THE PREPARATION AND PROPERTIES OF PURE INSULIN

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INTRODUCTION

In a previous paper[1] the difficulties encountered in the preparation of absolutely pure insulin, giving a horizontal solubility curve, were described. Only one out of numerous attempts to prepare material of absolute purity met with success, but this favourable result could not be reproduced.

It has now been recognised that there is a slight decomposition of insulin, when it is dissolved in dilute acid, sufficiently large, however, to give an aberration of the solubility curve. To determine this curve, the insulin samples were dissolved in 0.1 N acetic acid and subsequently adjusted to p_H 5 with 0.1 N sodium acetate. During the dissolution in the acid, the insulin becomes dishomogeneous, and this change, on prolonged standing, may be irreversible.

Unfortunately, very pure crystals dissolve very slowly in 0.1 N acetic acid and this period of about 24 hours may suffice to give a slight permanent decomposition, which has already a profound influence on the solubility curve. By equilibrating the insulin crystals with acetate buffer of p_H 5, the contact with acid can be avoided and the result is more reliable.

PREPARATION OF THE MATERIAL

The object was not in the first place to obtain as high a yield of pure material as possible, but to get this material in a relatively simple way in quantities, sufficient for our experiments. Therefore from the point of view of yields no claims are made and the method may be subject to improvement.

The starting material was crystalline insulin, prepared according to ROMANS, SCOTT, and FISHER[2]. The purity of this material was 24–25 international units per mg. With 3 g of this insulin, the crystallisation was repeated. The very thin layer of amorphous material which separated on top of the crystals on prolonged centrifuging was carefully removed. The wet precipitate was taken up in 100 ml 0.025 N HCl and immediately after dissolution 2 g of NaCl were added. This throws out the denatured material and some insulin. The solution was centrifuged at once and to the clear supernatant another 8 g of NaCl were added. After centrifugation the precipitate was dissolved in 500 ml of water and the p_H adjusted to 5. The precipitate was crystallised from acetate buffer as described by the Toronto workers. The crystals were repeatedly washed with water. No amorphous material was observed on prolonged centrifuging. The crystals were dried in vacuo over sulphuric acid. Yield 1.3 g. From another batch of 5 g, 3.1 g of pure crystals were obtained.

This technique is the outcome of many vainless attempts with other methods of purification. Repetition of the crystallisation procedure alone does not lead to the desired result. On the contrary, judged by the slope of the solubility curve, the im-

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pression is gained that, after some crystallisations, the material becomes less pure than it was originally.

THE SOLUBILITY CURVE

From one batch of pure crystals two solubility curves were determined, one according to the old technique, by first dissolving in acetic acid and subsequently bringing the p_H back to 5. For the other, different amounts of material were equilibrated with 0.1 M acetate buffer p_H 5, for 24 hours. In preliminary experiments it had been found that the amount dissolved under these circumstances became constant after 2 hours and remained so for at least 48 hours. WINTERSTEINER and ABRAMSON[3] working under almost identical conditions, found no differences in the amount dissolved after 24 and 48 hours. In our previous work[1] it has been demonstrated that, with the old procedure of determination of the solubility curve, equilibrium is very quickly established. This result has again been confirmed with pure crystals. The values obtained after one and after 48 hours equilibration are identical.

An example of the new procedure will be given here: 75 mg of pure insulin are shaken with 30 ml of 0.1 M acetate buffer p_H 5.05. In a few minutes a homogeneous suspension is formed. From this suspension, varying quantities are pipetted into a series of flat bottom flasks and diluted with 25 ml of the acetate buffer solution. A glass bead is added to each flask and they are closed by means of rubber stoppers. To reach equilibrium, they are rotated for 24 hours at about 10 revolutions per minute. The filtrate is obtained through analytical grade filterpaper. The nitrogen is determined by Micro Kjeldahl.

All glassware is steamed out before use. The rubber stoppers were boiled in distilled water. A blank was run of the acetate buffer. This blank was always negative.

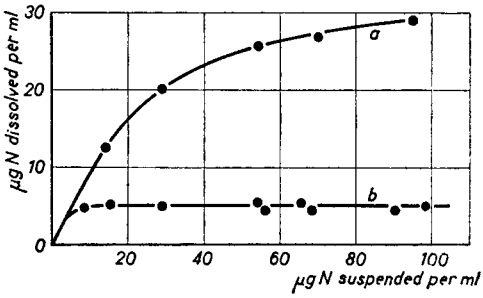

Fig. 1. Solubility curves of insulin. a. 75 mg insulin dissolved in 10 ml 0.1 N acetic acid, 20 ml 0.1 M sodium acetate added and the suspension further diluted with 0.1 M acetate buffer p_H 5. b. Same insulin equilibrated at once with 0.1 M acetate buffer p_H 5

The results of these experiments are represented in figure 1. There is a great difference between the solubility curves, depending on the procedure of the determination. Apparently, the insulin is pure but in the case where it has been in contact with the acetic acid, some has deteriorated and the decomposition product gives an aberration. For insulin of less purity, the two curves can almost be made to coïncide by shortening the time of exposure to the acid as much as possible.

THE STABILITY IN 0.1 N ACETIC ACID

The kinetics of the deterioration in 0.1 N acetic acid cannot be investigated easily. During the process of dissolution, the insulin concentration in the solution slowly builds up to the final value. But meanwhile the reaction proceeds. Taking the moment all insulin has gone in solution as zero time is an injustified simplification of the state of affairs. Nevertheless, it would be useful to have some idea of the losses incurred by this decomposition in an approximate way. The loss of activity cannot be determined directly. To assay insulin, it has to be dissolved in dilute acid. By dissolving it, some of the activity is lost if it is assumed that the decomposition product is inactive or less

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active than the original material. Besides, it can hardly be assumed that the loss will be great and the determination of a difference of a few percents in activity by biological assay cannot be seriously considered.

Some idea of the loss involved can be obtained in a more or less indirect way. 75 mg of insulin were dissolved in 10 ml 0.1 N acetic acid in the course of 24 hours. There after 20 ml of 0.1 N sodium acetate were added, bringing the p_H to 4.98 and the precipitate filtered off after one hour. The filtrate had a nitrogen content of 28 μg per ml. If no inactivation had taken place, this value would correspond to 4.7 international units per ml. On biological assay on 20 rabbits, 3.7 units per ml were found, corresponding to a loss of 4 % of the total activity present. In an acid solution precipitated after 72 hours, exactly identical values were found, again on 20 rabbits.

The actual loss may be somewhat greated than 4 %, as some of the decomposed material may be present in the precipitate. The reaction apparently does not proceed any further for the amount soluble at p_H 5 was found not to change if the acid solutions were kept for a fortnight at room temperature or for 48 hours at 35° C.

From the facts already observed it is clear that a solubility curve of pure insulin in acid solutions of sodium sulphate will not be horizontal. In fact, in an actual experiment it showed a considerable slope.

PROPERTIES OF PURE INSULIN

The biological activity of the pure insulin is particularly interesting. It is at once obvious that this activity determined in acid solution, will be somewhat low, owing to the described decomposition.

In a preliminary assay on 60 rabbits against our usual substandard an activity of 27.4 international units per mg was calculated. The final assay on 90 rabbits against the international standard gave a value of 26.8 $\pm$ 0.8 international units per mg. The procedure followed in the standardisation was that of DE JONGH, LENS, and SPANHOFF[4]. If no use was made of the predetermined regression line, the result was identical*.

Under the electronmicroscope, ordinary commercial insulin dried from acid solutions shows some fibrous inactivated material as described by WAUGH[5] to be formed on heating. According to our experience, small quantities of this fibrous form are to some extent always present, even in unheated samples. The absolutely pure material does not show these fibrils**. The nitrogen content of our preparation is 15.9 %. The zinc content, determined by titration with dithizone is 0.36 %.

DISCUSSION

As early as 1931, SJÖGREN and SVEDBERG[6] with the aid of the ultracentrifuge were able to show that insulin dissociates at a p_H less than 4.2. Their results were confirmed by GUTFREUND and OGSTON[7]. This dissociation is not completely reversible if the p_H comes far beyond the borders of the stability region. This observation has always been ignored in all published preparative methods for the purification of insulin and as a matter of fact it is hard to imagine a purification process completely avoiding the p_H

* The biological assays were performed by Dr J. HARTKAMP.

** The electronmicroscopic photographs were made at the Institute for Electronmicroscopy at Delft.

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range where insulin is stable according to these authors. Besides it is obvious that of the many reactions, resulting in loss of potency which the insulin molecule may undergo, this is quantitatively a minor one. However, if it comes to preparing absolutely pure material it cannot be neglected altogether.

In a previous paper[1] we described a horizontal solubility curve, once obtained with crystals, first dissolved at p_H 3. This observation is apparently in contradiction to our later results. But as a matter of fact, the crystals in this case had been dissolved from the wet state, without preceding desiccation. Under these conditions they dissolve much quicker and the importance thereof was not realised at the time of the experiments. The percentage contamination caused by the decomposition counts less in impure insulin, where already considerable amounts of other contaminants are present. Besides, impure crystals dissolve much quicker and therefore the effect is rather small in less pure material.

Crystalline insulin of constant solubility has also been obtained by COHN *et al*[8] by subjecting crystals to repeated equilibrations with water. The final solubility of the insulin was 0.014 g per l. In our investigation a solubility of 0.030 g per l in 0.1 M acetate buffer was observed. WINTERSTEIN and ABRAMSON[3] on the other hand claim solubilities as low as 0.004 g per l in 1/30 M acetate buffer, a value almost agreeing with the *nitrogen* content of our solutions and not with the insulin content. The zinc content of our preparation is in complete accordance with the value reported by COHN *et al*[8].

SUMMARY

1. A method is given for the preparation of absolutely pure insulin with a horizontal solubility curve.

2. At p_H 3 there is some decomposition of insulin. Therefore the curve must be determined at p_H 5 by equilibrating the crystals with buffer solutions.

3. The activity of pure insulin amounts to 26.8 $\pm$ 0.8 international units per mg.

RÉSUMÉ

1. Une méthode a été décrite pour la préparation d'insuline absolument pure avec courbe de solubilité horizontale.

2. A p_H 3 l'insuline se décompose légèrement. C'est pourquoi la courbe doit être déterminée en travaillant à p_H 5 et en traitant les cristaux de solutions tampon.

3. L'activité de l'insuline pure est de 26.8 $\pm$ 0.8 Un. intern. par mg.

ZUSAMMENFASSUNG

1. Eine Methode zur Bereitung von absolut reinem Insulin mit einer horizontalen Löslichkeitskurve wird gegeben.

2. Bei p_H 3 tritt etwas Zersetzung auf. Daher muss die Kurve bei p_H 5 bestimmt werden, und zwar indem man die Kristalle mit Pufferlösungen ins Gleichgewicht bringt.

3. Die Aktivität reinen Insulins beträgt 26.8 $\pm$ 0.8 internationale Einheiten pro mg.

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