A STUDY OF THE INSULIN-LIKE ACTIVITY IN THE PANCREATIC JUICE OF THE SHEEP

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by

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INTRODUCTION

It is generally believed that insulin enters the blood stream after secretion from the beta cells of the islets of Langerhans. Before insulin can come in contact with the peripheral cells it must traverse the capillary basement membrane, the plasma membrane and the cytoplasm of the capillary endothelium upon entering and leaving the capillary. Then it seems reasonable that some insulin would traverse the pancreatic acinar cells and be released in the exocrine pancreatic secretion.

Further reasons for this hypothesis are that both the exocrine and the endocrine cells of the pancreas originate from the same area of the primitive foregut and secretions from both of these cell types are controlled in part by certain gastrointestinal hormones. The fact that insulin is widely distributed in various tissues and secretions supports this hypothesis.

If insulin is in the pancreatic juice it would raise several questions. What is its purpose for being in the pancreatic juice, how does it act in this regard, and is there any relationship to diabetes mellitus?

Pancreatic juice was collected from sheep by a new surgical procedure. The new procedure was devised since it was felt that existing procedures would not yield the desired unactivated pancreatic juice. The pancreatic juice was assayed

for insulin-like activity by a bioassay method. It was felt that the choice of a biological method of assay was desirable since some insulin-like activity of plasma is not assayable immunologically.

REVIEW OF THE LITERATURE

The Rat Diaphragm Method of Insulin Assay

The use of the isolated rat diaphragm to study the enhancement of glucose uptake by insulin was first introduced by Gemmill (1940). Since then many investigators have used the isolated rat hemi-diaphragm (HD) to estimate small quantities of insulin *in vitro* (Vallance-Owen and Hurlock, 1954; Randle, 1956; Wright, 1957; Cunningham, 1962; Jervell, 1965; Gjedde, 1968). The principle of the rat diaphragm method of insulin assay resides in the fact that rat HDs incubated in a balanced salt solution containing glucose and insulin have a greater glucose uptake than rat HDs incubated in the same medium lacking insulin. The increase in the glucose uptake by the HDs in the insulin containing medium as compared with the HDs in the medium lacking insulin has been termed the "insulin effect" (Vallance-Owen and Wright, 1960; Cunningham, 1962). By comparing the glucose uptake of HDs incubated in a solution containing a known amount of insulin with the uptake by HDs incubated in a solution with an unknown amount of insulin, the insulin-like activity (ILA) of the unknown solution can be calculated.

Specificity

More glucose uptake occurred in a balanced salt solution as the insulin concentration was increased (Vallance-Owen and Hurlock, 1954; Wright, 1960; Cunningham, 1962; Poffenbarger

et al., 1968; Gjedde, 1968). The addition of anti-insulin serum (AIS) inhibited the insulin effect (Poffenbarger, *et al.*, 1968; Gjedde, 1968). However, when serum was used in place of the salt solution, serum continued to exert ILA after the addition of AIS. The ILA that persists after the addition of AIS has been termed non-suppressible insulin-like activity (NSILA) by Froesch *et al.* (1963, 1966), and Solomon *et al.* (1967). The specificity of the rat diaphragm technique is dependent on unknown physiochemical properties of NSILA.

Albumin has been implicated in the promotion of glucose uptake by the rat HD and in the antagonism of the effects of insulin on the rat HD. Albumin prepared by trichloracetic acid-ethanol extraction from human serum promoted glucose uptake by the rat HD (Keen, 1963; Cameron *et al.*, 1964). The stimulatory effect of this albumin preparation was almost completely neutralized by AIS (Keen, 1963). He therefore concluded from this observation that the stimulatory effect of the albumin was due to insulin. Randle and Taylor (1958) prepared the albumin fraction of normal human serum by zone electrophoresis and column chromatography. Again AIS negated the stimulatory effects of albumin. Albumin has been reported to be antagonistic to the effects of insulin on the rat HD (Vallance-Owen *et al.*, 1958; Jervell, 1965). Jervell (1965) found that albumin extracted from normal human plasma by four commonly used methods, including trichloracetic acid-ethanol, were all antagonistic to the effects of added

insulin on the rat HD.

Other plasma components have been implicated from time to time in promoting glucose uptake by the rat HD. Randle (1954) reported plasma from acromegalic patients stimulated a greater glucose uptake than did plasma from normal subjects. Randle and Young (1956) reported that plasma from intact cats treated with growth hormone enhanced glucose uptake, whereas plasma from pancreatectomized cats that had been treated with growth hormone did not have this effect. This was interpreted as indicating that growth hormone promoted pancreatic insulin secretion. Taylor and Randle (1959) reported that the effects of insulin on glucose uptake were potentiated by adding the following agents _in vitro_: prolactin, corticotropin, normal guinea pig serum, and an albumin preparation from the serum of hypophysectomized rats. However, these agents alone did not promote glucose uptake.

The pancreatic proteases have been reported to have an effect on the metabolism of the rat diaphragm similar to insulin. Reiser and Reiser (1964) reported that insulin, trypsin, and chymotrypsin enhanced the uptake of D-xylose. Also, insulin, chymotrypsin, and ribonuclease were found to stimulate 3-O-methyl glucose uptake. Chymotrypsin stimulated the uptake of D-xylose and 3-O-methyl glucose considerably greater than did insulin, even though both insulin and chymotrypsin were present in the same molar concentration. The molar concentration was equal to 0.5 U insulin/ml. The

inactivation of chymotrypsin by diisopropylflurophosphate resulted in a loss of much of this stimulant effect. Also, all of the pancreatic proteases promoted glycogen deposition in the rat HD with trypsin as effective as insulin. Hines et al. (1965) reported finding an increased D-xylose uptake by the rat HD with high concentrations of chymotrypsin and trypsin. When the molar concentration of these enzymes was approximately equal to the molar concentration of physiological insulin, this did not occur. Pruitt et al. (1966) found that only chymotrypsin of the various pancreatic proteases and zymogens tested significantly enhanced glucose uptake when present in a molar concentration equal to 1.0 U insulin/ml. The stimulatory effect of chymotrypsin remained after the addition of AIS, thereby eliminating the possibility that the stimulatory effect might have been due to insulin contamination. Barnett and Whitney (1965) reported that trypsin and chymotrypsin did not enhance glucose uptake by the rat HD when these enzymes were present in a molar concentration equal to 0.5 U insulin/ml.

Randle and Smith (1958) found that the rat HD had a greater glucose uptake under anaerobic than aerobic conditions. They found that the glucose uptake from a medium anaerobically incubated with no insulin present was not different from a medium similarly incubated with 1000 μU insulin/ml. The following cell poisons were found to promote glucose uptake: 2:4-dinitrophenol, sodium arsenite, sodium arsenate,

sodium cyanide, and sodium salicylate.

Certain substances in a hyperosmolar concentration have been reported to promote glucose uptake by the rat HD. Kuzuya et al. (1965) reported that hyperosmolar concentrations of sucrose, mannitol, and saline enhanced glucose uptake to an extent equal to 1000 μU insulin/ml. Urea did not have this effect. Since urea is known to freely penetrate cells, this led the authors to conclude that the stimulatory effect by these other agents was due to an osmotic effect.

Increasing concentrations of glucose have been associated with an increased glucose utilization by the rat diaphragm (Gemmill and Hamman, 1941; Stadie et al., 1951). These authors demonstrated that increasing glucose concentrations were associated with greater glycogen deposition in the rat HD. Oyama and Grant (1960) reported that the mouse HDs had greater glucose uptakes as the glucose concentration rose from 0.5 to 4.0 mg glucose/ml incubation medium.

Materials and methods of other investigators

Vallance-Owen and Wright (1960) stated with regards to the rat diaphragm method that: "Only by rigorous adherence to a standardized procedure has it been possible to obtain reproducible results." There are two apparent reasons peculiar to this assay system that warrant this statement. First, diaphragms incubated in buffer without insulin have

a large glucose uptake when compared with the increase in glucose uptake caused by the insulin. Secondly, large changes in insulin concentrations result in a small biological effect.

The synthetic incubation media most extensively used have been Gey and Gey (1936) and Kreb's-Ringer bicarbonate buffer (KRB) (Umbreit *et al.*, 1964). These two incubation media have a mineral composition closely resembling blood plasma and are buffered like plasma by a sodium bicarbonate carbonic acid system. Oyama and Grant (1960), using mouse HD tissue to assay insulin, demonstrated that increasing the bicarbonate concentration of the incubation medium resulted in an increase in glucose uptake, but increases in potassium or magnesium concentration had a depressing effect on glucose uptake.

The pH of the incubation media was normally 7.4 (Kuzuya *et al.*, 1965; Davidson and Goodner, 1966; Gjedde, 1968). Gassing of KRB with 95 percent O_2 and five percent CO_2 was found to yield a pH of 7.4 (Umbreit *et al.*, 1964). Jervell (1965) observed that lowering of the pH below 7.4 resulted in a decrease in both basal and insulin stimulated glucose uptake.

Albumin and gelatin have been used as carriers for insulin in the incubation media. Wiseman and Baltz (1961) demonstrated that ^{131}I-insulin readily adsorbed to glassware, but human serum albumin prevented this loss. Twenty and

nine-tenths of the ^{131}I-insulin in a solution was found to adhere to glass beakers, but a threefold drop in this loss was found by using human serum albumin at a concentration of 0.1 mg/ml. Little additional protection was found above a concentration of 0.5 mg human serum albumin/ml where 95.8 percent of the ^{131}I-insulin was recovered. Cunningham (1962) found that the addition of 2.0 mg gelatin/ml to a solution containing insulin resulted in a greater glucose uptake. The gelatin must have prevented the adsorption of insulin to the glassware since the gelatin had no effect upon basal glucose uptake.

The glucose concentration normally used was 3.0 to 4.0 mg/ml incubation media (Vallance-Owen and Hurlock, 1954; Devlin and Flahavan, 1963; Ashton, 1965; Gjedde, 1968).

Either a crystalline ox insulin (Cunningham, 1962) or a crystalline porcine insulin (Shaw and Shuey, 1963; Gjedde, 1968) were generally used as an insulin standard. An insulin stock solution was prepared by dissolving the insulin in a weak acid. This was regarded as stable for three weeks (Wright, 1957) to several months (Cunningham, 1962). Serial dilutions were made from this stock solution for use in the assay. These serial dilutions were either made up fresh immediately before each assay (Wright, 1957; Randle and Smith, 1958) or were regarded as stable for several days (Gjedde, 1968).

Rats of the same sex and approximate weight were

generally used. Many investigators used a male albino rat in the weight range of 110 to 150 g that had been starved 14 to 24 hours before used (Shaw and Shuey, 1963; Jervell, 1965; Gjedde, 1968). Vallance-Owen and Wright (1960) stated that the diaphragms of larger rats were found to be less sensitive to insulin, weight for weight, than those of smaller rats. A variance was found in the glucose uptake between the diaphragms of two strains of Wistar rats (Wright, 1957).

The apparatus for incubating the flasks containing the HDs has been either a Warburg apparatus (Wright, 1957; Devlin and Flahavan, 1963) or a Dubnoff metabolic shaker (Shaw and Shuey, 1963; Kuzuya *et al.*, 1965; Davidson and Goodner, 1966).

Chronological procedure

The rats were killed and the diaphragms were dissected out immediately with a minimum of trauma. Since blood clots on the diaphragms appeared to result in variable glucose uptakes, the diaphragms were dissected with care.[1] Gjedde (1968) gave a detailed description of the dissection of the diaphragm. He first removed the falciform ligament and then pierced the central tendon of the diaphragm which allowed the diaphragm to balloon out. Finally he cut the diaphragm free from its costal insertions, using a pair of iris

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[1] Walter N. Shaw, Eli Lilly and Co., Indianapolis, Indiana. Bioassay of insulin. Private communication. 1969.

scissors, while the HD was fixed with a fine pair of forceps. Some investigators further subdivided the HDs (Krahl and Cori, 1947; Groen et al., 1952). Groen et al. (1952) found quarter diaphragms had a greater glucose uptake than HDs, weight for weight, when insulin was not present, but HDs had a greater glucose uptake than quarter diaphragms in the presence of insulin. In contrast Reiser and Reiser (1964) used the intact diaphragm attached to its costal attachments to measure glucose uptake which they called "caged" diaphragms.

Following excision the HDs were placed in a solution for a period of time called preincubation. Brown et al. (1952) demonstrated that soaking the HDs in a Kreb's-Ringer bicarbonate buffered solution with a glucose concentration of 1.4 mg/ml at $0^{\circ}C$ for a period of 15 minutes resulted in a glucose uptake during incubation that was twice as great as unsoaked tissue. Others preincubated the HDs in an ice cold buffer without glucose (Groen et al., 1952; Wright, 1957; Cameron et al., 1964; Kuzuya et al., 1965; Gjedde, 1968). The length of preincubation has been specified as lasting 15 minutes (Kuzuya et al., 1965; Gjedde, 1968).

At the end of preincubation the HDs were transferred to the incubation media. During transferal Gjedde (1968) blotted and weighed the HDs, but others waited until the end of the incubation period to weigh the HDs (Wright, 1957; Randle and Smith, 1958; Cunningham, 1962). Vallance-Owen

and Hurlock (1954) contended that drying the HDs at 105°C for two hours prior to weighing allowed for a more accurate determination of glucose uptake. However, Randle (1956) and Bingle (1963) found a good correlation between wet and dry weights of the HDs which did not support the contention that drying allowed for a more accurate determination of glucose uptake. Vallance-Owen and Wright (1960) stated that diaphragms incubated in a synthetic buffered media contained 75 percent (weight/weight) water.

A difference in glucose uptake has been reported between the two halves of a diaphragm. The right HD in the presence of insulin was found to have a significantly greater glucose uptake than the left HD (Bingle, 1963; Davidson and Goodner, 1966). In basal media they found that the right HD again had a greater glucose uptake than the left, but the difference was not significant. In contrast to this, Devlin and Flahavan (1963) found that the left HD had a greater glucose uptake than the right HD in basal media, but the difference was not significant. The fact that this difference has been reported should influence schemes for distributing HDs to the incubation solutions.

Different schemes for distributing the HDs to the incubation solutions have been employed. Stadie _et al_. (1951) and Keen (1963) incubated one half of each diaphragm in a buffer plus insulin or the material to be assayed. The other half of the diaphragm was incubated in a buffered

media. In these instances the insulin effect was determined by subtracting the glucose uptake of the HD which was incubated in the buffered media from the glucose uptake of the HD which was incubated either in the insulin containing buffer or the material to be assayed. This distribution scheme paired one half of the diaphragm against the other half of the diaphragm. Wright (1960) and Gjedde (1968) distributed the HDs without respect to pairing with the stipulation that no two HDs from the same rat were to be incubated in the same solution. Cunningham (1962) distributed six HDs in basal media and six HDs in the solutions to be tested without respect to origin. Davidson and Goodner (1966) incubated each HD serially in both the basal and insulin solution. In this way each HD acted as its own control. Groen _et al_. (1952) distributed eight quarter diaphragms from eight different rats to each solution.

The flasks containing the diaphragms were normally gassed with a mixture of gas containing 95 percent O_2 and five percent CO_2 for a period of two to five minutes immediately before incubation. The manner of gassing has varied. Jervell (1965) gassed the Erlenmeyer flasks through their stoppers by way of injection needles, whereas Randle and Smith (1958) and Gjedde (1968) gassed the incubation media before stoppering the flasks.

The duration of incubation has varied. Randle and Smith (1958) used a 60 minute incubation period; but

Cunningham (1962), Cameron _et al_. (1964), Kuzuya _et al_. (1965), and Jervell (1965) used a 90 minute incubation period. On the upper extreme Randle (1956) and Gjedde (1968) used a 180 minute incubation period. Brown _et al_. (1952) found by transferring the diaphragms to successive media every 30 minutes that the glucose uptake with and without insulin decreased with time. At the end of 90 minutes the insulin treated diaphragms were still consuming more glucose than the non-insulin treated diaphragms. However, Devlin and Flahavan (1963), in carrying out a similar experiment over 210 minutes, found that the insulin stimulant effect was over in 90 minutes.

All investigators agitated the flasks during incubation. Brown _et al_. (1952) found that the glucose uptake increased in a linear fashion with an increased shaking rate. Generally the shaking rate has varied with the investigator, but in most cases it was in the range of 80 to 110 oscillations/minute (Cameron _et al_., 1964; Jervell, 1965; Kuzuya _et al_., 1965; Gjedde, 1968).

Automated determination of glucose has been used with acceptable precision. Cameron _et al_. (1964) and Davidson and Goodner (1966) reported that duplicate determinations agreed within two percent using the alkaline ferricyanide-reduction method of Hoffman (1937) as adapted for the AutoAnalyzer.[1]

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[1]Technicon Corp., Tarrytown, N.Y.

Comparison of glucose uptake values

Comparison of uptake figures given by various authors is difficult not only because of the variations in technique, but also because of the difference in units used. Randle (1956) and Cunningham (1962) reported glucose uptake values per unit of weight per hour irrespective of the period of incubation used, assuming that glucose uptake occurs at a constant rate. Others report glucose uptake values per unit of weight per 90 minutes (Cameron *et al.*, 1964; Wright, 1957; Jervell, 1965) or 180 minutes (Gjedde, 1968). Both dry and wet weights of the HDs have been used in calculating the glucose uptake, but a comparison of these uptake values was possible assuming that HDs incubated in a buffered media contained 75 percent water (Vallance-Owen and Wright, 1960).

Cunningham (1962) stated that basal uptake generally ranged from 2.0 to 2.8 mg glucose/g diaphragm/hour. Bingle (1963) reported a similar value with a 90 minute period of incubation. These two authors reported the lowest basal glucose uptake values. On the upper extreme Devlin and Flahavan (1963) reported a basal glucose uptake of 12.25 μg glucose/mg diaphragm/90 minutes. There are several authors that reported a basal glucose uptake between these two extremes. Vallance-Owen and Wright (1960) referencing unpublished results by P.H. Wright reported a basal glucose uptake of 7.75 mg glucose/g diaphragm/90 minutes. This value is comparable to values reported by Gjedde (1968) who

reported a basal glucose uptake of 6.67 mg glucose/g diaphragm/180 minutes and Jervell (1965) who reported a basal glucose uptake of 6.43 mg glucose/g diaphragm/90 minutes. Wright (1957) reported that strain A Wistar rat had a basal uptake of 7.25 µg glucose/mg diaphragm/90 minutes, whereas strain B had a basal uptake of 8.35 µg glucose/mg diaphragm/90 minutes.

There was a reported daily difference in basal glucose uptake. Vallance-Owen and Hurlock (1954) stated that day to day variation in the basal glucose uptake made construction of a standard bioassay curve impossible. However, Vallance-Owen _et al_. (1955) stated that although there was considerable day to day variation in the basal glucose uptake, the difference in glucose uptake when no insulin was added compared to a known quantity of added insulin was more constant. This difference was the insulin effect.

Vallance-Owen and Wright (1960), referencing unpublished results by P. H. Wright, reported that the insulin effect at 100 µU insulin/ml was 3.40 mg glucose/g diaphragm/90 minutes and the insulin effect at 1000 µU insulin/ml was 6.08 mg glucose/g diaphragm/90 minutes. Gjedde (1968) reported an insulin effect at the same two insulin concentrations respectively of 3.57 µg glucose/mg diaphragm/180 minutes and 6.62 µg glucose/mg diaphragm/180 minutes. Cunningham (1962) reported an insulin effect using the same insulin concentrations respectively of 2.24 mg glucose/g

diaphragm/hour and 4.24 mg glucose/g diaphragm/hour. So a large increase in insulin concentration produced a relatively small increase in the insulin effect.

Precision and sensitivity

The rat diaphragm method of insulin assay appears to be fairly sensitive but not precise. Several investigators have reported they could effectively assay 10 μU insulin/ml (Vallance-Owen et al., 1955; Wright, 1957; Cunningham, 1962; Cameron et al., 1964). Groen et al. (1952) found that variations in sensitivity were encountered so that the minimum detectable concentration varied from 5 μU/ml to 500 μU/ml. Randle (1956) reported that the sensitivity varied with the minimum detectable concentration varying between less than 125 to 500 μU/ml. The variation in sensitivity has been reported to have been seasonal with the rat least sensitive in the summer (Groen et al., 1952; Vallance-Owen and Hurlock, 1954). Randle (1956) concluded on the basis of marked deviations in slope and in the index of precision that the rat diaphragm method of insulin assay is not very precise. Gjedde (1968) excluded 42 percent of his experiments because the index of precision exceeded 0.35 while using the rat diaphragm method of insulin assay.

The Distribution of Immunoreactive Insulin and Insulin-Like Activity

ILA has been reported to be present in various tissues and secretions other than plasma or serum. Solomon et al. (1967) reported ILA and NSILA to be present in rat tissue extracts. Their method of ILA assay was the rat epididymal fat pad method and NSILA was measured in the presence of an excess of AIS. Rat pancreas, lung, kidney, gastrointestinal tract, brain, diaphragm, heart, spleen, liver, and thymus all had measurable amounts of ILA and NSILA. NSILA from human serum was added to control tissues that were subsequently extracted to quantitate recovery procedures. Recovery of the NSILA was found to be quite variable (25 to 35 percent). This precluded the quantitative comparison of the content of NSILA in the various tissues. They did state that skeletal muscle and liver contained the greatest amount of NSILA. The radioimmunoassay of insulin on these various tissue extracts revealed immunoreactive insulin (IRI) to be present in all tissues except the thymus and the spleen.

Partamian et al. (1966) found ILA in rat lymph using the rat epididymal fat pad method. They also reported that IRI was present in rat lymph. However, using the intraperitoneal assay, measuring the incorporation of $^{14}C_1$-glucose into diaphragm and adipose tissue failed to reveal ILA to be present in rat lymph. Rasio et al. (1965) found pooled

rat lymph samples contained only 30 percent as much ILA as corresponding pooled rat serum samples, but 80 percent as much IRI was found in lymph pools as serum pools. IRI increased promptly and similarly in both serum and lymph following a rapid intravenous injection of glucose, but the ILA only rose significantly in the serum.

IRI and NSILA have been reported to be present in bile. Daniel and Henderson (1967) reported gallbladder bile from the rabbit contained a mean of 1780 μU IRI/ml bile while hepatic bile contained a mean of 35.8 μU IRI/ml bile. This was a concentration of IRI in gallbladder bile of nearly 50 times, while other constituents in gallbladder bile are normally three to four times as concentrated. On this basis they stated that this suggested that either the gallbladder secreted insulin or some differential process of reabsorption was taking place. Lopes-Quijada and Goñi (1967) reported IRI to be present in the bile of the following species: man, cat, dog, ox, rabbit, rat, swine, sheep, and chicken. They reported that the gallbladder bile in the rabbit had almost nine times as much insulin as did the hepatic bile. They found that IRI in bile rose markedly following glucose administration in both the dog and rabbit. Quijada and R-Candela (1967) reported NSILA to be present in the bile of rabbits. Although this NSILA was not absolutely quantitated, it was reported to be less than 50 μU/ml bile.

ILA (Lieberman, 1962) and IRI (Rubenstein <u>et al</u>., 1967)

have been reported to be present in urine. Rubenstein et al. (1967) reported the mean IRI level in the urine of 28 normal human subjects to be 215 μU insulin/hour. Two hours following a glucose tolerance test the level rose to a median of 834 μU insulin/hour.

IRI has been reported to be present in small concentrations in the aqueous humor and in the cerebrospinal fluid of the rabbit and the rhesus monkey (Daniel and Henderson, 1967).

The Interrelationship of Intestinal Factors with the Exocrine and Endocrine Pancreas

It is generally accepted that the normal stimulus for insulin synthesis and release in non-ruminants is glucose perfusing the islets of Langerhans (Field, 1964; Williams and Ensinck, 1966). Metz (1960) demonstrated that ILA output as measured by rat diaphragm method is a continuous positive function of the concentration of glucose in the blood. An approximate fourfold increase in blood glucose (70 to 300 mg/100 ml) resulted in the original ILA output of 7 mU/minute from the pancreaticoduodenal vein being increased to 70 mU/minute.

Orally administered glucose has been found to result in greater plasma insulin levels than intravenous dosage of the same amount of glucose (Perley and Kipnis, 1965). Elrick

et al. (1964) found higher blood glucose levels resulted from the intravenous administration of glucose as opposed to the same dosage given intragastrically, but intragastric glucose prompted a greater and more sustained rise in plasma insulin levels. McIntyre et al. (1965) found that the intrajejunal administration of glucose produced higher plasma insulin levels as compared with the intravenous administration of glucose. The glucose was purposely infused into the jejunum so that there would not be a higher concentration of glucose reaching the pancreas than other parts of the circulation. This suggested that factors other than circulating blood glucose are involved in plasma insulin release. As evidence for an intestinal factor being involved in glucose metabolism, Dupré (1964) found secretin injections accelerated the disappearance of intravenous glucose.

Short chained volatile fatty acids have been found to stimulate insulin release in sheep (Manns et al., 1967 Horino et al., 1968; Hertelendy et al., 1969). Short chained volatile fatty acids did not stimulate insulin release in non-ruminants (Horino et al., 1968). Normal rumen metabolites such as propionate, butyrate, valerate, and isovalerate when infused peripherally at a rate in the range of the normal volatile fatty acid influx significantly increased plasma insulin levels (Horino et al., 1968). This happened without producing a concomitant significant increase in the blood glucose levels. In fact, glucose was

found to be much less effective in raising plasma insulin levels in sheep than were the volatile fatty acids. Manns *et al*. (1967) found glucose infusions which raised the blood glucose to the maximum normal range for sheep did not produce significant increases in insulin levels. They also reported that blood glucose increased over a two to ten hour post feeding period, but in only one of four identical studies was the blood glucose increase accompanied by a significant increase in insulin concentrations. Manns *et al*. (1967) reported that infusions of butyrate and propionate into the anterior mesenteric vein of sheep, thereby allowing direct access to the pancreas, caused significant increases in plasma insulin levels. Hertelendy *et al*. (1969) found that the functional monogastric newborn lamb responded to propionate and valerate infusions by releasing insulin. This indicated that the response to the volatile fatty acids in sheep is a constitutional characteristic present at birth.

Several investigators have reported that certain gastrointestinal hormones are betacytotropic (Unger *et al*., 1967; Jarrett and Cohen, 1967; Meade *et al*., 1967; Baile *et al*., 1969). Rapid endoportal injections of secretin, pancreozymin, and gastrin in dogs produced striking increases in the plasma insulin concentration from the pancreaticoduodenal vein (Unger *et al*., 1967). This increase was of a one minute duration. Likewise, human subjects were found to respond to secretin injections with a brief, significant rise in plasma

insulin levels (Jarrett and Cohen, 1967). Meade et al. (1967) found that pancreozymin administered peripherally to dogs produced higher plasma insulin concentrations than glucose dosages that raised the blood glucose to a mean of 396 mg glucose/100 ml plasma. Pancreozymin itself had no effect on blood glucose levels. Goats failed to respond to secretin injections by increasing serum insulin levels, but serum insulin levels did significantly increase in goats following cholecystokinin-pancreozymin administration (Baile et al., 1969). However, pancreozymin, secretin and gastrin failed to stimulate insulin release from rat pancreatic slices (Lazarus et al., 1968).

One of the difficulties in trying to assess the effects of the gastrointestinal hormones is that their physiological blood levels are unknown (Lazarus et al., 1968). Another difficulty is relating the weight of the hormone to its activity. For example, a highly purified secretin preparation has been estimated to contain anywhere from 4330 to 17,500 U/mg.

The role of insulin in intestinal transport of sugars is inconclusive. One U insulin/ml added to the serosal side of rabbit jejunum *in vitro* facilitated the transport of 3-O-methyl glucose from the mucosal to the serosal side (Fromm et al., 1968). The transport was facilitated to a 25 percent greater extent than in controls. However, there was no facilitation of transport reported using rabbit

ileum, nor did insulin promote L-alanine transport in either membrane. Singleton (1969) in his review article concluded that insulin appears to have little effect upon intestinal absorption in physiologic doses and only in special *in vitro* conditions and in greatly increased dosages does insulin have an effect on intestinal glucose transport.

Harper (1959) concluded in his review article that evidence for neural influences on the exocrine pancreas is incomplete and inconclusive. Taylor (1962) found that stimulation of the cervical vagus in the anesthetized or decerebrate sheep caused a brief increase in the fluid secreted by the pancreas, but section of one vagus and stimulation of its central end did not result in an increase in pancreatic secretion. Section of both vagi in the anesthetized sheep did not result in a fall in the pancreatic secretion. Pilocarpine nitrate injections increased sheep pancreatic secretions (Magee, 1961; Taylor, 1962), and this effect of pilocarpine was blocked by atropine sulfate (Taylor, 1962).

Wang and Grossman (1951) demonstrated that the pancreatic response in digestion is dominated by secretin and pancreozymin. They transplanted pancreases in dogs from their normal location to a subcutaneous location. These preparations responded to normal pancreatic stimulants placed in the duodenum such as acid, fats, and peptone with great increases in pancreatic juice secretion. The response to these stimulants was not blocked by atropine. Likewise

ruminants responded with increased pancreatic secretion to the intestinal hormones. Intravenous secretin promoted pancreatic secretion in sheep (Magee, 1961; Taylor, 1962). Goats responded with dramatic increases in pancreatic juice secretion following intravenous secretin and cholecystotinin-pancreaozymin (Baile et al., 1969). The importance of the intestinal phase of digestion on pancreatic secretion is emphasized by the finding that prevention of the abomasal contents in sheep from entering the duodenum resulted in a 30 percent decrease in pancreatic juice secretion (Taylor, 1962).

Waddell et al. (1968) spoke of the portion of the gut immediately above and below the ampulla of Vater, including the antrum, duodenum, and pancreas, as one endocrine organ. He cited three features the endocrine products from this area have in common. The hormones from this area are all small peptides which are concerned with the regulation of digestion, absorption, and utilization of food with all of the endocrine secretory cells of this region arising from a limited area of the primitive foregut.

Collection and Composition of Sheep Pancreatic Juice

Sheep, along with goats and deer, have the anatomical peculiarity in that the pancreatic secretion is directed into the duodenum through the common bile duct (Mann et al., 1919-

1920). The sheep pancreatic duct is about 0.1 to 0.3 cm in diameter and is embedded in pancreatic tissue. The pancreatic duct joins the common bile duct about 6 cm from the opening of the latter into the duodenum. No accessory pancreatic ducts were found in sheep (Taylor, 1960). The common bile duct in sheep is the result of unification of the hepatic and cystic ducts. It was found to have a median length of 10.6 cm and an average diameter of 4.6 mm (Mann et al., 1920)

Direct cannulation of the common bile duct in sheep yields a mixture of bile and pancreatic juice. Therefore, collecting pancreatic juice in sheep necessitated ligating the common bile duct above its junction with the pancreatic duct and providing an alternative channel for the bile (Hill and Taylor, 1957; Taylor, 1960; Magee, 1961; Phaneuf, 1961). Taylor (1960) constructed an alternative channel for bile by transecting the jejunum and anastomosing the caudal cut end of the jejunum with the gallbladder. The anastomosis was effected by a screw type device. The side-arm of the jejunum carried the bile away by its peristalic movements. Continuity of the intestine was restored by an oblique-to-side anastomosis of the jejunum. Hill and Taylor (1957) constructed a side-arm of jejunum to reroute the bile as Taylor (1960) did, except they connected the gallbladder with the side-arm of jejunum by means of a length of polythene tubing. Phaneuf (1961) drained the bile from the gallbladder

into the abomasum by means of a polyethylene cannula secured in both structures. This cannula was the weakness in his procedure as it ultimately became obstructed with chyme from the abomasum and consequently bile leaked from the gallbladder. Kuimov (1953 cited in Phaneuf, 1961) cannulated the gallbladder of sheep with much success. He drained bile directly to the exterior before the bile was rerouted into the duodenum.

The placement of cannulas for collection of pancreatic juice has been done two ways. Taylor (1960) placed a cannula in the duodenum in a position opposite the opening of the common bile duct. A flexible catheter was passed through the exterior opening of this cannula into the opening of the common bile duct for collection of pancreatic juice. The catheter was connected by tubing to a container strapped to the animal's side for collection. A screw type cap was placed over the end of the duodenal cannula when pancreatic juice was not being collected. Phaneuf (1961) isolated a short segment of the duodenum receiving the common bile duct and placed a rubber cannula in this segment. The ends of this isolated segment were made into stumps and the continuity of the gastrointestinal tract was reestablished by an end-to-side anastomosis of the remaining duodenum. Another rubber cannula was placed in the reestablished duodenum for retrieving uncollected pancreatic juice. The ends of the rubber cannulas were exteriorized, and were

connected by glass tubing for returning pancreatic juice. A centrifuge bottle connected to the cannula draining the isolated duodenal segment was suspended under the sheep's neck for collection of pancreatic juice.

The measurement of ILA in sheep pancreatic juice in this study involved a comparison of the glucose uptake by the half of the diaphragm which was incubated in sheep pancreatic juice versus the glucose uptake by the other half of the diaphragm which was incubated in KRBG. Therefore, it is relevant to compare the electrolyte composition of these two solutions (Table 1).

Table 1. Comparison of the electrolyte composition of sheep pancreatic juice and Kreb's-Ringer bicarbonate

	Sheep pancreatic juice[a] (mEq/L)	Kreb's-Ringer bicarbonate[b] (mEq/L)
Na	135 - 165	144.1
K	3.9 - 5.4	6.0
Cl	110 - 126	128.8
HCO_3	15 - 30	25.1
Ca	4.0 - 5.7	5.1
Mg	0.66 - 1.52	2.49

[a]Data obtained from Taylor (1962).

[b]Calculated from Umbreit et al. (1964).

The relative proportions of the proteins in bovine pancreatic juice were given by Keller et al. (1958). A ten percent unidentifiable protein fraction was reported after quantitation of the various enzymes. Comparable literature was not found which discusses the complete protein composition of sheep pancreatic juice.

MATERIALS AND METHODS

Collection and Handling of Sheep Pancreatic Juice

Description of the cannulas

A pancreatic, gallbladder, and an abomasal cannula were prepared (Figure 1). The gallbladder and the abomasal cannulas were constructed by attaching a modified Zeman (1966) type cannula to the tubing. The ends of these cannulas were usually fenestrated so that obstruction to flow would be less likely.

Vinyl tubing[1] (0.067" ID and 0.107" OD) was used for the pancreatic cannula. It had a length of approximately 50 cm. A rectangular piece of Silastic® [2] sheeting was sutured to the cannula immediately posterior to a short fenestrated portion. The sheeting was used to hold the cannula in place. A stylet of vinyl tubing marked for the length of the pancreatic cannula was made and inserted into the pancreatic cannula.

The abomasal cannula consisted of Silastic® [2] tubing (0.125" ID and 0.250" OD) which had a modified Zeman (1966) type cannula attached. The modified Zeman (1966) type cannula had a length of 4.75 cm. In the first sheep prepared the Silastic® [2] tubing did not protrude beyond the cannula,

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[1] Becton, Dickinson and Company.

[2] Dow Corning, Medical Products Division, Midland, Mich.

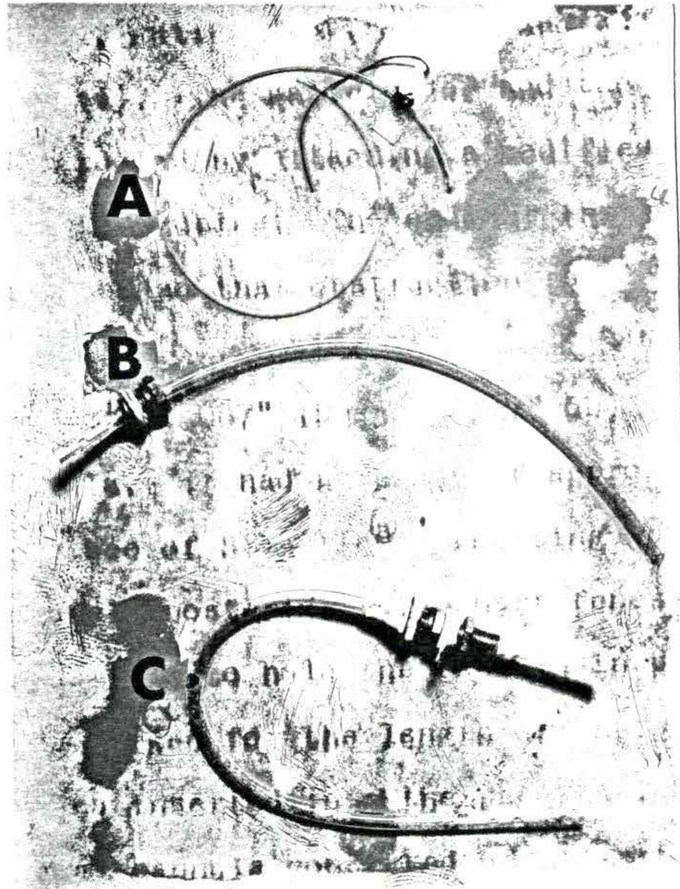

Figure 1. The surgical cannulas

A. Pancreatic cannula

B. Gallbladder cannula

C. Abomasal cannula

but in the second sheep that was prepared the tubing went through the cannula and beyond for a distance of 36 cm. The Silastic® tubing was secured to the cannula by Medical Silastic® 382 Elastomer[1] with approximately 7.0 cm of tubing left before the entrance into the cannula.

The gallbladder cannula consisted of vinyl tubing (0.250" ID and 0.375" OD) that was secured to a modified Zeman (1966) type cannula by white silicone rubber[2]. The fenestrated end of the vinyl tubing protruded 4.0 cm beyond the end of the cannula. In this instance the modified Zeman (1966) type cannula was shorter than the one used in the abomasal cannula. It had a length here of 1.5 cm.

Preparation of the sheep for surgery

Crossbred ewes were purchased locally. They were housed on concrete and fed pelleted alfalfa with corn and alfalfa hay.

The sheep were fasted for 24 to 48 hours before surgery. Approximately 40 mg of chlorpromazine[3] were given intravenously as a preanesthetic agent 15 to 30 minutes before surgery. Sodium pentobarbital was given to induce anesthesia and afterwards the animal was placed in left lateral recumbency. The anesthetic plane was maintained by giving

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[1]Dow Corning, Medical Products Division, Midland, Mich.

[2]General Electric, Waterford, New York.

[3]Thorazine®, Smith, Kline and French, Philadelphia, Pa.

sodium pentobarbital through a polyethylene cannula that was placed in the jugular vein. When the anesthetic agent was not being given, isotonic saline with approximately 250 mg of oxytetracycline[1]/L saline was slowly infused. Finally, an endotracheal tube and a stomach tube were fixed in position.

Surgery

The abdomen was opened by a long paracostal incision in the right flank approximately 2.5 cm posterior to the last rib. The viscera was retracted and the gallbladder located. A purse string suture was placed in the fundic end of the gallbladder using 2-0 silk. Care was taken in placing the purse string suture so as not to enter the lumen of the gallbladder. An incision was made in the middle of the purse string suture large enough to accommodate the modified Zeman (1966) type cannula. After placement of the cannula, the purse string suture was tightened and tied. The stay apparatus was pushed down and the burr tightened on the cannula. Next the common bile duct and pancreas were found. At a point immediately above the pancreas the common bile duct was dissected free of adjacent tissue. Here the common bile duct was doubly ligated. Upon ligating the common bile duct, bile was then forced to flow through the alternative channel that had been constructed.

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[1]Liquamycin®, Charles Pfizer and Company, Inc., New York, New York.

The pancreatic cannula was then placed in position. The entrance of the pancreatic duct into the common bile duct is obscured by pancreatic tissue so placement of the cannula for collection of pancreatic juice was performed as close to the entrance of the common bile duct into the duodenum as was feasible. This location was approximately 2 to 3 cm above the duodenum. At this point enough of the adjacent tissue was carefully dissected away from the common bile duct to allow for passage of a small pad of the Silastic® sheeting around the duct. A small incision was made into the lumen of the duct large enough to accommodate the pancreatic cannula. The fenestrated end of the pancreatic cannula was placed through this incision approximately 3 to 4 cm into the lumen of the common bile duct. The cannula was secured in place by the Silastic® pad that was fixed around the common bile duct. The two ends of the pad were sutured together in such a fashion that the pad was closely adhered to the common bile duct. A ligature was placed around the common bile duct directly behind the pad to force all pancreatic juice into the cannula. Finally, the stylet was removed from the pancreatic cannula to allow pancreatic juice to flow.

The abomasal cannula was the last cannula to be placed in position. The abomasum was draped so as to prevent contamination of the surgical site following a small incision in that organ. A purse string suture was placed in the abomasum (approximately 10 cm from the pylorus) using 2-0

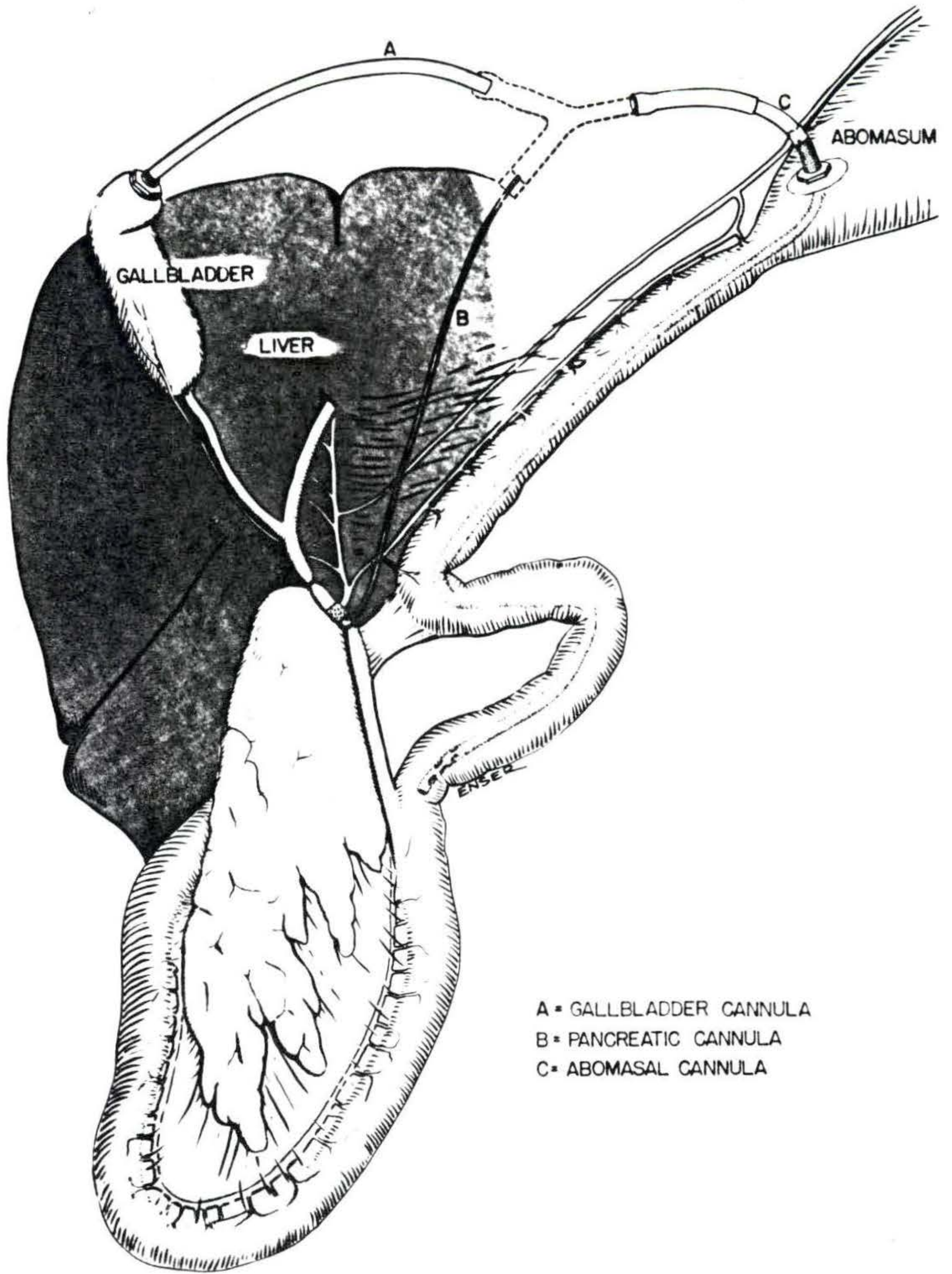
silk. A small incision was made in the middle of this purse string suture and a modified Zeman (1966) type cannula was secured in place. The Silastic® tubing that went beyond the cannula for a distance of 36 cm was used in the second sheep. This tubing was threaded through the pylorus and into the duodenum where the tip came to rest just distal to the double loop in the duodenum (Figure 2). The extension allowed for the secretions to enter in an area of less resistance, the duodenum, as compared to the abomasum. A stab wound was made in the body wall adjacent to the normal position of the part of the abomasum that held the cannula. The end of the modified Zeman (1966) cannula with the attached Silastic® tubing was brought through this incision.

Stab wounds were also made to bring the pancreatic cannula and the tubing part of the gallbladder cannula to the outside. The exterior location of these cannulas was slightly anterior and slightly posterior respectively to the paracostal incision. These two cannulas also came to the exterior more dorsally on the sheep than did the abomasal cannula (Figure 3).

Immediately below the skin the cannulas passed through a fenestrated circle of Silastic® sheeting. The Silastic® sheeting had a small circle of Silastic®[1] sponge glued on it. This device was intended to retard the migration of microbes

[1] Dow Corning, Medical Products Division, Midland, Mich.

Figure 2. Surgical placement of the cannulas


A
C
ABOMASUM
GALLBLADDER
B
LIVER
ENSER
A = GALLBLADDER CANNULA
B = PANCREATIC CANNULA
C = ABOMASAL CANNULA

to the interior.

The paracostal incision was closed by layers using number one or number two gut for the peritoneum and muscles. Vetafil®[1] was used to close the skin. A Y-type connecting tube was used to connect the cannulas. The lower part of the Y tube was connected to the abomasal cannula. The pancreatic and bile cannulas were connected to the two upper parts of the Y tube. In this way bile and pancreatic juice entered the upper parts of the Y tube and were returned to the gastrointestinal tract through the abomasal cannula.

Collection of pancreatic juice

The techniques of collecting pancreatic juice were a modification of techniques used by Butler et al. (1960). Pancreatic juice was collected in sterile 100 ml plastic tubes[2] (Figure 4). These tubes were rubber stoppered with two short glass tubes inserted into the rubber stopper. One glass tube was connected to the pancreatic cannula by means of adapters to allow for inflow of pancreatic juice. The other glass tube simply allowed for displaced air to escape. The collecting tube was chilled by being partially immersed in ice. The ice was contained in a plastic bag which was secured to the sheep by a strap. The duration of collection was usually two to five hours.

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[1]Bengen and Company, Hanover, Western Germany.

[2]The Nalge Company, Inc., Rochester, New York.

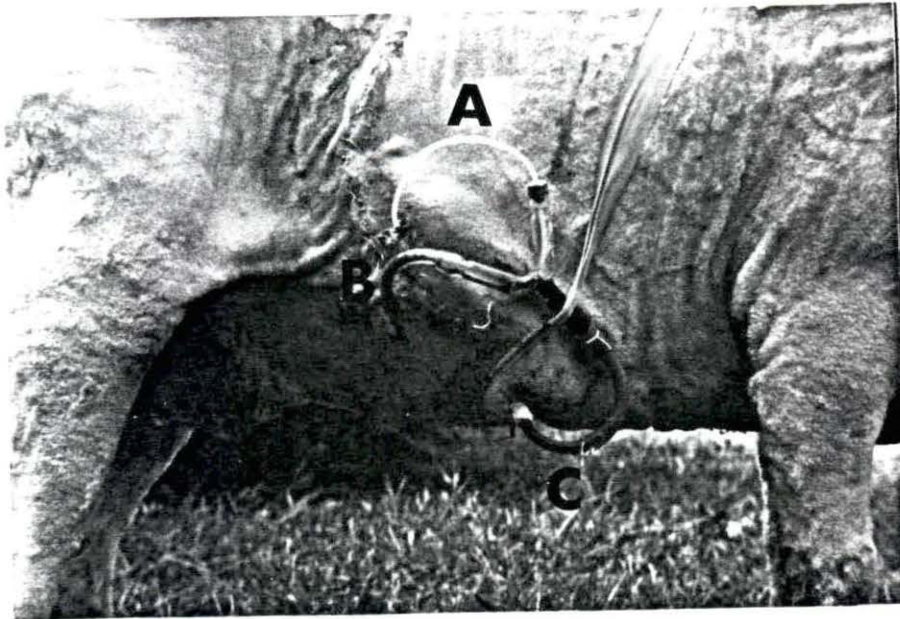

Figure 3. Exterior location of the surgical cannulas on the sheep
A. Pancreatic cannula
B. Gallbladder cannula
C. Abomasal cannula

Figure 4. Method of collecting pancreatic juice

Handling of pancreatic juice

At the end of the collection period the pH and volume of the pancreatic juice was measured and tested for proteolytic activity according to Sunderman and Sunderman (1961) (Table 2). The test is based on the principle that a gelatin solution under refrigeration will not harden in the presence of active proteolytic enzymes. The pancreatic juice

Table 2. Test for proteolytic activity

Tube	$NaHCO_3$ (5% solution) ml	Pancreatic Juice ml	Gelatin (7.5% solution) ml
Test[a]	1.68	0.32	2.0
Control[a]	2.00	0.00	2.0

[a]Incubate at 37°C for one hour. Then refrigerate 24 hours before reading.

was held in frozen storage a few days until approximately 60 ml had been collected. The pancreatic juice was lyophilized when this volume had been collected. The Department of Veterinary Microbiology furnished the equipment necessary for lyophilization. This equipment consisted of a commercial vacuum pump with ancillary parts that had been fabricated at Iowa State University. The lyophilized product was weighed, and the weight of the solids/ml pancreatic juice was deter-

mined. The pancreatic juice solids were then stored in screw cap jars at room temperature.

The pancreatic juice was reconstituted on the day of the assay. To reconstitute pancreatic juice a glucose solution (3.0 mg/ml) was used. One ml of glucose solution was added to pancreatic juice solids which was equivalent to one ml of pancreatic juice. The reconstituted juice was filtered with coarse filter paper either before or after the addition of 1.0 mg bovine fraction V albumin[1]/ml. In later experiments the pancreatic juice was filtered before the addition of albumin because it had been observed that the pancreatic juice passed much more quickly through the filter paper before albumin was added. The pH of the pancreatic juice was adjusted to a pH of 7.4 with a glass electrode before use by using HCl and/or NaOH (0.1 N and/or 0.01 N).

Reconstituted pancreatic juice was also tested for the presence of active proteolytic enzymes on two occasions according to Sunderman and Sunderman (1961).

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[1]Nutritional Biochemical Corp., Cleveland, Ohio.

The Rat Diaphragm Method of Insulin Assay

Rats

Spraque-Dawley male rats were purchased.[1] These were described by the company as being free of Salmonella, Bartonella, and epizootic pneumonia. On arrival these rats usually weighed between 70 to 95 g. They were used in the insulin bioassay when they weighed between 120 to 170 g after being fasted 18 to 24 hours. Other rats used in the bioassay of insulin were raised in our colony. These were male rats that originated from crosses between Spraque-Dawley males and Charles River*[2] females. Both groups of rats were fed a standard diet.[3]

Incubation media

Kreb's-Ringer bicarbonate (KRB) solution (Umbreit et al., 1964) was the balanced salt solution used for incubation. Glass distilled water and reagents that meet American Chemical Society specifications were used to make the KRB. All glassware that was used had been previously washed with a dilute solution of HCl and H_2NO_3 and rinsed several times with distilled water. It was found convenient to make up

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[1]Carworth, Division of Becton, Dickinson and Co., Kalamazoo, Michigan.

[2]The Charles River Breeding Laboratories, Wilmington, Massachusetts.

[3]Teklad Rat/Mouse Diet, Winfield, Iowa.

stock solutions of the various salts five times as concentrated so that they could be stored for several months in this form (Umbreit et al., 1964). These stock solutions were then diluted and combined in the prescribed manner to make the KRB (Table 3). A mixture of 95 percent O_2 to five percent CO_2 was passed through the KRB for about forty-five minutes to saturate the KRB with the gas mixture. The pH of the KRB was then checked with a glass electrode. It was generally found that the pH of the KRB was 7.4 at this time, but if it were not 7.4, the pH was adjusted to 7.4 using HCl or NaOH (0.1 N and/or 0.01 N). The KRB was placed in a glass stoppered volumetric flask and stored in a refrigerator for a maximum period of five days before use.

Immediately before use the KRB was again gassed with 95 percent O_2 to five percent CO_2 for about 30 minutes. Also, at this time enough five percent glucose (volume/volume) was added to the KRB to give a glucose concentration of approximately 3 mg glucose/ml KRB. After the addition of glucose, the medium was then known as Kreb's-Ringer bicarbonate glucose (KRBG). Finally, bovine fraction V albumin was added to give a concentration of 1.0 mg albumin/ml KRBG. The pH of the KRBG was checked after the addition of the albumin and again adjusted to a pH of 7.4 if necessary.

Insulin standards

A crystalline pork insulin standard[1] (lot PJ-5589) with an activity of 22.1 ± 2.8 I.U. by the 48 rabbit test and 22.7 ± 1.1 I.U. by the radioimmunoassay method of assaying insulin was the source of insulin. At four week intervals 10.0 mg of the crystalline insulin were dissolved in 10.0 ml of water made acid by adding three drops of 0.1 N HCl. This was known as the stock solution and was stored at 4°C. By appropriate dilution of the insulin stock solution with KRBG on each assay day the desired insulin concentration for each point of the standard curve was obtained (Table 4). Plastic test tubes[2] were used in the dilution of the insulin stock solution. The transfer from one test tube to another in the dilution of insulin was performed using glass pipettes which had been rinsed with KRBG plus albumin. The use of plastic test tubes and "coated" pipettes was an attempt to prevent loss of the insulin to the containers.

Procedure

The rats were decapitated and exsanguinated. Then they were placed on their dorsum and secured to a board. A small test tube was placed under the thoracic region to aid in exposure while dissecting out the diaphragms. The abdominal cavity was widely opened and the falciform ligament was

[1]Eli Lilly and Co., Indianapolis, Indiana.

[2]The Nalge Co., Inc., Rochester, New York.

Table 3. Reagents and concentrations for making Kreb's-Ringer and Kreb's-Ringer bicarbonate

Stock solutions	Concentration
NaCl	45.0 gm/L
KCl	5.750 gm/100 ml
$CaCl_2 \cdot 2H_2O$	8.080 gm/100 ml
KH_2PO_4	10.550 gm/100 ml
$MgSO_4 \cdot 7H_2O$	19.1 gm/100 ml
$NaHCO_3$[a]	1.300 gm/100 ml

Instructions for mixing KR	Amount mixed
60 ml of stock NaCl and dilute to 300 ml	300 ml
3 ml of stock KCl and dilute to 15 ml	12 ml
2 ml of stock $CaCl_2$ and dilute to 10 ml	9 ml
2 ml of stock KH_2PO_4 and dilute to 10 ml	3 ml
2 ml of stock $MgSO_4 \cdot 7H_2O$ and dilute to 10 ml	3 ml
	327 ml

In making the KRB, 48 ml of $NaHCO_3$ (1.300 gm/100 ml) were diluted to 300 ml with the KR

[a]Mixed just before making the KRB.

Table 4. Preparation of Insulin Standards

Insulin Dilution	Approximate Activity
1. 0.1 ml stock[a] into 10 ml KRBG[b] A.	221,000 μU/ml
2. 0.1 ml of A. into 11 ml KRBG B.	2,000 μU/ml
3. 10.0 ml of B. into 10 ml KRBG C.	1,000 μU/ml
4. 7.0 ml of C. into 7 ml KRBG D.	500 μU/ml
5. 3.0 ml of D. into 12 ml KRBG E.	100 μU/ml
6. 5.0 ml of E. into 5 ml KRBG F.	50 μU/ml
7. 2.0 ml of F. into 8 ml KRBG G.	10 μU/ml

[a]Insulin stock solution has an approximate activity of 2,210,000 μU insulin/0.1 ml.

[b]Kreb's-Ringer bicarbonate glucose.

cut. The central tendon of the diaphragm was pierced on each side at its muscular attachment which allowed the diaphragm to balloon out. Non-serrated thumb forceps were used to hold the HDs while they were cut away from the central tendon and from their costal attachments with iris scissors.

The HDs from each rat were then soaked in a beaker of KRB that was in an ice bath. This soaking period was called preincubation, and it lasted 15 minutes. During preincubation 95 percent O_2 to five percent CO_2 was constantly bubbled through the KRB. Following preincubation the HDs were spread on a piece of filter paper to remove excess moisture.

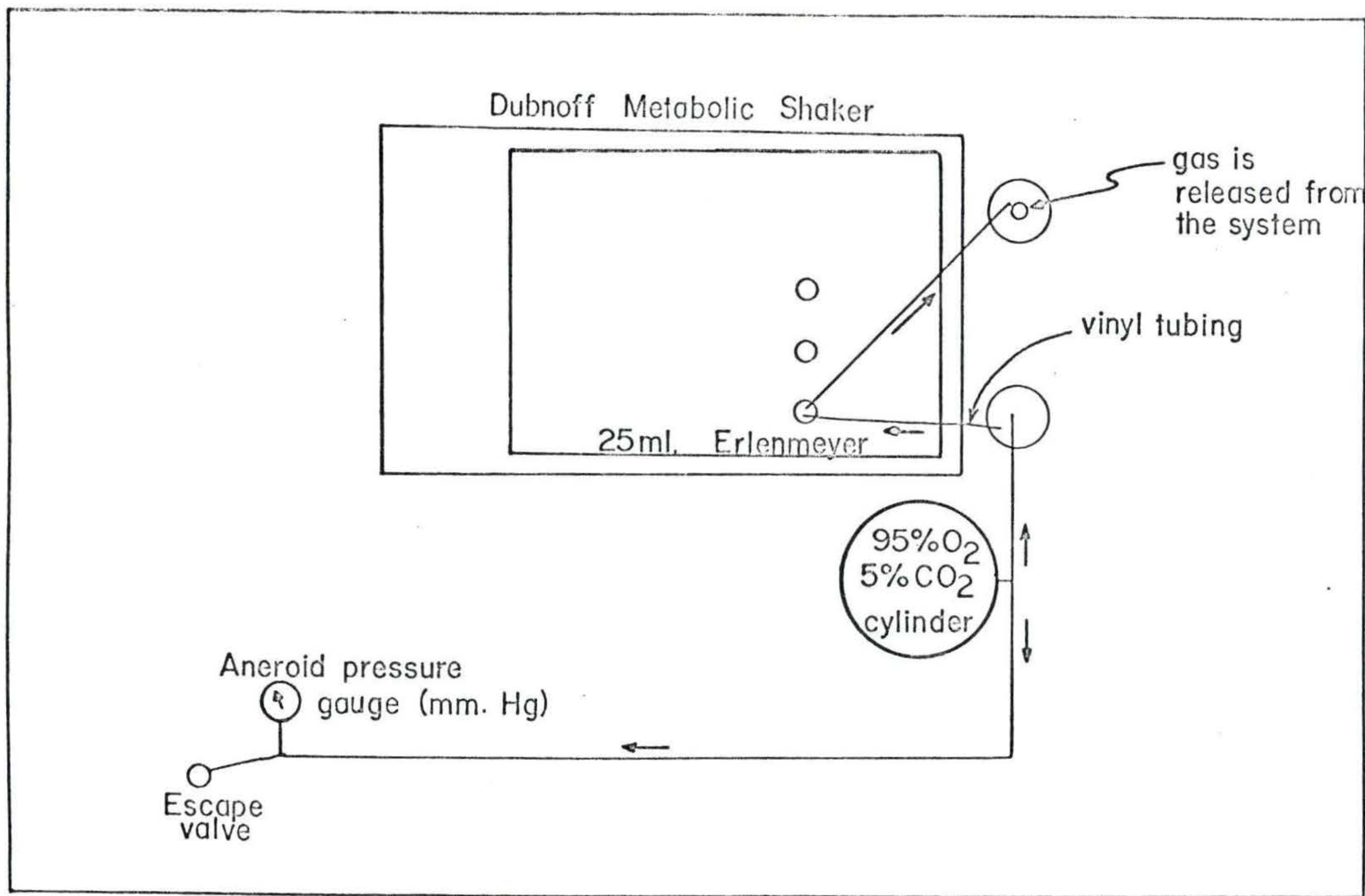

Figure 5. Method of gassing

The HDs were then transferred to 25 ml Erlenmeyer flasks into which 2.0 ml of the appropriate incubation medium had been pipetted.

On HD from each rat was incubated in KRBG without insulin (basal medium) and the other HD was incubated either in KRBG plus insulin or in pancreatic juice. In this way each treatment observation had as its control the opposite HD from the same rat.

Each flask was gassed for five minutes with 95 percent O_2 to five percent CO_2 after they were put on the Dubnoff metabolic shaker. The shaking rate was approximately 110 cycles/minute with an amplitude of 5 cm. The gas was placed into the flasks by way of injection needles which were connected to the source of gas by tubing (Figure 5). The gas was allowed to escape after circulating through the flasks through similar needles and tubing. Pressure in the system was maintained at approximately 4 mm Hg by observation of the pressure gauge. At the end of gassing the gas was shut off to each flask by a screw type clamp. Following gassing incubation was continued for 90 minutes at 37°C.

The HDs were weighed after incubation. In the process of being removed from the flasks both sides of the HDs were carefully wiped on the top of the flask to allow excessive moisture to return to the flasks. After being removed from the flasks the HDs were blotted between tissue paper and weighed on an electronic balance to the nearest 0.1 mg. The

flasks containing the residual incubation media were then closed with rubber stoppers to prevent evaporation.

Glucose determinations

Glucose was determined according to Hoffman (1937) as adapted for the AutoAnalyzer.[1] The reaction involves the reduction of alkaline ferricyanide with a consequent loss of color. Duplicate determinations were performed on each sample and the average of these two values was used to calculate glucose uptake. Agreement of the duplicate determinations was usually within 2.0 mg and never greater than 4.0 mg.

The glucose concentration from control flasks that did not contain a HD were used to establish the preincubation glucose level. The glucose concentration in each flask following incubation was subtracted from the preincubation glucose concentration to establish the glucose uptake. The glucose uptake was then expressed as mg glucose/100 ml incubation media. The glucose uptake was then converted to glucose uptake/2.0 ml, since 2.0 ml was the volume of incubation media used. The glucose uptake in mg was then converted to µg. The weight of the rat diaphragm that was expressed in mg was divided into the glucose uptake to give µg glucose uptake/mg rat diaphragm.

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[1] Technicon Corp., Tarrytown, New York.

RESULTS AND DISCUSSION

Basal Uptake

The glucose uptake of every HD incubated in either pancreatic juice or insulin had as its control the opposite HD, from the same rat, which was incubated in basal medium, KRBG. A total of 140 basal glucose uptake observations were made in 17 experiments. The mean glucose uptake from basal medium was 7.28 μg glucose/mg rat diaphragm ± 1.72 S.D. The mean basal glucose uptake value reported here is comparable to that reported by Wright (1957) for his strain A rats, Vallance-Owen and Wright (1960) and Gjedde (1968). A least squares analysis of variance was calculated to see if basal glucose uptake varied with the experiment (Table 5).

Table 5. The effect of experiments on basal glucose uptake

Source	Degrees of freedom	Mean square	F
Experiments	16	9.8334	4.74**
Error	123	2.0759	

**Significant at the one percent level.

The significant experiment effect indicates that the basal glucose uptake varied with the experiment. Vallance-Owen and Hurlock (1954) and Wright (1957) have reported

similar variations in the basal glucose uptake.

An experiment was conducted using only basal media to see how much variance could be expected when comparing the basal glucose uptake of two HDs from the same rat. The mean glucose uptake in this experiment was 7.09 μg glucose/mg rat diaphragm $\pm$ 0.37 S.D. This small standard deviation reflected the small difference in basal glucose uptake found when one half of the diaphragm was compared with the other half. Another experiment was conducted to see what effect anoxia would have on basal glucose uptake. These flasks were not gassed with 95 percent O_2 to five percent CO_2. The mean basal glucose uptake due to anoxia was found to be 11.40 μg glucose/mg rat diaphragm $\pm$1.46 S.D. A t-test was calculated to see if there was a significant mean difference between the basal glucose uptake under aerobic and anaerobic conditions. The calculated t-value in this case was 9.04 which was significant (one percent level). This confirmed the observation by Randle and Smith (1958) that anoxia stimulated glucose uptake.

Anoxia may have produced an artificial increase in glucose uptake in certain instances, although it is impossible to say which, if any, observation might have been involved. Large basal glucose uptakes, greater than two S.D. from the mean, were observed in five cases. These could be dismissed as normal biological variation considering that this many aberrant observations occurred in a total of 140 observa-

tions. However, if anoxia contributed to some large basal glucose uptakes, then there was certainly some chance of anoxia influencing the glucose uptake by HDs incubated in a media to which a treatment had been added.

A least squares analysis of variance was calculated to determine if diaphragm weight varied significantly with experiments and if glucose uptake could be correlated with diaphragm weight. The weights of the HDs that were used for determination of basal glucose uptake were used in this calculation. A significant F (one percent level) resulted indicating that the weight of HDs varied significantly with the experiment. The difference in the weights of the HDs between experiments can be attributed to the use of rats of different weights in some experiments. A correlation coefficient of -0.2074 was calculated between basal glucose uptake and diaphragm size. Thus a negative correlation existed between diaphragm size and basal glucose uptake.

Comparison of Glucose uptake from Sheep Pancreatic Juice with Glucose uptake from Basal Media

Nine experiments were conducted in which a total of 40 observations were made on the insulin effect of pancreatic juice. The insulin effect attributable to pancreatic juice resulted from the comparison of glucose uptake by HDs incubated in pancreatic juice with the glucose uptake by their control HDs which were incubated in basal media. The mean

insulin effect from pancreatic juice in these experiments was -0.45 μg glucose/mg rat diaphragm ± 2.65 S.D. This negative mean insulin effect from pancreatic juice indicated that there was less glucose uptake from pancreatic juice than there was from the basal media. Considering the slightly negative mean insulin effect found from the pancreatic juice and the S.D. from this mean, there then were some positive and some negative insulin effects.

A positive insulin effect resulted from all observations on pancreatic juice in experiments nine and ten (Table 6). In these two experiments an insulin effect that was greater than 1.0 μg glucose/mg rat diaphragm was observed in every instance. The mean insulin effect in these eight observations was 3.28 μg glucose/mg rat diaphragm with a range of 1.92 to 5.37. Other than these eight observations there were only two other observations on pancreatic juice where a positive insulin effect occurred that was greater than 1.0 μg glucose/mg rat diaphragm. These two observations occurred in experiment six. However, two other observations on pancreatic juice in this experiment resulted in negative insulin effects. There were five more observations in three other experiments which resulted in positive insulin effects, but these insulin effects were less than 1.0 μg glucose/mg rat diaphragm. These five observations could have resulted from the normal variance in glucose uptake expected between the two halves of a diaphragm. So a total

of 15 observations on pancreatic juice resulted either in a slight or a considerable positive insulin effect.

Table 6. The mean and the range of the insulin effect from pancreatic juice[a]

Experiment number[b]	Number of observations	The mean insulin effect[c]	Range[c]
6	4	0.34	-2.31 to 3.23
7	4	-0.22	-0.76 to -0.02
8	4	-0.26	-1.84 to 0.54
9	4	4.27	2.85 to 5.37
10	4	2.57	1.92 to 2.70
11	3	-0.10	-2.11 to 0.80
15	6	-0.47	-1.36 to 0.67
16	7	-3.92	-5.40 to -2.71
17	4	-2.34	-2.68 to -2.25
9	40	Total	

[a]See Appendix Tables 13-26.

[b]The experiments are in chronological order.

[c]µg glucose/mg rat diaphragm.

Therefore by difference a negative insulin effect resulted from 25 out of the 40 observations on pancreatic juice. Relatively large negative insulin effects consistently resulted from observations on pancreatic juice in experiments

16 and 17 (Table 6) which represented a total of 11 observations. The mean of the negative insulin effects in these two experiments was -3.48 μg glucose/mg rat diaphragm with a range of -5.40 to -2.25. There was a consistently negative insulin effect found from all observations on pancreatic juice in experiment seven (Table 6), but all of the values in this experiment were relatively close to zero.

The insulin effects produced by pancreatic juice in the majority of experiments (five out of nine) were either consistently negative or consistently positive. Pancreatic juice produced only one positive insulin effect in experiments 11 and 15 and these two observations were only slightly positive. The insulin effect produced by pancreatic juice in any experiment was of a narrower range than the range in insulin effects found from all the observations on pancreatic juice.

A least squares analysis of variance was calculated to see if there was a significant difference in glucose uptake from pancreatic juice between experiments (Table 7). This significant experiment effect indicated that the glucose uptake from pancreatic juice varied with the experiment.

Some of the significant experiment effect of the glucose uptake from pancreatic juice could have been related to the pH of the pancreatic juice. Jervell (1965) reported that a depression of the pH of the incubation media below 7.4 had an adverse effect upon glucose uptake by the isolated

rat diaphragm. The pH of the reconstituted pancreatic juice was always above 8.0, whereas the pH of fresh pancreatic juice was generally below 8.0. It seemed more difficult to adjust the pH of the pancreatic juice to 7.4 in some experiments than it did in other experiments. Consequently in some experiments it was necessary to use more acid or base to adjust the pH to 7.4. This could have altered the electrolyte composition or altered the buffering capacity of the pancreatic juice to the extent that this was either detrimental to glucose uptake or favored glucose uptake.

Table 7. Variation in glucose uptake from sheep pancreatic juice between experiments

Source	Degrees of freedom	Mean square	F
Experiments	8	28.3358	18.90**
Error	31	1.4929	

**Significant at the one percent level.

The experiment effect due to basal glucose uptake (Table 5) and the experiment effect due to pancreatic juice (Table 6) are both significant at the 0.005 level. However, the ratio of $\frac{\text{Experimental F Value}}{\text{Table F Value}}$ was much greater with pancreatic juice as compared to basal media (5.28 versus 2.00). The reason for this larger experiment effect found

with pancreatic juice may have been due to less constancy in the pancreatic juice from experiment to experiment than that possessed by the KRBG. This may have been a function of changing the ionic concentrations in the pancreatic juice from experiment to experiment by using different quantities of acid and/or base to adjust the pH to 7.4.

The techniques involved in the assay were not different from experiment to experiment with the exception of when albumin was added to pancreatic juice. It was observed that the pancreatic juice passed much faster through the coarse filter paper if albumin had not been added. Starting in experiment 10 (Table 6) 1.0 mg albumin/ml was added to the filtered pancreatic juice rather than adding the albumin to the pancreatic juice before filtration.

An experiment was conducted to see if albumin had an effect on basal glucose uptake. One half of the diaphragms was incubated in KRBG containing 10.0 mg of albumin/ml and the other half of the diaphragms was incubated in KRBG containing 1.0 mg of albumin/ml. A t-test was calculated to determine if there was a significant mean difference in the glucose uptake between the two treatments. A t-value of 1.740 was calculated which was not significant. It was concluded from this calculation that albumin would not significantly affect glucose uptake. Therefore, if albumin were lost to some extent in filtration, it should not affect the glucose uptake values.

The gross appearance of the reconstituted pancreatic juice was not unlike that of the freshly collected pancreatic juice. Both fresh and reconstituted pancreatic juice had a clear appearance with what appeared to be areas of uneven refractoriness. Lowering the pH of the reconstituted pancreatic juice in preparation for assay had the effect of precipitating some constituents from the pancreatic juice. The appearance of the juice then became opaque and flocculent. ILA that may have been present in the pancreatic juice could have been precipitated by this action and removed by filtration through the coarse filter paper.

A t-test was applied to the least squares analysis of variance presented in Table 7. to see if there was a significant mean difference between the glucose uptake from the basal media and pancreatic juice. A t-value of -0.532 was calculated which was not significant. Therefore, using this assay technique, ILA was not detected nor was there statistical evidence for the presence of an inhibitor being present in the pancreatic juice of sheep.

An experiment was conducted to examine the insulin effect that would result from doubling the concentration of pancreatic juice solids in the incubation media. The reconstituted pancreatic juice in this experiment then had twice the concentration of pancreatic juice solids/ml incubation media as did the pancreatic juice that was used as incubation media in other experiments. It was thought that

doubling the concentration of pancreatic juice solids/ml might produce a positive insulin effect by increasing the concentration of any ILA that might have been present. In addition to this deviation from procedure the reconstituted pancreatic juice was not filtered and albumin was not added.

A total of five observations made in this experiment resulted in a mean insulin effect of 2.76 µg glucose/mg rat diaphragm ± 2.26 S.D. Three of these observations revealed large positive insulin effects, one a slightly positive insulin effect, but one observation revealed a slightly negative insulin effect.

The mean positive insulin effect found in this experiment can be interpreted two ways. First, there might have been a low concentration of ILA present in the pancreatic juice which only became present in assayable concentrations after doubling the amount of pancreatic juice solids in each ml of incubation media. This may have been the situation, since the assay system was only sensitive to a minimum of 100 µU insulin/ml incubation media. Secondly, the mean positive insulin effect found in this experiment could have resulted from a hyperosmolar concentration of the incubation media. Kuzuya _et al_. (1965) reported that certain hyperosmolar solutions stimulated glucose uptake by the isolated rat diaphragm. It would appear that doubling the concentration of pancreatic juice solids/ml incubation media would produce a hypertonic solution with respect to

the rat diaphragm since pancreatic juice appeared to have an isotonicity that would not appreciably differ from KRB; KRB was isotonic with rat serum (Umbreit et al., 1964).

A Comparison of the Insulin Effects produced by Pancreatic Juice and Insulin Standards

The insulin effect produced by pancreatic juice was compared to the insulin effect produced by 100 and 1000 μU insulin/ml KRBG. The lowest insulin concentration used in this comparison was 100 μU insulin/ml as 10 μU insulin/ml could not be routinely assayed. The data for this comparison was taken from 11 experiments. Five of these experiments contained only insulin standards and six experiments had observations on both insulin standards and pancreatic juice. In these 11 experiments there were 29 observations using 100 μU insulin/ml KRBG, 30 observations using 1000 μU insulin/ml KRBG, and 25 observations using pancreatic juice as the incubation media. The pancreatic juice observations in this comparison were only taken from those experiments where insulin standards were included. The mean and S.D. of the insulin effects produced by 100 μU insulin/ml, 1000 μU insulin/ml and pancreatic juice are given in Table 8. The mean insulin effects produced by 100 and 1000 μU insulin/ml are similar to values reported by Vallance-Owen and Wright (1960) and Gjedde (1968).

Table 8. The mean and standard deviation of the insulin effects from 100 μU insulin/ml, 1000 μU insulin/ml, and pancreatic juice

	Mean	S.D.
100 μU insulin/ml	4.55	2.57
1000 μU insulin/ml	6.50	2.83
pancreatic juice	0.43	2.10

A least squares analysis of variance was calculated to examine the effect of experiments, treatments, and the interaction between experiments and treatments on glucose uptake (Table 9). In this least squares analysis of variance the experiments were adjusted for treatments, and the treatments were adjusted for experiment. This was necessary because some experiments had observations only on insulin standards.

There was a significant amount of interaction between treatments and experiments (five percent level). The effect of interaction means that the slope of the lines found by plotting the treatment levels against the insulin effects were not parallel from experiment to experiment. The significant experiment effect indicates that the insulin effect varied with the experiment. The significant experiment effect and the significant interaction effect suggest a lack of control of unknown factors. Randle (1956) and Gjedde (1968) found that the diaphragm method of insulin assay was not

precise. A contributing cause to this lack of precision may have been too few observations on each treatment level. There were usually two to three observations on each treatment level in each experiment. Then if the insulin effect in any observation were markedly different from the mean, this would most certainly contribute to the lack of precision.

Table 9. The effect of treatments, experiments, and their interaction upon the insulin effects

Source	Degrees of freedom	Mean Square	F
Experiments	10	11.9097	2.56*
Treatments	2	199.9579	43.02**
Experiments x treatments	15	10.4265	2.24*
Error	56	4.6486	

*Significant at the five percent level.

**Significant at the one percent level.

A t-test was applied to the least squares analysis of variance (Table 9) to see if the insulin effect of pancreatic juice in these experiments differed significantly from basal glucose uptake (zero added insulin). This was calculated assuming that these were a random set of experiments. The mean insulin effect of pancreatic juice in these exper-

iments was 0.43 μg glucose/mg rat diaphragm (Table 8). A t-value of 0.666 was calculated which was not significant. So the insulin effect produced by pancreatic juice did not differ significantly from basal glucose uptake.

A t-test was applied to the least squares analysis of variance (Table 9) to see if there was a significant mean difference between the insulin effect produced by 100 μU insulin/ml KRBG and the insulin effect produced by pancreatic juice. The mean insulin effect produced by 100 μU insulin/ml KRBG was 4.55 μg glucose/mg rat diaphragm and the mean insulin effect produced by pancreatic juice was 0.43 μg glucose/mg rat diaphragm (Table 8). A t-value of 4.923 was calculated which was significant (one percent level). Therefore while the insulin effect due to pancreatic juice did not differ from a mean of zero, it did significantly differ from the insulin effect of 100 μU insulin/ml KRBG.

A t-test was applied to the least squares analysis of variance (Table 9) to see if there was a significant mean difference between the insulin effect produced by 100 and 1000 μU insulin/ml KRBG. The mean insulin effect produced by the former was 4.55 μg glucose/mg rat diaphragm and the insulin effect produced by the latter was 6.50 μg glucose/mg rat diaphragm. A t-value of 2.321 was calculated which was significant (five percent level). The fact that there was a significant difference between the insulin treatments

gave validity to the assay system.

The Sheep Surgery

Unactivated pancreatic juice was successfully collected from two sheep. This procedure was developed since it was felt that unactivated pancreatic juice could not be collected according to methods used by Taylor (1960) or Phaneuf (1961). Their methods involved the pancreatic juice coming in contact to some extent with the gastrointestinal tract and therefore becoming activated.

Three sheep were surgically prepared and two recovered from the immediate effects of the surgery. The first sheep to recover lived 12 days and the second sheep lived seven days. In both cases the sheep had to be euthanized because of problems with the gallbladder cannula. The modified Zeman (1966) type cannula tore the gallbladder in the first sheep. In the second sheep the adhesive that was holding the modified Zeman (1966) type cannula to the vinyl tubing of the gallbladder cannula loosened when the external part of the tubing caught on the fence. Consequently the vinyl tubing came out of this sheep.

Bile and pancreatic juice could be returned naturally to the second sheep by way of the abomasal cannula (Figure 2). The extension on the abomasal cannula in this sheep allowed for these secretions to enter the duodenum. There was apparently less resistance for these secretions to enter

the animal here as compared to the abomasum. The abomasal cannula in the first sheep did not have this extension, and it was observed that these secretions could not be naturally returned to the abomasum. Apparently there was too much pressure in the abomasum to accept these secretions from this cannula design.

The Pancreatic Juice

Reiser and Reiser (1964) reported an enhancement of D-xylose and 3-O-methyl glucose uptake by various active pancreatic proteases. According to the test for proteolytic enzyme activity given by Sunderman and Sunderman (1961), neither fresh pancreatic juice nor reconstituted pancreatic juice used in these experiments revealed the presence of active proteolytic enzymes. This eliminated the possibility that any insulin effect could be attributed to these enzymes.

A control test was conducted to see if pancreatic juice itself had an effect upon the glucose concentration. Glucose determinations were made at different time intervals on the reconstituted pancreatic juice to which had been added approximately 3 mg glucose/ml pancreatic juice. Glucose determinations at 15, 30, 60, and 120 minutes following reconstitution of the pancreatic juice were all within 1 mg/100 ml. The glucose concentration at 15 minutes was 292 mg glucose/100 ml pancreatic juice and at 120 minutes it was 293 mg glucose/100 ml pancreatic juice. Therefore,

it appeared as though pancreatic juice did not affect the glucose concentration.

The weight of the pancreatic solids/ml pancreatic juice was very similar in the two volumes of pancreatic juice that were used for the assay of ILA. One volume (57 ml) contained 23.7 mg solids/ml pancreatic juice and the other volume (150 ml) contained 23.2 mg solids/ml pancreatic juice.

Duplicate determinations[1] for Na and K were made on one quantity of reconstituted pancreatic juice. The average K concentration was 4.5 mEq/L and the Na was 129 mEq/L. The K concentration was within the range of 3.9 to 5.4 mEq/L for sheep pancreatic juice given by Taylor (1962) (Table 1). The Na was slightly below the given range of 135 to 165 mEq/L. These values in turn were roughly comparable to the calculated values for the electrolyte composition of KRB (Table 1). Therefore pancreatic juice from the standpoint of ionic concentration appeared to be a satisfactory medium for incubation of rat diaphragms.

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[1]Technicon's AutoAnalyzer method files N-20b from Technicon Corp., Tarrytown, New York.

SUMMARY

Unactivated pancreatic juice was collected from two sheep. A newly devised surgical procedure was used for the collection. In this procedure pancreatic juice was collected by means of an indwelling cannula in the common bile duct. The bile was rerouted by another cannula so as to permit collection of pancreatic juice from the common bile duct.

Pancreatic juice was assayed for ILA by the rat diaphragm method of insulin assay by a paired HD technique. Some observations on pancreatic juice resulted in relatively large positive insulin effects while other observations resulted in relatively large negative insulin effects. However, the mean insulin effect produced by pancreatic juice did not significantly differ from basal glucose uptake (zero added insulin). The non-significance of this difference was calculated by applying t-tests to two different least squares analysis of variance. Insulin standards, 100 and 1000 μU insulin/ml KRBG were incorporated in most experiments. The mean insulin effect produced by pancreatic juice was significantly different from the insulin effect produced by 100 μU insulin/ml KRBG. Therefore it was concluded that sheep pancreatic juice contains no ILA as was determined by the lower limits of sensitivity of this assay method.

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APPENDIX

Table 10. A study of basal glucose uptake

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a] minus avg. residual glucose conc.	μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	glucose uptake (μg/mg)
1	268.0	26.25	525.0	70.6	7.44
2	272.5	21.75	435.0	59.9	7.26
3	267.5	26.75	535.0	79.6	6.72
4	266.5	27.75	555.0	75.9	7.31
5	268.5	25.75	515.0	77.0	6.69
6	272.0	22.25	445.0	69.6	6.39
7	268.0	26.25	525.0	74.0	7.06
8	263.0	31.25	625.0	84.1	7.43
9	259.0	35.25	705.0	93.9	7.51
10	260.5	33.75	675.0	95.8	7.06

[a]Average preincubation glucose concentration was 294.25 mg/100 ml.

Table 11. The effect of anoxia

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a] minus avg. residual glucose conc.	µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)
1	261.0	35.25	705.0	74.3	9.49
2	247.0	49.25	985.0	80.1	12.30
3	262.5	33.75	675.0	74.1	9.11
4	254.5	41.75	835.0	75.3	11.09
5	247.0	49.25	985.0	70.8	13.91
6	255.5	40.75	815.0	65.4	12.54
7	256.5	39.75	795.0	72.1	11.03
8	246.0	50.25	1005.0	80.4	12.50
9	248.0	48.25	965.0	85.0	11.35
10	260.0	36.25	725.0	68.8	10.66

[a]Preincubation glucose concentration was 296.25 mg/100 ml.

Table 12. The effect of albumin on glucose uptake

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1. 10.0 mg[c]/ml	265.5	30.5
2. 1.0 mg[d]/ml	252.0	48.0
3. 10.0 mg[c]/ml	266.0	29.5
4. 1.0 mg[d]/ml	271.5	28.5
5. 10.0 mg[c]/ml	267.5	29.0
6. 1.0 mg[d]/ml	262.5	37.5
7. 10.0 mg[c]/ml	256.0	40.0
8. 1.0 mg[d]/ml	246.5	53.5
9. 10.0 mg[c]/ml	255.0	41.0
10. 1.0 mg[d]/ml	268.0	32.0
11. 10.0 mg[c]/ml	270.0	26.0
12. 1.0 mg[d]/ml	272.5	27.5
13. 10.0 mg[c]/ml	267.5	28.5
14. 1.0 mg[d]/ml	269.5	30.5

[a]10.0 mg bovine fraction V/ml preincubation glucose concentration was 296.0 mg/ml.

[b]1.0 mg bovine fraction V/ml preincubation glucose concentration was 300.0 mg/ml.

[c]10.0 mg bovine albumin fraction V/ml.

[d]1.0 mg bovine albumin fraction V/ml.

μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (μg/mg)	Insulin effect (μg/mg)
610.0	77.6	7.68	-3.90
960.0	82.9	11.58	
590.0	72.6	8.13	1.18
570.0	82.0	6.95	
580.0	73.6	7.88	-1.13
750.0	83.2	9.01	
800.0	101.2	7.91	-3.26
1060.0	94.9	11.17	
820.0	88.8	9.23	1.18
640.0	79.5	8.05	
520.0	79.4	6.55	-1.49
550.0	68.4	8.04	
570.0	71.7	7.95	-0.70
610.0	70.5	8.65	

Table 13. Experiment one

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a] minus avg. residual glucose (mg/100 ml)
1. 100 μU	247.0	52.75
2. control	274.0	25.75
3. 1000 μU	232.0	67.55
4. control	266.5	33.25
5. 100 μU	244.0	55.75
6. control	274.0	25.75
7. 1000 μU	221.0	78.75
8. control	269.0	30.75
9. 100 μU	249.5	50.25
10. control	268.0	31.75
11. 1000 μU	250.5	49.25
12. control	263.0	36.75

[a]KRBG preincubation glucose concentration 299.75 mg/100 ml.

µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)	Insulin effect (µg/mg)
1055.0	94.7	11.14	4.95
515.0	83.2	6.19	
1351.0	83.2	16.23	8.50
665.0	86.0	7.73	
1115.0	88.4	12.61	6.46
515.0	83.7	6.15	
1575.0	100.9	15.61	8.20
615.0	83.0	7.41	
1005.0	81.9	12.27	5.60
635.0	95.2	6.67	
985.0	88.5	11.13	2.60
735.0	86.2	8.53	

Table 14. Experiment two

Flasks		Avg. residual glucose (mg/100 ml)	Preincubation[a] minus avg. residual glucose (mg/100 ml)
1.	100 μU	240.0	58.88
2.	control	271.0	27.88
3.	1000 μU	239.5	59.38
4.	control	263.0	35.88
5.	100 μU	238.5	60.38
6.	control	272.5	26.38
7.	1000 μU	227.5	71.38
8.	control	277.5	21.38
9.	100 μU	251.5	47.38
10.	control	266.0	32.88
11.	1000 μU	236.5	62.38
12.	control	270.5	28.38

[a]KRBG preincubation glucose concentration 298.88.

µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)	Insulin effect (µg/mg)
1177.6	95.3	12.36	5.45
557.6	80.7	6.91	
1187.6	81.3	14.61	6.75
717.6	91.3	7.86	
1207.6	94.3	12.81	6.23
527.6	80.2	6.58	
1427.6	92.5	15.43	9.16
427.6	68.2	6.27	
947.6	82.9	11.43	3.96
657.6	88.0	7.47	
1247.6	93.0	13.42	7.56
567.6	96.8	5.86	

Table 15. Experiment three

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a] minus avg. residual glucose (mg/100 ml)
1. 10 μU	256.5	41.5
2. control	274.5	23.5
3. 1000 μU	240.5	57.5
4. control	268.5	29.5
5. 100 μU	261.0	37.0
6. control	275.0	23.0
7. 10 μU	266.5	31.5
8. control	274.5	23.5
9. 1000 μU	244.5	53.5
10. control	273.5	24.5
11. 100 μU	261.5	36.5
12. control	276.5	21.5
13. 10 μU	265.0	33.0
14. control	281.0	17.0
15. 1000 μU	244.0	54.0
16. control	274.0	24.0
17. 100 μU	255.0	43.0
18. control	270.0	28.0
19. 10 μU	262.0	36.0
20. control	279.5	18.5

[a]KRBG preincubation glucose concentration 298.0 mg/100 ml.

µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)	Insulin effect (µg/mg)
830.0	75.8	10.95	3.83
470.0	66.0	7.12	
1150.0	74.9	15.35	8.04
590.0	80.7	7.31	
740.0	63.1	11.73	6.37
460.0	85.9	5.36	
630.0	86.1	7.32	0.39
470.0	67.8	6.93	
1070.0	70.0	15.29	8.08
490.0	68.0	7.21	
730.0	66.9	10.91	4.88
430.0	71.3	6.03	
660.0	74.7	8.84	3.65
340.0	65.5	5.19	
1080.0	74.7	14.46	6.98
480.0	64.2	7.48	
860.0	65.1	13.21	5.91
560.0	76.7	7.30	
720.0	84.2	8.55	2.38
370.0	60.0	6.17	

Table 16. Experiment four

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a] minus avg. residual glucose (mg/100 ml)
1. 10 μU	269.0	27.75
2. control	265.0	31.75
3. 1000 μU	246.0	50.75
4. control	266.0	30.75
5. 100 μU	241.5	55.25
6. control	267.0	29.75
7. 10 μU	261.0	35.75
8. control	270.0	26.75
9. 1000 μU	234.5	61.25
10. control	272.5	24.25
11. 100 μU	265.0	31.75
12. control	248.0	48.75
13. 10 μU	269.0	27.75
14. control	274.5	22.25
15. 1000 μU	237.5	59.25
16. control	256.5	40.25
17. 100 μU	246.0	50.75
18. control	275.5	21.25

[a]KRBG preincubation glucose concentration 296.75 mg/100 ml.

µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)	Insulin effect (µg/mg)
555.0	80.0	6.94	-2.83
635.0	65.0	9.77	
1015.0	70.0	14.50	6.23
615.0	74.4	8.27	
1105.0	101.6	10.88	3.81
595.0	84.2	7.07	
715.0	74.8	9.56	2.91
535.0	80.4	6.65	
1225.0	91.0	13.46	7.39
485.0	79.9	6.07	
635.0	82.6	7.69	-3.33
975.0	88.5	11.02	
555.0	65.5	8.47	0.44
445.0	55.4	8.03	
1185.0	75.4	15.72	5.41
805.0	78.1	10.31	
1015.0	107.1	9.48	4.40
425.0	83.6	5.08	

Table 17. Experiment 7

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1. 1000 μU	239.0	60.25
2. control	269.5	29.75
3. PJ[c]	272.5	26.75
4. control	265.0	34.25
5. 100 μU	250.0	49.25
6. control	268.0	31.25
7. 1000 μU	213.5	85.75
8. control	264.0	35.25
9. PJ[c]	273.5	25.75
10. control	269.0	30.25
11. 100 μU	234.0	65.25
12. control	274.0	25.25
13. PJ[c]	279.0	20.25
14. control	272.5	26.75
15. PJ[c]	271.5	27.75
16. control	273.0	26.25

[a]Pancreatic juice preincubation glucose concentration 301.5 mg/100 ml.

[b]KRBG preincubation glucose concentration 299.25 mg/100 ml.

[c]Pancreatic juice.

μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (μg/mg)	Insulin effect (μg/mg)
1250.0	84.4	14.81	7.10
595.0	77.2	7.71	
535.0	87.8	6.09	-0.76
685.0	100.0	6.85	
985.0	89.2	11.04	4.47
625.0	95.1	6.57	
1715.0	104.7	16.38	8.70
705.0	91.8	7.68	
515.0	85.4	6.03	-0.02
605.0	100.0	6.05	
1305.0	100.0	13.05	6.88
505.0	81.9	6.17	
405.0	85.6	4.73	-0.41
535.0	104.0	5.14	
555.0	95.0	5.84	-0.26
525.0	86.1	6.10	

Table 18. Experiment 8

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1. 1000 μU	243.5	51.5
2. control	268.0	27.0
3. PJ[c]	262.5	32.5
4. control	260.0	35.0
5. 100 μU	256.0	39.0
6. control	269.0	26.0
7. PJ[c]	263.0	32.0
8. control	267.5	27.5
9. 1000 μU	244.0	51.0
10. control	262.0	33.0
11. PJ[c]	278.0	17.0
12. control	270.5	24.5
13. 100 μU	258.0	37.0
14. control	271.0	24.0
15. PJ[c]	275.0	20.0
16. control	271.0	24.0
17. 1000 μU	253.0	42.0

[a]Pancreatic juice preincubation glucose concentration 303.5 mg/100 ml.

[b]KRBG preincubation glucose concentration 295 mg/100 ml.

[c]Pancreatic juice.

μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (μg/mg)	Insulin effect (μg/mg)
1030.0	76.1	13.53	5.53
540.0	67.5	8.00	
650.0	73.6	8.83	-1.26
700.0	69.4	10.09	
780.0	54.8	14.23	6.21
520.0	64.8	8.02	
640.0	80.0	8.00	0.54
550.0	73.7	7.46	
1020.0	80.0	12.75	4.32
660.0	78.3	8.43	
340.0	65.7	5.23	-0.97
490.0	79.0	6.20	
740.0	82.6	8.96	2.00
480.0	69.5	6.96	
400.0	68.5	5.84	-1.84
480.0	62.5	7.68	
840.0	78.2	10.74	3.58

Table 18 (Continued).

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
18. control	269.0	26.0
19. 100 µU	264.0	31.0
20. control	271.0	24.0

μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (μg/mg)	Insulin effect (μg/mg)
520.0	72.6	7.16	
620.0	69.3	8.95	2.55
480.0	75.3	6.40	

Table 19. Experiment 9

Flasks		Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1.	1000 μU	243.5	50.00
2.	control	266.0	27.50
3.	PJ[c]	244.0	48.25
4.	control	269.0	24.50
5.	100 μU	255.0	38.50
6.	control	264.5	29.00
7.	PJ[c]	256.0	36.25
8.	control	268.0	25.50
9.	1000 μU	242.5	51.00
10.	control	261.0	32.50
11.	PJ[c]	255.5	36.75
12.	control	264.5	29.00
13.	100 μU	257.0	36.50
14.	control	262.5	31.00
15.	PJ[c]	270.5	21.75
16.	control	274.5	19.00

[a]Pancreatic juice preincubation glucose concentration 292.25.

[b]KRBG preincubation glucose concentration 293.5.

[c]Pancreatic juice.

µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)	Insulin effect (µg/mg)
1000.0	77.7	12.87	5.73
550.0	77.9	7.14	
965.0	86.0	11.22	5.37
490.0	83.7	5.85	
770.0	84.2	9.14	1.81
580.0	79.1	7.33	
725.0	85.7	8.46	2.85
510.0	90.9	5.61	
1020.0	84.7	12.04	4.13
650.0	82.2	7.91	
735.0	70.0	10.50	3.06
580.0	78.0	7.44	
730.0	67.3	10.85	2.27
620.0	72.3	8.58	
635.0	67.5	9.41	5.25
380.0	91.3	4.16	

Table 19 (Continued).

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
17. 1000 μU	244.0	49.50
18. control	270.5	23.00
19. 100 μU	271.0	22.50
20. control	260.5	33.50

µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)	Insulin effect (µg/mg)
990.0	82.3	12.03	6.55
460.0	83.9	5.48	
450.0	78.1	5.76	-4.66
670.0	64.3	10.42	

Table 20. Experiment 10

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1. PJ[c]	235.0	39.0
2. control	265.0	22.5
3. PJ[c]	243.0	31.0
4. control	259.5	28.0
5. basal	257.5	30.0
6. basal	261.5	26.0
7. PJ[c]	236.5	37.5
8. control	261.0	26.5
9. basal	259.0	28.5
10. basal	262.0	25.5
11. PJ[c]	241.5	32.5
12. control	265.0	22.5
13. basal	251.0	36.5
14. basal	262.5	25.0
15. basal	267.5	20.0
16. basal	260.0	27.5

[a]Pancreatic juice preincubation glucose concentration 274 mg/100 ml.

[b]KRBG preincubation glucose concentration 287.5 mg/100 ml.

[c]Pancreatic juice.

μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (μg/mg)	Insulin effect (μg/mg)
780.0	93.6	8.33	1.92
450.0	70.2	6.41	
620.0	54.1	11.46	2.70
560.0	63.9	8.76	
600.0	65.5	9.16	
520.0	58.5	8.89	
750.0	73.5	10.20	2.58
530.0	69.6	7.62	
570.0	77.6	7.35	
510.0	81.2	6.28	
650.0	76.6	8.49	2.53
450.0	75.5	5.96	
730.0	55.9	13.06	
500.0	54.8	9.12	
400.0	71.1	5.63	
550.0	85.1	6.46	

Table 21. Experiment 11

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1. 1000 μU	253.5	43.0
2. control	246.5	53.0
3. PJ[c]	243.0	41.5
4. control	243.5	53.0
5. 100 μU	242.5	54.0
6. control	263.0	33.5
7. PJ[c]	254.0	30.5
8. control	268.0	28.5
9. 1000 μU	244.5	52.0
10. control	263.0	33.5
11. PJ[c]	252.5	32.0
12. control	250.0	36.5
13. 100 μU	250.5	36.0
14. control	273.0	23.5
15. 1000 μU	235.0	61.5
16. control	269.0	27.5

[a]Pancreatic juice preincubation glucose concentration 284.5 mg/100 ml.

[b]KRBG preincubation glucose concentration 284.5 mg/100 ml.

[c]Pancreatic juice.

μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (μg/mg)	Insulin effect (μg/mg)
860.0	89.2	9.64	-1.61
1060.0	94.2	11.25	
830.0	83.1	9.99	-2.11
1060.0	87.6	12.10	
1080.0	92.1	11.73	3.48
670.0	81.2	8.25	
610.0	69.7	8.75	0.58
570.0	69.8	8.17	
1140.0	92.5	12.32	4.31
670.0	83.6	8.01	
640.0	63.6	10.06	0.80
730.0	78.8	9.26	
720.0	79.6	9.05	3.23
470.0	80.7	5.82	
1230.0	85.9	14.32	7.43
550.0	79.8	6.89	

Table 22. Experiment 12

Flasks		Avg. residual glucose (mg/100 ml)	Preincubation[a] minus avg. residual glucose (mg/100 ml)
1.	1000 μU	250.0	47.55
2.	control	267.5	29.75
3.	100 μU	255.0	42.25
4.	control	263.5	33.75
5.	1000 μU	250.0	47.25
6.	control	269.0	28.25
7.	100 μU	258.0	39.25
8.	control	269.5	27.75
9.	1000 μU	232.0	65.25
10.	control	277.0	20.25
11.	100 μU	259.5	37.75
12.	control	270.5	26.75

[a]KRBG preincubation glucose concentration 297.25 mg/100 ml.

µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)	Insulin effect (µg/mg)
951.0	85.5	11.12	3.92
595.0	82.6	7.20	
845.0	69.9	12.09	3.76
675.0	81.0	8.33	
945.0	83.1	11.37	4.30
565.0	79.9	7.07	
785.0	80.6	9.74	2.40
555.0	75.6	7.34	
1305.0	69.1	18.89	14.70
405.0	96.7	4.19	
755.0	69.7	10.83	2.65
535.0	65.4	8.18	

Table 23. Experiment 15

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1. PJ[c]	265.5	20.5
2. control	271.0	23.5
3. 1000 μU	253.0	41.5
4. control	268.0	26.5
5. PJ[c]	264.0	22.0
6. control	272.0	22.5
7. 100 μU	255.5	39.0
8. control	272.5	22.0
9. PJ[c]	268.0	18.0
10. control	275.5	19.0
11. 1000 μU	253.0	41.5
12. control	280.0	14.5
13. PJ[c]	268.5	17.5
14. control	281.0	13.5
15. 100 μU	267.0	27.5
16. control	280.5	14.0

[a]Pancreatic juice preincubation glucose concentration 286 mg/100 ml.

[b]KRBG preincubation glucose concentration 294.5 mg/100 ml.

[c]Pancreatic juice.

μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (μg/mg)	Insulin effect (μg/mg)
410.0	54.6	7.51	-1.36
470.0	53.3	8.87	
830.0	62.9	13.20	4.98
530.0	64.5	8.22	
440.0	60.2	7.31	-1.21
450.0	52.8	8.52	
780.0	61.0	12.79	5.69
440.0	62.0	7.10	
360.0	59.1	6.09	-0.54
380.0	57.3	6.63	
830.0	56.7	14.64	8.06
390.0	59.3	6.58	
350.0	63.1	5.55	0.67
270.0	55.3	4.88	
550.0	51.4	10.70	5.20
280.0	50.9	5.50	

Table 23 (Continued).

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
17. PJ[c]	268.0	18.0
18. control	277.5	17.0
19. PJ[c]	269.0	17.0
20. control	275.5	19.0

µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)	Insulin effect (µg/mg)
360.0	61.3	5.87	-0.20
340.0	56.0	6.07	
340.0	59.4	5.76	-0.23
380.0	63.4	5.99	

Table 24. Experiment 16

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1. PJ[c]	265.0	16.5
2. control	277.5	21.5
3. PJ[c]	265.5	16.0
4. control	270.5	29.0
5. PJ[c]	266.0	15.5
6. control	270.5	28.5
7. PJ[c]	270.5	11.0
8. control	271.0	28.0
9. PJ[c]	265.5	16.0
10. control	275.0	24.0
11. PJ[c]	265.0	16.0
12. control	274.0	25.0
13. PJ[c]	266.0	15.5
14. control	276.5	22.5

[a]Pancreatic juice preincubation glucose concentration 281.5 mg/100 ml.

[b]KRBG preincubation glucose concentration 299.0 mg/100 ml.

[c]Pancreatic juice.

μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (μg/mg)	Insulin effect (μg/mg)
330.0	48.4	6.82	-5.40
430.0	35.2	12.22	
320.0	49.2	6.50	-2.71
580.0	63.0	9.21	
310.0	61.4	5.05	-4.78
570.0	58.8	9.83	
220.0	55.9	3.94	-5.08
560.0	62.1	9.02	
320.0	50.5	6.40	-4.36
480.0	44.6	10.76	
320.0	68.6	4.71	-3.35
500.0	62.3	8.06	
310.0	67.9	4.63	-2.72
450.0	61.2	7.35	

Table 25. Experiment 17

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1. PJ[c]	306.0	11.0
2. control	266.0	17.5
3. PJ[c]	304.0	13.0
4. control	261.0	22.5
5. PJ[c]	304.0	13.0
6. control	263.5	20.0
7. PJ[c]	302.5	14.5
8. control	257.0	26.5

[a]Pancreatic juice preincubation glucose concentration 317.0 mg/100 ml.

[b]KRBG preincubation glucose concentration 283.5 mg/100 ml.

[c]Pancreatic juice.

µg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (µg/mg)	Insulin effect (µg/mg)
220.0	59.5	3.70	-2.25
350.0	58.8	5.95	
260.0	54.2	4.80	-2.68
450.0	60.2	7.48	
260.0	64.8	4.01	-2.42
400.0	62.2	6.43	
290.0	65.4	4.43	-2.57
530.0	75.7	7.00	

Table 26. Pancreatic juice was twice as concentrated

Flasks	Avg. residual glucose (mg/100 ml)	Preincubation[a,b] minus avg. residual glucose (mg/100 ml)
1. PJ[c]	244.5	22.0
2. control	283.0	4.0
3. PJ[c]	247.0	19.5
4. control	269.5	17.5
5. PJ[c]	239.0	27.5
6. control	274.5	12.5
7. PJ[c]	238.5	28.0
8. control	273.0	14.0
9. PJ[c]	240.5	26.0
10. control	262.0	25.0

[a]Pancreatic juice preincubation glucose concentration 266.5 mg/100 ml.

[b]KRBG preincubation glucose concentration 287.0 mg/100 ml.

[c]Pancreatic juice.

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μg glucose uptake/2.0 ml	Diaphragm wt. (mg)	Glucose uptake (μg/mg)	Insulin effect (μg/mg)
440.0	61.0	7.21	5.50
80.0	46.8	1.71	
390.0	60.2	6.48	1.04
350.0	64.3	5.44	
550.0	63.5	8.66	4.44
250.0	59.3	4.22	
560.0	68.1	8.22	3.07
280.0	54.4	5.15	
520.0	93.6	5.56	-0.28
500.0	85.6	5.84	