TABLE II (*Continued*)

p-Substituent (frequencies in cm.$^{-1}$)					
-H	$-OCH_3$	$-CH_3$	-Cl	$-NO_2$	Assignment
795s		793s	790s		
783m	782m	786s	784ssh	785nish	
		772s			
757s					Monosubst. C_6H_5
744s				740msh	
727s	734m	723s	725s	725mbr	
720s					Monosubst. C_6H_5
702s	706wsh	706s	702m	707m	
694s		697m	699m	698m	
654m		655w	665wbr		
637w		633w	635w		

[a] s, strong; m, medium; w, weak; vw, very weak; sh, shoulder; br, broad; o, overlap.

The strong absorption at 1518 cm.$^{-1}$ in the nitroporphine derivative is assigned to the antisymmetric N–O stretching vibration, while the band at 1345 cm.$^{-1}$ is attributed to the symmetric N–O stretching vibration. In *p*-nitrobenzaldehyde these absorptions occur at 1535 and 1345 cm.$^{-1}$, respectively. The shift to a lower frequency in the porphine of the first band is undoubtedly due to greater conjugation in the porphine molecule. Numerous weak and medium phenyl and substituted phenyl vibrations occur in the 1225–950 cm.$^{-1}$ region, in accordance with observations on other compounds by Bellamy,[13b] Colthup[15] and others. A few of these bands have been assigned in Table II. Characteristic *para*-substitution vibrations which usually occur from 1125–1090 cm.$^{-1}$ have been assigned to the bands observed in the frequency range 1100–1105 cm.$^{-1}$. Sharp, strong out-of-plane C–H deformations characteristic of monosubstituted benzene occur within 730–770 cm.$^{-1}$ and also near 700 cm.$^{-1}$. The strong bands of tetraphenylporphine at 757 and 720 cm.$^{-1}$ are assigned to monosubstituted benzene. *para*-Disubstitution is characterized by sharp vibrations in the 800–860 cm.$^{-1}$ range. The sharp bands occurring near 800 cm.$^{-1}$ are assigned to this mode of vibration.

In pyrrole, three sharp bands of increasing intensity occur at 1076, 1046 and 1015 cm.$^{-1}$. The three bands at approximately 965, 980 and 990 cm.$^{-1}$ are common to all porphyrins and are believed to be due to the same mode of vibrations even though they are closer together and at a lower frequency. The influence of the large resonating ring may account for the lower frequency observed in this research. Randall, Fowler, Fuson and Dangl[14a] have assigned the three bands in pyrrole to C–H rocking vibrations. The stretching absorption of the C–Cl bond reportedly occurs at 600–800 cm.$^{-1}$, but interaction with an aromatic ring raises the frequency to 845 cm.$^{-1}$.[13c] If the C–Cl band occurs at the low frequency range, it is most likely obscured by the overlapping of stronger phenyl and other absorptions in the low frequency region. If not, the frequency of 941 cm.$^{-1}$ may possibly be assigned to the C–Cl stretching vibration.

(15) N. B. Colthup, *J. Opt. Soc. Amer.*, **40**, 397 (1950).

WORCESTER, MASS.

[CONTRIBUTION FROM THE CHEMICAL LABORATORY, SANTA BARBARA COTTAGE HOSPITAL RESEARCH INSTITUTE]

Reaction Products of Insulin in Urea Solution[1]

BY FRITZ BISCHOFF AND ABOLFATH KHAN BAKHTIAR

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On the basis of differences in solubility and bioassay by the hypoglycemic response, five reaction products were isolated when amorphous or crystalline insulin was permitted to react in concentrated aqueous urea solution under various conditions of temperature and time. Insulin OU is formed when the insulin is precipitated immediately, from the urea solution, by the addition of a large volume of water. It is insoluble at *p*H 7.0 and its suspension has a delayed biologic response while its acid solution has a normal response. Insulin IU is formed when the reaction in urea solution is prolonged. It is soluble at *p*H 6.5 and has the same biologic response as regular insulin. Insulin IIU, the next reaction product, is insoluble in 0.03 *N* sulfate at *p*H 3, is soluble at the *p*H of the body fluids and has a delayed biologic response even when given intravenously. Its formation follows first-order kinetics. The energy of activation is about 33,000 cal. per mole, the free energy of activation 26,000 cal. per mole at 38° and the entropy change of activation 18–27 cal. per degree per mole. Insulin IIIU, formed on further prolonging the time in urea solution, differs from insulin IIU in that it is insoluble at *p*H 2.0, dilute HCl, and has half the biologic activity of insulin IIU. It also has a delayed response. Insulin IVU, the next reaction product, is devoid of biologic activity. When heated at 99°, *p*H 2.0, insulin IIU forms both the classic heat precipitate and a precipitate soluble at *p*H 8.4. The heat precipitate of insulin dissolves in concentrated urea solution at 99°, thereby restoring its biologic activity.

The delayed resorption of insulin[2] and certain of the gonadotropins[3] from a subcutaneous depot produces an augmentation effect, and simulates more nearly the physiologic state. Until recently, delayed acting insulins used clinically have depended upon the injection of an insoluble insulin combination or the injection of a solution forming an insoluble insulin complex at the injection site. By intra-

(1) Financed in part by the Carbon P. and Bertha E. Dubbs Foundation.

(2) L. C. Maxwell and F. Bischoff, *Am. J. Physiol.*, **112**, 172 (1935).

(3) F. Bischoff, *ibid.*, **121**, 765 (1938); U. S. Patent 2,121,900 (June 28, 1938).

venous injections of these so-called depot insulins, no delay in lowering the blood sugar is manifested.[4,5] The production of a delayed action insulin, which does not form an insoluble precipitate at the *p*H of the body and yet produces a delayed effect when given either by the intravenous or subcutaneous routes, was achieved by treating regular insulin in concentrated aqueous urea solution under specific conditions of time and temperature.[6]

The present report is concerned with the characterization and isolation of the reaction products of insulin in concentrated urea solution and includes a study of the reaction kinetics and the relation of the urea treated insulin to the heat precipitate.

Experimental

Zinc insulin crystals, supplied by Armour and Company and Eli Lilly and Company, assayed, respectively, 24.3 and 27 units per mg. The amorphous insulin was prepared by precipitating a crystalline insulin at the isoelectric point, washing the precipitate with ethanol and air drying the washed precipitate to a glassy mass.

The bioassays were performed by cross matching in 10 to 12 rabbits. These procedures with the statistical treatment were previously described.[5] The micro sugar determination was a combination of the Nelson–Somogyi procedures.[7]

Solubility determinations were performed by equilibrating for 1 hour and re-equilibrating the solute for 24 or 48 hours. Unless otherwise stated these values checked within the analytical error. Insulin concentration was determined by the Folin–Ciocalteu reaction.

Step I and II, Insulin OU and IU.—Crystalline or amorphous insulin dissolves rapidly to form a 5 to 10% solution in aqueous 40% urea solution, *p*H 6.4 at 15°, provided the insulin is not permitted to clump. If clumping, due to inadequate mixing, occurs the insulin forms a glassy mass and does not dissolve. On adding water to insulin recently dissolved in 40% urea, a colloid, colloid plus precipitate, or a precipitate forms depending upon the amount of water added and the amount of insulin dissolved in the urea. On the addition of 19 volumes water to a 5 to 10% solution of either crystalline or amorphous insulin, 97% of the insulin is precipitated on standing at 12°, 12 to 24 hours. The addition of 4 to 11 volumes of water produces a colloid and precipitate, which precipitate may comprise 60 to 86% of the original insulin. The colloidal suspension, if in low concentration, dissolves on the addition of NaCl. However, on cautiously adding $^1/_{1000}$ *N* NaOH to an insulin colloid the colloidal suspension remained intact at *p*H 7.5. Insulin OU can be crystallized readily.

It is of interest to note that the solubility of crystalline insulin in 40% urea at 25° exceeds 50 mg. per ml. on equilibrating only 15 minutes. When such a solution is diluted with water to 20% urea concentration, the solubility of insulin falls to 6.8 mg. per ml. On the other hand the solubility of insulin in 20% urea obtained by equilibration with the solid crystalline phase is only 1.4 mg. per ml. (same value at 5 and 30 minutes).

The reprecipitation of the insulin from urea solution by water is abolished after a proper interval of reaction time and at a proper reaction temperature. After 141 hours at 3° the precipitate still forms, while at 55° it no longer forms after 1.5 hours. The failure of the precipitate or colloid to form is not due to a change in *p*H, which has been very carefully controlled, but must be regarded as a change in the insulin molecule (IU) brought about by the urea. The reaction may be studied semi-quantitatively by reading the optical density of the aqueous suspension at different time intervals. Thus at 38° the optical density at 8 hours is one-fourth what it was at 10 minutes.

A phenomenon was encountered, which has not been further investigated, namely, that at 55° the 40% urea solution of insulin turns opalescent after 20 minutes at this temperature and subsequently clarifies.

(4) F. Bischoff, *Am. J. Physiol.*, **117**, 182 (1936).

(5) B. B. Longwell and A. Ravin, *ibid.*, **117**, 453 (1936).

(6) F. Bischoff, *ibid.*, **168**, 37 (1952); U. S. Patent 2,704,737 (March 22, 1955).

(7) M. Somogyi, *J. Biol. Chem.*, **195**, 19 (1952).

Bioassay of Insulin OU.—Insulin OU when dissolved and administered at *p*H 3.0 was found to have the same hypoglycemic response as regular amorphous or crystalline insulin solutions administered at the same *p*H (cross matches in 12 rabbits, intramuscular route). However, when administered as a washed suspension of the original precipitate a delay in hypoglycemic response comparable to that produced by a suspension of regular insulin crystals was observed. Thus when given intramuscularly a suspension of insulin crystals produced, respectively, a 44 ± 7 and 16 ± 8 mg. per 100 ml. lower blood sugar the 6th and 9th hours after dosage than the regular soluble insulin solution. On a comparable basis the suspension of insulin OU produced 54 ± 4 and 19 ± 8 mg. per 100 ml. lower blood sugars. It should be emphasized that the precipitate of insulin OU as administered appeared amorphous.

Solubility of Insulin OU.—Solubility determinations of the 0 hour urea insulin (insulin OU) are given in Table I. In the case in which both crystalline and amorphous insulins were used as the starting materials 0.25% phosphate or acetate greatly increased the solubility in water or 1.5% glycerol. In the case of the material made from crystalline insulin the solubility in water and in 1.5% glycerol is less than 0.07 mg. per ml., because this concentration contained some colloid. The solubility would be less than 1 biologic unit per ml. At *p*H 7.3, on equilibrating 48 hours the solubility in 1.5% glycerol, 0.05% acetate was quite high, 0.46 mg. per ml. On re-equilibrating the insoluble residue another 48 hours at the same *p*H the solubility fell to 0.03 mg. per ml. This proves that the insulin in the 0 hour precipitate is not homogeneous. Seventy per cent. of the insulin has the low solubility value.

TABLE I

SOLUBILITY OF INSULINS OU AND IIU AT 22–24°

Insulin	Solvent	*p*H	Solubility, mg./ml.
OU (from amorphous insulin)	2% urea	6.0	0.06
	Water	6.0	0.07
	1.5% glycerol	6.1	0.12
	1.5% glycerol	7.0	0.06
	0.25% Na acetate	6.7	>1.0
	0.25% Na phosphate	6.9	>1.0
OU (from crystalline insulin)	Water	6.9	<0.07[a]
	1.5% glycerol	6.7	<0.08[a]
	0.25% Na acetate	7.0	0.53
	0.25% Na phosphate	7.0	>1.2
	1.5% glycerol, 0.05% Na acetate	7.3	0.46
	1.5% glycerol, 0.05% Na acetate[b]	7.3	0.03
IIU	0.01 HCl	2.0	Over 1.0
	0.03 *M* citric, 2.2% urea	2.0	Over 1.4
	H_2O	6.1	Over 0.6
	0.9% NaCl	6.2	Over 1.0
	0.9% NaCl	2.5	Precipitated
	2.2% urea, 0.02 *M* citric, 0.03 *N* Na_2SO_4	2.7	0.058
	2.2% urea, 0.02 *M* citric, 0.03 *N* Na_2SO_4	2.9	.015
	4.4% urea, 0.02 *M* citric, 0.03 *N* Na_2SO_4	3.0	.090
	0.020 *M* citric, 0.03 *N* Na_2SO_4	2.57	.018
	0.020 *M* citric, 0.03 *N* Na_2SO_4	3.05	.016

[a] Colloid. [b] Re-equilibration of solute.

Solubility and Preparation of Insulin IU.—Insulin IU can be isolated by allowing the reaction in 40% urea to proceed to the point where all the insulin OU has disappeared and the minimum amount of insulin IIU has formed. Twenty-four hours at 38° or 1.5 hours at 55° would be suitable. Insulin IIU is removed by precipitating in 0.03 *N* Na_2SO_4 concentration in the *p*H range 2.7–3.0. To 0.5 part 40% urea insulin solution is added 4.5 parts water, 2 parts 0.1 *M* citric acid and 3 parts 0.1 *N* Na_2SO_4 solution. In the concentration of urea attained, 2.2%, the solubility of IU exceeds 1.7 mg. per ml. Insulin IU is separated from the sulfate filtrate by isoelectric precipitation and redissolved in dilute citric acid (volumes reduced by one-third) and again subjected to a concentration of 0.03 *N* Na_2SO_4. This removes all but a trace of the 0.05 mg. per ml. of insulin IIU which is the solubility of this insulin in 0.03 *N* Na_2SO_4 and 2.2% urea. The solubility of insulin IU in 0.03 *N*

TABLE II

FORMATION OF INSULINS OU, IU, IIU, IIIU AND IVU IN 40% AQUEOUS UREA SOLUTION[a]

T °C.	Hour	OU,[b] %	IU,[c] %	k (sec.$^{-1}$) × 10^3	IIU,[d] %	k (sec.$^{-1}$) × 10^4	IIIU,[e] %	IVU,[f] %
3	141	+			<5			
24	24	69	31	0.42	<5			
	72	+			10	0.0039		
	141	−			19	0.0042		
38	0	98	−					
	3	47	53	6.9				
	6	34	66	5.0				
	8	22	78	5.3	<5			
	12	6	78	6.4	16	0.040		
	22	−	70		30	.044		
	48	−			66	.061		
	70	−			79	.061	−	
	141	−			94	.056	+	
	336	−			40		30	(30)
55	1.5	−	79		21	0.44	−	
	3.0	−			44	.56	−	
	5.0	−			68	.64	−	
99	0.08				84	61	−	
	0.25				59		32	(9)
	0.5				27		44	29

[a] 20 mg. crystalline Armour insulin per ml. 40% urea. [b] Insulin OU was determined by diluting the urea solution with water, 1 to 10. The optical density was compared with a 0 hour standard, using a clear solution as the blank. [c] Insulin IU was the difference between insulin OU and IIU. [d] Insulin IIU was determined by dilution of the reaction mixture with water 1 to 10, and precipitation as the sulfate in a 1 to 20 dilution, buffered with citric acid to give a *p*H 2.7 to 2.9 and a 0.03 *N* sulfate concentration. A correction was made for solubility in this environment. Insulin in the filtrate was determined by the Folin–Ciocalteu reaction. The sulfate precipitate is soluble in 0.02 *N* HCl. [e] Insulin IIIU is the sulfate precipitate insoluble in 0.02 *N* HCl, and determined by the Folin–Ciocalteu reaction. [f] Insulin IVU was the biologically inactive fraction in the sulfate filtrate.

Na_2SO_4, *p*H 2.8, was found to be 0.8 mg. per ml. It is thus 40 times as soluble as insulin IIU under comparable conditions.

Step III, Insulin IIU.—The reaction in 40% urea solution forms a product which is soluble in dilute acid (0.01 *N* HCl or 0.03 *M* citric) in the *p*H range 2.0 to 3.0, but is precipitated by a concentration of 0.03 *N* Na_2SO_4 and by 0.9% NaCl. The solubility of the sulfate precipitate in water is quite low, 0.018 mg. per ml. at *p*H 2.57 and is increased by a 2.2% concentration of urea to 0.058 mg. per ml. at *p*H 2.7. Solubility data are given in Table I.

The formation of insulin IIU with time and temperature is given in Table II. Determinations were made of the insulin remaining in the sulfate filtrate, and also as a check in some instances of the precipitate. A correction for solubility is made for the data presented in Table II.

Insulin IIU is formed in high yields by heating the 40% urea solution 5 minutes at 99° or 100 hours at 38°. It is separated from insulins OU and IU by its insolubility in 0.03 *N* Na_2SO_4, *p*H 2.7-3.0, and from insulin IIIU by its solubility in 0.02 *N* HCl.

The biologic response of insulin IIU is given in Table III, exp. I, demonstrating a significant delayed response even on intravenous injection.

Detailed information on the biologic response of preparations containing 80 to 90% of this product has been published.[6] It should be stressed that the 25 mg. per 100 ml. difference in blood sugar on intravenous injection of insulin IIU or preparations containing 80 to 90% of IIU is highly significant by statistical analysis and that such a difference is equivalent to increasing the dose of native insulin 50%.

Step IV, Insulin IIIU.—When the treatment in concentrated urea solution is prolonged, a fraction of the insulin forms which is insoluble in dilute citric acid or HCl, *p*H 2 to 3. This property distinguishes it from the fraction which is soluble in dilute citric or hydrochloric acid but is precipitated by 0.03 *N* Na_2SO_4 in the acid range, and also from regular insulin. The dilute acid insoluble insulin is soluble in dilute $NaHCO_3$ solution and remains soluble on adding dilute HCl to *p*H 6.0. The formation of this fraction is best illustrated by heating a 2% insulin solution in 40% urea at 99° for 30 minutes, separating it, IIIU, and insulin IIU as the sulfate. IIU is then separated by solution in 0.02 *N* HCl.

The dilute acid insoluble insulin is further characterized by an apparent partial loss in biological activity, with a delayed action effect. See Table III, exp. II.

Regular insulin on standing in solution for long periods of time also forms an insoluble sulfate at *p*H 3.0. This fraction does not, however, show a delayed biologic response. See Table III, expt. III.

TABLE III

BIOASSAY OF UREA-TREATED INSULINS AND A 20-YEAR OLD REGULAR INSULIN SOLUTION BY THE INTRAVENOUS INJECTION ROUTE WITH CROSS MATCHING IN 10 TO 12 RABBITS

Exp.	Insulin source	Blood sugar in mg./100 ml. 1.5 hr.	3.0 hr.	4.5 hr.	5.0 hr.	6.0–7.5 hr.
I	Regular	40	60	89		103[a]
	Insulin *IIU*	44	50	65		93
		−4 ± 3.8	10 ± 3.3	24 ± 6.5		10 ± 4.3
II	Regular	46		95		102[b]
	Insulin IIIU, double dose	55		75		87
		−9 ± 2		20 ± 4.4		15 ± 6
III	Regular	30	67		88	
	20-yr. old insulin, 0.03 *N* sulfate ppt., double dose	34	65		88	
		−4 ± 2.0	+2 ± 6.4		0 ± 1.8	
IV	Regular	24	56		89	
	Heat ppt. dissolved in 40% urea	31	47		83	
		−7 ± 1.8	+9 ± 2.3		+6 ± 3.4	
V	Insulin IIU	28	40		81	
	Insulin IIU heated 50 min. at 99°	31	45		81	
		−3 ± 0.8	−5 ± 2.1		0 ± 3	

[a] 6 hours. [b] 7.5 hours.

Step V, Insulin IVU.—It will be noted in Table II that the sum of the changed insulin fractions at 15 minutes 99° is 91% and at 30 minutes 99° is only 71%; the decrease in the sulfate insoluble insulin IIU is 32%, while the increase in the dilute acid insoluble insulin is only 12%. This change is not due to the re-formation of regular insulin, because the acid soluble, sulfate soluble fraction has now lost more than 75% of its biologic activity. In the bioassay this insulin was compared with $^1/_4$ to $^1/_5$ its equivalent of regular insulin. At 1.5 hours, regular insulin produced a blood sugar of 60, 4 to 5 times this dose of the reaction product produced a blood sugar of 87. At 5 hours the sugar values were in the normal fasting range indicating no delayed action. This fourth type reaction product cannot be ascribed directly to the effect of the urea for on heating aqueous urea at 99° for 30 minutes the *p*H exceeds 8.5 due to the formation of ammonia and the inactivation may therefore be the result of the well known inactivation of insulin in alkaline solution. The point to be noticed is that the urea reaction products do not escape this fate.

Heat Precipitate.—The heat precipitate of insulin, which was discovered[8] in this Laboratory by accident, has been the subject of intensive investigation. It may be regarded as the stable form of insulin to which the biologically active instable form gradually reverts at room temperature, more rapidly in acid solution at higher temperatures. Waugh[9] has shown the heat precipitate is made up of fibrils in the form of spherites. At room temperature the spherites go to the fibrils at *p*H 11.0–11.5 and the fibrils break up above *p*H 12.0. The heat precipitate is completely devoid of biologic activity, which is regained by resolution at *p*H 12.0. The biologic activity is also regained by solution in 20% HCl,[10] and in phenol[11] and as we have found in the present study in 40% urea at 99°.

Influence of Heat on Urea-treated Insulin (Insulin IIU).—The washed sulfate was dissolved in 0.02 *N* HCl (1.2 mg./ml.) and heated for 50 minutes at 99°. No heat precipitate formed and there was no change in biologic activity (see Table III, exp. V). In 2.5 hours, however, 43% precipitated with no change in *p*H and in 8 hours 85% was precipitated. Ninety per cent. of this precipitate was soluble at *p*H 8.4 in dilute $NaHCO_3$ solution, differentiating it from the classic heat precipitate of regular insulin, which does not dissolve until a *p*H of 12 is reached. In another experiment in which crystalline insulin, *p*H 2.1, 1 mg./ml., was compared with the urea-treated insulin under strictly comparable conditions, the results on heating at 99° for three hours follow. Both the regular and urea-treated insulins formed 25% heat precipitate, which was insoluble, *p*H 8.7, soluble in *N*/10 NaOH. In addition, the urea-treated insulin formed 51% precipitate, which was soluble at *p*H 8.7, the regular insulin formed 4%. While the 4% could conceivably be occluded regular insulin, it is unlikely that 50% of the urea-treated insulin could be occluded by 25% precipitate. In each case the original heat precipitate was washed twice with water before solution in $NaHCO_3$. The $NaHCO_3$ soluble heat precipitate was not devoid of biologic activity.

Influence of Urea on the Heat Precipitate.—The heat precipitate was formed by dissolving 25 mg. of crystalline insulin in 5 ml. of 0.1*N* H_2SO_4, *p*H 1.3, and heating 30 minutes at 99°. The precipitate was separated by centrifugation and washed once with water. The precipitate remained insoluble in 40% urea solution after 7 days at 12° or after 2 hours at 50°, but dissolved at 99° in 10 minutes. The original heat precipitate was devoid of biologic activity when given to four rabbits, two of which received a double dose. After solution in 40% urea, the original biologic activity was restored with evidence of formation of some delayed acting insulin. See Table III, exp. IV.

Discussion

In the present state of knowledge it cannot be deduced whether insulin OU has suffered a change in molecular rearrangement or whether its insolubility in water at a neutral *p*H is due to a somewhat stable precipitate of native insulin. The insolubility and hence delayed action of the precipitate cannot be explained on the basis of crystalline form or zinc content. The precipitate was amorphous and the zinc content was less than 0.5% in all the starting material. The higher solubility in 20% urea solution further distinguishes it from crystalline insulin.

While the solubility properties of and the biologic response to insulin IIU are well characterized, the mechanism by which a delayed hypoglycemic response is produced is not established. It is not a depot insulin, on the basis of its high solubility at *p*H 7.4, and the fact that it produces a delayed action on intravenous injection. Insulin IIU should be of interest in its relation to the formation of anti-bodies and anti-insulin as compared with regular insulin and clinically to that type of diabetes typified by a resistance to regular insulin. That the molecule has suffered a change in orientation is amply demonstrated by the kinetic studies.

The reaction rates for the formation of insulins IU and IIU from insulin OU are calculated as a first-order reaction and given in Table II. The maximum variation would be accountable by an over-all 5% error in the analytical procedures, which would be reasonable for the micro analytical technique used. The result at 99° should be regarded as semi-quantitative, since the short time at this temperature does not allow for the initial period required to reach temperature equilibrium.

ΔE, the energy of activation, may be calculated by the Arrhenius equation from the above reaction rates.

$$\Delta E = \frac{4.575 T_1 T_2}{T_2 - T_1} \log \frac{k_2}{k_1}$$

ΔE for insulin IU is estimated as 34,750 cal. per mole for the temperature range 24–38°.

ΔE for insulin IIU is estimated as 33,800 cal. per mole for the temperature range 24–38° and 30,800 cal. per mole for the temperature range 24–55°.

The heat of activation, ΔH, may be calculated from the relation

$$\Delta H = \Delta E + RT$$

and the free energy of activation from the Eyring equation

$$k = \kappa \frac{T}{h} e^{-\Delta F/RT}$$

where k is the reaction rate, κ is Boltzmann's constant, and h is Planck's constant.

$$\Delta F = \Delta H - T\Delta S$$

The free energy of activation, ΔF, for insulin IU is calculated as 24,300 cal. per mole at 38°, and the entropy change of activation, ΔS, as 36 cal. per degree per mole.

The free energy of activation, ΔF, for insulin IIU is calculated as 25,800 cal. per mole at 38° and the entropy change of activation, ΔS, is 18–27 cal. per degree per mole.

The free energy of activation already has been determined for other protein hormone reactions in 40% urea solution.[12] For the mare serum gonadotropin, the chorionic gonadotropin, and its

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

(9) D. T. Waugh, THIS JOURNAL, **68**, 247 (1946).

(10) V. du Vigneaud, *J. Biol. Chem.*, **92**, liv (1931).

(11) J. Lens, *ibid.*, **169**, 313 (1947).

(12) F. Bischoff, *ibid.*, **165**, 399 (1946).

two reaction products, ΔF at 37° was, respectively, 25,200, 22,800, 25,300 and 27,400 cal. per mole. These figures are of the same order as that obtained for the formation of insulins IU and IIU in the present studies. For these gonadotropins the respective entropy changes of activation varied from +133 to −17 cal. per degree per mole. The energy changes in the urea insulin reaction are therefore akin to those found for the other protein hormones studied. In the case of the mare serum gonadotropin, the reaction product was devoid of biologic activity, while in the case of the chorionic gonadotropin a product of decreased biologic activity was obtained. The qualitative biologic response of the latter compound was unchanged. If the urea reaction is an unfolding of the protein molecular chain, biologic activity must be dependent upon the spacing of active groups in relation to each other.

Waugh, *et al.*,[13] studied the ability of insulin to form fibrils or crystals after solution in concentrated urea solution (8 *M*) with subsequent recovery. Two of their experiments cover the approximate temperature range, *p*H and concentrations used in our experiments. Waugh, *et al.*, found that 24 hours at 0°, *p*H 7.0, abolished neither the ability to form fibrils nor form crystals. We found that 141 hours at 3° produced no measurable amount of insulin IIU, and that insulin OU remained intact. Waugh, *et al.*, found that 24 hours at 37° abolished completely the ability to crystallize but not to form fibrils. We found that under these conditions insulin OU has disappeared and 30% of insulin IIU has appeared. The failure to crystallize must therefore appear as a property of insulins IU and IIU, and the ability to crystallize is retained by insulin OU. The ability of insulin IIU to form fibrils and of insulin OU to crystallize has been observed in this Laboratory.

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

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, ILLINOIS INSTITUTE OF TECHNOLOGY]

Infrared Investigation of Bound Water in Hydrates

BY PETER J. LUCCHESI AND WILLIAM A. GLASSON

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The infrared absorption spectrum of water bound in crystalline salt hydrates has been studied for a variety of systems in the region 4000 to 800 cm.$^{-1}$, by the KBr pellet technique. The positions of the fundamental absorption bands of water were found to vary slightly with the nature of the hydrate. The variation appears to be different for each of the three bands studied. The effects of the salt on the 3425 cm.$^{-1}$ band are in most cases reversed with respect to the 1616 cm.$^{-1}$ bands whereas the position of the absorption in the 2915 cm.$^{-1}$ region is not significantly altered.

Introduction

Several studies have been reported on the Raman[1] and infrared[2-5] spectra of hydrates, both in solution and in the solid phase. The positions of the absorption bands of water in such studies have been used to gain insight into the nature and strength of binding of the water in the hydrate.[6-8] With the development of the KBr pellet technique by Stimson[9] and Schiedt,[10] it has become possible to measure infrared spectra of solid samples in pressed KBr pellets with elimination of scattering losses and background interference, and with a considerably improved separation and sharpness of bands. In the present study, this technique was employed to measure the infrared absorption spectrum of various salt hydrates and to determine whether any systematic shifts in the water bands could be observed with changing nature of the salt.

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Experimental

Materials and Apparatus.—All salts and salt hydrates were of AR grade. Pressed KBr pellets of these materials, at the weight concentrations listed in Table I, were prepared with the Perkin-Elmer evacuable dye and a Loomis 20-ton hydraulic press. Spectra were recorded on the Perkin-Elmer Model 21 Recording Spectrophotometer, with the pellets mounted on the pellet holder and microcell adapter supplied by the manufacturer. All spectra were measured with CaF_2 optics and wave length calibrations were made using the known bands of water vapor and CO_2.

Sample Preparation.—Since some of the materials are extremely hygroscopic, it was necessary to work under conditions as dry as could be obtained. The pellets were pressed under vacuum for two minutes under 23,000 pounds total load. All materials were sifted through a 300-mesh screen. All sampling and mixing operations were carried out in a moisture free dry box in an atmosphere of dry nitrogen. Thus, the pellets were actually exposed to atmospheric water vapor only during the time required to scan the spectrum. Repeating the scanning showed that during this time the intensity or position of the water bands was not altered significantly.

It was found that, provided 300-mesh material was used throughout, the position of the bands could be reproduced within the limits given in the next section. Within these limits, the positions could be reproduced for different periods of grinding and mixing. However, this reproducibility could only be obtained when the samples were prepared under anhydrous conditions.