MATERIALS AND METHODS

During the period between March to September each year, a large number of snake bite subjects were admitted to the Burdwan Medical College and Hospital, Burdwan. These victims comprise of viper, cobra, krait and non-poisonous snake bite. All the patients were clinically examined and through laboratory investigations were done to establish the diagnosis and to evaluate the prognosis of the patient.

Experimental Design

The study was carried out on 755 snake bite patients who were admitted at Burdwan Medical College & Hospital during the year 1981-86. Different biochemical, histological and electro-microscopical studies were done. The common biochemical parameter such as Blood urea, Serum Creatinine, Serum Sodium and Potassium and the enzymes such as alanine amino transferase, Aspertate amino transferase Cholinesterase, Aldolase, Phosphohexoseisomerase and Creatine phosphokinase isomerase and lipid profiles such total lipid, Phospholipid, Triglyceride and Free fatty acid were measured in the blood and serum of these patients. These results were computed to find out any diagnostic clue to differentiate between poisonous and non-poisonous bite or if it is poisonous then victim attacked by Elapidae or Viperidae group.

The histological studies were also made in the following tissue heart, kidney, brain and liver after post mortem to see the extent of lession in the different tissues amounting death of the patients.

SEM were also done in all these tissues to have a thorough look regarding the surface ultrastructural status of the tissue following snake bite.

Selection of Patients

Patients were selected among the snake bite patients those were admitted into the Hospital within zero to 3rd day of the bite. The collection of serum were made on the date of admission i.e. '0' day and subsequently as per experimental schedule i.e. on 3rd day and 7th day.

Collection of Serum

Blood samples were collected from the snake bite patients directly into ice cold test-tube. After clotting the blood, serum was separated by centrifugation at $0^{o}C$. Biochemical investigations were carried out as early as possible on the day of collection.

Estimation of serum sodium and serum potassium (Flame photometric method)

0.1 ml serum was diluted to 10 ml with distilled water. The flame-photometer was standardised and inserted the sodium

filter to estimate the value of sodium. The test solution (diluted serum) was brought under the capillary inlet and the reading of the indicator was taken where it rests automatically. The same operations are carried out for potassium by placing the potassium filter in position and diluting the serum 50 times i.e. 0.2 ml was diluted to 10 ml with distilled water.

Calculation

The serum sodium and potassium contents were calculated from the calibration curve prepared by the stock standard.

Preparation of reagents :

1) Stock solution of sodium (100 milliequivalents per liter) : 2.9225 gm of properly dried NaCl was dissolved in water and made the volume to 500 ml with water.

2) Stock solution of Potassium (2.5 milliequivalents per litre) : 0.1865 gm of dried KCl was dissolved in 1000 ml distilled water.

3) Mixed Standard of Sodium and Potassium : 3.75 ml of stock solution of sodium and 12.0 ml of stock solution of potassium was mixed in a 250 ml volumetric flask and made up the volume with water. This mixed standard

corresponds to 150 milliequivalents of sodium and 6 milliequivalents of potassium per litre of serum which had been diluted during experiment 100 times for sodium and 50 times for potassium.

Determination of aspartate aminotransferase (GOT) (EC 2.61.1) (Reitman and Frankel, 1957) :

In two tubes marked 'Test' and 'Control' 0.5 ml of buffered substrate was taken and preheated for 5 minutes then 0.1 ml of serum was taken in the tube marked as 'Test'. Both the tubes were incubaed for exactly 60 minutes at 37°C and 0.5 ml of DNPH solution was added to each of them followed by the addition of 0.1 ml of the serum to the 'Control' tube. Mixed and waited for 20 minutes and then added 5 ml of 0.4 N NAOH to each tube. Mixed and allowed to stand for further 10 minutes at room temp. and then the readings of 'T' was taken against 'Control' at 520 mμ in a Spectronic-20 spectrophotometer. Two other test tubes marked as standard and 'Blank' were taken. In the tube marked 'Blank' 0.5 ml buffered substrate was taken while in the tube marked standard working pyruvate standard and buffered substrate were taken in measured quantity to make the total volume as 0.5 ml. To each tube 0.1 ml of water and 0.5 ml of DNPH solution were added and mixed well. After 20 minutes 5 ml of 0.4 N NaOH was added to the tubes. Mixed and

waited for further 10 minutes and the reading of standard was taken against 'Blank' at 520 mμ.

Calculation :

I.U. per litre under the experimental conditions at 37°C i.e., micromoles of pyruvate formed/min/litre = $\frac{U}{S}$ X 33.3

Preparation of reagents :

1. Phosphate buffer (0.1 M, pH 7.4) : (a) 14.2 g of anhydrous disodium hydrogen phosphate was dissolved in water and made to 1000 ml. (b) 3.49 of potassium dihydrogen phosphate was dissolved in water and made to 250 ml.

   (a) and (b) were mixed together to get the required buffer.

2. Buffered substrate (200 mM aspartic acid 2 mM Oxoglutoric acid) : 2.66 g of DL-aspartic acid and 0.0292 g of -oxoglutaric acid were dissolved in 20-30 ml of phosphate buffer (slight heating might be necessary) and 2.5 N (10%) sodium hydroxide was added to bring the pH to 7.4 (about 8 ml is necessary). The volume was made to 100 ml with phosphate buffer. It was stored at -15°C in small fractions so that a portion once taken out and

partly used, may not have to be stored again. The unused excess was discarded.

3. Pyruvate standard : (a) Stock standard solution : 110 mg of sodium pyruvate/100 ml was dissolved in phosphate buffer. It was stored at -15°C in 2 ml portions or prepared freshly every week. This contains 1 millimole/100 ml.

   (b) Working standard solution : Stock standard was dilluted 50 times with phosphate buffer. Dilution was done just before use. THis contains 20 µ mole/100 ml.

4. 2:4 dinitrophenyl hydrazine (DNPH - 1 mM) : 19.8 mg of dinitrophenyl hydrazine was dissolved in 10 ml of conc. HCl and made to 100 ml with water. It was stored in a brown bottle.

5. Sodium hydroxide (2.5 N - 10%) : 10 g of NaOH was dissolved in water and made to 100 ml.

6. Sodium hydroxide (0.4 N) : 16 ml of 2.5 N NaOH was diluted to 100 ml with water.

Determination of alanine aminotransferase (GPT) (EC. 2.6.1.2) (Reitman and Frankel, 1957) :

The same procedure was followed as in GOT, except that the time of incubation was 30 minutes in this estimation.

Calculation :

I.U./litre, i.e. the micromoles of pyruvate formed/min/lit

$$= \frac{U}{S} \times 66.66$$

Preparation of reagents :

All the reagents (1.3,4,5 and 6) needed for GOT were also required for this except the buffered substrate, preparation of which was as follows :

2. Buffered substrate (200 mM alanine; 2 mM - Oxoglutaric acid) : 1.8 g of alanine and 0.0292 g of - oxoglutaric acid were dissolved in 20-30 ml of phosphate buffer and 2.5 N NaOH was added to bring the pH to 7.4. The final volume was made to 100 ml with phosphate buffer. The buffered substrate was preserved at -15°C.

Estimation of aldolase activity (EC. 4.1.2.13)

(Sibley and Lehninger, 1949)

To each of two tubes marked 'Test' and 'Blank' 1.4 ml veronal buffer, 0.2 ml hydrazine sulphate and 0.2 ml test sample were added. Placed in both at 37°C for 5 minute and 0.2 ml sodium-fructose, 1.6 diphosphate was added to one (the Test). These are incubated exactly for 30 minutes at 37°C and then to both the tubes 2 ml 10% TCA was added followed by

0.2 ml substrate to the second tube (the 'Blank') mixed, centrifuged, then to 1 ml of the supernatent from each tube 1 ml NaOH was added and allowed to stand at room temperature for 10 minutes. Then 1 ml DNPH was added, mixed and incubated at 37°C exactly for 10 minutes, added 7 ml 0.75 N NaOH and after standing at room temperature for 5 minutes. The optical density of the test solution was measured against the blank at 540 nm in a Spectronic-20 spectrophotometer (Bausch and Lomb, USA).

Preparation of reagents :

1. Fructuse 1.6-diphosphate substrate, 50 m mol/l : It was prepared by dissolving 0.243 g Na-fructose 1.6-diphosphate in 10 cc water. It should be stored at 0-5°C.

2. Veronal buffer (pH - 7.4) : 1.236 g sodium diethyl barabiturate was dissolved in water and made upto 100 ml.

3. 2-4-Dinitrophenyl hydrazine (DNPH) solution : 1 g was grinded with small volume of 2 mol/l HCl. Filtered into 1 litre volumetric flask and made to the mark with the same acid.

4. 10% TCA

5. NaOH solution (750 m mol/L or 30 g/L).

6. Hydrazine sulphate solution (56 m mol/l or 7.29 g/L) : The pH was adjusted to about 7.4 with dilute NaOH.

Estimation of phosphohexose isomerase (EC 5.3.1.9) (Slein, 1955) :

To 0.1 ml of test sample, 0.14 ml of water, 0.2 ml substrate (Glucose-6-phosphate) and 0.06 ml of tris buffer were added. This was incubated at 30°C for 10 minutes. After incubation, 1 ml 50% TCA was added and centrifuged. 0.5 ml supernatant was taken in another tube and to it 3.5 ml of 8.3 M HCl and 1 ml 0.1% resorcinol was added and heated at 80°C for 10 minutes.

For blank, same protocol was followed except that 0.2 ml of glass distilled water was taken instead of the substrate.

The optical density of test solution was measured against the blank at 540 nm in a Spectronic-20 colorimeter (Bausch and Lamb, USA).

Preparation of reagents :

1. Glucose-6-phosphate : 13 mg was dissolved in 5 ml of glass distilled water and kept in a refrigerator with a rubber stopper.
2. Tris buffer 0.06 M, pH 9.1
3. TCA - 50%

4. HCl - 8.3 M.

5. Resorcinol 0.1% : 0.1 gm resorcinol was dissolved in 100 ml alcohol and kept in freeze.

Determination of serum cholinesterase (EC 3.1.1.8)

(Ellman *et al.*, 1968) :

An incubation mixture was prepared by adding tris buffer (pH-8) 0.3 ml, substrate 0.05 ml and the test sample 0.05 ml in a tube marked 'Test'. Incubated at 37°C for 30 minutes and then 0.1 ml of 20% TCA was added to stop the reaction. In case of control ; the test sample was added after the addition of TCA. Both test and control were centrifuged and 0.1 ml of supernatant was taken in other tubes respectively. Then 4 ml of DTNB solution was added to each of them and readings were taken within 2-3 min at 420 nm.

Preparation of reagents :

1. Buffer - Tris - HCl : 0.1 M - pH - 8 ; 0.1 M - pH - 7.4.

2. Dithio Bis-nitro benzoic acid (DTNB) : 39.6 mg DTNB and 15 mg Na_2CO_3 was dissolved in 10 ml of 0.1 M Tris (pH-7.4) buffer and was diluted 150 fold before use.

Determination of creatine phosphokinase activity (EC 2.7.3.2) (Swanson *et al.*, 1972) :

Procedure

To a series of cuvets having a 1 cm light path, 2.6 ml of the buffer reagent mixture and 0.1 ml each of the NADP solution, thiol solution and enzyme solution, were mixed and warmed in a water both to 30°C for about 5 minutes and then added 0.1 ml of the supernatant, mixed and incubated at 30°C. Absorbances was measured after 5 minutes at time intervals of 30 sec. or 1 min for 5-10 minute and average absorbance change per minute was calculated.

Calculation

The activity is then calculated in the usual way as follows :

$$\text{units /L} : \frac{\Delta A}{\text{min}} \times \frac{100 \times 300}{0.22 \times 0.1} = \frac{\Delta A}{\text{min}} \times 4823$$

Preparation of reagents :

1. Imidazole buffer 115 m moles/L, pH-6.7, 7.49 gm imidazole was dissolved in glass distilled water. pH was adjusted to 6.7 by the addition of 6 moles/L hydrochloric acid (Conc. HCl diluted - with an equal volume of water) ; then diluted - to one litre and mixed well. This buffer was kept in refrigerator.

2. Buffered reagent mixture

In 1 dl of the buffer magnesium acetate tetrahydrate. 246 mg glucose 415 mg : creatine phosphate disodium salt, hydrate, 1.13 gm Adenosine monophosphate (AMP), sodium salt - 103 mg were dissolved. After these chemicals had dissolved, the pH should be checked and adjusted if necessary. The solution is not too stable and is best preserved by pipetting 2.6 ml aliquots in small tubes, tightly stoppering these, and freezing. The tubes are then thawed as needed.

3. NADP solution, 4.6 mg/ml in buffer

This was prepared as needed, which is best done by adding 1.1 ml of buffer to a pre weight vial containing 5 mg NADP.

4. Thiol solution

980 mg N-acetyl cysteine was dissolved in 10 ml of buffer. This solution may be stored in the refrigerator for a few days.

5. Enzyme solution

A suspension of hexokinase was diluted to a concentration of 50 units/ml (50,000 units/L) and a suspension of glucose-6-phosphate dehydrogenase to a concentration of 30

units/ml (30,000 units/L), then equal volumes of the two solution were mixed. The resulting mixture is stable for about a week when kept in refrigerator.

Estimation of total lipids (Folch *et al.*, 1957)

0.2 ml of serum was taken in a tube to which 4.8 ml concentrated H_2SO_4 was added and the mixture was boiled for 10 minutes. After cooling 0.2 ml of it was taken in another tube and 3.8 ml of orthophosphoric acid and 1 ml of vanillin was added to it. After 10 minutes, the pink colour developed and was measured at 530 mμ in a Spectronic-20 spectrophotometer against the blank which contained the mixture of 4 ml orthophosphoric acid and 1 ml vanilin.

The total lipid content was measured from the standard curve.

Preparation of reagents :

1. Vanillin reagent : 600 mg vanillin was dissolved in 100 ml water (with aid of few drops of alcohol).

Estimation of phospholipids (Fiske and Subbarow, 1925)

6 ml of ether-alcohol mixture was taken in a 20 ml hard glass test tube and 0.2 ml serum was slowly added to it with

constant shaking. A rubber stopper was put tightly and the tube was shaken vigorously for one minute releasing the vapour carefully every 15 sec. Allowed to settle. Filtered through a dry filter paper without pouring the precipitate and collecting the filtrate in a dry test tube marked at 15 ml. Another 5 ml of the solvent was added to the ppt, shake and filtered as before collecting the filtrate in the same tube. The procedure was repeated with another 4 ml of ether-alcohol mixture and made up the volume of the confined filtrate to 15 ml with the same solvent.

10 ml of it was transferred into another tube and evaporated to dryness in an oven maintained at 70-75°C. The residue was heated with 0.5 ml of 10(N) H_2SO_4 slowly over a microburner in a fume cupboard, keeping the test tube in a slanting position. When white fumes started coming from the dark digest, it was cooled and one drop of conc. nitric acid was added to it and heated again till the solution was clear and colorless. If any brown tinge persists, it needs repeated heating with another drop of nitric acid. Then the digest was dissolved in water and made to 10 ml. 5 ml of the solution was transferred in another test tube and 0.5 ml of ascorbic acid was added to it. Then with the start of stop watch, 0.5 ml of ammonium molybdate was added to it and mixed. Reading

was taken against water blank at 5th, 10th and 15th minutes at 640 mμ in Spectronic-20 spectrophotometer (Bausch and Lomb, USA). The readings were plotted against time and the reading at zero muinute was denoted as 'U'.

In another similar test tube, 0.5 ml of the working standard, 0.5 ml of ascorbic acid and 0.5 ml of ammonium molybdate were taken in the same way as the test solution and readings were taken similarly. From the above readings, the reading at zero minute was calculated and was denoted as 'S'.

Calculation :

$$\text{mg of phosphorus/dl} = \frac{U}{S} \times 15$$

Preparation of reagents :

1. Ether-alcohol mixture (1:3)
2. Sulphuric acid, 10(N) analytical reagent - 10 ml of concentrated acid was diluted to 36 ml with water.
3. Conc. nitric acid.
4. Ascorbic acid solution - 1%
5. Ammonium molybdate solution - 1% in 0.05 N sulphuric acid - 1 gm of pure ammonium molybdate was dissolved in 50-60 ml of water with the aid of heat and transferred to a 100 ml

volumetric flask quantitatively. 10 ml of 0.5 N H_2SO_4 was added to it and diluted to the mark with water.

6. Standard solution of phosphate - Stock standard - 10 mg phosphorous/1 ml. 2 ml of stock standards was added to 2.5 ml of 10 N H_2SO_4 in a 100 ml volumetric flask and the volume was made up with water.

Determination of free fatty acid

(Chakrabarty *et al.*, 1969) :

Total lipid materials serum were extracted and washed following the method of Folch *et al.*, as described earlier. Then to 0.2 ml serum, 4 ml benzene was added and shaken vigorously until white colour appeared. Then the mixture was filtered and the filtrate evaporated to dryness. Again 4 ml benzene and 2 ml Rhodamin G-6 was added to it. After 30 minutes the readings were taken at 530 nm in a Spectronic-20 spectrophotometer against the blank prepared by mixing 4 ml benzene and 2 ml Rhodamin G-6. The free fatty acid content was calculated from standad curve plotted using palmitic acid as standard.

Preparation of reagents :

1. Rhodamin G-6 reagent : 25 mg of Rhodamin G-6 was dissolved in 25 ml of phosphate buffer and immediately extracted with

500 ml of benzene. The aqueous layer was discarded and the clear orange yellow coloured benzene layer was transferred into a throughly cleaned dried amber coloured glass bottle and preserved over solid caustic soda in the dark.

2. Phosphate buffer (0.2 M KH_2PO_4 + 0.2 M NaOH) : 27.2 gm KH_2PO_4 + 8 gm NaOH in 1000 CC water.

3. Standard solution of palmitic acid : 51.2 mg palmitic acid was dissolved in 10 ml benzene.

Histological Methods :

Human tissues were collected after post mortem of snakebite bodies and an unnatural death body as control within 6 hrs of death to avoid any histological changes due to mutilation. The tissues, brain, heart, liver and kidney were separated and fixed in alcoholic (70%) Bouins fixative for 24 hours at room temperature. The tissues were washed properly by several changes of 70% alcohol and dehydrated by routine dehydration procedure i.e. they are passed through 90% and absolute alcohol and then the tissue were placed in cedar wood oil. In the cedar wood oil the tissues can be kept for several months. The tissues from cedar wood oil were cleared in xylol. Now the tissues were ready for impregnation with molten paraffin. The tissue were then placed in a mixture of half xylol and half paraffin (melting point 52^{o}C) for 30 minutes. The tissues were finally infiltrated with pure paraffin at 54^{o}C for two hours with two changes in pure paraffin. After through impregnation with paraffin, the tissues were placed in a paper mould filled with molten paraffin and paraffin block were then prepared in usual manner.

Section cutting

The paraffin blocks were trimmed properly and microtome sections were cut at 5 microns thickness. All the tissues

under study were sectioned transversely. Sections were then stretched on glass slides coated with Mayer's albumin on hot plate and made ready for histological staining and observations. Prior to staining, the tissues were first deparaffinized in xylene which was changed twice during the period of 10 minutes. For histological studies eosin and hematoxylin staining technique was followed. For eosin and hematoxylin staining technique, the usual method described in Carletons Histological Technique (4th ed) was followed which involved the following steps.

1. Sections were passed through graded alcohol i.e. absolute, 90%, 70%, 50%, 30% and brough down to water.

2. Those were stained in Hematoxylin for 1-2 minutes.

3. Washing was done in running tap water for 2-3 minutes.

4. Differentiation was done in 1% acid water.

5. Slides were then kept in running tap water for at least 30 minutes.

6. Slides were that dehydrated through 30%, 50%, 70% alcohol and then stained in alcoholic eosin (90%).

7. Washed in 90% alcohol.

8. Dehydration was completed in absolute alcohol.

9. Cleared in xylene and mounting was done in D.P.X. The slides were observed under light microscope.

Scanning Electron Microscopic Studies

1. The brain, liver, kidney and heart tissues of human dead bodies of snake bite both viper and cobra and unnatural death, were collected and washed in physiological saline solution (0.9%).

2. The tissues were immediately fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2) for 4 hrs.

3. Washed in buffer 4 to 5 times.

4. Post fixed in 2% OSO_4 in 0.1 M Na-Cacodylate buffers for 3 hrs.

5. Washed in same buffer 4 to 5 times.

6. Subsequently the preparation were dehydrated in alcohol of adsending concentration (30%, 50%, 70%, 90% absolute alcohol) while the dehydration time in each grade of alcohol was 30 minutes.

7. The tissues were passed through the mixture of absolute alcohol and amylacetate in the ratio of 3:1, 1:1 and 1:3 for 30 minutes in each with 2 changes and then placed in pure amylacetate for one hour with two changes.

8. The tissues were then dehydrated in critical point dehydration (CPD). This technique projects the surface morphology intact.

9. The preparation were coated with gold, the coating layer being 400 A^{o}. The SEM examination were performed with Hitachi S-530 scanning electron microscope.

10. SEM photographs were done with Mamiya 6x7 under a range of magnifications.