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The reaction of p-nitrophenyl acetate with lysine hydrochloride and poly-1-lysine hydrobromide

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APPROVAL OF THESIS

THE REACTION OF p-NITROPHENYL ACETATE WITH

LYSINE HYDROCHLORIDE AND POLY-L-LYSINE HYDROBROMIDE

by

EMMANUEL PLANGE MATTEER

for

DEPARTMENT OF CHEMICAL ENGINEERING

NEWARK COLLEGE OF ENGINEERING

by

FACULTY COMMITTEE

APPROVED: _____

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1972

THE REACTION OF p-NITROPHENYL ACETATE WITH LYSINE HYDROCHLORIDE AND POLY-L-LYSINE HYDROBROMIDE

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ABSTRACT

The reactions of p-nitrophenylacetate with L-lysine hydrochloride and poly-L-lysine hydrobromide as a function of pH were investigated.

The results indicate that both compounds react with the substrate in the pH range 7.0-10.0 with an apparent optimum pH in the region near 9.65.

Poly-L-lysine reacts more rapidly over the entire pH range investigated than does L-lysine.

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INTRODUCTION

Hydrolysis can be defined as a hydrogen-ion catalysed reaction of an ester with water to yield alcohol and acid. The reaction is reversible and the reverse is termed esterification. Hence, starting the reaction with ester and water, the same equilibrium state can be reached.

The acid catalysts suitable for esterification are just as effective in bringing about a hydrolysis. A method of hydrolyzing an ester consists of refluxing the substance with excess water containing hydrochloric or sulfuric acid.

$$RCOOR' + HOH \text{ (excess)} \rightleftharpoons RCOOH + R'OH$$

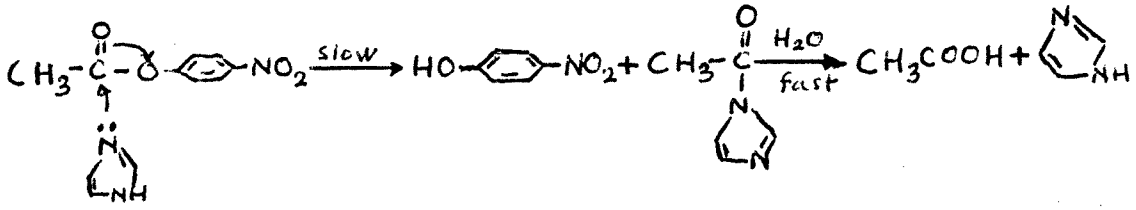
The mechanism and intermediates of the hydrolytic reaction are the same as those for esterification.

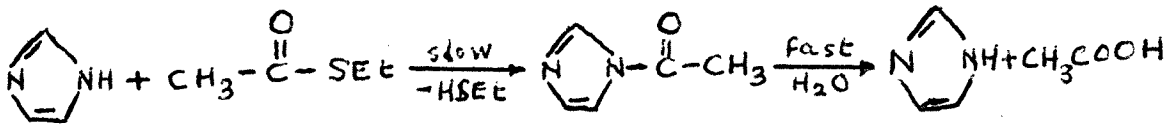
Hydrolysis of p-Nitrophenyl Acetate (pNPA) by Simple Organic and Inorganic Ions.

Hydrolysis of pNPA with various species as catalysts have shown to be nucleophilic reactions.

A nucleophile is a term used to describe an atomic center which has a strong tendency to donate an electron-pair. Likewise, the term nucleophilic catalysis can be used to describe a catalyzed that proceeds via donation of an electronpair from the catalyst to the substrate if this "nucleophilic attack" either partially or completely governs the reaction.

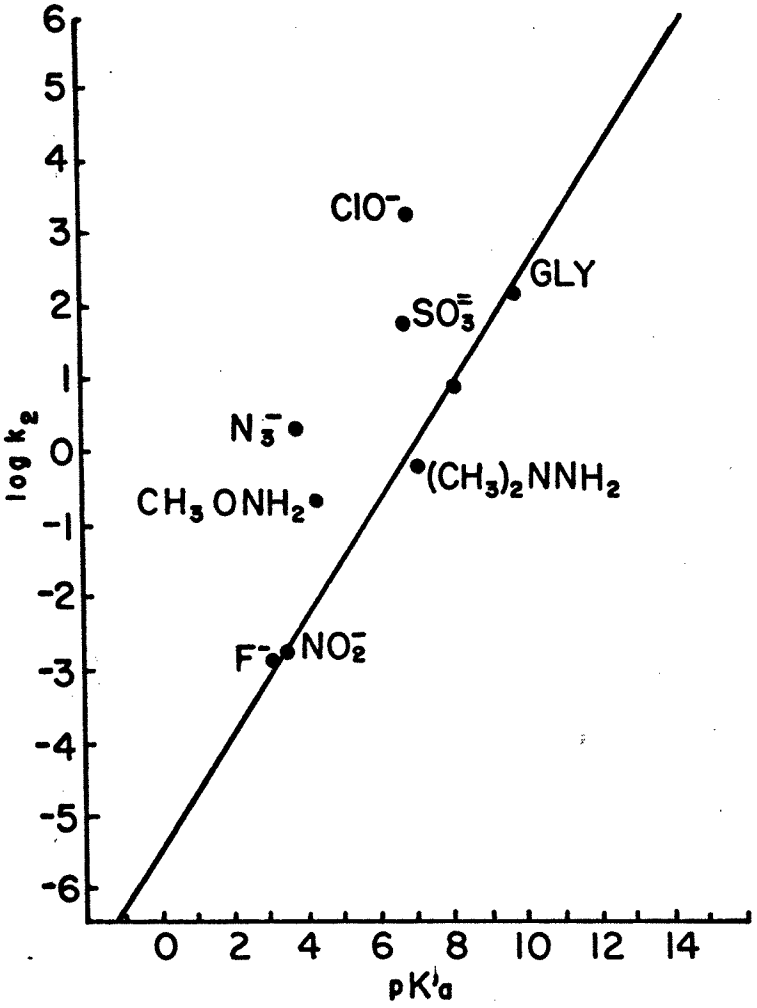
The following are some examples of nucleophilic catalysis.[1]

a) 

b) 

In Figure I the rates of nucleophilic reactions with p-nitrophenyl acetate in aqueous solution at 25° have been plotted against the basicity of the attacking reagent.

Figure 1

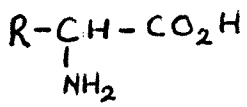


Rates of nucleophilic reactions with p-nitrophenyl acetate in aqueous solution at 25° plotted against the basicity of the attacking reagent. Abbreviation: GLY, glycine.

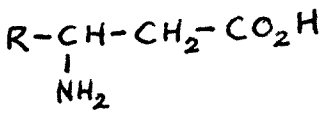
Source: T.C. Bruice and S. Benkovic, Bioorganic Mechanisms, Benjamin, New York, p. 38

Characteristics of L-lysine

L-lysine is one of the twenty-odd amino acids which commonly occurs in proteins. An α-amino acid is one in which the amino group and the carboxyl group are attached to the same carbon atom.

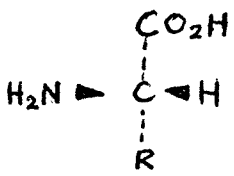


an α-amino acid

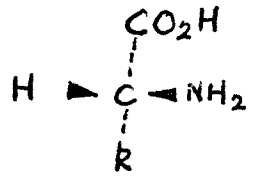


a β-amino acid

The "L" designation refers to the stereochemistry of the asymmetric carbon which bears both the amino and carboxyl groups. All of the amino acids found in proteins have the "L" configuration, although some small polypeptides contain some "D" amino acids.



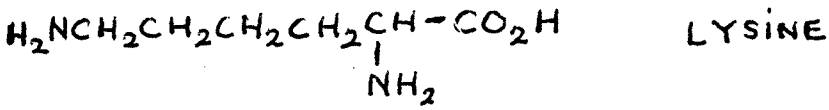
L-amino acids



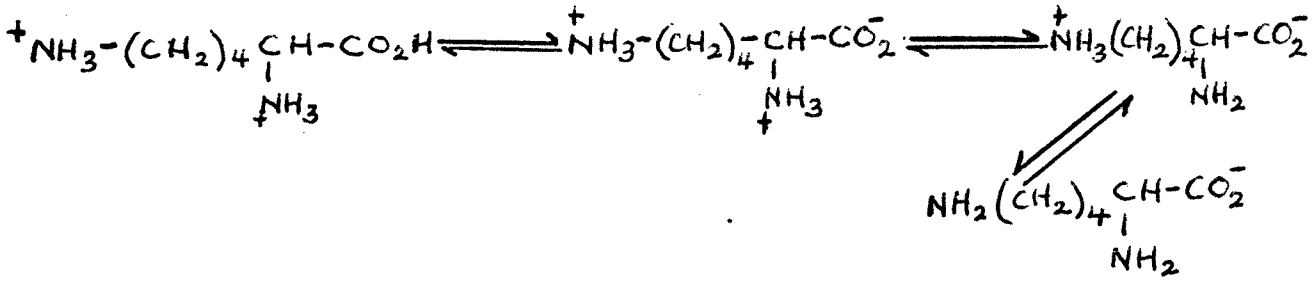
D-amino acids

Lysine and arginine are the only amino acids found in proteins which contain basic side chains (i.e. "R" groups). These basic groups confer great polarity and hydrophilicity to proteins which contain lysine and

arginine. The basic side chain of the lysine molecule consists of a second primary amino group attached to a four carbon chain.



Lysine has three groups which can act as acids, two amino groups and one carboxyl group. The pKa_s are 2.18, 8.95 and 10.53 for the carboxyl, the α-amino group and the ε-amino group, so that the following forms predominate at different values.



The pI of L-lysine is 9.74.[2] Lysine is an essential amino acid. The minimum daily requirement for a human is 0.8 gram per day.[3]

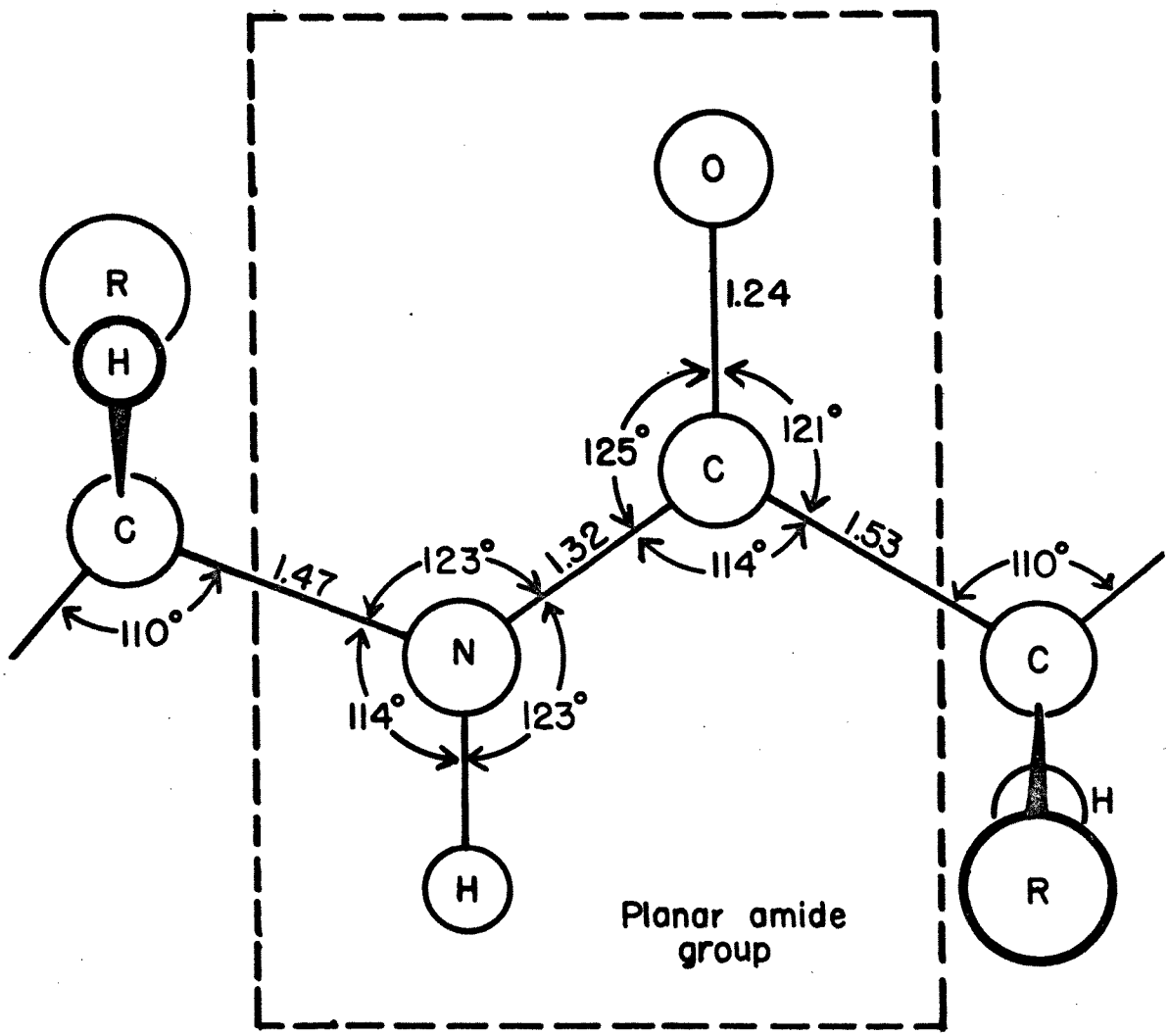
STRUCTURES OF PROTEINS

Peptides are composed of amino acids bound together by peptide linkages. The geometry of the peptide linkage is as shown in figure IA. The short C-N distance, presumably indicative of significant "double bond character" would lead one to expect that the amide linkage would be planar and the crystallographic studies of Pauling and Corey[4] confirm this expectation. There is, however, opportunity for rotation about the C-C bond, thus the polypeptide chain may assume a random coil configuration characteristic of linear polymers in good solvents.

Polypeptides also have the ability to form a hydrogen-bonded helical configuration, the α-helix. The α-helix, as proposed by Pauling, Corey and Branson[5] has 3.6 amino-acid residues per turn of the helix, with each amide hydrogen being hydrogen bonded to the carbonyl oxygen of the third following amide group. The residues R of figure 1A extend radially from the helix. This helical configuration can exist not only in the solid state but also in solution.

In 1957, Doty, Wado, Yang and Blout[6] demonstrated that, by altering the degree of ionization of poly-L-glutamic acid in water-dioxane solution or by altering the temperature, a reversible transformation between the α-helix and random-coil configuration could be induced. They observed the transition by determining the concomitant changes in optical rotation and viscosity corresponding to the change in the shape of the molecule. Similarly, Doty and Applequist[7] in 1961 investigated poly-L-lysine, the R group of figure 1 being $-(CH_2)_4-NH_2$. In the neighborhood of room temperature and at pH less than 8 when the $-NH_2$ groups are almost completely protonated the polymer in aqueous solution exists in the random-coil form. As the pH is increased, there is a gradual transition proceeding presumably through a series of states involving alternating helical and coil regions along a single chain until, at pH 12, when the $-NH_2$ groups are virtually unchanged, the transition from coil to helix is essentially complete.

FIGURE 1A



Bond angles and bond distances of polypeptide chains as derived from x-ray analysis of amino acids and simple peptides.

Reactions of Acetate Esters with Amino Acids and Polyamino Acids.

The shapes of polypeptides vary with pH and temperature, thus causing a variation in the number of active sites formed by the different interactions of the amino acid residues. Therefore, only a small fraction of the amino acid residues play a role in these reactions. The use of synthetic polyamino acids composed of simple combinations of amino acids as catalysts for the hydrolysis of esters may be the best method for investigating the mechanisms of the catalytic action of enzymes (which are themselves polyamino acids) because it is easier to relate the charge and conformation of synthetic polypeptides to their catalytic activity. These synthetic molecules serve as models of enzyme molecules.

The rates of nucleophilic reactions of glycine and glycyl-glycine with phenyl acetate[8] and PNPA[9] in aqueous solutions were found to correspond to their basicities.

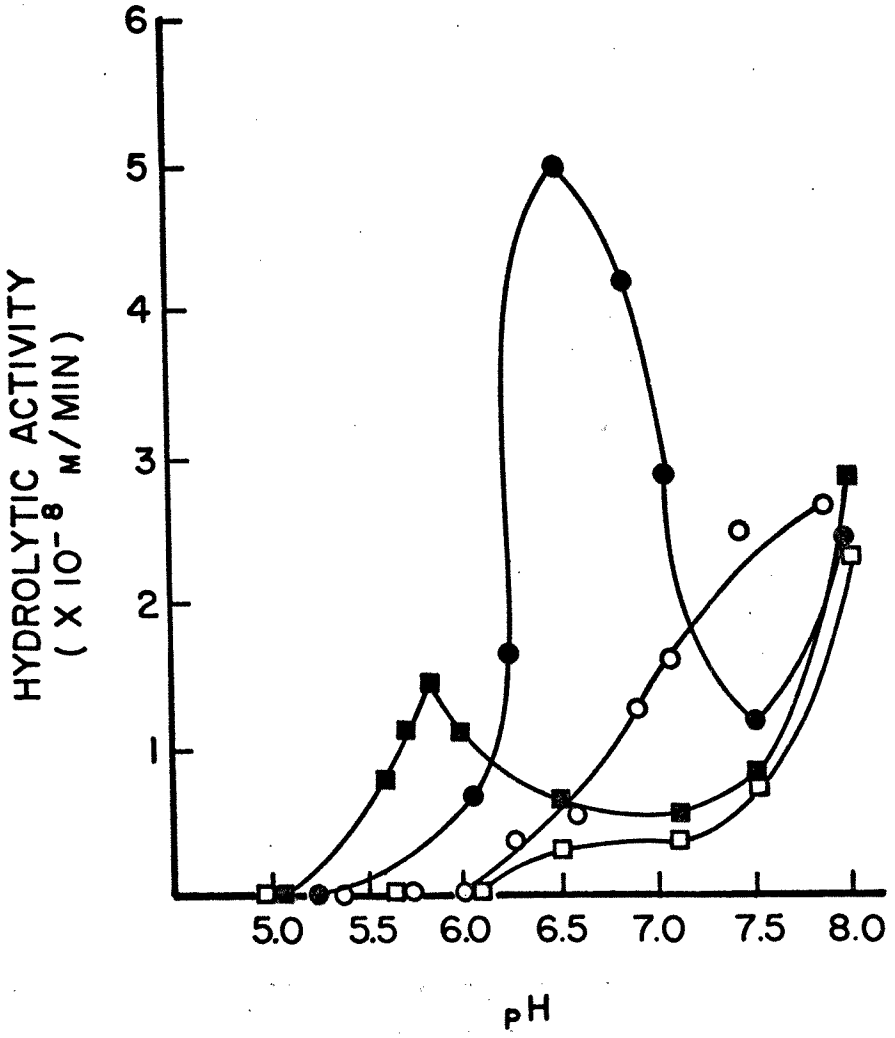
Copolymers of aspartic acid and serine, aspartic acid and threonine, glutamic acid and serine, and glutamic acid and threonine displayed activities toward acetate esters which increased with either an increase in pH or an increase in temperature. Incorporation of alanine into the aspartic

acid polymers resulted in materials which showed an optimum temperature, 50° and 47.5° respectively for their activity. Incorporation of alanine into the glutamic acid polymers resulted in materials which had both an optimum pH, 6.5 and 5.9 respectively and an optimum temperature, 45° and 37.5° respectively. Copolymers of glutamic acid with either tyrosine, tryptophan or cysteine showed maximal activity near pH 6.0 and temperatures near 40°. On the other hand, polymers of aspartic acid and either tryptophan, tyrosine or cysteine showed no catalytic activity.[10]

Several enzymes with quite diverse functions possess a lysine residue, in addition to other amino acids residues at their active sites. Hydrolytic enzymes such as ribonuclease,[11] lysozyme[11] and aldolase,[12] enzymes involved in group transfers such as glutamate-aspartate transaminase and enzymes involved in the breaking of carbon-carbon bonds, such as acetoacetate decarboxylase appear to require a lysine residue for their activities.

The investigations into the nature of the interactions between substrate and enzyme molecules can be divided into two types. The first, which has been mentioned, is the use of model compounds. The second area is well-defined and involves a study of the kinetics of the reaction between the enzyme and substrate.

FIGURE 2



pH Dependence of NPA Hydrolysis at 40°C.

Symbol	Copolymer
○	Copoly (L-Ser, L-Glu)
●	Copoly (L-Ser, L-Glu, L-Ala)
□	Copoly (L-Thr, L-Glu)
■	Copoly (L-Thr, L-Glu, L-Ala)

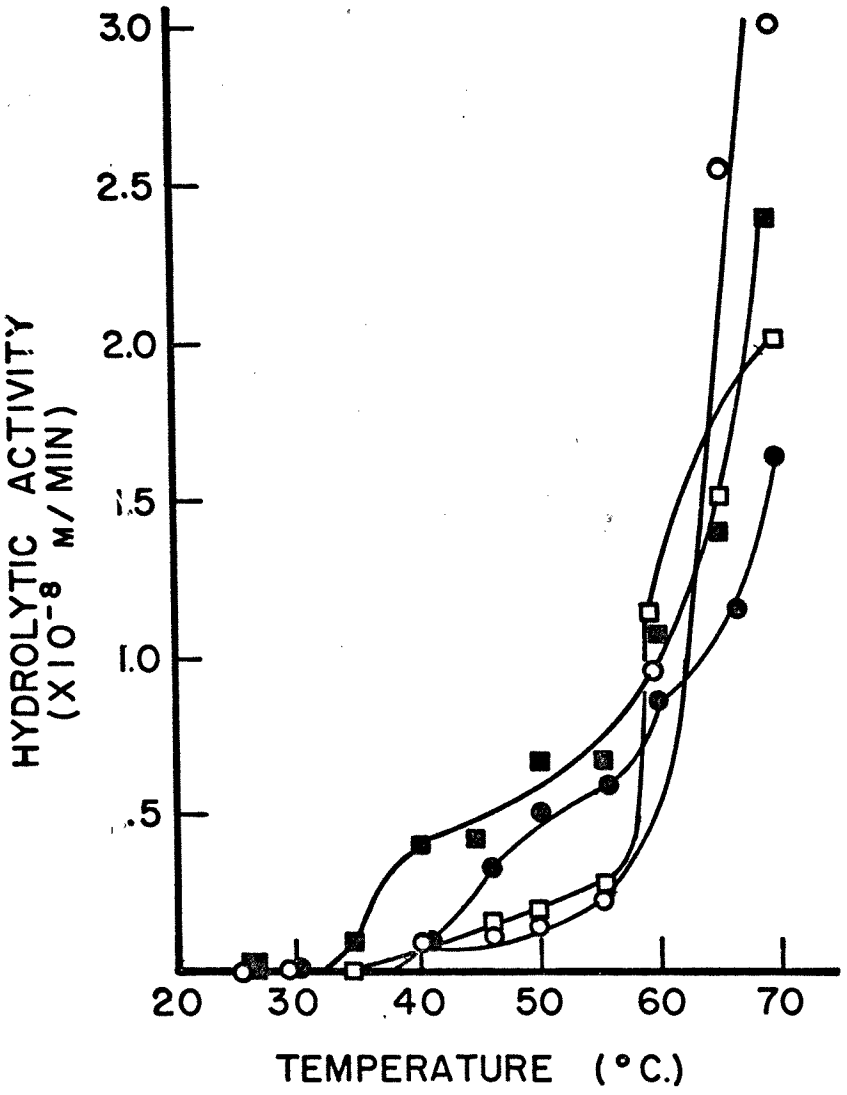


Fig. 3 - Temperature dependence of NPA hydrolysis. ○, copoly (L-Ser, L-Asp); ●, copoly (L-Ser, L-Asp, L-Ala); □, copoly (L-Ser, L-Glu); ■, copoly (L-Ser, L-Glu, L-Ala) at pH 6.50

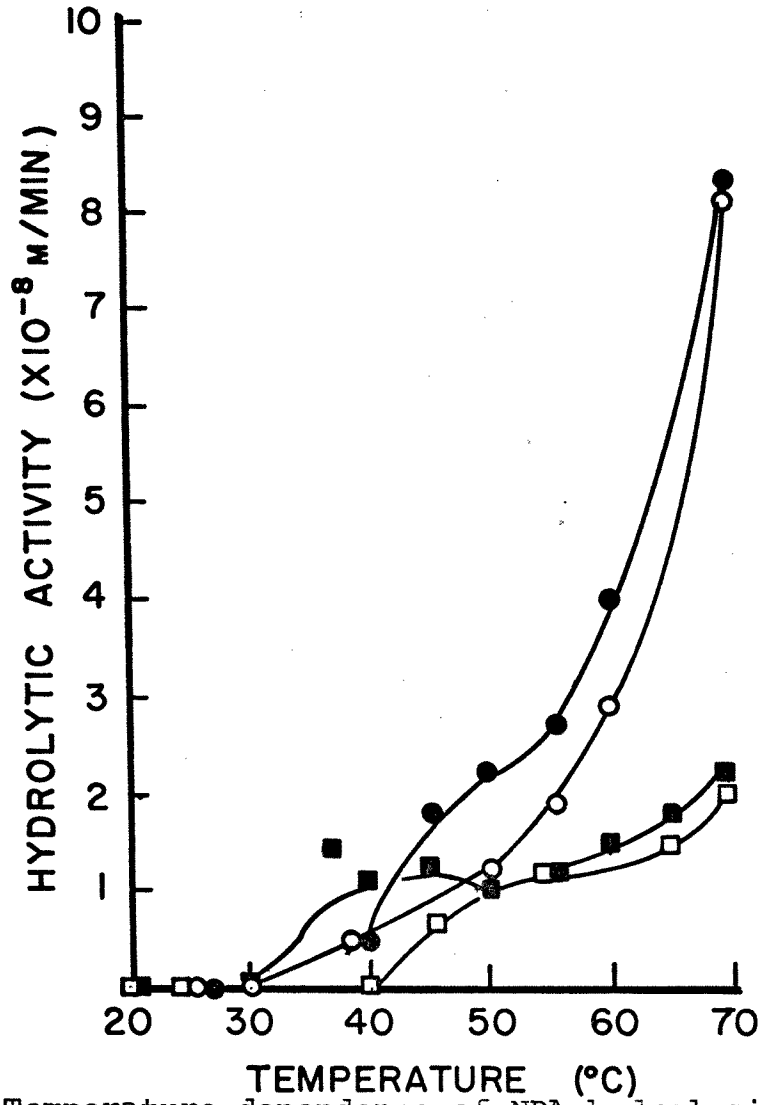


Figure 4 Temperature dependence of NPA hydrolysis.

○, copoly (L-Thr, L-Asp); ●, copoly (L-Thr, L-Asp,L-Ala); □, copoly (L-Thr, L-Glu); ■, copoly (L-Thr, L-Glu, L-Ala) at pH 5.90.

The Mechanism of Enzyme Reactions

The first serious study of the mechanism of enzyme action was made by Fischer in 1894 and he called it the "lock and key mechanism". The enzyme is pictured as the lock which can only be selectively "opened" by the substrate, the "key". He demonstrated this by the hydrolysis of acetyl-L-phenyalanine methyl ester enhanced by the enzyme chymotrypsin. The D-isomer was found to be inert in the presence of chymotrypsin.[13] In 1902, Brown observed that the absolute amount of sucrose hydrolyzed per unit time in the enzyme-catalyzed reaction was independent of the initial concentration of sucrose in the reaction system (zero order reaction with respect to sucrose).

In proposing a mechanism to account for this phenomena, he postulated the formation of an enzyme-substrate complex which subsequently decomposes to products and free enzyme.

$$E + S \longrightarrow ES \longrightarrow P_1 + P_2 + \ldots\ldots + P_n + E$$

where E = Enzyme

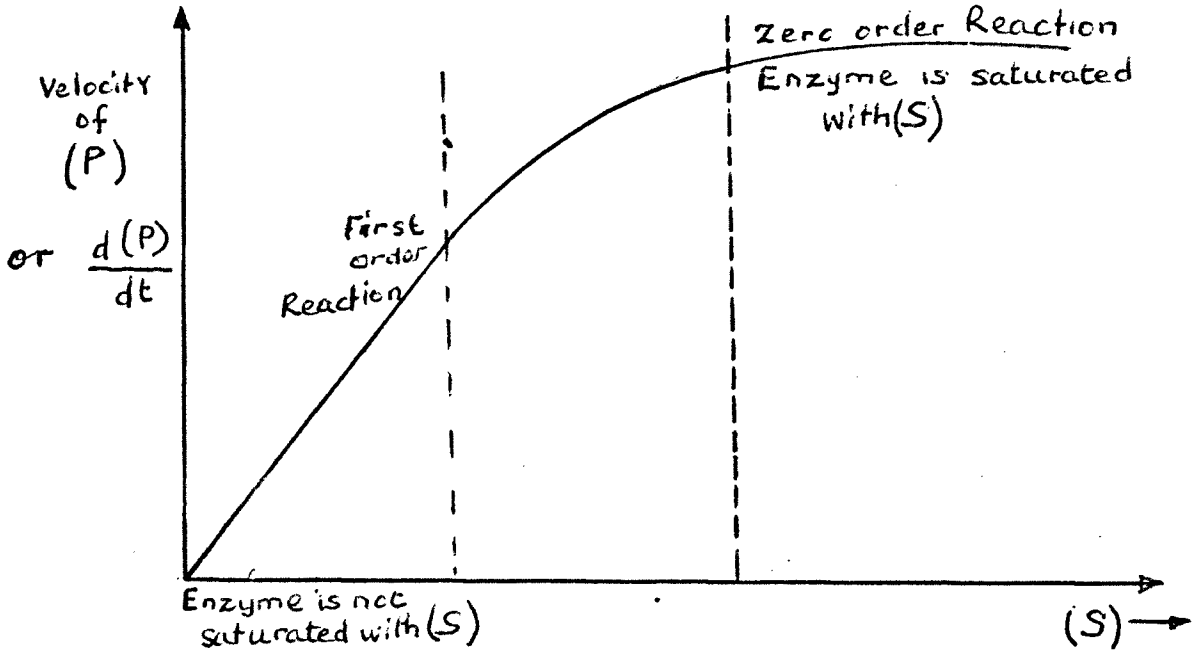
S = Substrate

ES = Enzyme-Substrate Complex

Pn = Products n

The above model made the following predictions:

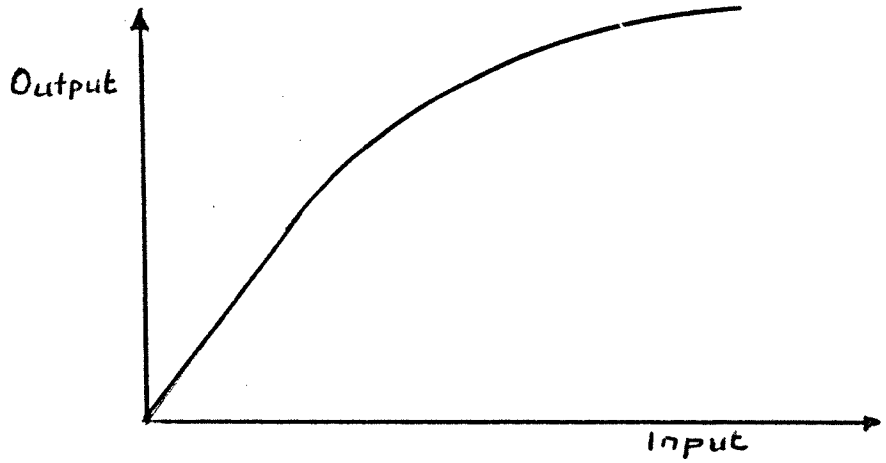
1. The existence of an intermediary complex.
2. That the enzyme cycles continuously.
3. For high concentrations of substrate, almost all of the enzyme would be tied up in the enzyme-substrate complex.
4. Therefore, if the formation of ES were more rapid than its decomposition, the rate of formation of the product would be the "rate-limiting" step. This velocity would be proportional only to the concentration of ES and be independent of the substrate concentration. Therefore, changes in S, when S is large, would have no effect on the velocity of the product formation. Moreover, Brown correctly predicted that when S is reduced to small concentrations, the rate of formation of P would be proportional to S:



Here (S) and (P) refer to concentrations of substrate and product respectively, and t=time. Brown experimentally verified the first order of linear behavior of (S) in the sucrose-enzyme system.

Mathematical Models of Enzyme Mechanism

Probably the first enzymatic mathematical model in a hyperbolic form i.e.



was due to Henri in 1902. This equation is now referred to as integrated form of the "Michaelis-Menten" equation. Based on the chemical scheme

$$E + S \rightleftharpoons ES \longrightarrow P + E$$

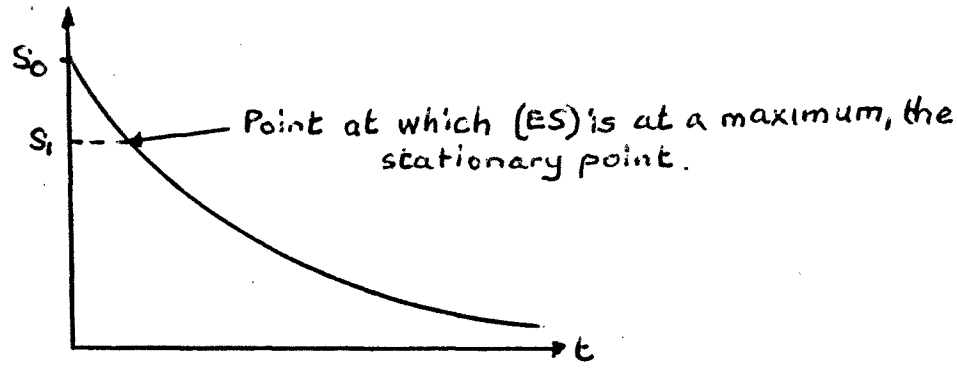
his equation assumes that:

1. The initial enzyme concentration is very small with respect to the initial substrate concentration, i.e.

$$(E)_o << (S)_o$$

The assumption was made in order that (S) + (P) would be approximately constant and equal to $(S)_o$.

2. In addition, to integrate the Michaelis-Menten equation legitimately, it must be assumed that $(S)_1$ is nearly equal to $(S)_o$:



3. Pre-equilibrium is assumed. That is $E + S \rightleftharpoons ES$ can be considered apart from $ES \longrightarrow P + E$.

Finally, this equation can be written in terms of (P) and t as

$$\frac{2.3}{t} \text{Log}_{10} \frac{(S)_o}{(S)_o - (P)} = \frac{V}{K_m} - \frac{1}{K_m} \frac{(P)}{t}$$

where Km = Michaelis constant

V = maximum velocity of formation of product.

Michaelis-Menten Law

Michaelis-Menten in 1913 proposed the equation

$$V = \frac{Vmax \quad (S)}{K_m \quad +(S)}$$

to describe the dependence of the rate of an enzymatic reaction on the substrate concentration. This equation is derived from the Michaelis-Menten theory which essentially is a modification of the original theory suggested by Brown in 1902.

The essence of the Michaelis-Menten theory is that enzyme reactions proceed in two well defined steps. The first is the formation of the complex between enzyme and substrate,

(1) $E + S \rightleftharpoons ES$

and the second is the decomposition of the complex into the products of the reaction, the enzyme being regenerated in this step. (2) $ES \longrightarrow P + E$

Michaelis-Menten equation confirms the Brown's predictions that at very low substrate concentrations most of the enzyme molecules are in the free state and only a small fraction being combined with substrate. Under

these conditions the amount of complex formed is proportional to the amount of substrate, so that the rate of formation of products, that is the rate of reaction is proportional to the concentration of substrate. At high substrate concentrations, on the other hand, the enzyme becomes saturated with substrate; practically all the enzyme being in the form of the complex. Increase in the substrate concentration can therefore no further increase in the concentration of complex so that the rate, proportional to the concentration of complex is now independent of the substrate concentration.

Since the work to be described will show that L-lysine and poly-L-lysine (here-after referred to as lysine and polylysine) may serve as models of enzyme activity it would be appropriate here to discuss some of the parameters concerning enzyme kinetics.[14] The basic assumption of Michaelis is that the enzyme forms a complex with the substrate. Consider the idealized model of a single intermediate

(1) $E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_3} P + E.$

where E = Enzyme

S = Substrate

ES = Enzyme - Substrate

P = Product

Ki = Rate constant i; k = 1, 2, 3

From this postulate, the Michaelis-Menten equation can be easily derived. Following the Law of Mass Action we can write the following differential equations to describe the model in (1) as

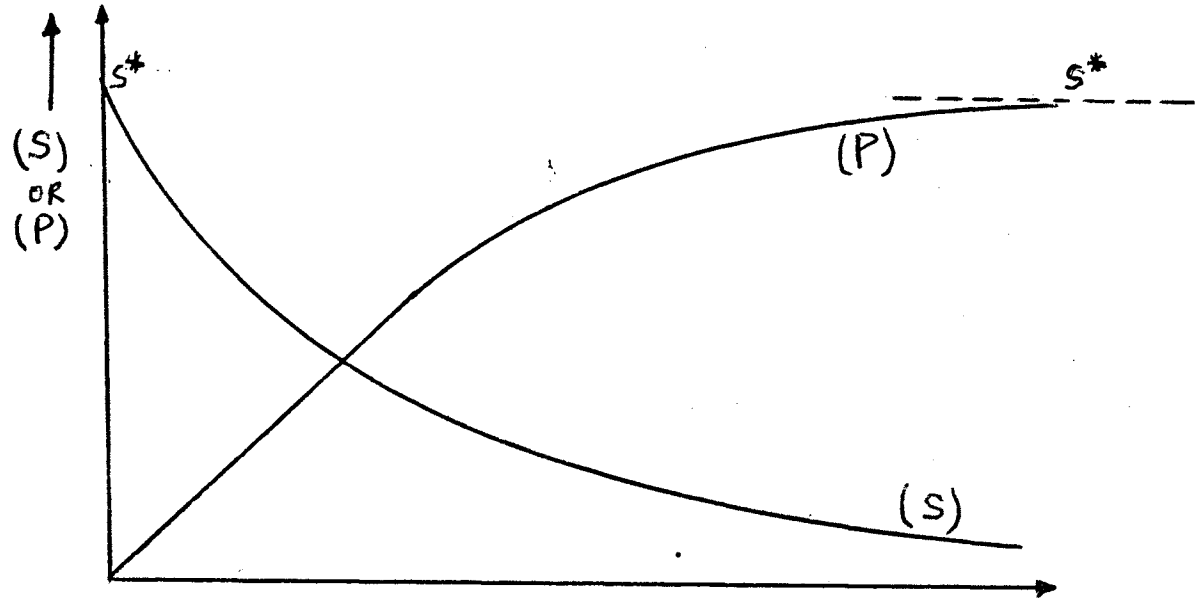
(2) $\frac{d(S)}{dE} = k_1 (E)(S) - k_2 (ES)$; $S(t=o)=S(o)=S^*$

(3) $\frac{d(E)}{dt} = k_1 (E)(S) + (k_2 + k_3)(ES)$; $E(o) = E^*$

(4) $\frac{d(ES)}{dt} = k_1 (E)(S) - (k_2 + k_3)(ES)$; $ES(o) = 0$

(5) $\frac{dP}{dt} = k_3 (ES)$ Initial condition $P(o) = 0$

These equations describe the complete time trajectories which look like the following graph:



The Stationary - State assumption becomes:

$$\frac{d\ (ES)}{dt} = 0$$

or: $\frac{d\ (ES)}{dt} = 0 = k_1\ (E)\ \ (S) - (k_2 + k_3)\ \ (ES)$

or: $(k_2 + k_3)\ (ES) = k_1\ (E)\ \ (S)$ (6)

Combining (3) and (4)

$$\frac{d\ (E)}{dt} + \frac{d\ (ES)}{dt} = 0 = \frac{d}{dt}\ \left[(E) + (ES)\right]$$

Hence $(E) + (ES) = E^*$

Substituting (7) in (6) we obtain:

$$(k_2 + k_3)\ \ (ES) = k_1\ (E^* - (ES))\ \ (S)$$

or: $\left[(k_2 + k_3) + k_1\ (S)\right]\ \ (ES) = k_1\ E^*\ \ (S)$

$$(ES) = \frac{E^*\ (S)}{\frac{k_2 + k_3}{k_1} + (S)}$$

Substituting the equation into the differential equation for $\frac{d\ (P)}{dt}$, we obtain the reduced model:

$$\frac{d\ (P)}{dt} = \frac{(k_3\ E^*)\ \ (S)}{\frac{k_2 + k_3}{k_1} + (S)}$$

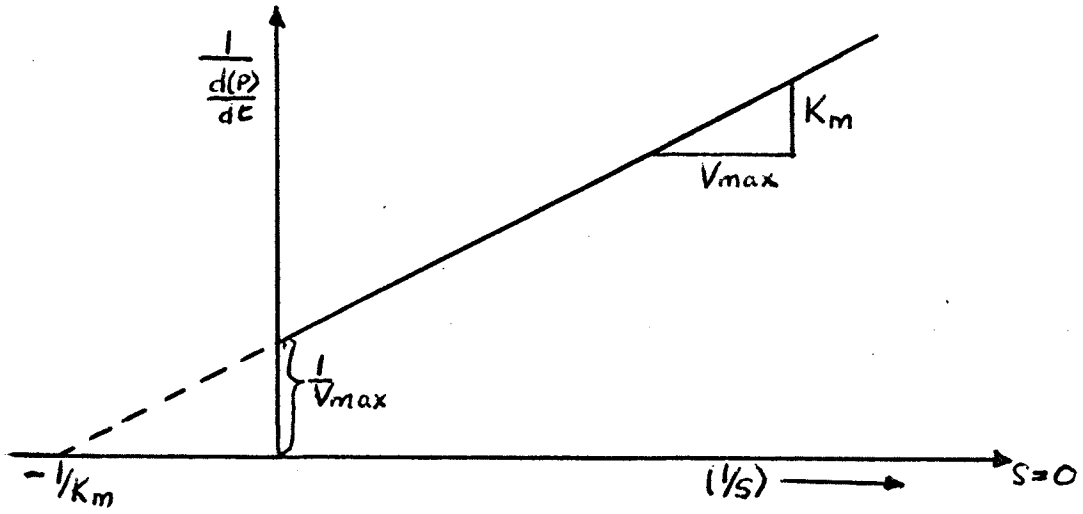
or: $\frac{d\ (P)}{dt} = v = \frac{Vmax\ (S)}{KM + (S)}$

where: $Vmax = k_s\ E^*$

and: $Km = \frac{k_2 + k_3}{k_1}$ = Michaelis-Menten constant

The Michaelis-Menten equation can be simplified and the data linearized to give the Lineweaver-Burk plot.

$$\frac{1}{\frac{d(P)}{dt}} = \frac{1}{v} = \frac{Km}{Vmax}\left(\frac{1}{S}\right) + \frac{1}{Vmax}$$



This plot gives relatively easily the two parameters Km and V to describe the system.

Finally, when (S) = Km

$$v = \frac{Vmax\ (S)}{Km + (S)} = \frac{Vmax\ Km}{Km + Km} = \frac{Km}{2KM}\ Vmax$$

$$v = \frac{Vmax}{2} \quad \text{when} \quad (S) = Km$$

The Michaelis-Menten equation was not applied to this work. It would, however, be a simple matter to select the optimum pH and then vary pNPA concentration in order to determine the maximum velocity of the reactions and the Michaelis constant for both lysine and polysine.

By obtaining these parameters, a better understanding of the nature of the interaction between polylysine and pNPA would emerge, thus making polylysine a more useful enzyme model.

EXPERIMENTAL

The reaction was initiated by injecting p-nitrophenylacetate solution into buffered L-lysine or poly-L-lysine solutions. Progress of the reaction was followed spectrophotometrically by observing the absorption of the product p-nitrophenolate ion at 400mμ as a function to time. L-lysine, poly-L-lysine and buffer solutions were prepared using distilled water. The p-nitrophenylacetate solution had to be prepared using an organic solvent to ensure that no hydrolysis of this ester would occur until the desired initiation (injection) time.

All chemicals were either of reagent grade quality or were purified before their use. They were:

a) Poly-L-lysine HBr of viscosity average molecular weight of 100,000 from Pilot Chemicals.

b) L-lysine HCl from Aldrich Chemicals.

c) P-nitrophenylacetate from Aldrich Chemicals which was re-crystallized from carbon tetrachloride to remove residual p-nitrophenol.

d) Buffers from Fischer Chemical.

The first phase of this project consisted of finding a suitable solvent for the p-nitrophenylacetate. The initial choice was acetone.

Although the ester was readily soluble in acetone, a problem arose when the pNPA solution was injected into the buffered poly-L-lysine solutions: The polymer precipitated out. Attempts were made to alleviate this problem by increasing the ionic strength with a neutral salt to such an extent that the poly-L-lysine would remain in solution even in the presence of the organic solvent.

The addition of LiBr did reduce the precipitation effect to a considerable extent, but did not entirely eliminate the problem as some cloudiness remained. This approach was therefore rejected.

Next, several solvents were investigated in order to find one with the following suitable characteristics:

a) high solubility of pNPA

b) high solubility of poly-L-lysine, and

c) negligible enhancement of the reaction rate.

Unfortunately, no one solvent exhibited all three parameters. Dimethylsulforide and Dimethylformamide accelerated the reaction to such an extent that it could not be followed in a practical manner. Although Dimethoxyethane had no appreciable effect on the reaction rate, the

solubility of the Poly-L-lysine was not sufficiently high. Among all the solvents investigated, methanol best satisfied the requirements of solubility and rate effect.

The second phase of the project was concerned with the investigation of the rate of both the catalyzed and uncatalyzed reactions. The reactions were carried out in spectrophotometric cells and followed as previously mentioned by observing the absorption due to p-nitrophenolate ion at 400mμ. Preliminary measurements were made using the Bausch and Lomb Spectronic 20. This instrument had no temperature control with the result that the temperature changed several degrees during the time interval that measurements were taken. The resulting data was considered unacceptable and thus this instrument could not be used.

The Beckman B Spectrophotometer was the next instrument to be utilized. Although it had no temperature control, the temperature variation stayed within such reasonable limits that the effect of the temperature variation could be discounted. At lower buffer pH the reaction was slow enough that it could be monitored accurately, but at higher pH the rate of change of absorption was so rapid that the instrument could not be used to accurately follow the re-

action. It was, therefore, concluded that the reactions would have to be run below room temperature and carefully controlled.

As a result, measurements were shifted to the Beckman D.U. Spectrophotometer which had a thermostated cell compartment. The temperature was now maintained at 20°C throughout the experiments.

The procedure for a typical run was as follows: Each of 3 cells was filled with the desired amount of Buffer and catalyst solutions (or water in case of the uncatalyzed reaction). The cells were then placed into the thermostated compartment and 30 minutes time was allowed to pass to ensure that thermal equilibrium had been attained. The reaction in each cell was initiated by injecting the appropriate amount of p-nitrophenylacetate solution, the cell contents were mixed well, and the absorption was measured as a function of time. At low pH values when the rates were slow, all three reactions were initiated one after the other and measurements were taken simultaneously. At the higher pH's the reaction in the first cell was initiated and measurements were taken until the hydrolysis

was complete. Then the reaction in cell two was initiated and the absorption measurements taken. Finally the reaction in cell three was initiated and followed to completion. The exact volumes of buffer, catalyst and pNPA solutions added to each cell are listed in the table below.

Cell 1	2	Buffer	1	Distilled Water	10 μl	PNPA
Cell 2	2	Buffer	1	L-lysine (.175mg/ml)	10 μl	PNPA
Cell 3	2	Buffer	1	Poly-L-lysine (.2mg/ml)	10 μl	PNPA

The quantities and concentrations of L-lysine HCl and Poly-L-lysine HBr remained constant throughout the project, while for a selected concentration of p-nitrophenylacetate the pH was varied. The data obtained are presented in tables 1 and 2 (pages 29 to 33).

TABLE 1 - OPTICAL DENSITY VERSUS TIME DATA AT 20°C.

Buffer pH = 8.0 Concentration of pNPA = 0.2 mg/mL

Cell 1

Time (min)	2.50	4.00	11.78	18.333	27.100	28.75	32.50
Optical Density	0.153	0.157	0.163	0.165	0.174	0.177	0.182

Cell 2

Time (min)	1.050	2.450	4.967	7.550	10.883	12.967	20.133
Optical Density	0.156	0.168	0.173	0.178	0.182	0.189	0.193

Cell 3

Time (min)	2.367	3.667	6.400	10.667	18.333	27.667	33.917	38.583
Optical Density	0.274	0.274	0.279	0.288	0.293	0.307	0.312	0.316

Buffer pH Concentration of pNPA = 0.2mg/mL

Cell 1

Time (min)	1.083	2.033	3.033	4.233	6.00	7.533	10.667	13.833	17.50
Optical Density	0.333	0.337	0.338	0.347	0.353	0.359	0.369	0.379	0.387

Cell 2

Time (min)	1.167	1.883	3.667	5.833	6.967	8.583	10.250	14.417	18.417
Optical Density	0.374	0.384	0.388	0.403	0.410	0.418	0.422	0.443	0.470

Cell 3

Time (min)	1.433	2.350	3.933	5.350	7.100	9.183	13.433	21.350
Optical Density	0.365	0.375	0.388	0.400	0.414	0.429	0.460	0.508

TABLE I continued

Buffer pH = 9.18 Concentration of PNPA = 0.2 mg/ml

Cell 1

Time (min)	0.700	1.383	2.133	3.083	3.833	6.000	7.167	10.083	12.417
Optical Density	0.113	0.134	0.144	0.153	0.162	0.185	0.202	0.237	0.248

Cell 2

Time (min)	0.833	1.400	1.900	2.717	3.907	5.100	6.30	8.167	9.667
Optical Density	0.15	0.16	0.169	0.181	0.206	0.223	0.24	0.270	0.295

Cell 3

Time (min)	0.900	1.733	2.833	4.067	5.233	6.567	7.867	10.40	11.900
Optical Density	0.144	0.166	0.193	0.221	0.251	0.279	0.301	0.352	0.374

For Buffer pH = 9.65 Concentration of pNPA = 0.2mg/ml

Cell 1

Time (min)	0.900	1.667	2.233	2.750	3.467	4.000	4.700	5.333	6.167	6.833
Optical Density	0.344	0.410	0.449	0.482	0.523	0.552	0.581	0.608	0.638	0.655

Cell 2

Time (min)	0.933	1.500	2.067	2.550	3.417	4.467	6.733	8.750	10.917	13.333
Optical Density	0.349	0.404	0.445	0.488	0.524	0.574	0.650	0.697	0.735	0.760

Cell 3

Time (min)	1.133	1.667	2.133	3.233	3.967	4.733	5.633	7.217	9.383
Optical Density	0.418	0.474	0.515	0.638	0.674	0.710	0.755	0.800	

TABLE I Continued

Buffer pH = 10.4 Concentration of PNPA = 0.2 mg/ml

Cell 1

Time (min)	9.783	1.250	1.867	2.333	2.667	3.100	4.033	5.340	6.950
Optical Density	0.32	0.398	0.475	0.520	0.550	0.582	0.625	0.680	0.712

Cell 2

Time (min)	1.067	1.667	2.100	2.700	3.450	5.200	6.583	8.450	10.00	12.400
Optical Density	0.370	0.442	0.487	0.540	0.595	0.683	0.715	0.750	0.765	0.785

Cell 3

Time (min)	1.083	1.517	2.033	2.533	4.533	5.200	8.150	9.450	13.067
Optical Density	0.374	0.438	0.498	0.500	0.661	0.686	0.748	0.755	0.767

For Buffer pH = 8.0 Concentration of pNPA = 0.968 mg/ml

Cell 1

Time (min)	0.733	1.900	3.833	7.367	13.633	17.133	18.833	20.333	25.250
Optical Density	0.327	0.337	0.344	0.363	0.402	0.419	0.431	0.444	0.470

Cell 2

Time (min)	1.167	2.500	4.117	5.767	10.600	14.733	20.067	24.033	27.283
Optical Density	0.362	0.377	0.392	0.408	0.449	0.483	0.528	0.562	0.582

Cell 3

Time (min)	0.683	2.383	4.000	5.583	8.383	10.850	12.383	15.317
Optical Density	0.457	0.473	0.487	0.492	0.522	0.530	0.542	0.564

At this point the cooling water circulation pump broke down. The experiment could not be continued at the constant temperature of 20°C. A comparison of the temperature before and after the experiment revealed the variation to be small.

TABLE 2 - OPTICAL DENSITY VERSUS TIME
Temperature varies from 24.1 to 26°C.
Buffer pH - 6.86 Concentration of pNPA = 0.968 mg/ml

			Cell 1					
Time (min)	1.133	4.300	9.367	14.583	19.667	26.750	30.750	36.417
Optical Density	0.273	0.277	0.278	0.286	0.294	0.296	0.305	0.317
			Cell 2					
Time (min)	1.667	3.133	7.417	11.333	27.667	34.467	42.500	51.667
Optical Density	0.292	0.294	0.300	0.302	0.325	0.336	0.351	0.372
			Cell 3					
Time (min)	1.200	13.400	20.033	28.200	37.533	46.783	59.617	68.200
Optical Density	0.357	0.370	0.380	0.396	0.402	0.406	0.415	0.428

TABLE 2 continued

Buffer pH = 7.41 Concentration of pNPA = 0.968 mg/mL
Temperature varied from 22.2 to 24.2°C

			Cell 1					
Time (min)	1.335	2.750	5.170	7.160	10.850	15.950	18.700	20.380
Optical Density	0.334	0.337	0.346	0.353	0.357	0.370	0.382	0.387
			Cell 2					
Time (min)	1.200	2.565	3.870	5.650	10.450	15.450	20.500	27.250
Optical Density	0.382	0.386	0.395	0.407	0.425	0.425	0.460	0.483
			Cell 3					
Time (min)	1.085	3.370	6.100	8.590	11.050	17.80	25.10	33.20
Optical Density	0.477	0.482	0.488	0.498	0.506	0.525	0.550	0.580

For Buffer pH = 6.2 Concentration of pNPA = 0.968 mg/mL
Temperature varied from 24.2 to 25.2°C

			Cell 1					
Time (min)	1.333	2.417	5.250	12.667	15.833	18.333	23.083	28.400
Optical Density	0.022	0.023	0.024	0.024	0.026	0.027	0.027	0.027
			Cell 2					
Time (min)	1.333	3.750	8.417	13.267	16.333	31.500	45.500	58.833
Optical Density	0.034	0.034	0.036	0.034	0.036	0.041	0.044	0.044
			Cell 3					
Time (min)	1.833	4.833	20.167	33.917	47.333	60.917	72.167	75.500
Optical Density	0.159	0.164	0.171	0.172	0.172	0.174	0.174	0.177

The data are presented graphically in figures 7 to 15. For purposes of comparison the initial rates are to be obtained and thus only the first few points of a given run have been plotted. In each case the data points not shown are those which curve off the linear region and approach a limiting value of adsorption at infinite time indicative of the reaction having gone to completion. The initial rates for the control and catalyzed reactions are summarized in Table 3 on page 35.

TABLE 3 INITIAL RATES OF REACTION

Buffer pH	Concentration of pNPA (mg/mL)	Temperature (°C)	Reaction Rate (O.D./Minute)		
			Control	Lysine HCL	Polylysine HB
8.0	0.200	20.0	0.9×10^{-3}	2.0×10^{-3}	1.6×10^{-3}
8.55	0.200	20.0	3.8×10^{-3}	5.9×10^{-3}	8.2×10^{-3}
9.18	0.200	20.0	13.1×10^{-3}	18.4×10^{-3}	22.5×10^{-3}
9.65	0.200	20.0	55×10^{-3}	70×10^{-3}	76×10^{-3}
10.4	0.200	20.0	100×10^{-3}	100×10^{-3}	100×10^{-3}
8.0	0.968	20.0	6.1×10^{-3}	8.8×10^{-3}	7.3×10^{-3}
7.41	0.968	22.2-24.2	2.5×10^{-3}	4.8×10^{-3}	3.5×10^{-3}
6.2	4.84	22.0	$.9 \times 10^{-3}$	1.7×10^{-3}	$.9 \times 10^{-3}$

Figure 7

Buffer pH = 8.0 Concentration of pNPA = 0.2mg/ml
Temperature = 20°C

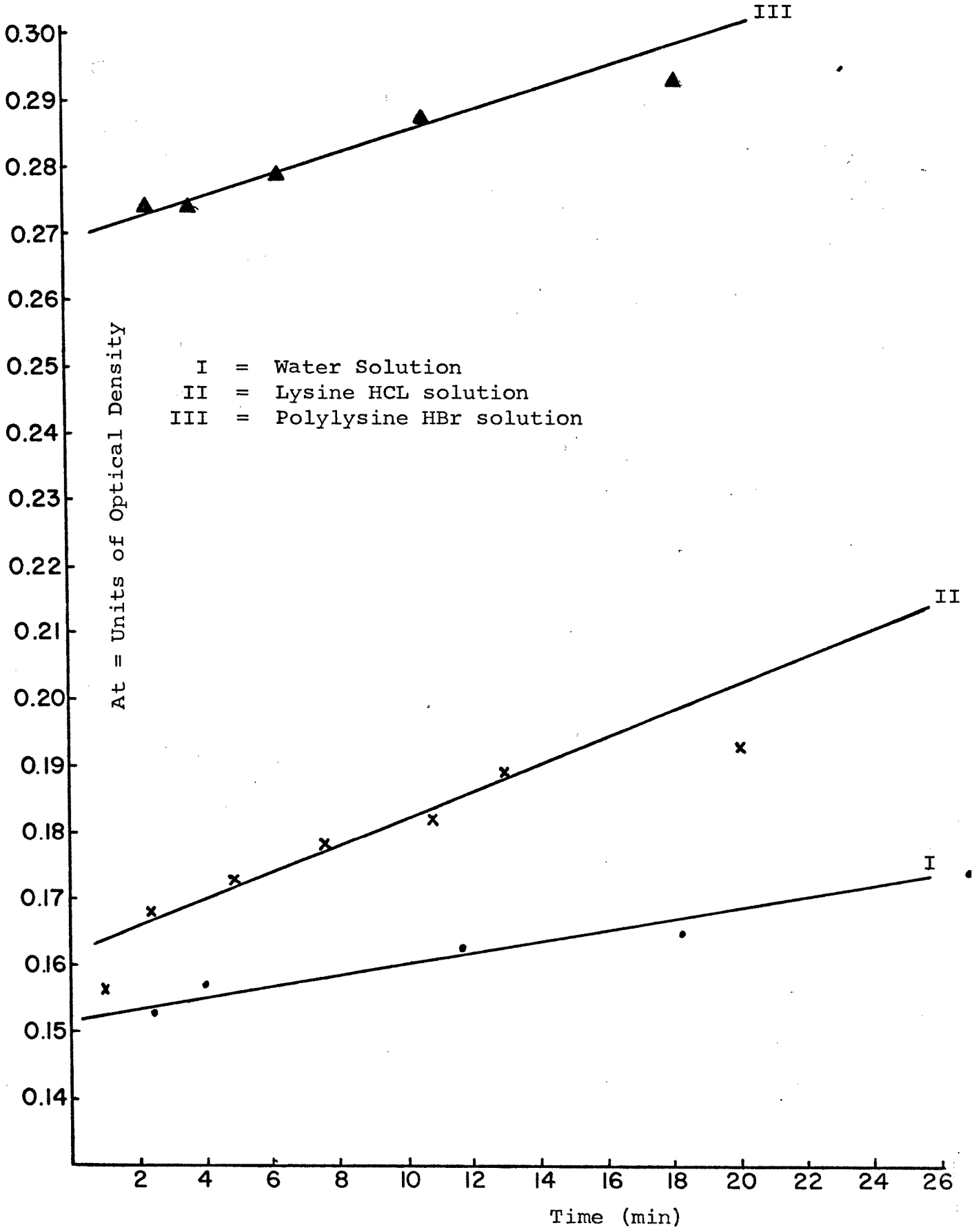


FIGURE 8

Buffer pH = 8.55 Concentration of pNPA = 0.2 mg/ml

Temperature = 20°C

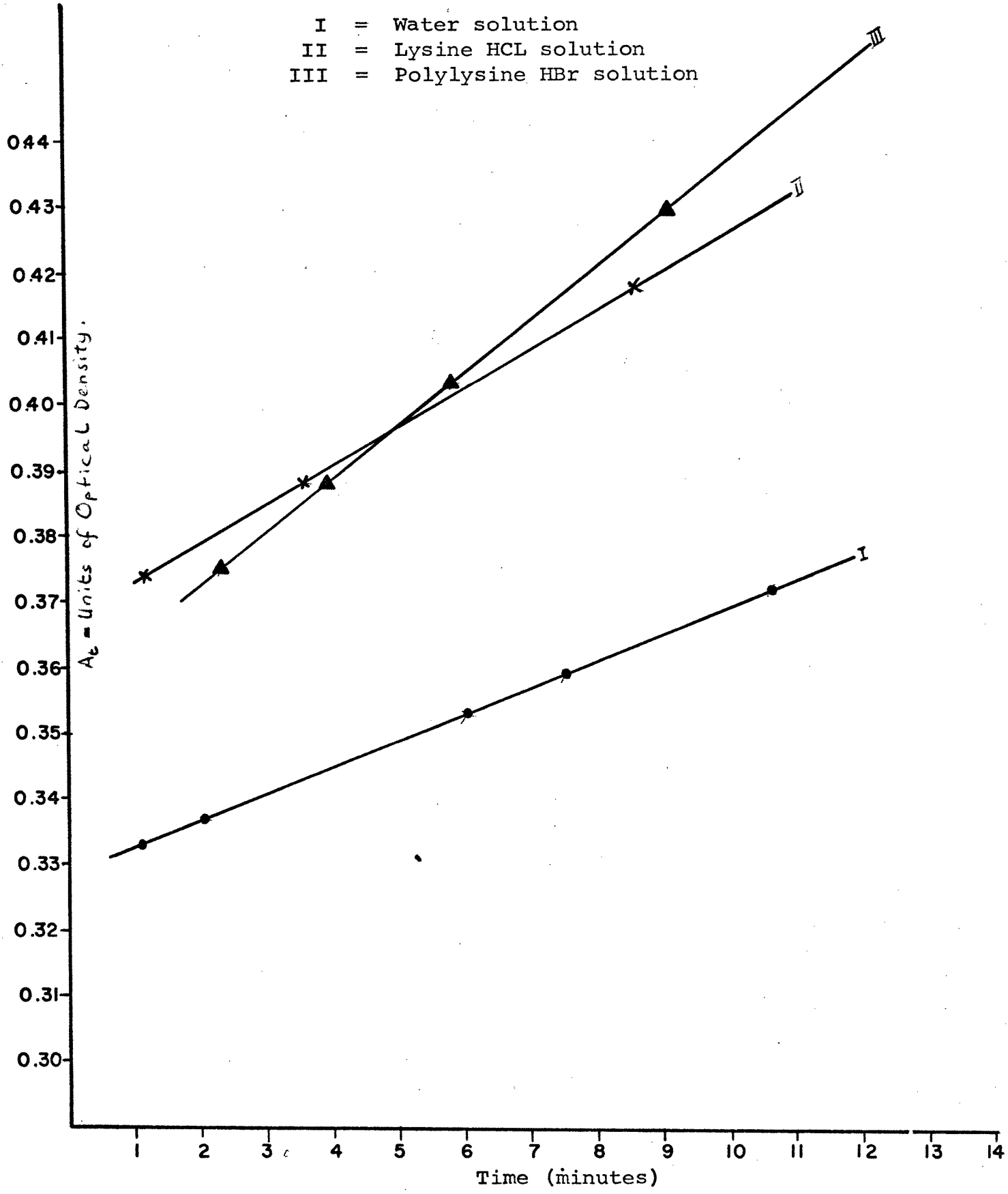


FIGURE 9

Optical Density versus Time at 20°C
for
Buffer pH = 9.18 Concentration of pNPA = 0.2 mg/ml

I = Water solution
II = Lysine HCL solution
III = Polysine HBr solution

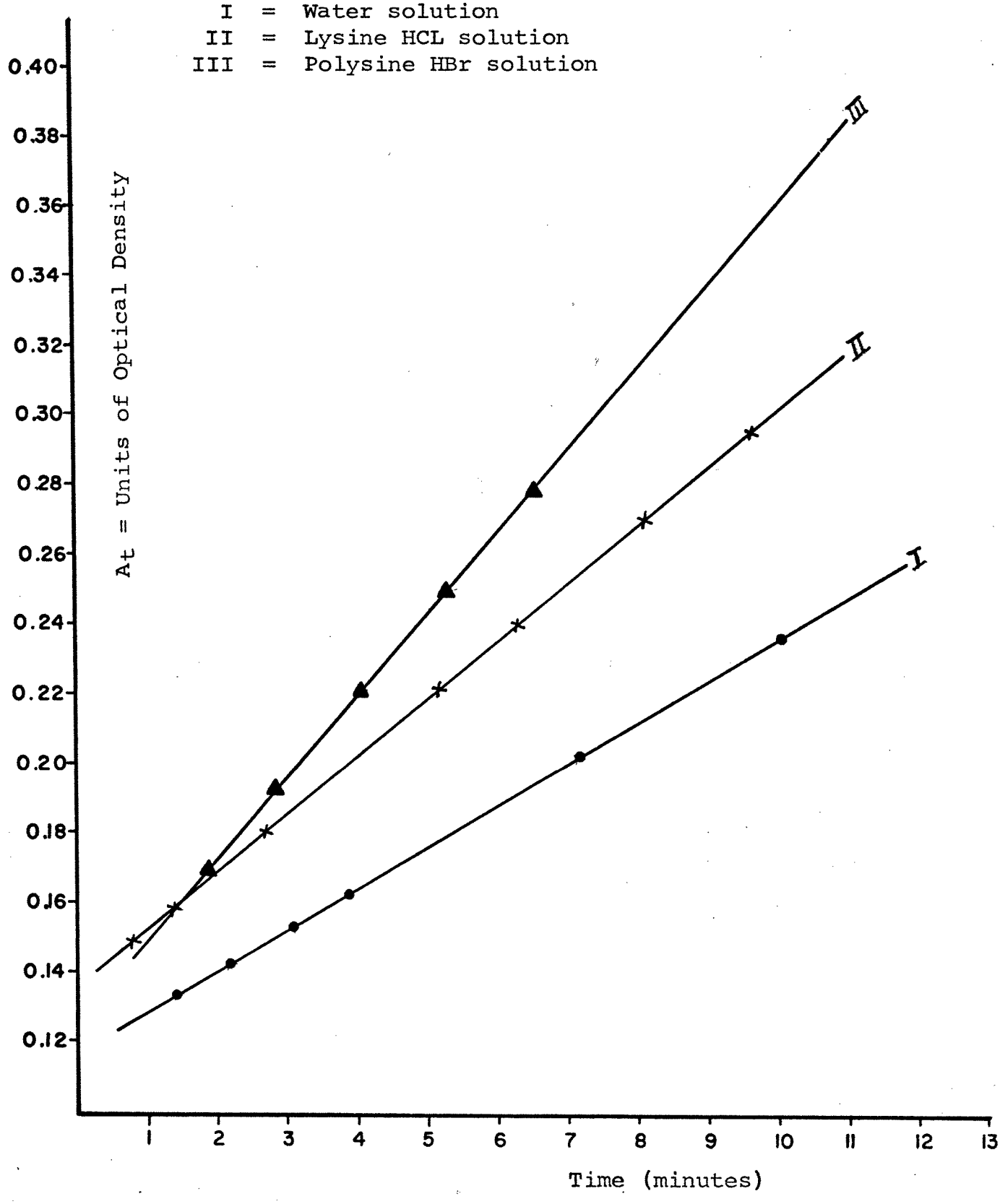


FIGURE 10

Buffer pH = 9.65 Concentration of pNPA = 0.2 mg/ml
Temperature = 20°C

I = Water solution
II = Lysine HCL solution
III = Polylysine HBr solution

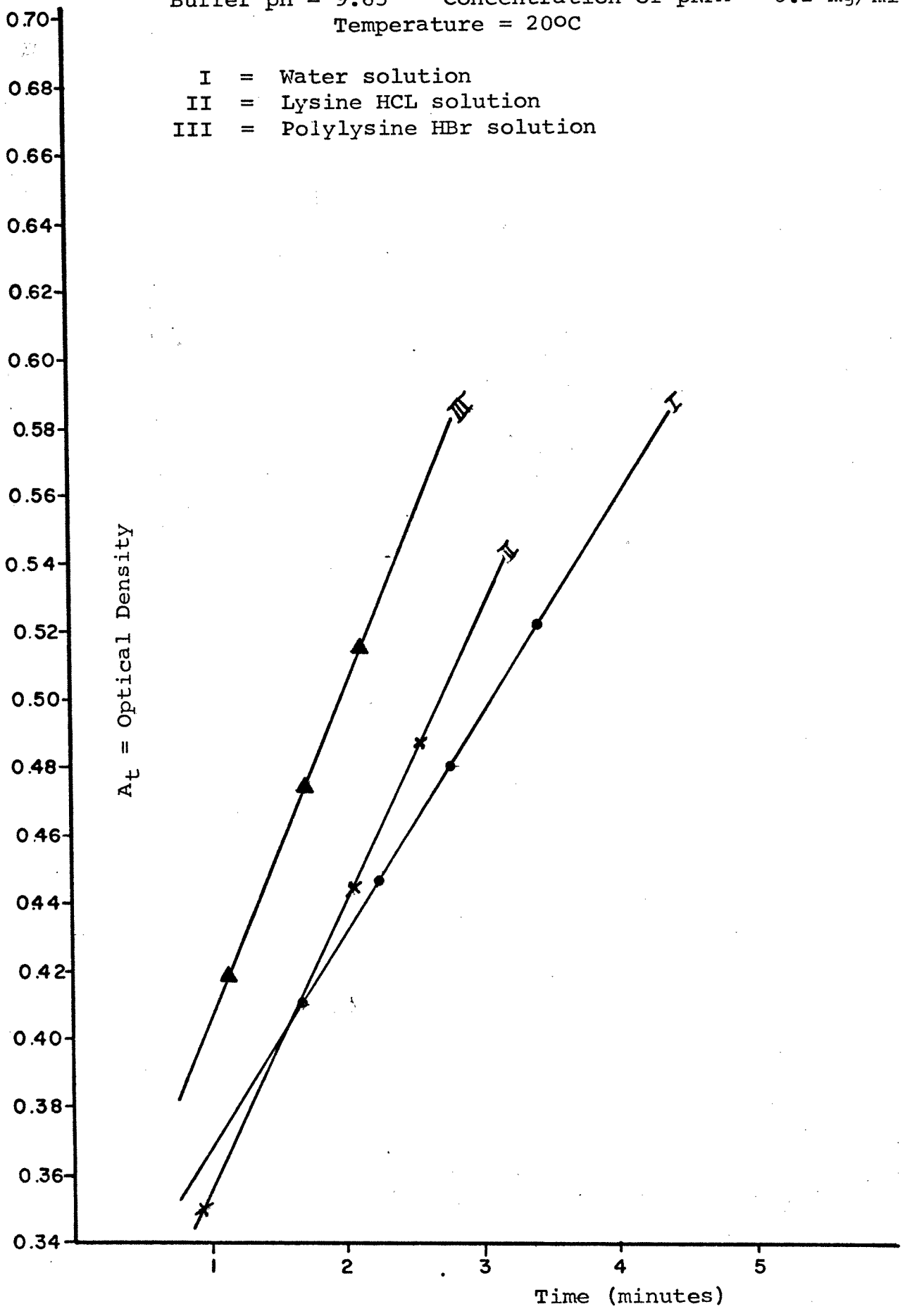


FIGURE 11

Buffer pH = 10.4
Concentration of pNPA = 0.2 mg/ml
Temperature = 20°C

I = Water Solution
II = Lysine HCL solution
III = Polylysine HBr solution

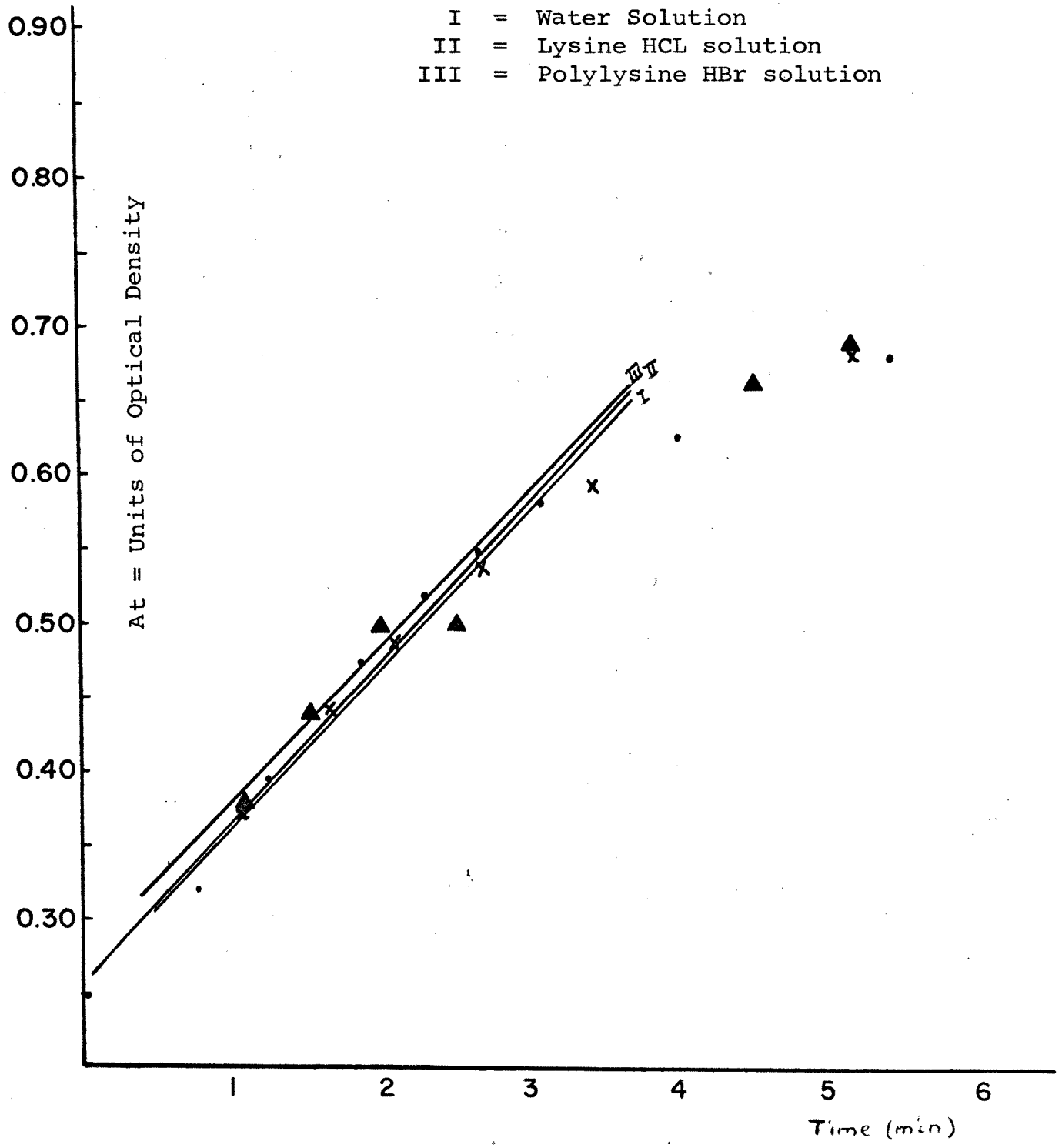


FIGURE 12

Buffer pH = 7.41 Concentration of pNPA = 0.968 mg/ml
Temperature varied from 22.2 to 24.2°C

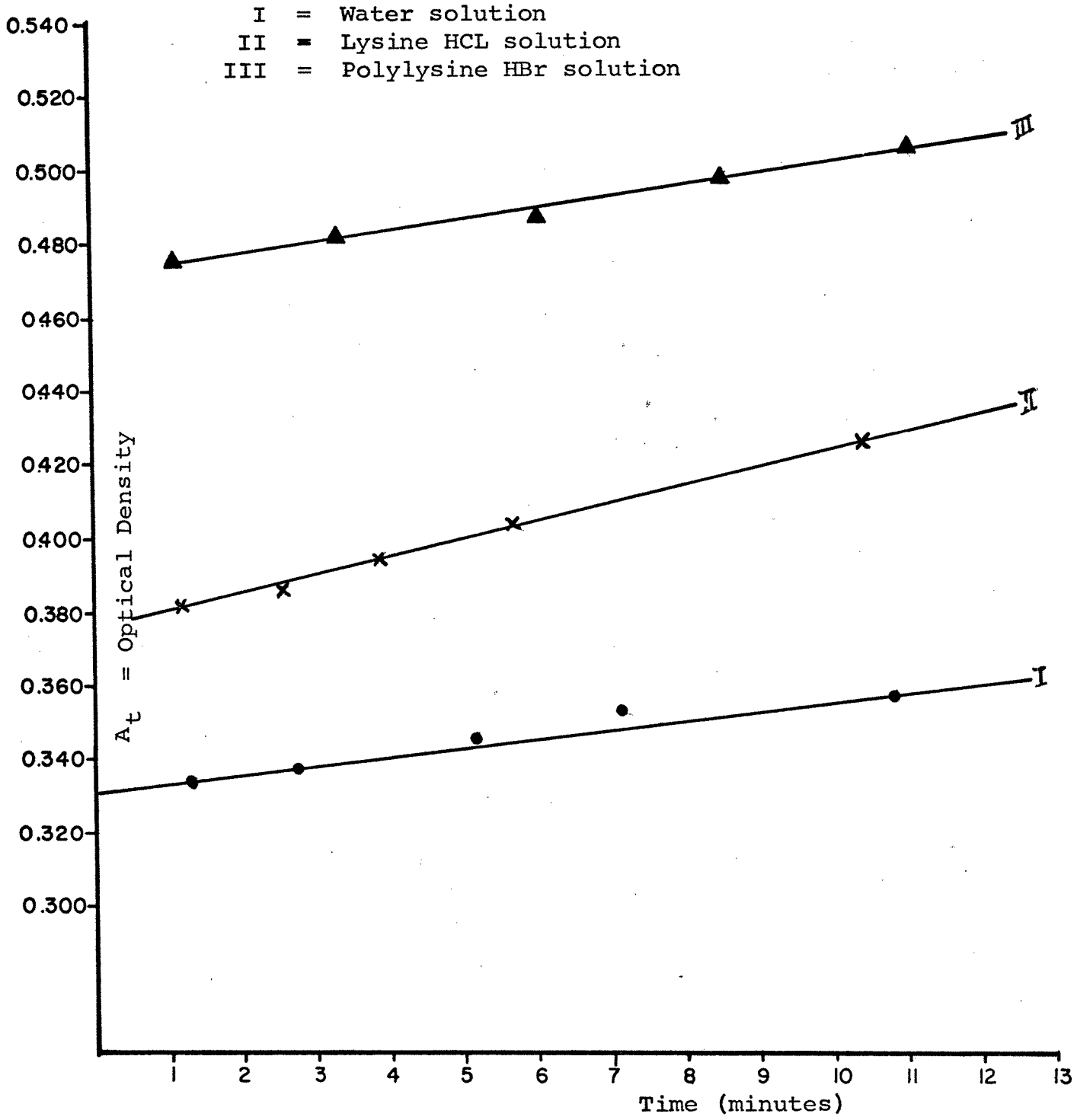


FIGURE 13

Buffer pH = 8.0 Concentration of pNPA = 0.968 mg/ml

I = Water solution
II = Lysine HCL solution
III = Polylysine HBr solution

Temperature = 20°C

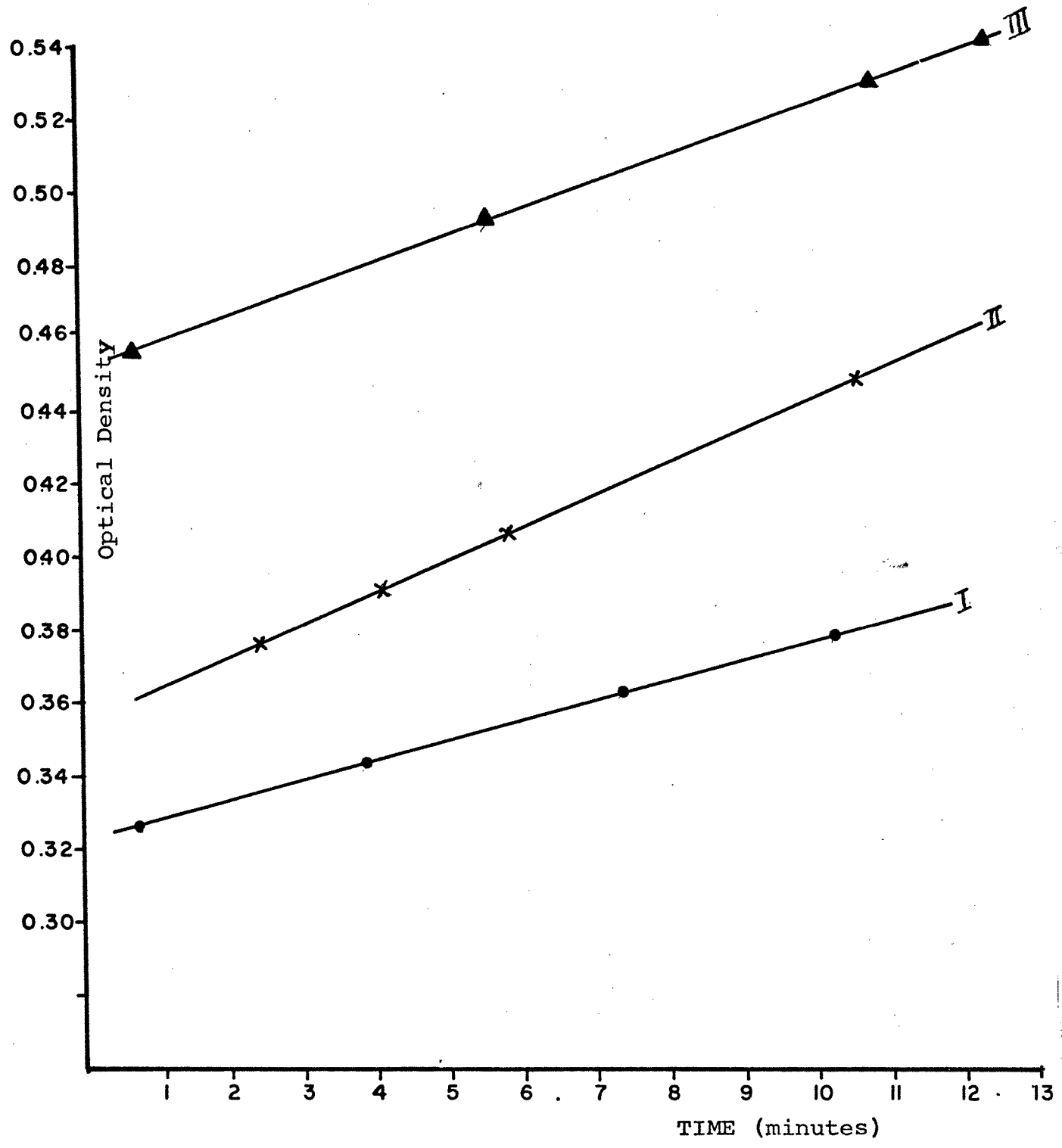


FIGURE 14

Buffer pH = 6.2 Concentration of pNPA = 4.84 mg/ml

I = Water solution
II = Lysine HCL solution
III = Polylysine HBr solution

Temperature = 22°C

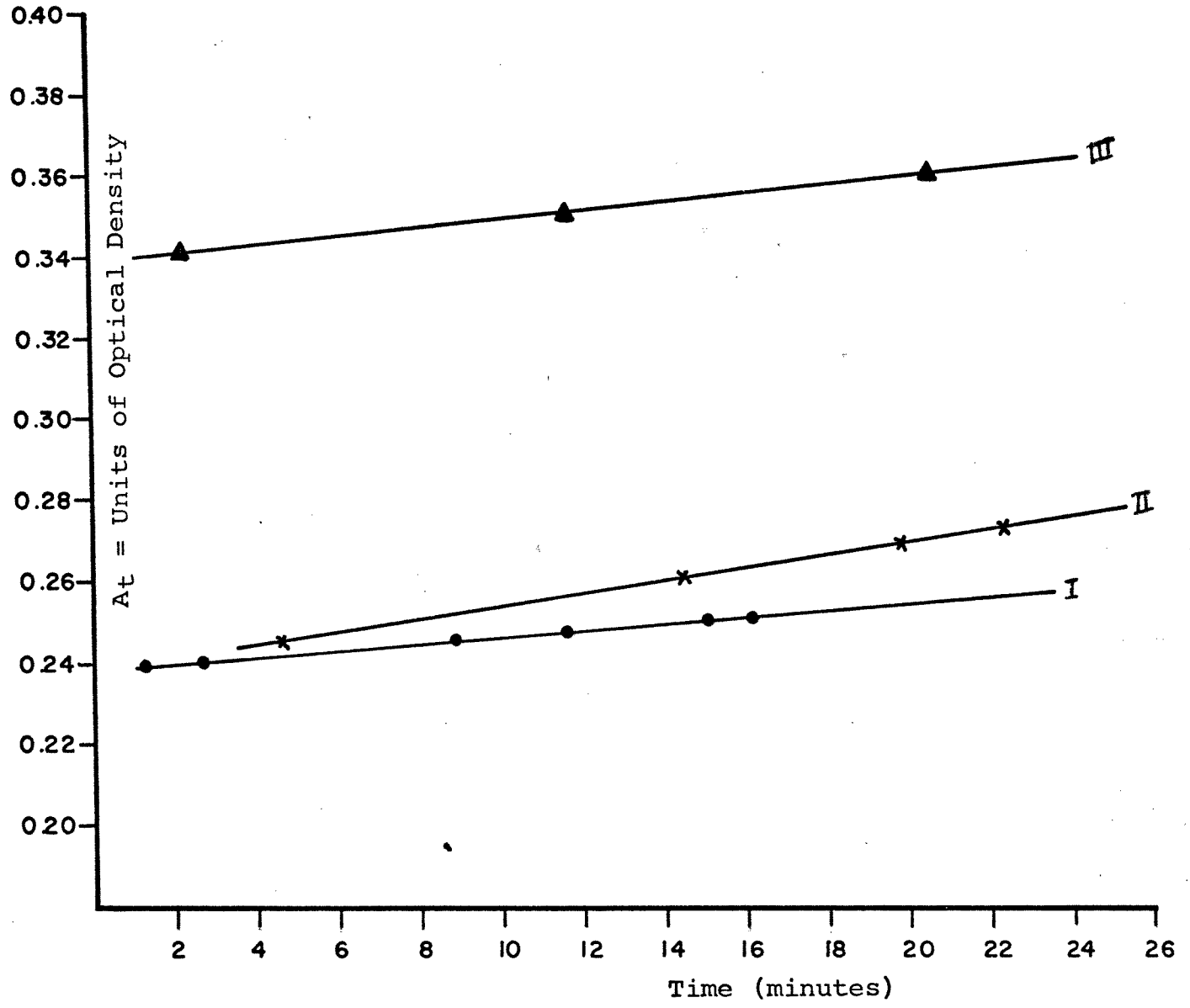
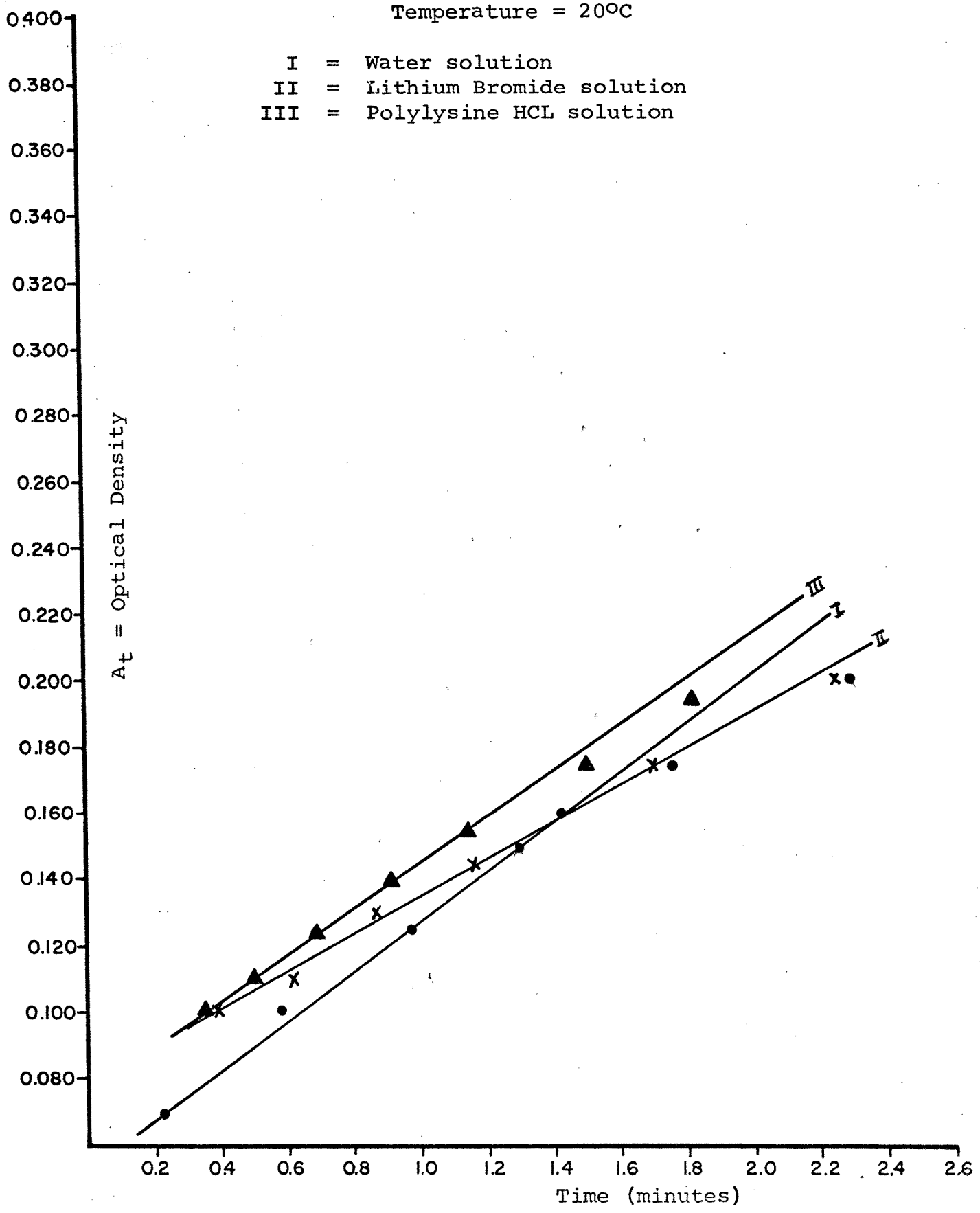


FIGURE 15

Buffer pH = 10.4 Concentration of pNPA = 1.0 mg/ml
Temperature = 20°C

I = Water solution
II = Lithium Bromide solution
III = Polylysine HCL solution



DISCUSSION

Little work has been done on the catalytic activity of lysine and lysine peptides. In a study of hydrolytic enzyme models[15] it was observed that a polymer composed almost completely of lysine residues exhibited no catalytic activity at acidic pH's. This investigation has shown that the reaction of pNPA is catalyzed by the presence of either polylysine hydrobromide or lysine hydrochloride in alkaline pH's. In the range of pH's which was investigated, the two catalysts exhibited similar behavior.

In general, the rate of reaction, both catalyzed and uncatalyzed, increases with increasing pH. As can be seen in Table 3, the uncatalyzed reaction increases by a factor of over 100 between pH 8.00 and 10.4. In this same pH range the lysine and polylysine reaction rates increased by factors of 50 and 60 respectively.

To facilitate discussions of catalytic effects it is convenient to introduce the term "rate of reaction" defined as the difference between the catalyzed and uncatalyzed reaction rates, as follows:[16]

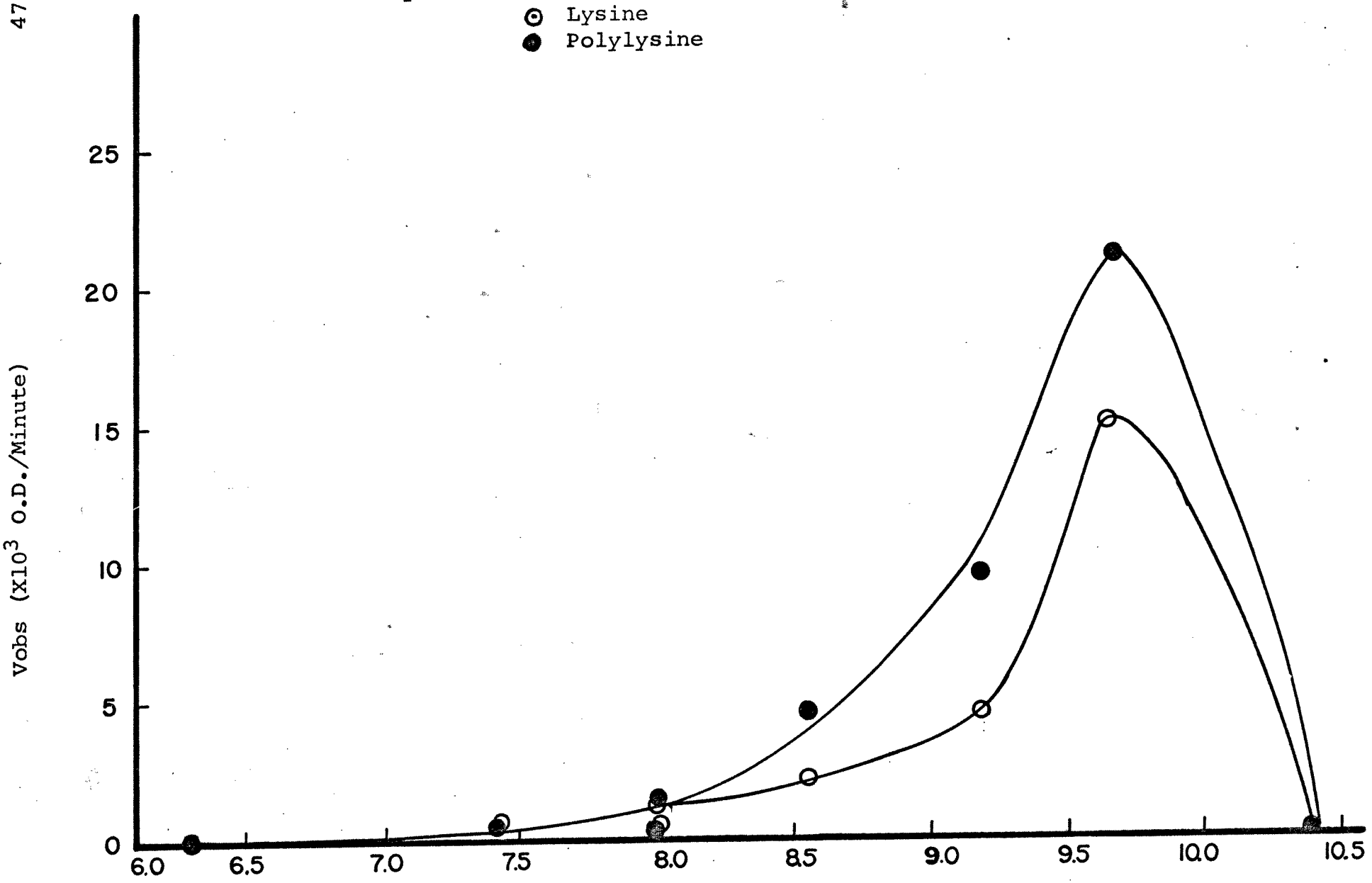
$$V_{observed} = V_{measured} - V_{blank}$$

where Vmeasured was the rate of reaction by either lysine or polylysine and Vblank was the rate of reaction without catalysis. The rates are optical density units per minute. Similar behavior was observed for lysine and polysine in the pH range 8.0 to 10.4. Figure 7 shows that at pH 8.0 catalytic activities are small; as the pH increases the catalytic activities for both increase to a maximum near pH 9.6; further increase of pH to 10.4 causes destruction of the catalytic effect. It appears that polylysine is a more efficient catalyst. At the optimum pH of both lysine and polylysine, 9.65, the polylysine is 40 percent more active than lysine.

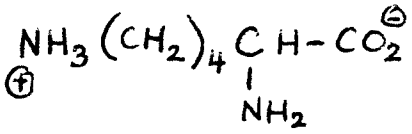
Preliminary examination of the reaction at pH's below 8.0 revealed feeble catalysis. In order to magnify any effect, the concentration of pNPA was raised by a factor of 5 at pH's 7.41 and 8.0, and by a factor of 24 at pH 6.2. Comparisons of the data obtained at these higher concentrations of pNPA required correction by dividing the rates by an appropriate factor so that all rates correspond to a concentration of 0.2 mg/ml of pNPA. This correction was deemed reasonable because the rates of the reactions at the two different pNPA concentrations at pH 8.0 showed that rates were essentially proportional to the pNPA concentration (see Table 3).

FIGURE 6

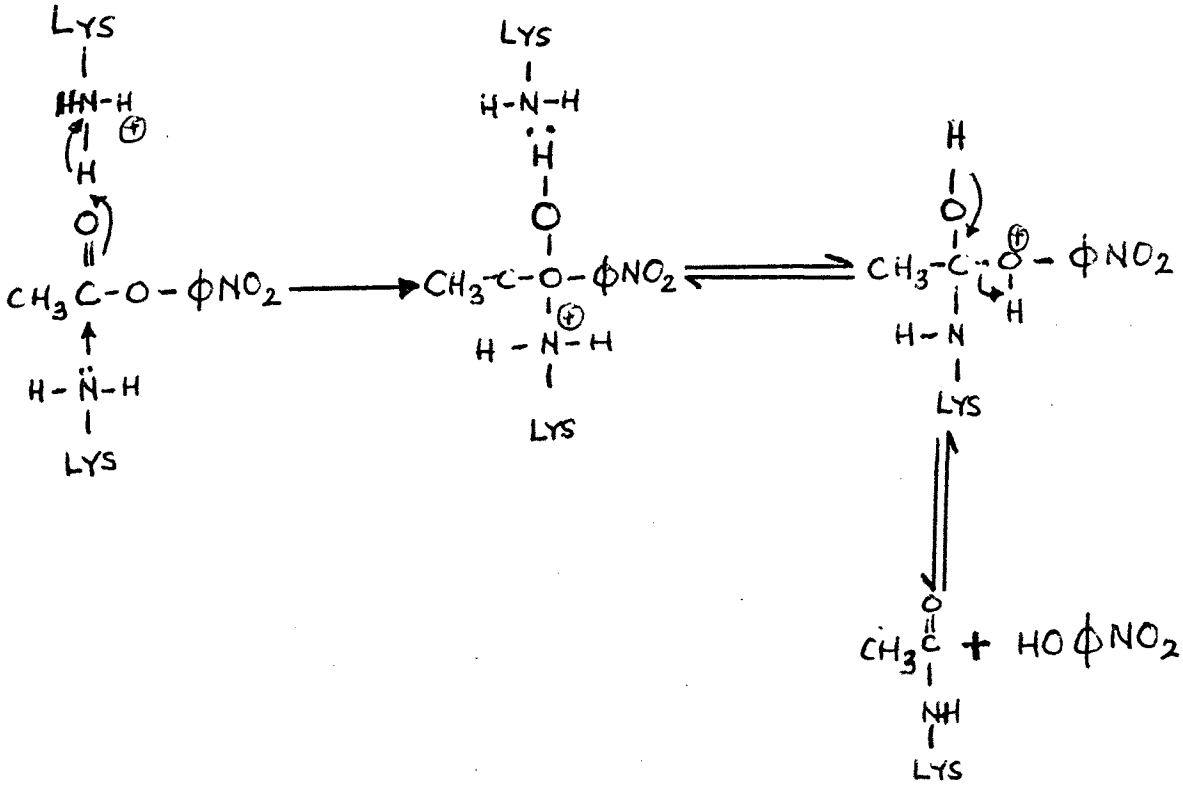
Catalytic Effect as a Function of pH



These results are incorporated into figure 6. The observation that the rate of reaction of pNPA with lysine exhibits a maximum near pH 9.7 correlates with the known isoelectric pH of lysine, 9.74.[17] The predominant form of lysine at this pH is the neutral species.



It should be noted that in this form both positive and neutral amino groups are present in equal quantities. A reasonable mechanism for the reaction of pNPA with lysine is as follows:

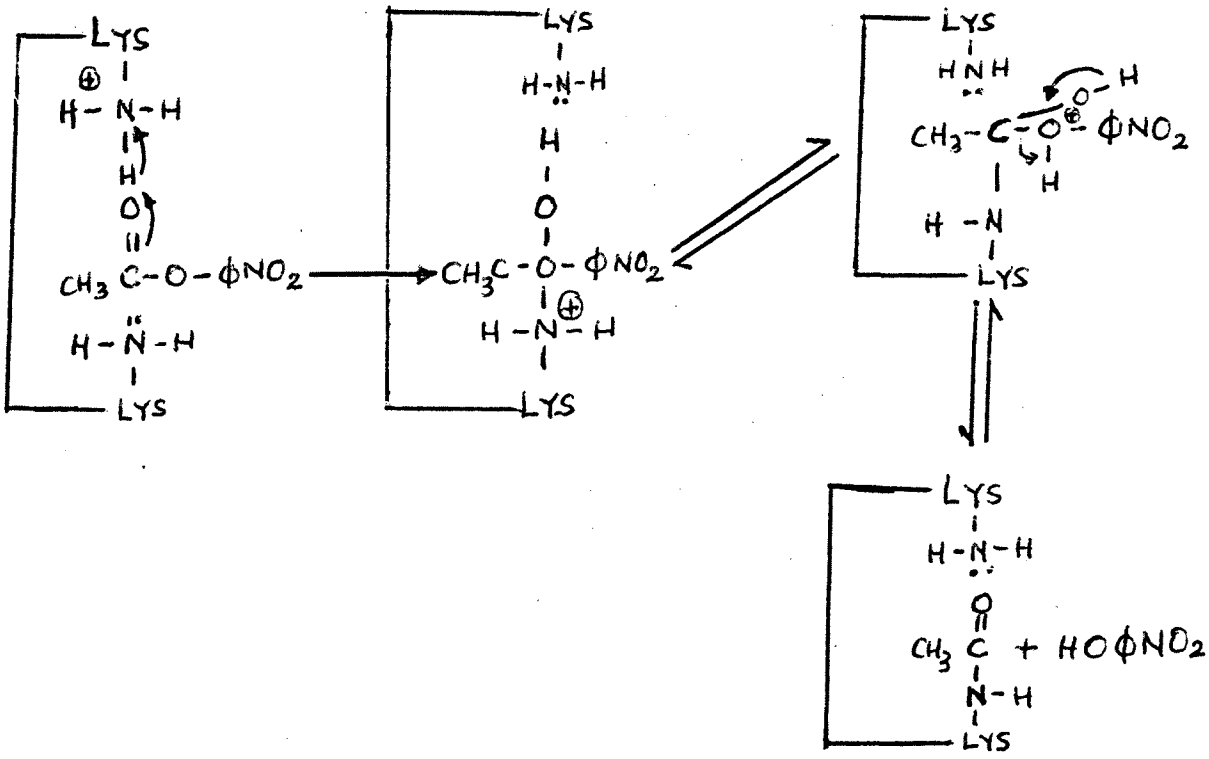


In this mechanism the charged amino group protonates the pNPA, making it more susceptibel to nucleo-philic attack by the neutral amino group of a second lysine molecule. It would therefore be expected that the rate of reaction would be greatest at a pH where both neutral and positive amino groups of lysine exist in significant numbers, that is at the isoelectric point.

It has been observed that at pH's below 8.0 polylysine exists predominantly in a random coil and its side chain amino groups are fully positively charged. The polylysine becomes half-charged near pH 11.2. However, at this pH the molecule exists as a tightly wound helix. As a tightly wound helix, polylysine would not readily be able to complex with the substrate molecule of pNPA because this would require breaking of the intro-molecular hydrogen bonds which had stabilized this helix. The energy of this hydrogen bond is about 8 kcal/mole, a barrier which might be sufficient to retard reaction. In fact, the reaction was not catalyzed at pH 10. A random coil is flexible enough so that any two lysine residues could engage in reaction. In consideration only of the helical versus random coil configurations, maximum catalytic activity might then be

expected at pH less than 8.0 where the polylysine is essentially completely in a random coil. This would permit all of the lysine residues to engage in complexing with the substrate without the necessity of breaking of hydrogen bonds. The optimum pH for reacting with pNPA might be expected to be at a pH where there is both significant numbers of random coil segments together with significant numbers of uncharged side chain amino groups. A compromise pH between 11.2 (all uncharged amino groups) and 8.0 (all random coil) would be a pH of 9.6*. While more data would better sustain the tentative conclusion that has been drawn concerning the activity of polylysine, it appears significant that the optimum pH for activity of polylysine is near pH 9.6. The cooperative effect of lysine residues which are attached by the polypeptide backbone may account for the enhanced catalytic activity of polylysine versus lysine. In the latter case it would always be necessary to involve two different lysine molecules which would require a higher entropy of activation. A mechanism for reaction of pNPA with polylysine would be almost identical to the one for the reaction with lysine, except that the lysine residues are attached to each other.

*This is the pH at which approximately 50% of the lysine units are charged. See reference 18.



In summary, L-lysine and poly-L-lysine have been shown to react with pNPA at an optimum pH of 9.6. Poly-L-lysine is a more efficient reactant that lysine.

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