INTRODUCTION

Role of ANS in Blood Sugar Regulation :

Brain is the principal site where diligent activities of a living system are controlled. The two divisions of Brain are central nervous system (CNS) and peripheral nervous system. CNS is the main division which apart from its myriad neurophysiological functions, also controls the metabolic and hormonal functions as a whole. Hypothalamus is the main sphere in CNS which regulates the level of glucose and other metabolites. Hypothalamus also has various nuclei with varying functions which makes it an imperious endocrine gland.

There are two major centres in Hypothalamus, Ventro-medial hypothalamus (VMH) and Lateral hypothalamic area (LHA). VMH is the "Satiety centre" which judges the blood sugar level and transmits the signals accordingly to LHA which is the "Feeding centre". VMH has glucose receptor cells in them, which in a hypoglycemic condition remains unsaturated, the message gets conveyed to LHA for proper and immediate action. Many brain sites possess specific insulin receptors which are located in areas devoid of tight capillary junctions that constitute the blood brain barrier (BBB). Certain regions devoid of BBB are medial basal hypothalamus, median eminence, and area postrem. These areas are probably related to ANS. Areas with efficient BBB cannot be reached by insulin while those devoid of them are exposed to this hormone. This show that hypothalamus could judge the level of glucose and insulin which in turn singly or in combination may conceivably influence the overall CNS regulation of feeding and blood sugar level. Historically the study of control of carbohydrate metabolism by CNS was initiated by C.Bernard (1848) who

Fig : 1. The Autonomic System :

Sympathetic	-	Red
Parasympathetic	-	Violet
Central conduction paths	-	Blue
Preganglionic fibres	-	Solid lines
Postganglionic fibres	-	Broken lines

(Adapted from : Anatomy and Physiology by Thibodeau G.A.)

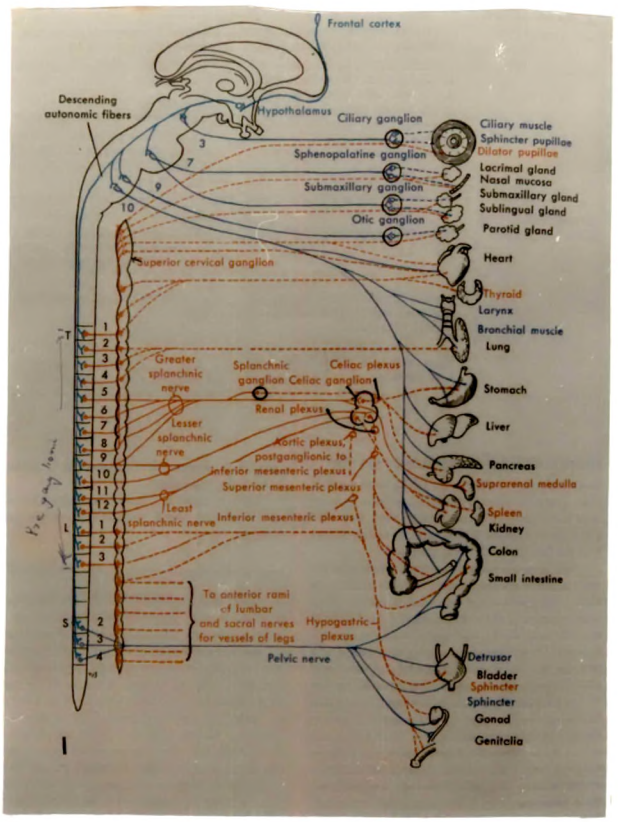
Frontal cortex
Descending autonomic fibers
Hypothalamus
Ciliary ganglion
Ciliary muscle
Sphincter pupillae
Dilator pupillae
Sphenopalatine ganglion
Lacrimal gland
Nasal mucosa
Submaxillary ganglion
Submaxillary gland
Sublingual gland
Otic ganglion
Parotid gland
Superior cervical ganglion
Heart
Thyroid
Larynx
Bronchial muscle
Lung
Greater splanchnic nerve
Splanchnic ganglion
Celiac plexus
Celiac ganglion
Stomach
Renal plexus
Liver
Lesser splanchnic nerve
Aortic plexus
postganglionic to
inferior mesenteric plexus
Pancreas
Suprarenal medulla
Superior mesenteric plexus
Least splanchnic nerve
Inferior mesenteric plexus
Spleen
Kidney
Colon
Small intestine
To anterior rami of lumbar and sacral nerves for vessels of legs
Hypogastric plexus
Pelvic nerve
Detrusor
Bladder
Sphincter
Sphincter
Gonad
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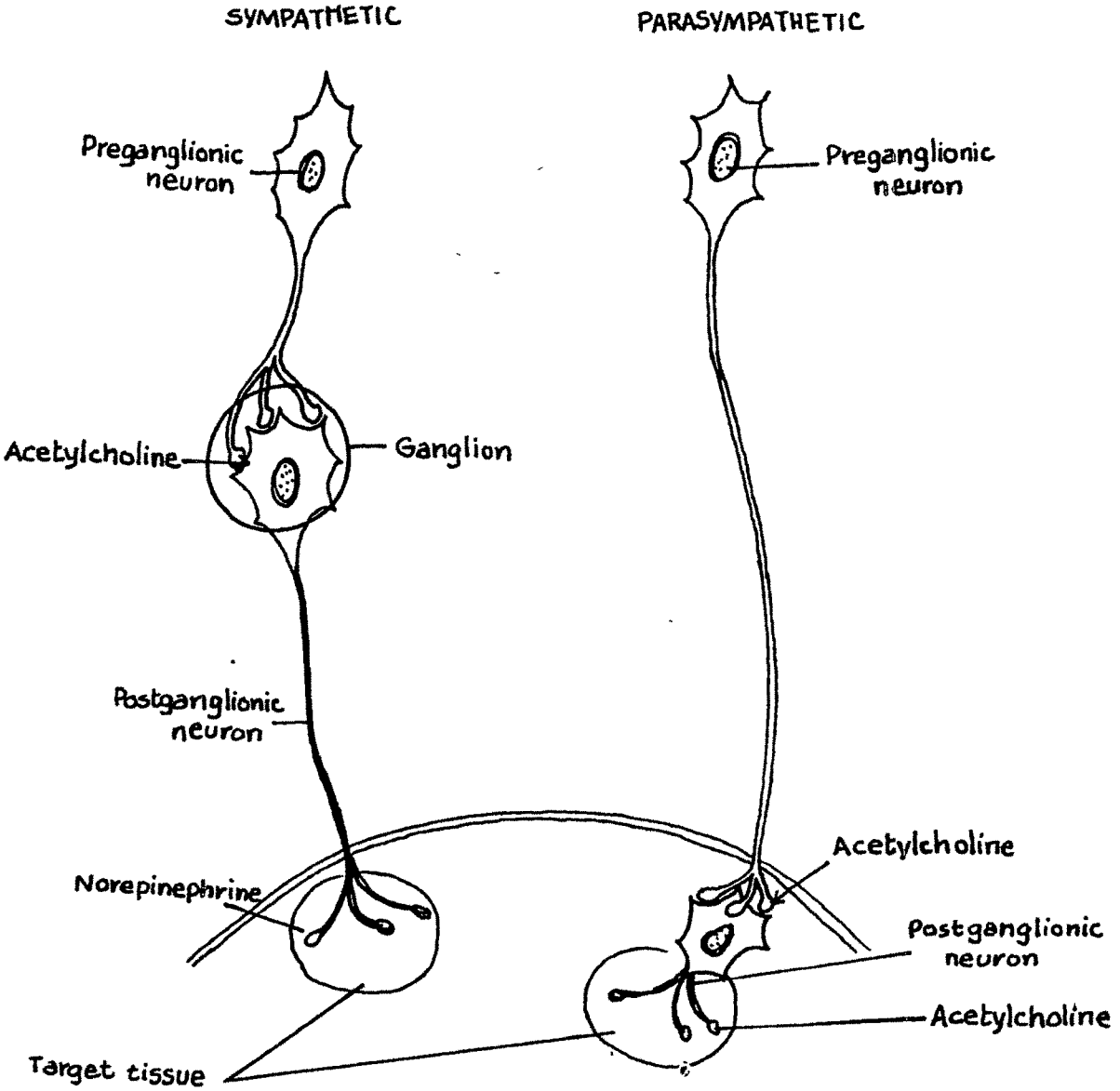
Fig : 1. A. **ACTION OF CHOLINERGIC AND ADRENERGIC FIBRES**



showed that a puncture in the floor of IVth ventricle produced hyperglycaemia. Hypothalamus regulates its activities through autonomic nervous system (ANS)(Fig 1). ANS has sympathetic nervous system (SNS) and parasympathetic nervous system (PNS)(Fig 1A) from which adrenergic and cholinergic fibres respectively innervate liver, kidney, pancreas, G-I tract, adrenal, thyroid, skin, etc. and regulates their activities independently as well as by counter balancing the effects of each other. Cholinergic fibres secrete acetyl choline while post synaptic fibres of SNS secrete norepinephrine at the nerve endings.

Responses of pre-absorbtive changes which modify metabolic functions are known as "anticipatory reflex". They prepare and sensitize the release of those factors which allow metabolism of injested substances and could be utilised. Early reflex phase of insulin or glucagon is a part of 'anticipatory reflex' and may be defined as "autonomic endocrine reflex" which is triggered by the contact of incoming nutrients. For eg. early phase of insulin secretion is brought by the administration of oral glucose, by feeding animals with carbohydrate free diet. These reflexes may have important physiological relevance which may be labelled as "priming" or "preparing" the endocrine pancreas. This priming effect is explained as follows : when an early cephalic phase of insulin secretion displays a well characterized first and second phase secretory pattern, hyperglycaemia is moderate and returns to basal values faster. In contrast when early phase insulin is absent (denervation of pancreas) subsequent insulin secretion does not retain a biphasic pattern. The first

peak of insulin secretion is missing, subsequent rise in plasma insulin is delayed and ultimately reaches values that are higher than those seen in animals showing early phase insulin secretion. Since insulin is secreted into pancreaticoduodenal veins and reaches the liver immediately; it has been proposed that early phase insulin secretion would favour hepatic glucose utilization. Such disposal of glucose would contribute to decreasing meal-induced hyperglycaemia. On the contrary, lack of this phase would bring hyperglycaemia. Thus this phase helps in minimizing the subsequent insulin requirement for maintaining the normal glucose tolerance.

Dysfunction of autonomic nervous system (ANS) manifested by a variety of cardiovascular, genitourinary and alimentary abnormalities are complications of type I and type II diabetes mellitus (DM) finally leading various pathologies. The peripheral nervous system is frequently involved in diabetes that neuropathy, diabetic nephropathy and diabetic retinopathy constitute a clinical triad.

Autonomic Neuropathy :

Involvement of autonomic nervous system is common in diabetes and usually accompanies some type of peripheral neuropathy, but may also occur as sole manifestation of neuropathy. Postural hypotension is frequently observed. The causation of diabetic peripheral nerve disease remains controversial. Diabetic polyneuropathy has been considered as an ischemic process. Disturbance in carbohydrate metabolism is an alternative cause for diabetic neuropathy. The neuropathy is generally

more prevalent in patients having severe, long standing disease. Another theory is of deficient endogenous insulin at metabolic site of the peripheral nerve. An exogenous supply of insulin facilitates oxygen and glucose consumption. Thus proper signals are transferred to the brain to elicit or evoke control on various physiological response.

Influence of ANS on Renal Metabolism :

Earlier studies have shown that ANS innervates kidney, liver and many other visceral organs (Pilo et al., 1984). Many studies also focussed on involvement of kidney in regulating the blood sugar level through ANS (Mehta and Pilo, 1985). The role of kidney in blood sugar regulation was considered through its capacity to release glucose into blood stream. Since kidney has low glycogen deposits, during starvation or stress, glucose released through glycogenolysis will last for only a short time. In such conditions kidney utilizes its gluconeogenic capacity of converting reabsorbed metabolites and amino acids into glucose.

Gluconeogenesis is an adaptive mechanism concerned with production of glucose from non-carbohydrate sources and is initiated during stress and starved conditions.

Hormones like glucocorticoids, glucagon, catecholamines, thyroxine, insulin etc. help in metabolic adaptive regulations. Gluconeogenesis is a regulated process on a "need" basis; both short term and long term regulations are encountered in liver and kidney. Metabolic regulation is carried out on a short-term basis by activation or inactivation of certain enzymes. Long-

term regulation on the other hand involves induction or repression of enzyme synthesis i.e. long-term regulations are carried out by recruiting more enzyme molecules or down-regulating the existing number of molecules. Allosterically influenced enzymes are regulated by the supply of substrates. Some key enzymes are activated or inhibited by phosphorylation / dephosphorylation mechanisms. Protein kinases are activated either by cAMP or calcium. The alterations in the concentrations of these could influence the hormonal metabolism. Although several metabolic hormones could stimulate gluconeogenesis, there could be subtle differences in the degree of activation by each hormone in a given tissue. Gluconeogenic precursors join the pathway at different points. For e.g. : lactate undergoes oxidation, glycerol joins the pathway at triosephosphate level; amino acids such as alanine and aspartate are converted into ketoacids and ultimately form oxaloacetate and join the gluconeogenic pathway. Starvations could induce release of glucagon and catecholamines. Administration of catecholamines enhances gluconeogenic activity. It is reported that avian kidney utilizes precursors such as glycerol, alanine, aspartate etc. while in mammals liver had "higher storage of these than in kidney (Watford et al., 1981; Ogata et al., 1982).

Avian and Mammalian Kidney - Certain Differences :

Morphologically and physiologically avian and mammalian kidney have lot of differences between them. Main differences seen in peculiarities are in terms of (1) arrangement of renal tissues into lobules, (2) presence of

two types of nephrons, (3) arrangement of interlobular and perilobular connecting tubules, (4) presence of renal portal system draining to cortical area, (5) presence of peculiar medullary cone draining into secondary branch of ureter. Physiological difference is seen in terms of nitrogenous waste products excreted and mechanism by which urine is concentrated. Presence of inducible cytosolic form of phosphoenol pyruvate carboxy kinase (PEPCK) equip the avian kidney in utilizing precursors such as alanine, aspartate and other amino acids and pyruvate to synthesize glucose. Due to these reasons avian kidney has a high gluconeogenic capacity which is an adaptive mechanism concerned with glucose homeostasis.

Diabetogenic : Involvement of ANS and Cisplatin :

Vagal fibres help in maintaining peristaltic movements, sphincter relaxation, dilatation of blood vessels etc. When these activities are maintained effector reactions are vastly enhanced. Denervation of vagal fibres caused a constriction of sphincter muscles, which blocked the passage of food and resulting effect was a "distended stomach". Vagotomy also stopped peristaltic movements and caused nausea in animals. While **ocarding** for an experimental model to study diabetogenic autonomic neuropathy, several reports came to the notice about an anti-cancer drug Cisplatin, which caused side effects some which were similar to that of vagotomy. It was immense interest to know whether the mechanism by which cisplatin was effecting this toxicity was through the vagal fibres directly or indirectly.

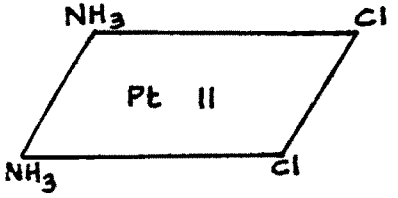
The fact that vagotomy caused hyperglycaemia (Pilo et al., 1984) indicated that autonomic neuropathy, especially that of PNS could produce diabetic condition. Vagotomy could not effectively duplicate autonomic neuropathy as vagotomy removes both afferent and efferent fibres.

Cisplatin [Cis diammine dichloro platinum-II; CDDP] is an anti neoplastic drug which has been widely used in treatment of cancer. This compound, a neutral species of platinum complex, was found by Resiet (1844), Peyrronnes (1845) and its biological application was formulated by Rosenberg (1965).

Therapeutic administration of this drug brought an effect in 24 hours time. Platinum bound to plasma proteins was primarily detected in kidney, liver, muscle and skin. Major drawbacks of this drug was its cytotoxicity and renal toxicity. Toxicity of cisplatin lead to glycosuria, proteinuria, shedding of renal enzymes into urine, severe haemmorhage, enterocolitis, hypocellularity, weight loss, decreased food intake, audiotoxicity, nausea, vomiting etc.

Cisplatin treatment brought necrosis at renal proximal tubular area. Administration of hypertonic saline decreased the lethality. Goldstein et al. (1981) carried out a dose dependent study of nephrotoxicity and observed that after two days of drug administration there was an increase in K^+ ion excretion. Glycosuria with hyperglycaemia, an increased blood urea and nitrogen (BUN), a decreased glomerular

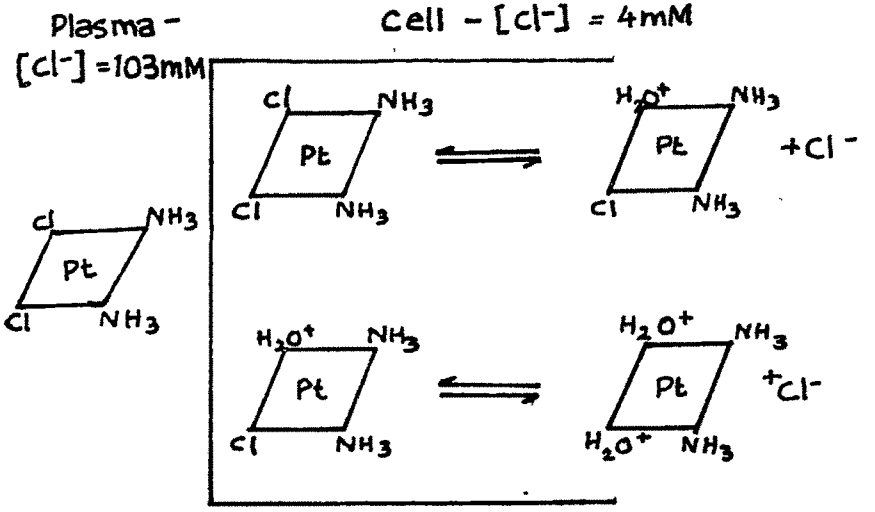
CISPLATIN



cis - PDD

STRUCTURE

Central platinum atoms and relative position of the ligands to one another. Active platinum compounds must have two sides to interact with intracellular targets in Cis-configuration (i.e., adjacent to one another rather than trans-configuration (or opposite to each other).

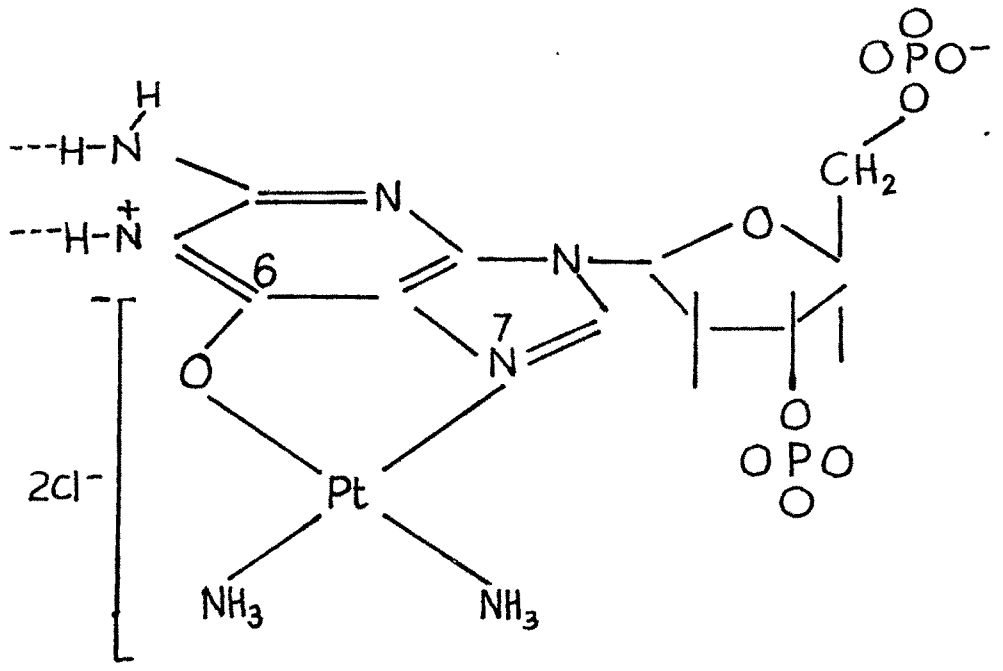


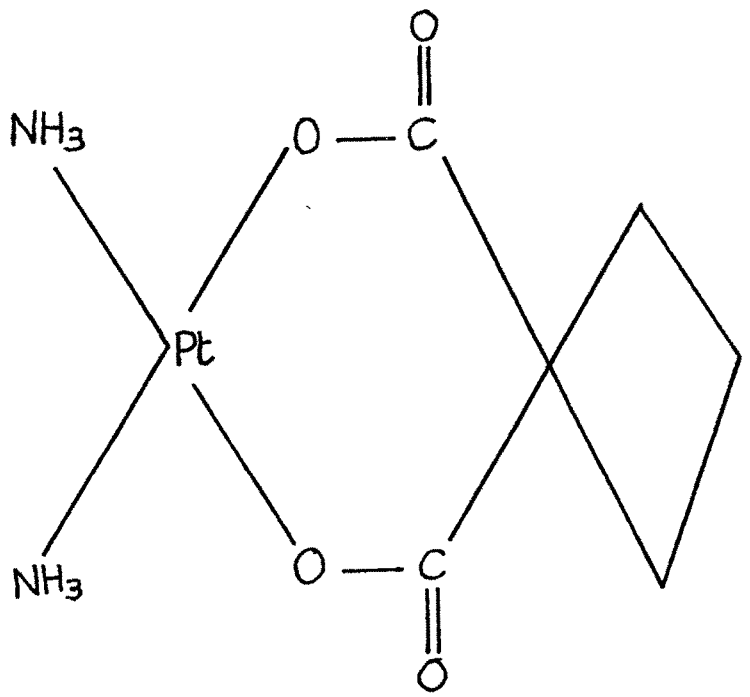
AQUATION OF SPECIES

The chloride concentration in circulating blood is high (103 mM) while it is lower inside the cell (4 mM). Thus Cl^- leaving groups are replaced by water molecules resulting in positively charged aquated platinum species which can react with nucleophilic sites within the cell in a manner analogous to that of bifunctional alkylating agents. NH_3 ligands are unlikely to participate in cis-platinum's reaction with biomacromolecules.

(as adapted from Zwelling and Kohn, 1979)

PROPOSED METHOD OF CDDP
BINDING TO GUANINE





CARBOPLATIN _ CBDCA

filtration rate (GFR) and an increased urine output unaccompanied by increased intake of fluid were also noted. Transport enzyme activities were effected which interferred with mitosis and cytokinesis. The alterations in level of enzyme activities could influence the kidney viability and function.

The role of autonomic nerves, especially parasympathetic nerves in the metabolic activities of the kidney could be partially studied with the help of studies on effect of vagotomy on various enzymes. A parallel set of enzyme studies were carried out in platinum compound treated kidney too.

Role of Calcium in Mitigationary Measures on Toxicity :

It is reported that cisplatin toxicity could be, to a certain extent masked if calcium gluconate is administered before or during cisplatin treatment. Most of the metabolic effects seen in vagotomized and cisplatin treated animals are invariably due to alteration in Ca^{++} movements in and out of cells. $CaCl_2$ administration in both the experimental groups resulted in preventing alterations in activities in many enzymes compared to control values. Impairment of calcium conductance can also bring about poor neural influence on effector cells.

Cisplatin is believed to cause efflux of calcium (Ca^{2+}) ions from mitochondria (Aggrawal and Menon, 1981) thereby arresting the cellular functionings.

A number of physiological and biochemical functions such as blood clotting, bone formation nerve excite ability, muscle contraction, intracellular communication, membrane permeability, hormone secretion etc. are particularly sensitive to even modest changes in extracellular Ca^{2+} concentration.

The operation of Na^{+} / Ca^{2+} exchange in basolateral membrances have important implications for transport activity of cells, which is depended on the coupling stimulus. In diabetes mellitus (DM) (Braten et al., 1974) and in VMH lesioned rats (Moltzetal, 1979) parodoxical glucagon responses were initiated which was thought to be operated by Ca^{2+} ions effecting the A cells. This effect suggests that an active adrenergic functioning can result in altered Ca^{2+} movements, thereby releasing glucose. With parasympathectomy and CDDP treatment such a similarity can be expected. Hence it was thought to induce exogenously calcium and study the cell functioning.

Carboplatin (Diammine cyclobutane - diacarboxylat platinum; CBDCA) was found to have subcellular mechanisms similar to that of cisplatin, although clinical spectrum of side effects were different. Carboplatin was 45 times less toxic than cisplatin. Peak levels were at 6-12 hours later to that of CDDP. CBDCA further was reported to be free from neurotoxicity or nephrotoxicity. Hence the administration would cause less derangements in metabolic pathways.

Autonomic dysfunction has been investigated by many workers in our laboratory. The study involved mainly the Avian system as a model and specifically domestic pigeons. Birds were used as a model as they have a higher glucose tolerance level.

In mammals the normal glucose tolerance is 120 mg/100 ml of blood. In birds it ranges from 220 - 260 mg/100 ml of blood. This shows they have basically a higher level of circulating glucose in them. They possess a higher amount of glucagon and catecholamines which probably is helping them to maintain such a high glucose release. Glucagon stimulates gluconeogenesis (Exton and Park, 1968; Feliu et al., 1976). Many reports are at hand suggesting the influence of glucagon on renal metabolism. Glucagon does exert regulatory actions of kidney function in mammals (Baily et al., 1980). Diabetogenic and gluconeogenic actions in mammals are brought by glucocorticoids (Ingle, 1952; Welt et al., 1952) catecholamines stimulate glucose production and simultaneously decrease lactate and pyruvate formation (Blair et al., 1973; Clark et al., 1974; Kneer et al., 1974; Pilkis et al., 1976; Rogunstad, 1976; Foster and Blair, 1978; Kneer et al., 1979; Yip and Lardy, 1981). All the above mentioned reports brought out the role of gluconeogenesis in the renal tissue.

The role of hormones in regulating avian kidney gluconeogenesis were studied by Mehta (1985).

The Theme of Present Study :

The series of investigations were designed to compare the effects and routes of the cholinergic pathway involved in renal metabolism after a vagal denervation with that of cisplatin and carboplatin administrations in mammals (Part I) and birds (Part II). The studies centered around -

1. Glycogen metabolism
2. Glycolysis
3. Glucose uptake
4. Glucose release
5. Gluconeogenesis

Effect of Vagotomy and Platinum Treatment on Renal Metabolism in Mammals :

Male rats belonging to Charles Foster strain were chosen. They were subjected to sub-diaphragmatic vagotomy, cisplatin and carboplatin administration (Chapter 1). Various biochemical pathways were analysed to evaluate the carbohydrate metabolism after vagal denervation and CDDP treatment.

Enzymes related to glycogenolysis, glycolysis and gluconeogenesis were studied (Chapter 2). The parameters studied were glycogen levels glycogen - synthetase, G-6-Pase, phosphorylase, aldolase, LDH, SDH and pyruvate carboxylase in the kidney.

The activities of transaminases and phosphatases were measured in rats (Chapter 3). Pyruvate transaminases (GPT) conversions were found to be

more in rat kidney compared to that of oxaloacetate formations (GOT).

The metabolic effects after administration of carboplatin was studied in (Chapter 4). All the above mentioned enzymes were analysed. In addition, $CaCl_2$ was given to vagally denervated and cisplatin treated rats to see the protective functioning of the system.

In mammalian tissue glycogenolysis and gluconeogenesis were partially activated, while glycolysis was enhanced. The transmination through pyruvate pathways were high. Transport activities were completely inhibited $CaCl_2$ brought a reverse in response in most of the enzymes, thereby bringing a protective effect. Since carboplatin was less toxic, the derangements in metabolic activities too were less.

All the enzyme activities were functioning at moderate levels. Hyperglycemia though persisted was at a lower index.

Effective Pathways of Renal Metabolism in Birds :

Adult domestic blue rock pigeons (Columba livia, GMELIN) were used for the study. Parameters were designed in a similar fashion to that of mammals. Birds were cervically vagotomised in one set and cisplatin was administered to the second set. The third set received carboplatin.

Enzyme parameters chosen for biochemical pathway remained similar as explained for mammals. General carbohydrate metabolism revealed

(Chapter 5) decrease in glycogenolysis, increased glycolysis and increased gluconeogenesis. Non-specific phosphatases and transaminases (Chapter 6) showed that oxaloacetic transaminase conversions (GOT) were more than pyruvate (GPT) conversions.

Carboplatin administration did not evoke an adversity totally. As seen in mammals, though hyperglycemia was observed, the glycemic level was much lower compared to that of vagotomy and CDDP treatments. $CaCl_2$ administration to a certain extent was effectively masking the adverse side effects evoked by vagotomy and cisplatin treatment.

CHAPTER - 1

MATERIALS AND METHODS

SECTION A : Surgical Methods and Various Treatments

SECTION B : Biochemical Analysis

PART-I (Mammals) : (Chapter 2,3,4)

Experimental Protocol :

Male albino rats (Rattus Norvegicus Albinus) of the Charles Foster strain weighing between 250-300 gms were used for the study. The animals were acclimatized for three weeks under laboratory conditions and were handled regularly before the experiments. They were divided into ten groups, each group consisting of 6 animals.

Group - I : Vagotomized (VgX) :

The animals of the group were subjected to bilateral sub-diaphragmatic vagotomy according to the surgical method of Snowdon and Epstein (1970). The animals were anaesthetized with ether, a 3 cm midventral incision was made directly posterior to the Xiphosternum and a piece of vagus from both sides of the oesophagus was snipped off. The animals were considered to be vagotomized only after the observation that at 72 hours the stomach was distended with partially digested food. Appropriate postoperational care was given.

Group - II : Sham Operated :

Controls were appropriately sham-operated with the vagi being only lifted at the sub-diaphragmatic level and then allowed to drop back intact in its normal position.

Group - III : Cisplatin Administration :

The animals were injected with cisplatin [cis-diammine-dichloroplatinum (II) or CDDP, Sigma Chemical Co., USA]. The drug in powdered form was dissolved in 0.9% physiological saline (7 mg/10 ml) by gentle warming. It was established early by the NCI screening test (Wolpert - DeFilippes, 1979) that the drug is most effective when administered either as an intraperitoneal (ip) or intravenous (iv) injection. Subcutaneous and intramuscular injections were less effective, and oral administration was not effective at all. The therapeutic dose is 7 mg kg^{-1} body weight which is less than L D_{10} (Rosenberg, 1984). Hence, the rats received a single ip dose of 7 mg kg^{-1} body weight.

Group - IV : Saline Treated :

Control animals received 0.9% sodium chloride.

Group - V : Vagotomized + $CaCl_2$

Subdiaphragmatic vagotomized rats received 1.3% calcium chloride injections (ip). Calcium was given (1 ml) twice daily (morning and evening) at the same time each day until the day of sacrificing, i.e. 72 hours.

Group VI : Sham operated + $CaCl_2$

Sham operated rats were given $CaCl_2$ similarly.

Group - VII : Cisplatin + $CaCl_2$

Cisplatin treated rats received similar dose of $CaCl_2$ (1 ml) twice daily, until the day of sacrificing.

Group - VIII : Control + $CaCl_2$

Saline treated rats received similar dose of $CaCl_2$.

Time (in h) of calcium chloride : ($CaCl_2$ injections)

0 h, 12 h, 24 h, 36 h, 48 h and 60 h.

Group - IX : Carboplatin Treatment, (CBDCA) :

Rats received a single dose of carboplatin (Diamminecyclo butane - dicarboxylatoplatinum II - CBDCA; Sigma Chemicals CO., USA). The drug in powdered form was dissolved in 5% sucrose.

Group - X : Sucrose Treatment :

Controls were treated with 5% sucrose.

PART - II (Birds) : (Chapter 5,6,7)

Experimental Protocol :

Adult domestic pigeons (Columba livia, Gmelin) weighing between 250-300 gms were chosen for the present study. Birds were procurred from a local dealer and were acclimated to laboratory conditions for about 3 weeks. They were divided into 10 groups consisting of 6 birds in each.

Group - I : Vagotomized :

The animals of the group were subjected to bilateral cervical vagotomy. An incision of 3 cm was made on the dorsal side at the cervical region, the piece of vagus adjacent to jugular veins were traced and snipped off from both sides. Postoperational care was given.

Group - II : Sham operation :

Controls were sham operated, with the vagi being lifted at the cervical level and then allowed to drop back intact in its normal position.

Group - III : Cisplatin Treatment :

Cisplatin was administered (ip) at a dose of 5 mg/kg body weight in 0.85% saline (avian saline).

Group - IV : Saline Treatment :

Birds were treated with 0.85% saline and served as controls.

Group - V : Vagotomized + $CaCl_2$:

Birds subjected to cervical vagotomy received 1.3% $CaCl_2$ 1 ml twice daily as described earlier.

Group - VI : Sham + $CaCl_2$:

Sham operated birds received $CaCl_2$ in a similar way.

Group - VII : Cisplatin + $CaCl_2$:

Cisplatin treated birds received $CaCl_2$ (1 ml) twice daily.

Group - VIII : Control + $CaCl_2$:

Saline treated birds received $CaCl_2$ 1 ml twice daily.

Group - IX : Carboplatin Treatment :

Birds received carboplatin at a dose of 50 mg kg^{-1} body weight in 5% sucrose.

Group - X : Sucrose Treatment :

Sucrose (5%) was administered to birds and served as controls.

All the groups were pairfed, water was provided ad libitum. After appropriate treatment to each group the animals were starved overnight. They were sacrificed by exsanguination under mild anaesthesia. Kidneys from both sides were excised, weighed on an electronic Mettler Balance Known weight of tissue was transferred to 30% KOH for estimating glycogen content, rest of the tissue was homogenized in various buffers for enzyme assays - described in Section B.

Preparation of Drugs :

1) Cisplatin was dissolved in respective saline by slightly warming it (0.85% for birds, 0.9% mammals) (Sigma Chemicals Co., St.Louis, USA).

2) Carboplatin was dissolved in sucrose (Sigma Chemicals Co., St.Louis, USA).

Indebtess to Prof.S.K.Aggarwal, Michigan State University, USA for providing the drug.

SECTION - B : BIOCHEMICAL ANALYSIS

Analytical Methods :

1. Protein :

The protein content was analysed using 0.1 ml of aliquot of enzymes which is used for analysis. The medium contained 2% Na_2CO_3 in 0.1 N NaOH, 0.5% $CuSO_4$ $5H_2O$ in 1% sodium tartarate. The colour developed on addition of Folin ciocalteus reagent and was measured at 660 μ on Klett Summerson Colorimeter. Bovine Serum Albumin was used as standard and units expressed as gram % age of wet tissue / 30 mts. The method followed was of Lowry et al. (1951).

2. Glycogen :

Roughly about 100 mg tissue (kidney) was used for estimation of glycogen, employing Anthrone method described by Seifter et al. (1950). The tissue was digested in hot KOH and glycogen was precipitated in alcohol. The precipitate was dissolved in known volume of water and was treated with Anthrone in sulphuric acid. The optical density was measured at 620 μ on (Spectronic Photo Colorimeter; Spec-20) (Spectrophotometer 106 MKII) and expressed as mg glycogen / 100 mg tissue.

Enzymes :

Enzymes lie on the borderline where physical and biological sciences meet. Life depends on a complex network of chemical reactions brought about by specific enzymes. They act as catalyst and fascinate fields of scientific investigation.

The first clear recognition of an enzyme was made by Payen and Persoz (1833). Later in 1898 Duclaux proposed the usage of suffix - 'ase' to be attached to the root indicating nature of substance on which enzymes act, thus providing a base for nomenclature of enzymes. In 1878 W.Kunne suggested the name 'enzyme' - which states that something occurs that exerts this or that activity. The relation between enzyme and substrate was found by Emil Fischer - who gave the famous lock and key analogy for this interaction. In the present century enzymology has reached a very exciting stage, where a rich harvest is beginning to be reaped from the large amount of patient investigation that has been done over many years, and from the convergence of many different lines of study.

However, enormous amount of work remains to be done in the field. It is probably true to say that enzymology has to be probed further scientifically.

Enzyme Assays :

1. Glycogen Synthetase (E.C. 2.4.1.11) :

UDPG 1,4-glucan glucosyl transferase :

Enzyme was assayed by using 0.1 ml of aliquot according to method of Lefoir and Goldenberg (1962). The assay medium contained 0.75 u glycine buffer pH 8.5, EDTA 0.25 u, 0.05 M glucose-6-phosphate sodium salt, UDPG 25 moles/ml, glycogen 40 mg/ml, KCl 0.4 m; 0.01 m PEP, $MgSO_4$ 0.1 m, pyruvate kinase, 0.1% DNPH. Reaction is stopped using 10 N NaOH and 95% ethanol. Activity was measured by analysing the amount of

UDP formed from UDPG in presence of glycogen and glucose-6-phosphate. UDP estimation was carried out using a preparation of pyruvate kinase which catalyses phosphate from phosphopyruvate. Pyruvate liberated was estimated colorimetrically at 520 μ on Spec-20. Enzyme activity was expressed as μ moles of UDP formed / mg protein / 10 minutes.

2. Glucose-6-phosphatase (E.C. 3.1.3.9)

D-glucose-6-phosphatase phospho hydrolase

This method was described by Harper (1963) using disodium salt of glucose-6-phosphate. Phosphate released was estimated according to method of Fiske and Subbarow (1925) and the enzyme activity was expressed as μ moles of phosphate released / mg protein / 15 mts.

The assay medium contained 1 μ citrate buffer , pH 6.5, G-6-P-sodium salt (0.08 M). Reaction was stopped using TCA 10%. After centrifugation phosphate released was measured using 1 ml of the filtrate. Acidic ammonium molybdate 0.002 N and alpha naphtho sulphonic acid (ANSA) was used as reducing agent. The colour developed was measured in Klett Summerson Colorimeter at 660 μ wavelength.

3. Phosphorylase (E.C. 2.4.1.1)

1,4-alpha-D-glucan : orthophosphate α D-glucosyl transferase

The method is modified version of method of Cori et al. (1943) by Cahill et al. (1957). Assay medium contained 0.1 μ citrate buffer pH 5.9, potassium flouride 0.8% and g-1-p dipotassium salt as substrate.

Reaction was stopped by TCA and centrifuged to collect the supernatent. Phosphorus liberated was estimated using 1 ml filtrate, into which acidic ammonium molybdate was added, alpha naphtho sulphonic acid was used as reducing agent. This method was demonstrated by Fiske and Subbarow (1925). The colour developed was measured at 660 μ on Klett Summerson Colorimeter and was expressed as μ moles of phosphorous released / mg protein / 10 mts.

4. Aldolase (E.C. 4.1.2.13)

D-fructose-1,6-biphosphate, D-glyceraldehyde 3 phosphate lyase

Enzyme assayed by the method of Bergmeyer as described in methods of enzymatic analysis (1963). Activity is measured at 550 μ in Klett Summerson Colorimeter by the amount of fructose-6-Diphosphate (FDP) formed. The triose phosphates formed from fructose-6-diphosphate from action of aldolase are trapped with hydracine. After deproteinization with TCA they are hydrolysed by NaOH. The free trioses are treated with 2,4-dinitrophenyl hydrazine, both the compounds dissolve in alkali. The activity is expressed as μ moles of FDP cleaved / mg protein / 15 mts. Assay medium consisted of collidine buffer pH 7.4, 0.1 M FDP disodium salt as substrate, 0.56 M hydrazine and 0.1% DNPH. Reaction was stopped using TCA and 1 ml of filtrate was used to measure the amount of FDP formed.

Transport Enzymes (Assay of non-specific phosphatases)

5. Acid phosphatase (E.C. 3.1.3.2)

Orthophosphoric monoester phosphohydrolase (Acid optimum)

This method was according to Bergmeyer (1963) from methods of Enzymatic analysis. Assay medium consisted of 0.05 M citrate buffer pH 4.8 and 0.005 M paranitrophenol as substrate. Reaction was stopped using 0.1 N NaOH. The activity was measured at 410 μ on the Spectrophotometer MK 10611, and was expressed as μ moles of paranitrophenol released / mg protein / 30 mts.

6. Alkaline phosphatase (E.C. 3.1.3.1)

Othophosphoric monoester phosphohydrolase (Alkaline optimum)

The method followed was of Bergmeyer (1963) from methods of enzymatic analysis. Assay system consisted of glycine buffer 0.05 M, pH 10.5, 0.005 M paranitrophenol as substrate. Reaction was stopped using 0.02 N NaOH. The activity was measured at 410 μ on Spectrophotometer MK 10611 and was expressed as μ moles of paranitrophenol released / mg protein / 30 mts.

7. Sodium potassium ATPase (Na^+-K^+-ATPase) (E.C. 3.6.1.3)

Na^+-K^+-ATPase was assayed according to the method described by Post and Sen (1967). Using Ouabain as an inhibitor and substracting the value obtained from Mg^+-ATPase activity. The inorganic phosphate released was measured according to the method of Fiske and Subbarow (1925).

The readings were taken at 660 nm on Klett Summerson's photoelectric colorimeter and the enzyme is expressed as µg phosphorus released / mg protein / 10 minutes.

Transaminases

8. Glutamate oxaloacetic transaminase (E.C. 2.6.1.1)

Aspartate amino transferase

Method described by Bernt and Bergmeyer (1963) in methods of enzymatic analysis. The assay system consisted 0.1 M phosphate buffer pH 7.4, 0.002 M alpha-ketoglutarate as substrate and 0.1 M L-aspartate, incubated for 1 hr. Ketone reagent 0.001 M was added and kept at room temperature for 20 mins. Reaction stopped by 0.4 N NaOH. Amount of hydrazone formed by oxaloacetate was measured photometrically at the wavelength of 545 µ with an increase in oxaloacetate along with alpha ketoglutarate decrease. Resulting increase in absorbance is proportional to pyruvate or oxaloacetate that is produced. Optical density of colour developed was read in (Spec.20). Karmen units was taken from standard tables. The enzyme activity was measured and expressed as Karamen units / mg protein / minute.

9. Glutamate pyruvate transaminase (E.C. 2.6.1.2)

Alanine amino transferase

The method is described in "Methods of Enzymatic Analysis" by Bergmeyer (1963). The assay system consisted of 0.1 M phosphate buffer pH 7.4, 0.002 M alpha-ketoglutarate as substrate and 0.2 M DL-alanine.

The tubes were incubated for 30 mins, 0.001 M ketone reagent was added and kept in room temperature for 20 mins. Reaction is stopped by using 0.4 N NaOH. Amount of hydrazone formed by pyruvate in the enzyme reactions was measured photometrically at the wavelength of 545 μ, with increase in pyruvate along with alpha-ketoglutarate decrease. Resulting increase in absorbance is proportional to pyruvate that is produced. Optical density of the colour developed was read in Spectrophotometer MK 10611. Karmen units was taken from standard tables. Enzyme activity was measured as karmen units / mg protein / minute.

Dehydrogenases

10. Lactate dehydrogenase (E.C. 1.1.1.27)

L-lactate NAD^+ oxidoreductase

The assay is based on colorimetric method of King (1971) as described by Varley (1975) using sodium lactate as substrate and NAD^+ as cofactor. Optical density was read at 440 u on Spectrophotometer MK 10611 and the enzyme activity expressed as u moles of lactate oxidized / mg protein / 15 mins. The assay system consisted of 0.1 M glycine buffer pH 10.0, 0.02 M NAD as substrate and 2-4 DNPH as colouring reagent. The enzyme was incubated in a waterbath for 30 mins. The reaction was stopped using 0.4 N NaOH. The colour developed was read at 440 μ.

11. Succinic dehydrogenase (E.C. 1.3.99.1)

Succinate (acceptor) oxidoreductase

The activity was measured according to method of M.Nachlas et al. (1959) using INT [2-(4-iodophenyl)3-4 nitrophenyl 5 phenyl tetrazolium

chloride] as electron acceptor. The assay system consisted phosphate buffer pH 7.4, INT as substrate which is dissolved in dimethyl formamide (DMF). The enzyme is incubated at 37°C for 1 hr. Reaction is stopped using 95% acetone. The colour developed is due to the amount of formazan produced. Specific activity was measured at 540 μ on Klett Summerson colorimeter and expressed as ug formazan formed / mg protein / 60 minutes.

12. Pyruvate carboxylase (E.C. 6.4.1.1)
Pyruvate carbondioxide ligase (ADP forming)

Method was described in Methods in Enzymology Vol.1, originally published in Jr. of Biol. Chem. Vol.241, by M.C.Scrutton, M.R.Olmsted, M.F.Utter (1965). The medium consists of Tris-HCl 0.5 M (pH 7.8). Tris pyruvate 0.5 M, disodium ATP 0.05 M pH 7.0, neutralized with Tris, Acetyl CoA 0.2 mM (from Sigma Chemicals Co., USA), MDH 10 mg/ml (Sigma Chemicals Co., USA), NAD 4.5 mM (freshly prepared). The enzyme was prepared in 1 M sucrose containing 0.1 M phosphate and 0.06 M ammonium sulphate. Control tube was run without Acyl CoA at 20° - 25°C. The assay was carried out in first 5 mins. and the activity measured spectrophotometrically at 340 μ and expressed as units/mg protein.

Statistical Analysis :

Data was analysed by student 't' test. When the analysis gave a significant F, differences were evaluated using Fisher's LSD tabels

(1950). A P value $\leq$ 0.05 was considered statistically significant.

Table 1 : Abbreviations and units of parameters used in the present study :

Parameters	Abbreviations	Units
Glycogen		mg/100 mg wet tissue
Protein	-	mg/100 mg wet tissue
Glycogen synthetase	-	umoles UDP formed/mg protein/10 minutes.
Phosphorylase	-	ug phosphorous released / mg protein/10 minutes.
Glucose-6-phosphatase	G-6-Pase	u moles phosphate released/mg protein/minute.
Lactate dehydrogenase	LDH	umoles lactate oxidised / mg protein / 15 minutes.
Aldolase	-	u moles FDP cleaved / ug protein / 15 minutes.
Succinic dehydrogenase	SDH	ug formazan formod / mg protein / 60 minutes.
Pyruvate carboxylase	-	units/mg protein
Sodium-potassium adenosine triphosphatase	Na^+-K^+-ATPase	ug phosphorous released/ mg protein/10 minutes.
Alkaline phosphatase	Alkaline Pase	u moles p-nitrophenol released/mg protein/30 mins
Acid phosphatase	Acid Pase	umoles p-nitrophenol released/mg protein/30 mins.
Glutamate oxaloacetate transaminase	GOT	Karmen units/mg protein/ minute
Glutamate pyruvate transaminase	GPT	Karmen units/mg protein/ minute.