ON THE PREPARATION OF INSULIN.

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The procedure for the preparation of insulin outlined by us in an earlier abstract (1) was a modified form of the method first used successfully by the discoverers of insulin as described by Collip and coworkers (2). Retaining the use of alcohol for extraction the three important points added by us, and as a result of which great increases in yield and of purity were attained, were: (a) the use of much strong acid during the alcohol extraction, (b) the precipitation of the active substance from the crude aqueous solutions by ammonium sulfate, and (c) the precipitation of the insulin from the semipurified solutions by adjusting the reaction to pH 5 to 6.

The use of strong acid insures the solution of the insulin and prevents its destruction by proteolytic enzymes during extraction and subsequent evaporation. The precipitation by half saturation with ammonium sulfate accomplishes a complete separation from about nine-tenths of the accompanying proteins contained in the first extract and concentrates the activity in any desired volume. By precipitation of the insulin by adjusting the reaction to about pH 5, preparations were obtained of such activity that about 0.2 mg. of substance per kilo caused typical hypoglycemia and convulsions in rabbits weighing about 2 kilos. The yield of material of this or greater activity was by this procedure at least 500 “per kilo rabbit units” from each kilo of beef pancreas.

The purpose of this paper is to report the further purification of the product prepared by the method previously described.
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and as later improved, and to review the process of preparation in the light of the properties of the "insulin-protein." Since the activity expressed by the word "insulin," is (when obtained from beef pancreas at any rate) constantly associated with what appears to be a single, fairly well characterized protein, it becomes possible to formulate somewhat more rational methods for its extraction and purification than could be done before the physical and chemical properties of the product were known.

In consequence of the reviews of the literature contained in recent papers on this subject, we may omit detailed comment on other methods of insulin preparation. Attention may be called especially to the papers by Collip and coworkers, 1922 (2), Best and Macleod, 1923 (3), Murlin and associates, 1923 (4, 5), Best and Scott, 1923 (6), Witzemann and Livshis, 1923 (7), Dudley, 1923 (8), and Fisher, 1923 (9).

The "Insulin-Protein."

Although it would be premature to claim that our most active product is insulin, it behaves as an individual, fairly well characterized substance, which so far we have been unable to fractionate. Different preparations, purified by different methods, have the same properties and substantially the same degree of activity. Furthermore, we have not encountered active insulin preparations from which we were unable to isolate the characteristic substance. The last statement must be limited to preparations from beef pancreas; we have not used other material. For insulin of pancreas, the activity appears to be associated only with a single constituent. The purified insulin to which we refer is a protein, giving distinct biuret and Millon reactions, but negative glyoxylic acid reaction for tryptophane. Its activity is indicated by the fact that on injection into rabbits about 0.05 mg. (a conservative estimate) of dry substance per kilo of body weight causes convulsions and marked hypoglycemia, and on this basis about 0.03 mg. may be taken as 1 standard Toronto unit. (For a 2 kilo rabbit, \( \frac{0.05 \times 2}{3} = 0.033 \) mg. for 1 unit.) In view of the above facts and of the rapid destruction of the activity by proteolytic enzymes (7, 8) we are
inclined to the view that the activity is a property of this protein, and not of a still more potent admixture.¹

If the view is correct that the activity is a property of "insulin-protein," it should be of great importance in affording a basis for the chemical assay of the substance. The present methods of physiological assay are tedious, difficult, and uncertain for quantitative purposes, and the ideal method is obviously the isolation and weighing of the active principle. Although we feel that our data alone are insufficient to establish the identity of insulin, we have been strongly impressed with the quantitatively very similar, if not identical, activity of our different "pure" preparations of "insulin-protein," purified by different methods, and with the fact that these different preparations appear to have otherwise the same properties.

As will be explained below we have separated from the "isoelectric insulin-proteins" described in our first report, two distinct proteins of less or no activity, and it is possible that the substance which we now call "insulin-protein" is still composed of more than one protein, only one of which is active. We have no evidence to disprove such a hypothesis. Whether or not insulin is identical with the "insulin-protein" can be determined only by the result of further efforts to separate and concentrate the activity.

The view above expressed (and suggested in our first report), that insulin is a protein, is in line with the conclusion of Dudley (8), but is contrary to the opinion first stated by Best and Macleod (3), and more recently by Murlin (5), who base their opinion that

¹ If insulin is not a protein and is present only as an admixture in purified "insulin-protein" it must have very high activity indeed. It could scarcely be present in amount more than 5 per cent of the weight of the "insulin-protein." That fraction of the quantity of "pure" "insulin-protein" which frequently gives convulsions in 1 kilo rabbits (0.01 to 0.02 mg.) would be 0.0005 to 0.001 mg. and this when distributed in the blood and tissues of the animal would give a concentration of the active principle of about 1 in 1 or 2 billion. This would be five to ten times the remarkable activity observed by Abel and coworkers (10) with their "pituitary tartrate," 0.01 mg. of which is required to produce rise of blood pressure on injection into cats. By the same sort of calculation the effective convulsive concentration of the "insulin-protein" itself would be of the order of 1 in about 50 million. Considering that it is a convulsive dose, either concentration expresses a very high degree of potency.
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insulin is not a protein chiefly on the fact that some of their active solutions failed to give the biuret reaction. That evidence is, however, not necessarily conclusive for the reason that the test for activity considerably exceeds in delicacy the biuret reaction. Moderately pure preparations of "insulin-protein," diluted so that about 1 cc. is required to produce marked hypoglycemia and convulsions in rabbits (0.01 per cent or less of protein), are too dilute to show the biuret reaction. We are therefore inclined to suspect that the conclusions of Macleod and Best and of Murlin on this point have resulted from their use of too dilute solutions for the protein tests. In concentrations of 0.02 per cent (which corresponds to at least 5 "units" per cc.) or more, our purest (i.e. most active) preparations give an unmistakable biuret reaction, and according to analyses to be reported later by one of us, are beyond question protein in nature. Whether it is a derived protein or is present as such in the pancreas it is impossible at present to decide, though we incline to the latter view.

Much the most useful property of the "insulin-protein," for purposes of its separation from other substances and its purification, is its relative insolubility at a reaction of approximately pH 5, reported in our earlier communication (1). Following a partial purification of the crude extracts, this property affords an ideal separation, which has been adopted by the manufacturers (11, 12).2

In the absence of more than traces of salts, the range of its precipitation zone is between about pH 4.4 to 5.8, with optimum flocking out near pH 5, which doubtless represents approximately

2 When this point was first communicated by one of us (P. A. S.) to Messrs. C. H. Best and D. A. Scott and, a few minutes later to Dr. G. H. A. Clowes, at Toronto, on December 28, 1922, it was unknown in the Toronto laboratory, though Dr. Clowes stated that it had been independently discovered in the laboratory of Eli Lilly and Co. (see outline of Lilly process, J. Am. Med. Assn., 1923, lxxx, 1851). Recent examination (December, 1923) of two vials of "Insulin-Lilly," "H-10" from the stock of the Barnes Hospital, showed it to contain 0.07 mg. of solids (non-volatile at 110°C. and leaving no weighable ash on combustion) per labelled unit. The solids consisted of "insulin-protein," together with some of the "pH 4-proteins." These preparations were, therefore, according to our standards, of a high degree of purity, being about half as active as our best "insulin-protein".
its isoelectric point. It dissolves clear at pH 4 and at pH 6, and beyond this zone on either side, presumably in the form of acid and alkali salts.\textsuperscript{3}

In the presence of even low concentrations of inorganic salts, especially sulfates, the range at which it precipitates is modified by being extended on the acid side. Solutions at pH 4 or at somewhat more acid reactions, which in the absence of salts are clear, are at once precipitated on the addition of small amounts of sodium or ammonium sulfate. Other salts have a similar, though less marked, effect and because of this effect salts must be removed for separation of the "insulin-protein" from the other "isoelectric proteins" to be described later. Alkali salts do not show such effects on the alkaline side of the isoelectric point, although at pH about 6 their presence may interfere with the separation just mentioned because of extending the precipitation zone of a less acid protein to be mentioned below. Moderate concentrations of salts have also the effect of slightly increasing the solubility of the "isoelectric proteins" within the precipitation zone.

Higher concentrations of salts, one-third to one-half saturation with ammonium sulfate and saturation with sodium sulfate or chloride, cause practically complete precipitation of the "insulin-protein" (and also of some other accompanying proteins) even at acid reactions far above its isoelectric range.

In alcoholic solutions, up to about 80 per cent, the purified "insulin-protein" is also quite soluble, but only in the form of its acid or alkali salts. At reactions near its isoelectric point, that is, in the form of isoelectric protein, it is little more soluble in dilute alcohol than in water. If a moderately strong aqueous solution is either slightly acidified with HCl or made alkaline with NaOH, or NH\textsubscript{4}OH, it may be diluted with alcohol up to about 80 per cent without causing precipitation. From such clear alcoholic solutions the insulin is promptly precipitated on the addition of dilute alkali or acid to a reaction near the isoelectric point. On further increasing the alcohol concentration or adding ether, precipitation takes place even in the presence of an excess of acid, the completeness of precipitation depending upon the amount of acid excess as well as upon the alcohol concentration. If precipitated

\textsuperscript{3} As pointed out by Dudley (8), it is precipitated also by 3.3 per cent HCl. It is even less soluble in about 5 N H\textsubscript{2}SO\textsubscript{4} than in HCl.
at pH 5 in aqueous solution, the addition of alcohol does not dissolve it. The activity is quite stable in the form of its acid salt and is less stable or unstable in the presence of excess alkali (7, 8).

Further information concerning the reactions and composition of the “insulin-protein” will be given in a later paper by Doisy and Weber.

**Contaminating Isoelectric Proteins.**—On adjusting the reaction to about pH 5, there separates from solutions of crude insulin (such as obtained by the Collip method or by dissolving in water the first half saturation ammonium sulfate precipitate) a precipitate which usually contains, in variable amounts, at least three proteins. One is the “insulin-protein” above described, and the other two are very similar in behavior except that each has, in the absence of salts, especially sulfates, a different range of precipitation, presumably due to different isoelectric points. All are precipitated at pH 5, especially in the presence of sulfates, contained in the solution referred to. One, which we term the “pH 8-isoelectric,” has its optimum precipitation, in absence of salts, about pH 7 or 8 at which reaction both the “insulin-protein” and the third, the “pH 4-isoelectric,” are quite soluble. Since low concentrations of alkali salts have little effect upon the solubility range on the alkaline side of the isoelectric points, the “pH 8-isoelectric” protein may be easily removed by adjusting the reaction with dilute ammonia to pH 7 or 8, at which reaction it precipitates, while the others remain in solution, even in the presence of some sulfates.

The purified “pH 8-protein” is not active on injection into rabbits. Different lots of the separated “pH 8-protein” have been injected in amounts from 0.17 to 4.3 mg. per kilo without causing symptoms or convulsions. (In a series of twelve rabbits, one which received 0.3 mg. had a convulsion after 3 hours.)

In the presence of sulfates, the ranges of precipitation of the “pH 4-isoelectric” and of the “insulin-protein” are so fused that their separation by fractional precipitation is difficult. But if both be precipitated together from such solutions at pH 5, the salt may be removed by washing by centrifugation several times with water at pH 5. On dissolving the combined precipitate in dilute acid (HCl or acetic) the “pH 4-isoelectric” precipitates on bringing the reaction to pH 4, at which reaction a large part of the “insulin-
protein” remains in solution. The substance which separates at pH 4 is, however, active, in all probability due to its carrying down some of the “insulin-protein.” From such precipitates by repeated fractionation we are able to separate typical “insulin-protein,” though we have not so far succeeded in removing all sugar lowering activity from the “pH 4-isoelectric” fractions. From the fact that such “acid” fractions appear to decrease considerably in their activity, we are inclined to suppose that the activity is due to contamination with the “insulin-protein.”

We shall describe in a later section the procedures which we find preferable for the separation of the isoelectric proteins.

The Alcohol-Soluble Protein.

The aqueous solutions left after the evaporation of the alcohol from the first extracts of the pancreas hash, and after filtration from the fats and fatty acids, contain besides the “isoelectric proteins” described above, a protein which is in part precipitated with them by half saturation ammonium sulfate and is characterized by its ready solubility in alcohol up to about 80 per cent, and its insolubility in higher concentrations of alcohol. It is not precipitated from water or dilute alcohol on adjusting the reaction and thus may be separated from the “isoelectric proteins.”

When present in relatively high concentration in solutions from which the “insulin-protein” is to be precipitated, it, however, considerably interferes with and renders incomplete that precipitation. This is often the case with solutions of the first (NH₄)₂SO₄ precipitate and especially with preparations obtained by the Collip method which consist chiefly of “alcohol-protein.” (The success of the Collip method largely depends upon the fact that precipitation by strong alcohol of this “alcohol-protein” carries down with it varying amounts of the active “insulin-protein.”) In such cases it is advantageous to reprecipitate once or twice by 0.4 saturation ammonium sulfate, which each time carries down substantially all the “insulin-protein” with progressively smaller amounts of the “alcohol-protein.” In this way we have often obtained considerable amounts of “insulin-protein” of typical behavior and activity from solutions which failed to yield much precipitate on first adjusting the reaction.
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If the relative amount of "alcohol-protein" is not too great, simple dilution of the solution often increases markedly the yield of "isoelectric proteins."

The purified solutions of this "alcohol-protein" carry some activity, but its activity is relatively small and is not more than can be explained as due to the presence of traces of the "insulin-protein" which it is difficult or impossible to remove completely. It is not unlikely that this "alcohol-protein" is a group of proteins, and may include the toxic substance described by Fisher (9). It requires further study.

The Choice of Acid for Extraction.

As already noted, the use of much strong acid during the alcohol extraction of pancreas hash is essential to high yield. To this the Toronto workers agree (6). In our earliest preparations we added sufficient mineral acid (HCl, H₂SO₄, H₃PO₄, or HNO₃) to keep the alcoholic liquid acid to Congo red paper during the extraction. To this fortunate step is doubtless due the fact that from the first our preparations were remarkably active. The amounts of acid used were 10 to 40 cc. of 10 N for each kilo of pancreas. A large part of the acid is absorbed by the undissolved protein, with the formation of acid protein salts, and only by providing an excess can the desired reaction of the solution be secured. From the properties of the "insulin-protein" above described, it is now evident that to insure its solution the acidity should be safely above pH 4 and far above in the presence of salts. In order to secure a pH of about 3, at least 200 cc. of N acid are required for each kilo of pancreas. The buffer action of the proteins is so great that considerably more will be absorbed before the reaction becomes very strongly acid. In our earlier abstract we recommended 40 cc. of 10 N acid per kilo. This is unnecessarily great, half or three-fourths that quantity being sufficient and preferable. Less than this amount, according to our experience, is insufficient for good yield.

Table II contains data showing that the yield of activity with 2.5 cc. of 10 N H₂SO₄ was less (probably much less) than one-eighth as high as with 20 cc. of 10 N acid per kilo of hash. With the smaller amounts of acid only very small amounts of "isoelectric
proteins" and of activity were obtained; and with increasing amounts of acid the yield of "isoelectric protein" increased, parallel with the increase of activity. 20 to 30 cc. of 10 N acid per kilo of pancreas appear to be the optimum amount.

Of the common mineral acids, sulfuric is, according to our experience, the best. It causes minimum swelling of the hash with resulting rapid filtration and large volume of filtrate. Hydrochloric and phosphoric acids are objectionable because of their swelling effect, making filtration slow and yield of filtrate small. This difficulty can be in large part avoided by neutralization of the acid before filtration. Neutralization, however, greatly increases the salt concentration, and in theory, at least, endangers loss of insulin by precipitation. This danger can probably be avoided by neutralization to pH 7 or 8, on the alkaline side of the precipitation zone. But in this there is the practical difficulty that the combined acid of the undissolved acid protein salts is liberated slowly with consequent constant change of the reaction of the liquid. Furthermore, the solubility of other inactive proteins is changed on neutralization and extracts prepared with HCl (and alcohol), followed by neutralization, contain larger amounts of protein with corresponding difficulty in later purification of the active fraction. In spite of these objections successful preparations can be made with HCl, and in our earlier preparations with this acid the product was considerably lighter in color than those from the use of sulfuric acid. It is our impression, however, that the yield of activity with HCl, due to the necessity of neutralization, is apt to be lower than with H$_2$SO$_4$. With suitable quantities of H$_2$SO$_4$ filtration is quite rapid without neutralization and by its omission not only may the danger of loss of activity by precipitation be avoided, but less of other contaminating protein is contained in the first acid extract and the whole process is considerably simplified.

Filtration without neutralization and the evaporation of the acid extract has another important advantage, besides those above mentioned. During the alcohol extraction a very considerable saponification of fat occurs, due doubtless to the lipase and the favorable effect of the alcohol on the solution and dispersion of the fat. If the extracts be evaporated at nearly neutral reaction, some of the fatty acid separates as alkali or protein
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soaps which emulsify the remaining fat and proteins and thus make very troublesome their separation and filtration. But if the extracts remain rather strongly acid (pH ± 3) during evaporation, the fat and fatty acids separate in a form which allows their subsequent complete removal by simple filtration through wet paper, and leaves the "insulin-protein" together with minimum admix-

### Table I.

Nitrogen Content of Pancreas Extracts and Insulin Preparations at Different Stages.*

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>10 N acid per kg.</th>
<th>Alcohol used for extraction.</th>
<th>Filtered crude extract after evaporation of alcohol.</th>
<th>First (NH₄)₂SO₄ precipitate.</th>
<th>&quot;Isoelectric protein&quot; precipitate.</th>
<th>Acidity of first crude extract after evaporation of alcohol (electro-metric).</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cc.</td>
<td>vols.</td>
<td>mg. N</td>
<td>mg. N</td>
<td>mg. N</td>
<td>mg. solids</td>
</tr>
<tr>
<td>94</td>
<td>20 HCl</td>
<td>1.5</td>
<td>3,950</td>
<td>273</td>
<td></td>
<td></td>
</tr>
<tr>
<td>97</td>
<td>15 H₂SO₄</td>
<td>1.5</td>
<td>2,600</td>
<td>117</td>
<td>14</td>
<td>93†</td>
</tr>
<tr>
<td>95</td>
<td>20 &quot;</td>
<td>1.5</td>
<td>3,780</td>
<td>245</td>
<td>21</td>
<td>140†</td>
</tr>
<tr>
<td>98</td>
<td>30 &quot;</td>
<td>1.2</td>
<td>2,140</td>
<td>294</td>
<td>28</td>
<td>185†</td>
</tr>
<tr>
<td>96</td>
<td>45 &quot;</td>
<td>1.2</td>
<td>4,110</td>
<td>23</td>
<td>153†</td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>20 &quot;</td>
<td>1.5</td>
<td>2,430</td>
<td>182</td>
<td>17</td>
<td>114†</td>
</tr>
<tr>
<td>118</td>
<td>0</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>111 a</td>
<td>2.5 H₂SO₄</td>
<td>1.5</td>
<td>2,550</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>111 b</td>
<td>5 &quot;</td>
<td>1.5</td>
<td>2,840</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>111 c</td>
<td>10 &quot;</td>
<td>1.5</td>
<td>2,410</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>111 d</td>
<td>15 &quot;</td>
<td>1.5</td>
<td>2,250</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>111 e</td>
<td>20 &quot;</td>
<td>1.5</td>
<td>2,090</td>
<td>182</td>
<td>8†</td>
<td>53</td>
</tr>
<tr>
<td>112</td>
<td>20 &quot;</td>
<td>1.5</td>
<td>1,554</td>
<td>171</td>
<td>7†</td>
<td>48</td>
</tr>
<tr>
<td>113</td>
<td>20 &quot;</td>
<td>1.5</td>
<td>1,554</td>
<td>157</td>
<td>5.4†</td>
<td>36</td>
</tr>
<tr>
<td>114</td>
<td>20 &quot;</td>
<td>1.5</td>
<td>3,400</td>
<td>230</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>117</td>
<td>30 &quot;</td>
<td>1.5</td>
<td>3,190</td>
<td></td>
<td>19</td>
<td>126</td>
</tr>
<tr>
<td>116</td>
<td>22 acetic.</td>
<td>1.5</td>
<td>3,600</td>
<td></td>
<td></td>
<td>Very small.</td>
</tr>
</tbody>
</table>

* Results expressed per kilo of pancreas hash.
† Calculated from nitrogen determinations, assuming 15 per cent nitrogen, or vice versa.

ture of other proteins in the perfectly clear filtrates. For this reason, if the first extracts are neutralized, they should be acidified (to Congo red paper) before evaporation. Unless the acidity of the solution at the time of filtration is pH 3 or greater, a considerable part of the "insulin-protein" is undissolved and is removed with the fat.
Table I contains data showing the reaction of the first extracts, after evaporation of alcohol and filtration from fat, for different amounts of acid used in the extraction of the pancreas hash. The extracts were made as described later (p. 43) except for the varying amounts of acids used. The determinations of pH were made by the electrometric method. It will be seen that when no acid is added, the reaction of the first crude aqueous extract is pH 5.6; with 5 cc. of 10 N H$_2$SO$_4$, pH = 5.0; and with 10 cc. of 10 N acid, pH = 4.4. With 22 cc. of 10 N acetic acid, used by the Toronto laboratory as described by Best and Scott, the reaction was pH 4.5. All these reactions are within the zone of precipitation of the "insulin-protein," and as one would expect from this fact, the yield of "insulin-protein" and activity is with these amounts of acid relatively small. The "insulin-protein," obtained with the smaller amounts of sulfuric acid and with acetic acid, was not weighed, but from its volume on precipitation and centrifugation, was manifestly very small. This was the case even with 22 cc. of 10 N acetic acid.

The results, given in Table II, of injecting the crude extracts into rabbits, indicate the relative yield of activity from these preparations. An amount of extract equivalent to 4 gm. of pancreas per kilo of rabbit weight of the 2.5 cc. of 10 N acid preparation, to 2 gm. of the 5 cc. of 10 N acid preparation, and to 1.5 gm. of the 10 and 15 cc. of 10 N acid preparations, showed only little, although increasing activity, while an amount of isolated "insulin-protein" (0.026 mg.) representing only 0.5 gm. of pancreas of the preparation with 20 cc. of 10 N acid gave convulsions with blood sugar of 0.047 and 0.048. The pancreas used was from the same batch in all these preparations, and it is clear from the data that the amount of acid used, and the resulting hydrogen ion concentration, were the main factors responsible for the different yields. The low yield with small amounts of acid is probably due both to poor extraction from the pancreas and to precipitation during evaporation and loss with the fat on filtration. We did not determine separately the amount removed with the fat, and, therefore, cannot decide at which stage the greater loss occurs. Both during alcohol extraction and later filtration of aqueous extract the reaction was doubtless unfavorable for the solution of the "insulin-protein."
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The other data of Table I concern the amount of nitrogen contained in extracts and fractions at different stages of the preparation. The first crude aqueous solutions after the evaporation of alcohol and filtration contain from 1.5 to 4.0 gm. of nitrogen per kilo of hash. Duration of extraction, concentration of alcohol, and the amount of acid added, all influence the amount of protein extracted. The total amount of protein extracted bears no constant relation to the yield of "insulin-protein" or of activity. The

**TABLE II.**
**Illustrating the Effect of Amount of Acid Used for Extraction upon Yield of Activity.**

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>10 N H₂SO₄ per kg. pancreas.</th>
<th>Rabbit No.</th>
<th>Amount injected per kg. rabbit weight.</th>
<th>Blood sugar.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Equivalent of pancreas.</td>
<td>1 hr.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isoprotein.</td>
<td>gm.</td>
<td>mg.</td>
</tr>
<tr>
<td>111 a</td>
<td>2.5</td>
<td>74</td>
<td>3,270</td>
<td>4.0</td>
</tr>
<tr>
<td>111 b</td>
<td>5.0</td>
<td>400</td>
<td>3,600</td>
<td>2.0</td>
</tr>
<tr>
<td>111 c</td>
<td>10.0</td>
<td>300</td>
<td>2,960</td>
<td>1.5</td>
</tr>
<tr>
<td>111 d</td>
<td>15.0</td>
<td>70</td>
<td>2,770</td>
<td>1.0</td>
</tr>
<tr>
<td>111 e</td>
<td>20.0</td>
<td>36</td>
<td>2,720</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>991</td>
<td>2,720</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>91</td>
<td>3,080</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>43</td>
<td>2,120</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
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<td>80</td>
<td>1,800</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>2,550</td>
<td>0.01</td>
</tr>
</tbody>
</table>

All prepared as described on page 43, except for different amounts of sulfuric acid in extraction of pancreas hash. C indicates typical hypoglycemic convulsions.

first crude extracts obtained by the procedure recommended in a later section yielding high activity, commonly contain 2 to 3 gm. of nitrogen; while as much or more nitrogen may be contained in extracts having only very little activity.

On precipitating the first crude extract by half saturation ammonium sulfate, the nitrogen content of the precipitate (after removal of ammonia by boiling with MgO and alcohol) is usually about one-tenth or less of the original, 0.15 to 0.25 gm. of nitrogen.
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per kilo of hash. The solution of this precipitate yields on adjusting the reaction to pH 5 (from successful preparations) about 0.05 to 0.16 gm. of total "isoelectric proteins," equivalent to about 0.007 to 0.025 gm. of N. By the successive use of ammonium sulfate and isoelectric precipitation, the activity is thus concentrated in less than 1 per cent of the material contained in the first alcoholic extract.

In this first precipitate the "insulin-protein" is contaminated with the accompanying "isoelectric proteins." When they are more or less completely removed, as later described, there should be left at least 0.05 gm. of fairly pure "insulin-protein" for each kilo of pancreas. The figures given in Table I for the amount of crude "isoelectric proteins," include for preparations Nos. 94 to 108, considerable "pH 4-" and "pH 5-isoelectric proteins," while the amounts stated for later preparations, Nos. 111 to 117, represent the yield of fairly pure "insulin-protein" by extraction and precipitation of the first crude precipitate by buffers of known pH as described on page 45.

Taking 0.05 gm. as a conservative estimate of the amount of "insulin-protein" obtained from 1 kilo of beef pancreas, we may calculate that the content of "insulin-protein" in pancreas is 0.005 per cent.

Method of Preparation.

It may be stated in connection with the following description of the process for the preparation of insulin that we have used only common laboratory facilities, and have not had at our disposal high vacuum stills, continuous centrifuges, or other aids which are doubtless of importance for large scale production. Nevertheless, the process is smooth and simple and can be depended upon to yield without difficulty highly active preparations. (We are using it as a class exercise with medical students.)

Fresh beef pancreas is finely hashed by passing twice through a meat grinder. We commonly add 20 or 30 cc. of 10 N H₂SO₄ to each kilo of hash, and mix by stirring, before passing the second time through the grinder. If there is much delay in grinding, this is probably desirable. With a rapid motor-driven grinder it is not necessary. If the acid has already been added, add 1,500 cc. of 95 per cent alcohol; otherwise add that amount of alcohol and 20 or 30 cc. of 10 N acid per kilo of hash. Mix well
Preparation of Insulin

by stirring. After standing at room temperature for 4 to 12 hours, with occasional stirring, the mixture (without neutralization) is poured on large filters. The filtration is rapid, though it is convenient to allow it to drain overnight. The residue on the filters is pressed in a hand-press and the press-liquid filtered. A reextraction of the residue with 60 to 70 per cent alcohol, slightly increases the final yield. The combined filtrates (total without reextraction, about 2,100 cc. per kilo) are evaporated at low temperature. We have used a horizontal tunnel through which a current of warm air (40-45°C.) is blown by a motor fan, and in which the filtrates are placed in glass photographic development trays. The temperature of the liquid during evaporation in our apparatus is 25-30°C.

When the odor of alcohol is gone, and the volume reduced to one-tenth or less, the liquid is poured on moistened filter paper. (It is sometimes necessary to add water during evaporation to avoid too great concentration before all alcohol is gone.) Filtration is rapid and the separated fats and proteins are thus easily removed. The trays and filter are washed repeatedly with small amounts of water until the clear filtrate has a volume of about 200 cc. for each kilo of pancreas used.

4 This apparatus does not provide for recovery of alcohol, which is blown to the outside air. (Its evaporation in a closed room caused an explosion.) Since only 1.5 to 2 liters of alcohol are used per kilo of pancreas, yielding 1,000 to 2,000 “units” of insulin, the alcohol, if tax-free or denatured, represents only a small item in the cost of production.

5 At this dilution the subsequent precipitation by ammonium sulfate carries down less “alcohol-protein” than when the extract is more concentrated. Because of its effect in interfering with the later precipitation of the “isoelectric proteins,” it is desirable to remove most of the “alcohol-protein”, and for this purpose a second precipitation with somewhat smaller concentration of (NH₄)₂SO₄ is advantageous.

6 The grayish brown precipitate on the filter, consisting chiefly of fat and fatty acid with some protein, contains also some “insulin-protein” which with high acid extraction is scarcely worth recovering. If recovery is desired the precipitate is well stirred with water acidified (Congo red paper) with HCl or H₂SO₄ and warmed to about 60°C. The mixture is then cooled in the ice box and filtered cold through moistened paper. The filtrate is precipitate with ammonium sulfate, the precipitate dissolved, and the isoelectric proteins are separated at pH 5, as described for the main fraction of the extract. The material recovered in this way appears to be less active than the main fraction, and we prefer not to combine them. Whether the lower activity is due to greater contamination or to injury of the insulin is undecided.
To the clear, although colored, filtrate (which has a reaction between pH 2.5 and 3.1) add 40 gm. of (NH₄)₂SO₄ for each 100 cc. and dissolve with stirring. On standing for some hours in the ice box the precipitate congeals and sticks to the walls and stirring rod, and allows the liquid to be poured off without loss. The brown gummy precipitate is dissolved in water and the liquid diluted to about 100 cc. for each kilo of pancreas hash, and is again precipitated by the addition of two-thirds its volume of saturated (NH₄)₂SO₄ solution. On standing some hours in the ice box the precipitate (much smaller in amount than before) congeals and sticks to the walls, the liquid is poured off, and if necessary, is centrifuged to avoid loss of small particles of the gummy material. The precipitate is dissolved in water with the addition of enough 0.1 N NH₄OH to make the reaction just distinctly yellow to methyl red (pH 6 to 8), which dissolves the "insulin-protein," but, if the reaction is not too alkaline, leaves undissolved any "pH 8-protein" which may accompany it. The solution is centrifuged and poured off from the dark colored precipitate, the latter being reextracted with water if desired. The combined solutions are diluted to about 100 cc. for each kilo of pancreas. On adding dilute acetic acid to about pH 5 (about midway of the color change of methyl red), a flocculent precipitate forms, which after standing some hours, is centrifuged out, washed with water at pH 5, and dissolved in a slight excess of 0.1 N HCl. From the mother liquor on standing for some days in the ice box, and by adding more acetic acid, more precipitate usually forms which is active, and is removed, dissolved, and added to the main fraction. This solution, although considerably colored and containing some admixed "pH 4-protein" is probably sufficiently pure for experimental or clinical use. From 50 to 100 mg. of this material are obtained from each kilo of pancreas hash, the larger amounts probably containing more of the "acid protein."

It is preferable to purify further the material as follows: The above mentioned precipitate, formed after adding acetic acid to about pH 5, after washing with water by centrifugation once

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7 Prior to February, 1923, such preparations were used with diabetic patients in the St. Louis Children's and Barnes Hospitals, without evidence of any objectionable effect.
or twice to remove sulfates, is dissolved in a measured volume of \( \pm 0.1 \text{ N acetic acid} \) (5 or 10 cc. for each kilo of pancreas represented by the precipitate). Add exactly 20 per cent of the equivalent of NaOH. This mixture (acetic acid, one-fifth neutralized) gives a pH close to 4, at which reaction the "insulin-protein" is soluble while the "acid protein" in large part precipitates. After standing in the cold room some hours the precipitate is centrifuged out and washed by a second centrifugation with a smaller amount of water. (The buffer solution remaining in the precipitate suffices to maintain the pH of the wash water.)

To the combined, often opalescent, supernatant solutions add an additional amount of NaOH corresponding to exactly one-half the equivalent of the acetic acid used. This gives a solution of acetic acid, seven-tenths neutralized, the pH of which is close to 5. At this reaction the "insulin-protein" separates at once and (after some days in the ice box if greatest possible recovery is desired) is removed by centrifugation, washed once or twice with distilled water, and dissolved in water by the addition of a few drops of dilute HCl.

The material obtained in this way, although of high activity or purity, probably contains some "pH 4-isoelectric" protein. Its solutions in slight excess of HCl are usually slightly opalescent, at pH 4 are dissolved but not quite clear, and in fairly concentrated solution are more or less colored. By removing the "acid" fraction by precipitation at pH 4.3 instead of 4.0, the final solutions are less colored, and more nearly free from the "pH 4-protein," although the product gains but little in activity, at the cost of much loss of material. Repeating the fractionation at pH 4 is less costly. The activity of the buffer-purified "insulin-protein" is indicated by the data in Table III, from which it would appear that 0.03 to 0.05 mg. per kilo represents a unit dose; for a 2 kilo rabbit (\( 2 \times 0.03 \text{ or } 0.05 \text{ mg.} \) \( \times \frac{1}{2} = 0.02 \) or 0.033 mg. as 1 standard Toronto unit. On this basis the yield may be estimated as from 1,500 to 2,500 "units" per kilo of pancreas.

By fractional precipitation from 80 to 95 per cent alcohol by addition of small amounts of acid or alkali, lighter colored (colorless) and apparently more active preparations have been secured. Table IV gives data to illustrate the activity of such alcohol-
Somogyi, Doisy, and Shaffer

purified preparations, from which it would appear that even 0.01 mg. per kilo is perhaps a unit dose. From the fact that such preparations were colorless and dissolved clear at pH 4 and 6, they were doubtless purer than those precipitated only from water, though we hesitate to conclude how much the activity was increased.

We are not disposed to urge the acceptance of these estimates of the amounts which represent a "unit," but prefer to leave the data to the reader's judgment. The great variability in the resistance of different rabbits to insulin has made its quantitative assay in our hands very uncertain. Only by closest attention in the selection and care of the rabbits, and by discarding those not in good condition, can fairly concordant results be obtained. Many of our animals were used repeatedly at intervals of a week or two, and at various times some had a respiratory infection ("snuffles") which seems to decrease resistance. All were fasted 24 hours before the injections. Rabbits fresh from the country are apt to be more resistant than after they have been kept for a time in the laboratory, even though they gain in weight and seem to be in good condition. In the face of these variations we venture no very exact statement as to how much "insulin-protein" represents a "unit," and are willing that the reader reach his own opinion from the data given in the tables. When, as above, definite quantities are mentioned in relation to units, it is to be understood that this qualification applies.
### TABLE III.

*Activity of "Insulin-Protein," Purified by Extraction at pH 7 and pH 4.*

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Blood sugar. at 3.0 hrs. 40 C 53
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* C indicates convulsions; G, subcutaneous injection of glucose; and S, symptoms such as prostration or marked excitement. All blood determinations by the Shaffer-Hartmann method.
TABLE III—Concluded.

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<tr>
<td>74</td>
<td>8</td>
<td>2,740</td>
<td>0.069</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Preparation of Insulin
TABLE IV.

Rabbit Assay of "Insulin-Protein," Purified by Fractionation from Alcohol.*

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Rabbit No.</th>
<th>Weight (gm.)</th>
<th>Amount injected (mg.)</th>
<th>Blood sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per kg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D x 1</td>
<td>93</td>
<td>1,140</td>
<td>0.018 0.016 45 S</td>
<td>48 111</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>1,290</td>
<td>0.020 0.016 55 C</td>
<td>70 37</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>1,360</td>
<td>0.022 0.016 92 55 C</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>2,830</td>
<td>0.036 0.013 52 81</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2,120</td>
<td>0.065 0.03 52 35</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>1,330</td>
<td>0.016 0.012 68 70</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>1,430</td>
<td>0.017 0.012 70 70</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>93</td>
<td>1,190</td>
<td>0.014 0.012 62 78</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>1,150</td>
<td>0.012 0.012 65 80</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>1,250</td>
<td>0.015 0.012 78 (?)</td>
<td>80 115</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2,240</td>
<td>0.022 0.01 36 42</td>
<td>94 118</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>2,200</td>
<td>0.022 0.01 66 66</td>
<td>82 118</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>2,710</td>
<td>0.027 0.01 67 47</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>2,600</td>
<td>0.026 0.01 50 52</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>989</td>
<td>2,640</td>
<td>0.015 0.006 66 66</td>
<td>80 118</td>
</tr>
<tr>
<td>D x 2</td>
<td>992</td>
<td>2,810</td>
<td>0.028 0.01 58 70</td>
<td>100 55</td>
</tr>
<tr>
<td></td>
<td>336</td>
<td>2,510</td>
<td>0.075 0.03 48 35</td>
<td>35</td>
</tr>
<tr>
<td>D x 3</td>
<td>F</td>
<td>2,600</td>
<td>0.09 0.035 104 72</td>
<td>45 S 44 C 44 C</td>
</tr>
<tr>
<td></td>
<td>M1</td>
<td>2,620</td>
<td>0.09 0.034 120 50</td>
<td>30 C 40 C 70</td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>2,750</td>
<td>0.09 0.033 162 110</td>
<td>64 70</td>
</tr>
<tr>
<td></td>
<td>31</td>
<td>2,830</td>
<td>0.06 0.02 59 35 C</td>
<td>C 115</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>2,900</td>
<td>0.029 0.01 55 72</td>
<td>91 115</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2,450</td>
<td>0.015 0.006 83 58</td>
<td>83 108</td>
</tr>
</tbody>
</table>

*C refers to convulsions; S, to symptoms such as prostration or marked excitement; and G, subcutaneous injection of glucose.
<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Rabbit No.</th>
<th>Weight</th>
<th>Amount injected</th>
<th>Blood sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gm.</td>
<td>mg.</td>
<td>1 hr.</td>
</tr>
<tr>
<td>59 a P</td>
<td>992</td>
<td>2,680</td>
<td>0.27</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>336</td>
<td>2,450</td>
<td>0.13</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2,340</td>
<td>0.12</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>2,340</td>
<td>0.07</td>
<td>36 S</td>
</tr>
<tr>
<td></td>
<td>321</td>
<td>2,770</td>
<td>0.08</td>
<td>40</td>
</tr>
<tr>
<td>F 94</td>
<td>1,230</td>
<td>0.02</td>
<td>0.017</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>93</td>
<td>1,050</td>
<td>0.019</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>1,200</td>
<td>0.018</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>94</td>
<td>1,100</td>
<td>0.017</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>980</td>
<td>0.013</td>
<td>55</td>
</tr>
<tr>
<td>F 93</td>
<td>1,190</td>
<td>0.012</td>
<td>0.01</td>
<td>70</td>
</tr>
<tr>
<td>F 92</td>
<td>1,120</td>
<td>0.011</td>
<td>0.01</td>
<td>74</td>
</tr>
<tr>
<td>901</td>
<td>2,500</td>
<td>0.025</td>
<td>0.01</td>
<td>60</td>
</tr>
<tr>
<td>988</td>
<td>2,450</td>
<td>0.025</td>
<td>0.01</td>
<td>37</td>
</tr>
<tr>
<td>31</td>
<td>2,720</td>
<td>0.035</td>
<td>0.013</td>
<td>53</td>
</tr>
<tr>
<td>984</td>
<td>2,750</td>
<td>0.035</td>
<td>0.013</td>
<td>62</td>
</tr>
<tr>
<td>983</td>
<td>2,690</td>
<td>0.035</td>
<td>0.013</td>
<td>42</td>
</tr>
<tr>
<td>F 95</td>
<td>1,260</td>
<td>0.01</td>
<td>0.008</td>
<td>94</td>
</tr>
<tr>
<td>Same, 3 mos. lat.</td>
<td>74</td>
<td>2,080</td>
<td>0.10</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>1,640</td>
<td>0.065</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>81</td>
<td>2,140</td>
<td>0.084</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>1,830</td>
<td>0.055</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>1,450</td>
<td>0.03</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>1,500</td>
<td>0.03</td>
<td>60</td>
</tr>
<tr>
<td>59 b P</td>
<td>12</td>
<td>2,120</td>
<td>0.20</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2,120</td>
<td>0.10</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>2,350</td>
<td>0.11</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>2,700</td>
<td>0.08</td>
<td>58</td>
</tr>
</tbody>
</table>
TABLE V.

Summary of Assay of Some Early Preparations, Purified by Repeated Solution and Precipitation at about pH 5.

<table>
<thead>
<tr>
<th>Preparation No.</th>
<th>Times reprecipitated from water solution at pH 5.</th>
<th>Injected mg. per kg. of rabbit weight.</th>
<th>No. of rabbits injected.</th>
<th>No. of rabbits showing:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>Crude.</td>
<td>0.25 to 0.3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>55 P</td>
<td>5</td>
<td>0.4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 to 0.25</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.18</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.13</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>D 20</td>
<td>6 mos. later.</td>
<td>0.10 to 0.20</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2 to 0.3</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15 to 0.19</td>
<td>14</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.13</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>60-1</td>
<td>10</td>
<td>0.2 to 0.25</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>60-1</td>
<td>4</td>
<td>0.16 to 0.28</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6 mos. later.</td>
<td>0.12 to 0.20</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>60-2</td>
<td>4</td>
<td>0.17 to 0.21</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>

Undoubtedly contained "pH4-" and "pH 8-proteins" as well as "insulin-protein."

The following few examples will indicate the activity of different preparations. Table V illustrates the activity of the proteins separated by adjusting the reaction to about pH 5, of solutions prepared by the method outlined in our earlier abstract (1). The data given for Nos. 55 and 59 show that the activity of the isoelectric precipitate persists through its repeated solution and reprecipitation. The figures also appear to indicate that simple reprecipitation without a separation of proteins at particular reactions, accomplishes but little purification. On evidence of the sort contained in Table V, we based our earlier estimate of about 0.25 mg. per kilo as the rabbit unit. That estimate was probably overly conservative, for in a few cases the injection of much smaller amounts gave convulsions and very low blood sugar. The product from these early preparations undoubtedly contained...
contaminating "isoelectric proteins." At the time these preparations were worked with, we had not clearly realized the existence of different proteins in the "isoelectric protein" fraction. Later preparations by improved methods showed activity from much smaller amounts, as illustrated in Table III, and we are disposed to believe that the difference is due in large part to smaller contamination with inactive "isoelectric proteins," and in part to our failure to inject smaller amounts of the early preparations. The products obtained by our improved method of preparation (which contain very little "acid" or "alkaline proteins") are smaller in amount as well as considerably more active than from the earlier method. It will be noted from Table I that while 140 mg. of crude "isoelectric proteins" were obtained per kilo of pancreas, representing perhaps 500 to 1,000 units, from the earlier preparations (No. 95), only 40 to 70 mg. are obtained with the same amount of acid by the process as now carried out (Nos. 111 to 114). The latter amounts represent, however, at least as great activity, 1,000 or more units, depending upon the amount taken as a "unit."

By purification of the early material, by fractionation from alcohol (as described under preparation No. 59), considerably more active fractions were obtained, as shown in Table IV. As little as 0.01 to 0.03 mg. per kilo fairly regularly gave convulsions and low blood sugar. By this method we have obtained our most active material.

Preparation No. 54 b.—Extraction by 1.5 volumes of 80 per cent alcohol and 40 cc. of 10 N HCl per kilo and neutralization before filtration. Residue after evaporation of alcohol, including fat, precipitated by half saturation with ammonium sulfate. The precipitate was extracted with 60 per cent alcohol acidified with HCl, filtered, and precipitated by strong alcohol. This precipitate was dissolved in water and the isoelectric protein separated by adjusting reaction to about pH 5. The solution "No. 54 b iso" contained 104 mg. of protein in 20 cc. for 1 kilo of pancreas hash. This solution was diluted and amounts varying from 0.1 to 1 mg. per kilo were injected into ten fasting rabbits, weighing 0.8 to 1.5 kilos, all of which had convulsions in from 1½ to 4 hours. Blood sugar was determined only in four in which cases it was 30 to 60 mg. after 3 hours. Since the smallest
dose gave convulsions, the per kilo dose is perhaps 0.1 mg. or less. Taking a more conservative estimate of double this amount the yield would be 800 units per kilo of pancreas, 

\[
\frac{(0.2 \times 2) \times 2}{3} = 0.13 \text{ mg. per "unit,"}
\]

104 mg. \( \div \) 0.13 = 800 units per kilo.

*Preparation No. 55.*—Made in the same way as No. 54, 100 cc. of “isoelectric protein” solution contained 860 mg. of protein from 8 kilos of pancreas or 107 mg. per kilo. Of this 0.23 to 0.4 mg. per kilo injected into four 1 kilo rabbits gave convulsions in 2 or 3 hours, while 0.17 mg. in one rabbit gave no symptoms. Blood sugar was not determined.

This “crude” material was precipitated, dissolved, and reprecipitated with dilute NaOH and HCl five times, after which it (“No. 55 P”) was injected into twenty-five rabbits with the results shown in Table V. It seems at any rate doubtful whether any increase in activity resulted from the purification. Doses of 0.13 mg. per kilo lowered the blood sugar to 79, but showed no symptoms; 0.18 mg. gave definite symptoms in five cases out of nine, but the blood sugar values are not very low, 39 to 60 mg. With 0.22 mg. and more the convulsions are more constant, and where determined the blood sugar was lower. About 0.2 mg. would be taken as the per kilo unit of activity.

6 months later the same solution was again injected into three rabbits, and even 0.1 mg. per kilo gave 49 mg. of blood sugar and convulsions. From this it might be supposed that the activity had increased, but it is more likely due to variations in susceptibility of the animals. The purified as well as the original solution doubtless contained a good deal of the “pH 4-” or “pH 8-protein,” or both, which were carried through the “purification.”

*Preparation No. 59.*—Made in the same way as No. 54, the crude total “isoelectric proteins” were precipitated, dissolved, and reprecipitated from water ten times. 0.3 mg. per kilo of the crude material caused convulsions in three rabbits, while about 0.2 mg. failed to cause symptoms in five rabbits. Blood sugar was not determined. From the “crude” solution a fraction was separated by precipitation at pH 6.5 to 6.8 which was later recognized as the distinct “pH 8-isoelectric” and which was inactive in amounts up to 0.37 mg. per kilo. (One rabbit receiving this amount had convulsions after 3 hours.) After re-
peated precipitation of the material freed from the "pH 8-protein," 0.2 to 0.25 mg. caused convulsions in three out of four rabbits. This material was considerably further purified by fractionation from alcohol as follows: It was again precipitated by addition of dilute alkali to about pH 5 and centrifugated. The precipitate was dissolved in 25 cc. of water by cautious addition of dilute alkali (pH 6 to 7) and 150 cc. of 95% per cent alcohol. Dilute HCl was then added cautiously first to opalescence and then to a flocculent precipitate. Judged by the color of methyl red the reaction was on the alkaline side of the optimum point of precipitation in aqueous solution. The solution was filtered after 1 hour in the ice box, and the precipitate (containing the "pH 8-protein") set aside. To the filtrate a little more acid was added, together with 100 cc. more alcohol, and the solution was placed in the ice box at 0°C. overnight. The precipitate which formed was removed by centrifuge, dissolved in acidified water, and reprecipitated twice, then dissolved in water with a few drops of dilute HCl. This solution, "No. 59 a P," contained 9.2 mg. per cc. and on injection into rabbits showed the activity indicated in Table IV.

To the alcohol mother liquor from which "No. 59 a" had separated, 1 drop of 0.1 N HCl and 100 cc. of ether were added. After 2 days in the ice box, a precipitate had formed which was removed by centrifuge, dissolved in acid water, and precipitated twice by cautious addition of dilute NaOH. A solution of the final precipitate was dissolved in water with addition of dilute HCl and labelled "No. 59 b P." The solution contained 3.6 mg. per cc. and after dilution was injected into rabbits as recorded in Table IV. Both Nos. 59 a and 59 b are quite active, a being more active than b. As little as 0.01 mg. per kilo of a caused convulsions and blood sugar of 40 after 1½ hours. The smallest dose injected of b, 0.03 mg. in one rabbit, caused symptoms in 3 hours, the lowest blood sugar being 0.058. The other preparations recorded in Table IV were purified by similar fractionation from alcohol.

SUMMARY.

A simplified method is described for the preparation of insulin from beef pancreas. The activity of insulin is probably a property of an individual protein, "insulin-protein," the solubility
of which is described. The method of extraction from pancreas and its purification is considered from the point of view of the properties of the "insulin-protein." It has been separated from two other proteins which also are precipitated at their isoelectric points, and which are included in the preparations of insulin, purified by "isoelectric precipitation," as earlier described by the authors.

Data are given to indicate the activity of the purified "insulin-protein."

Addendum.—A paper by Shonle and Waldo, from the laboratory of the Eli Lilly and Company, has just appeared (Shonle, H. A., and Waldo, J. H., J. Biol. Chem., 1924, lviii, 731) reporting upon certain reactions and analyses of purified insulin preparations. They "conclude that the pancreatic substance containing insulin appears to be a complex mixture of proteoses . . . . from which it has been as yet impossible to isolate a simple substance." Of the eight different purified preparations cited by these authors, only one, No. 72,723, has about the same activity (0.0337 mg. per unit) as our better preparations, while most of their others are considerably less active. They report even more active material from a dialysate, 0.002 mg. of nitrogen of which corresponded to 1 unit. Assuming 18 per cent of nitrogen, this would represent 0.011 mg. of substance per unit, which is similar to the activity of our preparations purified by fractionation from alcohol. The table on page 734 indicates that all their other preparations contained very large proportions of ash (40 to 200 times as much ash as organic substance!) and this fact makes one suspect that these preparations, in spite of their purification by precipitation at isoelectric points, were contaminated with inactive isoelectric proteins and were far from "pure." They describe five of their most active preparations as having been purified by precipitation "at the isoelectric points or with trichloroacetic acid". We should not expect a general protein precipitant like trichloroacetic acid to be a favorable reagent for the separation of proteins having such similar behavior as have the isoelectric proteins of pancreas extracts.

BIBLIOGRAPHY.

Preparation of Insulin


ON THE PREPARATION OF INSULIN
Michael Somogyi, Edward A. Doisy and Philip A. Shaffer

J. Biol. Chem. 1924, 60:31-58.