XLII. THE PURIFICATION OF INSULIN AND SOME OF ITS PROPERTIES.

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THE discovery of Banting and Best [1922] of the pancreatic hormone, insulin, which affects the metabolism of the normal animal so profoundly, and which has proved to be of such great practical importance in the treatment of human diabetes, has aroused widespread interest.

The present communication describes attempts to obtain the active principle in as pure a condition as possible, and experiments which have been made with the hope of determining some of its general chemical characteristics.

The insulin employed in this research has been made from fresh ox pancreas by a method which is essentially that of Collip [1923] with certain slight modifications which we have found advantageous, and is described in detail in the experimental section of this paper. The active fraction, which we may call "crude insulin," is obtained in the form of a white, hygroscopic powder, very readily soluble in water, in which it dissolves to form a pale straw-coloured solution which is slightly acid to litmus. The yield varies considerably from preparation to preparation, but the average yield from 21 batches, in which a total weight of 103·6 kilos. pancreas was worked up, was 0·718 g. per kilo. The estimation of the activity of these preparations has been based on the original "Toronto rabbit-unit," *i.e.* the dose necessary, when injected subcutaneously, to lower the blood sugar of a 2 kilo. rabbit, which has fasted for 16–24 hours, from a normal value of about 0·1 % to about 0·04 % and to cause typical hypoglycaemic convulsions within four hours.

Our own experience confirms the observations of McCormick, Macleod, Noble and O'Brien [1923] that the rabbit test is by no means quantitative. We find that rabbits, even after the same preliminary preparation by starving, vary greatly in sensitiveness to similar doses of insulin. The same rabbit will usually, though certainly not always, suffer about the same fall in blood sugar whenever it is given identical doses of insulin.

It is, therefore, possible, by using large numbers of animals, and particularly animals whose reactions are well-known from repeated experiments, to form a fairly accurate opinion as to the relative activities of various insulin preparations, though a really quantitative method of estimation is not yet available.

The dose of "crude insulin" necessary to cause hypoglycaemic convulsions in a 2 kilo. rabbit has been, in our most active preparations, 5 mg. or less, but more usually it is about 10 mg., and we get, on an average, a "rabbit unit" from about 15 g. pancreas.

Over 50 % of "crude insulin" consists of inorganic salts, and it gives positive reactions for chlorides and phosphates.

The following reactions are also positive: biuret, Millon (tyrosine), glyoxylic acid (tryptophan), Pauly (iminazole ring), Molisch (carbohydrate) and Seliwanoff (fructose). A solution in caustic soda, to which lead acetate has been added, blackens on heating, indicating the presence of sulphur.

It is obviously a very complex mixture, and it is impossible to associate any of its reactions with the physiologically active material.

The hormone is precipitated from an aqueous solution of "crude insulin" by a variety of reagents, such as uranium acetate and phosphotungstic acid, but the most practically useful precipitant is picric acid, which removes it from dilute aqueous solution quantitatively. The picrate, which is an amorphous, lemon-yellow powder, weighs from one-twelfth to one-fourteenth of the amount of crude insulin taken. It is only very slightly soluble in water but can be dissolved in dilute ($N/15$) di-sodium hydrogen phosphate solution for testing on rabbits. A rabbit unit of the picrate is about 1 mg.

It has been found to be quite easy to convert this insoluble picrate into a soluble hydrochloride. It is ground up with a little absolute alcohol until a uniformly fine suspension is obtained. A small quantity of an alcoholic solution of hydrochloric acid is then added and the mixture is stirred for a few minutes and dry ether is finally added. The hydrochloride separates out as an almost white powder which may be washed free from picric acid with dry ether. It is a white, perfectly non-hygroscopic and stable powder, readily soluble in water, and is obtained in a yield of 75–80 % of the picrate. The rabbit unit of this material falls generally between 0.5 and 1 mg.

The yield of hydrochloride is therefore 1 g. from 20–25 kilos. pancreas and approximately 6 % of the "crude insulin." Within the limits of determination possible by the admittedly inaccurate rabbit test, we have not been able to detect any loss of activity in preparing the hydrochloride from "crude insulin."

"Crude insulin" contains some material which causes a moderate, though transient, fall in the blood pressure of an anaesthetised animal. This substance is completely eliminated in the purification effected by means of picric acid, and the hydrochloride consequently displays no such effect.

The chemical reactions of the hydrochloride are interesting; in contrast to "crude insulin" its solution no longer gives an immediate positive test for phosphate. Further, after oxidising the solid with a mixture of nitric and sulphuric acids the test for phosphoric acid is negative, and indicates the absence of organically combined phosphorus.

This confirms the observation of Doisy, Somogyi and Shaffer [1923] made on "insulin" purified by a different procedure. Moreover, it no longer gives

Seliwanoff's reaction for fructose. This therefore disposes of the suggestion of Winter and Smith [1923] that these reactions may be associated with the active principle.

It no longer gives any reaction for tryptophan, and the Millon reaction (tyrosine) is now very faint and atypical. It still gives a positive Molisch (carbohydrate) reaction, but it is well-known that this reaction is almost always present in protein-containing material. The biuret reaction is given strongly, the Pauly reaction is intense, and the organic sulphur reaction is quite marked. It is, in fact, a substance, exhibiting the characteristics of a protein derivative, in which, of the common tests applied in such cases, the biuret and those for the iminazole ring (histidine) and for sulphur are the only striking reactions. It should be noted that Best and Macleod [1923] state that insulin prepared from the ox and pig pancreas gives a biuret reaction, an orange colour by the xanthoproteic test and a faint purple with glyoxylic acid. They fail, however, to produce any of these reactions with insulin prepared from skate pancreas by alcoholic extraction and heat. Doisy, Somogyi and Shaffer attribute a distinct biuret reaction, faint glyoxylic acid and doubtful Millon reactions to their purest preparation.

None of these investigators appears to have tried the Pauly and sulphur tests which are the characteristic reactions of the preparation from ox pancreas; the others, with the exception of the biuret, vanish or become insignificant when insulin is purified by precipitation with picric acid. It may be mentioned, in passing, that small amounts of insulin were prepared in the course of this investigation from the pancreas of the dog-fish and of the skate by the method described in this paper, and in both cases the "crude insulin" gave a very definite biuret reaction.

Whether the sulphur is present as cystine, or as some other organic sulphur-containing compound, is at present uncertain. After hydrolysis with hydrochloric acid under suitable conditions a solution is obtained which, after reduction, gives the sodium nitroprusside reaction, carried out according to Hopkins' instructions [1921], with considerable intensity. Hydrolysis with stronger acid, however, apparently destroys the substance which gives this reaction. Further experiments are being performed in this connection. It is impossible to obtain a positive nitroprusside reaction in a reduced solution of insulin hydrochloride itself. The sulphur in the intact substance is therefore not reducible, as it is, for example, in glutathione.

Doisy, Somogyi and Shaffer [1923] have shown that insulin may be precipitated from aqueous solution at what is called its "isoelectric point" by adjusting the p_H of the solution to about 5. If a solution of the hydrochloride be treated in this manner a precipitate is formed which has a rabbit unit value of about 0.3 mg.

The whole of the active substance, however, is not precipitated, at any rate from a 2 % solution of the hydrochloride. This precipitate, like the hydrochloride, gives strong Pauly and sulphur reactions.

The hydrochloride dissolves in water giving a solution with a p_H of about 4, and the "isoelectric point" precipitate is produced by adding alkali to such a solution. A precipitate is also formed by adding strong acid to the hydrochloride solution, until it contains about 3.3 % HCl. A flocculent white precipitate is formed which is somewhat more active than the "isoelectric point" precipitate. 0.25 mg. of this substance proved to be a rabbit unit, and it is the most potent preparation so far obtained.

In this case also the precipitation of the active principle is not complete. The precipitate, again like the hydrochloride, gives strong Pauly and sulphur reactions.

Experiments have been made to ascertain the stability of insulin to acid and alkali. It proves to be rather surprisingly stable to acid. If an aqueous solution of "crude insulin" is heated to 100° for 10 minutes in a boiling water-bath a great deal of its activity is destroyed. But in $N/10$ H_2SO_4 it will stand this treatment for 30 minutes without detectable loss of activity. After one hour of such treatment it is obvious that some destruction has taken place, and still more after two hours, but even then a considerable amount of the active principle remains.

Very different is its behaviour to alkali. Heating to 37° for $1\frac{1}{2}$ hours in $N/10$ NaOH destroys its activity completely, and the same destruction is effected by heating to 100° in a boiling water-bath for 10 minutes in $N/10$ Na_2CO_3 . It is more slowly inactivated by digestion in $N/10$ Na_2CO_3 at 37° , retaining a considerable amount of activity after two hours under these conditions.

It is noteworthy that not only is "insulin" readily destroyed by trypsin, thus confirming the original supposition of the Toronto workers, on which they based their methods for its isolation, but it is also decomposed with remarkable ease and rapidity by pepsin.

An attempt to filter a solution of "crude insulin" through a collodion sack under pressure showed that only minute amounts of the active principle passed through.

As before mentioned, addition of uranium acetate to a solution of crude insulin precipitates the active principle completely. A point of some practical importance was established when it was found that the insulin could be washed off the precipitate by means of weakly alkaline solutions of disodium hydrogen phosphate. It is evidently adsorbed on the precipitate of uranyl phosphate in virtue of its colloidal nature. For human use insulin has to be sterilised by filtration through Berkefeld filters, and serious losses of activity had been experienced when solutions of "crude insulin" were so filtered. These solutions are faintly acid, with a p_H not far removed from the "isoelectric point." In consequence of the uranium acetate experiment it was found that if the p_H of a solution of insulin is first adjusted to approximately 7.5 it may be filtered through a candle without detectable loss. It is obvious that filtration of acid solutions is bound to be a risky procedure in view of

the formation of precipitates, not only at the "isoelectric point," but also in stronger acid.

A review of the results obtained enables us to draw certain general conclusions.

The *complete* precipitation of insulin from dilute solution by picric acid, its ready adsorption, its failure to pass through an ultra-filter, its rapid digestion by trypsin, and particularly by pepsin, all indicate that it is most probably a substance having a very complex structure and protein-like in its nature. The hope of its isolation as a chemically pure substance becomes slender, and of its synthesis very remote, by methods at present at our disposal.

It is certainly much more complex than the active principles of the pituitary posterior lobe, probably even more so than "secretin." It is more analogous in complexity to the toxins, to ricin, or to Vaughan's toxic protein derivatives.

It is obvious that the administration of insulin to patients by the mouth is impossible on account of its rapid destruction by the digestive enzymes, and the relatively large size of its molecule makes it unlikely that absorption from mucous surfaces, such as that of the nasal passages, a method which has recently proved useful in controlling diabetes insipidus with pituitrin, will be practically useful in the case of insulin.

The fact that great physiological activity is associated with both the "isoelectric point" precipitate and the strong hydrochloric acid precipitate, described in this paper, suggests that the active principle is present in such a preparation as the "insulin hydrochloride" in quite indeterminate amount, associated with other substances of approximately similar physical properties. In this case it is impossible to decide whether the characteristic Pauly and sulphur reactions bear any relation to the actual active principle. It is, however, intriguingly interesting that the Pauly reaction should be so strongly positive. It occurs so frequently in connection with preparations of highly physiologically potent substances, such as those of the oxytocic and pressor principles of the pituitary gland, that one is tempted to suspect that derivatives of histidine are apt to be endowed with various intense physiological activities; but, at present, this is pure speculation.

EXPERIMENTAL.

Preparation of "Crude Insulin."

Five kilos. of perfectly fresh ox pancreas are minced through a sausage machine into 5 litres of 95 % alcohol, previously cooled to -3° . Not more than two hours should elapse between the slaughtering of the animals and the mincing of the tissue into alcohol. It is advantageous to strain off the minced tissue on a fine sieve, squeeze out as much of the alcohol as possible by hand, remince the tissue into the alcohol and repeat the mincing a third time in order to get as good

disintegration as possible. The mixture is then allowed to stand for two hours at room temperature with frequent stirring. It is then filtered through large folded filters of thick "Chardin" paper. As soon as the main bulk of the liquid, which may be slightly turbid, is filtered, the thick paste on the paper is transferred to a suitable press and as much liquid as possible is squeezed out. The press liquor and filtrate are mixed. This filtration is tedious, but is completed in three to four hours. Approximately 5.5 litres of filtrate are collected. $1\frac{1}{2}$ volumes (*i.e.* 8.25 litres) of 95 % alcohol are now added. A white precipitate appears and the liquid is placed in a cold room at -3° overnight.

The liquid is then filtered through "Chardin" folded filters in the cold room. This filtration proceeds without difficulty, giving a perfectly clear, yellow solution. The filtrate is transferred in quantities of $2\frac{1}{2}$ –3 litres to a battery of five 5-litre round-bottomed flasks, and the alcohol is distilled off *in vacuo*, the temperature of the water-baths being kept at 45° .

When most of the alcohol has been removed the liquid becomes turbid owing to the separation of fat. The residual liquid in the five flasks is collected in one flask and further distilled *in vacuo* with the water-bath at 45° . This removes the remainder of the alcohol, and the fat usually separates in buttery lumps. The distillation is continued until the volume of the aqueous residue is about 250 cc. It is then filtered from fat on a Buchner funnel and the main bulk of the fat is shaken out of the flask and squeezed as free from solution as possible. (Occasionally the fat does not solidify but remains floating in the concentrated aqueous solution as a thick, yellow oil. When this happens, the entire contents of the flask are transferred to a separating funnel and shaken with a small quantity of light petroleum. This dissolves the fat readily, and the aqueous layer can be tapped off without loss.) The aqueous solution is then made up to 80 % with absolute alcohol; that is to say, to every 100 cc. of solution are added 400 cc. of absolute alcohol. A yellow syrup separates in fine drops and falls to the bottom of the solution which is then placed in the cold room overnight.

After standing, the syrup and a certain amount of solid matter adhere to the bottom of the containing vessel, preferably a tall, narrow cylinder, and the supernatant liquid is decanted without difficulty and made up to 93 % by the addition of two volumes of absolute alcohol. A somewhat scanty white precipitate forms and the liquid is placed in the cold room for 15–20 hours. The precipitate has now settled to the bottom as a white granular powder, the supernatant liquid being almost, if not quite, clear. The latter is removed by means of a siphon except for a small amount left covering the precipitate, which is then stirred up with the residual alcohol and poured into centrifuge tubes. The precipitate is centrifuged down, washed once or twice with absolute alcohol and once or twice with dry ether in the centrifuge. After pouring off the ether the tubes containing the precipitate are quickly transferred to a vacuum desiccator and dried over sulphuric acid. A yield of from 3–4 g. is obtained in the form of a white, hygroscopic powder.

Preparation of the Active Picrate and Hydrochloride.

A 1.5 % aqueous solution of "crude insulin" is made. It is freed from any small amount of suspended matter by centrifuging—filtration, even through paper, is apt to be attended by loss of activity—and then half its volume of a saturated aqueous solution of picric acid is added. An immediate flocculent, amorphous precipitate is formed which settles down rapidly to the bottom of the liquid, leaving a fine suspension in the supernatant fluid. After standing for one or two days at ordinary room temperature the supernatant liquid becomes quite clear and is poured off. A little water is then added to the precipitate which is now adhering to the bottom of the beaker. The picrate is rubbed up with a glass rod and the suspension is then transferred to a centrifuge tube and spun down. It is washed with repeated additions of water in the centrifuge until finally the supernatant liquid displays a slight turbidity on the addition of a few drops of saturated aqueous picric acid. This indicates that the picrate is beginning to go into solution. It is then suspended in a small volume of water and filtered on a small Buchner funnel and finally placed on porous pot and dried in a vacuum desiccator over H_2SO_4 . A lemon-yellow amorphous powder is obtained in a yield of about 7.5 % of the crude insulin taken.

The precipitation should be carried out in dilute solution as indicated, although it is permissible to use a 2 % solution of "crude insulin," adding equivalently larger amounts of picric acid solution. If precipitation from strong solutions is attempted other picrates are precipitated also which are inert physiologically.

The mother liquor from the precipitation of the picrate has been examined in numerous batches for blood-sugar lowering material. It has been evaporated *in vacuo* to a convenient bulk, then faintly acidified with hydrochloric acid, the picric acid shaken out with ether, and finally injected into a rabbit. We have never succeeded in finding a trace of insulin in this way. For instance, 1 g. of "crude insulin," which had a rabbit unit of 10 mg. was precipitated with picric acid in the manner described. Half the filtrate, representing 0.5 g. of the original powder, or 50 rabbit units, was concentrated *in vacuo* to about 10 cc. It was then acidified with 2 cc. $N \text{ H}_2\text{SO}_4$ and the picric acid was removed by shaking out with ether. The ether was removed from the solution *in vacuo* and then, after adding 2 cc. $N \text{ Na}_2\text{CO}_3$, the whole solution was injected into a 2.4 kilo. rabbit subcutaneously. The blood sugar values, taken every hour, were as follows:

Blood sugar, normal	0.096 %
" " 1 hour after injection	0.093
" " 2 hours " "	0.101
" " 3 " " "	0.105

It will be seen that there is no fall in the blood sugar value, indicating that the removal of insulin by means of picric acid is quantitative.

The proper washing of the picrate is of great importance. It is more practicable to do the washing in a centrifuge tube since the picrate tends to clog on a filter paper. If any mother liquor remains in the wet precipitate it will be found that, on drying, the picrate, instead of remaining pale yellow and easily powderable, forms a hard, dark brown mass, rather like shellac. In this condition it is quite unsuitable for conversion into the hydrochloride. If a batch of picrate is found in this state after drying it should be dissolved in $N/10$ Na_2CO_3 and the clear solution should then be acidified with HCl until just acid to litmus. After the addition of a small amount of picric acid solution the reprecipitated picrate is washed thoroughly and recovered as described above. It may be noted that on reprecipitation in this manner the picrate is in a somewhat more easily filterable condition and may be collected and washed on a filter paper.

If a sufficient quantity of picrate is available this reprecipitation is to be recommended as giving a slightly purer product, which is also in better physical condition for the subsequent transformation into hydrochloride.

This is accomplished by grinding up 1 g. of picrate with 6 cc. absolute alcohol in a mortar until a very fine uniform suspension is obtained. It is then transferred to a centrifuge tube of about 40 cc. capacity and washed in with 2 cc. absolute alcohol. 4.5 cc. of a solution of dry HCl (5 % by weight) in absolute alcohol are added to the suspension which is stirred for about five minutes. About 30 cc. of dry ether are then added and the hydrochloride settles rapidly as a white amorphous powder. After centrifuging, the clear liquid is poured off and the precipitate is washed twice with dry ether. It is then transferred in the tube to a vacuum desiccator containing sulphuric acid and the residual ether is removed by cautious evacuation. The hydrochloride, an almost white, non-hygroscopic powder, is obtained in a yield of about 75–80 % of the picrate.

In a typical preparation 4.64 g. of "crude insulin" were taken. A rabbit unit of this material was 15 mg. 0.3281 g. picrate was obtained, of which 1 mg. was a rabbit unit. This yielded 0.2461 g. of hydrochloride which on first testing caused convulsions in a rabbit in a dose of 0.5 mg. On retesting, this dose just failed to produce convulsions although the blood sugar approached the convulsant level, while a rabbit receiving 1 mg. had severe convulsions. The total number of rabbit units in the "crude insulin" was 309. The assay of the picrate indicated 328 "units," while if 0.75 mg. be taken as the "rabbit unit" of the hydrochloride the yield from the picrate was quantitative.

Elimination of Depressor Constituent of "Crude Insulin" by means of the Picrate Purification.

In this experiment a portion of a batch of "crude insulin" which had a rabbit unit of 10 mg., was converted into the hydrochloride of which 0.75–1.0 mg. proved to be a rabbit unit.

A cat, with vagi intact, under ether anaesthesia, was used to determine the effect of the "crude insulin" and the hydrochloride on the blood pressure.

Fig. 1.

The following injections into the femoral vein were given

A.	At 4.0	p.m.:	2 mg.	hydrochloride.
B.	" 4.2	"	20 "	" "crude insulin."
C.	" 4.6	"	5 "	hydrochloride.
D.	" 4.8	"	20 "	" "crude insulin."
E.	" 4.13	"	50 "	" "crude insulin."

At *A*, *B* and *D*, therefore, two rabbit units of insulin were given and at *C* and *E* five rabbit units.

The hydrochloride, then, under these conditions, had no effect on the blood pressure, while corresponding doses of "crude insulin" produced marked falls in blood pressure.

The "Isoelectric Point" Precipitate.

0.1045 g. of "insulin hydrochloride" was dissolved in 4 cc. of water in a centrifuge tube. 0.4 cc. $N Na_2HPO_4$ was added, drop by drop. It was found that after this amount had been added no further precipitate was formed. The p_H of the solution was 5.7. The precipitate was formed very promptly; standing in the cold room overnight produced no more. It was centrifuged off, washed with absolute alcohol and finally with dry ether. It was then dried by placing it in a vacuum desiccator over H_2SO_4 and evacuating cautiously. The dry precipitate, a white powder, weighed 0.0353 g.

It was dissolved in $N/15 Na_2HPO_4$ solution for testing on rabbits. The rabbit unit of the hydrochloride was 0.5 mg. and that of the precipitate 0.33 mg., so that 50 % of the activity had been removed from the solution and the precipitate was about $1\frac{1}{2}$ times as active as the hydrochloride from which it had been prepared.

This ratio was checked by testing the residual activity of the solution from which the precipitate was obtained.

The Precipitate formed by the Addition of strong Hydrochloride Acid to a Solution of "Insulin Hydrochloride."

0.13 g. of hydrochloride was dissolved in 10 cc. water. 10 % hydrochloric acid was added gradually until no further precipitate was formed. This required 5 cc. of the acid. After standing in the cold room overnight the precipitate was centrifuged down, washed with a little 3.3 % HCl, and dried in a vacuum desiccator. The weight of the precipitate was 0.02 g.

It was dissolved in water for the rabbit test. It was not quite completely soluble in water, a small amount of flocculent material remaining suspended in the liquid.

The mother liquor was nearly neutralised with NaOH and then treated with aqueous picric acid solution and the residual material recovered as picrate. It weighed 0.0815 g.

The rabbit unit of the hydrochloride was 0.5 mg., and that of the precipitate 0.25 mg. The precipitate was therefore twice as active as the hydrochloride from which it had been prepared, and 32 % of the total activity had been precipitated.

Stability of Insulin to Acid.

A batch of "crude insulin" of which the rabbit unit was 10 mg. was used in these experiments.

A solution of the "crude insulin" containing 10 mg. per cc. was made in $N/10$ H_2SO_4 and divided into 1 cc. portions. These were heated in test-tubes in a boiling water-bath for various periods, and then cooled, neutralised with $N/10$ Na_2CO_3 , and injected subcutaneously into rabbits. The following results were obtained:

Ten minutes' heating. Rabbit weighed 1.6 kilo.:

Blood sugar, normal	0.107 %
" " 1 hour after injection	0.047
" " 2 hours " "	0.023

The animal had convulsions in 2 hours and was recovered by injecting glucose.

Thirty minutes' heating. Rabbit weighed 2.35 kilos.:

Blood sugar, normal	0.111 %
" " 1 hour after injection	0.036
" " 2 hours " "	0.029

Animal had convulsions in 2 hours and was recovered by injecting glucose.

One hour's heating. Rabbit weighed 1.1 kilo.:

Blood sugar, normal	0.090 %
" " 1 hour after injection	0.061
" " 2 hours " "	0.056
" " 3 " " "	0.065

Two hours' heating. Rabbit weighed 1.1 kilo.:

Blood sugar, normal	0.088 %
" " 1 hour after injection	0.055
" " 2 hours " "	0.072
" " 3 " " "	0.093

The last two results show that slow destruction of the active principle is occurring.

An aqueous solution of the same batch of "crude insulin" containing 10 mg. per cc. was heated to 100° for ten minutes. 1 cc. of the solution was then injected subcutaneously into a rabbit weighing 1.75 kilo.:

Blood sugar, normal	0.108 %
" " 1 hour after injection	0.092
" " 2 hours " "	0.069
" " 3 " " "	0.081
" " 4 " " "	0.090

Evidently in such a faintly acid solution—the p_H is about 6.1—insulin is quite unstable at 100°.

Stability to Alkali.

The same batch of insulin was used as in the experiments on stability to acid.

10 mg. were dissolved in 1 cc. $N/10$ NaOH and heated to 37° for 1½ hours. The solution was then neutralised with 1 cc. $N/10$ HCl and injected into a rabbit weighing 1.5 kilo.:

Blood sugar, normal	0.101 %
" " 1 hour after injection	0.105
" " 2 hours " "	0.101

No trace of activity is left.

10 mg. were dissolved in 1 cc. $N/10$ Na_2CO_3 and heated to 37° for one hour. After neutralisation with 1 cc. $N/10$ HCl the solution was injected into a rabbit weighing 1.5 kilo.:

Blood sugar, normal	0.114 %
" " 1 hour after injection	0.066
" " 2 hours " "	0.048
" " 3 " " "	0.035

The animal had convulsions in three hours and was recovered by the injection of glucose.

10 mg. were dissolved in 1 cc. $N/10$ Na_2CO_3 and heated in a boiling water-bath to 100° for ten minutes. After neutralisation the solution was injected into a rabbit weighing 1.5 kilo.:

Blood sugar, normal	0.113 %
" " 1 hour after injection	0.107
" " 2 hours " "	0.113
" " 3 " " "	0.129

This result indicates complete destruction of the active principle.

Action of Trypsin.

0.5 g. of a commercial sample of trypsin was dissolved in 10 cc. $N/10$ Na_2CO_3 and 20 mg. of the same "crude insulin" which was used in the previous experiments on the stability to acid and alkali was dissolved in 2 cc. water.

The insulin solution was divided into two equal portions of 1 cc. each and to one of them was added 1 cc. of the trypsin solution, and to the other 1 cc. of a portion of the trypsin solution which had previously been heated to 100° for 30 minutes. A drop of chloroform was added to each solution.

After incubation at 37° for two hours they were injected into two rabbits.

(1) Trypsin experiment. Rabbit weighed 3.5 kilos.:

Blood sugar, normal	0.092 %
" " 1 hour after injection	0.104
" " 2 hours " "	0.101
" " 3 " " "	0.104

(2) Heated trypsin control. Rabbit weighed 3.25 kilos.:

Blood sugar, normal	0.101 %
" " 1 hour after injection	0.059
" " 2 hours " "	0.066
" " 3 " " "	0.074

Trypsin, therefore, promptly destroys insulin.

Action of Pepsin.

1st experiment. 0.3 g. of a commercial pepsin preparation was dissolved in 50 cc. *N*/500 HCl. A portion of this solution was heated to 100° for 30 minutes.

20 mg. "insulin hydrochloride," of which the rabbit unit was 0.8 mg., were dissolved in 5 cc. water.

(1) To 2.5 cc. of the insulin hydrochloride solution were added 2.5 cc. of the pepsin solution.

(2) To 2.5 cc. of the insulin hydrochloride solution were added 2.5 cc. of the heated pepsin solution.

To each of the solutions were added a few drops of chloroform. They were then placed in the hot room at 37° for 16 hours.

1 cc. of (1) (*i.e.* 2 mg. insulin hydrochloride) was injected into a rabbit weighing 2.4 kilos.:

Blood sugar, normal	0.123 %
" " 1 hour after injection	0.129
" " 2 hours " "	0.124

1 cc. of (2) (*i.e.* 2 mg. insulin hydrochloride) was injected into a rabbit weighing 2.3 kilos.:

Blood sugar, normal	0.109 %
" " 1 hour after injection	0.077
" " 2 hours " "	0.041

The animal had convulsions 2½ hours after the injection and was recovered by injecting glucose.

The experiment shows a complete destruction of insulin by pepsin.

2nd experiment. It was desired, if possible, to demonstrate the action of pepsin in a shorter time than in the previous experiment.

A 1 % solution of commercial pepsin of a different source from that employed in the first experiment was made in *N*/10 HCl. A portion of this solution was heated to 100° for 30 minutes. A solution of the same specimen of "insulin hydrochloride" containing 4 mg. per cc. was prepared.

(1) To 3 cc. of the insulin hydrochloride were added 3 cc. of the pepsin solution.

(2) To 2 cc. of the insulin hydrochloride were added 2 cc. of the heated pepsin solution.

A few drops of chloroform were added to the solutions, which were then placed in the hot room at 37°.

After one hour 1 cc. of solution (1) was removed, neutralised with Na_2CO_3 and injected into a rabbit weighing 2.2 kilos. After two hours a similar sample was withdrawn and injected into a rabbit. As the destruction of the insulin was found to be complete at the end of the first hour only the blood sugar record of the first rabbit is given:

Blood sugar, normal	0.107 %
" " 1 hour after injection	0.113
" " 2 hours " "	0.114
" " 3 " " "	0.126

After four hours a 1 cc. sample was withdrawn from the control experiment (2) and, after neutralisation, injected into a rabbit weighing 1.9 kilo.:

Blood sugar, normal	0.120 %
" " 1 hour after injection	0.074
" " 2 hours " "	0.063

The animal was then given an injection of glucose and the experiment discontinued.

The insulin was completely destroyed by pepsin in one hour.

Berkefeld Filtration of "Crude Insulin."

1.04 g. of crude insulin was dissolved in 100 cc. water. The p_H of this solution was 6.1.

It was then sucked through a kieselguhr candle, except for a small quantity (6.5 cc.) which was held back for tests. Owing to traces of alkali in the candle the p_H of the filtered solution had risen to 6.6. The filter was washed by drawing 50 cc. $N/10 \text{ Na}_2\text{HPO}_4$ through it.

1 cc. of the unfiltered solution was injected into a rabbit weighing 1.6 kilo.:

Blood sugar, normal	0.111 %
" " 1 hour after injection	0.046
" " 2 hours " "	0.030

The animal had convulsions $1\frac{1}{2}$ hours after the injection and was recovered by injection of glucose after the 2nd hour blood sample had been taken.

1 cc. of the filtered solution was injected into a rabbit weighing 2.3 kilos:

Blood sugar, normal	0.115 %
" " 1 hour after injection	0.085
" " 2 hours " "	0.071
" " 3 " " "	0.087
" " 4 " " "	0.102

0.5 cc. of the sodium phosphate solution was injected into a rabbit weighing 2.5 kilos:

Blood sugar, normal	0.106 %
" " 1 hour after injection	0.041
" " 2 hours " "	0.031
" " 3 " " "	0.032

The animal recovered from convulsions without requiring an injection of glucose.

It is apparent that nearly all of the active material was adsorbed by the kieselguhr filter, and that the adsorbed substance was recovered by passing the alkaline solution of sodium phosphate through the filter.

Berkefeld Filtration of "Crude Insulin" in Alkaline Solution.

0.547 g. of the preparation used in the previous experiment was dissolved in 54.7 cc. $N/10$ Na_2HPO_4 . The p_{H} of this solution was 7.2.

A portion was reserved unfiltered for control tests and the rest was passed through a kieselguhr filter.

The p_{H} of the filtered liquid was 7.4 owing to traces of alkali in the candle. The filtrate was found to be sterile by bacteriological tests.

1 cc. of the unfiltered solution was injected into a 2.5 kilo. rabbit.

Blood sugar, normal	0.090 %
" " 1 hour after injection	0.040
" " 2 hours " "	0.047

The animal had convulsions $1\frac{1}{2}$ hours after the injection and was recovered by injection of glucose.

1 cc. of the filtered solution was injected into a rabbit weighing 2.1 kilos.:

Blood sugar, normal	0.116 %
" " 1 hour after injection	0.052
" " 2 hours " "	0.046

The animal had convulsions $1\frac{1}{2}$ hours after the injection and was recovered by injection of glucose.

All the rabbits used throughout these experiments were starved for 16-24 hours before the tests were made.

The blood sugars were estimated by the method of Shaffer and Hartmann [1920].

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SUMMARY.

1. A method of purifying crude preparations of insulin is described. It consists in precipitating the active material with picric acid and converting the insoluble picrate into a soluble hydrochloride by means of an alcoholic solution of hydrochloric acid.

2. This hydrochloride is an almost white powder of which 0.5-1 mg. will lower the blood sugar of a 2 kilo. rabbit, which has been starved for 24 hours, to about 0.04 % and cause typical hypoglycaemic convulsions.

3. The characteristic reactions given by this substance are the biuret, Pauly and organic sulphur tests. The glyoxylic acid reaction is negative, and the Millon test is very faint and atypical. It contains no phosphorus.

4. From its solutions two precipitates may be obtained; one by the addition of acid and the other by the addition of alkali. The first is about twice and the second about one-and-a-half times as active as the original hydrochloride.

5. It is shown that both pepsin and trypsin destroy insulin.

6. Insulin is relatively stable to acid, but is decomposed with ease by alkali.

7. Insulin, in faintly acid solutions, is very readily adsorbed, but it may be filtered through Berkefeld filters without appreciable loss if the solution is first made weakly alkaline.

8. Insulin appears to be a very complex protein derivative.

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