Materials and Methods
Maintenance of animals

Male albino rats (Wistar strain) of 6-8 months old age were procured from Indian Institute of Science, Bangalore, India. The rats were maintained on commercial animal feed, Annie diet (Hindustan Lever Ltd), and water ad libitum. The animals were acclimatized to laboratory conditions for a minimum of 15 days, before experimentation.

Rats were fasted for 18 hrs before sacrifice, unless stated otherwise, with free access to water.

Chemicals

Tetraiodothyronine (Thyroxine) (gift from Sreenivas, A., IISc., Bangalore), Glucose-6-phosphatase (disodium salt), oxaloacetate, 2-oxo-glutarate, inosine triphosphate, cacodylic acid, glucose oxidase, peroxidase, o-dianisidine, all are from Sigma Chemical Company, St. Louis, U.S.A.

2,4-Dinitrophenylhydrazine, p-hydroxydiphenyl, calcium oxide, diethyldithiocarbomate, EDTA and all the chemicals which are used belongs to AnaLaR quality.

Solutions

The solutions of solids in liquid were prepared on W/V basis, and of liquids in liquids on V/V basis, unless otherwise stated.
METHODS

Treatment of animals

Induction of hyperthyroidism

Animals were given 30 μg T_{4}/100 g body wt., subcutaneously, consecutively for 5 days (Malbon et al., 1978). Animals were sacrificed on 6th day after 18 hrs fasting.

Control rats were treated similarly except that 0.2 ml of vehicle (0.9% NaCl containing 5 mM NaOH pH 8.2) was given.

In vitro treatment

The hormone T_{4} was added to the incubation medium at appropriate concentrations as indicated.

Renal gluconeogenesis in vitro

Rats were sacrificed by cervical dislocation and the kidneys were quickly removed and placed in Krebs-Henseleit (1932) medium. Slices of kidney cortex of about 0.3 mm thickness were prepared by the method of Deustch (1936). One slice from each rat which served as control, was incubated in 4 ml Krebs-Henseleit (1932) medium without any added substrates. The slices were incubated under
identical conditions along with controls but containing the substrates. The final concentration of substrate used was 10 mM. Succinate, glutamate, pyruvate and glycerol were used as substrates for studying renal gluconeogenesis. Incubation procedures and other details of glucose estimation were essentially as described by Krebs et al. (1963, 1966).

The kidney cortical slices weighing approximately 50 mg wet wt. were incubated in 4 ml Krebs-Henseleit medium in a 25 ml Erlenmeyer flask which was closed with a rubber cork carrying an inlet and outlet tubes. The flasks were gassed with carbogen (O₂:CO₂=95:5) for 2 min to replace the air inside, the inlet and outlet were closed with glass stoppers. The contents of the flasks were incubated for 1 hr in a metabolic shaking water bath at 38°C with 40-60 oscillations per minute. At the end of incubation the slices were removed and dried at 110°C to a constant weight. The medium was deproteinized with 0.1 volumes of 3 N HClO₄ and centrifuged. The glucose in the supernatant was determined by glucose oxidase method of Huggett and Nixon (1957) as adopted by Krebs et al. (1963). Results were expressed as μ moles of extra glucose produced/g dry wt/h.

Estimation of blood constituents

Rats were sacrificed by cervical dislocation and
blood was collected in heparinized tubes by cardiac puncture, and was used immediately. Plasma was separated by centrifugation and used immediately.

**Estimation of glucose**

Glucose was estimated by the method of Huggett and Nixon (1957) as adopted by Krebs et al. (1963).

To 1 ml of sample, 4 ml of glucose oxidase reagent [125 mg of glucose oxidase, 4 mg of peroxidase in 100 ml of phosphate buffer, 0.5 M, pH 7.0, and 0.5 ml 1% o-dianisidine in 95% ethanol (prepared fresh every day)] was added and incubated at 37°C for 1 hr. Standard solutions of glucose containing 10-50 µg were run simultaneously under similar conditions. The extinction was measured at 440 nm in ELICO Digital Spectrophotometer.

**Blood pyruvate estimation**

Blood pyruvate was estimated by the method of Friedeman and Haugen (Varley, 1968).

Blood was deprotenized with T.C.A. and the pyruvate in the supernatant was allowed to react with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone. The reddish compound formed by reacting phenylhydrazone with strong alkali was estimated colorimetrically.
Blood was deproteinized with 1:4 volumes of cold solution of 10% T.C.A. 3 ml of clear supernatant was mixed with 1 ml of 1% 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid and allowed to react at room temperature for 5 minutes. The phenylhydrazone formed was extracted with 3 ml of xylene. The lower aqueous layer was removed and 6 ml of 10% sodium carbonate was added to the xylene extract to extract the phenylhydrazone. 5 ml of sodium carbonate extract was transferred to 5 ml of 1.5 N sodium hydroxide and mixed. The colour developed was read at 520 μm, 5-10 minutes after adding the alkali against reagent blank.

Standard of pyruvate solution containing 10 to 50 μg of pyruvate was used to prepare a standard curve.

**Blood-lactic acid**

Blood-lactic acid was estimated by the method of Barker and Summerson (1941). The proteins were precipitated with T.C.A. and the glucose in protein free filtrate was removed by treating with copper sulphate and solid calcium oxide. Lactic acid was converted to acetaldehyde by heating with conc. H₂SO₄, the amount of which was measured by means of purple colour given by p-hydroxydiphenyl in the presence of copper.

Blood was deproteinized with 1:4 volumes of a cold solution of 10% trichloro acetic acid. An aliquot containing
20–100 μg of lactic acid was added to 1 ml of 20% copper sulphate solution in a glass stoppered centrifuge tube and made up to 10 ml with water. 1 g of powdered calcium oxide was added, stoppered and shaken vigorously. It was allowed to stand for 60 min with intermittent shaking. At the end of 60 min, centrifuged and 1 ml of clear supernatant was taken and added 0.05 ml of 4% CuSO₄·5H₂O and cooled in ice. To this ice cold solution 6 ml of conc. H₂SO₄ was added slowly mixing the contents. The tubes were kept in a boiling water for 5 minutes, removed and cooled to below 20°C. 2 drops of p-hydroxydiphenyl reagent (1.5% in 0.5% NaOH) was added and mixed immediately. The tubes were allowed to stand at room temperature for 30 min and the excess p-hydroxydiphenyl was destroyed by keeping the tubes in boiling water bath for 90 sec. and then cooled. The extinction was read at 560 μm in a digital ELICO Spectrophotometer against a reagent blank treated similarly. For standards, lithium lactate solution containing 5–25 μg of lactate were taken and treated similarly along with test solutions.

The results were expressed as mg of lactic acid per 100 ml of blood.
Blood free amino acid nitrogen estimation

Amino acid nitrogen in blood was estimated by the method of Russell (1944) as described by Varley (1975).

1 Vol. of blood was deproteinized with 4.5 ml of each 5% ZnSO₄ and 0.3 N NaOH. Mixed and centrifuged at 2000 rpm for 10 min. Suitable aliquot of this supernatant was taken for amino acid nitrogen estimation.

The sample was made alkaline with 0.1 N NaOH with phenolphthalein as indicator, mixed with 1 ml of 2% borax solution and 1 ml of 0.5% sodium β-naphthoquinone-4-sulfonate and kept in a boiling water bath for 10 min, and cooled. The final volume was made to 13 ml with water and 1 ml of acid formaldehyde reagent (3 ml of 40% formaldehyde in a litre of 0.3 N HCl) and 1 ml of 0.05 N sodium thiosulphate were added and mixed. The tubes were kept at room temperature for 15 min and extinction was read at 480 nm against reagent blank. Standards containing known concentrations of amino acids were run simultaneously under similar conditions along with unknown solution.

The results were expressed as mg of amino acid nitrogen/100 ml of blood.
Estimation of plasma free-fatty acids

Plasma free fatty acids were estimated by the method of Itaya and Ui (1965). The FFA were extracted by chloroform and complexed with cupric ions which was soluble in chloroform. The copper in the complex was allowed to react with diethyldithiocarbamate and the golden yellow coloured complex was measured in spectrophotometer.

To 6 ml of chloroform in a glass stoppered test tubes was added 1 ml of 0.5 M phosphate buffer (pH 6.5) and 0.2 ml of plasma. The tubes were shaken for 90 seconds, allowed to stand for 15 minutes and centrifuged. The chloroform layer was transferred to a stoppered tube. 3 ml of copper-triethanolamine solution (90 ml of 1 M triethanolamine + 10 ml of 1 N acetic acid + 100 ml of 6.45% Cu(NO₃)₂·3H₂O) was added, mixed and shaken for 30 min. These tubes were allowed to stand for 15 min and centrifuged. 3 ml of the chloroform layer was taken and 0.2 ml of 0.4% sodium diethyldithiocarbamate in butanol was added to the filtrate, mixed, made up to 10 ml with chloroform and extinction was read against reagent blank at 440 μm. Standards containing 0.08 μEq. - 0.4 μEq. of palmitic acid were treated similarly along with unknown.
Glucose-6-phosphatase (EC 3.1.3.9)

Glucose-6-phosphatase was estimated by the procedure of Beginski et al. (1974).

To temperature equilibrated assay mixture of 0.3 ml containing 62.5 mM sucrose, 0.25 mM EDTA, 25 mM G-6-p, 25 mM cacodylate buffer (pH 6.5), 0.1 ml of 5% homogenate of kidney cortex in 0.25 M sucrose was added and incubated at 37°C for 10 minutes. The reaction was terminated by addition of 2.0 ml of ascorbic acid - T.C.A. (2% - 10%) mixture. Mixed and centrifuged for 30 min at 3000 xg and Pi liberated was estimated.

To 1 ml of the supernatant, 0.5 ml of 1% ammonium molybdate was added, mixed and then 1 ml of arsenite-citrate (2% each) was added and the tubes were allowed to stand for 15 minutes at room temperature. The extinction was read at 770 nm in ELICO Digital Spectrophotometer against blank.

Standards containing known quantity of phosphate was run simultaneously along with unknown sample.

Controls were done for every test by adding the homogenate after the addition of ascorbic acid - T.C.A. mixture.

The results were expressed as n moles of Pi liberated/min/mg protein.
L-Alanine-2-oxoglutarate amino-transferase (EC 2.6.1.2)

The enzyme was assayed colorimetrically by the method of Reitman and Frankel (Bergmeyer and Bernt, 1974). The amount of pyruvate formed from L-alanine and 2-oxoglutarate was determined colorimetrically, by converting to hydrazone with 2,4-dinitrophenylhydrazine and treating with alkali.

A 5% homogenate of the tissue in 0.01 M pH 7.4 phosphate buffer was centrifuged at 3000 rpm for 10 min and supernatant fraction was used for assay.

1 ml of buffered substrate solution (0.1 M phosphate buffer pH 7.4, 0.2 M L-alanine, 2 mM 2-oxoglutarate) was incubated with 0.2 ml of supernatant for 30 min at 37°C. The reaction was arrested by the addition of 1 ml of 0.45 mM 2,4-dinitrophenylhydrazine and left at room temperature for 30 minutes. 10 ml of 0.4 N NaOH was added, mixed well and read the extinction against blank at 550 nm in ELICO Digital Spectrophotometer.

Controls were done for every test by adding the enzyme after the addition of 2,4-dinitrophenylhydrazine solution.

Standards containing pyruvate 10-50 µg were treated similarly.
The results were expressed as μ moles of pyruvate formed/min/100 mg protein.

*L-Aspartate; 2-oxoglutarate amino transferase (EC 2.6.1.1)*

The enzyme was assayed colorimetrically by the method of Reitman and Frankel (Bergmeyer and Bernt, 1974). The oxaloacetate formed from aspartate was decarboxylated to pyruvate which was estimated colorimetrically as hydrazone.

The method followed was same as outlined for ALT except 0.2 M aspartate was used instead of alanine.

The results were expressed in n moles of pyruvate formed/min/100 mg protein.