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

Studies of the Nucleation and Growth Reactions of Selected Types of Insulin Fibrils

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RECEIVED DECEMBER 15, 1952

The characteristics of the growth and nucleation reactions, and the reversion of insulin fibrils are examined. At appropriate values of ionic strength, temperature and time the growth reaction (native insulin) can be carried essentially to completion in the presence of many mineral acids (FN), at least 9.6, 8.3, 6.7 and 5.6 *M* formic, acetic, propionic and butyric acids (*p*H < 2.0), in the presence of 6.0 *M* urea at *p*H 1.6 or at *p*H 8.0 and in 70% ethyl alcohol. In the absence of urea at *p*H 8.0 growth is absent. In contrast to their effects on growth organic acids and urea (or phenol) suppress the nucleation reaction. Reduction of one disufide linkage per M.W. = 12,000 abolishes growth. Addition of organic acids or phenol causes FN to revert rapidly to give a product resembling native insulin. After sufficient time, however, new fibrous systems, identical with FN in X-ray diffraction patterns and electron micrographs, will develop. These are termed FN–FN(O) and FN–FN(P) and are accounted for by an increase in the availability of bonding groups. Quantitative fibril nucleation and growth are observed with insulins modified to give, per M.W. = 12,000, twelve ester groups (FE), six acetylated amino groups (FA), and eight to eleven phenylazo and *p*-carboxyphenylazo groups (FD). Nucleation and growth proceed readily in forming FE and FD but with difficulty in forming FA. The growth reactions of FA at *p*H 2.0 and, in contrast to FN, at *p*H 7.0 are similar. FA and FN–FN(O) revert in alkali in the same manner as FN while FD, FE and FN–FN(P) revert slowly or insignificantly. Conclusions are (a) essentially undistorted monomeric insulin is involved, (b) covalent, electrostatic and hydrogen bonds play modifying roles but are not primarily involved in inter-insulin linkage and (c) linkage may be ascribed to the secondary valence interactions between non-polar side chains.

In recent years the transformations between protein molecules (or corpuscles) and fibrils have been recognized as ubiquitous reactions. Insulin,[1] muscle proteins (ref. 2, has a summary of recent papers), serum albumin,[3] and fibrin,[4] are representatives. Certain of these transformations are known to be reversible (insulin, muscle proteins) or to give rise to reversion products similar to the starting proteins (serum albumin, fibrin). Systems such as collagen, previously thought to consist of continuous extended polypeptide chains, possibly are also made up of corpuscular units (for review of evidence see 5). Reversible corpuscle fibril transformations are probably responsible for many of the changes in physical properties which take place in cells and tissues.

The particular type of insulin fibril obtained depends upon the conditions of fibril formation as well as the type of insulin used. The set of insulin fibrils represent structures of varying stability, certain of which may be reverted to give insulin as in 1c. It is felt that a study of the conditions under which insulins form fibrils will contribute not only to an understanding of this particular protein but also to other proteins and of the way in which stable and ephemeral fibrils may be formed *in vivo* in the organism.[6]

The present communication examines the occurrence of nucleation, growth, and, to a lesser extent, reversion of insulin fibrils under chosen sets of experimental conditions. Fibrils from four types of insulin are considered: native insulin (FN), esterified insulin (FE), acetylated insulin (FA) and diazotized insulin (FD). The present information will be used to select the secondary valence attractive forces between non-polar side chains as being primarily responsible for inter-insulin linkage.

Certain types of insulin fibrils form from FN under conditions where FN revert to insulin. These fibrils will be treated in more detail separately as will the mechanism of fibril reversion, an X-ray diffraction analysis of insulin fibrils, and the reaction kinetics of insulin fibril formation.

Materials and Methods

Insulin.—The experiments to be reported here were carried out over an extended period of time. For a few of them crystalline zinc insulin was used directly. For most the insulin was made essentially zinc free either by precipitating insulin from acidic solution with acetone[7] or by twice salting out a 1% solution of insulin in 0.1 *N* hydrochloric acid with 15% sodium chloride and then dialyzing against running distilled water at *p*H 5 to 6 and 4° for 36 hours. All insulins used had activities of 23 to 26 International units per mg.

Samples of crystalline insulin from different sources, and from the same source but of different lots, show variations in their properties. For example, the rates and extents of fibril formation, the conditions under which maximum crystallizations are obtained, and the ratios of maxima to minima in the ultraviolet absorption spectra may be quite different. None of the procedures given above produces a standardized insulin. The experiments described here do not require insulin of entirely reproducible properties. A procedure for standardizing insulin will be detailed under kinetic studies for which it was devised.

Preparation of Standard Insulin Gel (Fibrils).—A 2% solution of insulin in 0.05 *N* hydrochloric acid (*p*H 1.6–1.8) is sealed in acid-washed siliconed Pyrex glass tube ampules 4 mm. internal diameter, about 2 cc. per ampule. The tubes are heated (2 to 10 min.) at 80–100° until faint flow double refraction appears but heating is stopped before any spherite formation occurs.[1b] The ampule is now cooled and frozen rapidly by immersion in solid CO_2–acetone solution. It is thawed under running tap water and reheated (80–100°) for approximately two minutes. The procedure of freezing and thawing and heating is repeated until, on disturbance, the contents of the tube show intense static double refraction. Reasons for proceeding in this fashion have been given.[1c] After disturbance the tube is given a final 5 min. treatment at 80° to ensure complete fibril growth.

Seeding Fibrils.—Where insulin is to be removed by adding fibrils as seeds the rate of removal of insulin per mg. of fibrous insulin may be enhanced by rapidly freezing and thawing (but not reheating) standard insulin gel. The long fibrils of the gel are broken into segments, the ends of which act as centers for growth (see ref. 8).

Assay by Double Refraction.—When fibrous systems develop in the same fashion, semi-quantitative data may be

(1) (a) D. F. Waugh, THIS JOURNAL, **66**, 663 (1944); (b) **68**, 247 (1946); (c) **70**, 1850 (1948).

(2) D. F. Waugh, *Ann. Rev. Physiol.*, **14**, 13 (1952).

(3) M. Jaggi and D. F. Waugh, *Federation Proc.*, **9**, No. 1, 66 (1950).

(4) P. Erlich, S. Shulman and J. D. Ferry, THIS JOURNAL, **74**, 2258 (1952). This source gives reference to other publications.

(5) R. S. Bear and H. J. Rugo, *Ann. N. Y. Acad. Sci.*, **53**, 627 (1951); R. S. Bear, *Advances in Protein Chem.*, **6**, 69 (1952).

(6) D. F. Waugh, *Anat. Record*, **101**, 656 (1948).

(7) D. A. Scott and A. M. Fisher, *Biochem. J.*, **29**, 1048 (1935).

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

obtained by observing the development of flow and static double refraction in the reaction ampule. The contents of the tube are observed during flow, or after disturbance, between crossed Polaroid screens.

Fibrous precipitates may be differentiated from amorphous precipitates by means of the petrographic microscope.[1b]

Assay by Centrifugation.—In view of the fact that crystallizations and bioassays are time consuming, complicated, and frequently subject to large errors, the extent of fibril formation and fibril reversion were examined by centrifugation.

A 0.2- to 1.0-ml. aliquot of the reaction mixture was diluted with 5.0 ml. of 0.01 *N* hydrochloric acid. In those cases where fibril centrifugation was difficult (organic acids), the diluting acid was made 0.07 *N* with sodium chloride. The diluted mixture was centrifuged in machined polystyrene tubes (to avoid the ultraviolet absorbing materials which leach out of celluloid) at 144,000 × *g* for 2 hours. Precipitates were dissolved in 2 ml. of 0.1 *N* sodium hydroxide for 2 min. at room temperature and then brought to *p*H 2.0. Ultraviolet absorption of supernatants and dissolved precipitates was measured at λ 2760 Å.

With very few exceptions (see Urea) fibrils can be removed quantitatively from a suspension of short fibrils made by freezing and thawing standard gel. Native insulin can be recovered quantitatively immediately after addition to such a suspension of fibrils. The adsorption of insulin to fibrils under these assay conditions is therefore negligible.

Assay by Crystallization.—Further details of this procedure are more appropriately reserved for a subsequent paper. See, however ref. 1c.

Reversion.—A standard technique for regenerating insulin from FN has been described.[1c]

Esterification.—Three procedures have been used for esterification. Procedure 1 follows the technique of Carr, *et al.*,[9] and employs 80% alcohol, 8 *N* with respect to hydrogen chloride. Procedure 2, Fraenkel-Conrat and Olcott,[10] esterifies by using 98% alcohol and 0.02–0.1 *N* hydrochloric acid. Procedure 3, Mommaerts and Neurath,[11] consists in treating insulin by suspending it in 1% concentration in alcohol made 0.1–0.01 *N* with respect to hydrogen chloride. After the reaction the insulin was completely precipitated by adding anhydrous acetone. Recoveries of 75–90% were obtained.

All esterified insulins are similar in their fibril forming properties.

Diazotization.—A variety of groups have been added to insulin by treating the protein with the appropriate diazonium salt. In general the procedures of Reiner and Lang[12] were used. Sufficient diazonium salt is added to cover a specified number of groups. Coupling proceeds at *p*H 8.0 and room temperature over a period of 1.5 hours. Recoveries were about 80%.

Acetylation.—Two techniques have been used. Procedure 1 follows closely that of Herriott and Northrop[13] using ketene. Procedure 2 is that of Olcott and Fraenkel-Conrat[14] using acetic anhydride. Acetylated insulins prepared by both methods are similar in nucleation and growth. In procedure 2 which has been generally used the reaction is carried out at *p*H 6.0 to 7.5 and 0° using a reaction time of 1 hour. The acetic anhydride is added dropwise to a buffered solution containing 25 mg. of insulin per ml. Recoveries were 80 to 90%.

Fibrils Formed from Native Insulin (FN)

Solutions of insulin in acid (*i.e.*, *p*H 2.0) are relatively stable for long periods of time at temperatures below 20°. On being heated, the insulin spontaneously transforms into a population of fibrils of variable lengths and diameters (over-all reaction). Certain of the general characteristics of fibril formation, and particularly the further aggregation of fibrils into spherites or floccules, have been described.[1b] The largest of the resulting fibrils are hundreds of ångström units in diameter, and many thousands of ångström units long as indicated previously[1a] and shown in the electron micrographs of Farrant and Mercer.[15] At 90° a 2.0% solution of insulin at *p*H 1.6 will become completely fibrous in approximately 30 minutes.

Once having been formed the insulin fibril can act as a locus for the further recruitment of insulin molecules at temperatures at which the over-all reaction is extremely slow (growth reaction). This can be shown by seeding a few preformed fibrils into a solution of insulin at room temperature or below. The fibrils thus seeded elongate and can remove the insulin quantitatively.[8] At 90° 2 mg. per ml. of seeding fibrils will remove the insulin from a 2% insulin solution at *p*H 1.6 in less than 3 minutes. Reversion experiments suggest that fibrils formed at higher temperatures and at lower temperatures through the growth reaction are structurally similar.

The kinetics of fibril formation will be treated in detail elsewhere. The following is given as an aid in interpreting the results of nucleation and growth experiments in solutions of organic acids and urea and in interpreting the reformation of insulin fibrils in solutions in which phenol or organic acids revert FN.

The over-all and growth reactions of insulin fibril formation[16] suggest that the initiation of a fibril (nucleation reaction) requires the simultaneous interaction of several insulin monomers. The degree to which the monomers are prepared for bonding and are approximated is temperature sensitive. On this basis nucleation was expected and has been found to vary with some power of the insulin concentration, the exponent (as high as four) being a function of the minimal number of monomer units required to produce a stable nucleus. Once having been formed the surface of the fibril presents a configuration which is a composite of the properties of the individual molecules. During growth collision between a single insulin monomer and the surface of the fibril brings the monomer into association with the requisite number of neighbors to produce a stable linkage. Thus the growth of a fibril was expected and has been found to be a function of the surface area of the fibril population and the concentration of insulin in solution. Nucleation and growth have been found to vary differently with respect to changes in ionic strength, *p*H, etc.

Mineral Acids.—Side reactions, such as spherite formation which is enhanced by polyvalent anions,[1b] interfere seriously with kinetic studies, reversion experiments, and the reaction experiments described here. A number of acids were examined for their effects on fibril formation and particularly their ability to produce a clear gel free of spherites.

(9) F. H. Carr, K. Culhane, A. Thomas-Fuller and S. W. F. Underhill, *Biochem. J.*, **23**, 1010 (1929).

(10) H. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

(11) F. W. H. M. Mommaerts and H. Neurath, *ibid.*, **185**, 909 (1950).

(12) L. Reiner and E. H. Lang, *ibid.*, **139**, 641 (1941).

(13) R. M. Herriott and J. H. Northrop, *J. Gen. Physiol.*, **18**, 35 (1934–1935). See also K. G. Stern and A. White, *J. Biol. Chem.*, **122**, 371 (1937–1938).

(14) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 151 (1947).

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

(16) D. F. Waugh, *Federation Proc.*, **5**, 111 (1946).

In the present work the insulin was first precipitated from solution in the appropriate acid by adding an excess of acetone and the acid salt thus obtained was dissolved to give a 2% solution at a *p*H of approximately 1.8. The resulting solutions were heated continuously at 100° or were subject to the fibril forming procedure used in making standard gel. It was found that insulin fibrils would form in all of the acids tested: phosphoric, nitric, hydrobromic, hydrochloric, sulfamic, sulfuric and hydriodic acids (the last being used only in seeding experiments at lower temperatures). None was found to be superior to hydrochloric acid, which has been generally used.

A variety of sulfonic acids have been tested without forming the acid salt of insulin. Those which will yield a low *p*H and will dissolve insulin (*e.g.*, *p*-toluenesulfonic acid and diphenyl-*p*,*p*'-disulfonic acid) supported fibril formation with the formation of spherites.

Organic Acids.—The possibility that the inter-insulin linkage is the result of the combination of carboxyl groups through the formation of hydrogen bonds suggested that the nucleation and growth of insulin fibrils be studied in concentrated organic acids.[17] Experiments of essentially three types have been performed. These in which (I) the growth reaction was examined by seeding 10% by volume of seeding fibrils or standard gel into solutions of native insulin in organic acids; (II) the over-all reaction (nucleation and growth) was examined by heating solutions of native insulin in organic acids, and (III) the reversion of fibrils prepared in hydrochloric acid was examined by adding organic acid to standard insulin gel. Assay by centrifugation was used.

Formic, acetic, propionic and butyric acids have been examined in final concentrations up to 9.1, 8.3, 6.7 and 5.6 *M* (all about 50% by volume). Similar effects are observed with this group. Caproic acid, 4.3 *M*, produces few of the observed effects. In view of the uniformity of the former group, representative rather than complete data are given.

Results of the three types of experiments are illustrated in Tables I, II and III. Table I, which examines growth in the presence of 8.3 *M* acetic acid, illustrates the general growth retardation produced by organic acids compared to hydrochloric acid as described under FN. This retardation is offset by an increase in ionic strength, obtained by addition of sodium chloride. In all organic acids studied, and at a variety of ionic strengths and temperatures, growth can proceed to completion as indicated for acetic acid in Table I. There is no evidence that a measureable equilibrium between monomer and fibrous insulin can be established in organic acids. This is particularly interesting in view of the reversion experiments described below.

As shown in Table II increasing concentrations of acetic acid retard the over-all reaction, which is dependent upon nucleation. In 8.3 *M* acetic acid nucleation is effectively absent at 50°, an added ionic strength of 0.04, and over a reaction time of 18 hours. Increasing the ionic strength to 0.11 increases the rate of nucleation as does an increase in temperature. The sensitivity of nucleation will be treated further in the discussion.

(17) S. C. Commerford, Masters' Thesis, The Massachusetts Institute of Technology, Cambridge, 1952.

TABLE I

GROWTH REACTION

Acid, *M*	Native insulin, mg./ml.	Fibrous insulin, mg./ml.	*p*H	Ionic strength	Temp., °C.	Time, hr.	Fibril formation
8.3	10	2	1.6	0.04	25	56	0
8.3	10	2	1.6	.11	25	56	99
8.3	10	2	1.6	.04	50	4.7	52
8.3	10	2	1.6	.11	50	4.7	97
8.3	10	2	1.6	.11	80	4.7	95
8.3	20	2	1.6	.02	50	17.5	92
8.3	20	2	1.6	.08	60	6.3	97
8.3	20	2	1.6	.08	60	6.3	99

TABLE II

OVER-ALL REACTION (NUCLEATION AND GROWTH)

3.3	10	0	1.6	0.04	50	18	33
5.8	10	0	1.6	.04	50	18	4
8.3	10	0	1.6	.04	50	18	0
8.3	20	0	1.6	.04	25	56	0
8.3	20	0	1.6	.04	50	38	7
8.3	20	0	1.6	.04	80	15	42
8.3	20	0	1.6	.11	25	56	0
8.3	20	0	1.6	.11	50	16	84
8.3	20	0	1.6	.11	80	4.7	72

TABLE III

FN REVERSION BY ORGANIC ACIDS

A. 8.3 *M* acetic acid, *p*H 1.5, 10 mg. per ml. fibrils, added ionic strength 0.02, and $T = 25°$

Time, hr.	0	0.5	1.3	3.3	6.2	12.0	24.0
Reversion, %	4	9	15	22	15	21	17

B. 5.4 *M* butyric acid, *p*H 1.5, 10 mg. per ml. fibrils, added ionic strength 0.13, and $T = 80°$

Time, min.	0	1	2	4	10	40	120	770
Reversion, %	30	70	77	71	74	51	27	0

Reversion of insulin fibrils may be observed when standard gel is mixed with organic acids. A similar effect is not generally observed when standard gel is diluted with hydrochloric acid at the same or at a lower *p*H for the fibrils may be removed quantitatively by centrifugation (see Methods). The extent of reversion in organic acids is dependent upon acid concentration, temperature, ionic strength and time. Table III, which refers to the effects of acetic and butyric acids, illustrates the effects observed with formic, acetic, propionic and butyric acids, but not with caproic acid which produces little or no reversion. As shown in Table III part A, at 25°, 8.3 *M* acetic acid produces a reversion which increases with time and becomes 15 to 20% over the time range 1.3 to 24 hours. The extent of reversion at 25° is independent of the fibril concentration over the range examined, 2.8 to 10 mg. fibrous protein per ml.

Part B of Table III shows that at 80°, 5.4 *M* butyric acid produces a reversion which reaches a maximum of 70 to 80% in 10 minutes. During

reversion there is a striking decrease in flow double refraction. If the reversion time be extended beyond 10 minutes there is a gradual return of protein to the fibrous structure as evidenced by the return of flow double refraction and spherite formation. The return is quantitative within 12.8 hours. The extent of reversion is temperature sensitive. Thus at 60°, and other conditions as in Table III-B, butyric acid produces a maximum reversion of about 60% in 20 minutes. At 50° a maximum reversion of about 50% is observed in 30 to 60 minutes. At these temperatures reconversion to the fibrous form is quantitative if the time is extended sufficiently. The return to the fibrous form is achieved in 1.3 hours with 8.3 *M* acetic acid at 80°. Formic acid in 9.1 *M* concentration (*p*H approximately 0.55), an added ionic strength of 0.13 *M*, and a temperature of 80° produces a maximum of 77% reversion in 2 minutes. At 2 hours the reversion has decreased to 27%, a complete return being registered within 13 hours. Organic acid stable fibrils prepared in this way will be termed FN–FN(O).

TABLE IV

EFFECTS OF UREA AND GUANIDINIUM CHLORIDE ON NATIVE INSULIN

*p*H	Temp., °C.	Recovery, %	Growth reaction, %	Crystallization, %
A. 8 *M* urea, 10 mg. per ml. insulin, 24 hours treatment time				
2	0	35	100	75
7	0	75	90	90
9	0	86	90	75
2	37	84	60	90
7	37	87	100	0
9	37	90	90	3
B. 8 *M* guanidinium chloride, 10 mg. per ml. insulin, 24 hours treatment time				
2	0	75	100	75
7	0	102	70	55
9	0	124	100	80
2	37	96	95	0
7	37	96	100	70
9	37	96	65	10

All of the precipitates obtained with organic acids have been examined with a petrographic microscope and have been found to be fibrous in structure, either spherites or tactoids.[1b]

In the experiments illustrated here the reversion of FN by organic acids is not quantitative, the maximum reversions being approximately 70 to 80%. Standard alkaline reversion of FN–FN(O), obtained free of organic acid by dialysis, is accomplished to the same extent as reversion of FN. The bonds operating in the formation of FN–FN(O) therefore appear to be similar to but slightly stronger than those operating in the formation of FN. The reversion–reconversion phenomena found in organic acids are similar to those found in phenol (see phenol) in which case the transformation is designated FN–FN(P). Much stronger bonds are indicated in the latter type of fibrous modification for FN–FN(P) revert, if at all, only at much higher alkali concentrations than FN.

Insulins obtained from the reversion of FN by organic acids or the alkaline reversion of FN–FN(O) will, when dissolved in hydrochloric acid, form characteristic FN quantitatively. Thus, the structural requirements necessary for the formation of fibrous insulin have not been affected by treatment with organic acids. In view of the basic similarity of all insulin fibril structures, as determined by X-ray diffraction techniques,[18] it is suggested that the limited reversions and complete reconversions as found in organic acids may be due to (1) discontinuities in the layers of insulin molecules making up FN (where such a discontinuity occurs a given molecule may not be bonded to the requisite number of neighbors to produce a linkage stable in the new environment) and (2) the mobilization of bonding groups in addition to those operating in the formation of FN. Reversion and reconversion of fibrils will be treated in greater detail in a subsequent publication.

Urea.[17,19]—Urea has long been recognized as an agent which dissociates proteins: possibly through the formation of hydrogen bonds,[20] possibly through the formation of loose complexes mediated by other than hydrogen bonds. Urea[21] is known to inactivate "irreversibly" certain proteins. An examination has been made of certain conditions under which native insulin can be recovered after treatment with urea and guanidinium chloride without losing its capacity for fibril growth and crystallization.[19] Concentrations of urea and guanidinium chloride up to 8 *M* were examined using 10 mg. per ml. insulin at *p*H's of 2, 7 and 9 and temperatures of 0 and 37°. The treatment time was 24 hours. Insulin was recovered in four fractions: initial dialysis (precipitate was fraction 1), adjustment of the *p*H to 5.35 and centrifugation (precipitate was fraction 2), second dialysis (precipitate was fraction 3) and lyophilizing the supernatant (residue was fraction 4). Fibril growth was examined by seeding a 2% solution of the recovered fraction dissolved in 0.05 *N* hydrochloric acid so that the final solution contained 5 mg. of seeding fibrils for each 10 mg. of treated insulin. The reaction mixture was allowed to stand at 37° for 48 hours before being assayed by a modified centrifugation technique.

The pertinent data are summarized in Table IV. The relatively uniform effects observed with the several fractions have been combined in column 4 (fibril growth) and column 5 (crystallization). The values given are estimated to be accurate to 15%. It is evident from Table IV that fibril growth is more resistant to the effects of both reagents than crystallization since the latter can be abolished selectively at *p*H's 7 and 9 at 37° using urea and at *p*H's 2 and 9 at 37° using guanidinium chloride. None of the chosen conditions produce changes sufficiently drastic to abolish fibril growth. Insulin has been treated with 5.3

(18) W. Koltun, D. F. Waugh and R. S. Bear, to be published.

(19) M. L. Sackler, Master's Thesis, The Mass. Inst. of Tech., Cambridge, 1945.

(20) L. Pauling, "The Nature of the Chemical Bond," Cornell University Press, Ithaca, N. Y., 1945.

(21) H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

M urea at *p*H 1.6 for 1.5 hours at 80°. The protein recovered after dialysis was found to be capable of complete fibril formation (growth or over-all reaction). From the data which follow it is evident that fibril growth will occur in the presence of concentrated urea at higher temperatures.

The effects of high concentrations of urea on the growth and over-all reactions of insulin fibrils, and the reversion of FN, are examined in Table V.[17] All reaction mixtures were prepared by mixing insulin solution or standard gel and urea in solution. Growth experiments were conducted by seeding with standard gel.

TABLE V

EFFECTS OF UREA ON FIBRIL GROWTH, THE OVER-ALL REACTION AND FIBRIL REVERSION

Urea, *M*	*p*H	Native insulin, mg./ml.	Fibrous mg./ml.	Temp., °C.	Time, hr.	Growth, %	Reversion, %
			Growth reaction				
5.3	1.6	9	2	25	18	69	..
5.3	1.6	9	2	25	18	72	..
5.3	1.6	9	2	80	1.5	57	..
4.6	1.6	12	2	25	7	7	..
5.3	8.0	10	2	25	18	0	..
4.6	7.6	12	2	60	7	40	..
5.3	8.0	10	2	50	18	100	..
6.0	7.0	10	2	50	20	100	..
			Over-all reaction				
5.8	1.6	10	0	25	18	1	..
5.8	1.6	10	0	25	18	0	..
7.8	1.6	10	0	80	1.5	7	..
5.3	7.2	10	0	25	18	0	..
5.3	7.2	10	0	50	18	0	..
4.6	7.6	12	0	60	7	0	..
			Reversion reaction				
7.8	3.2	0	6.7	25	18	...	1
7.8	1.6	0	6.7	25	18	...	1
0	1.6	0	6.7	25	18	...	30
7.8	1.6	0	6.7	25	18	...	26
0	7.3	0	6.7	25	20	...	49
0	7.3	0	6.7	50	20	...	30
7.5	7.0	0	6.0	25	7	...	27
8.0	7.3	0	6.7	25	20	...	42
8.0	7.3	0	6.7	50	9	...	6
8.0	7.3	0	6.0	50	20	...	7
7.5	7.0	0	6.7	60	7	...	19

TABLE VI

REDUCTION OF 0.5% INSULIN WITH 1% SODIUM CYANIDE

Time, min.	1	5	30	90	180
SH groups, %	0	0	5	10	20

The results of studies at *p*H 1.6 and *p*H 7 to 8 are shown under Growth reaction. At either *p*H the growth reaction takes place in the presence of 5.3 *M* urea. The growth rate increases with temperature and the growth reaction becomes more nearly complete as time is increased. At *p*H 1.6 the growth rate approximates that found in hydrochloric acid at the same *p*H. The most striking effect of urea is its ability to support growth of fibrils at *p*H 7.0 (see Discussion). In contrast to its effect on growth, urea suppresses nucleation markedly. The lack of nucleation in 1.5 hours at 80° and *p*H 1.6 is striking (Table V, Over-all reaction). Thus, although urea induces fibril growth at *p*H 7.0 to 8.0 it will not support nucleation at the same *p*H, and will suppress nucleation under conditions where it normally takes place.

The data of Table V, Reversion reaction, suggest that at 25°, 7.8 *M* urea has little effect on the structure of FN as shown in the first two lines. The third line, chosen purposely, refers to a sample which at zero urea concentration shows 30% of the insulin in the non-centrifugable state. The same amount of insulin (26%) is found after treatment with 7.8 *M* urea as indicated in line 4. The 30% "reversion" recorded in line 3 is occasionally obtained on diluting a preparation of standard gel and is probably due to an aberration in the over-all reaction which leads to the formation of numerous small fibers which are not removed by centrifugation. On changing the conditions of standard gel, particularly a change in *p*H,[22] limited reversions are frequently obtained. Thus, the reversions of 49 and 30% shown in lines 5 and 6 as found with zero urea concentration at *p*H 7.3 are expected. In these cases 0.01 *N* alkali was added to the gel. When the gel is brought to *p*H 7.0 by dialysis reversion is generally 30%. Examination of Table V, Reversion reaction lines 7 to 11, shows that at *p*H 7 to 7.3 the presence of 7.5 to 8.0 *M* urea produces considerably *less* reversion than that obtained by *p*H change alone (lines 5 and 6). A return of protein to the fibrous structure, consistent with the growth experiments cited above, is evident. The absence of even limited reversion with urea is to be contrasted with the effects of organic acids.

Acid–Alcohol.—The fact that growth may be carried out in 70% acid–alcohol is shown by an extension of an *in vitro* assay for insulin[8] to include alcoholic pancreatic extracts. Thus, the growth reaction takes place not only in the presence of 70 to 80% alcohol but in the presence of large amounts of non-insulin material and initial insulin concentrations near 0.04 mg. per ml.

Phenol.—Phenol and compounds similar to phenol have been found to be excellent reverting agents,[23] which under appropriate conditions will lead to a recovery of over 95% of the insulin in crystalline form (10% phenol, 60°, *p*H 1.6, and 15 minutes). If the reversion time in phenol be extended a new fibrous system, similar in electronmicrographs and X-ray diffraction pattern to FN, will develop in periods of 0.5 to 2.0 hours. The transformation is designated FN–FN(P) (see also organic acids). FN–FN(P) are reverted by alkali only with difficulty, if at all.

In solutions of native insulin at *p*H ~ 2.0 and temperatures of 80–100° phenol in concentrations above ~6% will progressively retard normal fibril nucleation.

(22) Many changes in variable, *e.g.*, addition of phenol or organic acids at constant *p*H or a change in *p*H, lead to the appearance of some soluble, crystallizable insulin in solution. The phenomenon will be considered further under reversion.

(23) D. F. Waugh, M. J. Smith and D. F. Fearing, *Federation Proc.*, 7, 131 (1948).

Reduced Insulin.[16]—du Vigneaud, *et al.*,[24a] have shown that strong reduction of insulin with cyanide leads to a loss of all characteristic properties including the formation of the heat precipitate (fibril formation, 1). Later, White and Stern[24b] using thioglycolic acid, showed that a reduction of 1 to 2 disulfide bonds per molecule of 36,000 led to a loss of 50% of the biological activity, complete loss occurring when 30% of the disulfides had been reduced. In view of the fact that urea and guanidinium chloride can produce an insulin which will engage in the growth reaction but will not crystallize it was desirable to find the minimum reduction associated with the loss of fibril growth.

Table VI indicates the extent of reduction produced by varying treatment times using the minimum conditions described by du Vigneaud, *et al.*, as leading eventually to the complete inactivation of insulin. The reaction mixture contained 5 mg. per ml. of insulin and 10 mg. per ml. of sodium cyanide and was incubated at 30°. The extent of reduction in the reaction mixture was determined by a calibrated nitroprusside reaction. Insulin was recovered from the reaction mixture by dialyzing against distilled water in an atmosphere of nitrogen. The dialysate was precipitated at *p*H 5.3 and the precipitate washed and dried *in vacuo*. The sulfhydryl groups reoxidized during the washing and drying procedure. Five minutes reaction time yielded a precipitate which would not crystallize but 25% of which formed fibrils as tested for by the growth reaction (5 mg. per ml. fibrils, 10 mg. per ml. insulin, *p*H 1.6, 37°, and 48 hours). After 30 minutes reaction time (5% average reduction of disulfide groups in the reaction mixture) the fibril forming capacity of the recovered precipitate was essentially eliminated (4% fibril growth). Not all of the protein precipitates at the *p*H 5.3 precipitation step. Protein was therefore recovered from the supernatant. It amounted to 34% of the initial protein and was 88% effective in fibril growth. If all of the 5% reduction were confined to the precipitated protein, about 7 to 8% reduction in this fraction would be calculated. The actual reduction is greater than that calculated for reduction continued during the dialysis step. The extent of reduction in the reaction mixture at 90 minutes is 10% (Table VI). The conditions of dialysis removed the cyanide in a period of 30 to 60 minutes. On including dialysis, the maximum reduction in the precipitate is therefore approximately 14%. The reduction of one disulfide per molecule of M.W. = 12,000 would require a 16% conversion of disulfides to sulfhydryls. It can be concluded that the reduction of one disulfide per molecule of M.W. = 12,000 will lead to complete loss of fibril growth.

Chemically Modified Insulins

Fibrils Formed from Esterified Insulin (FE).—The carboxyl groups were esterified with methyl alcohol using procedure 3, room temperature, 0.1 *N* HCl, and a reaction time of 24 hours. Under such conditions all of the 12 available carboxyl groups per molecule M.W. = 12,000[11,25] are converted. Fibril nucleation and growth (over-all reaction) were examined by dissolving the esterified insulin in over 2% concentration in acid and adjusting aliquots of this solution to several *p*H values with alkali, the final protein concentration being 2.0%. These solutions were then heated at 80 to 100°, and compared with native insulin adjusted in the same way. The growth of preformed fibrils was examined by seeding and heating at 100°, the final conditions being 1 mg. per ml. of seeding fibrils (standard gel) and 18 to 20 mg. per ml. of methylated (or native) insulin. The rate of appearance of double refraction was used to compare the methylated and native insulin systems. The termination of the over-all reaction with methylated insulin at *p*H 1.5 (10 min., 80°) was assayed by centrifugation. Fibril formation was found to be over 95% complete.

When uncontrolled variations in ionic strength are taken into account, the appearance of double refraction at *p*H 1.5 (80°) and *p*H 2.8 (100°) suggests that native and esterified insulins are comparable with respect to the over-all (10 to 30 min. at 100 to 80°) and growth (<3 min. at 100°) reactions. At *p*H 4.1 the growth reaction proceeds nearly equally (6 *vs.* 9 min. at 100°) for methylated and native insulins but the over-all reaction is considerably faster for methylated insulin. At *p*H 4.6 methylated insulin will form a clear gel showing brilliant static double refraction in 3.6 hours at 100° or in less than 1.5 hours at 100° using the growth reaction. The behavior of native insulin at *p*H 4.6 requires comment. Only a portion of the 18 to 20 mg. per ml. of native insulin is in solution. On heating for 1 hour at 100° the character of the precipitate changes from white floccules to translucent granules. Examination with the petrographic microscope shows that these granules have little double refraction and are therefore clearly different from the spherites and tactoids which form in systems undergoing the monomer–fibril transformation.[1b] The fact that the translucent granules do not revert under standard alkaline conditions also clearly differentiates them from FN spherites.

The rapidity of the growth reaction, the fact that growth may be obtained in the presence of 30% acid-alcohol (to suppress hydrolysis), the stability of the ester linkage at the higher *p*H values used, the clear differentiation of native insulin from esterified insulin at *p*H 4.6 and the fact that FE revert in alkali about one-hundreth as fast as FN, leaves little doubt that esterified insulin is capable of forming insulin fibrils.

Mommaerts and Neurath[11] who also describe fibril formation with esterified insulin remark that esterified insulin requires the same high acidity as native insulin to form fibrils. The results just described show that esterified insulin will form fibrils under conditions where native insulin proceeds to form a different type of precipitate.

Esterified insulin will be discussed after the effects obtained with acetylated insulin have been presented.

(24) (a) V. du Vigneaud, R. R. Sifferd and R. R. Sealock, *J. Biol. Chem.*, **102**, 521 (1933); (b) A. White and K. G. Stern, *ibid.*, **117**, 95 (1937).

(25) C. Fromageot, *Cold Spring Harbor Symposia. Quant. Biol.*, **14**, 49 (1949).

Fibrils Formed from Acetylated Insulin (FA).—Insulin was acetylated as described using procedure 2 and 127 moles of acetic anhydride per 12,000 g. of insulin. All of the six available amino groups, two lysine[25] plus four terminal amino groups per M.W. = 12,000, should be covered by this technique. In acid solution[26] phenol acetates are labile suggesting that the products examined here are largely amino-acetylated. When acetylated to this extent insulin becomes less soluble in acidic solution, but in the *p*H range 1.5 to 2.0 solutions of 2% protein may be prepared.

The over-all reaction was examined by preparing a 1.6% solution of acetylated insulin at *p*H 1.6 and heating at 80 to 100°. On continuous heating the solution generally becomes an opalescent gel having a much greater rigidity than a similar gel made with native insulin. Such gels form in about 4 hours at 80° and exhibit faint static double refraction which can be increased only slightly by disturbing the gel. When examined with the petrographic microscope gel fragments also show faint static double refraction. Conversion to the fibrous state may be demonstrated, and the rate of conversion increased by a factor of about four, either by subjecting the reaction mixture to a repeated freezing (Dry Ice–acetone)–thawing–heating cycle, the heat periods being 20 minutes, or by seeding and heating continuously (20 mg. per ml. of acetylated insulin, 2 mg. per ml. of seeding fibrils). In either case the solution may develop strong flow or static double refraction. Assay by centrifugation indicates complete conversion to the fibrous form. The gels obtained with acetylated insulin are most probably cross linked as compared to FN gels which depend upon electrostatic repulsion for their gel characteristics. For example dilution will convert a gel of FN to a uniform dispersion of fibrils but will not so convert a gel of FA. FA revert in alkali in a manner similar to FN.

There can be no doubt that the thoroughly acetylated molecule becomes refractory to both nucleation and growth. Out of the twelve possible positive groups in native insulin, acetylated insulin retains the two guanidinium and four imidazolium groups per molecule of M.W. = 12,000. At *p*H values below approximately four, the repulsive forces between acetylated insulin molecules should be much less than those between native insulin molecules. If the structures of the two molecules in acid solution were similar an increased rate of fibril nucleation and growth with acetylated insulin would be predicted, for acetylation would be expected to have an effect similar to an increase in ionic strength. Our finding that the opposite is the case suggests that the structures available to the two types of molecules are different. It is suggested that the $\sim+12$ net charge possible when the carboxyl groups of native insulin are uncharged leads to an instability of internal structure such that bonding groups may be presented externally for inter-insulin linkage. When the acetylated molecule is in acid the forces inducing instability are decreased since the net charge is now $\sim+6$ and the bonding groups may make intramolecular associations thus rendering them less available for intermolecular linkage. The rates of the nucleation and growth reactions with acetylated insulin are thus decreased markedly.

Esterification, which allows the same $\sim+12$ net positive charge to occur at higher *p*H values, allows the over-all and growth reactions to proceed at *p*H values excluded from the range associated with native insulin.

The above suggestions receive support from the observation that on using 2 mg. per ml. of fibrils (standard gel) as seed, a 2% solution of acetylated insulin at *p*H 7.0 may be converted to a doubly refracting gel in which over 90% of the acetylated insulin is in the gel structure. In this case instability is attributed to the net negative charge of approximately -10. The growth reactions of acetylated insulin at *p*H $\sim$ 2.0 and *p*H $\sim$ 7.0 are similar.

Fibrils Formed from Azo-insulin (FD).—The diazonium salts of aniline and *p*-aminobenzoic acid have been employed as described under methods using sufficient diazonium salt to cover 1 to 11 groups per molecule of M.W. = 12,000. In general, studies have been made of insulins having 8 to 11 modified groups. According to Fraenkel-Conrat and co-workers (see ref. 25) the imidazolium and tyrosine groups of insulin are highly reactive and about equal in reactivity. Since there are eight tyrosine and four imidazolium groups per molecule of M.W. = 12,000, essentially complete coverage is expected for those insulins most extensively modified. The reaction kinetics of fibril formation have been extensively examined with these modifications. With the above azo-insulins the rates of over-all and growth reactions are slower than those obtained with native insulin under the same conditions (to be described elsewhere). Within 60 to 90 minutes at 90 to 100° a solution of 2% azo-insulin in 0.05 *N* hydrochloric acid will develop intense double refraction. The reaction goes to well over 90% of completion as determined by assay by centrifugation. Azo-insulin prepared as described may or may not yield a gel as does native insulin. At times a population of shorter fibrils develops which exhibits intense flow double refraction. The fibril suspensions remain clear and show little tendency to form spherites. Such FD are relatively stable to the standard alkaline reversion procedure.

Other diazonium salts have been coupled with insulin and the resulting products examined for fibril formation. Experience with *p*-aminobenzenesulfonic acid is illustrative. This agent progressively reduces the over-all reaction as the number of groups added increases. Even in acidic solution, the strength of the sulfonic acid radical introduces negative groups and may have the same effect that an increase in *p*H has on native insulin. It was noted however that the insulin became insoluble as the number of substituted groups increased from 4 to 8 per molecule of M.W. = 12,000.

Discussion

The Type of Native Insulin Molecule Which Can Enter into Fibril Formation.—Oncley, *et al.*,[27] have

(26) H. Fraenkel-Conrat, in "Amino Acids and Proteins," D. M. Greenberg, Editor, Charles C Thomas, Springfield, Ill., 1951.

(27) J. L. Oncley, D. Ellenbogen, D. Gitlin and F. R. N. Gurd, *J. Phys. Chem.*, **56**, 85 (1952).

shown that the dissociation of the trimer form of insulin in 1% solutions containing 0.1 *M* sodium chloride takes place between *p*H ~ 5.0 and *p*H 2.0 at 20°, the solution containing units of M.W. = 12,000 at the latter *p*H. In comparing native insulin with acetylated insulin it was found here that fibril nucleation and growth with native insulin are slow at a *p*H of 4.1 and absent at a *p*H of 4.6 or above. At *p*H's below 4.1 the rate of nucleation and growth increase as the *p*H decreases in spite of the accompanying increased repulsion due to net charge. It has been pointed out that the increased net positive charge may be introducing an instability which makes available bonding groups. The increased rates observed as the *p*H is decreased below 1.5 to 2.0, the *p*H at which a maximum net positive charge is expected, are probably due to the increase in ionic strength which accompanies a decrease in *p*H. It can be concluded that the monomeric form of insulin, molecular weight 12,000[27,28] or possibly 6,000,[29,30] is involved.

Fibril formation does not require the same structural precision as crystallization for complete fibril formation can be obtained with insulins which will not crystallize, *i.e.*, insulin treated with guanidinium chloride and urea. Insulins which have been altered by reagents which break covalent linkages, however, will no longer form fibrils. Thus, the reduction of one disulfide linkage per unit of M = 12,000 leads to such a product as does the hydrolysis of insulin by enzymes and strong acids and alkalies.

Possibly when the insulin molecule enters the fibril association it exists much as it does in acidic solution; perhaps as a member of a family of molecules having slightly different structures. Alternatively, since several molecules are required to stabilize the inter-insulin linkage, the coöperative effects of the molecules already in the fibril structure may induce in the entering molecule an unusual structural change. At present the structural alterations attending fibril formation are felt to be small for the following reasons: (a) fibril growth will take place at low temperatures; (b) any structural changes produced during fibril formation must be reversible for crystalline insulin may be quantitatively recovered from FN; (c) the disulfide bonds must remain intact, a circumstance expected to limit the extent of permissible unfolding; (d) the frictional ratios of insulin in acidic solutions[27] suggest that there is no extensive unfolding; (e) any extensive unfolding would require the rupture of many of the hydrogen bonds assumed to be responsible for the stabilization of the fold or helix of the polypeptide chain[31] and (f) any induced structural changes must involve the entire molecule, *i.e.*, be transmitted to the fibril surface. Thus, the suggestion of Ambrose and Elliott[32] that the insulin fibril contains insulin "denatured" and in the β or extended chain structure seems improbable. An X-ray diffraction analysis of several types of insulin fibrils has been in progress for some time.[18] Such studies also suggest that a complete β-structure is unlikely. At the same time, the transformations FN–FN(O) and FN–FN(P) show that more than one bonding configuration is possible; the FN bonding (assumed to be studied by Ambrose and Elliott) being much *less* stable than the FN–FN(P) bonding. As will be discussed elsewhere certain small structural changes are felt to be required by the kinetics of fibril formation.

The Inter-insulin Linkage.—The formation of stable inter-insulin linkages at low temperatures, the sensitivity of the fibril-forming reaction to slight reduction of the disulfide linkages in insulin, the fact that several insulin monomers must cooperate in establishing a stable nucleus and the fact that fibril formation takes place with insulins which have had their carboxyl, amino, tyrosine and imidazolium groups blocked by chemical modification (FE, FA, FD), effectively eliminates the possibility that covalent bonds are operating.

Fibril formation takes place readily under conditions where the native insulin molecule is maximally positively charged, where negative charges are largely absent and where the protein is normally highly soluble. Indeed, with native insulin fibril nucleation and growth become negligible at *p*H's near the isoelectric point where the interaction of positive and negative charges becomes probable. Further, fibril formation will occur when those groups responsible for the introduction of positive or negative charges have been largely blocked by chemical modification. For these reasons linkages involving the interaction of positively and negatively charged groups are considered unlikely.

It should be remembered here that charged groups play a modifying role. For example, an increase in ionic strength increases the rates of nucleation and growth while inhibiting, in the correct concentration, fibril reversion.[10] Likewise, a shift in *p*H toward the isoelectric point can block reversion by reagents, such as phenol, which do not operate directly through charged groups.

Of the bonds of secondary valence type the most prominent possible with proteins are hydrogen bonds[20] and interactions of the type associated with hydrocarbons and responsible for stabilizing soap micelles (van der Waals forces[33]). In considerations of protein structure[31] an important role has been assigned to the hydrogen bond. The experiments with high concentrations of urea and organic acids were formulated with the objective of testing fibril formation under conditions where competition with strong hydrogen bond forming agents in excess would be expected to reduce or abolish fibril nucleation and particularly growth if this type of bond were primarily involved. Fibril growth takes place in the presence of 8.3 *M* acetic acid, other organic acids in high concentration, with 5.3 to 6.0 *M* urea at *p*H 1.6 or *p*H 7.0, with insulins which have had their amino, carboxyl, tyrosine and imidazolium groups modified, and in acidic solutions containing high concentrations of

(28) H. Gutfreund and A. G. Ogston, *Biochem. J.*, **40**, 432 (1946); H. Gutfreund, *ibid.*, **42**, 544 (1948).

(29) E. Fredericq and H. Neurath, THIS JOURNAL, **72**, 2684 (1950). See also F. Tietze and H. Neurath, *J. Biol. Chem.*, **194**, 1 (1952).

(30) E. J. Harfenist and L. Craig, THIS JOURNAL, **74**, 3087 (1952).

(31) L. Pauling, R. B. Corey and H. R. Branson, *Proc. Nat. Acad. Sci. U. S.*, **37**, 205 (1951).

(32) E. J. Ambrose and A. Elliott, *Proc. Roy. Soc.* (*London*), **A208**, 75 (1951).

(33) P. Debye, *Ann. N. Y Acad. Sci.*, **51**, 573 (1949).

ethyl alcohol. The weight of such evidence is felt to eliminate hydrogen bonds as *primary* bonding agencies. Hydrogen bonds are expected to have a modifying influence under conditions where they can form. As will be considered elsewhere the reversion of fibrils by phenol(s) and the submaximal reversions obtained with organic acids suggest that hydrogen bonds are important in this respect.

It is felt that a bond involving the interaction of non-polar side chains, a possibility previously suggested,[1a] will account for the properties of insulin fibrils presented here and is consistent also with the reversion and reaction kinetic experiments to be presented subsequently. The relative insensitivity of the non-polar residue to chemical modification suggests that such a bond be selected partly by elimination and partly on the basis of the general properties of insulin fibrils. Of the side chains of insulin over 30% (valine, tyrosine, leucine, isoleucine, phenylalanine) may be classified as non-polar. A non-polar bond will itself be insensitive to *p*H changes. Thus, in the absence of negatively charged groups, as in methylated insulin, alkaline reversion is suppressed and the inter-insulin linkage is stable. Once formed, such a bond should also be insensitive to the modification of reactive groups, unless, as in insulin treated with the diazonium salt of aniline, additional non-polar groups are introduced. As expected, FD of this type are unusually stable toward alkaline reversion.

A consideration of the occurrence of non-polar groups and the net charge suggests that the utilization of all non-polar groups would result in a linkage stable over the entire *p*H range used. The formation of FN would therefore require only a fraction of the total non-polar residues, and the transformations FN–FN(O) and FN–FN(P) can be accounted for on the basis of the participation of additional groups.

The single exception so far found to the observation that with native insulin fibril nucleation and growth require the solution to have an acid *p*H is the fact that growth can take place at *p*H 7.0 to 7.5 in the presence of 6.0 *M* urea. Although the multiple effects of urea are not understood it is interesting that Redlich, *et al.*,[34a] and Schlenk and Holman[34b] have found urea complexes to occur with a variety of hydrocarbons. Since the urea solutions used here are similar to those employed elsewhere[34] one would expect a complexing which might well offset electrostatic interactions and by forming loose complexes with non-polar residues aid in establishing the bonding configuration.

It is concluded that non-polar secondary valence interactions are primarily involved. This bond type, and the modifying effects of hydrogen bonds and electrostatic interactions, will be discussed further after a consideration of reversion.

Nucleation and Growth.—Several instances have been noted in which specific components of reaction mixtures could effectively eliminate the nucleation reaction while allowing the growth reaction to proceed, within the experimental error, to completion. Such components as described here are: organic acids in high concentration in the absence of added salt, high concentrations of urea at *p*H 1.6 or *p*H 7.0 and high concentrations of phenol.

The selective sensitivity of the nucleation reaction to changes in the reaction mixture is felt to reside in the physical complexity of the nucleation process as compared to the growth process. Thus, a change in variable which introduces an increment in, for example, the energy barrier preventing close approach of molecules will have its greatest effect on nucleation which requires the nearly simultaneous interaction of three or four monomeric units.

Acknowledgment.—The authors express their appreciation to the Research Division and the Armour Laboratories of Armour and Company, Chicago, Ill., for generous support of this research.

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(34) (a) O. Redlich, C. M. Gable, A. K. Dunlop and R. W. Millar, THIS JOURNAL, **72**, 4153 (1950); (b) H. Schlenk and R. T. Holman, *ibid.*, **72**, 5001 (1950).