2-amino-4-chlorothiophenol was dissolved in pyridine and one mole of aldehyde added dropwise to the warm solution. The mixture was finally heated from two to thirty minutes on the water-bath, the aromatic and the higher aliphatic aldehydes requiring the longer heating time.[9] The benzothiazoline was precipitated by acidifying the mixture; yields, 70–90%.

Oxidation of Benzothiazolines to Benzothiazoles.—The alkyl benzothiazolines could be crystallized unchanged from various solvents. 2-Phenyl-5-chlorobenzothiazoline, however, was converted to the benzothiazole upon two or three crystallizations from alcohol. Similarly 2-(*o*-chlorophenyl)-5-chlorobenzothiazoline was converted to the benzothiazole upon crystallization from acetone or carbon tetrachloride. Any of the benzothiazolines are oxidized to the benzothiazole in good yield by warming for a few minutes with a slight excess of ferric chloride in alcohol.

Summary

1. Aldehydes condense with 2-amino-4-chlorothiophenol to give benzothiazolines.
2. Benzothiazolines are readily oxidized to benzothiazoles.

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[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

THE MOLECULAR WEIGHT OF INSULIN

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The eminent physiological importance of the pancreas hormone insulin has in recent years, and especially since the isolation of crystalline insulin by Abel,[1] made it the subject of numerous chemical investigations. The experimental material so far collected decidedly indicates that insulin is of protein nature.[2] It gives several of the characteristic protein reactions,[3] its empirical composition resembles that of the proteins,[3] it is an amphoteric electrolyte possessing an isoelectric point at about P_H 5,[3] which is in the same region where the isoelectric points of many proteins are situated and it shows a light absorption in the ultraviolet at exactly the same place as most of the proteins with a maximum at 270 $m\mu$.[4]

At the suggestion of Dr. H. Jensen of The Johns Hopkins University, Baltimore, we have undertaken an ultracentrifugal study of insulin along the same lines as already followed in this Laboratory for the determination

[9] The formaldehyde employed was a 30–35% aqueous solution; the other aldehydes used were dried in the process of purification.

[1] J. J. Abel, *Proc. Nat. Acad. Sci.*, **12**, 132 (1926).

[2] H. Jensen and A. M. De Lawder, *Z. physiol. Chem.*, **190**, 262 (1930).

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

[4] W. Graubner, *Z. Ges. Exp. Medizin*, **63**, 527 (1928).

of molecular weight and PH-stability region of a considerable number of proteins.[5]

Material Used.—A quantity of 0.25 g. of crystalline insulin was kindly put at our disposal by Dr. Jensen and this small sample proved quite sufficient for a rather complete ultracentrifugal study. According to Dr. Jensen the material was prepared from commercial beef pancreas insulin of Squibb and Sons by the usual method of crystallization. The first crystalline product obtained had been recrystallized twice without the addition of brucine.

Specific Volume.—For the calculation of the molecular weight from ultracentrifugal data it is necessary to know the partial specific volume of the substance in solution. Two determinations were carried out at 20°, as described in previous communications. The data are given in Table I.

TABLE I

PARTIAL SPECIFIC VOLUME OF INSULIN AT 20°

Insulin concn., %	Solvent	PH of soln.	Partial sp. vol.
0.90	0.067 *M* in Na_2HPO_4	8.1	0.748
.45	.033 *M* in Na_2HPO_4 .033 *M* in KH_2PO_4	6.8	.750

The solution of *P*H 6.8 was prepared from that of *P*H 8.1 by addition of KH_2PO_4. The two determinations give the same result within the limits of error. As shown by the ultracentrifugal study insulin is (reversibly) dissociated at *P*H 8.1. This circumstance therefore does not influence the specific volume. A similar behavior has already been observed for several proteins.

The mean value 0.749 of the specific volume of insulin is the same as that of almost all the proteins so far studied in this Laboratory.

Determination of Sedimentation Constant and *P*H Stability Region.—The high-speed oil turbine ultracentrifuge was used for measuring the sedimentation constant in the *P*H range 3.5 to 12.3. The temperature of the solution was between 20 and 23° during centrifuging, the time varied from three to four hours and the speed was about 40,000 r. p. m. in all the runs. The insulin solutions were prepared from the dry material immediately before starting a run. In the *P*H region 6.4 to 7.4 where it is comparatively difficult to bring insulin into solution, the dry material was first dissolved by means of Na_2HPO_4 and then a suitable amount of KH_2PO_4 added. The solution of *P*H 4.2 was made up by dissolving insulin in acetic acid and adding sodium acetate. The determinations are summarized in Table II.

The sedimentation is independent of *P*H from about 4.5 to about 7.0 and has a mean value of 3.47×10^{-13}. This *P*H range therefore represents the stability region of insulin. Special ultracentrifugal tests showed that

[5] Svedberg and co-workers, THIS JOURNAL, 48–52 (1926–1930).

TABLE II

SEDIMENTATION VELOCITY MEASUREMENTS ON INSULIN

No.	Solvent, *M* HAc	NaAc	KH_2PO_4	Na_2HPO_4	NaOH	*P*H of soln.	Concn. of insulin, %	$s_{20°} \times 10^{13}$
1[a]	0.1	..	..	..	..	3.5	0.16	1.27
2[b]	.074	0.026	..	..	..	4.2	.14	3.10
3	..	..	0.086	0.047	..	6.4	.12	3.46
4	..	..	.080	.053	..	6.6	.22	3.41
5	..	..	.080	.053	..	6.6	.20	3.51
6	..	..	.060	.073	..	6.7	.32	3.55
7	..	..	.067	.067	..	6.8	.19	3.43
8[c]	..	..	.053	.080	..	7.0	.25	3.21
9[d]	..	..	.027	.107	..	7.4	.17	2.84
10[e]	..	..	..	.133	..	8.1	.20	2.87
11[f]	..	..	..	.032	0.001	8.9	.16	2.93
12[g]	..	..	..	..	.025	12.3	.30	0.73

[a] 20% of non-centrifugible products. [b] 10% non-centrifugible products. [c] 5% of non-centrifugible products. [d,e] 8% of non-centrifugible products. [f] 10% of non-centrifugible products; solution 1% NaCl. [g] 50–55% of non-centrifugible products; solution 1.5% in NaCl.

the dissociation of the insulin molecule into products of low molecular weight, as demonstrated by the rapid fall in the sedimentation constant outside this range, is reversible both on the acid and the alkaline side, if the *P*H of the solution is not too far removed from the stability region and provided that the solution is not kept too long outside the stability region. It is of interest to note that similar experiments have been made regarding the physiological activity of insulin. This fact seems to indicate that the activity is destroyed when the dissociation products lose their ability to reconstruct the insulin molecule.

The sedimentation constant is within the limits of experimental error identical with the value previously found for egg albumin[6] and for Bence-Jones protein,[7] *viz.*, 3.54×10^{-13} and 3.55×10^{-13}, respectively. The *P*H stability range is decidedly narrower for insulin than for egg albumin and for Bence-Jones protein. In the case of insulin it has an extension of only about 2.5 *P*H units, while egg albumin is stable over 5 *P*H units (*P*H 4 to 9) and Bence-Jones protein over 4 units (*P*H 3.5 to 7.5). The isoelectric point of insulin is situated at a higher *P*H than that of egg albumin. The very low solubility of insulin near the isoelectric point has not permitted the determination of this constant accurately enough to allow of a comparison with Bence-Jones protein which, as a matter of fact, has its isoelectric point situated in the same region as insulin (*P*H 5.2).

Determination of Molecular Weight.—When the sedimentation equilibrium method is used, the molecular weight M is given by the relation

[6] T. Svedberg and J. B. Nichols, THIS JOURNAL, **48**, 3081 (1926); B. Sjögren and T. Svedberg, *ibid.*, **52**, 5187 (1930).

[7] T. Svedberg and B. Sjögren, *ibid.*, **51**, 3594 (1929).

$$M = \frac{2RT \ln (c_2/c_1)}{(1 - V\rho)\omega^2 (x_2^2 - x_1^2)}$$

where R is the gas constant, T the absolute temperature, V the partial specific volume of the solute, ρ the density of the solvent, ω the angular velocity and c_2 and c_1 are the concentrations at the distances x_2 and x_1 from the center of rotation.

Three equilibrium runs were performed within the stability region of insulin at a temperature of 20.1° and a speed of 10,500–11,000 r. p. m. Table III gives the complete data of a typical run and in Table IV are summarized the results of the three runs.

The mean value for the molecular weight 35,100 is within the limits of error identical with the values previously obtained for egg albumin[6] and Bence-Jones protein,[7] *viz.*, 34,500 and 35,000, respectively. Table III shows that there is no drift in the molecular weight values with distance from the center of rotation and that accordingly insulin is homogeneous with regard to molecular weight.

TABLE III

SEDIMENTATION EQUILIBRIUM RUN ON INSULIN

Concn., 0.10%; phosphate buffer, P_H 6.8 (0.067 M in KH_2PO_4, 0.067 M in Na_2HPO_4); V = 0.750; ρ = 1.012; T = 293.2; length of col. of soln., 0.47 cm.; thickness of col., 0.80 cm.; dist. of outer end of soln., from axis of rotation, 5.95 cm.; speed, 10,500 r. p. m. ($\omega = 350\pi$); light absorption standard, M/1200 in K_2CrO_4, with a thickness of layer of 0.40 cm.; source of light, mercury lamp; light filters, chlorine and bromine; aperture of objective, F:36; plates, Imperial Process; time of exposure, 30, 60 and 90 seconds; exposure made after 31, 36 and 44 hours of centrifuging

Distances, cm. x_2	x_1	Mean concn., % c_2	c_1	Numbers of exposures	Mol. wt.
5.88	5.83	0.110	0.097	10	35,900
5.83	5.78	.097	.086	10	34,600
5.78	5.73	.086	.076	10	35,900
5.73	5.68	.076	.067	10	36,900
5.68	5.63	.067	.059	8	37,600
				Mean	36,200

TABLE IV

SUMMARY OF SEDIMENTATION EQUILIBRIUM MEASUREMENTS ON INSULIN

Solvent, M KH_2PO_4	Na_2HPO_4	P_H of soln.	Concn. of insulin at start, %	Mol. wt.
0.060	0.073	6.7	0.10	33,900
.067	.067	6.8	.20	35,200
.067	.067	6.8	.10	36,200
			Mean	35,100

By means of the values for the molecular weight, M = 35,100, and the sedimentation constant, $s = 3.47 \times 10^{-13}$, we are now able to deduce the value for the molar frictional constant, $f = [M(1 - V\rho)]/s$, and find 2.54×10^{16}. The calculation of the molar frictional constant of a spherical molecule of the same molecular weight and the same specific volume as insulin

calculated from the formula $f_s = 6\pi\eta N(3MV/4\pi N)^{1/3}$, where η is the viscosity of the solvent, gives the same value. Accordingly the insulin molecule must be regarded as spherical. The radius, calculated from the formula $r = (3MV/4\pi N)^{1/3}$, is 2.18 mμ. According to previous determinations[6,7] the egg albumin molecules and the Bence-Jones protein molecules are also spherical in shape with values for the radius of 2.17 and 2.18 mμ, respectively.

The fact borne out by the above investigation that the molecules of crystalline insulin are, with regard to mass, shape and size, almost identical with those of egg albumin and Bence-Jones protein and that, within the P_H region 4.5–7.0, the insulin solutions are built up of molecules of equal mass, strongly support the view that insulin is a well-defined protein. It has a P_H-stability region which is decidedly narrower than the stability regions for egg albumin and Bence-Jones protein, it has a much lower solubility and also in other respects differs chemically from those two proteins. These circumstances seem to indicate that we are justified in assuming that the physiologically active principle is not a low-molecular substance accidentally adsorbed on some high-molecular material but is the insulin molecule itself or some special group within it.[8]

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Summary

1. The molecular weight and P_H stability region of insulin have been determined by means of ultracentrifugal methods.

2. Insulin is stable from a P_H of about 4.5 to about 7.0 with a molecular weight of 35,100. At lower and higher P_H values the insulin molecule is broken up into smaller units. Near the borders of the stability region this dissociation is reversible.

3. Within the stability region the sedimentation constant of insulin is 3.47×10^{-13} and the molar frictional constant 2.54×10^{16}. The molecules are spherical with a radius of 2.18 mμ.

4. The molecular weight, sedimentation constant, molar frictional constant and molecular radius of insulin are within the limits of error identical with the corresponding constants for egg albumin and Bence-Jones protein. This circumstance makes it extremely probable that insulin is a well-defined protein and that the physiological activity of this hormone is a property of the insulin molecule itself or some special group within it.

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[8] Compare H. Jensen and A. M. De Lawder, Ref. 2.