A New Principle for Large Scale Production of Insulin

ERIK JORPES, VIKTOR MUTT and SELAHADDIN RASTGELDI

Chemistry Department II, Karolinska Institutet, Stockholm, Sweden

The conventional method for large scale manufacture of insulin starts with an extraction of the pancreatic glands with acidified ethyl alcohol and subsequent evaporation of the alcohol *in vacuo* at low temperature. The authors present a technique of adsorbing the insulin from the alcoholic extract at pH about 3 on an ion exchanger, alginic acid, with subsequent elution of the insulin by means of 0.2 N HCl.

The use of a cumbersome and expensive distilling equipment is thereby made superfluous. Fats are not adsorbed. An increased yield of insulin is obtained if the traces of tryptic enzymes still present in the extract are destroyed by heating the alcoholic extract at pH 3.2 to 70°C before adsorbtion.

Since the very first beginning, when insulin in 1921—1922 became available for clinical use through the endeavours of the University of Toronto group, the manufacturing process has included extraction of the pancreatic glands with alcohol, acidified with hydrochloric or sulphuric acid, evaporation of the alcohol *in vacuo* at low temperature, removal of fats from the aqueous residue and precipitation of proteins with sodium chloride followed by isoelectric precipitation of the insulin from water at pH 5.2 and subsequent crystallization. During all these years, no major changes have been made in this procedure.

Since no information was available on the successful use of ion exchangers for recovery of the insulin from the alcoholic extract, we in 1954 took up experiments along such a line. As a result the Swedish manufacturer of insulin, Vitrum AB, Stockholm, could in 1956 start to apply a new technique, which now has been in use for the last three years. Since no distillation at low temperature is necessary, the large-scale production has been greatly facilitated.

Historical. The technique of adsorbing bases on inorganic ion-exchangers was introduced in biochemistry in 1917 by Otto Folin, who adsorbed ammonia on Permutite. The same reagent was later used extensively for the adsorption of organic bases, basic proteins of low molecular weight and peptides [1].

From 1950 on, the carboxylic acid ion-exchange resin Amberlite IRC-50 was used by different authors to study the homogeneity of basic proteins of low molecular weight, such as cytochrome c[2], ribonuclease [3], lysozyme [4] and chymotrypsinogen [5]. Up to 1953, the ion-exchange resins had been used only

for chromatographic analysis of small quantities of purified proteins, the applicability being limited to low-molecular basic proteins.

When Peterson and Sober [6] in 1956 recommended cellulose ion-exchangers for the chromatography of proteins, they stated that "the application of chromatography to the separation of proteins has not met with the general success obtained with smaller, simpler molecules. The large size of the protein molecule prevents its penetration into the adsorbent particle, therefore only the adsorbing sites located on the exterior can be utilized."

Boardman [7] tried in 1955 to increase the surface of a sulphonated polystyrene ion-exchanger by precipitating the resin on the surface of kieselguhr (Celite 545), and used the product for the study of the purity of crystalline insulin.

The only successful attempt at using ion-exchangers on a technical scale for the reversible adsorption of proteins was that of Astwood, Raben, Payne and Grady [8] of 1951. They adsorbed the basic peptide hormone, corticotropin, on oxycellulose from dilute aqueous acetic acid.

No attempts seem to have been made to take up the insulin from the acidic alcoholic extract on ion-exchangers. As an illustration, it can be mentioned that as late as June 1956, Samsonov and Faddajeva [9] published a study of the possibility of adsorbing insulin on an ion-exchange resin, SBS-1, having a particularly suitable porosity. Elution was performed with 1 N ammonia. They applied their technique to the residual aqueous extract after distilling off the alcohol.

Own work. Since the previous discussion dealt with ion-exchange chromatography in aqueous solution and, furthermore, was confined to more or less basic proteins of low molecular weight, removal of the acidic protein insulin from a crude alcoholic extract offered new aspects. The use of ordinary cation-exchangers of the Dowex-50 type was excluded because of the size of the insulin molecule, which does not allow a diffusion into the inner spaces of the resin. Moreover, if adsorption is to be carried out at an acidic reaction, pH below 4, making the insulin a cation, the most commonly used adsorbent, Amberlite, IRC-50, is only sparingly ionized and less efficient as an adsorbent. An ion-exchanger with easily accessible ionizable groups is necessary.

We found alginic acid to be a highly suitable ion exchanger for this purpose *. Insulin is taken up quantitatively by the alginic acid from a 65 % ethanolic solution having an acidity of pH 2.8—3.4. The bulk of fats remains in the alcoholic extract from which the alcohol is recovered in an ordinary rectifying column. The insulin is eluted from the alginic acid with 0.2—0.3 N aqueous hydrochloric acid. The stability of the molecule of the alginic acid, its chain-like structure [19] and the strong ionization of the carboxylic groups of this polyhydroxyacid at pH 3.4 [11] could explain its usefulness in this respect.

Since the previous technique of manufacturing insulin required an elaborate distillation plant with evaporators, condensers, vacuum pumps and a heavy cooling machinery, demanding both manpower, steam and electricity

---

* Jorpes, J. E., Mutt, V. and Rastgeldi, S. Swedish patent 157, 387 Oct. 18, 1956. Priority date Feb. 9, 1955, Swed. patent 162, 669 Jan. 23, 1958, Britt. patent 797, 959 Oct. 29, 1958, U.S. patent 2, 878, 159 March 17, 1958.

to run it, the possibility of taking up the insulin directly on an ion-exchanger completely altered the large-scale manufacturing process.

EXPERIMENTAL

The minced frozen glands are extracted with the ordinary amount of ethyl alcohol, acidified with oxalic acid (30 g per kg pancreas, dissolved in water), citric acid (70 g per kg) or preferably phosphoric acid (15—20 ml conc. per kg).

In this procedure as in the old one ammonia is added to pH 8.0 and the proteins precipitated are removed. The ammonium ions present would prevent the adsorption on the alginic acid and are therefore to be removed. This is achieved by means of Dowex-50 in the hydrogen form, added step-wise until pH 3.2 is reached. The ion-exchanger is collected for regeneration with acid.

Before use the alginic acid is washed free of metallic ions with 0.3 N hydrochloric acid and then with water. It is usually stored slightly moistened at −15°C. If dry it must be moistened with water before adding it to the alcoholic extract. *The capacity of new lots of alginic acid to adsorb insulin was constantly low, about 50 % of normal, and increased to an optimum level after 2 regenerations.* Per kg pancreas 30 g of alginic acid is taken, to be left in contact with the extract during 4 h at room temperature under constant stirring. The acid is collected through filtration or centrifugation and washed with 95 % and absolute ethanol in order to remove fats, and the alcohol is removed by aeration. Eluation of the insulin is performed with 0.2—0.3 N hydrochloric acid at room temperature. The alginic acid is washed with water and used again for at least 10, possibly 15 times.

Elimination of proteolytic enzymes. In using calf pancreas the procedure worked excelently. When it was applied to ordinary frozen ox pancreas the yield of insulin slowly dropped, until on the sixth to eighth time the alginic acid was in use, no insulin at all precipitated on adding sodium chloride to the eluate. The different behaviour of the calf and the ox pancreas could hardly be due to anything else than a destruction of the insulin through enzymes still present in the extract and accumulated on the alginic acid during its repeated use. As most clearly demonstrated by Birgitta Werner [12] in 1948, the enzyme-producing apparatus of the pancreas is, in man, not yet fully developed in the newborn.

We therefore together with Mr Gunnar Lindén of Vitrum AB after removal of the Dowex-50 heated the alcoholic extract of the ox pancreas at pH 3.2 before addition of the alginic acid. The yield of insulin then even exceeded the expected level and the product crystallized readily. This also applies to the hog pancreas. After introducing this procedure of raising the temperature of the alcoholic extract up to 70—75°C in the course of 30 min, and cooling it to 30°C during a further 30 min — a procedure which destroys the proteolytic enzymes — the manufacturing process ran smoothly.

The yield of insulin depends not only upon the manufacturing process, but also upon the gland material available. Old dairy cows, an important source of glands in some countries, may have as low an insulin level in the pancreas as 400—500 units per pound, whereas the corresponding figure for young cattle is about 2 000 units. The collection and the storage of the glands also influence the yield. The heaviest losses are of course made during the concentration of the alcoholic extract, particularly if the distilling equipment is less efficient. The new procedure eliminates this risk, thereby increasing the yield of insulin with at least 20—30 %. The simultaneous gain through the saving of laboratory space, equipment, power consumption and labour add further to the value of the technique.

REFERENCES

1. Nachod, F. C. *Ion exchange.* Acad. Press, New York 1949.
2. Paleus, S. and Neilands, J. B. *Acta Chem. Scand.* 4 (1950) 1024.
3. Hirs, C. H. W., Moore, S. and Stein, W. H. *J. Am. Chem. Soc.* 73 (1951) 1893.

4. Tallan, H. H. and Stein, W. H *J Biol Chem* **200** (1953) 507.
5. Hirs, C. H. W. *J. Biol. Chem.* **205** (1953) 93.
6. Peterson, E. A and Sober, H. A *J. Am. Chem. Soc.* **78** (1956) 751.
7. Boardman, N. K. *Biochim. et Biophys. Acta* **18** (1955) 290.
8. Astwood, E. B., Raben, M. S., Payne, R. W. and Grady, A. B. *J. Am. Chem. Soc.* **73** (1951) 2969.
9. Samsonov, G. V. and Faddajeva, M. D. *Biokhimiya* **21** (1956) 403.
10. Percival, E. G. V. *Structural Carbohydrate Chemsitry*, London 1950.
11. Heming, A. E. and Flanagan, Th. L. *Ann. N. Y. Acad. Sci.* **57** (1953) Art. 3, p. 246.
12. Werner, B. *Acta Paediat.* **35** (1948) Suppl. 6.