THE DEMONSTRATION OF GLUCOSE-6-PHOSPHATE PHOSPHATASE ACTIVITY IN THE INTESTINAL MUCOSA OF THE BULLFROG *Rana catesbeiana* (Shaw)

by

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SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF ARTS

in the

GRADUATE SCHOOL

of the

UNIVERSITY OF MISSOURI

1958

The undersigned, appointed by the Dean of the Graduate Faculty, have examined a thesis entitled

THE DEMONSTRATION OF GLUCOSE-6-PHOSPHATE PHOSPHATASE ACTIVITY IN THE INTESTINAL MUCOSA OF THE BULLFROG *Rana Catesbeiana* (Shaw)

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ACKNOWLEDGMENT

The writer wishes to express his gratitude to Dr. Warren R. Fleming, under whose direction this problem was done, for his interest, advice, and encouragement. The writer is also indebted to Dr. Robert S. Campbell who contributed greatly in reading the manuscript and who offered many constructive criticisms.

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INTRODUCTION

In those animals in which food is taken into an alimentary tract and then digested, the products of such a process must cross the walls of the alimentary canal in order to be used by the tissues and cells. This introduces the question of how the products cross the wall, or, in other words, how are they absorbed. Since digestion converts the large complex foods into diffusible compounds of much smaller molecular dimensions it makes possible the passage of these substances across the wall by diffusion. It was logical then, that the early investigators in this field would apply the laws of physics and chemistry in the hope that they would explain the mechanism of absorption. While the laws of diffusion and osmosis are important factors in explaining intestinal absorption, there remained facts which could not be explained on any simple physicochemical basis.

Nagano (1902), one of the pioneers in this field, was probably the first to demonstrate carefully that sugar absorption could not entirely be explained by simple diffusion. By using a Vella fistulae he showed that the sugars were absorbed according to the series galactose>glucose>fructose>mannose>xylose>arabinose.

This field of study remained dormant for over two decades until Cori et al (1925 a, b) confirmed Nagano's results by obtaining precisely the same results. Since then, several workers have compared the rate of absorption of various sugars and generally the rates are the same as those found by Nagano (1902). The information gained from these observations was summed up by Verzar and McDougall (1936) when they said: "In the absorption of certain substances there is a special selective advantage which seems to be completely contrary to physical laws. These "favored" substances are ones which are especially used in the metabolism of the body. Such a specific selectivity is the result of active life processes in the mucosal cells."

In an attempt to explain this non-physicochemical phenomenon Wilbrandt and Laszt (1933) originally suggested that the reason hexose sugars were absorbed more readily than pentoses was the fact that hexoses are able to combine with phosphate. They believed that sugars were first phosphorylated in the mucosal cells and then dephosphorylated as the sugars were absorbed into the blood from the intestine. This same idea was given in more detail by Wilbrandt and Rosenburg (1951) who added that the glucose was first phosphorylated at the luminal border of the cell. The phosphorylated glucose passes through the cytoplasm by diffusion and

is dephosphorylated and extruded into the blood stream at the opposite border. Figure 1 represents this hypothetical scheme. Such an idea is also supported by the fact that both the phosphorylation and dephosphorylation reactions are generally common to cells and are present in the mucosal cells.

The evidence in favor of the theory has been many-sided. To cite only a few recent papers which support the phosphorylation mechanism, Ota and Shibata (1954) showed that the specific activity of the inorganic phosphate, acid soluble phosphate, and barium soluble phosphate increased following the injection of phosphorus^{32}. Ponz and Queralto (1955) studied the rate of excretion of inorganic phosphate, ester-phosphate, and lipid phosphate from the mucosa to the lumen of the intestine. Their data showed that the excretion of inorganic phosphate increased with glucose concentration while the movement of the ester phosphate was depressed under such conditions. In addition, Papadopoulos and Roe (1957) reported that in the case of fructose, the rate of disappearance of fructose corresponded to the rate of appearance of fructose esters in the rat mucosa. These results suggest that phosphorylation and dephosphorylation are fundamental steps in the absorption of fructose at least.

Although these papers suggest strongly that phos-

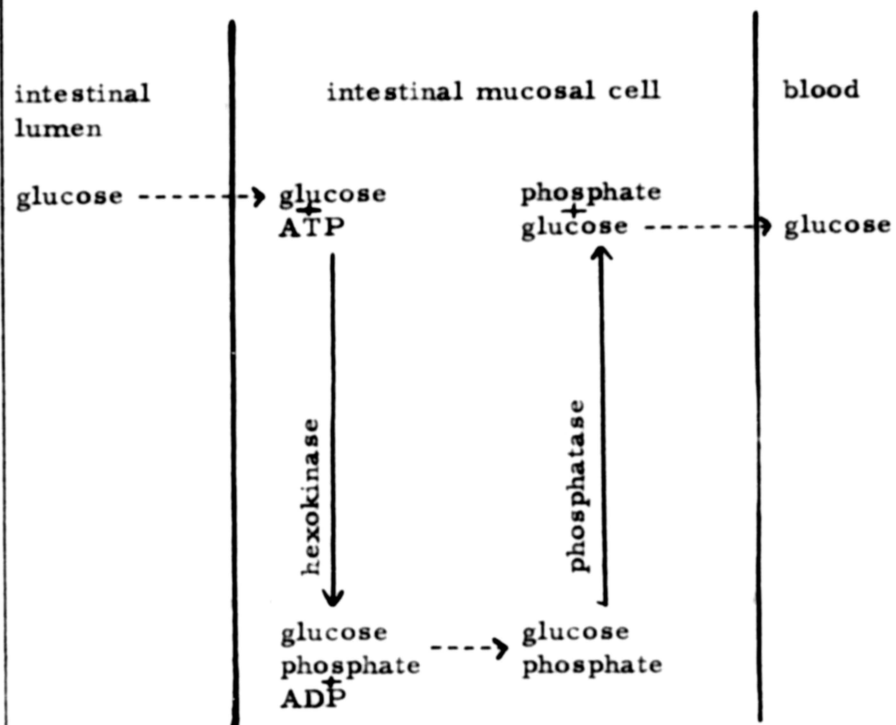

Figure 1 A hypothetical diagram showing absorption of glucose across the intestinal wall. Dotted lines indicate diffusion. Solid lines indicate chemical reaction.

phorylation plays a role in the absorption of glucose from the intestine, there is a considerable body of evidence to the contrary. Sols (1955), for instance, found no correlation between phosphorylation by hexokinase and the absorption of sugars from the intestine which suggest that phosphorylation is not part of the mechanism of sugar absorption. Probably the paper which is most damaging to the phosphorylation hypothesis is that of Crane and Krane (1956). In this paper they observed that 1-desoxyglucose and 6-desoxyglucose are actively transported by isolated loops of the small intestine of the hamster. Obviously, in the case of these two compounds at least, phosphorylation cannot be involved in the transport mechanism or mechanisms. These papers suggest strongly that some other mechanism or mechanisms are responsible for the transport of glucose across the intestinal wall.

Thus, while it is known that glucose is transported across the mucosa, the underlying mechanisms remain obscure. It was felt that the study of Crane and Krane (1956) did not eliminate the phosphorylation mechanism because of the possibility of alternate pathways of transport. It seemed that a critical study either would demonstrate that the activity of one of the enzyme systems postulated by Wilbrant and Lazt (1933) was correlated with transport of a specific molecular species, or would show that transport

could be inhibited without inhibition of the enzyme system. Though de Duve et al (1949) reported that glucose-6-phosphate phosphatase, the enzyme required for the specific splitting of glucose esters, was found in the intestinal mucosa of the rat his paper described only the characteristics of glucose-6-phosphate phosphatase as present in the liver. The only other paper which reports glucose-6-phosphate phosphatase in the intestinal mucosa was Maeda (1954). His paper is open to criticism, however, as he did his histochemical study at pH 7.2 which is a full pH unit above the optimum as found in this study for glucose-6-phosphate phosphatase activity. In addition, Hers (1951) reported that glucose-6-phosphate phosphatase was found only in the liver and kidney. In view of the uncertainty of the presence of glucose-6-phosphate phosphatase in the intestinal mucosa, it was first necessary to determine if this enzyme is indeed present. If not, it would seem impossible to construct a phosphorylation mechanism which would be specific for glucose.

The objective of this thesis then, is to determine if the enzyme glucose-6-phosphate phosphatase can be demonstrated in frog intestinal mucosa, and to determine the optimum conditions for its study.

CHAPTER 1

REVIEW OF THE LITERATURE

This review consists of two parts: the first part dealing with the literature related to the problem of the transport of sugars in the small intestine, the second with literature concerning the enzyme glucose-6-phosphate phosphatase.

Part One: Transport of Sugars in the Small Intestine

During the development of the various concepts of absorption across the intestinal tract, the question of whether or not absorption takes place in the intestine by a simple process such as diffusion through a membrane, divided the early workers into two groups. Those who believed that absorption could be explained by a simple physical process such as diffusion constituted the mechanists' camp while those who thought that an active process was involved were desiganated vitalists.

In the early literature it was emphasized by various authors that diffusion is the main force in the absorption of sugars. In fact, even until about the beginning of this century, the belief of most workers in this field was centered around a physical and

physico-chemical thesis as the mechanism of absorption.

In 1902, Nagano published his investigation which clearly showed preferential absorption of certain sugars. This paper may be viewed as a turning point in the study of absorption of sugars in the small intestine because of two things: (1) it was the first work which showed the preferential absorption of certain sugars, and (2) it has pioneered investigations of the mechanisms of sugar absorption by the small intestine from that time to date. Nagano (1902) obtained his results by introducing sugar solutions into Vella fistulas of the small intestine of dogs. He left the sugars in contact with the intestine for one hour and found the following order of the rate of absorption of sugars: galactose>glucose>fructose>mannose>xylose>arabinose. Hoeber (1899), in a similar experiment also on dogs, had shown that glucose and galactose were absorbed more rapidly than sucrose or lactose. He contended, however, that these results supported his theory that absorption ran parallel to diffusibility. Hedon (1900) also obtained similar results with rabbits. He found that glucose and galactose were absorbed by the intestine at a faster rate than arabinose. Hewitt (1924), using cats, found the following order of the rate of absorption of sugars: glucose>galactose>fructose. He also observed that these different rates of absorption disappeared if the mucosa

was destroyed with sodium fluoride or hot water. Full confirmation of the results of Nagano (1902) was made by Cori (1925 a, b). In his experiments the sugars under investigation were fed by a stomach tube. By studying the same sugars as Nagano (1902), his order of the rates at which the sugars were absorbed agreed precisely with the results obtained by Nagano. Cori also showed that 50% solutions of glucose, galactose, fructose, and mannose were absorbed at a constant rate even up to the point of 70% absorption of the sugars. Since 1925, numerous investigators have carried out experiments showing the relative rates of absorption of the different sugars. Table 1 gives the results of these studies. Though the results are not fully in agreement, they are ample proof that different sugars are absorbed at different rates from the small intestine. Further, these papers suggest strongly the posibility that the mechanism of sugar absorption is the same in most species.

Rogmann and Nagano (1903) reported that the relative absorption rates of dissacharides were sucrose>maltose> lactose. They also reported that absorption rates of sugars decrease progressively towards the caudal end of the small intestine. London and Polowzowa (1906) disagreed with this but it has been supported by von Frey (1909), Omi (1909), Westenbrink (1936 a), Verzar and Wirz (1937), Lium and Forey (1939), Dividson and

AUTHOR	ANIMAL	RELATIVE RATES OF ABSORPTION
Hober (1899)	Dogs	Galactose=fructose
Hedon (1900)	Rabbits	Glucose>galactose>arabinose>rhambose
Nagano (1902)	Dogs	Galactose>glucose>fructose>mannose> xylose>arabinose
Hewit (1924)	Cats	Glucose>galactose>fructose
	Rabbits	Glucose>fructose
Cori (1925)	Rats	Galactose>glucose>fructose>mannose> xylose>arabinose
McCance and Madders (1930)	Man and rat	Xylose>arabinose>rhambose
Maclead, Magee, and Purves (1930)	Rabbits and cats	glucose>xylose
Miller and Lewis (1932)	Rats	Glucose>xylose
Wilbrandt and Laszt (1933)	Rats	Galactose>glucose>fructose>pentoses

Table 1 Relative rates of absorption of monosaccharides from the small intestine.

AUTHOR	ANIMAL	RELATIVE RATES OF ABSORPTION
Westenbrink (1936b)	Rats	Galactose>glucose>fructose>mannose>xylose>arabinose
	Pigeons	Galactose>glucose>fructose>mannose>xylose>arabinose
Westenbrink and Gratame (1937)	Frogs	Galactose>glucose>mannose>fructose>xylose>arabinose
Green (1937)	Man	Galactose>glucose>fructose
Klinghoffer (1938)	Rats	Glucose>xylose
Davidson and Gary (1939)	Rats	Galactose>glucose>fructose>xylose
Davidson and Gary (1940)	Cats (distal end)	Glucose>galactose>fructose
Sols and Ponz (1947)	Rats	Galactose>glucose>fructose>xylose sorbose>arabinose>rhamnose
Darlington and Quanstel (1953)	Guinea Pigs	Galactose>glucose>fructose>sorbose

Table 1 (con't) Relative rates of absorption of monosaccharides from the small intestine.

AUTHOR	ANIMAL	RELATIVE RATES OF ABSORPTION
Cordier, Maurice, and Chanel (1954)	Fish	Glucose=galactose>fructose>xylose
Ota and Shibata (1955)	Rabbits	Galactose>glucose>fructose>ribose>xylose>arabinose
Cordier and Chanel (1957)	Fish	Glucose=galactose>arabinose>xylose>arabinose
Cordier and Maurice (1957)	Fish	Galactose>glucose>arabinose>xylose>fructose

Table 1 (con't) Relative rates of absorption of monosaccharides from the small intestine.

Garry (1940), and Hele (1953b).

Studying absorption rates of sugars Cori (1926) found that mixtures of sugars interfere with each other's absorption rates. Sobotaka and Reiner (1930) and Cajori and Karr (1935) confirmed Cori's results. Further, Hele (1953a), showed that sugars in mixtures interfere with each other's phosphorylation rates.

Interesting results were found by Gelhorn and Skupa (1933), who, by perfusing a frog intestine, showed that potassium salts increased, while calcium salts decreased, the absorption of glucose. This observation was confirmed by Gradver and Burget (1938). Both groups of investigators concluded that this could very possibly be a mode of controlling sugar absorption by the intestine for these findings agree well with the known effects of these ions on cell permeability.

In 1933, Wilbrandt and Laszt, presented evidence that iodoacetate, which is known to interfere with phosphorylating enzyme activity, reduces the absorption of glucose to about one third of the normal rate. The rate of absorption of galactose was also greatly reduced, that of fructose only slightly. The absorption rates of xylose and arabinose were not affected. They suggested that this was the reason why hexoses were absorbed more readily than pentoses. They added further that the physiologically im-

portant sugars required phosphorylation in order to bring about their rapid, selective absorption, and that other sugars are absorbed at a slower rate by ordinary physical means.

Westenbrink (1936) challenged the results of Wilbrant and Laszt (1933) on the grounds that, with rats, doses of monoiodoacetic acid given inhibited not only the absorption of glucose, but also the absorption of xylose. He maintained that this was due to the disturbing effect of iodoacetic acid on the circulatory system rather than a direct effect on the metabolism of the intestinal mucosa. Further, Westenbrink found that monoiodoacetic acid which did not kill pigeons in two hours did not effect glucose absorption.

Klinghoffer (1938) investigating the same problem by injecting monoiodoacetic acid subcutaneously, found that xylose and sodium chloride absorption, as well as that of glucose, was inhibited. This led Klinghoffer to point out that since there is no evidence that the absorption of xylose and sodium chloride are in any way associated with the phosphorylation process, plus the fact that the intestinal damage was severe, it was apparent that the inhibition of glucose absorption by monoiodoacetic acid could not be due to a specific effect on phosphorylation. Ohnell and Hober (1939) also felt that no conclusions could be drawn from the poisoning effect of monoiodoacetic acid, the reason being that even through

selective absorption of sugars were abolished, there was in most cases a severe and irreversible damage to the intestine. This poisoning effect was apparent particulary in those cellular functions in which energy is required whether phosphorylations are directly concerned or not. The idea of Wilbrant and Laszt (1933) was supported though, by similar experiments done by Nakazawa (1922), Lundsgaard (1933) and Wertherner (1933). In these experiments it was shown that phlorizin prevents phosphorylation and also the selective absorption of hexoses.

Laszt and Sullman (1935) were the first to show that during sugar absorption there is an accumulation of phosphorylated sugars in the intestine. Verzar and Sullmann followed up this report in 1937. They found that the intestinal mucosa of rats contained more acid soluble organic phosphate when glucose, fructose, galactose, mannose, and glycerol had been in the intestine than when the animals received a salt solution. Also the order on the amount of acid soluble organic phosphate formed by the different sugars was: Fructose>galactose>glucose>mannose. This of course, is different from the order of absorption rates of different sugars. Furthermore, in animals poisoned with iodoacetic acid there was not an increase in acid soluble organic phosphate of the mucosa on absorption of fructose.

Lundsgaard (1939) confirmed these findings and found higher phosphate accumulations than had been obtained by Laszt and Sullmann (1935). The higher values obtained in the case of fructose were interpreted as due to slower dephosphorylation, thus accounting for the slower absorption of this sugar. The highest values were obtained in extracts of intestinal mucosa from rats. Lundsgaard found similar accumulation in hexose absorping intestines from cats and rabbits. Reiser (1940) obtained corresponding values for swine during absorption of glucose.

Laszt (1940) found that during sugar absorption from isolated loops of the small intestine, but not during absorption of sodium chloride or amino acids, inorganic phosphate appears in the intestinal lumen. Also, with selectively absorbed sugars the rate of appearance and reabsorption are different from those with other sugars. Kjerulf-Jensen (1942) also demonstrated that sugar phosphates accumulated in the intestinal mucosa during the absorption of sugars by the intestines.

Beck (1942), using phlorizin, was unable to demonstrate an accumulation of hexose phosphates in the intestine of rats fed glucose plus phlorizin comparable to the increase found in rats fed glucose alone. In the animals given phlorizin there was a simultaneous decreased glucose absorption. These and other find-

ings led Beck to point out a strong possibility that the inhibition of glucose absorption was due to interference by phlorizin in the process of phosphorylation of the sugar.

Naito (1944) found that ribose is absorbed in the intestinal tract of rats (fasted 48 hours) at a rate different from the other pentoses. He stated that absorption of ribose is selective and is associated with a phosphorylation process which can be inhibited by iodoacetic acid. In ribose absorption the inorganic phosphate decreases and acid-soluble P increases in the intestinal mucosa. Naito (1944) reported also that the selective absorption of galactose, glucose, ribose, and fructose is associated with an increase in the rate of a glycogen-like substance in the intestinal mucosa. This increase is greater the higher the absorption rate and is confined to the region of the mucosa where the sugar is absorbed. It disappears when the absorption is inhibited by iodoacetic acid.

Sols and Ponz (1946) by circulation of fluid through a cannulated intestinal tract of rats and dogs found inactivation of the secreted phosphatases by heat and recirculation of the fluid produces a decrease sugar absorption. Also cadmium, copper, and phlorhizin leads to inhibition of the phosphatases and of the selective absorption of sugars. Further, addition of phosphatases

to the fluid, results in a concomitant increase of sugar absorption.

Bogdanove and Barker (1950) posutlated that there was specific phosphorylating enzyme. This was based on their findings that phlorizin inhibited the absorption of glucose, galactose and possibly mannose and sorbose but not fructose in rats.

Bissegger and Laszt (1951) found that the activity of intestinal hexokinase on various monosaccharides parallels their rate of absorption.

2, 4-dinitrophenol when given even in a toxic concentration had no retarding effects on the absorption of a galactose-sorbose or glucose-xylose mixture in ligated intestinal loops of the rat, though phlorizin did retard. This conflicting report of the action of 2, 4-dinitrophenol was made by Bruckner (1951). It is repudiated by Darlington and Quastel (1953).

Wilbrandt and Rosenburg (1951) postulated a phosphorylation theory for the mechanism of transport of sugars across the intestinal wall which is generally the one accepted today. In this theory they visualized glucose first being phosphorylated at the luminal border then the phosphorylated glucose diffuses to the opposite border. There, the ester is dephosphorylated and enters the blood stream, by diffusion (refer to figure one).

In 1952 Ponz and Larralde observed that glucose

absorption by intact rat intestine was depressed by monoiodoacetic acid, sodium fluoride, and by phlorizin when they were administered in the glucose solution. They postulated since cell permeability is effected only at much greater concentrations the effect of these drugs appeared to be on enzymes affecting selective absorption of glucose.

Sapiro (1952a) from work with isolated fresh rat intestine suggested the possibility that what was regarded as actively absorbed glucose being transported as glucose across the intestinal membrane actually wasn't the case. He presumed that glucose is really metabolized to some other substance and re-transformed to sugar elsewhere, presumably in the liver.

In 1953 Hestrin-Lerner and Shapiro published the first of their two highly controversial papers. Their work was initiated with the intention of establishing whether or not an active transfer of glucose could be demonstrated in the isolated rat intestine. By starting with identical solutions inside and outside the loop a concentration gradient was rapidly developed. The magnitude of the uptake of glucose was of the same order of magnitude as that found *in vivo* in similar solutions. The difficulty came when they found that the glucose disappearing from the inner solution could not be recovered in the outer solution nor could it be

extracted from the mucosa. The sugars which were not actively absorbed *in vivo* were not absorbed in the isolated intestine and phlorizin did inhibit the uptake of glucose. To follow up, similar experiments were performed with carbon ^{14} glucose. In these experiments they found that a metabolite accumulated in the intestinal tissue and diffused into the outer solution. Only a negligible amount of the glucose was oxidized to carbon dioxide. This metabolite was isolated, purified and analyzed and it could not be idenified with any of the known intermediary metabolites of glucose. From these results they gave three alternative explanations: (a) the formation of the metabolite is not related to the transport mechanism and is the result of a high rate of intestinal metabolism of glucose, (b) the transport mechanism involves, to a large extent, the transfer of glucose across the intestine as a metabolite, with reformation of glucose, and (c) the formation of the metabolite, is an artifact, due to the peculiar conditions in these *in vitro* experiments. Hestrin-Lerner and Sapiro (1954) in their second paper made the same experiments *in vivo.* In one series of tests with rats the portal-arterial differences in glucose concentrations were studied during the resorption of known amounts of glucose. In a second series, the intestine was perfused *in situ* with blood of known composition and the entire blood perfusing the intestine was collected

from the portal vein and analyzed. As was found in their first paper, actively absorbed glucose is transported into the blood in the form of a metabolite. Lactic acid found in the perfusion fluid, accounted for about one fourth of the glucose which disappeared. Oxidation accounted for only a negligible part of the glucose which disappeared from the lumen. It was their idea that glucose is not transported as such although they admitted that there is a preferential uptake of glucose from the lumen of the intestine. They pointed out the work of Lundsgard (1935) who found that the phosphatase enzyme is concentrated near the luminal border contrary to the hypothesis of Wilbrandt and Rosenburg (1951). Also they attacked the idea that the correlation found between the inhibition of absorption and the poisoning of the postulated enzymatic processes by phlorizin could be used as evidence for the phosphorylation hypothesis. In support of these views, they cited the four following papers: (1) Gomore (1939) who found that the concentration of phlorizin, sufficient to cause complete inhibition of glucose absorption in the kidney, has no measurable effect on glucose phosphorylation, (2) Shapiro (1947) who showed that phlorizin in the kidney inhibits the formation of high energy phosphate bonds which decreases the available chemical energy; in other words, any process requiring energy, regardless of the

mechanism involved, might thus be poisoned by phlorizin, and (3) that phlorizin is not a specific inhibitor as showed by Lambrechts (1937) by the fact that phlorizin inhibits the reabsorption of various dyes in the kidney, and White (1940) who showed that phlorizin inhibited the secretion of diodrast by the kidney tubules.

In a series of two papers Hele (1953a, b) reported that different monosaccharides have different phosphorylation rates and that this determines the relative absorption rates of the various sugars. He postulated that the effect of phlorizin and iodoacetic acid upon sugar absorption might be simply due to the lowering of available ATP in the mucosa which would decrease the rate of hexokinase activity. Further, when the sugar concentration is not the limiting factor in hexokinase activity, the rate of absorption varies with the concentration of ATP. Also, sugars in mixtures show a depression in phosphorylation rate below that of single sugar controls, and that the rate of the hexokinase reaction in the intestinal mucosa is more that sufficient to account for the phosphorylation of all the sugars absorbed in the experiments done *in vivo*.

By perfusion of a isolated guinea pig intestine Darlington and Quastel (1953) found that fructose is absorbed by a transformation to glucose. Contrary to the report of Brucker

(1951) they found that 2, 4-dinitrophenol exercises, at low concentrations, a highly inhibitory effect on active absorption of glucose. Glucose, galactose, and fructose were selectively absorbed in relative rates similar to those which have been observed. They confirmed Verzars (1935) work that sorbose enters the intestine only by diffusion. They reported that there was no concomitant transfer of phosphate, and that the phosphate was not required in the perfusion.

Wilson (1953) perfused the upper small intestine of the rat *in vitro* with a glucose solution. Of the glucose which is absorbed more than 80% of it appeared on the serosal side as lactic acid. Another surprise was finding the fall of the bicarbonate ion concentration on the mucosa side. He argued that the production of lactic acid in one direction suggests that lactic acid may play a role in the absorption of glucose. In 1956 Wilson repeated this and found practically the same thing.

Ota and Shibata (1954) reported that phosphorylation appeared to be involved in the absorption since the specific activity of inorganic phosphorus, acid soluble phosphorus, and barium soluble phosphorus fractions of the intestine following the injection of sodium monobasic phosphate or the perfusion of intestinal segments with radioactive sodium monobasic phosphate

was about twice as high in the presence of glucose as in the control. Moreover, the magnitude of the barium-soluble phosphate and the adenosine-triphosphate fractions of the intestinal segments was increased and that of the inorganic phosphate fractions was decreased upon the addition of glucose. Also reagents such as 2, 4-dinitrophenol, phlorizin, or monoiodoacetate, which interfered with the phosphorylation process by the imposition of anaerobic conditions, caused the rate of absorption of glucose, galactose, and fructose to fall to that of arabinose. They suggested that since during the absorption, most of the phosphate in either the portal blood in the *in situ* experiments or in the outer perfusion blood in the *in vitro* experiments was in inorganic form and there was not an increase in the specific activity of this phosphate above that in controls without sugar, then sugars undergo phosphorylation during absorption, and phosphorus must be split off as the sugar passes from the intestine into the blood or into the outer perfusion fluid.

In contrast to the paper of Ota and Shibata (1954), Sols (1955), found that when he compared the rates of phosphorylation by hexokinase, no correlation could be demonstrated. This, he reasoned, ruled out hexokinase as primarily related to the mechanism of active sugar absorption and also suggested that

phosphorylation is not a part of the process. Crane and Krane (1956) also came to the conclusion that the phosphorylation process is not involved in intestinal absorption of sugars. They arrived at this conclusion when they obtained active transport of 1-Deosyglucose and 6-Deoxyglucose by using an isolated, everted hamster intestine. This should not occur if phosphorylation is essential. The reason being that these compounds lack the hydroxyl groups at carbon atoms one and six and are therefore incapable of undergoing phosphorylation.

Ponz and Queralto (1955) showed that the excretion of inorganic phosphate increased with the glucose concentration while the movement of the ester phosphate was depressed by these conditions in agreement with Ota and Shibata (1954).

Cordier and Worbe (1955) found that prolonged oxygen deficiency retards absorption of glucose and galactose in the intestine of the live frog but has no influence on absorption of fructose and xylose. They concluded that a possible reason for this is that phosphorylation of glucose and galactose is inhibited.

Klingmuller and Schweiger (1955) reported that they confirmed the results of Hestrin-Lerner and Shapiro (1953, 1954) when they found that glucose, absorbed by the internal surface of the intestine, was only recovered in part from the external sur-

face of the intestine; was only recovered in part from the external surface, even when high concentrations of glucose were used. Their results were not confirmed by Taylor and Langdon (1956) however. These authors observed that when they administered glucose-1-C^{14} by stomach tube to rats that over 90% of the absorbed glucose found in the liver glycogen was apparently absorbed by the intestine and transported to the liver as a 6-carbon chain. Glucose which was changed during the process of intestinal absorption and transported to the liver is of minor significance.

Fridhandler and Quastal (1956) using the isolated guinea pig intestine, reported that the amount of fructose absorbed by the gut increases with a corresponding increase of fructose in the perfusion fluid. They found, however, that there is a limiting value for fructose absorption and that the same is true of sorbose and sucrose. They demonstrated further that fructose appeared on the serosal side principally as glucose, using low concentrations of fructose in the mucosal solution, but mainly as fructose when high concentrations were used. In addition they showed that glucose formation from fructose was inhibited by 2, 4-dinitrophenol and anaerobic conditions whereas phlorizin did not so inhibit. The appearance of fructose on the serosal side was not inhibited by any of the three conditions. They maintained that these results sup-

ported the conclusion that there is no "active" absorption of fructose and that glucose formation from fructose in the intestine is controlled by enzymes.

Kiyasu et al (1956) challenged the results of Hestrin-Lerner and Shapiro (1953, 1954). They followed the absorption of C^{14} glucose from a loop of small intestine isolated with ligatures and prepared so as to make possible the collection of all the venous blood drain from this loop. Analysis of the venous blood and the intestinal mucosa accounted for practically all the C^{14}. They found that 82-92% of the C^{14} was glucose, 4-16% as lactate, and 1-5% as alanine in the portal plasma. These authors pointed out that though these results were not in agreement with Hestrin-Lerner and Shapiro (1953, 1954) it should be noted that the conditions of the two experiments were different. Alkinsom et. al. (1957) also found that when glucose labled with C^{14} was placed in a segment of a small intestine in an anesthetized dog, the venous blood drained from the dog contained the following: (1) 70-80% glucose, (2) 7-17% lactic acid, and (3) insignificant amounts as pyruvic acid, alanine, and carbon dioxide.

Still another paper disagreeing with Hestrin-Lerner and Shapiro (1953, 1954) was published by Hawkins and Wills (1957). By administering glucose-1-C^{14} by stomach tube and sampling the

blood they found little degradation of the glucose molecule while it was being absorbed. Also doing *in vitro* experiments with intestinal sacs filled with glucose-1-C^{14} and incubated in Krebs-Ringer phosphate, they observed that practically all of the radioactivity appearing in the outside fluid could be accounted for as glucose. They suggested that the major portion of glucose is normally absorbed *in vivo* without degradation and the remainder is metabolized to provide energy for absorption. *In vitro*, there can be considerable degradation without transport.

In an interesting paper Wilson (1957) making *in vitro* studies by using the everted small intestine sac technique did not obtain glucose transport across the intestinal wall of the following fish: sea robin (*Prinotus carolinus*), scup (*Shenotomus*), toadfish (*Opsanus tau*), or puffer. Galactose and 6-deoxy-D glucose were also tested with the puffer gut preparation and were not transported.

Papadoporlos and Roe (1957) reported a large increase in fructose phosphate esters in the intestinal mucosa after fructose administration to a fasted rat. This, they interpreted to indicate that phosphorylation of sugars in absorption serves a more extensive function than to initiate glycolysis for normal metabolism of mucosa cells. In addition, the data suggested that phosphorylation and dephosphorylation are functional steps in the absorption

of fructose from the small intestine.

Nagasawa (1957) studied the mechanism of absorption of sugars by observing the phosphate esters which were formed. He injected a solution of glucose, fructose, or galactose, and $P^{32}O_4$ into the stomach of starved rats and after a period of one hour determined the amount of phosphate esters in the intestinal mucosa. He found the following phosphate esters: glucose-1-phosphate, glucose-6-phosphate, and fructose-1, 6-diphosphate after injecting glucose; glucose-1-phosphate, glucose-6-phosphate, galactose-1-phosphate, and galactose-6-phosphate after injecting galactose; and glucose-6-phosphate, fructose-1, 6-diphosphate, and fructose-1-phosphate after fructose. Further, the absorption of glucose-1-phosphate, glucose-6-phosphate, and fructose-1, 6-diphosphate were not effected though glucose and fructose were reduced about 40% when dinitrophenol was added.

Part Two: Glucose-6-phosphate Phosphatase

The indentification of the enzyme glucose-6-phosphate phosphatase has been relatively recent, though the investigation which led eventially to the characterization of this enzyme was done in the 1930's. The interest in the enzyme developed out of studies concerning the production of glucose in the liver from

glucose phosphate. Papers, such as the ones published by Cori and Shine (1936) and Goda (1937) who reported that hexose-6-phosphates were dephosphorylated by liver pulp were the beginning. A year later Cori and Cori (1938) even suggested that glucose of the liver and blood is formed by dephosphorylation of glucose-phosphates.

From these studies there was obviously an enzyme in the liver catalysing the reaction which produces glucose from glucose phosphate. Accordingly, there has been several investigators who have studied the reactions in liver extracts in order to determine the nature of the phosphatase which splits glucose phosphates.

Though two non-specific phosphatases had already been identified, a paper published in 1945 by Fantl and Rome was the first to hint strongly of a specific phosphatase which would split glucose-6-phosphate. By using fresh extract of rabbit liver they postulated that in addition to the two nonspecific phosphomonoesterases, a specific enzyme which causes the breakdown of glucose-6-phosphate into its components occurred in this tissue. They justified this conclusion by the observation that the pH activity curve for the breakdown of hexose-mono-phosphates differ from the pH activity curves obtained when other phosphoric acid esters

were used as the substrate. Also they demonstrated that glucose-6-phosphate phosphatase is less stable than phosphoglucomutase, isomerases, and the non-specific phosphatases by dialysis experiments. They further observed that glucose-6-phosphate phosphatase reacts to inhibitors in much the same manner as the non-specific phosphatases, sodium fluoride inhibiting both to the same degree although phlorizin inhibited glucose-6-phosphate phosphatase more than the other enzymes.

de Duve et al (1949) also reported evidence for the existence of specific glucose-6-phosphate phosphatase. They separated the enzyme from acid phosphatase and did not obtain any activity on B-glycero-phosphate or in the absence of phosphoglucomutase of glucose-1-phosphate. de Duve's group claimed that glucose-6-phosphate phosphatase was found in the intestine, the liver, and the kidney. This, they argued, supported the hypothesis that both tubular reabsorption and intestinal absorption of glucose occurred through a phosphorylation mechanism. They suggested further that the glucose-6-phosphate phosphatase reaction was the mechanism by which glucose entered the blood stream.

Possibly the paper which most completely characterizes the enzyme was published by Swanson (1950). In this paper it was again observed that there is a specific enzyme,

glucose-6-phosphate phosphatase, which had a very high affinity toward glucose-6-phosphate and a low affinity toward glucose-1-phosphate and fructose-1-phosphate. There was a high degree of inactivation by even a very short exposure to heat. This she maintained, is the reason why some workers had all three hexose phosphates split at the same rate. She challenged also, Fantl and Rome's (1945) observation that glucose-6-phosphate phosphatase could be inactivated while the mutase and isomerase were still active. By pointing out that whole homogenates hydrolyze glucose-6-phosphate so much more rapidly than glucose-1-phosphate she also concluded that a specific glucose-1-phosphatase is not present in the liver. As had other workers, she noted that the pH at which there was an optimum activity for acid and alkaline phosphatase was different from the pH at which there was the greatest glucose-6-phosphate phosphatase activity. She maintained that this showed that glucose-6-phosphate phosphatase is not a part of either of the two other groups of phosphatases. There was also a high activity of glucose-6-phosphate phosphatase in the soluble proteins and large granules of the cell. Carrying out inhibition studies, she found that there was no inhibition by calcium, magnesium, arsenite, iodoacetate, cyanide, and phlorizin up to 0.5% but there was inhibition by molybdate and fluoride.

Hers et. al. (1951) reported that the activity of glucose-6-phosphate phosphatase of the liver and kidney was confined largely to the microsomes.

In the first of three papers, Beaufay and de Duve (1954a) showed that glucose-6-phosphate phosphatase of the rat liver microsomes lost its activity when incubated at pH 5.0. At pH 6.5 it reacted readily with glucose-6-phosphate but had little or no action of fructose diphosphate and phosphoric esters of gluconic acid, glycolic acid, amino ethanol, and morpholinoethanol. Beaufay et. al. (1954b) reported a pH of 6.0 for the optimum activity of glucose-6-phosphate phosphatase. Also in this paper they presented a series of results concerned with the inhibition, activation, and protection of the enzyme from inactivation. Their results did not agree fully with Swanson's (1950). They reported that low concentrations of calcium ions slightly activated but higher concentrations of calcium ions as well as any concentration of magnesium ions inhibited. Also, the enzyme is irreversibly inactivated by zinc and cuprous ions, and alloxan. Sodium fluoride partially inhibits with maximum effect at 0.02 M. The substances which protected the enzyme from inactivation were proteins, citrate, oxalate, sulfides, cyanides, and especially, versene. The final contribution of this paper was that preparations of

glucose-6-phosphate phosphatase may be dialyzed against dilute sodium bicarbonate solution without loss of activity. In the final paper, Beaufay and de Duve (1954c) made an attempt to determine the composition of the enzyme. By adding ribonuclease, or by the extraction of the nucleic acid using appropriate solutions, hydrolysis of the microsome nucleic acid was effected. This procedure, though did not effect the activity of the enzyme. When they treated the microsomes with chymotrypsin, glucose-6-phosphate phosphatase was inactivated but not liberated. Also testing with lecithinase they obtained about 75% inactivation in the same time required to hydrolized all the choline phospholipid. The remainder of the enzyme was made more sensitive to denaturation by heat. From these experiments they postulated that glucose-6-phosphate phosphatase is a lipo-protein and was very strongly bound to the microsomes.

In an interesting paper, Maeda (1954) showed by histochemical studies that glucose-6-phosphate phosphatase activity is very active in the liver cells, kidney epithelia, small intestinal mucosa, and the cartilage tissue of mice. His paper is open to criticism however, for his studies were done at the pH of 7.2 whereas the optimum for glucose-6-phosphate phosphatase activity is the pH of 6.0 (Beaufay et. al. (1954b).

It is generally accepted that there is a mechanism for the preferential uptake of glucose, galactose, and fructose by the small intestine. Also, as has been shown, there has been work suggesting that the hypothesis of Wilbrandt and Laszt (1933) is one such possible mechanism for the absorption of glucose. The results of Ota and Shibata (1954) and Nagasawa (1957) also suggest that fructose and galactose are phosphorylated and dephosphorylated during absorption. Darlington and Quastel (1953) interpreted their results to indicate that fructose is transported by a transformation into glucose. Later, Fridhandler and Quastel (1956) obtained results which supported Darlington and Quastel (1953). From the above there is a possible implication of a specific phosphatase which will split glucose-6-phosphate. Fantl and Rome (1945) and others since have observed a specific enzyme in the liver, glucose-6-phosphate phosphatase, which seemingly fits these qualifications, but its presence in the intestine is uncertain. Therefore, a study clarifying the presence of glucose-6-phosphate phosphatase in the intestine, would suggest further, the possibility of the construction of a phosphorylation mechanism which would be specific for glucose and vice versa.

The experiments reported here were carried out with the purpose of possibly demonstrating glucose-6-phosphate

phosphatase in the intestinal mucosa of the bullfrog, *Rana catesbeiana*.

CHAPTER 2

MATERIALS & METHODS

All the work was done on the bullfrog *Rana catesbeiana* (Shaw), obtained from commercial sources and collected locally. As soon as they were received they were placed in a battery jar which contained 800 cc. of water. They were kept at a temperature of 20°C to 23°C for at least a week before they were used. These animals were considered as starved.

A starved animal was pithed and three cc. of a glucose solution (5000 micrograms/milliliter) was injected just posterior to the phloric sphincter in the intestine. Animals which served as controls were pithed and the abdomen opened. At the end of a four-hour period the intestine was removed and was rinsed by injecting 20 milliliters of cold distilled water just posterior to the pyloric sphincter. The intestine was opened and the mucosa was removed by scraping the mucosa off with a scapel. The mucosa was placed directly into two to five milliliters of cold distilled water to be homogenized. This procedure was carried out at 2°C to 4°C. The mucosa was kept cold in an ice water bath.

The sample was homogenized using a Vir Tis "45"

Homogenizer.

The assay method used to measure the activity of glucose-6-phosphate phosphatase is based primarily on that of Swanson (1950). The incubation mixture contained 0.1 milliliter of glucose-6-phosphate (0.075 M) and 0.3 milliliter of 0.076 M Tris buffer added to a cone shaped centrifuge tube and placed in a warm water bath (37°C). At least a period of five minutes was allowed for the above to reach the temperature of 37°C. One-tenth milliliter of the homogenate was then added and incubated for a period of 30 minutes at the temperature of 37°C. At the end of the incubation period the enzyme activity was stopped by placing in a boiling water bath for one and a half minutes. Controls were boiled at the beginning of the incubation time. The sample was centrifuged until the tissue was separated from the supernatent. Assays were run in duplicate.

By using an alloquot of the supernatent the glucose split was measured directly by the glucostat method (Worthington Biochemical Corporation).

The activity of the glucose-6-phosphate phosphatase is expressed in micrograms of glucose which were released from glucose-6-phosphate by a sample of homogenate which contained one milligram of nitrogen.

The procedure of Pulley (1954) was used to determine total nitrogen. In this procedure 0.2 milliliters of homogenate is added to 3 milliliters of digestive fluid (50% concentrated sulfuric acid) and heated between 400°C and 450°C. This was done on a Micro Kjeldal Apparatus and was continued until the solution was clear. Seven and five-tenths milliliters of 5 normal sodium hydroxide was added and 0.5 milliliter alloquot was added to 10 milliliters of distilled water in an ice water bath. After five minutes 1.5 milliliters of Nessler Reagent (Hartman-Leddon Co.) was added. The color intensity was determined at 450 millimicrons after 15 minutes. This was compared to a standard ammonium chloride solution. A Bausch and Lomb Spectronic 20 was used in all the spectrophotometric determinations.

Acid phosphatase and alkaline phosphatase assays were run with each glucose-6-phosphate phosphatase assay. This was necessary since acid phosphatase and alkaline phosphatase also split glucose-6-phosphate.

CHAPTER 3

EXPERIMENTAL

Since the presence of glucose-6-phosphate phosphatase in the small intestine is uncertain, it was necessary to first of all demonstrate the presence of the enzyme.

To demonstrate the presence of glucose-6-phosphate phosphatase in the intestinal mucosa the results of the following papers were used. Fantl and Rome (1949) concluded that a specific glucose-6-phosphate phosphatase was present in the liver as they obtained a pH activity curve for the breakdown of hexose-monophosphates that was different from the pH activity curves obtained when other phosphoric acid esters were used as the substrate. By similar experiments, de Duve et. al. (1949) and Swanson (1950) reported that this enzyme had a pH optimum of 6. 5 while Beaufay et. al. (1954b) reported a pH optimum of 6. 0. These observations were all obtained with liver homogenates.

The demonstration of glucose-6-phosphate phosphatase activity in the small intestinal mucosa could be attempted by using these reports as a guide. Using tris buffers at different pH ranges, and mucosal homogenates, pH activity curves were

found for the different substrates, p-nitrophenol phosphate and glucose-6-phosphate.

A modification of the sigma serum acid phosphatase determination was used for determining the pH activity curve using p-nitrophenol phosphate as the substrate. The following changes were made: (1) 0.5 milliliters of tris buffer at pH 6.0 was substituted for 0.5 milliliters of citrate buffer at pH 4.8 and (2) six milliliters of 0.1 normal sodium hydroxide instead of four milliliters of 0.1 normal sodium hydroxide was added to stop the enzymatic reaction.

Figure 3 shows a pH activity curve obtained by substracting the activity obtained using p-nitrophenol phosphate as the substrate from the activity using glucose-6-phosphate as the substrate (figure 2). The resulting pH activity curve shows the activity of glucose-6-phosphate phosphatase. This curve is drawn on the assumption that (1) the curve obtained by using p-nitrophenol phosphate as the substrate is the result of the activity of the two groups of nonspecific phosphatases (acid and alkaline) because glucose-6-phosphate phosphatase being specific for glucose-6-phosphate supposedly will not split p-nitrophenol phosphate, and (2) the curve for glucose-6-phosphate used as the substrate is a representation of the activity of glucose-6-phosphate phosphatase

(a) Glucose-6-phosphate as Substrate

Frog	pH used						
	5.0	5.4	5.75	5.95	6.15	6.45	6.85
1	313	250	278	333	319	270	250
2	260	292	292	290	247	233	220
3	185	172	193	193	193	178	157
average	253	238	261	272	253	227	209

(b) p-nitrophenol Phosphate as Substrate

Frog	pH used						
	5.2	5.55	5.90	6.00	6.075	6.3	6.45
1	13.9	12.4	9.2	9.2	9.6	8.0	6.4
2	13.9	13.2	10.0	9.6	9.6	8.4	6.8
average	13.9	12.8	9.6	9.4	9.6	8.2	6.6

Table 2: pH activity curves using (a) glucose-6-phosphate and (b) p-nitrophenol phosphate as the substrate. The activity of (a) is in micrograms of glucose release per milligram nitrogen and of table (b) is in alkaline sigma units per milligram nitrogen X50.

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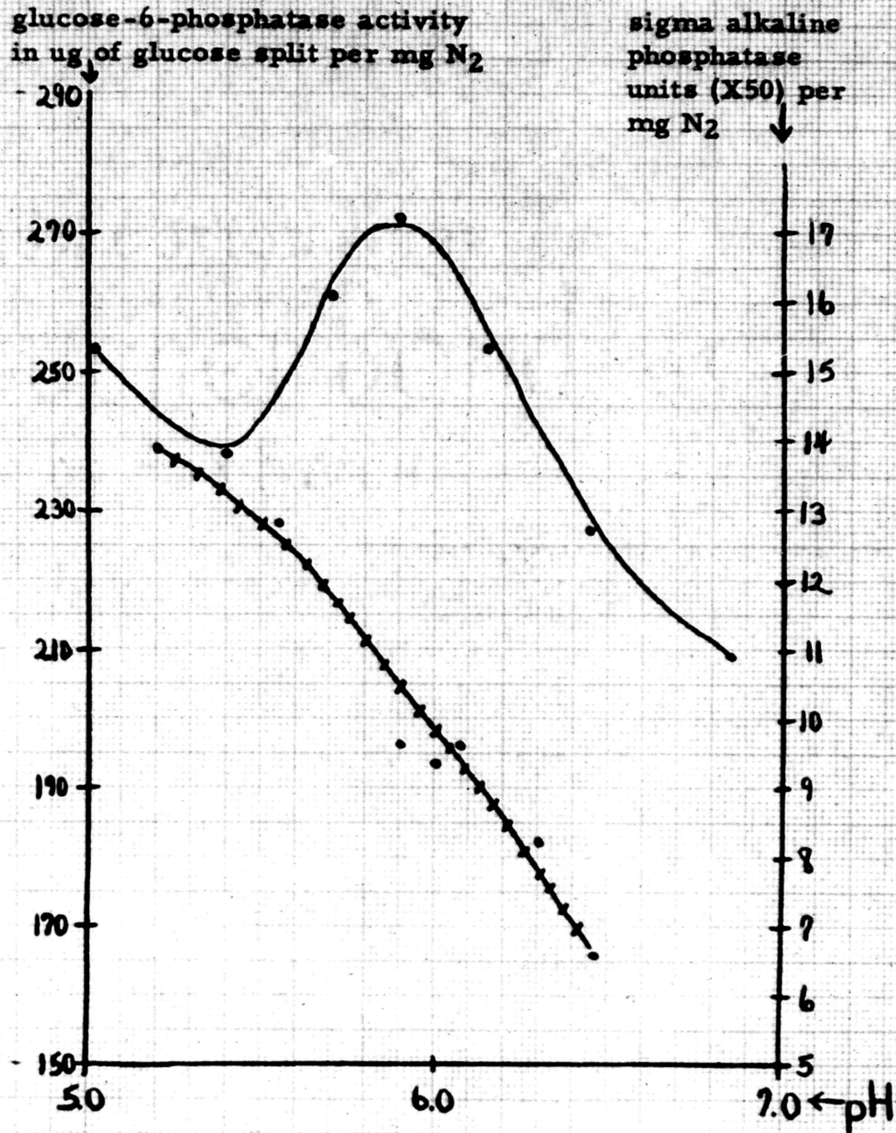

Figure 2: (a) pH Activity Curve with Glucose-6-phosphate As Substrate and (b) pH Activity Curve with p-nitrophenol Phosphate As Substrate

Curve (a) ——

Curve (b) -+-+-+-

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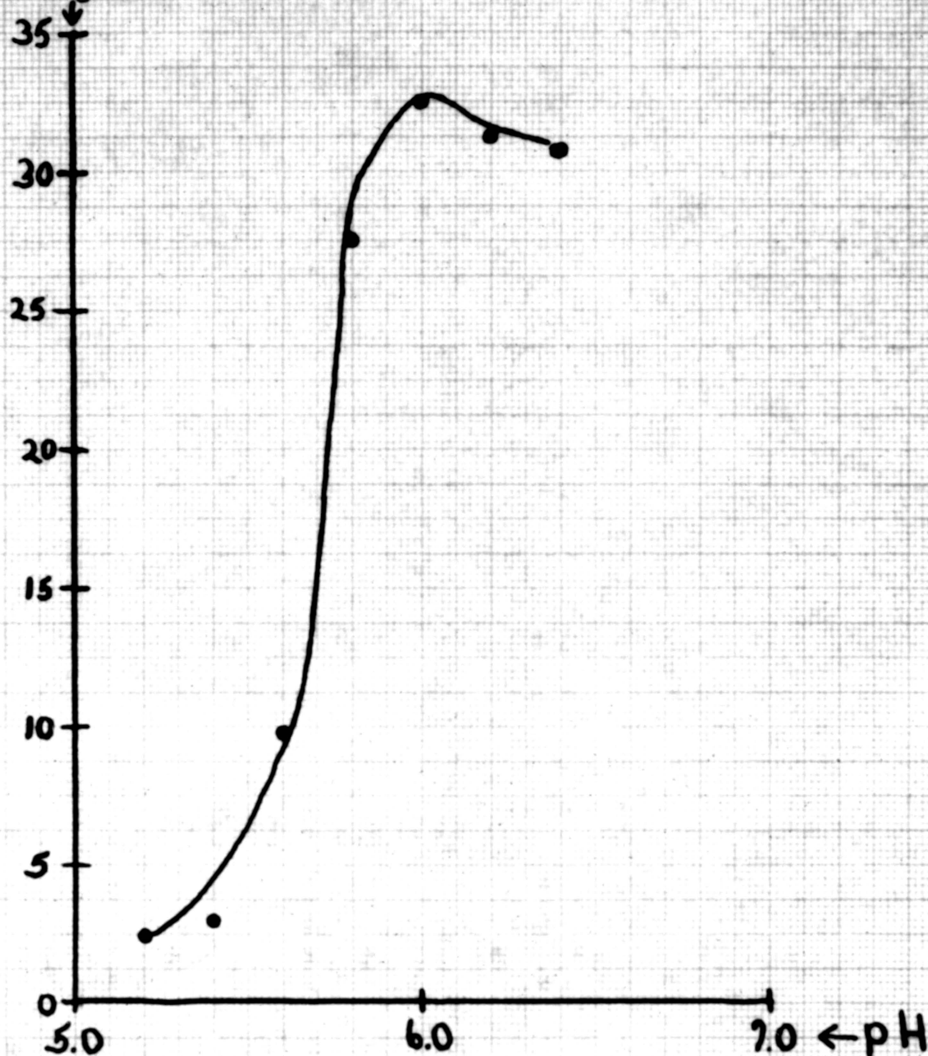

Figure 3: pH Activity Curve for Glucose-6-phosphate Phosphatase

as well as the activity of acid and alkaline phosphatase. Therefore, if glucose-6-phosphate phosphatase is present, the subtraction of the former pH activity curve from the latter would show the activity of this enzyme.

The data presented (figure 2 and 3 and table 2) show that there is an enzyme in the intestinal mucosa which has an optimum activity at approximately pH 6.0, and will split glucose-6-phosphate but p-nitrophenol phosphate only slightly or not at all. These observations correlate well with the previous reports concerning the enzyme glucose-6-phosphate phosphatase of the liver and it seems safe to suggest that the enzyme reported by Fantl and Rome (1945) is also present in the intestinal mucosa.

Characteristics Determined for the Developement of a Satisfactory Assay Method for Glucose-6-phosphate Phosphatase

The previous data have demonstrated the presence of glucose-6-phosphate phosphatase in the intestinal mucosa. It was then necessary to work out the optimum conditions for the study of enzyme in order to develop a satisfactory assay method.

Two additional characteristics necessary for the developement of a standard assay method for glucose-6-phosphate phosphatase of the small intestine are: (1) the determination of the

concentration of glucose-6-phosphate (substrate) which would favor optimum enzymatic activity and (2) the buffer which would allow for optimum activity.

Assays of glucose-6-phosphate phosphatase activity were run using different concentrations of substrate (glucose-6-phosphate). As seen in table 3 and figure 4 as the concentration of the substrate used in the assay is lowered the percentage of the substrate split is increased. From this, it was decided that the lowest possible concentration which could be used and still maintain reliability for the assay method was a 0.075 molar glucose-6-phosphate solution.

Examination of table 4 shows phosphate buffer is inhibitory when compared to maleate and tris buffers. This is probably due to a mass law effect. Tris buffer was chosen for the assay of glucose-6-phosphate phosphatase activity.

Effect of the Presence of Glucose in the Intestinal Lumen on the Activity of Glucose-6-phosphate Phosphatase

Since the presence of glucose-6-phosphate phosphatase has been demonstrated in the small intestine and a satisfactory assay method determined, a study directed at uncovering the function of this enzyme in the intestine would be relevant. It

Frog	Concentration of Glucose-6-phosphate Used				
	0.0323	0.05395	0.0898	0.1495	0.249
1	7.68	5.52	3.76	3.35	2.27
2	8.32	6.73	4.29	2.78	2.91
3	6.22	5.22	4.78	3.55	2.35
average	7.76	5.82	4.31	3.39	2.51

Table 3: Activity of glucose-6-phosphate phosphatase at different substrate (glucose-6-phosphate) concentrations. Figures are in percent glucose released out of the total amount possible.

was decided that if the presence of glucose in the small intestine would result in increase in glucose-6-phosphate phosphatase activity, it would suggest a possible role of glucose-6-phosphate phosphatase in the transport of glucose in the small intestine. To study this hypothesis, the following experiment was carried out.

Frogs which were starved and had been conditioned to 4°C were used. These animals were run in groups of four with two being injected with three milliliters of a glucose solution in the small intestine and two serving as controls. Alkaline phosphatase assays were done at the pH 10.5, and acid phosphatase assays at the pH 4.8 and 6.0 as well as the glucose-6-phosphate phosphatase assays at the pH 6.0. Sigma's alkaline and acid phosphatase assay

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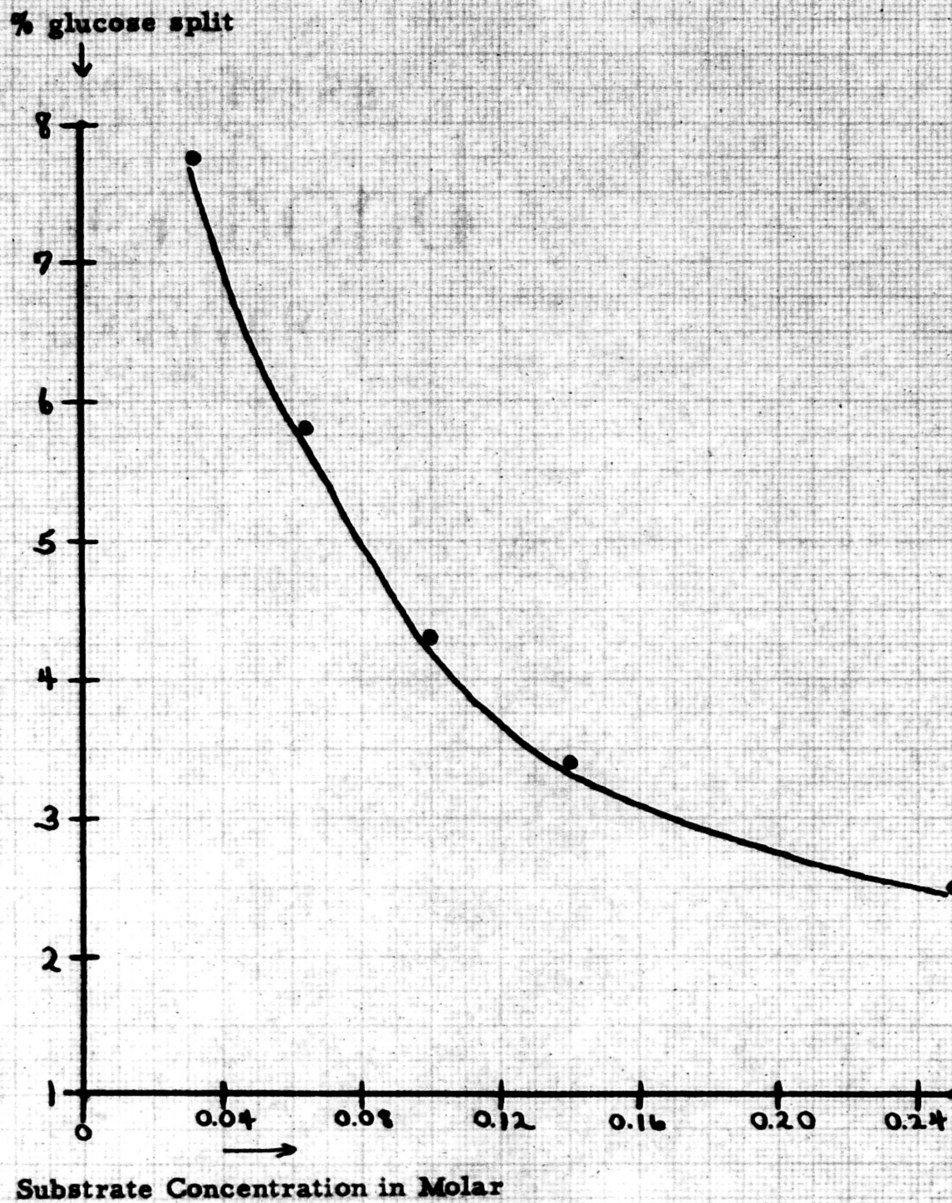

Substrate Concentration in Molar

Figure 4: Activity of Glucose-6-phosphate Phosphatase at Different Substrate Concentrations

		Maleate Buffer	
Frog		pH Used	
	6.6	8.7	11.4
1	300	250	200
2	260	220	175
average	280	235	188
		Tris Buffer	
		pH Used	
	5.95	6.45	9.2
1	333	270	220
2	305	258	195
average	319	264	208
		Phosphate Buffer	
		pH Used	
	5.9	6.4	7.15
1	200	187	197
2	180	178	182
average	190	183	190

Table 4: Glucose-6-phosphate phosphatase activity in the presence of different buffers (figures are in micrograms of glucose split per milligram nitrogen).

(a) Glucose Injected in the Small Intestine

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
1	76.0	6.12	0.843	13.50
2	128.5	5.13	0.206	20.60
3	145.5	7.66	0.691	13.85
4	91.4	2.32	0.261	12.00
5	124.0	6.15	1.025	12.45
6	177.0	5.68	0.902	15.25
7	152.0	6.76	0.896	7.62
8	143.6	4.74	0.782	11.25
average	129.6 ±23.95	5.57 ±1.14	0.701 ±0.173	14.61 ±0.860

Table 5: The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 4°C and (a) with an injection of glucose in the small intestine. Figures representing glucose-6-phosphate phosphatase activity are in micrograms of glucose released per milligram nitrogen, acid and alkaline phosphatase activity are in sigma alkaline phosphatase units per milligram of nitrogen X50.

(b) Control Animals

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
9	155.0	7.72	1.410	10.25
10	114.0	6.44	0.616	13.40
11	91.2	11.00	1.250	12.20
12	127.0	3.51	0.050	16.20
13	86.5	9.02	1.785	26.30
14	173.0	8.54	1.673	32.20
15	78.2	5.10	0.868	5.46
16	99.4	5.36	0.740	11.90
average	115.5 ±27.1	7.09 ±1.97	1.049 ±0.469	15.99 ±1.91

Table 5 (con't): The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 4°C and (b) control. Figures representing glucose-6-phosphate phosphatase activity are in micrograms of glucose released per milligram nitrogen, acid alkaline phosphatase activity are in sigma alkaline phosphatase units per milligram of nitrogen X50.

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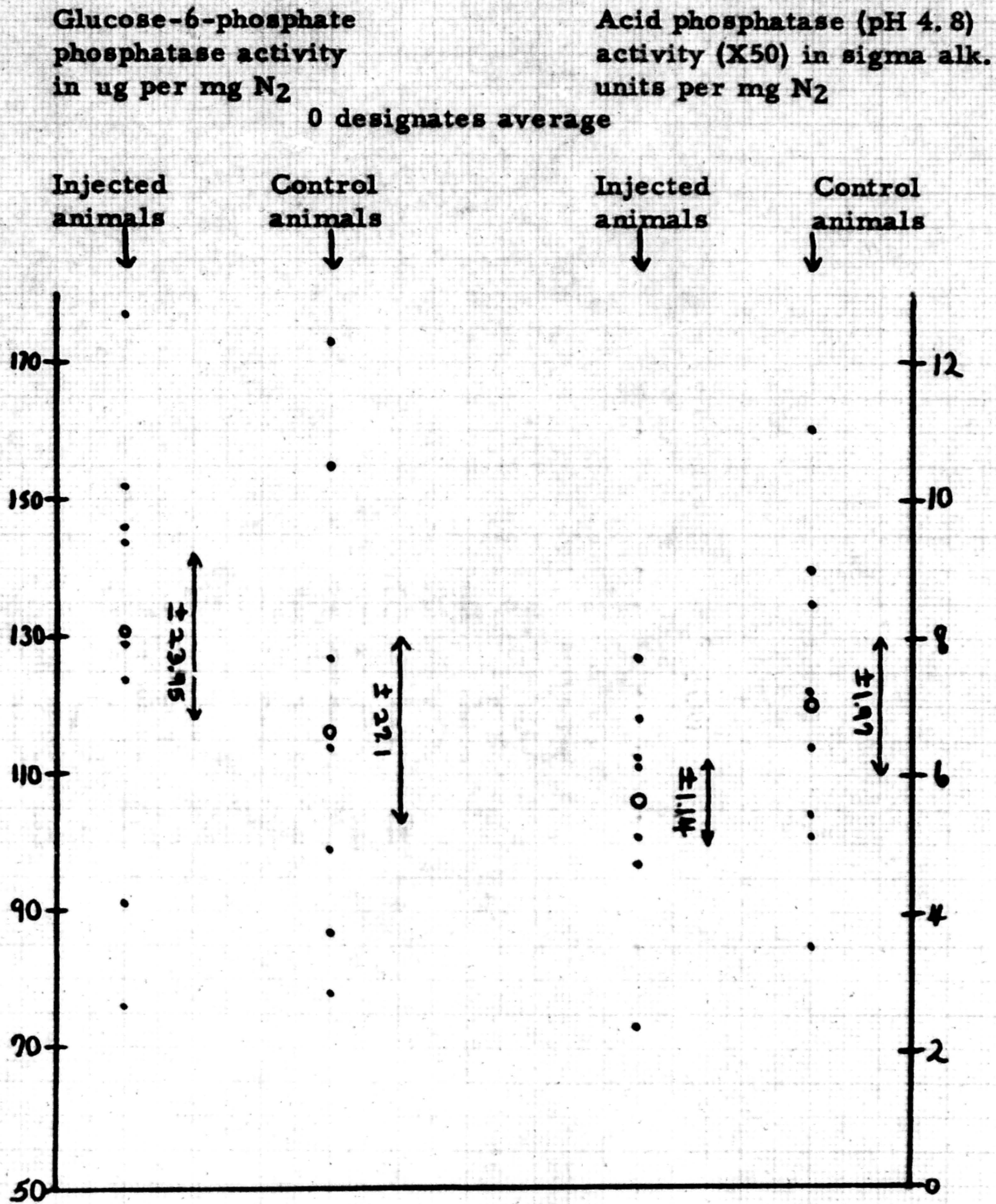

Figure 5: The Effect of the Presence of Glucose on the Activity of Glucose-6-phosphate Phosphatase in the Small Intestine of the Bullfrog Conditioned to 4°C

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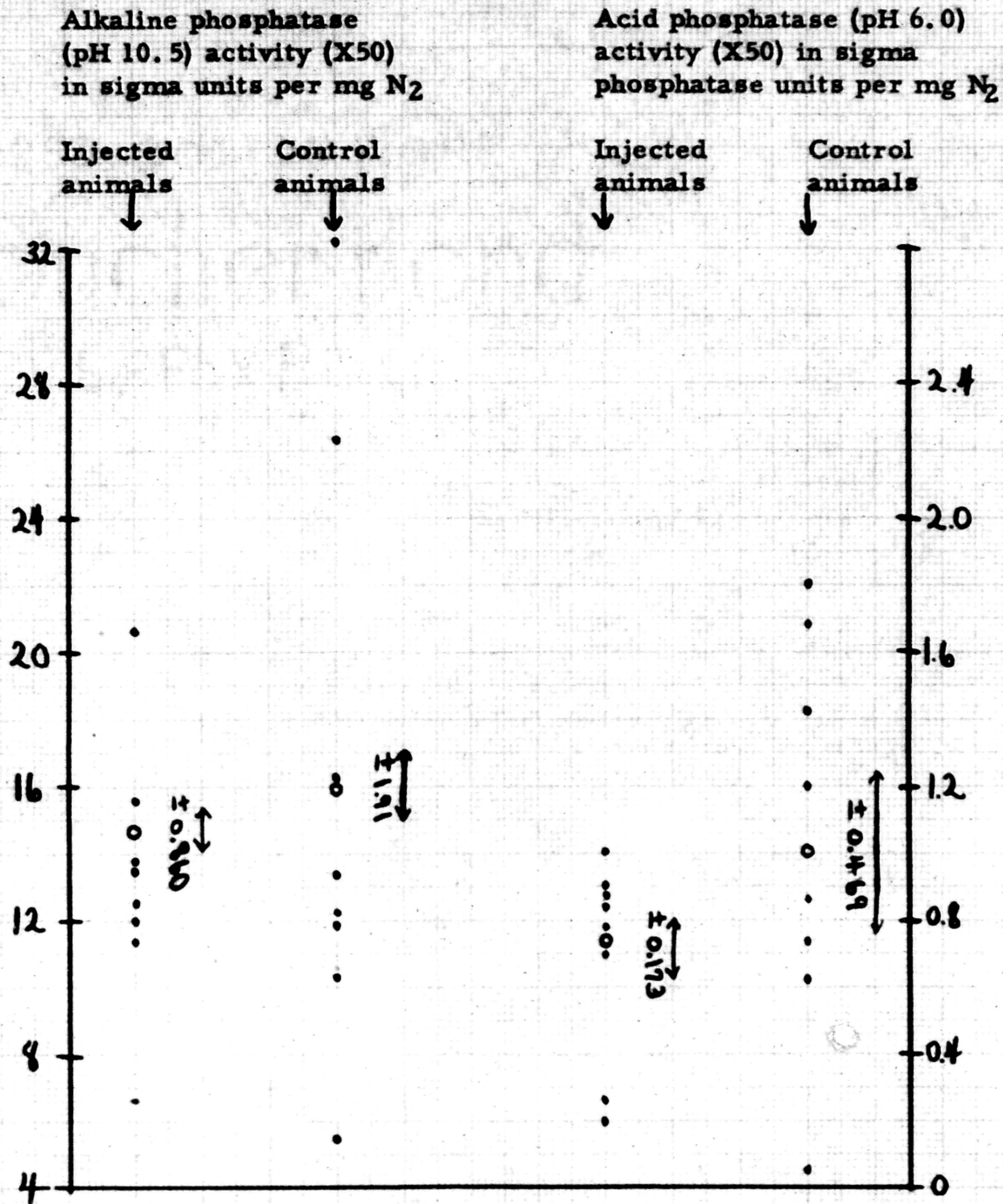

Figure 5 (con't): The Effect of the Presence of Glucose on the Activity of Glucose-6-phosphate Phosphatase in Small Intestine of the Bullfrog Conditioned to 4°C

(a) Glucose Injected Into the Small Intestine

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
1	266	5.76	0.562	15.40
2	108	5.62	0.654	14.80
3	256	7.82	1.075	13.79
4	243	6.69	1.255	8.82
5	293	8.88	1.180	7.05
6	348	8.14	1.020	19.55
7	175	5.63	0.283	2.00
8	235	8.07	1.380	6.92

Table 6: The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 20°C-23°C and (a) with an injection of glucose into the intestine. Figures representing glucose-6-phosphate phosphatase are in micrograms of glucose released per milligram of nitrogen. Acid and alkaline phosphatase activity are in sigma alkaline phosphatase units per milligram of nitrogen X50.

(a) Glucose Injected Into the Small Intestine

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
9	117	4.85	0.868	11.50
10	261	5.10	0.821	18.00
11	231	7.56	1.320	10.20
12	113	5.20	1.110	10.05
13	223	5.98	1.015	7.65
14	163	5.08	1.065	10.35
15	226	5.24	1.290	15.75
16	187	3.50	1.000	9.86
average	209 $\pm$36.5	6.20 $\pm$0.748	0.994 $\pm$0.194	11.40 $\pm$2.74

Table 6 (con't): The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 20°C-23°C and (a) with an injection of glucose into the intestine. Figures representing glucose-6-phosphate phosphatase are in micrograms of glucose released per milligram of nitrogen. Acid and alkaline phosphatase activity are in sigma alkaline phosphatase units per milligram of nitrogen X50.

(b) Control Animals

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
17	100.0	8.31	1.86	13.65
18	70.5	3.38	0.70	13.80
19	202.0	7.78	1.56	15.80
20	126.0	8.30	1.65	20.35
21	113.0	7.08	1.18	16.75
22	133.0	5.82	0.50	19.20
23	132.0	5.35	1.23	18.60
24	109.0	6.08	1.14	11.80

Table 6 (con't): The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 20°C-23°C and (b) controls. Figures representing glucose-6-phosphate phosphatase are in micrograms of glucose released per milligram of nitrogen. Acid and alkaline phosphatase activity are in sigma alkaline phosphatase units per milligram of nitrogen X50.

(b) Control Animals

Frog	Glucose-6-phosphate phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
25	94.0	6.31	0.91	13.45
26	109.0	5.10	0.88	11.20
27	141.0	6.34	1.25	9.88
28	110.0	4.32	0.89	10.95
29	80.0	3.50	0.93	8.77
30	101.0	3.94	0.85	12.85
31	89.0	7.56	1.13	6.88
32	123.0	10.3	1.72	20.60
average	115.0 ±19.2	6.22 ±1.53	1.15 ±0.222	14.04 ±3.67

Table 6 (con't): The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 20°C-23°C and (b) controls. Figures representing glucose-6-phosphate phosphatase are in micrograms of glucose released per milligram of nitrogen. Acid and alkaline phosphatase activity are in sigma alkaline phosphatase units per milligram of nitrogen X50.

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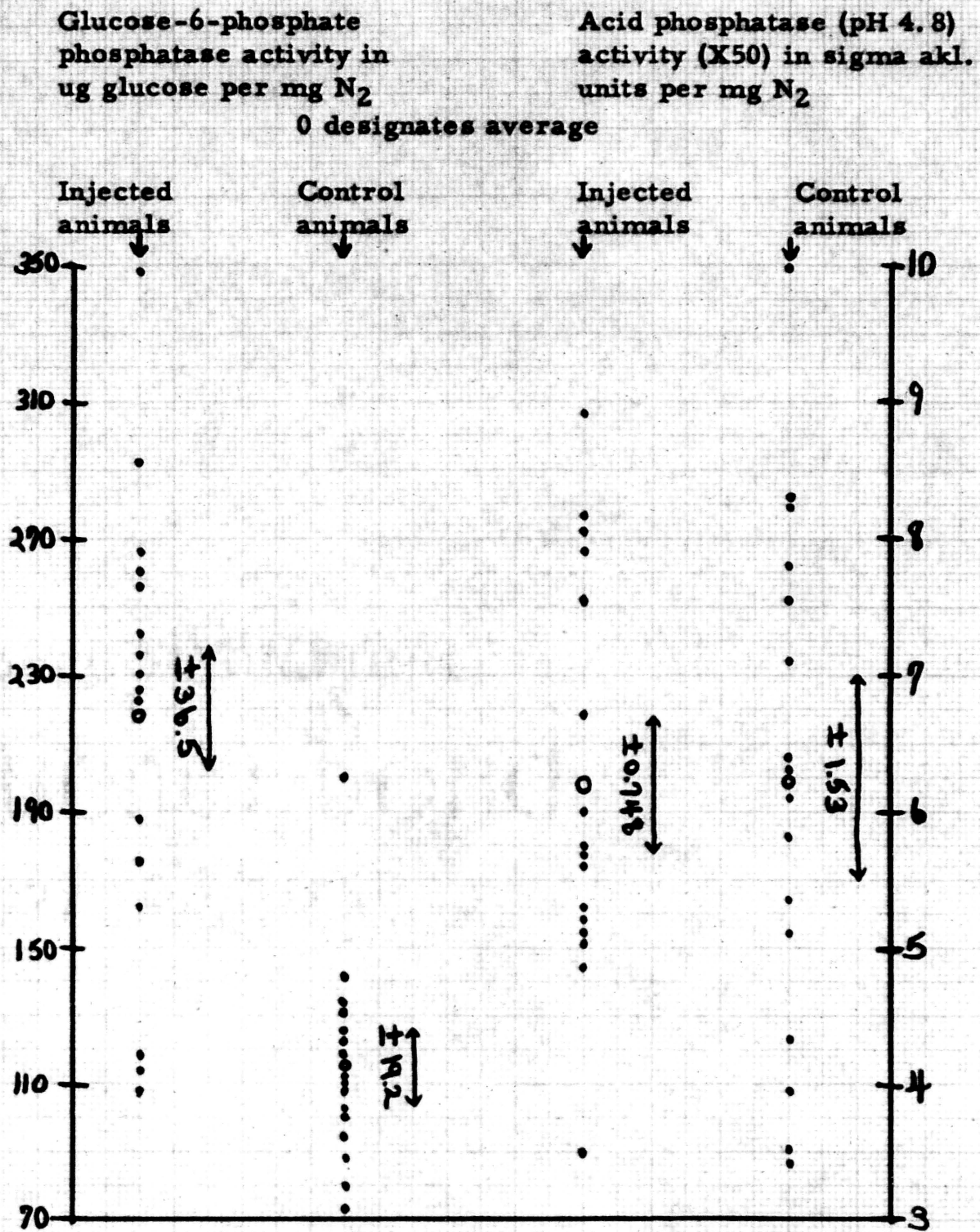

Figure 6: The Effect of Presence of Glucose on the Activity of Glucose-6-phosphate Phosphatase in the Small Intestine of the Bullfrog Conditioned to 20°C-23°C

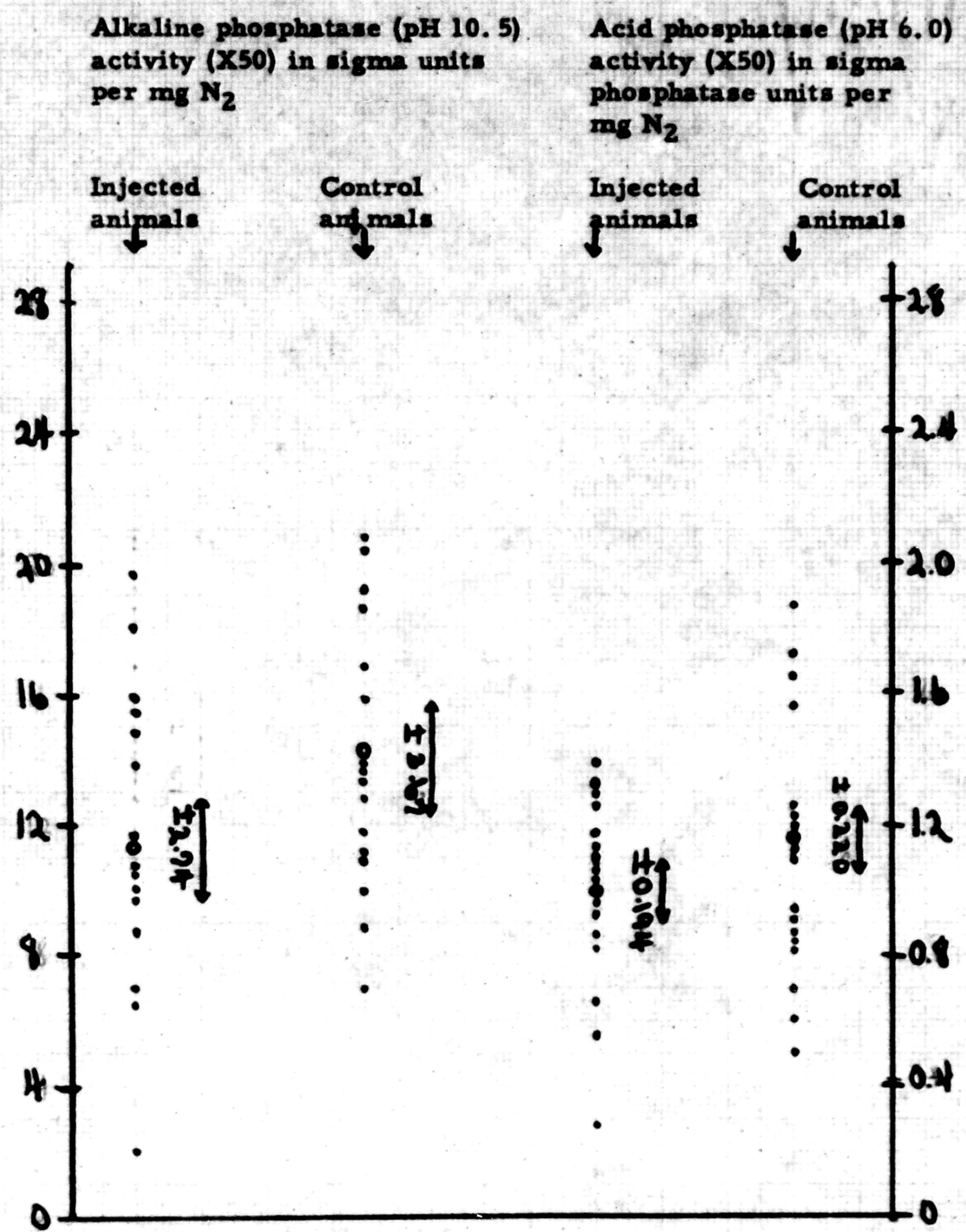

Figure 6 (con't): The Effect of Presence of Glucose on the Activity of Glucose-6-phosphate Phosphatase in the Small Intestine of the Bullfrog Conditioned to 20°C-23°C

methods were followed with the exception that tris buffer at pH 6.0 was used instead of the regular buffer.

The results, shown in table 5 and figure 5, indicate that the presence of glucose did not affect the activity of glucose-6-phosphate phosphatase in the small intestine. This does not support the idea that glucose-6-phosphate phosphatase is involved in the mechanism of sugar transport in the intestine.

It was felt that it would be worthwhile to repeat this experiment using animals conditioned at 20°C-23°C instead of those conditioned at 4°C. This was done, and as seen in table 6 and figure 6, very significant differences were obtained.

These data show nearly a 90% increase of the average glucose-6-phosphate phosphatase activity with the animals having glucose present in the small intestine over the average glucose-6-phosphate phosphatase activity of the animals which were starved. The activity of acid phosphatase (at pH 4.8 and 6.0) and alkaline phosphatase (at pH 10.5) in the same animals remained constant. This shows that in some way the introduction of glucose in the small intestine of a starved bullfrog causes an increase in the activity of glucose-6-phosphate phosphatase while the activity of acid and alkaline phosphatases remained constant.

Further, this supports the previous finding that

there is a specific enzyme, glucose-6-phosphate phosphatase, present in the small intestinal mucosa. These data also support the observation that glucose-6-phosphate phosphatase has low affinity or none at all for p-nitrophenol phosphate as compared to glucose-6-phosphate as the substrate for this enzyme.

The next problem which is suggested by this experiment is the explanation of the effect of glucose upon the activity of glucose-6-phosphate phosphatase in the small intestine between animals conditioned to 4°C to animals conditioned to 20°C-23°C (data contained in tables 4 and 5). It is shown that there is an increase in glucose-6-phosphate phosphatase activity in the animals at 20°C-23°C while none is found in the 4°C animals. The most obvious difference between a bullfrog at 4°C and one at 20°C-23°C is that the metabolism of the former is very low compared to the latter animal. This implies that energy is probably necessary for the increase of glucose-6-phosphate phosphatase activity in the small intestine of the animals conditioned at 20°C-23°C.

Effect of Chloramphenicol on the Apparent Increase in the Activity of Glucose-6-phosphate Phosphatase in the Small Intestine of the Bullfrog at 20°C-23°C

As a follow-up of the observation that there is an

increase in the activity of glucose-6-phosphate phosphatase when glucose is present in the small intestine of a starved frog at 20°C-23°C, the effect of chloramphenicol was studied. The basis for the use of chloramphenicol is that since it was reported by Gale and Folkes (1953) to be an inhibitor of protein synthesis and that one possible explanation for the necessity of energy for the increased activity of glucose-6-phosphate phosphatase is a synthesis of a particular protein, in this case glucose-6-phosphate phosphatase, the presence of chloramphenicol might inhibit the increase of glucose-6-phosphate phosphatase in the glucose injected intestine of the bullfrog maintained at 20°C-23°C.

In this experiment the animals were run in groups of six, two not injected, two with a solution of glucose, two with a solution of glucose and chloramphenicol, and two controls. Chloramphenicol was found to have no effect (table 7 and figure 7). These results, though, still do not rule out the possibility that there is a synthesis of glucose-6-phosphate phosphatase molecules.

One possible reason why chloramphenicol did not have an effect is that possibly the chloramphenicol molecules were not absorbed into the mucosal cells of the small intestine. To check this possibility, it was decided that the same experiment should be repeated only with the injections made into the dorsal

(a) Chloramphenicol Plus Glucose Injected

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
1	121	5.88	1.06	16.84
2	102	4.88	0.73	12.50
3	105	4.29	0.56	14.84
4	218	3.26	0.30	16.36
5	113	8.8	2.86	11.36
6	110	7.0	1.92	11.60
7	179	9.3	1.10	12.68
8	114	7.0	0.59	16.24
average	133	6.3	1.14	14.02

Table 7: The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 20°C-23°C and (a) injection of chloramphenicol glucose solution in the small intestine. Activity of glucose-6-phosphate phosphatase expressed in micrograms of glucose released per milligram of nitrogen. Acid and alkaline phosphatase are expressed in sigma alkaline phosphatase units per milligram nitrogen X50.

(b) Glucose Injected Animals

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
9	148	7.5	0.80	18.52
10	83	4.82	1.50	16.60
11	142	7.5	1.56	13.70
12	129	7.6	0.46	14.58
13	105	2.7	1.15	10.20
14	117	7.2	1.50	14.6
15	180	7.1	1.00	15.02
16	162	5.5	0.73	10.30
average	133	6.2	1.09	14.13

Table 7 (con't): The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 20°C-23°C and (b) injection of glucose solution in the small intestine. Activity of glucose-6-phosphate phosphatase expressed in micrograms of glucose released per milligram of nitrogen. Acid and alkaline phosphatase are expressed in sigma alkaline phosphatase units per milligram nitrogen X50.

(c) Control Animals

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
17	104	6.0	0.38	12.24
18	84	8.5	2.70	12.94
19	87	7.3	0.25	17.06
20	63	3.2	0.56	11.18
21	56	4.2	1.59	19.62
22	82	11.2	2.63	12.42
23	113	6.9	0.93	18.96
24	131	5.2	1.07	18.96
average	89.7	6.60	1.26	15.39

Table 7 (con't): The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 20°C-23°C and (c) controls. Activity of glucose-6-phosphate phosphatase expressed in micrograms of glucose released per milligram of nitrogen. Acid and alkaline phosphatase are expressed in sigma alkaline phosphatase units per milligram nitrogen X50.

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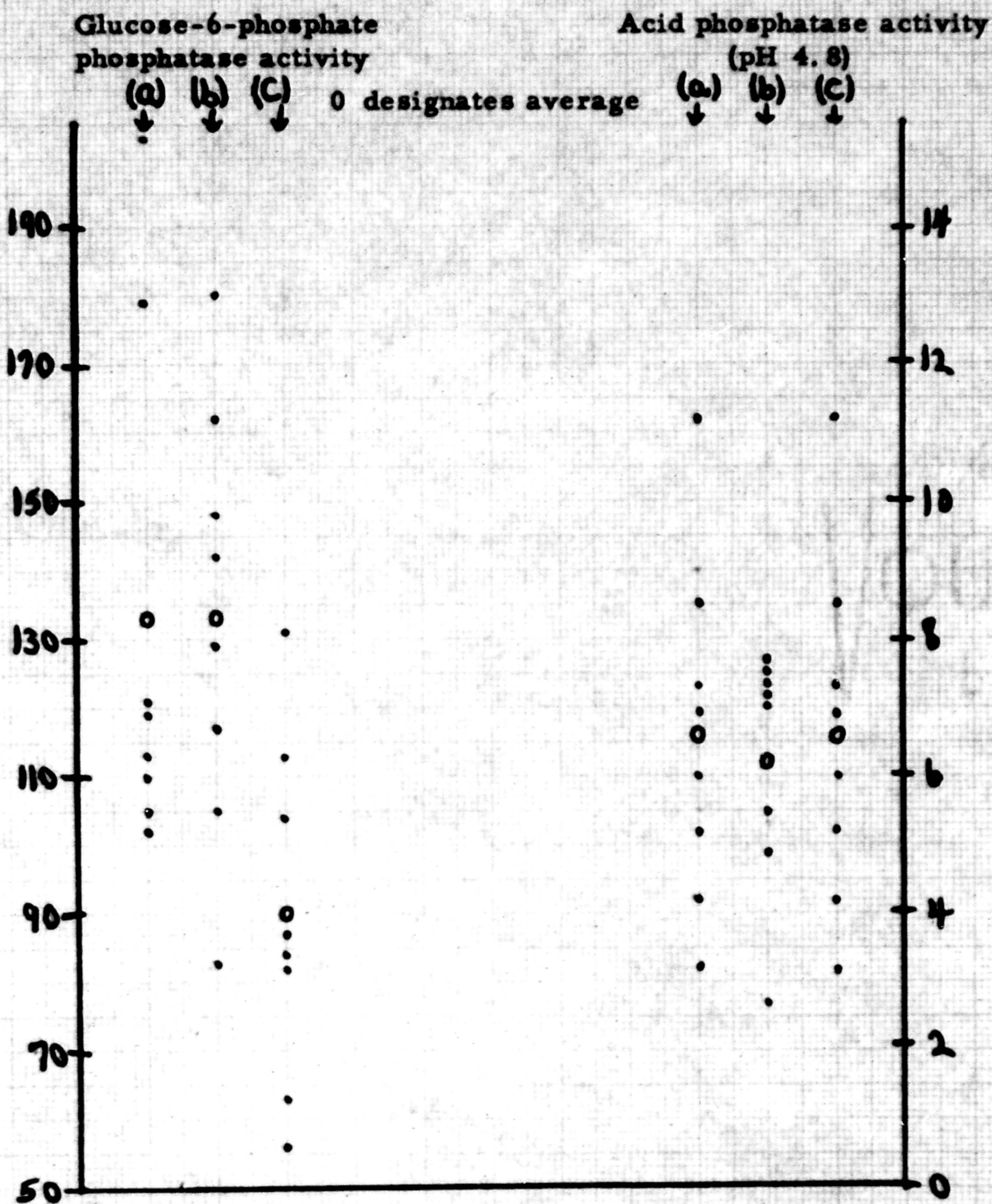

Figure 7: The Activity of Glucose-6-phosphate Phosphatase in the Small Intestine of Bullfrogs Conditioned to 20°C-23°C and (a) Chloramphenicol-glucose Solution and (b) Glucose Solution Injected into Small Intestine and (c) Control

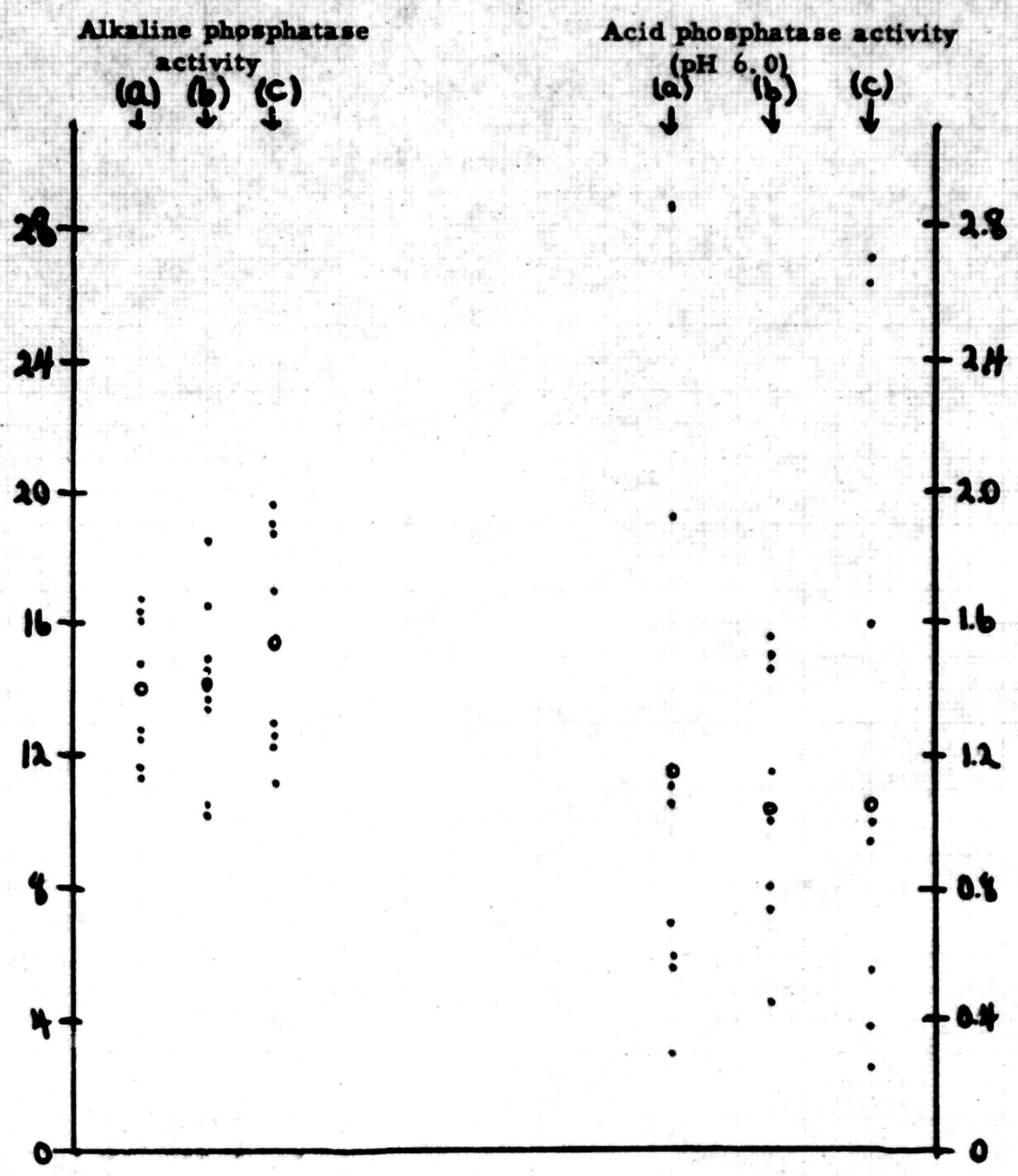

Figure 7 (con't): The Activity of Glucose-6-phosphate Phosphatase in the Small Intestine of Bullfrogs Conditioned 20°C-23°C and (a) Chloramphenicol-glucose Solution and (b) Glucose Solution Injected into Small Intestine and (c) Control

(a) Chloramphenicol+Glucose Injected

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
1	113	6.98	1.18	14.06
2	114	5.84	1.18	16.36
3	116	5.40	0.99	12.72
4	120	6.52	1.53	14.40
average	116	6.44	1.22	14.39
(b) Glucose Injected				
5	138	10.30	1.09	13.90
6	125	4.91	1.67	15.88
7	88	3.18	0.61	9.92
8	137	8.40	1.23	17.68
average	122	6.52	1.15	14.32

Table 8: The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 20°C-23°C and (a) with an injection of glucose-chloramphenicol solution into the dorsal lymph sac, (b) injection of glucose into the dorsal lymph sac, and (c) controls. Activity of glucose-6-phosphate phosphatase expressed in micrograms of glucose released per milligram of nitrogen. Acid and alkaline phosphatase are expressed in sigma alkaline phosphatase units per milligram nitrogen X50.

(c) Control Animals

Frog	Glucose-6-phosphate Phosphatase Activity	Acid Phosphatase Activity at pH 4.8	Acid Phosphatase Activity at pH 6.0	Alk. Phosphatase Activity at pH 10.5
9	113	8.17	1.07	18.60
10	135	4.68	1.66	9.46
11	137	3.57	0.68	9.68
12	91	7.62	1.25	19.20
average	119	6.01	1.17	14.24

Table 8 (con't): The activity of glucose-6-phosphate phosphatase in the small intestine of bullfrogs conditioned to 20°C-23°C and (a) with an injection of glucose-chloramphenicol solution into the dorsal lymph sac, (b) injection of glucose into the dorsal lymph sac, and (c) controls. Activity of glucose-6-phosphate phosphatase expressed in micrograms of glucose released per milligram of nitrogen. Acid and alkaline phosphatase are expressed in sigma alkaline phosphatase units per milligram nitrogen X50.

lymph sac instead of the small intestine. Ten milliliters of solution (either glucose and chloramphenicol or glucose) were injected each time. It was thought that with the presence of chloramphenicol in the blood bathing the cells that there was a better chance of the chloramphenicol molecules entering the cells. However, there was not even an increase in the activity of glucose-6-phosphate phosphatase in the small intestine of the glucose injected bullfrogs (Table 8). Therefore nothing further could be added to the previous observation that chloramphenicol apparently does not effect the increase glucose-6-phosphate phosphatase activity in the small intestine.

CHAPTER 4

DISCUSSION

Other investigators, in their descriptions of glucose-6-phosphate phosphatase, have separated this enzyme from acid and alkaline phosphatases by the use of two criteria. These criteria are (1) glucose-6-phosphate phosphatase is specific for the splitting of glucose-6-phosphate with very little or no affinity for other phosphoric acid esters which are split by acid and alkaline phosphatases and (2) the pH optimum for glucose-6-phosphate phosphatase activity has been reported to be between 6.0 and 6.5 while that of acid phosphatase is approximately 4.8 and alkaline is 10.5. These criteria were used by Fantl and Rome (1945) in their first description of glucose-6-phosphate phosphatase in the liver. Later, their observations were confirmed by de Duve (1949) and Swanson (1950).

The findings (table 2 and figure 2) presented in this thesis have demonstrated that there is an enzyme present in the intestinal mucosa which has optimum activity at pH 6.0 and a higher affinity towards glucose-6-phosphate than p-nitrophenol

phosphate. It may be concluded that this enzyme is glucose-6-phosphatase since it meets the criteria just described for that enzyme by other workers.

This conclusion that glucose-6-phosphate phosphatase is present in the intestinal mucosa is in disagreement with Hers and de Duve (1950). This paper maintains that glucose-6-phosphate phosphatase is present only in the liver and kidney and was definitely not found in the small intestine. On the other hand, it supports Maeda (1954) though the validity of his paper is in question.

The presence of a glucose molecule present in the mucosal cells might be explained three ways: (1) absorption by osmosis from the lumen into the blood stream, (2) to provide energy for the intestinal mucosal cell, and (3) active transport from the lumen to the blood stream.

Of these three possibilities, the first may be discounted on the grounds that it is not plausible that an enzyme would have a role in a physical process such as osmosis.

Though there seems to be no logical place for glucose-6-phosphate phosphatase in the production of energy from glucose in the small intestine, it is still possible that it might be involved in glycogen storage. If this were so then this enzyme would only serve to prevent the metabolism of glucose by the

mucosal cells.

The function of a phosphatase in active transport (figure 1) has been hypothesized by Wilbrandt and Laszt (1933). Further, Darlington and Quastel (1953) and Fridhandler and Quastel (1954), imply that the phosphatase could not be alkaline and acid, for fructose is only absorbed via the glucose mechanism. This suggests that glucose-6-phosphate phosphatase is used. It is at least possible, therefore, that the enzyme functions in glucose transport.

The function of glucose-6-phosphate phosphatase in glucose transport would be further suggested by an increase in the activity of the enzyme when glucose was in the lumen of the small intestine. Such an increase was clearly demonstrated (table 6 and figure 6). These results, however, only suggest that the purpose of glucose-6-phosphate phosphatase is that it participates in the transport of glucose across the intestinal mucosa, because glucose transport was not actually measured.

It is surprising that glucose injected into the dorsal lymph sac does not activate glucose-6-phosphate phosphatase. One may speculate that the glucose which enters from the intestinal lumen has been changed into some form which then serves as an activator of glucose-6-phosphate phosphatase whereas

apparently glucose which enters via diffusion from the blood stream has not been changed in form and therefore does not activate glucose-6-phosphate phosphatase.

It is evident that the finding of glucose-6-phosphate phosphatase in the small intestinal mucosa and the demonstration that the presence of glucose will activate it has only pointed the way for further studies. Such studies, which would involve (1) the essential characteristics of the molecule which activates the enzyme i.e. effect of other sugars, sugar phosphates, etc., (2) the correlation between glucose-6-phosphate phosphatase and glucose transport, i.e. the measurement of glucose absorption and enzyme activity _in vivo_, and (3) the effect of inhibitors on glucose-6-phosphate phosphatase and glucose transport _in vivo_, are planned.

SUMMARY

1. The presence of glucose-6-phosphate phosphatase has been demonstrated in the intestinal mucosa of the bullfrog, Rana catesbeiana (Shaw).

2. The activity of glucose-6-phosphate phosphatase was higher when tris and maleate were used as buffers than when a phosphate buffer was used in the assay.

3. The presence of glucose in the intestinal lumen of bullfrogs conditioned at 4°C did not cause an increase in glucose-6-phosphate phosphatase activity over the controls while it did increase the glucose-6-phosphate phosphatase activity of frogs conditioned at 20°C-23°C.

4. A glucose solution injected into the dorsal lymph sac of bullfrogs conditioned at 20°C-23°C did not increase the activity of glucose-6-phosphate phosphatase in the intestinal mucosa.

5. Chloramphenicol did not inhibit the increase in the activity of glucose-6-phosphate phosphatase in the intestinal mucosa of bullfrogs conditioned at 20°C-23°C when injected with the glucose solution into the small intestinal lumen.

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