CHAPTER 4

INFLUENCE OF OUABAIN AND PHLORIZIN ON GLUCOSE TRANSPORT IN THE LIVER OF DOMESTIC PIGEON UNDER *IN VITRO* CONDITIONS

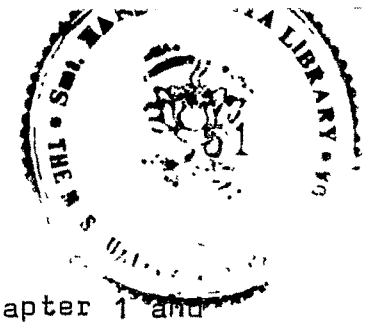
Each tissue takes up glucose through one or two specific transport mechanism(s). In certain tissues like kidney and intestine, the transport of sugar is characterized by (1) high specificity of sugar substrate i.e., critical dependence on certain hydroxyl groups, (2) high sodium dependence, and (3) active transport against a concentration gradient. In others, sugar enters through simple diffusion or through a carrier mediated transport. Some exhibits a chemiosmotic 'pull' to take up glucose into the cell interior, a mechanism which involves a stepped up glucose disposal (by converting it into glycogen or fat or by metabolising it). Tissues such as intestine and kidney also have a flow coupled glucose transport, a mechanism that is coupled essentially to the active transport of sodium out of the cell. In erythrocytes, this may be the only way glucose is taken up into the cell interior. Liver being a 'centre' of metabolic homeostasis, exhibits several types of glucose transport. The predominance of one or the other type of sugar transport in the liver, depends on dietary adaptations, glucagon/insulin ratio, receptor kinetics of the liver, speed and degree by which pancreatic hormones respond to glucose induced direct or neurally mediated stimuli and the degree of innervation of autonomic fibres in the liver. The pigeon liver is reported to be a tissue where insulin independent flow coupled and acetyl-

choline (vagal) induced sugar transport mechanisms predominate (Pilo and Patel, 1978). Sodium dependent flow coupled glucose transport is an active process probably involving Na^+-K^+-ATPase (pump ATPase). In <u>in vitro</u> conditions even alterations in the extracellular ionic gradients was found to induce glucose uptake by liver cells (Chapter 2). While sodium or potassium hypertonicity attenuated insulin induced glucose uptake, these cations accelerated the acetylcholine induced uptake (Chapter 3). Calcium ions together with acetylcholine stimulated maximum sugar transport into the liver (Chapter 3) indicating a synergism between calcium and acetylcholine. Probably both are activating the same sugar transport mechanism.

To understand, whether or not acetylcholine mediated transport of sugar is based on the flow coupled transport principle, the liver slices were incubated in the presence of ouabain and phlorizin which are selective inhibitors of sodium transport and sugar transport respectively. The fact that phlorizin inhibits glucose transport across the kidney tubule and the intestinal mucosa has been known long before (Crane, 1962). It has also been shown that phlorizin inhibits the insulin stimulated glucose uptake by muscle (Battaglia <u>et al.</u>, 1960) and it blocks the ability of insulin to enhance the permeation of galactose into muscle both <u>in vivo</u> (Keller and Lotspeich, 1959) and <u>In vitro</u>. Ouabain inhibits the active transport of sodium in a variety of cells. It also inhibits the Na^+-K^+-dependent membrane ATPase (pump ATPase) which is thought to be involved in the active cation transport

(Glynn, 1964). It is reasonable to assume that cell membrane is the site of these inhibitory effects. Ouabain which has a very low lipid-water distribution ratio and thus cannot readily penetrate the cell produces the same inhibitory action on the cation transport and on the membrane ATPase (Csaky and Hara, 1965). Active cation transport by the sodium pump involves a cyclic Na^+-dependent phosphorylation of the enzyme by intracellular ATP, and hydrolytic dephosphorylation of the phosphoenzyme, stimulated by K^+. In human red blood cells (Garrahan and Glynn, 1967) skeletal muscle (Caldwell et al., 1960) and squid axons, replacement of extracellular K^+ by Na^+ results in a ouabain sensitive efflux of Na^+ coupled to an influx of extracellular Na^+. There is apparently no net Na^+ movement (Garraham and Glynn, 1967) nor net hydrolysis of ATP (Garraham and Glynn, 1967). The rate of Na^+-K^+ exchange is stimulated by increased levels of ADP (Glynn and Hoffman, 1971) and exchange transport is not observed in cells totally depleted of intracellular ATP. These characteristics suggest that the biochemical mechanism underlying the Na^+ exchange mode of Na^+ Pump involves phosphorylation of the enzyme by ATP (which requires intracellular Na^+) followed by its dephosphorylation by ADP. Such a reaction has been observed in a partially purified Na^+-K^+-ATPase from a variety of sources (Fahn et al., 1966; Stahl, 1968; Benerjee and Wong, 1972) and its dependence on Na^+ concentration has been described (Wildes et al., 1973).

The present chapter reports the effect of ouabain and phlorizin with or without the presence of insulin or ACh on the glucose uptake by liver slices.



MATERIALS AND METHODS

The slices were prepared as described in Chapter 1 and were incubated in the KRB medium containing following additives.

(1) 5 ml KRB Medium + D-Glucose (3 mg/ml) + Albumin (2 mg/ml) + Ouabain (100 μM)

(2) 5 ml KRB Medium + D-Glucose (3 mg/ml) + Albumin (2 mg/ml) + Ouabain (100 μM) + Insulin (1 unit/ml)

(3) 5 ml KRB Medium + D-Glucose (3 mg/ml) + Albumin (2 mg/ml) + Ouabain (100 μM) + ACh (15 mg/ml)

(4) 5 ml KRB Medium + D-Glucose (3 mg/ml) + Albumin (2 mg/ml) + Phlorizin (100 μM)

(5) 5 ml KRB Medium + D-Glucose (3 mg/ml) + Albumin (2 mg/ml) + Phlorizin (100 μM) + Insulin (1 unit/ml)

(6) 5 ml KRB Medium + D-Glucose (3 mg/ml) + Albumin (2 mg/ml) + Phlorizin (100 μM) + ACh (15 mg/ml).

After the incubation, the slices were quickly washed with chilled KRB medium and were digested with KOH for glycogen estimation. The glycogen was estimated in slices removed from liver as well as in slices after incubation as per the method described in chapter 1. The glucose concentration was determined in the medium before and after incubation and the differences were calculated. The glucose was estimated as per the method given in Chapter 1.

RESULTS

The data on the uptake or release of glucose by liver slices in the presence of ouabain or phlorizin alone or in combination with insulin or ACh are presented in Table 4-1 and Figs. 4-1 and 4-2.

It is clear from the data that both ouabain and phlorizin inhibited glucose uptake by liver slices. Instead, both induced a release of glucose into the medium. The release of glucose was more when the medium contained phlorizin than when it contained ouabain. Even in the presence of insulin, both ouabain and phlorizin could effectively inhibit glucose uptake. So was the case when ouabain or phlorizin was present in the medium along with ACh.

DISCUSSION

Many reviews explain exhaustively the different models which have been formulated to explain the Kinetic phenomenon associated with transmembrane glucose transport in erythrocytes (Kotyk, 1973; Lieb and Stein, 1972). It is important to note that the kinetics of sugar transport in the liver are entirely consistent with those observed for sugar transport in the red cells except that there is an active Na^+ dependent electrogenic transport instead of facilitated Na^+ dependent diffusion. The mobile carrier mechanism for glucose transport in red cells cannot be excluded on the basis of current kinetics. However experimental evidence against such mobile transporters come from studies

Fig. 4-1. Effect of phlorizin and ouabain alone or in combination with insulin or acetylcholine (ACh) on glucose uptake by pigeon liver slices under *in vitro* conditions.

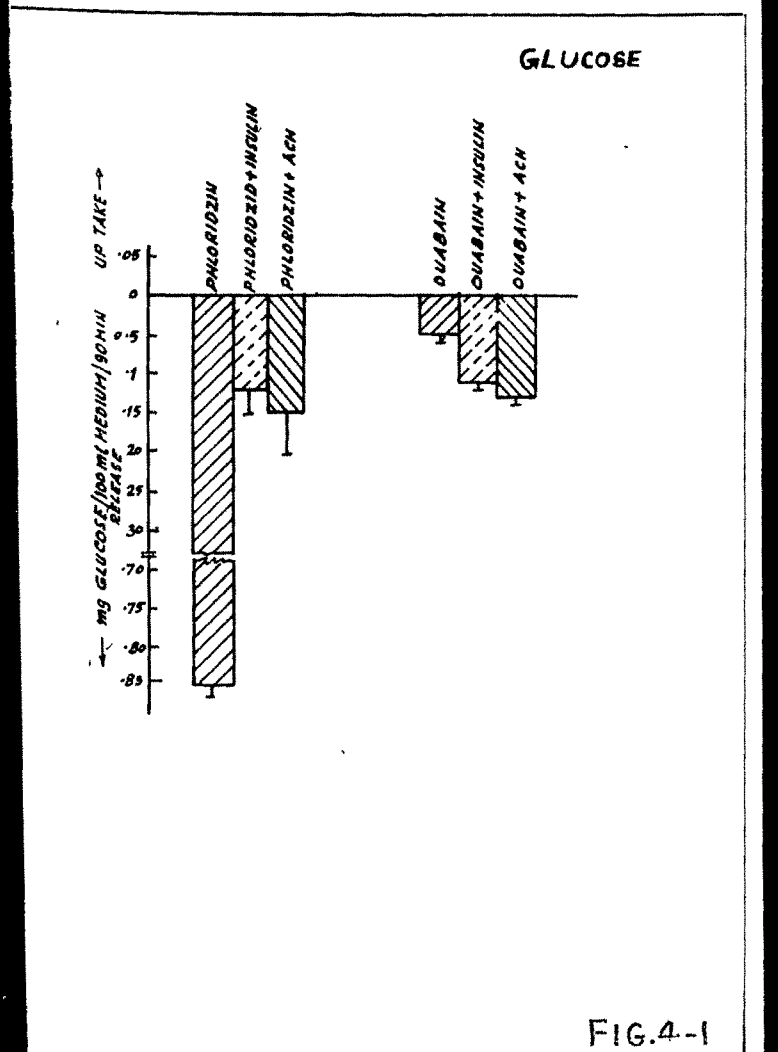

GLUCOSE
PHLORIDZIN
PHLORIDZID+INSULIN
PHLORIDZIN + ACH
OUABAIN
OUABAIN+INSULIN
OUABAIN + ACH
UP TAKE →
RELEASE
mg GLUCOSE/100 ml MEDIUM/90 MIN
·05
0
·5
1
1·5
2·0
2·5
3·0
7·0
7·5
8·0
8·5

FIG.4-1

Fig. 4-2. Effect of phlorizin and ouabain alone or in combination with insulin or acetylcholine (ACh) on glycogen content in pigeon liver slices under *in vitro* conditions.

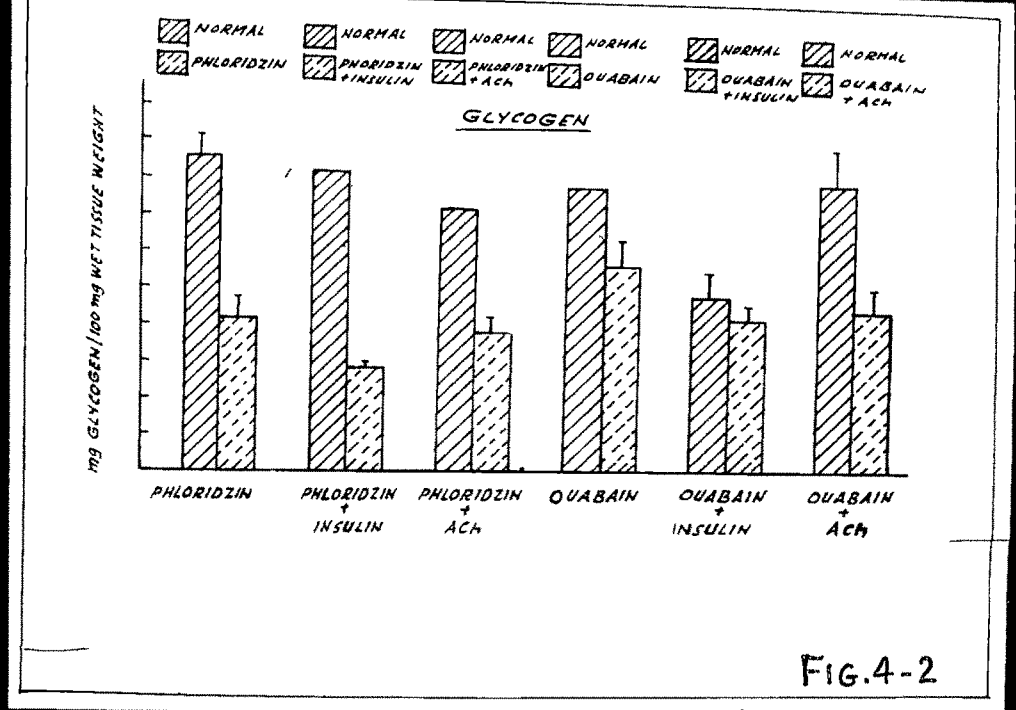

NORMAL
PHLORIDZIN
NORMAL
PHORIDZIN + INSULIN
NORMAL
PHLORIDZIN + ACh
NORMAL
OUABAIN
NORMAL
OUABAIN + INSULIN
NORMAL
OUABAIN + ACh
GLYCOGEN
mg GLYCOGEN/100 mg WET TISSUE WEIGHT
PHLORIDZIN
PHLORIDZIN + INSULIN
PHLORIDZIN + ACh
OUABAIN
OUABAIN + INSULIN
OUABAIN + ACh

FIG. 4-2

Table 4-1

Effect of phlorizin and ouabain, alone or in combination with insulin or acetylcholine on the uptake or release of glucose by pigeon liver slices under *in vitro* conditions.

Additives	Glucose		Glycogen Depletion(2)
	Uptake (1)	Release (1)	
Phlorizin	-	0.8700 ±0.0451	2.2520 ±0.0550 ***
Phlorizin + Insulin	-	0.1267 ±0.0129	2.6785 ±0.1959 ***
Phlorizin + ACh	-	0.1550 ±0.0262	1.6872 ±0.2211 ***
Ouabain	-	0.0550 ±0.0117	1.0834 ±0.1731 ****
Ouabain + Insulin	-	0.1172 ±0.0106	0.2783 ±0.0612 ***
Ouabain + ACh	-	0.1367 ±0.0105	1.6916 ±0.2587 ***

(1) Mg glucose taken up or released by 100 mg liver.

(2) Mg glycogen depletion/100 mg liver. *** $P < 0.01$ **** $P < 0.001$

of active transport of Na^+ and K^+ via an ATPase enzyme extracted from renal medulla (Kyle, 1974). It appears that Na^+ and K^+ movements occur along channels formed by protein subunits, spanning the width of the membrane which do not diffuse through or rotate across the membrane during a transport cycle (Kyle,1975). Transport protein spanning the bilayer undergo partial conformational changes so that they exhibit carrier behaviour.

In the present study the glucose transport system of liver stimulated by both insulin and ACh was found to be Na^+-K^+ dependent and inhibited by phlorizin and ouabain. This phenomenon is also explained using brush border vesicle of intestine (Hopfer et al., 1973). When liver tissue is preloaded with glucose, the stereospecific uptake of glucose was increased. Interpretation of this kinetic model suggests that it is an example of counter flow. Both competitive exchange diffusion and counter flow are natural consequences of the saturation equation. In principle active sugar transport across the liver cell membrane could result from direct coupling of the transporter to the metabolic energy of the cell. Such primary active transport system for sugar carrier are generally found in more primitive organisms (Kotyk, 1973). There are evidences to support the idea that sugar transport in liver is not very primary, but occurs by a cotransport process in which its movement is linked to the transmembrane flux of another actively transported chemical. The activity of Na^+ and K^+ dependent ATPase, which is localized exclusively in the membrane, also seen in kidney and intestine (Fijitz et al., 1972), results in an

active Na^+ extrusion from cells. This creates low intracellular Na^+ concentration and in turn establishes a gradient of Na^+ concentration across the membrane. The sugar transport in liver is dependent on Na^+ as in the case of intestine (Riklis and Quastel, 1958). Ouabain when added to the medium, inhibited glucose transport in liver, a phenomenon also seen in serosal surface of intestine (Csaky and Hara, 1965). When phlorizin was added in the medium it also exerted its inhibitory action on the transport of glucose across the liver cell membrane as observed by other workers in intestinal mucosal surface. Ouabain inhibits D-Glucose and a-methyl D-glucopyranoside uptake in kidney cortex slices (Kleinzeller et al., 1967). In the intestine it was demonstrated that uptake of sugar occurs only in the presence of external Na^+ (Bihler et al., 1962). The so called Na^+ dependent conformation of carrier molecule was found to be highly sensitive to inhibition by phlorizin, while the Na^+ independent state exhibits low phlorizin sensitivity (Silverman, 1976). The transport of sugar is blocked by phlorizin at a dose of 100 μM and this inhibition by phlorizin can be reversed by probenecid (Silverman et al., 1970). Phlorizin administration inhibits the interaction of glucose with the membrane. Insulin increases the glucose transporters but when phlorizin is also present glucose interaction to its transporters is disturbed, hence no glucose uptake take place even though insulin is present. When ACh was taken along with phlorizin the inhibitory action of phlorizin dominated over the stimulatory actions of ACh. In the presence of insulin and ACh, the sensitivity of phlorizin exhibited a difference. When compared the sensitivity of many other sugars

to phlorizin, Silverman and Black (1975) found that D-Glucose transport was the most sensitive to inhibition by phlorizin. Thus we know now that phlorizin is competitive inhibitor of D-Glucose transport even in the presence of glucose-uptake-inducers such as insulin and ACh. It is crucial to make note of the fact that phlorizin has a major inhibiting action on oxidative metabolism. Any studies using concentration of phlorizin in excess of 1000 μM can be expected to give atleast 50 % inhibition of active sugar transport not by virtue of any membrane effect of the drug but because of metabolic inhibition.

Phlorizin seems to have approximately 1000 times greater affinity for glucose transporters than glucose itself under in vivo conditions. Phlorizin molecule has several points of attachment to glucose transport receptor, (1) via the glucoside moiety, and (2) via hydroxyl groups on the aromatic A and B rings (Diedrich, 1966). Phlorizin binding to a transporter is dependent upon the outside Na^+ concentration. The binding of different functional hydroxyl groups on the phlorizin molecule to the glucose transporter (Burgen et al., 1975) might occur sequentially rather than simultaneously. In studies on the rat diaphragm muscle, in vitro, it has been reported that phlorizin at a concentration of 1×10^{-3} M causes about a 50 % inhibition of the insulin induced uptake of monosaccharide. This is definitely supported by the present data. In the present investigation also it is observed that insulin induced glucose uptake is

inhibited by phlorizin. Battaglia et al., (1960) have reported that phlorizin at a concentration of 3×10^{-3} M produces a 60 % inhibition of the glucose uptake induced by insulin in isolated rat diaphragm muscle. So the data obtained here also suggest that the action of phlorizin is by the interference with the specific carrier mediated transport of glucose across the cell membrane. None of the evidences contradict the conclusion drawn by other workers (Crane, 1962). If we accept the premise that both insulin and ACh act upon liver tissue to increase the number of active carriers for the transport of glucose across the cell membrane, then one may consider that phlorizin is competitively inhibiting the action of these carriers. The data also show that both insulin and ACh activate a phlorizin sensitive carrier (glucose transporter) for the transport of glucose into liver cells. However, the degree of activation of these specific carriers may vary for insulin and ACh. Moreover, both of them may also activate other types of glucose transport in the liver.

The recognition of the interrelationship between sugar and Na^+ pump and the fact that ouabain inhibits active Na^+ transport, prompted this investigation into the possible effect of ouabain on the active transport of glucose into liver. It was found that ouabain in 100 uM concentration depresses the active glucose transport in liver tissue. It was also observed by Csaky et al., (1961) in the isolated intestine of grass frog. Thus, a functioning Na^+ pump is essential for the activation of the

sugar pump. Hence, ouabain has to act on the membrane where the Na^+ pump is also situated to inhibit the sugar transport. Secondly, an optimum concentration of sodium has to be maintained for the functioning of pump ATPase, which indicates that this enzyme may be involved in the active transport of both Na^+ and sugar. Ouabain competes with K^+ in the Na^+ K^+ exchange pumps and that the entry of K^+ through the membrane may regulate the Na^+ permeability of the membrane. This is also seen in the epithelium of the toad bladder (Essig et al., 1963). Ouabain interferes with the Na^+ pump located in the membrane, thereby inhibiting the active glucose transport. Yaremenko et al. (1981) also showed that the action of ouabain on serous surface of the rabbit gall bladder epithelial cells was to decrease Na^+-K^+-ATPase and HCO_3-ATPase activities. Thus, it is possible to surmise that sugar transport and Na^+ pump could be so linked, that in tissues like liver and intestine, any stimulus or action that leads to ionic movement could result in glucose uptake. In intestine even the Na^+ diffusion due to concentration gradient difference suffices to induce glucose movement across the membrane. In liver, glucose uptake against a concentration gradient could be induced by insulin as well as ACh. Both must be affecting the ionic permeability of the hepatocyte membrane which in turn triggers Na^+ pump and the subsequent sugar transport. Added to this, on mammalian liver, insulin could also stimulate the hexokinase which is sensitive to insulin. Since the hexokinase of avian liver is not very sensitive to insulin, the rate of insulin induced uptake of glucose is much

low. Moreover, the glucose induced insulin release is sluggish in birds (Hazelwood, 1973). The profound cholinergic innervation of liver, thus, could assume greater role in glucose uptake. ACh secreted by cholinergic parasympatheitic fibres, could induce membrane permeability changes and through ionic movement could bring about glucose uptake. The action of ACh is probably through Ca^{++} as ACh and Ca^{++} together could induce maximum glucose uptake (Chapter 3). Ca^{++} released from membrane bound state or that enters through Ca^{++} channels in response to ACh, in turn induces membrane permeability, ultimately leading to glucose uptake. This was evident from the fact that both phlorizin and ouabain could inhibit glucose uptake by liver cells even in the presence of ACh. In the avian liver, insulin must be also mediating the glucose uptake through a flow coupled transport as both ouabain and phlorizin could inhibit the insulin action. Probably insulin induces the Na^{+} and K^{+} channels to open. Insulin in the presence of Na^{+} or K^{+} could induce much more glucose uptake than in presence of Ca^{++} (Chapter 3). Thus an interplay of ionic movements, Na^{+}-K^{+}-ATPase and active transport of Na^{+} out of the cell and K^{+} into the cell could be the underlying mechanism that results in the net glucose uptake under the influence of insulin and ACh.