C H A P T E R II

MATERIALS AND METHODS

MATERIALS AND METHODS

		Page No.
1.	Experimental Design	140 - 145
2.	Diet Compositions	145 - 152
3.	Chemicals	152
4.	Separation of Brain Tissue	152 - 153
5.	Processing of Tissue for Biochemical Assays	153
6.	Determination of Moisture Content	154
7.	Estimation of Brain Proteins	154 - 156
8.	Estimation of Brain DNA	156 - 159
9.	Estimation of Thiamine Content in Liver and Brain	160 - 169
10.	Estimation of Brain acetylcholine esterase Activity	169 - 172
11.	Estimation of Brain Choline Acetylase Activity	172 - 179
12.	Estimation of Brain Acetylcholine Content	179 - 184
13.	Measurement of Rate of Synthesis of Labelled ACh in the Brain	184 - 189
14.	Statistical Treatment	189

+ + + + + +

MATERIALS AND METHODS

1. Experimental Design :

As mentioned in the earlier section, the present studies were mainly concerned with the effects of nutritional deficiencies during prenatal, postnatal and postweaning periods on the acetylcholine metabolism in the brain of albino rats. Different experiments were carried out to study the effects of deficiencies of calorie, protein and thiamine. The details of experimental procedure are given below :-

EXPERIMENT - I : Prenatal undernutrition :

Prenatal undernutrition was brought about by feeding low protein diet (5 % protein) to the females of proved fertility (after the first parity). The dietary regimen was instituted from the day of conception (as judged by presence of sperms in vaginal lavages) and continued throughout gestation and lactation (G^-L^-). The progeny of these animals were compared with those born of mothers reared on normal, balanced diet - 20% protein (G^+L^+).

EXPERIMENT - II : Neonatal undernutrition :

In the initial studies the neonatal undernutrition was brought about by increasing the litter size. The control

group had a litter size of 8 (control SL) and the undernourished group of 16 (LL, large litter size). For this purpose, pups born on the same day were randomally distributed to forster mothers in litters of 8 or 16 till 21 days of age. The mothers were given food and water ad lib. throughout the period of experiment.

In the next studies the neonatal undernutrition was achieved by feeding low protein (5% protein)diet to mothers from the day of partus ($G^{+}L^{-}$). The food and water was provided ad lib. The control group was reared on 20 % protein diet ad lib.

EXPERIMENT - III : Rehabilitation following early undernutrition :

The pups born of undernourished females either from conception and/or partus were transferred to individual cages at 21 days of age and fed 20 % protein diet ad lib. for 5 weeks.

EXPERIMENT - IV : Postweaning undernutrition and protein deficiency :

The weanling rats were matched for age, sex and body weights. They were divided into groups of 8 each. They were housed individually and given food and water ad lib., except where restriction of diet was studied.

The first three groups were fed 5,8 and 20 % protein diet ad lib. The next three groups were fed restricted 20 % protein diet ~~ad lib~~. In the case of the restricted groups the amounts were adjusted at 100, 50 and 25 per cent of the ad lib. food intake of animals fed the 5% protein diet. The regimen was continued for a period of 6 weeks.

EXPERIMENT - V : Long range protein deficiency :

Since no changes were observed in the activities of brain enzymes in the experiment No. IV, the protein deficiency after weanling period was continued further for 65 weeks. The food and water ~~was~~ were given ad lib.

EXPERIMENT - VI : Prenatal thiamine deficiency :

Female rats were fed 20 % protein diet without any thiamine in the diet from the 7th day of conception. The food and water were given ad lib. The regimen was continued till the end of lactation. The control females were fed 20 % protein diet with thiamine. The food intake of the controls was restricted to match that of the deficient group. The progeny were compared at different ages.

EXPERIMENT - VII : Neonatal thiamine deficiency :

Female rats were fed 20 % protein diet without any thiamine in the diet from the day of partus. The food and water were given ad lib. The control group was pair-fed with the deficient group. The progeny of both groups were compared at different ages.

EXPERIMENT - VIII : Rehabilitation of early thiamine deficiency :

Since thiamine deficiency during pre- and post-natal periods resulted in significant deficits in brain ACh levels the rehabilitation of the deficient animals was attempted by feeding normal diet from the 29th day of age. At the end of 5 weeks of feeding the normal diet, rats were sacrificed for evaluation of reversibility of various deficits.

EXPERIMENT - IX : Post-weaning thiamine deficiency and ACh levels :

Weanling rats, matched for age, sex and body weights, were divided into six groups of eight each. The six groups were fed 20 % protein diet with 100 % thiamine ad lib., 20 % protein diet with 100% thiamine pair-fed to 0 % thiamine group, 50% thiamine, 25 % thiamine, 10% thiamine and

0 % thiamine. Except the first group, all groups were pair-fed to sixth group (0 % thiamine). The water was provided ad lib. At the end of 5th-6th week these rats were sacrificed to estimate ACh levels in the brain. The special attention was given to see that the deficient rats had developed frank symptoms of neurological dysfunction.

EXPERIMENT - X : Post-weaning thiamine deficiency and brain enzymes :

Weanling rats, matched for age, sex and body weights, were divided into three groups of eight each. The groups were fed normal diet (20 % protein), thiamine deficient diet and normal diet pair-fed to the deficient group for a period of six weeks. At the end of this period, rats were sacrificed to study moisture and thiamine content in different regions of the brain and enzyme activity in the whole brain.

EXPERIMENT - XI : Studies on the incorporation of label form 1-^{14}C acetate and ^{3}H-(methyl) choline into brain ACh of the deficient rats :

For this study three groups of rats were reared separately on protein-deficient, thiamine-deficient and pair-fed controls to the second group for a period of five weeks

from weaning. At the end of the experiment, the precursors of ACh were intravenously injected and rats killed at different time intervals to study the profiles of incorporation in these groups.

In all these experiments rats were individually caged. Ten hours light period followed by 14 hours dark period was approximately maintained. Water and food were given ad lib. except where restriction of food intake was desired. Records were kept for body weight changes during treatment. Food intake was recorded wherever possible.

Experiments related to postnatal and post-weaning protein deficiency and post-weaning thiamine deficiency and brain ACh levels were conducted at Biochemistry Department of Baroda University. The rest of the experiments were carried out at Haffkine Institute, Bombay. The first series of diets mentioned in the following pages were used at Baroda and the second series at Haffkine Institute. In the former case albino rats of the Charles Foster strain were used whereas in the latter studies rats of Haffkine Institute strain were utilized for the study. There was somewhat less of body weight gain in the latter strain.

2. Diet Composition :

The animals were reared on the semisynthetic experimental diets or a stock diet. The compositions of

these diets are shown in Tables 9 and 10.

Table-9 : Composition of the stock diet.

Ingredients		Amount (g)
Wheat flour	...	350
Bajra flour	...	100
Bengal gram flour	...	110
Milk powder	...	210
Sprouted legumes	...	160
Groundnut oil	...	70
Dark green leafy vegetables	...	60-80

This diet was found to be a very well balanced diet as ascertained by the growth curves of weanling rats and reproductive performance of adult female rats. As per the assessment, this diet was comparable to 20 % protein diet.

Table-10 : Composition of low and high protein diets.

		% Dietary Protein					
		1			2		
		5	8	20	5	8	20
Washed casein	(g)	6.0	10.0	24.0	6.0	10.0	24.0
Vitamin mixture	(g)	2.0	2.0	2.0	2.0	2.0	2.0
Salt mixture	(g)	4.0	4.0	4.0	4.0	4.0	4.0
Groundnut oil	(g)	7.0	7.0	7.0	7.0	7.0	7.0
Sucrose	(g)	18.0	-	-	20.0	20.0	20.0
Sago	(g)	63.0	73.0	63.0	-	-	-
Potato starch	(g)	-	-	-	61.0	57.0	43.0

1 = In the initial experiments these diets were used with additional oral feeding of shark liver oil once a week to each animal providing 70-100 µg vitamin A.

2 = In the next series of experiments, these diets were used with additional feeding fat-soluble vitamins in place of shark liver oil. Vitamin A,D,E and K were dissolved in groundnut oil and appropriate amounts were mixed in the diet.

i) Casein :

(A) In the 1st series of diets, the edible casein obtained from Amul Dairy, Anand, was washed first with alcohol and then washed free of alcohol with tap-water and finally with

distilled water. The washed casein was sun-dried, seived and used. Each lot was analysed for nitrogen content by the Micro-Kjeldahl method and protein content was calculated as N X 6.25.

(B) In the second series of diets, edible casein (Holland make) was defatted by refluxing 5 kg lot with n-hexane (petroleum ether 60-80) in a Soxhlet apparatus. The fat free casein was dried at 60°C and seived before used. Each lot was analysed for nitrogen content.

ii) Starch :

In the first series of diets, Sago was used as a source of starch. Commercially available sago prepared from Tapioca flour (Manihot utilissima) was ground, seived and used. It contained only 0.2 per cent protein and no more than traces of vitamins and minerals. During the process of preparation of sago, starch in the same is believed to be rendered in the readily available form (Booher, Behan and McMeans, 1951).

In the second series of diets, potato starch (Universal, India) was used as a source of starch. It was found to contain negligible amounts of vitamins and minerals. The protein content was estimated to be less than 0.05 %.

iii) Vitamin Mixture :

In the both series of diets the following water-soluble vitamin mixture was used for maternal diet :

Table- 11 : Composition of the vitamin mixture.

	Amount per kg of the diet.
Thiamine hydrochloride (mg)*	4.0
Riboflavin (mg) *	4.0
Pyridoxine hydrochloride (mg)	1.0
Niacin (mg)	15.0
Calcium-d-pantothenate (mg)	10.0
Choline chloride (mg)	750.0
Inositol (mg)	200.0
Para-amino benzoic acid (mg)	10.0
Folic acid (mg)	1.0
Cyanocobalamin (µg)	5.0
Biotin (µg)	1.0

Powdered sugar approximately 19 g so as to make a total of 20 g.

* For the studies on postweaning experiments 1.5 mg of thiamine and 2.5 mg of riboflavin were used.
Stored in amber coloured bottles in the refrigerator.

The water soluble vitamin mixture used was mormulated (Rajalakshmi and Ramakrishnan, 1969) on the basis of the allowances suggested by Brown and Sturtevant (1949), recommendation made by NAS-NRC (1962) and reviewed by Mitchell (1964).

In the second series of diets, the fat soluble vitamin mixture had the following composition (Jacob and Forbes, 1970; Spaeth and Schneider, 1974).

		Per kg diet.
Vitamin A (mg)	....	4.0
Vitamin D (ug)	....	31.25
Vitamin E (mg)	....	300.00
Vitamin K (mg)	....	5.0

Dissolved in groundnut oil and mixed with the diet.

iv) Salt Mixture :

The salt mixture used was the modified Hawk-Oser salt mixture No.4 (Hawk and Oser, 1931; Oser, 1976). The composition of this mixture is given in Table 12 & Table 13.

Table-12 : Composition of the salt mixture A.

	(g)
$FeNH_4$ citrate, USP	91.41
$CuSO_4.5H_2O$	5.98

contd...

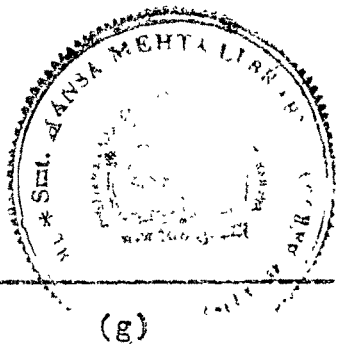


Table-12 (contd...)

	(g)
NaF	0.76
$MnSO_4.2H_2O$	1.07
KA $(SO_4)_2.12H_2O$	0.54
KI	0.24
Total	100.09

Table-13 : Composition of the salt mixture No.3

	Amount (g)
Salt mixture A	16.7
Tricalcium dicitrate, $4H_2O$	308.2
$Ca(H_2PO_4)_2H_2O$	112.8
K_2HPO_4	218.7
KCl	124.7
NaCl	77.0
$CaCO_3$	68.5
$MgSO_4$ (anhydrous)	38.3
$MgCO_3$. $Mg(OH)_2.3H_2O$	35.1

The salt mixtures were prepared in bulk and stored in air-tight containers.

The diets were prepared once a week and the vitamin mixtures and groundnut oil were added at the time of feeding. All the components of the diets were mixed in mortar and pestle to bring to necessary homogenity.

3. Chemicals :

The chemicals used for the present study were of research grade purity and obtained either from the British Drug House Ltd., Sarabhai Chemicals or Merck (India) Ltd. All the solvents were freshly distilled before use. The source for fine chemical is listed in the individual methods discussed later.

The following radioactive compounds were obtained from the source indicated below :

^{14}C-acetate -- B.A.R.C.
^{14}C-Acetyl-CoA -- Amersham, U.K.
3-H-methyl choline- Amersham, U.K.

All the fine chemicals were stored in the dessicator at room temperature or in the refrigerator or -20^{o}C in the deep freezer as necessary.

4. Separation of brain tissue :

The rats were decapitated and the brains with olfactory lobes were removed immediately. The brains were

dipped in cold normal saline, the adhering blood vessels removed by pressing the tissue gently between folds of filter paper and weighed.

The different areas of the brain were dissected out on cold petri dishes stationed on ice-salt mixture. The areas dissected out as per the description of Zeman and Innes (1963) were - cortex, cerebellum, and medulla + pons (with stem). The areas were weighed individually.

Before processing the tissue for analysis, it was stored in aluminium foil on ice blocks.

5. Processing of tissue for biochemical analysis :

The weighed tissue was homogenized in 0.02 M phosphate buffer pH 7.0 with the help of all glass homogenizer and teflon pestle. The clearance between the tube and pestle was 2-3 mm. The tissue was homogenized for 90 seconds with a gap of 30 seconds in between. The speed of the teflon pestle was 4000 r.p.m. with a stroke speed of 20 per minute. The 10 % homogenate (w/v) was stored under ice-cold condition before actual use or further centrifugation as necessary for a particular estimation. The liver homogenate was also prepared likewise.

6. Determination of moisture content :

For this purpose the tissue was quickly weighed after separation and placed in a stainless steel planchet. The tissue and the planchet were weighed together. For drying the planchets were placed in an oven with a mild vacuum at 60°C. The weights of the dry tissue were recorded on three different days until there was no further loss in the weight. Deduction of the dried tissue plus planchet from that of wet tissue plus planchet served as an index for loss of moisture. Moisture as % of tissue weight was calculated accordingly.

7. Estimation of brain protein :

Protein content in the brain was estimated by the method of Lowry et al. (1951).

Principle :

The method consists of measuring the colour produced by the reaction of alkaline copper tartrate between phenolic groups on protein and Folin-Ciocalteau reagent. The colour produced is measured at 660 nm.

Reagents :

1) Folin-Ciocalteau Reagent : A mixture of sodium tungstate 100 g, sodium molybdate 25 g, distilled water 700 ml,

concentrated hydrochloric acid 100 ml and phosphoric acid 50 ml were refluxed for 10 hours. After cooling to room temperature lithium sulphate 50 g and distilled water 50 ml were added. The content shaken vigorously after adding few drops of liquid bromine. The flask was boiled for 5 minutes in fuming chamber to expell out excess bromine. The final reagent was golden yellow in colour. This was titrated to 1 N and stored in dark amber coloured bottle at $4^{o}C$. It was diluted 1:1 with water before use.

2) Alkaline copper tartarate reagent : This was prepared freshly. Fifty ml of 2 % sodium carbonate (anhydrous) in 0.1 N NaOH was mixed with 1 ml of 1:1 mixture of one percent solution of copper sulphate (hydrated) and 2 % solution of sodium potassium tartarate.

3) Standard protein solution : Ten mg of bovine serum albumin (Cohn Fraction V, Sigma Chemicals, St. Louis, USA) were dissolved in 0.1 N NaOH and diluted to 100 ml with the same to give final concentration of 100 µg/ml.

Procedure :

0.2 ml of 10 % brain homogenate prepared in 0.02 M phosphate buffer pH 7.0 was precipitated with 1 ml of 10 % TCA. The supernatant was removed by centrifugation and

precipitate dissolved in 10 ml of 0.1N NaOH. 0.2 ml aliquot of this solution was taken and made to 1 ml with 0.1N NaOH. To this solution 5 ml of alkaline copper tartarate reagent were added and mixed well on Vortex mixture. After exactly 15 minutes 0.5 ml of diluted Folin-Ciocalteau reagent was added and mixed well on Vortex mixture. The colour developed in next 30 minutes was measured at 660 nm against blank which consisted of 1 ml 0.1N NaOH, 5 ml of alkaline copper tartarate reagent and 0.5 ml Folin-Ciocalteau reagent.

A standard graph was obtained by developing colour similarly in protein solution containing graded concentrations (10 to 100 µg) of bovine serum albumin.

The protein content of the brain was calculated from standard graph and expressed as grams per 100 grams of fresh brain.

8. Estimation of Deoxyribonucleic acid (DNA) in Brain :

DNA was separated from other tissue compounds by the method of Schneider (1945). Estimation of DNA was carried out by the method of Dische (1930) as described in details by Schneider (1957).

Principle :

The procedure for separation of DNA from other tissue compounds is based on the finding that nucleic acids can be separated by their preferential solubility in hot trichloroacetic acid. The isolated nucleic acids are then quantitated by means of colorimetric reaction involving the pentose components of the nucleic acids with that of diphenylamine. The colour intensity so developed is measured at 600 nm.

Reagents :

1) 5 % Trichloroacetic acid (TCA) : Five grams of TCA (Riedel, W. Germany) were dissolved in glass distilled water and the final volume was made upto 100 ml.

2) 10 % TCA : Ten grams TCA were dissolved in glass distilled water and the final volume made upto 100 ml.

3) 95 % Ethanol : Ethanol was distiled at 78.5°C and collected over sodium. 95 ml of distilled ethanol and 5 ml of glass distilled water were mixed to get 95% ethanol.

4) Diphenylamine reagent : One gram of diphenylamine (BDH, AR) was dissolved in 100 ml of glacial acetic acid

and to this 2.75 ml of concentrated sulphuric acid was added.

5) Standard DNA solution : DNA (NBC, U.S.A.) was used for this purpose. The concentration used was 100 µg/ml in 0.1N NaOH.

Procedure :

A) Separation of DNA from brain homogenate :

1) One ml of 10% brain homogenate in 0.02 M phosphate buffer, pH 7.0 was taken in glass stoppered centrifuge tube. To this solution, 2.5 ml of 10% TCA was added, mixed and allowed to stand for 15 minutes. Then the mixture was centrifuged at 8,000 X g for 20 minutes.

2) The residue was washed twice with 2.5 ml of 10% TCA and the washings were discarded.

3) The residue was suspended in 5 ml of 95% ethanol and allowed to stand for 10 minutes and then centrifuged. One more wash was carried out with 95% ethanol.

4) The residue was suspended in 2.5 ml of 5% TCA and heated at 90°C for 15 minutes, cooled and

centrifuged at 2,000 r.p.m. for 10 minutes.

The supernatant was taken for DNA estimation.

B) DNA estimation :

1) Standard DNA graph : Standard DNA solution was taken at the following concentrations : 20, 40, 60, 80 and 100 µg. The volume was made upto one ml with 5% TCA. To this 2 ml of diphenylamine reagent were added.

2) Brain DNA sample : One ml of supernatant from the processed (as above) brain homogenate was taken in duplicate and to this 2 ml of diphenylamine reagent were added.

After addition of diphenylamine reagent, the tubes were kept in boiling water-bath for 10 minutes. After cooling, the intensity of blue colour is measured at 600 nm on Spectrophotometer (Carlzeiss, PMQ II).

The linear correlation was obtained between the DNA concentration in standard DNA solution and optical density readings. DNA concentration per gram brain was calculated from the optical density reading of the supernatant compared with that of standard DNA solution.

9. Estimation of Thiamine by Thiochrome Method :

For estimating thiamine content in heart, liver and different areas of the brain, thiochrome method was used as detailed by the Association of Vitamin Chemists, Inc. (1966).

Principle :

This method is mainly based on the oxidation of thiamine to thiochrome which fluoresces in ultra violet light. Under standard conditions and in absence of other fluorescing substances, the fluorescence is proportional to the thiochrome present, and hence the thiamine originally present in the solution. In the case of animal tissues, the thiamine is freed from interfering substances by treatment with adsorbents which retain thiamine, but not the impurities. Subsequent elution usually provides an extract sufficiently pure for analysis.

Reagents :

1) Anhydrous sodium sulphate granular.

2) 15 % sodium hydroxide solution : 15 g. of sodium hydroxide were dissolved in glass distilled water and diluted to 100 ml.

3) 1 % Potassium ferricyanide solution : 1.0 g of potassium ferricyanide dissolved in glass distilled water and diluted to 100 ml.

4) Alkaline potassium ferricyanide solution : 5 ml of 1% potassium ferricyanide were diluted to 100 ml with 15% NaOH solution. This reagent was prepared freshly.

5) 0.1N Hydrochloric acid : 8.5 ml Analar grade concentrated HCl were diluted to 1 litre with glass distilled water.

6) 0.1N Sulphuric acid solution : 2.8 ml of Analar grade concentrated H_2SO_4 were diluted to 1 litre with glass distilled water.

7) 2.5 M Sodium acetate solution : 205 g of anhydrous sodium acetate were dissolved in glass distilled water and diluted to 1 litre.

8) Isobutyl alcohol : Analar grade isobutyl alcohol was treated with excess of sodium hydroxide pellets and the aqueous layer was removed by siphoning in the separating funnel. After separation, isobutyl alcohol was shaken with activated charcoal to remove traces of fluoroscence, if any, it was further purified by freshly redistilling using all-glass apparatus and collected at 105-108^{o}C.

9) Enzyme solution : 6 g of Taka Diastase (Bios Laboratories, U.S.A.) were dissolved in 2.5 M sodium acetate solution and diluted to 100 ml with the same solution. This solution was freshly prepared and stored in ice-bath. The enzyme source was found to contain very negligible amount of thiamine, for which proper deductions were made while calculating thiamine content of the tissues.

10) 25 % Potassium chloride solution : 250 g of KCl dissolved in glass distilled water and diluted to one litre.

11) Acid 25% potassium chloride : 8.5 ml concentrated HCl were diluted to 1 litre with 25% KCl solution.

12) Activated decalso : A mixture of equal parts of 30-50 mesh and 60-80 mesh Decalso (E.Merck, West Germany) was used for isolation of thiamine. The entire procedure for activation of Decalso was carried out in a Buchner funnel. To 100 g of Decalso 250 ml of hot 3% acetic acid was added and allowed to remain in contact with Decalso for 15 minutes. Then acetic acid was drained off using mild vacuum. This acid wash was repeated twice. The similar wash was given using hot 25% KCl solution once and followed by hot 3% acetic acid. After draining off the wash solution, the remaining KCl was flushed out with hot glass distilled water till the washings were free of chloride ions as indicated by failure to

give a precipitate with 1 % $AgNO_3$ solution. Chloride free Decalso was dried at room temperature and stored in wide-mouth amber coloured bottle.

13) Stock thiamine solution : Thiamine hydrochloride (WHO Reference Standard) was dried over phosphorus pentaoxide in a dessicator for 24 hours prior to actual use. 100 mg of dried thiamine hydrochloride were dissolved in 25 % ethanol to make final volume of 1 litre. Stored at $4^{o}C$.

14) Intermediate thiamine solution : Five ml of prewarmed (to room temperature) stock thiamine solution were diluted to 100 ml with glass distilled water.

15) Working thiamine solution : 4.0 ml of the intermediate thiamine solution was mixed with 75 ml of 0.1 N H_2SO_4 and 5 ml of sodium acetate solution and the volume was made upto 100 ml with glass distilled water. The final concentration of thiamine was 0.2 micrograms per ml. This solution was freshly prepared.

16) Stock Quinine sulfate solution : Hundred mg of quinine sulphate were dissolved in 0.1N H_2SO_4 and diluted to one litre with the same solution. The solution was stored in dark amber coloured bottle at $8-10^{o}C$.

17) Working Quinine Sulphate Solution : Three ml of stock quinine sulphate solution were freshly diluted to 1 litre with 0.1N H_2SO_4.

18) 3 % Acetic acid : Thirty ml of glacial acetic acid were diluted to 1 litre with glass distilled water.

Procedure :

Glasswares used for this estimation were specially washed with chromic acid, liquid detergent and distilled water. Extra precaution was taken to keep the glassware free from any contamination.

(A) Extraction :

1. Accurately weighed tissue samples, containing approximately less than 30 micrograms of thiamine, were taken in 100 ml conical flasks. To these samples, 75.0 ml of 0.1N HCl were added and heated in boiling water-bath with occasional shaking for 30 minutes.

2. The extracts were cooled to room temperature, pH adjusted to 4.5 with 2.5M sodium acetate solution and 5.0 ml of freshly prepared enzyme suspension was added and incubated at 37°C for 24 hours.

3) The extracts were cooled to room temperature and the volume was made upto 100 ml with glass distilled water. The solution was filtered through Whatman No.1 filter paper.

(B) Purification :

1) All glass columns of 25 ml reservoir capacity with a stem length of 6-8 cm and width of 5-6 mm were used for packing Decalso.

2) Glass wool was cleaned by boiling in mild acidic solution and then by glass distilled water till free of acid. A plug of glass wool was placed at the end of each column.

3) Four to six grams of activated Decalso in water were allowed to settle by gravity reaching upto the lower tip of the reservoir. Decalso was repeatedly washed with glass distilled water and then drained until no water was visible above Decalso.

4) Twenty five ml of sample filtrate were pipetted into the reservoir and allowed to pass slowly through the Decalso. The filtrate coming out of column was discarded. The reservoir and the column were washed successively three times with hot water. The washings were discarded.

5) After washing the column, 10 ml of acid KCl were pipetted in the reservoir and the effluent collected in a 25 ml volumetric flasks. A second 10 ml portion of acid KCl was added and the effluent collected in the same flask. After the second portion drained into the flask, the volume was made upto 25 ml with acid KCl.

6) All above five steps were repeated with a new column using 25 ml of working thiamine solution containing 0.2 micrograms of thiamine per ml in place of the sample aliquot.

7) In the initial stages, enzyme suspension after heating in boiling water-bath for half an hour and filtering through Whatman No.1 filter paper, was treated in the above manner to detect the presence of thiamine.

(C) Conversion to Thiochrome :

1) Five ml of the acid KCl eluate were taken in glass tube (of 50 ml capacity).

2) Three ml of alkaline ferricyanide were added to the above solution and mixed gently.

3) Fifteen ml of isobutyl alcohol were added and the tubes were shaken vigorously for 90 seconds.

4) The above three steps were repeated for working thiamine eluate.

(D) Separation of Thiochrome solution :

The aqueous layer (lower) was siphoned out with the help of a pipette. To the remaining alcohol portion 2-3 g of anhydrous sodium sulphate was added. The tubes were shaken for 30 seconds and allowed to stand until sparkling clear.

(E) Measurement of Thiochrome :

1) The photofluorometer (Coleman, U.K.) was used with respective primary and secondary filters for estimating the fluoroscence. The instrument was allowed to stabilize by keeping it on for 1/2 hour prior to use.

2) The galvanometer deflection was adjusted with working quinine sulphate and isobutyl alcohol. These settings were checked and adjusted in between the readings for samples.

3) Ten ml clear portion of isobutyl alcohol solutions of samples and working thiamine solution were decanted in the matched cuvettes and the fluoroscence was determined in 15 seconds.

(F) Preparation of Blanks :

A second 5 ml aliquot of acid KCl eluate was placed in glass tube and treated it identically except that 15% NaOH was added in place of alkaline ferricyanide. The samples, standard and blanks were run simultaneously.

(G) Calculation :

Thiamine content of the tissue sample was calculated as follows :

$$\text{Micrograms of thiamine per g tissue} = \frac{U - UB}{S - SB} \times \frac{1}{5} \times \frac{25}{V} \times \frac{100}{\text{Wt. of sample}}$$

where,

U = Deflection of unknown.

UB = Deflection of the unknown blank.

S = Deflection of standard.

SB = Deflection of standard blank.

v = Volume of solution used for the adsorption on Decalso.

1/5 = This factor converted the readings to micrograms per ml.

25/V = Since the final volume of eluate was 25 ml this factor corrected for volume changes during adsorption and eluation.

This method was found to give accurate results with a variation of $\pm$ 2.5 % of the mean in a duplicate runs.

The recovery experiment with the addition of known standard thiamine to the sample yielded satisfactory results.

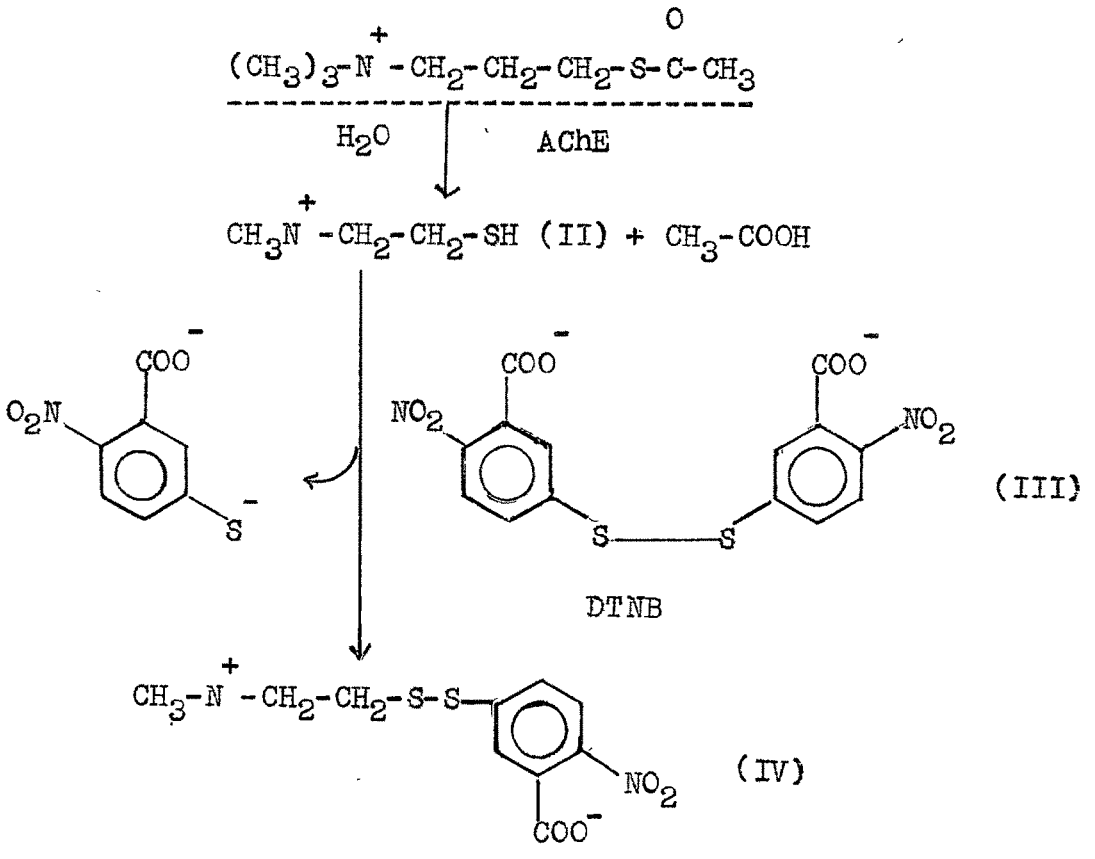
10. Assay of Acetylcholinesterase Activity :

This enzyme assay was carried out by the method of Ellman et al. (1961).

The supernatant obtained at 10,000 g for 30 min. (Spinco, 12,000 r.p.m., rotar No. 30) for 10% brain homogenate prepared in 0.02 M phosphate buffer pH 7.0 was used for the assay. Prior to centrifugation the homogenate was treated for 1/2 hour with Triton-X-100 (Koch-Light, U.K.) at the concentration of 0.5% w/v.

Principle :

Acetylcholinesterase (Acetylcholine hydrolase, E.C. 3.1.1.7) hydrolyzes acetylthiocholine (I), the substrate, into acetate and thiocholine (II); the latter combines with 5,5-dithiobis-2-nitrobenzoate ions (III) to produce the yellow coloured anion of 5-thio-2-nitrobenzoic acid (IV).



The rate of colour production is measured at 412 nm in a spectrophotometer. The reaction with thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme activity and in the concentration used does not inhibit the enzymatic hydrolysis.

Reagents :

1. Phosphate buffer, 0.1M, pH 8.0.

   A. 0.2M solution of monobasic sodium phosphate (27.8 g in 1000 ml).

   B. 0.2M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4 . 7H_2O$ in 1000 ml).

5.3 ml of A were mixed with 94.7 ml of B so as to get pH 8.0 using Metzer pH meter; it was diluted to 200 ml with glass distilled water.

2. Acetylthiocholine iodide (Substrate), 0.075M :

21.67 mg of acetylthiocholine iodide (BDH, U.K.) were dissolved in 1 ml of glass distilled water.

3. 5,5'-Dithiobis-2-nitrobenzoic acid (DTNB),0.01M :

39.6 mg of DTNB (BDH, U.K.) were dissolved in 10 ml phosphate buffer, pH 7.0, 0.1 M and 15 mg of sodium bicarbonate were added.

Procedure :

The actual enzyme reaction was carried out in Quartz Cuvettes at 25°C. The assay system contained :

Phosphate buffer, 0.1M, pH 8.0	...	3.0 ml
DTNB solution, 0.01 M	...	0.1 ml
Enzyme (supernatant at 1000 X g)	...	0.1 ml
Acetylthiocholine iodide,0.075M	...	0.02 ml
		3.22 ml

The blank for such a run consisted of buffer, substrate, DTNB and water. The reaction was started by adding substrate solution and mixing the assay system. The

change in optical density per minute was recorded at 412 nm on the Spectrophotometer (Carlzeiss, PMQ II) with a slit width of 0.8. Wherever necessary, supernatant was diluted with the buffer to obtain the required activity.

The change in the optical density per minute was computed from the graph of OD vs time and used for further calculations as follows :

$$\text{Activity of the Enzyme} = \frac{\mu\text{moles of substrate hydrolysed per minute per g brain}}{} = \frac{\triangle 412/\text{min.} \times 3220 \times 10^6}{1.36 \times 10^4 \times 10^3 \times Co}$$

where,

412/min = Change in optical density at 412 mu/min.

Co = Concentration of the brain homogenate (mg/ml).

11. Assay of Cholineacetyl Transferase Activity :

The activity of choline acetyltransferase (choline acetylase, acetyl CoA-choline O-acetyltransferase, ec: 2,3,1.6) was measured by the radioactive method of Fonnum (1966b).

Principle :

The rate of conversion of preformed ^{14}C-acetyl CoA to ^{14}C-acetylcholine by this enzyme was estimated. The preformed ^{14}C-acetyl CoA was prepared in the assay system by using the acetone dried preparation of pigeon liver and ^{14}C-acetate. From the assay system ^{14}C-acetylcholine was selectively precipitated by sodium tetraphenylborate and processed further for counting the radioactivity on liquid scintillation system.

Reagents :

1. Enzyme preparations : Acetone-dried pigeon liver was prepared essentially by the method of Kaplan and Lipmann (1948). The activity of this preparation was estimated by the method of Lipmann and Truttle (1958) and found to be of the order of 1 µmole of acetyl CoA formed per hour per 70 mg of acetone-dried powder. Accordingly, the aliquot of the preparation was taken for further procedure.

The powder was distributed in number of screw capped test tubes and stored in the desiccator at -20°C.

b) Brain homogenate : 10% homogenate of the whole brain or the regions of the brain was prepared in 0.02 M

phosphate buffer (pH 7.0). The homogenates were treated with Triton X-100 at the level of 0.5 % (w/v) and used for estimation after 1/2 hour.

2. Assay system :

a) Sodium acetate, 0.2M, 1-^{14}C-acetate 350,000 cpm : 1.640 g of sodium acetate were dissolved in glass distilled water. To this 1-^{14}C acetate (B.A.R.C.) with the specific activity of 47.6 mCi/m mole was added in such an amount so as to give final counts of 350,000 cpm per 0.1 ml after dilution of the solution to 100 ml.

b) Choline chloride, 0.2M : 2.792 g of choline chloride (anhydrous, preserved under vacuum) were dissolved in glass distilled water and diluted to 100 ml.

c) Sodium chloride, 5 M : 29.2 g of sodium chloride were dissolved in glass distilled water and the volume made upto 100 ml.

d) Potassium chloride, 0.1 M : 0.745 g of potassium chloride were dissolved in glass distilled water and the volume made upto 100 ml.

e) Sodium fluoride, 0.1M : 0.419 g of sodium fluoride were dissolved in glass distilled water and the volume made upto 100 ml. This solution was freshly prepared.

f) Potassium borohydride, 0.025M : 0.53 g of potassium borohydride (BDH, U.K.) were dissolved in glass distilled water and the volume was made upto 100 ml. This solution was freshly prepared.

g) Coenzyme-A, 0.0025 M : 2.02 mgms of Coenzyme-A, sodium salt, grade I (Sigma, U.S.A.) were dissolved in one ml of glass distilled water. This solution was freshly prepared.

h) Adenosine-5'-triphosphoric acid, 0.4 M : 221 mg of ATP (Disodium salt, E.Merck, West Germany) were dissolved in one ml of glass distilled water. This solution was freshly prepared.

i) Eserine sulphate, 0.0025 M : 6.5 mg of eserine sulphate (Sigma, U.S.A.) were dissolved in one ml of glass distilled water.

3. Hydroxylamine hydrochloride, 1M, pH 6.5 : 6.949 g of hydroxylamine hydrochloride were dissolved in glass distilled water and pH adjusted to 6.5 with 1.0 N sodium hydroxide. The final volume was made upto 100 ml. This solution was freshly prepared.

4. Trichloroacetic acid (TCA), 100 % w/v : 100 g of TCA (Riedel, W.Germany) were dissolved in glass distilled water and made up the final volume to 100 ml. To this solution 666.6 mg of o-acetylcholine perchlorate were added to obtain the final concentration of 2 mg per 0.3 ml.

5. Sodium tetraphenyl borate, 0.1 M : 342.23 mg of sodium tetraphenyl borate (E.Merck, W.Germany) were dissolved in 10 ml of glass distilled water at the time of actual use.

6. Ethanol : Ethanol was freshly distilled at 78.5°C and collected over sodium.

7. Ether : Ether was freshly distilled at 34-35°C and collected over sodium.

8. Benzyl alcohol : Benzyl alcohol was freshly distilled at 202-206°C and collected over sodium or activated sodium sulphate.

9. Acetonitrile : Acetonitrile was freshly distilled at 80-82°C and collected over sodium or activated sodium sulphate.

10. Scintillator : Freshly distilled toluene at 110°C was used. One litre of toluene contained -

a) 5 g (0.5 %, w/v) 2,5-Diphenyloxazole (Koch-Light, U.K.) ;

b) 0.2 g (0.02 %, w/v) 1,4-bis-(4-methyl-5-phenyl-oxazol-2-yl)benzene (E.Merck, W.Germany).

Procedure :

The incubation mixture contained :

Solution	Molarity M	Volume ml	Concentration mM
1. Sodium acetate	0.2	0.1	8.0
2. Choline chloride	0.2	0.1	8.0
3. Sodium chloride	5.0	0.1	200.0
4. Potassium chloride	0.1	0.1	4.0
5. Sodium fluoride	0.1	0.1	40.0
6. Potassium borohydride	0.025	0.05	0.5
7. Co-A	0.0025	0.05	0.05
8. ATP	0.4	0.1	16.0
9. Eserine sulphate	0.0025	0.1	0.1
10. Pigeon liver (Acetone powder)*	-	-	70 mg/ml
11. Glass distilled water - to adjust the final volume to 2.5 ml			

* Pigeon liver acetone powder was either added as such or an appropriate extract in cold 0.05M potassium bicarbonate and 10,000 X g supernatant was used as source for acetate activating enzyme. The activity by either of the methods was not found to be rate limiting.

Before incubation, the assay system was adjusted to pH 7.2 with dilute sodium hydroxide solution using Metzer pH meter. The assay system was incubated for 10 minutes at 37°C with slow shaking (Techno, water-bath with shaking speed of 6-8 strokes per minute). For this purpose, glass stoppered centrifuge tubes with 15 ml capacity were used. In some cases the estimation was carried out on micro-scale with same concentrations of all the components with a final volume of 0.25 ml. For the micro-assay, Eppendorf centrifuge tubes of 1.8 ml capacity were used.

At the end of 10 minutes of incubation, brain homogenate was added and the incubation was continued for 60 minutes. Boiled enzyme (for 15 minutes in boiling water-bath and cooled) served as a control.

At the end of 60 minutes of incubation, the reaction was stopped by adding 1.0 ml of M-hydroxylamine hydrochloride adjusted to pH 6.5. Then the tubes were placed in ice-bath, cooled for 10 minutes and 0.3 ml of 100 % TCA (w/v) containing 2 mg of acetylcholine perchlorate was added.

In the next step, the mixture was centrifuged and 3.0 ml of clear supernatant were added to 1.0 ml of aqueous 0.1M sodium tetraphenyl borate in glass stoppered centrifuge

tubes. The precipitate that formed after one hour was collected by centrifuging at 8,000 X g for 10 minutes. The pellet was washed twice by resuspending in 0.3 ml of ethanol and then adding 10 ml of ether.

Finally, the pellet was transferred to the scintillation vial by dissolving it in 3.0 ml of acetonitrile and 3.0 ml of benzyl alcohol. To this 10 ml of scintillator fluid was added. The radioactivity was counted at 2,500 V with window setting 7.5, 0.5 and 3.2, 0.5 in ECIL (LSS34) liquid scintillation counter. Under these conditions the counting efficiency was 75 %.

For the calculations of the enzyme activity the counts per minute were taken into account after deducting the control values, adjusting for % efficiency and chemical quenching; cpm were converted to μ-moles of ACh formed per hour per gram of brain.

12. Estimation of Brain Acetylcholine (ACh) :

ACh levels in the brain were estimated using the rectus abdominis muscle of the frog as an isolated tissue preparation. The method is essentially the same as detailed in "Pharmacological Experiments on Isolated preparations" (by the staff of the Department of Pharmacology, University

of Edinburgh, E & S Livingstone, London, 1970).

Principle :

The rectus abdominis muscle of the frog responds to acetylcholine by giving slow contracture. This contraction is proportional to ACh concentration in the organ bath. With the help of levers, the contraction is magnified and recorded on the kymograph. The unknown sample is assessed for the response and from the heights of the known standards, ACh concentration in the unknown is calculated.

Reagents :

1. Clark-Frog-Ringer solution : One litre of solution contained the following :-

Sodium chloride (g)	....	6.5
Potassium chloride (g)	....	0.14
Calcium chloride (g)	....	0.12
Sodium dihydrogen phosphate (g)	....	0.01
Sodium bicarbonate (g)	....	0.2
Glucose (g)	....	2.0
Eserine sulphate (mg)	....	0.10

2. Standard Ach solution : Acetylcholine bromide (BDH, U.K.) was used as a reference standard. A 10 µg/ml ACh bromide was freshly prepared in glass distilled water.

3. 10 % TCA : 10 g of trichloroacetic acid dissolved in water and diluted to 100 ml.

4. Fixing solution for Kymograph : Commercially available gum resin, 500 g, was dissolved in a mixture of methanol and ethanol (1:1).

Procedure :

A) The frog rectus abdominis preparation : Frogs weighing roughly 20-40 g were kept in tap water for 3-4 weeks without feeding. The frog was stunned and the spinal cord was destroyed by inserting a needle through cervical vertebrae. The frog was pinned out on a dissecting board and the skin of the abdomen was cut from above the sternum down to the fork. The skin was then laterally cut and the abdomen muscles were exposed and washed with frog-Ringer solution. The rectus muscles were freed from the xiphisternum and detached with sharp scissors. The muscles were transferred to a petridish containing frog-Ringer solution and dissected out through the midline.

Two threads were sutured through the each end of the rectus muscle. The lower end of the muscle was tied to the pin in the organ-bath and other one to the lever. One gram of load was placed on the lever to relax the tissue for

half an hour. The tissue was aerated by air-bubbles released in Frog-Ringer solution in the organ bath.

The capacity of the organ bath used was about 15-20 ml. It had a three way outlets for draining, filling and bubbling the solution by ordinary fish-tank aerater. Responses were taken by a frontal writing lever on a smoked kymographic paper. The lever was perfectly balanced and gave an approximate 10-fold magnification. The aerater itself served as a suitable vibrator.

B) Standard Responses : After relaxation for 30 minutes, the 1 g load was released from the lever and the base line was recorded on the kymograph for 2 minutes. This was followed by the addition of known amount of ACh by syringe into the middle of the organ-bath. The response was recorded for 2 minutes and the motor of the kymograph was switched off. The solution was drained out and replaced by fresh frog-Ringer. The tissue was washed thrice with Frog-Ringer and allowed to stretch for 10 minutes by placing the one gram load on the lever. The same cycle of operations was repeated with the different concentrations of ACh.

C) Extraction of Brain ACh : The method of Toru and Aprison (1966) was used for extraction of ACh from the brain.

After the rat was decapitated, the brain was quickly removed, weighed and transferred to the mortar containing ice-cold 10% TCA. The mortar was kept chilled in ice-salt mixture. The brain was homogenized for 20 minutes, the volume recorded and centrifuged at 10,000 X g for 30 minutes. The supernatant was adjusted to pH 3.6 with 1N NaOH and heated in a boiling water bath for 10 minutes. After cooling, the supernatant was divided into two parts. In one part, the pH was adjusted to 7.0 with 1N NaOH and this solution was used as a test solution. The other half was adjusted to pH above 10 with 5N NaOH and brought back to 7.0 with 5N H_2SO_4. This solution was used as a control

D) Responses of the Test solution :

After recording the responses of different concentrations of known ACh, the known aliquot of the test solution was added into the organ-bath and the response was recorded for two minutes. This was followed by a response of the standard solution to give approximately same response as that of the test solution. The response of the test solution was repeated, followed by that of the control solution. The latter evoked no response.

The above method was slightly modified so as to facilitate handling of large number of samples. Instead of

conventional method followed in evaluation for drug testing by T_3 or T_4 assay the test response was followed by the standard response, the control response and then by the next test response and so on. The results of this modification were quite comparable to that of T_3 or T_4 assay.

E) Calculations : The semilogarithmic graph of the heights of response vs ACh concentration was plotted. The concentration of ACh in the brain homogenate was calculated from the response of the test solution by comparing the logarithmic height on the graph of standard response vs. ACh concentration.

13. Measurement of rate of Synthesis of ACh in vivo :

The method used for this purpose was essentially the same as described by Schuberth et al. (1969). This method was slightly modified to suit our laboratory conditions.

Principle :

The labelled choline (or acetate) was injected intravenously, the rats were killed at different time intervals,

brains were homogenized in cold TCA and the homogenate was processed so as to get renickates of ACh and Ch; on chromatographic separation the inrorporation of label into ACh and Ch was estimated using liquid scintillation counter.

Reagents :

1. Sodium acetate 1-^{14}C, sp.activity : 46.15 mCi/mole, purchased from Isotope Division, B.A.R.C., Bombay.

2. 3H(Methyl)choline chloride, sp.activity, 10.1 Ci/m mole; radiochemical purity, 98%; purchased from the Radiochemical Centre, Amersham, England.

3. 7% Trichloroacetic acid : 7 g of TCA dissolved in glass distilled water to the final volume of 100 ml.

4. Saturated ammonium renickate solution in glass distilled water, freshly prepared at the time of actual use.

5. Ether : Freshly distilled over sodium in vacuo.

6. Acetone : Water (1:1 v/v).

7. Dowex 2-X 8 in chloride form, packed in a column of 7 X 80 mm.

8. n-Butanol:Ethanol:Water (5:1:4), freshly prepared.

9. Scintillation solution : 50 g naphthalene, 7 g PPO, 150 mg POPOP, 50 ml ethanol, 100 ml toluene to make final volume to 1000 ml with dioxan. For aqueous solutions/ suspension sample the above scintillation solution was used with thixotrophic gel (CAB-O-SIL) 40 g/L.

Procedure :

As mentioned earlier, the original method was suitably modified. One of the modification was the reduced dose of labelled precursors used. 40 µCi each of 3H(methyl)-choline and (1-^{14}C acetate) were injected intravenously through tail vein. A special care was taken to standardise this method using trial injections of neutral dye on discarded animals. Rats were sacrificed by decapitation and the brain quickly removed and transferred to mortar containing ice-cold 7 % TCA containing 4 µmoles of each unlabelled choline and acetylcholine. The brain was thoroughly ground to get a homogenous liquid paste which was transferred to a pre-weighed centrifuge tube. The tubes were then weighed again to determine the actual weight of the brain. By this method of determining the brain weight, the time required was considerably reduced so as to minimize post-mortem changes in concentration of ACh and Ch. The tubes were left in the refrigerator for 30 ' after which they were centrifuged at 12,000 X g at 4^{o}C for 5 minutes in Spinco refrigerated centrifuge. The

supernatant was collected and the pellet was re-extracted with cold 7 % TCA without added carrier. The tubes were then centrifuged at 12,000 X g for 5 minutes. Both the supernatants were combined and adjusted to pH 4.0 using 1N NaOH. A known aliquot of the extract was removed to determine the total radioactivity in the brain. It was extracted six times with 3 ml portion of solvent ether to remove the TCA. To remaining sample 2 ml of freshly prepared saturated solution of ammonium renickate was added. The precipitate formed were separated by centrifugation and dissolved in minimum quantity of acetone:water (1:1 v/v). This was applied to a column of anion exchanger Dowex 2X 8 Cl^- (70 X 8 mm) and eluted with acetone-water (1:1 v/v) mixture. The first 7 ml of the eluate were collected and evaporated to dryness in vacuo. The residue was dissolved in 0.1 -0.5 ml of water. Choline and acetyl choline were separated by circular paper chromatography on Whatman No.1 filter paper. The chromatograms were developed using the solvent system n-butanol-ethanol-water (5:1:4 v/v). Choline and ACh were visualized on the chromatograms by exposing them to iodine vapours. The typical separation of the two is given in Figure-5 (schematic), which consist of ACh moving faster than Ch.

After visualizing the spots were marked and cut and put at the bottom of counting vial. To this 10 ml of scintillation fluid was added and radioactivity in each was

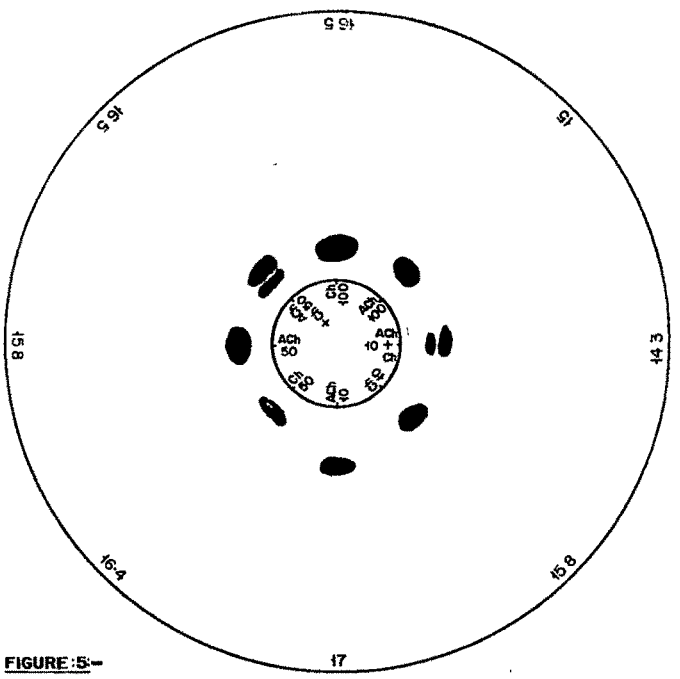


FIGURE:5:-

CIRCULAR CHROMATOGRAPHY FOR SEPARATION OF ACETYLCHOLINE AND CHOLINE FROM BRAIN EXTRACTS.

(SOLVENT SYSTEM: n-BUTANOL: ETHANOL: WATER, 5:1:4)

determined using liquid scintillation counter. For aqueous suspension sample, thixotrophic gel in naphthalene dioxan scintillation solution was used and others either naphthalene dioxan or toluene scintillation solutions were used. The necessary corrections were applied for volume changes, quenching and spill-over.

14. Statistical Treatment :

All the results are expressed as Mean ± S.E.M. The data were processed for this purpose by the statistical treatment as described by Armitage (1971) and based on the t- tables of Fisher and Yates (1963).