Material and Methods

Adult male albino rats (*Rattus norvegicus albinus*) of Charles Foster strain weighing between 150-170gms were used for all the experiments in the present study. The rats were maintained in standard laboratory conditions with *ad libitum* food and water and 12hrs:12hrs of dark:light photoperiod regime. Before using the rats for the experiments, they were acclimatized to human handling for 2-3 weeks. Later, rats were divided in twelve groups with ten animals in each group for various treatments. After respective treatments, biochemical analysis, hormone assay and glucose tolerance test were executed.

SECTION A: Surgical methods and drug treatments
SECTION B: Biochemical analysis
SECTION C: Hormone assay
SECTION D: Glucose tolerance test (GTT)

SECTION A

I Vagotomy (VGX)

Animals of this group were operated for total subdiaphragmatic vagotomy according to the surgical methods of Snowdon and Epstein (1970). Ether anaesthetized animals were given a 3cm midventral incision posterior to the xiphisternum, after removing fur from the abdominal region on the ventral side. Liver and intestine were gently displaced to provide access to the lower oesophagus. Stomach and oesophagus were drawn out through the incision and vagus nerves present on the either side of the oesophagus were located. With
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utmost care a 3mm piece from both the vagus nerves were sectioned. Caution was taken for not damaging liver, stomach or oesophagus. After sectioning the nerves, stomach and oesophagus were replaced back to the original place and incision was sutured. All the animals recovered consciousness within 30 minutes. Following surgery postoperative care was taken. In these rats, parasympathetic component of autonomic nervous system is suppressed whereas sympathetic component and adrenal gland remain intact.

II Sham Vagotomy (VGS)
In the animals of this group, same surgical procedure was followed as in the experimental animals but vagi were only lifted at the subdiaphragmatic level and allowed to drop back intact in their original positions without any damage. So, in these rats parasympathetic component is functional.

III Adrenalectomy (ADX)
Animals of this group were surgically operated for bilateral adrenalectomy according to the method of Bern and Nandi (1970). In ether anaesthetized animals 2cm dorsolateral incisions were made on both the sides posterior to the rib cage, the skin was laterally retracted to the right and with a pair of fine pointed forceps, a small hole was made through the thin muscle wall just anterior to the upper pole of the kidney. Caution was taken not to pierce any of the large vascular organs in this region, such as liver and kidney. The adrenal, recognized by its orange or reddish yellow colour, was surgically removed and the incision was closed by suturing. 0.9% saline was given to the animals after operation. These rats were thus incapable of releasing any of adrenal hormones.

IV Sham Adrenalectomy (ADS)
In sham operated animals incisions were made as in the operated animals. The adrenals were lifted and allowed to drop in their original positions without damaging any tissue. So, in this group rats, adrenals were intact and there is a normal supply of adrenal hormones for the maintenance of metabolic homeostasis.

V Vagotomy + Adrenalectomy (VGX + ADX)
Vagotomy and adrenalectomy were performed simultaneously/together one after another, by employing the techniques as in group I and III. Both the vagi and adrenals were sectioned
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with care. Thus, these rats were devoid of both parasympathetic component and adrenal hormones, while sympathetic nervous system is unaffected. After operation, animals were supplied with 0.9% saline.

VI Sham Vagotomy + Sham Adrenalectomy (VGS + ADS)
In these animals incisions for sham vagotomy and sham adrenalectomy were given same as in the experimental animals of group V. Adrenals and vagi were only lifted up and allowed to drop to their original positions. There is no effect on the regulatory function of vagus and adrenal glands.

VII Chemical Sympathectomy (CSX)
This group of rats were injected with Guanethidine sulphate (Sigma Chemical Co., U.S.A.). Intraperitoneal injections of guanethidine sulphate (50mg/kg body weight; pH:10.2) was given daily for four weeks/28 days. Drug dissolved in a physiological saline as vehicle was injected regularly at 10.00am. Guanethidine destroys the adrenergic neurons and suppresses the sympathetic system completely at pH:10.2. It has no effect on parasympathetic component and adrenal glands.

VIII Control Chemical Sympathectomy (CSS)
Animals of this group served as controls for chemical sympathectomy and were injected with 0.9% saline (i/p) daily for four weeks at 10.00am.

IX Chemical Sympathectomy + Adrenalectomy (CSX + ADX)
This group of rats were injected intraperitoneally with guanethidine sulphate (50mg/kg body weight; daily for 28 days). On 26th day along with injections of guanethidine sulphate surgically bilateral adrenalectomy was also performed. Both the adrenals were removed with care. In these rats, sympathetic component of autonomic nervous system and adrenal hormone secretion is suppressed leaving only parasympathetic component intact. After operating for adrenalectomy, animals were supplemented with 0.9% saline. After operation, these rats were maintained in a stress free environment.

X Control Chemical Sympathectomy + Sham Adrenalectomy (CSS + ADS)
Rats of this group were injected with 0.9% saline for four weeks and on 26th sham operation
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for adrenalectomy was performed. Operation was done same as in the experimental animals and adrenals were kept intact.

XI Chemical Sympathectomy + Vagotomy (CSX + VGX)
This group rats were injected intraperitoneally with guanethidine sulphate (50mg/kg body weight) for four weeks (28 days). In the same animals, 48hrs prior to the completion of the drug treatment duration (on 26th day), surgical subdiaphragmatic vagotomy was performed. Both the vagi were sectioned. All the rats regained conscious within 30 minutes after the operation. Guanethidine injections continued on 27th and 28th day after the operation also. Post operation care were taken.

XII Control Chemical Sympathectomy + Sham Vagotomy (CSS + VGS)
Rats were injected with 0.9% saline (ip) for four weeks and 48hrs prior to the completion of saline injections (i.e. on 26th day) sham operation for vagotomy was performed. Incisions were made same as in sham vagotomized rats of group II. And, both the vagus nerves were lifted and replaced back in the original position without sectioning. After operation saline injections continued till 28th day.

SECTION B

Analytical Methods

Blood glucose
Blood glucose level was estimated by GOD/POD method described by Trinder (1969) using glucose-oxidase kit. To 0.02ml serum 1.5ml working glucose reagent and 1.5ml distilled water is added. This mixture was incubated for 15 minutes. The optical density of the coloured solution was read at 510nm against distilled water and was expressed as mg glucose/100ml of blood.

Glycogen
For the estimation of tissue glycogen content method of Seifter et al. (1950) was employed using anthrone as colour reagent. A preweighed piece of liver was dissolved in 30% KOH and glycogen was precipitated with 95% alcohol. Precipitates were dissolved in water and treated with anthrone reagent. The resultant colour is obtained by keeping the tubes for 4 minutes in boiling water-bath and then cooling it immediately to room temperature. The glycostatic
content in the hepatic tissue is measured spectrophotometrically at 620 nm and expressed as mg glycogen/100mg wet tissue.

**Protein**
The total protein content of the liver was assayed by the method of Lowry et al. (1951) using Bovine serum albumin (BSA) as standard and Folin phenol as colour reagent. To the homogenate 5ml of reagent C (5ml reagent A + 0.1ml reagent B) was added and after 10 minutes add 0.5ml Folin phenol was added. Following 30 minutes the optical density of the colour obtained was read at 620 nm as mg protein/100mg wet tissue.

**Lipid**
Total lipid was extracted employing the method of Folch et al. (1957). A preweighed piece of liver was kept in an oven at 60°c for complete drying. The tissue was weighed regularly till constant weight is achieved. The dried liver was then powdered and a mixture of chloroform:methanol (2:1 ratio; v/v) was added. These test-tubes were kept overnight undisturbed. After centrifuging the supernatant is taken in known weight of test-tube. This process is repeated twice or thrice. Collected supernatant is allowed to evaporate. The extracted lipid was measured gravimetrically and expressed as mg/100mg non fat dry tissue (NFDT).

**Enzymes**

*Glucose-6-phosphatase (G-6-Pase)*

*E.C. 3.1.3.9 D Glucose-6-phosphate Phosphohydrolase*

Activity of G-6-Pase was assayed by the method of Harper (1963) using 0.1U citrate buffer (pH:6.5) and 0.08M disodium salt of glucose-6-phosphate as the substrate. The reaction was stopped using 10% TCA. The mixture was centrifuged and the supernatant was used to estimate inorganic phosphate released employing the method of Fiske and Subbarow (1925). The assay medium contains 0.002N acidic ammonium molybdate solution and ANSA as reducing agent. The colour developed was measured on Spec-20 at 660 nm and the enzyme activity was expressed as μmoles of phosphate released/mg protein/15 minutes.
Material and Methods

Glycogen Synthetase
E.C. 2.4.1.11 UDPG 1-4 Glucan glycosyl transferase
Method of Leloir and Goldemberg (1962) was employed for the assay. The assay medium contains glycogen, glycine buffer (pH:8.5), G-6-P and UDPG. After incubating for 10 minutes, heated in boiling water-bath for 1 minute, PEP and Pyruvate Kinase were added. Following the incubation of 15 minutes DNPH was added. After 5 minutes 10N NaOH and ethanol were added. The enzyme activity was measured by estimating the amount of UDP formed from UDPG in presence of glycogen and G-6-P (disodium salt) by using a preparation of pyruvate kinase which catalysis the transfer of phosphate from phosphopyruvate. The pyruvate liberated was measured colorimetrically at 250 nm and the enzyme activity was expressed as \( \mu \text{moles of UDP formed/mg protein/10 minutes} \).

Glycogen Phosphorylase
E.C. 2.4.1.1 1,4 alpha-D-Glucan-orthophosphate, alpha-D-Glucosyl transferase
Phosphorylase activity was estimated by the modified version of Cori et al (1943) as followed by Cahill et al (1957) using 0.1\( \mu \) citrate buffer (pH:5.9), 0.8% potassium fluoride and dipotassium salt of G-1-P as the substrate. The reaction was stopped using 10% TCA and the mixture was centrifuged. In the supernatant inorganic phosphate produced was assayed by the method of Fiske and Subbarow (1925) using 10N \( \text{H}_2\text{SO}_4 \), 2.5% ammonium molybdate and ANSA as reducing agent. The colour developed was measured on UV spectrophotometer at 660nm and the enzyme activity was expressed as \( \mu \text{moles of phosphorous released/mg protein/10 minutes} \).

Lactate dehydrogenase (LDH)
E.C. 1.1.1.27 L-Lactate NAD\(^+\) oxidoreductase
This enzyme activity was assayed based on colorimetric method of King (1971) as described by Varley (1975). The medium comprises 0.1M glycine buffer (pH:10.0), sodium lactate was used as substrate, 0.002M NAD\(^+\) as cofactor and 2-4-dinitrophenyl phosphate (DNPH) as the colouring reagent. After incubating for 15 minutes, the reaction was stopped using 0.4N NaOH. The optical density of the colour obtained was read at 440 nm and the enzyme activity was expressed as \( \mu \text{moles lactate oxidized/mg protein/15 minutes} \).
**Material and Methods**

**Succinate Dehydrogenase (SDH)**

*E.C. 1.3.99.1 [Succinate (acceptor) oxidoreductase]*

SDH activity was estimated employing the method of M. Nachlas *et al.* (1959) using INT [2-(4-iodophenyl) 3-4 nitrophenyl 5-phenyl tetrazolium chloride] as electron acceptor, dissolved in DMF. The enzyme activity as assayed in medium comprising 0.1M sodium fumarate, 0.2M sodium succinate, 1% gelatin and 0.2% INT. After incubating for 5 minutes, homogenates prepared in 0.1M phosphate buffer (pH:7.7) and 0.8% PMS was added. Following the incubation of 15 minutes the reaction was stopped by 0.25M HCL and the amount of formazan formed was measured at 540nm on a spectrophotometer. The enzyme activity was expressed as µg formazan formed/mg protein/60 minutes.

**Acid Phosphatase**

*E.C. 3.1.3.2 Orthophosphoric monoester phosphohydrolase*

Acid phosphatase was estimated by the method of Linhardt and Walter (1963) using 0.05M citrate buffer (pH:4.8) and 0.005M PNP (paranitrophenyl phosphate) as substrate. Reaction was stopped by adding 0.1N NaOH. The activity of the enzyme was measured spectrophotometrically at 410nm and was expressed as µmoles of PNP released/mg protein/30 minutes.

**Alkaline Phosphatase**

*E.C. 3.1.3.1 Orthophosphoric monoester phosphohydrolase*

Alkaline phosphate activity was assayed by the method of Lindart and Walter (1963) using 0.05m glycine buffer (pH:10.5) and 0.005M PNP as substrate. By adding 0.02N NaOH the reaction was stopped and the optical density of the colour obtained was read at 410nm as µmoles of PNP released/mg protein/30 minutes.

**Sodium-Potassium-Activated Adenosine Triphosphatase (Na⁺-K⁺ ATPase)**

*E.C. 3.6.1.3*

The assay was based on the calorimetric method of Post and Sen (1967). The rate of inorganic phosphate released from ATP in the presence of Mg²⁺ and ouabain is subtracted from the rate of inorganic phosphate released from ATP in presence of Na⁺, K⁺ and Mg²⁺. The amount of inorganic phosphate released was measured according to the method of Fiske
and SubbaRow (1925) at 660nm on photoelectric colorimeter and the enzyme activity was
expressed as µg phosphorous released/mg protein/10 minutes.

**Aspartate Aminotransferase**  
*E.C. 2.6.1.1*

The method of Bergmeyer and Bernt (1963) was followed for the estimation of aspartate
aminotransferase activity. The assay system consisted of 0.1M phosphate buffer (pH: 7.4),
0.1M aspartate and alpha-ketoglutarate as a substrate. Following the incubation of 1hr 0.001M
ketone reagent was added and allowed to remain at room temperature for 20 minutes.
Thereafter, the reaction is stopped by 0.4N NaOH. The amount of oxaloacetate formed in the
enzymatic reaction was measured by estimating the hydrazon formed from oxaloacetate with
an increase in oxaloacetate and a concomitant decrease of alpha ketoglutarate, the resulting
increase in absorbance is proportional to oxaloacetate that is produced. Optical density of the
resultant colour was read on a spectrophotometer at 546nm and the equivalent Karman units
was taken from standard tables. The enzyme activity was expressed as Karman units/mg
protein/minute.

**Alanine Aminotransferase**  
*E.C. 2.6.1.2*

Its activity was assayed according to the method described in methods of enzymatic analysis
by Bergmeyer and Bernt (1963) using 0.1M phosphate buffer (pH:7.4), 0.002M alpha-
ketoglutarate as a substrate and 0.2M D-L-alanine. Incubating for 30 minutes 0.001M ketone
reagent was added. This mixture is kept at room temperature for 20 minutes and then the
reaction is stopped by adding 0.4N NaOH. The amount of hydrazon formed from pyruvate
in the enzymatic reaction was measured photometrically at the wavelength of 546nm on a
spectrophotometer. With the increase in pyruvate along with alpha-ketoglutarate decrease, the
resulting increase in absorbance is proportional to pyruvate that is produced. For the optical
density read spectrophotometrically, equivalent Karman units were taken from standard tables
and expressed as Karman units/mg protein/minute.
Material and Methods

Acetylcholinesterase (AChE)
E.C. 3.1.1.7

Activity of acetylcholinesterase was estimated by the calorimetric method of Ellman (1961). Triton 100X was used as surfactant and acetylthiocholine iodide (AChI) as a substrate. Tissue is homogenized in phosphate buffer (pH:7.4) using triton 100X. The enzyme activity was measured on UV spectrophotometer at 412nm after adding DTNB and expressed as µmoles of acetylcholine iodide hydrolysed/mg protein/60 seconds.

In the present study Enzyme immunological test for the quantitative determination of serum insulin, triiodothyronine (T₃) and thyroxine (T₄) and thyroid stimulating hormone (TSH) was carried out using tube ELISA kit from Boehringer Mannheim, Germany. Enzyme immunoassays combine the specificity of antibodies with the sensitivity of simple spectrophotometric enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme that also possess a high turnover number. ELISA may be used for assaying antigens by either a competitive or a double antibody method and for assaying a specific antibody by an indirect method.

Insulin

Insulin concentration in the serum sample was estimated by the tube ELISA ki using phosphate buffer as incubating solution and chromogen solution as substrate. The resultant colour was read on spectrophotometer at 420nm. A calibration curve was obtained by plotting the mean absorbance against the respective concentrations of the standards. Insulin concentration (µU/ml) of the samples were obtained from the calibration curve by reading off against the respective absorbance value.

Triiodothyronine (T₃)

Enzyme immunological test for the quantitative determination of total triiodothyronine (T₃) in vitro was done by the tube ELISA kit. Conjugate buffer was used as incubating solution and chromogen solution as a substrate. Absorbance of the colour obtained was read on a spectrophotometer at 420 nm. Plot the mean absorbance against respective concentrations.
of the standards on graph paper. Draw the calibration curve through the points and obtain \( T_3 \) concentrations (ng/ml or nmol/l) of the samples from the calibration curve by reading off against the respective value.

**Thyroxine (T\(_4\))**

Estimation of serum \( T_4 \) level was carried out by ELISA kit. Mixture of buffer for incubation + thyroxine-POD conjugate was used as incubating solution and chromogen solution as substrate. Absorbance of coloured solution was measured on a spectrophotometer at 420 nm. A calibration curve was plotted by the mean absorbance against the respective concentrations of the standards. Serum \( T_4 \) concentrations (\( \mu g/dl \) or nmol/l) of the samples were calculated from the calibration curve by reading off against the respective value on Y axis.

**Thyroid Stimulating Hormone (TSH)**

Serum TSH level was estimated by ELISA kit of Boehringer Mannheim using incubating solution (Buffer for incubation + anti-TSH-POD conjugate). Chromogen solution was used as substrate. Absorbance of resultant tube content was read on a spectrophotometer at 420 nm. By plotting the mean absorbance against respective concentrations of the standards a calibration curve was obtained. Serum TSH concentration (\( \mu U/ml \)) of the samples were obtained from the calibration curve by reading off absorbance against the respective value.

**SECTION D**

**Glucose Tolerance Test (GTT)**

Glucose tolerance after performing various surgeries and drug treatments was evaluated by GTTest. From the overnight fasted rats blood was drawn by puncturing orbital sinus using heparinized capillary tube. Immediately after the first sampling, the animals were injected with glucose load (1gm/kg body weight) intraperitoneally. Blood samples were collected from orbital sinus at 15, 30, 60, 90, 120 and 150 minutes from the time of glucose load administration. The analysis of the samples were done using glucose oxidase kit at 660nm and the blood glucose level was expressed as mg glucose/100ml of blood.
Material and Methods

STATISTICAL ANALYSIS

The results are expressed as mean ± standard error of mean (SEM). The data were subjected to Student’s ‘t’ test with a 95% confidence limit.