CXXXI. CRYSTALLINE INSULIN.

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(Received March 4th, 1935.)

IN a previous communication from these Laboratories [Scott, 1934] attention was directed to the importance of certain metals in the crystallisation of insulin. It was shown that zinc was a constituent of insulin crystals prepared by the pyridine-brucine method of Abel, or by the saponin method of Harington and Scott, and it was found that good yields of crystals were obtained when the chlorides of zinc, nickel, cobalt or cadmium were added to the insulin solution. In fact, it was evident that in cases where the amorphous insulin was free from ash, the addition of one of these metals was essential for the formation of crystals. Moreover, it was suggested that the success of the previously published methods for crystallising insulin depended in part upon the fact that zinc is present in pancreas, in commercial insulin preparations and as an impurity in saponin. We have recently been engaged in the determination of the ash content of samples of insulin crystallised from ammonium acetate buffers to which one of the above metals had been added. The results of this work, together with a discussion of some observations already recorded by one of us (D. A. S.), are presented in the present communication.

In the method of crystallisation previously reported it was shown that insulin crystals can be readily obtained from amorphous preparations of insulin dissolved in phosphate, sodium acetate or ammonium acetate buffers if traces of certain metals are present. There are however a number of minor differences between these buffers which should be borne in mind. In the first place it was found that the addition of about 10 % of acetone to the phosphate or sodium acetate buffers facilitated crystallisation. With ammonium acetate however the best yields of crystals were obtained when the buffer was free from acetone. In fact the addition of this reagent caused the formation of an amorphous, rather than a crystalline, precipitate. Secondly, insulin crystallises best from a phosphate buffer solution at acidities between p_H 6.0 and 6.2 whereas, using ammonium acetate buffer, crystallisation proceeds most readily between p_H 5.8 and 6.0. Thirdly, a phosphate buffer is the most convenient one to use when the work is such that it extends over a lengthy period, in that quantities of this solution can be stored for several months without any change in p_H . Ammonium acetate buffers involve the neutralisation of acetic acid solutions of insulin with ammonium hydroxide; since the strength of the latter solution does not remain constant, considerable manipulation is necessary to reach the requisite acidity of the neutralised insulin solution. Fourthly, insulin crystals prepared by the phosphate buffer method are generally sharper in outline than those separated from an ammonium acetate solution. On the other hand ammonium acetate buffers have a decided advantage over phosphate buffers in the crystallisation of insulin by means of heavy metals in that the former yield crystals of lower ash content; this may perhaps be due to the greater solubility of the acetates of heavy metals as compared with the phosphates. Since it is desirable to have crystalline insulin with as low an ash content as possible, it is therefore advisable

to subject the material to a final recrystallisation from ammonium acetate. We have found it convenient to conduct the initial crystallisation of insulin in a phosphate buffer solution containing zinc chloride and acetone, and to recrystallise this material from ammonium acetate without acetone or the further addition of metals. Insulin crystals prepared in this manner have an ash content of 0.65 % when zinc is the metal used to aid in the first crystallisation.

It was previously shown that a large part of the ash could be removed from insulin crystals by precipitating the insulin from aqueous 0.1N HCl with the aid of alcohol and ether. Dissolving and reprecipitating the insulin as the hydrochloride a second time however did not materially decrease the ash content. Since zinc chloride is reasonably soluble in acid alcohol, and since a large proportion of the zinc is removed from crystalline insulin by this solvent, it is difficult to understand why an ash-free product is not obtained by this treatment. We recently carried out a great number of experiments to clarify this problem. In this work we dissolved insulin containing 0.7 % ash in various concentrations of hydrochloric acid and precipitated it as the hydrochloride with alcohol and ether, acetone, methylethylketone or dioxan. The ash content of the resultant product was usually 0.2 %. On one lot of crystals an ash content of 0.05 % was regularly obtained when acetone was used. We are at present unable to offer a satisfactory explanation for this phenomenon. It may be that in certain commercial preparations of insulin there is some other metal the chloride of which is not soluble in high concentrations of alcohol and ether, or in acetone. This metal may or may not aid in the formation of insulin crystals. That it is not essential for crystallisation however is shown by the fact that an insulin hydrochloride having an ash content of only 0.02 % crystallises readily in the presence of traces of zinc, nickel, cobalt or cadmium.

In the experiments recorded in the previous paper it was noted that after electro dialysis and precipitation of the insulin as the hydrochloride by alcohol and ether, there was a slight loss in insulin activity. Recent experiments have shown that the loss in activity did not occur during electro dialysis but did occur during the preparation of the insulin hydrochloride. Carr *et al.* [1929] were the first to observe that acid solutions of insulin were inactivated by alcohol and suggested that the inactivation was due to esterification. In our experiments on the precipitation of insulin as the hydrochloride by means of alcohol and ether, it seemed scarcely possible that inactivation was due to esterification since the precipitation of the insulin was effected almost immediately after solution. Nevertheless, to eliminate the possibility of such a reaction, all our recent experiments have been conducted by precipitating the insulin as the hydrochloride by means of acetone or methylethylketone. The acetone was first purified by boiling for 20 hours with potassium permanganate and was then distilled. From a large number of experiments on the precipitation of insulin as the hydrochloride with acetone, varying results have been obtained. In about 60 % of the experiments there was a loss in potency of approximately 15 % during precipitation. In two experiments a 20 % increase in activity was obtained. This material however after being kept in a dry state for 2 months, lost 50 % of its activity. In the remaining experiments no change in activity was detectable. When inactivation occurred, attempts were made to reactivate the insulin with alkali. Some measure of success was attained but the original activity of the crystalline insulin was never secured. These facts again raise the question of the inactivation of insulin with various solvents. We do not believe that it is due to the reducing action of traces of metals since the same insulin can be kept in an aqueous acid solution for several weeks without any loss

in activity, nor are we inclined to believe that it is due to esterification since no evidence of such has been found by analytical procedures [Charles and Scott, 1931]. Moreover, inactivation takes place with such solvents as acetone and methylethylketone where esterification is impossible. This inactivation may be due to some molecular rearrangement such as a tautomeric change or to the opening or closing of a ring structure. The proof of such theories however must wait until more is known of the chemical structure of insulin.

The observation was made in the former publication that the insulin after electrodialysis did not flocculate until p_H 6.3 was reached when neutralised with sodium hydroxide. We have verified this observation many times during the past few months and have found that the acidity at which an electrodialysed ash-free product flocculates is between p_H 6.1 and 6.3. Wintersteiner and Abramson [1932-33] found that insulin crystals suspended in $M/30$ sodium acetate buffers are isoelectric at p_H 5.0 and have an isoelectric point of p_H 5.3 as determined by electric mobility experiments using insulin absorbed on quartz in $M/30$ acetate. The difference between the isoelectric point of insulin as found by these authors and the point of maximum precipitation as observed in our experiments may be due to a different concentration of electrolytes. However, another view might be considered. It is possible that after neutralising the dialysed insulin with sodium hydroxide or ammonium hydroxide the amorphous sodium or ammonium salt of insulin is formed. We have repeatedly found that the best acidity for the crystallisation of insulin in the presence of certain metals is between p_H 6.0 and 6.2. If crystallisation is due to the combination of these metals with the free carboxyl groups of insulin, then this process should take place more readily on the alkaline side of the isoelectric point. In other words, crystalline compounds of insulin with various metals should have the characteristics of a substance having an isoelectric point more alkaline than that of the ash-free material. Similarly, if the sodium or ammonium salt of insulin is formed on neutralising a solution of insulin after dialysis, the point of maximum precipitation would be on the alkaline side of the isoelectric point of insulin.

During the past few months a great amount of experimental work has been done on insulin hydrochloride of low ash content. It was felt that should crystalline insulin be a complex compound of two or three chemical entities (such as proteins) bound to each other by combination with a metal, then, after the removal of the metals, there might be a possibility of obtaining a more active insulin by fractional precipitation. Using insulin with an ash content of 0.05 % we have tried fractionation experiments with alcohol and with acetone. This work has been carried out over a wide range of acidities, using either sulphuric acid or hydrochloric acid. Fractionation was also attempted at the isoelectric point with these solvents. In some cases, the solvents were added to insulin solutions which had been made alkaline with sodium hydroxide or with ammonium hydroxide. None of these experiments resulted in a successful fractionation of the insulin.

We have also determined the ash content of insulin crystals formed by the recrystallisation of an ash-free insulin. The metals used were zinc, cadmium and cobalt. The experimental work is presented in detail below.

EXPERIMENTAL.

For the purposes of this research, a large quantity of crystalline insulin was prepared. The first crystallisation was conducted in a phosphate buffer solution containing zinc chloride. The crystals were removed, washed and dried and

then recrystallised from ammonium acetate at room temperature without acetone or the further addition of metal. The final product had an ash content of 0.7 % and contained 25 i.u. of insulin per mg.

From this material an ash-free insulin was obtained by the following treatment. 0.5 g. of the crystals was placed in each of two 250 ml. centrifuge-tubes. To each tube were added 10 ml. of 0.1 *N* HCl. As soon as the crystals had dissolved, 200 ml. of acetone were added to each. The insulin readily precipitated. The tubes were placed in a refrigerator for 2 hours. The mixtures were then centrifuged, the acetone decanted and the precipitates combined by immediately dissolving them in 500 ml. of water containing 2 ml. of *N* HCl. The solution was then transferred to a litre beaker and electro dialysed using the type of apparatus formerly described. The solution in the beaker was vigorously stirred with a mechanical stirrer. Dialysis was continued for 1½ hours, during which time 35 litres of acid water, p_H 2.3, circulated through the thimbles. After the completion of dialysis, the solution was filtered into a 600 ml. beaker and very slowly neutralised with *N* NH₄OH until maximum precipitation was reached. The mixture was placed in the refrigerator overnight. The supernatant liquid was then decanted and the precipitate centrifuged in a 250 ml. tube. The clear solutions were combined and the p_H was determined and was found to be 6.2. The precipitated insulin was then dissolved in 15 ml. of 0.1 *N* HCl. After the addition of 100 ml. of acetone, the solution was divided into two equal parts and the insulin was precipitated by adding 150 ml. of acetone to each sample. The mixtures were then placed in the refrigerator overnight. After centrifuging, the supernatant liquids were discarded and the precipitates dried *in vacuo*. Samples of the dried insulin hydrochloride were then removed for ash estimations which gave the values of 0.01 and 0.03 %. The test for zinc was faintly positive. A physiological assay in which 300 mice were used showed that the material contained 23 i.u. per mg. From this material the various crystalline metallic salts were prepared and their respective ash contents were determined.

Samples of this ash-free insulin hydrochloride were crystallised by means of various metals from ammonium acetate. Ammonium acetate was chosen in preference to phosphate buffer for reasons already stated. Acetic acid, which yielded no residue on evaporation, and freshly distilled ammonium hydroxide were used to prepare *N*/3 solutions. 100 mg. of the insulin powder were weighed into a dry beaker and 50 ml. of *N*/3 acetic acid were added. After the insulin had dissolved, 5 ml. samples of the solution were measured into each of ten 15 ml. centrifuge-tubes previously treated with cleaning solution and washed several times with distilled water. 0.1 ml. of 0.5 % ZnCl₂ was added to each sample. The acidity was adjusted to approximately p_H 5.9 with *N*/3 NH₄OH. The tubes were inverted to ensure thorough mixing (but were not scratched) and kept at room temperature for 2 hours. They were then put in the refrigerator overnight. At the same time a control tube was prepared containing 0.5 ml. of the zinc chloride solution in the same volume of buffer, but no insulin. The following morning each tube except the control contained well-defined crystals. About 5 ml. of each supernatant liquid were decanted and the precipitates from every two tubes mixed, centrifuged and the supernatant liquids discarded. The five crystalline precipitates thus obtained were allowed to drain for 15 minutes by inverting the tubes over filter-paper. 2 ml. of distilled water were then measured into each tube and the crystals washed by gentle shaking. After centrifuging, the tubes were allowed to drain for 15 minutes over filter-paper. This washing process was repeated three times; during this washing, any amorphous material was carefully removed from the crystals. After the final washing

and draining, the precipitates in each of the five tubes were dried *in vacuo*, and the ash content of each sample was determined. These values are reported in Table I.

Table I. *Ash estimations of zinc-insulinate.*

Weight of crystals mg.	Weight of ash (ZnO) mg.	ZnO %	Zn %
8.09	0.048	0.60	0.48
8.92	0.060	0.67	0.54
12.31	0.089	0.72	0.58
7.71	0.050	0.65	0.52
7.84	0.043	0.55	0.44
10.61	0.070	0.70	0.56
Mean			0.52

The insulin crystals which were formed with cadmium and cobalt were prepared in a similar manner except that 0.1 ml. of 0.5 % CdCl_2 or 0.1 ml. of 0.5 % CoCl_3 was added to each tube instead of the zinc chloride. The crystals were washed as previously described. The ash contents are shown in Tables II and III.

Table II. *Ash estimations of cadmium-insulinate.*

Weight of crystals mg.	Weight of ash (CdSO_4) mg.	CdSO_4 %	Cd %
8.14	0.118	1.45	0.78
8.49	0.113	1.33	0.72
6.88	0.104	1.51	0.81
Mean			0.77

Table III. *Ash estimations of cobaltous-insulinate.*

Weight of crystals mg.	Weight of ash (Co_3O_4) mg.	Co_3O_4 %	Co %
5.40	0.033	0.61	0.45
7.91	0.049	0.62	0.46
8.01	0.044	0.55	0.41
Mean			0.44

Further quantities of crystalline insulin were prepared in the manner described above, using zinc chloride. After washing and drying, a sample of the material was removed and found to contain 0.7 % ash. The remainder was recrystallised from an ammonium acetate buffer without further addition of metal. The ash of this material was also 0.7 %.

Ash determinations were made by burning a known weight of the crystals in a current of oxygen [Emich-Schneider, 1932]. Difficulty was encountered in determining cadmium as the oxide because of the volatility of this substance. Hence it was estimated as the sulphate by adding one or two drops of strong sulphuric acid to the sample before ashing. The ash from the zinc experiments gave a negative chloride test.

We have also determined the nitrogen contents of a number of samples of insulin crystallised from phosphate and from ammonium acetate buffers using zinc chloride. A series of eight samples of crystals was prepared from sodium phosphate and a similar series from ammonium acetate. In the former series, the buffer was neutralised with $N/3$ NaOH instead of NH_4OH . After centrifuging, the crystals were washed and dried as already described. The average of eight estimations on each series showed that the crystals isolated from the phosphate buffer contained 14.0 % N while those prepared from the ammonium acetate buffer contained 14.7 % N.

DISCUSSION.

From a quantity of insulin hydrochloride having an ash content of 0.02 % and containing 23 i.u. per mg., several crystalline preparations of insulin were made. Crystallisation of the insulin was conducted in an ammonium acetate buffer solution containing traces of either zinc, cobalt or cadmium. All the crystals had the same microscopic appearance. The ash contents of the various kinds of crystals are reported in Tables I, II and III, from which it will be observed that the agreement between the ash values of different preparations of the same insulin salt is within the limits of experimental error and further that the percentage of ash varies with the metal used to aid in the crystallisation of the insulin.

The problem of the formula weight of insulin has been discussed by at least two groups of workers. Basing their estimate on data obtained by chemical treatment, Freudenberg and Dirscherl [1931] have advanced the suggestion that the formula weight of insulin may be 10,000 or 20,000. Accepting the latter figure, and using the data of Tables I, II and III in the present paper, a simple calculation shows that each formula weight of insulin contains 1.6 formula weights of zinc, 1.4 formula weights of cadmium or 1.5 formula weights of cobalt. If however we assume double the value suggested by these authors, then the metallic content of the crystals, in formula weights, can be expressed as whole numbers within the limits of experimental error. Using the ultracentrifuge, Svedberg [1931] estimated the formula weight of insulin to be 35,100. However, our knowledge of the chemistry of insulin may still be advanced without having an exact value for its formula weight.

Crystalline preparations of insulin containing either zinc, cadmium or cobalt have been described in this paper. It was found that the ash content of each sample was proportional to the formula weight of the metal which it contained. Furthermore, this ash value seemed to be characteristic in that it was not decreased by recrystallisation. These facts suggest that the metal occurs as a chemical constituent of the crystals and not merely as an impurity. The manner in which each metal is combined cannot however be decided. One might advance the suggestion that a metallic salt had been formed through the carboxyl groups. The large proportions of the dibasic amino-acids, glutamic acid and cystine in this hormone [Jensen and Wintersteiner, 1932] seem to bring this idea within the realm of possibility. If the formula weight of insulin is 40,000, the metallic content of the crystals could account for six carboxyl groups. This represents but a small number of the possible free carboxyl groups from the dibasic acids in insulin. On the other hand, the formation of protein salts through free carboxyl groups has been questioned by Robertson [1924]. The tendency of zinc, cadmium and cobalt to form many complex salts with amines should not be overlooked. There also remains the possibility of formation of a ring structure by combination of these metals with both amino- and carboxyl-groups.

In our work on the crystallisation of insulin it was found that crystals prepared from an ammonium acetate buffer had a higher nitrogen content than those obtained from a sodium phosphate buffer. In the past this fact has been attributed to traces of ammonium acetate adhering to the crystals. However, since repeated washing of the crystals failed to decrease the nitrogen content, we are inclined to believe that the nitrogen is chemically combined. Here again, combination through carboxyl groups could be suggested. It is also reasonable to suppose that the ammonia has formed a molecular complex with the metal in the insulin, for it is well known that certain salts of cobalt form as many as six molecular complexes with ammonia.

SUMMARY.

A quantity of insulin hydrochloride having an ash content of only 0.02 % was prepared. Different samples of this insulin were crystallised from an ammonium acetate buffer solution by means of zinc, cobalt or cadmium. Ash estimations made on different samples of the crystalline products showed constant values for the respective metals. Further, the average ash content of each insulin salt was proportional to the atomic weight of the metal which it contained. This fact strongly indicates that crystalline insulin contains the metals as chemically combined constituents and not as impurities. The percentage ash values are discussed in respect to certain observations regarding the chemistry of insulin. The crystallisation of insulin and certain other aspects of earlier work on this problem are also considered.

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