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

# Regeneration of Insulin from Insulin Fibrils by the Action of Alkali

BY DAVID F. WAUGH

Insulin may be converted into highly asymmetric fibrils having lengths up to tens of thousands of ångström units and widths averaging about 150 Å.[1] Aggregation of these fibrils into spherites and the further clumping of spherites accounts for the visible heat precipitate of insulin.[2] Several investigations in which fibrils are produced by the unfolding of globular proteins and the aligning of polypeptide chains thus produced have been reported.[3] Evidence from monolayers, which showed that films of native insulin and fibrous insulin had the same properties[4] and the fact that mild treatment with alkali would greatly decrease the high viscosity of a suspension of fibrils indicated the possibility that the insulin particle (molecule) was *not* unfolded to any extent during fibril formation. This implies the linkage of corpuscular or globular units, a circumstance to be demonstrated in this publication. The reversible linkage of corpuscular units, along with the relative stability of the fibril, place insulin in a rather unique position of considerable importance.

Additional indications of reversion came from early studies of the "heat precipitate" of insulin.[5,6,7,8] In these it was claimed that the heat precipitate, inactive in itself, could be treated with alkali to give a product having at least 80% of the original activity. Greenstein, however, indicates that insulin may be opened up to expose disulfide groupings without loss of biological activity[9] and Rothen, *et al.*, report that surface films of insulin are active.[10] Thus reversion to the native state cannot be assumed on the basis of biological activity alone.

The present communication considers the reversion of insulin fibrils by alkali and compares the reversion product with native insulin. During the process of reversion of fibrils (or regeneration of insulin) the system contains fibrils and a reversion product which will be referred to as r-insulin. Native insulin will be referred to as n-insulin, insulin treated with alkali as a-insulin, and fibrous insulin as f-insulin.

(1) D. F. Waugh, THIS JOURNAL, **66**, 663 (1944); *Am. J. Physiol.*, **133**, 484 (1941).

(2) D. F. Waugh, *ibid.*, **68**, 247 (1946).

(3) H. P. Lundgren, *Silk J.*, **23**, No. **269**, 48 and No. **270**, 32 (1946); *Textile Research J.*, **15**, 335 (1945); THIS JOURNAL, **63**, 2854 (1941); G. C. Nutting, M. Halwer, M. J. Copley and F. R. Senti, *Textile Research J.*, **16**, 599 (1946); G. C. Nutting, F. R. Senti and M. J. Copley, *Science*, **99**, 328 (1944); F. R. Senti, C. R. Eddy and G. C. Nutting, THIS JOURNAL, **65**, 2473 (1943); K. J. Palmer and J. A. Galvin, *ibid.*, **65**, 2187 (1943).

(4) I. Langmuir and D. F. Waugh, *ibid.*, **62**, 2771 (1940).

(5) N. R. Blatherwick, F. Bischoff, L. C. Maxwell, J. Berger and M. Sahyun, *J. Biol. Chem.*, **72**, 57 (1927).

(6) V. du Vigneaud, E. M. K. Geiling and C. A. Eddy, *J. Pharmacol.*, **33** 497 (1928).

(7) T. D. Gerlough and R. W. Bates, *ibid.*, **45**, 19 (1932).

(8) V. du Vigneaud, R. H. Sifferd and R. R. Sealock, *J. Biol. Chem.*, **102**, 521 (1933).

(9) H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944), p. 185.

(10) A. Rothen, B. F. Chow, R. O. Greep and H. B. Van Dyke, *Cold Spring Harbor Symposia Quant. Biol.*, **9**, 272 (1941).

## Preparation of Fibrous Insulin

Quantitative knowledge concerning r-insulin and the process of reversion of f-insulin necessitated finding methods by which insulin could be converted completely into freely suspended insulin fibrils.

The following method is satisfactory. Crystalline zinc insulin 2% by weight in 0.035 to 0.05 *N* hydrochloric acid (*p*H about 1.8) is sealed in acid washed glass ampules, 2 cc. per ampule. The ampules are immersed for about five to twelve minutes in a water-bath at 100°. During this period weak flow double refraction and, in some cases, incipient flocculation appear.[11] At this time the ampule is cooled in tap water, dried, and frozen by immersion in solid carbon dioxide and acetone. After two minutes the tube is withdrawn, thawed, and reimmersed in water at 100° for five to eight minutes.

The initial heating period produces small numbers of relatively long fibrils. The effect of the freezing–thawing is to break such elongated fibrils into short segments. Each of these acts as a new center for elongation. Since fibril elongation is quite rapid at 100°[12] lateral alignment and spherite formation[2] do not take place. The tube gels within a few seconds and may remain clear except for light scattering associated with highly asymmetric particles. After orienting the fibrils (initially oriented at random) the tube shows intense interference colors when viewed between crossed polaroids. Gels prepared in this way will be referred to as standard fibrous gels.

**Extent of Fibril Formation in Standard Gel.**—The amount of n-insulin and material incapable of forming fibrils present in a standard fibrous gel may be estimated from the following.

1. Filtration through no. 50 Whatman paper removed over 95% of the nitrogen (fibrils) from the original gel. An ultra-fine Buchner funnel removes 99% of the nitrogen even after repeated washings. The great lengths of insulin fibrils may be appreciated from their inability to pass through such a filter paper.

2. It has been shown that insulin fibrils, once formed, will elongate rapidly at room temperature.[12] After mixing 2 ml. of 2% n-insulin and 0.8 ml. 2% fibrous insulin at 25° 86% of the n-insulin was converted into fibrils in forty-six hours. The reaction, which is pseudo monomolecular, has a $Q_{10}$ (temperature coefficient) of about 4.0. If this material had been heated at 100° for six minutes, assuming a temperature coefficient of 4 over the range between 25 and 100°, less than 0.02% would have remained in the n-insulin form.

Under the conditions given for preparing a standard gel it is therefore estimated that conversion to fibrils is better than 99% and that unconverted n-insulin may be neglected. The possibility that n-insulin is adsorbed to fibrils is treated in the discussion.

## Crystallization Procedure

A modification of the final crystallization of Romans, Scott and Fisher[13] was used. The preparation to be

(11) Flow double refraction is observed by tilting the vial between crossed polaroids. The flow thus produced aligns only the longer fibrils.

(12) D. F. Waugh, *Proceedings of Federation of Societies for Experimental Biology*, **5**, No. 1, 111 (1946).

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

crystallized is brought to *p*H 5.3–5.5 with 0.3 *N* acetic acid and centrifuged for fifteen to twenty minutes with a clinical centrifuge (*ca.* 400 g.). The precipitate is dissolved in sufficient 0.327 *N* acetic acid to give a theoretical insulin concentration of 0.2 to 0.4%. A volume of 0.31 *N* ammonium hydroxide equal to the volume of acetic acid is added and the *p*H adjusted to 8.0. For each 10 ml. solution is then added 0.15 ml. of zinc acetate in 0.3 *N* ammonium acetate buffer at *p*H 6.0–6.3 containing 0.25 mg. of zinc per ml. The *p*H is then adjusted to 5.9–6.1 with 0.31 *N* ammonium hydroxide and crystallization is allowed to proceed for six days, after which the crystals are assayed.

Slight variations, mainly concerned with protein concentration, will be indicated in the text.

**Assay of Crystals.**—Amounts of crystals were obtained directly by washing in distilled water, drying and weighing. In most instances, however, samples of 10 mg. or less were used. Rapid and effective assays of such crystalline materials were obtained by transferring from the crystallizing vial to an assay tube consisting of a 10 ml. test-tube with a heavy-walled capillary 4 cm. long and 1.3 mm. in internal diameter sealed on the end. The assay tube was centrifuged on a clinical centrifuge for two to three minutes after which the length of capillary tube occupied by the crystals was measured. Insulin crystals, having a density considerably higher than any other materials present, pack first and the upper boundary of the crystalline material may be seen quite easily. The lengths of capillary occupied by the crystals may be converted to mg. of n-insulin in the crystallizing vial or to final mg. crystals with some degree of accuracy as shown in Table I and Table II. In Table I the first column gives the mg. of initial n-insulin crystallized in a final volume of about 10 ml.

TABLE I

THE CAPILLARY ASSAY OF CRYSTALLIZED INSULIN

| *n*-Insulin, mg. | Crystal size | Capillary assay, mm. | Calculated capillary assay, mm./10 mg. |
|---|---|---|---|
| 1 | Small | 1.5 | 15 |
| 2 | Medium | 3.0 | 15 |
| 3 | Large | 4.0 | 13.5 |
| 4 | Large | 5.5 | 13.7 |
| 5 | Large | 6.5 | 13.0 |
| 10 | Large | 13.5 | 13.5 |
| 10 | Large | 13.5 | 13.5 |
| 10 | Small | 13.5 | 13.5 |
| 10 | Medium | 13.5 | 13.5 |
| 10 | Large | 12.8 | 12.8 |
| 10 | Large | 13.5 | 13.5 |
| | | Av. | 13.4 ± 0.1 |

Crystals from last six determinations weighed 38 mg. after careful washing which reduced the 80.3 mm. recorded above to 79.8 mm.

TABLE II

RELATIONSHIPS BETWEEN WEIGHT AND CAPILLARY ASSAY

| Insulin type | Crystal size | Capillary assay mm. crystals | Crystal weight, mg. | M. crystals mm. assay |
|---|---|---|---|---|
| n-Insulin | Mixed | 79.8 | 38.0 | 0.48 |
| a-Insulin | Mixed | 109.5 | 53.5 | .49 |
| a-Insulin | Small | 49.5 | 25.0 | .50 |
| r-Insulin | Large | 82.5 | 47.0 | .57 |
| r-Insulin | Mixed | 126.5 | 64.5 | .51 |
| a-Insulin | Small | 55.5 | 27.0 | .49 |
| | | | Av. | .51 ± 0.2 |

Medium crystals are between 30 and 80 $\mu$. Large and small are on either side of this range.

The second column indicates crystal size, the third mm. crystals as measured in the assay capillary and the last column calculated mm. assay column for 10 mg. n-insulin. The first two values in the last column are probably too high due to the fact that small amounts were being assayed in a capillary which had a somewhat rounded end. The remaining figures average 13.4 mm. for each 10 mg. of n-insulin dissolved and crystallized.

Table II shows the relationship between total length in the capillary and the weights of these same crystals. In these, crystals from similar experiments were pooled. The first row of Table II represents the pooling of the last six samples of Table I. In column 1, Table II, a-insulin is insulin which has been first treated with alkali and r-insulin is material obtained after the reversion of fibrils. The second column gives crystal size, the third and fourth mm. in assay tubes and mg. dry weight of the same crystals and the last column the ratio, f, of mg. weight and mm. in assay tubes. The last column averages f = 0.5.

## Effects of Alkali on Insulin

Sufficiently strong alkali irreversibly inactivates insulin. At the same time crystallizing potency is lost. These effects, for a given reagent, may be minimized by working at low temperature. Thus, at 36°, 0.033 *N* sodium hydroxide not only inactivates in three hours but, after twenty-five hours, 0.5% hydrogen sulfide appears.[14] At 0–4° the molecule is not appreciably affected after ten hours. According to Jensen and Geiling[15] insulin may be treated with 0.01 *N* alkali at 0° for forty-eight hours without noticeable loss in physiological activity.

The effects of alkali on the crystallizing ability of n-insulin have been examined. Figure 1 and Table III summarize the results. In all cases 10 mg. of n-insulin dissolved in 0.5 ml. of 0.035 *N* hydrochloric acid were added to 5.0 ml. of alkali of appropriate concentration at 0°. After the times indicated, the alkali was neutralized to *p*H 5.35 with 0.3 *N* acetic acid and the precipitate centrifuged and crystallized. The crystals within certain groups shown in Table III were pooled,

TABLE III

THE EFFECT OF 0.03 *N* SODIUM HYDROXIDE ON NATIVE INSULIN AT 0°

| Time, min. | Crystal size | Crystal assay, mm. | Group |
|---|---|---|---|
| 0 | Large | 11.25 | |
| 60 | Small | 14.35 | |
| 105 | Small | 14.2 | I |
| 180 | Small | 12.0 | |
| 300 | Medium | 6.0 | |
| 1380 | Mixed | 10.2 | |
| 14 | Small | 14.5 | |
| 19 | Small | 14.4 | II |
| 30 | Small | 14.5 | |
| 45 | Small | 15.5 | |

Group I, total mm. 49.5; total weight, 25.0 mg.; *f* = 0.50. Group II, total mm. 55.5; total weight, 27.0 mg.; *f* = 0.49.

(14) K. Freudenberg and A. Münch, *Z. physiol. Chem.*, **263**, 1 (1940).

(15) H. Jensen and E. M. K. Geiling, *J. Pharmacol.*, **35**, 511 (1928).

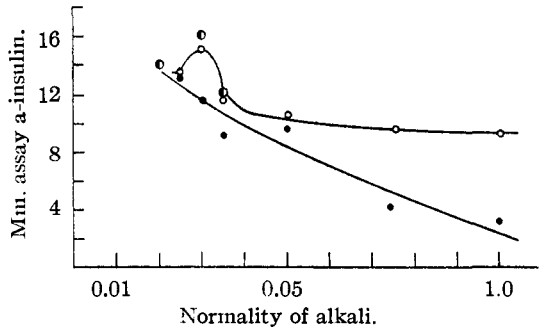



Fig. 1.—The effect of sodium hydroxide treatment on the crystallizing ability of *n*-insulin: O, 345 min.; ◐, 405 min.; ●, 1380 min.

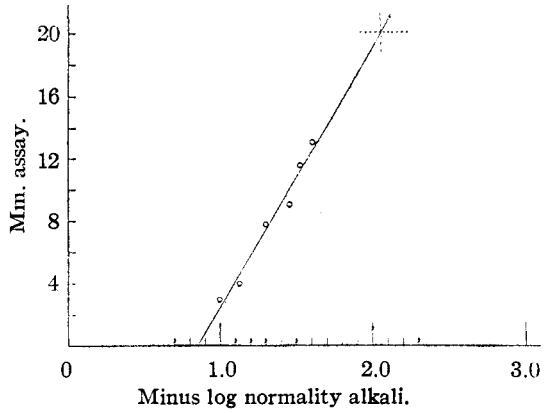



Fig. 2.—Recovery of insulin after 1380 minutes of treatment with sodium hydroxide of different concentrations. The intercept at 0 mm. indicates that 0.141 *N* alkali will abolish crystallization in this time. The abscissa corresponding to 20 mm. recovery indicates that 0.0085 *N* alkali is the maximum concentration which will have no effect in 1380 minutes (twenty-three hours).

washed with distilled water, dried *in vacuo* and weighed. The total mm. crystals and mg. dry weight are given at the base of the table for each group.

The data for 345, 405 and 1380 minutes are represented in Fig. 1. Recovery of crystals in mm. is plotted as ordinate against the normality of the alkali used. It seems that the shorter treatment times with alkali concentrations near 0.03 *N* "condition" the insulin so that a larger yield of crystals is obtained. Thus, although a yield of 13.5 mm. crystals is obtained with no alkaline treatment (see Table I), treatment with 0.03 *N* sodium hydroxide may increase this value to as much as 16 mm. as shown by the double circle of Fig. 1, the maximum for 405 minutes. However, the "conditioning" effect of alkali is not reproducible as seen by comparing the data for 0.03 *N* alkali at 300 and 180 min. in Table III with the values for 0.03 *N* alkali in Fig. 1.[16]

The longest time used, 1380 minutes, indicates a gradual destruction of insulin over the entire alkali range used. Thus one may assume that "conditioning" effects have been maximal at some previous time and that the expected slow destruction of insulin is taking place. Figure 2 represents a plot of the data for 1380 minutes using mm. recovery *versus* minus log normality. A small but undetermined displacement of the curve to higher abscissas and subtraction from 14 would bring these latter values in close correspondence with the *p*H's of the solutions. The alkaline destruction of insulin appears as a linear function of *p*H, thus indicating proton dissociation as being an important step. Extrapolation indicates first, that 0.141 *N* sodium hydroxide would reduce crystallization to 0 mm. in 1380 minutes, and second, that 0.0085 *N* sodium hydroxide is the highest concentration of alkali which will exert no effect on crystallization (recovery of 20 mm.) in the same length of time. Isolated values in the literature are in agreement with those predicted in this way.

The data of Fig. 1 and Table III indicate that 0.03 *N* sodium hydroxide is best for regeneration purposes. Table III, which shows slow destruction with times greater than one to two hours, suggests that regeneration be accomplished within sixty minutes. All recoveries are dependent upon crystallizing procedure.[16] It is felt that improvements in this technique will not appreciably alter these conclusions.

## Alkaline Regeneration of Insulin from Fibrils

**General Effects of Alkali on Spherites and Fibrils.**—As indicated previously: alkali will disperse spherites into their constituent fibrils at *p*H's between 11.0 and 11.5. Above a *p*H of 11.5 the fibrils disappear, more rapidly as the *p*H increases. Thus at *p*H 12 to 14, fibrils disappear within a few hours to a few minutes. To minimize the inactivating effects of alkali experiments were performed at 0°. *p*H values as such were not measured but, as in the case of alkali-treated n-insulin, a standard procedure for treating insulin fibrils was used. Thus 0.5 ml. (containing 10 mg. protein) of the fibril preparation was mixed with 5.0 ml. of carbonate free alkali previously cooled to 0°. After standing the solution was brought to *p*H 5.35 with 0.3 *N* acetic acid, centrifuged, and the precipitate crystallized as described.

**Initial Treatments of Standard Fibrous Gel.**—As a preliminary, portions of standard gel were treated with 0.02, 0.25 and 0.03 *N* sodium hydroxide for eighteen and sixty-six hours. Even after sixty-six hours flow double refraction was present which decreased in the order 0.02, 0.025, 0.03 *N* alkali. Table IV shows typical results of crystallization. The first column shows

(16) The yield of crystals is a function of the crystallizing procedure as well as the alkaline pretreatment. Thus the "conditioning effect" of alkali might be expected to disappear under those conditions where a quantitative yield of crystallizable protein is obtained. One would expect 20 mm. crystals per 10 mg. insulin. This indicates that the crystallizing technique used recovers about 70 to 80% of the potentially crystallizable protein. It is probable that short alkaline treatments cause a more rapid crystallization, thus increasing relative yields. This is under investigation.

alkali concentration, the second time in hours and the third mm. crystals per 10 mg. of fibrils. With the exception of 0.02 *N* sodium hydroxide the longer treatment times destroy part of the r-insulin for the recovery falls. The low yields of r-insulin with 0.02 *N* sodium hydroxide indicated that longer treatment times would not be particularly effective. Methods were sought by which the rate of liberation of r-insulin could be increased.

TABLE IV

REGENERATION OF r-INSULIN FROM STANDARD FIBROUS GEL WITH ALKALI AT 0°

| Alkali normality | Time in alkali hours | Mm. crystals per 10 mg. fibrils mm. |
|---|---|---|
| 0.02 | 18 | 2.2 |
| .02 | 66 | 8.2 |
| .025 | 18 | 9.9 |
| .025 | 66 | 3.7 |
| .03 | 18 | 7.7 |
| .03 | 66 | 5.5 |

**Further Treatments of Standard Gel.**—Among others, it was considered that the reversion of fibrils by alkali might depend upon the number of available fibril ends rather than on the total fibril surface area. Thus, that fibrils disaggregate in a sequence about the reverse of that in which they form. This was tested by freezing standard gel in solid carbon dioxide–acetone and thawing just before subjecting the fibrils to alkaline treatment.

As indicated previously[1] the effect of freezing and thawing is to break the longer fibrils into short segments. Such segments are quite active and will unite again in a matter of minutes at room temperature. Therefore the frozen-thawed material is treated immediately. Standard gels have been subject to multiple freezing-thawing cycles and have been treated with a number of alkali concentrations in the range 0.025 to 0.05 *N*. The results with 0.03 *N* sodium hydroxide are typical. With two freezing-thawing cycles and alkali exposure times of 30, 60, 300 and 1380 minutes assays showed 12.0, 11.0, 10.0 and 8.2 mm. crystals indicating, as found previously, that exposure times of sixty minutes or less are most effective.

Table V shows a typical experiment using forty-five min. exposure times with 0.03 *N* sodium hydroxide at 0°. The samples were frozen-thawed as shown in the first row, the row below giving recoveries, in mg., calculated from mm. assay. The sudden rise in regeneration yield with the first cycle and the smaller but significant rise with the second cycle are typical. Little is gained after two freezing–thawing treatments. This agrees with visual observation for, after two cycles, the insulin fibrils are clumped and are not further broken up.

TABLE V

FREEZING–THAWING CYCLES AND REGENERATION YIELDS

These data were obtained with a modified crystalline technique

| Freezing–thawing cycles | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| Calculated recovery, g. | 0 | 5.2 | 7.2 | 7.2 | 7.6 |

Essentially the same results as those described have been obtained with mechanical methods for breaking fibrils (homogenization) thus eliminating any specific effects of freezing–thawing as increasing regeneration yield and indicating that the numerical increase in fibril ends is the responsible factor.

Table VI shows typical recoveries for 2, 3 and 4 freezing–thawing treatments using 0.3 *N* sodium hydroxide and times, at 0°, of ten, twenty, thirty and forty-five minutes. The averages shown in the last row for thirty and forty-five minutes indicate that about 12.0 mm. crystals may be realized from the reversion of 10 mg. fibrils. This figure may be compared with a figure of 14.5 mm. obtained after a similar alkaline treatment of native insulin (Table III and Fig. 1, 0.03 *N* alkali). If the crystallization properties of a-insulin and r-insulin are the same, 75 to 83% of the insulin in the fibrils may be recovered in crystalline form.

TABLE VI

REGENERATION YIELDS OF r-INSULIN AS AFFECTED BY TIME AND FREEZING–THAWING CYCLES AT 0° USING 0.03 *N* SODIUM HYDROXIDE

| Freezing–thawing cycles | Mm. crystals per 10 mg. fibrils after treatment (time, min.) 10 | 20 | 30 | 45 | Row |
|---|---|---|---|---|---|
| 2 | 6 | 10.5 | 12.0 | 12.0 | 1 |
| 2 | 9.8 | 12.2 | 11.0 | 12.7 | 2 |
| 3 | 10.2 | 12.0 | 12.0 | 12.5 | 3 |
| 3 | 10.2 | 12.0 | 12.5 | 12.7 | 4 |
| 4 | 8.5 | 10.5 | 12.2 | 10.8 | 5 |
| 4 | 10.8 | 12.2 | 12.1 | 12.2 | 6 |
| Average | 9.2 | 11.6 | 12.0 | 12.1 | 7 |

Total crystals recovered from rows 2 and 3 weighed 47 mg.; from rows 4, 5, and 6, 64.5 mg.

The treatments described do not transform all of the fibrous material. The unreverted fibrils, after the crystallization following the first alkali treatment, may be recovered, washed, and subjected to another reversion treatment. In this way an additional 1–1.5 mm. of crystalline material may be obtained bringing the total recovery to 13 to 13.5 mm. or about 85 to 90% of the original protein. This is in good agreement with determinations of biological activity after alkaline treatment of the heat precipitate[5,6,7,8] in which reactivations of 80 to 100% have been claimed for biological activity and with our more recent regenerations by a variety of reagents.

From the foregoing information it appears that crystalline recoveries of 85 to 90% may be obtained by treating twice frozen–thawed fibrils with 0.03 *N* sodium hydroxide at 0° for forty-five minutes with a ratio of 0.5 ml. of 2% fibrils to 5.0 ml. alkali.

**The Effect of Sodium Chloride on Alkaline Reversion of Fibrils.**—Reversion has been carried out with the procedure described at the end of the preceding section with the addition of sodium chloride to the alkali. The results are summarized in Fig. 3 which shows mg. of recovery of crystalline r-insulin (calculated from mm. of assay and using 10 mg. of starting fibrils) plotted against the normality of sodium chloride present in the alkali. The resulting curve, an average of several values, shows that inhibition of reversion starts with salt concentrations somewhat in excess of 0.01 *N* and is marked at 1.0 *N*. With 2.0 *N* sodium chloride complete inhibition is obtained. These results will be considered in the discussion.

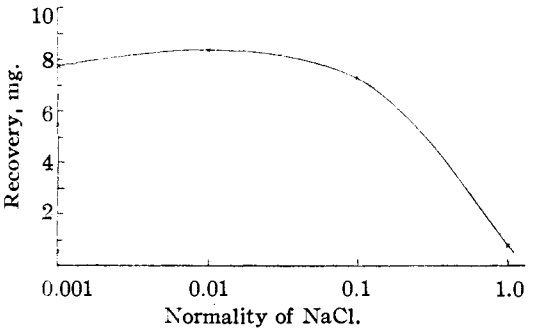



Fig. 3.—Regeneration of insulin from fibrils when various amounts of sodium chloride (abscissa) have been added to the regenerating alkali. Recoveries are calculated from mm. assay using $f = 0.5$.

## Properties of r-Insulin Regenerated from Fibrils by Alkali

**Crystallization.**—In agreement with the properties of n-insulin, crystallization of r-insulin will not proceed in the absence of zinc. Other metal ions which may be substituted for zinc have not been tested. In these experiments relatively zinc free preparations were made by dialyzing an acid solution of r-insulin and by precipitating an acid solution with organic solvents according to Scott.[17] Recrystallization of the same preparations in the presence of zinc produced typical crystals.

r-Insulin and n-insulin have the same crystal form although r-insulin generally gives somewhat larger crystals than n-insulin. A comparison of recrystallized n-insulin and r-insulin with a petrographic microscope reveals the two types of crystals described by Abel, Geiling, Rouiller, Bell and Wintersteiner.[18] The type showing well-defined negative double refraction seemed more numerous in n-insulin samples, r-insulin containing proportionately more crystals having well defined edges with little double refraction.

The recoveries of r-insulin and n-insulin were not significantly different on recrystallization.

**Biological Activity.**—Crystals of r-insulin and n-insulin were washed with distilled water and dried *in vacuo*. Biological assays arranged by Dr. R. E. Thompson[19] gave

r-insulin, 20.2 units per mg. ± 5.9%
n-insulin, 22.1 units per mg. ± 7.8%

The small difference between regenerated and native insulins is not considered significant and is probably due to inert protein, the drying procedure, or some other uncontrolled factor.

**Ultracentrifuge Determinations.**—Recrystallized n-insulin was converted to standard fibrous gel and r-insulin was regenerated. The resulting r-insulin crystals were carefully washed and a clear lot isolated. This amounted to 42.4% by weight of the initial insulin. Dr. J. L. Oncley[20] has compared the behavior of n-insulin and r-insulin in the ultracentrifuge.

Dr. Oncley writes, "Solutions of insulin crystals obtained from r-insulin containing 1 and 2% of protein were studied in an air-driven ultracentrifuge[21,22] equipped with a modified Philpot schlieren optical system.[23] The measurements were made in a cell 1.5 cm. high, 1.0 cm. thick, whose center was 6.5 cm. from the axis of rotation. A speed of 54,000 r.p.m., equivalent to centrifugal forces of from 200,000 to 240,000 times gravity, was used and the average temperature was about 24°. Values of sedimentation constant have been reduced to the value in a solvent of the density and viscosity of water at 20°. A phosphate buffer of 0.1 ionic strength, *p*H 7.2, was used as a diluent. The protein solutions were made by dissolving dried insulin crystals in a small volume of dilute hydrochloric acid and neutralizing with a phosphate buffer calculated to yield a final ionic strength of 0.1 and *p*H 7.2. A solution made from ordinary crystalline insulin (from beef)[24] was used for comparison. Sedimentation diagrams of unmodified crystalline insulin and the crystals obtained from r-insulin were compared and found to be practically identical. The sedimentation constant usually assigned to crystalline insulin at this *p*H was obtained, that is, about 3.3 to 3.6.[25,26,27] The diagrams from the r-insulin showed no more evidence of faster moving components than is usually obtained from commercial insulin crystals, and the 'apparent diffusion constant' obtained by analysis of the sedimentation diagrams was only slightly larger than the observed diffusion con-

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

(18) J. J. Abel, E. M. K. Geiling, C. A. Rouiller, F. K. Bell and O. Wintersteiner, *J. Pharmacol.*, **31**, 65 (1927).

(19) The author is indebted to the Chemical Research and Development Laboratories of Armour and Company for these assays. Many others confirm the conclusions drawn here.

(20) Great appreciation is expressed to Dr. J. L. Oncley of the Department of Physical Chemistry, Harvard Medical School, for this analysis.

(21) J. H. Bauer and E. G. Pickels, *J. Exp. Med.*, **65**, 565 (1937); also in T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford Press, 1940.

(22) E. G. Pickels, *Rev. Sci. Instruments*, **9**, 358 (1938); **13**, 426 (1942).

(23) J. St. L. Philpot, *Nature*, **141**, 283 (1938).

(24) Obtained from Eli Lilly & Company.

(25) B. Sjögren and T. Svedberg, THIS JOURNAL, **53**, 2657 (1931).

(26) G. L. Miller and K. J. I. Andersson, *J. Biol. Chem.*, **144**, 459 (1942).

(27) H. Gutfreund and A. G. Ogston, *Biochem. J.*, **40**, 432 (1946).

stant, again indicating the homogeneity of the materials."

In addition the ultracentrifuge data suggest that r-insulin is able to go through the same cycle of reversible fragmentation as n-insulin.

**Sensitivity to Alkali.**—Crystallization of the isoelectric precipitate obtained after treating 0.5 ml. of 2% crystalline r-insulin (10 mg.) with 5.0 ml. of 0.03 *N* sodium hydroxide at 0° for times of 0 to 400 minutes gave consistent yields of about 95% ± 5% up to times of 200 minutes after which the recovery fell to 86% and at 400 minutes 84%. Up to 200 minutes, therefore, r-insulin seems less sensitive to alkali treatment than n-insulin since treatment of n-insulin with 0.03 *N* alkali usually gives yields of about 80% with times up to 200 minutes (Table III). This difference, at present, cannot be interpreted as showing significant differences between the effects of alkali on r- and n-insulins but could be due to the crystallization procedure which may handle r-insulin somewhat better than n-insulin.[16]

**Labile Ammonia.**—It has been considered generally that liberation of ammonia accompanied the heat precipitation of insulin although du Vigneaud, Sifferd and Sealock[8] found no relationship between amounts of ammonia liberated and heat precipitate formed. Insulin fibrils seeded into solutions of n-insulin will elongate at room temperature and transform the n-insulin into fibrils.[12] One can therefore test for liberation of ammonia at 25°. n-Insulin and n-insulin seeded with short fibrils (80 mg. n-insulin, 20 mg. short fibrils) were dialyzed against 10 ml. 0.01 *N* hydrochloric acid. All dialysis fluids, by Nesslerization, had the same nitrogen contents within the experimental error. The liberation of 0.03% nitrogen could have been detected.

**Disulfide and Sulfhydryl.**—The nitroprusside reaction[8] was used to test for free sulfhydryl groups. This has been shown to be negative in n-insulin and fibrous insulin.[28] Free disulfides, as shown by the cyanide–nitroprusside reaction, do not increase on fibril formation. This test is not particularly indicative since n-insulin gives a strong cyanide–nitroprusside reaction equivalent to about 25–30% of the total sulfur present.

In agreement with others[8] reduction with cyanide abolishes the heat precipitate and fibril formation. The status of the disulfide linkage will be treated in the discussion.

**Fibril Formation with r-Insulin.**—When dissolved in 2% concentrated at *p*H 1.6–1.7 (0.05 *N* hydrochloric acid) n-insulin remains quite stable at 20° or below and shows evidence of fibril formation only after many weeks, and, in instances, months.

Unpurified products of alkaline reversion precipitated at *p*H 5.35 and taken up in acid show rapid fibril formation at room temperature or in the ice chest. Treatment with alkali, however, usually does not revert quantitatively since the presence of unreverted fibrils may be demonstrated by double refraction of flow or other methods. These act as active centers,[12] elongate at low temperatures, and under proper conditions give rise to products ranging from the heat precipitate to a clear fibrous gel. In order to compare r-insulin and n-insulin these unreverted fibrils must be removed. Recrystallization has been used with success. The r-insulin after the first recrystallization differs from n-insulin in forming a fibrous gel having brilliant double refraction in two to three minutes at 100° and in showing slow but appreciable fibril formation at room temperature over a period of several days. The insulin was dissolved in 2% conc. in 0.05 *N* hydrochloric acid (*p*H 1.6–1.7). Under the same conditions twice recrystallized r-insulin, washed many times with distilled water, shows rapid fibril formation at 100° but at room temperature very faint flow double refraction only was detected after several weeks. After a third recrystallization the product, representing 46% by weight of the initial fibrous insulin, showed no double refraction or alterations in other properties, such as viscosity and clarity, over a period of fourteen weeks. At 100° the thrice-recrystallized r-insulin seems to show fibril formation slightly faster than commercial, untreated n-insulin. However, fibril formation is sensitive to a number of variables including salt concentration, *p*H, anions, etc. Thus n-insulin will show varying rates of fibril formation, particularly at higher temperatures, after manipulations such as recrystallization. The rates of fibril formation obtained with thrice recrystallized r-insulin at 100° are well within the normal range.

Repeated crystallization, therefore, leads to an r-insulin which approaches n-insulin in the characteristics of fibril formation at low and high temperatures.

## Discussion

The physical, chemical and biological tests applied cannot thus far differentiate between r-insulin and n-insulin. These tests include crystallization, ultracentrifuge analysis, biological activity, changes in labile groups such as amino and disulfide, and fibril formation. Before an analysis is undertaken consideration should be given to the extent to which n-insulin enters into the basic fibril forming reaction. Thus, high yields of r-insulin of 80–90% do not rule out the possibility that 10% of the n-insulin irreversibly unfolds and gives rise to a fiber "skeleton" while the remaining 90% is physically adsorbed to this skeleton as n-insulin. Several lines of evidence exclude this possibility. First, the adsorption of n-insulin onto fibrils has been examined in connection with reaction kinetics. Adsorption becomes important only at *p*H's well above 3.0. Second, fibrils form and elongate at *p*H's which render insulin highly soluble and in which strong repulsive forces would be

(28) M. L. Sackler, Chemical Modifications of Insulin and Their Relation to Fibril Formation, Master's Thesis, 1945, Massachusetts Institute of Technology, Cambridge, Mass.

expected to occur between the molecules.[2] Third, washing a fibrous gel with acid does not produce elution. Fourth, the fibril once initiated can elongate at room temperature or below.[12] Insulin shows its maximal stability at *p*H's close to 2.0 at low temperatures and would not be expected to show spontaneous unfolding under these conditions. Fifth, the fibril, in alkali, liberates r-insulin selectively from its ends and not uniformly over the entire surface as one might expect from a skeleton containing 4 to 9 times its weight of adsorbed material. It seems reasonable to conclude that all of the n-insulin enters the basic fibril structure.

Native insulin may be defined on the basis of certain intrinsic properties such as crystallizability, biological activity and fibril forming capacity. Although there is evidence that these characteristics may be dissociated to some extent (*i. e.*, an insulin which will form fibrils but will not crystallize), certain series are recognized (*i.e.*, if an insulin crystallizes it also has biological activity and shows fibril formation). In this way, crystallization emerges as a delicate test for insulin, other properties requiring less precise structure.

A number of modifications of insulin have been reported. Such modifications may be divided into those involving mainly surface groups and those in which internal structure has been obviously altered. Examples of the first type are the acetylation of insulin with ketene[29] reported by Stern and White and the linking of insulin with diazonium salts reported by Reiner and Lang.[30] In instances these "surface" modifications retain their biological activity and form crystals. A number of reagents, used primarily for surface group modification, are suspected of causing internal structural changes. In these cases both biological activity and crystal formation are irreversibly lost. Directed changes in internal structure have been quantitatively studied thus far by reduction of some of the disulfide groups (of which seven or eight are usually available out of a total of 23). Reduction of an average of 1 or 2 groups per molecule leads to a 50% loss in biological activity.[31] On *reoxidation* the activity decreases to about 1% of original. More drastic reduction[8,28,32] causes a complete loss of crystallizability, biological activity and fibril formation. It should be mentioned here that an extensive unfolding of the protein would be expected to involve the breaking of several disulfide linkages. On the basis of evidence mentioned a refolding with the formation of new disulfide linkages should lead to a product having none of the intrinsic characteristics of insulin. Mild alkaline treatment[28] may lead to a simultaneous loss of biological activity and crystallizability while treatment with concentrated solutions of guanidine and urea may produce a complete loss of crystallizing ability with only a slight diminution in fibril forming capacity.

Thus it would appear that demonstrable changes in internal structure lead to irreversible loss of one or more of the characteristic properties. r-Insulin retains these labile characteristics completely. From this and the fact that fibril elongation may proceed below 20° it is concluded that fibril formation involves the endwise linkage of globular (corpuscular) units which are not appreciably unfolded in the process.

**A Note on "Denaturation."**—According to most definitions the stable fibrous form of insulin would represent a denatured protein, for the fibrils retain few of the properties of n-insulin. Regeneration, however, shows that the insulin molecules have not lost these properties irreversibly. It seems obvious that four general factors determine the over-all reversibility of this system. These are the strength of the intermolecular bond, the ability of a regenerating agent to overcome this bond, and the ability of the fibrils and reversion product to withstand the damaging action of the reverting (regenerating) agent. The rates of the several reactions enter as important variables. A somewhat stronger inter-insulin bond would require more drastic alkaline treatments. These would effect reversion but the r-insulin liberated would be inactivated and irreversibly altered during the process. If the sequence of events were not clearly recognized, one might conclude that the n-insulin had suffered irreversible changes during the process of fibril formation itself. Many coagulations have been considered irreversible. Evidence should be presented which shows that the structure of the protein has been irreversibly altered during the process of coagulation and not subsequently.

**Mechanism of Reversion.**—Calculations from amino acid analyses and titration data[33,34] show that a molecule of insulin of molecular weight 40,000 contains about 80 free carboxyl groups, 40 free amino groups, and 50 hydroxyl groups, some of which are available.[9]

Fibril formation and fibril aggregation or heat precipitation[2] have been shown to be dependent to a marked degree on mechanisms which change the repulsive forces between the molecules. It seems clear that acid *p*H's are necessary to produce a variety of insulin which can enter the fibril forming reactions. However, once this condition has been fulfilled the addition of neutral salt, such as sodium chloride, greatly increases the rate of fibril formation, presumably by causing a decrease in electrostatic repulsion. In acid solution, *p*H's below 3.0, one would expect the carboxyl groups to be largely un-ionized and thus a quantity of insulin corresponding to mol. wt. 40,000 would have

(29) K. G. Stern and A. White, *J. Biol. Chem.*, **122**, 371 (1938).
(30) L. Reiner and E. H. Lang, *ibid.*, **139**, 641 (1941).
(31) K. G. Stern and A. White, *ibid.*, **117**, 95 (1937).
(32) O. Wintersteiner, *ibid.*, **102**, 473 (1933).
(33) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943.
(34) C. R. Harrington and A. Neuberger, *Biochem. J.*, **30**, 809 (1936).

about 40 positive charges. The effect of salt would be to decrease the repulsive forces due to these charges and allow a greater proportion of the molecular collisions to be effective in bonding.

At the regenerating *p*H of about 12.3 one would expect the amino groups to lose their charge while the carboxyl groups and some hydroxyl groups would now ionize and become charged. Thus the net charge would change from an average of roughly +40 to over −80. It seems clear that the great increase in net charge in going from acid to alkali would set up stronger repulsive forces which would aid in disrupting the inter-insulin bond. Evidence in support of this mechanism as being part of the process comes from the action of salt, which decreases reversion as shown in Fig. 3, and those experiments which indicate that reversion proceeds at the ends of the fibrils where the inter-insulin bonds would be weakest since fewer insulin units would be engaged in their stabilization. Reversion and the inter-insulin bond are receiving further attention.

**Acknowledgment.**—The author wishes to acknowledge the generous support which Armour and Company has given to this and other research.

## Summary

Insulin fibrils may be reverted by treatment with alkali to give a crystalline product similar to native insulin: the alkali has therefore regenerated an insulin termed r-insulin. Limiting conditions for regeneration procedure were determined by studying the effect of alkali on native insulin. Using 0.5 ml. of 2% insulin (10 mg.) and 5.0 ml. of sodium hydroxide experiments indicated 0.03 *N* alkali, 0°, and forty-five minute treatment time would be optimal. This was found to be the case. In addition reversion of fibrils is greatly accelerated by increasing the number of available fibril ends suggesting that disaggregation occurs mainly at these positions. Sodium chloride in the alkali inhibits reversion by 90% in 1.0 *N* concentration. Thus, the repulsive forces between similarly charged groups may play a part in the mechanism of disaggregation.

The crystalline product from reverted fibrils is not significantly different from native insulin in certain intrinsic properties such as: crystallization, in which r-insulin will not crystallize in the absence of zinc, and recrystallization recovery; biological activity (20 I.U. per mg.); ultracentrifuge pattern (sedimentation constant 3.3–3.6); and fibril formation (at 20 and 100°). Tests for changes in labile groups, such as amino and disulfide, have been negative.

The absence of changes in labile groups, the retention by r-insulin of the characteristic properties of insulin, the known sensitivity of these characteristic properties to structural changes, and the fact that fibril elongation may take place at low temperatures in the *p*H region of maximum stability, are interpreted as showing that only small structural changes take place during fibril formation and that the process is therefore one in which globular or corpuscular units are linked endwise.

CAMBRIDGE 39, MASSACHUSETTS
RECEIVED NOVEMBER 18, 1947

---

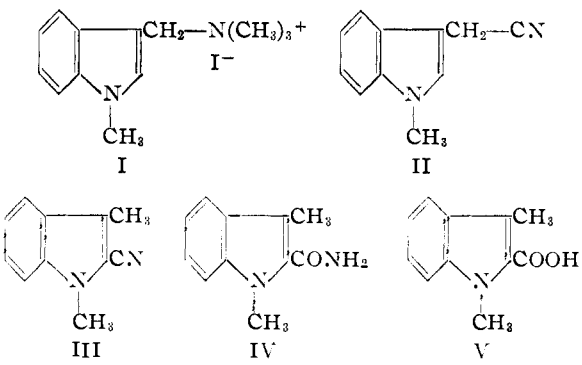
[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

# An Allylic Rearrangement in an Alkylation by a Quaternary Ammonium Salt

BY H. R. SNYDER AND ERNEST L. ELIEL

In a previous communication[1] it was stated that the reaction of 1-methylgramine methiodide (I) with aqueous sodium cyanide produced not only 1-methyl-3-indoleacetonitrile (II) but also an isomer of this nitrile. This isomer has now been identified as 1,3-dimethyl-2-cyanoindole (III). The pure isomer (III) was isolated from the reaction mixture in 4.3% yield, but in view of the difficulties encountered in the purification it is believed to have been formed in an appreciably larger amount, perhaps to the extent of 10–15%.

Comparison of the infrared absorption spectra of (II) and its isomer revealed a shift of the CN-absorption band of 36 cm.$^{-1}$ toward smaller wave numbers in the case of the isomer (III) (see the figures), indicating conjugation of the cyano group with one of the double bonds of the rings. Alkaline hydrolysis of the isomeric nitrile (III) yielded mainly the corresponding amide and only very




small amounts of the acid, probably because of steric hindrance of the nitrile function. The acid was finally obtained in poor yield by increasing the concentration of alkali and extending the reaction time in the hydrolysis.

The acid (V) was synthesized by a known

(1) Snyder and Eliel, THIS JOURNAL, **70**, 1703 (1948).