CHAPTER TWO

MATERIAL AND METHODS

I MATERIAL

Male Albino mice were used for the present investigation. Mice were bred in the departmental animal house. Breeding pairs were obtained from Hindustan Antibiotics, Pune. They were housed in aluminium cages in group of 4 to 5 and were supplied with Gold Mohur mouse feed (Lipton India) and drinking water ad libitum.

Hundred mice of one and two months of age weighing 9 to 15 gms and 22 to 29 gms respectively were used. Mice were fasted overnight before operation. Operation tray was cleaned with soap water, sun dried and then scrubbed with 95 % alcohol. Scissors, forceps, needles, thread were autoclaved.

Sialoadenectomy was carried out under mild ether anaesthesia. Operations were carried out in between 9 A.M. to 10 A.M. Operated mice were maintained in animal house with proper care for one month. Controls were sham operated. Operated mice from both groups were sacrificed by cervical dislocation and following muscles were dissected out -

1) Recutus abdominis
2) Gastrocnemius
3) Soleus

and were subjected to the following investigations -

1) Blood glucose level
2) Glycogen content in muscles,

3) Estimation of proteins from muscles,

4) Estimation and separation of lactate dehydrogenase from muscles,

5) Estimation of Alkaline Phosphatase from muscles.

The results obtained were recorded and mean and standard deviations were calculated and statistical evaluation of the data was made by means of student 't' test, P-value < 0.05 was considered as significant.

II METHODS

1) Estimation of blood glucose : (Folin and Wu, 1920) :

Principle : Protein free blood filterate when treated with alkaline copper sulphate, using special tube for preventing reoxidation, cuprus oxide was formed which treated with phospho-molibdic acid, blue colour was formed, compared with standard colour produced by glucose from standard.

Reagents

1) 2/3 N H_2SO_4 :
   36 ml H_2SO_4 raised to 100 ml distilled water.
2) 10 % sodium tungstic acid
3) Glucose solution -
   20 mg glucose in 100 ml of distilled water.
4) Alkaline copper sulphate solution -
   20 gm sodium carbonate + 200 ml distilled water + 3.25 gm

tartaric acid + 2.25 gm of copper sulphate mixed with distilled water volume was raised to 500 ml.

5) Phosphomolibdic acid -

3.5 gm molybdic acid + 0.5 gm sodium tungstate + 20 ml of 10 % NaOH + 20 ml distilled water. Boiled for 20' to 30', cooled, diluted to about 35 ml with distilled water. 12.5 ml 85 % phosphoric acid was added.

Procedure :

1 ml of blood was transferred to a flask having a capacity at least 15 times more than that of a volume taken. Diluted it with 7 ml distilled water, mixed well and 1 ml of 10 % sodium tungstate solution was added. Again mixed well and finally slowly with shaking 1 ml of 2/3 N H_2SO_4 was added. The mixture was allowed to stand for ten minutes. The precipitate formed was dirty chocolate. It was filtered through a dry filter paper and colourless filterate obtained was taken for glucose estimation.

2 ml of above tungstic acid blood filtrate was taken in a folin-wu tube graduated to 25 ml. The second folin-wu tube was blank, containing 2 ml distilled water. Standard was formed of glucose solution. To each tube 2 ml Alkaline copper sulphate was added. The tubes were mixed well and placed in a boiling waterbath for eight minutes. Test tubes were cooled under running water without shaking. To each test tube 2 ml phospho-

molybdic acid reagent was added. After one minute aliquots were diluted to the mark (25 ml) with distilled water and mixed well. While mixing, care was taken to get uniform colour, because greater part of blue colour formed was in the bulb of the tube. Colour intensity was measured at 420 nm by adjusting the colorimeter to zero with the blank.

Calculations :

$$\frac{\text{\% transmittance of standard} \times \text{Amount of standard}}{\text{\% transmittance of sample} \times \text{Amount of sample}} \times 100 = \text{mg of glucose/ 100 ml of blood}$$

ii) Estimation of glycogen : (Pfleiderer, 1957)

Principle : Glycogen is usually determined by hydrolysis to glucose, which is then estimated chemically.

Reagents :

1) Ethanol 96 %
2) Sulphuric acid 2 N
3) Sodium hydroxide 2 N
4) Potassium hydroxide
5) Alkaline copper sulphate solution, described earlier.
6) Glucose solution -

   20 mg glucose in 100 ml distilled water.
7) Phosphomolybdic acid, described in previous section.

Procedure :

After removing, muscles were quickly weighed on a torsion balance and placed in test tubes containing 2 ml of 30 % KOH. A glass bulb was placed over the mouth of each tube to minimize evaporation and the tubes were then heated in a boiling water bath for 15 minutes. Then cooled. 3.25 ml of 96 % ethanol was added. Again the tubes were heated in boiling water bath until the mixture began to boil. It was then cooled in an ice water bath for 15 to 30 minutes and centrifuged at 3000 rpm for 15 minutes. The supernatant was decanted and the precipitated glycogen was treated with 3 ml ethanol, mixed well, centrifuged and supernatant was decanted and the inverted tubes were allowed to drain. The glycogen thus purified was hydrolyzed by addition of 2 ml of 2 N H_2SO_4 into the test tubes provided with a glass bulb and the tubes were heated for 120 minutes in a boiling water bath. The glycogen was hydrolysed to glucose. Cooled to room temperature, neutralized (pH 7) with 2 N NaOH. And diluted to 10 ml with distilled water. 2 ml of this solution was used for the glucose estimation. Glucose was estimated by Folin Wu method described in early section.

Calculations:

$$\frac{\text{\% transmittance of standard} \times \text{Amount of Standard}}{\text{\% transmittance of sample} \times \text{Amount of sample}} \times 100 = \text{mg of glucose/ 100 mg of wet tissue}$$

iii) Estimation of Protein : (Lowery et al., 1951)

Homogenization of the muscles (rectus abdominis, gastrocnemius and soleus) was carried out using refrigerated glass morter and pestle. Tissues were crushed at the bottom of the morter for instantaneous freezing and gradual thawing with cold distilled water. The perfectly uniform homogenates were centrifuged at 10°C at 5000 rpm for 10 minutes. The supernatant was used for estimations of protein.

Reagents:

1) Reagent A -

2 % Na_2CO_3 in 0.1 N NaOH

2) Reagent B -

0.5 % $CuSO_4$ in 1 % Na K tartarate

3) Reagent C -

was prepared at the time of use (50 ml A + 1 ml B).

4) Reagent D -

Folin ciocalteau phenol reagent.

5) Standard protein solution -

Bovine serum albumin 13 mg in 250 ml distilled water.

Folin Ciocalteau-phenol reagent :

100 gm Sodium tungastate $Na_2WO_4 . 2H_2O$ and 25 gm Sodium molybdate $Na_2MO\ O_4 . 2H_2O$ were dissolved in 700 ml distilled water. 100 ml HCl and 50 ml 85 % Phosphoric acid were added and the mixture was refluxed for 10 hrs in glass apparatus. There after added 150 gm of lithium sulfate followed by 50 ml distilled water and a few drops of Br_2. Boiled for 15 minutes to remove excess bromine, cooled diluted upto 1000 ml and then filtered. The reagent was golden yellow colour, stored in refrigerator. This was stock reagent and was diluted with equal volume of water just before use.

Procedure :

0.5 ml supernatant was taken in test tube that test tube was labelled as sample. Standard tube was prepared using bovine serum albumin. Additons were made as follows :

	Sample	Standard	Blank
Sample	0.5 ml	-	-
Bovine serum albumin	-	0.5 ml	-
Distilled water	-	-	0.5 ml
Reagent C (A+B)(50+1)	5 ml	5 ml	5 ml
STAND FOR 10 MINUTES AT ROOM TEMP.			
Reagent D (Phenol reagent)	0.5 ml	0.5 ml	0.5 ml
WAIT FOR 30 MINUTES			

Reading were taken at 660 nm on spectronic 20 colorimeter adjusting to zero with blank.

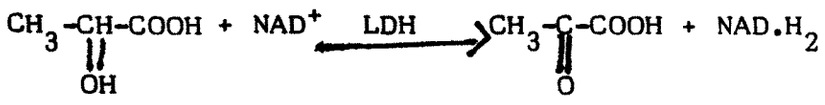
Reaction :The final colour production was a result of biuret reaction of protein with copper ions in an alkaline medium and reduction of phosphomolybdic phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein.

Calculation :

$$\frac{\text{Amount of standard} \times \text{Optical density of sample}}{\text{Optical density Standard} \times \text{Amount of Sample}} \times 100 = \text{mg/100 mg of the wet tissue.}$$

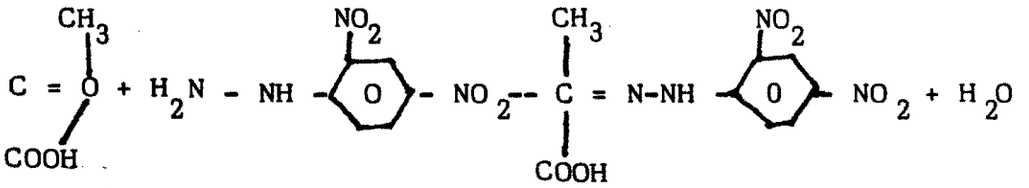
iv) Estimation of Lactate dehydrogenase: (Sevela and Tovorek, 1989)

Principle : This method was based on the measurement of the pyruvate formation rate during the lactate oxidation, reaction is assisted by lactate dehydrogenase of the sample.



Lactate Pyruvate

Pyruvate reacts with 2,4 dinitrophenylhydrazine in an alkaline medium to yield a hydrazone of red brown colour, whose intensity was presumed to be proportional to the ketoacid concentration.



Pyruvate | 2,4 dinitro phenyl hydrazine | 2,4 dinitrophenyl hydrazone pyruvate

Reagents :

1) 0.45 M Sodium lactate -

10.8 ml was raised to 100 ml with distilled water.

2) 0.03 M Sodium phyrophosphate -

0.334 mg dissolved in 25 ml distilled water, adjusted to pH 8.8 using 1 M hydrochloric acid and diluted with distilled water to 250 ml.

3) 0.4 M Sodium hydroxide -

1,600 gm was dissolved in 100 ml distilled water.

4) .3 µg/ml solution of NAD^+

3 mg NAD^+ was dissolved in 100 ml distilled water. From this solution 10 ml was taken and raised to 100 ml.

5) 0.2 % 2,4 dinitrophenylhydrazine

200 mg 2,4 dinitrophenylhydrazine dissolved in 100 ml, 1 M hydrochloric acid.

6) 11 µg/ml Sodium pyruvate -

11 mg Sodium pyruvate was dissolved in 100 ml distilled water from this solution 10 ml was taken and diluted to 100 ml.

Procedure :

Homogenization of different muscles was carried out as described for the estimation of protein in the muscles. 0.1 ml of the sample and 0.3 ml NAD^+ solution were transferred to a test tube and placed it in a water bath to warm at 37°C for five minutes.

Transferred 0.8 ml of sodium pyrophosphate solution and 0.2 ml of sodium lactate solution to another test tube and warmed in the water bath at 37°C for 5 minutes.

Both the test tubes were in the water bath, content of the latter test tube was poured into the former, stirred briskly with a glass rod, and the time was noted. The reaction was allowed to run for 25 minutes and then stoped it by adding 0.5 ml of 2,4 dinitrophenylhydrazine. The test tubes were kept aside for 20 minutes at room temperature (to allow the hydrozoan to form). Poured 5 ml of sodium hydroxide solution into the test tube, stirred briskly with the glass rod and waited 10 minutes to allow the coloration to develop. Measurement of the absorbance was taken for sample solution against blank solution on the Spectronic 20 Colorimeter.

At 520-560 nm wavelength using 1 cm thick cells. The control solution was made in a manner similar to that for the sample solution, the only distinction being that sample was added

after incubation.

Calculation:

The activity of enzyme was estimated by making use of an analytical curve the conditions for its plotting are summarized in following table. The absorbance values are plotted as ordinate versus the corresponding LDH activity units (in m mol h^{-1} $litre^{-1}$) as abscissa.

Sr. No.	Sodium pyruvate ml	Sodium pyro-phosphate ml	Distilled water	Pyruvate content in the sample mole	LDH activity m mole h^{-1},l^{-1}	Absor-bance
1	0.1	0.8	0.5	0.01	1.2	
2	0.2	0.8	0.4	0.02	2.4	
3	0.4	0.8	0.2	0.04	4.8	
4	0.6	0.8	-	0.06	7.2	
5	0.8	0.6	-	0.08	9.6	

v) Estimation of Alkaline Phosphate : (Linhardt, K. and Walter, K. 1965).

Principle :

P-nitrophenyl phosphate is used as the substrate for the determination of activity of alkaline phosphatase. After 30

minutes incubation the alkaline phosphatase activity was inhibited by NaOH and the P-nitrophenol liberated by the phosphatase forms a yellow anion. The phosphatase activity is directly proportional to the amount of P-nitrophenol liberated per unit time.

Homogenization :

Homogeniztion of the muscles (rectus abdominis, gastrocnemius and soleus) was carried out using refrigerated glass morter and pestle. Tissues were crushed at the bottom of the morter for instantaneous freezing and gradual thawing with cold distilled water. The perfectly uniform homogenates were centrifuged at 10°C at 5000 rpm for 10 minutes. The supernatant was used for estimations of alkaline phosphatase.

Reagents :

1) Alkaline buffer substrate solution -

(0.05 M glycine buffer; 5.5 X 10^{-3} M P-nitrophenyl phosphate; pH 10.5).

Dissolve 375 mg glycine + 10 mg $MgCl_2.6H_2O$ and 165 mg Na-P-Nitrophenyl Phosphate were dissolved in 42 ml of 0.1 N NaOH and diluted to 100 ml with doubly distilled water. The pH was checked with glass electrode.

Procedure : In all six tubes were prepared. Three were for

sample and another three were for control. Addition were made as follows -

	Sample	Control
Buffer substrate solution	0.1 ml	0.1 ml
Equilibrated 5 - 10 minutes (37°C)		
Sample (Supernatant)	1.0 ml	

The contents of the tubes were mixed by inverting tubes several times. After incubation for 30 minutes at 37°C, following additions were carried out.

0.02 N NaOH	10 ml	10 ml
Sample (Supernatant)	-	0.1 ml

After inverting the tubes several times, the optical density of the sample was noted on the Spectronic 20 Colorimeter, adjusting zero with control at 400 nm wavelength.

Calculations :

$$\frac{\text{Optical density X 11.82}}{\text{mg tissue used for estimation}} \times 1000 = \text{µ mols of P-nitrophenol/mg wet weight}$$

With an essay volume of 11.1 ml, 0.1 ml serum and an incubation period of 30 minutes. A unit corresponds to an optical density of 0.084 at 400 m therefore.

$$\frac{E_{400}}{0.084} = E_{400} \times 11.82$$

vi) Electrophoresis :

Polyacrylamide gel elecctrophoresis (PAGE) was performed in cylindrical glass tubes (Davis, 1964; Ornstein, 1964). Each sample was run on a separate rod, for an accurate comparison of different samples. Conditions were identical for all rods throughout the experiment. The glass tubes had identical dimensions precisely vertical and contain the same length of gel and that the upper surfaces of the gels were accurately flat so that identical running condition occur in all gels. The glass tubes used for gel preparation were having 5 mm dimension and 8 cm length.

Gel Preparation :

5 % PAG :

Acrylamide	4.75 gm
Bisacrylamide	0.25 gm
TEMED	0.05 ml
(N', N', N', N', tetramethyl ethylene diamine)	
RiboFlavin	0.01 gm

Prepared in 100 ml separation gel buffer No stacking gel was used.

Electrode chamber buffer :

At Anode :

0.26 M Tris buffer pH (9.1)

Tris (Hydroxy methyl - aminomethane)	25.2 gm
EDTA (Ethylenediamine tetra-acetic acid)	2.5 gm
Boric acid	1.9 gm
Distilled water to	1000 ml

9.1 to 9.2 pH was checked with digital pH meter (Systronic, Ahmedabad)

At Cathode :

A barbital buffer pH 8.6

Sodium diethyl barbiturate	5.15 gm
Diethyl barbituric acid	0.92 gm
Distilled water to	1000 ml

Separtion gel buffer - (pH 7.5)

Tris (Hydroxy methyl)	0.856 gm
1 M HCl	6 ml

Dissolved in 100 ml doubly distilled water.

Preparation of the sample :

Homogenization of the muscles (retus abdominis, gastrocnemius and soleus) was carried out using refrigerated glass morter and pestle. Tissues werre crushed at the bottom of the morter for instantaneous freezing and gradual thawing with cold distilled water. The concentration of the muscle was 50 mg/ml distilled water. The perfectly uniform homogenates were centrifuged at 10°C at 5000 rpm for 10 minutes. The supernatant was used for electrophoresis.

Sample dye :

1 ml supernatantt + 50 mg sucrose + 0.3 ml glycerol + 0.001 % Bromophenol blue. 20 µl. sample dye was loaded for a single gel rod with help of micro syring.

Electric supply : The voltage was kept constant at 150 volt during the operation of electrophoresis and 3 mA current per rod was employed. During the first five minutes only 2 mA per rod was employed to avoid diffusion. The separation time was 30 to 40 minutes. Electrophoresis was stopped when dye front reached the tip leaving 5 mm distance. The mobility was from cathode to anode.

Gel Staining :

1) For lactate dehydrogenase :

Staining mixture -

substrate

1) 1 M Buffered Substrate (lactate)

1 ml of 60 % Sodium lactate was diluted to 10 ml with M/15 phosphate buffer pH 7.5 (M/15 phosphate buffer was prepared by addition of 84.1 parts anhydrous Na_2HPO_4 + 15.9 parts KH_2PO_4 to one litre).

2) Nitroblue tetrazolium (N.B.T.) -

1 mg/ml in water.

3) Phenazine methosulphate (P.M.S.) -

1 mg/ml made up in water.

Working solution :

a) 1 ml buffered substrate (lactate)

b) 3 ml N.B.T.

c) 0.14 ml P.M.S.

d) 1 ml LDH buffer

e) 10 mg NAD^+

The gels were put in container, containing staining mixture. Incubated at 37°C until bands were resolved. This took about 30 minutes. Then gels were washed with 10 % acetic acid. And stored in 7 % acetic acid.

Gel Photography and Scanning :

The photographs of gel were made to record the observations. the scanning of gel of lactate dehydrogenase were done using Shimadzu UV - 240 mode spectrophotometer. The gels of lactate dehydrogenase were scanned at 400 nm.

vii) Statistical Methods (Fisher, 1950, Gupta, 1991).

a) $\bar{X}$ = Arithmatic mean of x independent variable

$$\bar{X} = \frac{\Sigma X}{N}$$

where, X = independent variable

N = number of observations

b) S.D. = Standard deviation

$$S.D. = \sqrt{\frac{\Sigma(X - \bar{X})^2}{N}}$$

c) SE = Standard Error for a mean of N variable.

$$SE = \frac{S.D}{\sqrt{N}}$$

d) S = Combined standard deviation

$$S = \sqrt{\frac{\Sigma(X_1 - \bar{X}_1)^2 + \Sigma(X_2 - \bar{X}_2)^2}{N_1 + N_2 - 2}}$$

where, $\bar{X}_1$ = mean of the first group

$\bar{X}_2$ = mean of the second group

N_1 = number of observation of first group

N_2 = number of observation of second group

$N_1 + N_2 - 2$ = degree of freedom

e) Student 't' test

$$t = \frac{\bar{X}_1 - \bar{X}_2}{S} \times \sqrt{\frac{N_1 \times N_2}{N_1 + N_2}}$$

where, S = Combined standard deviation

f) Significance

The probability P, of obtaining 't' value at least as greater as the calculated one for a given number of degree of freedom is found in Fisher's tables. The P-values are signified according to the following conventions.

P	> 0.05	Non-significant
P	< 0.01	Significant
P	< 0.05	Almost significant
P	< 0.001	Highly significant

viii) CHEMICALS :

Name of the Chemical	Batch No.	Source
Acrylamide	8252433	MERCK
Amino black 10B	55936	Fluka A.G.
Bovine serum albumin	140	Romali
Bromophenol blue	110	Romali

Name of the Chemical	Batch No.	Source
Barbiton sodium	25527	Dofoer
Copper sulphate	F. 30705	MERCK
Di-sodium hydrogen phosphate 2-hydrate Na_2HPO_4 $2H_2O$	L 6, 22500	MERCK
Glucose	0084 4711	Qualigens
Hydrochloric acid	F_8 37281	MERCK
N', N', N', N' tetra Methyl ethylene diamine (TEMED)		Kolch-Light England
N',N' methylbis Acrylamide	29227	BDH
Nitro Blue Tetrazolium	94060	S.D.Fine CHEM LTD.
NAD^+ Phenazine metho sulfate	813446	SRL
Sodium hydroxide	NL 0501 5009	Qualigens
Sodium lactate	665603	Boehringer ingel heim
Sodium pyruvate	0393-793-270211	S.D.FINE CHEM LTD
Sodium sulphate	37170	MERCH
Sodium dihydrogen phosphate		MERCK
Sodium carbonate		SAR
Sucrose	NL 0301 4910	Qualigens
Sodium pyrophosphate	7910 659 115/01-166395	MERCK

Name of the Chemical	Batch No.	Source
Tris (Hydroxy methyl) methyl amine	NL 0263 4909	Qualigens
Molibdic acid	14 34311	MERCK
Potassium sodium (+) tartarate	5/1192/1192/ 11111	S.D.-FINE CHEM LTD.
Tartaric acid	5608 3803	AnalaR