42. The Detection of Insulin in Urine

By Margery Cutting,* *From the Biochemical Department, King's College Hospital, London and Horton Emergency Hospital, Epsom*

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The presence or absence of insulin in the urine of either normal or diabetic persons is still a matter of dispute, for the findings of the various investigators are conflicting. Best & Scott [1923] claimed that insulin was eliminated by the kidneys under certain conditions. They found that the equivalent of 800 ml. of normal urine, when injected into a depancreatized dog, depressed the blood sugar considerably over a long period, but they were unable to recover any insulin from the urine of diabetic patients unless they were having insulin treatment. Partos [1929] cited evidence that insulin was a normal component of both human and rabbit urines though he could not detect it in urine from diabetic patients. Weiler *et al.* [1934] have confirmed these findings and Kozuka [1927] isolated a blood sugar-depressing substance (which he believed to be insulin) from normal urine but could not extract it from diabetic urine unless the patient was receiving insulin and not excreting sugar. Brugsch & Horsters [1930] found an insulin-like substance in the urine of both normal and diabetic subjects.

In contradiction to the above series of findings Lawrence *et al.* [1930] and Uberrack & Zell [1931] were unable to recover any insulin from the urine of either normal or diabetic persons, although they employed the same method as Partos. Fisher & Noble [1923] were also unable to detect any insulin in normal urine or the urine of diabetic patients (excepting those who had been treated with insulin), though they demonstrated that the kidney was capable of excreting it by injecting large amounts (40 units) into the circulation of normal dogs under veronal anesthesia and recovering almost the entire quantity from the urine. Bruger & Friedman [1938] have performed similar experiments on rabbits and their results are in agreement with those of Fisher & Noble, as also are those of Goadby & Richardson [1940] who injected 40 units of insulin into rabbits and later withdrew samples of blood at varying time intervals. This blood when injected into another fasting rabbit was found to depress the blood sugar. They considered that this action was due to the injected insulin and found that the hypoglycaemic activity gradually disappeared, being absent after 90 min., although when the kidneys were excluded from the circulation the activity lasted much longer.

These conflicting opinions may well be due to the fact that only a few of the workers have investigated sufficiently critically the methods they have employed to detect the presence of insulin in urine and in only one instance (Goadby & Richardson) do experiments appear to have been carried out to determine the smallest amount of insulin that could be detected by the method adopted. The present paper describes attempts made to find out under what conditions insulin can be recovered from urine and how far such methods can be made quantitative.

EXPERIMENTAL

Detection of hypoglycaemic activity in extracts

A rabbit that had been starved for at least 18 hr. was removed to a box in the laboratory, where it was allowed to rest for 45 min. before the experiment was commenced. The usual precautions were taken to avoid fluctuations of the blood sugar as far as possible.

* Halley Stewart Research Fellow during the early part of this work.

Two 0.1 ml. samples of blood were collected from the marginal ear vein and a known volume of the extract under investigation was injected intravenously. Further 0.1 ml. samples of blood were collected at 10, 20, 30, 45, 60, 75 and 90 min. intervals after the injection and the blood sugars subsequently estimated by the method of Hagedorn & Jensen [1923].

The blood sugar curves given in this paper represent the average response of three or more rabbits. Unfortunately, owing to the war, the number of animals available for this work has been rather limited. If the response to any important preparation was being investigated or if the results were indecisive more rabbits were used. The encircled figure adjoining each curve denotes the number of animals employed.

The smallest quantity of insulin that will give a satisfactory depression curve was found to be 0.2 unit. The response in this case was, on the average, a depression of 25 mg./100 ml. (min. 18, max. 36) in 20 or 30 min. from the time of injection, followed

Fig. 1. Curve A. Blood sugar curve resulting after injection of 0.2 unit insulin directly. Curve B. Blood sugar curve resulting after injection of 5 ml. of 10 ml. extract prepared from normal urine. 0.4 unit of insulin was added to the 10 ml. extract.

by a return to fasting level in 90 min. The average response of 7 rabbits is shown in Fig. 1, curve A. Smaller quantities of insulin do not produce satisfactory results, it being unwise to attach much significance to a fall in blood sugar of less than 20 mg./100 ml., as the fasting level may well vary within ± 10 mg. during the 90 min. of the experiment, even when no injection has been made. All the results have been compared with this curve.

Preparation of extracts for injection

Since the presence of insulin in urine is uncertain, in all preliminary experiments small quantities of the crystalline material were added to the urine before commencing the preparation of the extract.

Direct injection of urine. 10 ml. samples of urine (containing 2–10 units insulin per 100 ml.) were injected directly into a number of rabbits. This method was found to be unsatisfactory and was abandoned because the toxic substances present produced an initial rise in blood sugar. The rise was usually followed by a depression to a figure below that of the fasting level. The significance of this fall after the preliminary rise was uncertain and it was deemed that the method would not be a very satisfactory one for detecting small amounts of insulin.

Alcoholic extraction. Insulin is soluble in 80 % aqueous alcohol [Collip, 1923] and this fact was used by Partos [1929] as the basis of his method for extracting it from urine. In

the present investigation extracts were prepared by this method from 100 ml. of urine to which 10 units of insulin had been added. When the entire extracts were injected they produced a marked fall in the blood sugar of the experimental rabbits, but when smaller quantities of the extract were injected it became apparent that only a portion of the insulin had been recovered.

Precipitation of the insulin with ammonium sulphate. Doisy *et al.* [1923] showed that insulin could be fairly completely precipitated from aqueous solution by half-saturation with ammonium sulphate and they made use of this fact in their method for isolating it from fresh pancreas. Fisher & Noble [1923] employed this principle for extracting insulin from urine, but unfortunately their paper does not include any details of technique. This type of method has one considerable advantage in that glucose is not adsorbed on to the precipitate when extracts are prepared from diabetic urines containing sugar. If, however, an alcoholic extraction process such as that of Partos is employed with a urine containing sugar, the sugar is extracted as well and must be removed by fermentation or other means before the fluid can be tested for its power to lower the blood sugar. To assist the precipitation of small amounts of insulin the author added a small quantity of freshly collected rabbit's plasma to the urine so that a bulky precipitate formed which it was hoped would carry down the insulin by entrainment.

Experiments on these lines proved successful and became the basis of the procedure finally adopted which is described in detail below.

Detailed description of method

Reagents and apparatus: Ammonium sulphate (crystalline). A.R. HCl. Boric acid buffer of pH 8.0 (prepared by taking 0.62 g. boric acid, 8 ml. *N*/10 NaOH and 65 ml. 0.85% NaCl and diluting to 200 ml.) [modified from Cole, 1933]. Bromocresol green indicator. Lovibond comparator and disk (for bromocresol green, range 3.6–5.2). Rabbit's plasma freshly collected. Hagedorn and Jensen blood sugar reagents.

The urine was first adjusted to pH 5.0 with HCl, using a Lovibond comparator and bromocresol green as indicator. 0.5 ml. of rabbit plasma was added to a 100 ml. sample of urine and this was half-saturated with ammonium sulphate by the addition of 32 g. of the crystalline salt. The mixture was stirred well to bring the salt into solution and the urine was kept in a warm place (about 37°) in order that the resulting precipitate should flocculate.

The precipitate was collected by filtering the urine through a no. 42 Whatman filter paper on a Büchner funnel and allowing to dry (on the filter paper) in a desiccator. This precipitate, which contained the insulin, could be left in the desiccator for at least 4 days without losing its activity. Before assay it was washed off the filter paper with 10 ml. of a borate buffer solution at pH 8.0 and then ground up well with this solution in a mortar. Some of the solid material did not dissolve and was removed in the centrifuge. The supernatant fluid containing the active insulin was injected into the test animals.

The effect of pH on precipitation and elution. The isoelectric point of insulin is near to pH 5.0 [Harrow & Sherwin, 1934]. It was expected therefore, that the optimum pH for precipitation would be in the region of that point and this was found to be so in practice. Fig. 2 shows the curves obtained when the precipitation was carried out at varying pH. In each case the extract was made from 100 ml. of urine to which 2 units of insulin had been added and 5 ml. of the final 10 ml. extract were injected into the rabbit. It will be seen that the response resulted in a smaller depression of the blood sugar as the pH of the urine was lowered.

Elution was effected with a buffer solution at pH 8.0. It was considered that this pH was sufficiently far removed from that at which precipitation had taken place to dissolve the insulin. Satisfactory results were obtained, but acidification of the solution produced

a small precipitate and it seemed that this might be a possible way of removing further impurities. Accordingly nine extracts were prepared from different normal urines and each dissolved in 10 ml. of buffer solution (pH 8.0); 0.4 unit of insulin was added to every

Fig. 2. Blood sugar curves obtained from extracts prepared from urines of differing pH . In each case 2.0 units were added to 100 ml. urine before extracting and 5 ml. of the 10 ml. extract injected. Curve A. pH 5.0. Curve B. pH 4.0. Curve C. pH 3.0.

Fig. 3. Blood sugar curves obtained from extracts of differing pH . 0.4 unit insulin added to 10 ml. extract prepared from 100 ml. urine and 5 ml. of the extract injected in each case. Curve A. pH 8.0. Curve B. pH 4.0. Curve C. pH 3.0.

one and then three were adjusted to pH 4.0, three to pH 3.0 and the remaining three were kept as controls. After these adjustments the extracts were kept for 30 min. and were then centrifuged. 5 ml. of the supernatant fluid were used for assay in each case. The

results, which are given in Fig. 3 show that a better response is obtained without acidification. It is probable that a small amount of insulin is carried down with the precipitate especially at pH 4.0. The extracts were always left on the filter paper in the desiccator until the day of assay and then they were dissolved immediately before injection. In this way any loss that might occur following the dissolving of the insulin in an alkaline solution was eliminated as far as possible.

Quantitative aspects of insulin recovery

Normal urine without added insulin. If Partos and other workers who think as he does are correct, insulin is a normal component of urine and the extent of recovery of added insulin cannot be ascertained without some knowledge of the total amount present in the original specimen. Extracts were prepared in the above manner from many samples of

Fig. 4. Blood sugar curve resulting from extracts prepared from normal urine to which no insulin had been added.

urine collected from normal subjects to which no insulin had been added. In some instances 24-hr. specimens were used and at other times the extract was prepared from a fresh 2- or 3-hr. sample. The blood sugar curve representing the average response of 13 rabbits is shown in Fig. 4. The curves almost invariably showed a small rise during the first 20 min. and in only one instance was there any depression of the blood sugar. From these results it seems unlikely that there is any insulin in normal urine.

Normal urine with added insulin. Small quantities of protein and other substances in the extract besides insulin might alter the response of the rabbit to 0.2 unit insulin, but in practice this was not found to be the case. 0.2 unit was added to half (5 ml.) the extract prepared from 100 ml. normal urine and this was injected into a rabbit. The average response obtained from three such experiments differs little from the effect of injecting 0.2 unit directly (Fig. 1, curves A, B) and either curve can be used for comparison with those resulting from attempts to recover insulin added to urine.

Experiments were now carried out in order to assess the amount of insulin that was recovered by this method when 2 units were added to 100 ml. of urine. Extracts were prepared in the manner described above and the volume of fluid for injection made up to 10 ml. Then 5, 2 and 1 ml. (equivalent to theoretical recoveries of 1, 0.4 and 0.2 unit respectively) were injected into different rabbits. Fig. 5 shows the responses obtained and

if the curve *C* is compared with those in Fig. 1 it is apparent that most of the insulin has been recovered. However, it was hoped that it would be possible to recover quantities smaller than 2 units/100 ml. and so specimens of urine containing 0.5 and 0.25 unit/100 ml. were taken and extracts prepared from them. Half the extract from the former (i.e. equivalent to 0.25 unit) and the entire extract from the latter (made up to 5 ml. only in

Fig. 5. Blood sugar curves resulting after injecting varying quantities of a 10 ml. extract prepared from 100 ml. urine to which 2.0 units insulin had been added. Curve A. 5.0 ml. Curve B. 2.0 ml. Curve C. 1.0 ml.

Fig. 6. Curve A. Blood sugar curve obtained after injecting half extract prepared from 100 ml. normal urine to which 0.5 unit insulin had been added. Curve B. Blood sugar curve obtained after injecting entire extract prepared from 100 ml. urine to which 0.25 unit insulin had been added.

this case) were used for assay. Thus, in theory, each should contain the same amount of insulin. The results are shown in Fig. 6 and it will be seen that the two curves are very similar, the response in the first case being a maximum depression of 28 mg. and in the second case 26 mg., reached in 30 and 20 min. respectively. Again, these two curves are very similar to those shown in Fig. 1 and suggest that most if not all, of the insulin is recovered by this technique and, furthermore, that it is possible to detect the presence of insulin in urine, even when it is there in quantities as small as 0.25 unit/100 ml.

Stability of insulin in urine

In all the experiments so far described, the extracts have been prepared immediately after the addition of insulin to the urine. When collecting specimens from human subjects it is not always possible to commence the extraction immediately after the specimen has been voided and this is, of course, particularly the case when 24 hr. specimens have to be examined for the presence of insulin. The question arises as to whether the insulin becomes inactivated on long standing in urine. If this were so it would be a possible explanation of the varying results obtained by the earlier workers.

This possibility has been examined in the following ways. Several 24 hr. specimens were collected by different normal subjects but each specimen that was passed was halved immediately. To one half insulin was added straight away (the amount varied from 0.5 to 2 unit/100 ml. in the different collections). No insulin was added to the other half, which was used as a control. Extracts were prepared from both portions as soon as the 24 hr. collection had been completed. When half the extract from the control was injected the blood sugar did not fall, whereas when the other extract was investigated by employing an amount for injection that would theoretically contain 0.25 unit, the rabbit's blood sugar showed on the average a fall of 25 mg. within 30 min. (see Fig. 7, curve A, which shows a typical response) indicating that no apparent loss of insulin occurs during the collection of a 24 hr. sample when this is analysed immediately. Some of these specimens were left in the ice chest for 5-7 days and then a second extract prepared, of which the same quantity was used for injection. Fig. 7 shows the result of one such experiment. In this case the original specimens contained 0.5 unit/100 ml., and the extracts were made up to 10 ml., of which 5 ml. were used for injection. Curve A shows the effect when the extract was prepared immediately, whereas curve B shows the response that is obtained if the specimen is kept in the ice chest for 5 days before commencing the extraction and it is apparent that some loss has taken place.

In other experiments in which varying quantities of insulin (0.2-2 units/100 ml.) have been added to specimens of normal urine, which have been kept for 24 hr. at room temperature before commencing the extraction of insulin, there does not appear to be any measurable loss of activity. This is probably the maximum length of time that it is safe to leave the specimen before commencing the ammonium sulphate precipitation and subsequent extraction of insulin.

Finally there is a possibility that insulin may be destroyed in the urine while in the bladder, where the higher temperature might lessen its stability. Accordingly specimens

Fig. 7. Curve A. Blood sugar curve resulting from injection of 5 ml. of 10 ml. extract prepared from 24 hourly specimen of urine containing 0.5 unit insulin per 100 ml. Curve B. Response after injecting 5 ml. of 10 ml. extract prepared from the same urine 5 days later.

of urine containing 2 units/100 ml. were incubated at 37° for 1 hr. before commencing the extraction. Assays were made and showed that there is little likelihood of any loss due to this cause *in vivo*, as curves identical with those shown in Fig. 5 were obtained.

CONCLUSIONS

The experiments described above have shown that it is quite possible to detect the presence of 0.25 unit of insulin in 100 ml. urine even 24 hr. after it has been voided. On the other hand when extracts were prepared from normal urine to which no insulin had been added and injected into fasting rabbits, no fall in blood sugar resulted. This suggests that insulin is not a component of normal urine, or if it is, must be present in quantities smaller than 0.25 unit/100 ml.

Up to the present it has not been possible to investigate the urine of either untreated diabetic persons, or diabetics receiving insulin, excepting one patient who was very resistant to insulin therapy and was having 240 units of insulin per day. Two 24 hr. specimens were collected from this patient and examined in the manner described above but no insulin was detected in either. It is not possible to draw any conclusions from this one case but it is hoped it will be possible to carry out more work along these lines and make this the subject of a future communication.

SUMMARY

1. A method for detecting the presence of insulin in urine has been described and investigated.
2. It has been shown that it is possible to detect the presence of 0.25 unit insulin per 100 ml. when this is added *in vitro*.
3. Insulin added to urine could be recovered 24 hr. later without apparent loss of activity, nor was the activity destroyed by incubating at 37° for 1 hr.
4. No insulin could be detected in normal urine and, if present, must be there in amounts less than 0.25 unit/100 ml.

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