MATERIALS AND METHODS
1. The subjects for the work were selected from rabbits. Ten male rabbits were selected with weights, 1325 gms., 1437 gms., 1413 gms., 1586 gms., 1529 gms., 1665 gms., 1604 gms., 1648 gms., 1382 gms. and 1696 gms. These were kept on wet grams, 50 gms. to each rabbit with fresh grass cleansed throughly with boiled distilled water and water ad lib. Their weights were recorded daily for 10 days and the weights were found to remain more or less constant. Experiments were next started. Fasting blood glucose values were noted for 3 days successively and mean values were recorded.

2. Glucose tolerance test was performed on the ten normal rabbits by feeding 1.5 gr. of glucose dissolved in 10 ml. of distilled water by means of a rubber tube fitted with a 10 ml. syringe. Fasting blood glucose value as well as blood glucose values every half an hour up to two hours following the oral administration of glucose solution were carefully noted. The experiments were done in triplicate and the mean values recorded.

3. These normal ten rabbits were now alloxenised by successive injections of alloxan monohydrate (E. Merck Ag. Darmstadt.) 2% in doses of 2 ml., 4 ml. and 6 ml. given intramuscularly. The weights were recorded daily. Determinations of blood glucose were made in fasting conditions prior to alloxan injections and 24 hours after the injections. Each dose was injected for
three successive days and the mean values of fasting blood glucose before and after the injections were noted. By these injections a diabetic state was produced as evidenced from the degree of hyperglycaemia and the weight loss and presence of glucose and acetone in the urine.

4. Glucose tolerance tests were also performed on six pre-diabetic rabbits. These rabbits were the offspring of one male and one female rabbit whose fasting blood glucose values were taken and found to be within the normal range. These were made diabetic by injections of alloxan monohydrate 2% solution (E. Merck AG, Darmstadt) in doses of 2 ml., 4 ml., and 6 ml., each dose being given for three successive days intramuscularly. Their weights were carefully recorded daily and the fasting blood glucose values were taken after 24 hours of the injections. The weight loss and the degrees of hyperglycaemia as well as presence of glucose and acetone in the urine confirmed the establishment of the diabetic state. These alloxanised diabetic male and female rabbits were then mated and kept in different cages. Similarly, another pair of male and female rabbits were also similarly alloxanised and mated and kept in different cages. The female rabbit of the first pair gave birth to three male and two female rabbits of weights 28.7 gm., 37.9 gm., 37 gm., 30.5 gm. and 34.5 gm. respectively. Of these five, two female rabbits died within two hours of birth,
IMMATURE FOETUS OF RABBIT IN HYPERGLYCEMIC CONDITION AFTER INJECTION OF ALLOXAN MONOHYDRATE.
one of which is preserved for further study. The three male issues gradually developed into adult male rabbits. After the birth and death of the two female issues, the three male issues were put on to the breast for feeding. Breast feeding was done two times daily. This was continued for three weeks when they were segregated from the mother and put on to 5 to 10 gms. of wet grams and grasses thoroughly cleansed with boiling water and water ad lib. Gradually as they developed, amount of wet grams with grasses is also increased from 10 gms. to 50 gms. daily. The same procedure was adopted for the second pair. The female rabbit of the second pair gave birth to three male rabbits of weights 28.7 gms., 32 gms. and 34.5 gms. respectively. Three male rabbits of the first pair and three male rabbits of the second pair i.e. total six rabbits have been utilised for the present study. As these six rabbits have been obtained from alloxanised diabetic parents, these were utilised as pre-diabetic rabbits. Their weights were carefully recorded.

5. Glucose tolerance tests were done on six male pre-diabetic rabbits obtained as stated above in the following manner. 1.5 gm. of glucose dissolved in 10 ml. of distilled water were given orally after the fasting blood glucose values were recorded. Estimations of blood glucose were done for the next \( \frac{1}{2} \) hour, 1 hour, \( 1 \frac{1}{2} \) hour and 2 hours. The experiments were done in triplicate and their mean values were recorded.
6. Glucose tolerance tests were similarly performed in ten male alloxanized (diabetic) rabbits by feeding each 1.5 gm. of glucose dissolved in 10 ml. of distilled water. The fasting blood glucose values were recorded prior to the oral feeding. Estimations of blood glucose were done every half an hour for two hours. Experiments were done in triplicate and their mean values were as recorded.

7. Urine was collected in the three series, normal, pre-diabetic and alloxanized (diabetic) by means of a narrow bore sterilised rubber catheter and a qualitative study made. The qualitative study involved detection of albumin, acetone and glucose.

8. Histopathological Study:

(a) Two normal male rabbits of weights 1432 gms. and 1510 gms. were taken, kept in a separate cage on wet grass with grass and water ad lib. For one week, daily estimation of fasting blood glucose value was made. These were found to be within the normal range. They were then sacrificed on the 8th day by decapitation and immediately the eyeballs were taken out and kept in 10% formol saline solution. The following tissues were collected viz. liver, kidney, adrenal and pancreas and kept in 10% formol saline solution. The paraffin blocks were prepared of the different tissues and section made in 5 μ and stained suitably. After 24 hours the eyeball was taken
out from the formal saline and after removal of the lens, cornea, aqueous and vitreous humour, the three coats of the eye ball were cut into small segments. These were divided into ten segments. Five of these were kept for paraffin blocks and the remainder five were utilised for Trypsin Digest Study. Sections were made from the blocks (5 μ) and stained suitably.

(b) Similar procedures were adopted in the case of pre-diabetic and alloxenised (diabetic) rabbits. The tissues were collected and the eye balls were similarly treated.

9. Trypsin Digest Study (Kuwabara, T. and Cogan, D.G., 1960) -

The eyes of the three series of normal, pre-diabetic and alloxenised diabetic rabbits are fixed in 10% formal saline and kept over night. After 24 hours, these were taken out and washed with distilled water and the retina is cut into segments and placed in a glass jar and washed over night in running tap water. Next morning these were then incubated at 37°C in a solution of 3% Trypsin (Difco) and 0.1M Tris buffer for 6 hours and washed for any cloudiness to develop. When the media became cloudy the segments were transferred to water. The internal limiting membrane was peeled off and the specimens were gently shaken to free network of retinal vessels. These were then washed in distilled water and mounted on a clear slide and allowed to dry. After this, these were stained with
P.A.S. and Haematoxylin as well as P.A.S. and 1% Toluidine blue, dehydrated and cleared in xylol and mounted in Canada Balsam.

10. Chemical Methods:

(A) Determination of Blood Sugar by the method of Hagedorn and Jensen (1923)

Procedure: Into a test tube (15 x 150 mm.) 1 ml. of 0.1 N NaOH and 5 ml. of 0.45% Zinc Sulphate solution carefully placed with the help of two pipettes. A gelatinous precipitate of Zinc hydroxide formed. 0.1 ml. of blood is taken from the marginal ear vein of the rabbit. With the help of a capillary pipette and placed inside the mixture, the pipette being washed out twice with the mixture and blown empty. The tube was placed now in a boiling water bath for three minutes. It was allowed to cool and then filtered on a funnel 4 cm. diameter which had been tipped with a small filter of washed moistened cotton gently placed into the funnel and filtered into a clean test tube (30 x 90 mm.). The funnel was washed with 3 ml. portions of distilled water twice. The filtrate was absolutely clear. It was then treated with 2 ml. of alkaline potassium ferricyanide solution and placed in a boiling water bath for 15 minutes. The tube was then cooled down and 3 ml. of iodide-sulphate solution and 2 ml. of 3% acetic acid solution added to it. The mixture was now titrated
with 0.005 N sodium thiosulphate from a microburette using as an indicator two drops of 1% solution of soluble starch in saturated sodium chloride solution. The point at which the blue colour just disappeared was the end point when the burette reading was taken. Similar procedure was carried out separately taking all the reagents as stated above and adding 0.1 ml. of distilled water as blank. The glucose value of the blank was subtracted from the glucose value of the blood and the actual figure of blood glucose was obtained. For every estimation the whole procedure was repeated for 3 times to get the mean value of blood glucose in mgms%.

Reagents:

(1) 0.45% Zinc Sulphate Solution - 45 gms. of Zinc sulphate (E. Merck) were dissolved in 100 ml. of distilled water and kept in a stock. 1 ml. of this solution was diluted to 100 ml. in a 100 ml. measuring flask. 1 ml. of this solution was diluted upto 100 ml. measuring flask to obtain 0.45% Zinc sulphate solution.

(2) 0.1 N NaOH - 40 gms. of NaOH Crystal (B.D.H. Analar grade) dissolved in a litre of cooled boiled distilled water in a litre measuring flask and the stopper kept tightly fitted. 10 ml. of this solution was diluted upto 100 ml. in a 100 ml. measuring flask to obtain 0.1 N NaOH.
(3) Alkaline Ferricyanide Solution - 1.65 gm. of potassium ferricyanide and fused sodium carbonate 10.6 gm. were dissolved in 100 ml. of distilled water and kept in a blue coloured file to protect from light.

(4) Iodide – Sulphate – Chloride Solution - Potassium iodide (E.Merck) 5 gms, Zinc sulphate (E.Merck) 10 gms, sodium chloride (E.Merck) 50 gms. were dissolved in distilled water and the volume made upto 200 ml. The solution was filtered through thick filter paper.

(5) Acetic Acid Solution - 3 ml. of iron free acetic acid (B.D.H. Analar grade) added to distilled water and made upto 100 ml.

(6) Starch solution - 1 gm. of soluble starch (B.D.H. Analar grade) dissolved in 100 ml. of saturated sodium chloride solution.

(7) Sodium Thiosulphate solution - 0.7 gm. of sodium thiosulphate (E.Merck) dissolved in distilled water and the volume made upto 500 ml.

(8) 0.005 N Potassium Iodate - 0.3566 gm. of Potassium iodate (E.Merck) water free dissolved in water and the volume made upto 2000 ml.

(B) Qualitative Analysis of the Urine:

Urine was collected in all the three series of rabbits, normal pre-diabetic and alloxenised (diabetic) groups by means
of a narrow bore of sterilised rubber catheter end examined for albumin, acetone and glucose.

(1) Albumin - Heller's Nitric Acid Test.

5 ml. of concentrated nitric acid is placed in a test tube and by means of a pipette a few drops of urine allowed to flow down slowly by the side of the tube and any white zone of precipitate at the zone of contact was carefully observed for the presence of albumin, waiting for 5 minutes in individual case. To eliminate the chance of uric acid or urates producing white ring in all the cases showing the white ring urine was diluted four times and then the test repeated. If the white fluffy ring now appeared it was taken to denote the positive presence of albumin.

(2) Glucose - Benedict's Test.

5 ml. of Benedict's reagent was taken in a test tube and 8 drops of urine added to it, thoroughly mixed and then kept in a boiling water bath for 5 minutes and then removed and allowed to cool in the air and development of the precipitate green, yellow or red in colour was carefully observed when the positive presence of glucose was noted. In the absence of any of the above precipitates and the solution remaining perfectly clear with the colour of the reagent, it was taken as negative.

(3) Acetone - Legal's Test.

To 3 ml. of urine 5 drops of freshly prepared 5% sodium nitroprusside solution added and mixed up carefully concentrated
Ammonium hydroxide added down the side of the test tube to form a layer over the sample. A purple ring at the zone of contact was taken as indication of the presence of acetone bodies. In the absence of any such ring, it was taken to be negative.

11) Histopathological Staining Technique:

(a) Haematoxylin and Eosin Stain:

Staining Technique:

(1) The sections of 5 μ obtained from the paraffin blocks of the tissues, liver, kidney, adrenal and pancreas were treated with xylol for 1 minute and noting whether the paraffin was dissolved and the sections were transparent. In some cases, the sections if cloudy were treated for the second time with xylol for another 1 minute to remove the last trace of paraffin.

(2) The deparaffinised sections were treated with absolute alcohol for 1 minute and then successively with 90%, 75%, 50% Alcohol for ½ min. with each grade of alcohol.

(3) The sections were now brought to water by treating with distilled water.

(4) The sections were now stained with haematoxylin (Ehrlich) for 5 to 7 minutes and then washed with freely flowing tap water.

(5) The sections were allowed to dry and then observed under the microscope to note the prominence of the nuclei of the cells. In case of excess of haematoxylin, the sections were
treated with acid alcohol for 2 to 3 seconds and then washed under tap water and observed under the microscope when the nuclei appeared very prominent. These were now treated with 50%, 75% and 95% alcohol for half minute in each and stained with alcoholic eosin for 2 minutes.

(6) The sections were now treated with absolute alcohol for half minute to remove the excess eosin.

(7) The sections were now placed in cloves oil for 1 minute.

(8) These were finally transferred to xylol for 1 minute.

(9) Canada Balsam was put on a cover slip which was inverted and carefully placed over the stained sections.

Staining Reagents:

1. Harlich's Alum Haematoxylin stain 2%

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxylin</td>
<td>6 gms.</td>
</tr>
<tr>
<td>Absolute alcohol</td>
<td>300 ml.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>300 ml.</td>
</tr>
<tr>
<td>Glycerol</td>
<td>300 ml.</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>30 ml.</td>
</tr>
<tr>
<td>Potassium alum</td>
<td>in excess</td>
</tr>
</tbody>
</table>

Put in a bottle and exposed to sun for maturation.
2. Alcohol Eosin stain 1/2

Spirit soluble eosin
Alcohol 96%

Put in a well stoppered bottle.

3. Acid Alcohol.

Alcohol 96%
Distilled water
Conc. Hydrochloric acid

(b) P.A.S. and Haematoxylin Stain.

Staining Technique:

1. The sections were brought to water
2. These were oxidised for 5 to 10 minutes in 1% aqueous periodic acid.
3. These were then washed in running tap water for 5 minutes and rinsed with distilled water.
4. The sections were treated with Schiff reagent for 30 minutes.
5. After this, these were transferred directly to first sulphite rinse for 1 minute.
6. Again transferred directly to the second sulphite rinse for 2 minutes.
7. Next transferred directly to the third sulphite rinse for 2 minutes.
8. These were washed for 10 minutes in running tap water.
9. Counterstained with haematoxylin 2%.
10. Dehydrated, cleared and mounted in Canada balsam.
Staining Reagents:

Barger and De Lamerè's Schiff reagent.

1. 1 gm. of basic fuchsin was dissolved in 400 ml. of distilled water with occasional heating.

2. 1 ml. of thionyl chloride added and the flask was stoppered and after shaking, allowed to stand for 12 hours.

3. 2 gms. of activated charcoal added and shaken and filtered immediately and placed in a refrigerator.

(C) P.A.S. and Toluidine Blue stain.

Staining Technique.

1. The sections were brought to water.

2. These were oxidised for 5 to 10 minutes in 1% aqueous periodic acid.

3. These were then washed in running tap water for 5 minutes and rinsed with distilled water.

4. The sections were treated with Schiff reagent for 30 minutes.

5. After this, these were transferred directly to first sulphite rinse for 1 minute.

6. Again transferred directly to the second sulphite rinse for 2 minutes.

7. Next transferred directly to the third sulphite rinse for 2 minutes.

8. These were washed for 10 minutes in running tap water.
9. Counterstained with Toluidine blue 1%.
10. Dehydrated, cleared and mounted in Canada balsam.

Staining Reagents:

1 gm. of Toluidine Blue (Toluidin bleu 0,E,Merck, Ag. Darmstadt) dissolved in 100 ml. of distilled water and filtered.

12). Sub-total Pancreatectomy:

Two rabbits of weights 1440 gms. and 1435 gms. were taken and maintained on a diet of 50 gms. of wet grams and grass cleansed thoroughly in boiled water with water ad lib. The fasting blood glucose values were estimated daily for 10 days and the mean values were recorded. The weights were taken daily and the rabbits maintained a more or less constant weight with the diet as given above. The fasting blood glucose values were within the normal range (75 to 80 mgs%).

Operation Technique:

The rabbits were anaesthetised with ether and a mid-line incision given. The coils of the intestine were removed gently and the stomach lifted up. The pancreas was visible and about 3/4th of this was removed from left side. The vessels were sutured up and complete haemostasis was ensured. The abdominal cavity was mopped clean and the coils of intestine returned back
to its original position and the stomach placed in its initial position. The skin united by continuous ligature with catgut. After the shock period was over, the rabbits were maintained with antibiotics like penicillin crystalline G(Squibb) 1000 units intramuscularly every 6 hours. Vitamin C (Redoxen, Roche) 25 gms. twice daily by intramuscular injections. During the post-operative period they were kept on boiled distilled water ad lib.

When the rabbits recovered fully from the effect of the operation they were given to start with grasses only. From the 5th day onwards, wet grams were given in increasing amount from 10 gms. gradually to 50 gms. daily. This could be given from the 7th day onwards. The weights were recorded daily from the 7th day onwards and fasting blood glucose estimations were made from the 10th day and the values were recorded. The urine was also examined from the 10th day onwards qualitatively for albumin, glucose and acetone which have been shown in table Nos.15 and 16.

These two rabbits were sacrificed and their eyeballs removed and treated in the same procedure as described under histopathological study(Item No.8) and histopathological staining technique(Item No.11).