LXXXV. STUDIES ON CARBOHYDRATE METABOLISM.

II. ON THE PREPARATION OF AN ANTI-DIABETIC HORMONE FROM YEAST. PART I.

BY HENRY BROUGHAM HUTCHINSON, WILLIAM SMITH
AND LEWIS BLAND WINTER.

*From the Distillers Company Research Laboratory, Hammersmith, and the
Biochemical Laboratory, Cambridge.*

(Received July 30th, 1923.)

THE importance of phosphorus in the metabolism of carbohydrate is well known. Since insulin relieves the symptoms of diabetes, and carbohydrate-phosphorus compounds are formed by the action of yeast, it seemed possible that a substance similar to insulin might be obtained from this source. It has been shown [Winter and Smith, 1923, 1] that it is possible to extract from yeast a substance which has a similar effect to insulin in causing a lowering of the blood sugar of normal animals. Though this extract, like crude insulin, contains organic phosphorus, Dudley [1923] has shown that insulin purified by the picrate method contains no phosphorus, and that the phosphorus in the crude product has no significance in this connection. A beneficial effect was observed when the extract of yeast was injected into persons suffering from diabetes mellitus [Winter and Smith, 1923, 2]. Later Collip [1923, 1] obtained an anti-diabetic substance from a variety of vegetable sources, but he apparently found that yeast was not a satisfactory source, for he reported twenty failures before an active extract was obtained. Recently Funk and Corbitt [1923] have met with similar variation when yeast was used. We soon found that different samples of yeast gave extracts which differed widely in their activity, some being quite inactive.

In a private communication from Dr H. H. Dale we were informed that from experiments performed in his laboratory no confirmation of our results could be obtained; as much as 400 mg. of the extract was injected into a rabbit without any lowering of the blood sugar being obtained.

The following is a description of some experiments which we have undertaken to determine the conditions under which an anti-diabetic substance could be obtained from yeast. Collip [1923, 1] has suggested the name *gluco-kinin* for anti-diabetic hormones obtained from sources other than pancreas, and this name will be adopted in the following account.

EXPERIMENTAL.

The method employed for the extraction of the yeast was essentially that devised by Collip [1922] for the preparation of insulin from pancreas, and modified by Dudley [1923].

In our first experiments 2 kilos. of fresh baker's yeast were mixed with an equal volume of 95 % alcohol, and ground up with sand in a mortar. The extraction was allowed to continue at room temperature for two hours. The mixture was then filtered through a coarse pleated paper. Filtration was readily effected by this means. The remaining steps in the process are the same as in the pancreatic preparation, but unlike the extraction from pancreas, there is no difficulty caused by the separation of fat when the concentration of the alcoholic extract is nearly complete. Light petroleum was rarely necessary. The final product is a hygroscopic white powder readily soluble in water. For injection into animals a known weight of this was dissolved in normal saline. The injections were given subcutaneously, rabbits and rats being used as experimental animals. Food was withheld for 24 hours previous to injection. The blood sugar estimations were carried out by Bang's old method [1913].

The local reaction caused by injection of this extract was much less than in the case of insulin. This difference was particularly noticeable when cases of diabetes in man were being treated. The amount of extract necessary to lower the blood sugar to a similar level was much greater than that required when insulin was used. The effect of glucokinin would appear however to be more prolonged (see Fig. 1, p. 692). The action on the blood sugar may be seen from the following example.

Rabbit 2.2 kilos.

Time						Blood sugar %
10.00	...	...	...	...	...	.14
10.10	injection of	glucokinin	.06 mg./g.	body weight		
11.30	...	...	...	...	...	.10
2.20	...	...	...	...	...	.06
4.30	...	...	...	...	...	.07
6.30	...	...	...	...	...	.09

Though the weight of extract from a given quantity of yeast varied, the same standard dose was always given to each animal (0.06 mg. per g. body weight).

When it was found that certain samples of yeast gave little activity it seemed possible that the properties of glucokinin might be slightly different from those of insulin, and that some modification in the alcoholic process might be necessary. Small samples of the same yeast were taken and extracted with different strengths of alcohol, and in the ensuing stages precipitation was carried out in 70 or 80 % alcohol. No appreciable difference in the activity was observed. The acidity derived from the yeast would appear to be sufficient to ensure the stability of the active principle, assuming that

its properties are similar to those of insulin, since no advantage was derived from the addition of acetate buffer, p_H 4.5, during concentration of the filtrate. Insulin is fairly stable in acid solution but is rapidly destroyed by alkali (ten minutes in $N/10$ sodium carbonate at 100°) [Dudley, 1923].

It seemed possible, however, that glucokinin might be present in the cells as a precursor from which it could be liberated by the action of alkali. Yeast which yielded very little active principle by the ordinary method was extracted with alcohol in alkaline solution, and acidified before concentration. No increase of activity in these cases was observed.

The final precipitation with 80 % alcohol causes a separation of a yellow oily liquid. A small loss occurs at this stage, some of the glucokinin being precipitated. The greater part, however, of the glucokinin remains in solution to be precipitated in 95 % alcohol at -7° .

The disintegration of the cells by grinding the yeast with sand in a mortar being obviously incomplete, the possibility of splitting the cells by rapid freezing and thawing was tested with a view to increasing the yield from samples of yeast deficient in glucokinin. Through the kindness of Mr W. B. Hardy, we were able to carry out the freezing at a temperature of -19° in the Low Temperature Station, Cambridge. The yeast was spread out in a thin layer on trays. When thoroughly frozen it was rapidly thawed under warm alcohol in a water bath. Little success resulted, and we were forced to conclude that some factor was lacking in the samples of inactive yeast, and that no more complete extraction would increase the yield of glucokinin. The following is an example.

Rabbit 2.1 kilos.					Rabbit 2.9 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.00	...	...	...	.14	10.10	...	...	...	.13
10.15	injected	(usual process)	...	.12	10.30	injected with extract ob-	tained from frozen yeast	...	.12
12.30	...	...	...	.12	12.45	...	...	...	.11
2.30	...	...	...	.13	2.45	...	...	...	.12
4.30	...	...	...		4.40	...	...	...	

Up to the present we had used only samples of baker's yeast obtained from the same source locally. Since it was possible that the freshness of the yeast might be of importance, samples of baker's yeast were obtained as fresh as possible, and immediately placed in alcohol. Variability was experienced as before, and no definite evidence could be brought to show that the freshness or otherwise of the yeast was responsible for this.

Experiments were next carried out to determine whether brewer's yeast might be more suitable as a source of glucokinin. Samples of yeast were skimmed from beers of different gravity, and placed as soon as possible under alcohol. Some samples were taken at the usual period (end of fermentation), others were collected when the fermentation was proceeding most rapidly. These were necessarily small as considerable quantities of the fermenting fluid had to be filtered before the necessary quantity of yeast could be collected.

Both top and bottom yeasts were also tested. The activity was usually poor, and in the case of a sample of bottom yeast the blood sugar rose. The following are some results obtained from brewer's yeast.

Rabbit 2.5 kilos.					Rabbit 2.3 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.15	...	...	...	...	11.00	...	...	...	...
10.30	injected	with	extract	of	11.15	extract	of	bottom	yeast
	top	yeast			2.00	...	...	...	...
12.20	...	...	...	...	5.00	...	...	...	...
2.30	...	...	...	...	7.00	...	...	...	...
5.00	...	...	...	...					

Rabbit 1.6 kilos.

Time									Blood sugar %
10.00	...	...	...	...	...	...	...	...	...
10.15	extract	of	yeast	collected	at	height	of	fermentation	...
12.30	...	...	...	...	...	...	...	...	...
2.00	...	...	...	...	...	...	...	...	...
4.00	...	...	...	...	...	...	...	...	...

It was found that when the final precipitation was carried out (in 95 % alcohol at -7°) the greater part of the precipitate usually settled overnight. The remainder being finely divided took some days to settle. These two fractions were roughly separated. No variation in the activity occurred as will be seen from the following figures.

Rabbit 2.2 kilos.					Rabbit 2.3 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.15	...	...	...	...	10.45	...	...	...	...
10.30	first	fraction	injected	...	11.00	second	fraction	given	...
11.30	...	...	...	...	12.30	...	...	...	...
2.00	...	...	...	...	2.30	...	...	...	...
3.30	...	...	...	...	5.00	...	...	...	...

A comparison was next made between the amounts of glucokinase obtainable from samples of the same brand of commercial yeast obtained on different dates. These were treated in the same way, and gave such very divergent results as may be seen from the following figures.

Rabbit 2.7 kilos.					Rabbit 2 kilos.				
Time				Blood sugar %	Time				Blood sugar %
11.45	...	...	...	...	10.00	...	...	...	...
12.15	injected			...	10.30	injected			...
2.00	...	...	...	...	12.30	...	...	...	...
3.30	...	...	...	...	2.30	...	...	...	...
5.00	...	...	...	...	5.15	...	...	...	...
6.00	...	...	...	...					
7.00	...	...	...	...					
7.30	convulsions	occurred							
7.45	40 cc. 10 %	glucose	injected						
8.15	appeared	normal,	eating						
9.00	...	...	...	...					

The batch which caused the convulsions in the above experiment was used in treating hospital cases.

It seemed possible that the activity of poor samples of yeast might be renewed by suitable treatment. It was also possible that a considerable

quantity of the active principle might reach the fermenting liquor as an exudate when the yeast was grown in a suitable medium. Fermentation experiments were carried out using glucose or fructose. Phosphate was added in some experiments in order to increase the rate of fermentation, for it was not impossible that the presence of glucokinase might be associated with the rapid metabolism of carbohydrate which was taking place. The reaction was stopped after different periods by the addition of alcohol. The mixture was then filtered, and the filtrate was made up to 80 % alcohol. The process was completed in the usual manner. Negative results were obtained in every case, confirming the experiment shown above when the yeast had been skimmed at the height of fermentation. We concluded therefore that fermentation was not necessarily associated with the production of increased amounts of glucokinase. Cultivation of the yeast was then attempted under conditions in which the growth and fermentation would be slower. It seemed possible that the presence of glucokinase might be associated with a certain nutritional state of the yeast cell, such as its content of glycogen. The unusual course was adopted of mixing the yeast with potatoes as a source of nutrition; it was hoped that by not supplying the yeast with preformed reducing sugar some other process possibly associated with the production of glucokinase might be brought into prominence. In case potatoes might also be a source of glucokinase, uncooked potatoes were extracted with alcohol and the usual white powder obtained. No effect was observed when this was injected into a normal rabbit. For the cultivation of the yeast the potatoes were boiled, mashed, cooled and mixed with water before the addition of the yeast. In these experiments as little water as possible was added in order to economise alcohol, but sufficient to make the mixture liquid.

Experiment. 500 g. of yeast were added to 500 g. of potatoes in 700 cc. water and well mixed. The mixture was placed in an incubator at 26° and allowed to remain 24 hours. A similar experiment was carried out with the addition of 20 g. di-sodium hydrogen phosphate. At the end of the period of incubation the action was stopped by the addition of alcohol and the process completed as before. The products obtained were tested by injection into rabbits with the following results.

Rabbit 3 kilos.					Rabbit 2.2 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.15	...	...	...	...	10.15	...	...	...	...
10.30	injected (+ phosphate)	...	...	...	10.45	injected (no phosphate)	...	...	...
12.30	...	...	...	...	12.45	...	...	...	...
2.30	...	...	...	...	2.40	...	...	...	...
3.45	...	...	...	...	4.00	...	...	...	...
5.30	...	...	...	...	6.00	...	...	...	...
				·13					·14
				·09					·13
				·10					·12
				·12					·14
				·14					·13

The original yeast as received was almost inactive. The result of similar experiments has shown that the addition of phosphate increased the yield of glucokinase. A sample of French yeast was divided into two parts. The first

was treated by the ordinary method, the other was cultivated with potatoes in the presence of phosphate. The extracts had the following activity.

Rabbit 1.7 kilos.					Rabbit 1.9 kilos.				
Time				Blood sugar %	Time				Blood sugar %
10.00	...	...	...	...	10.15	...	...	...	...
10.15	injected	(+ phosphate)	...	...	10.30	injected	(original yeast)	...	...
11.00	...	...	...	...	12.00	...	...	...	...
12.20	...	...	...	...	2.00	...	...	...	...
12.30	convulsed	...	...	...	4.30	...	...	...	...
12.40	...	...	...	...					
1.00	40 cc. 10 % glucose injected	...	...	...					
1.15		animal normal and eating	...	...					
2.00	...	...	...	...					

The varying effect on different animals is shown in the following experiment in which the sample which convulsed the animal in the foregoing experiment was tested on another rabbit. Only a moderate fall in the blood sugar occurred.

Rabbit 2.1 kilos.					Blood sugar %				
Time									
9.45	...	...	...	...	...	...	...	...	...
10.00	injected	...	...	...	...	...	...	...	...
11.30	...	...	...	...	...	...	...	...	...
12.30	...	...	...	...	...	...	...	...	...
3.00	...	...	...	...	...	...	...	...	...
5.00	...	...	...	...	...	...	...	...	...

These two experiments again emphasise the difference of effect which may occur when the same dose is given to animals of the same species, and this must always be a difficulty when tests of substances depend on their action on different animals.

In the above experiments very little grinding of the yeast was possible owing to the admixture with potatoes. It is probable that only very imperfect extraction of the cells by the alcohol took place, as it is well known that substances are extracted from cells only with great difficulty unless the cell membrane is thoroughly disintegrated. The active principle obtained in the above experiments may have been mainly derived from that which had already passed from the cells into the surrounding liquor during fermentation. Bacterial action cannot, of course, be excluded, but the fact that the temperature of incubation was 26° and that incubation was only continued for one day renders it likely that the glucokinase arose from the dominant organism, that is the yeast. Experiments were then carried out to determine whether rupture of the cell membrane would increase the yield of glucokinase. The yeast was mixed with potatoes as before, with and without phosphate. The mixture was then incubated at 26° for one day, and afterwards placed in the cold room at -19° until thoroughly frozen. It was rapidly thawed under alcohol as before. No increase of activity resulted. The above series of experiments tended to show that the presence of glucokinase was not associated with ordinary growth or fermentation. Similar experiments in which the

mixture was maintained at different temperatures, such as 10° and 15°, gave negative results.

Yeast was allowed to autolyse at 45° for two days. The liquid was then treated in the usual manner. There was no evidence of the presence of glucokinase as a result of this variation. This was to be expected since insulin is destroyed by trypsin and pepsin [Dudley, 1923]. Although glucokinase may not be identical with insulin, it is reasonable to expect that its behaviour towards proteolytic enzymes would be similar. Some of the same yeast was dried in a current of warm air. It was then extracted with alcohol. The product obtained was without action on the blood sugar of a rabbit.

It was possible that rapid aeration might influence the formation of glucokinase. Yeast was mixed with an equal weight of potatoes to which phosphate was added. Enough water was then added to make the mass sufficiently liquid for aeration. A rapid current of air was drawn through the liquid overnight, the liquid being maintained at 26°. A sample of the same yeast was treated in a similar manner but without aeration. The results obtained from the two samples were as follows.

Rabbit 2.1 kilos.					Rabbit 1.8 kilos.				
				Blood sugar					Blood sugar
Time				%	Time				%
10.20	...	...	...	·14	10.30	...	...	...	·13
10.45	injected (without aeration)				11.00	injected (with aeration)			
12.30	...	...	...	·10	12.45	...	...	...	·13
2.00	...	...	...	·10	2.15	...	...	...	·12
4.30	...	...	...	·13	4.45	...	...	...	·13

Since the original yeast had a small content of glucokinase which was increased by incubation of the yeast with potatoes in the presence of phosphate, it might be concluded that the effect of aeration is to destroy any glucokinase which may originally be present. The destruction may be caused by oxidation, or it may be that the metabolism of yeast in the presence of excess of oxygen is altered, and carried out on different lines, which do not necessitate the formation of glucokinase. Since the precise conditions under which glucokinase is formed by the cells, and the effect of glucokinase on carbohydrate metabolism is unknown, it is impossible to come to any conclusion on this point at the present time. The result may also be explained on the basis that the responsible organism was not a yeast but some other type (possibly anaerobic) which was suppressed by aeration.

The foregoing experiments on the effect of fermentation, mixing with potatoes, temperature, and drying, were all made with yeast from the same distillery, which if extracted without additional treatment gave only small amounts of glucokinase. The results showed that the yield could not be increased to a marked extent: the only factor which appeared to have any influence was the presence or absence of phosphates, as seen in the cultivation experiments with potatoes. When phosphate was added the extract was sufficiently potent to cause convulsions in a rabbit. The similar treatment without phosphates gave no increase of activity. This fact merely serves to

emphasise the importance of phosphate in questions connected with carbohydrate metabolism, but the mechanism at work in the increased production of glucokinin is unknown. Harden and Young [1908] showed that the rate of alcoholic fermentation was increased by the addition of phosphate due to increased formation of hexosephosphoric acid being possible. Since rapid fermentation had no effect on the yield of glucokinin, the increased formation of hexosephosphate would appear to be unconnected with the formation of the active principle. It is possible that the effect of phosphates may not be the same on different yeasts as regards their glucokinin content, and that the effect may vary with other cultural conditions.

It seemed probable that samples of commercial yeast from different sources might vary in their content of glucokinin. Samples were obtained from different distilleries and tested. Further samples were obtained at intervals, and it was found that in most cases the yield of glucokinin was small, but that in one case there was constant activity, which however was not so great as that of the yeast used in our first experiments. It was possible that there might be some factor of nutrition which was only present in the process employed in this distillery, or that there might be present a small amount of a certain strain of yeast which was almost the sole origin of the glucokinin.

Experiments have been carried out on these lines, with the result that a strain of yeast has been isolated, which gives a yield of glucokinin greater than that ever obtained previously. This property seems to be maintained, as successive batches have continued to prove active.

An investigation into possible means of increasing the yield of glucokinin from such yeast, and on the factors which lead to its formation, is in progress. The results will be given in Part II of this paper.

DISCUSSION.

The fact that an extract of yeast causes a fall in the blood sugar of normal animals affords no proof that it contains an anti-diabetic hormone. In those experiments in which only a small fall of blood sugar occurred, it would be still more difficult to maintain that it was due to a hormone of this type, and not to the large amount of other substances which are also present in the extract, such as breakdown products of proteins. For this reason we have refrained from injecting large quantities of yeast extract, and from continuing the blood sugar determinations for longer than nine hours after the injection. The effect of starvation might possibly affect the condition of the animal to such a degree that the effect of the foreign substances, other than glucokinin, in the injection might begin to manifest themselves. We have emphasised the delayed action of glucokinin as compared with insulin, but this delay (as measured by the time when convulsions occur) we have never found to be more than eight hours.

In a recent paper Collip [1923, 2] gives some results of injecting an extract of yeast into normal animals. His results appear to be very different from those

which we have obtained. He uses several different processes (among them freezing) in obtaining his extract, but the action on the blood sugar appears to have been extremely delayed. No injection figures are given, but from the fact that one animal received nine injections in the course of twenty hours and another six in fourteen hours, it would appear that very large quantities of extract were administered. During the whole of this time the animal was without food. In the cases in which convulsions are recorded the animals died. It is necessary to emphasise here that in every case in which convulsions have occurred we have found complete recovery of the animal to ensue after injection of glucose. It would seem therefore that the anti-diabetic hormone which Collip claims was present in his extracts must be of a totally different type from the one present in our extracts, or that his extracts were so weak that vast quantities were necessary to obtain an effect. In this case the quantities of other substances present would probably have an injurious effect on the animal after more than a day's starvation with repeated withdrawals of blood. The Schaffer and Hartmann [1920] estimation of blood sugar was used by Collip. This method requires 1 cc. of blood for an estimation. We have always used Bang's old method, because of the small quantity of blood that is required, and the ease of obtaining it on an absorbent paper without serious inconvenience to the animal.

There appears to be little doubt that by using a suitable yeast a genuine anti-diabetic hormone can be obtained. It has been found [Winter and Smith, 1923, 2] that following administration of yeast extracts to diabetic patients, excretion of sugar is stopped, and the ratio copper reducing power to polarimetric value of the blood sugar is altered in the same way as after injections of insulin [Forrest, Smith and Winter, 1923].

MacLeod [1923] has suggested that such extracts may merely stimulate the secretion of insulin by the pancreas when injected into normal animals. The results with diabetic cases [Winter and Smith, 1923, 2] would appear to show that just as beneficial an effect may occur as when pancreatic insulin is used; and that therefore, in our opinion, the evidence is strongly in favour of a true anti-diabetic hormone being obtainable from suitable yeasts.

SUMMARY.

1. It is shown that a substance may be extracted from yeast which has an effect similar to that of insulin on the blood sugar of normal animals.
2. When convulsions occur as the result of the injection of this substance, the animals may be recovered by injections of glucose.
3. Samples of yeast obtained from different sources yield varying amounts of glucokinin.
4. By allowing the yeast to ferment under different conditions, it is found that only in the case of an originally active yeast is the glucokinin formation increased under certain cultural conditions.

We wish to express our grateful thanks to Professor F. G. Hopkins for his continued interest in this work, and for providing the large amount of material used in this necessarily expensive research.

Our thanks are due to Messrs D. and D. F. Scanlan for much help during the course of this work, to Dr H. H. Dale for keeping us informed of the progress of the work on insulin which has been carried on in his laboratory, to Messrs Bishop and Brooke for obtaining for us regular supplies of yeast from different sources, and to Messrs H. and G. Simonds, Reading, for giving us every help in the investigation of brewer's yeast.

One of us (W. S.) is indebted to the Scientific and Industrial Research Board, and one (L. B. W.) to the Medical Research Council for grants held during the course of this research.

Fig. 1. Curves of blood sugar values of rabbits: I, after insulin (pancreatic); II, after yeast extract. At A the injection was given. The thick lines denote the period during which convulsions occurred. At the end of this period the animals were recovered by injections of glucose. The delayed action of the yeast extract is shown. Time in hours.

REFERENCES.

- Bang (1913). *Der Blutzucker*, Wiesbaden.
 Collip (1922). *Trans. Roy. Soc. Canada*.
 — (1923, 1). *Proc. Soc. Exp. Biol. Med.* **20**, 321.
 — (1923, 2). *J. Biol. Chem.* **56**, 513.
 Dudley (1923). *Biochem. J.* **17**, 376.
 Forrest, Smith and Winter (1923). *J. Physiol.* **57**, 224.
 Funk and Corbitt (1923). *Proc. Soc. Exp. Biol. Med.* **20**, 422.
 Harden and Young (1908). *Proc. Roy. Soc. Lond. B.* **80**, 299.
 MacLeod (1923). Address to International Congress, Edinburgh.
 Schaffer and Hartman (1920). *J. Biol. Chem.* **45**, 365.
 Winter and Smith (1923, 1). *J. Physiol.* **57**; *Proc.* xl. Feb. 17th.
 — (1923, 2). *Brit. Med. J.* **1**, 711.