EXPERIMENTAL
II. EXPERIMENTAL

A. Materials

1. Reagents used for ammonium sulphate fractionation of tissue homogenate: Ammonium sulphate was obtained from Sarabhai chemicals, Baroda. Sephadex G-25 was purchased from Pharmacia Fine Chemicals Uppsala, Sweden.

2. Reagents used for enzyme assay: Reagents used for the estimation of L-amino acid activity were DL aspartic acid (B.D.H., England), \( \alpha \)-ketoglutaric acid (Fluka, Switzerland), alanine (Sisco, Laboratories, Bombay), sodium pyruvate (Loba Chemie Indoaustranol Co., Bombay), 2,4-dinitrophenyl hydrazine (Loba Chemie Indoaustranol Co., Bombay).

Disodium phosphate (B.D.H., England), 4-aminopyrine (B.D.H., England), potassium ferricyanide (Loba Chemie Indoaustranol Co., Bombay) and Phenol (B.D.H., India) were used in the estimation of alkaline phosphatase activity. Reagents used for estimation of acid phosphatase activity were citric acid (Sarabhai Chemicals, Baroda), L(+) tartaric acid (Sarabhai Chemicals, Baroda). Others were as in alkaline phosphatase.

Benzonic acid (Sarabhai Chemicals, Baroda), soluble starch (E. Merck, India), potassium iodide (B.D.H., India),
sublime iodine (E. Merck, Germany) and potassium fluoride (B.D.H., India) were used in the determination of amylase activity.

3. Reagents used for estimation of protein: Reagents used for estimation of protein were Na-K tartarate (Glaxo Laboratories), copper sulphate (Sarabhai Chemicals, Baroda), sodium tungstate (B.D.H., India), sodium molybdate (Veb Gen Pharm, Germany), Lithium sulphate (B.D.H., India), orthophosphoric acid (B.D.H., India), liquid bromine.

4. Others reagents used: Other reagents used were disodium hydrogen phosphate (Sarabhai Chemicals, Baroda). Potassium dihydrogen phosphate (Merck, India), sodium hydroxide (B.D.H., India), sodium carbonate (Glaxo Laboratories), sodium bicarbonate (B.D.H., India), hydrochloric acid (Sarabhai Chemicals, Baroda).

All glass double distilled water was used throughout.

B. Methods

1. pH measurement: pH of solution was measured either on an Elico LI-10 pH meter using Elico glass and calomel electrodes or with EC combination electrode. The pH meter was calibrated
with 0.05 M potassium hydrogen phthalate buffer, pH 4.0, at 25°C in an acidic pH range and with 0.01M sodium tetraborate buffer, pH 9.2, at 25°C in the basic pH range.

2. **Optical measurement:** Absorbance of solutions in the visible range was determined on photochem colorimeter, model G-110.

3. **Determination of protein concentration:** Protein concentration was measured by the method of Lowry et al. (97) using bovine serum albumin as standard.

  Folin-phenol reagent was prepared according to the method of Folin and Ciocalteu (98). The copper reagent was prepared by mixing 4% sodium carbobate, 2% sodium-potassium tartarate and 2% copper sulphate in the ratio of 100:1:1 in the sequence to avoid precipitation.

  To 1 ml of protein solution, 5 ml of copper reagent was added. After 10 minutes 1 ml of Folin-phenol reagent was added and the solution was kept for 30 minutes for the development of the colour. Colour intensity was read at 650 or 700 nm against a blank in which instead of protein solution, 1 ml water or buffer was taken.

  The protein concentration of the solution was then
determined from the calibration curve obtained with bovine serum albumin.

4. Assay of transaminases: The activity of transaminase was measured by the method of Reitman and Frankel (99).

a) Principle: The pyruvate produced by transamination by ALT reacts with 2,4 dinitrophenyl hydrazine (DNPH) to give a brown coloured hydrazone, which is measured in colorimeter at 510 nm. The oxaloacetate formed in the reaction with AST decarboxylates spontaneously to pyruvate which is again measured by hydrazone formation.

b) Reagents: i) 0.01 M phosphate buffer pH 7.4; ii) AST substrate (200 mM-DL-aspartic acid; 2 mM alpha ketoglutarate) - For the preparation of AST substrate 13.3 g of DL aspartic acid was dissolved in minimum amount of 1 N sodium hydroxide and the pH was adjusted to 7.4 by addition of sodium hydroxide. Now 0.146 of alpha Ketoglutaric acid was dissolved in it and the pH was again adjusted to 7.4 with 1 N sodium hydroxide. Finally the volume was made upto 500 ml with phosphate buffer. It was stored frozen at -15°C.

iii) ALT substrate (200 mM alanine; 2 mM alpha ketoglutarate) - For the preparation of ALT substrate 9 g alanine was dissolved in 90 ml water with the addition of about 2.5 ml of 1 N sodium
hydroxide to adjust the pH to 7.4. Now 0.146 g of alpha ketoglutaric acid was dissolved in it and the pH was adjusted to 7.4 with 1 N sodium hydroxide. Finally the volume was made upto 500 ml with phosphate buffer. It was stored frozen at -15°C.

iv) Stock pyruvate standard: (20 mM) - For the preparation of stock pyruvate standard 220 mg of sodium pyruvate was dissolved in 100 ml of phosphate buffer. It was stored at -15°C in 1 ml aliquots.

v) Working pyruvate standard (4 mM).

vi) 2-4-, dinitrophenyl hydrazine (1 mM) - For the preparation of 2-4-DNPH, 19.8 g of 2,4-DNPH was dissolved in 10 ml concentrated hydrochloric acid and was made upto 100 ml with water. It was stored in a brown bottle at room temperature.

vii) 0.4 N sodium hydroxide.

c) Method for Estimation of AST: To 0.5 ml of the substrate (previously warmed at 37°C for 3 minutes) was added 0.1 ml of sample, mixed gently and incubated for 60 minutes at 37°C and then 0.5 ml of DNPH solution was added with gentle mixing. After incubation for 20 minutes at room temperature, 5 ml of 0.4 N sodium hydroxide was added. This was incubated for another 10 minutes and the colour intensity was measured at
510 nm. To prepare a control, sample was added after the addition of DNPH. For the preparation of blank, water was added instead of sample. For the preparation of standard, 0.1 ml of working pyruvate standard was mixed with 0.4 ml of substrate and 0.1 ml of water and the rest is similar to test sample.

(d) Method for Estimation of ALT: It was same as for AST determination except for using ALT substrate and reducing the incubation time to 30 minutes.

(e) Calculation: The specific activity of the enzyme was calculated from the following formula:

\[
\text{Specific activity} = \frac{\text{Optical density}_{\text{test}} - \text{Optical density}_{\text{control}}}{\text{Time of incubation (hr).} \times \text{Protein concentration of sample (mg)}}
\]

The enzyme activity in serum can also be calculated in the following way.

The pyruvate formed by the sample was responsible for the difference between the optical density of test and control \((\text{OD}_T - \text{OD}_C)\).

The pyruvate in 0.1 ml of the working standard (0.4 umole) produced the difference between the optical density of
standard and blank (OD\textsubscript{S} - OD\textsubscript{B}). So the pyruvate formed in 60 minutes by AST with 0.1 ml of samples was:

\[
\frac{\text{OD}\textsubscript{T} - \text{OD}\textsubscript{G}}{\text{OD}\textsubscript{S} - \text{OD}\textsubscript{B}} \times 0.4 \text{ mole.}
\]

Thus the pyruvate formed per minute per litre of sample by AST was:

\[
\frac{\text{OD}\textsubscript{T} - \text{OD}\textsubscript{G}}{\text{OD}\textsubscript{S} - \text{OD}\textsubscript{B}} \times 0.4 \times \frac{1}{60} \times \frac{1000}{0.1} = \frac{\text{OD}\textsubscript{T} - \text{OD}\textsubscript{G}}{\text{OD}\textsubscript{S} - \text{OD}\textsubscript{B}} \times 67 \text{ umole.}
\]

Similarly for ALT, the pyruvate formed per minute per litre of sample was:

\[
\frac{\text{OD}\textsubscript{T} - \text{OD}\textsubscript{G}}{\text{OD}\textsubscript{S} - \text{OD}\textsubscript{B}} \times 0.4 \times \frac{1}{30} \times \frac{1000}{0.1} = \frac{\text{OD}\textsubscript{T} - \text{OD}\textsubscript{G}}{\text{OD}\textsubscript{X} - \text{OD}\textsubscript{B}} \times 133 \text{ umole}
\]

In both the cases, the calculated pyruvate was converted into I.U./L from the chart given by Wooton (107).

5. **Assay of alkaline phosphatase:** The activity of alkaline phosphatase was measured by the method of Kind and King (100).

(a) **Principle:** Phenol released by enzymatic hydrolysis from phenyl phosphate, under defined conditions of time, temperature and pH is estimated colorimetrically.
(b) **Reagents:**

1) **Carbonate buffer pH - 9.9.**

2) **Substrate (0.01 mole/L disodium phenyl phosphate):** for the preparation of the substrate 2.18 g of disodium phenyl phosphate was dissolved in 1 L of water. The solution was boiled quickly to kill any organisms followed by rapid cooling. It was preserved with a little chloroform at 4°C.

3) **Stock phenol standard (1 mg/ml) -** For the preparation of stock phenol standard, 1 g pure crystalline phenol was dissolved in 1 litre 0.1 mol/L hydrochloric acid. It was kept at 4°C in a brown bottle.

4) **Working phenol standard (1 mg/100 ml).**

5) **Sodium hydroxide (0.5 mole/L)**

6) **Sodium bicarbonate (0.5 mole/L)**

7) **4-amino antipyrine -** For the preparation of 4-amino-antipyrine solution, 6 g of it was dissolved in a litre of water. It was stored in a brown bottle at room temperature.

8) **Potassium ferricyanide:** For the preparation of potassium ferricyanide solution, 24 g of it was dissolved in a litre of water. It was also stored in a brown bottle at room temperature.
c) **Method:** To a mixture of 1 ml buffer and 1 ml substrate (previously warmed at 37°C for 3 minutes), 0.1 ml sample was added, mixed gently and incubated exactly for 15 minutes at 37°C and then 0.8 ml of sodium hydroxide (0.5 mol/L) was added with gentle mixing.

To it, 1.2 ml of sodium bicarbonate (0.5 mol/L) was then added followed by addition of 1 ml of 4-aminooantipyrine solution and 1 ml of potassium ferricyanide solution in sequence with proper mixing after each addition.

The reddish brown colour was compared immediately at 510 nm. To prepare the control, sample was added after the addition of sodium hydroxide. Blank was prepared by taking 1.1 ml buffer with 1 ml of water and the rest is similar to test sample. For the preparation of standard 1.1 ml of buffer was mixed with 1 ml phenol standard (1 mg/dl) and the rest is similar to test sample.

(d) **Calculations:** The specific activity of the enzyme was calculated by the formula as in the transaminases.

The enzyme activity in serum can also be calculated in the following way.

The amount of phenol present in the standard tube was 10 µg. Thus the phenol produced in 15 minutes in the test was
\[
\frac{\text{OD}_T - \text{OD}_C}{\text{OD}_S - \text{OD}_B} \times 10 \text{ mg}, \text{ Hence 100 ml of sample would liberate}
\]

\[
\frac{\text{OD}_T - \text{OD}_C}{\text{OD}_S - \text{OD}_B} \times 10 \text{ mg of phenol. Since 1 KA is the production of 1 mg of phenol in 15 minutes under the conditions of the test.}
\]

Alkaline phosphatase activity (KA/dl) \[
\frac{\text{OD}_T - \text{OD}_C}{\text{OD}_S - \text{OD}_B} \times 10
\]

6. Assay of Acid Phosphatase: The activity of acid phosphatase was determined by the method of King and Jegatheesan (101).

(a) Principle: - It is same as described in alkaline phosphatase.

(b) Reagents: All are same as in alkaline phosphatase except the buffer and the tartarate.

1) Citrate buffer (pH 4.9): For the preparation of citrate buffer, 42 g of crystalline citric acid was dissolved in water. To it 376 ml of 1 mol/L sodium hydroxide was added and made upto 1 litre with water. pH was checked and prepared with a few drops of chloroform at 4^\circ\text{C}.

ii) Tartarate solution (1 mol/L): For the preparation of tartarate solution, 15 g of tartaric acid was dissolved in
about 70 ml of water. 18.5 ml of 10 mol/L sodium hydroxide was then added and the pH was adjusted to 4.9. Finally the volume was made up to 100 ml with water. It was stored in a dropping bottle at 4°C.

c) Method:— For total acid phosphatase, 1 ml of citrate buffer was mixed with 1 ml of substrate in a test tube and placed in an incubator at 37°C for 3 minutes. Then 0.2 ml of sample was added, mixed gently and incubated for 1 hour exactly at 37°C and then 1 ml of 0.5 mol/l sodium hydroxide was added.

To it, 1 ml of 0.5 mole/L sodium bicarbonate was added followed by addition of 1 ml of 4-amino antipyrine and 1 ml of potassium ferricyanide solution in sequence with proper mixing after each addition.

The reddish brown colour was then compared immediately at 510 nm.

For the determination of the prostatic fraction (tartarate labile), a second tube was prepared exactly in the same manner except for adding a drop of tartarate solution (1 mole/L) before pipetting the serum. To prepare the control, sample was added after the addition of sodium hydroxide.

Blank was prepared by taking 1.2 ml buffer with 1 ml of water and the rest is similar to test sample. For the
preparation of standard, 1.2 ml of buffer was mixed with 1 ml of phenol standard (1 mg/dl) and the rest is similar to test sample.

d) **Calculation:** The specific activity of the enzyme was calculated by the formula as in the transaminase. The enzyme activity in the serum can also be calculated in the following way.

The amount of phenol present in the standard tube was 10 µg. Thus the phenol is the test was

\[
\frac{\text{OD}_T - \text{OD}_C}{\text{OD}_S - \text{OD}_B} \times 5 \text{ mg of phenol.}
\]

Since 1 KA unit is the production of 1 mg of phenol in 60 minutes under the conditions of the test.

\[
\text{Acid phosphatase activity (KA/dl)} = \frac{\text{OD}_T - \text{OD}_C}{\text{OD}_S - \text{OD}_B} \times 5
\]

To obtain the tartarate labile phosphate, the above formula was used to calculate the results of the two tests (one without and the other with added tartarate solution). The difference between the results represented the phosphatase which had been inactivated by tartarate.
7. **Assay of amylase:** The activity of amylase was measured by the iodometric method of Caraway and Wendell (102).

(a) **Principle:** Starch forms a blue colloidal complex with iodine in solution and the intensity of the colour is directly proportional to the concentration of the starch. The blue colour produced by the starch substrate when combined with iodine, is measured after incubation with sample and compared to a control. The decrease in colour is proportional to the amylase activity.

(b) **Reagents:**

i) **Buffered starch substrate (pH 7):** For the preparation of buffered starch substrate 13.3 g of dry anhydrous disodium hydrogen phosphate and 4.3 g benzoic acid were dissolved in 250 ml water. It was brought to boiling. A separate solution was made with 0.200 g of soluble starch in 5 ml of cold water in a beaker and was added to the boiling mixture, rinsing the beaker out with additional cold water. Boiling was continued for 1 minutes followed by cooling to room temperature and diluted to 500 ml with water finally. It was stored at 4°C.

ii) **Stock Iodine Solution (0.1 N):** To prepare stock iodine solution, 13.5 g of pure sublimed iodine was dissolved in a solution of 24 g of potassium iodide in about 100 ml water.
and the final volume was made up to 1 litre with water.

iii) **Working iodine solution (0.01 N):** For its preparation 50 g of potassium fluoride was dissolved in a little water. To it, 100 ml of stock iodine solution was added and the final volume was made up to 1 litre with water. It was stored in a brown bottle at 4°C.

c) **Method:** The sample was diluted 1 in 10 with 0.9% saline. To 1 ml of buffered starch substrate (previously warmed at 37°C for 3 minutes), 0.1 ml of the diluted sample was added, mixed gently and incubated for exactly 15 minutes at 37°C. Now 0.4 ml of working iodine solution was added, mixed well followed by addition of 8.5 ml of water with proper mixing. For the control 1 ml of buffered substrate, 8.6 ml of water and 0.4 ml of iodine were mixed in sequence.

The colours were compared immediately at 660 nm using water as a blank.

d) **Calculations:** The specific activity of the enzyme was calculated by the formula as in the transaminases.

The activity of the enzyme in serum can also be calculated in the following way. The control tube contained 0.4 mg starch. The amount of starch which had been digested was therefore:
The amylase unit is the amount of enzyme digesting 5 mg of starch in these conditions. The amount of enzyme present in 0.01 ml of the sample was:

\[
\frac{OD_C - OD_T}{OD_C - OD_B} \times 0.4 \text{ mg}
\]

8. **Extraction of protein from various tissues for enzyme assay:**

Protein was extracted from liver, kidney, skeletal muscle, heart muscle, brain and lung tissues of buffalo, goat and rabbit.

Each tissue (20 g) was first washed with ice cold water and then peripheral membrane and connective tissues were removed followed by cutting into small pieces. It was then homogenized in phosphate buffered saline in a homogenizer for 10-15 minutes. The homogenate was centrifuged at 7,000 rpm for 1 hour to remove the debris.
The supernatant was collected and was saturated with 30% ammonium sulphate.

After incubation for 2 hours, this was centrifuged at 6000 rpm for 30 minutes and clear supernatant was collected.

To remove ammonium sulphate, the supernatant was passed on Sephadex G-25 column. Sephadex G-25 column (20 x 1 cm) was packed and equilibrated with phosphate buffered saline. Void volume of the column was determined by passing 1 ml of (5 mg/ml) blue dextran. The void volume was found to be 30 ml. The column was operated at a flow rate of 40 ml/hour. The supernatant obtained from ammonium sulphate precipitation was passed on this Sephadex G-25 column and protein eluting in the void volume was collected and used to determine enzyme activity.

Before reusing, the column was washed extensively with phosphate buffered saline to remove ammonium sulphate.

Human serum and serum from buffalo, goat and rabbit were used as such for enzyme activity estimation.

C. Statistical Evaluation

(a) As the study group is large, observations in this study were statistically evaluated by using 'Z' test. The mean and
standard deviations were calculated with the help of an electron­
tronic calculator which provided the above two data directly

\[ Z = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}} \]

Where \( n_1, \bar{X}_1 \) and \( S_1 \) are respectively the number of items, mean
and standard deviation of the values of first series, \( n_2, \bar{X}_2 \)
and \( S_2 \) are respectively the number of items, mean and standard
deviation of the values of second series.

'p' value was calculated from the probability tables
by comparing the value of 'Z'.

(b) Statistical evaluation of the observations was also done
using student's 't' test.

\[ t = \frac{\bar{X}_1 - \bar{X}_2}{S \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \]

The value of \( S \) was calculated before hand by the formula:

\[ S = \sqrt{\frac{(n_1-1) S_1^2 + (n_2-1) S_2^2}{n_1 + n_2 - 2}} \]
Where \( n_1, \bar{X}_1 \) and \( S_1 \) are the number of items, mean and standard deviation of the values of first series respectively, \( n_2, \bar{X}_2 \) and \( S_2 \) are the number of items, mean and standard deviation of the values of second series respectively.

The value of 't' thus obtained was compared with the probability tables given by Fischer and Yates and values of 'p' noted. p value less than 0.05 is significant.