CHAPTER - II

EXPERIMENTAL

Materials.

The following materials were used in this study without further purification.

Hen egg white lysozyme (3X Crystalline lot Nos. 110B-078, 15B-8530) from Sigma Chemical Company; Trypsin (3X crystalline) from Nutritional Biochemicals; FDNB (A grade Calbiochem; m.p. 25-26°); Urea (E. Merck); GU-HCl (Fluka); NAG (Koch-light).

Standard DNP-amino acids from Mann Laboratories.

High specific activity FDNB-UL^{14}-C (1 mc/7.75 µg) from N.V. Philips.

Tyrosine-glutamic acid polypeptide $(1:12)_{n=48}$ (Poly, Tyr-Glu) was a gift from Dr. Littauer of Weizman Institute, Israel. Lyophilized M. lysodeikticus cells from Worthington Biochemical Corporation (C606-23); CM-cellulose or CM-cellex from Calbiochem; Sephadex G-100 and G-200 from Pharmicia-Uppsala, Sweden; IRP-64 Carboxylic resin from Rohm and Haas, Philadelphia, U.S.A.; Resin of particle size -200, +350 mesh was used.

N, N, N', N' - Tetramethylethylene diamine (TEMED) Batch No. 3682; and acrylamide and methylene bisacrylamide from Canalco Industries Corporation, Rockville, M.D., USA. Starch hydrolysed (for gel electrophoresis) from Connaught Medical Research Laboratories; University of Toronto, Canada (Lot No. 271-1); Aluminium lactate was prepared according to the method

of Jones (137).

Methods.

Dinitrophenylation:

Dinitrophenylation of lysozyme was done at two pH values namely 7.2 and 9.1. Lysozyme solution in distilled water was first dialysed for 24 hrs at 4° against distilled water, the outside solution being stirred with a magnetic stirrer. For experiments at pH 7.2, the following procedure was used - Lysozyme (0.2 - 2.5 μ mole) in 2.0 ml of 0.02 M tri-ethanolamine-HCl buffer pH 7.2 was allowed to react with varying equivalents (mole/mole 100-600) of FDNB in 0.5 ml of 95% ethanol (final concentration of ethyl alcohol 19%) at 15° or 26° for varying intervals of time. Experiments at pH 9.1 were done as follows - Lysozyme 0.2 - 1.0 μ mole in 2 ml of 0.1 M sodium carbonate-bicarbonate buffer, pH 9.1 was allowed to react with varying equivalents (mole/mole 18-312) of FDNB in 0.5 ml of 95% ethanol (final concentration of ethyl alcohol 19%) at 15° or 26° for varying intervals of time.

The reaction mixture was stirred magnetically and was protected from light by wrapping the reaction vessel with black paper. At the end of the required interval of time the mixture was cooled in an ice bath. Unreacted FDNB was removed by extraction several times with eight volumes of ether, till the extract was colorless. The aqueous phase was then acidified with 0.1N HCl (pH between 3 and 4) and extracted again as above until the ether extract was colorless (138). A control sample

of lysozyme was subjected to the same treatment without the addition of FDNB. Alcohol neither precipitated the protein nor had any effect on its enzyme activity. The DNP-protein solution was diluted with the appropriate buffer for physico-chemical studies or dialysed against water for DNP-amino acid analysis, heat stability experiments and denaturation studies.

Determination of the extent of Dinitrophenylation.

The extent of dinitrophenylation of the protein was estimated by measuring O.D. at 360 mμ (133, 138) in 0.2M acetate buffer pH 5.4, and using a molar extinction coefficient of 17,600 for DNP group (133, 134).

For the determination of the number of DNP-groups taken up per molecule of protein, FDNB (UL-^{14}C) (1 μc/7.75 μg) was used along with the unlabelled FDNB in the dinitrophenylation described above (133).

In control experiments the same amount of unlabelled FDNB was used and dinitrophenylation carried out for the same interval of time. At the time of extraction of unreacted FDNB with ether the same amount of ^{14}C labelled FDNB was added and extracted with ether as described above.

An aliquot of the acidified aqueous protein solution was plated on a stainless steel planchet, dried and counted in a Tracer lab counter with a counting efficiency of 6% for ^{14}C. The measurements were made with both the experimental and control samples.

Protein Concentration.

Native lysozyme concentration was determined both by Folin's (139) method and by spectrophotometric method by measurement of the absorbance of the solution at 280 mμ in a 1 cm cell and using a value of 26.35 for the absorbance of 1% solution at 280 mμ in acetate buffer of pH 5.4 and 0.2M (140). The optical densities were read on Hilger and Watts ultraviolet spectrophotometer.

The concentration of the DNP-lysozymes was determined only by Folin's method since DNP group interferes in absorption measurements at 280 mμ. It has been shown that DNP does not interfere in the protein estimation by Folin's method (130, 133).

Determination of enzyme activity.

Lysozyme activity was measured by the following methods (141).

An aliquot of 3.9 ml of lyophilised M. lysodeikticus cells (0.3 mg/ml) suspended in 0.1M phosphate buffer pH 6.8 was brought to 37° and 0.1 ml of the enzyme solution containing 0.5 - 4.0 μg of native or DNP-lysozyme in the same buffer was added. The mixture was then incubated for 10 min at 37° in a shaking water bath. At the end of the incubation period the tubes were cooled in an ice bath and the O.D. of the suspension was measured at 450 mμ in a Unicam spectrophotometer. The control had the cell suspension in the same volume of buffer without enzyme and was incubated simultaneously. The difference in O.D. between the control and the experimental was taken as a measure of the lytic activity of enzyme. The specific enzyme activity of 0.10 O.D. difference is equivalent

to 100 units.

The rate of lysis was measured in the following way. The cell suspension (3.4 ml) was pipetted into a spectrophotometer cell of 1 cm optical path and the spectrophotometer was set at 450 mμ. The enzyme solution (0.1 ml) containing 1.8 - 10 μg of the enzyme in the same buffer was added and the O.D. read every 30 seconds till 5 or more min. The control was 3.4 ml of the cell suspension containing 0.1 ml of the buffer.

Effect of pH on enzyme activity.

A suspension of M. lysodeikticus cells (0.3 mg/ml) in different buffers at different pH's was taken. Enzyme assay was carried out as described above at 37° for 10 min. The following buffers were used: for the pH range 5.0 - 8.0, phosphate buffer; for pH 6.8 - 9.6 Veronal-HCl; for pH 8.0 - 10.0 diethanol amine-HCl; for pH 8.6 - 10.0 glycine-sodium hydroxide.

Effect of molarity of buffer on enzyme activity.

With 0.3 mg/ml suspension of substrate, M. lysodeikticus, in different buffers of varying molarity, the enzyme activity was measured both at 25° and 37°.

Assay of enzymatic activity in the presence of inhibitors.

The cell suspension in 0.1 M phosphate buffer, pH 6.8, containing the appropriate concentration of the inhibitors, NAG or Poly Tyr-Glu was used for assay at 25° and 37°.

The effect of denaturing agents on the enzyme activity.

(a) When enzyme activity was examined in the presence of 4 M urea, the cell suspension was prepared in 0.1 M phosphate buffer, pH 6.8 containing 4M urea and used immediately for assay. When the reversible effect of denaturing agents on activity was determined the enzyme (0.5%) was left in contact with 4M urea or 2M Gu-HCl solution in 0.1M and 0.03 M phosphate buffer (pH 6.8) respectively at 25° for varying intervals of time. It was then diluted with the buffer so that the final concentration of the denaturing agent was negligible and the activity measured as described above. (b) The effect of urea and GU-HCl on the enzymic activity of lysozyme dissolved in buffers of different molarity was also measured immediately after preparation of substrate in denaturing agent.

Heat Stability.

Heat stability of native and DNP-lysozyme was determined in the following way. Aliquots of enzyme solution (0.21%) in distilled water were heated for 10 min at various temperatures from 40-90°. At the end of the heating period the solutions were chilled immediately, diluted with 0.1 M phosphate buffer, pH 6.8 and enzymatic activity determined at 37°.

Absorption spectra of N-lysozyme and DNP-lysozyme.

Known concentrations of N-lysozyme and DNP-lysozyme were taken in acetate buffer (0.2M, pH 5.4) or in phosphate buffer (pH 6.8, 0.1M) and O.D. in the U.V. range (220-350 mμ) was measured in a Hilger U.V. spectrophotometer model H-700 and in the range

350-420 mμ in a Unicam spectrophotometer.

Ultracentrifuge experiments.

(a) Sedimentation velocity (142):- The measurements were made with a Spinco model ultracentrifuge fitted with phase plate schlieren optics. The experiments were made at room temperature (25°). Solutions (0.5 - 1.0%) were used. Sedimentation velocity experiments were conducted at the maximum speed, 59,780 rpm. Pictures of the sedimentation velocity patterns were taken at different time intervals of centrifugation on Kodak metallographic plates. The plates were measured with a Gaertner microcomparator which has a two way movement. From the value of X obtained as a function of time, plots of lnx vs t were made and the sedimentation coefficient calculated with the equation

$$S = \frac{dx/dt}{w^2 x}$$

Here w is the angular velocity in radians per sec and is given by the equation

$$w^2 = (2\pi \text{ rps})^2$$

where rps is the number of revolutions per second. The calculated sedimentation coefficient, S, was reduced to the standard value of $S_{20,W}$ with the equation

$$S_{20,W} = S \frac{\eta}{\eta_{20,W}}$$

Here η is the viscosity of the solvent at the temperature of the experiment, and $\eta_{20,W}$ is the viscosity of water at 20°.

(b) Molecular weight determination. Molecular weight was determined by the Archibald method (142). A standard 12 mm 4° sector duraluminium cell centre piece was used. 0.1 ml of fluorocarbon oil (Fc 43) was used to create a false bottom and this obviates certain optical difficulties associated with the location of the true cell bottom. It was observed that the fluorocarbon oil did not cause precipitation of the protein and thus had no deleterious effect on the protein. The cell was then centrifuged at a known constant speed and the schlieren photographs were taken at regular intervals of time. The bar angle was kept at 80°. During the experiment the rotor was maintained at 25° ± 0.1° with the RTIC unit. The temperature of the rotor was also measured during the run.

The photographic plate was then measured with Gaertner micro comparator. The equation for the calculation of molecular weight used was

$$M = \frac{RT\ dc/dx}{(1-\bar{V}\rho)\ W^2 x\ C}$$

where M is the molecular weight; R, is the gas constant (= 8.3144×10^7 ergs per degree per mole); T, the absolute temperature; $\bar{V}$, the partial specific volume of protein; ρ, the density of the solution, W, the angular velocity; X, the radius of rotation corresponding to the top or the bottom of the liquid column; dc/dx the concentration gradient at the top or the bottom and C, is the total concentration.

This equation which is the same as the sedimentation equillibrium equation, is valid at only two planes, namely, the

top (air-liquid meniscus) and the bottom (liquid-fluorocarbon meniscus).

The schlieren optical system used in the model E ultracentrifuge registers directly a quantity proportional to the concentration gradient, $dc/_{dx}$. The corresponding concentration C is obtained in terms of original solute concentration, C_o with the following equations.

$$C_{x_o} = C_o - \frac{1}{x_o^2} \int_{x_o}^{x} x^2 \cdot \frac{dc}{dx} \cdot dx$$

$$C_{x_b} = C_o + \frac{1}{x_b^2} \int_{x}^{x_b} x^2 \cdot \frac{dc}{dx} \cdot dx$$

where X_o and X_b are the radius corresponding to the top and the bottom meniscus respectively and X is the radius of any plane in the 'plateau-region' where $dc/_{dx} = 0$.

The determination of C_o in terms of refractive index units was made by doing a separate experiment with a synthetic boundary cell using a cell centre piece of the type described by Kegeles(142) or of the 'Filled-Epon' capillary type (Spinco part No. 5094). The solution and solvent (buffer solution) are filled into separate compartments in such a cell. Solvent layers itself on the solution without convective mixing when the cell is centrifuged at low speed. The schlieren pattern of such a boundary was photographed at the same bar angle (namely 80°).

The values of $dc/_{dx}$ with the concentration boundary were read at regular intervals, say 0.025 cm. The sum of these values

multiplied by 0.025 gives a numerical value proportional to C_o. In the Archibald pattern, $dc/_{dx}$ values were read at 0.025 cm intervals starting from top meniscus to the 'plateau' region. The same procedure was repeated from 'plateau' region to the bottom meniscus. The distance of the mid point of the cell to the axis of rotation in the ultracentrifuge is 6.5000 cm. Knowing the magnification factor of the optical system the true radius corresponding to the top and bottom meniscus of the liquid column and also other points where $dc/_{dx}$ values are read can be calculated. The RHS of the equations can be computed with these values.

At either of the two radii X_o or X_b, $dc/_{dx}$ values cannot be read with precision directly on the micro-comparator because of optical distortion. The values at these menisci have to be obtained by an extrapolation procedure. To avoid uncertainty in extrapolation of steep gradients, experiments were carried out at moderate speeds and setting up of large gradients was avoided.

For the calculation of molecular weight using equation the partial specific volume of lysozyme has been assumed to be 0.722 (72).

Column Chromatography.

Gel filtration.

Sephadex G-100 or G-200 was allowed to swell by soaking in excess of distilled water for 3 days. The fines were removed by repeated washing and decantation. The gel was then equilibrated with the appropriate solution (0.1 M acetic acid or 0.1M potassium nitrate); equilibration being achieved by washing

with several changes of solution. The gel in the form of a slurry was poured into a glass column and allowed to settle under gravity. The column was washed repeatedly with the solvent solution till the column height was constant. A filter paper circle placed on the top of the gel column protected the top surface from disturbance during sample application. Protein solution containing a known amount of the protein was loaded on the column and allowed to be absorbed. The column was then eluted with the solvent solution and 2 ml fractions of the eluent were collected on a Gilson Medical Electronics (GME) automatic fraction collector. The runs were made at 25°.

The O.D. of the fractions were read at 280 mμ for protein and 360 mμ (DNP group) using 1 cm cell after dilution of the fractions to 3.5 ml with 0.1M acetic acid or 0.1M potassium nitrate. The gel filtration pattern was obtained by plotting optical density versus fraction number. The specific enzymic activity of fraction was also measured.

Chromatography on CM-cellulose.

CM-cellulose was soaked in water and fines removed. It was then treated with 0.5N sodium hydroxide and washed free of alkali (as tested with phenolphthalein). It was then regenerated with 0.5N HCl and washed free from HCl (as tested for chloride ion). The regenerated CM-cellulose was equilibrated with 0.05M sodium acetate buffer pH 5.4 and then packed under gravity into a column. On the column the resin was washed 2-3 times with the same buffer. A known amount of the protein in acetate buffer was

loaded on the column and absorbed. The elution of the protein was done using a linear salt gradient as follows. The reservoir contained 190 ml of acetate buffer of pH 5.4 and 0.05M containing 0.45M sodium chloride and the mixing chamber contained 190 ml of acetate buffer of pH 5.4 and 0.05M. Thorough mixing in the chamber was achieved by using a teflon-coated magnetic stirring bar with a magnetic stirror. 3 ml fractions were collected and O.D. of the fractions were measured at 280 mμ and 360 mμ.

Chromatographic experiments were also done using both salt and pH gradients with phosphate buffer. The regeneration and packing of the resin was done as described above. On the column the resin was washed 2-3 times with the starting sodium phosphate buffer, pH 6.8 and 0.05M. The reservoir contained 100 ml of sodium phosphate buffer, 0.2M pH 8.0 and the mixing chamber contained 100 ml of 0.05M pH 6.8. 3 ml fractions were collected and the O.D. of the fractions measured at 280 mμ and 360 mμ. Thorough mixing was obtained in the mixing chamber by using a teflon coated magnetic bar and a magnetic stirrer. Chromatographic runs were done at 25°.

Chromatography on Amberlite IRP-64.

The method of Tallan and Stein (143) was followed for the preparation of resin. Resin of particle size -200, +350 mesh was used. The fines were removed by repeated decantation after stirring. The regenerated resin IRP-64 was equilibrated with 0.2M sodium phosphate buffer pH 7.18 and then packed under gravity into a column 1.1 x 28 cm. The resin was washed on the column

5-6 times with the same buffer until the pH of the inflowing buffer and the buffer emerging out of the column had the same pH value. A filter paper circle placed on the top of the resin column protected the top surface from disturbance during sample application. Protein solution was dialysed against the same buffer which was used for elution at 4° for 24 hrs. The dialysed protein solution containing a known amount of protein was loaded on the column and allowed to be absorbed. The column was eluted with the same buffer (0.2M sodium phosphate buffer pH 7.18). 2 ml fractions of the eluant were collected on a Gilson Medical Electronics (G.M.E.) automatic fraction collector. The flow rates were maintained at 6-8 ml/hr. The runs were made at 25°.

The O.D. of the fractions were read at 280 mμ for protein and 360 mμ (DNP-group) using cells of 0.5 cm optical path. The protein content of the fractions was measured by Folin method (139). The specific enzyme activity of fractions was also measured. The chromatographic pattern was obtained by plotting O.D. versus fraction number. The resin was regenerated by washing the column with 0.2M sodium phosphate buffer pH 7.18 for the repeated runs.

Disc electrophoresis with polyacrylamide gel.

This technique permits the resolution of mixtures of proteins or peptides with samples as small as 50 μg within a short time. This method achieves this by concentrating the components of dilute samples into thin starting zones and by utilising the frictional properties of the gel to aid separation by molecular sieving and charge separation. Disc electrophoresis was carried out by the method of Ornstein and Davies as modified by Reisfeld et al (144, 145).

It was performed in small columns of polyacrylamide gel consisting of two sections (1) a large pore gel in which electrophoretic concentration of protein takes place and (2) a small pore gel in which electrophoretic separation of protein is accomplished.

Preparation of the column and electrophoresis of the sample.

The following stock solutions were prepared and used for pH 8.9 gels.

A)	1N HCl	..	4.8 ml
	Tris	..	3.66 g
	TEMED	..	0.023 ml
	Water to (pH 8.9)	..	10.0 ml
B)	1N HCl	..	4.8 ml
	Tris	..	0.598 g
	TEMED	..	0.046 ml
	Water to (pH 6.7 - 6.8)	..	10.0 ml
C)	Acrylamide	..	6.0 g
	Bis-acrylanide	..	0.04 g
	Water to	..	10.0 ml
D)	Acrylamide	..	1.0 g
	Bisacrylamide	..	0.25 g
	Water to	..	10.0 ml
E)	Riboflavin	..	4.0 mg
	Water to	..	100 ml

F) Sucrose .. 40 g

Water to .. 100 ml

Working solutions.

Small pore gel		Large pore gel	
Small pore solutions		Large pore solution	Large pore solution for washing
I	II		
1 part A	28 mg	1 part B	1 part B
2 parts C	Of ammonium presulfate to 10 ml of water	2 parts D	1 part E
1 part water		1 part E	6 parts water
		4 parts F	
(pH 8.9)		(pH 6.7 - 6.8)	
Mixed equal volumes of I and II solutions			

The following stock solutions were prepared and used for pH 4.3 gels.

A) N KOH .. 4.8 ml

Glacial acetic acid.. 1.72 ml

TEMED .. 0.4 ml

Water to .. 10 ml

(pH 4.3)

B) N KOH .. 4.8 ml

Glacial acetic acid.. 0.287 ml

TEMED .. 0.046 ml

Water to .. 10 ml

(pH 6.7 - 6.8)

Solutions C, D and E are same as in pH 8.9 gels.

Working solutions.

Small pore gel Small pore solutions		Large pore gel Large pore solution
I	II	
1 part A 2 parts C 1 part water	28 mg of ammonium persulfate to 10 ml of water	1 part B 2 parts D 1 part E 4 parts water
(pH 4.3)		(pH 6.8)
Mixed equal volumes of I and II solutions.		

Composition of tray buffers.

Components	pH 8.9 gels	pH 4.3 gels	
	pH 8.3 [144]	pH 4.5 [145]	pH 3.1 [137, 146]
Tris (gm)	6.0		
Glycine (gm)	28.0		
β-alanine (gm)		31.2	
Glacial acetic acid (ml)		8.0	
Aluminium lactate (gm)			4.9
Lactic acid (ml)			5.2

Diluted to litre with distilled water.

The stock tray buffers were further diluted 1 ⟶ 5 and used.

In the case of urea gels the stock buffer was further diluted 0.75 ⟶ 1.25.

Glass tubes.

Glass tubes 0.55 x 7.5 cm (i.dia) were tightly closed at one end with rubber caps (pencillin bottle stopper). Tubes were kept in vertical position with the aid of the stoppers and were filled at room temperature with 1.3 ml of small pore gel solution. The solution was carefully overlaid with one drop of distilled water and allowed to polymerise for 15-20 min. The purpose of water layer is to prevent from air oxidation and evaporation of solution ~~xx~~ of gel at top. The water layer was removed and the tube was rinced with large pore solution to attain the upper gel pH and for removal of water. Then 0.2 ml of large pore gel solution was added and a drop of distilled water overlaid. A day light fluorescent tube (20 watts) was placed behind the tubes at a distance of 1 to 2 inches. The large pore solution became opalescent and polymerised within 15-20 min. Then the water layer was removed.

The protein sample was dissolved in 20% sucrose final concentration and layered on the upper gel. The sucrose solution was used to dissolve the protein to get a dense solution and to prevent mixing with tray buffer. Tray buffer solution was introduced into the column above the protein solution. The lower cap was removed and the tube with the sample layer in the upper most position was attached to anode buffer compartment, which was then filled with the tray buffer. A hanging drop was attached to the bottom of each tube to avoid trapping of air bubbles. The tubes were placed in such a way

that they remained immersed about ¼" into the buffer solution contained in the cathode compartment. In the anode compartment methyl green was used as marker. 110 ml of electrode buffer was used in each compartment. Platinum electrodes were used. In pH 8.9 gel runs also the upper buffer compartment was connected to the anode and lower buffer compartment to cathode, since isoelectric point of lysozyme is 10.4 and at pH 8.9 it would be positively charged.

Then a current of 5-6 m. amps per tube was applied. Electrophoresis was performed for 70-90 min. at a constant temperature of 4°. The gels were immediately removed with the help of a syringe needle and fixed and stained with 1% amidoblack in 7% acetic acid for 30 min. Background stain was removed by repeated washing with 7% acetic acid. The destained gels were finally stored in stoppered vials and photographed. These photographs were read on a chromoscan micro-densitometer (Joyce Loebl & Co. Ltd., Gateshead, England) with reflection attachment.

Starch gel electrophoresis.

The method comprises of zone electrophoresis with a starch gel as the supporting medium. In starch gel electrophoresis resolution occurs both due to charge (movement in the electric field) and shape (rate of filtration through the sieve like gel matrix) (147, 148).

The following buffers were used:-

(1) 0.05M sodium phosphate buffer pH 7.0 for gel preparation and for sample. 0.066M sodium phosphate buffer pH 7.0 for bridge solution.

(2) 0.05M acetate buffer pH 4.1 for gel preparation and for sample. 0.066M acetate buffer pH 4.1 for bridge solution.

Starch gel preparation.

33 g. of starch were weighed into 220 ml of buffer contained in a Buchner flask to get a final concentration of 15% starch. The suspension was mixed thoroughly to get a uniform suspension. The flask was fitted tightly with rubber cork and heated gently over a ~~soft~~ Bunsen flame while continuing the swirling action of the flask. As the solution is heated, it becomes viscous. On further heating, discrete grains of starch can no longer be seen and the suspension becomes a semi-solid, opaque mass. When it was opaque the heating was stopped, and the flask was evacuated for 1-2 min. to remove the dissolved air bubbles. As the vacuum builds up, the flask contents boil. This boiling removes air bubbles from the gel. Then the gel was poured on a gel forming plate which had been previously smeared with liquid paraffin. Then a glass plate which had been coated with liquid paraffin and heated to 60° was used to cover the gel plate without allowing any air bubbles to enter. Then the lead weights were kept on the plate and the gel was allowed to settle at room temperature for 3-4 hr.

After gel setting, the upper glass cover was removed slowly without disturbing the gel. The gel was trimmed by scrapping the starch sticking to the sides, with the help of a spatula. The sample slots were cut and sample inserted over filter paper. Then the gel was covered with a mixture of histo-

pathowax (m.p. 52-54°) and white vaseline. After wax covering, the starch gel tray was kept in the tank and at each end Whatmann 3 MM filter paper was fitted with their free ends dipping into the buffer solution contained in the platinum electrode chambers. The tank was closed and a voltage gradient of 5-6 volts/cm was applied. The electrophoretic run was conducted at 4° in a refrigerator.

500 ml of buffer was used in each buffer compartment. Methyl green was used as a marker. After the run (16-20 hr) was over, the wax cover and filter papers were removed and the gel trimmed by cutting the gel. The gel was cut into half by a thin wire. The cut gel was stained for 20 min in 1% solution of amidoblack in methanol:acetic acid:water (50:10:50 v/v). Then it was destained with the same solvent mixture.

Starch gel electrophoresis at pH 4.1 was carried out according to the method of Murray (149). The gels prepared in acetate buffer pH 4.1 (0.05M) at 15% gel concentration and acetate buffer pH 4.1 (0.066M) as bridge solution were used.

Starch gel electrophoresis was also done at pH 7.0 using sodium phosphate buffer of 0.05M. A 15% starch concentration and phosphate buffer (pH 7.0 and 0.066M) as bridge buffer were used.

Rate of tryptic hydrolysis.

Tryptic hydrolysis of native and DNP-lysozymes was carried out as follows. The N-lysozyme or DNP-lysozyme (300-500 µg) was taken in a 0.05 - 0.1 ml of distilled water and

made upto 0.5 ml with sodium phosphate buffer (0.01M; pH 8.0) and to this a required volume of trypsin solution in water was added and incubated at 37° in a shaking water bath for different time intervals. For each time point a set of 4 tubes was used. A blank containing only trypsin solution was also run; the other 3 tubes contained lysozyme and trypsin in the ratio of 25 to 1 (W/W). At the end of reaction time 0.05 ml of 0.1N HCl was added "to stop" the tryptic hydrolysis and stored at -20° until further estimations were performed. Incubation was performed upto 30 hr at 4 hr intervals.

Out of the 4 tubes, trypsin control and two other tubes were used for ninhydrin estimation and the fourth tube for enzyme activity measurements. The enzyme activity was measured ~~out~~ at 37°. Ninhydrin estimation was carried out according to the method of Moore and Stein (150, 151). To 0.5 ml of the hydrolysed samples or trypsin control 0.5 ml of citrate buffer pH 5.0 was added followed by 1.0 ml of 2% ninhydrin solution. The tubes were covered with aluminium foil and heated in a boiling water bath for 20 min. To this 3.9 ml of diluent (equal volumes of distilled water and reagent grade n-propanol) were added to make up to 6.0 ml. Then the tubes were cooled to room temperature and the O.D. of the solution was read at 570 mμ. Correction for blank was made by using trypsin control. The diluent was used as the spectrophotometer blank.

Gel filtration of trypsin hydrolysed N-lysozyme and DNP-lysozyme.

The tryptic hydrolysis was carried out by using about 8-10 mg of N-lysozyme or DNP-lysozyme in water and by adding the required

amount of trypsin in the ratio of 25 to 1 of lysozyme to trypsin (W/W). The pH was then adjusted between pH 7 and 8 by the addition of ammonium carbonate. The reaction mixture was incubated at 37° for 12 hrs in a shaking water bath. After incubation the samples were centrifuged to remove the precipitate. The supernatant was loaded on Sephadex-G-100 and eluted with 0.1M acetic acid, 2 ml fractions were collected and O.D. reading at 280 and 360 mμ for DNP-lysozyme and at only 280 mμ for N-lysozyme were taken. The specific enzyme activity of fraction was also measured.

Amino acid analysis.

Acid hydrolysis of DNP-protein and extraction of DNP-amino acids wasedone as follows. The DNP-lysozymes were hydrolysed with 5.7 N HCl at 105° for 24 hr. The hydrolysates were diluted to 1N HCl with water and the ether-soluble DNP-amino acids were recovered by extraction four to five times with 20 volumes of ether. The water soluble DNP-amino acids were recovered by extraction four to five times with 20 volumes of n-butanol. The ether and butanol soluble fractions were concentrated at 40°, on a heating mantle, under reduced pressure. The hydrolysates were then analysed by paper chromatography in various solvent systems (138).

Paper chromatography of DNP-amino acids.

DNP-amino acids from the hydrolysate of the DNP-lysozyme were detected by one dimensional or two dimensional chromatography (138). Standard DNP-amino acids were used as reference

substances. Di-DNP-lysine which is extracted in ether was identified by one dimensional paper chromatography on Whatman No.1 using the solvent system, toluene-2-chloroethanol; pyridine:0.8N ammonia (30:18:9:18 V/V). DNP-lysine was identified by one dimensional paper chromatography on Whatman No. 1 using the solvent systems (a) Butanol-Acetic acid-Water (4:1:5 V/V); (b) tertiary amyl alcohol saturated with 0.05N phthalate buffer pH 6.0 using Whatman No.3 mm. It was also detected by two dimensional paper chromatography on Whatman No. 1 using solvent systems Butanol:Pyridine:water (1:1:1 V/V) in one direction and 80% phenol saturated with 0.8N ammonia in the other direction. DNP-amino acids were located by the visual yellow colour of DNP-amino acids as well as by spraying the paper with 0.2% ninhydrin in acetone, in order to find out whether there is any O-DNP-tyrosine and imidazole-DNP-histidine (152). A known aliquot of the solution containing standard DNP-amino acids was spotted as reference amino acid. The chromatography runs were conducted in dark at 25°.

Other experimental techniques.

(a) pH measurements.

The pH of various buffer solutions used were measured with a Metrom pH meter fitted with a combined calomel electrode and glass electrode. The pH measurements were made at room temperature. The instrument was standardised with 0.05M potassium hydrogen phthalate solution (pH 4.0) or with a standard phosphate buffer solution of pH 6.86. The reading accuracy of the pH meter was ± 0.05 pH unit.

(b) Centrifugation.

Mistral 6 centrifuge, supplied by Measuring and Scientific Equipment Co., England, was used. Where necessary, refrigeration was also used.

(c) Dialysis.

The dialysis experiments were conducted in a cold room. Dialysis bags prepared from seamless visking sausage tubings were used. The solution surrounding the dialysis bag was kept stirring with a magnetic stirrer.

(d) Reagents.

All the reagents used in these studies were of analytical reagent quality. Solutions of these were prepared in distilled water. Where accuracy of a high order was needed in the preparation of solutions, calibrated volumetric flasks and pipettes were used. For other purposes, graduated glassware supplied by reputed firms were used.

(e) When experiments were done with DNP-lysozymes, the column was wrapped with black paper and the fractions were stored in dark till O.D. and enzyme activity measurements were made since DNP-protein is light sensitive.