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On the possible iodometric estimation of insulin.

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It is generally agreed that insulin is destroyed by trypsin and other proteolytic enzymes. Recently,[1] we suggested that the physiologically inactive addition compound, which is formed between insulin and trypsin before the digestion of insulin starts, is probably the so-called intermediate compound between enzyme and substrate. Up to the present, conclusions as to the existence or non-existence of the enzyme-substrate compound have been drawn almost exclusively[2] from the analysis of kinetic studies. Further progress in the investigation of the insulin-enzyme reaction depends in part on the development of a suitable method for the chemical estimation of insulin.

Certain earlier observations[3] induced us to attempt an iodome-

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[1] Brand, E., and Sandberg, M., PROC. SOC. EXP. BIOL. AND MED., 1925, xxii, 428.

[2] *Cf.* Bergmann, M., *Liebigs Ann.*, 1925, cdiliii, 223.

[3] In 1922, one of us (B.) carried out with Dr. Benno Brahn some experiments on the iodometric estimation of epinephrin in acid solution. We shall report on these studies in the near future.

tric estimation of insulin. Our present knowledge of the chemical constitution of insulin is not opposed to the feasibility of such a method. We know from studies on the iodization of proteins[4] that iodine reacts with various groups of the protein molecule, among others with the lead-blackening sulphur. The presence of lead-blackening sulphur in the molecule of insulin seems to have been demonstrated by Abel and Geiling.[5] Whether the insulin molecule contains other groups that react with iodine cannot yet be decided. It has been recommended that the iodometric estimation of certain inorganic groups be carried out in the presence of neutral buffer. We have noticed that phenols, glucose, cyclic aminoacids, and other substances, which are supposed to react with I_2-KI solution only at an alkaline pH, take up iodine also in the presence of neutral buffer.[6]

For the iodization and iodometric estimation of organic compounds the use of neutral-buffered I_2-KI solution offers an additional method, which may be of considerable value both preparatively and analytically. It will also be interesting to study bromine oxidations by using bromine-neutral buffer instead of bromine-alkali or bromine-alkali carbonate.

In acid solution, insulin (20 units per mg.) immediately forms a reddish brown voluminous precipitate with I_2-KI solution (formation of a periodide?). In neutral-buffered or alkaline solution no precipitation takes place at first. After standing for some time and with slight heating a yellow precipitate appears. After iodization in neutral-buffered or alkaline solution the test for lead-blackening sulphur is no longer positive.

An iodometric estimation of insulin can be attempted successfully only with material of a certain degree of purity. At lower stages of purification, groups that are present, though not belonging to insulin proper, will also absorb iodine. The majority of the preparations we used seem to contain approximately 15 to 20 units of insulin per mg. The iodine value of an older preparation containing only 8 units per mg., however, is irregular and far too high.

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[4] *Cf.* Blum, F., and Strauss, E., *Z. physiol. Chem.*, 1921, cxii, 111.

[5] Abel, J. J., and Geiling, E. M. K., *J. Pharmacol. and Exp. Therap.*, 1925, xxv, 422. *Cf.* Taylor, T. C., Braun, C. E., and Scott, E. L., *Am. J. Physiol.*, 1925, lxxiv, 539.

[6] We shall report on iodization and iodometric estimation of organic compounds in the presence of neutral buffer in another publication.

The method consists of permitting an excess of iodine (20 cc. of 0.01 N iodine solution) to react with insulin (twice precipitated at the isoelectric point in order to remove the preservative) in the presence of a neutral buffer (M/5 phosphate buffer pH=6.8) under standard conditions (17 hours, 37° C.), and estimating by titration (in 0.1 normal acid solution) the amount of iodine used. We find that the amount of iodine consumed by the insulin is directly proportional to the number of units present. Different batches of insulin from different manufacturers require approximately the same amount of iodine for an equal number of units. The iodine values of different preparations of insulin agree, as closely as can be expected, from material which has been standardized physiologically. Forty clinical units of insulin use 2.05 (±0.05) cc. of 0.01 N iodine solution, but this cannot yet be considered as the final value.

The iodine value of insulin at an alkaline reaction is higher than in neutral-buffered solution. But the alkaline iodine value

Insulin Preparation	cc. 0.01 N iodine.					
	pH = 6.8 (3 cc. M/5 phosphate buffer)			alkaline (1 cc. 2N NaOH)		
	20 units	40 units	60 units	20 units	40 units	60 units
Squibb U 20 Lot 29376	1.15	2.30	3.35	1.80	3.20	—
Lilly U 20 Lot 774002 16.7 units per mg.	1.02	2.00	3.00	1.64	2.90	4.01
Lilly U 40 Lot R-511	1.10	2.10	—	1.60	2.80	—
Lilly U 440 Lot 769999 20 units per mg.	(22 un.) 1.11	(44 un.) 2.17	(66 un.) 3.18	(22 un.) 1.75	(44 un.) 3.45	(66 un.) 5.12
Toronto U 40 Special Nov. 3, 1925.	1.03	2.12	3.12	2.37	4.20	5.90
Toronto U 20 Lot 318-25	1.06	2.12	3.17	1.80	3.15	4.25
Toronto U 10 Lot 317-48	1.07	2.08	—	1.70	2.98	—
Lilly—powdered Lot 441146 8 units per mg.	1.78	3.51	5.43	2.72	4.87	6.65

is not as nearly proportional to the unitage as in neutral-buffered solution. Moreover, preparations of insulin with the same neutral values have different alkaline iodine values.

The higher alkaline iodine value of insulin may be due to different causes:

(1) Hydrolysis may change the insulin preparation in such a way that more iodine can react. Our experiments show, however, that after hydrolysis with 2N NaOH or 2N HCl (48 hours 37°C.) the original neutral and alkaline iodine values are but slightly changed.

(2) Certain groups may be present that react with iodine at only an alkaline reaction. These groups may belong either to the insulin molecule itself or to impurities. It is, therefore, possible that pure insulin preparations will show the same iodine value in neutral and alkaline solution.

(3) An alkaline iodine solution may oxidize the same groups more strongly than does a neutral-buffered iodine solution.

It cannot yet be decided which of the suggestions offered under (2) and (3) is correct.

Our results seem to indicate that if the insulin preparations are of sufficient purity the number of units can be established iodometrically, using a neutral-buffered I_2-KI solution. The value of chemical estimations of physiologically active substances has been sufficiently discussed in the case of epinephrin.[7]

It seems possible that the iodometric estimation of insulin will be of some value in the standardization and further purification of insulin preparations. It is to be hoped that it will soon be feasible to decide some of the questions discussed in this paper with the aid of crystallized insulin, which has been recently isolated by Abel.[8]

NOTE: We wish to express our thanks to Eli Lilly and Co. and to the Connaught Laboratories, Toronto, for providing us with samples of insulin and with detailed information, regarding each.

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[7] *Cf.* Maiweg, H., *Biochem. Z.*, 1923, cxxxiv, 292. Frowein, B., *Biochem. Z.*, 1923, cxxxiv, 559.

[8] Abel, G. G., Pasteur lecture given before Chicago Institute of Medicine, January 22, 1926.