CHAPTER 12

EFFECT OF CHOLINE CHLORIDE ADMINISTRATION ON Na^+-K^+-ATPase AND PHOSPHOLIPID CONTENT OF THE LIVER OF VAGOTOMIZED PIGEONS

Phospholipids were considered until recently, as "inert structural substances, "whose major reason for existence was to provide recognizable boundaries for cells and organelles" or "to provide a convenient matrix to support more important biochemical substances, such as membrane bound enzymes, transporters or receptors" (Farese, 1983). Phospholipids are viewed now as neither "inert" nor simply "structural". Many phospholipids have very high rates of turnover, and can profoundly alter membrane function. Moreover, most hormones and neurotransmitters have now been shown to provoke rapid and dramatic changes in phospholipid metabolism. These changes in phospholipid metabolism have been correlated to changes in cellular functions. The phospholipid metabolic changes are getting recognized as important mediators of many hormones and neurotransmitters and function both before and after the generation of the "second messengers", Ca^{++} and cyclic nucleotides (Farese, 1983). The most striking changes in the cellular phospholipid metabolism are those concerned with phosphatidic acid (PA) synthesis and phosphatidyl inositol (PI) formation from PA. The PA formation is mediated by phospholipase C and results in the release of Ca^{++}, while PI formation binds Ca^{++}. The PI hydrolysis is stimulated by agonists such as α- adrenergic agent, acetyl-

choline, insulin and many others while PA de novo synthesis is stimulated by ACTH, LH, PTH etc. (Farese, 1983). The phospholipase C is reported to be a membrane bound enzyme which could be activated by receptors bound to agonists. The PI-PA cycle is believed to be involved in several cellular responses. Classical example is the secretogogue function in B cells. Cholinergic, α-adrenergic agonists and glucose can induce the phospholipid turnover in islet B cells and through Ca^{++} release, they ultimately induce the release of insulin (Best and Malaisse, 1983). Insulin can also directly influence the synthesis of phospholipids in many tissues. Some of these phospholipids can activate phosphodiesterase and pyruvate dehydrogenase. Apart from such second or third messenger functions, the phospholipid changes may influence the activity of membrane bound enzymes, receptors or transmitters, either directly or indirectly by alteration in the membrane configuration, fluidity or ion binding (Farese, 1983). Phospholipid composition changes in the membrane especially that of mitochondria could alter the mitochondrial enzyme activity (Vidal et al., 1983). In the light of these observations, it was deemed worthwhile to undertake a preliminary study on phospholipid content of the liver, and the activity of membrane bound enzyme (Na^{+} - K^{+} - ATPase) after glucose, choline chloride and glucose + choline chloride administration in vagotomized pigeons.

MATERIAL AND METHODS

Adult domestic pigeons (Columba livia) weighing around 200 - 250 grams, maintained in laboratory conditions with

standard diet were used for the experiments. Birds were divided into 3 groups and each group was divided into 5 sub-groups having 5 birds each. The sub-groups were:

(1) Normal overnight starved
(2) Sham operated 48 hr. starved
(3) Vagotomized 48 hr. starved
(4) Sham operated 72 hr. starved
(5) Vagotomized 72 hr. starved.

Each main group was injected with glucose (70 mg/100 gram body weight), choline chloride (15 mg/animal) and glucose + choline chloride (70 mg/100 gram body weight + 15 mg/animal) and at regular interval of 30 minutes (i.e. 0, 30, 60, 90 and 120 minutes) after injection, the animals were sacrificed and Na^+-K^+-ATPase and phospholipid contents quantitatively estimated as per the methods described in Chapter 1.

RESULTS

The data on phospholipid content and activity of Na^+-K^+-ATPase in the liver of overnight starved, sham operated (48 hr. and 72 hr.) and vagotomized (48 hr. and 72 hr.) pigeons after administration of glucose, choline chloride and glucose + choline chloride are presented in tables 12-1 to 12-6 and Figs. 12-1 to 12-2.

FIG. 1. EFFECT OF GLUCOSE, CHOLINE CHLORIDE OR GLUCOSE + CHOLINE CHLORIDE ADMINISTRATION ON PHOSPHOLIPID CONTENT IN THE LIVER OF NORMAL, SHAM OPERATED (SHAM) OR VAGOTOMIZED (VGX) PIGEONS.

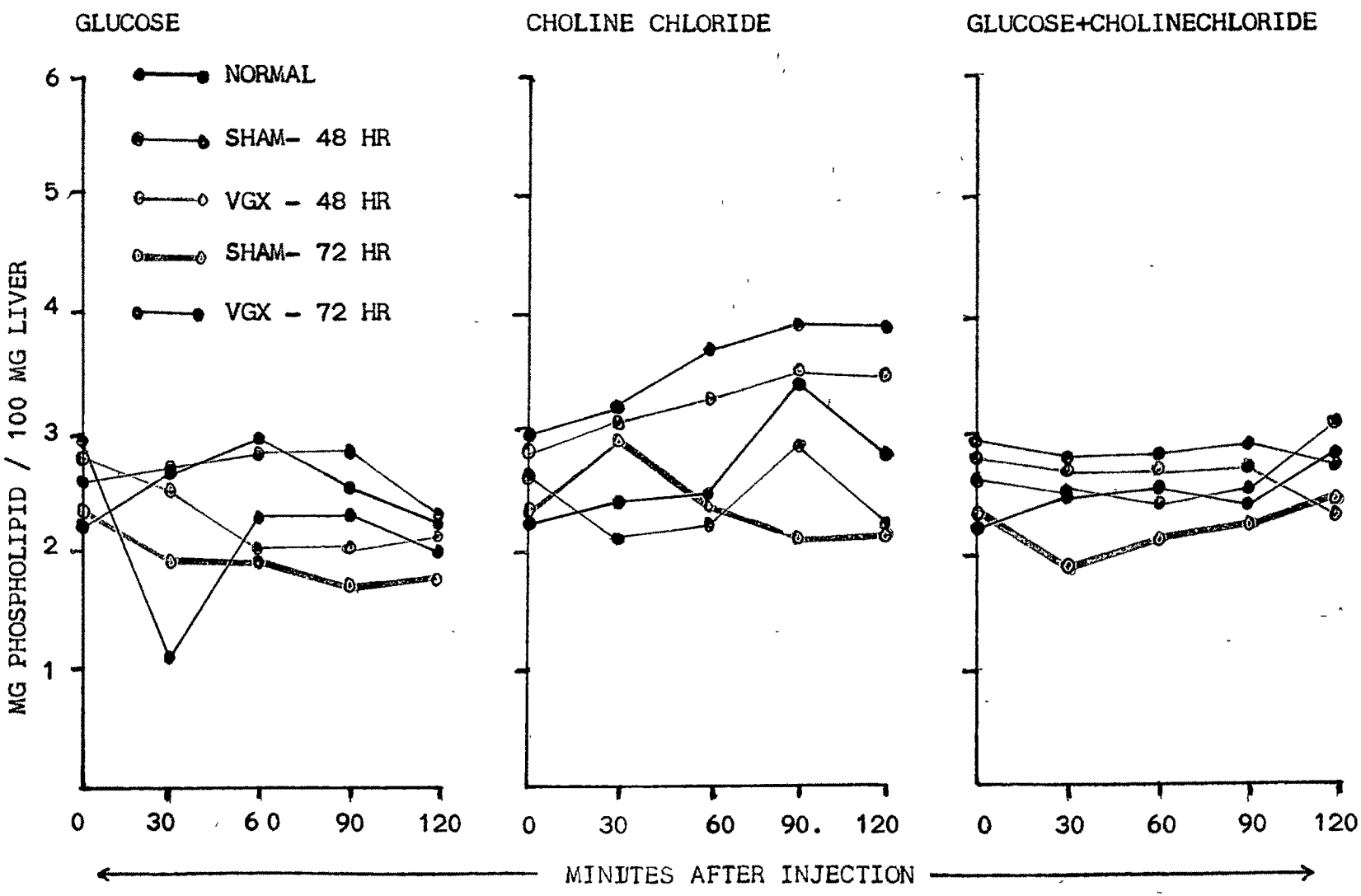

FIG. 12-2. EFFECT OF GLUCOSE, CHOLINE CHLORIDE AND GLUCOSE + CHOLINE CHLORIDE ADMINISTRATION ON $Na^{+}K^{+}$ATPASE ACTIVITY IN THE LIVER OF NORMAL, SHAM OPERATED (SHAM) OR VAGOTOMIZED (VGX) PIGEONS.

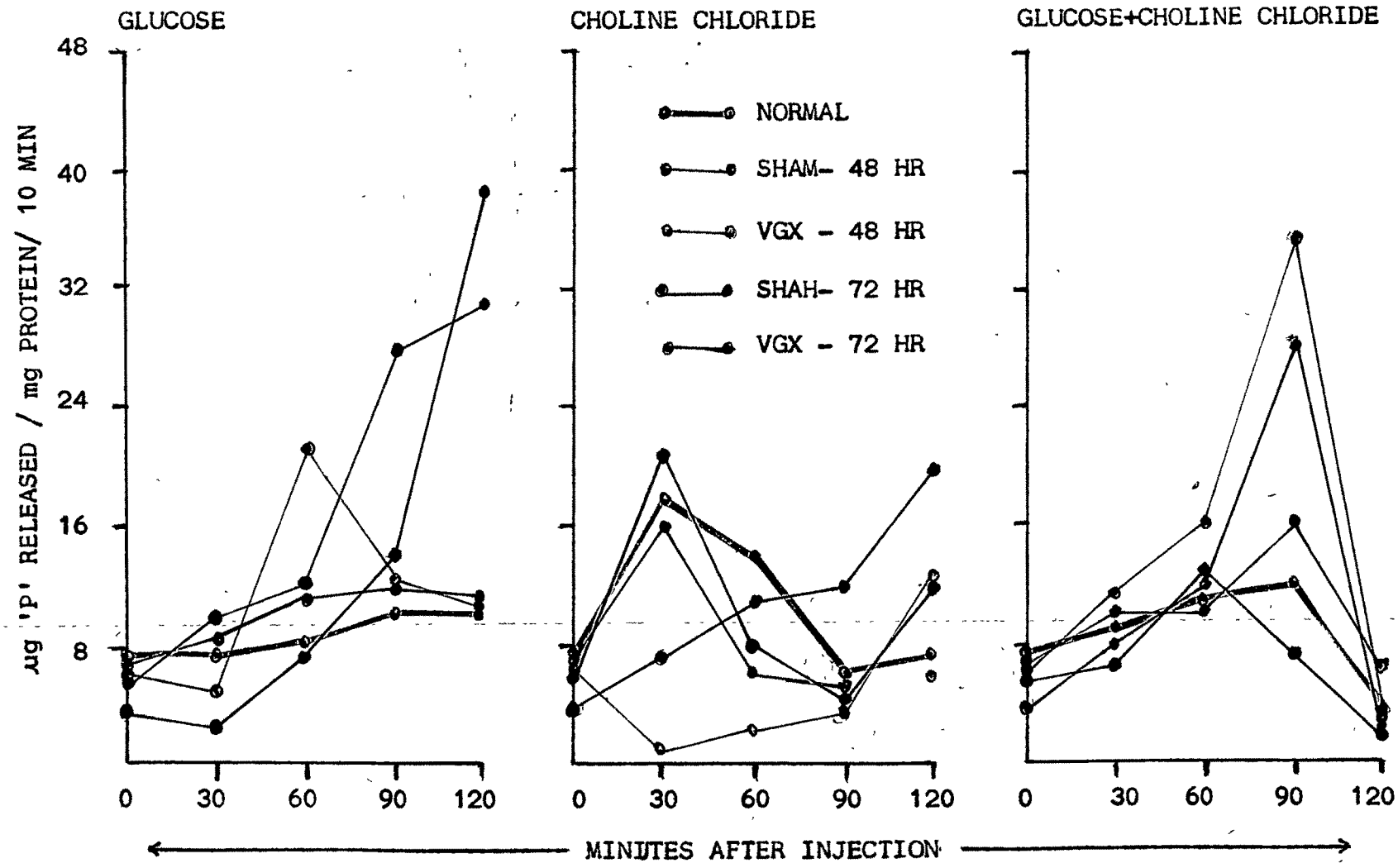

Table 12-1

Effect of glucose administration on phospholipid content of the liver of normal, sham operated and vagotomized pigeons.

(Values are expressed as mg phospholipid/100 mg fresh tissue weight. Mean ± S.E.M).

Time Interval in Minutes	Normal	Sham-operated (48 hrs)	Vagotomized (48 hrs)	Sham-operated (72 hrs)	Vagotomized (72 hrs)
0	2.321 ±0.341	2.582 ±0.321	2.824 ±0.408	2.247 ±0.282	2.985 ±0.823
30	1.940 NS ±0.210	2.698 NS ±0.203	2.520 NS ±0.394	2.730 NS ±0.187	1.125* ±0.062
60	1.904 NS ±0.026	2.861 NS ±0.019	1.998 NS ±0.013	2.931 NS ±0.07	2.268 NS ±0.115
90	1.696 NS ±0.186	2.854 NS ±0.022	2.072 NS ±0.025	2.534 NS ±0.159	2.327 NS ±0.175
120	1.771 NS ±0.192	2.229 NS ±0.106	2.110 NS ±0.043	2.226 ±0.094	2.044 NS ±0.029

NS - Not significant, * $P < 0.05$.

Table 12-2

Effect of glucose administration on phospholipid content of the liver of normal, sham operated and vagotomized pigeons.

(Values are expressed as Mg phospholipid/100 mg fresh tissue weight. Mean ± S. E. M)

Time Interval in Minutes	Normal	Sham-operated (48 hrs)	Vagotomized (48 hrs)	Sham-operated (72 hrs)	Vagotomized (72 hrs)
0	2.321 ±0.521	2.582 ±0.321	2.824 ±0.408	2.247 ±0.282	2.985 ±0.823
30	2.956 NS ±0.046	2.093 NS ±0.191	3.091 NS ±0.062	2.375 NS ±0.176	3.194 NS ±0.099
60	2.364 NS ±0.175	2.219 NS ±0.054	3.297 NS ±0.179	2.395 NS ±0.190	3.726 NS ±0.183
90	2.178 NS ±0.065	2.973 NS ±0.028	3.499* ±0.143	3.417* ±0.103	3.937* ±0.11
120	2.158 NS ±0.101	2.192 NS ±0.322	3.480* ±0.324	2.855 NS ±0.209	3.901* ±0.094

NS - Not significant, * $P < 0.05$.

Table 12-3

Effect of glucose + Choline chloride administration on phospholipid content of the liver of normal, sham operated and vagotomized pigeons.

(Values are expressed as Mg phospholipid/100 mg fresh tissue weight Mean ± S.E.M)

Time Interval in Minutes	Normal	Sham-operated (48 hrs)	Vagotomized (48 hrs)	Sham-operated (72 hrs)	Vagotomized (72 hrs)
0	2.321 ±0.521	2.582 ±0.321	2.824 ±0.408	2.247 ±0.282	2.985 ±0.823
30	1.844 NS ±0.209	2.539 NS ±0.179	2.683 NS ±0.253	2.538 NS ±0.157	2.813 NS ±0.12
60	2.083 NS ±0.045	2.462 NS ±0.195	2.722 NS ±0.11	2.639 NS ±0.139	2.839 NS ±0.181
90	2.257 NS ±0.115	2.507 NS ±0.291	2.682 NS ±0.175	2.396 NS ±0.032	2.940 NS ±0.024
120	2.457 NS ±0.16	3.101 NS ±0.16	2.371 NS ±0.202	2.832 NS ±0.156	2.686 NS ±0.176

NS - Not significant.

Table 12-4

Effect of glucose administration on Na^+-K^+-ATPase activity in the liver of normal, sham operated and vagotomized pigeons.

Values are expressed as µg phosphorus released/Mg protein/10 minutes.
Mean ± S.E.M.

Time Interval in Minutes	Normal	Sham-operated (48 hrs)	Vagotomized (48 hrs)	Sham-operated (72 hrs)	Vagotomized (72 hrs)
0	7.42 ±4.51	7.30 ±4.42	6.87 ±0.867	6.23 ±0.07	3.91 ±0.858
30	7.657 NS ±0.42	8.169 NS ±3.51	5.003 NS ±1.11	9.985* ±1.17	2.546 NS ±0.45
60	8.203 NS ±0.26	11.447 NS ± 1.94	21.915*** ± 3.34	12.672* ± 1.59	7.475 NS ±0.39
90	10.124 NS ± 0.62	12.859 NS ± 1.19	12.629* ± 0.86	28.223*** ± 2.24	14.385*** ± 0.81
120	10.812 NS ± 1.313	11.699 NS ± 1.19	11.4897* ± 2.06	31.779**** ± 6.40	39.666**** ± 5.50

NS - Not significant, * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, **** $P < 0.001$.

Table 12-5

Effect of choline chloride administration on Na^+-K^+-ATPase activity in the liver of normal, sham operated and vagotomized pigeons.

Values are expressed as µg phosphorus released/mg protein/10 minutes.

Mean ± S.E.M.

Time interval in Minutes	Normal	Sham-operated (48 hrs)	Vagotomized (48 hrs)	Sham-operated (72 hrs)	Vagotomized (72 hrs)
0	7.42 ±4.51	7.30 ±4.42	6.87 ±0.867	6.23 ±0.07	3.91 ±0.858
30	18.403** ± 2.882	16.387*** ± 1.765	1.355* ±0.141	21.862** ± 0.541	7.1599* ±2.870
60	14.391* ± 1.002	6.227 NS ±1.143	2.294 NS ±0.173	8.353 NS ±0.806	11.699** ± 0.527
90	6.411 NS ±1.192	5.115 NS ±0.779	2.966 NS ±0.033	4.227 NS ±0.780	12.618*** ± 0.614
120	7.694 NS ±0.711	6.334 NS ±0.607	13.894 NS ± 1.221	13.042** ± 1.113	20.238*** ± 2.963

NS - Not significant, * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$,

Table 12-6

Effect of glucose + Choline chloride administration on Na^+-K^+-ATPase activity in the liver of normal, sham operated and vagotomized pigeons.

Values are expressed as µg phosphorus released/100 mg protein/10 minutes. Mean ± S.E.M.

Time Interval in Minutes	Normal	Sham-operated (48 hrs)	Vagotomized (48 hrs)	Sham-operated (72 hrs)	Vagotomized (72 hrs)
0	7.42 ±4.57	7.30 ±4.42	6.87 NS ±0.867	6.23 ±0.07	3.91 ±0.858
30	9.422 NS ±0.24	10.439 NS ± 1.36	11.532 NS ± 1.50	6.292 NS ±0.96	8.135 NS ±0.71
60	12.199 NS ± 1.23	11.283 NS ± 1.24	16.412* ± 1.02	13.856* ± 0.64	12.446* ± 0.24
90	12.121 NS ± 0.63	16.209* ± 1.49	35.075*** ± 9.1	7.452 NS ±0.81	28.989*** ± 3.12
120	6.862 NS ±1.30	6.332 NS ±0.83	3.248 NS ±0.46	1.995* ±0.294	3.705 NS ±0.24

NS - Not significant, * $P < 0.05$, ** $P < 0.02$, *** $P < 0.01$, **** $P < 0.001$.

Phospholipid

Glucose injection (Table 12-1; Fig.12-1)

Glucose injection produced a gradual decrease in the phospholipid content of the liver in normal overnight starved pigeons, while in the sham operated 48 hr. and 72 hr. pigeons there was a slight decrease by 120 min. In vagotomized 48 hr. pigeon liver, glucose injection produced not much variation except at 120 min., whence a slight decrease was observed. In vagotomized 72 hr. pigeon liver, glucose injection caused an initial (30 min.) drop in the content.

Choline chloride injection (Table 12-2; Fig. 12-1)

Normal overnight starved or sham operated 48 hr. and 72 hr. pigeon liver showed no significant changes in phospholipid content after administration of choline chloride. However, vagotomized (48 hr. and 72 hr.) pigeon liver responded to choline chloride by showing significant increase by 90 min.

Glucose + Choline chloride injection (Table 12-3;Fig.12-1)

When both glucose and choline chloride were injected together, there was no variation in the phospholipid content of the liver in any group of pigeons, except a slight initial dip in the normal (overnight starved) pigeon liver.

$Na^+ - K^+$ ATPase

Glucose injection (Table 12-4; Fig.12-2)

The enzyme exhibited a gradual rise in the normal pigeon liver when glucose was injected. In the sham operated 48 hr. pigeon liver also, the enzyme showed a gradual increase. But in the sham operated 72 hr. pigeon liver, the enzyme activity showed a steep rise by 90 min. The vagotomized 48 hr. liver on the other hand showed a sharp peak at 60 min., while in vagotomized 72 hr. pigeon liver, the activity showed a steep rise by 120 min.

Choline chloride injection (Table 12-5;Fig.12-2)

Choline chloride activated $Na^+ - K^+$ ATPase in normal and sham operated (both 48 hr. and 72 hr.) pigeon liver by 30 min. However, in vagotomized 48 hr. pigeon liver, choline chloride brought about an increase in the enzyme activity only by 120 min., while in vagotomized 72 hr. pigeon liver it brought about a gradual rise up to 90 min. and a steep one at 120 min.

Glucose + Choline chloride injection (Table 12-6;Fig.12-2)

In the normal (overnight starved) pigeon liver, administration of both glucose and choline chloride together produced a maximum increase of $Na^+ - K^+$ ATPase during 60 and 90 min. intervals. Sham operated 48 hr. pigeon liver showed a peak at 90 min., while in sham operated 72 hr. pigeon liver, the peak was at 60 min. In vagotomized 48 hr. and 72 hr. pigeon liver, the activity increased sharply at 90 min.

DISCUSSION

Both glucose and choline agonists which are secretogogues to B cells, are shown to induce enhanced ^{32}p labelling of phosphatidic acid (PA) and phosphatidyl inositol in islet cells (Best and Malaisse, 1983a, 1983b). Several other tissues were also shown to respond to cholinergic agonists with altered phospholipid metabolism (Farese, 1983). The accelerated phospholipid metabolism was pertaining to PA and PI. In this study, the phospholipids were measured as a class and not individually. Hence, changes seen in phospholipid contents in the liver in response to glucose, choline chloride or both these combined were not very striking. Moreover, the subtle variation that are detectable were masked by individual variations. Hence only a few generalized statements are all that could be put forth. Glucose administration did not result in any significant variations in the phospholipid contents in the liver of control or experimental (vagotomized) pigeons. Choline chloride administration showed significant increase in phospholipid content at 90 min. in all groups, the response of vagotomized pigeons being slightly higher. When both glucose and choline chloride were given together, all groups showed a resistance to react. The only conclusion that could be given at this juncture is that a glucose induced response of phospholipid is absent in the liver inspite of the presence of choline chloride whether vagus is intact or transected.

However, that glucose or choline chloride or both together induced some changes in the membrane integrity or in the membrane

bound mechanism, is clear from the data on $Na^+ - K^+$ ATPase. $Na^+ - K^+$ ATPase is a membrane boumd enzyme and it showed a significantly increased activity when the pigeons were injected with glucose or glucose + choline chloride. Interestingly, 72 hr. starved sham operated pigeons showed high sensitivity to glucose, as was the case with vagotomized pigeons (48 hr. and 72 hr.). The sensitivity of this enzyme became much more sharp and well defined in vagotomized pigeon liver when both glucose and choline chloride were administered together. The response of the enzyme to choline chloride injection was more dramatic in normal (overnight starved) and sham operated pigeons. Vagotomy, in other words, suppressed the response of $Na^+ - K^+$ ATPase to choline chloride, while it accelerated the response to glucose. Increased phospholipid content was observed in vagotomized pigeons administered with choline chloride. Probably an inverse correlation could be extended with respect to vagotomy and choline chloride administration as far as the hepatic response in the phospholipid content and $Na^+ - K^+$ ATPase activity are concerned.

These preliminary observations, at best could point to a possible effect of glucose and choline chloride on the phospholipid and membrane bound $Na^+ - K^+$ ATPase in the liver of pigeon and also the possible influence of vagal fibres on the modulation of these parameters in response to glucose or even other hormones. It is probably premature to explain the permeability changes, Ca^{++} involvement and PA and PI metabolism in the membrane of hepatocytes in response to glucose or cholinergic agonists, unless more specific phospholipid measurements and radio PI labelling etc. are made.

release took place. When Na^+ or K^+ was taken in combination with Ca^{++}, glucose uptake was stimulated, but Na^+ and K^+ together did not stimulate glucose uptake. Sodium ions can move into the cell if the extracellular concentration of Na^+ is in hypertonic range or when membrane permeability is altered. When extrusion of Na^+ and reuptake of K^+ are taking place, glucose could move into the cell. The carrier couples with K^+ and glucose and transfers them towards the inner side of the membrane. The experiments proved that, the movement of Na^+ into the cell, either induced by a gradient difference or by effecting the permeability of the membrane, brings about a corollary uptake of glucose by liver cells.

Chapter 3

In vitro studies suggest that glucose is transported across membrane of several cells by carrier mediated facilitated diffusion. Insulin increases glucose transport by increasing the number of available carriers. Insulin stimulates glucose uptake which is amplified by the synergistic participation of nervous elements and their neurotransmitters (Mondon and Burton, 1974). That ACh participates in the transport of glucose across hepatic cell membrane was suggested earlier by the observation that the sinusoidal lining of hepatic cords of birds have high activity of AChE. In this chapter the physiological action of insulin and ACh individually as well as in combination with ions, on glucose uptake is reported. Insulin increases glucose

transport, a well established fact, was also evidenced in the present work. When ACh was taken alone in the medium it also stimulated glucose uptake. When insulin or ACh was taken in combination with Na^+, K^+ or Ca^{++}, it stimulated glucose uptake by liver cells. The results indicate that part of the insulin and most of the ACh actions in the uptake of glucose by liver cells, could be influenced by the cation concentration differences in the medium.

Chapter 4

Phlorizin is known to inhibit glucose transport across the kidney and the intestinal mucosa. It has been shown that phlorizin inhibits insulin stimulated glucose uptake by muscle and blocks the ability of insulin to enhance the permeation of galactose into muscle both *in vivo* and *in vitro*. Since insulin markedly increases the rate of glucose utilization, it was deemed worthwhile to investigate the action of phlorizin upon this process. Ouabain inhibits the active transport of sodium in a variety of cells. It also inhibits the Na^+-K^+ dependent membrane ATPase which is thought to be involved in active cation transport. Cell membrane is the site where inhibitory action on cation tramsport and membrane ATPase takes place. When ouabain and phlorizin were taken in the incubation medium as additives, alone as well as in combination with insulin and ACh, they actively inhibited glucose uptake by liver cells, indicating that at least in the liver, both insulin and ACh enhance glucose uptake through a membrane bound mechanism, part of which is coupled to ionic movements.

Chapter 5

Thyroid hormones are closely related with oxidative reactions and regulation of metabolism in the body. Many conflicting mechanisms have been proposed to explain the multiple biological actions of thyroid hormones, especially since the discovery that administration of large doses of thyroxine or direct addition in vitro leads to uncoupling of oxidative phosphorylation in liver mitochondria. Some studies show the direct action of thyroid hormone on mitochondria or mitochondrial permeability as seen by their swelling and contraction. In the present experiment, when thyroxine was added (10 µg/ml) in the incubation medium, under in vitro condition, it stimulated glucose uptake whether alone or in combination with insulin or ACh. Thyroxine affects the permeability of the hepatocyte membrane and thereby increases the glucose uptake without involving either the insulin stimulated or the ACh stimulated glucose uptake mechanism. Thyroxine does not stimulate glucose uptake under in vivo conditions, there it acts as a hyperglycemic agent. The difference in the action of thyroxine under in vivo and in vitro conditions, is that in the in vivo condition, hyperglycemic agents such as glucagon and catecholamines could also be present. In the absence of hyperglycaemic agents, thyroxine stimulates glucose uptake, especially under in vitro conditions. Some of the enzymes such as Na^+-K^+ ATPase, SDH and alkaline phosphatase showed response to thyroxine alone in the medium, and thyroxine in combination with insulin or ACh. The enzyme responses were more or less conforming to the gluconeogenic actions of thyroxine. However, thyroxine due to its action

on membrane permeability, probably through its influence on Na^+-K^+ ATPase could also induce glucose uptake.

Chapter 6

Glucagon is the major carbohydrate regulator in aves, while insulin plays only a supportive role. The insulin sensitives membrane components which are involved in glucose transport are very sparsely distributed in the avian liver. The glucose stimulated insulin release is also very sluggish in birds. Due to all these reasons, the influence of glucagon has assumed an over all importance in carbohydrate metabolism in birds. Glucagon has specific binding sites in many cells and the glucagon-receptor complex activates the adenyl cyclase enzyme present in the plasma membrane. The consequent rise in cAMP concentration, which in turn triggers off a series of reactions culminating in phosphorylase activation ultimately leads to the release of glucose from liver cells. This characteristic action of glucagon was readily seen in the in vitro studies with liver slices. Glucagon alone in the medium effected glucose release from the liver slices. This action of glucagon was countered by both insulin and ACh. The alkaline phosphatase activity was found to be high irrespective of the fact that insulin or ACh was present in the medium along with glucagon. This and the fact that acid phosphatase activity did not show any variation, were mainly responsible for preventing insulin from inducing glucose uptake as much as it usually did when present alone in the medium. Thus, inspite of the fact that glucagon could

induce metabolic reactions in the liver, as it always does, glucose release was inhibited by both insulin and ACh.

Chapter 7

Apart from the influence of ACTH on insulin secretion, ACTH also causes a rapid increase in phosphorylase activity in liver, and this stimulatiom of hepatic phosphorylase resembles that by glucagon. ACTH also increases glucose uptake by liver slices when present in the medium. Thus it can be said that ACTH has some direct effect on the liver functions. Actually ACTH receptors are present on the adrenals which are the target organs. In the present study liver is an experimental tissue which is not the target of ACTH action. But extra-target organ actions can be expected to occur when sufficient concentration of tropic hormone is present which exerts same or similar effects on basic cellular processes there. The action of ACTH on glucose uptake could not be through any effect on membrane permeability because it failed to increase glucose uptake in the presence of ACh further than what was observed when it was present alone in the medium. Since the action of ACTH on glucose uptake was additive in the presence of insulin, it is possible to believe that the action of ACTH was very similar to what was induced by insulin. Probably the action of ACTH may be through its action on Ca^{++} movement into the cells or its release from the bound state. The decreased SDH activity and the increased LDH activity in the liver slices in presence of ACTH either alone or in combination

with insulin or ACh indicate a general reduction in aerobic metabolism while anaerobic reactions are activated.

Chapter 8

Glucocorticoid also plays a major role in maintaining blood glucose level and most of its actions are manifested due to the presence of large number of receptors on liver cells. Dexamethasone disodium phosphate (DXM), a synthetic glucocorticoid, decreases glucose uptake by tissues. The administration of glucocorticoid induced insulin resistence in man and rats. There was also a decreased insulin stimulated glucose oxidation and decreased insulin binding capacity. The ability of DXM to inhibit glucose uptake is believed to be through its inhibition of hexokinase. The decrease in the rate of glucose transport could also be due to depression in carrier synthesis or reversible conversion of carrier to an inactive apo-carrier. When dexamethasone was taken along with insulin and ACh, both insulin and ACh showed an ability to reverse the DXM induced inhibition of glucose uptake. Alkaline phosphatase which is mainly involved in glucose release from the liver was activated by DXM. The action of DXM on the release of glucose could be thus through its stimulatory effect on alkaline phosphatase. The post-receptor steps by DXM involve activation of alkaline phosphatase and ATPase, and inhibition of acid phosphatase, SDH and LDH in the liver slices. Probably all the enzymes in the glycolytic pathway are suppressed by glucocorticoid and hence the failure of insulin or ACh to manifest

a glucose uptake rate as high as in conditions when they are present alone.

Chapter 9

AChE inhibitors such as monocrotophos (MCP), acothione and prostigmine have been chosen for the assessment of the biochemical effects of these on glucose transport by the pigeon liver slices. MCP and acothione are organophosphorus compounds, highly toxic and they affect the living system tremendously. Prostigmine is an inhibitory drug, which inhibits AChE thereby increasing ACh levels. The organophosphorus compounds are potent inhibitors of ChE and the mechanism of action on ChE enzyme is fairly well understood. The organophosphorus pesticide works like a substrate analogue for ChE and inhibits ChE as enzymic proprotein gets phosphorylated thus becoming stable and hence does not remain capable of effecting usual hydrolysis. Previous *in vivo* studies have shown elevation of ChE activity in liver, following glucose administration in both pigeon and rats, suggesting participation of ACh in the transport of glucose across hepatic cell membrane. MCP, acothione and prostigmine when present in the medium, induced an uptake of glucose when they were taken alone as well as in combination with insulin and ACh. As a result of AChE inhibition, ACh accumulates in the liver cells which influences the uptake of glucose through its action on permeability of the membrane. Prostigmine, although could inhibit AChE as effectively as organophosphorus compounds such as MCP and acothione, failed to

increase the uptake of glucose as much as them even in presence of ACh. There was no increased metabolic activities in the cells as evident from the decreased ATPase, SDH and LDH activities, which could account for increased uptake of glucose. Thus glucose entered the hepatic cells mainly due to permeability changes of the hepatocyte membrane with the resulting flow coupled transport.

Chapter 10

Parasympathetic stimulation could induce glucose uptake by liver cells. The parasympathetic action is mediated by ACh and the sympathetic action by nor-epinephrine. To a certain extent, ACh mimics the action of insulin, especially in the induction of liver cells to take up more glucose. In birds, vagotomy lowered the glucose level in blood but when injected with glucose, hyperglycaemia prevailed for a longer time, a situation similar to diabetes in mammals. Vagus is now known to be involved in several glucoregulatory processes: (1) efferent fibres affecting the liver directly (2) efferent fibres inducing insulin secretion from B-cells and inhibiting the A-cell secretion (glucagon) and (3) afferent fibres signal the glycaemic state of the liver to hypothalamus. Moreover, ACh is shown to act synergistically with insulin in the uptake of glucose by liver cells. In the present study an attempt was made to see the action of ACh, choline chloride, insulin, glucagon and DXM on the glycaemic levels in vagatomised pigeons. When injected in vagotomized 48 hr. starved

pigeons, choline chloride caused a highly significant increase in glucose levels at all the intervals. In vagotomized 72 hr. starved pigeons, the glucose level showed an increase at 30, 60, and 90 min. after injection of choline chloride, but by 120 min. the level decreased to the pre-injection level. When ACh was injected in vagotomized 48 hr. starved pigeons, ACh produced an increased glucose level compared to that at the pre-injection period. In vagotomized 72 hr. pigeons, ACh administration brought about a significant increase of blood sugar level right from the 30 min. interval onwards.

Insulin exhibited hypoglycaemic action in all the groups of pigeons. In vagotomized 48 hr. pigeons, glucagon administration produced an increase in glucose level at 30 and 60 min., but thereafter the level fell drastically below the pre-injection level. In vagotomized 72 hr. pigeons, glucagon injection produced significant increase in the glycaemic level but the pre-injection level was attained only at 120 min.

The synthetic glucocorticoid in normal pigeons, produced an increase in the glucose level by 60 min. but caused a decrease at the successive intervals. In vagotomized 48 hr. pigeons, DXM increased the glycaemic level significantly at all the intervals. Although vagotomized 72 hr. pigeons too showed more or less similar response to DXM, the increase was significant at 60 min. only.

The data exhibit some curious phenomena. ACh or choline chloride injections apparently cause an increase in glucose level in the blood probably by increasing sympathetic action. The 48 hr. starved and 72 hr. starved pigeons responded to hormones differently. The metabolic state of the birds, particularly that of liver, modulates the influence of various hormones.

Chapter 11

The nature of neural control of liver function and glucose homeostasis is only getting slowly understood. The antagonistic actions of parasympathetic and sympathetic nerves are also seen in their effects on glucose homeostasis and liver functions. While parasympathetic fibres induce glucose uptake, the sympathetic action induces liver to release glucose. The parasympathetic action is mediated by ACh and the sympathetic actiom by norepinephrine. Vagal stimulation increased glycogen deposition in the liver, through the activation of glycogen synthetase. Vagotomy reduced glycogen deposition in the liver and choline chloride injection restored the rate of deposition. Choline chloride, when administered along with glucose could suppress the adverse effect of vagotomy on the glycaemic level. This was probably through activating glucose uptake and glycogen deposition mechanisms in the liver. Both glycogen synthetase and acid phosphatase were activated at a proper time by choline chloride during glucose loading, thereby the hyperglycaemic effect was not observed even in the vagotomized pigeons.

Chapter 12

Choline chloride, the metabolic product of acetylcholine degradation by acetylcholinesterase is known to affect the liver especially in the uptake of glucose and deposition of glycogen. The stimulatory effect of choline chloride on these metabolic activities is believed to be mediated through changes in the membrane, resulting in permeability alterations, activation of membrane bound receptors and enzymes, and release of 'second' or 'third' messengers. To understand the effect of choline chloride on the membrane systems, phospholipid content and $Na^+ - K^+$ ATPase were estimated in the liver after administration of glucose, choline chloride and both together in normal, sham operated (48 hr. and 72 hr. starved) and vagotomized (48 hr. and 72 hr starved) pigeons. The results imdicate that vagal transection caused a cognizable variation in the response of the liver to glucose, choline chloride or both together. Vagotomy suppressed the $Na^+ - K^+$ ATPase response to choline chloride while accelerated the response to glucose. Choline chloride produced an increased phospholipid content in the liver of vagotomized pigeons. The data also indicate an inverse relationship between liver responses to choline chloride and glucose.

of glucose from the liver. The latter is probably through inactivation of glycogen phosphorylase. The parasympathetic nerves act synergistically with insulin. The parasympathetic response may be blocked by concomitant catacholamine or glucagon activation of glucose release (Lautt, 1980).

The role of afferent (sensory) nerve fibres

Although not conclusively proved, the evidence (See Lautt, 1980) seems to strongly indicate that there are likely to be hepatic receptors for atleast sodium, glucose, osmotic pressure and oncotic pressure, with some degree of overlap between the various receptor systems. The afferent fibres are known to be projected to hypothalamus and hence may have important role to play in the regulation of blood sugar level, food intake, water intake and osmotic pressure.

More recent literature on the role of hepatic nerves are reviewed by De Wulf and Carton (1981), Shimazu (1981), Shimazu (1983) and Lautt (1983).

Most of the studies on hepatic nerves are confined to mammals. There are only few investigations that are pertaining to birds, mainly carried out by Pilo and associates (Pilo and Patel, 1977, 1978a, 1978b; Patel and Pilo, 1977, 1978; Pilo et al., 1982, 1984). Birds are hyperglycemic compared to mammals. Birds have more A cell predominant islets in the pancreas, and

Although a general action of acetylcholine or choline chloride in hepatic glycogen deposition and glucose uptake were shown (Pilo and Patel, 1977), the mechanism by which these cholinergic agonists induced them was not completely elucidated. The present investigation is an attempt to bridge this gap.

Since acetylcholine or choline chloride has to induce glucose uptake, the logical site of action would be the hepatocyte membrane. Glucose transport across cell membrane could take place through facilitated diffusion, chemiosmotic "pull" mechanism or carrier mediated transport. In intestine and kidney, glucose is also transported through a flow coupled transport which is Na^+ dependent. This flow coupled transport of glucose as also seen in liver, essentially utilizes the same carrier molecules, and energy involved in Na^+ and K^+ transport. The extracellular concentration of ions, thus invariably influences this type of glucose transport. When liver slices were incubated in Krebs Bicarbonate medium containing hyperosmotic Na^+ concentration, glucose uptake resulted (Chapter 2). Neither K^+ nor Ca^{++} in hypertonic concentrations induced glucose uptake. However, when Ca^{++} was present along with Na^+ or K^+, uptake was significantly increased. The result indicates that Na^+ movement into the liver cells, either induced by hypertonic extracellular concentration or produced by Ca^{++} in presence of hypertonic cation concentration, induces liver cells to take up more glucose. The movement of glucose into the cells thus is somewhat closely related to ionic movements.

When liver slices were incubated with insulin, acetylcholine or choline chloride, invariably glucose uptake was observed (Chapter 3). The action of both acetylcholine and insulin increased when the medium also contained Na^+ or K^+ or Ca^{++}. ACh + Ca^{++} induced the maximum glucose uptake. A possible explanation is that both ACh and insulin must be affecting the permeability of the hepatocyte membrane, more so when the medium contained hypertonic cations. Ca^{++} could act as 'second messanger' of both ACh and insulin probably through the activation of phosphodiestrase that degrades cyclic AMP (Rasmussen and Waisman, 1983). The data presented in Chapter 3 show that glucose uptake response of liver slices induced by ACh or insulin is through a Ca^{++} dependent Na^+ and/or K^+ movement across the plasma membrane. In other words, the flow coupled transport of glucose is induced by both ACh and insulin to a certain extent. Flow coupled transport is an active transport and Na^+-K^+-ATPase is involved in the mechanism. When insulin or ACh was present in the medium along with either ouabain(an active transport (Na^+-K^+- ATPase) inhibitor) or phlorizin(a specific glucose transport inhibitor), no glucose uptake was observed (Chapter 4); which clearly indicates that in pigeon liver atleast, the major glucose transport mechanism is coupled to sodium pump. Thus, the <u>in vitro</u> effect of ACh and insulin is an increased glucose uptake by liver cells. The uptake is initiated by a change in permeability of the membrane resulting in an increased release or influx of Ca^{++}. These changes may open up Na^+ channels in the membrane. The influx of Na^+ ions stimulates active

transport of Na^+ (sodium pump) probably involving $Na^+ - K^+$-ATPase. During Na^+ extrusion by the pump, K^+ as well as glucose move into the cell (flow coupled transport). Both ACh and insulin are capable of inducing this flow coupled glucose uptake, more so by ACh than insulin. A high extracellular Na^+ concentration, or hypertonic Ca^{++} with high K^+ or Na^+ levels can also induce glucose uptake, indicating the interrelationship of ionic concentration and glucose uptake. Ca^{++} could enhance the action of ACh or insulin in the uptake of glucose by liver cells.

The *in vitro* effects of ACh or insulin may not be seen in *in vivo* conditions, where several other chemical, physical or physiological parameters will have modifying effects. To understand such interrelationship or modifying influence of other hormones on ACh and insulin actions, pigeon liver slices were incubated with thyroxine (Chapter 5), glucagon (Chapter 6), ACTH (Chapter 7), and glucacorticoid (Chapter 8) either alone or in combination with ACh or insulin. Thyroxine acts as hyperglycaemic agent in *in vivo* conditions (John *et al.*, 1983), but in the present *in vitro* condition, thyroxine (alone) induced a glucose uptake response in the liver slices. In the presence of ACh or insulin, thyroxine produced the same response. Thyroxine must be affecting permeability of the membrane as indicated by the increase in $Na^+ - K^+$ ATPase. However, thyroxine also induced a general increase in the activity of enzymes. In all probability, thyroxine may be facilitating the

action of other hormones. Thus when the medium contained ACh or insulin along with thyroxine, glucose uptake response was enhanced. If the medium contained glucagon or catachlamines, thyroxine would have induced glucose release. This facilitative action of thyroxine was reported by John et al. (1983) who showed that thyroidectomized diabetic rats showed a very high glycaemia which could be reduced by insulin treatment but not by thyroxine alone.

Glucagon is the major pancreatic hormone in birds and hence liver must be having large number of receptors of glucagon. Glucagon produces its effects on liver through cAMP which in turn activates the enzymes such as phosphorylase and G-6-Pase. Thus, the hormone in in vitro condition (Chapter 6) produced a significant glucose release from the liver slices. However, both ACh and insulin could inhibit the glucose release action of glucagon, ACh being more effective. Hence, one of the major responsibilities of vagal cholinergic fibers in the liver could be to counter act the action of glucagon. That corticotrophin (ACTH) has hypoglycaemic action was demonstrated by Westermeyer and Roben (1954) and Egel and Egel (1955) even in adrenalectomized mice and rats. In the present in vitro experiments (Chapter 7) also, ACTH produced a glucose uptake influence on the liver slices. This action of ACTH was enhanced when the medium also contained insulin or ACh. ACTH + insulin induced more glucose uptake than ACTH + ACh. Since the enzyme reactions were similar to that produced by insulin it could be reasoned that ACTH more

or less mimics the insulin action.

Although ACTH may have hypoglycaemic action, glucocorticoids have the opposite action. When dexamethasone (DXM), a synthetic glucocorticoid was taken in the medium, glucose uptake was inhibited (Chapter 8). This hormone also inhibited glycolytic pathway and activated gluconeogenic pathway. However, inspite of such metabolic activities, insulin and ACh could suppress the action of DXM.

Thus, the vagal cholinergic fibers in the liver, through ACh, could not only induce glucose uptake by liver cells, but also effectively counteract the actions of glucagon and glucocorticoids. Both thyroxine and ACTH could also synergistically induce glucose uptake together with ACh. In many respects, ACh could act like insulin on the liver. However, ACh may not be able to induce its action on liver cells for longer duration as insulin, since ACh is putative in action as well as it is quickly inactivated by cholinesterase. When acetylcholinesterase inhibitors (such as MCP, acothione, or prostigmine) were present in the medium along with ACh, glucose uptake was stimulated in the liver slices (Chapter 9). These inhibitors not only inhibited AChE but also must be affecting permeability of the membrane, as these chemicals alone in the medium induced glucose uptake.

The data presented in Chapters 2-9, thus, clearly indicate the role of acetylcholine in glucose uptake in the liver. In other words, vagal stimulation could induce glucose uptake and glycogenesis. In mammals also vagal stimulation has the same effect (see Lautt, 1983; Shimazu, 1983). Vagotomy in pigeons produced a prolonged hyperglycaemia, and increased gluconeogenesis and lipolysis (Verma, 1982). This effect of vagotomy was explained as due to the activation of sympathetic tone. To understand whether ACh, choline chloride or insulin could counteract the effect of vagotomy, these were injected in the vagotomized pigeons (Chapter 10). Curiously, only insulin could bring about a hypoglycaemic action in vagotomized pigeons, while ACh or choline chloride increased the glycaemia, just as glucagon or glucocorticoid. It is believed that administration of ACh or choline chloride only increased the sympathetic activity in the vagotomized pigeons.

However, when choline chloride was administered along with glucose, the glycaemic level was effectively brought down (Chapter 11). Either the action of choline chloride was enhanced in presence of a glucose load or the glucose load effectively reduced the sympathetic action. A glucose load could also reduce the release of glucagon and catecholamines. Choline chloride administration along with glucose in vagotomized pigeons could effectively check the advent of a hyperglycaemic condition. Choline chloride also could induce membrane permeability changes in liver as evidenced by the changes in

phospholipid content and Na^+ K^+ ATPase activity in the liver (Chapter 12).

The present investigation highlights the following roles of acetylcholine, choline chloride, or vagal fibers in the avian liver.

1. Prepare the liver for glucose influx.
2. Stimulate glucose uptake through a Na^+ dependent, Ca^{++} stimulated membrane permeability changes.
3. Effectively interact and complement the actions of insulin.
4. Effectively counteract the action of sympathetic nerves, glucagon and glucocorticoids.
5. Activate enzymes involved in glycogenesis and inactivate enzymes involved in glycogenolysis and glucose release.

The confirmation of these speculative suggestions comes from the studies on vagotomy. Vagotomy abolished most of the actions of vagal nerves as well as enhanced the actions of sympathetic system. Choline chloride administration along with glucose could counteract the effect of vagotomy.

If parasympathetic cholinergic nerves could influence the liver carbohydrate metabolism and thereby control blood sugar level and vagotomy could produce hyperglycemia on glucose loading, the cause of some diabetic conditions, especially maturity onset diabetes, could be the parasympathetic neuropathy.

Lautt (1980) also suggested such eventuality. If this speculative line of reasoning is accepted, the post-prandial hyperglycaemia of the non-obese, maturity onset diabetes could be readily controlled by preprandial administration of a cholinergic agonist such as choline chloride, thereby overcoming the dysfuctional hepatic parasympathetic action.