CHEMICAL MODIFICATIONS OF INSULIN AND THEIR RELATION TO FIBRIL FORMATION***

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I INTRODUCTION

Insulin, the internal secretion of the islets of the pancreas, was first isolated by Banting and Best in 1921-22 by extraction of normally enzyme-free macerated pancreas of foetal calves with Ringer's solution. By using acidified alcohol which inhibits enzyme action more aged pancreas could be utilized (1). Collip (2), observing that insulin is soluble in 80% but insoluble in 90% alcohol, obtained more potent and less toxic extracts with this medium.

Present day methods of purification are based upon extraction at acid or alkaline pH with water or organic solvents followed by fractional precipitation. The relatively pure material is then crystallized in the presence of zinc.(3)

Some of the properties of insulin, of interest in connection with the work to be discussed, are tabulated as follows:

1. Crystallization

Insulin was first obtained in crystalline form by Abel and his associates (4) in 1926. In agreement with Dudley (5) they regarded sulfur as an integral part of the molecule. Guided by this observation they isolated

the hormone in crystalline form from those fractions having a high labile sulfur content.

Although soluble in acid and alkali, insulin is particularly insoluble in distilled water near its isoelectric point. Crystallization, however, must be carried out within a pH unit alkaline to the isoelectric point. Solution of the problem of crystallization, aside from special features to be mentioned, involved finding conditions under which usable concentrations (about 0.2%) of insulin could be brought into solution (supersaturated with respect to crystallization) at the correct pH. The solubility of insulin increases with increasing salt concentration and, up to 20-30%, with the presence of some organic solvent such as acetone or alcohol. Highly buffered solutions, in some instances with the addition of acetone, are therefore used for crystallizing the protein (6,7,8). In most cases acetate or phosphate buffers at pH 5.8-6.5 have been used although earlier experimenters resorted to a variety of organic reagents (brucine, saponine, or digitonin) (4,9). Pure acetate or phosphate buffers are usually utilized for recrystallization.

Scott (10) observed that insulin would only crystallize in the presence of such divalent ions as zinc, cobalt, nickel, or cadmium. The crystalline protein always con-

tains traces of these elements ranging from 0.3% (usual ash content) to 0.06% (11,12) depending upon the pH of crystallization. In the presence of ample metal ion and at pH 5.9-6.0 X-ray diffraction data (38) show that 3 zinc ions per molecule of insulin are combined in the crystal.

The zinc saturation value of insulin, obtained by precipitating insulin at pH 6.5 from solutions containing various amounts of zinc is 2.72-2.48% or 29.2-37.4 equivalents of zinc per mole of protein (assuming M=35,000) (13). After crystallization the value does not exceed 1.73%. Since 30 to 35 free carboxyl groups are estimated for insulin, it is considered that zinc combines with these (13). The crystalline product is, therefore, a zinc insulinate.

2. Molecular Weight

The most recent, and probably most accurate estimates of the molecular weight of insulin come from the original sedimentation data of Sjögren and Svedberg (14) and a recent determination of the diffusion constant by Polson (15). From the combined data Polson published a molecular weight of 41,000. Redetermination of both the sedimentation constant and diffusion constant by Miller and Andersson (16) indicated a molecular weight of 46,000. Crowfoot, and Crowfoot and Riley (17,18,19) from X-ray diffraction data

calculate a rhombohedral unit cell of a=44.3 Å, α=115° and a molecular weight of 39,500 (dry) and 52,400(wet).

3. Shape

The X-ray diffraction data given above indicate a low asymmetry and calculations by Neurath (20) based on diffusion data indicate an axial ratio of about 3.3.

4. Amino Acid Composition

The elementary composition is similar to that of the average protein except for the unusually high sulfur content-3.2% accounted for by 12.5% cystine. Phosphorous has been shown not to be a constituent. Du Vigneaud (21) describes the constitution of crystalline insulin as follows:

Constituent	Percent	Constituent	Percent
zinc	0.52	glutamic acid	30.00
lysine	2.26	leucine	30.00
arginine	3.22	cystine	12.50
histidine	8.00	phenylalanine	?
tyrosine	12.20	proline	?
ammonia	1.65		

Ultraviolet absorption studies show a characteristic absorption band between 2500-2900 Å. This band has been

ascribed to the cystine and tyrosine content of the hormone. Crammer and Neuberger (22) have utilized the changes in the ultraviolet absorption spectrum with pH to establish the pK values of the phenolic group of tyrosine in diiodotyrosine alone and in various proteins. In insulin it is shown that the phenolic groups are free to ionize and that the pK is displaced towards higher values due to the negative charge of the molecules in alkaline solution.

The number of peptide bonds (23) per nitrogen atom is 0.75 $\pm$ 0.005. The nitrogen content of ash-free, moisture-free insulin is calculated to be 15.54% (24).

Careful measurements by Wintersteiner and Abramson (25) and by Howitt and Prideaux (26) establish the isolectric point of crystalline insulin at pH 5.3-5.35. Scott and Fisher (11) have shown that the optimum pH for the crystallization of insulin lies over the range of pH 5.8-6.3 varying in different solutions.

From electrometric titrations in aqueous and alcoholic solutions Harrington and Neuberger (27) have calculated the acid binding capacity of insulin to be 43 $\pm$ 2 groups per molecule and the base binding capacity to be 60-70 groups per molecule. Similar values for the acid binding capacity of ash-free amorphous insulin have been

determined by Harvey, Howitt and Prideaux (28).

5. Chemical Reactivity

Insulin undergoes most of the reactions of a typical protein (29). It is precipitated from aqueous solutions by such reagents as tannic, picric, tungstic, trichloracetic, phosphoric, and flavianic acids. Positive tests and reactions include the Biuret, Millon, Pauly, ninhydrin and Sullivan test for cystine. Negative tests include the tryptophane reaction (Viosonet, Hopkins-Cole, Acree) sodium nitroprusside test for free sulfhydryl groups and the Molisch test. After reduction sulfhydryl groups make their appearance.

Insulin may be acetylated, coupled with methyl iodide, diazotized (30), or treated with formaldehyde with either little inactivation or partially reversible inactivation. Reactions such as iodination, reduction, oxidation, and treatment with strong alkali irreversibly inactivate the protein.

(a) Effect of Reducing Agents

The effect of cysteine and glutathione on crystalline insulin was first studied by du Vigneaud et al (40), who felt reason to believe that the action of cysteine was fairly specific for the reduction of the disulfide group-

ing. They observed that reduced insulin was physiologically inactive and did not give the characteristic heat precipitate. Reoxidation did not restore either of these properties. Since then several investigators (33,45,46,47) have extended this work to other reducing agents with similar results. Wintersteiner (45) and White and Stern (46) concur in the opinion that inactivation is diminished by 50% after the reduction of 1 to 3 disulfide groups with complete physiological inactivation after the reduction of 30% of these linkages. Miller and Anderson (47) in an ultracentrifuge study of insulin reduced with thioglycollate observe the following physical changes with increased reduction: 1) gradual transformation into an alkali soluble, acid insoluble derivative and 2) increasing numbers of insoluble particles of much greater size than the native protein as well as a few small fragments. Insulin and serum albumin, both having a high cystine content, show similar physical changes after reduction while egg albumin which has a low proportion of cystine remains unchanged. These data suggest that the denaturing action is a result of reduction and not a side effect of the reagent (47).

(b) Effect of Alkali

The effect of dilute alkali on insulin has been

studied by three groups of investigators with special reference to the relationship between the physiological inactivation and the splitting out of hydrogen sulphide. Jensen and Geiling (42) observed that insulin treated with 0.01N NaOH at 0°C. for 15 to 48 hours produced no marked change in activity, while 0.03N NaOH at 0°C. for 15 hours produced a 30% decrease. After 3 hours there was no detectable hydrogen sulphide while after 35 hours the solutions gave a strongly positive test. They found no relation between inactivation and the appearance of hydrogen sulphide. Jensen, Evans, Pennington and Shock (43) studied insulin treated with 0.033N NaOH at 34° for 3 hours. These investigators found the protein had been irreversibly inactivated with the splitting out of ammonia. They also observed a decrease in cystine to one-half its original value. Insulin treated with 0.033N NaOH at 36° for 1.5 hours was reported by Freudenburg and Munch (44) to be completely inactive with a portion of the available ammonia split out. They observed no relation between the appearance of hydrogen sulphide and inactivation. They also observed that the rate of ammonia liberation was not associated with either of the other two phenomena.
Du Vigneaud, Sifferd and Sealock (33) found that insulin treated with 0.04N NaOH at 25°C. for 12 hours was completely inactive and did not yield a heat precipitate.

(c) Effect of Urea and Guanidine Hydrochloride

The denaturing effect of urea and guanidine hydrochloride on proteins has been studied extensively with reference to their effect upon the availability of titrable groups. The proteins investigated have been categorized by Neurath (41) as follows:

1. Proteins which give no test for sulfhydryl groups but do so after treatment with concentrated guanidine hydrochloride: serum albumin (48), egg albumin (49), edestin (50), excelsin (50), horse globin (50), and tobacco mosaic virus (51).

2. Proteins which give tests for sulfhydryl groups neither in the native state nor after treatment with concentrated guanidine hydrochloride: amandin (50), insulin (29).

3. Proteins which contain free sulfhydryl groups, whose number is increased after treatment with concentrated guanidine hydrochloride: myosin (51), and urease (52).

4. Proteins containing free sulfhydryl groups the number of which is the same after treatment with

concentrated guanidine hydrochloride: liver nucleoproteins of several mammalian species (53).

Denaturation of serum albumin by urea liberates disulfide groups corresponding to nearly all of the cystine-cysteine content of the protein (48). Sulfhydryl groups are not liberated by urea treatment but are liberated by guanidine hydrochloride. The disulfide groups seem to be more easily exposed than the sulfhydryl groups and may be unmasked by reagents not sufficiently strong to similarly affect the sulfhydryl groups.

Free sulfhydryl groups may be abolished by the addition of glycine or ammonium salts to solutions of native myosin (51).

In all of the proteins in which guanidine hydrochloride liberated sulfhydryl groups the maximum proportion of groups is exhibited in 5 Molar solution; higher concentrations produce no further effect. Urea, in similar concentrations, produces a much smaller effect, and in some instances no effect. The proportion of sulfhydryl groups observed is independent of the protein concentration (50,51). Egg albumin treated with urea or guanidine hydrochloride exhibits the maximum number of sulfhydryl groups, for each concentration of these reagents, within half an hour at 25°C. Further standing up to 3 hours

shows neither an increase nor a decrease in the number of these groups (49).

Egg albumin is soluble in urea and guanidine hydrochloride at the isoelectric point but when these reagents are removed or water is added the protein becomes insoluble. A correlation is shown between the solubility and the liberation of sulfhydryl groups. When insufficient urea is added to liberate all of the groups only a portion of the protein is insoluble (39).

The corpuscular proteins all possess higher viscosity in urea solutions than in water. Solutions of myosin and tobacco mosaic virus are considerably less viscous in urea solutions than in water. Changes in hydration are negligible in comparison with changes in molecular asymmetry. It is believed that the changes in viscosity and frictional ratio are largely, if not wholly, due to changes in molecular shape (41).

Solution of a number of proteins in urea produces changes in molecular weight from that observed in water alone, while some show no observable difference. There is no obvious correlation, however, between the liberation of sulfhydryl groups and the changes in molecular weight. (For references see (41), p.210-221.)

Greenstein (41) has recently reported that solutions of pure amorphous insulin concentrated by negative pressure

dialysis to 2% gave a negative cyanide-nitroprusside reaction but that a positive reaction was obtained after previous treatment with high concentrations of urea or guanidine hydrochloride. The author has observed a positive cyanide-nitroprusside reaction in all 2% solutions of amorphous or crystalline insulin amounting to 30% of the total sulfur. Jensen also reports a positive cyanide-nitroprusside reaction for insulin ((29) p. 42,50).

6. The Heat Precipitate of Insulin

The formation of a precipitate during heating in dilute acid was independently observed by Blatherwick et al (31) and du Vigneaud, Geiling and Eddy (32). The latter found that a 0.5% solution of insulin in 0.1N hydrochloric acid formed a flocculent precipitate at 100°C. Precipitation was complete after 1.5 to 2.0 hours. Both groups of workers found that treating the precipitate with dilute alkali yielded a product which had 80% or more of the activity of the original protein. Gerlough and Bates (8) and du Vigneaud, Sifferd and Sealock (33) have published extended observations on the conditions under which the heat precipitate forms.

Using slightly different conditions, Langmuir and Waugh (34) reported that a 2% solution of amorphous in-

sulin at pH 2.0 after heating for 30 minutes at 100°C. formed a clear thixotropic gel which showed intense static double refraction. After dilution of the gel flow double refraction appeared. From double refraction measurements Waugh (35) concluded that insulin had been modified to form highly asymmetric fibrils. More recently, electron micrographs of the doubly refracting precipitate by C. E. Hall, of this department (36), confirm the presence of fibrils having average lengths and widths of about 16,000 Å and 140 Å.

Waugh (37) has demonstrated the connection between the heat precipitate and fibrils. The former is shown to be composed of spherocrystals constructed of radially oriented fibrils. The spherite structure is relatively stable in acid and at pH's up to 11.0 but rapidly disperses into fibrils at pH's above 11.5. Knowing the structure of the heat precipitate in terms of fibrils, indicates that determinations made on the heat precipitate are in reality determinations of fibril forming capacity and enables one to point out the significance of certain data of Gerlough and Bates (8) and du Vigneaud, Sifferd and Sealock (33) with respect to fibril formation. When added to the unpublished data of Waugh this indicates that fibril formation is a reaction which takes place

without any demonstrable chemical change in the protein molecule - that is, without the liberation of ammonia or sulfhydryl groups, or the appearance of degradation products. Reversed spherites (8,33) show about 80% of the activity of the original insulin. In addition (37), carefully reversed fibrils may be crystallized to yield crystals having the same physical and chemical characteristics as native insulin. Such experiments suggest that an insulin, quite similar to the native protein, may be released from the fibrous modification.

Insulin fibrils are stable at pH's at which native insulin and crystalline insulin are quite soluble and in cases unstable, indicating that some alteration in structure may have taken place or that those groups which contribute to the lability of the molecule are now strongly bound in the fibrous form. This pronounced alteration in solubility further indicates that the bonding between insulin "molecules" is not of crystalline character (presumably zinc ions binding carboxyl groups on adjacent insulins (38)) but is considerably stronger and more acid and alkali stable. The experimental data to be reported furnish further evidence in this connection.

II EXPERIMENTAL TECHNIQUES *

(1) Fibril Formation

Fibril formation was estimated by two methods - the 100° method and the 37° method. In the earlier experiments the ability of treated insulin to form fibrils was measured by dissolving a known weight of the material in 0.04N hydrochloric acid in 2.0% concentration. The solution was sealed into a pyrex tube 200 x 8 mm. and heated in a water bath at 100°C. By rocking the tube, so that the solution flowed from one end to the other, static or flow double refraction could be observed when the cooled sample was held at a 45° angle between vertical crossed polaroids.

Waugh (37) observed: 1) Freezing and thawing gradually diminished the length of fibrils formed at 100°C. and 2) when a 2.0% solution of native insulin in 0.04N hydrochloric acid to which a small amount of short fibrils had been added was allowed to stand for 48 hours at 37°C. all of the added native protein was converted into fibrils. The author has found that 5 mg. of short fibrils would convert 10 mg. of native insulin into fibrils if allowed to stand for 48 hours at 37°C. The fibrils could then be separated quantitatively from any protein which had not been converted.

*Crystalline beef insulin (Squibb) was used in this study.

Short fibrils were prepared by heating a 2.0% solution of insulin in 0.04N hydrochloric acid at 100°C. in a sealed ampule for 4 to 10 minutes, or until faint flow double refraction could be demonstrated. The cooled ampule was immersed in a freezing mixture of acetone and dry ice for 2 minutes. The frozen material was then thawed and reheated at 100°C. for 4 minutes. This process produces a clear gel containing long fibrils. After cooling the gel was again frozen for two minutes and thawed and this cycle repeated. 5 mg. portions of short fibrils were added immediately to 10 mgs. of test material dissolved in 0.5 ml. of 0.04N hydrochloric acid in a weighed centrifuge tube and placed at 37°C. for 48 hours. After the reaction had been completed the fibrils were separated as follows: 3 ml. of pH 5.8 acetate buffer was added to each tube and allowed to stand for one minute. The buffer causes the fibrils to clump strongly. 9 ml. of 0.001N hydrochloric acid was then added with stirring and the tubes allowed to stand for ten minutes. The tubes were centrifuged, throwing the clumped fibrils down firmly and the supernatant discarded. The material was washed three times more with 0.001N hydrochloric acid for ten minutes each time to remove soluble insulin or other soluble non-fibrous ma-

terial. After the final washing the tubes were dried in a vacuum dessicator and weighed. The weight of fibrils was recorded. Control experiments showed that of 5 mg. of short fibrils treated as above 4.1 mg. could be recovered. Therefore, 4.1 was subtracted from the total milligrams recovered after drying, the difference being the milligrams of active protein converted. In those experiments where no fibrils were formed from the test samples a much lower figure was obtained varying from 3.0 to 4.1 mg. This may be due to physical interference by the non-fibril forming molecules inhibiting the free association of the short fibrils into longer ones. Where all of the test material was converted into fibrils control experiments indicated an average recovery of 13.3 mg. after drying.

2. Crystallization

Crystallizing ability was estimated by the method of Waugh (37) a variation of the method of Romans, Scott, and Fisher (3). 10.0 mg. of insulin were dissolved in 1.0 ml. of 0.04N hydrochloric acid, 5.0 ml. of 0.33N acetic acid were added and then, rapidly, enough 0.033N ammonium hydroxide to reach pH 7.5-8.0. Four drops (0.13 ml.) of zinc solution (containing 0.25 mg. of zinc, as zinc chloride, per ml.) in pH 5.9 acetate buffer were

added slowly and the pH of the final solution slowly adjusted to 5.9-6.0. The solution was allowed to stand undisturbed for two days at room temperature and then placed in the ice chest for from 3 to 5 days. The yield of crystals was measured in calibrated capillary tubes, especially constructed for the purpose. Control experiments with 10 mg. of native insulin yielded a constant amount of crystals (13.5 mm.) as measured along the length of the capillary tubes (37). Weighed samples (37) indicate a conversion factor between millimeters and milligrams as follows: $(mm.)(\frac{1}{2}) = mg.$

3. Cyanide-nitroprusside Reaction

The presence of unrestricted disulfide linkages was estimated by a semi-quantitative cyanide-nitroprusside test. 5 mg. of test material was reduced with 1 ml. of 5% aqueous sodium cyanide over a period of 10 minutes. The color produced by the addition of 0.5 ml. of 1% aqueous sodium nitroprusside was compared immediately with previously prepared color standards. It was found that a mixture of Tashiro's indicator and neutral red indicator in acid solution produced a color which matched fairly well that produced by insulin in the presence of sodium cyanide and sodium nitroprusside. Standards were prepared which corresponded to the amount

of cystine equivalent to that contained in varying amounts of insulin. 12.5% of cystine and a molecular weight of 40,000 were used for calculations.

It is felt that the method was dependable within about ± 4 percent units and served as a suitable standard for comparison of the changes in availability of the disulfide groups. Frequent controls with known quantities of native insulin always yielded the same result using reagents up to 30 hours old. Mirsky (39) has observed a correlation between the sulfhydryl groups reacting with sodium nitroprusside and those reacting with ferricyanide from which ferrocyanide equivalent to the number of titratable sulfhydryl groups is formed. This is evidence of the acceptability of the nitroprusside reaction.

III RESULTS

1. Effect of Reduction (Chart I)

Solutions were used containing 5 mg./ml. of insulin and 10 mg./ml. of sodium cyanide. Reductions were carried out at 30°C. from 5 to 180 minutes. These conditions were described by du Vigneaud, Sifferd and Sealock (33) as the lower limit for inactivation of crystalline insulin, the reaction going to completion after 180 minutes. The protein-cyanide solution was dialyzed against distilled water in an atmosphere of nitrogen to minimize reoxidation. The dialysate was precipitated at pH 5.3 and the precipitate dried in vacuo after washing with distilled water and centrifugation. It should be pointed out that dialysis added a constant time increment to each treatment estimated to be 30 minutes. Short treatments should, therefore, be carried out by some other method such as isoelectric precipitation after the cyanide treatment. All tests were performed on the dried material.

At five minutes crystallization had been destroyed and only 24.5% of the sample could be converted into fibrils. Longer treatment completely eliminated fibril formation. Of the starting material, 25 to 30%

CHART I

EFFECT OF SODIUM CYANIDE ON CRYSTALLINE INSULIN

Treatment				Start. Mater.	Recovery				Crystallization				Fibril Formation						Disulf. as alk. lab. Sulf.	Sulf-hydryl as
													37°C				100°C			
Conc. of Reag.	Time	Temp.	pH	Start. Mater.	Total Recov.	Fract. Num.	Fract. Wgt.	% of Total Recov.	Aliquot	Crystals	Crystals based on 10 mg. Aliquot	% of Norm.	Aliquot	Total Fib.	Converted prot.	% of norm.	Heat. Time	Double Refraction		
%	hrs.	°C		mg.	mg.		mg.	%	mg.	min.	min.	%	mg.	mg.	mg.	%	min.		%	%
Preliminary Experiment																				
1	180	30		50	30	II											60	neg.		
Later Experiments																				
1	5	30		50	26	II			10	0	0	0	4.5	5.2	0.4	24.5			5	0
1	30	30		50	25	II			10	0	0	0	4.0	3.0	0	0			5	0
1	30	30		100	81.8	II	50.7	62.0	10	0	0	0	10	4.5	0	4.0				
						III	2.9	3.4												
						IV	28.3	34.4	10	0	0	0	2.5	6.3	1.5	88				
1	90	30		50	25	II			10	0	0	0	4	3.2	0	0			5	0
1	180	30		50	25				10	0	0	0	2.5	3.7	0	0			5	0

Fraction I - Precipitated on dialysis with distilled water

Fraction II - Precipitated at pH 5.3

Fraction III - Precipitated on re-dialysis with distilled water

Fraction IV - Obtained from drying final dialyzed supernatant.

was recovered by precipitation at the isoelectric point. In one experiment the clear supernatant liquid was dialyzed to remove the salt produced by the isoelectric adjustment and an additional 3.4% precipitated after this step. The resulting supernatant was dried in vacuo and an additional fraction recovered which represented 34.4% of the total recovery. This latter fraction, while not possessing the ability to form crystals, showed 76-88% fibril formation.

Nitroprusside tests on all fractions precipitated at pH 5.30, washed, and dried were negative. Standard cyanide-nitroprusside tests revealed the presence of 5% of the sulfur, as disulfide, available to the reagents. However, nitroprusside tests performed on the original protein cyanide solution showed that 20% of the disulfides had been reduced to sulfhydryl after 180 minutes (Chart II). During washing and drying there is evidently a re-oxidation of sulfhydryl groups to disulfide groups, the dried material yielding fewer available disulfides than native insulin.

2. Effect of Alkali (Chart III)

All reactions were carried out with 0.2% concentration of the protein. After completion of the reaction the pH was adjusted to 5.3 and the resulting precipitate

CHART III

EFFECT OF ALKALI ON CRYSTALLINE INSULIN

Treatment					Recovery				Crystallization				Fibril Formation						Disulf. as alk. lab. Sulf.	Sulf-hydryl as
													37°C				100°C			
Conc. of Reag.	Time	Temp.	pH	Start. Mater.	Total Recov.	Fract. Num.	Fract. Wgt.	% of Total Recov.	Aliquot	Crystals	Crystals based on 10 mg. Aliquot	% of norm.	Aliquot	Total Fib.	Con-verted prot.	% of norm.	Heat. Time	Double Refraction		
Norm.	hrs.	°C		mg.	mg.		mg.	%	mg.	min.	min.	%	mg.	mg.	mg.	%	min.		%	%
Preliminary Experiments																				
	15	30	10.5	60	55.8	II			10	12.5	12.5	96	10	15	10.2	111				
	15	37	10.5	30	26.4	II											34	flow		
	15	37	10.5	30	21.9	II											26	sl.flow		
																	71	stat.st. flow		
0.03	1.5	37		30	14.7	II											51	neg.		
0.05	5	0		30													49	static		
0.05	5	30		30														neg.		
0.05	27	0		30													89	static		
0.05	27	30		30														neg.		
0.05	49	0		30														neg.		
0.075	27	0		30	14.0													neg.		
Later Experiments																				
0.075	1	0		50					10	12.5	12.5	96	10	12	7.9	86			5	neg.
0.075	1	0		50	45.6				10	11	11	81	10	15.8	117	127				
0.075	2	0		50					10	12	12	90	10	13.4	9.3	101			10	neg.
0.075	4	0		50	42.4				10	10.5	10.5	78							10	neg.
0.075	4	0		50	43.9				10	11	11	81	10	15.3	11	122				
0.075	8	0		50	30.8				10	12	12	90	7.2	9.3	5.2	78			10	neg.
0.075	8	0		50	44.7				10	10	10	74	10	14.5	10.4	113				
0.075	16	0		50	27.5				10	7	7	52	6.5	4.9	.7	10.9			10	neg.
0.075	16	0		50	38.6				10	3	3	22	10	11.8	7.7	83.5				
									10	5	5	37	10	14.9	10.8	117	4	sl.stat.		
0.075	16	0		150	188.5	II	119.9	63.7	10	5.5	5.5	41	10	10.1	6.0	65	10	str.static		
						III	6.7	3.5	6.7	3	4.5	33								
						IV	61.9	32.8	10	0	0	0	10	5.2	1.1	12				
0.075	36	0		50	30.8	II			10	3	3	22	7	4.6	.5	7.6			10	meg.
0.075	40	0		50	28.0				10	0	0	0	10	11.2	7.1	77				

Fraction I - Precipitated on dialysis with distilled water

Fraction II - Precipitated at pH 5.3

Fraction III - Precipitated on re-dialysis with distilled water

Fraction IV - Obtained from drying final dialyzed supernatant.

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CHART 3

centrifuged and dried in vacuo. In some cases, the supernatant was dialyzed against distilled water and dried in vacuo.

In one set of experiments using 0.075N sodium hydroxide at 0°C. for 1 to 40 hours the percentage of fibril formation maintained lower values than the percentage of crystallization. In another later experiment using the same conditions the values for crystallization were lower than those for fibril formation. In both sets of experiments, however, the rate of loss of fibril forming capacity paralleled the rate of loss in crystallizing capacity (Fig. 1). No great reliance, other than pointing out the concommitant disappearance of crystallization and fibril formation, is placed on these experiments.

According to Freudenburg and Munch (44) insulin at pH 10.5 and 37°C. for 15 hours becomes inactive without the liberation of hydrogen sulphide or ammonia. The author has repeated this experiment at 37°C. and 30°C. At 30°C. the resulting product had normal crystallizing and fibril forming capacity. Since all known crystalline insulins have biological activity this material would be expected to be fully active. An earlier experiment, at 37°C., produced a material having fibril forming capacity. Crystallization was not measured.

CHART II

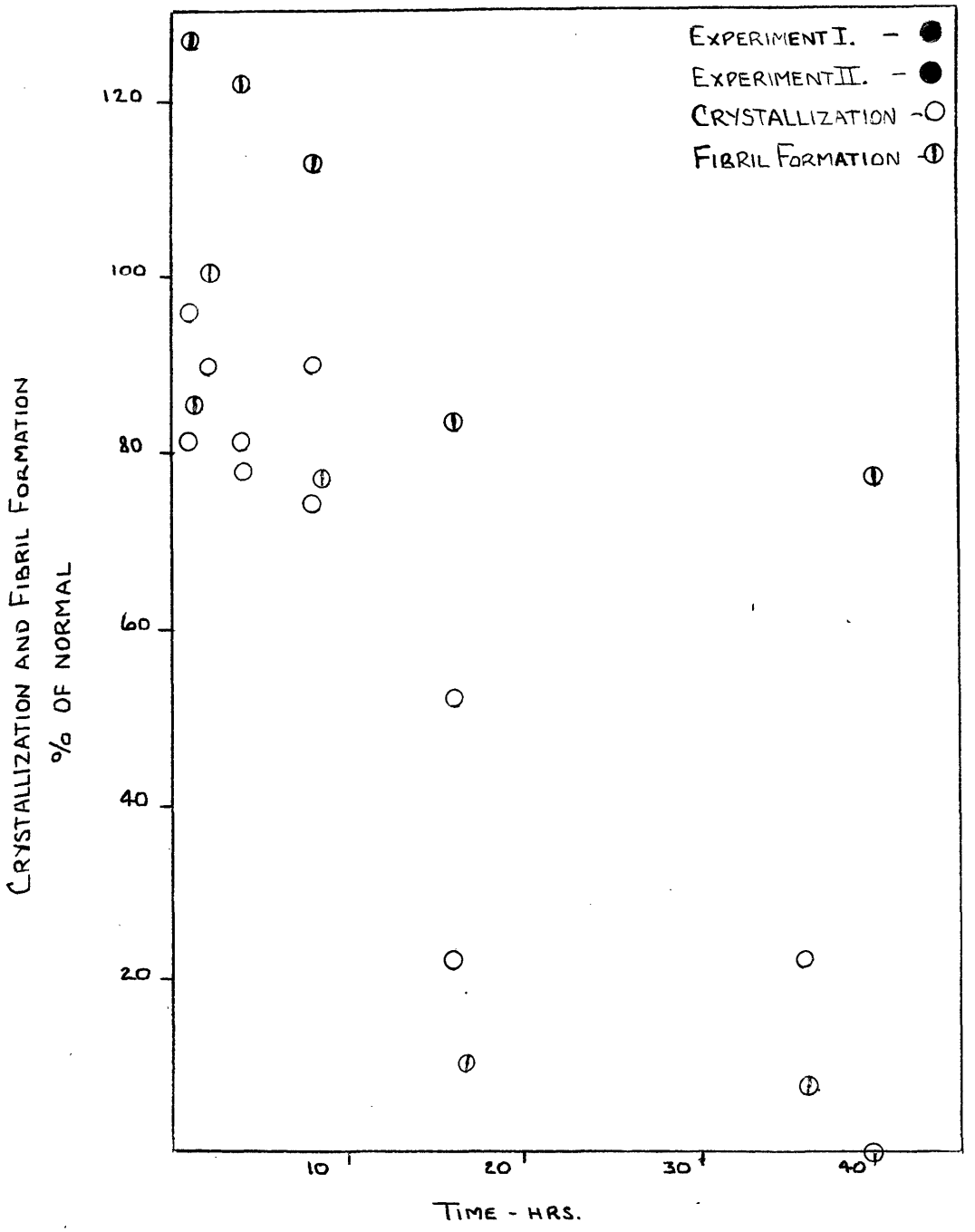
REDUCTION WITH 1% SODIUM CYANIDE

0.5% protein solution tested in the presence of the reagent.

Time	Sulfhydryl as alk. labile sulfur
min.	%
1	0
5	0
30	5
90	10
180	20

FIGURE I.

EFFECT OF ALKALI ON CRYSTALLIZATION AND FIBRIL FORMATION WITH TIME



3. Effect of Guanidine Hydrochloride (Chart IV)

Reactions were carried out with 1% concentration of the protein for 24 hours. After completion of the reaction the solutions were dialyzed against distilled water to remove the reagent. Any precipitate formed on dialysis was centrifuged and dried (fraction I). The supernatant was adjusted to pH 5.3 and the precipitate removed and dried (fraction II). Subsequent alterations in pH failed to yield any further precipitate from the frequently opalescent supernatant. The latter was dialyzed to remove salt, in instances yielding a small third precipitate (fraction III). The final supernatant was evaporated to dryness in vacuo (fraction IV).

Preliminary experiments were run with 2,4,8 and 11M guanidine hydrochloride at pH 5.2 (the pH of the guanidine plus protein, the pH of guanidine alone ranging from 4.1 to 2.5 with increasing concentration) at 30°C. and 24 hours. In all of the later experiments 8M guanidine hydrochloride at 24 hours was used at two temperatures (0°C. and 37°C.) and three pH's (pH 2.0, 7.0, and 9.0).

At pH 5.2 and 30°C. and guanidine hydrochloride concentrations ranging from 2 to 11M from 60 to 92% of the

CHART IV

EFFECT OF GUANIDINE HYDROCHLORIDE ON CRYSTALLINE INSULIN

Treatment					Recovery				Crystallization				Fibril Formation							
													37°C				100°C		Disulf. as alk. lab. Sulf.	Sulf-hydryl as
Conc. of Reag.	Time	Temp.	pH	Start. Mater.	Total Recov.	Fract. Num.	Fract. Wgt.	% of Total Recov.	Aliquot	Crystals	Crystals based on 10 mg. Aliquot	% of norm.	Aliquot	Total Fib.	Converted prot.	% of norm.	Heat Time	Double Refraction		
Mol.	Hrs.	°C		mg.	mg.		mg.	%	mg.	min.	min.	%	mg.	mg.	mg.	%	min.		%	%
Preliminary Experiments																				
2	24	30	5.2	40	46.2	I			10	11	11	81					50	neg. sl. spherite	10	neg.
4	24	30	5.2	40	45.2	I			10	13	13	96					50	sl. stat. spherites	25	neg.
8	24	30	5.2	40	29.3	I			10	13	13	96					50	str. stat. str. spherite	25	neg.
11	24	30	5.2	40	33.7	I			10	15	15	111					50	str. stat. str. flow	25	neg.
Later Experiments																				
8	24	0	2	50	36.8	I	12.0	32.6	5	5	10	74								
						II	22.0	59.8	10	13	13	96	11	14.3	10.2	100				
						IV	2.8	7.6												
8	24	0	7	50	51.9	I	27.2	52.5	5	1.5	3	22	10	8.5	4.4	48				
						II	2.7	5.2												
						III	4.8	9.3					4.8	8.4	4.3	98				
						IV	17.2	33.2	5	7.0	14	104	5.6	9.4	5.1	100				
8	24	0	9	50	62.6	I	26.1	41.8	9	11	12.2	90	10.	14.2	10.1	110				
						II	4.2	6.7					4.2	6.5	2.4	62				
						III	12.4	19.8	5	5.5	11	81	7.4	10.4	6.3	93				
						IV	19.9	31.8	10	10	10	74	10	14.3	10.2	111				
8	24	37	2	50	47.9	I	45.2	94.2	10	0	0	0	10	13.2	9.1	99				
						IV	2.7	5.7												
8	24	37	7	50	48.7	I	28.1	57.5	10	10	10	74	10	13.4	9.3	101				
						II	3.4	7.0					3.4	7.0	2.9	93				
						III	4.9	10.0	4.9	5.5	11.2	83								
						IV	12.3	25.2					6.3	10.7	6.6	114				
8	24	37	9	50	47.7	I	8.9	18.6					5.0	8.7	4.6	100				
						II	4.8	10.0					4.8	5.4	1.3	29.6				
						III	10.0	21.0	5	2.5	5	37								
						IV	24.0	50.4	5	0	0	0	10.5	98	5.7	59				

Fraction I - Precipitated on dialysis with distilled water

Fraction II - Precipitated at pH 5.3

Fraction III - Precipitated on re-dialysis with distilled water

Fraction IV - Obtained from drying final dialyzed supernatant.

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CHART 4

protein was recovered in the first fraction. This fraction showed normal crystallization and normal fibril formation, indicating the stability of the protein towards guanidine at the isoelectric point.

At pH 7.0 and 0°C. and 37°C. the bulk of the protein was recovered in the first and fourth fractions. All fractions showed normal fibril formation and normal or slightly reduced crystallization. One case at 0°C. showed a large reduction in both properties in the first fraction. Since the yield in this instance was very small the results may be attributed to poor separation and the co-precipitation of insoluble impurities which are expected to come down with the first fraction.

At pH's 2.0 and 9.0 and 37°C. the protein revealed its greatest reactivity. At 0°C. practically normal properties were exhibited at both pH's. At pH 2.0, 37°C. the first fraction, representing 94% of the total recovery, showed no crystallization and normal fibril formation. At pH 9, 37°C. the fourth showed no crystallization and 59% fibril formation. The first fraction showed 100% fibril formation while the second and third fractions showed a marked diminution in both properties.

4. Effect of Urea (Chart V)

Treatment was carried out with 1% protein solutions. Preliminary experiments involved 8M urea at 30°C. for 24, 48, and 96 hours and 12M urea at 30°C. for 24, 36, 51, 96, 144, and 192 hours. All preliminary experiments were at about pH 7.0. The results indicated that later treatments should be carried out with 8M urea at 24 hours. Experiments were performed at 0° and 37°C. with pH's 2.0, 7.0, and 9.0.

With 12M urea at pH 7 after 24 hours, 96% of the protein was recovered in fraction I which showed 74% crystallization and fibril formation at 100°C. After 36 to 192 hours the bulk of the protein appeared in fraction II in which no crystallization was observed but fibril formation appeared at 100°C.

With 8M urea, 24 hours at pH 2.0, 7.0, and 9.0, at 0°C. there was a small observable decrease in crystallization while fibril formation remained normal. At this temperature, as in the case of guanidine hydrochloride, there was no marked difference in reactivity among the pH's studied except for a different distribution in the fractions. Recovery was best at pH 7.0 and 9.0 because the bulk of the protein precipitated at pH 5.3, while at pH 2.0, 34% of the starting material

CHART V

EFFECT OF UREA ON CRYSTALLINE INSULIN

Treatment					Recovery		Crystallization						Fibril Formation						Disulf. as alk. lab. Sulf.	Sulfhydryl as
													37°C				100°C			
Conc. of Reag.	Time	Temp.	pH	Start Mater.	Total Recov.	Fract. Num.	Fract. Wgt.	% of Total Recov.	Aliquot	Crystals	Crystals based on 10 mg. Aliquot	% of norm.	Aliquot	Total Fib.	converted prot.	% of norm.	Heat Time	Double Refraction		
Mol.	Hrs.	°C		mg.	mg.		mg.	%	mg.	min.	min.	%	mg.	mg.	mg.	%	min.		%	%
Preliminary Experiments																				
8	24	30	7	40	39.5	I	7.5	19									6	Static		
						II	32.0	81	10	tr.	tr.	0								
8	24	30	7	80	19	I	19		10	14	14	104					3	static		
8	48	30	7	50	47.1	I	3.3	7.0												
						II	43.8	93.0	10	0	0	0							5-10	neg.
8	96	30	7	50	43.7	I			10	tr.	tr.	0					49	spherite	5	neg.
12	24	30	7	50	48	I			10	10	10	74					66	static	5	neg.
12	36	30	7	50	33.6	II			10	tr.	tr.	0					9	flow	5	neg.
12	51	30	7	50	32.5	II			10	tr.	tr.	0					9	str.flow	5	neg.
12	96	30	7	50	41.0	II			10	0	0	0					9	sl.flow	10	neg.
12	144	30	7	50	?	II			10	0	0	0					9	sl.flow	10	neg.
12	192	30	7	50	?	II			10	0	0	0					9	sl.flow	10	neg.
Later Experiments																				
8	24	0	2	50	17	I	11		10	10	10	74	7	10.8	6.7	104				
							Cloudy supernatant could not be precipitated													
8	24	0	7	50	37.6	I	7	18.6					7	10.8	6.7	104				
						II	30.6	81.4	10	5	5	37	10	11.6	7.5	81.5			10.0	
8	24	0	9	50	42.8	I	4	9.4					3.5	6.9	2.8	87				
						II	38.8	90.6	10	10	10	74	10	13.2	9.1	99			10.0	
8	24	30	7	100	59.4?	I	tr.													
						II	30?	50.5	10	0	0	0	10	13.4	9.3	101				
						*III	3.0	5.0												
						IV	26.4	44.5	5	1	2	15	10	13.2	9.1	99				
8	24	37	2	50	15	I	15	100	5	5.5	11	81	10	13.5	9.4	102				
							Cloudy supernatant could not be precipitated													
8	24	37	2	150	126.6	I	9.7	7.7	5	2	4	29.6	4.7	5.9	1.8	42				
						II	112.6	89.0	10	9	9	66.5	10	14.3	10.2	111				
									10	8.5	8.5	63.0	10	14.0	9.9	107	4	str.static	10.0	
						IV	4.3	3.3												
8	24	37	7	50	29.6	I	tr.													
						II	29.6		10	0	0	0	10	13.4	9.3	101				

*NOTE: 8M, 24 hrs., 30°C, pH7, fraction III precipitated at pH 8.5

25a
CHART 5

CHART V (cont'd)

EFFECT OF UREA ON CRYSTALLINE INSULIN

Treatment					Recovery				Crystallization				Fibril Formation						Disulf. as alk. lab. Sulf.	Sulf-hydryl as
													37°C				100°C			
Conc. of Reag.	Time	Temp.	pH	Start. Mater.	Total Recov.	Fract. Num.	Fract. Wgt.	% of Total Recov.	Aliquot	Crystals	Crystals based on 10 mg. Aliquot	% of norm.	Aliquot	Total Fib.	Con-verted prot.	% of norm.	Heat. Time	Double Refraction		
Mol.	Hrs.	°C		mg.	mg.		mg.	%	mg.	min.	min.	%	mg.	mg.	mg.	%	min.		%	%
8	24	37	7	150	144.6	I	1.9	1.3												
						II	47.2	32.8	10	tr.	tr.	0	10	12.9	8.8	96	4	str.static		
						IV	95.5	65.9	10	0	0	0	10	14.6	10.5	114			10	
8	24	37	9	50	35	I	tr.													
						II	35?		10	0	0		10	13.9	9.8	106			10.0	
8	24	37	9	150	145.7	I	2.6	1.5												
						II	136.1	93.5	10	1.0	1.0	7.4	10	9.5	5.4	59	4	str.static	10.0	
									10	1.0	1.0	7.4	10	13.3	9.2	100				
						IV	7.0	4.8												
8	48	30	7	100	108.6	I	15.0	13.8	5	5	10	74	10	13.4	9.3	101				
						II	39.6	36.5	10	0	0	0	10	13.2	9.1	99				
						IV	54.0	49.7	10	0	0	0	10	13.7	9.6	104				

Fraction I - Precipitated on dialysis with distilled water

Fraction II - Precipitated at pH 5.3

Fraction III - Precipitated on re-dialysis with distilled water

Fraction IV - Obtained from drying final dialyzed supernatant.

precipitated on dialysis and the remainder could probably have been recovered from the opalescent supernatant.

At pH 2.0 and 37°C., 89% of the recovery was in fraction II which showed 65% crystallization and normal fibril formation.

At pH 7.0 and 9.0, and 30 to 37°C., the greatest effects were observed. At pH 7.0, fraction I from two experiments, representing 14 to 23% of the recovery, showed normal fibril formation and in one case normal crystallization and in the other a slight diminution. In fractions II and IV, out of nine fractions, eight showed no crystallization and one a reduction to 15%. In seven out of the nine, normal fibril formation was measured, the other two samples being too small for estimation. The nine fractions, mentioned above, represent about 90% of the recovered protein and over 90% of the starting material.

At pH 9.0, 80 to 90% of the protein was precipitated in fraction II and gave 0 to 7.4% crystallization while fibril formation remained normal.

In no case using urea has fibril formation been seriously affected while there has been a complete loss in crystallization in many instances.

In experiments involving both guanidine and urea, it is unfortunate that the smaller fractions were not ob-

tained in sufficient amounts to have completed tests for crystallization and fibril formation.

5. Availability of Disulfide or Sulfhydryl Groups

Native insulin, 0.5%, dissolved in 5% sodium cyanide gives an immediate reaction with nitroprusside equivalent to 25-30% of the total sulfur present. With 1% sodium cyanide 5% of the disulfide groups appear after 30 minutes and this increases to 20% after 180 minutes.

Urea in all concentrations studied does not liberate any disulfide or sulfhydryl above that found in native insulin. Guanidine hydrochloride in 8M concentration makes all of the disulfide bonds available, but does not break any of them to sulfhydryl.

Insulin treated with sodium cyanide, alkali (0.075N for 1 to 36 hours), or urea, showed no sulfhydryl and 5-10% available disulfide groupings, after precipitation or dialysis, and drying. These quantities are somewhat smaller than those found in native insulin. The material resulting from guanidine hydrochloride treatment, 4 to 11 M, after dialysis and drying, yields 20-25% free disulfide groups, about equivalent to that found in native insulin. The material from 2M guanidine treatment has about 10% of the disulfides available, a decrease from native insulin similar to that found with sodium

cyanide, alkali, and urea.

All dried samples, when dissolved in urea or guanidine, yield disulfide groups of the same magnitude as those found with native insulin. Thus dried protein from 8M guanidine treatment, which had 100% of the disulfides available in the presence of the reagent, now exhibits the usual 25-30% if dissolved in 8M urea, and 100% if dissolved in 8M guanidine hydrochloride.

IV DISCUSSION

Ever since corpuscular proteins have been considered unique molecular entities, methods have been sought by which specific properties of these proteins could be altered without producing a general degradation. It has been hoped that such studies would give clues as to the internal structure of the protein molecule.

Hopkins (54) was the first to demonstrate the liberation of sulfhydryl groups by the action of concentrated urea without concurrent degradation of the protein. This seemed to be a valuable method of attack and the specific groupings such as sulfhydryl, disulfide, hydroxyl of tyrosine, and others were assayed before and after the action of urea and other mild reagents. Although the majority of work of this nature has been done on the disulfide and sulfhydryl groups, recent years have seen techniques developed for measuring changes in molecular weight, electrophoretic diffusion, molecular symmetry, solubility, and crystallization. A review of the important data in this connection is that of Neurath, Greenstein, Putnam and Erickson (41).

Insulin, an important corpuscular protein, is particularly suited to these studies whereby one hopes to correlate changes in properties with changes in struc-

ture. In addition to its characterization by biological activity, crystallization, chemical and physical properties, the insulin molecule demonstrates the intrinsic reaction of fibril formation. This reaction is specific to the extent that it does not take place after bonds, such as disulfide, have been broken; yet does take place after other demonstrable changes in structure have occurred. In the present study four reagents were used to effect structural changes, 1) sodium cyanide, 2) sodium hydroxide, 3) urea and 4) guanidine hydrochloride. In each case the resultant product was tested for deviation from the native insulin by the use of three standard reactions; fibril formation, crystallization, and availability of disulfide and sulfhydryl groups. This particular series of reagents was chosen for the following reasons: 1) Reduction by cyanide produces gross changes in which specific bonds are disrupted, 2) alkali produces large or small changes depending on the concentration and, in addition, its effects on many properties of insulin have been studied by others, 3) urea and guanidine hydrochloride in most cases produce slight changes involving shape, symmetry, and the availability of specific groupings.

Under appropriate conditions all of the reagents studied show profound effects on crystallization while

some at the same time appear to have little effect on fibril formation. These reagents are not only different with respect to the extent to which they will affect the properties of the insulin molecule but they have different modes of action. Alkali seems to cause crystallization and fibril formation to disappear at about the same rate (Fig.1). Guanidine hydrochloride in 8M concentration definitely abolishes crystallization with some evidence that under more drastic conditions fibril formation will disappear. Urea, on the other hand, with all concentrations, times, and pH's studied affects only crystallization leaving the fibril forming capacity unaltered. Thus the reagents are arranged in descending order of reactivity when crystallization and fibril formation are considered. If one were to consider the availability of disulfide groups a different order would be evident since 8M guanidine liberates all of the disulfides while 5% sodium cyanide and 8M urea reduce or make available 25-30% of the total disulfide linkages.

Some of the characteristic properties of each reagent follow.

Alkali: The concommitant disappearance of crystallization and fibril formation suggests that alkali, if it af-

fects a given molecule at all, produces sufficient change to eliminate both of these properties. Concentrated alkali is known to split out hydrogen sulfide from insulin. This therefore suggests that the above mentioned effects result from alterations in the disulfide groupings. ~~Concentrated alkali is known to split out hydrogen sulfide from insulin~~ (42, 43, 44).

Urea and Guanidine: In the native state a corpuscular protein is considered to be in a compact configuration from whose surface many hydrophyllic groups protrude and in which polypeptide chains of amino acids are stabilized by cross linkages of different types. Urea and guanidine apparently affect the molecule by loosening or breaking these cross linkages, allowing the protein to fold into new configurations where possibly new cross linkages are made (41). In many instances, groups not available in the native protein are made reactive by the action of these reagents. Some proteins undergo considerable changes in symmetry, with or without evidence of aggregation or disaggregation.

If the effects of urea and guanidine are the result of chemical reactions they should, unless they possess zero activation energy, be affected by temperature. They should also be influenced by the number and stability of

cross linkages, a factor which depends upon pH in many instances. Thus any given reagent would be expected to exert a greater effect if stabilizing forces in the molecule had previously been altered by acid or alkaline environment.

The extensive literature contains scanty reference to pH as an important variable. Mirsky (39) treated egg albumin with guanidine and measured the availability of sulfhydryl groups between pH's 4.4 to 7.8. The availability became slightly greater as one receded from the isoelectric point (pH 4.56).

In the present series of experiments pH and temperature are considered with respect to their effect on crystallization and fibril formation. It is interesting that insulin has its greatest stability in 8M guanidine hydrochloride at pH 5.3, the isoelectric point. At pH 7.0 it is still relatively stable, while at pH 2.0 it is unstable with respect to crystallization and at pH 9.0 unstable with respect to both crystallization and fibril formation. With urea, insulin is relatively stable at pH 2.0 and becomes unstable with respect to crystallization at pH 7.0 and 9.0. This reagent does not affect fibril formation at any of the pH's or temperatures studied. The data in general support the expectation

that a protein will be most stable at its isoelectric point.

Temperature exerts a noticeable effect on the rate at which the several reactions take place, with no apparent initiation of new reactions at the higher temperatures. The data are insufficient to allow one to calculate activation energies.

One of the most interesting features of the present study has been a separation of the treated protein into different fractions on the basis of solubility characteristics. After treatment with urea or guanidine hydrochloride, a portion of the protein, varying in quantity with concentrations, times, and temperatures, becomes insoluble after dialysis. A soluble fraction can be precipitated by adjusting the pH to 5.3. In instances, these two fractions represent only a portion of the total starting material. If the clear or opalescent supernatant obtained after the last pH adjustment is dialyzed to remove salt, a third, usually small, fraction may be obtained. Upon drying of the final supernatant, an appreciable precipitate can frequently be recovered.

In all experiments the four fractions represented approximately 90% of the starting material. The most

striking change from the native protein is to be observed in the fourth fraction. Native insulin is particularly insoluble in distilled water at the isoelectric point, while this fourth fraction is so soluble that it cannot be recovered by isoelectric precipitation after dialysis. In all instances this fraction showed a complete inability to crystallize, although fibril formation was normal.

These various fractions point to a progressive change in solubility properties of the molecule which, except for the fourth fraction, do not appear to be directly related to a loss in crystallization or fibril formation. The native protein which is insoluble in distilled water at pH 4 to 7.5, as observed during electrometric titrations, increases its solubility until it is soluble from pH 6 to 7, but can be precipitated at the isoelectric point, while another portion is soluble at all pH's and can be recovered only by evaporating the solvent.

No reliance is placed on the integrity of the fractions obtained. Without previous knowledge it seemed necessary to test each as if it were different from the the others. In general, any particular treatment divided the insulin into two fractions - an insoluble fraction (I or II) and a soluble fraction (IV). Guanidine

divided the protein into two main fractions I and IV while urea treatment converted the bulk of the protein into a single fraction either II or IV. In all cases fraction III was negligible.

Examination of the series of fractions resulting from individual treatments reveals a general trend in which the first more insoluble fractions have more normal properties than the later more soluble fractions. Thus if fraction II shows alterations, later fractions show the same alterations. Outside of this tendency there seems to be no absolute consistency of any one fraction with respect to crystallization and fibril formation. One can pick out examples from fractions II and IV which are normal (8M guanidine, pH 2.0, 0°C., fraction II; 8M guanidine, pH 7.0, 0°C. fraction IV) or which lack crystallization properties (8M urea, 37°C., pH 7.0,fraction IV). Consideration of the data obtained from material reduced with sodium cyanide reveals insoluble and soluble fractions which have neither crystallization nor fibril forming capacity.

Fibril Formation and the -S-S- Bond: It appears that the sulfur of the molecule is not directly involved in the fibril forming linkage. This conclusion stems from the following: 1) The breaking of one or two disulfide

bonds per molecule, out of a total of twenty-three bonds of which seven or eight are generally available, leads to an irreversible loss of physiological activity, even though reoxidation be allowed to take place (33,45,46). 2) No more free disulfides are demonstrable in fibrils than in native insulin. 3) Regenerated insulin fibrils, as "heat precipitate" (8,31,33) have the same physiological activity as native insulin. If any disulfide bonds had been broken, as indicated from reduction experiments, one would expect the regenerated material to be inactive.

In agreement with others (33) reduction of insulin by sodium cyanide abolished fibril formation. In addition, crystallization properties were lost. This suggests that fibril formation requires some special molecular configuration, but not necessarily that found in the native protein.

Reduction by cyanide, however, involves reduction of only 25-30% of the total disulfide groups. Treatment with guanidine, which makes available all of the disulfide groups, may not abolish fibril formation. Fibril formation, therefore, while it demands that disulfide linkages remain intact, does not demand that they maintain absolutely fixed positions within the framework of the insulin molecule.

It is felt that a further characterization of the main fractions with respect to crystallization and fibril formation and possibly biological activity together with measurements of molecular size, shape, and weight would yield data affording a more definitive solution to the problem of the internal structural changes in the molecule.

V SUMMARY

The protein insulin has been studied with reference to the effect of four mild reagents: 1) sodium cyanide, 2) sodium hydroxide, 3) urea, and 4) guanidine hydrochloride; on three characteristic properties: 1) fibril formation, 2) crystallization, and 3) availability of disulfide and sulfhydryl groups. A quantitative method for the estimation of fibril formation as well as a semi-quantitative adaptation of the cyanide-nitro prusside reaction has been developed. The material recovered from each treatment has been fractionated by solubility methods and studies made on the dried fractions.

Alkali seems to abolish crystallization and fibril formation at about the same rate. After urea treatment crystallization is frequently abolished with no concurrent affect on fibril formation. Guanidine affects crystallization immediately, fibril formation diminishing much later. Those fractions insoluble at the isoelectric point have been changed least while the more soluble fractions, obtained by drying the supernatants, have undergone the greatest change.

The initial position of the disulfide bonds in the molecule does not seem to be necessary for fibril forma-

tion but the latter reaction is absent in those instances where free sulfhydryl groups are formed by reduction.

Loss of crystallization and fibril formation, and the appearance of sulfhydryl groups, measure the succession of minor changes occurring during the first stages of structural modification. Crystallization is the most delicate known indicator of change, yet all of the disulfide bonds may be made available with 8M guanidine without destroying this property. Fibril formation, an intrinsic property of the protein, may occur after crystallization has been abolished. This reaction, which does not take place after reduction of disulfides, indicates the second stage of molecular change.

It is hoped that simultaneous determinations of other factors, such as change in size and symmetry, will enable one to understand more precisely the succession of events which lead to the ultimate abolition of all unique properties of the protein.

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