PURIFICATION AND CRYSTALLIZATION OF GLUCAGON

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Shortly after the discovery of insulin, Kimball and Murlin (1) postulated the existence of a hyperglycemic pancreatic factor, which they named glucagon. The factor was believed to be responsible for the initial transient hyperglycemia following parenteral administration of pancreatic extracts of insulin preparations. Several workers attempted to isolate and purify the active principle. A decade after Kimball and Murlin’s original observations, Bürger and Brandt (2) were able to prepare a partially purified material having glucagon activity. They, however, experienced great difficulties in trying to obtain a substance free from insulin. The investigators hence concluded that insulin and glucagon must be closely related, especially since the chemical properties of the preparations they obtained were very similar to those of insulin itself. In more recent years other workers, Sutherland et al. (3) and de Duve and Vuylsteke (4), obtained small amounts of partially purified preparations having significant glucagon activity. Unfortunately, they were unable to obtain sufficient amounts of material to permit extensive characterization.

Our continuing interest in insulin and in its effects on carbohydrate metabolism led us to an interest in this substance, which had an opposite effect to insulin with respect to blood sugar levels. Characterization of the hyperglycemic factor with respect to both physical and biological properties and exploration of possible relationships to insulin necessitated isolation in an essentially pure form. The successful preparation of a crystalline material for which we accepted the name glucagon was announced previously (5) and is reported in detail in this paper.

Methods

The biological activity of glucagon was assayed in cats as described previously (6). The mouse convulsion assay was used for estimating the insulin activity of the various fractions.

The zone electrophoresis experiments were carried out essentially according to the procedure described recently by Fønss-Beech and Li (7). The starch† was washed thoroughly with the buffer employed for each particular experiment. The pH of the wet starch usually is somewhat different from

that of the buffer used to prepare the starch paste. It also drifts slightly during the course of the electrophoresis. The pH indicated for each experiment represents the average value of all the segment eluates.

Glucagon was hydrolyzed with glass-distilled 20 per cent HCl for 16 to 48 hours at 105° in sealed ampuls filled with nitrogen. Redfield's (8) method was used for the two-dimensional amino acid chromatography. Prior to analytical determinations, glucagon was dried for 48 hours at 40° in vacuo over phosphorus pentoxide.

**Purification Procedure**

An amorphous fraction obtained during the commercial purification of insulin was used as the starting material. It contained approximately 4 per cent glucagon and 7 per cent insulin. Denaturation of protein and hence inactivation of the biologically active components insulin and glucagon were prevented by using mild purification procedures. Thus, it was possible to follow the removal of insulin during purification by insulin assays. The hyperglycemic activity of the protein remaining dissolved in the supernatant fluids of Steps 1 to 3 was negligible.

**Step 1. Acetone Fractionation**—40 gm. of the starting material are dissolved in 1000 ml. of water by adjusting the pH to 3.5 with 10 per cent hydrochloric acid. The clear solution is cooled to 0-4° in an ice bath; 3.16 volumes of ice-cold acetone are added slowly from two or three separatory funnels with tips of small diameter. The suspension is equilibrated for 15 minutes, and the precipitate is collected by centrifugation; yield, approximately 20 gm. (dry weight).

**Step 2. Fractional Precipitation at pH 4.3**—Sodium acetate buffer is prepared by dissolving 90 gm. of anhydrous sodium acetate and 340 ml. of glacial acetic acid in 30 liters of water. The pH of this solution is adjusted to 4.3 with 10 per cent sodium hydroxide (approximately 40 ml.). The material resulting from Step 1 is dissolved to prepare a 1 per cent protein solution. After adjusting the pH to 3.3 with 10 per cent hydrochloric acid, the solution is placed in a Visking cellophane membrane and dialyzed in the cold (4-7°) against the acetate buffer for 24 to 48 hours. The colloidal precipitate that formed slowly during dialysis is collected by centrifugation at high speed (30 to 50 minutes at 20,000 X g). It is re-suspended in approximately 250 ml. of water and is dialyzed for 24 hours against 0.025 N hydrochloric acid, and thereafter for 24 to 48 hours against water at 4-7°. The final solution contained 6 to 6.5 gm. of protein.

**Step 3. Fractional Precipitations at pH 2.5**—All dialysis operations described in this step are carried out in the chill-room at 4-7°. The dilute acid used is 0.025 N hydrochloric acid. The phosphate solution is prepared by dissolving 15 gm. of KH₂PO₄ per liter of water and adjusting the pH to 2.5 with hydrochloric acid.
The solution resulting from Step 2 is diluted with water (approximately 350 ml.) to obtain a protein concentration of 1 per cent. After adjusting the pH to 2.5 with 0.2 per cent hydrochloric acid, the solution is placed in a Visking cellophane membrane and is dialyzed for 24 hours against 30 liters of the phosphate solution. The colloidal precipitate is, as in Step 2, collected by centrifugation at high speed and is redissolved in 500 ml. of dilute acid. The solution is dialyzed, first for 3 hours against 0.025 N hydrochloric acid and thereafter for 24 hours against the phosphate solution. The collected precipitate is redissolved and dialyzed once more in the same manner. The salt is removed from the final precipitate by dissolving it in 250 ml. of dilute acid and by extensive dialysis (40 to 48 hours) of the solution against 0.025 N acid followed by water. The solution, virtually free of phosphate, is lyophilized. Yield, 1.3 to 1.8 gm. of a slightly yellow powder.

Step 4. Crystallization of Highly Purified Glucagon—1 gm. of the lyophilized material from Step 3 is dissolved in 75 ml. of water, 25 ml. of 4 M urea are added, and the pH of the mixture is adjusted to 8.9 with 0.2 per cent sodium hydroxide. The volume is brought to 150 ml. with 0.1 M glycine buffer of pH 8.5 and the solution (pH 8.6) is allowed to stand in the refrigerator. Crystallization starts after a few hours and is completed within 24 to 48 hours. The crystals are collected by gentle centrifugation in a clinical centrifuge; they are washed three times with a cold 0.01 per cent sodium chloride solution and dried subsequently in vacuo over phosphorus pentoxide. The colloidal precipitate which has formed during the crystallization process is removed by centrifugation at high speed. It contains 40 to 50 per cent glucagon, part of which can be recovered in crystalline form by repeating the crystallization step.

Recrystallization of glucagon is performed by using essentially the same procedure as described above. For example, 100 mg. of crystalline glucagon are dissolved in 14.5 ml. of water by adjusting the pH to approximately 11. 870 mg. of urea and 29 mg. of glycine are added. The solution is centrifuged and the clear supernatant fluid is readjusted to pH 8.5 with a small amount of 10 per cent hydrochloric acid. This operation precipitates some of the protein in amorphous form; its transformation into crystals, however, takes place upon standing in the refrigerator for 24 to 48 hours. The crystals are collected, washed, and dried in the described manner. Washing of once, or especially twice, recrystallized glucagon with a cold 0.01 per cent sodium chloride solution is essential, since the fragile crystals are often transformed into a gel if washed with electrolyte-free water. The specific biological activity of the residual protein, isolated from the mother liquor after recrystallization of glucagon, is identical with that of the crystals. Thus, within the limits of the biological test, no additional purification is accomplished by repeated recrystallization. The
protein recovery and distribution of biological activities for each purification step are summarized in Table I.

**Characterization and Properties of Glucagon**

The glucagon crystals belong to the isometric system and appear as the sharply defined rhombic dodecahedra shown in Fig. 1.

**Table I**

*Distribution of Protein and Biological Activity in Inactive and Active Fractions of Each Purification Step*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Inactive protein fraction</th>
<th>Active protein fraction</th>
<th>Overall recovery of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Recovered protein per cent</td>
<td>Glucagon content* per cent</td>
<td>Insulin units per mg.</td>
</tr>
<tr>
<td>Acetone fractionation</td>
<td>50</td>
<td>0.6-1</td>
<td>2.5</td>
</tr>
<tr>
<td>Fractional precipitation in acetate buffer at pH 4.3</td>
<td>65-70</td>
<td>-1.3</td>
<td>1 -1.3</td>
</tr>
<tr>
<td>Fractional precipitation in phosphate solution at pH 2.5</td>
<td>1st dialysis</td>
<td>70-75</td>
<td>1.5-2</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Crystallization</td>
<td>29†</td>
<td>48‡</td>
<td>40 -50</td>
</tr>
</tbody>
</table>

* Based on the assumption that crystalline glucagon is 100 per cent pure.
† Recovered from supernatant fluid.
‡ Colloidal precipitate formed during crystallization.

The crystalline powder is generally white, but occasionally has a bluish tint owing to trace contaminations of copper and cobalt ions. Emission-spectroscopic analysis revealed that the glucagon crystals obtained by the crystallization described contain less than 0.01 per cent of zinc. The moisture content is on the average 12.8 per cent; it can be removed by drying the crystals for 20 to 24 hours in vacuo at 100° over phosphorus pentoxide. The crystalline protein is relatively insoluble in water, especially in the presence of electrolytes. It, however, is soluble at alkaline and acid pH values, e.g. between pH 9.5 and 10.5 and below pH 4. The solubility is partly dependent on the amount and type of electrolyte present. Highly purified glucagon tends to form fibrils rather easily in acid aqueous solution. Fig. 2 shows an electron micrograph of glucagon fibrils.
The fact that no additional purification was accomplished by repeated recrystallization suggested that the crystalline glucagon preparations were of respectable purity. In addition, end-group analysis provided evidence that the crystalline product contained no detectable amounts of contaminating proteins. Sanger's dinitrophenyl procedure (9) yielded only 1 N-terminal residue, namely didinitrophenyl-histidine. The purity of glucagon has also been studied by zone electrophoresis with starch as a supporting medium. Crystalline and recrystallized preparations were tested under varying experimental conditions. Curve A of Fig. 3 represents the distribution of 6 mg. of once recrystallized glucagon after electrophoresis for 40 hours (160 to 170 volts, 13 to 15 ma.) at pH 8.8 in starch buffered with 0.075 M glycine. It is evident that the protein separated into two distinct peaks. The faster moving component comprises approximately 11 per cent of the total glucagon applied. The slight adsorption of protein at the point of application is probably due to the relatively low pH of this particular experiment. Adsorption did not occur in a similar experiment at pH 9.86 (Curve B). The more alkaline pH caused the protein to migrate somewhat faster, but had no effect on the proportion or separation of the two components. Repeated recrystallization of glucagon failed to remove the small constituent. Curve C illustrates the distribu-
tion of a glucagon sample recrystallized three times subjected to zone electrophoresis at pH 10.3 for 40 hours. The curve demonstrates that the two components are still present in approximately the same proportion.

Fig. 2. Electron micrograph of glucagon fibrils (20,000 ×)
Analysis of the minor component reveals that its qualitative amino acid composition is identical with that of the main peak and the crystalline protein itself. It is biologically about half as active as crystalline glucagon. These data suggested that the faster moving protein might be derived from glucagon itself, rather than being an extraneous impurity. This hypothesis was tested by reelectrophoresis of the protein isolated from the main peak of two large scale experiments, 20 mg. of crystalline glucagon being employed for each run. The electrophoresis (150 volts, 13 to 15 ma.) was carried out in a 60 cm. long trough at pH 10.2. The starch was washed with a glycine buffer (0.075 M) of pH 11.5. The time of electrophoresis was extended to 65 hours to insure separation of the two constituents. The shaded area in Fig. 4 indicates the portion which was used for the isolation of the main component. The recovered protein was crystallized and its electrophoretic behavior studied under the same conditions as employed for Curve B (Fig. 3). The result recorded in Curve D of Fig. 3 shows that reformation of the faster moving constituent has taken place in

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**Fig. 3.** Zone electrophoresis of crystalline glucagon on starch. The arrows indicate the point of application of the protein. Curve A, glucagon recrystallized once, 0.075 M glycine buffer, pH 8.80; Curve B, glucagon recrystallized once, 0.075 M glycine buffer, pH 9.86; Curve C, glucagon recrystallized three times, 0.05 M Na₂CO₃ solution, pH 10.3; Curve D, rerun of crystalline glucagon isolated from main peak (see Fig. 4), 0.075 M glycine buffer, pH 9.68.
about the same proportion. This result is consistent with the interpretation that the small component is derived from glucagon itself. The evidence obtained from these experiments suggests strongly that crystalline glucagon is a protein of high purity, although further studies are necessary to prove its purity conclusively.

The elementary composition of twice recrystallized glucagon is summarized as follows: C 50.11, H 6.42, S 0.77, N 17.45 per cent. Glucagon may be distinguished from insulin by its relatively high nitrogen and low sulfur content. It should be noted, however, that all the analytical values presented are comparable to those found for most typical proteins. Two-

![Optical Density vs. Migration of Glucagon](http://www.jbc.org/content/232/1/626/suppl/DC1/fig4)

Fig. 4. Large scale zone electrophoresis of crystalline glucagon on starch. The arrows indicate the point of application of the protein.

dimensional paper chromatography of glucagon hydrolysates revealed the following components: methionine, tryptophan, arginine, histidine, lysine, phenylalanine, tyrosine, leucine, valine, alanine, threonine, serine, glycine, aspartic acid, glutamic acid, and ammonia. The amino acid composition of glucagon differs significantly from that of insulin with respect to several amino acids. Cystine, proline, and isoleucine could not be detected by chromatographic procedures even when relatively high concentrations of glucagon hydrolysates were used. The protein, however, contained considerable amounts of methionine and tryptophan; both of these amino acids are absent in insulin. Quantitative determinations of methionine showed that it accounts for all the sulfur found in crystalline glucagon; this confirms the finding that no other sulfur-containing amino acids are present.
Parenteral administration of crystalline glucagon produces a rapid increase in blood sugar, which is presumably due to the liberation of glucose from glycogen stores in the liver (10). The minimal effective dose is dependent on factors such as animal species, route of administration, and nutritional status. The hyperglycemic response of near minimal doses of crystalline glucagon in cats, rabbits, and mice is illustrated in Figs. 5 and 6.

The blood sugar Curves A, B, and C of Fig. 5 illustrate the hyperglycemic responses in cats obtained with varying amounts of glucagon injected intravenously and subcutaneously. The curves clearly show that glucagon is considerably less effective when administered by the subcutaneous route than when given intravenously. Subcutaneous injection of glucagon up to 3 γ per kilo of cat body weight produced no significant hyperglycemia, whereas by intravenous administration as little as 0.05 γ per kilo caused a blood sugar rise of approximately 30 mg. per cent. This difference in activity is not observed in the rabbit. The hyperglycemia achieved
by 1 $\gamma$ of crystalline glucagon per kilo, given by either route, is of the same order of magnitude. Fig. 5 reveals, furthermore, that the rabbit is less sensitive to glucagon than the cat. The former requires about 10 to 15 times more glucagon to achieve a comparable rise in blood sugar. The sensitivity appears to differ from species to species. White mice of a standard strain are relatively insensitive to glucagon. The blood sugar rise produced by a dose of 0.1 $\gamma$ per mouse (25 to 30 gm.) is not significant even when injected intravenously. The same dose on a kilo basis (3 to 4 $\gamma$ per kilo) would cause a pronounced hyperglycemia in cats or rabbits. As illustrated in Fig. 6, a significant hyperglycemia can be obtained in mice by administering larger amounts of glucagon, e.g. 1 to 2 $\gamma$ per mouse. Anesthetized cats not only are more sensitive to glucagon than either rabbits or mice, but they also show less variation in sensitivity. It was found that the standard error of the mean in mice for groups of fifteen to seventeen animals varied between 20 and 30 per cent, whereas, for the same number of cats, it never exceeded 10 per cent.

In regard to stability it is evident from earlier work (2, 10) that glucagon is more resistant to alkali than insulin. This difference can be explained
on the basis of the amino acid composition of the two proteins. The insulin monomer (mol. wt. 6000) contains 3 molecules of cystine, an amino acid that is very susceptible to alkali. Even partial rupture of the disulfide linkages results in a total loss of the hypoglycemic activity (11, 12). The absence of cystine in glucagon is probably responsible for its relatively high stability in the presence of alkali. Although alkaline glucagon solutions are slowly inactivated as shown in Fig. 7, they can be kept under refrigeration for at least a few weeks without noticeable loss in biological activity. Glucagon dissolves readily by adding small amounts of alkali to crystal suspensions. These solutions can be kept at alkaline pH values of 10.5 to 11.5 for several hours without endangering the biological activity. Like insulin, glucagon appears to be more stable at acid pH values. A 0.1 per cent glucagon solution in insulin-diluting fluid at pH 3 was kept in the chill-room (3-4°) for 7 months without ensuing loss of hyperglycemic activity. Dry crystalline glucagon is relatively resistant to heating. Its biological activity remained unchanged after a heating period of 48 hours in vacuo at 105°.

DISCUSSION

Many of the earlier reports dealing with the action of crude insulin preparations suggested that the hyperglycemic effect might be an inherent property of insulin (13, 14) or be due to an artifact created during its
Bürger and Brandt (2) were first to study the properties of purified glucagon preparations. Their data suggested, in agreement with Kimball and Murlin's hypothesis, that the hypo- and hyperglycemic activities of pancreatic extracts are due to two different substances. Bürger and Brandt's preparations were active in rabbits at a dose level of 20 γ per kilo of body weight (given intravenously). It seems unlikely, however, that the purity of these samples exceeded 10 per cent, since 1 to 2 γ of crystalline glucagon per kilo of rabbit produced a comparable response. On this basis it is not surprising that the properties of crystalline glucagon described in this paper differ significantly in many respects from those described by the German investigators. Their preparations, for example, contained 2.7 per cent sulfur, whereas crystalline glucagon contains only 0.77 per cent. The low sulfur content of glucagon is explained by the absence of cystine which is present in considerable amounts in impure glucagon preparations or in insulin. Furthermore, the pK of glucagon is, contrary to Bürger and Brandt's observation, higher than that of insulin. Preliminary experiments indicated that the isoelectric region of glucagon is approximately between pH 7.5 and 8.5. The chemical and physico-chemical properties of the crystalline protein prove conclusively that glucagon is indeed an entity distinctly different from insulin. The possibility of its being a degradation product of the latter is eliminated by the amino acid composition of glucagon.

Mohnike and Boser (16) recently reported the preparation of a crystalline hyperglycemic, glycogenolytic substance. It appears to be a nucleoprotein and differs markedly from the crystalline glucagon described in this paper. Glucagon is free of carbohydrate and has all the properties of a simple protein. It contains methionine and threonine, two amino acids which are absent in the nucleoprotein. On the other hand, isoleucine and proline, two constituents of Mohnike and Boser's preparation, are missing in glucagon. The difference in biological activity between the two proteins is remarkable. 500 γ of Mohnike and Boser's preparation are necessary to achieve a minimal blood sugar rise in a rabbit weighing 3.5 kilos; a comparable response is obtained with as little as 2 to 3 γ of crystalline glucagon. Moreover, the biological activity of the nucleoprotein disappears rapidly, even when stored as a dry powder, whereas glucagon under these conditions is stable. In view of these facts, it is concluded that the two substances are entirely different.

Weitzel et al. (17) in a recent publication reported on the relationship between zinc content and hyperglycemic effect of several crystalline insulin preparations. The hyperglycemic activity of insulin, within certain limits, appeared to be a function of the zinc content. Insulin samples of low zinc content showed little or no hyperglycemic activity; however, considerable
biological activity was noted for preparations containing 0.9 to 1.4 per cent of zinc. Removal of part of the zinc from such samples produced insulin crystals essentially free of hyperglycemic activity. Based on these data, the investigators speculated that the hyperglycemic factor might possibly be inactive by itself, gaining its full potency only through formation of a complex with zinc and insulin. This hypothesis, however, is unlikely, since the biological activity of crystalline glucagon, containing at the most traces of zinc and insulin, is higher than that of any preparation described in the literature. Addition of either one or both of these agents has no potentiating effect on the biological activity. The protein, as far as it is known, contains no non-protein prosthetic groups. The hyperglycemic effect of glucagon, therefore, must be an inherent property of the polypeptide. Although this work clearly demonstrates that the formation of a complex with either insulin or zinc is not a prerequisite for biological activity, the possibility that these substances may form association products or complexes as described by Tanford and Epstein (18) is not eliminated.

Based on quantitative determinations carried out for some of the amino acids, it is possible to make an estimation with respect to the molecular weight of glucagon. Two-dimensional paper chromatography of dinitrophenyl-glucagon hydrolysates revealed no free histidine. Furthermore, no separate ninhydrin-positive spot, possibly due to colorless imidazole-dinitrophenyl-histidine was detected. The N-terminal histidine residue is therefore the only one occurring in the polypeptide chain and accounts for all of the 3.7 per cent histidine found in crystalline glucagon. This conclusion is in agreement with molecular ratio determinations and sequence studies presently in progress. The molecular weight of glucagon calculated from these data is 4200; the same value was obtained on the basis of the methionine content. This suggests that the polypeptide is composed of approximately 28 to 32 amino acid residues.

SUMMARY

The isolation and crystallization of glucagon, the hyperglycemic-glycogenolytic factor of the pancreas, are described. Physical and chemical studies indicate that the crystalline protein is of high purity. The amino acid composition revealed that glucagon is a distinct entity with no apparent relationship to insulin. Histidine is the N-terminal amino acid. Glucagon is entirely different from the hyperglycemic substance recently described by Mohnike and Boser. Crystalline glucagon virtually free of zinc is biologically highly active; its activity is not potentiated by addition of either zinc or insulin.

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