CHAPTER - XI

IN VITRO STUDIES ON THE INFLUENCE OF INSULIN, ACETYLCHOLINE, GLUCAGON AND ADRENALIN ON TRANSPORT OF GLUCOSE, AND GLYCOGEN LEVELS IN LIVER AND MUSCLE SLICES OF NORMAL AND PINEALECTOMISED WILD PIGEONS, *COLUMBA LIVIA*

The hormones having influence on carbohydrate metabolism of liver and peripheral organs are insulin and glucagon, the pancreatic hormones and to a certain extent adrenalin and other hormones such as thyroxine and glucocorticoid. Insulin lowers blood glucose level by promoting glucose uptake and utilization by peripheral tissues and uptake and glycogenesis by liver. Glucagon has the reverse influence and accordingly induces glycogenolysis principally in liver and thereby elevates blood glucose level. Adrenalin is also hyperglycemic in nature and induces glygenolysis in liver and muscle. Though insulin has a cardinal role in glucose metabolism of mammals, it is suggested to play only a secondary role in birds (Hazelwood, 1973). Some of the recent reports have indicated the participation of neural elements especially the parasympathetic secretions in glucose transport and metabolism (Shimazu, 1967; Mondon and Burton,1971). As the metabolic requirements vary under different conditions, the secretion and actions of the hormones are also bound to undergo quantitative and qualitative alterations. Previous studies from this laboratory had shown definite alterations in carbohydrate metabolism with reference to seasonal breeding

activities in wild pigeons (Patel, 1982). The same study also recorded distinct alterations in the tissue glycogen contents and glycemic level of pinealectomised birds from those of intact birds. Such alterations were again demonstrated to be having a seasonal influence with the result the changes observed being different during the breeding and non-breeding periods. These observations have tended to indicate the involvement of pineal in regulating carbohydrate metabolism. Pertinently, there are also a few other reports suggesting the participation of pineal in carbohydrate metabolism (Delahunty et al., 1978; Mihail and Giurgea, 1979).

Based on the findings in wild pigeons Patel (1982) had tentatively hypothesized and anti-insulinic role for pineal during the breeding season. Studies on response to infusion of glucose in terms of glycemic level carried out during both the breeding and non-breeding seasons as a sequal to the above studies have tended to strengthen the suggested anti-insulinic role of pineal (Chapter-9). Further, evaluation of glycemic levels in response to injections of glucagon and adrenalin in normal and pinealectomised birds has also indicated some subtle seasonal alterations (Chapter-10). In the wake of these observations, an in vitro study on the ability of liver and muscle slices of intact and pinealectomised wild pigeons in terms of uptake and release of glucose, and deposition and depletion of glycogen, in presence of insulin, acetylcholine glucagon and adrenalin was deemed necessary and is undertaken herein.

MATERIAL AND METHODS

Healthy adult pigeons were procured from the local animal dealer during the breeding season (March-May) and maintained in the aviary on a diet of grains and water ad libitum. After acclimating them to the laboratory conditions, 18 pigeons were divided into three groups (C, PN and PX) of six each. The PX and PN groups of birds were pinealectomised and sham pinealectomised respectively. The C group of birds represented the intact controls. After 30 days post-pineal ablation/sham operation, the birds of all three groups were sacrificed under mild anaesthesia. Pieces of liver and muscle were excised and slices weighing about 30-50 mg were cut and washed in chilled Krebs-ringer bicarbonate medium (KRB). The slices were incubated in the KRB medium (previously gased with air mixture for 15 minutes) containing 0.2 mg bovine albumin per ml. The pH was adjusted to 7.4 and incubation carried out for 90 minutes at 37° C in a shaker. The liver and muscle slices of all the three groups of pigeons were incubated in media containing :

5 ml KRB medium + glucose (3 mg/ml)

5 ml KRB medium + glucose + insulin lent (1 unit/ml)

5 ml KRB medium + glucose + acetylcholine chloride (1.5 mg/ml)

5 ml KRB medium + glucose + glucagon (0.002 mg/ml)

5 ml KRB medium + glucose + adrenalin (0.018 mg/ml)

The glucose concentration in the medium prior to and after incubation was estimated by the method of Folin and Malmros (1929). The difference in glucose concentration was taken to represent the uptake (insulin, acetylcholine) or release (glucagon, adrenalin) of glucose by the slices as the case may be. The glycogen content of the slices was estimated after incubation as per the method of Siefter et al., (1950) and the difference from the content estimated in a slice of the liver sample without incubation was taken to represent the amount of glycogen deposited (insulin, acetylcholine) or depleted (glucagon, adrenalin) as the case may be.

RESULTS

The results obtained are represented in tables 1 and 2.

GLUCOSE UPTAKE AND GLYCOGEN DEPOSITION

Definite glucose uptake by the slices of both liver and muscle was clearly evidenced by the reduction in concentration of glucose in the incubation medium in presence of both insulin and acetylcholine. Glycogen depletion was the feature in all the media containing insulin or acetylcholine or not containing either of the two. However, the presence of insulin or acetylcholine was found to reduce the depletion of glycogen. The difference in glycogen depletion occurring in presence and absence of insulin or acetylcholine was taken to represent the

resistance offered by insulin or acetylcholine to glycogenolysis and hence indirectly the glycogen deposited. Worth recalling in this context is the report of Mortimore et al. (1967) of loss of glycogen from isolated perfused rat liver and the sparing effect of added insulin on glycogen loss. Again it has been shown by Jeejeebhoy et al. (1980) that contrary to the findings of short term studies on perfused liver, insulin is necessary and is capable of maintaining glucose transport and glucogenesis on longer periods of time ($>$ 12 hrs). From this it may be presumed that whereas the initial influence of insulin on short term in vitro experiments is visible in the form of resistance to glycogen loss, the long standing experiments are necessary to assess the direct glucose uptake and glycogenetic responses to insulin in in vitro studies. From the tables, it is evident that glucose uptake in presence of insulin in C and PN group of birds was 0.925 mg/100 mg and 0.880 mg/100 mg respectively by liver and 1.014 mg/100 mg and 1.283 mg/100 mg respectively by muscle. In the PX condition, while the liver showed no glucose uptake, the muscle showed an enhanced glucose uptake (2.351 mg/100 mg). Expressed in terms of percentage glucose taken up from the medium, the C and PN bird liver slices did not show much variation while the PX bird's muscle showed a very high percentage uptake (12.62) in comparison to C and PN muscle (4.86 and 6.78 respectively) The glucose uptake by liver slice was apparently promoted better by acetylcholine as the values recorded were 1.426 mg/100 mg (5.18 %) for C and 2.447 mg/100 mg (6.66 %) for birds of PN group

TABLE-2 : *In vitro* uptake and release of glucose, and, deposition and depletion of glycogen by muscle slices in presence of insulin and adrenaline.

	Glucose uptake with Insulin		Glucose release with Adrenaline		Glycogen deposited with Insulin		Glycogen depletion with Adrenaline	
	Tissue uptake	% uptake	Glucose released from tissue	% released into the medium	Amount deposited	%	Amount depleted	%
C	1.014 ±0.14	4.86	2.706 ±0.60	11.5	.004 ±0.001	3.99	0.0052 ±0.001	4.99
PX	2.351 ±0.31	12.62	2.785 ±0.71	15.98	-	-	0.0265 ±0.008	29.02
PN	1.283 ±0.238	6.78	2.586 ±0.62	11.98	0.008 ±0.002	4.3	0.0206 ±0.009	11.07

C - Intact control , PX - Pinealectomised , PN - Sham operated.

with PX bird liver once again showing no uptake. The glycogen deposited in liver under the influence of insulin in C and PN birds was 0.0611 mg/100 mg (27.15 %) and 0.0964 mg/100 mg (18.54 %). In presence of acetylcholine, it was 0.133 mg/100 mg (7.5 %) and 0.0283 mg/100 mg (5.16 %) respectively. The liver of PX birds did not show any glycogen deposition either in presence of insulin or acetylcholine. The corresponding glycogen deposition in muscle of C, and PN birds in presence of insulin was 0.004 mg/100 mg (3.94 %) and 0.008 mg/100 mg (4.3 %) respectively with PX bird muscle too depicting no glycogen deposition.

GLUCOSE RELEASE AND GLYCOGEN DEPLETION

Both liver and muscle slices of all the three groups of birds showed glucose release as well as glycogen depletion in response to the presence glucagon as well as adrenalin. In intact birds the liver showed a release of 4.375 mg/100 mg and 4.557 mg/100 mg glucose in response to glucagon and adrenalin respectively. The corresponding percentage values being 12.66 and 11.14. Glycogen depletion shown by liver under the influence of the two hormones in intact controls was 0.026 mg/100 mg (15.9 %) and 0.020 mg/100 mg (12.25) respectively. Glucagon induced a release of 15.31 % glucose (4.507 mg/100 mg) from liver slice of PN birds and 15.32 % (4.697 mg/100 mg) from PX liver slice. The corresponding glycogen depletion was 17.8 % and 39.4 % respectively. The adrenalin induced responses were 12.79 % glucose release and

17.8 % and 39.4 % respectively. The adrenalin induced responses were 12.79 % glucose release and 13.22 % glycogen depletion from PN bird's liver and 20.14 % glucose release and 41.56 % glycogen depletion from PX bird liver. Finally, the adrenalin induced glucose release and glycogen depletion from muscle slices of C, PN and PX pigeons were 2.706 mg/100 mg of glucose (11.5 %) and 0.005 mg/100 mg glycogen (4.99 %), 2586 mg/100 mg of glucose (11.98 %) and 0.0206 mg/100 mg glycogen (11.07 %) and 2.785 mg/100 mg glucose (15.98 %) and 0.0265 mg/100 mg glycogen (29.02)%) respectively.

DISCUSSION

The results obtained in the present study which was principally carried out to substantiate some of the earlier contentions regarding altered hormonal responses/actions due to pinealectomy have proved confirmative. The most interesting aspect is the total insensitiveness of liver and muscle of pinealectomised birds to insulin or acetylcholine mediated glycogen deposition. Apart from that the liver failed to show even glucose uptake either in presence of insulin or acetylcholine, conditions under which liver of both the intact and sham operated birds did demonstrate definite glucose uptake. Whereas the insulin mediated glucose uptake by liver did not show any difference between C and PN birds, it did nevertheless show a distinct difference in favour of PN birds in

presence of acetylcholine. Apparently acetylcholine sensitivity and the glucose uptake mediated by it are stimulated more in sham operation induced surgical stress. It is also worth noting that in general the glucose uptake by liver was nearly double with acetylcholine in the medium rather than with insulin. Obviously, the parasympathetic system may concievably play an effective role in glucose transport across membranes of hepatocytes in pigeon liver which is in contrast to that of rats where insulin is an effective agent, though Mondon and Burton (1971) have shown an enhancing influence of acetylcholine on insulin induced glucose uptake and glycogen deposition of rat liver. The observations of Pilo and Patel (1978a) based on their in vitro studies of pigeon and rat liver are concurrent to the present studies. Though in the above studies the workers observed increased glucose uptake as well as glycogen deposition in presence of acetylcholine, the present study reveals the favourable influence of acetylcholine only on glucose uptake but not on glycogen deposition. Whereas the glycogen deposition expressed as percentage ranges from 18-27 % in insulin containing media, it was only about 5-7 % in acetylcholine containing media. This difference in results may be accredited to the species difference involved in the pigeons used in the two studies (domestic and wild). Nevertheless both the studies highlight the relative importance of acetylcholine in glucose uptake by the liver of birds. Since sugar transport can be mediated by flow coupled transport

(Wilbrandt, 1975) and as acetylcholine can induce alterations in membrane permeability and ionic movement (Augustensson,1950) with release of membrane bound Ca^{++} and increase in cAMP content by inhibition of phosphodiesterase (Rasmussen, 1975), the possible role of acetylcholine on hepatic uptake of glucose in birds can be reasonably guessed as was done by Pilo and Patel (1978b). The most glaring observation which is more relevant in the context of the present study is the inability of liver and muscle of pinealectomised pigeons (in the breeding season) to show the glycogenic response to insulin. The suggested anti-insulinic role of pineal in wild pigeons (Patel, 1982) and the observation of increased insulin sensitivity in pineal-ectomised pigeons (Chapter-9) are in the present context somewhat contradictory. It may be recalled that Patel (1982) had shown reduced hepatic glycogen content in PX birds during the breeding season, which supports the present observation. Another interesting and significant observation of the present study is the 2 to 3 fold increase in glucose uptake shown by muscle slices of pinealectomised pigeons in comparison to those from intact and sham pinealectomised birds, which could easily dispel the contradiction. Conceivably, the pinealectomy induced increased insulin sensitivity is directed towards enhanced peripheral utilization, a concept which has been already sounded in the earlier study (Patel, 1982). This increased peripheral utilisation can easily explain the posi-tive insulin sensitive response shown by PX birds (Chapter-9)

as well as the recorded hypoglycemia (Ramachandran et al.,1984).

The concurrent studies on the in vitro glycogenolytic and glucose efflux responses towards glucagon and adrenalin of liver and muscle of C, PN and PX birds though positive in all cases do nevertheless show certain interesting alterations. It is evident that both the hormones are capable of eliciting glucose efflux and glycogenolysis from the liver though by different molecular mechanisms (Park, 1981). This property common to both the hormones is of universal nature in the vertebrate series is borne out by the reported release of glucose from the liver slices of frogs and tadpoles even (Farrar and Frye, 1979). The glucagon and adrenalin induced glucose release though apparently similar in both C and PN bird liver slices, nevertheless was slightly more pronounced for glucagon in the liver slices of sham operated birds which was equalled by the PX bird liver too. The possibility of increased glucagon sensitivity post-surgical stress may be inferred in this context. The more significant revelation is the enhanced glucose release depicted by both liver and muscle in response to adrenalin which was approximately 66 % and 36 % respectively above the corresponding sham control levels. Obviously pinealectomy induces increased adrenalin sensitivity, which may be responsible for nullifying the increased insulin responses of the hepatic tissue of PX birds in the wake of the known antagonism between adrenalin and insulin observable not only in the

present *in vitro* studies but also in the already reported *in vivo* studies (Chapter-9). Corresponding to increased glucose release, the glycogen depletion also was slightly more in the set ups of PX birds containing glucagon or adrenalin. It appears from the results that the adrenalin induced glycogenolysis and glucose release are mediated by the insulin sensitised glucose uptake and glycogenesis in the muscle of PX birds. The most significant aspect of the present study is the evidence of altered insulin and adrenalin sensitivity of the muscle in PX pigeons. It is likely that the increased sensitivity to muscle glycolysis shown by PX birds (due to adrenalin action) may be responsible for the high glucose tolerance (Chapter-9) and insulin sensitivity shown by the pineal ablated pigeons (Chapters 9, 10). Some correlation in this respect comes from though an unrelated study of Espinal *et al.* (1983), on sensitivity to insulin of glycolysis and glycogen synthetase of isolated soleus muscle strip from exercise trained rats.

CHAPTER - I

Seasonal alterations and effect of pinealectomy on body weight and relative weights of gonads, adrenal and thyroid in normal, sham operated and pinealectomised domestic pigeons were studied. Pinealectomy resulted in gonadal regression during breeding and activation during non-breeding thereby suggesting progonadal as well as antigonadal roles of the pineal in the two seasons. The alterations induced by pinealectomy on the relative weights of gonads, adrenal and thyroid and the histological profiles are discussed in detail in the text. In the present study pinealectomy was noted to induce fall in weight of thyroid and adrenal during the breeding season and increase in weight during the non-breeding season. Since adrenal and thyroid are known to show cyclic variations with reproductive activities, the present observations on histophysiology of these two glands in the pinealectomised birds suggest either direct or indirect relation of pineal with adrenals and thyroid in conjunction with the pineal-gonad axis.

CHAPTER - II

Seasonal alterations and effect of pinealectomy on the weights of spleen, pancreas and uropygial gland of normal, sham operated and pinealectomised domestic pigeons were studied.

Pinealectomy in the breeding season resulted in decrement of spleen weight and increment in weight of the uropygial gland. The reverse set of changes were the feature in pigeons pinealectomised during the non-breeding season. Pinealectomy in either season brought about increase in weight of pancreas. Whereas the pineal-pancreas interactions are purported to be involved in the regulation of carbohydrate metabolism, the weight changes observed for spleen and uropygial gland are being discussed in terms of haemopoietic activity and as a target organ for steroid hormone respectively.

CHAPTER - III

Seasonal alterations and effect of pinealectomy on carbohydrate metabolism as revealed by tissue glycogen content and blood glucose levels of normal, sham operated and pinealectomised domestic pigeons have been studied. Pinealectomy induced increased hepatic and gonadal glycogen contents and reduced glycemic level, changes characteristics of intact birds during the non-breeding season. In contrast, pinealectomys performed during the non-breeding season induced decrement in the glycogen contents of liver and gonads and increase in blood glucose level, again changes characteristic of intact birds during the breeding season. The phase shift in carbohydrate metabolism noted to occur in response to pinealectomy is being discussed in terms of the phase shift in gonadal activation induced by pinealectomy.

CHAPTER - IV

Seasonal alterations in AA content of gonads, liver and muscle of normal, sham operated and pinealectomised domestic pigeons have been studied. Increased AA content in all the tissues during the breeding season and its depletion during the non-breeding period is indicative of faster mobilization and utilization of AA in the breeding season. Pinealectomy performed in the two seasons brought about differential set of alterations in all the three organs, and these changes are discussed in detail in the text in relation to gonadal structure and functioning.

CHAPTER - V

In order to understand the effect of annual cyclic reproductive activities and pinealectomy on lipid metabolism of the gonads, quantitative alterations in total lipids, phospholipids, cholesterol and glycerides have been assessed in the domestic pigeons, Columba livia. Seasonal alterations (in terms of breeding and non-breeding phases) of the various lipid fractions were not very drastic in the intact birds. The lowest levels were recorded during the early breeding period which tended to increase towards the late breeding phase and the levels remained moderately high during the non-breeding phase. The cholesterol content was higher during the

non-breeding season. Pinealectomy induced significantly higher levels of lipids in the breeding season and the pinealectomised birds during the non-breeding season depicted significantly reduced lipid contents in the gonads. These changes are taken to indicate the involvement of pineal in modulations of gonadal lipid metabolism in relation to the breeding cycle in domestic pigeons.

CHAPTER - VI

Changes in the total protein content and the activities of acid and alkaline phosphatases in the gonads have been evaluated in normal and pinealectomised domestic pigeons, Columba livia during the breeding and non-breeding phases. In general, increasing activity of both the phosphatases and decreasing protein content were the feature during the breeding phase. Pinealectomised birds during the breeding season tended to have increased protein content and reduced acid phosphatase activity, while pinealectomy in the non-breeding season brought about a decrease in protein content and increased activities of both the phosphatases. Involvement of phosphatases in gonadal activation and functioning during the reproductively active phases and the alterations induced by pinealectomy in relation to the progonadal and antigonadal roles of the pineal are discussed in detail in the text.

CHAPTER - VII

Ionic content (Na^+ and K^+) of the gonads during seasonal breeding activity as well as post-pinealectomy has been estimated in the domestic pigeon, Columba livia. Seasonal reproductive activity was marked by reduced Na^+ content and increased K^+ content in the gonads. Pinealectomy induced increased Na^+ content and decreased K^+ content during the breeding season and reverse changes during the non-breeding season. These changes are correlated with changing metabolic profile during normal annual reproductive cyclicity and the involvement of pineal thereof albeit indirectly.

CHAPTER - VIII

Histochemical alterations in sudanophilic lipids and neutral lipids together with the localization of steroid dehydrogenases (3β HSDH, 17β HSDH and 3 α HSDH) have been undertaken in the gonads of normal and pinealectomised domestic pigeons, Columba livia on a seasonal basis. The intact birds were characterised by decreased lipid contents and increased activity of steroid dehydrogenases during the breeding season and vice versa during the non-breeding season. Pinealectomy induced phase shift in these changes whereby pinealectomised birds depicted increased lipids with reduced activity of steroid dehydrogenases during the breeding season and vice versa during the non-breeding season. These changes are discussed

in terms of active steroidogenesis occuring in the gonads of intact birds during the breeding season and also in terms of the reported progonadal and antigonadal roles of the pineal in domestic pigeons.

CHAPTER - IX

In order to gain confirmatory evidence on an earlier observation of the possible anti-insulinic role of pineal in wild pigeons, glucose tolerance test (GTT) and insulin response test (IRT) on blood glucose levels of normal and pinealectomised wild pigeons have been undertaken on a seasonal basis *vis a vis* breeding activity. The tests have revealed increased glucose tolerance and increased insulin sensitivity in pinealectomised birds during the breeding season. In the non-breeding season both the intact and operated birds tended to show more or less similar response for both GTT and IRT. These changes are taken to confirm the anti-insulinic role of pineal of wild pigeons during the breeding season; and the possible significance of this pineal-pancreas interaction in terms of breeding adaptation is discussed.

CHAPTER - X

Since breeding and non-breeding seasons were observed to induce altered profiles of carbohydrate metabolism in both intact and pinealectomised wild pigeons, the glycemic response to single i.v. injection of glucagon and adrenalin in normal,

sham operated and pinealectomised wild pigeons have been assessed. On a comparative basis, the glucagon and adrenalin responses of intact birds were greater during the breeding season. Whereas the glucagon response was not much altered in either of the two seasons by pinealectomy, the adrenalin response was however better in the non-breeding season. The results obtained tend to indicate better glucagon and adrenalin sensitivities in intact birds during the breeding season and pinealectomy not having much influence on glucagon sensitivity while inducing some season specific alterations in adrenalin responses. These are related to the seasonal actions of pineal and are discussed in terms of possible pineal-pancreas-adrenal axis.

CHAPTER - XI

Apart from the anti-insulinic role of pineal, increased peripheral utilization of blood glucose was also suggested by the previous study in wild pigeons. In this wake, an *in vitro* study on the ability of liver and muscle slices of intact and pinealectomised wild pigeons in terms of uptake and release of glucose, and deposition and depletion of glycogen, in presence of insulin, acetyl choline, glucagon and adrenalin has been undertaken. The study has shown a total insensitiveness of liver and muscle of pinealectomised birds for glycogen deposition and insensitiveness of liver for glucose uptake and

increased adrenalin sensitivity in terms of liver and muscle for glucose release. The results obtained are discussed in detail in the text in terms of involvement of pineal in metabolic regulation associated with breeding activities.