CHAPTER - III

RESULTS AND DISCUSSION

The results of the lysozyme modification carried out at pH 7.2 are presented first; and the results of the similar study carried out at pH 9.1 will follow subsequently.

Effect of FDNB concentration on uptake of DNP and lysozyme activity.

Experiments were conducted to determine if the ratio of FDNB to enzyme had any effect on the extent of dinitrophenylation. The extent of dinitrophenylation was followed by measuring the absorbance at 360 mu where DNP-group absorbs; the loss in enzyme activity was also followed. The enzyme activity of the DNP-lysozyme as a function of FDNB to enzyme ratio was measured at 26°, the reaction time being 30 min. It can be seen from Fig. 2, that as the ratio increases there is a concurrent decrease in enzyme activity, reaching a value of 50% of the original activity at a ratio of 600 equivalents. On the other hand, the absorbance readings do not show any marked increase between the lowest and highest ratios used, namely 100 and 600 respectively. From these measurements it may be seen that the ratios higher than 300 cause the same extent of dinitrophenylation. Therefore in all the subsequent experiments ratios 300 and higher have been used to dinitrophenylate the enzyme.

Effect of time and temperature on the uptake of DNP and lysozyme activity.

The extent of dinitrophenylation and the residual enzyme activity of the DNP-lysozyme as a function of reaction time of lysozyme with FDNB at two temperatures, 15° and 26°, pH 7.2 are

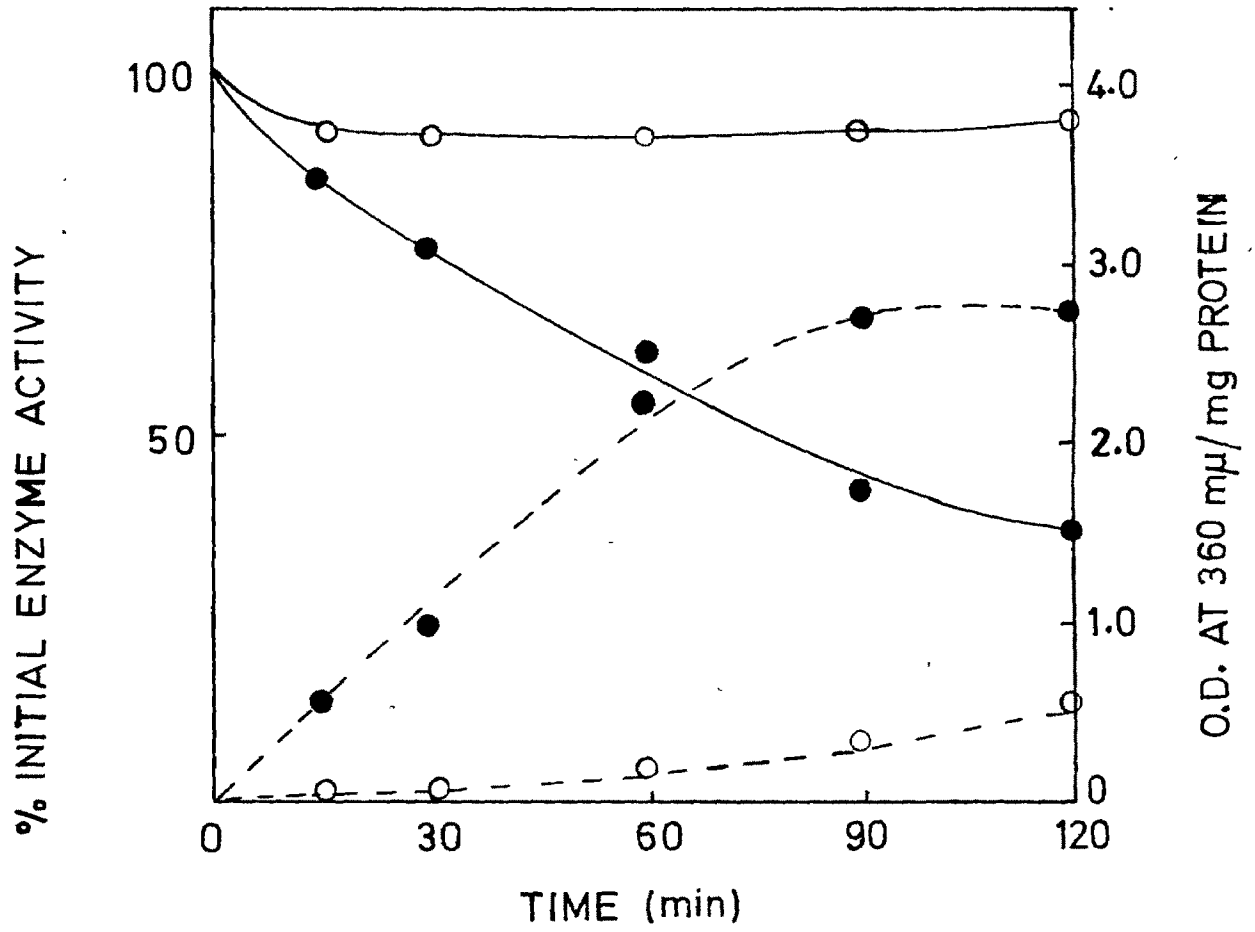
given in Fig. 3. These data were obtained by using FDNB to enzyme ratio of 312:1. The data show that lysozyme reacts with FDNB at 15° and the DNP-uptake increases only slightly with the time of reaction as indicated by the O.D. values at 360 mμ. However there is no loss in activity even after reaction for 120 min. at this temperature. The number of moles of DNP taken up per mole of lysozyme can be calculated from the absorbance reading. If we use the values of 17,600 for the molar extinction coefficient of DNP at 360 mμ and a value of 14,500 for the molecular weight of lysozyme the DNP mole per mole of lysozyme is 0.43 after 120 min. reaction. This suggests that the uptake is very low even after a reaction time of 120 min.

However, when the reaction was carried out at 26°, the extent of dinitrophenylation was higher. The O.D. values at 360 mμ increased linearly with the time of reaction for about 60 min. and then attained more or less steady values. Concomitantly there was a decrease in activity; the activity at the end of 120 min. reaction was only about 40% of that of the native enzyme. The moles of DNP per mole of enzyme calculated from absorbancy measurements is 2.35. As will be discussed later the extent of dinitrophenylation calculated from the absorbance values does not give a correct estimate. At any rate the results from absorbance measurements clearly show that by varying temperature and reaction time it is possible to get DNP-derivatives of lysozyme with varying DNP content and varying enzyme activity. It is clear from the above observations that at pH 7.2 reaction of lysozyme with FDNB depends on factors such as time,

Fig.2. The effect of FDNB to enzyme ratio (mole/mole) on enzyme activity and extent of dinitrophenylation at 26°; pH 7.2; reaction time 30 min. O——O relative enzyme activity; ●——● O.D. at 360 mμ.

Fig.3. The extent of dinitrophenylation of lysozyme as a function of time of reaction and its effect on enzyme activity at 15° and 26°; pH 7.2. O- - -O O.D. at 360 mμ; O——O relative enzyme activity at 15°. FDNB/Enzyme, 312:1, mole/mole. ●- - -● O.D. at 360 mμ; ●——● relative enzyme activity at 26°. FDNB/Enzyme, 312:1 mole/mole.

FIG. 3


100
50
0
30
60
90
120
TIME (min)
% INITIAL ENZYME ACTIVITY
4.0
3.0
2.0
1.0
0
O.D. AT 360 mμ/ mg PROTEIN

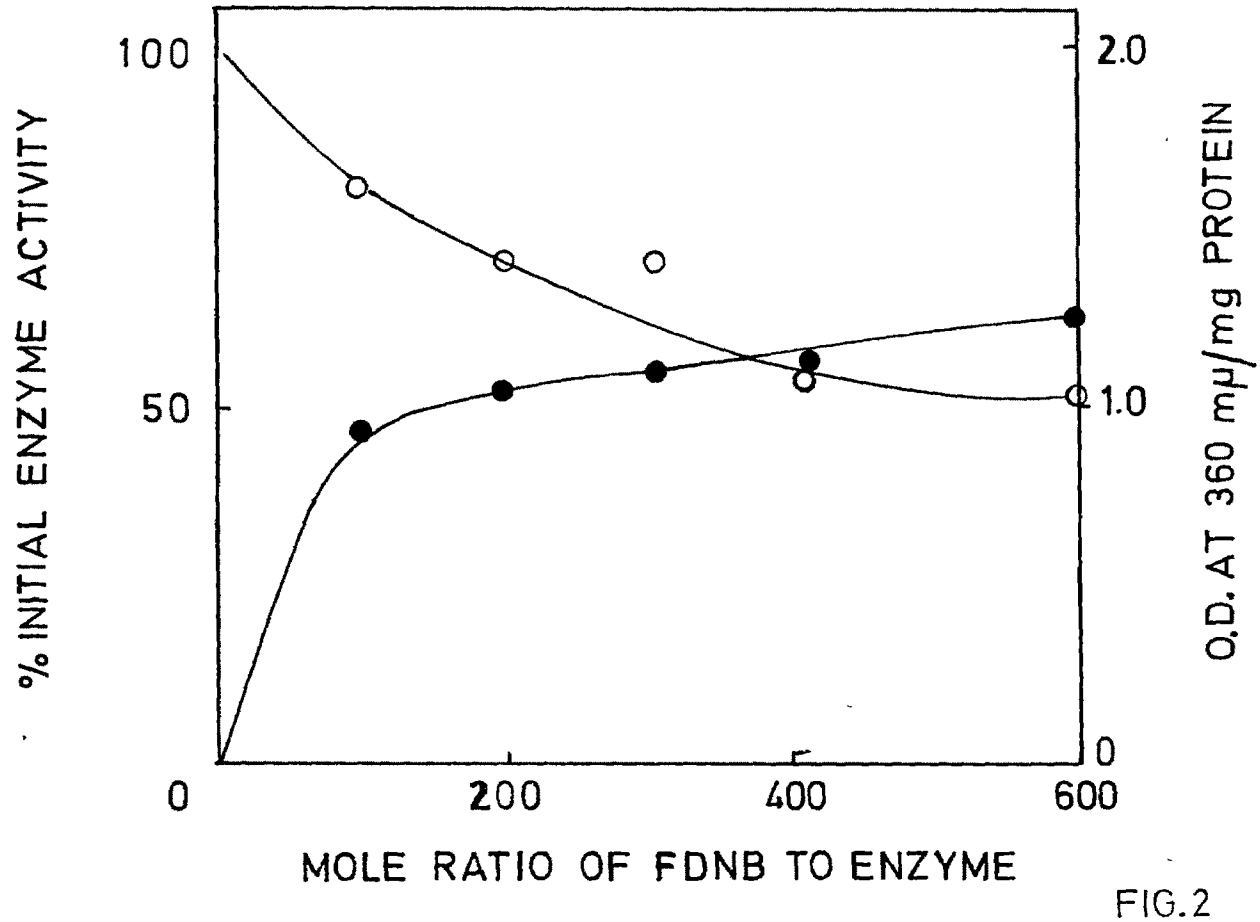

100
50
0
200
400
600
MOLE RATIO OF FDNB TO ENZYME
% INITIAL ENZYME ACTIVITY
2.0
1.0
0
O.D. AT 360 mμ/mg PROTEIN

FIG. 2

temperature and FDNB concentration. Increased DNP substitution could be obtained by changing the various parameters. Using this information the following derivatives were prepared for further investigations on the effect of modification on enzyme activity and physicochemical properties.

1) A derivative which retained full enzyme activity was obtained at 15° by reacting FDNB with the enzyme at a ratio of 312:1, for 120 min.
2) A partially active derivative with 55-60% of the original activity, was obtained at 26° by reacting FDNB with the enzyme at a ratio of 412:1, for 30 min.
3) A second partially active derivative with 40% of the original activity was obtained at 26° by reacting FDNB with the enzyme at a ratio of 312:1, for 120 min.

For brevity and to avoid repetition, these products will be referred to as P_1, P_2 and P_3 and native lysozyme as N-lysozyme.

DNP-Content of the derivatives.

The details of the method of determination of DNP-content by using labelled ^{14}C-FDNB are given in Table 4. The extent of dinitrophenylation determined by absorbance and isotope methods is presented in Table 5. The amino acid residues dinitrophenylated in each derivative (determined by the chromatography of the hydrolysates) are also indicated in Table 5. DNP content data obtained by isotope method shows that P_1, P_2 and P_3 preparations contained 1, 3 and 6 DNP groups per mole of the enzyme respectively. However, it may be seen that the values calculated by absorbance measurements are consistently lower than those determined by radio activity method

TABLE No. 4

EXPERIMENTAL DETAILS OF THE DETERMINATION OF DNP CONTENT OF LYSOZYME BY USING RADIO ACTIVE ^{14}C FDNB

DNP-derivative	% enzyme activity	Total counts added	FDNB in μ moles added	CPM/ μ mole of FDNB	Enzyme in μ moles	Total counts above control	μ mole of DNP taken = Counts in enzyme/Counts for μ mole of FDNB	DNP content in μ mole per μ mole of enzyme = DNP in μ mole/enzyme in μ mole
P_1	100	111615	215	520	0.6885	400	0.769	1.11
P_2	55-60	38950	100.6	387	0.2414	270	0.6971	2.89
	55-60	38950	402.4	97	0.9556	300	3.092	3.2
P_3	40	15600	220	71	0.702	312) 300	4.2	6.0
		15600	220	71	0.702	288)		

TABLE No. 5

COMPARISON OF THE DETERMINATION OF DNP CONTENT OF LYSOZYME BY ABSORBANCY AND RADIO ACTIVE MEASUREMENTS

DNP-derivative	% enzyme activity	DNP moles/mole of enzyme determined by		Amino acids dinitrophenylated
		Measurement of OD at 360 mμ	Radio-active method	
P_1	100	0.43	1.1	ε-DNP-lysine
P_2	55-60	0.8 - 1.0	3.0	ε-DNP-lysine and di-DNP-lysine
P_3	40	2.35	6.0	ε-DNP-lysine and di-DNP-lysine

Horecker and Coworkers (133, 134) in their experiments on dinitrophenylation of transaldolase obtained good agreement in the values of DNP content of the derivatives estimated by the two methods.

All the three derivatives (with increasing content of DNP and decreasing enzyme activity) appeared fairly homogeneous on ultra-centrifugation, gel filtration on Sephadex, disc electrophoresis at pH 3.1 and starch gel electrophoresis at pH 7.0 which would be described later.

DNP-amino acid analysis.

Following results were obtained on amino acid analysis of the hydrolysates of P_1, P_2 and P_3 (Table 5).

1) P_1 has only ϵ-DNP-lysine and does not show the presence of any other DNP amino acid. Since P_1 shows the definite presence of ϵ-DNP-lysine it is clear that this DNP-lysozyme must have atleast one amino group dinitrophenylated. The absorbance method gives a value of 0.43 DNP mole/mole of the enzyme. Since this product was fairly homogeneous (see discussion later) obviously the value of 0.43 cannot be correct. On the other hand, the value of 1 obtained by radioactivity measurements agrees with the conclusions of amino acid analysis.

2) Both P_2 and P_3 show the presence of ϵ-DNP-lysine and di-DNP-lysine. Obviously the di-DNP-lysine must have been derived from the N-terminal group which in egg white lysozyme is known to be lysine. Similarly, like P_1, P_2 by absorbance method shows a value of 0.8 to 1.0 DNP mole/mole of the enzyme, whereas amino acid analysis of this derivative shows the presence of both

di-DNP-lysine and ε-DNP-lysine. In that case the DNP content of P_2 should be atleast 3 groups per molecule. Here again the value found by the absorbance method is much lower than the value obtained either by isotope method or by amino acid analysis. Since the data obtained by amino acid analysis and the DNP content measured by isotope methods appear to give mutually consistent results we have used the values found by using labelled ^{14}C-FDNB as a correct estimate of the DNP-content of the different DNP-lysozymes. Whereas the absorbance method seems to give better results with other proteins, it does not appear to work well with lysozyme (133, 134). Further, only ε-DNP-lysine was obtained from P_1 derivative and not di-DNP-lysine, suggesting that under the conditions in which P_1 is prepared, N-terminal lysine does not react with FDNB. If the ε-DNP-lysine was derived from N-terminal lysine it would mean that the ε-amino group of N-terminal lysine is dinitrophenylated in preference to α-amino group. But this is unlikely as it has been shown that the primary amino group of the N-terminal lysine of lysozyme is more reactive than its ε-amino group (153). Therefore it is more probable that the single amino group dinitrophenylated in P_1 derivative may be derived from any of the (interior) lysine residues other than the N-terminal lysine. Venkatappa and Steinrauf (106) observed that when lysozyme is reacted with 4-iodo-2-nitrofluorobenzene at neutral pH a derivative, 4-iodo-2-nitrophenyl lysozyme is obtained which contains a single 4-iodo-2-nitrophenyl group. The lysine residue that had reacted with this modifying agent was identified as lys-13, which

appears to be more reactive than N-terminal lysine in the reaction of lysozyme with 4-iodo-2-nitrofluorobenzene. It is possible that there may be a similar difference in the reactivity of the various residues towards FDNB.

The chromatographic analysis of the hydrolysates of P_1, P_2 and P_3 do not reveal the presence of any amino acid residues other than the lysine residues. Besides, lysine the groups that can react with FDNB are sulfhydryl, imidazole and phenolic groups. Lysozyme does not contain any free SH group. The DNP-derivatives of imidazole give grey brown colour when sprayed with ninhydrin, and the O-DNP-derivative of tyrosine also gives the same colour (152). We have carefully examined with chromatograms obtained with the hydrolysates of DNP-lysozymes and have failed to observe any spots with this characteristic colour. Further some preliminary experiments on the amino acid composition of DNP-lysozyme done with an automatic amino acid analyser revealed the presence of histidine. It is fairly certain that under the experimental conditions we have used, only the lysine residues of lysozyme react with FDNB.

Effect of modification on enzyme activity.

The enzyme activity of native lysozyme and of P_2 was measured as a function of enzyme concentration at 37°. The data are given in Fig. 4. The plots obtained have the shape of a typical enzyme reaction with a region where enzyme activity increases linearly with enzyme concentration and another region of steady values, suggesting that the substrate is exhausted and that increasing the enzyme concentration has no effect on the activity. It may be seen that P_2 has consistently lower activity

Fig. 4. The enzyme activity of native lysozyme and P_2 as a function of enzyme concentration at 37°; O——O N-lysozyme; ●——● P_2.

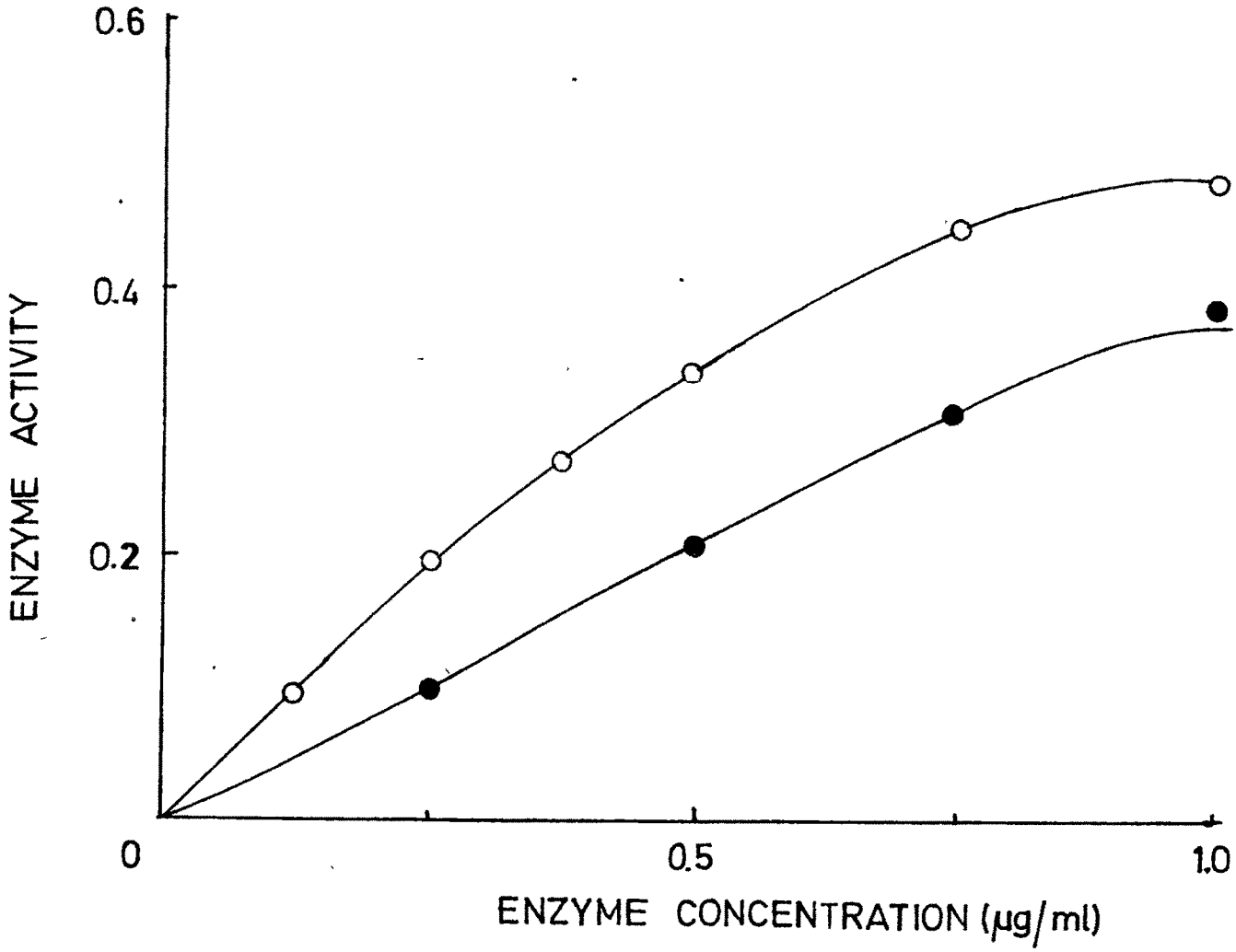


FIG. 4

than native lysozyme at all the enzyme concentrations used. From the linear portion of the plots the specific activities were calculated and it is found that the P_2 had about 55% of the activity of N-lysozyme.

Whereas P_1 in which one amino group had been blocked by DNP group had full enzyme activity, P_2 and P_3 in which 3 and 6 groups respectively had been blocked had 60% and 40% activity of N-lysozyme.

The substrate binding capacity of an enzyme will depend on the state of ionisation of ionisable groups in the protein as well as substrate molecules (1954). To determine if dinitriphenylation had caused any changes in the ionization characteristics of various groups in lysozyme, the activity of native lysozyme, P_2 and P_3 was determined as a function of pH. The relevent data are given in Fig. 5A. These measurements were made at different pH values in 0.1 M phosphate buffer. N-lysozyme has maximum activity between 6.2 and 6.8. The pH optimum observed with N-lysozyme is in agreement with that reported by other workers(11). P_2 and P_3 also show maximum activity in the same pH range. These results indicate that there is no change in the pH optimum of lysozyme due to modification of one to six lysine residues. Yamasaki et.al (71) have reported that the activity of lysozyme in the neutral pH range decreased as the number of amino groups acetylated increased. Further, the pH optimum sifted to acidic pH values and it depended on the number of groups acetylated. Acetyl lysozyme in which six amino groups had been acetylated had a pH optimum of 5.2 and at this pH, the activity

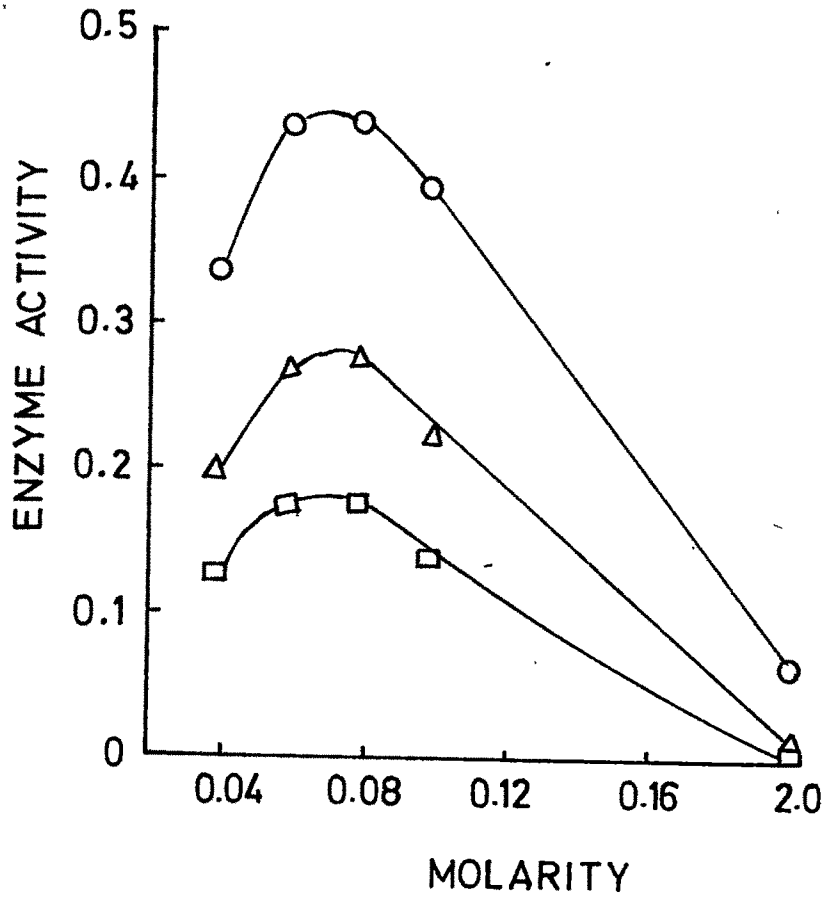
of N-lysozyme was the same as that of the acetylated lysozyme. As seen from the data presented above, a similar effect was not observed with DNP-derivatives, indicating that in the DNP-derivatives ionizing characteristics of various groups are not altered.

It was mentioned earlier that the activity of lysozyme is influenced by the molarity of the phosphate buffer. We have examined the activity of N-lysozyme, P_2 and P_3 as a function of phosphate molarity at pH 6.8 (Fig. 5B). In all the cases the activity increased to a maximum between 0.06 and 0.08 M; on either side of this molarity the activity is less. It is possible that the negatively charged phosphate ions may be strongly bound to the positively charged groups of lysozyme molecule and thus affect the enzyme activity. It is well known that the binding of small molecular weight substances may cause conformational changes in a protein molecule (155). It is probable that in the case of lysozyme phosphate ion binding may change the conformation in such a way that the enzyme has highest activity at phosphate concentrations of 0.06 to 0.08 M. At very high phosphate concentrations these ions may seriously interfere in enzyme substrate complex formation and thus reduce the activity of lysozyme. Other workers have observed that acetylation of lysozyme changes the activity-ionic strength profile. It has been reported that completely acetylated lysozyme was active at low buffer concentrations (156, 157). No such effect was observed with DNP-derivatives.

Fig. 5A. The enzyme activity of N-lysozyme, P_2 and P_3 as a function of pH. Phosphate buffer of 0.1M used throughout pH range; O——O N-lysozyme; △——△ P_2 and □——□ P_3; 0.5 µg enzyme/ml at 37°.

Fig. 5B. The enzyme activity of N-lysozyme, P_2 and P_3 at different molarity of phosphate buffer at pH 6.8. O——O N-lysozyme; △——△ P_2 and □——□ P_3; 0.5 µg enzyme/ml, at 37°.

FIG. 5B


0.5
0.4
0.3
0.2
0.1
0
ENZYME ACTIVITY
0.04
0.08
0.12
0.16
2.0
MOLARITY

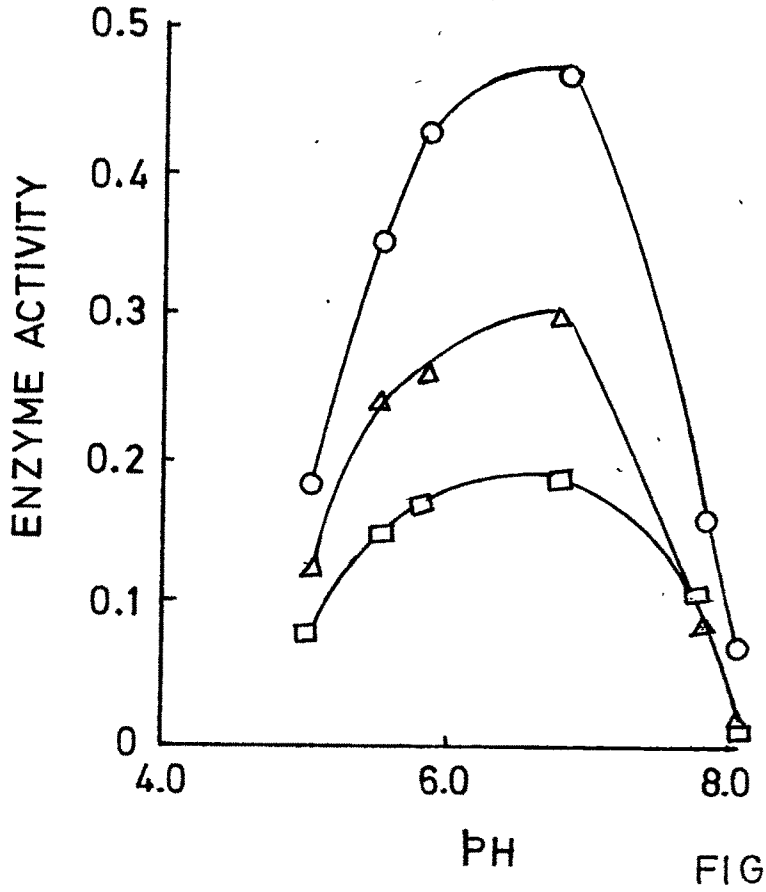

0.5
0.4
0.3
0.2
0.1
0
ENZYME ACTIVITY
4.0
6.0
8.0
PH

FIG. 5A

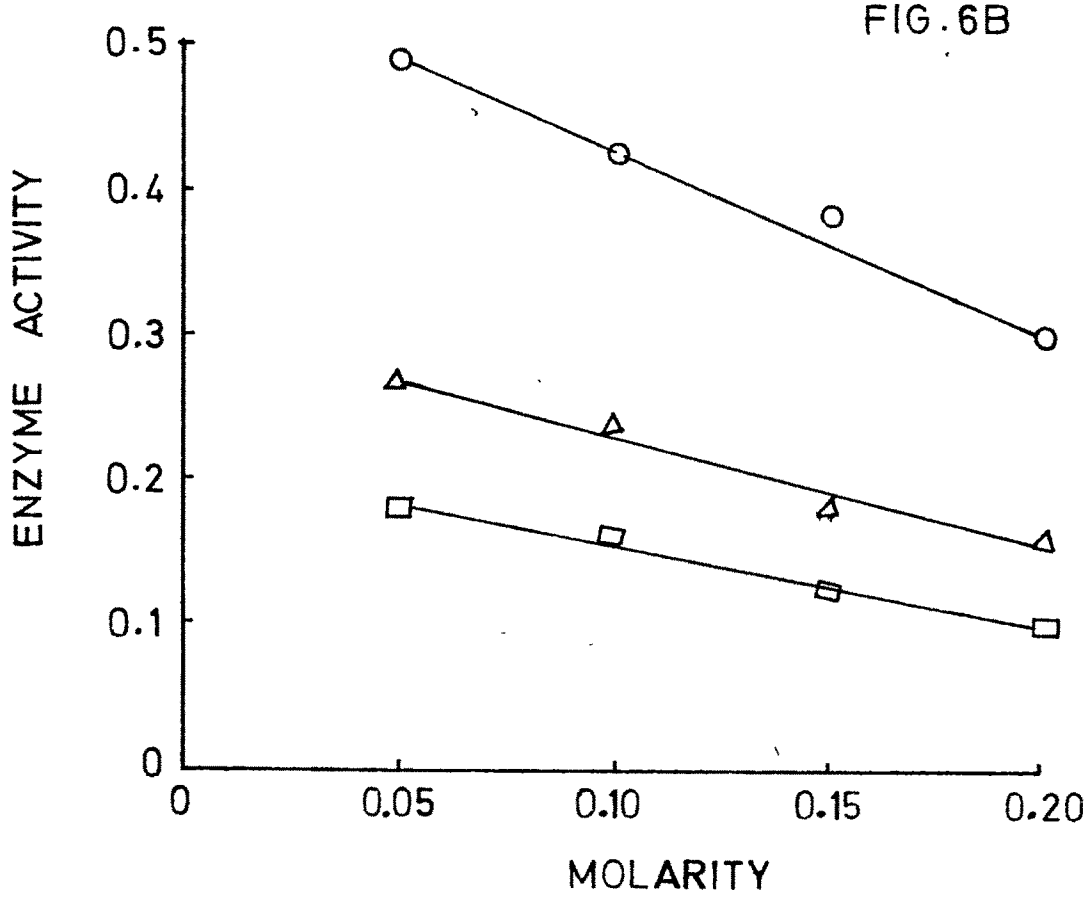
N-lysozyme has another pH optimum at pH 9.2 (156). The activity of lysozyme is more or less the same at the two pH optima, 6.8 and 9.2. We were therefore interested in finding out whether any changes occurred in the second pH optimum as a consequenteof modification of lysine residues. The activity of N-lysozyme, P_2 and P_3 as a function of pH in the pH range 8.6 - 10.0 has been measured in 0.1 M glycine-sodium hydroxide buffer, Fig. 6A. All the three lysozymes show maximum activity around pH 9.2. Thus in the case of the second pH optimum also there is no difference between native lysozyme, P_2 and P_3.

Since it was observed that at pH 6.8 the activity of native lysozyme, P_2 or P_3 was dependent on the molarity of phosphate buffer it was of interest to determine whether at pH 9.2 also activity depended on the molarity of the buffer. The results of the activity measurements at different molarities of glycine-sodium hydroxide buffer are given in Fig. 6B. The experiments were carried out in the same concentration range as was used with phosphate buffer, namely 0.05 to 0.2M. The measurements show that the activity of all the three enzymes decreases slightly as the molarity of the buffer increases. Further there is no molarity at which the activity passes through a maximum value. The decrease in activity with increase in molarity may be due to the increased binding of negatively charged glycinate and hydroxyl ions by the enzyme and thus a further reduction in the positive charge on lysozyme. It appears that the effect of phosphate ions observed at pH 6.8 could be a specific anion effect on the conformation of the enzyme. The first pH optimum of lysozyme (between pH 6.2 and 6.8) has been

Fig. 6A. The enzyme activity of N-lysozyme, P_2 and P_3 as a function of pH; glycine-NaOH buffer 0.1M used throughout pH range; O——O N-lysozyme; △——△ P_2; and □——□ P_3; 0.5 µg enzyme/ml, at 37°.

Fig. 6B. The enzyme activity of N-lysozyme; P_2 and P_3 at different molarities of glycine-NaOH buffer pH 9.2. O——O N-lysozyme; △——△ P_2; and □——□ P_3; 0.5 µg enzyme/ml, at 37°.

FIG.6B


ENZYME ACTIVITY
0.5
0.4
0.3
0.2
0.1
0
0
0.05
0.10
0.15
0.20
MOLARITY

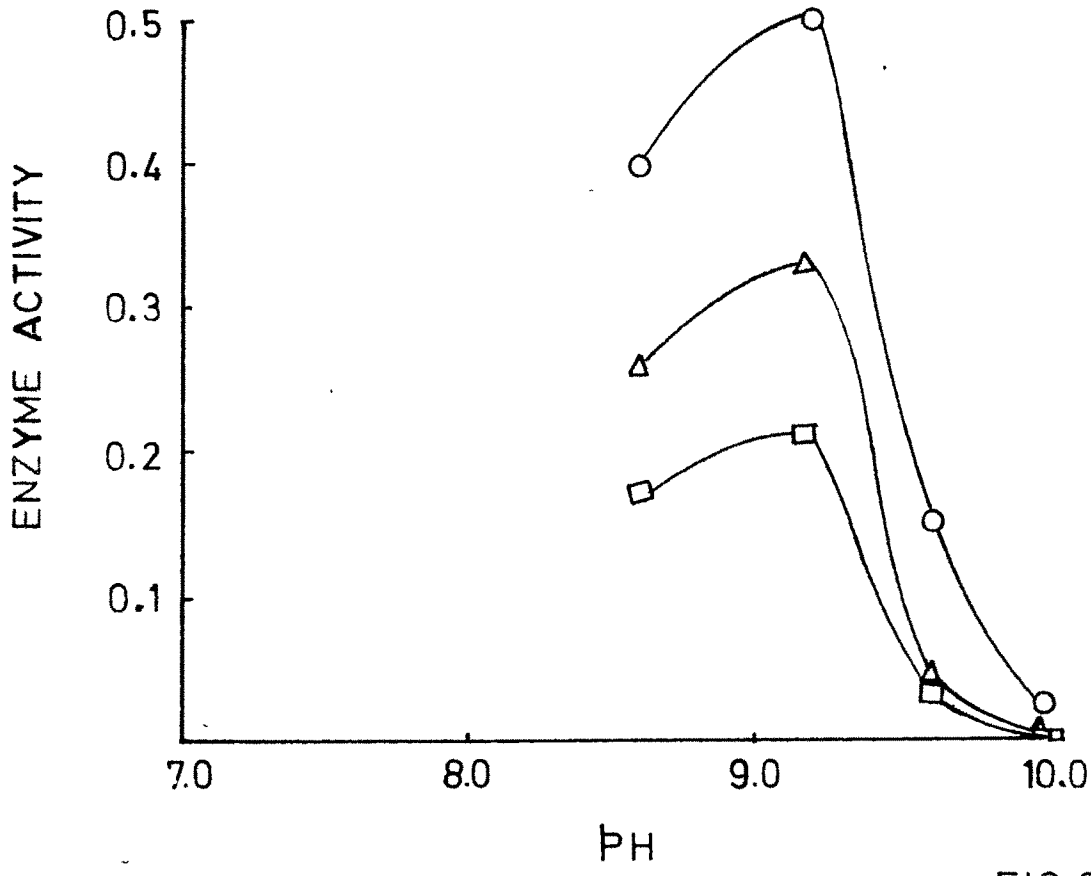

ENZYME ACTIVITY
0.5
0.4
0.3
0.2
0.1
7.0
8.0
9.0
10.0
PH

FIG.6A

reported to depend on the nature of buffer systems used to measure the activity. Whereas in phosphate buffer the optimum pH is between 6.2 - 6.8, in veronal buffer it is around pH 8.0 (157, 158). We have done similar experiments to examine if the activity of DNP-lysozyme also followed the same pattern. From the data presented in Fig. 7 it may be seen that in veronal-HCl buffer of 0.05 M N-lysozyme has pH optimum at pH 8.0; a similar value was given by P_2 and P_3 also.

We have also investigated if the second pH optimum is also dependent on the nature of buffer ions. The activity measured in the pH range 8-10.0 in diethanolamine-HCl system of 0.05 M are given in Fig. 8. It may be seen that the second pH optimum is abolished in this system and the activity decreases gradually from pH 8.0 to 10.0. N-lysozyme P_2 and P_3 behaved alike.

From the foregoing discussion on the variation of the activity as a function of pH, ionic concentration and ionic species, it may be concluded that the two DNP-lysozymes, P_2 and P_3 behave in the same manner as N-lysozyme.

The conformation of a protein molecule would, in part at least, be dependent on the interaction between various ionic groups in the enzyme molecule and their state of ionization. The latter would be governed by the pH of the solution. The effect of pH on the enzyme activity of N-lysozymes, P_2 and P_3 is similar suggesting that they have identical conformation.

The fact that the introduction of DNP-groups in the enzyme molecule has no effect either on activity-pH profile or on the

Fig. 7. The enzyme activity of N-lysozyme, P_2 and P_3 as a function of pH. Veronal-HCl buffer, 0.05M used throughout the pH range; O——O N-lysozyme; △——△ P_2 and □——□ P_3; 0.5 µg enzyme/ml, at 37°.

Fig. 8. The activity of N-lysozyme, P_2 and P_3 as a function of pH. Diethanolamine-HCl buffer; 0.05M used throughout the pH range; O——O N-lysozyme; △——△ P_2 and □——□ P_3; 0.5 µg enzyme/ml, at 37°.

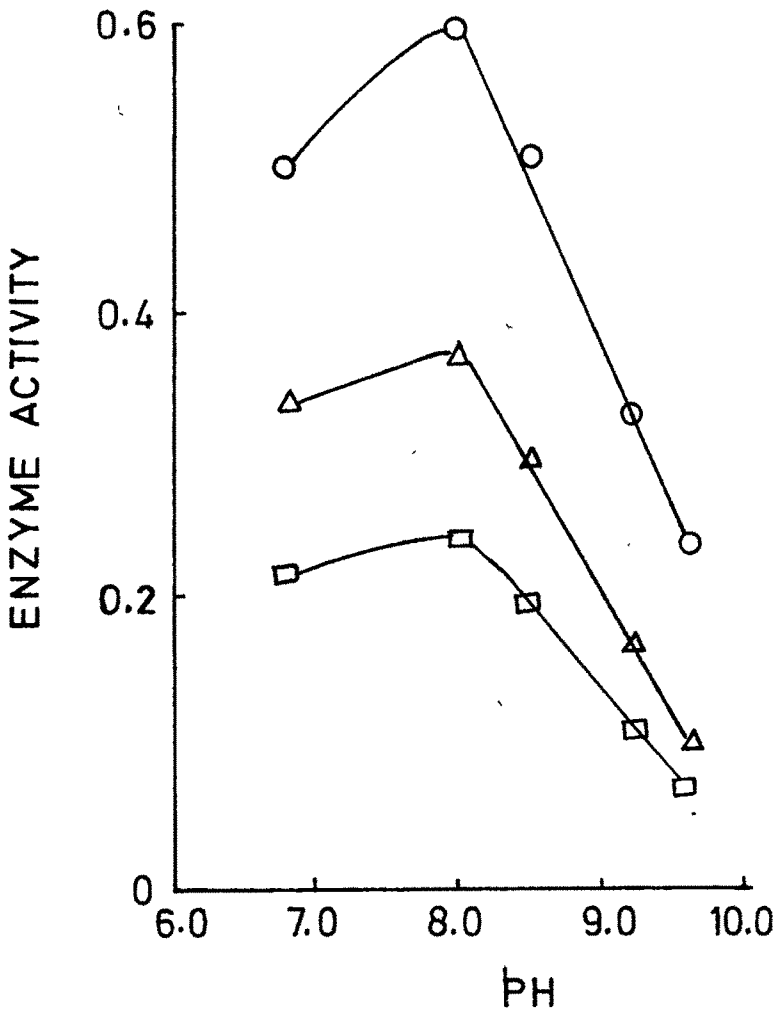

ENZYME ACTIVITY
0.6
0.4
0.2
0
6.0
7.0
8.0
9.0
10.0
PH

FIG. 7

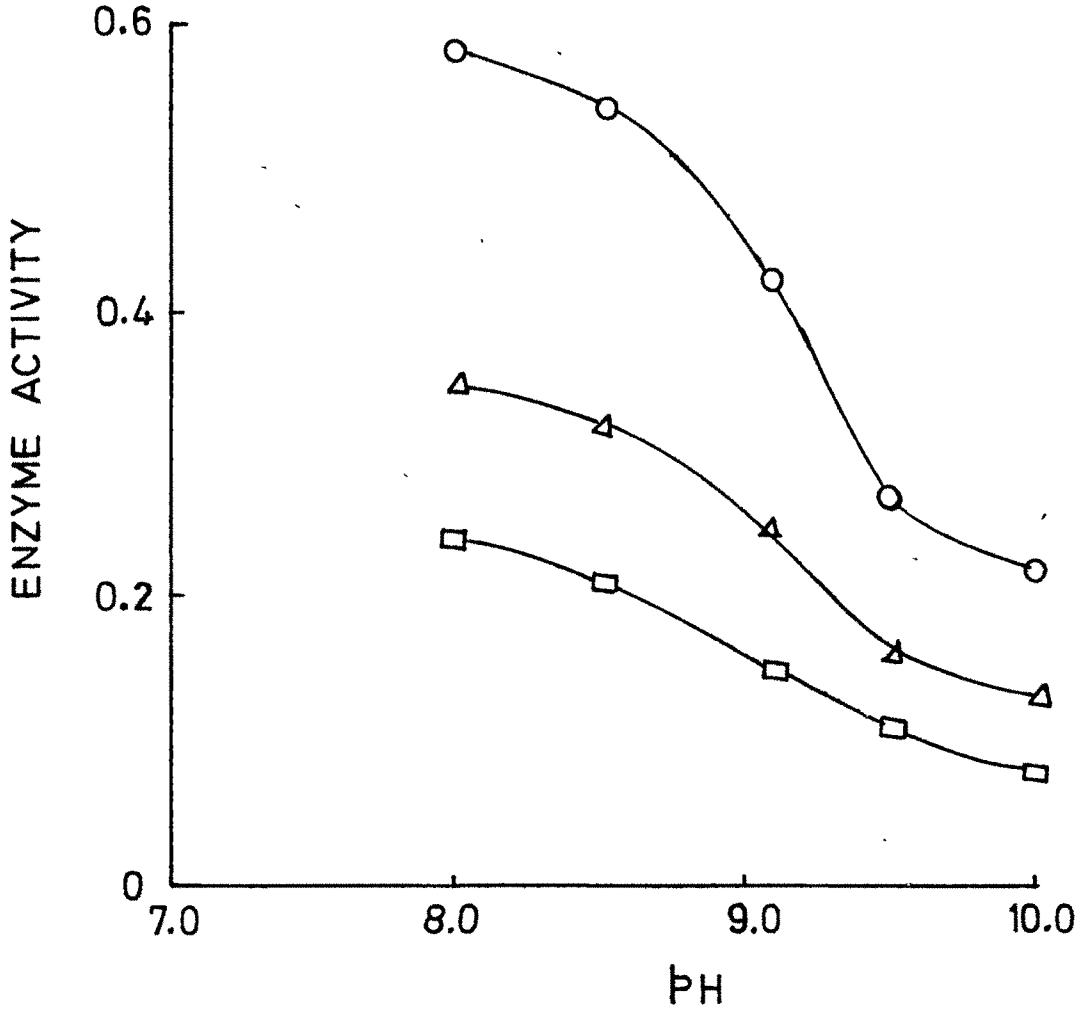

ENZYME ACTIVITY
0.6
0.4
0.2
0
7.0
8.0
9.0
10.0
PH

FIG. 8

activity-buffer concentration profile would suggest that the electrostatic interaction~~s~~ involved between enzyme and substrate are not affected to any appreciable extent. Furthermore, retention of the pH optimum both in acid and alkaline region is a supporting evidence that decrease in the positive charge probably does not affect the forces involved in the formation of the enzyme substrate complex.

The effect of the inhibitor, Tyr-Glu polypeptide on the enzyme activity of N-lysozyme and P_2 are shown in Fig. 9A. These data were obtained at an inhibitor to enzyme ratio of 32:1 (W/W). It may be seen that the activity of both the enzymes is inhibited to the same extent. The extent and the effectiveness of this inhibitor was also examined at various inhibitor to enzyme ratios (W/W) at 37°. Results are shown in Fig. 9B. It is seen that at each inhibitor concentration the partially active P_2 lysozyme is inhibited to the same extent as the native lysozyme. ~~Try-Gly~~ Tyr-Glu polypeptide is a nonspecific inhibitor for lysozyme and its ability to inhibit activity appears to arise in part by electrostatic interaction between the basic enzyme and the acidic compolymer (159). The observation that both N-lysozyme and P_2 are inhibited in a similar way suggests that the nature of charge distribution on P_2 is similar to that on native lysozyme. This charge distribution would undoubtedly depend on the conformation of the proteins. Since both the proteins appear to have similar charge distribution, perhaps they have similar conformation also.

Fig. 9A. The effect of inhibitor, tyr-glu acid polypeptide on the enzyme activity of N-lysozyme and P_2. Enzyme activity measured in the presence or in the absence of the inhibitor is plotted as a function of time; O——O N-lysozyme; □——□ P_2 in the absence of the inhibitor; ●——● N-lysozyme; ■——■ P_2 in the presence of the inhibitor. Inhibitor/Enzyme 32:1 (W/W); 0.5 µg. enzyme/ml, at 26°.

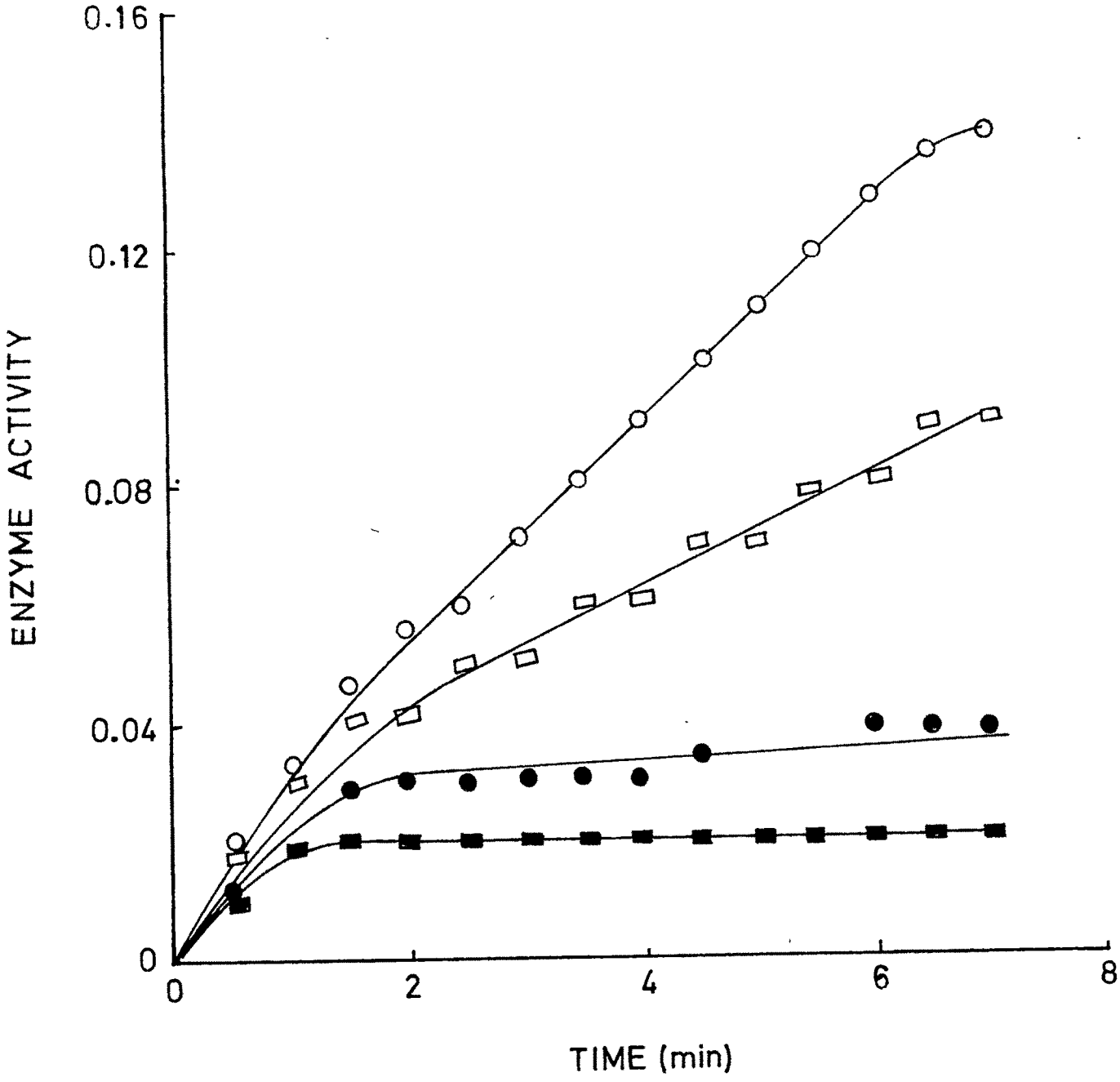


FIG.9A

Fig. 9B. The effect of tyr-glu, acid polypeptide concentration on the enzyme activity. The percent initial activity is plotted as a function of inhibitor enzyme ratio (W/W); O——O N-lysozyme; ●——● P_2.

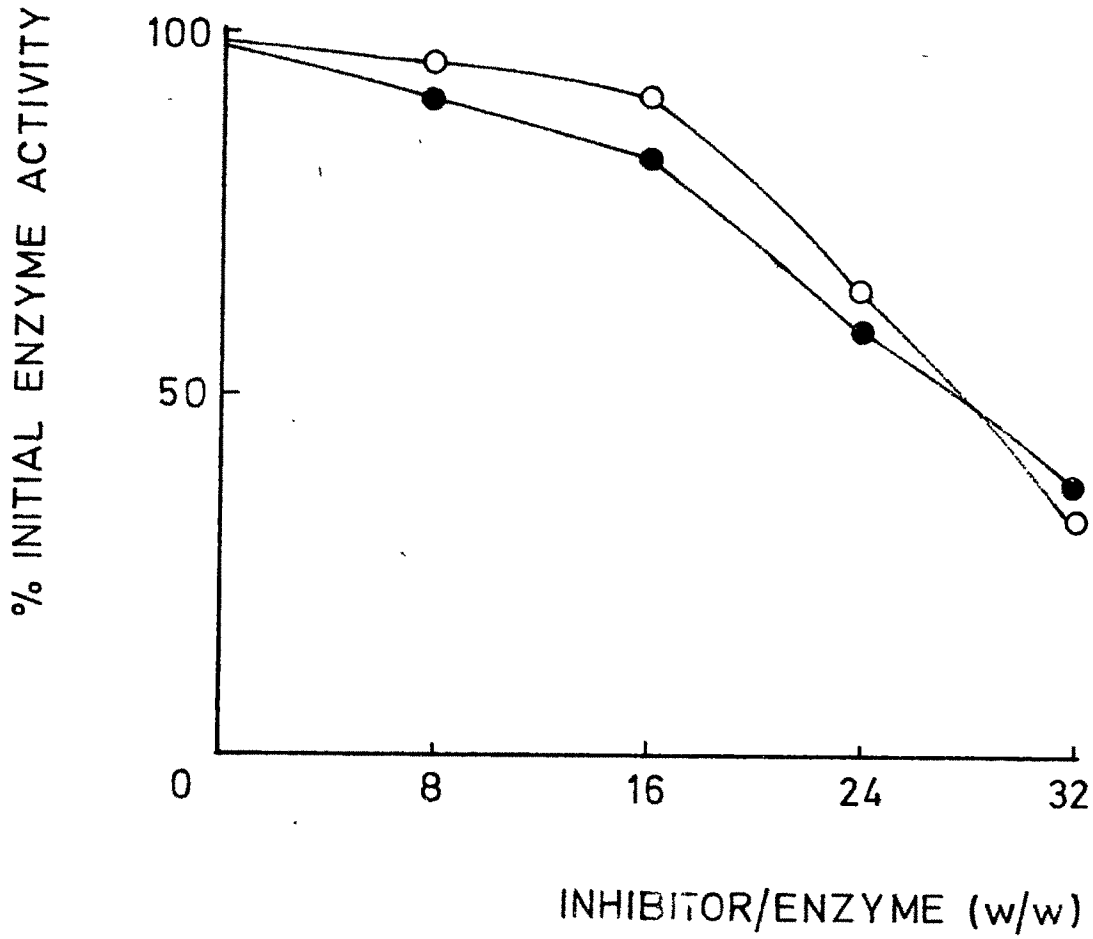

% INITIAL ENZYME ACTIVITY
100
50
0
8
16
24
32
INHIBITOR/ENZYME (w/w)

FIG. 9B

The effect of the competitive inhibitor, NAG on the activity of N-lysozyme, P_1 and P_2 is shown in Fig. 10. These data have been obtained at an inhibitor concentration of 0.15 M. Hereagain ~~in~~ all the three enzymes are inhibited to the same extent of about 60%. Since NAG is a competitive inhibitor the following conclusions may be drawn:- 1) the inhibitor binding site on the lysozyme molecule is not affected by dinitrophenylation. If the modified residues were in the inhibitor site the inhibitor would not be able to bind and cause any decrease in the activity of the enzyme; 2) the evidence presented earlier shows that it is only the amino groups of the lysine residues that have been dinitrophenylated by FDNB and therefore lysine residues are not part of the binding (inhibitor) site.

This conclusion is further supported by the following experiment in which dinitrophenylation was carried out in the presence of NAG. This experiment was performed at 26° for 30 minutes using FDNB to enzyme ratio of 412:1 and an inhibitor concentration of 0.15 M. The DNP-derivative that was obtained was identical to the one prepared in the absence of the inhibitor in its enzyme activity. On a mole to mole basis the concentration of the inhibitor is very high compared to the concentration of the FDNB. Even if the binding affinity of the enzyme for the inhibitor is low this high concentration of the inhibitor should be able to protect the inhibitor site from dinitrophenylation. The observation that the DNP-lysozymes prepared by interaction with FDNB in the presence or absence of NAG are identical in enzyme activity would suggest that the

Fig. 10. The effect of N-acetyl-D-glucosamine on the activity of N-lysozyme; P_1 and P_2. Enzyme activity measured in the presence or in the absence of inhibitor is plotted as a function of time; 0——0 N-lysozyme; △——△ P_1; and □——□ P_2; in the absence of NAG; ●——● N-lysozyme; ▲——▲ P_1 and ■——■ P_2 in the presence of 0.15M NAG; 2.9 µg/ml, at 26°.

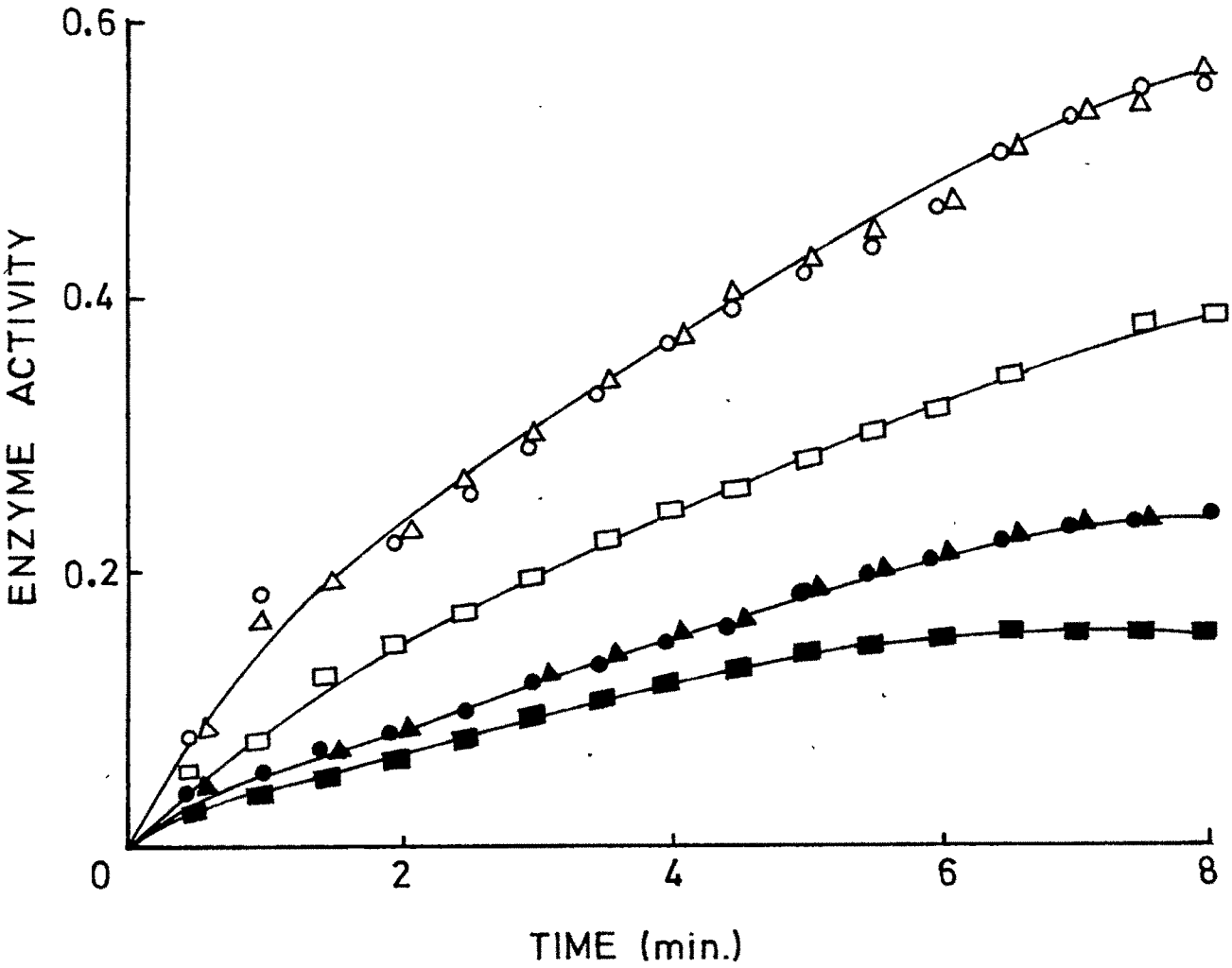


FIG.10

presence of the inhibitor does not protect the lysine amino groups that react with FDNB under the experimental conditions. This fact would by implication mean that lysine residues do not form a part of the binding site on the lysozyme molecule. This conclusion is in harmoney with that from X-ray studies in which it is shown that none of the six lysines is situated close to the area on the lysozyme molecule occupied by NAG molecule (76). The above results support the conclusions drawn from the data obtained from pH and buffer concentration studies that the introduction of DNP-groups does not effect the interactions involved in enzyme-substrate or enzyme-inhibitor complex formation.

The heat stability of N-lysozyme and P_2 are shown in Fig. 11A. There was no precipitation of the enzyme either during heating or chilling. In Fig. 11A the enzyme activity has been expressed as the ratio of the activity of the heated enzyme to that of the unheated enzyme as a function of the temperature. It may be seen that heating N-lysozyme or P_2 to temperatures upto 90° does not inactivate the enzyme. Thus the DNP-lysozyme like the native enzyme was found to be heat stable. The heat stability observed with N-lysozyme is in agreement with the observations of other workers (160). It is however significant that the DNP-lysozyme is also heat stable and its activity remains unchanged over the temperature range of 40 to 90°. It had been anticipated in these experiments that if dinitrophenylation had caused small changes in the conformation of lysozyme molecule, these might be amplified by heating. Since the DNP-derivatives of

lysozyme behaves like N-lysozyme, no important changes in the conformation of DNP-lysozyme appear to have occurred.

In Fig. 11B, C the effect of exposure of N-lysozyme P_1 and P_2 to either 4M urea or 2M GU-HCl for different intervals of time on the activity of enzyme is given. The measurements were made in 0.1M phosphate buffer of pH 6.8. The enzyme solution was prepared in the buffer solution containing 4M urea or 2M GU-HCl and exposed to the denaturing agents for durations shown in the graph. For the assay of enzyme activity the solutions were diluted with the buffer so that the final concentration of the denaturants was negligible. These experiments were carried out mainly to determine whether the enzyme activity could be reversed after the conformation of the protein molecule is changed in contact with denaturing agents. It may be seen that neither 4M urea nor 2M GU-HCl affect the initial activity of N-lysozyme, P_1 or P_2. Our observations with native lysozyme are in agreement with those of other workers (161, 162). The complete reversal of the enzyme activity would suggest that the conformation of the active site of P_1 and P_2 is not much different from that of N-lysozyme.

It is known both by O.R.D. measurements and by X-ray diffraction methods that lysozyme contains α-helix, β-pleated structure and random coil (18, 19). The first two structures arise out of hydrogen bonding. Urea is believed to rupture hydrogen bonds and thus disrupt the organised structure. Recent concepts suggest that urea may also act on the hydrophobic bonds (163). Since urea is unable to inactivate the enzyme irreversibly, it may mean that either the conformation of the

Fig. 11A. The effect of heat on the enzyme activity of N-lysozyme and P_2; O——O N-lysozyme; ●——● P_2.

Fig. 11B.C. The reversible effect of 2.0 M Gu-HCl or 4.0 M urea on the enzyme activity of N-lysozyme, P_1 and P_2.

11B. 2.0M guanidine HCl

11C. 4.0M urea

O——O N-lysozyme; △——△ P_1; and ●——● P_2.

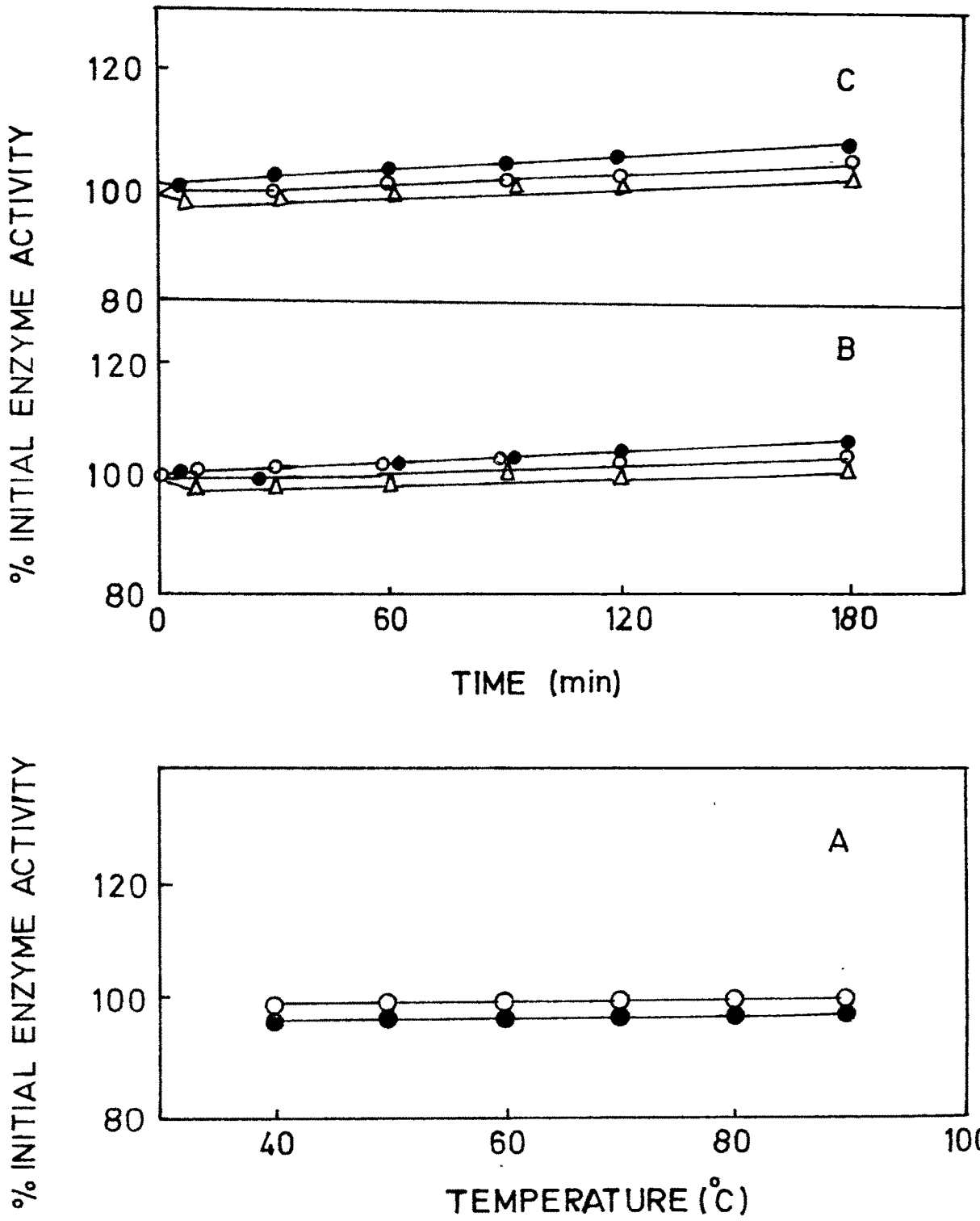

% INITIAL ENZYME ACTIVITY
120
100
80
C
B
0
60
120
180
TIME (min)
A
40
60
80
100
TEMPERATURE (°C)

FIG. 11

catalytic site is unaffected by urea or the regions of hydrogen bonded structure are shielded from urea. It is also probable that any unfolding of lysozyme that occurs in the presence of urea is completely reversed on removing the denaturant. That this interpretation is more probable is shown by the following experiments.

The effect of 4 M urea on the enzyme activity of N-lysozyme, P_1 and P_2 determined in phosphate buffer pH 6.8 and 0.1M is given in Fig. 12. In this series of experiments the solutions which were used for enzyme activity measurements contained 4M urea. The data are given as enzyme activity versus time of incubation. The activity of N-lysozyme and P_1 are identical. P_2 has approximately 55% of the activity of N-lysozyme. In the presence of urea the activities of all the three enzymes are reduced (unlike in phosphate buffer of pH 6.8 and 0.06M). The activity of P_1 decreased to the same extent as that of native lysozyme. In the case of P_2 also the extent of decrease was more or less the same. It may be pointed out that the data obtained in the presence of 4M urea are subject to some uncertainty because of the reduced activity of enzyme. These results would qualitatively suggest that the effect of urea on N-lysozyme and on the two DNP-lysozyme is the same.

There have been reports in literature that lysozyme is active in the presence of urea and is in fact reported to be more active in the initial stages of the reaction (164, 165). In view of these results we have made detailed studies on the effect of 4M urea at various molarities of phosphate buffer to

Fig. 12. The effect of 4.0M urea on the enzyme activity of the N-lysozyme, P_1 and P_2. O——O N-lysozyme; △——△ P_1; and □——□ P_2 in the absence of urea; ●——● N-lysozyme; ▲——▲ P_1; and ■——■ P_2 in the presence of 4.0M urea; 2.9 µg enzyme/ml, at 26°.

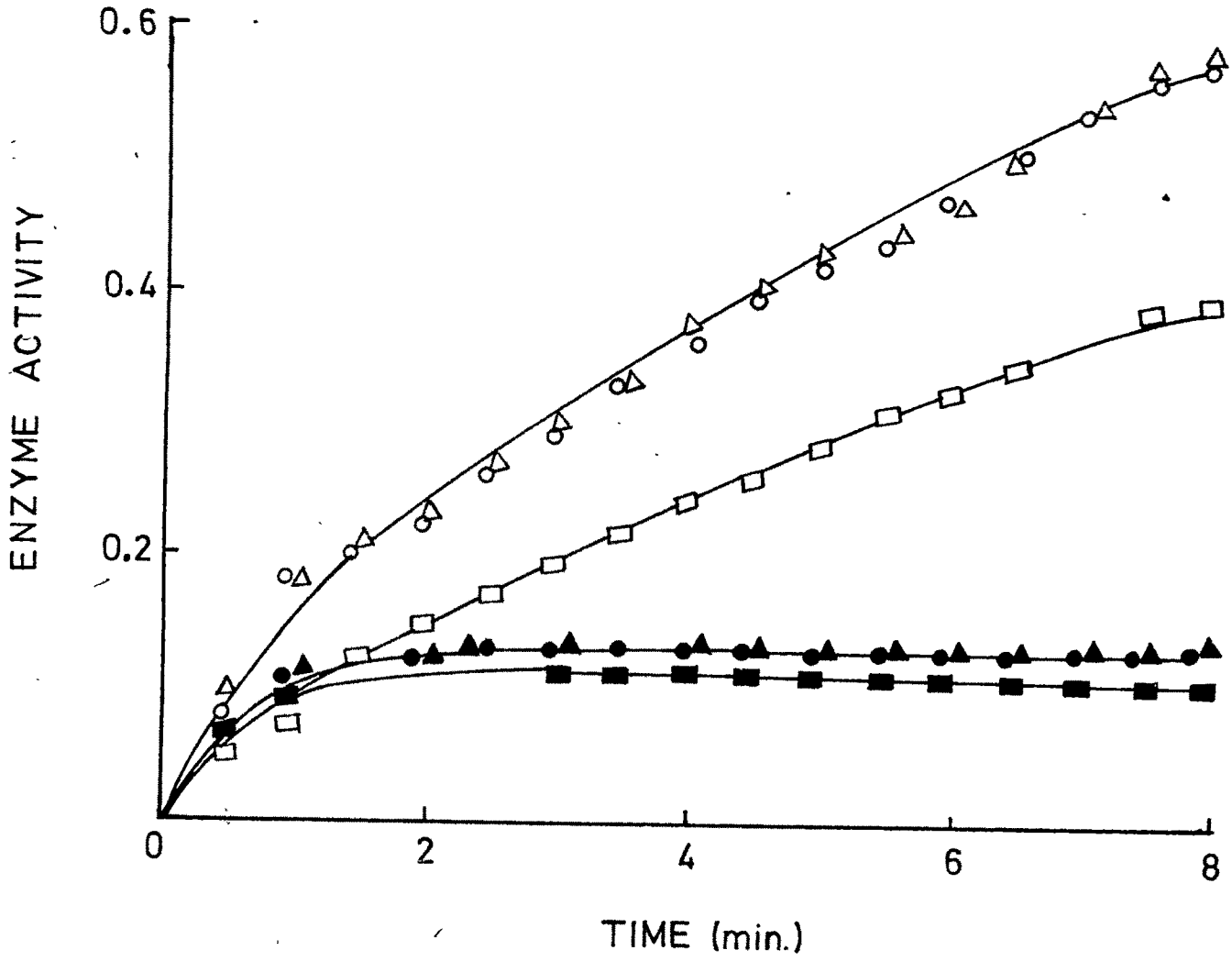


FIG.12

determine if the reduced activity we obtain in 0.1M phosphate buffer is due to the high concentration of the salt; earlier workers have used phosphate buffer of 0.03 M. In Fig. 13 the enzyme activity of native lysozyme measured as a function of time in phosphate buffer of different molarities with and without added urea is given. It may be seen that even in the absence of urea, molarity of phosphate buffer has an effect on the activity of lysozyme. The activity increases with phosphate molarity from 0.02M to 0.06M and then decreases. Further the effect of urea is also not constant. Whereas the addition of urea to enzyme and substrate in 0.02M buffer enhances the activity, in 0.03 M buffer it does not alter the activity and in 0.06 and 0.1 M buffer the activity actually decreases. Thus the decreased activity we have observed in 4 M urea in contradiction to earlier reports is no doubt due to the high molarity of phosphate buffer which we have used. The differing effects of urea at different phosphate molarities may be due to the enzyme having different conformations at different phosphate molarities.

It was also of interest to determine if GU-HCl which is known to be a more powerful denaturing agent than urea has a similar effect on the activity of N-lysozyme. The effect of various concentrations of GU-HCl on activity was determined at a single concentration of phosphate buffer 0.03 M. From the data given in Fig. 14 it is seen that low concentrations of GU-HCl slightly increases the activity of lysozyme and high concentrations decrease the activity appreciably. This effect may possibly be a result of the binding of the denaturant to the enzyme in such a way that it inactivates the enzyme. The observations that

Fig. 13. The enzyme activity of N-lysozyme in the absence or in the presence of 4.0 M urea at different molarities of phosphate buffer; pH 6.8. The activity is plotted as a function of time; ○ 0.02M; △ 0.03M; □ 0.06M; and ▽ 0.1M in the absence of urea; ● 0.02M; ▲ 0.03M; ■ 0.06M; and ▼ 0.1M in the presence of urea; 3.0 µg enzyme/ml, at 26°.

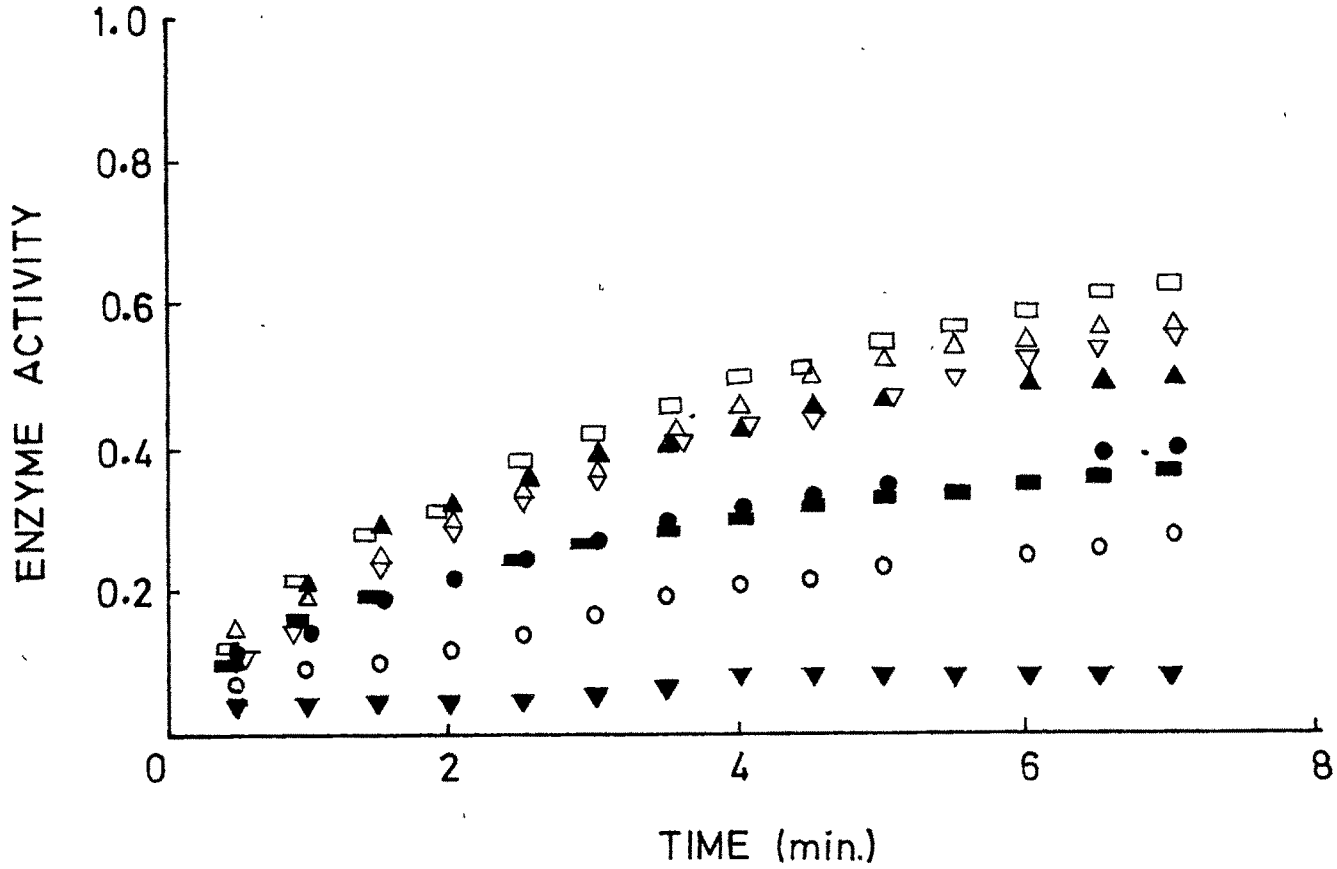


FIG.13

Fig. 14. The enzyme activity of N-lysozyme in the absence or in the presence of different molarities of guanidine HCl in 0.03M phosphate buffer pH 6.8. The activity measured is plotted as a function of time; O——O in the absence of guanidine HCl; ●——● 0.028M; △——△ 0.57M; ▲——▲ 0.1M; and □——□ 0.2M guanidine HCl. 3.1 µg enzyme/ml, at 26°.

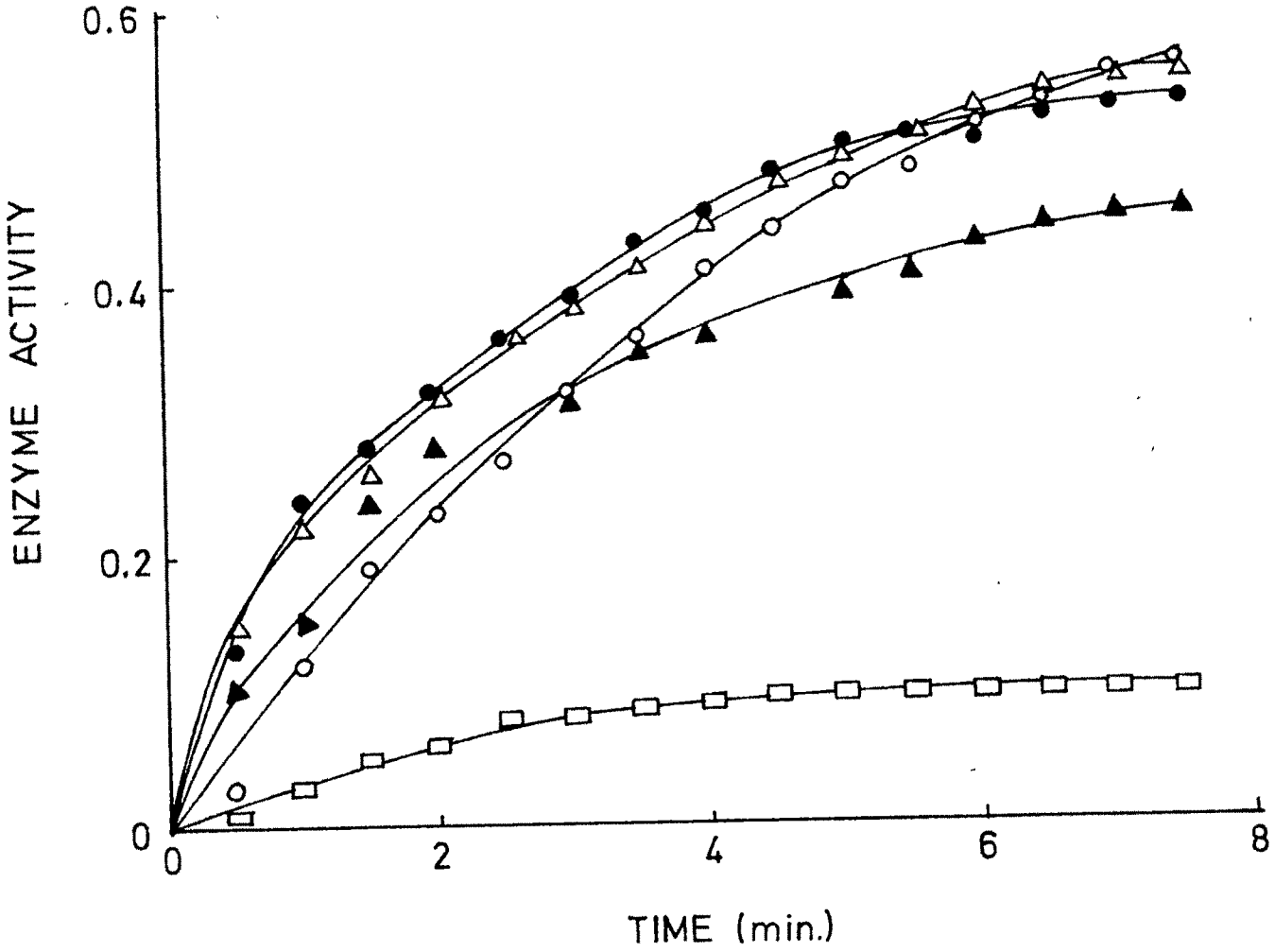


FIG.14

urea and GU-HCl have a similar effect on N-lysozyme, P_1 and P_2 would suggest that all the three have the same conformation.

It has been postulated that GU-HCl molecules may denature a protein by actually binding either to the aromatic amino acids or to the peptide bonds or both (166). Studies on the effect of GU-HCl on the fluorscence spectra of lysozyme support the postulate of GU-HCl binding by lysozyme (167).

The results on the enzyme activity of the lysozyme modified to varying extent have been discussed so far. It was observed that enzyme activity was reduced as the DNP-content of the molecule increased. However, the derivatives containing different amounts of DNP retain the characteristic properties of the enzyme. Thus the modified derivatives retained original pH and ionic strength optima, were inhibited by the inhibitors and were affected by the denaturing agents in the same way as N-lysozyme. Even the enzyme activity was easily reversible on removal of the denaturing agents. The pH, buffer concentration and denaturation studies also show that the conformation of the derivatives is not affected to any appreciable extent due to modification. Inhibitor studies show that the binding of the competitive and noncompetitive inhibitors is not blocked due to the presence of the bulky DNP-groups in the enzyme molecule. And from this it could be inferred that DNP-lysozyme binds the substrate, M. lysodeikticus, in the same way as the unmodified lysozyme. The affinity of the enzyme for the substrate is largely governed by the electrostatic interactions between the enzyme and the substrate. The present data show that these inter-

actions are not markedly affected by the introduction of the DNP-groups into lysozyme molecule. It can therefore be concluded on the basis of the above observations that the lysine residues neither participate in the enzyme activity nor are involved in the maintenance of the conformation. And the decrease in the enzyme activity could be due to some other factors.

The data lead to the suggestion that the introduction of DNP-groups into lysozyme molecule does not interfere with the first step of combination of the DNP-enzyme with the substrate. A reasonable explanation of the decrease in the enzyme efficiency appears to be that a stable (non-productive) complex may be formed between DNP-lysozyme and the substrate which is converted into the product and enzyme with difficulty. It seems probable that arylation of the lysozyme molecule increases its adsorbing power. It is perhaps relevent to mention here that most of the DNP-derivatives (of different proteins) (130, 168) are found to be adsorbed strongly on the resins usually employed for purification purposes. The process may be analogous in that the modified lysozyme binds the cellular substrate more tenaciously aided by the aryl-groups. This may result in the formation of DNP-lysozyme-substrate complex which is transformed only slowly and may be the cause of the reduced rate of the enzyme activity observed with the modified lysozyme.

Our data and those of other workers on different derivatives of lysozymes still leave us unclear regarding the role of lysine residues in the lysozyme molecule. X-ray studies show that all the six lysine residues are distributed on the surface of

the molecule. These residues carry basic functional group and it is probable that energetically these residues prefer chain-solvent contacts over chain-chain contacts. And it may be the function of lysine residue to interact with the solvent molecules to keep the protein molecule in solution. Otherwise perhaps a stable solution could not be achieved and a precipitate would result. It is frequently observed that when the lysine residues of lysozyme are blocked with any of the modifying agents (acyl, aryl, or others) the solubility of the protein is decreased. We have made a similar observation. DNP-derivatives when prepared at alkaline pH (to be reported later) easily precipitated in phosphate buffer solution and could be solubilized only either in acidified solution or in the 0.1 M acetic acid. DNP-derivative prepared at pH 7.2 which we have shown to retain the native conformation can also be slowly precipitated in alkaline buffers.

Several studies on the modification of lysine residues in lysozyme show that these residues are not important for either activity or conformation. However the solubility characteristics of such modified lysozymes are different from those of N-lysozyme suggesting that these residues may function mainly as solubilizing side chains.

Effect of modifications on the physicochemical properties of the derivatives:

In the results reported so far it was shown that the introduction of one to six DNP-groups in the lysozyme molecule did not affect its enzymatic properties. The fact that enzyme activity was reversible after the DNP-derivative was exposed to

either heat or the denaturing agents suggested that the introduction of DNP-groups at pH 7.2 did not affect the overall conformation of lysozyme molecule, or atleast the local conformation of the active centre to any appreciable extent. Limited conformational changes around the substituted residues, however, cannot be ruled out. Besides these studies could not indicate if the introduction of the DNP group into the enzyme molecule occured to the same extent and at the same amino acid residues in the entire population of lysozyme molecule. If, dinitrophenylation being a random reaction, resulted in a mixture of DNP-derivatives modified to varying extents at different positions, it would be difficult to account for the loss of activity due to the modification of one or more particular residue(s).

Studies on the three DNP derivatives, P_1, P_2 and P_3 substituted to varying extent (as described in the preceeding experiments) were therefore extended. Some physicochemical characteristics and homogenity of the derivatives were examined in detail by the following techniques. 1) Ultracentrifugation 2) UV-spectra, 3) gel filtration on Sephadex G-100, 4) Column chromatography on CM-cellulose and Amberlite IRP-64, 5) electrophoresis on starch gel, 6) disc electrophoresis with polyacrylamide gel and 8) by determining the rate of tryptic hydrolysis.

In Fig. 15A, B the absorption spectra of N-lysozyme, P_2 and P_3 in the range 220 to 420 mμ are shown. The spectrum of N-lysozyme is that of a typical protein with an absorption

Fig. 15A. Absorption Spectra of N-lysozyme and P_2, (in 0.2M acetate buffer, pH 5.4) O——O N-lysozyme (concentration: 0.1 mg/ml); ●——● P_2 (concentration: 0.1 mg/ml).

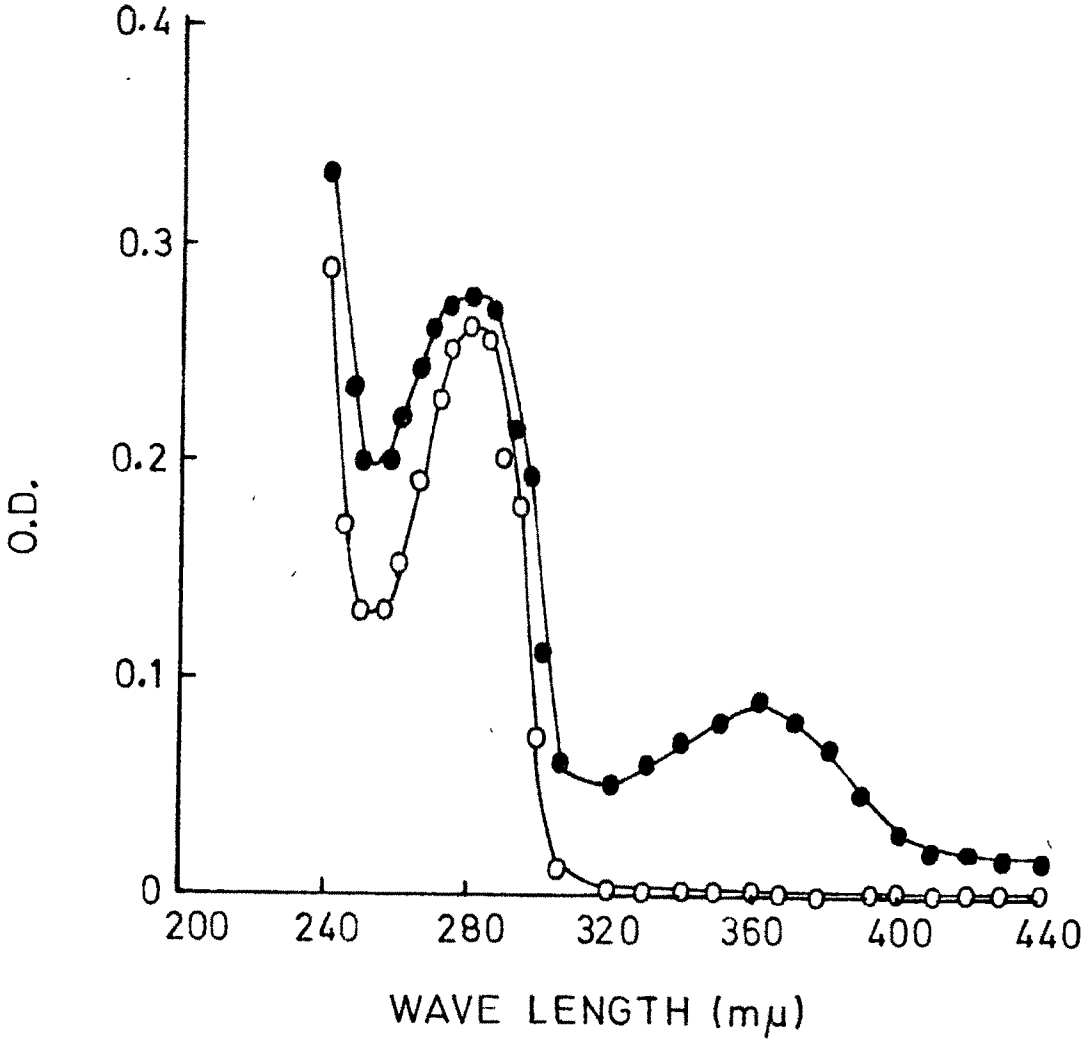


FIG. 15 A

Fig. 15B. Absorption Spectra of P_3 (in 0.2M acetate buffer, pH 5.4)
concentration: 0.2 mg/ml.

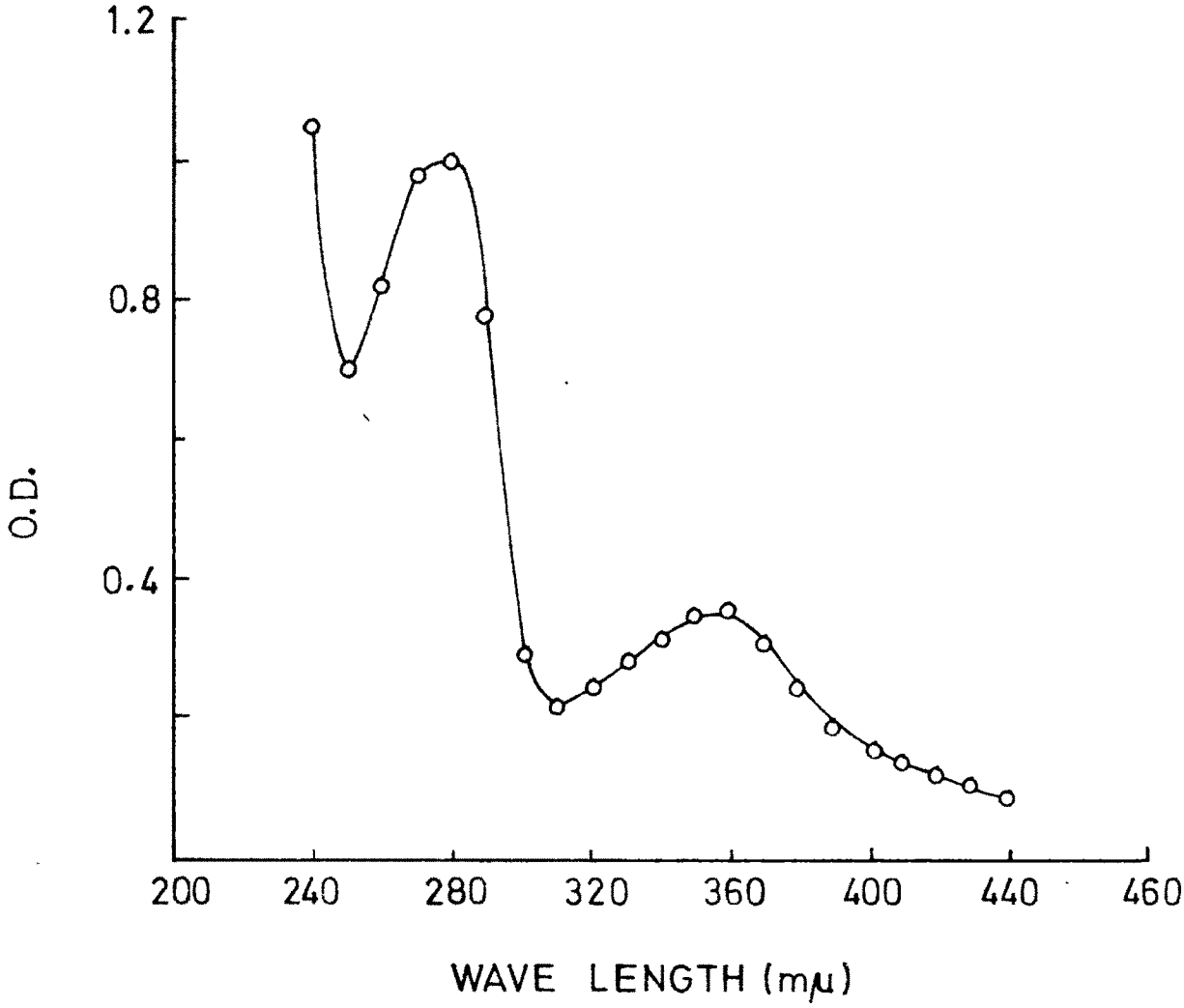


FIG. 15B

minimum at 250 mμ and an absorption maximum at 280 mμ. Both P_2 and P_3 give the same type of spectra. In addition they have another absorption maximum at 360 mμ which is due to the absorption by the dinitrophenyl groups (138). The absorption maximum of N-lysozyme, P_2 and P_3 occur at the same wavelength namely 280 mμ. We have also measured the absorption spectrum of N-lysozyme in presence of 8 M urea in phosphate buffer of pH 6.8 (0.06 M); under these experimental conditions the enzyme is inactivated to 80%. However, there is no change in the wavelength of maximum absorption. Perlmann has reported that when pepsin is denatured its absorption maximum shifts from 278 mμ to 276 mμ (169). The observation that the absorption maximum of P_2 and P_3 occur at the same wavelength as that of N-lysozyme seems to suggest that dinitrophenylation has not caused any change in the conformation of lysozyme which would affect its absorption spectra. It would have been desirable to determine the difference spectra of P_2 and P_3 with N-lysozyme as the reference material. Unfortunately, these experiments could not succeed for the following reasons:- 1) It was very difficult to adjust the concentrations of the proteins in the reference cell and in the experimental cell to identical values which is a prerequisite for taking different spectra; 2) dinitrophenyl group itself has absorption in the ultraviolet region and this vitiated the results. For the same reasons, O.R.D. measurements in the visible range could not also be made.

Therefore information on conformation was obtained indirectly by studying the effect of heat, urea and GU-HCl on the enzyme activity of N-lysozyme and of the DNP-derivatives. The data

obtained by measurements of the UV spectrum and by ultra-centrifuge studies further support the earlier observations.

The ultracentrifugal patterns of N-lysozyme, P_1, P_2 and P_3 are given in Figs. 16A, B, and C. In all cases single symmetrical peaks were obtained indicating that all the products were homogeneous with respect to molecular weight and shape. The sedimentation coefficients and molecular weights (determined by Archibald method) are given in Table 6. There is no significant difference between $S_{20,W}$ value of N-lysozyme and of DNP-lysozymes. The value of N-lysozyme is in good agreement with the reported values (140, 170). The molecular weight obtained is slightly higher than the value reported by sedimentation-diffusion technique and the value computed from the amino acid analysis data namely 14,600. Sophianopoulos and Van-Holde have reported that lysozyme undergoes a dimerisation reaction above pH 5.4 and the extent of dimerisation increases with the increase in pH (104). It is therefore likely that the slightly higher value that we have obtained is due to the tendency of lysozyme to dimerise. Narasinga Rao and Pandit have also reported a value of 18,000 at pH 7.0 from ultracentrifuge field relaxation technique (171). However, the more important point of interest to us is that there is no difference between the molecular weight of N-lysozyme and the DNP-lysozymes.

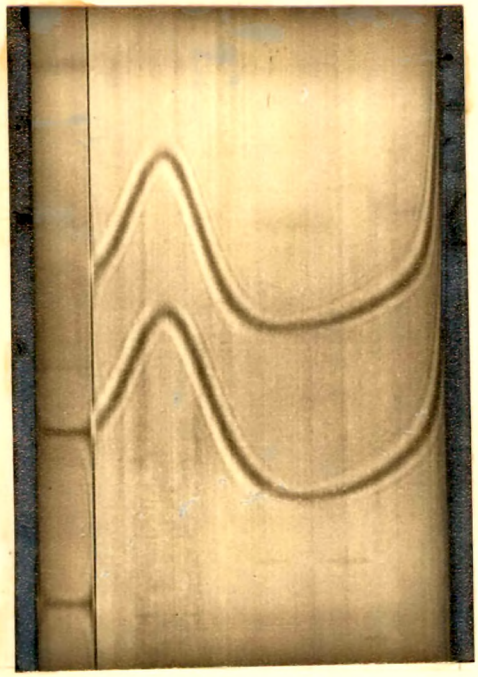
In Figs. 17A, B, the gel filtration patterns of N-lysozyme, P_1, P_2 and P_3 are given. In each case a single symmetrical peak without indication of any other major or minor peak is obtained. With P_1, P_2 and P_3 in addition to absorbance at 280 mμ, absorbance

Fig. 16. The sedimentation velocity pattern of N-lysozyme, P_1, P_2 and P_3

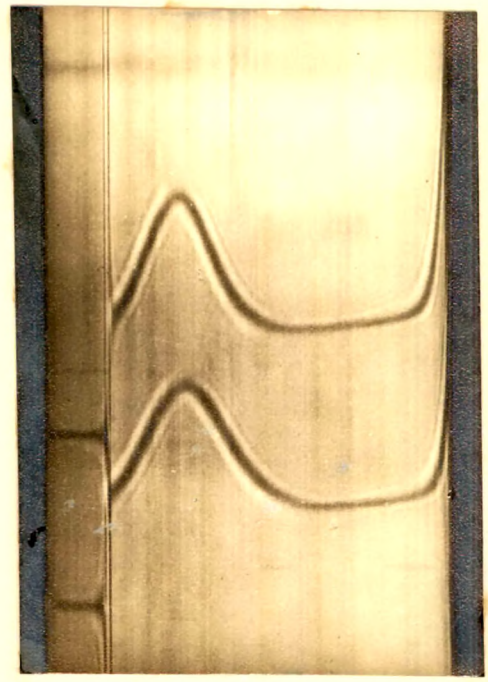
A. P_1; picture taken at 76 min.

B. Upper pattern N-lysozyme and lower P_2; picture taken at 80 min.

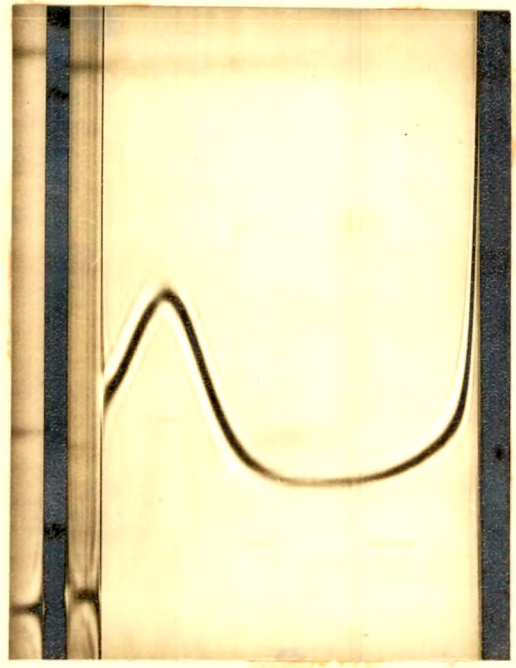
C. Upper pattern N-lysozyme and lower P_3; picture taken at 80 min. Centrifugation was done at 59, 780 rpm (sedimentation proceeds from left to right).



B.



C.



A.

FIG. 16

TABLE No. 6

SEDIMENTATION COEFFICIENT AND MOLECULAR WEIGHT OF NATIVE AND DNP-LYSOZYMES

	Buffer system	Native lysozyme	P_1	P_2	P_3
Sedimentation Coefficient ($S_{20,w}$)	0.2M Acetate buffer pH 5.4	1.9 - 2.0	-	-	2.2
	0.2M sodium phosphate buffer pH 7.2	1.9	1.98	2.0	-
Molecular weight	0.2M sodium phosphate buffer pH 7.2	18,400	18,200	17,700	-

Fig. 17A. The gel filtration pattern of N-lysozyme and P_2 on Sephadex G-200; column size 1.1 x 43 cm; eluant 0.1M acetic acid; N-lysozyme (load 17.0 O.D. at 280 mμ), P_2 (load 20.0 O.D. at 280 mμ); O——O O.D. at 280 mμ; ●——● O.D. at 360 mμ.

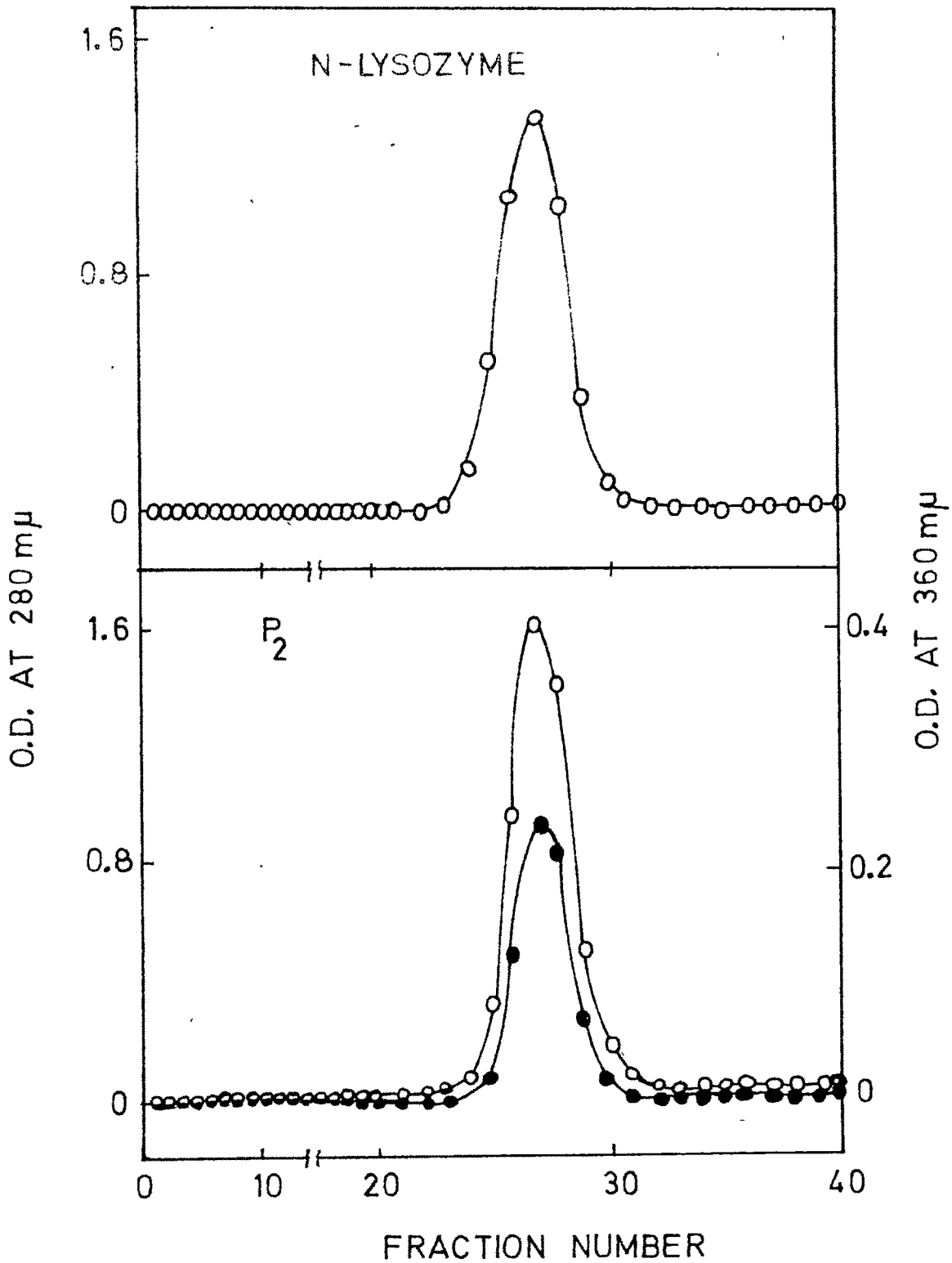

N-LYSOZYME
1.6
0.8
0
P2
1.6
0.8
0
0.4
0.2
0
O.D. AT 280 mμ
O.D. AT 360 mμ
0
10
20
30
40
FRACTION NUMBER

FIG. 17A

Fig. 17B. The gel filtration pattern of N-lysozyme, P_1 and P_3 on Sephadex G-100; Column size 1.2 x 46 cm; eluant 0.1M acetic acid; N-lysozyme (load 22.0 O.D. at 280 mμ); P_1 (load 22.5 O.D. at 280 mμ); P_3 (load 14.5 O.D. at 280 mμ); O——O at 280 mμ; ●——● O.D. at 360 mμ.

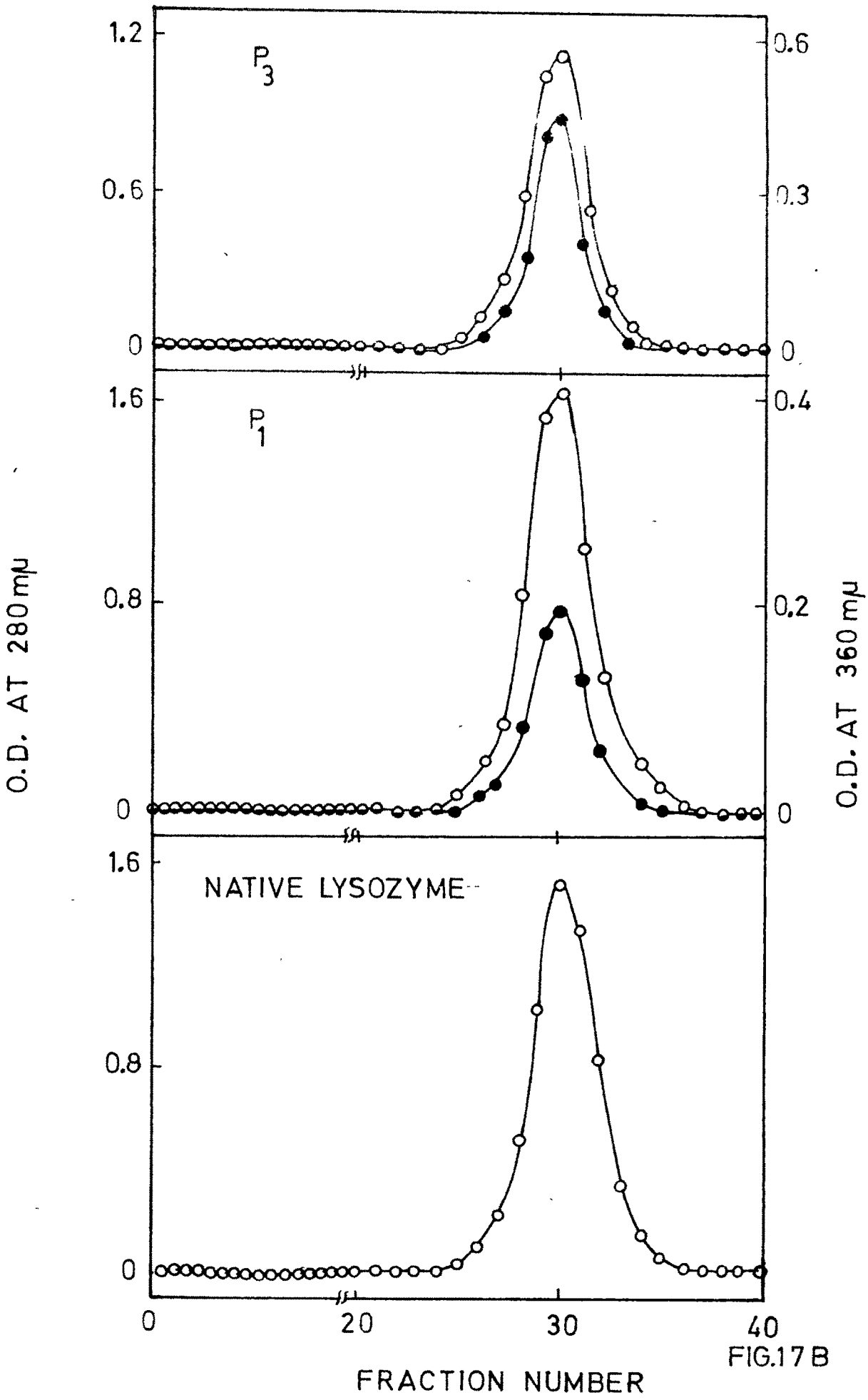

P3
P1
NATIVE LYSOZYME
1.2
0.6
0
0.6
0.3
0
1.6
0.8
0
0.4
0.2
0
1.6
0.8
0
0
20
30
40
O.D. AT 280 mμ
O.D. AT 360 mμ
FRACTION NUMBER

FIG.17 B

at 360 mμ has also been measured. The absorbance values at the two wavelengths follow the same order. In gel filtration technique separation of components takes place primarily on the basis of differences in molecular weight. This technique has been extensively used not only for the fractionation of proteins of different sizes but also for the determination of the molecular weights. On a column of given dimensions the elution volume of a protein depends upon its molecular weight (172) As the molecular weight decreases the elution volume increases. The fact that P_1, P_2 and P_3 elute at the same position as N-lysozyme shows that dinitrophenylation has not caused any aggregation or degradation of lysozyme. Although, gel filtration is primarily based on differences in molecular weight, shape factors also have some influence (172). From the gel filtration experiments it may be concluded that the DNP-lysozymes are homogeneous with respect to size and shape.

The rate of hydrolysis of protein substrates by proteolytic enzymes may be used to determine subtle conformational changes in the substrate molecule which are not detected by conventional physicochemical methods. Markus (173) has shown that low molecular weight ligands bound by bovine serum albumin and human serum albumin cause a change in the rate of hydrolysis of these substrates by a variety of proteolytic enzymes. He has interpreted these results as an effect of the ligands on the conformation of the substrate. Maurer and Sriram (174) found that trichloroacetic acid (TCA) treated bovine serum albumin was hydrolysed at a slightly higher rate by trypsin although Narasinga Rao *et al* (175) found no differences between the TCA

treated and native bovine serum albumin by physicochemical measurements. Gurnani et al reported that TCA-treated lysozyme is hydrolysed by trypsin at the same rate as native lysozyme and there is no change in conformation either (170). Rate of tryptic hydrolysis of N-lysozyme and P_2 was measured to detect any change in the conformation of these two proteins. The rate of tryptic hydrolysis has been measured by determining the ninhydrin value as a function of time of hydrolysis by trypsin. The data are given in Fig. 18. The rate of tryptic hydrolysis seems to be the same for both native lysozyme and P_2. It is also seen from the figure that lysozyme is resistant to the action of trypsin for nearly 10 hr and only after this time interval there is measurable hydrolysis. This observation is in agreement with the reports of other investigators (170). This data appeared to suggest that the P_2 has a conformation similar to that of N-lysozyme.

However, aliquots tested for enzyme (lytic) activity at varying intervals of hydrolysis (Fig. 18) showed that P_2 looses enzyme activity at a faster rate than the native lysozyme. Thus it indicates that the small conformational changes lead to a different cleavage pattern affecting the enzyme activity. Probably, the differences in the rate of hydrolysis were not apparent because those peptide bonds next to DNP lysines were perhaps not hydrolysed by trypsin (168, 176). Modification of lysine restricts the points of the tryptic cleavage of the polypeptide chains to bonds involving arginine residues. In the case of DNP-lysozyme only bonds involving arginine might be cleaved. That the cleavage pattern is different in the native and in the P_2 was confirmed when 12 hr tryptic hydrolysate was

Fig. 18. The rate of hydrolysis of N-lysozyme and P_2 by trypsin. The enzyme activity and ninhydrin value plotted as a function of the time of hydrolysis; O- - -O N-lysozyme activity; ●——● P_2 activity; △- - -△ N-lysozyme ninhydrin value; ▲——▲ P_2 ninhydrin value. Lysozyme to trypsin ratio 25:1 (W/W).

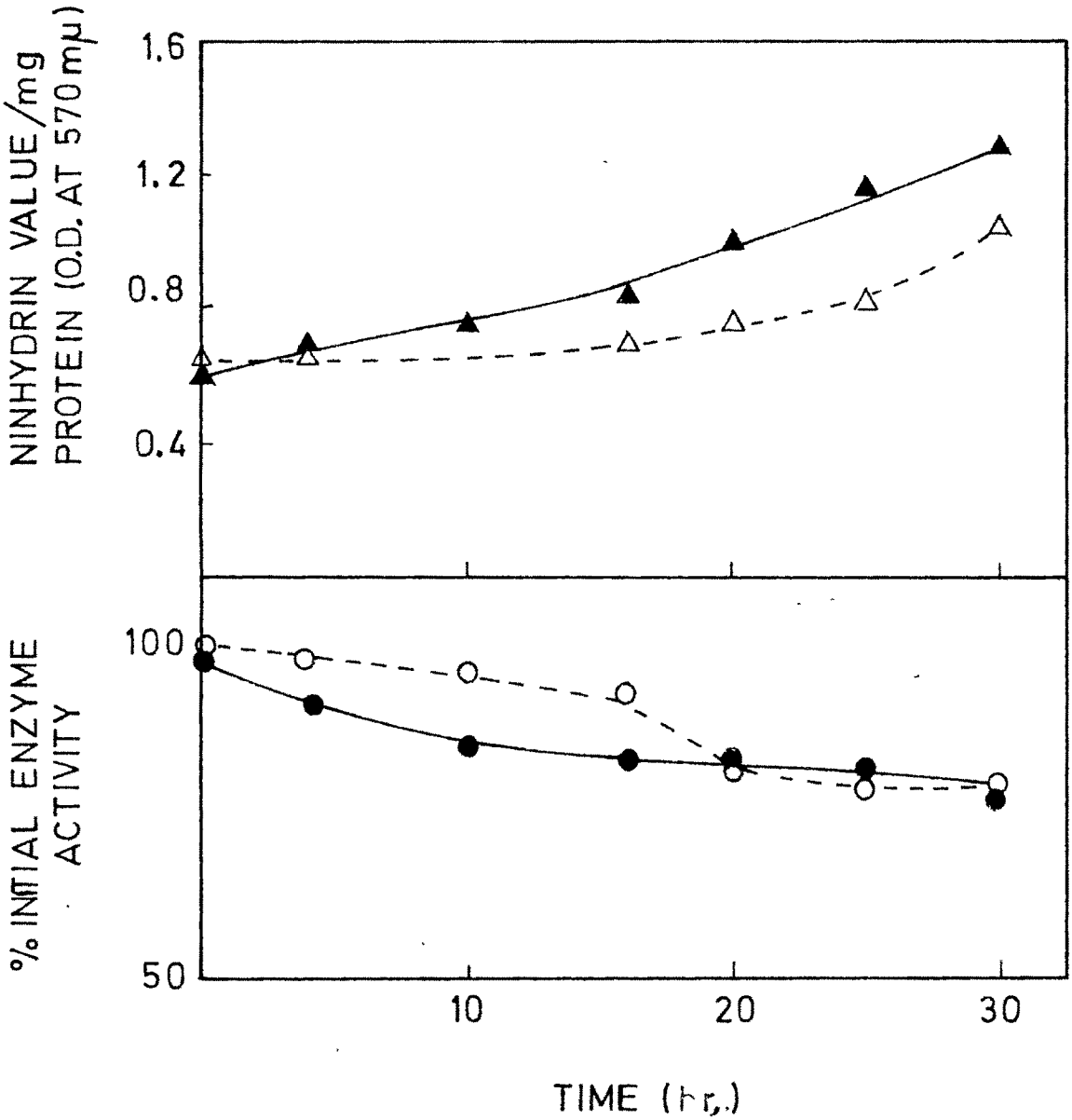

NINHYDRIN VALUE/mg PROTEIN (O.D. AT 570mμ)
1.6
1.2
0.8
0.4
% INITIAL ENZYME ACTIVITY
100
50
10
20
30
TIME (hr.)

FIG.18

analysed for cleavage fragments on a Sephadex G-100 column (Fig. 19A, B). As can be seen in this figure N-lysozyme is not cleaved by trypsin as extensively as P_2. The gel filtration pattern of hydrolysed P_2 is distinctly different from that of hydrolysed N-lysozyme. The 280 mμ absorption of the former shows the presence of two fragments not properly resolved. The earlier eluting peak shows enzyme activity while the latter peak which contains DNP group does not show much enzyme activity.

The tryptic hydrolysis data indicates that the substitution of the DNP-groups probably involves local small conformation changes. Although, these changes could not be detected by UV absorption, ultracentrifugation or reversibility (of the enzyme activity) experiments, they were detectable by the combined determination of the activity, rate of tryptic hydrolysis and analysis of the hydrolysate on the Sephadex column. It is interesting to note that such limited conformational changes do not affect the reversibility of the enzyme activity as described in detail in the preceeding pages.

Fractionation based on a different principle namely ion exchange phenomena was also used to determine the homogenity of the N-lysozyme and DNP-derivatives. In Fig. 20 the chromatograms obtained on CM-cellulose using salt gradients for elution are given. All the proteins were eluted from the column at a salt concentration of 0.2 to 0.30 M. N-lysozyme gives a single peak with a trailing edge indicating that the native lysozyme may not be very homogeneous. P_2 and P_3 also lead to peaks with diffuse trailing edges. Both 280 mμ and 360 mμ absorbance readings follow the same order. Apart from

Fig. 19A. Gel filtration patterns of N-lysozyme before and after tryptic hydrolysis, on Sephadex G-100. Column size 1.1 x 42 cm; eluant 0.1M acetic acid. N-lysozyme (load 18.0 O.D. at 280 mμ) and tryptic hydrolysate (load 18.0 O.D. at 280 mμ). O——O at 280 mμ. ×- - -× specific enzyme activity.

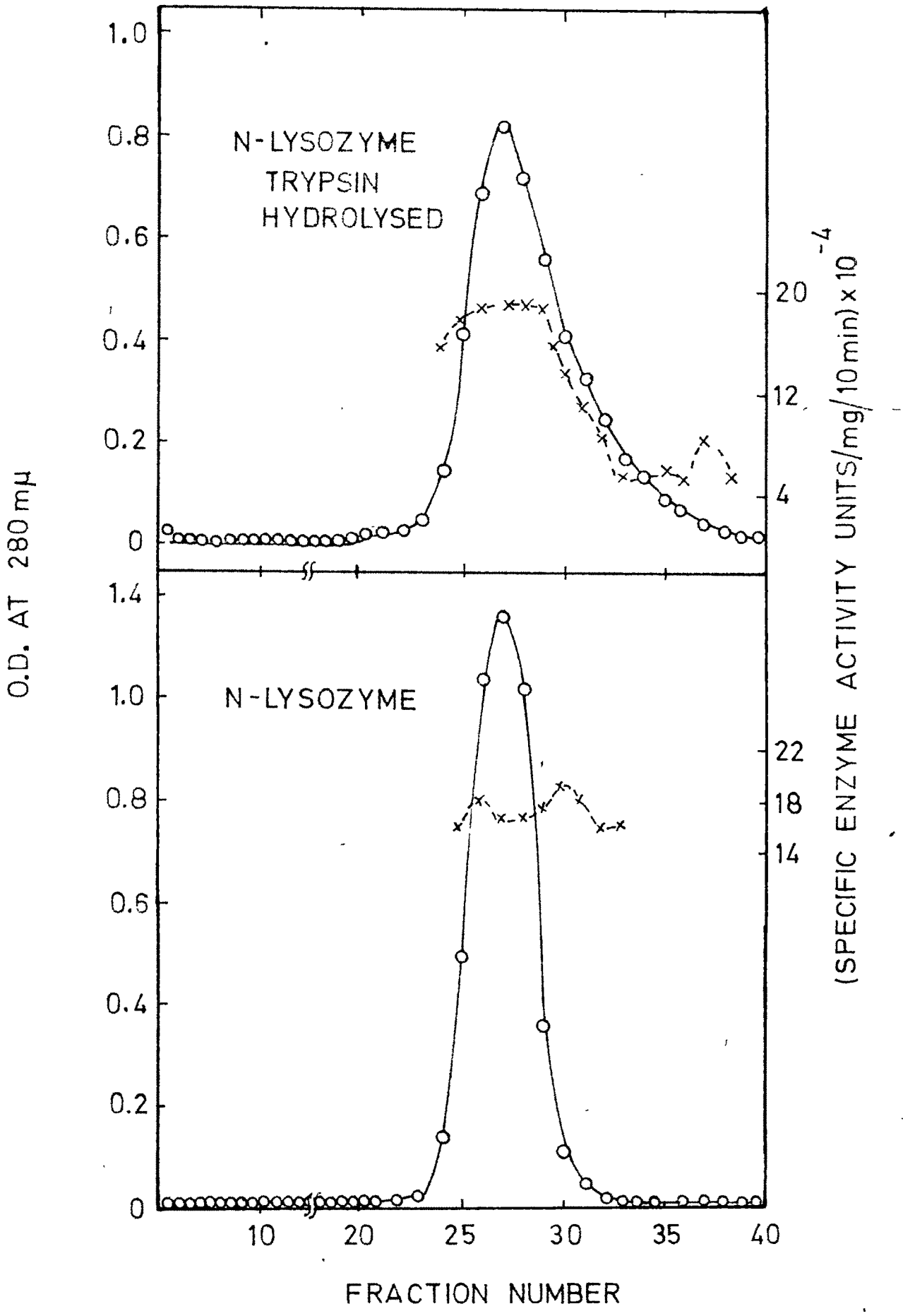

N-LYSOZYME
TRYPSIN
HYDROLYSED
N-LYSOZYME
O.D. AT 280 mμ
(SPECIFIC ENZYME ACTIVITY UNITS/mg/10 min) x 10 -4
FRACTION NUMBER
1.0
0.8
0.6
0.4
0.2
0
1.4
1.0
0.8
0.6
0.4
0.2
0
20
12
4
22
18
14
10
20
25
30
35
40

FIG.19A

Fig. 19B. Gel filtration patterns of P_2 before and after tryptic hydrolysis, on Sephadex G-100. Column size 1.1 x 42 cm; eluant 0.1M acetic acid. P_2 (load 11.5 O.D. at 280 mμ) and P_2 tryptic hydrolysate (15.0 O.D. at 280 mμ).

O——O O.D. at 280 mμ. ●——● O.D. at 360 mμ.

×--× Specific enzyme activity.

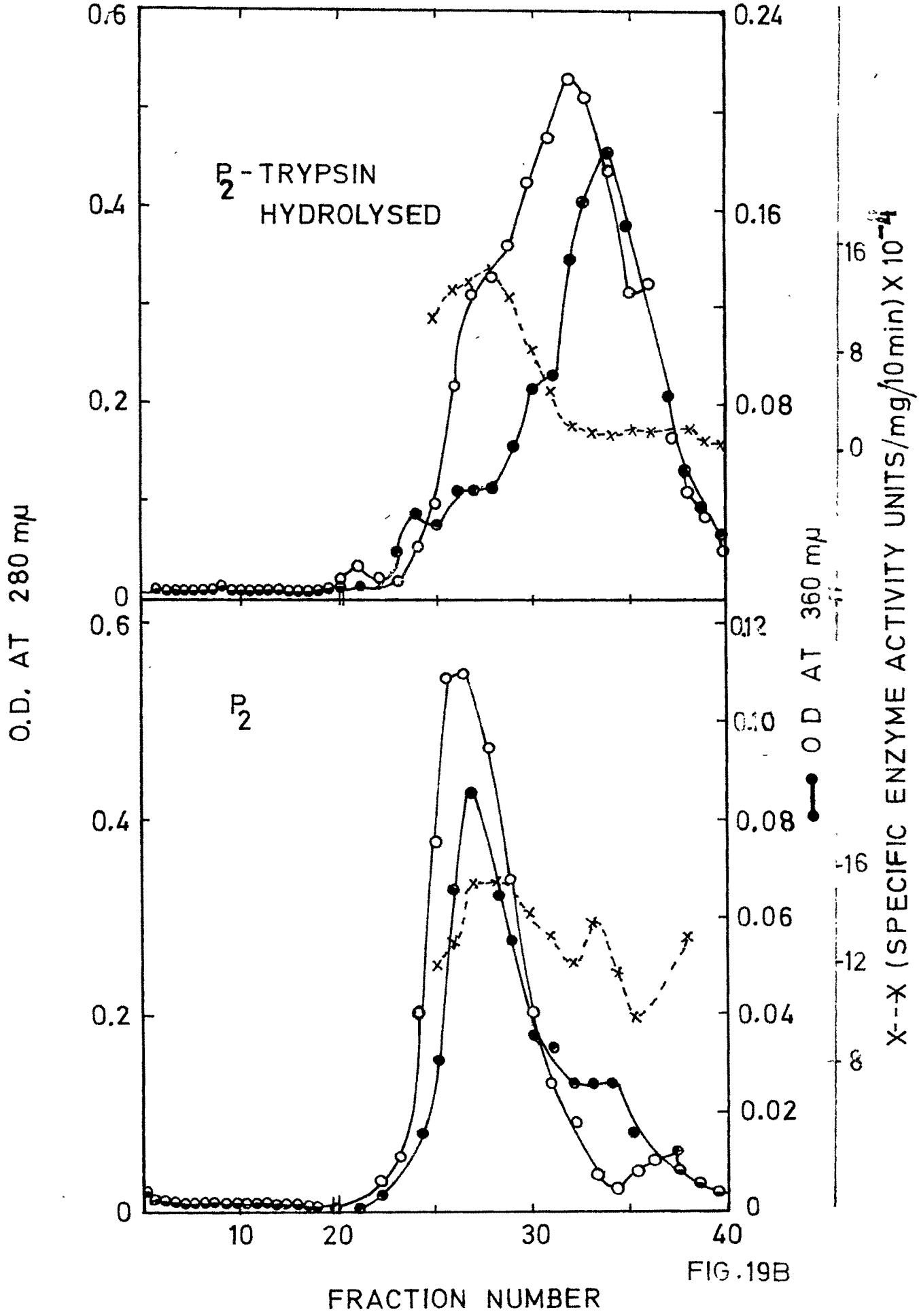

P2 - TRYPSIN HYDROLYSED
P2
O.D. AT 280 mμ
O D AT 360 mμ
X--X (SPECIFIC ENZYME ACTIVITY UNITS/mg/10 min) X 10⁻⁴
FRACTION NUMBER
0.6
0.4
0.2
0
0.24
0.16
0.08
0.12
0.10
0.08
0.06
0.04
0.02
16
8
0
-16
-12
-8
10
20
30
40

FIG. 19B

Fig. 20. Chromatography of N-lysozyme, P_2 and P_3 on CM-cellulose; Column size 1.3 x 20 cm; N-lysozyme (load 35.0 O.D. at 280 mμ); P_2 (load 40.0 O.D. at 280 mμ); P_3 (load 64.0 O.D. at 280 mμ); O——O O.D. at 280 mμ; ●——● O.D. at 360 mμ (a gradient of 0 to 0.45 M sodium chloride was used for elution).

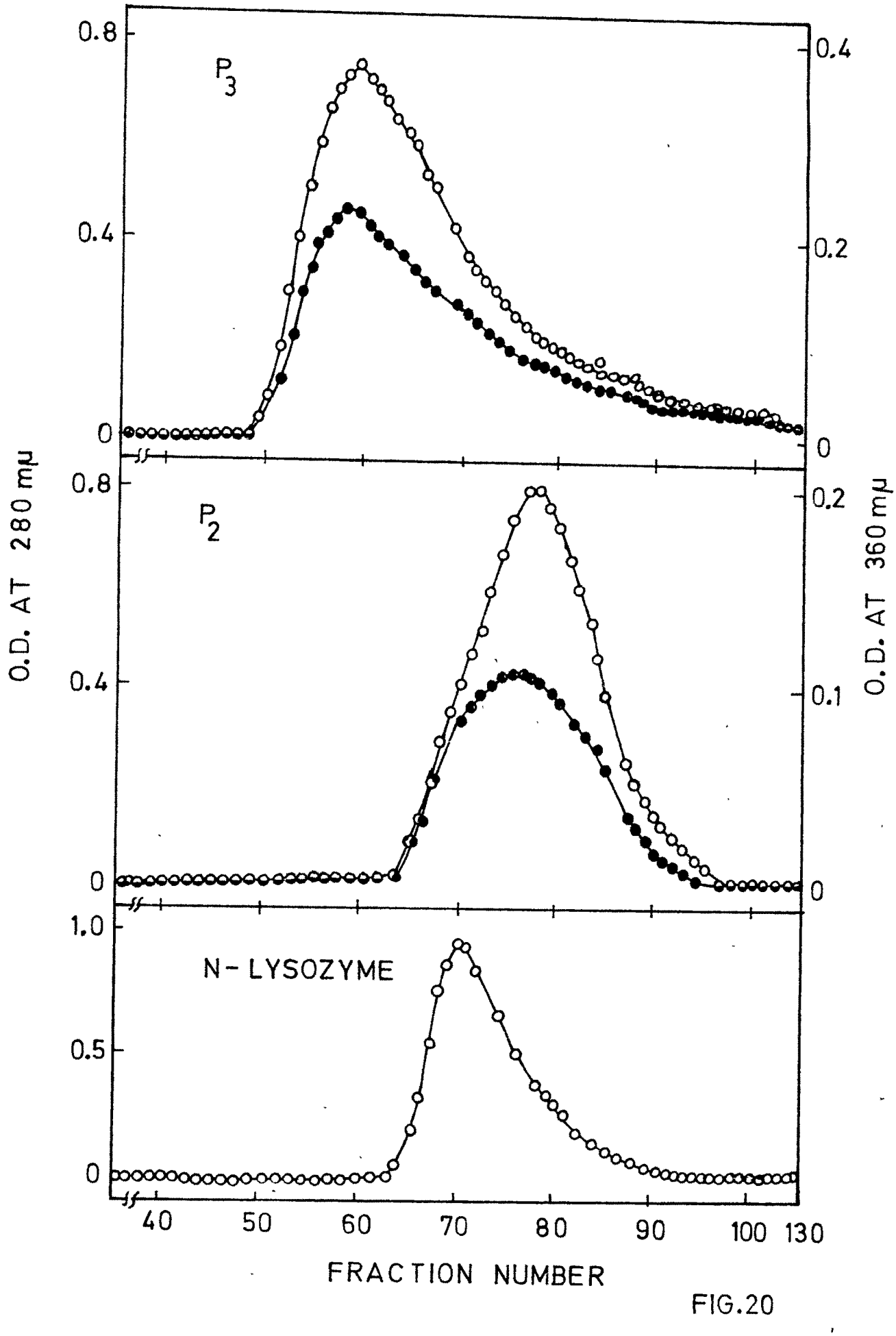

0.8
0.4
0
P3
0.4
0.2
0
0.8
0.4
0
P2
0.2
0.1
0
1.0
0.5
0
N-LYSOZYME
O.D. AT 280 mμ
O.D. AT 360 mμ
40
50
60
70
80
90
100
130
FRACTION NUMBER

FIG.20

the unsymmetrical nature of the major peak there is no indication of other heterogeneity. In the case of DNP-lysozyme the exaggerated unsymetrical pattern may be due to DNP-groups which are known to get strongly adsorbed on the resins. This has been shown with other DNP-protein such as DNP-RNase which was difficult to elute from the column (130, 168). The unsymmetry of the peak may also mean that the slight heterogeneity of P_2 and P_3 derivatives are not getting resolved on CM-cellulose. Similar patterns were obtained when separation was tried using combined salt and pH gradient between pH 5.4 - 8.5. From the chromatographic profiles on CM-cellulose it was felt that DNP-derivatives may or may not be as homogeneous as N-lysozyme itself. The results seem to suggest that during dinitrophenylation reaction the whole population of lysozyme molecules may not have been labelled uniformly with DNP-groups. It therefore appeared that dinitrophenylation of lysozyme may have resulted in a heterogeneous product due to the random nature of the reaction which may cause a nonuniform modification of the enzyme molecules. Modifications may have been such that these were not discriminated on the CM-cellulose chromatography. The derivatives were therefore further examined on starch and polyacrylamide gel electrophoresis using different buffers and pH values 3.0 - 8.3.

In Figs. 21A, B are shown electrophoretograms obtained by starch gel electrophoresis done at two pH values namely pH 7.0 (Fig. 21A) and pH 4.1 (Fig. 21B). From Fig. 21A it may be seen that N-lysozyme, P_2 and P_3 each gives a single band suggesting that the preparations are homogeneous. However, at

pH 4.1 (Fig. 21B) N-lysozyme gives a single band whereas P_2 and P_3 show some heterogeneity, but do not lead to well resolved bands, although the presence of about three distinct components can be distinguished. P_2 and P_3 moved slightly slower than native lysozyme as may be expected due to decrease in the positive charge on the derivatives.

The same conclusions can be drawn from disc electrophoresis experiments which were conducted at pH 8.3, 4.5 and 3.1 (Figs. 22A, B, C). All the three proteins give single band at pH 3.1 (Fig. 22A) at pH 8.3 N-lysozyme gives two well separated bands (Fig. 22B). At pH 4.5 (Fig.22C) N-lysozyme gives a single band, P_2 and P_3 show more than one band although these bands are diffused. The patterns are nearly similar to what was observed on starch gel at pH 4.1. Microdensitometer tracings do suggest slight unresolved heterogeneity (Fig. 22C). It may be of interest to mention that when the experiment at pH 4.5 is repeated in the presence of 8M urea all the 3 proteins, namely N-lysozyme, P_2 and P_3 give single but broad and diffuse bands (Fig. 22D). The reason why single band is obtained at pH 7.0 and multiple bands at pH 4.1 may be that pH 7.0 is nearer the isoelectric point of lysozyme and charge differences may be small. The same may be true for obtaining single bands at pH 3.1. At very acid pH the differences in the charge on DNP-derivative species may be negligible. A similar thing may be happening in 8M urea in which the conformation of the native lysozyme and that of the DNP-derivatives would be disrupted and then the various DNP-derivatives may not be distinguishable electrophoretically. Separation in starch and polyacrylamide

Fig. 21A. Starch gel electrophoresis patterns of N-lysozyme, P_2 and P_3 at pH 7.0 (phosphate buffer) 6 volts per cm.

N=N-lysozyme (1.44 mg)

P_2 (1.1 mg)

P_3 (1.0 mg)

Fig. 21B. Starch gel electrophoresis patterns of N-lysozyme, P_2 and P_3 at pH 4.1 (acetate buffer) 6 volts per cm.

N = N-lysozyme (1.44 mg)

P_2 (1.1 mg)

P_3 (1.0 mg)

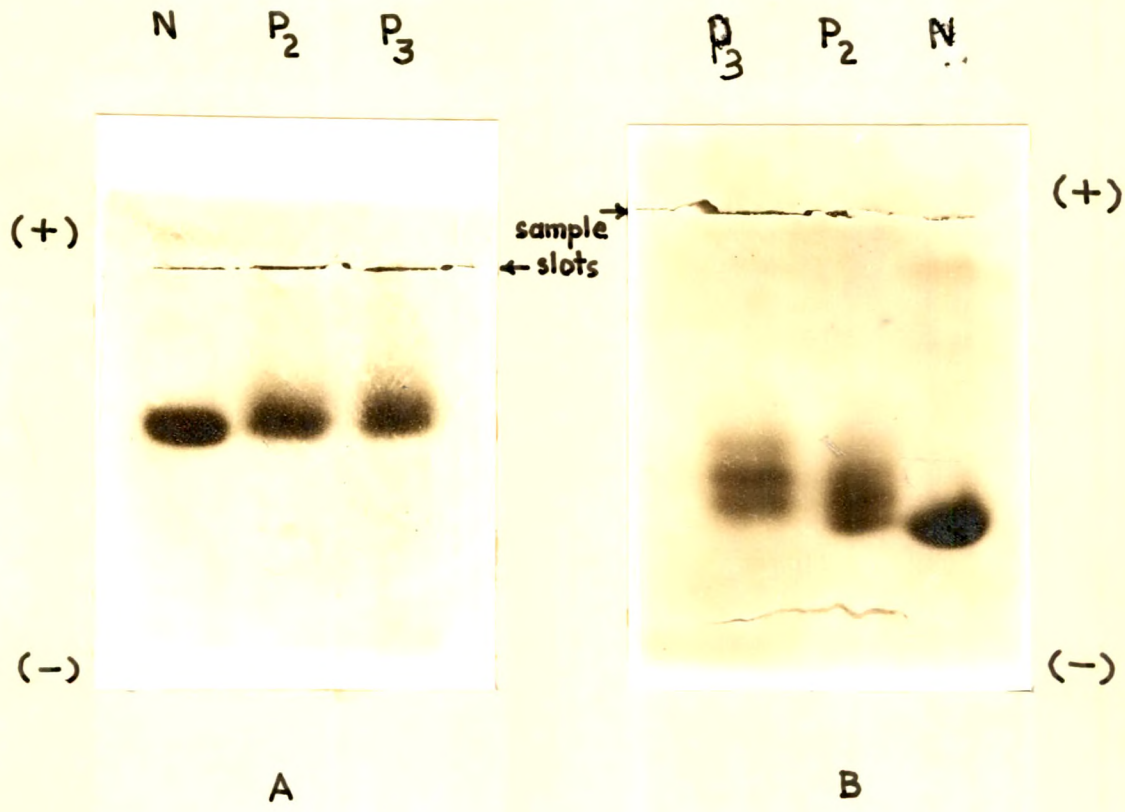


FIG. 21

Fig. 22A. Disc electrophoresis patterns of N-lysozyme, P_2 and P_3 on polyacrylamide gel; pH 4.3 gels and run at pH 3.1 (aluminium lactate-lactic acid buffer); period of electrophoresis 75 min. at 5 m. amps per tube.

N = N-lysozyme (40 µg)

P_2 (40 µg)

P_3 (40 µg)

Fig. 22B. Disc electrophoresis patterns of N-lysozyme, P_2 and P_3 on polyacrylamide gel; pH 8.9 gels and run at pH 8.3 (Tris-glycine buffer); period of electrophoresis 90 min. at 5 m. amps per tube.

N = N-lysozyme (40 µg)

P_2 (40 µg)

P_3 (40 µg)

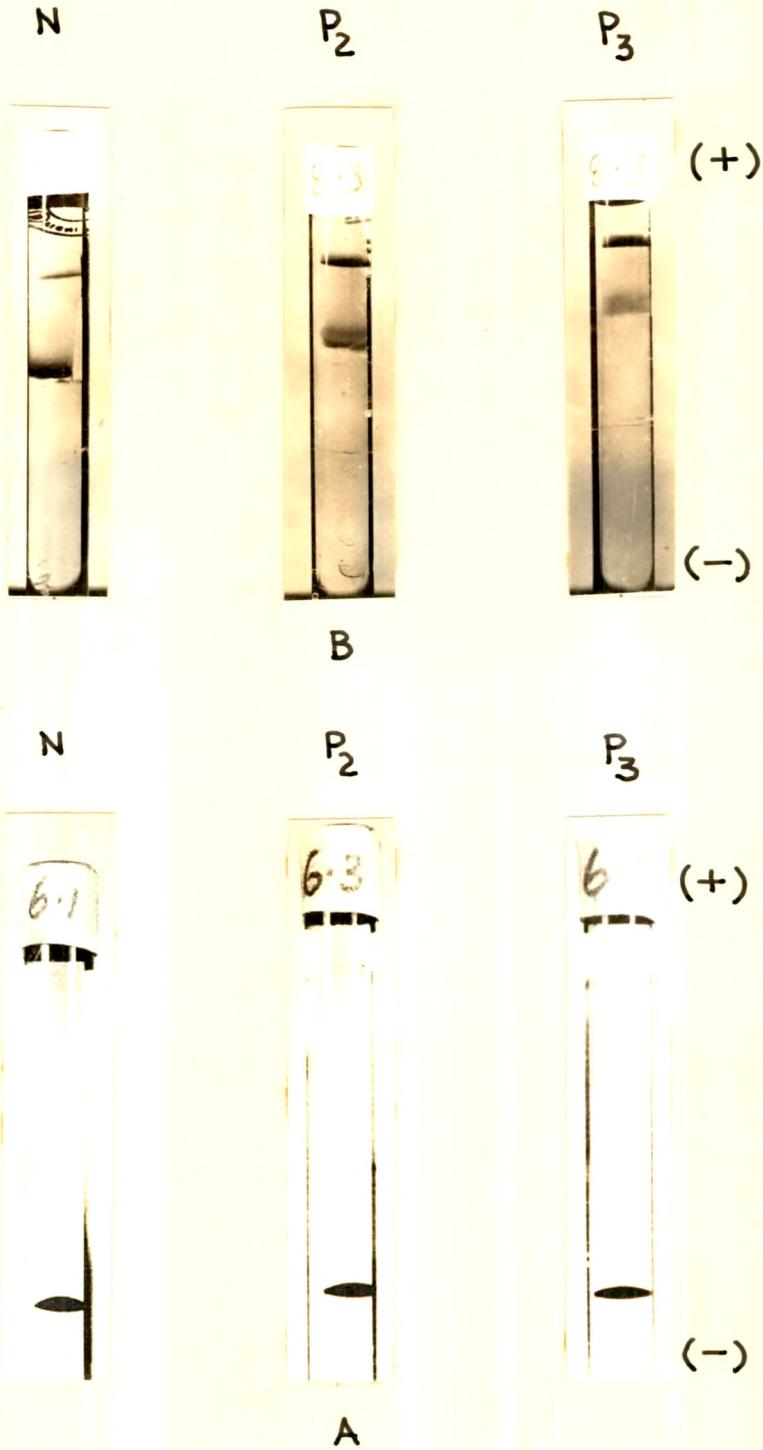


FIG 22

Fig. 22C. Disc electrophoresis patterns of N-lysozyme; P_2 and P_3 on polyacrylamide gel and their microdensitometer tracings. pH 4.3 gels and run at pH 4.5 (β-alanine-acetic acid buffer); period of electrophoresis 80 min. at 5 m.amps per tube.

N=N-lysozyme (40 µg)

P_2 (40 µg)

P_3 (40 µg).

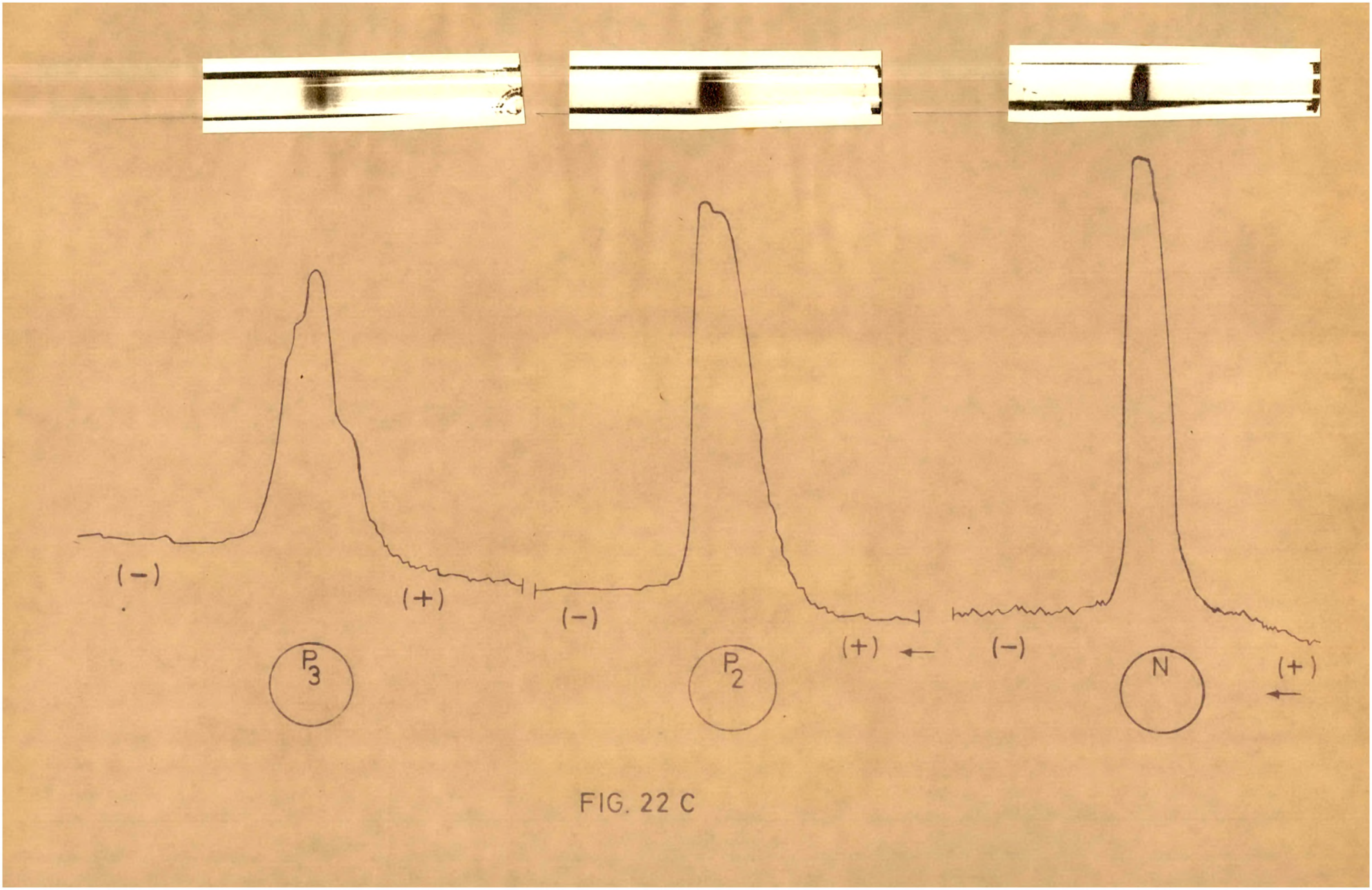


FIG. 22 C

Fig. 22D. Disc electrophoresis patterns of N-lysozyme; P_2 and P_3 on polyacrylamide gel, pH 4.3 gels containing 8.0M urea and run at pH 4.5 (β-alanine-acetic acid buffer with 8.0M urea); period of electrophoresis 75 min. at 5 m. amps per tube.

N=N-lysozyme (40 µg)

P_2 (40 µg)

P_3 (40 µg)

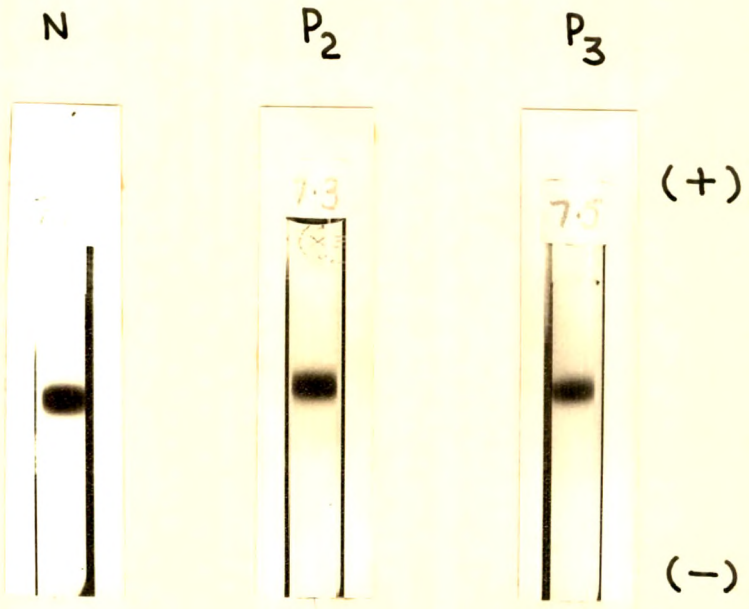


FIG 22 D

gel electrophoresis takes place both on the basis of molecular size and on the basis of net charge on the protein molecule (147, 148). The fact that distinct diffuse bands are obtained with P_2 and P_3 at pH 4.1 which are not well resolved may mean that these derivatives constitute a mixture of sub DNP-derivatives which differ only slightly in charge. It therefore appeared that P_2 and P_3 were not very homogeneous. It was now important to separate the derivatives for individual study and to establish in what way each sub DNP-derivative differed from the other. Once the sub DNP-derivatives species could be separated and obtained in relatively pure form the number and position of the dinitrophenylated lysine could be determined. Attempts were therefore made to separate the derivatives on another ion exchange resin. In this case we used Amberlite IRP-64. On a small column (1.5 x 6 cm) P_2 resolved into two components. When loaded on a long column (1.5 x 29 cm) it was resolved into three components (Fig. 23B). The ratio of the absorption at 360 mμ to that at 280 mμ decreased progressively whereas specific activity of the enzyme increased only slightly. The separation of this resin confirmed the heterogeneous pattern observed on electrophoresis. All the protein appears to be dinitrophenylated as it had absorption at 360 mμ. Further it was observed that there was no unreacted lysozyme since there was no peak at the position of N-lysozyme (Fig. 23A). However, chromatographic patterns varied from one experiment to other. Recovery of the DNP-protein from the column also varied. It is known that DNP-proteins often adsorb on the columns strongly. The lack of reproducibility in the chromatographic patterns and low recovery could be due to the varying adsorption of the DNP-derivatives on the column. Therefore, our attempts to isolate

Fig. 23A. Chromatography pattern of N-lysozyme on Amberlite IRP-64; Column size 1.1 x 28 cm; eluant 0.2M sodium phosphate buffer pH 7.18. N-lysozyme (load 50.0 O.D. at 280 mμ). O——O O.D. at 280 mμ. X- -X specific enzyme activity.

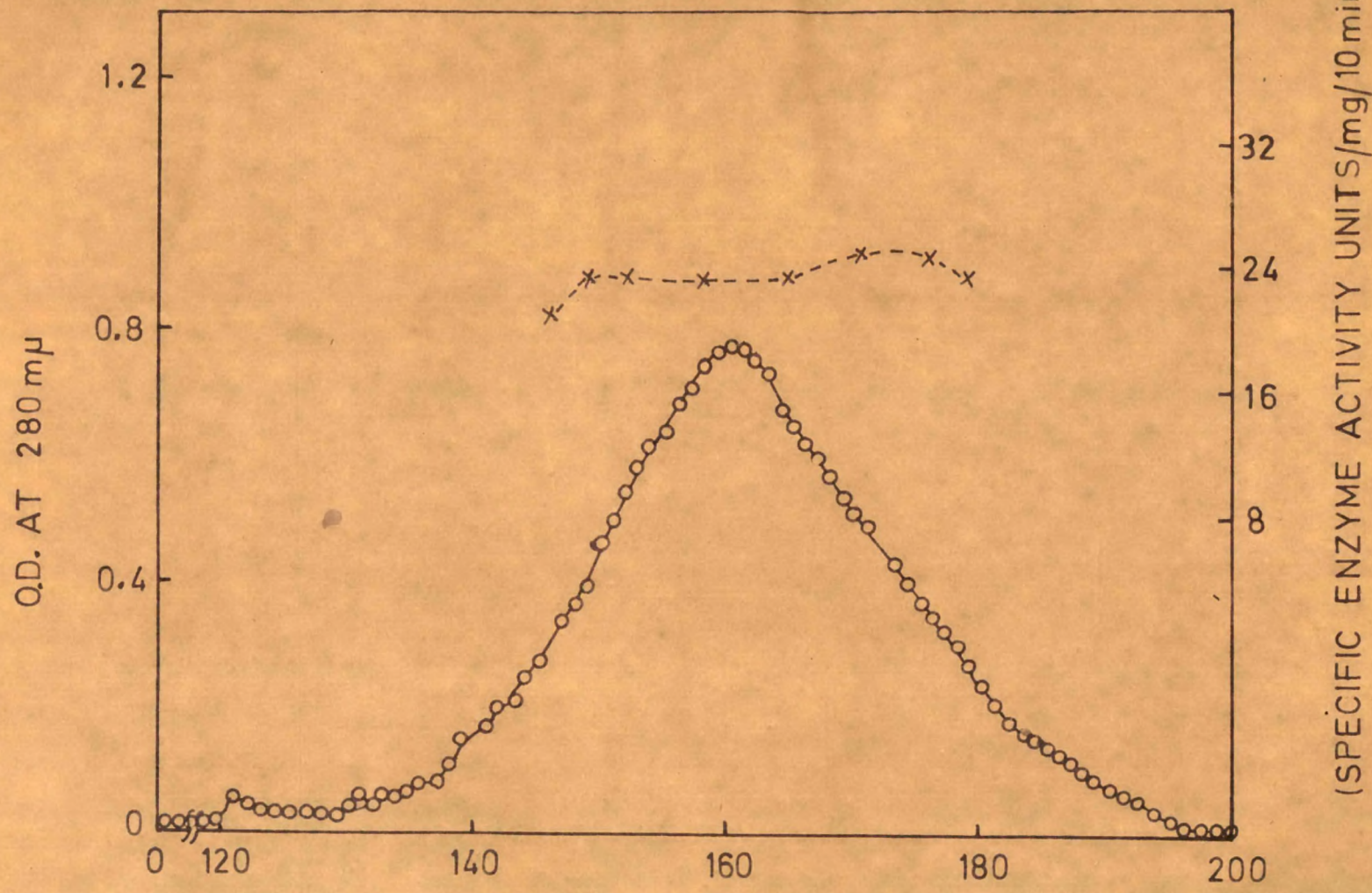

1.2
0.8
0.4
0
O.D. AT 280 mμ
0
120
140
160
180
200
FRACTION NUMBER
32
24
16
8
(SPECIFIC ENZYME ACTIVITY UNITS/mg/10 min) X 10⁻⁴

FIG. 23 A

Fig. 23B. Chromatography pattern of P_2 on Amberlite IRP-64; Column size 1.1 x 28 cm; eluant 0.2M sodium phosphate buffer pH 7.18. P_2 (load 70 O.D. at 280 mμ). O——O O.D. at 280 mμ. ●——● O.D. at 360 mμ. X--X specific enzyme activity.

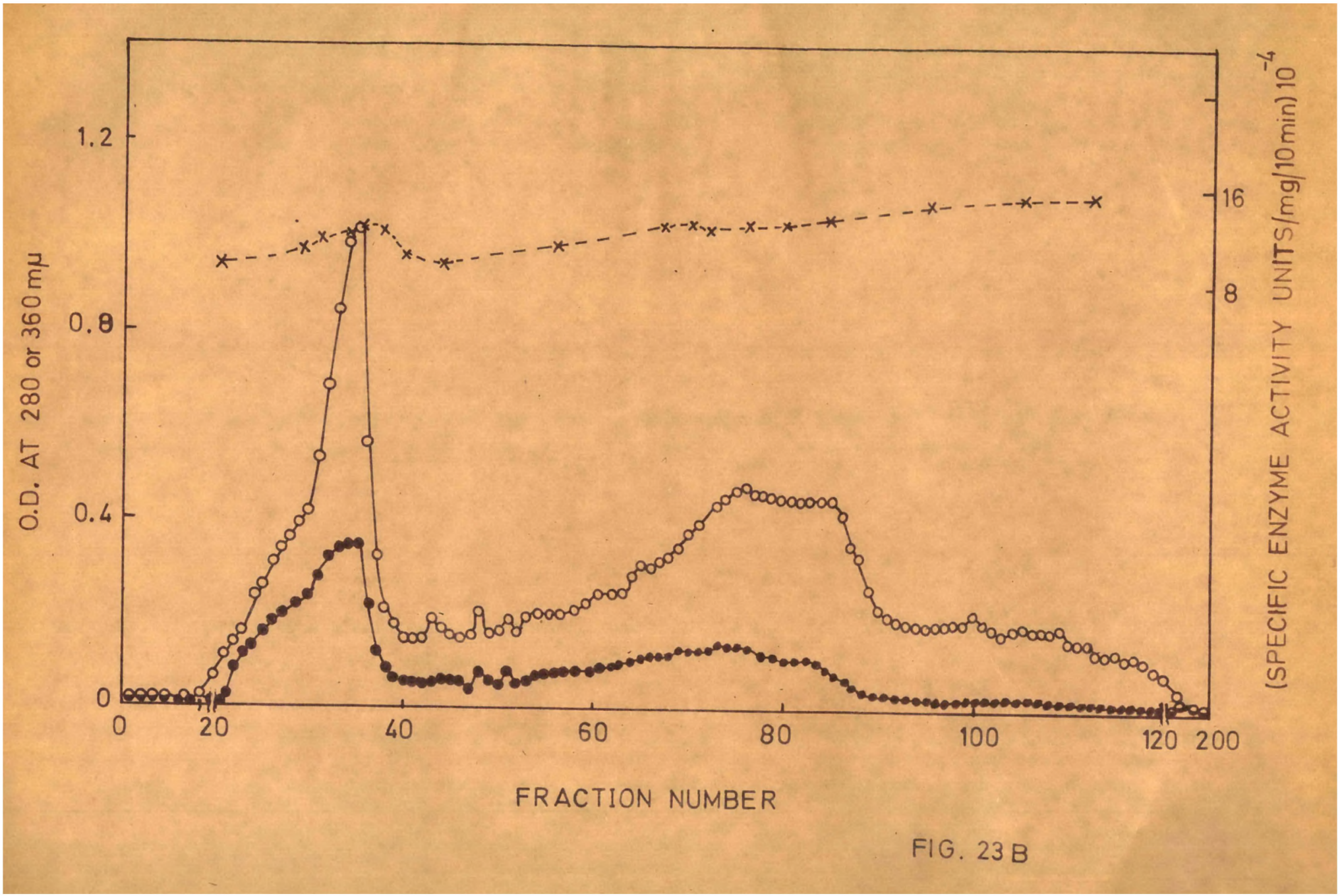

1.2
0.8
0.4
0
O.D. AT 280 or 360 mμ
16
8
(SPECIFIC ENZYME ACTIVITY UNITS/mg/10 min) 10^{-4}
0
20
40
60
80
100
120
200
FRACTION NUMBER

FIG. 23 B

the derivatives in sufficient quantity were not successful. However, in order to determine the position of the labelled lysine it would be desirable to separate the mixture of the DNP-derivatives. It is hoped that further attempts with some new approach will eventually resolve the question of the separation of the sub DNP-derivative species.

The results of the effect of modification on the physico-chemical characteristics of the DNP-lysozymes prepared at pH 7.2 were described. The data indicate that incorporation of one to six DNP-groups in the enzyme molecule do not change its shape or molecular size to any appreciable extent as shown by the UV spectral and ultracentrifuge experiments. No aggregation was found on gel filtration. However, tryptic hydrolysis studies showed that P_2 was hydrolysed much more than native lysozyme during 12 hr period, suggesting small conformational change in the derivative. The derivatives also showed the presence of more than one sub derivative on electrophoresis between pH 4.0 - 4.5. On either side of this pH, the derivatives moved on the electrophoresis column more or less as single species. Derivatives could not be resolved on CM-cellulose, using salt gradient, pH gradient or combined pH and salt gradient. On electrophoresis DNP-protein bands were diffuse and CM-cellulose column the derivatives emerged as unsymmanterical and broad peaks. DNP-proteins adsorbed strongly on Amberlite IRP-64 although they were resolved in three sub-derivatives. Results varied from experiment to experiment. DNP-content of the sub-derivatives decreased progressively from the first peak to the third peak whereas enzyme activity increased only

slightly.

It may be pointed out at this stage that the fact of micro heterogeneity in the DNP-lysozymes does not vitiate our conclusions mainly drawn from the enzyme activity studies regarding the role of lysine residues. It was shown that although DNP-content decreased from peak to peak, indicating different DNP-content in each peak, change in the activity was very slight. This means all the three peak proteins had on an average the same activity. Therefore, our conclusions regarding the role of lysine residues should still be valid.

However, in this phase of the present investigation, it is clearly shown that modification of the enzyme even if carried out under mild controled conditions can lead to small or limited conformational changes. The resulting modified derivatives may not be homogeneous or uniformly labelled. The use of only one technique to detect homogeneity or conformational changes may lead to wrong conclusions. Our results clearly show that the use of a combination of various techniques is imperative in such studies to get a more satisfactory picture.

Studies on DNP-lysozymes prepared by reaction with FDNB at pH 9.1.

As mentioned earlier dinitrophenylation studies were also conducted at pH 9.1. These experiments were carried out in sodium carbonate-bicarbonate buffer at 15° and 26°. It was observed that at this pH, inactivation of the enzyme occurred very rapidly (within about 10 min.) at FDNB to enzyme ratio of 312:1, both at 26° and at 15°. The product was completely inactive. However, when a lower ratio of FDNB to enzyme, namely 18:1, was used at 26° and the reaction was carried out for 15 min. a derivative with about 30% of the original activity was obtained.

Effect of time on dinitrophenylation at pH 9.1.

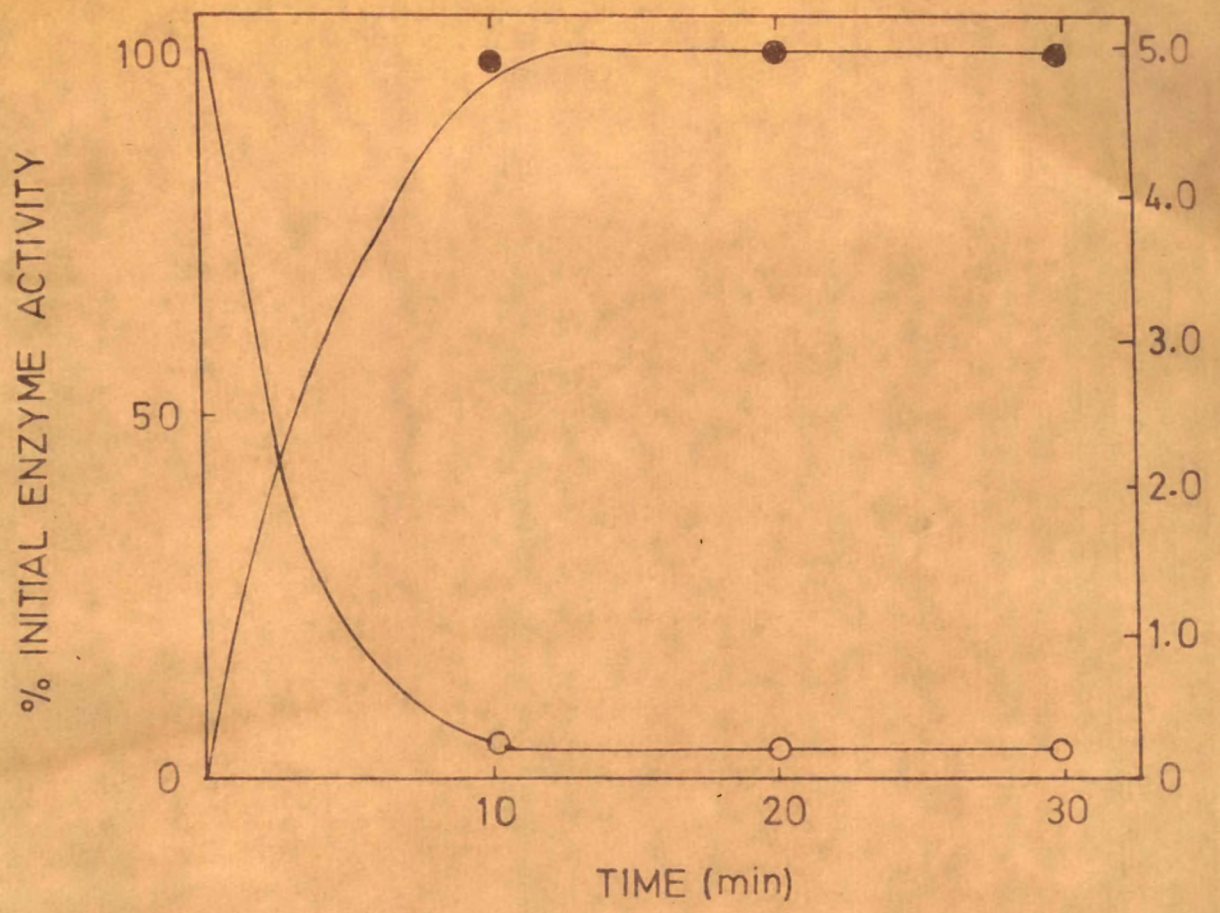
In Fig. 24 the effect of reacting lysozyme with FDNB for different intervals of time is shown. The data were obtained using FDNB to enzyme ratio of 18:1 at 26°. Both enzyme activity and absorbance at 360 mμ were measured. The increase in the DNP-uptake and the decrease in the enzyme activity proceed almost with a linear rate upto 15 min when the enzyme has 30% of its original activity. At the end of 30 min reaction there is an increase in DNP-content and the enzyme still retains 15% activity.

In Fig. 25 the effect of reacting lysozyme with FDNB for different intervals of time with FDNB to enzyme ratio of 312:1 is given. At 15° or 26° in about 10 min of reaction time the enzyme is completely inactivated. The absorbance at 360 mμ has been measured to determine the extent of dinitrophenylation. At 10 min of reaction time the enzyme was completely inactive

Fig. 24. The extent of dinitrophenylation of lysozyme as a function of time of reaction and its effect on enzyme activity of lysozyme at 26°; pH 9.1. ▲——▲ O.D. at 360 mμ and △——△ relative enzyme activity. [FDNB/Enzyme, 18:1 mole/mole]. 0——0 Activity of N-lysozyme.

Fig. 25. The extent of dinitrophenylation of lysozyme as a function of time of reaction and its effect on enzyme activity at 15° and 26°; pH 9.1. ●——● O.D. at 360 mμ; and 0——0 relative enzyme activity 15° or 26° [FDNB/Enzyme, 312:1 mole/mole].

FIG. 25


% INITIAL ENZYME ACTIVITY
100
50
0
10
20
30
TIME (min)
5.0
4.0
3.0
2.0
1.0
0
O.D. AT 360 mμ/mg PROTEIN

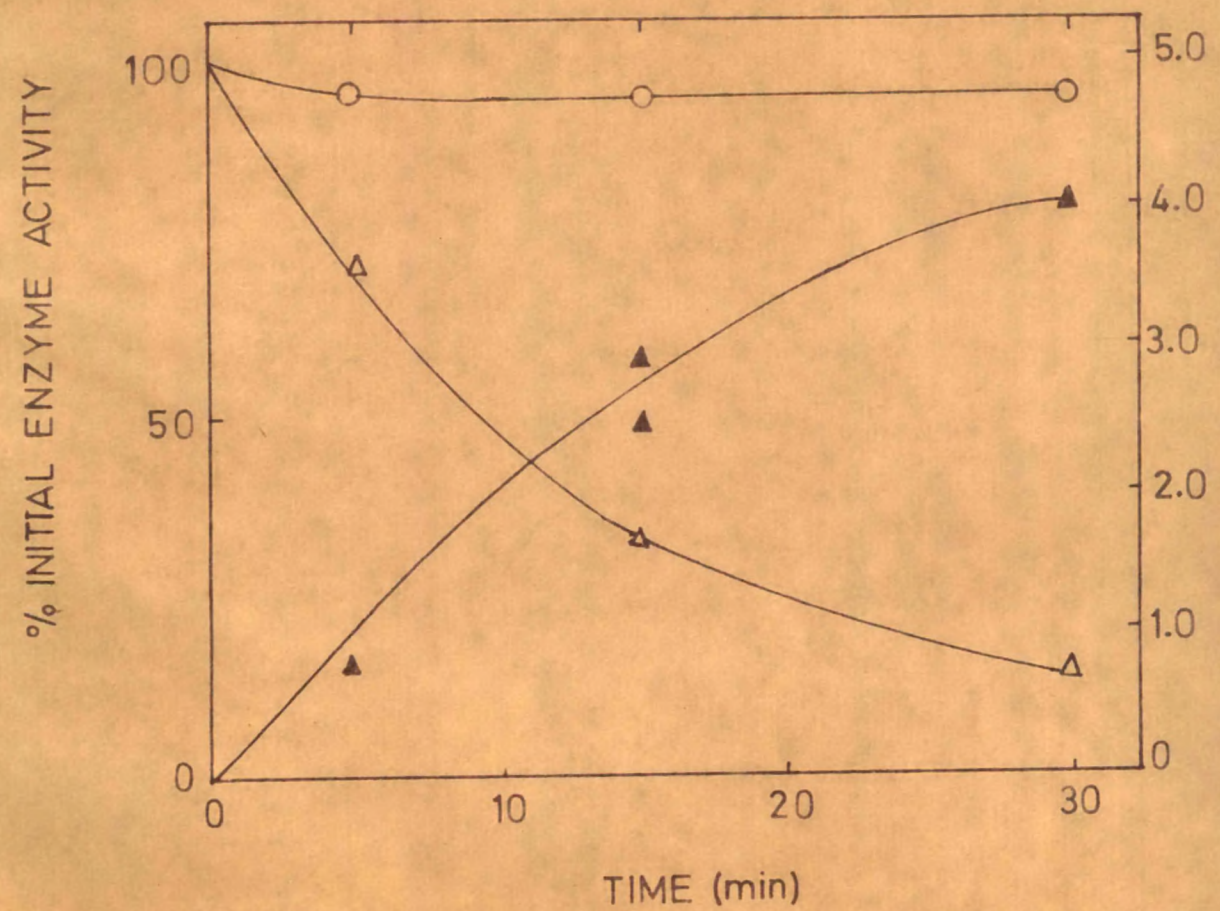

% INITIAL ENZYME ACTIVITY
100
50
0
0
10
20
30
TIME (min)
5.0
4.0
3.0
2.0
1.0
0
O.D. AT 360 mμ/mg PROTEIN

FIG. 24

and the DNP content reached a maximum value.

The following DNP-derivatives were prepared at pH 9.1. The effect of modification was then examined on the enzyme activity and physicochemical properties. It may be mentioned here that at pH 7.2 derivatives were obtained with minimal or no conformational change, whereas at pH 9.1 the derivatives obtained were almost drastically changed in conformation.

1) A derivative with 30% of its original activity was obtained at 26° by reacting lysozyme with FDNB for 15 min, using FDNB to enzyme ratio of 18:1.

2) A second derivative with less than 10% activity was obtained by reacting lysozyme with FDNB at 26° for 10 min using FDNB to enzyme ratio of 312:1.

3) A third derivative again with less than 10% residual activity was obtained by reacting lysozyme with FDNB at 15° for 10 min, using FDNB to enzyme ratio of 312:1.

DNP-amino acid analysis.

The amino acid analysis of the three preparations showed that the ether extract contained only di-DNP-lysine. The butanol extract (i.e. water soluble DNP-amino acids) of all the preparations, when run on, butanol-acetic acid-water system gave two yellow DNP-spots. Out of these two spots one coincided with standard DNP-lysine (R_f 64-68), the other, fast moving spot appeared to coincide with O-DNP-tyrosine (R_f 70-75). Since the second spot had bright yellow colour it did not appear likely to be O-DNP-tyrosine. The butanol extract was also run on a second solvent system namely phthalate buffer system. In

this also two yellow spots were obtained; one had R_f value similar to the standard ε-DNP-lysine (R_f 33-38) and the other fast moving yellow spot and had R_f value approximately close to the standard 2,4 dinitroaniline (R_f 85-90). The fast moving DNP-spot was eluted with 1% $NaHCO_3$ and the spectrum taken. The spectra of standard O-DNP-tyrosine, standard 2,4 dinitroaniline and the unknown fast moving DNP-spot are shown in Fig. 26. From the spectra it is clear that the unknown DNP-spot material is not O-DNP-tyrosine. But it corresponds with the spectrum of dinitroaniline. Therefore the unknown DNP-spot may be dinitroaniline or it may be some DNP breakdown product of DNP-amino acids produced during acid hydrolysis (177). The unknown DNP-spot is not due to dinitrophenol as tested by exposure to HCl. The yellow colour did not disappear after exposure to HCl. The occurrance of unknown DNP-spots in the hydrolysates of DNP-proteins has been reported by several workers (138). It may be mentioned here that no unknown spot was detected from the derivatives prepared at pH 7.2.

The order of DNP-amino acid spots in a given solvent was entirely reproducible but the absolute R_f values were not. Therefore for every chromatographic run standard DNP-amino acids were also run simultaneously.

In Table 7 the details of chromatographic runs in different solvent systems and the R_f values are given.

Solubility: All the three DNP-lysozymes described above were soluble in acidified aqueous solution but precipitated slightly

Fig. 26. Absorption spectra in U.V. range.

X—X standard O-DNP-tyrosine;

□—□ standard dinitroaniline;

O—O unknown DNP-spot (30% active hydrolysate);

●—● unknown DNP-spot (< 10% active hydrolysate).

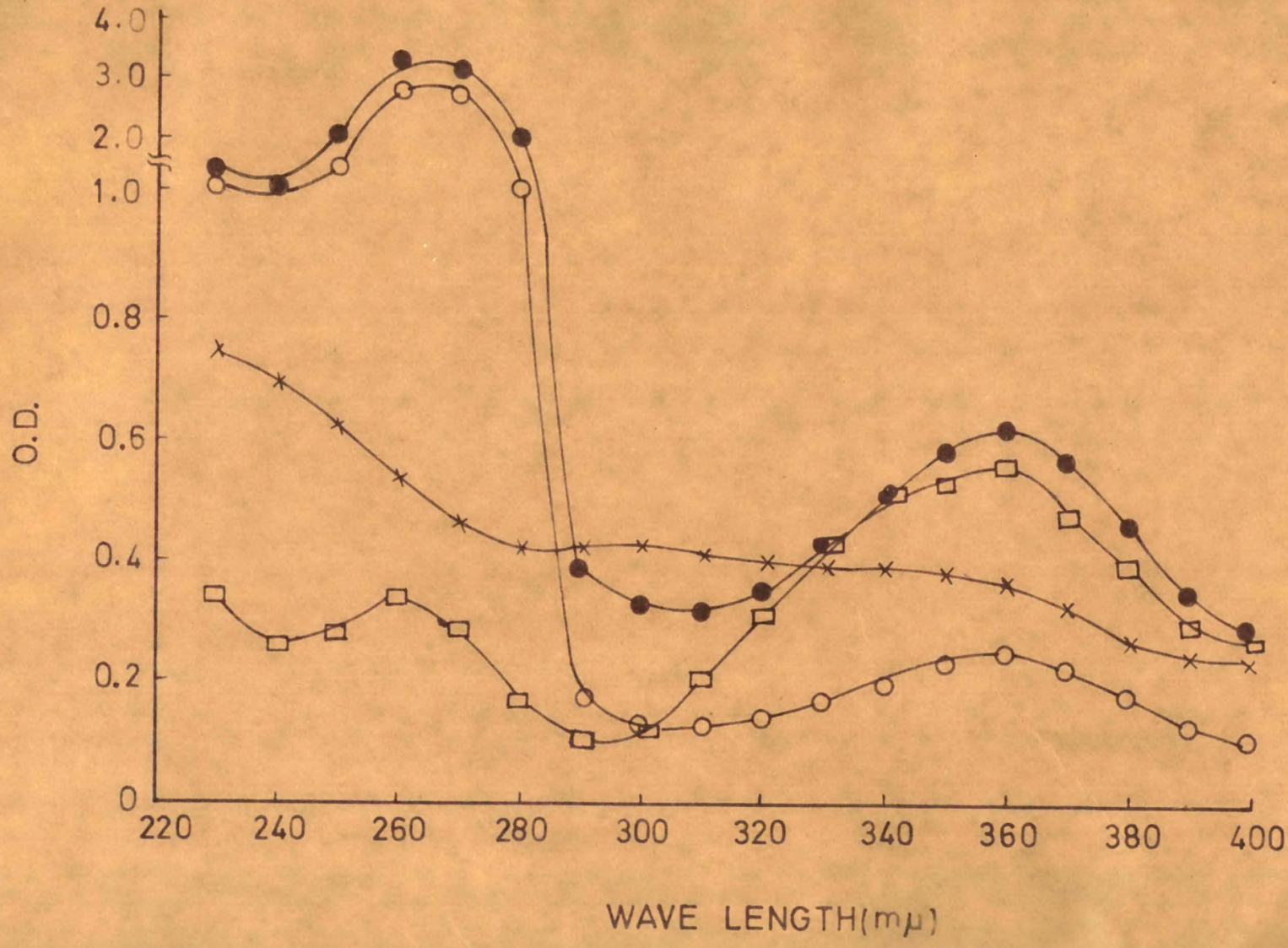

4.0
3.0
2.0
1.0
0.8
0.6
0.4
0.2
0
O.D.
220
240
260
280
300
320
340
360
380
400
WAVE LENGTH(mμ)

FIG. 26

TABLE No. 7

R_f VALUES OF DNP-AMINO ACIDS OBTAINED USING DIFFERENT SOLVENT SYSTEMS

Standard DNP amino acids or samples	Solvent system used	R_f values
Di-DNP-lysine	Toluene; 2-chloro-ethanol: Pyridine: ammonia	70-75
ε-DNP-lysine	Butanol:acetic acid:water	64-68
O-DNP-tyrosine	"	70-75
Unknown DNP-spot	"	70-75
ε-DNP-lysine	Phthalate system	33-38
O-DNP-tyrosine		50-53
2,4 dinitroaniline		90-94
Unknown DNP-spot		90
Di-DNP-lysine	Butanol:Pyridine:water and phenol	85-90

and slowly when dialysed against distilled water or 0.2 M phosphate buffer (pH 7.18). However, they were readily solubilized in 0.1M acetic acid. During the dialysis period under the above conditions N-lysozyme remained in solution. These DNP-lysozyme preparations could also be crystallized easily when the solutions were made 0.1 M in KNO_3, while native lysozyme under the same conditions remained in sdution.

The relative insolubility of these DNP-lysozymes in distilled water and phosphate buffer at nearly neutral pH and their ease of crystallization accompanied by complete or partial loss of the enzyme activity suggested a substantial change in the conformation as a consequence of the introduction of DNP-groups in the molecule at pH 9.1.

Because the solubility of the derivatives was rather low detailed experimentation was not possible. However, both gel filtration experiments and ultracentrifugation experiments revealed heterogeneity of these samples. In each case two peaks were observed, one which ~~showed~~ eluted from the column at the same place as N-lysozyme and the other of aggregates. The gel filtration patterns of 30% active DNP-lysozyme and two other almost completely inactive lysozymes are given in Fig. 27A, B.

It may be mentioned that the proportion of the material which emerged from the column earlier than N-lysozyme increases to some extent as inactivation increases. For example the proportion of the first fraction in the 30% active DNP-lysozyme

Fig. 27A. The gel filtration pattern of N-lysozyme and 30% active DNP-lysozyme on Sephadex G-100. Column size 1.1 x 47 cm; eluant 0.1M acetic acid; N-lysozyme (load 32.0 O.D. at 280 mu); 30% active DNP-lysozyme (load 22.0 O.D. at 280 mμ).

O——O O.D. at 280 mμ

●——● O.D. at 360 mμ.

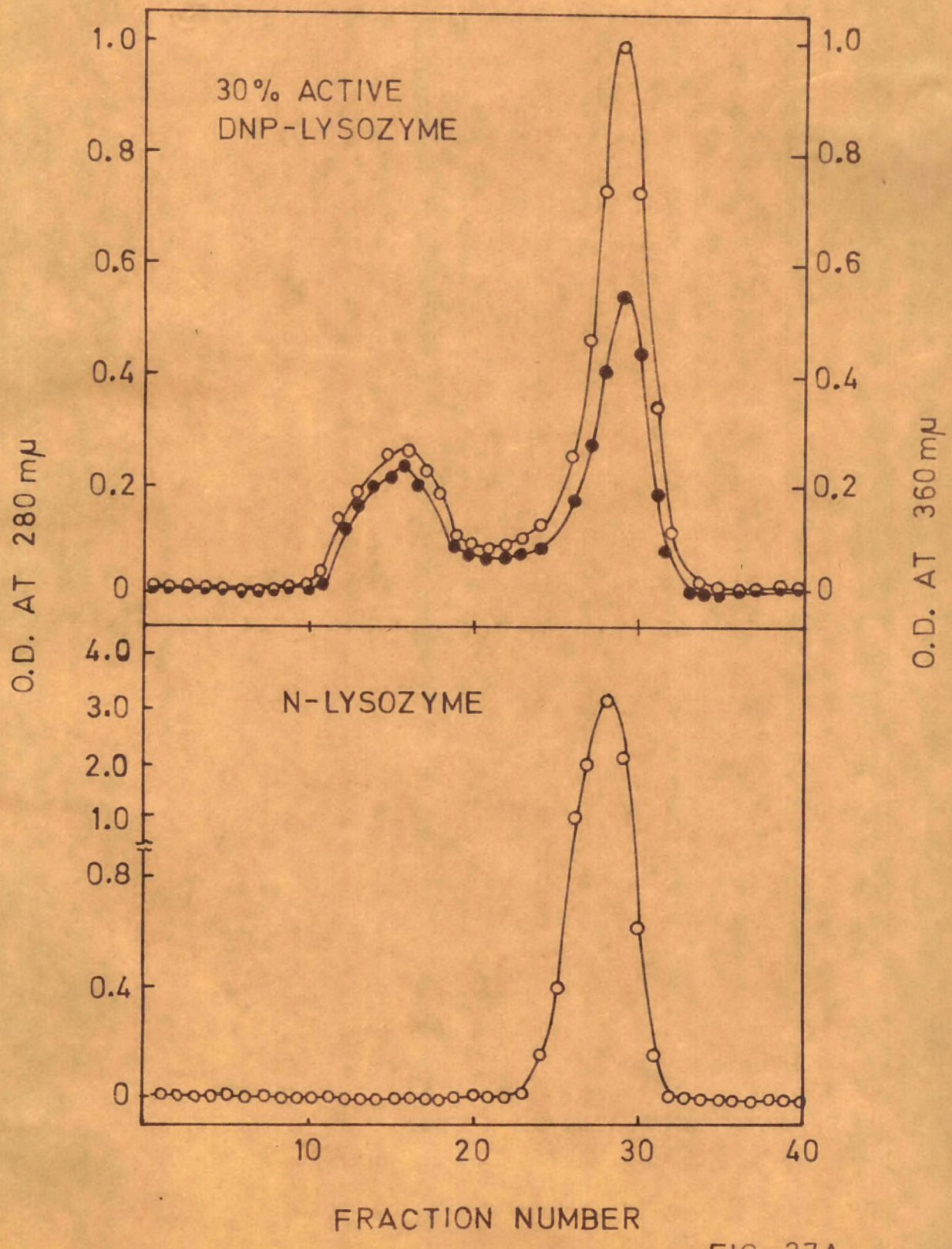

30% ACTIVE
DNP-LYSOZYME
N-LYSOZYME
O.D. AT 280 mμ
O.D. AT 360 mμ
1.0
0.8
0.6
0.4
0.2
0
4.0
3.0
2.0
1.0
0.8
0.4
0
10
20
30
40
FRACTION NUMBER

FIG. 27A

Fig. 27B. The gel filtration pattern of N-lysozyme and inactive DNP-lysozyme on Sephadex G-100. Column size 1.1 x 47 cm; eluant 0.1M acetic acid; N-lysozyme (load 22.0 O.D. at 280 mu) Inactive DNP-lysozyme, 26° preparation (load 21.5 O.D. at 280 mμ) and inactive DNP-lysozyme, 15° preparation (load 28.5 O.D. at 280 mμ).

O——O O.D. at 280 mμ

●——● O.D. at 360 mμ.

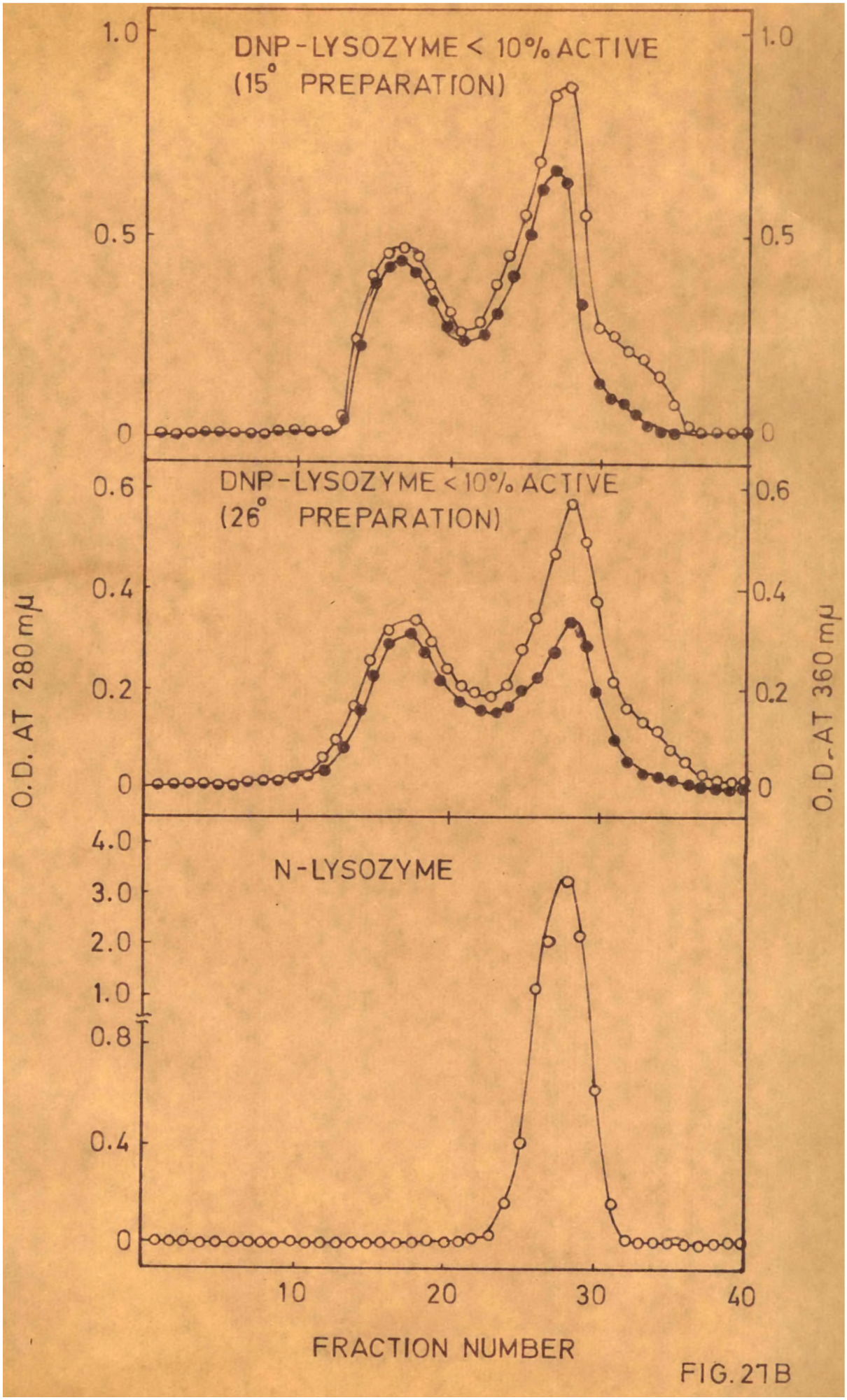

DNP-LYSOZYME < 10% ACTIVE
(15° PREPARATION)
DNP-LYSOZYME < 10% ACTIVE
(26° PREPARATION)
N-LYSOZYME
1.0
0.5
0
0.6
0.4
0.2
4.0
3.0
2.0
1.0
0.8
0.4
10
20
30
40
O.D. AT 280 mμ
O.D. AT 360 mμ
FRACTION NUMBER

FIG. 27B

is about 30% and in the completely inactive derivative it is about 40%. The first fraction in each case is nearly completely inactive (< 10%). However, the second fraction of the 30% active DNP-lysozyme was about 55-60% active whereas the second fraction of the inactive derivatives had negligible activity.

The ultracentrifugal measurements in acidified aqueous solutions (pH 3-4) gave a $S_{20,w}$ value of 11-16 for the fast moving component and a value of 1.8 to 2.0 for the slow moving component. In conformity with the gel filtration experiments the proportion of the 11-16S component is higher in the totally inactive DNP-lysozyme than in the partially-active lysozyme. Since the S value of the slow moving peak agrees with of N-lysozyme it may be concluded that the higher aggregate may have a molecular weight 15-16 times that of native-lysozyme. It may be mentioned that lysozyme undergoes a dimerisation reaction between pH 5.0 - 9.0 and above pH 9.0 higher aggregates of lysozyme are formed (104). Dinitrophenylation at pH 9.1 appears to favour the aggregation of the DNP-derivatives. Our results that the aggregates are also dinitrophenylated may mean that the sites of aggregation perhaps do not involve lysine residues which are modified. In view of the complexity introduced by aggregation it was not possible to draw any definite conclusions on the relationship between the activity of lysozyme and the number of lysine groups that are dinitrophenylated.

The data presented above show that dinitrophenylation of lysozyme at pH 9.1 is very fast at either temperature 15° or 26° although the extent of dinitrophenylation is still dependent on

FDNB concentration. Thus at a FDNB to enzyme ratio of 312:1 the enzyme was modified within 10 min.at the above two temperatures and the derivative was found to be completely inactive. These derivatives which were prepared at alkaline pH were less soluble, easily crystallisable and were aggregated. The aggregated and the monomer derivatives were detectable by ultracentrifuge experiments and were also resolved on gel filtration. From the above observations it was clear that the derivatives have conformation very much different from the native lysozyme. The change in the conformation of the derivatives results probably from aggregation. The aggregate appears to have molecular weight 15-16 times that of the native lysozyme.

Because of the above manifest properties detailed studies on the effect of modification on the activity and physicochemical properties were not possible. On the basis of the above findings it was concluded that the loss of the enzyme activity due to modification of lysine residues at alkaline pH is caused by the aggregation of the derivatives. Thus the inactivation of lysozyme due to dinitrophenylation appears to be different at the two pH values. Whereas at pH 7.2 there is little or no aggregation of lysozyme due to modification there is considerable aggregation at pH 9.1.

The data obtained from the studies conducted at pH 9.1 support the conclusions drawn from the modifications made at pH 7.2, in that the blocking of lysine residues reduces the solubility of the enzyme molecule and enhances its capacity to be absorbed on resins. In this case protein-protein interaction of the derivatives leads to the aggregation of the modified enzyme.

This interchain interaction is perhaps being enhanced by the DNP groups.

But it is not clear why the introduction of DNP-groups into the enzyme molecule at pH 9.1 leads to insolubilisation and aggregation of the derivatives but not at pH 7.2. There, probably, are many answers to this question. However, the following speculation may explain the phenomena partially. In either extreme environment, acidic or alkaline, enzyme molecule tends to expand due to electrostatic repulsions. If the modification is carried out when the molecule is in an expanded state the bulky DNP groups linked to the side chains may sandwitch in between the folded chains and loops and thus freeze the expanded state of the molecule by hindering the refolding of the molecule. The sandwitched DNP-groups could make interchain contacts with other side chains of amino acid residues making a sort of bridge between the polypeptide chains. The charge distribution on the expanded molecule would help these interactions. The result would be that the molecule may permanently remain slightly expanded in comparison to its original state. The above interactions of the DNP groups together with blocking of the basic residues (lysines) essentially required for the solubilization may probably enhance the insolubility of the protein molecule. Similar phenomena may explain the protein-protein interactions leading to the aggregation.