FACTORS INFLUENCING THE STABILITY OF INSULIN

BY MELVILLE SAHYUN, M. GOODELL, AND ARTHUR NIXON

(From the Biochemical Research Laboratory, Frederick Stearns and Company, Detroit)

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Before any given lot of insulin can be marketed, its potency and stability must meet certain requirements. Stability is arbitrarily determined by subjecting a sample of insulin to a "heat test." This test consists of exposing the carefully standardized insulin to a temperature of 52° for 10 days. The potency of the "heated" material is then compared either with a standard insulin or with a sample of the same preparation not exposed to the heat test. As a rule insulin thus treated loses some of its physiologic activity, but if there should be a considerable loss, e.g. 15 per cent or more, then such a preparation is not considered suitable for general use. The purpose of the heat test is to insure all insulin preparations for stability under adverse conditions. The causes of the loss of potency of the "heated" insulin have not, hitherto, been established. Traces of certain metals, particularly copper and iron, were found in some of the unstable preparations; it was therefore assumed that the presence of these metals might be a factor affecting deterioration either upon prolonged standing at room temperature or upon subjection to a heat test.

The deterioration of insulin upon standing is a problem of considerable importance not only to the manufacturer but to the physician, and in particular, to the diabetic patient. Thus it was deemed advisable to secure data on this problem. For this purpose it was considered desirable to study a preparation of insulin low in its ash content and practically free of such metals as copper, iron, and zinc.

This paper deals with (1) a method for the preparation of an insulin low in ash and free of copper, iron, and zinc; (2) the effect of temperature on the stability of such a preparation; and (3)
the effect of temperature on such a preparation to which known amounts of copper, iron, and zinc had been added.

Method of Preparation

The method for the preparation of an insulin low in its ash content and free of copper, iron, and zinc is as follows:

To 400 cc. of commercial insulin 100 units per cc. (40,000 units) 100 cc. of 1 M ammonium acetate and 60 cc. of acetone are added. The reaction is adjusted to about pH 6.5, and the mixture is allowed to stand overnight and filtered or centrifuged. The reaction of the clear filtrate is lowered to about pH 5.0 and allowed to stand in the refrigerator for 3 or 4 days, centrifuged, and the precipitate dissolved in dilute sulfuric acid to pH 2 or 2.5 and brought to a volume of 500 cc. The acidified solution is treated with 4 volumes of 95 per cent denatured ethyl alcohol and allowed to stand overnight. Any precipitate formed is removed by filtration through a hardened filter paper. The volume of the clear alcoholic filtrate is brought to 3.5 liters by the addition of absolute alcohol followed by 3.5 liters of ether. The mixture is allowed to stand for 2 days in a refrigerator. The clear alcohol-ether is siphoned off and the precipitate centrifuged.

The alcohol-ether precipitate is dissolved in lactic acid and filtered. The reaction of the solution is carefully adjusted to the isoelectric point with 1 N ammonium hydroxide. This process of precipitation with lactic acid and ammonium hydroxide is repeated twice. After the third precipitation, the precipitate is again taken up with sulfuric acid to pH 2 and to a volume of 500 cc., and 4 volumes of alcohol are added. Any precipitate formed is removed by filtration and the alcohol-soluble fraction is precipitated with alcohol-ether in the manner already described. The alcohol-ether precipitate is taken up in 100 cc. of distilled water and dissolved by the use of lactic acid. Then 50 cc. of 10 per cent trichloroacetic acid are added. The trichloroacetic acid precipitate formed is centrifuged and taken up in 90 per cent ethyl alcohol and filtered. An equal volume of absolute alcohol is added, followed by twice its volume of ether along with 1 cc. of 1 M ammonium acetate. The addition of ammonium acetate is very helpful in securing a complete flocculation of the insulin protein. After standing overnight the precipitate thus formed is
carefully centrifuged and washed twice with ether and dried in a vacuum.

Since the main object is to secure a preparation of low ash content, no attempt was made to secure any data on the amount of insulin lost. This process was followed on two different lots with the following results.

<table>
<thead>
<tr>
<th>Lot No.</th>
<th>Potency</th>
<th>Ash</th>
<th>Nitrogen</th>
<th>Copper, iron, zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units per 1 mg.</td>
<td>per cent</td>
<td>per cent</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22</td>
<td>0.09</td>
<td>13.71</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>0.006</td>
<td>13.72</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

**General Procedure**

*Assay*—The above preparation of low ash, zinc-free insulin was assayed according to the method of Sahyun and Blatherwick (1).

*Stock Dilution*—100 units per 1 cc. of this insulin were prepared as follows: A known amount of the dry insulin powder was accurately weighed and dissolved in 0.1 N hydrochloric acid. Enough of this acid was added to dissolve the insulin and to bring about a reaction of approximately pH 3. As a preservative, 0.1 cc. of tricresol was added to every 100 cc. This stock solution of 100 units of insulin in each cc. was kept in a refrigerator and later used in the various experiments described in this paper.

*Metals*—Separate solutions containing 1 mg. of the following metals were prepared: copper, iron, and zinc. Accurately weighed amounts of the purest metals available were dissolved in either hydrochloric or sulfuric acid and made up to a concentration of 1 mg. in each cc. Copper was dissolved in sulfuric acid; iron and zinc were dissolved in hydrochloric acid. The amount of each metal used is given in each experiment.

*Temperature*—In order to test the stability of insulin, the various samples were kept in an incubator carefully adjusted to about 52°. Samples were removed from the incubator once every week and made up to the proper dilution suitable for assay and kept in the refrigerator. This procedure was essential, as only one sample could be tested each day.

*Blood Sugar*—Sahyun's modification of Folin and Wu's method (2) was employed for blood sugar determination.
Stability of Insulin

EXPERIMENTAL

Experiment 1—25 cc. of the low ash, metal-free insulin, 100 units per cc., were introduced into each of a series of 50 cc. volumetric flasks. Sample A was used as a control. Sample B contained 2.5 mg. of copper, Sample C 2.5 mg. of iron, and Sample D 2.5 mg. of zinc. The contents of the flasks were next diluted to the 50 cc. mark with redistilled water, thoroughly mixed, stoppered, and kept in an incubator regulated at 52°. Once every week 2 cc. were removed from each sample for assay. This experiment lasted for 9 weeks and the results of the weekly assay of each sample, expressed in terms of per cent potency, are shown in Fig. 1.

It is apparent from the results shown in Fig. 1 that the control Sample A showed a loss of about 17 per cent of its potency 1 week after incubation at 52° and a 50 per cent loss of potency at the end of 9 weeks incubation. On the other hand, and contrary to expectation, the samples to which copper (Sample B) and iron (Sample C) had been added showed a more gradual decrease in potency than did the control sample. Finally, to our great surprise the sample containing 1 mg. of zinc per 1000 units showed a remarkable stability with no detectable decrease in potency after
incubation for at least 7 weeks and with only 10 per cent decrease at the end of 9 weeks incubation at 52°.

Fig. 2. The effect of insulin before incubation, with and without added zinc, on the blood sugar of rabbits.

Fig. 3. The effect of "heated" insulin, with and without added zinc, on the blood sugar of rabbits.

Experiment 2—This experiment consisted of a comparative study of the effect of insulin, prior to its incubation at 52°, with
and without the addition of zinc, on the blood sugars of twenty-four rabbits. The amount of zinc added was 1 mg. of the metal to every 1000 units of insulin. In each instance the insulin was diluted so that 1 cc. contained 2 units, and 0.5 unit per kilo was injected subcutaneously into each rabbit after a 24 hour fast. Samples of blood were withdrawn from the marginal ear vein at 0, 1.5, 3, 4, and 5 hours after injection. During the 1st week, twelve of the rabbits received the control insulin and the other twelve received the insulin to which zinc had been added; during the following week the same animals were used, but those previously receiving the control insulin were given insulin plus zinc and those receiving insulin plus zinc were given control insulin. The averages of blood sugars at the intervals indicated were next plotted, as shown in Fig. 2.

This experiment demonstrates that the addition of 1 mg. of zinc per 1000 units neither increases the efficiency nor tends to prolong the duration of the hypoglycemic action of insulin on the blood sugars of normal fasting rabbits.
Experiment 3—In this experiment a comparison was made between the effects of the “unheated” control against the “heated” insulin (at 52° for 9 weeks) with and without added zinc. The procedure described in Experiment 2 was followed on the same animals. The averages of blood sugars are shown in Fig. 3.

In studying the averages of blood sugars shown in Fig. 3 it is observed that the averages for the three initial blood sugars are at different levels. Since the amount of insulin subcutaneously administered was below the convulsive dose, its effectiveness in lowering the blood sugar at a given time might well be represented by the actual differences between the blood sugar at that given time and the initial blood sugar. Thus if the initial blood sugars are 111, 100, and 94 mg. per cent and the blood sugars at 1.5 hours are 64, 54, and 81 mg. per cent respectively, the actual decreases will be 47, 46, and 13 mg. respectively. Values thus obtained were calculated and plotted as shown in Fig. 4.

SUMMARY

A method for the preparation of an insulin low in ash and free of copper, iron, and zinc is described. Such a preparation was shown to be unstable when incubated at 52° for at least 1 week. At the end of 9 weeks incubation at this temperature the insulin lost 50 per cent of its physiologic activity.

When 1 mg. of copper or 1 mg. of iron was added to every 1000 units and incubated at 52°, not only was no greater loss in the physiologic activity of the insulin observed but in fact deterioration seemed to proceed at a slower rate (see Fig. 1).

The sample of insulin to which 1 mg. of zinc per 1000 units was added appeared to be quite stable at a temperature of 52° for 7 weeks. At the end of 9 weeks incubation at 52° the sample lost only 10 per cent of its physiologic activity.

Insulin to which 1 mg. of zinc per 1000 units was added did not cause either a delayed onset or a greater duration of its hypoglycemic effect (see Fig. 2).

Further work on the influence of ions of different metals on the stability of insulin is in progress.

BIBLIOGRAPHY

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Melville Sahyun, M. Goodell and Arthur Nixon


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