XIX. IMPROVEMENTS IN THE PREPARATION OF INSULIN. ALKALINE EXTRACTION OF PANCREAS.

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THE original method for preparing insulin from normal fresh pancreas, worked out by Collip [1923], depended on the extraction of the minced tissue with plain alcohol. Doisy, Somogyi and Shaffer [1923] found that they could obtain considerably larger yields of the active principle by extraction with strongly acid alcohol.

The fact that insulin in acid solution is readily adsorbed by a great variety of substances, while in faintly alkaline solutions this is not the case, [Dudley, 1923] suggested that possibly the yield of insulin might be increased by extracting the minced pancreas in an alkaline medium. The only danger which we anticipated was that in such a process we should have conditions favourable to the action of trypsin which is known rapidly to destroy insulin [Dudley, 1923]. Our fears in this respect proved to be groundless and by a very simple modification of the method of alcoholic extraction we have been able to obtain between four and five times the amount of insulin which we had been getting by the original method.

We carry out the process as follows. One kilogram of fresh ox pancreas is minced into one litre of 95 % spirit; 85 g. sodium bicarbonate are stirred into the mixture, which is then poured into the mincer and reminced; this procedure is repeated a second time. The mixture is then allowed to stand with frequent stirring for two hours at room temperature, or if desired it may be kept in the cold room overnight. It is then poured on to a stout cloth, which is squeezed as thoroughly as possible in a suitable press. The turbid filtrate which has a p_H of about 7.5 is treated with $1\frac{1}{2}$ times its volume of 95 % spirit and placed in the cold room (-3°) overnight. It is then filtered through a folded paper; 10 cc. of glacial acetic acid are added to the filtrate, which is then evaporated *in vacuo*, in a water-bath at $40-45^\circ$. When the volume of the residue has reached about 150 cc., the fat which has separated is removed by shaking out with light petroleum and the aqueous layer is freed from traces of this solvent by a few minutes' distillation under the conditions described above.

Four volumes of absolute alcohol are added to the aqueous solution, and after standing for about ten hours in the cold room, the alcoholic solution is carefully decanted from the precipitate which has formed and two volumes of absolute alcohol are added to it. After standing in the cold room for 12–24 hours the supernatant liquid is carefully decanted and the precipitate is washed into centrifuge tubes with absolute alcohol, washed with absolute alcohol and dry ether, and finally dried in a vacuum desiccator over H_2SO_4 as described in a previous paper [Dudley, 1923].

Altogether we have worked up 43 batches of 1 kilo. each by this method. In many of these experiments different amounts of sodium bicarbonate were used and other experimental variations were introduced with a view to finding the best conditions. The yields of crude insulin varied, of course, in these experiments, but, taking the good with the bad, the average yield of insulin over the whole of the series was 257 rabbit units per kilo.

It is fairer to judge the method by taking the results of our last eight batches in which the experimental conditions, described above, were constant. In these the yield of "crude insulin" varied from 1.95 g. to 2.4 g. and the rabbit units from 300 to 480, the average yield of "crude insulin" being 2.16 g. and of rabbit units 412 per kilo. of pancreas.

Our average yield of rabbit units per kilo. pancreas by the original method was 88, that is to say, slightly more than one-fifth of the yield obtained by the alkaline process. The method of acid extraction to which reference has been made above, has, in our hands, given us an average yield of 257 rabbit units per kilo., or about five-eighths of the yield obtainable by the method of alkaline extraction.

A rabbit unit of the crude insulin prepared by alkaline extraction is usually from 3 to 5 mg. In our experience the material obtained by the method of acid extraction is always much more highly pigmented than that prepared by either of the two other processes. An aqueous solution of "acid" crude insulin is a deep brown colour, while a corresponding solution of "alkaline" crude insulin is pale yellow and somewhat less coloured than that prepared by the original simple alcoholic extraction.

The highest yield of crude insulin which we have obtained by alkaline extraction is 508 rabbit units per kilo., and it is worthy of note that the pancreas used was ox-pancreas which was put into the cold room (-3°) about two hours after removal from the animal, and kept there for a week before it was worked up. It is therefore obvious that, provided it is frozen promptly, pancreas may be stored for a considerable time without risk of losing its insulin.

Improved method of purification of crude insulin.

The crude insulin obtained by alkaline extraction can be purified by precipitation with picric acid and conversion of the picrate into a soluble hydrochloride, as described in the paper previously mentioned [Dudley, 1923].

The technique of this method of purification has been considerably simplified and is now carried out in the following manner. The crude insulin is dissolved in a small volume of water and the flocculent, insoluble material is removed by centrifuging. The clear solution is then diluted with the requisite amount of water to give a 1.5 % concentration of the original material, making no allowance for the insoluble material which has been removed. The p_H of the solution is then adjusted to about 5. This may be conveniently accomplished by adding acid or alkali (according to the original p_H) until a faint turbidity is produced due to the formation of the "isoelectric" point precipitate [Doisy *et al.* 1923]. Half its volume of saturated aqueous picric acid is then added. After standing for a day or two the clear supernatant liquid is poured off. The lemon-yellow precipitate which has collected on the bottom of the beaker is rubbed up with a small amount of water and then $N/10$ sodium carbonate solution is added in small quantities at a time with constant stirring until a clear, or nearly clear, dark brown solution is obtained. As soon as addition of sodium carbonate solution fails to clarify the solution further it is filtered through a folded filter paper, which is well washed with distilled water on completion of the filtration. This process should be carried out with all reasonable speed and it is well to keep the temperature below 10° . The solution is then diluted to contain about 1.5 to 2 g. of picrate per litre. The necessary volume is calculated on the assumption that the amount of picrate obtained is about one-twelfth the weight of the original crude insulin. An amount of $N/10$ HCl, equivalent to the Na_2CO_3 used, is then added to the well-stirred liquid. An immediate precipitation of the picrate occurs. To each litre of solution is added 250 cc. of saturated aqueous picric acid. After standing for a day or two the precipitate has again collected on the bottom of the beaker and the clear supernatant liquor is poured away.

A solution containing 5 cc. of saturated aqueous picric acid in 100 cc. water is made. The picrate is rubbed up in a small quantity of this solution and is then filtered on a Buchner funnel. After adequate washing with the dilute picric acid solution to remove sodium chloride, the moist picrate is transferred to a beaker and stirred with a solution of hydrochloric acid in 75 % alcohol, prepared by mixing 25 cc. 3 N (aqueous) HCl with 75 cc. of absolute alcohol.

The picrate, after first forming thick, dark brown sticky drops, dissolves in this reagent, yielding a turbid, brown solution. It must be stirred until all the sticky drops have disappeared. The amount of the alcoholic-aqueous HCl reagent used is not important but from 10–15 cc. per g. of picrate is an appropriate quantity. Pure acetone is then added rapidly until no further precipitate appears. From 10–20 volumes are necessary. The white flocculent precipitate of "insulin hydrochloride" settles rapidly and is immediately filtered on a Buchner funnel, washed with acetone and finally with dry ether until perfectly free from picric acid. The funnel with its contents is quickly transferred to a vacuum desiccator and dried over sulphuric acid.

The material so obtained is a white, non-deliquescent, amorphous powder, which dissolves completely in water giving a colourless solution.

This method of handling the picrate obviates the necessity of preparing it in the dry state and, further, gives a hydrochloride which contains no trace of picric acid, a result which was difficult to attain by the original method. We have prepared from "alkaline" crude insulin a hydrochloride by this process of which 0.2 mg. proved to be a rabbit unit.

In the course of these experiments we have found that while the picrate is only very slightly soluble in water and practically insoluble in absolute alcohol it is quite freely soluble in mixtures of water and alcohol. It must therefore be emphasised that insulin cannot be precipitated satisfactorily by means of picric acid from aqueous solutions containing alcohol; in the absence of alcohol, however, it is precipitated quantitatively.

Wherever "rabbit units" are mentioned in this paper, we mean the original Toronto rabbit unit; *i.e.* the amount of insulin necessary, when injected subcutaneously, to lower the blood sugar of a 2 kilo. rabbit which has fasted for 16–24 hours, from a normal value of about 0.1 % to about 0.04 % and to cause typical hypoglycaemic convulsions within four hours.

SUMMARY.

1. By a simple modification of the original process of alcoholic extraction of pancreas, namely, by adding sodium bicarbonate to the mixture of minced pancreas and alcohol the yield of insulin is increased nearly fivefold. In one experiment 508 rabbit units were obtained (as finished hydrochloride) from 1 kilo. of ox pancreas.

2. An improved technique for the purification of crude insulin by precipitation with picric acid and subsequent conversion of the picrate into a soluble hydrochloride is described.

3. At least 90 % of the impurities in crude insulin are removed by this method.

4. By these means preparations have been obtained of which 0.2 mg. has proved to be a "rabbit unit."

We wish to express our best thanks to Mr H. P. Marks, who has carried out all the physiological assays of the insulin produced in this series of experiments.

REFERENCES.

- Collip (1923). *J. Biol. Chem.* 55; *Proc. Amer. Soc. Biol. Chemists*, xl.
Doisy, Somogyi and Shaffer (1923). *J. Biol. Chem.* 55; *Proc. Amer. Soc. Biol. Chemists*, xxxi.
Dudley (1923). *Biochem. J.* 17, 376.