EXPERIMENTAL PANCREATIC DIABETES IN THE CALF

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More than fifty years ago Minkowski and Weintraud suggested that there are species differences in the effects of pancreatectomy and that these seem to be associated with the natural dietary habits of the animal.[1] Recent research indicates that these differences, while correlated with dietary habits, are a result of the degree of activity of the anterior pituitary, adrenals, and possibly other endocrine organs.

Minkowski[2], Langendorff[3], and Weintraud[4], in qualitative studies on carnivorous birds, such as the hawk, falcon, buzzard, and raven found a marked glycosuria after pancreatectomy.

Since then, Nelson, Elgart and Mirsky[5], in a detailed investigation of the carnivorous owl, observed an extreme hyperglycemia following pancreatectomy, which could be varied with food intake, and a ketosis when the animal failed to eat well. Death ensued nine to twelve days after operation due to inanition. All types of carnivorous birds investigated showed severe diabetic symptoms after pancreatic ablation.

Studies of herbivorous birds indicate that they have a much milder diabetes after removal of the pancreas. Bernard[6], Minkowski[2], Weintraud[4], Kausch[7], and Giaja[8] did the early work on pigeons, geese, ducks, and chickens. They discovered that, with a few individual exceptions, these birds failed to develop glycosuria or hyperglycemia. More accurate and extensive work has been done since then.

Batt studied the carbohydrate metabolism of normal and depancreatized chickens and noted the mildness of the diabetic symptoms after pancreatectomy.

Ivy and his associates[11] observed, in depancreatized chickens, a temporary hyperglycemia accompanied by a glycosuria, after which the blood sugar returned to its preoperative level and the birds resumed their normal state except for digestive disturbances resulting from the loss of the external secretion of the pancreas. They suspected that the mildness of the symptoms might be due to the presence of extra pancreatic insulin producing tissues. They, therefore, determined that insulin was not extractable from the tissues of depancreatized chickens[12], whereas, it is extractable from the tissues of intact birds. They, thus, ruled out the possibility that extra-pancreatic insulin producing tissue was the agent ameliorating the diabetes.

Seitz and Ivy[13] found similar results in research on the duck, a transitory hyperglycemia and a subsequent return to normal. They also noted that pancreatectomy made little change in the fasting respiratory quotient in this species[14].

Sprague and Ivy[15], in a more complete paper, supported and partially explained these results. They concluded that there is some change in the mechanism of carbohydrate metabolism which enables the duck to dispense with insulin, or that the lack of metabolic changes may be due to some inherent deficiency in the anterior pituitary or adrenal cortex.

Mirsky, Nelson and Grayman[16] have done the most thorough work on the duck. They state that in normal ducks there is a considerable ketonemia after a thirty day fast. They contrast this with the dog and cat, carnivorous animals, which show little ketonemia after a long fast. Furthermore, pancreatectomy depresses fasting ketosis in the duck, which again is unlike the effect on the dog, the cat, and the owl mentioned above. They conclude that the tissues of the duck can utilize exogenous acetone bodies, also that death is a result of the loss of

the external rather than the internal secretion of the pancreas.

They confirmed and extended this evidence in another paper[17] where they reported studies on the weight loss after pancreatectomy and concluded definitely that insulin insufficiency is not the factor responsible for this loss. They also found no significant difference in the sugar tolerance curves of the normal and depancreatized birds. This paper emphasized the importance of the dietary factor and contained no evidence for the assumption that the anterior pituitary factor is the cause of the mildness of the diabetes. The birds failed to respond to anterior pituitary injections. The authors also fed ducks on a meat diet and found a hyperglycemia of slightly longer duration after pancreatectomy. This, however, is merely suggestive, and the idea that the anterior pituitary deficiency is not the cause of the mildness of the symptoms has been thoroughly negated in work since then.

Detailed studies have also been made on mammals. Minkowski[2] began this work with his observations of the effects of pancreatectomy in the dog. Since then the dog and cat have been frequently used for the investigation of pancreatic diabetes. The literature on these forms is

very extensive and well reviewed in other places[18-25]. This literature indicates that both dogs and cats develop a severe diabetes after the removal of the pancreas. The effects seem to be especially severe in the cat. In both animals, unless insulin is administered, death from acidosis will ensue within two weeks or less. Hyperglycemia, glycosuria, ketosis, high urinary nitrogen, and the other classic symptoms of severe diabetes are all present.

The ferret gives a similar response to pancreatectomy[26].

The intensive study of dog diabetes has, in part, been inspired because diabetes in the dog is so like that in man. Information on human diabetes has also been gained through the study of clinical cases. These, however, seldom involve complete loss of the pancreas. There are, due to obvious difficulties, few reported cases of the effects of total pancreatectomy in man. Sweeney[27], and Ricketts, Brunschwig and Knowlton[28], however, have had the opportunity to observe and report on such cases. Their observations sustain the theory that the dog and man have similar reactions to total removal of the pancreas.

The rat, despite its frequent and convenient use as a laboratory animal, is also a little used subject for the

study of the effects of total pancreatectomy. This is because the diffuse character of the rodent pancreas makes such removal impossible. Shapiro and Pincus[29] succeeded in a probable complete removal in this species and found a subsequent marked hyperglycemia which was maintained for the life of the animal. This is not conclusive, but the use of alloxan which destroys the islets of the pancreas has opened the possibility of study of pancreatic diabetes in the rat.

The monkey seems to develop much less marked diabetic symptoms than any of the above animals. The literature on pancreatectomy in this animal contains some conflicting results. Collip, Selye and Neufeld[30], and Chapman and Fulton[31] found that although the monkey suffers severe weight loss, even with the administration of insulin, it can survive pancreatectomy without insulin for several months. They noted that the symptoms exhibited by depancreatized monkeys were similar to those of the depancreatized-adrenalectomized, or depancreatized-hypophysectomized or "Houssay" dogs. Ketonuria was observed directly after operation, but disappeared eight to fourteen days later. The animals readily became hypoglycemic on fasting, but even then no ketone bodies

appeared in the urine. The blood sugars fluctuated widely and the fasting nitrogen excretion increased. Animals lost weight markedly and died of inanition and digestive disturbances. No animals died in acidosis.

Mirsky, Nelson and Elgart[32] had observations unlike those above. They could maintain their animals by careful diet and insulin without the severe weight losses noted in the above work. When insulin and food were withdrawn, there was a rapid development of acetonemia and the animals died in a diabetic acidosis, very similar to that observed in man.

There is little in the above papers to account for the disparity in the results. One, however, might expect, judging from the known dietary habits of the animal, that the work of Collip and Fulton and associates indicates the truest picture.

The rabbit, as one would expect, has also shown a mild diabetes after pancreatectomy. Greeley[33, 34] found that the rabbit would survive as long as four months without insulin. Death then resulted from digestive upsets, rather than from the loss of insulin. Hyperglycemia did occur in these animals after feeding, but there was neither ketonemia nor acetonuria, as indicated

by qualitative tests. The blood sugar level could be depressed by fasting.

Studies on the pig, an omnivorous animal, have been of interest in connection with carbohydrate metabolism. As Lusk says, it is the "supreme convertor of maize to animal fat". This function seems to be mediated in some way by the blood sugar.

Carlson and associates[35, 36] in rather incomplete studies, found a mild diabetes after pancreatectomy in the pig.

Eveleth and Eveleth[37,38] have worked out the normal carbohydrate metabolism of the pig.

Lukens[39] then reported more completely the effects of pancreatic removal in the pig. He found that the animals failed to live more than fifteen days, but death was from digestive disturbances rather than from acidosis. There was a marked ketonuria but little or no glycosuria. Lukens thought this indicated that, although the blood sugar is raised by pancreatectomy, glucose may be converted to fat with such facility that it escapes excretion, also that in the presence of disturbed carbohydrate utilization, fat is burned in excessive quantity, giving rise to the ketonuria. The pig, since it fails to produce

acidosis, must have an unusually effective mechanism for eliminating organic acids. He believed that the pig has some essential difference from the carnivore in the kind and degree of its anterior pituitary function and that the functional state of the anterior pituitary or some other endocrine activated thereby is responsible for the mildness of the porcine diabetes.

The goat, too, has a mild diabetes after pancreatectomy. Lukens[40] found that the symptoms observed after operation were similar to those in the pig. There was a low glucose excretion and a relatively low nitrogen excretion. The animal manifested a certain ability to utilize carbohydrate and showed, in contrast to the pig, little or no acetonuria.

The above literature indicates that there is a species difference involved in the variations of metabolic changes that follow pancreatectomy. This difference is correlated with the dietary habits of the animal. Removal of the pancreas in the herbivore results in a relatively mild diabetes while in the carnivore it results in a more severe diabetes. No reference to the study of the effects of pancreatectomy in the calf has been found in the literature. We, therefore, have undertaken some observations

on the character of carbohydrate metabolism in the intact and in the depancreatised calf. The results indicate that the calf affords another instance of a "mild" diabetes in an animal following total pancreatic ablation. They add further to the evidence that severe diabetes does not occur in the herbivorous animal.

General Procedure and Methods

Three bull calves were used for this problem. They were obtained from the Cornell University Farms when they were about four days of age. Data on their ages and weights at various stages of the experiment follows:

	Calf I	Calf II	Calf III
Breed	Holstein	Guernsey	Guernsey
Date of Birth	July 16	August 25	Sept. 8
Date of start of control experiments	Aug. 7	Sept. 9	Sept. 21
Date of Pancreatectomy	Sept. 2	Sept. 30	Oct. 12
Sacrificed	Sept. 4*	Oct. 19	Oct. 24

*Calf I died following pancreatectomy.

Weight in Pounds

Calf I		Calf II		Calf III	
July 22	110	Sept. 5	85	Sept. 11	72
Aug. 1	118	Sept. 11	88	Sept. 20	74
Aug. 16	130	Sept. 26	95	Sept. 26	85
Sept. 2	140	Oct. 1	95	Oct. 5	76
		Oct. 5	97	Oct. 11	90
		Oct. 11	92	Oct. 14	79
		Oct. 14	89	Oct. 21	80
		Oct. 19	82		

Feeding. The animals were normally fed a liquid consisting of a mixture of 2700 cc. of whole milk of water, and 500 cc. of lime water twice daily

and 2:00 P.M. The temperature of the resultant mixture was always raised to 95° F. to 98° F. On some occasions, however, it was necessary to vary the feeding routine. On the first day following fasting periods, the milk fed was reduced to one-half the usual daily volume and given in three feedings; similarly, it was reduced to three-fourths volume on the second day and was reinstated to the pre-fasting quantity on the third day. If this procedure was not carried out, the animals invariably developed "scours".

Immediately following pancreatectomy, the administration of food was started gradually. To alleviate the indigestion which might result from the loss of pancreatic juice in the intestine, two grams of pancreatin (Merck) were added to each quart of milk. Even with this precaution, the animals developed some digestive disturbances and required further modification of the feeding schedule to meet their individual conditions. It was usually possible, however, to get them adjusted to two daily feedings each of 2200 cc. of milk.

<u>Pre-experimental training</u>. During a preliminary period, the animals were kept in a square pen (7 feet x 7 feet) with straw litter. They remained in this pen while learning to drink from a pail. Following this, they were moved to a stanchion and given time to become accustomed to handling

and to their surroundings before the tests were started. This all required from ten to fourteen days.

The stanchion was built of wood with an elevated platform which contained a removable grate under which a jar could be placed for the continuous collection of uncontaminated urine (Plate 1). At its front, and on each side of the animal's neck, parallel uprights were provided to hold the calf in the stanchion and still permit it to lie down or stand comfortably. Under these circumstances, it could remain here continuously for many days. While the animal was standing in the stanchion, blood samples could be drawn from and glucose injections be made into the jugular vein with ease. After preliminary training, it was possible to do these without undue excitement of the animal.

Control tests. Preoperative control tests for blood glucose; urin glucose, acetone, and nitrogen; and sugar tolerance following the injection of 1 gm. of glucose per kgm. of body weight were conducted under normal and fasting conditions.

Preoperative care. The care of the animals in preparation for the operation was most important. Three days, at least, before pancreatectomy, the animals were removed from the stanchion and placed in the pen where they could exercise and have fair freedom of movement. The daily food

ration was reduced to one-half the usual level for two days and then omitted entirely on the last preoperative day. Water consumption also was reduced on this last day. With this procedure, the animals were in optimal nutritional and physical conditions at the time of the operation.

Anesthesia and Preparation for Operation. Nembutal was used as the anesthetic, dissolved in sterile water and injected into the jugular vein. A safe basal amount for cattle is 6.5 mg. per pound of body weight[41]. This was given slowly and the amount was increased as necessary. In these experiments each animal required at least 9 mg. per pound of body weight for perfect anesthesia.

Following the injection of the anesthesia the animal was prepared for the incision. The hair was clipped from an area on the right side extending well above and below the last rib from the middle of the back down past the ventral midline. This area was scrubbed with soap and water and sterilized with 70 o/o alcohol and then painted with metaphane.

By use of a specially constructed hinged table top, the calf was supported so that it rested partially on its back and partially on its left side. In order to facilitate drainage of the profuse salivation, its head was allowed to hang over the edge of the table.

The animal was draped with a sterile field cloth and towels so that only the area of incision was exposed. Aseptic technique was observed throughout the operation.

Anatomy.[42] "The pancreas is irregularly quadrilateral in form, and lies almost entirely to the right of the median plane. The dorsal surface is related to the liver, right kidney, crura of the diaphragm, posterior vena cava, and anterior mesenteric arteries; covered largely by peritoneum. It is attached to the liver at and lateral to the potal fissure, and to the crura of the diaphragm. Between these adhesions it is free and forms the ventral wall of the epiploic foramen. On the right side it extends backward beyond the caudate lobe of the liver between the layers of the mesoduodenum...here it is related dorsally to the right kidney and by its ventro-lateral border to the retrograde part of the duodenum. The ventral ... surface is in contact with the dorsal curvature of the rumen and intestine. There is a deep notch for the portal vein and hepatic artery ... here ... The left extremity ... is related dorsally to the crus of the diaphragm ... and is adherent ventrally to the rumen. The right part ... is often divided into two branches..."

Operative procedure. Calf I. A transverse incision was made just below the last rib on the right side of the animal. This approach was unsatisfactory.

Calf II and Calf III. A ventral mid-line incision similar to that usually employed in pancreatectomy in the dog was used. By blunt dissection, the pancreas was gradually freed from any adhering structures. It was possible to remove the organ almost intact as it was fairly well integrated in these animals. Since the portal vein gives off many small branches to the pancreas extreme care had to be taken to avoid excessive bleeding from these branches. It was not, however, necessary to ligate many of these vessels.

The operation required about two hours and the animals apparently experienced little or no disturbance due to the operation itself. Infection was absent in all animals.

Post mortem examination indicated that pancreatic removal was complete in each case.

Postoperative care. This is also very important. The animal was placed in the pen until it recovered from the anesthesia and seemed normal. This took about 24 hours. While under the anesthesia, the calf was kept warm and dry. In order to avoid circulatory complications and pulmonary congestion, it was at first turned from one side to the

other and then, as it regained consciousness, helped to stand at frequent intervals. Feeding was resumed gradually after a postoperative period of about 24 hours. As soon as the animals condition would permit, it was returned to the stanchion where urine and blood collections could be made.

<u>Tests and methods.</u>* The Somojyi-Shaffer-Hartmann method[43] was used for the determination of blood and urine glucose. Urinary nitrogen excretion was found by the Kjeldahl procedure[44]. The acetone content of the urine was determined by the method of Renvin[45]. Post mortem liver and muscle glycogen percentages were obtained by the Good-Kramer-Somojyi method[46]. Glucose tolerance tests were done with the injection of 1 gm. of glucose per kgm. of body weight. All tests except those for liver and muscle glycogen were run on both fed and fasting animals. Control tests with these methods were made prior to pancreatectomy.

*See appendix for details of methods.

Analysis and Discussion of Data

Blood glucose. The glycemic level is a balance or equilibrium established between the income of glucose into and the outgo of glucose from the blood. The sources of this income are absorption of glucose from the intestine, glycogenolysis in the liver and gluconeogenesis in the same organ. Following hepatectomy or complete destruction of the liver through disease, all sources of glucose income are absent in the fasting animal. Loss of blood glucose may occur as a result of glycogen storage either in the liver or muscles, conversion of glucose to fat (presumably in the liver), oxidation of glucose by the tissues for energy purposes, and at times excretion in the urine.

The average observed normal blood glucose for the calf was 81 mgm. per cent (per 100 ml. of blood). See Table 1, Plate 2. This is about 20 to 40 mgm. per cent higher than is found in normal adult cattle[47]. During fasting states in the normal calf, there was a drop of about 60 per cent in the blood sugar level by the 42nd hour. By the 67th hour of fasting the blood glucose level had commenced to rise. See Table 2. By the 90th and 114th hours in calves II and III, respectively, the blood sugar had fallen again. The rise noted after the initial drop in blood glucose level was due, in part at least, to the new-

formation of glucose from protein, gluconeogenesis. A second possible cause of this blood sugar rise would be a decrease in the ability of the animal's tissues to oxidize glucose.

Table 1

NORMAL BLOOD GLUCOSE
Preoperative

Calf I		Calf II		Calf III	
Date	Mg.o/o	Date	Mg.o/o	Date	Mg.o/o
8-7	78	9-6	90	9-21	66
8-8	85	9-7	80	9-22	68
8-9	84	9-8	81	9-23	82
8-10	84	9-15	86	9-25	75
8-11	84	9-23	86	9-26	77
8-12	82	9-26	92	9-29	84
8-16	83	9-27	88	10-1	77
8-21	81	Average	86	Average	76
8-30	75				
Average	82				

The normal blood glucose ranged from 66 mg. o/o to 92 mg. o/o. The average for the three calves was 81 mg. o/o.

Table 2

NITROGEN AND BLOOD GLUCOSE
Preoperative, Fasting

Gms. / kgm. / day fasting urinary nitrogen and glucose equivalent and Mg. o/o fasting blood glucose, in animals before pancreatectomy.

Hours Fast	Blood Glucose	Urine Nitrogen	Glucose Equivalent
	Calf I		
0	82	.13	.47
43	56	.20	.73
67	66	.26	.95
91	70	.26	.95
⊛	86	1.00	
	Calf II		
0	86	.23	.84
42.5	48	.27	.99
66.5	61	.32	1.17
90.5	53	.34	1.24
⊛	88	.34	
	Calf III		
0	76	.28	1.02
18.5	77	.28	1.02
42.5	42	.60	2.19
66.5	47	.60	2.19
90.5	52	.83	3.04
114.5	45	.37	1.35
138.5	41	.57	2.08

O Average Normal Values
⊛ Recovery

The accepted experimental method of estimating quantitatively the magnitude of gluconeogenesis from protein in the animal body involves the determination of the quantity of nitrogen excreted in the urine. Ultimately most amino acids are deaminized in the liver, the nitrogenous fraction being excreted in the urine as urea. Urine-nitrogen is thus accepted as a fair index to liver gluconeogenesis. In the case of fasting calves, the urine nitrogen level rose as the blood glucose level fell, (See plates 3, 4, 5) indicating an increased gluconeogenesis at a time when the animals were in danger of developing a low blood sugar as a result of decreased supplies from other sources. The urinary nitrogen increased 50 per cent, 67 per cent and 33 per cent, respectively, in calves I, II, and III during a 90 hour fast. A drop in urine nitrogen was then noted in calf III in which the fast was continued for a longer period. (See Table 2)

A theoretical analysis of the metabolic problem with which the animal is confronted and based on available evidence from various sources has proved enlightening. In this manner one is able to ascertain roughly the part played by each of the factors listed above in the maintenance of the blood sugar levels. Using the data for calf III (Table 2) so far as possible together with other generally accepted

data for the dog an example may be given as an illustration. The animal was fasting, thus eliminating alimentary absorption as a factor.

Bollman and Mann[40] have shown that the hepatectomised dog which had been previously fed an average mixed diet of carbohydrates, proteins, fats, etc. requires a continuous intravenous infusion of glucose at the rate of 0.25 gm. per kgm. body weight per hour (6 gms. per kgm. per day) to maintain the normal blood sugar level. This would indicate that the normal liver was supplying sugar to the blood at this rate and, if the blood sugar is being maintained at a constant level, that the extrahepatic tissues were utilising blood sugar at this same rate. Since the liver makes up about 5 per cent of the total body weight or 50 gms. per kgm., and its average glycogen content is 5 per cent by weight, its carbohydrate storage is approximately 1.5 gms. glucose equivalent per kgm. of the total body weight. For comparative purposes all calculations will be based on the kgm. body weight. If the liver glycogen was completely utilised and the sole source of blood sugar, it would enable the animal to maintain its blood sugar at the normal level for 6 hours only at the above rate of glucose utilization. Beyond this length of time, other sources of blood sugar

would be required for a fasting animal to prevent the development of hypoglycemia unless the rate of consumption was also decreased. However, the animal maintained a fairly good blood sugar level for over 138 hours (see Plate 6).

Another possible source of the blood sugar requirement to maintain the normal glycemic level is gluconeogenesis from protein by the liver. Using the Urinary nitrogen as an index to the magnitude of this process, the following equivalent values are now accepted as standard: 1 gm. of urinary nitrogen is metabolically equivalent to the catabolism of 6.25 gms. of protein. In phlorizinized dogs, in which it has been assumed that the total sugar synthesized from protein by the liver is excreted in the urine, the urinary glucose: urinary nitrogen ratio (G/N ratio) has been established as 3.65/1. Thus for every 6.25 grams of protein catabolized, an assumed maximum of 3.65 grams of glucose may be produced, in other words, 3.65/6.25 or 58 per cent of the protein catabolized may be converted to glucose. This is at best only an approximation, but is quite generally employed in metabolic studies where such calculations are desirable.

In the present experiments, the glucose equivalents of gluconeogenesis from protein were obtained by multiplying the

number expressing the urinary nitrogen in gms./kgm./day by 3.65 (Table 2). In calf III, which had the maximum values, the 24 hour fasting average was 1.85 gms. of urinary nitrogen. The animal was fasted for 138.5 hours. Thus the theoretical glucose production by gluconeogenesis for this period would be $\frac{1.85 \times 138.5}{24 \text{ hours}} = 10.6$ gms. while the theoretical glucose requirement to maintain the normal blood sugar level would be 0.25 gms. x 138.5 hrs. = 34.6 gms./kgm. for the same period.

The blood and extracellular body fluid are additional sources of metabolic glucose. Any change in the level of blood glucose indicates a disturbed equilibrium between income and outgo. Because of the ready diffusion of glucose between the blood and body fluid, a diffusion equilibrium tends to be established. Because of this the extracellular fluid glucose concentration may be considered to be the same as that of the blood. The total blood and tissue fluid volume into which glucose is readily equilibrated has been determined to be equivalent to ~~be~~ one third of the body weight or 333 ml. per kgm. of body weight[49]. Since the blood glucose level fell from 76 mgm. per cent to 41 mgm. per cent (35 mgm. per 100 ml. of blood), the sugar balance indicates a utilization of $\frac{333 \times 35}{100 \times 1000} = 0.117$ gms.

of glucose per kgm. body weight in excess of intake.

Other possible sources of blood glucose are muscle glycogen and body fat. That any great quantity of glucose available to the blood arises from these sources seems doubtful. However, certain assumptions may be made as theoretical possibilities. Muscle glycogen is never a direct source of blood sugar, glycogenolysis in muscles produces lactic acid, not glucose. Since the former is readily converted into glucose and/or glycogen by the liver, muscle glycogen may become an indirect but not a direct source of blood sugar. Muscle glycogen levels are fairly well maintained during starvation and even in diabetes in some animal species. In the two diabetic animals studied, these were within the normal limits, being 0.52 per cent and 0.70 per cent. Additional work needs to be done on this part of the problem, but assuming an extreme muscle glycogen loss of 0.5 per cent, there is still a marked deficit in glucose source.

The muscles of the animal body compose about 50 per cent of the body by weight or 500 gms. per kgm. body weight. Assuming an average muscle glycogen loss of 0.5 per cent, this would produce a negative imbalance equivalent to $\frac{500 \times 0.5}{100}$ = 2.5 gms. of glucose per kgm. body weight.

During the total fasting period of 138.5 hours, the animal's body weight decreased from 38 kgm. at the outset to 35 kgm. at its termination, a loss of 3 kgm. Assuming that 90 per cent of this was due to loss of body fat, the metabolic fat would then be $\frac{90 \times 3000}{100} = 2700$ gms. or $\frac{2700}{38} = 71$ gms. of fat loss per kgm. of initial body weight.

Since glycerol can be converted to glucose by the liver (gluconeogenesis) and body fats contain about 10 per cent of glycerol, the metabolic use of the above calculated body fat might produce a maximal quantity of $\frac{71 \times 10}{100} = 7.1$ gms. of glycerol and approximately 7.1 gms of glucose.

Summarizing the possible sources of glucose for the 138.5 hour fasting period, we have:

1. Liver glycogen -------------------- 1.5 gms.
2. Gluconeogenesis from protein -------10.6 gms.
3. Blood and body fluids ------------- 0.117 gms.
4. Glycerol from fats ---------------- 7.1 gms.
5. Muscle glycogen ------------------- 2.5 gms.

Total ----- 21.817 gms.

An analysis of the data shows clearly that even with the acceptance of these outside values there remains a marked deficit in the carbohydrate balance sheet for the total animal body. For at the theoretical glucose

utilization rate of the normal animal receiving a mixed diet the glucose requirement for a period of 138.5 hours would be 34.6 grams, the calculated carbohydrate sources would account for a maximum of 21.817 gms. Either some additional source or sources of glucose were available to the animal, or the consumption rate of glucose was lower than the theoretical level of 0.25 gm. per kgm. per hour. Evidence that a decrease occurs in the ability of the tissues of fasting animals to oxidise glucose is available from various sources[50]. It has been contended by some workers[51] that prolonged fasting results in almost if not complete loss of the ability of the tissues to so utilise carbohydrates. In the present experiments this trend was indicated in two ways: (1) in the secondary rise of blood sugar during fasting, and (2) in the glucose tolerance tests described below.

At the beginning of a period of fasting the ~~hydrolysis~~ phosphorolysis of liver glycogen (glycogenolysis) to glucose is considered to be the first physiological mechanism called into action to prevent the fall of blood sugar. In the normal animal this process never leads to complete exhaustion of the liver glycogen stores. Cori[52] has shown that long before such exhaustion takes place, counter mechanisms consisting

in shifts in favor of endogenous fat and protein metabolism occur. With the increased protein metabolism there is an accompanying increased gluconeogenesis. At the outset of fasting this initial blood sugar fall associated with utilization by the tissues is retarded by glycogenolysis in the liver. This is soon supplemented by gluconeogenesis from: (1) endogenous protein, (2) glycerol of endogenous fat metabolism possibly, and (3) lactic acid arising in the muscles. These combined glucose sources however, are inadequate to maintain normal blood sugar levels, the blood sugar falls. Definite decreased glucose tolerance curves are obtained by the fourth day of fast. This indicates deficient glucose utilization by the animals and is probably the primary factor concerned with the secondary rise in blood sugar during fasting. (See Table II and Plate 6) Since the urinary nitrogen excretion rises to a maximum at about this time (See Plates 3, 4 and 5), gluconeogenesis from protein may be a secondary factor although quantitatively it is inadequate to explain the total rise.

A result commonly spoken of as the Staub-Traugott phenomenon occurred in Calf II. Following the first feeding at the termination of the fast, a rather marked hyperglycemia and glycosuria developed. (See Table 7) This is frequently

spoken of as hunger diabetes and is related to the decreased rate of glucose utilization by the extra hepatic tissues. This is probably aggravated by an existing elevated gluconeogenesis. The two sources of blood sugar tend to summate and thus raise the blood sugar above the kidney threshold for the substance; hence the glycosuria.

Notes on blood glucose following pancreactectomy. Blood samples were taken at 8 A.M. with the animal in as nearly a postabsorptive state as possible. Other samples were taken at 6 P.M.

The animals were fed, unless otherwise indicated, at 8:15 A.M. and at 2:00 P.M., 2200 cc. of whole milk, 500 cc. of water, 500 cc. of lime water and 2 gm. of Merck pancreatin per liter of milk.

The following table indicates the effect of feeding on the blood glucose level. The fasting values were much lower than the non-fasting values.

The averages (Calf II, 151 mg.o/o; Calf III, 226 mg. o/o), for the animals on a non-fasting regime, of the morning samples which were taken before feeding were less than the averages (Calf II, 259 mg. o/o; Calf III, 350 mg. o/o) for the evening samples which were taken after the feedings.

<u>Diabetes in the calf.</u> Following pancreatectomy in the calf, the glycemia was relatively proportional to the food intake. (See Plate 7 and Table 3) During periods of feeding the blood sugar level rose to diabetic levels, while during periods of fasting hypoglycemia developed (See Plate 8 and Table 4). On the routine diet, however, the blood sugar level did not rise above 378 mgm. per cent, table 3 and plate 6. The primary factor in the production of diabetic hyperglycemia in the calf is obviously related to the diet. This might be due to the absorption of glucose *per se* or to exogenous glucogeneogenesis from dietary protein, etc. in the liver. Both probably play a part in this role, but the latter appears to be the major factor. Endogenous gluconeogenesis, although increased above the level found in normal animals, is by itself insufficient in magnitude to maintain even a normal blood sugar level as is evidenced by the hypoglycemia which develops when these animals are fasted.

A second factor which develops during the first 3 to 5 days following pancreatectomy is the relative decrease in the ability of the extrahepatic tissues to oxidize glucose, ie., to utilize carbohydrates. This is an important factor in determining the decreased glucose tolerance of the diabetic animal. A third factor is the relatively deficient

glycogen storage capacity of the liver. This, however, is not affected to the same extent in the diabetic calf as in diabetic dogs and cats in which this function of the liver appears to be almost totally lost. A combination of the three factors just mentioned, namely, increased gluconeogenesis, decreased glucose utilization, and decreased glucose storage in the liver, will adequately account for the hyperglycemia and glycosuria of the calf. Obviously the glycosuria is secondary to the hyperglycemia. There is no evidence that the glucose threshold of the kidneys is lowered in diabetes, but what evidence there is would indicate that it may be raised. It is impossible with the available data on the calf to ascertain, with any degree of certainty, the relative importance of the above mentioned factors in the control of carbohydrate metabolism and the disturbances associated with pancreatic diabetes in this species. Further analysis of the problem, however, may help to present a clearer picture of the processes involved.

The normal liver responds to ingested glucose, and glycogenic substances which tend to produce an increase in blood sugar, by decreasing its output of glucose. This output is effected through glycogenolysis and gluconeogenesis. Thus the exogenous sugar income temporarily depresses and

Table 3

BLOOD GLUCOSE
Postpancreatectomy

Number hours postoperative	Mg. o/o Glucose	Feed	Remarks
	Calf I		
0	82		
2.5	118		
9.75	121		
21	297	2½ L.	
	Calf II		
0	82		
21*	68		
31	57	-	
45*	165	1 L.	
55	230	3 L.	
69*	224	+	
93*	202	+	
105	334	+	
117*	235	+	1 gm./kgm. glucose inj. after this
127	378	+	last pre-fast Fed 6 P.M.
142*	251	-	Fast
151	171	-	Fast
165*	33	-	Fast
178	38	1 L.	Fed following this sample
189*	231	-	Fast
199	166	-	
213*	49	-	1 gm./kgm. glucose inj. after this
237*	217	+	
261*	213	+	Placed in pen
325*	237	+	
347*	205	+	
381	169	-	Fast
419*	119	-	
429	61		

Table 3 (continued)

Number hours postoperative	Mg. o/o Glucose	Feed	Remarks
	Calf III		
0	76		
33*	92	2 L.	
57*	110	4 L.	
67	238		
81*	139	4 L.	
91	280		
105*	200	+	Fast started 111 hours
129*	148		1 gm./kgm. glucose inj. 129+ hours
159	47	-	Fast
163	40	-	
177*	27	-	
225*	120	1 L.	

* A.M. sample

Table 4

BLOOD GLUCOSE
Postpancreatectomy Fasting

Calf II			Calf III		
Hours fast	Mg.o/o Glucose	Remarks	Hours fast	Mg.o/o Glucose	Remarks
0	378		0	200	
14	251		18.5	106	
24	171		29.5	47	
38.5	33	Fed 1 L.	51	40	Fed 1 L.*
51	36	Fed	66	27	
62.5	231				
71.5	168				
86	49				

*Blood glucose apparently rose after this feeding and decreased again before the next sample. (See Plate 8)

replaces the prevailing endogenous supply and meets the requirements of the tissues for glucose. If an exogenous source of glucose is thus made available at a time when the endogenous gluconeogenic process is proceeding at a high level, the two sources of sugar income into the blood tend to summate and produce a temporary hyperglycemia and at times glycosuria (Staub-Traugott phenomenon). An adjustment, however, is soon made, especially by repeated administration of exogenous carbohydrate sources, which in nature is one of decreased gluconeogenesis in the liver. The blood glucose then returns to normal. This function of the liver appears to depend upon the proper balance between the so-called diabetic principle or hormone of the anterior pituitary and insulin. Insulin depresses gluconeogenesis, the anterior pituitary factor or factors stimulate an increased gluconeogenesis in the liver. This action of the pituitary is, in part at least, due to its adrenotropic hormone, and thus indirectly through the hormones of the adrenal cortex. These hormonal influences are only regulatory, Soskins et al[53-55] have shown that the Houssay preparation may have a normal glucose tolerance. In the absence of both insulin and anterior pituitary hormones, the liver exercises its glycogenic function in fairly normal

fashion. Basically the liver is able to respond with an increased glycogenesis when the blood sugar is high and with an increased glycogenolysis when the blood sugar tends to fall. This is the so-called homeostatic function of the liver[56]. It is undoubtedly fundamentally important in determining the glucose tolerance of the animal body.

This regulatory function of the liver fails, however, following pancreatectomy after which the animal is deprived of its insulin supply which is normally under physiological control. In the diabetic state, the blood sugar must rise to hyperglycemic levels before it causes the liver to diminish its gluconeogenic function and drastic glycogenolysis. Small amounts of glycogen may be stored in this organ when the blood sugar is maintained at sufficiently high levels. Exogenous and endogenous glucose summate to yield higher blood sugar levels. The liver not only fails to cease production as a result of the increased exogenous supply, but may actually increase production through gluconeogenesis from glycogenic dietary substances.

The evidence for this increased gluconeogenesis in the diabetic calves of the present experiments is not wholly satisfactory. Although the nitrogen excreted in the urine was increased by 48 per cent in the case of calf II and by

fifteen (ane)

eighty-seven per cent in calf III following pancreatectomy (Table 5), these figures are not of sufficient magnitude to account for more than a small part of the available sugar. The preoperative glucose equivalents of the excreted nitrogen were calculated from a G/N ratio of 3.65:1, whereas the G/N ratio of depancreatised animals is usually lower than this figure. In the present experiments the available data were inadequate for the calculation of reliable G/N ratios, these, however, must have been considerably lower than 3.65:1 since evidence was obtained which indicated that the diabetic calf is still able to utilize appreciable quantities of carbohydrate (Tables 18 and 19). The above ratio is the accepted ratio where all of the theoretical glucose produced from protein is excreted.

Liver glycogenolysis may account for a small portion of the increase in blood sugar. Although the liver glycogen values of 2.28 per cent and 2.55 per cent in calves II and III, respectively (see Table 21) are somewhat low, they are still within the normal liver glycogen ranges of from 2 to 10 per cent, averaging about 5 per cent. Other factors, such as muscle glycogen, glycerol from fats and possibly fatty acids, probably have effects in the diabetic animal similar to those already discussed in the maintenance of the

preoperative blood glucose levels. The diabetic animal mobilises a larger portion of its body fat so as a source of glucose would differ from the normal in magnitude. Muscle glycogen values in calves II and III while in the diabetic state were 0.52 and 0.78 per cent respectively (Table 21). These are within the limits of normal values, hence this is not an important source of blood sugar in diabetic calves.

TABLE 5

NITROGEN

Gms./ kgm./ day urine nitrogen excretion following feeding before and after pancreatectomy.

Calf I	Calf II		Calf III	
Pre-op.	Pre-op.	Post-op.	Pre-op.	Post-op.
.14	.20	.30	.26	.41
.10	.20	.34	.25	.33
.15	.23	.35	.33	.23
.08	.28	.35	.28	
.16		.34		
Av. .13	Av. .23	Av. .34	Av. .28	Av. .32

As far as the effect of subnormal utilisation of glucose by the extrahepatic tissues is concerned, the final word cannot be said until further work is done. The effects of hepatectomy and of administration of the diabetogenic hormones of the anterior pituitary on carbohydrate utilization by the tissues should be studied. However, from the

apparent relatively low figures for urinary nitrogen and gluconeogenesis, it would seem that the principal factor in the production of the high blood sugar of pancreatic diabetes in the calf is that of diminished utilization. Additional support for this conclusion will be furnished in the analysis of the glucose tolerance data.

Table 6

NITROGEN AND BLOOD GLUCOSE
Postoperative, fasting

Gms./ kgm./ day fasting urinary nitrogen and Mg. 1/o blood glucose, in animals following pancreatectomy.

Calf II			Calf III		
Hours Fast	Blood Glucose	Urine Nitrogen	Hours Fast	Blood Glucose	Urine Nitrogen
0	378	.35	0	200	
14	251	.33	18.5	106)	.69
24	171		29.5	47)	
38.5	53	.31			
52	56*				
62.5	231				
71.5	168				
86	49	.23			

0 Level before fast
* Fed 1 liter of milk before this sample

Fasting also has a somewhat different effect on the blood glucose and urine nitrogen of the diabetic calf than upon that of the intact animal. See Tables 4, 5 and 6. The blood sugar level dropped to 35 mgm. per cent and 47 mgm. per cent in 58.5 and 29.5 hours respectively in calves II and III. This rapidly developing hypoglycemia was relieved at this time by feeding. The lowest fasting blood sugar observed in these fasting diabetic calves, namely, 27 mgm. per cent, was obtained in calf III (see Table 3). The secondary rise in blood glucose as observed in fasting normal calves was absent after pancreatectomy under otherwise similar conditions. Associated with this, however, was the noteworthy observation that there was a decrease in the urinary nitrogen during fasting instead of an increase which invariably appeared in the preoperated animal under similar conditions (Table 6). This would seem to indicate that endogenous production of glucose was low in the fasting diabetic calf and that the hyperglycemia which follows feeding might, in large part, be due to failure of the animal to utilise glucose. This low gluconeogenesis during fasting accounts for the hypoglycemia which develops during fasting. That the blood sugar falls below the kidney threshold during fasting is also evidence that the tissues are utilising blood glucose.

These results are in marked contrast with those found in carnivora and would seem to indicate that herbivora have a much less well developed mechanism for endogenous gluconeogenesis than the carnivora. This is due, no doubt, to some difference in the anterior pituitary and possibly the adrenal cortex in these species and may account somewhat for the mildness of the diabetic state in the calf.

Excretion of glucose in the urine. Glucose, although found in faint traces in the urine of both fed and fasted normal animals (Table 7), varied fairly directly with the food intake and similarly with the blood sugar level in depancreatized calves (Table 8). During the hypoglycemic stages of fasting, the urine contained little or no glucose, while excretion of this sugar rose to relatively high levels when the animals were fed the routine diet (Table 8). In calf II while fasting before pancreatectomy, the urine sugar rose markedly following the first feeding (Table 7). This is the so-called "fasting diabetes", or the "Staub-Traugott phenomenon". The explanation of this condition is presented on an earlier page of this thesis. The fall of urine glucose during fasting indicates further the failure of the diabetic calf to maintain blood sugar above the kidney threshold when endogenous gluconeogenesis is its sole source.

<u>Ketonemia and ketonuria in the diabetic calf.</u> The diabetic state in the calf is also indicated somewhat by the blood and urine ketone bodies. The total ketone body excretion was determined from the total urine acetone after the oxidative conversion of acetoacetic acid and beta-hydroxybutyric acid into this substance. For methods see the appendix of this thesis. In the preoperative animal no acetone was found in the urine under any of the conditions tested. Unlike the dog, cat, and other carnivores, the calf excretes insignificant amounts of ketone bodies following pancreatectomy when the diabetic state is full blown. The maximal excretion of these substances observed was 1.8 gm. per kgm. per day (Table 9). This no doubt accounts somewhat for the mildness of the diabetic symptoms in this species. It is believed that acetoacetic acid is the ketone body which stimulates the respiratory centers and depresses the higher brain centers and thus causes "air hunger" and diabetic coma. Acetoacetic acid is spontaneously oxidized to acetone in the blood stream, in the lungs, and in the urinary bladder. Thus urinary acetone is a fairly accurate index to the presence of acetoacetic acid in the blood and tissues.

Table 7

URINE GLUCOSE
Preoperative

Gms./ kgm./ day glucose excreted in the urine of normal and fasting animals before pancreatectomy.

	Normal Glucose	Fasting Glucose	Hours Fast
Calf I		.008	43
		.008	67
		.010	91
		.009	Average
Calf II	.009	.015	42.5
	.017	.009	66.5
	.021	.002	90.5
	.026	.450	Recovery
	.018	.080	
		.026	
		.009	Average
Calf III	.025	.012	42.5
	.056	.012	66.5
	.065	.005	90.5
	.035	.007	114.5
	.061	.002	138.5
	.054	.007	Average

Table 8

BLOOD AND URINE GLUCOSE
Postoperative

Blood glucose Mg. o/o, urine glucose gms./kgm./day in the animal following pancreatectomy.

	Date	Blood	Urine	Remarks
Calf II	10/2	165	.588	
	10/3	224	.847	
	10/4	202	1.860	19 hr.urine
	10/5	235	4.220	
	10/5*	378	4.960	
	10/6	251	.200	Fasting
	10/7	33	.068	Fasting
	10/7*	36	.636	Fed 1 L.milk
	10/9	49	.902	Fasting
	10/10	217	1.780	Fed
	10/14	237	3.880	
	10/15	205	.975	
	10/15*	159	.125	Fasting
	10/16*	169	1.020	Fed 1 L.
	10/17	32	.840	Fasting
	10/18	61	0	Fasting
Calf III	10/15	118	2.94	
	10/16	139	2.16	
	10/17	200	5.55	
	10/17*	106	4.11	
	10/18	47	0	Fasting
	10/20	27	0	Fasting
	10/22	110	1.30	Fed

Note the corresponding drop in blood and urine glucose on fasting and the rise following feeding.
*Samples taken 6 P.M. - All other samples taken at 8 A.M. before feeding.

Table 9

ACETONE

Gm./ Kgm./ day excreted in the urine of the animal following pancreatectomy

Calf II Acetone	Calf II Remarks	Calf III Acetone	Calf III Remarks
.71		.56	
.68		.45	
1.80		.70	
.60		.21	Fasting
.32	Fasting	0	Fasting
.84	Average (non-fasting)	.009	Fed 1 L.milk
		0	Fasting
		0	Fasting
		.54	Average (non-fasting)

Note: Decrease in acetone excretion on fasting.

This relative lack of acetonuria may indicate a low level of fat metabolism or an unusually high level of fat and ketone body utilization by the tissues of the diabetic calf. The urinary acetone in these diabetic animals dropped to zero upon fasting (Table 9). This would indicate a relatively low endogenous ketogenesis or a high ketone body utilization rate, or both. In depancreatized carnivora it would seem that large quantities of fat are metabolized to provide energy, presumably because the animal is incapable of utilizing sufficient quantities of carbohydrate to meet its energy requirements. With this increased fat utilization there is an increased rate of fat processing in the liver which results in a higher rate of ketone body production, a ketonemia and ketonuria. The last two conditions occur only when the ketone body production exceeds the ability of the extrahepatic tissues to utilize these metabolites. In the calf, however, there is a less marked imbalance between production and utilization of ketone bodies. Two possibilities exist, either the rate of fat metabolism is relatively low or the utilization rate is high. The former is most likely. This conclusion is in agreement with the fact that the diabetic calf seems to be better able to utilize carbohydrates than the carnivores. Diabetic calves

possess better liver and muscle glycogen stores and the hyperglycemic levels are lower than in the dog and cat. Carbohydrates are antiketogenic in the sense that they replace and decrease the quantity of fat metabolised.

Glucose tolerance tests. Analysis of the data obtained from glucose tolerance tests may also aid in understanding the underlying mechanisms of this diabetes. Glucose tolerance is the measure of the ability of the body to metabolise glucose. It is the amount of glucose that may be given without causing glycosuria. Ordinarily it is based on the administration of known amounts of glucose on an empty stomach. In the ruminants, however, it is impossible to measure tolerance accurately by this method, so it was done instead by injection of 1 gm. of glucose / kgm. of body weight into the jugular vein.

The hyperglycemia that follows this injection is an indication of the inability of the organism to cope with the influx of exogenous sugar. The excess sugar excreted in the urine also indicates this.

The tolerance of rabbit, dog and man for slowly injected sugar is about 0.8 - 0.9 gm. of glucose / kgm. of body weight/ hour. Only a small fraction of this excess is excreted.

The extend and duration of the hyperglycemia following the injection of glucose is a truer indication of the animal's tolerance for that sugar than is the amount of glucose excreted for the blood glucose indicates the tolerance reaction even at subglycosuria levels and eliminates the kidney as a possible variable.

In these calves 1 gm. of glucose/ kgm. of body weight was injected within a ten minute period (injected as 50 per cent glucose solutions).

Blood glucose samples were taken before the injection, thirty minutes following the end of the injection and every hour thereafter until the termination of the experiment. By thirty minutes following the end of the injection in the normal animal, the blood glucose rose to an average of 294 mg. per cent (Tables 10-16), a rise of 155 mg. per cent above the preinjection level. (See plate 9 and plate 10, figures 3 and 4) After this rise the curve fell with fair rapidity, utilisation being apparently proportional to the concentration of glucose. In from 1 to 3 hours, varying with the initial post-injection level, the blood glucose returned to normal. The higher the original level the longer it took to return to normal, thus the slopes of the normal curves were all similar. After reaching the normal, the curve continued to fall, going below the preinjection level.

It remained subnormal for about two hours or a little longer. This hypoglycemic reaction was apparently a result of overcompensation for the excessive influx of glucose. This hypoglycemic phase also occurred in the diabetic, but after a longer time and the return to normal was slower, in fact it was never observed in these tests even though one of them was carried on for an extended period. (See plate 11, figures 3 and 4)

Some investigators have ascribed this hypoglycemic phase to an overcompensation of insulin. Most evidence seems to indicate, however, that it is better attributed to a decreased gluconeogenesis caused by overcompensation of the anterior pituitary, or due to a delay in reestablishing this gluconeogenesis. However, it appears that the fundamental factor involved is the "homeostatic" effect of the liver described below.

Apparently normal livers respond to an increased blood sugar resulting from an influx of exogenous glucose by decreasing their output of sugar and the blood glucose returns to normal or until the liver has again readjusted goes below normal. This "homeostatic" function has been shown to depend on a sufficiency of insulin in the blood and upon the diabetogenic hormone of the anterior pituitary.

The curve for the preoperative calf taken after a five day fast indicated a decrease in the ability of the animal to utilize glucose. (See plate 10, figure 5) It was very similar to the curve for a diabetic. This prolonged curve following fasting is probably due to the inability of the liver to respond to the sudden influx of glucose because of the imbalance between the pancreas and the anterior pituitary and the decreased ability of the tissues to utilize glucose as a result of the fast. The postfasting curve failed to return to the preinjection level in the time itwas observed.

Following pancreatectomy a somewhat similar condition prevailed. The blood glucose rose to an average of 201 mg. per cent on the thirty minute sample (Tables 19 and 20). This was about a 30 per cent greater rise than that of the normal non-fasting calf. The curves were much prolonged and failed to return to normal until after the fourth hour of fast (Plate 11, figure 3 and 4). The hypoglycemic phase was greatly prolonged, a return to the preinjection level was not observed even though, in the case of calf [illegible], this curve was followed for 9 hours. From this point the diabetic calf seems to pass directly into fasting by poglycema. This prolonged curve indicates a markedly decreased glucose tolerance in the depancreatised animal

which is apparently due to the failure of the "homeostatic" effect of the liver and to the inability of the animal to oxidise the excess glucose adequately.

The curve for the pancreatectomised animal following fasting was even more prolonged and failed to return to the preinjection level before the termination of the experiment. See plate 11, figures 1 and 2; Tables 17 and 18. Here apparently the effects of the previous fast and of the diabetes summated to produce a further reduced tolerance.

The urine glucose excreted during these tests may also serve as an indication of tolerance. An average of 90 per cent of the glucose injected was utilized in the normal calf. See tables 11-13, 15 and 16. Following pancreatectomy the non-fasting utilization of injected glucose, as judged by urine (see Table 20) glucose excretion, dropped to 26 per cent. In the depancreatized fasting calf, by the same criterion the utilization appeared to be 69 per cent (see Table 19). Since this animal was fed before the collection of the postinjection urine sample the figure for utilization may be even higher than this. At any rate the diabetic calf apparently has a fair ability to utilize glucose.

Table 10

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Pre-operative

Calf - I
Test - I
Date - 8-16-44
Body weight - 54 kilos.
Glucose injected - 118 cc. 50 o/o; 59 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	83	0
1	300	0.75
2	230	1.25
3	96	2
4	70	3
5	52	4
6	72	5

Urine

Time	Glucose Mg./Kgm./day	Nitrogen Mg./Kgm./day	Acetone Mg./Kgm./day
Pre-inj.	8	70	0
Post-inj.	123	170	0

Pre-injection excretion	.450	gms./day
Post-injection	6.30	"
Difference	5.95	"
Gluc-injected	59.00	"
Apparent gluc-utilization	53.05	
Percentage	89 o/o	

Table 11

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Pre-operative

Calf - I
Test - II
Date - 8-21-44
Body weight - 60 kilos.
Glucose injected - 120 cc. 50 o/o; 60 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	81	0
1	180	0.5
2	116	1
3	62	2
4	60	3
5	78	4
6	76	5

Urine

Time	Glucose 24 Mg./kgm./hrs.	Nitrogen 24 Mg./kgm./hrs.	Acetone 24 Mg./kgm./hrs.
Pre-inj.	10	166	0
Post-inj.	84	163	0

Pre-injection excretion	.63 gms./day
Post-injection excretion	5.05 gms./day
Difference	4.42 gms./day
Gluc.-injected	60.00 gms./day
Apparent gluc-utilization	55.58 gms./day
Percentage	92 o/o

Table 12

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Pre-operative

Calf - I
Test - III
Date - 9-30-44
Body weight - 64 kilos.
Glucose injected - 120 cc. 50 o/o; 60 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	75	0
1	260	0.5
2	167	1
3	77	2
4	45	3
5	65	4
6	71	5

Urine

Time	Glucose Mg./Kgm./24 hrs.	Nitrogen Mg./Kgm./24 hrs.	Acetone Mg./Kgm./24 hrs.
Pre-inj.	10	109	0
Post-inj.	82	187	0

Pre-injection excretion	0.12 gms./day
Post-injection excretion	5.30 "
Difference	5.18 "
Gluc-injected	60.00 "
Apparent gluc-utilization	54.82 "
Percentage	91 o/o

Table 13

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Pre-operative

Calf - II
Test - I
Date - 9-12-44
Body weight - 40 kilos.
Glucose injected - 80 cc. 50 o/o

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	64	0
1	219	0.5
2	246	1
3	90	2
4	38	3
5	47	4
6	47	5

Urine samples lost through contamination

Table 14

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Pre-operative

Calf - II
Test - II
Date - 9-27-44
Body weight - 43 kilos.
Glucose injected - 90 cc. 50 o/o; 45 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	92	0
1	211	0.5
2	138	1
3	86	2
4	57	3
5	75	4
6	75	5

Urine

Time	Glucose 24 Mg./Kgm./hrs.	Nitrogen 24 Mg./Kgm./hrs.	Acetone 24 Mg./Kgm./hrs.
Pre-inj.	25	109	0
Post-inj.	105	372	0

Pre-injection excretion	1.08 gms./day
Post-injection excretion	4.50 "
Difference	3.42 "
Gluc-injected	45.00 "
Apparent gluc-utilization	41.58 "
Percentage	92 o/o

Table 15

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Pre-operative

Calf - III
Test - I
Date - 9-29-44
Body weight - 30 kilos.
Glucose injected - 80 cc. 50 o/o; 40 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	84	0
1	198	0.5
2	104	1
3	52	2
4	72	3
5	75	4
6	75	5

Urine

Time	Glucose Mg./Kgm./hrs.	Nitrogen 24 Mg./Kgm./hrs.	Acetone 24 Mg./Kgm./hrs.
Pre-inj.	140	263	0
Post-inj.	267	344	0

Pre-injection excretion	5.34	gms./day
Post-injection excretion	10.14	"
Difference	4.80	"
Gluc-injected	40.00	"
Apparent gluc-utilisation	35.20	"
Percentage	88 o/o	

Table 16

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Pre-operative - Following 138.5 hour fast

Calf - III
Test - Fasting
Date - 10-6-44
Body weight - 35 kilos.
Glucose injected - 80 cc. 50 o/o; 40 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	41	
1	265	0.5
2	224	1
3	176	2
4	151	3
5	125	4
6	100	5

Urine contaminated

Table 17

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Post-operative - Fasting

Calf - II
Test - Fasting
Date - 10-9-44
Body weight - 45 kilos.
Glucose injected - 90 cc. 50 o/o; 45 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	49	
1	297	0.5
2	250	1
3	210	2
4	191	3
5	182	4
6	171	5

Urine

Time	Glucose 24 Mg./Kgm./hrs.	Nitrogen 24 Mg./Kgm./hrs.	Acetone 24 Mg./Kgm./hrs.
Pre-inj.	302	122	0
Post-inj.	1780	040	0

Table 18

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Post-operative - Fasting

Calf - III
Test - Fasting
Date - 10-20-44
Body weight - 36 kilos.
Glucose Injected - 90 cc. 50 o/o; 45 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	27	
1	187	0.5
2	176	1
3	158	2
4	145	3
5	125	4
6	110	5
7	80	6
8	80	7
9	75	8

Table 19

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Post-operative

Calf - II
Test - I
Date - 10-5-44
Body weight - 44 kilos.
Glucose injected - 90 cc. 50 o/o; 45 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	255	
1	432	0.5
2	367	1
3	302	2
4	267	3
5	257	4
6	250	5

Urine

Time	Glucose 24 Mg./Kgm./hrs.	Nitrogen 24 Mg./Kgm./hrs.	Acetone 24 Mg./Kgm./hrs.
Pre-inj.	4220	35	0
Post-inj.	4960	35	0

Pre-injection excretion	190	gms./day
Post-injection excretion	224	"
Difference	34	"
Glucose injected	45	"
Apparent gluc-utilization	11	"
Percentage	26	o/o

Table 20

GLUCOSE TOLERANCE CURVE
Following I. V. Injection of Glucose
Post-operative

Calf - III
Test - II
Date - 10-23-44
Body weight - 36 kilos.
Glucose injected - 90 cc. 50 o/o; 45 gms.

Blood Glucose

Sample No.	Blood Glucose Mg. o/o	Time Hours
0	138	
1	343	0.5
2	224	1
3	220	2
4	187	3
5	169	4
6	154	5
7	130	6
8	112	7
9	91	8
10	70	9

Post Mortem Observations

Calves II and III were sacrificed on the 20th and on the 12th days following pancreatectomy. In both animals gross examination revealed that pancreatectomy was complete. The animals had lost weight probably due mostly to digestive disturbances resulting from the lack of the external secretion of the pancreas. They, however, seemed to have grown somewhat in stature. Their hair was rough and dull otherwise the animals were in fairly good condition and with cautious feeding and care no doubt could have been maintained for a long period. At no time was it necessary to use insulin to alleviate the diabetes of these animals. No animal showed symptoms of acidosis and no gross fatty changes were noted in the liver.

Quantitative analysis of liver and muscle glycogen indicated that the true glycogen values for muscle were .52 per cent and .78 per cent and the values for liver glycogen were 2.28 per cent and 2.55 per cent in calves II and III (See table 21). These values are all within the normal mammalian range although the liver glycogen is in the low part of normal. Unfortunately values for this in the calf have not been established so now it must be assumed that

these are normal. This is another indication of the mildness of the calf diabetes and of the ability of the pancreatectomized calf to establish a nearly normal equilibrium between income and outgo of glucose in the liver.

Table 21

POST MORTEM CHEMISTRY
Liver and Muscle Glycogen Determinations

Calf - II
Date - 10-19-44
Body weight - 37 kgm.

Fermentable Sugar Values

	Glucose equivalent	True glycogen values
Liver	2.45 o/o	2.28 o/o
Muscle	.56 o/o	.52 o/o

Calf III
Date - 10-24-44
Body weight - 35 kgm.

	Glucose equivalent	True glycogen values
Liver	2.76 o/o	2.55 o/o
Muscle	.85 o/o	.78 o/o

Summary

Experiments were carried out to determine the effects of pancreatectomy in the calf. Previous to operation normal control levels of the factors tested were established. This led to the conclusions that:

1. The average blood glucose for the normal calf was 81 mgm. per cent. This was lowered by fasting, but, due to the ability of the body to compensate for this rose slightly after the initial drop during the fasting period before its terminal fall.

2. Pancreatectomy caused a moderate rise in blood glucose. The level could be varied directly with food intake and could during fasting be dropped below the level for the fasting intact animal.

3. Urinary nitrogen excretion was not excessive in the pancreatectomized calf. It rose about 60 per cent above that for the normal animal. Nitrogen excretion in the normal calf increased as the blood glucose level decreased during a fasting period. It decreased, however, under similar circumstances in the pancreatectomized animal.

4. Ketonuria was totally absent before operation and only insignificant amounts of acetone were found in the urine following pancreatectomy.

5. Before removal of the pancreas slight traces of glucose were found in the urine. Following pancreatectomy urinary glucose excretion varied roughly with the food intake and could be reduced to zero during fasting.

6. Glucose tolerance, judged by tests following injection of 1 gm. of glucose/ kgm. of body weight, was lower in depancreatised than in normal animals. It was also lowered by fasting in both normal and operated animals. In the normal animal the utilisation seemed to be roughly proportional to the concentration of glucose. Ten per cent of the injected sugar was excreted by the normal animal, indicating almost total utilisation. Larger amounts were excreted by the operated and the fasting normal animals indicating decreased ability to utilise glucose following pancreatectomy and following fasting. These animals were, however, capable of utilising fair amounts of the injected glucose.

7. The animals suffered from digestive disturbances following operation and lost weight due to this. They seemed to be little disturbed by the pancreatectomy. They were sacrificed at about 2 and 3 weeks following operation for post-mortem analysis.

8. Terminal chemistry showed normal muscle and liver glycogen and no gross fat deposition in the liver.

9. These results indicate relatively low levels of endogenous protein and fat metabolism in this species following pancreatectomy. These effects are probably related to anterior pituitary function.

Appendix

APPENDIX OF METHODS

Somojyi - Shaffer - Hartmann method for glucose[45]

Reagents:

1. Zinc sulfate - sulfuric acid reagent:

   62.5 g. $ZnSO_4 \cdot 7 H_2O$

   156 cc. N H_2SO_4 (4.16 cc. conc. H_2SO_4)

   Dissolve and dilute to 5 liters with distilled water.

2. 0.75 N sodium hydroxide reagent.

   30 g. NaOH, dissolve and dilute to 1 liter with distilled water.

3. 1 o/o starch solution.

   1 g. soluble starch (Merck), dissolve and dilute to 100 cc. with saturated benzoic acid solution. Heat gently until starch is dissolved.

4. Potassium iodide - sodium oxalate reagent.

   2.5 g. KI

   2.5 g. Na-oxalate

   Dissolve and dilute to 100 cc. with distilled water.

5. Somojyi-Shaffer-Hartmann Reagent No. 50.[57]

   25 g. Na_2CO_3 (Anhyd.)

   25 g. Rochelle salt

   Dissolve in about 500 cc. distilled water, then add slowly with stiring -

20 g. $NaHCO_3$ (Anhyd.)

7.5 g. $CuSO_4 \cdot 5H_2O$/ Dissolve in about 100 cc. of water and add it to the above solution by a pipette or funnel that extends well below the surface of the liquid, stir while adding.

1 g. of KI, dissolve in water and add slowly with stirring.

Transfer to a volumetric liter flask and add:

200 cc. 0.1 N KIO_3 (this is the quantitative reagent).

Make up to 1 liter volume with distilled water.

Filter through washed dry filter paper.

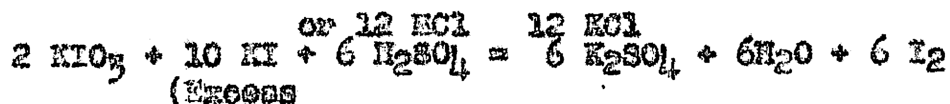
Notes on reaction and standardization of reagents:[43]

Blanks should titrate to end-point with 20 cc. of 0.005 N thiosulfate when 5 cc. of the reagent are used.

The KIO_3 is the most satisfactory reagent with which to standardize the thiosulfate reagent. Use Merck KIO_3.

200 cc. of 0.1 N KIO_3 per liter of reagent No. 50 permits analyses of glucose in 1:10 blood filtrates containing up to 400 mg. o/o of glucose; 250 cc. of KIO_3 up to 500 mg. o/o of glucose; 100 cc. of KIO_3 up to 200 mg. o/o. These solutions would be 0.020, 0.025, and 0.010 N KIO_3 in the final reagents.

The reaction involved is:



$$2\ KIO_3 + 10\ KI\ (\text{Excess}) + 6\ H_2SO_4\ (\text{or } 12\ HCl) = 6\ K_2SO_4\ (12\ KCl) + 6H_2O + 6\ I_2$$

1 liter of N iodate or biiodate contains 1/6 g. molecule of iodine.

KIO_3 = molecular weight 214.0
0.1 N = $\frac{21.4}{6}$ = 3.567 g. per liter

$KH(IO_3)_2$ = mol. wt. 389.97.
0.1 N sol. = $\frac{38.997}{12}$ = 3.250 g. per liter

Solution of either, if placed in a stoppered bottle and in a cool place, will retain its strength almost indefinitely.

Standardization of the 0.1 N thiosulfate.

$$2\ Na_2S_2O_3 + I_2 = 2\ NaI + Na_2S_4O_6$$

1 molecule of thiosulfate is oxidised by 1 atom of iodine, therefore a 1/10 molecular solution of thiosulfate is 0.1 N with respect to the relationship with I_2.

The molecular weight of crystalline thiosulfate is 248.2.

$$Na_2S_2O_3 \cdot 5H_2O$$

therefore: 24.82 g. of the salt dissolved and diluted to 1 liter makes a 0. 1 N solution.

Method of standardization.

To 25 cc. of the standard 0.1 N iodate or biiodate solution in an Erlenmeyr flask add about 10 cc. of a 10 o/o solution of KI and about 20 cc. of N H_2SO_4 or (HCl).

The thiosulfate is delivered into the acid iodate solution from a burette. When the iodine color has faded to a pale yellow 1 cc. of starch solution is added and the titration continued until the blue color of the starch-iodine has completely disappeared.

$$\frac{\text{cc. of the 0.1 N iodate}}{\text{cc. of thiosulfate}} = \text{Titration factor for 0.1 N thiosulfate}$$

Procedure for determining blood or urine glucose

Pipette the desired amount of blood or urine into a 50 or a 100 cc. Erlenmeyr flask. 5 cc. will yield more than enough filtrate for duplicate tests.

Add, for every 1 cc. of blood or urine, 8 cc. of the $ZnSO_4$-H_2SO_4 reagent, shake gently and let stand for five minutes.

Then add, for every 1 cc. of blood or urine 1 cc. 0.75 N NaOH, shake thoroughly (until the solution makes a "metallic" click against the side of the flask).

Filter. The filtrate should be water clear.

1. Pipette 5 cc. of the filtrate into a 25 cc. test tube with the pipette at the bottom of the tube.
2. 5 cc. of distilled water into a similar test tube in the same manner for a water blank. Run both the filtrate and the blank simultaneously as follows:
3. Pipette 5 cc. of Shaffer-Hartmann reagent No. 50, wash down the sides of the tube with this as it flows from the pipette.
4. Cover mouth of tube with a large marble.
5. Place the tubes in a wire compartment basket.
6. Place in a vigorously boiling water bath for exactly fifteen minutes.
7. Transfer basket and contents without agitation to a cold water bath, cool for 6 minutes, do not shake.
8. Add 2 cc. KI Na-oxalate solution - mix gently.
9. Add 5 cc. N H_2SO_4 and let stand for 5-10 minutes.
10. Titrate with . 005 N Thiosulfate solution, 5 cc. of 0.1 N thiosulfate diluted to 100 cc. with distilled water. Use a few drops of the starch solution for the indicator. During the titration keep well mixed by stirring with an up and down motion with a long stirring rod. Wash down the sides of the tube and rod with distilled water at intervals.

The end point is sharp, it occurs when the last trace of blue disappears.

Calculation

Reading of thiosulfate for blank	eg. 19.8 cc.
Less reading for unknown	eg. 16.2 cc.
Difference	3.6 cc. thiosulfate

Read from chart on following page = 84 mg. o/o

Somogyi modification- Shaffer-Hartman Blood Suger Method.
Filtrates 1-10; Zinc sulphate precipitates. J.A.D. 1/25/38.

	0	1	2	3	4	5	6	7	8	9
		2.4	4.8	7.2	9.6	12.0	14.4	16.8	19.2	21.6
1	24.0	26.3	28.6	30.9	33.2	35.5	37.8	40.1	42.4	44.7
2	47.0	49.3	51.6	53.9	56.2	58.5	60.8	63.1	65.4	67.7
3	70.0	72.3	74.6	76.9	79.2	81.5	83.8	86.1	88.4	90.7
4	92.0	94.2	96.4	98.6	100.8	103.0	105.2	107.4	109.6	111.8
5	114.0	116.2	118.4	120.6	122.8	125.0	127.2	129.4	131.6	133.8
6	136.0	138.2	140.4	142.6	144.8	147.0	149.2	151.4	153.6	155.8
7	158.0	160.2	162.4	164.6	166.8	169.0	171.2	173.4	175.6	177.8
8	180.0	182.2	184.4	186.6	188.8	191.0	193.2	195.4	197.5	199.8
9	202.0	204.2	206.4	208.6	210.8	213.0	215.2	217.4	219.6	221.8
10	224.0	226.2	228.4	230.6	232.8	235.0	237.2	239.4	241.6	243.8
11	246.0	248.2	250.4	252.6	254.8	257.0	259.2	261.4	263.6	265.8
12	268.0	270.2	272.4	274.6	276.8	279.0	281.2	283.4	285.6	287.8
13	290.0	292.2	294.4	296.6	298.8	301.0	303.2	305.4	307.6	309.8
14	312.0	314.2	316.4	318.6	320.8	323.0	325.2	327.4	329.6	331.8
15	334.0	336.2	338.4	340.6	342.8	345.0	347.2	349.4	351.6	353.8
16	356.0	358.2	360.4	362.6	364.8	367.0	369.2	371.4	373.6	375.8
17	378.0	380.2	382.4	384.6	386.8	389.0	391.2	393.4	395.6	397.8
18	400.0	402.2	404.4	406.6	408.8	411.0	413.2	415.4	417.6	419.8
19	422.0									

Formula for readings greater than 4 = (R x 2.2) + 4

Method for the Determination of Liver and Muscle Glycogen

Reagents:

1. 30 o/o KOH
2. 6 N HCl, use 498 cc. Conc. HCl per liter.

   83 cc. per liter of Conc. HCl = N HCl.
3. Phenol red -- 0.1 g. phenol red in 28.2 cc. 0.2 N NaOH diluted to 250 cc.
4. 0. 1 N $Na_2S_2O_3$; -- 25 g. of this salt in 1 liter of boiled distilled H_2O. Keep in dark bottle away from air CO_2. This can be used for titration in both glucose and glycogen determinations.

Procedure:

1. Weigh stoppered centrifuge tube containing 5 cc. 30 o/o KOH.
2. Drop about 2 g. of liver or muscle into the cold KOH; weigh quickly. The tissue should be cut up some and placed in KOH as soon as possible after removal from the animal.
3. Add 2 cc. of KOH for each g. of tissue over 2.5 g.
4. Hydrolyze in a boiling water bath until a homogenous solution is obtained.

5. Dilute about 2 times with water, mix, and then double the volume again with 95 o/o alcohol and mix thoroughly.
6. Heat carefully over open flame or in a boiling water bath until it just boils.
7. Let stand until contents come to room temperature.
8. Centrifuge for about 10 minutes.
9. Pour off supernatent fluid (waste).
10. Wash with 95 o/o alcohol, blow in from wash bottle, mix thoroughly.
11. Centrifuge again, pour off fluid (steps 10 and 11 may not be necessary).
12. Dissolve ppt. in hot water, using as little as convenient.
13. Add 6N HCl to make final concentration about 0.6 normal acid.
14. Hydrolyze in water bath for 2.5 to 3 hours. To prevent loss of volume through evaporation, use a straight tube condenser system, a tube about one foot long run through a rubber stopper is satisfactory.
15. Add 2 drops of phenol red as an indicator of pH.
16. Add 30 o/o KOH until phenol red just turns pink.
17. Titrate back to end point with 0.1 N HCl.
18. Transfer to 50, 100, 250 cc. volumetric flask (or larger if needed). Washings of tube must be added to flask.

19. Dilute to graduation mark of flask.

20. Take an aliquot - usually 5 cc. and run glucose determination.

21. Determine fermentable sugar by difference determination before and after yeast fermentation.

<u>Notes on calculation of muscle glucose and glycogen.</u>

Actual example:

Muscle sample ------------1.6 gms.
Dilution volume ---------50.0 cc.
Aliquot ---------------------- 5 cc.
Glucose determination - 80 mg. o/o *
(fermentable)

*Using table prepared for blood filtrates 1:10, and expressing values as mg./100 cc. blood. On basis of per 100 cc. of blood, this value is 10 x too high -- hence -

$\frac{\text{mg. o/o fer. glucose}}{10 \times 100 \text{ cc.}}$ = mg. fer. glucose per cc. of solution.

mg. glucose/cc. x 50 = total mg. fer. glucose in m. sample.

$\frac{\text{total glucose, mg.}}{1.6 \text{ gm. muscle}}$ = glucose in mg./ gram of muscle.

$\frac{\text{Total glucose, mg.} \times 100}{1.6 \text{ gm. muscle}}$ = mg. glucose/100 gms. muscle

$\frac{\text{Mg. glucose/100 gm. muscle}}{1000 \text{ mg./gm.}}$ = gms. glucose/100 gms. muscle or o/o glucose in muscle

Gm. Glucose x .927 = gms. glycogen

For these experiments this conversion was not necessary.

Putting these together in one formula:

$$\frac{\text{Mg. o/o for. glucose x Dil. vol., cc. x 100 gms. muscle}}{10 \times 100 \times \text{gms. muscle} \times 1000 \text{ mg./gm.}} =$$

o/o glucose in muscle.

$$\frac{80 \times 50 \times 100}{10 \times 100 \times 1.6 \times 1000} = 0.25 \text{ o/o glucose in muscle}$$

$$.927 \times .25 = 0.232 \text{ o/o glycogen in muscle}$$

or in short --

$$\frac{\text{Mg. o/o glucose x dil. vol. cc.}}{10 \times \text{wt. of muscle in mg.}} = \frac{80 \times 50}{10 \times 1600} = 0.25 \text{ o/o glucose in muscle}$$

If any other aliquot is used than 5 cc. then multiply by $\frac{5}{\text{aliquot}}$. If aliquot is 1 cc. $= \frac{5}{1}$ or $\frac{10}{2}$

$$\frac{80 \times 50 \times 5}{10 \times 1600 \times 1} = \frac{200}{160} = 1.25 \text{ o/o}$$

or more correctly if 1 cc. were used = 16 Mg. o/o

$$\frac{16 \times 50 \times 5}{10 \times 1600 \times 1} = \frac{400}{1600} = 0.25 \text{ o/o}$$

Yeast Fermentation Method[44]

Procedure:

1. Wash yeast as follows:

   Break up Fleishman's yeast in a suitable flask (about 1/4 pound is sufficient), add distilled water and mix thoroughly until the yeast is in a fine suspension. Transfer to centrifuge tubes. Centrifuge and decant the supernatant fluid. Repeat this washing several times. Thorough washing is essential!

2. Measure 6-7 cc. of a 20 o/o suspension of the washed yeast into a centrifuge tube.
3. Centrifuge.
4. Discard supernatant fluid.
5. Invert tube to drain for some second, and by means of a strip of filter paper remove the moisture adhering to the walls of the tube.
6. Introduce 12 - 14 cc. of the deproteinized filtrate to be fermented into the tube. Make total volume up to about 15 cc.
7. Stir with a glass rod and allow to stand at room temperature about 30 minutes.* Invert the tube occasionally to mix the contents.
8. Centrifuge.
9. Decant supernatant fluid at once into a clean receptacle save for tests.

This procedure without filtration yeilds over 10 cc. of perfectly clear sugar-free solution sufficient for two determinations of its reduction value.

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*Yeast and filtrate may be left for several hours at room temperature with no ill effects, but 10 minutes should be sufficient to cause fermentation of such quantities of sugar as are ordinarily encountered in the blood filtrates.

Kjeldahl Method for Determination of Nitrogen Content of the Urine[44]

Reagents:

1. 0.1 N H_2SO_4

   7 cc. of Conc. H_2SO_4 in 2.5 l of H_2O.

2. 0.1 N Na OH

   4.0005 g. NaOH per liter of water. Standardize by titrating with the 0.1 N H_2SO_4.

3. 20 o/o $CuSO_4$

   20 g. $CuSO_4$ in a 100 cc. volumetric flask, dilute to the mark.

4. Congo red

   Dissolve 2 g. Congo red in a little distilled water, dilute to 360 cc. Add 40 cc. ethyl alcohol.

Procedure:

1. Place 5 cc. of urine in a 500 cc. long-necked Pyrex Kjeldahl flask.
2. Add 20 cc. conc. H_2SO_4.
3. Add 2 cc. of the 20 o/o copper sulfate.
4. Boil in hood until the mixture becomes colorless, this requires about one hour.
5. Cool.

6. Dilute the contents of the flask with about 200 cc. of ammonia free water.
7. Add a little more of a concentrated solution of NaOH than is necessary to neutralize the H_2SO_4. The solution will turn dark brown at this point.
8. Add a few alumite chips to prevent "bumping" and a small piece of paraffin to prevent frothing.
9. Connect the flask with a condenser so arranged that the delivery tube passes into a receiving flask containing a few drops of Congo red indicator and a known volume of 0.1N H_2SO_4. The end of the delivery tube must reach beneath the surface of the fluid and the tube should be of large caliber to avoid "sucking back" of the fluid.
10. Shake the flask. Distill until the volume of the mixture has diminished to about half. (Until the flask begins to "bump".)
11. Titrate the partly neutralized 0.1 N H_2SO_4 solution with 0.1 N NaOH.

Calculation:

$$\frac{\text{cc. N } H_2SO_4 \text{ neutralized by } NH_3 \times .028 \times \text{total 24 hr. urine vol. in cc.}}{100 \times \text{Kg. body weight}} =$$

g. of nitrogen / kilo / day

Method for Determination of Acetone Bodies in the Urine[45]

Reagents:

1. 20 o/o $CuSO_4$
2. Calcium hydroxide powder
3. 50 o/o H_2SO_4 (18 N)
4. 0.02 o/o dichromate

   Dilute 20 cc. stock $K_2Cr_2O_7$ to 1 liter with water.
5. Salicylic aldehyde

   Dilute 20 cc. of salicylic aldehyde to 100 cc. with 95 o/o alcohol.
6. 40 o/o NaOH
7. Stock acetone

   1 gm. of C. P. acetone in a liter of distilled water.

Procedure:

1. Prepare filtrate:

   Add 50 cc. $CuSO_4$ to 50 cc. of urine, then add a few grams of soda lime and shake very thoroughly. Centrifuge.
2. Place an aliquot of the filtrate in a distilling flask.
3. Add 4 drops of 50 o/o H_2SO_4.

4. Distil for 3 - 4 minutes with the end of the condenser tube under 5 cc. of water in a 100 cc. volumetric flask used as a receiver.
5. Cautiously run 30 cc. of 50 o/o H_2SO_4 into the distilling flask through a dropping funnel.
6. Add dropwise 120 cc. of 0.02 o/o dichromate.
   The distilling time should be about 30 minutes and the dichromate should be all added by 10 minutes before the end of the distillation. The rate of addition of the dichromate and the height of the burner flame should be adjusted so that there are no "suck backs" and so that the receiving flask contains almost 100 cc. of the distillate at the end of 30 minutes.
7. Remove the receiving flask at the end of 30 minutes and dilute to the mark with washings from the condenser.
8. Using the 0.1 mg. per cc. acetone standard prepare "known" tubes with dilutions covering the probable range of the unknown, in this work 5 cc. of this standard was used per 100 cc. Thus giving a "known" value of 0.005 mg. of acetone per cc.
9. Place 5 cc. of the solution whose acetone content is to be determined in a test tube. Run the known standard in a similar manner simultaneously.

10. Add 4 cc. of 40 o/o NaOH.

11. Blow forcefully 1 cc. of salicylaldehyde into the tube.

12. Shake.

13. Place the tubes in a water bath at 45 - 50° C. for 20 minutes, shake occasionally.

14. Remove and cool for 1/2 hour.

15. Read rapidly on the Klett-Summerson photoelectric Colorimeter.

<u>Note</u> Since the color will continue to develop it is essential that the cooling time be standard and that the readings be done as rapidly as possible.

<u>Calculation:</u>

$$\frac{\text{Ru x 0.005 x 100 x vol. of urine}}{\text{Rs x 12.5 x Kg. body weight}} = \text{Mg. of acetone per kilo per day}$$

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