections taken together are somewhat more consistent with an orthorhombic unit cell having dimensions of 55 and 30.8 Å. normal to the fibril period.

With the exception of a 4.4 Å. equatorial reflection, which appeared only in those specimens containing glycerol, the spacings observed with fibrils prepared in the presence and absence of glycerol are the same. Glycerol does, however, contribute to background scattering and itself gives diffraction halos which, unfortunately, occur in those regions where the fibrils themselves diffract. The liquid halos thus tend to obscure some reflections. Indeed, the fibril meridional reflection of 16.1 Å., which is quite evident in glycerin-free fibrils, is almost completely obscured by general radiation diffraction from glycerol. However, the objectionable effects of glycerol are more than compensated for by the over-all improvement of the diffraction patterns.

Insulin Fibril Modifications.—Within limits set by inherent reflection diffuseness and incomplete orientation, the patterns obtained from air-dried FE (meth), FD (benzenediazonium chloride), FN–FN(P), and fibrils obtained by seeding are identical with those obtained from air-dried FN. It is interesting that the patterns obtained from FE are somewhat sharper than all others, which may reflect better packing of methylated monomers within the fibrils.

The pattern given by FA was very poor, showing only 4.78 Å. meridional and 30.9 and 15.9 Å. equatorial spacings. The latter is highly arced and diffuse and consists of several unresolved reflections. The lack of detail probably reflects the difficulties encountered in preparing oriented films from a cross-linked network. When mechanically disturbed the FA system compacts, making it impossible to align these fibrils.

Spherites.—The powder pattern of dry insulin spherites, likewise, shows no great structural detail. There are two diffuse rings consisting of several unresolved reflections at 10.5 and 3.8 Å., in addition to the sharp intense ring at 4.74 Å., which is prominent and characteristic of fibrous insulin.

Solvated Films.—As the relative humidity in contact with the dry fibrous film is increased the diffraction patterns show progessively less detail. With highly solvated films only spacings at 33.5 and 4.9 Å. remain. This extensive deterioration of the diffraction pattern and the relative increase in those spacings observed suggests that water is penetrating the fibrils themselves to such an extent that both molecular disorder and a physical separation of fibrils are produced.

Double Refraction of FN.—The form double refraction curve of FN, Fig. 2, indicates that insulin fibrils, like other fibrous proteins, possess both positive form and positive intrinsic double refractions. The major contribution comes from form double refraction, the intrinsic double refraction being 1.8×10^{-4}. The refractive index of the fibrils, as ascertained from the minimum in the immersion curve, is 1.55, a reasonable value for insulin.[11]

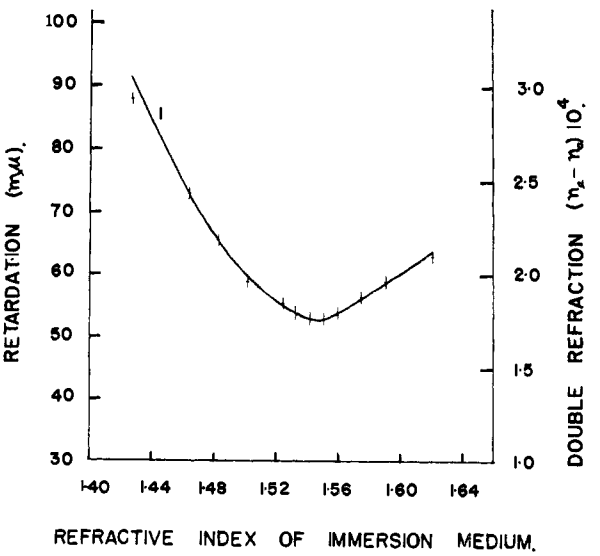

Fig. 2.—Double refraction curve of native insulin fibrils (FN).

(11) D. Crowfoot, *Proc. Roy. Soc.* (*London*), **A164**, 580 (1938).

Discussion

A consideration of all patterns obtained with fibrils prepared from native and modified insulins reveals: (a) the patterns have relatively small numbers of diffuse and highly arced reflections and (b) the patterns of all fibril types are similar. The diffuseness and arcing, present in spite of the elaborate procedures introduced to give good fibril orientation, are ascribed to irregularities in monomer packing. The similarity of diffraction patterns from FN, FE, FD, etc., means that extensive alterations of monomer side chains can be accommodated within the same basic fibril structure. The various insulins, therefore, do not form distinctly different fibrous structures, in spite of the aberrations which must be introduced, for example, in adding eight to eleven phenylazo groups per unit of 12,000 molecular weight. Rather, a family of fibrils is formed whose structural differences are reflected in a variability of strength of intermolecular linkage, in the characteristics and kinetics of fibril formation, and in their tendencies to undergo secondary aggregations (*e.g.*, spherite formation).

It has been suggested that the intermolecular linkage is due to the interaction of groups of the small non-polar side chains which predominate in insulin.[1,5] Such a linkage would not require a regular point-to-point approximation of surfaces but, since areas of interaction are involved, would through the shifting of bond regions relative to each other, account for packing irregularities. Along the same lines, the introduction of additional surface groups would modify local side-chain packing without requiring the introduction of a new type of monomer arrangement.

Insulin has been shown to crystallize in at least two forms. At *p*H 5.6–6.6, in the presence of Zn or other metals of the same group,[12] hexagonal crystals are formed having a unit cell containing three 12,000 units equivalent to a molecular weight of 36,000.[11,13,14] There appears to be no relation between hexagonal Zn-insulin crystals and insulin fibrils.

Low[15] has recently examined air-dried Zn-free insulin sulfate crystals prepared at *p*H 3.5. These crystals have an orthorhombic unit cell of a = 44 Å., b = 51.4 Å., and c = 30.4 Å., containing 4 molecules of molecular weight 13,500. Patterson projections indicated that the polypeptide chains are probably parallel to the a-axis. In the models suggested by Low the length of the molecule is 44 Å.

When insulin sulfate crystals are rapidly air-dried Low[16] finds superimposed upon the single crystal diffraction pattern a fiber pattern with maxima at 30.4, 9.6, 8.8, 6.4, 5.9, 4.8, 4.4, 4.05, 3.85 and 2.72 Å. The fiber lines at 9.6 and 4.8 Å. are oriented like the underlying (042) and (084) crystal reflections. Low[17] interprets these crystalline spacings and the corresponding fiber lines as being the first and second order maxima of interchain packing planes.

Although dissimilarities between the fiber patterns given by insulin sulfate crystals and insulin fibrils are evident, as discussed below, several pieces of evidence suggest a general correspondence in monomeric structure and packing. First, both insulin sulfate crystals and insulin fibrils form at *p*H's of 3.5 or below. Second, the fibrillar equatorial spacings of 55 and 30.8 Å. and the meridional repeat of 48.5 Å. are suggestively close to the unit cell dimensions of insulin sulfate crystals. Third, certain of the insulin sulfate fiber lines are close to spacings given by insulin fibrils, namely, 30.8, 9.5, 8.2, 6.9, 5.4 and 2.77 Å. (equatorial) and 4.12 and 3.80 Å. (meridional).

The possibility that Low's rapidly dried insulin sulfate crystals contained insulin fibrils, which would be responsible for the fiber pattern associated with the former, was examined by testing the crystals, kindly supplied by Dr. Low, for the presence of fibrils using essentially the sensitive seeding conditions as described elsewhere.[5] No fibrils or fibril nuclei were found. Thus, although the surface molecules[17] in a rapidly dried insulin sulfate crystal may be spatially situated as they are in the fibril, they are not bonded in the same way and therefore do not show FN properties. The fiber pattern given by orthorhombic insulin sulfate crystals might result from a displacement of molecules along the a-axis, a shift that would eliminate symmetry elements, which are indeed absent also in insulin fibrils.

If, as indicated above, there is a general correspondence in structure between the insulin sulfate crystal and the fibril, the polypeptide chain direction in the fibril would be parallel to the fiber axis. To a certain extent this is supported by the observed positive intrinsic double refraction. However, the value of the intrinsic double refraction is distinctly lower than that expected from a system of oriented extended polypeptide chains, 1.8×10^{-4} compared to 5×10^{-2} for silk fibroin.[18] A small intrinsic double refraction would be found when the polypeptide chains are folded, when only certain regions of the polypeptide chain are parallel to the fiber axis, or when a contribution to the intrinsic double refraction, negative in sign, is supplied by oriented side chains, the most obvious possibility being non-polar chains oriented perpendicular to the fiber axis.

The most characteristic reflection of insulin fibrils occurs as an intense meridional arc at 4.8 Å. (see Fig. 1) and has an unusual intensity distribution. This reflection is absent in insulin sulfate crystals, the closest similar spacing being a weak fiber line at 4.4 Å. corresponding to the (10.00) crystalline reflection. It should also be noted that Low's second-order packing plane reflection (an equatorial 4.8 Å. spacing) is absent in insulin fibrils. Thus it appears that the intense 4.8 Å. meridional spacing of insulin fibrils is not the second-order maximum of interchain packing planes

(12) D. A. Scott, *Biochem. J.*, **28**, 1592 (1934).

(13) D. Crowfoot, *Chem. Revs.*, **28**, 215 (1941).

(14) D. C. Hodgkin, *Cold Spring Harbor Symp. Quant. Biol.*, **14**, 65 (1949).

(15) B. W. Low, *Nature*, **169**, 955 (1952).

(16) B. W. Low, private communication. The authors express their appreciation to Dr. Barbara W. Low for helpful discussions.

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(18) A. Frey-Wyssling, "Submicroscopic Morphology of Protoplasm and Its Derivatives," Elsevier Publ. Co., New York, N. Y., 1948, p. 197.

but rather arises from the structural alterations attending intermolecular linkage.

On the basis of infrared spectroscopy of insulin fibrils and the assumption that the strong 4.8 Å. meridional reflection is a backbone spacing, Ambrose and Elliott[19] suggest that the polypeptide chains are in the β-configuration and lie perpendicular to the fibril axis. The data presented are not decisive and, as the authors note, could be interpreted also to indicate folded polypeptide chains parallel to the fibril axis. The X-ray diffraction pattern of insulin fibrils bears little resemblance to the cross β-pattern given by "denatured" insulin.[20] The 4.8 Å. meridional reflection is not a backbone spacing, as they assumed, since it is not only larger (4.8 *vs.* 4.65 Å.) but more intense than the usual backbone spacing. Rather for the reasons given above, we suggest that it is associated with the fibril period.

Moreover, the reversibility of the monomer–fibril transformation and the usual seeding conditions under which fibril elongation will take place suggest that monomeric insulin enters the fibril structure with, at most, slight distortion, and without the far reaching changes accompanying transformation to the β-form. It seems highly probable, therefore, that in insulin fibrils the polypeptide chains retain their normal folded condition, with axes predominantly parallel to the fibril axis.

Acknowledgment.—The work reported here was generously supported by Armour and Company, Chicago, Ill., and S. E. L. Manduro and Sons, Inc., of Chicago.

CAMBRIDGE, MASS.

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[DEPARTMENT OF MEDICINE, TULANE UNIVERSITY MEDICAL SCHOOL]

Mechanism of Desoxyribonuclease Depolymerization: Effect of Physical and Enzymatic Depolymerization on the Affinity of Methyl Green and of Desoxyribonuclease for Desoxyribonucleic Acid[1a]

BY N. B. KURNICK[1b]

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Desoxyribonucleic acid (DNA) was partially depolymerized by heat. The products were characterized by methyl green binding capacity, viscometry, dialysis and ultracentrifugation. The effect on the rate of depolymerization by desoxyribonuclease (DNase) was studied by means of methyl green affinity and viscometry. It was found that heat depolymerization of a degree insufficient to change the elementary composition of the DNA or to give rise to dialyzable products, greatly reduced the affinity for DNase. The change in enzymatic susceptibility paralleled the reduction in methyl green affinity produced by heat. Both DNase affinity and methyl green affinity are less extensively influenced by DNase depolymerization than by heat depolymerization, when the same reduction in viscosity of the DNA solution is produced by both. The implications as to the structure of polymerized DNA are considered.

The specific requirements in the desoxyribonucleic acid (DNA) molecule for optimal susceptibility to depolymerization by desoxyribonuclease (DNase) are not known. Tamm, *et al.*,[2] have suggested that alterations in the macromolecular structure of DNA, which do not involve change in atomic composition ("denaturation"), do not influence affinity for DNase. They report, however, that the removal of purine groups by acid hydrolysis results in marked reduction in enzyme affinity. The present study is concerned with the effect of "denaturation" on the susceptibility of the DNA molecule to depolymerization by DNase.

Previous studies on the action of depolymerizing agents on DNA have employed techniques to follow the reduction in viscosity, reduction in rate of sedimentation in the ultracentrifuge, increase in ultraviolet absorption, lowering of *p*H, formation of acid-soluble and dialyzable products. Because the affinity of DNA for methyl green appears to be influenced by the molecular size and steric configuration of the nucleic acid,[3,4] we have applied this tool, together with those mentioned above, to a study of the effect of "denaturing" agents on DNA.

Experimental

Materials. (a) Preparation of DNA.—The sodium desoxyribonucleate (DNA) used in these experiments was prepared by a modification of the method of Kay, Simmons and Dounce.[5] Fresh calf thymus (obtained directly from the slaughter-house) was blended in a Waring Blendor with approximately 5 volumes of a solution containing 0.14 *M* NaCl. and 0.01 *M* sodium citrate per liter, plus 1 volume ice (sufficient to keep the temperature at 0–2° during the blending). After blending 9 minutes, the sediment obtained on centrifugation at 2,000 r.p.m. (all procedures at 0–2°) was washed several times with cold sodium chloride–sodium citrate solution. The sediment was then resuspended in this solution, an equal volume of 2 *M* NaCl added, and blended. The very viscous, opalescent solution was centrifuged for 2–4 hours at 18,000 r.p.m. at 2° (Sorvall SS-2 centrifuge), the small sediment discarded, and the clear viscous supernatant treated 3 times with sodium dodecylsulfate according to Kay, Simmons and Dounce.[5] The fibrous DNA was collected, following precipitation by alcohol, washed with 95% alcohol and acetone. The acetone was evaporated off at room temperature, and the DNA stored in a $CaSO_4$ desiccator. It contained 8.1% P, 13.3% N (N/P =

(1) (a) This work was aided by grants from the American Heart Association; the National Heart Institute (H-714), United States Public Health Service; the American Cancer Society (recommended by the Committee on Growth, National Research Council) and the Life Insurance Medical Research Fund. Presented at the 123rd meeting of the Am. Chem. Soc., Abstract No. 58, p. 23C, March 15–19, 1953, Los Angeles. (b) V. A. Hospital, Long Beach 4, Calif.

(2) (a) C. Tamm and E. Chargaff, *Nature*, **168**, 916 (1951). (b) C. Tamm, H. S. Shapiro and E. Chargaff, *J. Biol. Chem.*, **199**, 313 (1952).

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(5) E. R. M. Kay, N. S. Simmons and A. L. Dounce, THIS JOURNAL, **74**, 1724 (1952).