EXPERIMENTS

on the

CHEMICAL BEHAVIOUR OF INSULIN.

Thesis

by

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Experiments on the Chemical Behaviour of Insulin.

Experimentation has been carried on for the past year and a half in an effort to isolate the hypo-glucemic active principle of insulin preparations as a chemical individual or else to obtain an insulin preparation containing a known percentage of this principle. The latter object might be attained without the former, if the quantitative estimation of a certain chemical grouping could be shown to parallel the hypo-glucemic activity as determined by rabbit testing. The only measure of insulin value that has been advanced as yet is based on the hypoglumemic action on a normal rabbit.

As purification proceeds, the apparent properties of the substance change, so that frequently the results secured with samples of a certain purity can not be quantitatively duplicated with a sample of different purity. The observations recorded are therefore, chiefly qualitative in nature. The kilo rabbit unit referred to in the text denotes the minimum amount per kilo of body weight required to produce the characteristic hypoglucemic convulsions in a normal rabbit.

The earlier part of this work was performed on extracts of cattle pancreas made according to the method outlined by Callip[1] with only slight modification. The latter part of the work was carried out with the commercial preparation of insulin marketed under the trade name "Iletin", prepared by the Eli Lilly Co.,

Indianapolis, from hog pancreas. There has been published several satisfactory methods for extracting insulin from the pancreas, notably those of Doisy, Somogyi, and Shaffer,[2] Best and Scott,[3] and Fenger and Wilson,[4] The method of Doisy, Somogyi, and Shaffer was tried during the course of this work, but led to no unusually satisfactory result.

The method of purification by repeated isoelectric precipitation reported by Doisy, Somogyi, and Shaffer [2] is of limited usefulness only, as it does not yield a very great amount of purification from accompanying organic materials. It is very useful in freeing the organic materials from all but traces of any inorganic salts, especially when ammonium sulfate is used in the preparation of the material. Isoelectric precipitation followed by a separation of the precipitate into 95% alcohol soluble and insoluble portions affords an easy method giving the greatest amount of purification that has been found possible in this work. By this means it has been possible to separate all the insulin preparations so far obtained into two fractions, one of which is about twice as active per unit as the other.

Using this method in connection with Collips[1] method of extraction of cattle pancreas, preparations were obtained having an insulin value of one kilo-rabbit-unit per 0.15 to 0.20 mg. of solid or 0.02 to 0.03 mg. N. Using this method on an Iletin preparation originally having one kilo-rabbit-unit per 0.012 mg. N. it was resolved into one portion having one kilo-rabbit-unit per 0.008 mg. N and another larger portion having one kilo-rabbit-unit per 0.017 mg.N.

This method of separation is especially useful when dry insulin preparations are desired, as the alcohol insoluble portion may be washed with ether and dried, while the alcohol soluble portion is completely precipitated by the addition of a few volumes of ether and the precipitate may then be washed with ether and dried. The preparation of highest purity as far obtained in this work was made by utilizing this general idea of fractionating by means of solubility in alcohol. Dry insulin preparations are extracted with absolute alcohol containing a small amount of tartaric acid and this extract precipitated with ether. This yields a product that is easily dried and has an insulin value of one kilo-rabbit-unit per 0.005 mg. N. from the preparations used.

The nitrogen content of all the preparations experimented with has been extremely constant, varying around 14-15% calculated on the dry weight of the substance when dried at room temperature over phosphous pentoxide. Free amino groups are probably not present in the unhydrolysed material as it has not been possible to obtain a measurable amount of nitrogen when treated with nitrous acid by the Van Slyke method. After acid hydrolysis of an insulin sample about 1.6% of the total nitrogen was amide nitrogen, about 20% of the total was diamino acids and basic nitrogen, the remainder representing the mono-amino acids present. The absence of amino nitrogen and the separation of the total nitrogen into groups is reported by Shonle and Waldo.[5]

A characteristic reaction paralleling the hypo-glucemic action

of the preparations used has not been found. No preparation has been obtained that does not give the characteristic protein reactions, the Buiret, Millon, Xanthoproteic, and reduced sulfur tests, if the tests are carried out with high enough consentrations of dissolved substance. The Molisch reaction is not given by purified preparations. The diazo reaction, positive but not specific for histidine and tyrosine, is given very strongly (one part yielding a coloration of the same intensity as 0.4 parts of histamine phosphate,) and appears as if it were indicative of the insulin activity of many preparations. This is not strictly the case however, as dialysed p ortions giving the same intensity of color with the diazo test do not have even approximately the same rabbit value. The purer preparation have no reducing action on sodium phosphotungstate or ferric chloride solution, which offers means for the quantitative estimation of adrenaline and similar substances.

Insulin is completely removed from solution by precipitation with Phosphotungstic, tannic, metaphosphoric, picric and picrolonic acids. The precipitates can usually be regenerated by dissolving in acid-alcohol and precipitating the insulin away from the acid present with ether. Regeneration of the precipitates by other methods, such as removal of phosphotungstic acid as the barium salt, most frequently causes total loss of insulin, presumably due to absorption by the precipitate formed in solution. It was found that considerable, if not total, loss in activity results from the precipitation of almost any substance from insulin active solutions. Salting out precipitants such as sodium chloride and ammonium sulfate cause complete precipitation

if used in sufficient concentrations. The insulin may be recovered without loss by filtering out the precipitate and dissolving in acidulated water. These and similar precipitation behavior have been reported by many others.[2,5]

Precipitation of aqueous solutions containing a purified insulin, only takes place with metallic salts when the reaction is neutral or alkaline. Under these conditions mercuric chloride, lead acetate, and silver nitrate cause precipitation with removal of the insulin activity, so far as can be. determined. The conclusions drawn as to the action of the metallic salts are not definite, however, as it is always necessary to remove the metal from solution by precipitation before testing physiologically. The simple removal of the metal by precipitation is very frequently accompanied by absorption of the activity upon the precipitate.

Purified insulin is soluble in water except over a small range of hydrogen ion concentration. The stability of the solution is dependent upon time, temperature and the reaction of the solution. In 0.1 N hydrochloric acid solution the insulin appears to be quite stable; room temperature for several days, 40°C. for an hour or heating on a boiling water bath for 15 minutes do not result in appreciable losses of insulin value. Behaviour similar to this has been reported by others.[4,5] Solutions that are alkaline in reaction are not very stable, concentrations of 0.01 N sodium hydroxide being quite destructive. Room temperatures for 24 hours or 40°C for one hour result in the destruction of at least one-half of the insulin value of the solution so treated, while heating on a boiling water bath for 15 minutes with this concentrate of base results in practically

complete in-activation. Witzeman and Livshis[6] have reported quite completely on the destruction of activity by basic substances in higher concentrations and the possibility of the return of activity on acidifying. In so far as can be observed, no change takes place in the optical rotation, hydrogen ion concentration, or basic titration value when the insulin is completely inactivated in mildly alkaline solution.

The optical rotation of an 0.5% solution of a purified insulin of neutral reaction is - 0.43'. In acid solution it is slightly greater than this value, and in basic solution slightly less. The specific rotatory power, calculated from this datum is about - 86'. The optical rotatory power of several different insulin samples was shown not to be a measure of rabbit activity. This was most definitely shown by the fact that the rotation does not change even though the insulin be inactivated by treatment with mild alkali.

Insulin is very readily absorbed from acid solution with fullers earth and a part of the activity may be recovered by extraction of the fullers earth with acid 95% alcohol. This amount recovered is not of unusual purity, however, and as a method of purification has not led to promising results as far as it has been tried. The absorption of insulin on charcoal and its reversal has been recently reported by Maloney and Findlay[7] who have secured some promising results in their work.

Acidic and basic solutions of insulin dialyze quite readily thru collodion membranes. In certain cases, probably dependent both on

the membrane and the purity of the material dialyzed, the activity passed through the membrane quite readily and a separation into two fractions of different activity per unit of nitrogen was obtained. The dialysis behaviour so far investigated is very variable and in two cases a large part of the activity apparently disappears, being neither on the inside nor on the outside and not readily recoverable by alcohol extraction from the membrane.

Iodine and hydrogen peroxide in slightly alkaline solution immediately destroy the insulin activity present. This is in accord with the observations reported by Shonle and Waldo[5]. Reduction by treatment of solutions with sulfur dioxide, hydrogen sulphide or even zinc and hydrochloric acid caused only a slight decrease in activity. This is contradictory to the observations reported by Shonle and Waldo[5] with regard to the action of reducing agents. An attempt to reactivate an oxidized insulin by reduction with zinc and hydrochloric acid failed.

From the work performed and from the work reported by others there is little reason to doubt that the active material is of a protein nature, although it may not be very complex. The fact that it does so readily dialyze seems to indicate a reasonably low molecular weight for the active principle and therefore the possibility of its eventual isolation as a chemical individual.

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