THE PREPARATION OF INSULIN.

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When the Insulin Committee suggested that we should report on the recent progress in the preparation of insulin an extensive review of the history of pancreatic extracts was at first contemplated. Macleod (1), Dale (2), and others, however, have recently reviewed certain parts of the literature, and since a study of that portion of this literature which describes the preparation of the extracts shows that many of them are of minor significance in the present connection, we have decided to refer only to those investigators whose work, in our opinion, led them very near to the solution of the problem.

Zuelzer (3), a German investigator, was one of the early workers in this field. He prepared extracts from pancreas in several ways, one of which is as follows: The pancreas was minced and sufficient sodium bicarbonate was added to produce a weak alkaline reaction. The mixture was then left to autolyze for several days. (This procedure in our experience results in a great loss of potency of the material. Zuelzer stated, however, that this step might be omitted.) The liquid was then pressed out and alcohol added until no more albuminous substances were precipitated. When the solution was free from albumin, it was filtered and concentrated in a vacuum still. The final product obtained, Zuelzer says, was a fine, dry, gray powder. This powder, he states, was easily soluble in water or dilute alkali; was free from ferments; and gave none of the known protein reactions. Insulin, as we know it at present, is less stable in alkaline than in acid solution. The purest preparation we have as yet obtained from mammalian pancreas gives a positive biuret reaction.

Zuelzer tested his preparation by determining to what extent it antidoted the hyperglycemia and glucosuria caused by administration of epinephrine. Von Fürth and Schwarz (4) and others have reported that many other substances counteract the effect of epinephrine.

Zuelzer's extract was administered to several diabetic patients, and in certain of these cases was partially successful, in his own hands, in alleviating the symptoms of diabetes. In the hands of others (Forschbach, 5), however, the toxic effects overshadowed the beneficial action to such an extent that further treatment was abandoned.

Although Zuelzer nearly discovered in 1908 the active pancreatic principle which we call insulin, his work in the light of subsequent events must be considered an abandoned research.

E. L. Scott (6), working on the hypothesis that later was independently formulated by Banting, narrowly missed demonstrating in 1912 the internal secretion of the pancreas. He was unable to secure complete atrophy of the acinous cells of the pancreas in dogs, after attempting to ligate the pancreatic ducts. He did not report the effect of administration of extract of this partially atrophied gland. Scott also tried to extract the internal secretion by means of alcohol, but in his endeavor to inhibit the action of the external secretion, he raised the concentration of alcohol to such a height that a large proportion of the active principle was undoubtedly never extracted from the gland. The alcoholic extract obtained from the gland was concentrated *in vacuo* and the residue extracted with ether. The ether extract was discarded. The residue was dissolved in 95 per cent alcohol. Since insulin from mammalian pancreas is only slightly soluble in alcohol of this concentration, Scott could have had very little of the active substance in his final extract. In the preparation of watery extracts Scott used a preliminary alcoholic extraction. The concentration of alcohol was approximately 85 per cent for the first extraction of the glands. At this concentration a part of the insulin would go into solution. This was evidently discarded. A large part of the active substance would be left behind, however, and should have been present in the second extract which was obtained by treating the glands with water. Possibly one reason why better effects were not obtained with this material was that an inhibiting substance which is at least partially precipitated by 80 per cent alcohol, must have been present in large amounts in the watery extracts.

Rennie and Fraser (7) in 1907 studied the effects on diabetic patients of the oral administration of the principal islets of certain bony fishes. In one case these investigators administered a saline extract of islet tissue by subcutaneous injection. No beneficial influence on the symptoms of diabetes was observed. Since insulin in a purified form has not as yet been administered successfully by mouth, we have not far to look for the cause of failure of those experiments in which the crude islet tissue was orally administered. The subcutaneous injection of the extract of the islet tissue produced very profound symptoms of toxicity.

Knowlton and Starling (8) hazarded the opinion that the pancreatic hormone "would be a body diffusible, soluble in water, unstable in alkaline solution, but more stable in slightly acid solution, and not destroyed immediately at the temperature of boiling water." It is interesting to note that these speculations (with the exception of that one relating to the diffusibility of the substance) are correct. We have not as yet obtained a preparation which dialyzes through parchment. The diffusibility of the active principle is a property which has to be retested every time a purer preparation is obtained. We have carried out the procedure used by these investigators in the preparation of their extract, and have demon-

strated the presence of insulin in the resulting mixture. The tests are much more conclusive, however, if the crude extract so obtained is purified in any one of the several ways which we will attempt to outline in the body of this paper. The work of Knowlton and Starling (8) and of Patterson and Starling (9) has been reviewed in several communications by the Toronto group (10). We merely wish to point out here that had these investigators used criteria other than the change in sugar consumption of the perfused mammalian heart to determine the potency of their material, more encouraging results might have been obtained.

Murlin and Kramer (11) in 1913, prepared alkaline extracts of pancreas. The administration of this extract reduced the degree of glycosuria in diabetic dogs. It was discovered, however, that this effect was produced by the administration of alkali alone. With the exception of experiments in which the respiratory quotients of several diabetic animals were studied, no further work was reported by Murlin and his collaborators, until after the publication of the initial experiments of the Toronto investigators.

Kleiner (12) showed that the administration to diabetic dogs of unfiltered watery extracts of fresh pancreas which had been diluted with 0.90 per cent sodium chloride, usually resulted in a marked decrease in blood sugar. A decrease in the amount of sugar excreted in the urine accompanied the reduction of the blood sugar level. Kleiner suggested that the diminished excretion of sugar might be partly due to the toxic renal effects. Kleiner's experiment was repeated and confirmed by Banting and Best (10). Anuria was observed, however, in the experimental animal.

Very significant results have been obtained by a group of workers who have attempted to demonstrate in blood or in pancreatic perfusates a substance necessary for the proper combustion of carbohydrates in the body. The most important of these contributions are those of A. H. Clark (13), Lepine (14), Drennan (15), Hédon (16), Forschbach (17), and Murlin and Kramer (11). Since we contemplate reporting on the demonstration of insulin in blood, a review of the work of these investigators will perhaps be more advantageously included in that communication.

Preparation of Earlier Extracts.

In July, 1921 (10), extracts were prepared in the Department of Physiology of the University of Toronto, which were shown to contain the internal secretion of the pancreas. In the preparation of this extract the degenerated pancreas was removed from dogs 10 weeks after ligation of the pancreatic ducts. The gland was obtained as soon as possible after the death of the animal and was sliced into a chilled mortar containing Ringer's solution. The mortar was placed in a freezing mixture and the contents were partially frozen. Sand was added and the tissue was completely pulverized. The temperature of the contents of the mortar was then raised and the resulting liquid filtered through paper. The filtrate was administered intravenously to diabetic dogs. The results of the injection of this material have been published (10). Extracts were prepared by

this simple procedure from the normal pancreas of the dog and from fetal calf pancreas. The normal pancreas did not yield as much of the active principle per gram of tissue as did the degenerated gland. The pancreas of the fetal calf, however, provided comparatively large quantities of the substance. It would be interesting to compare the amounts of active material obtainable from these sources, if the tissues were treated with a high acid extractive, as in our present procedure. This causes the immediate inactivation of proteolytic enzymes and a comparatively efficient extraction of the active principle.

Banting and Best (18) prepared active extracts from the normal pancreas of the ox by extraction of the gland with alcohol, which had been made acid by the addition of 0.2 per cent hydrochloric acid. The concentration of alcohol in the mixture was in some cases as high as 60 per cent. The liquid was removed from the glands by filtration and the filtrate evaporated either in porcelain dishes placed in a warm air current or by means of a laboratory vacuum still. The liquid was usually evaporated so that from 5 to 10 cc. contained one dose for a depancreatized dog. This material was administered to several diabetic dogs. One animal, in particular, Dog 33, which lived 70 days after pancreatectomy, received many injections of this preparation. Rigid aseptic precautions were not observed in the administration of the extract to this animal. However, so long as daily doses of active material were administered there was little or no suppuration at the sites of injection. Numerous abscesses developed when insulin was omitted. These disappeared, however, when the treatment was resumed. The improvement in the clinical condition of the animals receiving this material was very marked. The respiratory quotients of completely diabetic dogs were definitely raised and large amounts of glycogen[1] were deposited in the livers of the animals when glucose and insulin, which had been prepared by the above procedure, were administered. In some cases the filtrate referred to was evaporated to dryness and the residue extracted with toluene to remove the lipoid material. This procedure caused no loss in potency. *These investigators were able to demonstrate that the active principle contained in this residue was practically insoluble in 95 per cent ethyl alcohol.* They treated an aliquot portion of the dried residue with 95 per cent alcohol. The mixture was filtered and the filtrate evaporated. The residue was dissolved in saline solution. Administration of this solution produced no effect upon the blood or urinary sugar of a depancreatized dog. A saline solution of the material which did not dissolve in 95 per cent alcohol, however, definitely lowered the blood sugar and diminished the sugar excretion of the same animal. A watery solution of this material was passed through a Berkefeld filter, but a considerable loss of potency was observed. Tricresol in excess of that amount used as a preservative in biological products, such as diphtheria antitoxin, did not injure the active substance. Material prepared by these investigators from beef pancreas was administered to several diabetic

[1] *Tr. Roy. Soc. Canada*, 1922, xvi, 6.

patients in the clinic of the Toronto General Hospital. A decrease in blood sugar and a lowered sugar excretion resulted. A certain degree of local irritation was observed in some of the cases. This was probably due to the high percentage of protein present in the extract. The results of these experiments showed that insulin could be derived from a readily available source—beef pancreas; that a preparation could be secured from this source which was efficient in completely removing the symptoms of diabetes from depancreatized animals; and that these results, as far as the effects on hyperglycemia and glucosuria were concerned, could be duplicated in the clinic. The practical application of the results was very evident.

A method for the further purification of insulin was evolved principally by J. B. Collip who joined forces with the discoverers of insulin in December, 1921. This method has been reported (19). The details are briefly as follows: 95 per cent alcohol was added to freshly minced pancreas so that the volumes of extractive and glands were approximately equal. After an interval of a few hours, during which the mixture was stirred, the liquid was filtered off. 95 per cent alcohol was added to the filtrate to secure a concentration of approximately 80 per cent alcohol. The mixture was then filtered and the filtrate concentrated *in vacuo*. The vacuum was secured by the use of a laboratory water pump. When the liquid had been concentrated to a small volume the lipoid substances were removed by extraction with ether. The watery solution was then further concentrated to a pasty consistency. This material was treated with 80 per cent alcohol and the mixture centrifuged. The active principle was found to be contained entirely in the alcohol which formed the uppermost layer in the centrifuge tube. The alcoholic solution was removed and was added to several volumes of 95 per cent or absolute alcohol. The active principle, as mentioned above, was practically insoluble in alcohol of this concentration. The precipitate obtained by the above procedure was removed by a Buchner filtration, dissolved in distilled water, and concentrated to remove traces of alcohol and to secure the desired concentration of active substance. The preliminary clinical effects of this preparation have been reported (20). This method of purification worked out satisfactorily, for a short time, on a small scale. Larger scale experiments were not successful and subsequently it was found impossible to duplicate consistently the earlier results on any scale. For a period of 2 months scarcely any insulin was available. A method was then evolved, however, in this laboratory, with the assistance of various members of the Toronto group, which utilized the facts reported by Banting and Best and many of the details of Collip's procedure. The method gave consistent results, and furnished all the insulin used in Toronto for clinical and experimental work for a period of 3 months prior to the beginning of collaboration with Eli Lilly and Company. This method was as follows: Minced pancreas was extracted with an equal volume of 95 per cent acetone. A small amount of formic or acetic acid was added. Collip had previously found formic acid advantageous. The concentration never exceeded 0.1 per cent. (Higher acid was first used in this laboratory as a result of

a conversation with H. H. Dale, in which the acidity of the extractive was discussed.) The pancreas acetone mixture was allowed to stand for several hours and was then filtered. The filtrate was placed in enamel lined trays (500 cc. to each tray measuring 18 × 18 × 2.5 inches). The trays were placed in a tunnel through which a current of hot air was rapidly drawn. The 500 cc. were evaporated to approximately 50 cc. in about 1 hour. The temperature of the liquid never exceeded 35°C. The residue was removed from the trays, chilled to 0°C., and filtered. Lipoid material was largely eliminated in this manner. The filtrate thus obtained was treated with 95 per cent ethyl alcohol to secure a concentration of approximately 80 per cent alcohol. The mixture was then filtered and the filtrate added to 5 or more volumes of 95 per cent alcohol as in the previous method. The precipitate which formed was allowed to settle to the bottom of the alcoholic solution. From 24 to 48 hours were allowed for the precipitate to settle. The alcohol was then decanted off and the precipitate dissolved in distilled water. Traces of alcohol were removed by vacuum distillation. This was the method originally adopted and applied to larger scale production by Eli Lilly and Company when information regarding the production of insulin was communicated to them by the Toronto Committee.

One of the first contributions made by the scientific staff of Eli Lilly and Company was the employment of rotary high vacuum pumps which immediately made possible the efficient concentration of the original acetone or alcoholic filtrate and also of the filtrate after the 80 per cent alcoholic precipitation.

Benzoic Acid Method.

This method of preparing and purifying insulin was evolved in these laboratories by Moloney and Findlay (21). The principle of this process is based on the fact that certain substances readily adsorb insulin. The particular adsorbing substance used by these investigators was benzoic acid. The method briefly is as follows:

Minced pancreatic glands were extracted with alcohol and the filtrate was concentrated in an efficient vacuum still. To each liter of the crude aqueous concentrate 50 cc. of a 25 per cent sodium benzoate and 12.5 cc. of concentrated hydrochloric acid were used. These amounts were usually sufficient to saturate the solution with benzoic acid. However, these quantities can be increased or decreased proportionately depending on the amount necessary to cause a first lasting precipitate. Then to this saturated benzoic acid solution 40 cc. of 25 per cent sodium benzoate and 10 cc. of concentrated hydrochloric acid were added. The precipitate thus formed was allowed to settle and the solution filtered. This precipitate usually contained about two-thirds of the potent material. The filtrate was again treated with 40 cc. of sodium benzoate and 10 cc. of hydrochloric acid to secure a second precipitate. This precipitate was filtered off and the filtrate again treated if this was considered necessary. The benzoic acid precipitates were mixed and added to a small volume of

80 per cent ethyl alcohol which dissolved both the insulin and benzoic acid. Certain inert materials, however, settled out and the alcoholic solution was filtered. The filtrate was concentrated to dryness *in vacuo* and the benzoic acid dissolved by treatment with ether. This solution was transferred to a separatory funnel and a small volume of water was added. The insulin was contained in the aqueous layer.

The introduction of the benzoic acid method of purification marked a distinct advance in the production of insulin. By this process the large amounts of alcohol necessary for the fractional precipitation of the proteins and the final precipitation of the insulin in the previous methods were avoided. Chemically, it gave a product which was much freer from protein material, as determined by the nitrogen content, than anything we had hitherto been able to obtain. Clinically, the toxic and indurating effects which characterized all the earlier extracts were greatly reduced. The main disadvantage which this process possessed was that the separation of the benzoic acid precipitates often required long and tedious filtration. However, the benzoic acid method has been a very important factor in the production of insulin over a considerable period of time. This is shown by the fact that approximately 250,000 units of insulin made by that method under our direction in this laboratory were used clinically in Toronto in the autumn of 1922, with very satisfactory results.

Water Extracts.

In the past many attempts have been made to obtain the substance necessary for the utilization of carbohydrates in the body by watery extraction of the pancreas. Knowlton and Starling, as previously stated, prepared an extract from the pancreas by extracting the gland with acidulated cold water. Shortly after the original publication by the Toronto group, Sansum[2] was able to obtain a small quantity of insulin by hot water extraction of beef pancreas. Because of the possible economic significance of a watery extraction of the pancreas in the manufacture of insulin, this method has been investigated in our laboratories. Some 150 experiments using different modifications in the extraction, such as varying the time, the temperature, the acidity, etc., have been carried out. While all these experiments are of interest, we will report only a few which have given the most promising results.

2 pounds of minced beef pancreas were added to 300 cc. of distilled water which had been acidified with 4 cc. of concen-

[2] Sansum, W. D., Unpublished work.

trated sulfuric acid. After 20 minutes 1 liter of boiling water was added to the mixture, and the temperature raised to 80°C. by a jet of live steam. This temperature was maintained for a period of 2 minutes. The mixture was then poured into a flask and cooled quickly by connecting the flask to a high vacuum pump. The cooled contents were filtered. An almost colorless filtrate was obtained. After the completion of the filtration which usually took about ½ hour, the glands were reextracted with 1 liter of acidified water at room temperature for a period of 3 hours. The liquid was filtered off as in the first extraction. The insulin in the combined filtrates was purified either by the method elaborated by Banting, Best, Collip, and Macleod, or by the present method of purification used in our laboratories.

We were able to obtain equally satisfactory results by cold water extraction of the pancreatic glands. The method was as follows: 2 pounds of minced pancreas were added to 1,500 cc. of distilled water which was acidified with 3.5 cc. of concentrated sulfuric acid. The mixture, after extraction for 2 hours, was filtered through fluted filter paper. The filtrate was quite clear and had a pH of approximately 3.5. It is very essential that this pH be very close in the above value for two reasons. The acidity is outside the isoelectric range of many of the proteins in the pancreas, and at this hydrogen ion concentration there is obtained a mixture which filters readily and gives a clear filtrate. The glands were reextracted with acidified water, as above, for 2 hours and the liquid filtered off as in the first extraction. The insulin in the combined filtrates may be purified by any of the methods described in this paper.

Under conditions as described above we were able to obtain fairly satisfactory yields of insulin (see p. 720). The results, however, though very encouraging, have not as yet shown nearly as great a unitage per pound of pancreas as that obtained by the alcohol or acetone method of extraction under the best experimental conditions.

The Method of Doisy, Somogyi, and Shaffer (22).

These investigators have described a method of purification of insulin, the salient new features of which were the precipitation of insulin from watery solution with half saturation of ammonium sulfate and the so

called isoelectric precipitation. Ammonium sulfate in one-half saturation had been previously used in these laboratories by Moloney. Full details of the isoelectric precipitation of insulin from watery solutions containing the active substance were communicated to us by Prof. P. A. Shaffer, and almost immediately afterwards, by Dr. Clowes of Eli Lilly and Company. This method was evidently worked out independently in two laboratories at about the same time. We have profited by discussion of this method with Prof. Shaffer and Dr. Clowes on several occasions. The experimental work in the research laboratories of Eli Lilly and Company was carried out by G. Walden under the direction of Dr. Clowes. The crude material, to which the "isoelectric" method of purification was applied, was obtained by different procedures by the two groups of investigators. It appears from experiments we have carried out that insulin can be removed from watery solution at various hydrogen ion concentrations by procedures which cause a precipitate to settle out. For example, the addition of copper sulfate to obtain a concentration of 1 per cent, in a solution of insulin at pH 3.7 causes the separation of a precipitate which may contain much of the potent material. Similarly, if insulin is added to a solution of edestin and the hydrogen ion concentration adjusted to 6.89, the isoelectric point of this protein, a precipitate forms which may contain all the potency of the original solution.

The Present Method.

In our present process we have employed various steps of many of these methods. The precipitation of insulin from alcoholic solution by the addition of ether was suggested to us by an experiment performed by H. W. Dudley, in this laboratory. In this experiment Dudley demonstrated that the addition of an equal volume of ether to the alcohol used in the final precipitation of insulin in the procedure of Banting, Best, Collip, and Macleod, resulted in a much more efficient precipitation of the active principle than that obtained by the use of alcohol alone.

Fresh pancreatic glands from the ox are obtained from the abattoirs. After separating as much of the fat and connective tissue as possible the glands are placed in large containers which are collected every 3 hours and taken to the laboratory.

The glands are weighed. They are then run through a power meat chopper in which they are finely minced. This minced material is poured into large earthenware crocks which contain a weight of 95 per cent denatured alcohol (10 per cent methyl and the remainder ethyl), equal to that of the glands. The alcohol is acidified to 1.3 per cent with acetic acid. It is important that

a high hydrogen ion concentration be secured at this stage. It inhibits the action of proteolytic enzymes and affects the proteins in such a way as to facilitate separation of the solid and liquid materials at a later stage of the process. Sulfuric acid may be used in place of acetic acid, but, if so, a more highly colored filtrate is obtained. This color is difficult to remove at a later stage. The minced glands are extracted for 3 hours in this acid alcohol solution. During this time they are slowly agitated in order to facilitate extraction. At the end of 3 hours this alcoholic mixture is poured into a rotary centrifuge to separate the alcoholic extract from solid materials. After the completion of the centrifuging the solid material remaining in the centrifuge is reextracted for 3 hours with a volume of 60 per cent alcohol equal to that of the liquid removed after the first extraction. The alcoholic extract, after 3 hours, is separated by means of the centrifuge. The extracts from the first and second extractions are mixed, neutralized to litmus with sodium hydroxide, and chilled in a brine tank to 0°C. (the chilling may be omitted). During the chilling the filtrate becomes turbid due to the separation of lipoid and protein materials. The mixture is filtered through large glass funnels which have been fitted with fluted filter papers. The alcoholic extract thus obtained is almost colorless. The filtrate which contains the active principle is concentrated to about one-twentieth of its original volume in an efficient vacuum still. During the distillation the temperature is not allowed to rise above 30°C. The reasons for this are as follows: excessive heat will coagulate much of the protein material. This is undesirable at this stage because some of the insulin would be adsorbed on the precipitated proteins. Excessive heat over the period required for the concentration produces highly colored decomposition products which greatly increase the difficulty of purification of insulin. After the completion of the distillation the concentrate is quickly heated to 55°C. At this temperature lipoid and other materials rise to the surface and are readily skimmed off. This fatty mass which contains about one-quarter of the total potency of the concentrate is treated with sufficient ether to dissolve the lipoid material and is allowed to stand over night. The ether is then removed and the residue made up to 80 per cent with denatured alcohol. This mixture is filtered through paper.

Ammonium sulfate is added to the liquid portion of the concentrate to secure half saturation (37 gm. per 100 cc.). This mixture is stirred well and almost immediately protein material separates out and readily rises to the top of the liquid. After standing ½ hour the protein precipitate is skimmed off and allowed to drain on hardened filter paper for 3 to 6 hours. It is then added to sufficient 95 per cent alcohol to secure a final concentration of 75 to 80 per cent alcohol. The amount of alcohol added is usually very small, but varies with the amount of moisture held in the protein precipitate. Much of the protein material is precipitated by this concentration of alcohol and is removed by filtration. This filtrate is mixed with that obtained when the residue from the fatty mass (which was extracted with ether), is treated with 80 per cent alcohol, as described above. The active principle in these combined filtrates is precipitated by adding to them an equal volume of sulfuric ether. On standing over night this precipitate settles to the bottom of the flask and the ether-alcohol solution is decanted. The precipitate is brought to dryness *in vacuo* and is then treated with dilute ammonium hydroxide of such a concentration that the pH of the resulting solution is approximately 8. At this pH the insulin is completely soluble. The hydrogen ion concentration is then adjusted to a pH of 3.5. At this hydrogen ion concentration a precipitate containing dark colored material usually separates out. This is removed by filtration. The filtrate which is an aqueous extract containing the active principle may be pure enough for clinical use. However, it is advisable to purify it further either by the so called isoelectric precipitation (22), by Dudley's picrate method, or by the use of charcoal. This latter method of purification has been worked out by J. P. Moloney and D. M. Findlay in this laboratory and has been found very satisfactory. This method will be published by these investigators at an early date. The purified product is diluted with acidified water (pH 2.5) to the desired potency as estimated by the rabbit test.

After determining the strength of the insulin, 0.1 per cent tricresol is added, and the solution is passed through a Mandler filter. The insulin, after passing through the filter and before the vials are filled, is retested carefully to determine its potency. It is then diluted with sterile distilled water, pH 2.5, so that it

contains 10 or 20 units per cubic centimeter. The method of standardizing insulin has been described elsewhere (23). The tested insulin is poured into sterile glass vials with aseptic precautions, and the sterility of the final product thoroughly tested by approved methods.

Yields of Insulin.

The unit of insulin has recently been defined in several communications. It is one-third the amount of material required to lower the blood sugar of a 2 kilo rabbit, which has been fasted 24 hours, from the normal level (0.118 per cent) to 0.045 per cent over a period of 5 hours.

The earlier extracts obtained from the degenerated pancreas of the dog, normal dog's pancreas, or the pancreatic tissue of fetal calves, were tested upon diabetic dogs. It is difficult, therefore, since the relative susceptibility of depancreatized dogs and normal rabbits to insulin has not been accurately determined, to quote definite figures in rabbit units for the yield of insulin originally obtained per gram of these tissues.

Pork pancreas has consistently given us somewhat larger yields in experimental lots than has beef pancreas. Beef glands are, however, somewhat easier to process, because they have adherent a smaller amount of fat. They have been used exclusively in this laboratory for the production of larger quantities of material.

During the early part of April, 1922, the yield of insulin suitable for clinical use was approximately 15 units per kilo of pancreas. Later in the same month we were able to obtain about 40 units of purified material per kilo. Experimental lots at that time showed as high as 90 units of crude insulin per kilo. Our present procedure, as previously described, gives a yield of approximately 400 units of purified material per kilo. *The increase in acidity of the extractive has been the greatest single factor in improving the yields.*

The yields secured by watery extraction of the glands are extremely promising. The extraction with boiling water under the most favorable conditions gives a consistent yield of approximately 225 units per kilo. The results of extraction by cold, highly acidified water are even more interesting, at the present

time, than those from hot water. Extracts obtained by these procedures are at present more difficult to purify than those obtained by alcoholic extraction.

The highest yields we have as yet obtained were secured from beef pancreas by alcoholic extraction. In several experiments (15 pounds of pancreas were used in each experiment) we have been able to obtain approximately 900 units of purified insulin per kilo of pancreas. In the preliminary experiments of this series, however, large volumes of extractive were used, and we are not certain as yet that the procedure will be practical. The material was purified in some cases by the benzoic acid method, and in others by the ammonium sulfate and isoelectric method. Recent results tend to show that the volume of the extractive may be greatly diminished without lowering the yield if certain precautions are observed.[3]

DISCUSSION.

During the year 1922, those of us who were responsible for the preparation of insulin for clinical use had insufficient opportunities for the systematic investigation of the chemical properties of the material we were struggling to prepare for patients who were being treated by our clinical collaborators. Changes in the method of production were rapidly introduced, and in many cases were discarded after a brief trial. As our knowledge of the properties of the material has increased, improvements in the method have been introduced.

Alcohol was the extractive used by the original investigators in the University of Toronto in the preparation of insulin from beef pancreas. At many times in the past and especially very recently it has appeared that water would be a more economical solvent. To increase the number of units of insulin obtainable per kilo of pancreas or to introduce a cheaper extractive and thus to assist in lowering the cost of insulin is very desirable, but the question of yields must always be subsidiary to that of the purity of the product. In consideration of this latter point, we believe at the present time that alcohol is the most preferable extractive we have yet investigated.

[3] These experiments have been carried out with the assistance of W. J. Grant and will be reported shortly.

The active interest of the members of the Department of Physiology, Biochemistry, and Pharmacology, in our work has been a very important factor in our progress. We have benefited by the suggestions of the representatives of the British Medical Research Council who visited our laboratory. An ingenious method of purification evolved by Dudley[4] which has been extensively used in England, promises to be of use in the preparation of a dry powder. Insulin in this form seems to be very stable. The collaboration of the investigators mentioned in the body of this communication has been greatly appreciated. The research staff of Eli Lilly and Company has played a prominent part in the rapid development of efficient methods for large scale production.

It has been our intention to review the methods used in Toronto for the preparation of insulin, and not to discuss in detail the properties of this substance. However, certain obvious characteristics of the material are discernible from a study of the various procedures used in the preparation. The stability of insulin is of particular interest and suggests that further research may result in a more highly purified product being obtained.

We regret that we have not had the opportunity to test thoroughly various procedures for the preparation and purification of insulin which have been developed by Prof. T. Brailsford Robertson and Prof. A. B. Anderson of the University of Adelaide, Australia, and by Prof. August Krogh of Copenhagen University, Denmark. The details of these procedures were communicated to Prof. J. J. R. Macleod, and we are indebted to him for very promptly making them available to us.

We wish to express our thanks to Dr. J. G. FitzGerald and Dr. R. D. Defries for their helpful criticism and energetic support.

It is a pleasure to acknowledge our indebtedness to Mr. A. S. Wall and Miss Jessie H. Ridout, for their efficient assistance in our work.

[4] Dudley, H. W., Communicated to Insulin Committee. This method has recently been published (Dudley, H. W., *Biochem. J.*, 1923, xvii, 376).

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