XIII. *Chemical Properties of Insulin*

By P. J. MOLONEY, M.A., and D. M. FINDLAY, M.A.

Presented by J. G. FITZGERALD, M.D., F.R.S.C.

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One obvious end-point in the study of the chemistry of insulin would be the preparation of the physiologically active substance or substances in a chemically pure form. Unfortunately in such a problem there are no logical methods of attack, so that a successful outcome depends in a great measure on chance. Some of the results reported here may be in the light of future effort, of only negative value. Others, however, have already proved of practical importance, particularly in the preparation of products for clinical use.

The published literature (1) on this section is not large. The following are the salient facts. Insulin is soluble in water, is precipitated by ethyl alcohol of between 80 and 90 per cent. or by strong acetone. It is thermolabile, does not dialyze and shows the phenomenon of precipitation at an isoelectric point The isoelectric precipitate contains no phosphorus but does contain nitrogen and gives the biuret test. It should be noted that Macleod has reported the preparation of an active extract from fish islet tissue which does not give the biuret test.

The preliminary work which we are reporting here has to do with solubilities, reactions and stability relations of insulin. We have found insulin to be insoluble in chloroform, sulphuric ether, petroleum ether, ethyl acetate, butyl alcohol, methyl salicylate, carbon tetrachloride, ethyl benzoate and turpentine. Insulin is completely soluble in phenol and the cresols, glacial acetic acid, formamide and water of a suitable acidity. On adding pyridine to an aqueous solution a precipitate settles out between 50 and 75 per cent. pyridine which contains the potent material. When more pyridine is added this precipitate dissolves and from 75 per cent. to nearly 100 per cent. pyridine there is a region of complete solubility. However, if 100 per cent. pyridine is shaken with insulin in a dry powder form no appreciable potent material is dissolved. In acidified absolute ethyl alcohol insulin dissolves in part.

If phenol or the cresols are shaken with an aqueous solution of insulin, the insulin distributes until more than 90 per cent. of the

potency is contained in the phenol or cresol layers. By shaking the phenol or cresol layer with water and sufficient ether the insulin is distributed into the water layer. Chloroform or methyl salicylate may be used in place of ether. From formamide solutions, insulin is precipitated out solid by adding twenty volumes of butyl alcohol or it may be distributed into water by adding three volumes of butyl alcohol and washing with water. From acetic acid solution insulin may be ethered out solid or into a water layer. Pyridine or alcoholic solutions of insulin may be handled similarly.

Insulin is precipitated by dilute solutions of ferric chloride and also by half saturation with ammonium sulphate. Barium salts of weak acids, such as acetic, precipitate the potent material in part. Solutions of picric acid precipitate insulin completely (this observation has been independently made by Dudley in England). Further a dilute copper sulphate solution may be used as a solvent directly on the pancreas, holding back much inert material and dissolving out the insulin. Insulin is adsorbed from aqueous solutions by several reagents. Certain decolorizing carbons remove insulin completely from solution, but we have been unable to obtain good yields in attempts to redissolve the potent material. On the basic side of the isoelectric point we have used moist copper hydroxide to adsorb the potent material and on the acid side we have used benzoic acid. These two have this advantage that the adsorbing reagent can be readily dissolved, freeing the adsorbed material.

When a purified aqueous solution of insulin is frozen a jelly-like or solid precipitate comes out which dissolves but slowly. This well-known property of colloids seems to be independent of precipitation at the isoelectric point; the proportion settling out varies from 20 to 80 per cent. and is dependent on the procedure through which the insulin has gone prior to the freezing. The potency of this precipitate is very high, but it still gives a biuret test of the same order as the best isoelectric material. Using parchment shells we have been unable to detect the presence of insulin in the dialysate, which corroborates the results reported by Macleod.

We have verified the slow destruction of insulin by heat in various solvents. Benzoic acid on which the insulin is adsorbed or even with which it has been mixed and dried may be heated to the fusion point (approximately 120°C.) with no loss of potency. It has been heated in this way to 200°C. with only a partial loss in potency. This fusion does not always leave the properties entirely unchanged. Insulin is comparatively stable towards acids. It is precipitated with great loss in potency by 5 N H_2SO_4, which precipitate

is soluble in stronger acid. The solubility relations of insulin in this form are changed. In sodium hydroxide solution at room temperature insulin is completely destroyed at pH 14 in 2 hours, at pH 13 it is only partially destroyed in this time, while at pH 12 and 11 its sugar lowering power is not lost. Sixteen hours at pH 13 and room temperature completely destroys the insulin. To mild oxidation (H_2O_2) and reduction (sodium amalgam) an acid solution of insulin is relatively stable.

That insulin as we have it now is a colloid and at least closely associated with protein-like substances seems clear; it does not dialyze, it is precipitated at an isoelectric point, it exhibits the phenomenon of coagulation on freezing, and has various reactions characteristic of colloids and proteins. While these properties indicate a complex state, it may well be that the active material is a simple body closely associated with protein, in much the same way as thyroxin, which is bound up with protein and which Kendall has succeeded in isolating and synthesizing. There are several facts which point to an association of this kind. The partial solubility effects sometimes obtained, together with the variation of the physiological and chemical properties following certain procedures might indicate one simple chemical in combination with some inert material. Further, the difference in isoelectric point between insulin from hog pancreas and beef pancreas might be taken as an indication of association with two different proteins. Moreover Macleod has reported biuret-free skate insulin which is another hopeful sign.

Certain of our results have a distinctly practical importance, such as the precipitation with ammonium sulphate, the extraction with copper sulphate solutions of certain concentrations, the concentration of aqueous solutions by means of phenol, or by adsorption on benzoic acid.

The following is a procedure for concentrating insulin by adsorption on benzoic acid. To one litre of crude aqueous extract 50 cc. of a 25 per cent. sodium benzoate solution is added and the solution acidified with 12½ cc. of concentrated hydrochloric acid. This is usually sufficient to saturate the solution with benzoic acid and give a lasting precipitate; these quantities can be increased or diminished proportionately depending on the amount necessary to cause the first lasting precipitate. Then 40 cc. of 25 per cent. sodium benzoate and 10 cc. of concentrated hydrochloric acid are added, and after allowing sufficient time for complete precipitation the solution is filtered. This precipitate usually contains about 2/3 of the potent material. The filtrate is again treated with 40 cc. of 25 per cent. sodium benzoate

and 10 cc. of concentrated hydrochloric acid for a second precipitation. This is filtered and the resulting filtrate reprecipitated, if it still contains sufficient potent material. The benzoic precipitate containing the insulin may be washed on the filter paper with a saturated aqueous solution of benzoic acid. A small amount of potent material comes off in the wash water which may either be reprecipitated along with the mother liquor, or may be collected and reconcentrated separately. The insulin may be recovered in aqueous solution from the benzoic precipitate by various methods.

One procedure is to make up the moist precipitate to 80% with ethyl alcohol, which dissolves the insulin and the benzoic acid; certain inert materials are allowed to settle out and the solution filtered. The filtrate is boiled down under vacuum and the benzoic acid and traces of alcohol are removed by washing with ether.

Summary

Various reactions, stability and solubility relations of insulin have been given. The colloidal and protein-like characteristics have been discussed and one practical application of our results has been cited in detail.

Connaught Laboratories,
University of Toronto,
Toronto

References

(1) Banting, Best, Collip and Macleod. Trans. Royal Society of Canada, 1922.
(2) Doisy, Somagyi and Shaffer. Proc. Fed. Amer. Soc. Exper. Biol., December meeting, Toronto, 1922.
(3) Best and Macleod. Proc. Fed. Amer. Exper. Biol., December meeting, Toronto, 1922.