

PHYSICO-CHEMICAL STUDIES ON NITROGEN FIXATION
WITH SPECIAL REFERENCE TO THE
THERMODYNAMIC AND KINETIC PROPERTIES OF HYDROGENASE

A THESIS SUBMITTED FOR THE
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TO MY PARENTS,
GEORGE AND MUNIRA

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ABSTRACT

Hydrogenase of Azotobacter was purified to a large extent and in good yield. The enzyme is believed to be heterogeneous.

The purified hydrogenase was titrated at two temperatures and the number of ionizing groups and their respective pK's were determined. The titration of denatured protein shows that it unfolds to a great extent with release of many more titrable groups. Also the isoelectric pH of hydrogenase was determined using a free-boundary electrophoretic cell.

Spectral properties of hydrogenase are reported. The U.V., visible region and E.S.R. spectra were obtained.

A new method of assaying hydrogenase activity was devised and the kinetics and mechanism of hydrogenase-catalyzed reactions is put forward. This clearly shows ^{that the free radical} mechanism is unlikely.

The redox potentials of the enzyme system are reported and a discussion of the use of the data to elucidate the mechanism of nitrogen fixation is included.

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CHAPTER ONE

HISTORICAL INTRODUCTION

A - Introduction to nitrogen fixation

Although nitrogen fixation was discovered very early in the history of the world civilisation, and is as important as photosynthesis (carbon dioxide fixation) not enough work has been done to enable us to elucidate the mechanism of this process.

In this laboratory Azotobacter bacteria, which are capable of fixing nitrogen both in the soil and in vitro, are studied. The bacterium, gram-negative with spherical geometry, was discovered in 1901 (1).

The availability and application of isotopic nitrogen, ^{15}N and ^{13}N , in recent years made progress possible (2, 3). The production of cell-free extracts made the application of physico-chemical techniques possible (4).

Many mechanisms have been postulated, some are more likely than others, but none have been proved yet. However, it is almost certain (5, 6) that ammonia is the key product and not hydroxylamine as it has been suggested. Possible intermediates in the nitrogen cycle as obtained from inhibition studies will be discussed, and the reader is referred to Green's thesis which contains a full discussion of the subject (7).

Nitrous oxide was found to be a reversible competitive inhibitor (8, 9) of nitrogen fixation, and according to findings in this Laboratory, it is not utilized by Azotobacter (10). Compounds isosteric, and isoelectronic to nitrous oxide, such as carbon dioxide, azide,

and cyanate might be expected to have similar effects on nitrogen fixation (20), in fact carbon dioxide has not been fully investigated, and cyanate behaves quite differently. Azide, however, is very similar to nitrous oxide. To explain this the following hypothesis has been suggested; compounds like nitrous oxide combine in one way or the other (either chemically by bond formation or sterically by occupying the active site) with the enzyme system(s) thus inhibiting the reaction. Hyponitrous acid acts as a poison to *Azotobacter* (11), while nitramide is not utilized by it (12).

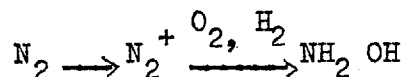
As it was mentioned above, hydroxylamine is not the end-product of nitrogen fixation (13). It was found that hydroxylamine (14) inhibited both ~~nitrogen fixation and bacterial respiration~~ though not to an equal extent. In the light of further evidence, Virtanen (15) discarded hydroxylamine as a major stage in the pathway of fixation. Hydrazine also inhibited nitrogen fixation in *Azotobacter* (16).

The basis of the hypothesis that ammonia is the end key-product of biological nitrogen fixation is its utilization with almost no lag time (2, 17, 18); compounds easily reducible to ammonia are also utilized at the expense of nitrogen fixation (18, 19). However, in the case of cultures trained to utilize ammonia, fixation and ammonia uptake occur simultaneously (21).

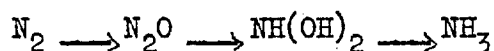
Equally, at high concentration of hydrogen, nitrogen fixation is inhibited (22). To explain this effect both Shug (22) and Winfield (23) proposed different mechanisms in which hydrogenase and 'nitrogenase' interacted.

To sum the above information three mechanisms for nitrogen fixation will be written:

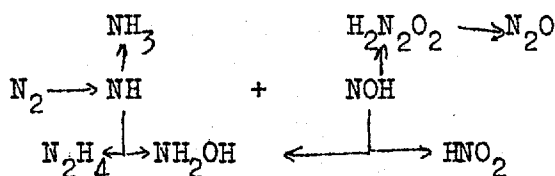
Virtanen (13):



Wilson (2):



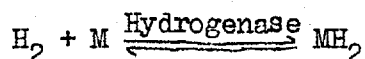
Roberts (24, 25):



Many other mechanisms based on theoretical grounds have been proposed (26 - 30), but their merits will not be discussed here.

B - Introduction to the system hydrogenase

Hydrogenase is the enzyme responsible for hydrogen metabolism in biological systems.



Hydrogenase is found in many systems; some fixing nitrogen, some not fixing nitrogen and even some that do not have a normal hydrogen metabolism. A list of these organisms may be found in Callendar's thesis (31).

It was noticed that the properties of hydrogenases, extracted from two different sources, are not identical (32, 33). Some hydrogenases reduce compounds that can be poisonous to others (34, 35).

Although the hydrogenases of *E. Coli* and *Azotobacter vinelandii* are similar (36, 37), we can not say that hydrogenase is a general reductase.

Hydrogenase has been assayed by different methods:

- 1 - reduction of methylene blue (39),
- 2 - reduction of ferrocyanide (40),
- 3 - evolution of hydrogen (41),
- and 4 - D_2 - exchange reaction (75).

Any one system may be used, but no correlation in activity as measured by the different methods, can be arrived at. Even ^{on} the addition of a cofactor that increases the rate of the reaction with ferrocyanide, no appreciable change in the reactivity towards the deuterium-exchange reaction (37, 38) has been observed. It was suggested that tritium should replace D_2 , but no reports of such work have appeared (42).

Oxygen (36), and carbon monoxide (43, 44) inhibit the catalysis of methylene blue and ferrocyanide reductions. Nitrogen and nitrous-oxide (45) inhibit the evolution of hydrogen from viologen blue. Oxygen inhibition has been explained on the basis of oxidised sulphhydryl groups (51, 52). The other effects have been combined with the following:

- 1 - activation of the enzyme after being inhibited may be achieved by storing the enzyme under H_2 in the cold (46),
- 2 - bacteria grown on nitrate or ammonium nitrogen show low hydrogenase activity (47, 49, 74),

and 3 - that hydrogen inhibits nitrogen fixation (49, 50), and a hypothesis claiming that the enzymes hydrogenase and 'nitrogenase' are identical. A summary of reasons against this theory, as given in Green's thesis follows (7):

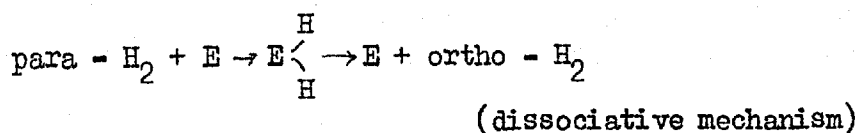
- 1 - Other organisms unable to fix nitrogen contain hydrogenase
- 2 - Vanadium ions inhibit fixation while they have no effect on hydrogenase activity.

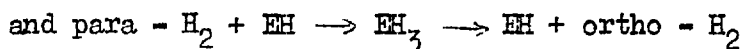
3 - A deficiency of iron causes inhibition of both 'nitrogenase' and hydrogenase, whereas a molybdenum deficiency reduces the first and does not affect the latter.

It has been stated more than once that hydrogenase is a highly-particulate metallo-enzyme (7, 51). Extracts of the enzyme when centrifuged at high g-values give very low activity figures for the supernatant and very high figures for the precipitate. Whether this is true or an artifact of the method of separation, we will try to show in this work.

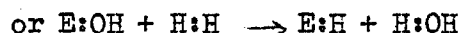
The metal ion involved in the enzyme has been proved to be iron (31, 53 - 58). It is note-worthy that transition metal ions in complexes or in the solid state catalyze hydrogen reductions (60 - 62).

The hydrogenase of *Desulfovibrio desulfuricans* has been purified and studied by Rittenberg and co-workers (57, 59), while that of *Azotobacter* was studied by the Burris-Wilson group (75). Mechanisms explaining the mechanism of hydrogenase have been put forward (63 - 66).



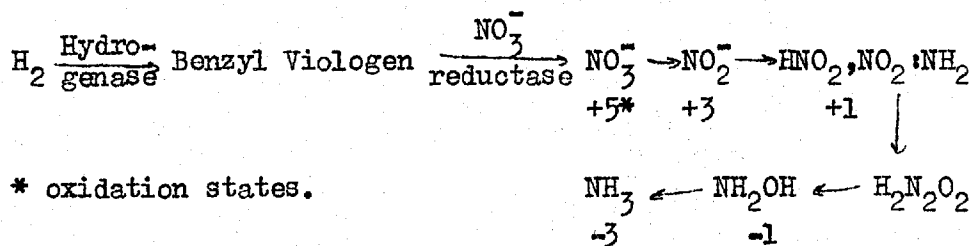


(exchange mechanism)

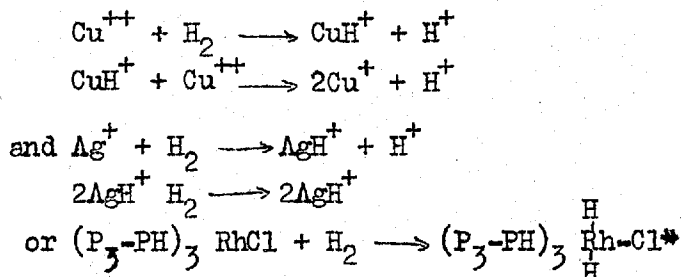


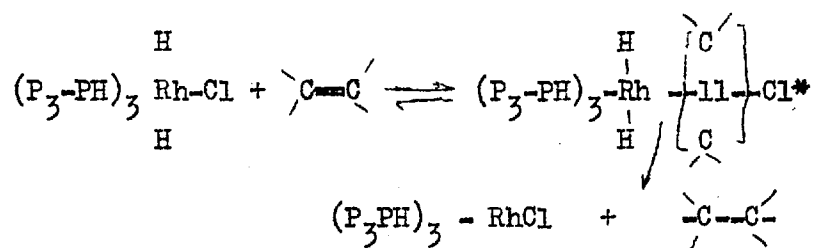
Callendar's work on *Desulfovibrio desulfuricans* showed that its hydrogenase is capable of reducing highly ring-substituted azo compounds (31). Stewart's work on the hydrogenase of *Azotobacter vinelandii* shows that the enzyme can reduce some derivatives of hydrazine (76).

Work on other reductases have been done, specially on nitrate, nitrite, and hydroxylamine reductases, and a mechanism for the enzyme action has been postulated (67 - 69).



Work on synthetic materials catalyzing the reduction with hydrogen may throw light on the possible mechanism of hydrogenase (70 - 73).





$\text{P}_3\text{-PH}$ = triphenyl phosphine

* intermediates have been isolated and N.M.R. spectra obtained.

CHAPTER TWO

Extraction and Purification of hydrogenase

A - Introduction

It is agreed that no significant and precise physico-chemical studies can be made on a protein unless it is highly pure. Hydrogenases did not receive all the attention they deserve; only one, that of *Desulfovibrio desulfuricans*, has been purified to a certain extent (57). Callendar purified the hydrogenase of *Azobacter vinelandii* but the preparation was not very satisfactory.(31).

Hydrogenase has been described as a high molecular weight protein that tends to form aggregates easily sedimented. Many workers realized that this could be due to their methods of extraction and often tried to change them.

Most of the published work on hydrogenase purification methods shows that chemically harsh methods such as: heating, addition of acetone, butanol, acid, and several stages of salting-out etc., have been used. Consequently, and although, they obtained highly purified hydrogenase, their yields were infinitesimal. It is the author's belief that these methods should be replaced by more sophisticated and gentle methods.

In this work, molecular sieve chromatography coupled with physical methods have been used to purify the hydrogenase of *Azobacter vinelandii*.

B - Techniques

i - Bacteriological examination

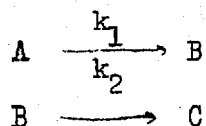
Bacteria prepared by Stewart in 1962 have been used. Before extraction they were examined under a microscope, and it was found that they were not contaminated. The same technique as that of Stewart (76) has been used.

ii - Ultrasonic disruption

The bacteria were disrupted by irradiation, for 20 minutes, by Ultrasonic waves (20 Kc/s). After centrifugation (Spinco Ultracentrifuge Model L) at 30,000 xg for one hour at 0°C, the solution was examined again under the microscope, the remaining cells have been removed by filtration through a glass-filter No. 5 or Oxoid filters. Both of these methods have been found to be satisfactory.

In an attempt to understand the reason why the activity of a disruptate falls down after a continuous increase of activity (76), the following hypothesis is put forward. These waves break the cell walls of bacteria, the content is then dissolved in the medium. It was suggested that ultrasonic waves might have a denaturing effect on protein (77) so it might be possible to explain the above mentioned observed effect as follows:

Let A, B, and C, represent cells, protein, and denatured proteins, then the process can be written as



the rate of production of B should be

$$(B) = A e^{k_1 t}$$

if no denaturing effect exists.

By plotting the results of Stewart we obtain a curve fitting the first-order kinetic law. Fig. II.1.

To study the second part of the process, a solution of hydrogenase ^{purified} as described later, was subjected to ultrasonic waves and a plot of these results showed that the process is a zero order reaction as we might have expected; Table II.1., Fig. II.2.

iii - Nitrogen content

The nitrogen contents of solutions of hydrogenase have been measured by the micro-kjeldhal method. The results were within $\pm 0.5\%$ error.

iv - Iron Content

The method of Cameron (78) for the assay of Fe in biological compounds, has been used in this work.

v - Enzyme Assay

Hydrogenase was assayed by measuring the uptake of hydrogen in a Warburg flask. The reduced species used was Methylene blue (39, 7).

vi - Buffers

Phosphate buffer solutions have been prepared according to the buffer recipe of Gomori (79).

C - Theory of Chromatography.

Molecular sieve chromatography is a newly developed tool. Many a theory has been proposed to explain the operational mechanism by which separation of proteins of different molecular weights is achieved. Some of these fit the experimental data and two will be discussed here.

TABLE II.1.Effect of Ultrasonic waves on Cell disruption (Stewart)

t (minutes)	0	5	10	15
Biuret protein mg/ml	476	2.88	3.22	3.73
Q_{H_2} $\mu\text{l/mg/hr}$	105	108	115	172
% Q_{H_2}	100	104	110	164

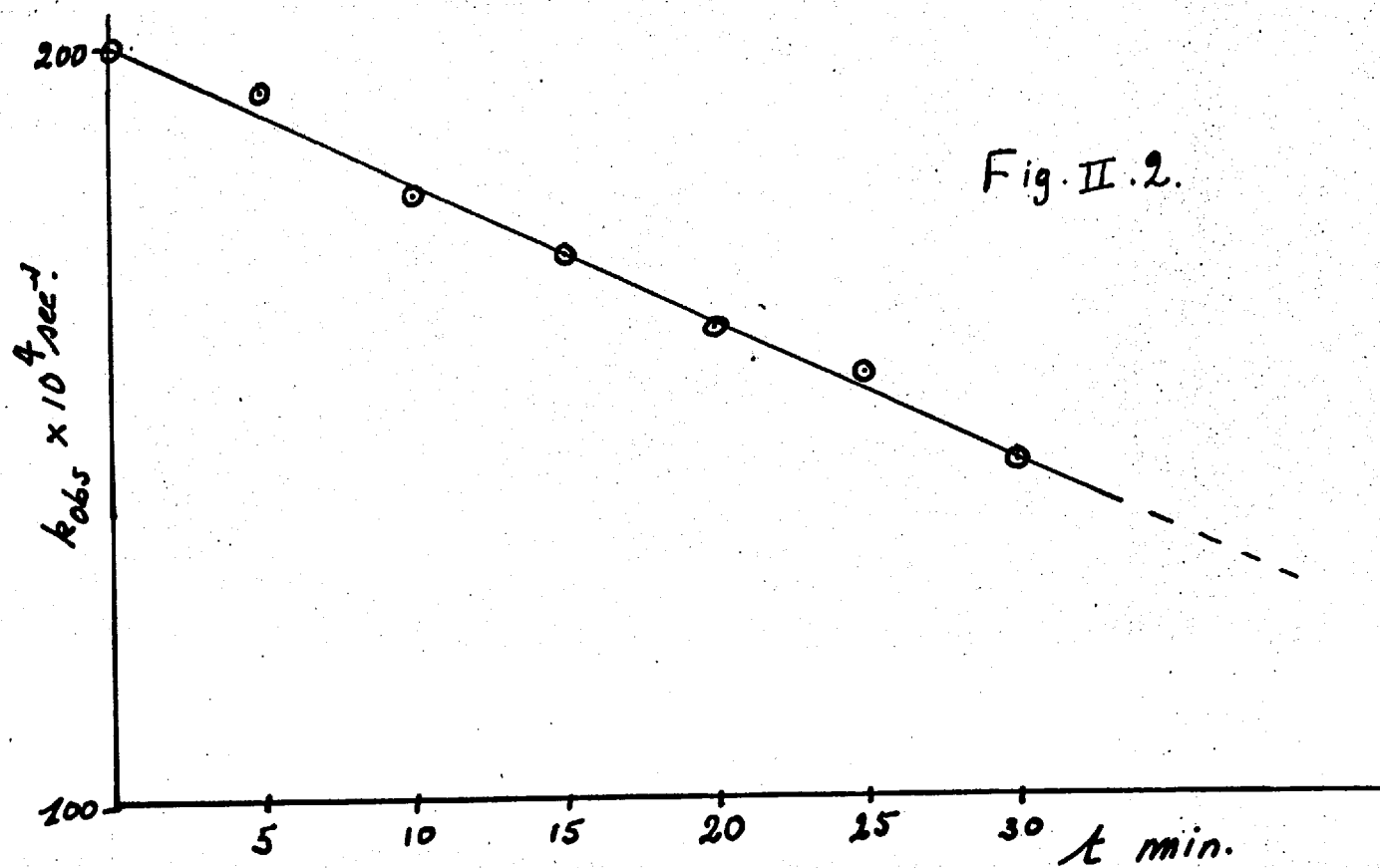
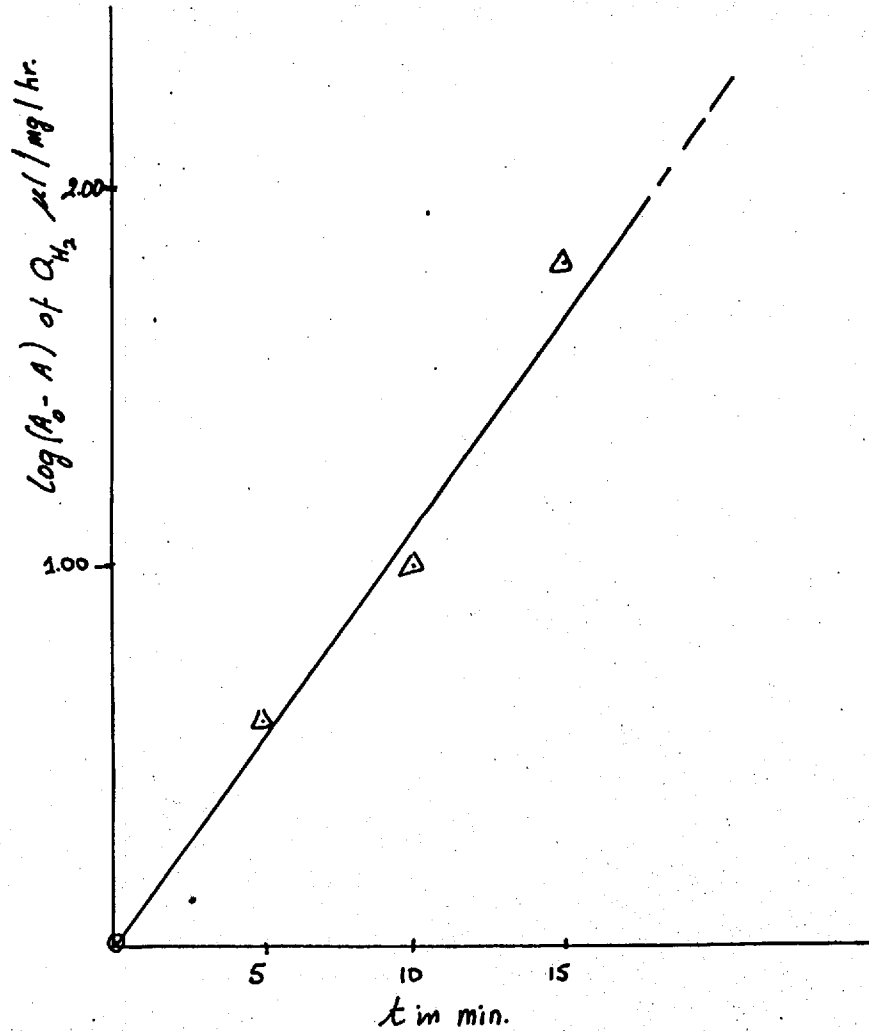
TABLE II.2.Effect of Ultrasonic waves on a purified hydrogenase solution

t (minutes)	$k_{\text{obs}} \times 10^4 \text{sec}^{-1}$	% Activity
0	200	100
5	194	97
10	180	90
15	172	86
20	162	81
25	156	78
30	144	72

Conditions: Hydrogenase = $30 \times 10^{-6} \text{ gm N}_2 / \text{ml}$

$[\text{NO}_2^-]$ = $5 \times 10^{-4} \text{ M}$

and PH ~ 6 .



i - Flodin Theory (80)

A new development in the field of Molecular sieves followed the preparation of dextran gels by Flodin. These gels allow molecules of certain size, thus molecular weight, to penetrate through their microscopic structure. The molecules in question distribute themselves between the solid phase (beads of gels) and the mobile phase (eluant). Let this distribution coefficient be K_D . A molecule that has a very large molecular weight (such as T.M.V., or Blue Dextran, etc.,) will be completely excluded and the volume of the gel then will be equal to their elution volume. This volume, usually called the void volume, will be denoted by V_0 and can be measured from the elution volumes of anyone of the above mentioned macromolecules. Equally, very small molecules (such as KCl, Li Cl, or even better D_2O) will be completely free to diffuse through the gel. This will give us the internal volume of the gel, V_i .

The elution volume of any macromolecule should be given by the following expression:

$$V_i = V_0 + K_D V_i$$

$$\text{or } K_D = \frac{V_e - V_0}{V_i}$$

We notice that K_D is independant of the flow rate and the physical dimensions of the column.

Several empirical relations (81, 82) have been published correlating the molecular weight to K_D or $\frac{V_e}{V_i}$

ii - Ackers' Theory (83)

On the other hand Ackers assumes that molecular sieving is merely a restricted diffusion process, in which the macromolecule has to cross a barrier. This reduces the flow rate and a preferential flow of low or high molecular weight macromolecule is achieved.

For a system, where the gel has been added to the macromolecule, K_D is defined by:

$$K_D = V_P/V_i$$

where V_P = non-excluded volume,

and V_i = internal volume.

For a system at equilibrium with a total volume V_t and a liquid phase volume V_l , the penetrable volume V_P is given by

$$V_P = \frac{Q_P}{C_P} = \frac{Q_t - Q_l}{C_P} = \frac{Q_t - C_o V_P}{C_P}$$

where Q_P is the amount of macromolecules in the gel,

Q_t is the total amount of macromolecules in the system,

Q_l is the amount of macromolecules in the liquid phase,

C_P is the concentration of macromolecules in the gel,

C_o is the concentration of macromolecules in the liquid phase,

and so

$$K_D = \frac{Q_t - C_o V_l}{C_P V_P}$$

For a thermodynamically reversible process (not applicable for a column),

$$C_P = C_o$$

$$\text{and } K_D = \frac{Q_t/C_o - V_l}{V_i}$$

For a column operating entirely by the molecular exclusion principle and under non-equilibrium conditions, K_D should be identical to that of Flodin and the flow-equation should be:

$$V_e - V_o = \frac{Q_t}{C_o} - V_l$$

K_D obtained from equilibrium and non-equilibrium processes on G-75 are identical; on G-100, slightly different; and on G-200 completely unreconcilable.

This led to the postulation of a restricted diffusion process, where K_D is interpreted as a parameter depending on the size and diffusion coefficient of the macromolecule. This is summarized as follows:

1 - For a molecule of radius a , which is smaller than the pore radius r , penetration is only possible if its centre passes by the pore at $r-a$. For a small particle this is possible, but the larger the molecule the lower is the probability of penetration due to steric hindrance.

2 - A molecule that enters a pore will encounter a higher hydrodynamic pressure than its surroundings, and its diffusion coefficient is decreased due to the increased frictional coefficient. This effect is generally more marked in large molecules than in small ones.

3 - Renkin equation (84) which relates the Stokes radius a , of a macromolecule diffusing within a restrictive barrier of effective pore radius r , to the equivalent free cross-sectional pore area A_0 , and effective actual area A_r available for diffusion, is equated to K_D .

$$\frac{A_r}{A_0} = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104 \frac{a}{r} + 2.09 \left(\frac{a}{r}\right)^3 - 0.95 \left(\frac{a}{r}\right)^5 \right]$$

The Stokes radius of a molecule can then be calculated from the value of K_D .

Once the Stokes radius is estimated, the molecular weight can be determined by the well known hydrodynamic relation:

$$M = \frac{4\pi N a^3}{3\bar{v}}$$

where $\bar{v}$ is the partial specific volume:

$$\bar{v} = \left(\frac{\partial \Delta V}{\partial M} \right)_T$$

and is usually 0.73 for proteins.

Assuming that the viscosity coefficient is close to unity at infinite dilution, the frictional and diffusion coefficients will be:

$$f_0 = 6\pi\eta_0 a$$

$$\text{and } D = \frac{kT}{6\pi\eta_0 a}$$

D - Extraction of hydrogenase and Results

Proteins and nucleic acids combine to form complexes of high molecular weight. This combination is due to:

- 1 - the hydrophobic character of proteins and nucleic acids,
- and 2 - the interactions between the large number of partial charges on both macromolecules.

The hydrogenase of *Azotobacter vinelandii* forms such a complex with nucleic acids and many workers reported that hydrogenase is highly particulate (7, 51). Protamine sulfate was used to remove the nucleic acids, but this removed all the proteins that were attached to them. Different media of extraction have been used without great success (43, 56, 57)

It was thought that guanidine hydrochloride should be an ideal compound to break these complexes, since it is known to be a strong H-bond competitor (86). After disruption of *Azotobacter* bacteria, enough guanidine hydrochloride was added to the solution and left in the cold (4°C) for 24 hours. The concentration of guanidine hydrochloride was 2M . The solution was then centrifuged and dialyzed against a buffer. At the end of a 4-hour dialysis, the buffer solution turned yellowish. The buffer solution was analyzed for sulphur and iron and they were detected. The dialysate showed no hydrogenase activity. We conclude that guanidine hydrochloride is a strong denaturant.

Potassium chloride was chosen then for two reasons:

- 1 - It increases the ionic strength without salting-out the proteins.
- 2 - It broadens the double-layer and thus makes any combination of protein-nucleic acid impossible.

Azotobacter vinelandii bacteria were dissolved in a buffer saturated with KCl, and disrupted. The solution was allowed to equilibrate at 4°C for 24 hours, then centrifuged and dialyzed. An increase in activity was observed and all further purifications were treated similarly: Table II.

The extracted solutions were put on a Sephadex G-25 and G-50 chromatography columns.

Chromatography on G-25* The chromatography eluent showed 4 peaks,

* The author would like to mention the help and assistance of Miss L. Kotite in the early chromatography experiments.

three of which showed absorption at a wave-length of 410 mμ. $K_D = 0.01$, $K_D = 0.73$, $K_D = 0.61$, $K_D = 0.55$. However, at a K_D value greater than unity, a green fluorescing pigment appeared (see Harris (85)).

It was then decided that a 50% saturated ammonium sulfate for 1 hour should be followed by the addition of solid sephadex G-25. The 50% saturated ammonium sulfate extraction is used to decrease the high molecular weight proteins and nucleic acids ($K_D < 0.10$). The addition of solid G-25 has a two-fold advantage.

1 - Concentration of the protein (84),

and 2 - removal of the yellow-green pigment.

Chromatography on G-50. The extract was then passed through a G-50 column. The element showed two peaks, one in the molecular weight region of 1×10^6 and the other at about 7×10^4 . It was decided to eluate the extract through a G-75 column.

Chromatography on G-75. The eluant showed three peaks at

$$K_D = 0.037$$

$$K_D = 0.114$$

$$K_D = 0.310$$

Fig. II, I, 2

Using a calibration curve, (courtesy of Mr. B. A. Allsopp) the molecular weights of these three proteins were estimated.

$$M.W.(1) = 1.1 \times 10^5$$

$$M.W.(2) = 7.9 \times 10^4$$

$$M.W.(3) = 2.6 \times 10^4$$

These fractions were found equally active in terms of hydrogen uptake per mg. of nitrogen (see table) and it was noticed that their molecular weights are in the ration of 5:3:1.

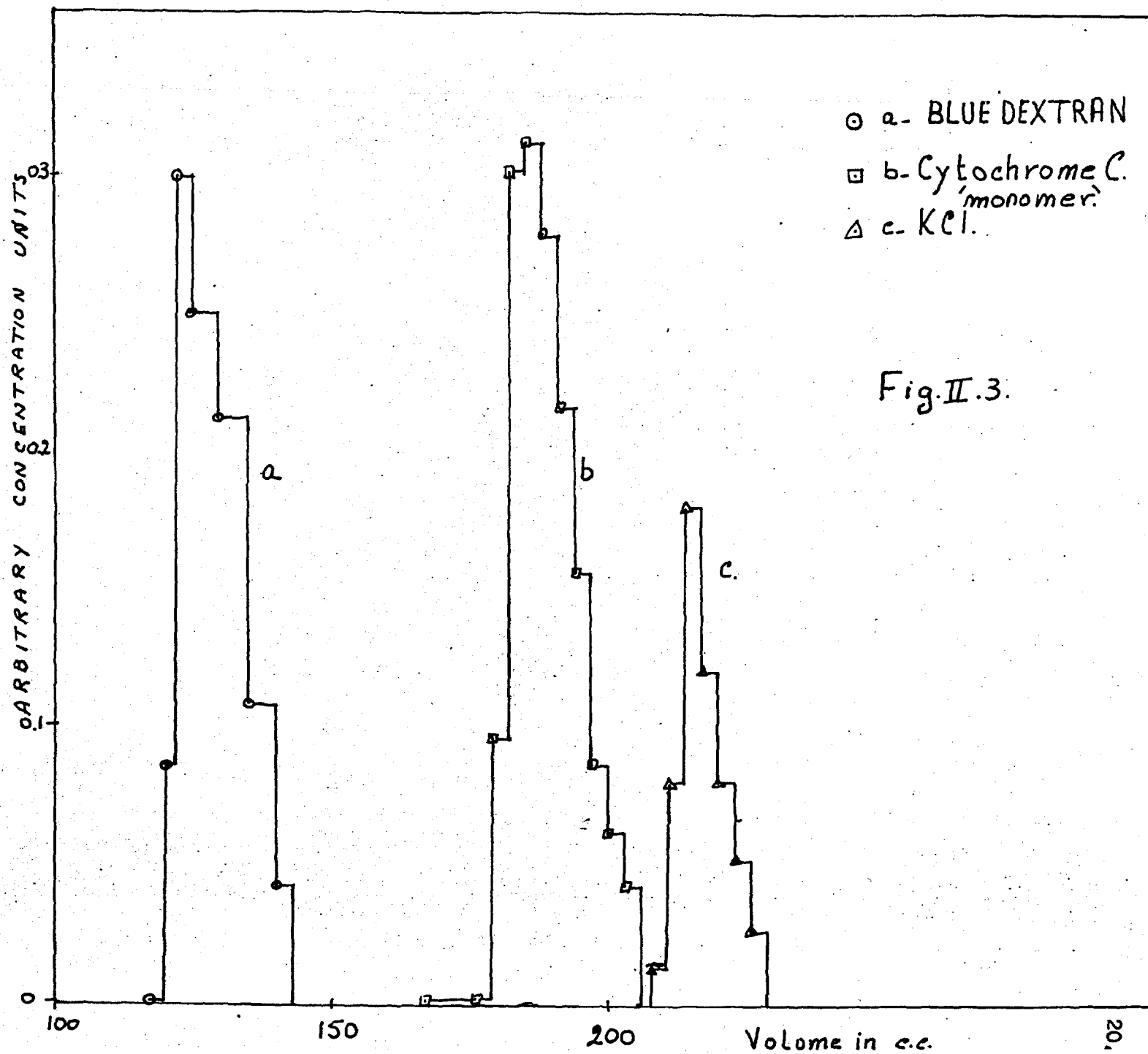
Their radii, frictional coefficients, diffusion coefficients are listed in table II.3.

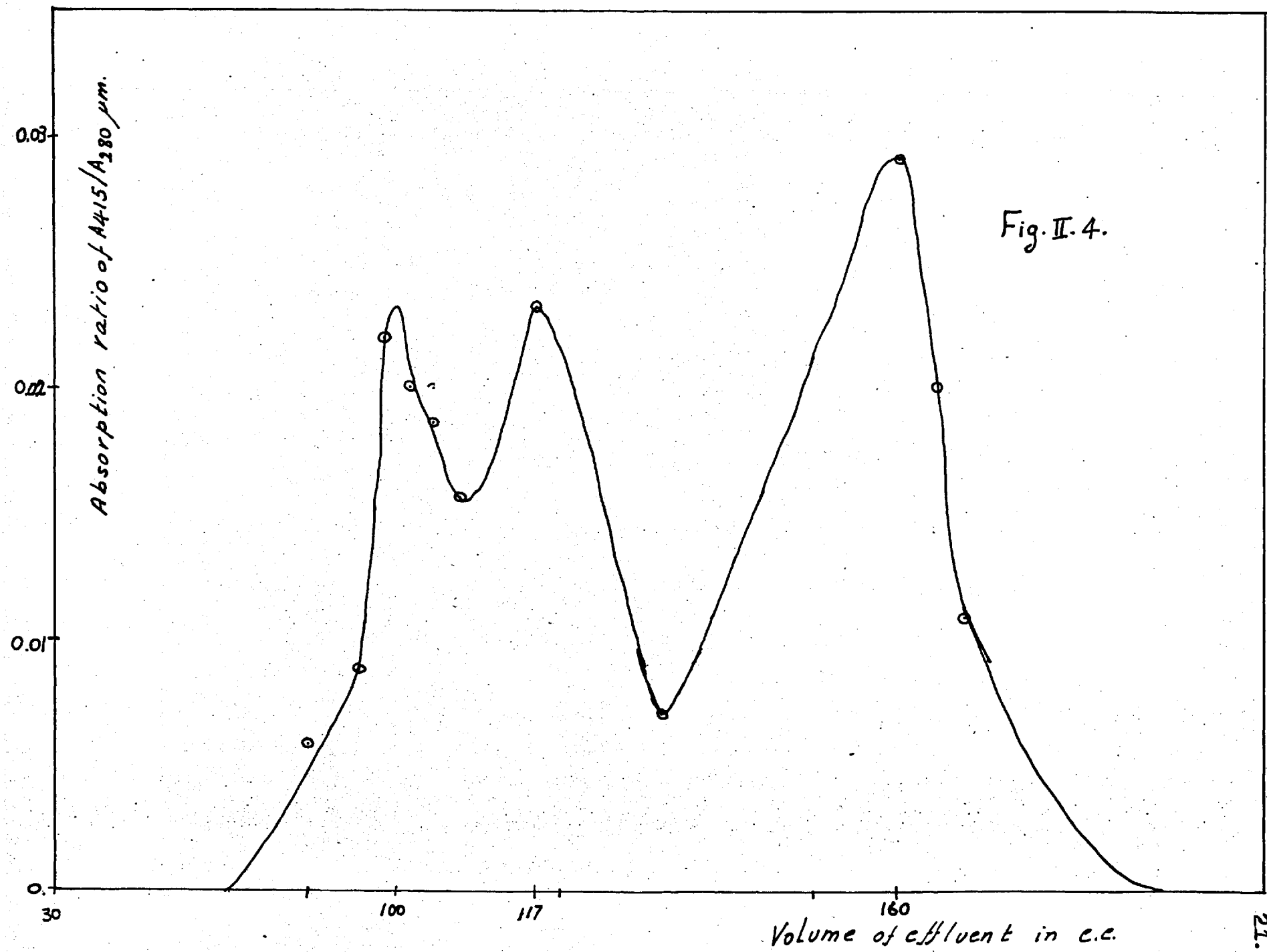
TABLE II. 2.Activities of hydrogenase solutions on purification

	$A \times 10^3$ μlH ₂ /ml	mgN ₂ /ml	$Q_{H_2} \times 10^3$	Yield%	Purifi- cation
Cells	7.65	3.74	2.04		
Disruptate	13.0	3.77	3.44		
Supernatant (Centrifugate)	4.34	2.33	1.89	100	1
Supernatant + KCl	11.0	2.00	5.55		
Ammonium sulfate 50%	10.0	0.50	20.0		
G-25	9.83	0.13	75.6		
G-75	9.70	0.03	324.	37.2	170
G-75 component	1	2	3		
$A/\mu l \times 10^4$	1.0	5.0	9.0		
mgN ₂ /ml	0.31	1.49	2.90		
$Q_{H_2} \times 10^5$	3.22	3.36	3.10		

TABLE II. 3.Hydrodynamic constants of hydrogenase

Component	1	2	3
K_D	0.310	0.110	0.037
Molecular Weight (Flodin)	2.6×10^4	7.9×10^4	1.1×10^5
Molecular Weight (Ackers)	6.5×10^4	2.9×10^5	7.1×10^5
Stokes radius ($\text{\AA}^\circ$)	26.6	43.9	58.8
$f_0 \times 10^6$	5.0	8.3	11
$D \times 10^9$	7.9	4.8	3.6





CHAPTER THREE

Thermodynamic properties of hydrogenase

A - Hydrogen equilibria in Hydrogenase

a- Introduction

Amino acids polymerize in a certain fixed pattern to form proteins. A protein molecule in solution is a highly complex but ordered structure. Since many amino acids, even in the combined state, have one or more freely ionizing groups, these protrude into the medium and equilibrate with the available hydrogen ions.

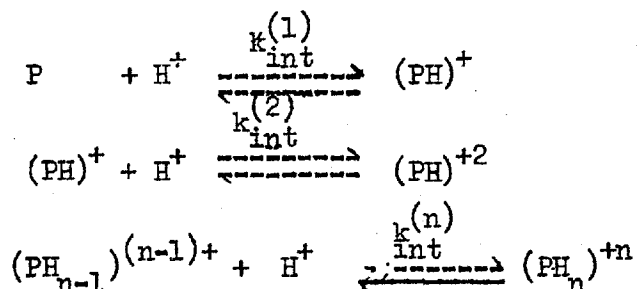
The ionization constant of any uncombined group of an amino acid, will differ from the pK of the same group when the amino acid is in a protein. This effect is explained on the basis of the electrostatic theory in macromolecules of Kirkwood (89). Many peculiar aspects of proteins and some of the mysteries of enzyme chemistry have been solved using models based on this theory, in particular haemoglobin reactions and the Bohr and haeme effects (90,91).

The importance of hydrogen ion equilibria is two-fold. First, a knowledge of the number of freely ionizing groups in a protein and their respective dissociation constants can be obtained. Secondly, a knowledge, although small, of the structure of the protein in aqueous solutions can be arrived at. The amino acid groups in a protein are usually hydrogen-bonded to other groups thus forming the helix main body (tertiary structure) which in turn is responsible for the quaternary structure (97). If hydrogen ions are added to such a solution, these compete with the hydrogen-bonds and break them; the helix unfolds bringing about changes in the tertiary and quaternary structure of the protein(92). This is usually described as a 'reversible denaturation' but should be termed more correctly as 'reversible changes in the configuration'

Although solid protein structure has been extensively studied using X-rays (93,94), no detailed intensive studies have been made on protein in solutions. It is the author's belief that the latter is much more important since all reactions of biological significance involving proteins and enzymes occur only in the aqueous phase.

b- Theory of ion association (95,96).

Titration curves and more generally association-dissociation curves may be treated as follows:



P, representing the protein; H^+ , hydrogen ions, and n is the number of combining sites and $k_{\text{int}}^{(1)}$ being:

$$k_{\text{int}}^{(1)} = \frac{[PH]}{[P][H]} \quad (1)$$

$$k_{\text{int}}^{(2)} = \frac{[PH_2]}{[PH][H]} \quad (2)$$

$$k_{\text{int}}^{(n)} = \frac{[PH_n]}{[PH_{n-1}][H]} \quad (3)$$

The subscript 'int' refers to intrinsic ionization constants. The constants ' k_{int} ' are not thermodynamic association constants, since effects of solvents molecules, ionic strength, etc., have not been included.

The concentration of the intermediate species, P, PH, PH_2 , ..., PH_n , are not experimentally determinable. However, when any amount of $[H^+]$ is added or taken from the solution, we can calculate what percentage of it reacted with the protein. Let α be this percentage, then α can be expressed as:

$$\alpha = \frac{\text{Mole combined of } [H^+]}{\text{Total protein}} \quad (4)$$

$$\alpha = \frac{[H^+]_T - [H^+]_F}{[P]} \quad (4')$$

where $[H^+]_T$ represents the total concentration of hydrogen ions and $[H^+]_F$ is the concentration of the free hydrogen ions in solution.

Let us for simplicity's sake represent $k_{int}^{(i)}$ by k_i and so on, then using equations (1), (2), and (3) we can express α in terms of the n association constants as follows:

$$[PH] = k_1 [H] [P] \quad (5)$$

$$[PH_2] = k_1 k_2 [H]^2 [P] \quad (6)$$

$$[PH_n] = k_1 k_2 k_3 \dots k_n [H]^n [P] \quad (7)$$

and α can be expressed by:

$$\alpha = \frac{k_1 [H] + 2k_1 k_2 [H]^2 + \dots + nk_1 k_2 \dots k_n [H]^n}{1 + k_1 [H] + k_1 k_2 [H]^2 + \dots + k_1 k_2 \dots k_n [H]^n} \quad (8)$$

and for an identical, and non-interacting groups system equation

$$(8), \text{ may be rewritten as } \alpha = \frac{\sum_{i=1}^n i \left\{ \prod_{j=1}^i \left(\frac{n-j+1}{j} \right) \right\} k^i [H]^i}{1 + \sum_{i=1}^n \left\{ \prod_{j=1}^i \left(\frac{n-j+1}{j} \right) \right\} k^i [H]^i} \quad (9)$$

The product appearing in equation (9) becomes:

$$\prod_{j=1}^i \left(\frac{n-j+1}{j} \right) = \frac{n!}{(n-i)! i!} \quad (10)$$

Using the binomial theory

$$(1 + k[H])^n = 1 + \sum_{i=1}^n \frac{n!}{(n-i)! i!} (k[H])^i \quad (12)$$

differentiating equation (12) with respect to $(k[H])$

$$\begin{aligned} (k[H]) \frac{d(1 + k[H])^n}{d(k[H])} &= k[H] n (1 + k[H])^{n-1} \quad (13) \\ &= \sum_{i=1}^n i \frac{n!}{(n-i)! i!} (k[H])^i \end{aligned}$$

Equations (12), and (13) are seen to be identical to the denominator and numerator of equation (11) and the latter becomes:

$$\text{Equation (9) becomes: } \alpha = \frac{\sum_{i=1}^n i \frac{n!}{(n-i)! i!} (k[H])^i}{1 + \sum_{i=1}^n \frac{n!}{(n-i)! i!} (k[H])^i} \quad (11)$$

$$\nu = \frac{k[H]n(1+k[H])^{n-1}}{(1+k[H])^n} = \frac{nk[H]}{1+k[H]} \quad (14)$$

rearranging equation (14) we obtain:

$$\frac{\nu}{n-\nu} = k[H] \quad (15)$$

$$\log \frac{\nu}{n-\nu} = \log k[H] \quad (16)$$

$$\text{and } \log \frac{n-\nu}{\nu} = pK + pH \quad (17)$$

Thus a plot of $(n-\nu)/\nu$ versus pH should yield a straight line, the intercept of which is pK.

The above treatment assumes that these reacting sites or groups are all identical and non-interacting. But this as it might be realised is a highly hypothetical situation.

However, most proteins have five different types of ionizing groups (97),

- 1- Carboxyl groups,
- 2- Imidazole groups,
- 3- Sulphydryl groups,
- 4- Ammonium groups,

and 5- Phenolic groups.

Fortunately the ionization of these groups are quite separated from each other that they can be treated separately; but no justification can exist for treating these ionizations as non-interacting.

If we assume that the interaction is of purely electrostatic nature, and this is a fairly close approximation, we can write the Gibbs free energy of the system as follows:

$$\Delta F = \Delta F_{\text{int}} + RT \phi(Z) \quad (18)$$

$$\text{or } k = k_{\text{int}} e^{-\phi(Z)} \quad (19)$$

and equation (15) should be written as

$$\frac{\gamma}{n-\gamma} = K_{\text{int}} e^{-\phi(Z)} [H] \quad (20)$$

which in the logarithmic form is :

$$\log \frac{n-\gamma}{\gamma} = \text{pH} + \text{p}K_{\text{int}} - 0.434 \phi(Z). \quad (21)$$

It is a good approximation to assume that $\phi(Z)$ equals the electrostatic free energy W_{el} , which can be calculated in the following manner. Suppose that d ions of charge z_i are added to the protein which has Z charges on it, then

$$\phi(Z) = W_{\text{el}} = 2 \Lambda N z_i \frac{Z}{RT} = 2 w z_i Z \quad (22)$$

$$\text{where } w = \frac{\Lambda N}{RT} \quad (23)$$

Λ being a constant, and N, R , and T have their usual meanings.

A more detailed study (98) expresses w as :

$$w = \frac{\epsilon^2}{DRkT} \left(1 - \frac{R}{1 + \kappa d} \right) \quad (24)$$

and the more extended treatment following the lines of Hermans and Overbeek (99) as:

$$w = \frac{\epsilon^2}{5DR} (1 + 0.6 R \kappa_e + 0.4 \kappa_e^2 R_e^2)^{-1} \quad (25)$$

D, ϵ , and κ , have their usual meanings; R , is the radius as determined from the molecular weight and density, and R_e is the effective radius.

Values of w can then be obtained and used in conjunction with equation (21).

B- Technique, results and analysis.

A solution containing the enzyme was titrated by successive additions of 10 μ l. of acid (HCl) or base (NaOH). The concentration of the titrant was chosen such that the change of volume at the end of each titration was not greater than 5%. pH readings were taken after each addition of titrant, once a constant pH-

value was observed. Temperature control was achieved by placing the vessel containing the enzyme in a thermostated bath.

The results of the titrations were plotted by a computer, Fig. 111.1-5. The computer programme is included. Fig. 111.1, and 4 represent the acid (HCl) titrations at 23°C and 0°C respectively. Fig. 111.3, and 5, represent the base (NaOH) titrations at 23°C and 0°C respectively. Fig. 111.2, represents the titration of the denatured protein at 23°C.

In order to analyse our results equation (15) is inverted and rearranged to yield:

$$\frac{1}{v} = \frac{1 + k[H]}{kn[H]} = 1/n + \frac{1}{kn[H]}$$

A plot of $1/v$ versus $1/H$ should yield a straight line the intercept of which on the $1/v$ axis is $1/n$, and the slope of which is $1/kn$.

However, Scatchard (108) believes that such plots do not show the non-linearity of the process; he suggests the use of:

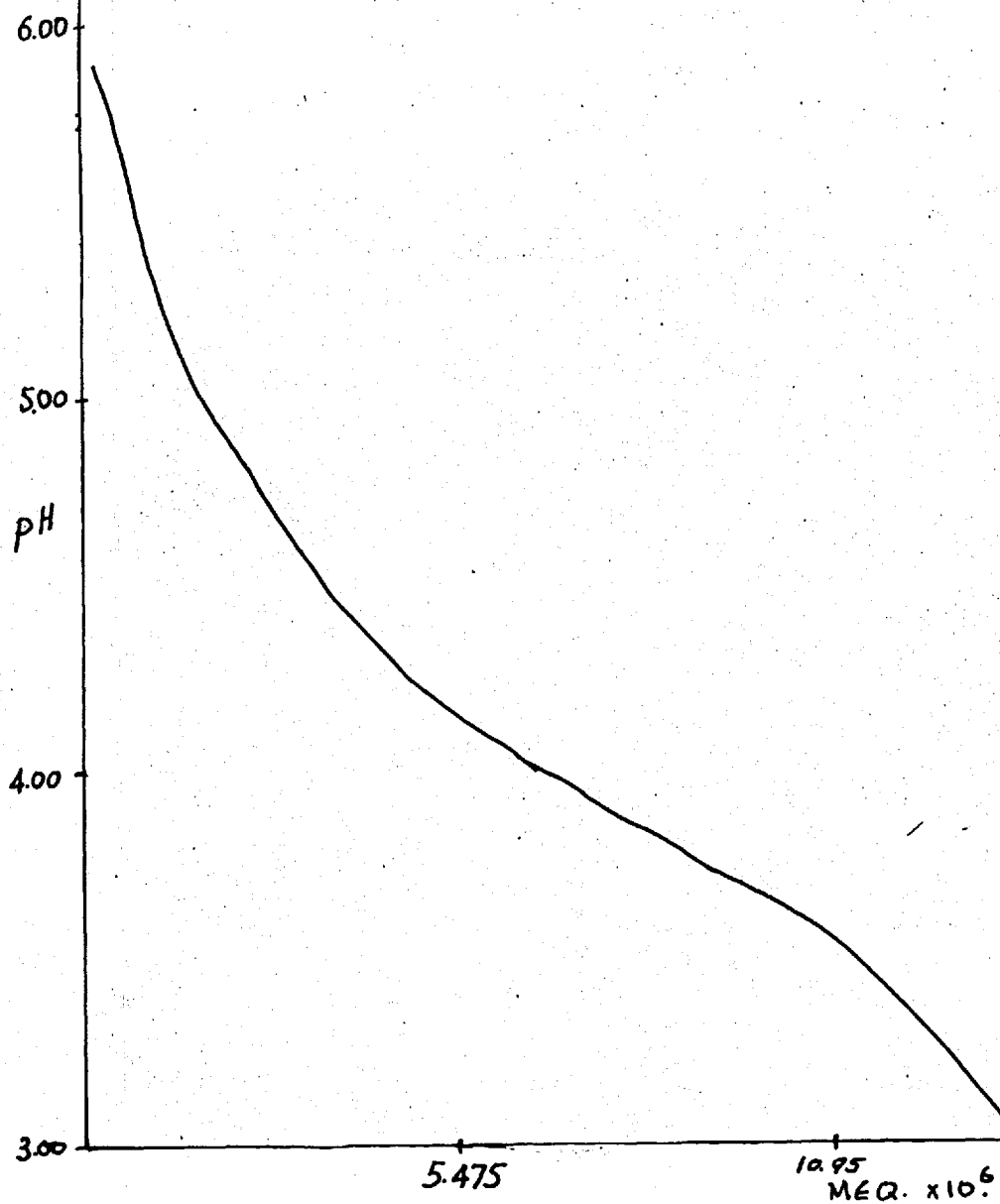
$$\frac{v}{[H]} = k(n - v)$$

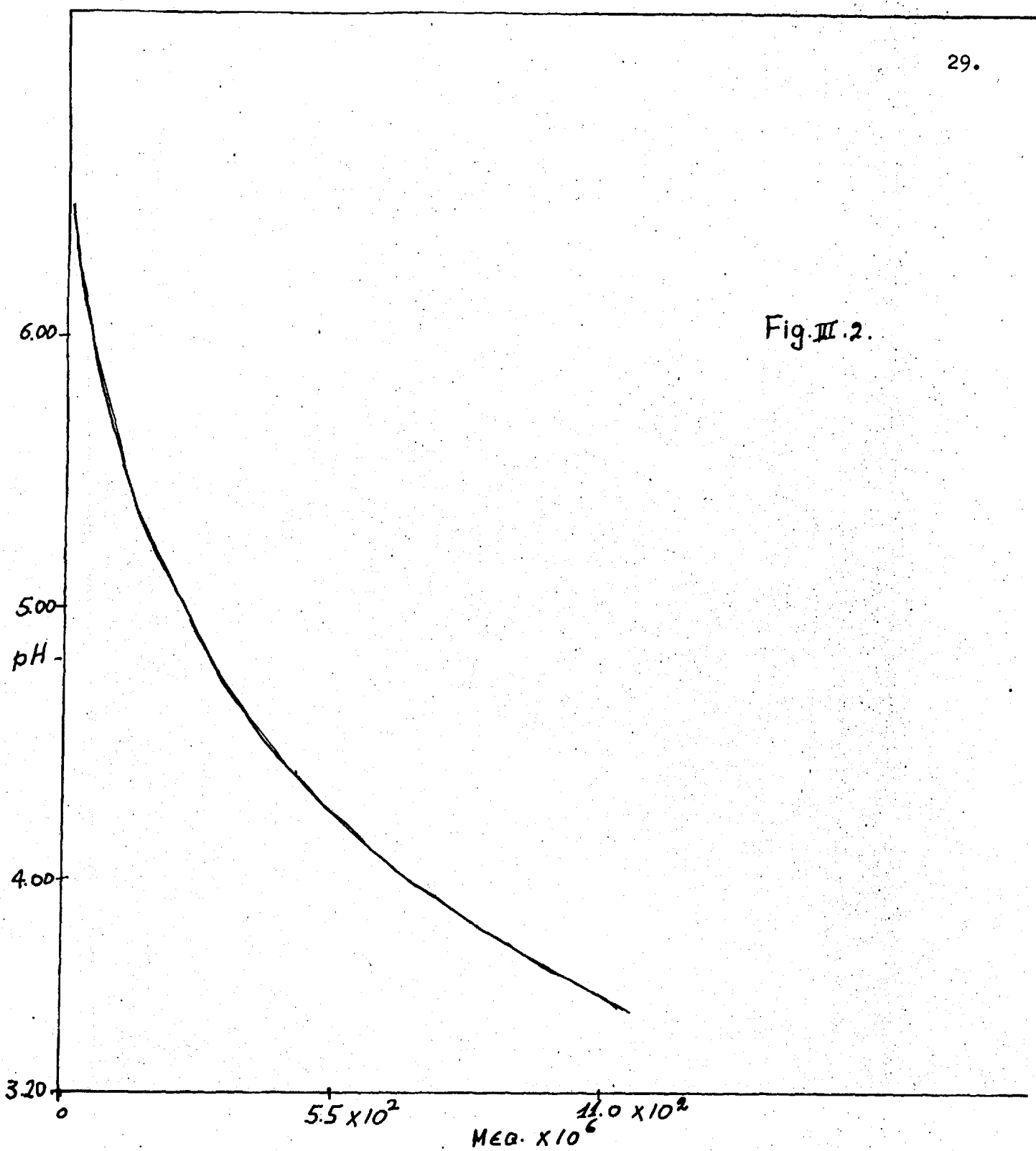
where plots of $v/[H]$ versus v should yield straight lines the intercepts of which on the $v/[H]$ and v axes are kn and n respectively.

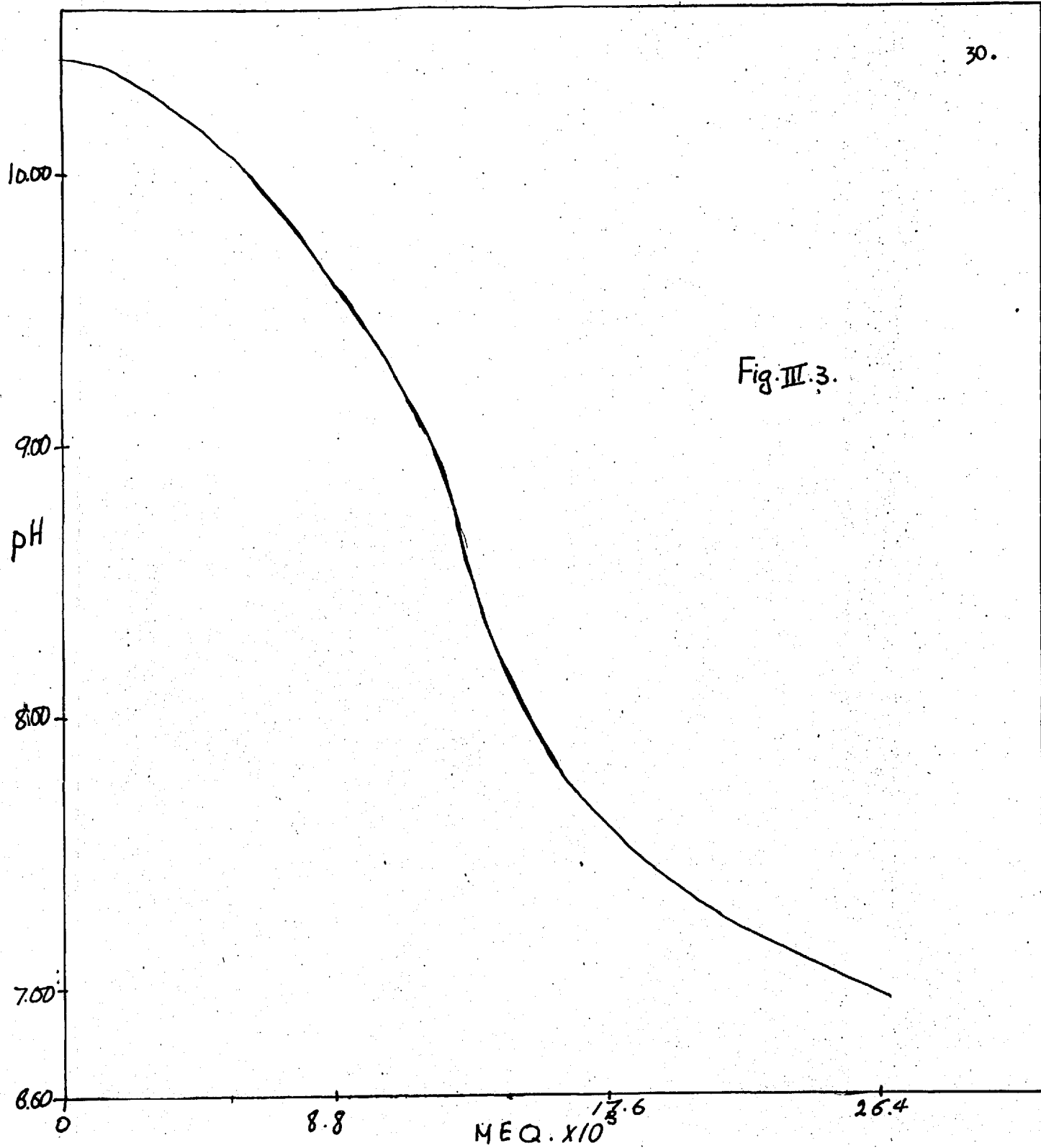
Fig. 111.6, 8, 11, and 13 are the analyses of Fig. 111.1 and 3; Fig. 111.9, 10, 14, and 15 are the analyses of Fig. 111.4 and 5; while the analysis of Fig. 111.2 is shown in Fig. 111.7 and 12.

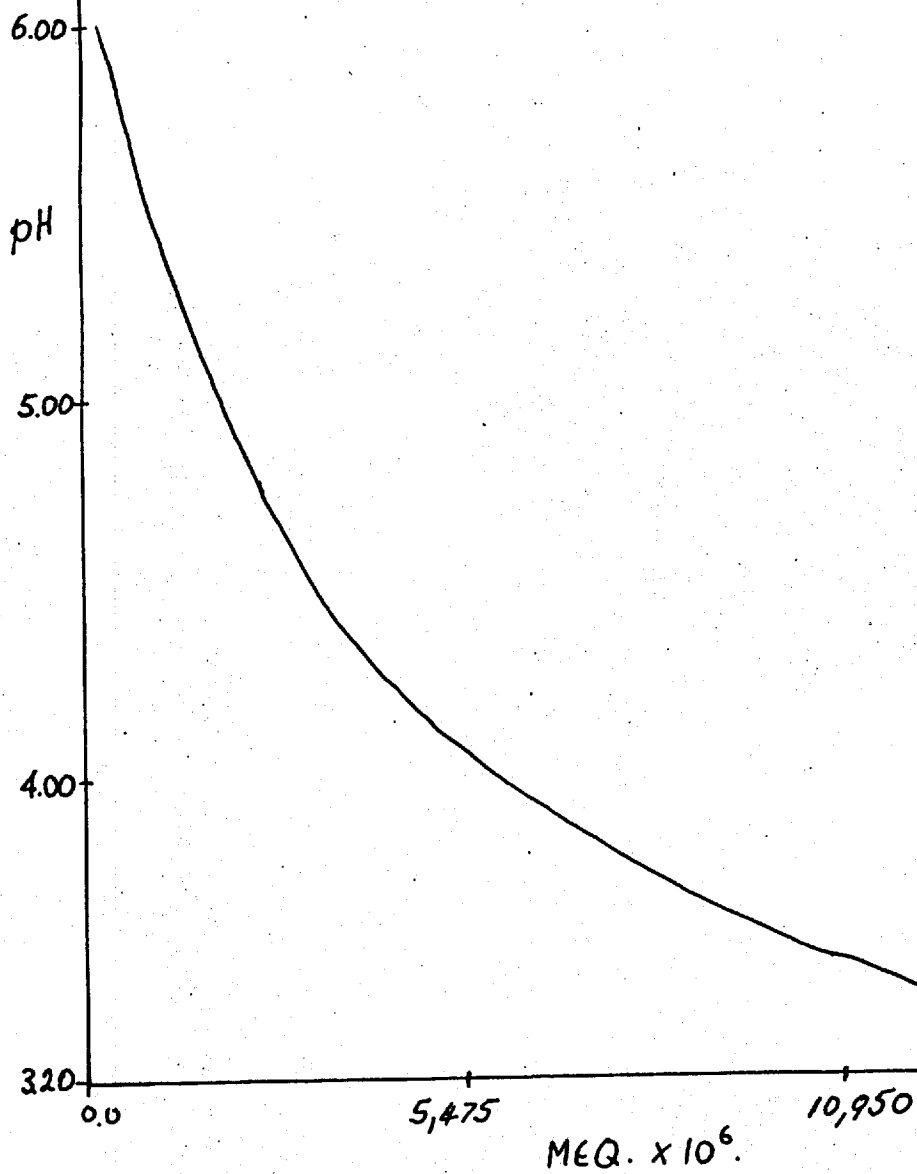
A table (Table 111.1) showing a specimen of the calculations is included. Table 111.2. shows the results of the analyses.

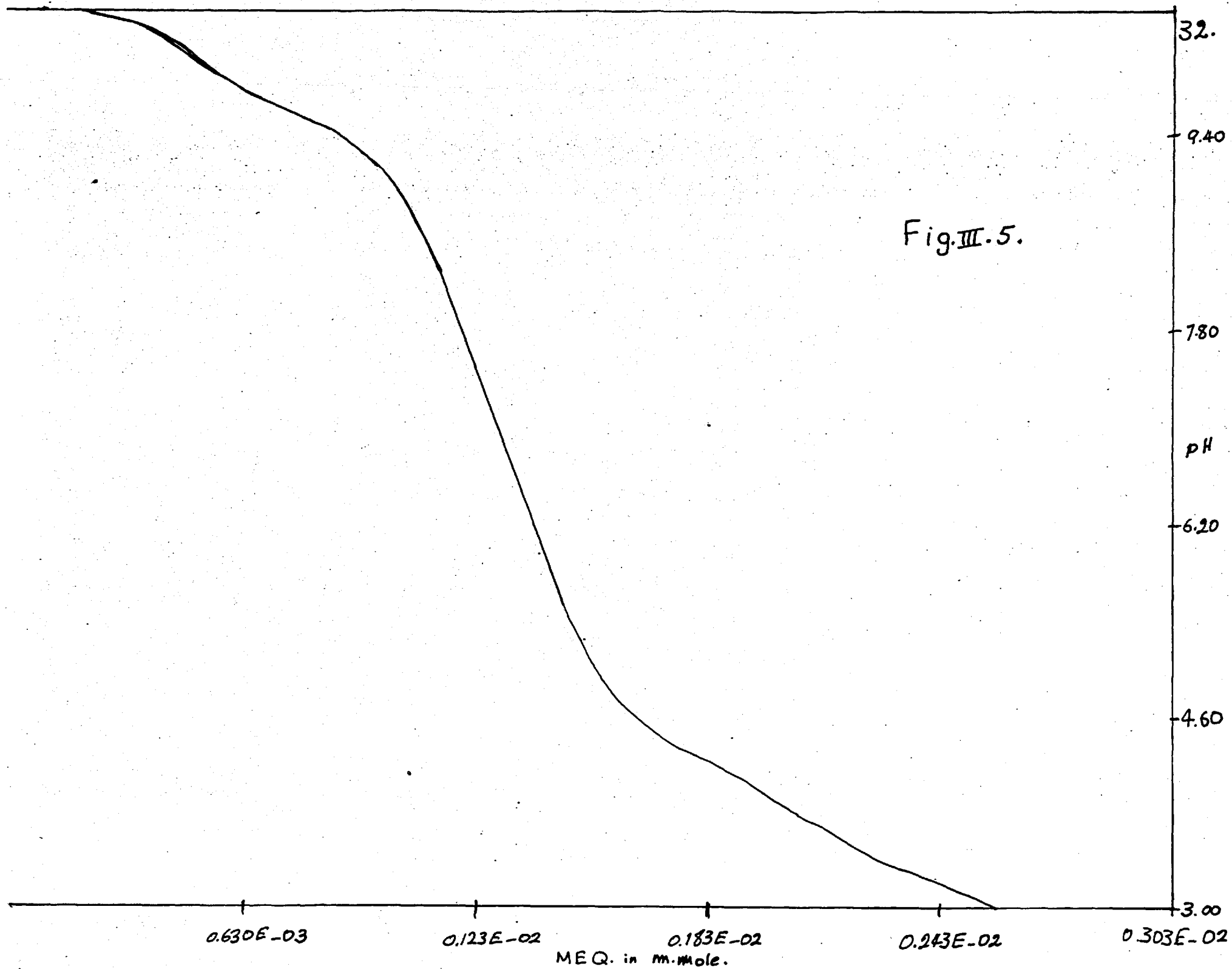
Fig. III. 1.











\$JOB XCCC0287 JTKLASCHKA IBJOB 6.0 2000 SUB TEST 1
 \$EXECUTE IBJOB
 \$IBJOB FIOCS
 \$IBFTC MAIN

```

    DIMENSION A(20,120),NP(10),T(60),XN(6),YN(36)
    DO 21 L=1,16
      IF(L.LT.16) K=1
      IF(L.EQ.16) K=5
      NFAM=K*2-1
      KSIX=K*6
      READ(5,2) (NP(I),I=1,K)
2  FORMAT(13)
      READ(5,3) (T(I),I=1,KSIX)
3  FORMAT(6A6)
      READ(5,4) (XN(I),I=1,6),(YN(I),I=1,36)
4  FORMAT(6A6,36A1)
      DO 20 I=1,NFAM,2
        IA=I+1
        N=NP(I)
        DO 20 J=1,N
20  READ(5,1) A(I,J),A(IA,J)
      1 FORMAT(2E7.3)
21  CALL PLOT(A,NP,K,T,XN,YN)
    STOP
    END

```

\$IBFTC GRAPH DECK

```

    SUBROUTINE PLOT (A,NP,K,T,XN,YN)
    DIMENSION A(20,120),P(121),C(30),AM(10),AX(10),D(4),NP(10),
    IUY(6),UX(6),T(60),XN(6),YN(36),MA(20)
    DATA(C(I),I=1,3,2)/1H*,1H+/, (C(I),I=21,29,2)/1HD,1H ,1HX,1HI,1H-/
    1(D(I),I=1,4)/3HMIN,5HSHRANGE,1HX,1HY/
    DATA (C(I),I=5,19,2)/1H3,1H4,1H5,1H6,1H7,1H8,1H9,1H0/
    M=K*2-1
    KK=2*K
    NX=101
    NY=51
    DO 6 I=1,KK
      AM(I)=A(I,1)
      AX(I)=0.
      IFACT=(I-1)/2.
      IF(MOD(I,2).EQ.1) N=NP(IFACTION+1)
      DO 6 J=1,N
        IF(A(I,J).GT.AX(I)) AX(I)=A(I,J)
6  IF(A(I,J).LT.AM(I)) AM(I)=A(I,J)
      IF(K.LT.2) GO TO 29
      DO 7 I=3,M,2
        IF(AM(I).LT.AM(I)) AM(I)=AM(I)
        IF(AX(I).GT.AX(I)) AX(I)=AX(I)
        IF(AM(I+1).LT.AM(2)) AM(2)=AM(I+1)
7  IF(AX(I+1).GT.AX(2)) AX(2)=AX(I+1)
29 ID=1
25 DO 8 I=1,2
      IF(ID.EQ.1) AP=AM(I)
      IF(ID.EQ.-1) AP=AX(I)

```

```

      IF(ID.EQ.1.AND.AP.LT.10.E-8) GO TO 20
      IF(AP.LT.10.E-8.OR.AP.GE.10.E12) GO TO 21
      DO 9 J=1,20
      X=10.** (J-9)
      Y=10.** (J-8)
      9 IF(AP.LT.Y.AND.AP.GE.X) GO TO 22
      22 IF(ID.EQ.-1) X=Y
      DO 10 J=1,11
      XL=X+X*0.1 *10.** ((1+ID)/2)*FLOAT(ID*(J-1))
      XH=XL+X*0.1 *10.** ((1+ID)/2)
      10 IF(AP.LT.XH.AND.AP.GE.XL) GO TO 23
      20 XL=0.
      23 IF(ID.EQ.1) AM(1)=XL
      IF(ID.EQ.1) AX(1)=AX(1)-AM(1)
      8 IF(ID.EQ.-1) AX(1)=XH
      IF(ID.EQ.-1) GO TO 24
      ID=-1
      GO TO 25
      24 IF(NX.EQ.51.OR.NX.EQ.101) GO TO 42
      WRITE(6,2)
      2 FORMAT(1H0,6X,16HFORMAT INCORRECT)
      RETURN
      42 DO 30 I=1,6
      UX(1)=AM(1)+AX(1)*FLOAT(I-1)/5.
      30 UY(1)=AM(2)+AX(2)*FLOAT(6-I)/5.
      IF(K.EQ.1) GO TO 35
      WRITE(6,12) K
      12 FORMAT(1H1,6X,13,2X,19HCURVES IN THIS PLOT?)
      DO 38 L=1,19,2
      38 MA(L)=(L-1)/2+1
      WRITE(6,106) (MA(L),C(L),L=1,19,2)
      106 FORMAT(1H0,1X,17HPLOTTING SYMBOLS,, 7(6H CURVE,13,2H, ,A2)/2X,3(6H
      1CURVE,13,2H, ,A2),4X,48HSYMBOL D INDICATES TWO OR MORE COINCIDENT
      2POINTS)
      35 DO 32 I=1,K
      NS=6*(I-1)+1
      NF=NS+5
      IF(K.EQ.1) WRITE(6,108) I,(T(NT),NT=NS,NF)
      32 IF(K.GT.1) WRITE(6,31) I,(T(NT),NT=NS,NF)
      31 FORMAT(1H0,6X,8H CURVE NO,13,2X,6A6)
      108 FORMAT(1H1,6X,8H CURVE NO,13,2X,6A6,4X,48HSYMBOL D INDICATES TWO OR
      1 MORE COINCIDENT POINTS)
      IF(K.GT.1) WRITE(6,12) K
      DO 40 IY=1,NY
      DO 41 IX=1,NX
      41 P(IX)=C(23)
      Y=AM(2)+((AX(2)/FLOAT(NY-1))*FLOAT(NY-IY))
      DO 13 I=1,M,2
      IFACT=(I-1)/2
      N=NP(IFACT+1)
      DO 13 J=1,N
      IF(A(I+1,J).EQ.10.**13) GO TO 13
      IF(A(I+1,J)-Y) 13,27,27
      27 CONTINUE
      A(I+1,J)=10.**13

```

```

DO 11 IX=1,NX
X=AM(1)+((AX(1)/FLOAT(NX-1))*FLOAT(IX-1))
IF(A(I,J).EQ.10.**13) GO TO 11
IF(A(I,J)-X) 28,28,11
28 IF(P(IX).NE.C(23)) P(IX)=C(21)
IF(P(IX).EQ.C(23)) P(IX)=C(1)
A(I,J)=10.**13
GO TO 12
11 CONTINUE
13 CONTINUE
26 DO 4 IX=1,NX
IR=0
CALL STEP(IY,IR,NY)
IQ=IP
CALL STEP(IX,IQ,NX)
AC=C(23)
IF(IQ.EQ.4.OR.IQ.EQ.3) AC=C(25)
IF(IQ.EQ.2.AND.IR.EQ.0) AC=C(27)
IF(IQ.EQ.2.AND.IR.EQ.2) AC=C(29)
IF(P(IX).NE.C(21).AND.P(IX).NE.C(1)) P(IX)=AC
4 CONTINUE
IF(IR-1) 33,34,34
33 IF(IY.LT.8.OR.IY.GT.43) WRITE(6,100) (P(IX),IX=1,NX)
IF(IY.GT.7.AND.IY.LT.44) WRITE(6,101) YN(IY-7), (P(IX),IX=1,NX)
GO TO 40
34 IYFACT=(IY-1)/10
IF(IY.LT.8.OR.IY.GT.43) WRITE(6,102) UY(IYFACT+1), (P(IX),IX=1,
1NX)
IF(IY.GT.7.AND.IY.LT.44) WRITE(6,103) YN(IY-7), UY(IYFACT+1),
1(P(IX),IX=1,NX)
100 FORMAT(1H ,15X,101A1)
101 FORMAT(1H ,2X,A1,12X,101A1)
102 FORMAT(1H ,5X,E9.3,1X,101A1)
103 FORMAT(1H ,2X,A1,2X,E9.3,1X,101A1)
40 CONTINUE
WRITE(6,104) (UX(I),I=1,6)
104 FORMAT(1H0,6(11X,E9.3))
WRITE(6,105) (XN(I),I=1,6)
105 FORMAT(1H0,48X,6A6)
RETURN
21 WRITE(6,3) D(1),D(IDIR)
3 FORMAT(1H1,6X,2A6,23HOUT OF RANGE OF PROGRAM)
RETURN
END

$IBFTC AINC DECK
SUBROUTINE STEP(I,K,J)
DIMENSION N(11)
DO 20 L=1,6
N(L)=L
IF(L.EQ.1) N(L)=1
IF(L.GT.1) N(L)=(L-1)*(J-1)/5+1
IF(I.EQ.N(L)) K=K+1
20 CONTINUE
IF(1.EQ.N(1).OR.1.EQ.N(6)) K=K+1
RETURN
END.

```

Fig. III.6

0.310E 05

 $1/\nu$

0.210E 05

0.110E 05

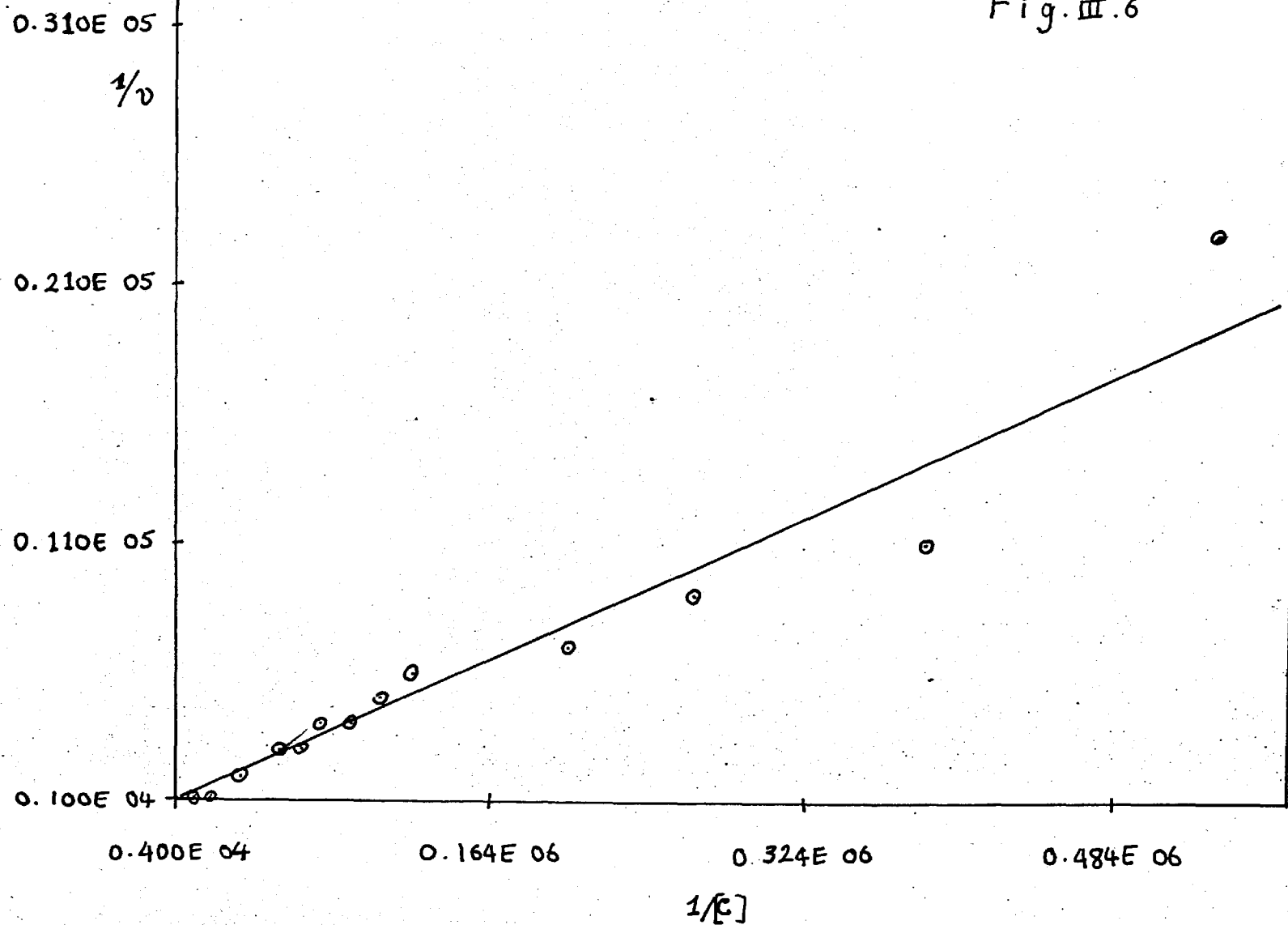
0.100E 04

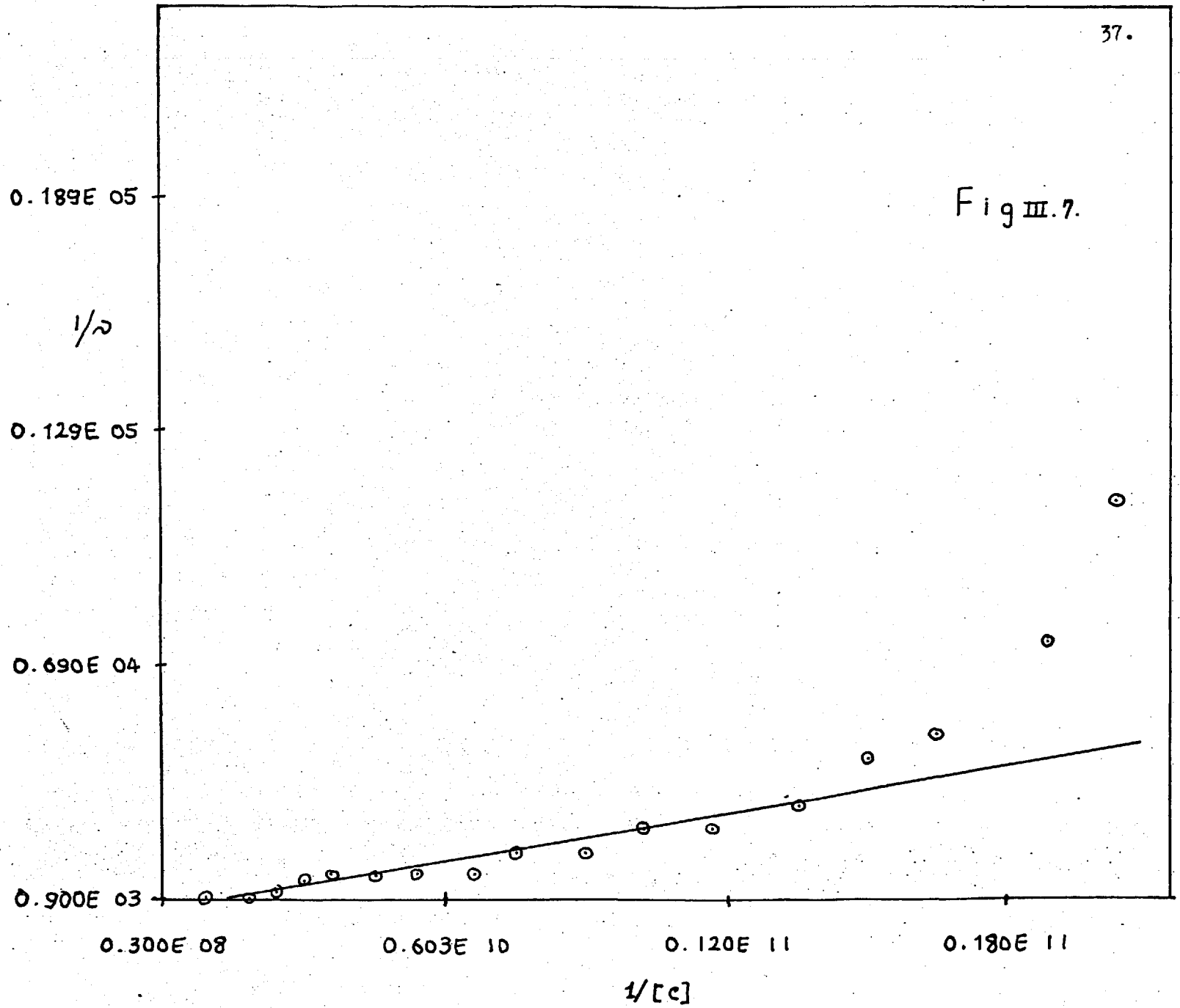
0.400E 04

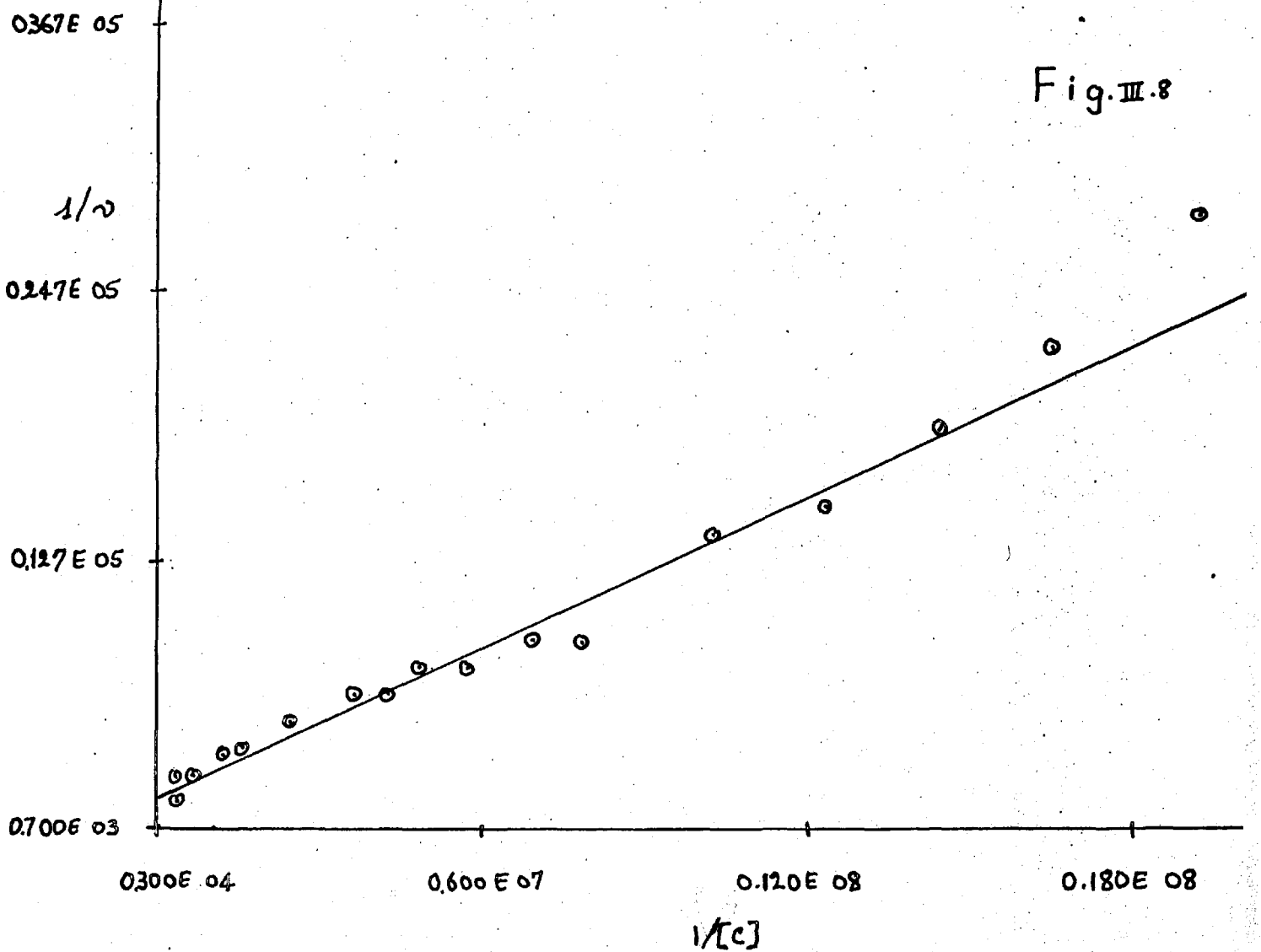
0.164E 06

0.324E 06

0.484E 06

 $1/[C]$ 





0310E 05

 $\frac{1}{2}$

0.210E 05

0410E 05

0100E 04

0200E 04

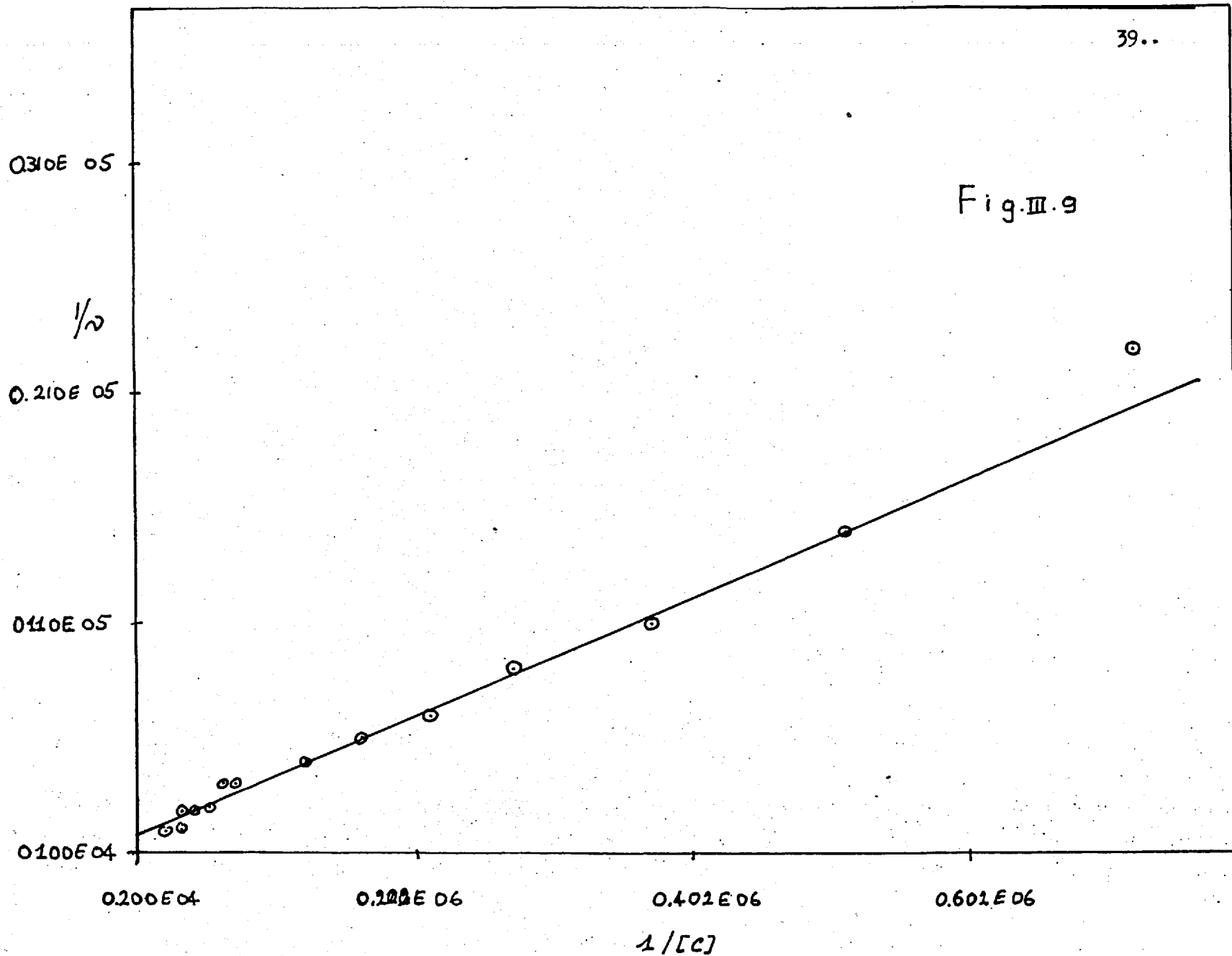
0.120E 06

0.401E 06

0.601E 06

 $\lambda / [c]$

Fig. III.9



0.590E04

0.410E04

 $\frac{1}{2}$

0.230E04

0.500E03

0.100E04

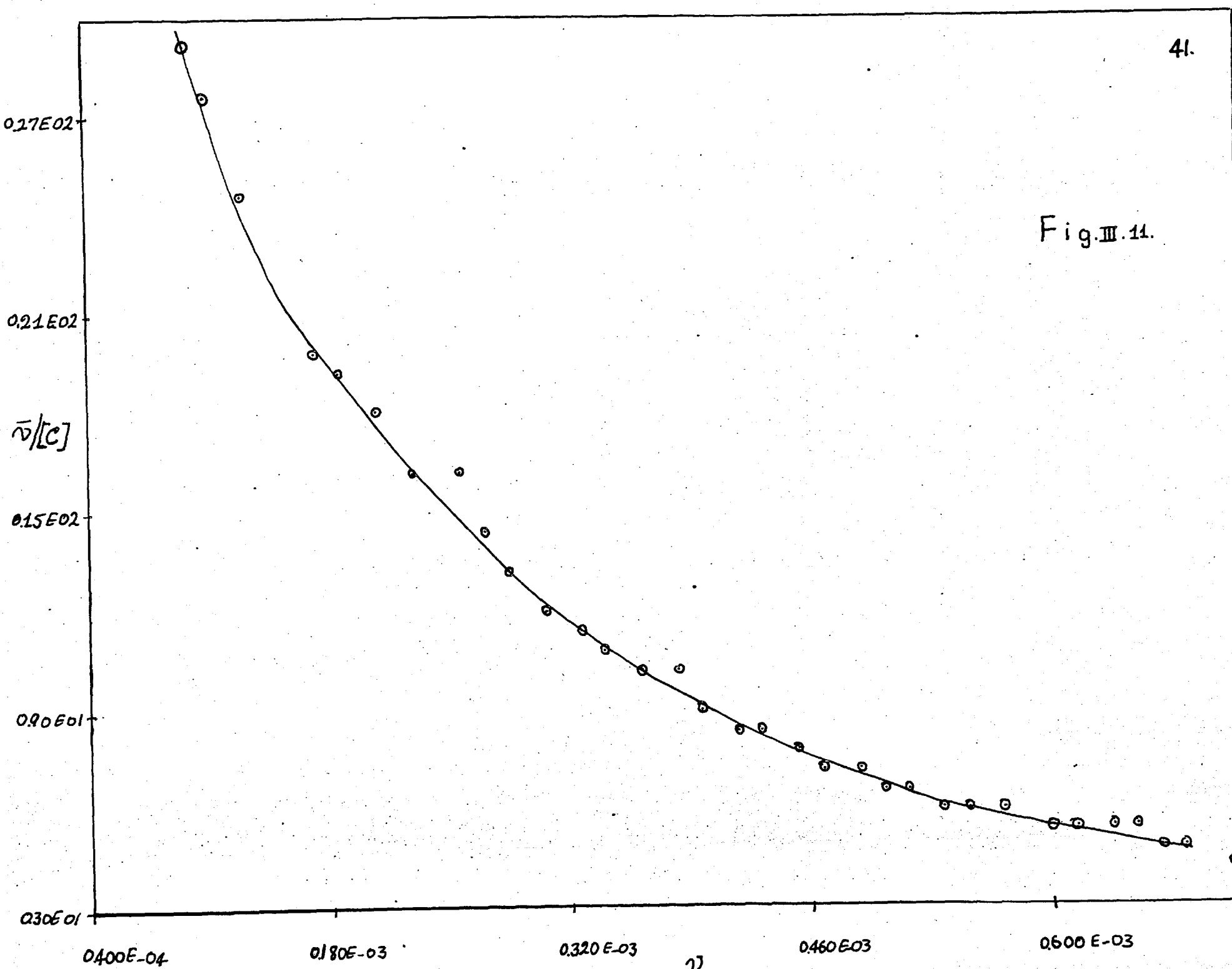
0.600E10

0.120E11

0.180E11

 $1/[c]$

Fig. III.10.



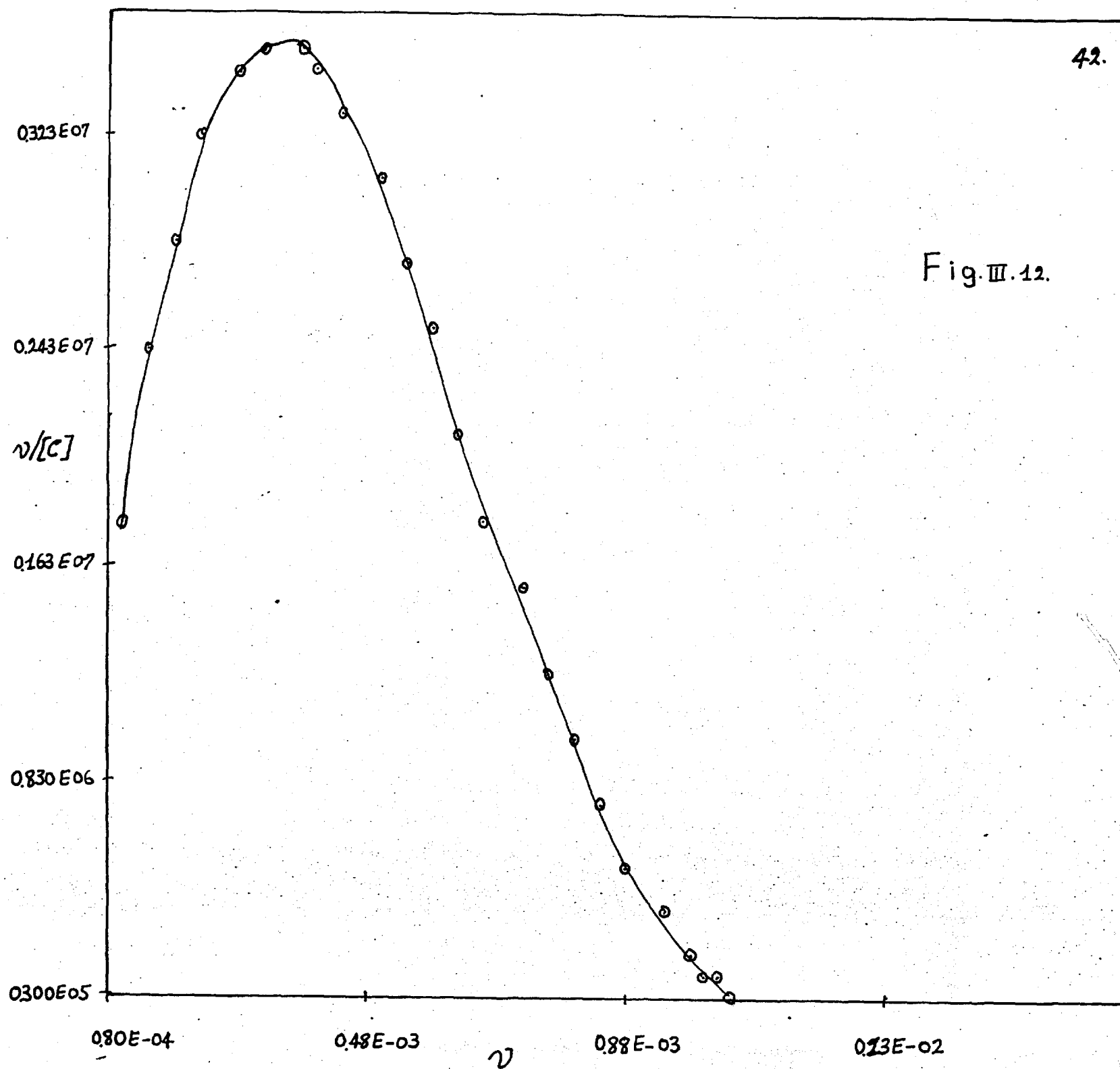


Fig. III.13.

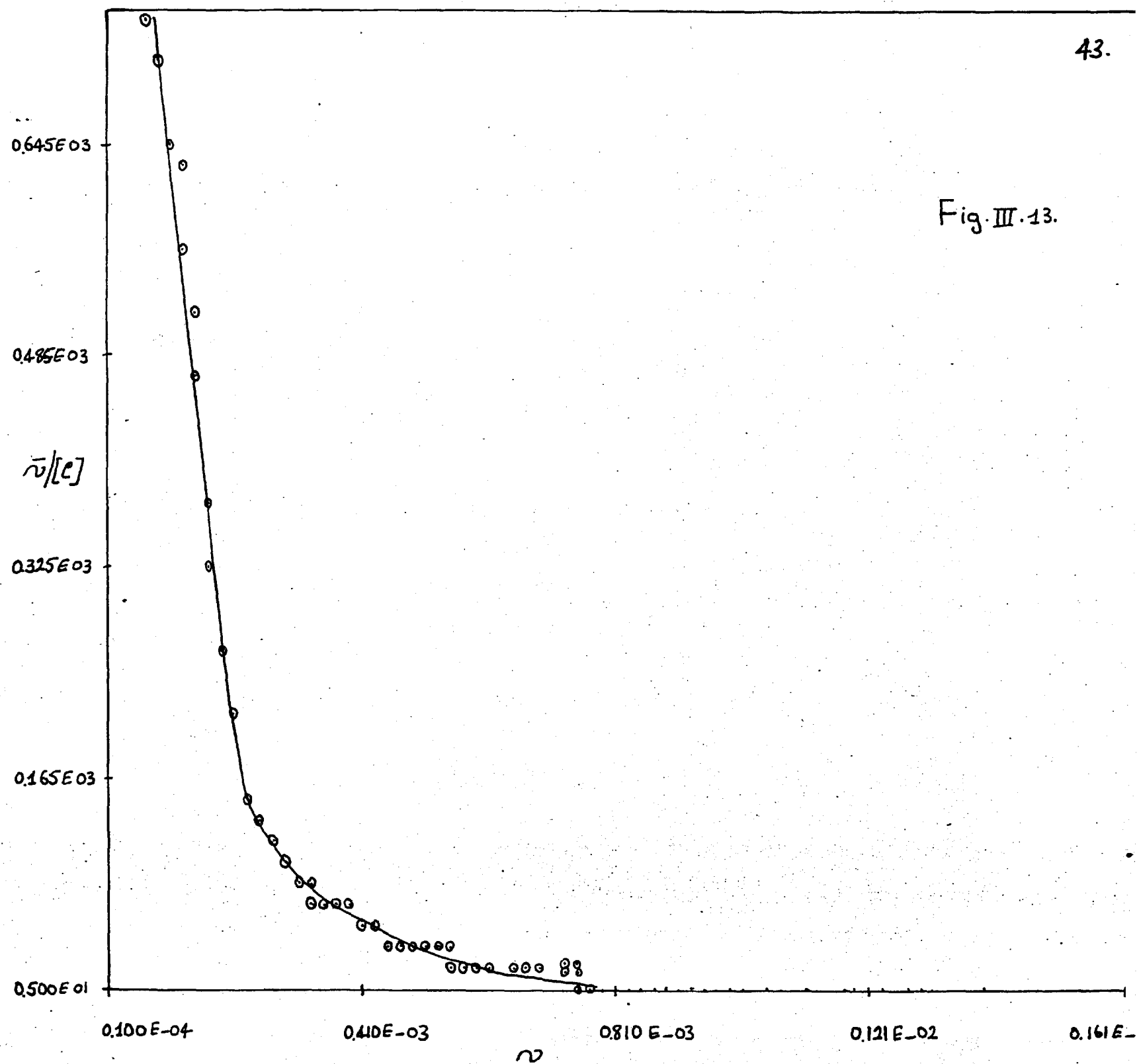
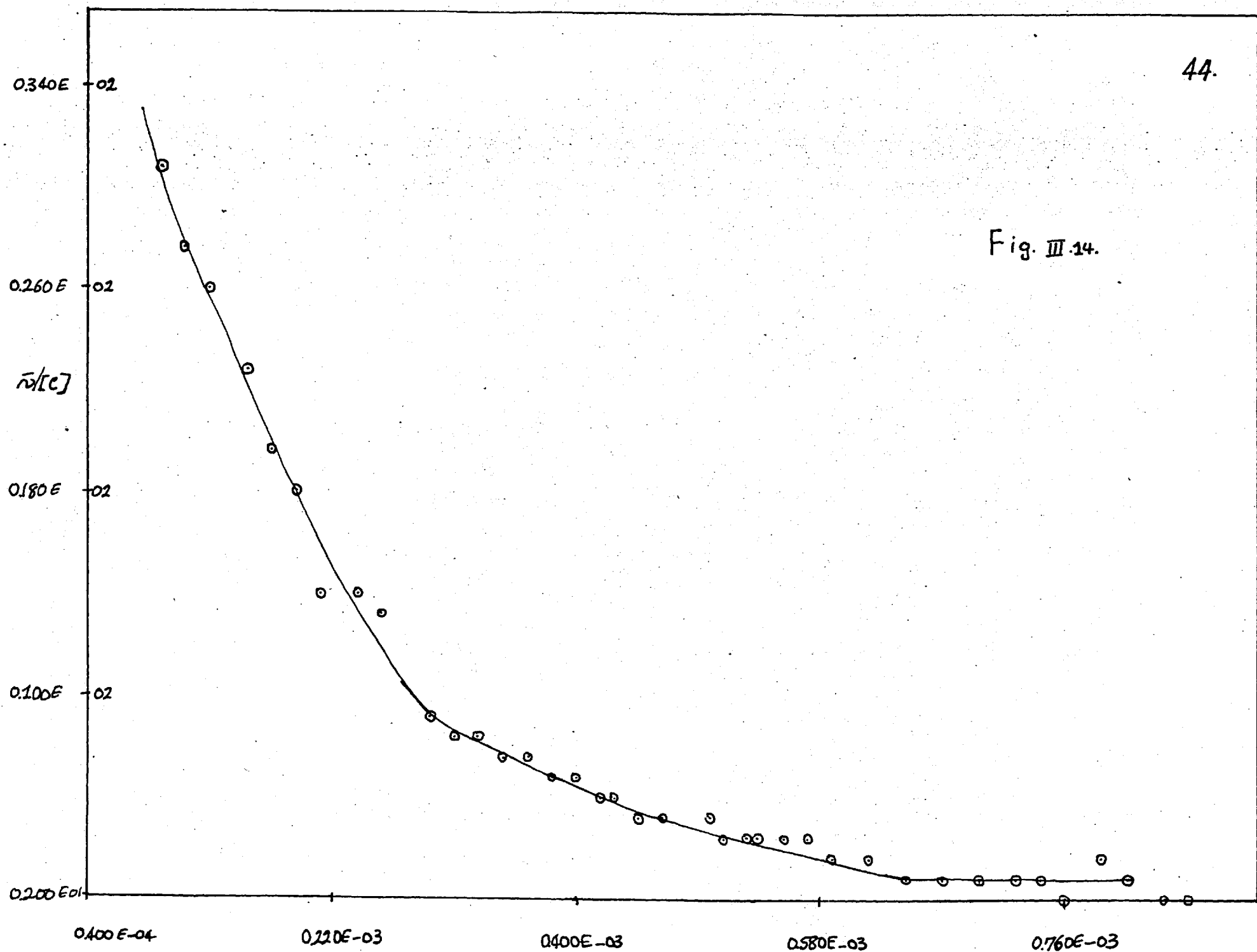


Fig. III.14.



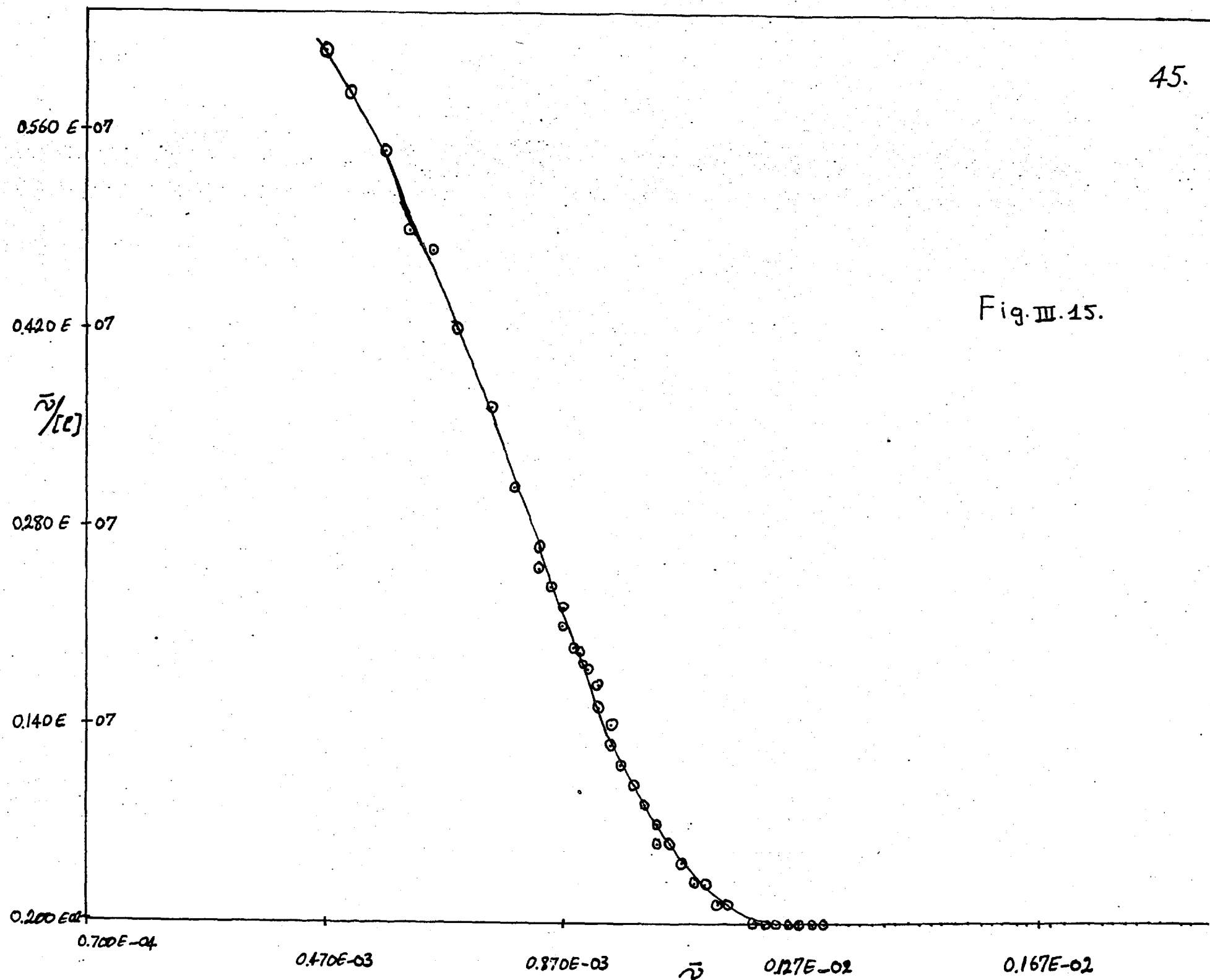


TABLE.III.2.Thermodynamic properties of hydrogenase.

T	-COOH			-NH ₃		
	K _d × 10 ⁵	pK	n	K _d × 10 ⁹	pK	n
23.0°C	2.86	4.34	40	1.06	8.98	20
0.6°C	1.08	4.96	20	5.53	8.28	7

The pK of the denatured protein at 23°C = 4.48.

n of Carboxyl groups = 70.

ΔF ₁	5.91 Kcal/ mole	12.23 Kcal/ mole
ΔF ₂	6.25 "	10.44 "
ΔH	10.5 "	-11.45 "
ΔS	15.6 Cal/ deg./mole	- 80 Cal/ deg./mole

TABLE 111.2

		Specimen of calculations used in the analyses of the data.					
MEM	pH	$C_t^{H^+} \times M$	$1/C_t^{H^+}$	$C_t^{H^+} \times 10^5$	$\bar{v} \times 10^4$	$1/\bar{v} \times 10^4$	$\bar{v}/c$
10 ⁶							
—	6.061	8.66×10^{-7}	1.15×10^6	—	—	—	—
219	5.895	1.27×10^{-6}	7.87×10^5	228	0.218	4.65	1.69
219	5.731	1.86	5.37	449	0.430	2.32	23.0
219	5.582	2.62	3.82	866	0.840	1.19	32.0
219	5.425	3.75	2.66	1085	1.05	0.955	27.9
219	5.298	5.03	1.98	1304	1.25	0.797	25.0
436	5.083	8.25	1.21	1742	1.66	0.602	20.1
219	5.012	9.72	1.02	19.61	1.86	0.532	19.2
219	4.942	1.14×10^{-5}	8.77×10^4	21.80	2.07	0.484	18.1
219	4.895	1.38	7.24	23.99	2.26	0.442	16.4
219	4.803	1.57	5.36	26.18	2.56	0.390	16.3
219	4.737	1.83	5.46	28.37	2.65	0.376	14.5
219	4.674	2.12	4.71	30.56	2.84	0.351	13.3
219	4.617	2.42	4.13	32.57	3.02	0.331	12.5
219	4.562	2.74	3.64	34.44	3.22	0.310	11.8
219	4.502	3.14	3.18	37.13	3.44	0.294	10.8
219	4.462	3.58	2.79	39.22	3.57	0.279	10.4
219	4.421	3.61	2.77	41.51	3.79	0.263	10.2
219	4.362	4.35	2.29	43.70	3.94	0.254	7.05
219	4.328	4.70	2.12	45.89	4.12	0.242	8.75
219	4.290	5.12	1.95	48.08	4.30	0.232	8.40
219	4.253	5.58	1.79	50.27	4.47	0.223	8.00
219	4.220	6.02	1.66	52.46	4.64	0.215	7.71
219	4.186	6.50	1.53	54.65	4.82	0.205	7.41
219	4.152	7.04	1.42	56.84	4.98	0.200	7.07
219	4.113	7.73	1.29	59.03	5.13	0.194	6.64
219	4.090	8.12	1.23	61.22	5.31	0.188	6.55
219	4.069	8.50	1.17	63.44	5.49	0.182	6.25
219	4.039	9.12	1.09	65.60	5.65	0.177	6.19
436	3.981	1.04×10^{-4}	9.61×10^3	69.96	5.96	0.167	5.73
219	3.955	1.11	9.00	72.15	6.11	0.163	5.54
219	3.941	1.14	8.77	74.34	6.25	0.158	5.51
219	3.917	1.21	8.26	76.53	6.44	0.155	5.33
219	3.894	1.27	7.87	78.72	6.60	0.151	5.20
219	3.870	1.35	7.40	80.91	6.74	0.148	4.99
657	3.785	1.64	6.09	86.48	7.01	0.142	4.32
219	3.758	1.74	5.74	88.67	7.13	0.140	4.10
1,095	3.670	2.14	4.67	92.77	6.83	0.146	3.20
1,095	3.600	2.18	4.58	90.86	6.91	0.144	3.17

C- Electrophoresis.

A- Introduction.

Electrophoresis is the phenomenon of ion migration due to an electric field. Paper as a supporting medium for electrophoretic migration was first suggested by König (100) in 1939. The first analytical application of this new tool was the isolation of a yellow pigment from snake venom (101). Since then, paper electrophoresis has become one of the most widely used analytical methods in protein chemistry.

Free-boundary electrophoresis was however, the first to be observed, and Tiselius (102) pioneered in this field. All theoretical treatments of electrophoretic mobilities are much simpler, although by no means simple and thorough, for the case of free-boundary than paper, gel or other types of electrophoresis. This is because structural considerations of the matrix used need not be taken into account.

Many texts have been written on the subject and Bier's treatise (103) is an excellent one.

b- Theory.

Ions subject to an electric field move towards the positive or negative electrode according to the sign of their charges; the speed with which they move is determined by the net total charge on the ions.

In free-boundary electrophoresis, the movement of these ions is due to the chemical and electrical potentials. Guggenheim (104) pointed out the impossibility of separating these two, and used,

for the first time, the term electrochemical potential n_i .

$$n_i = \mu_i + z_i e \phi \quad (1)$$

where μ_i is the chemical potential, z_i is the charge on the i ion, e is the charge of an electron, and ϕ is the electrical potential.

The evaluation of the electrical potential is achieved by assuming that the distance (x) from the interface, is connected with the average potential $\psi(x)$ by a Boltzmann equation.

$$n_i(x) = n_i(\infty) \exp. \left(- \frac{z_i e \psi(x)}{kT} \right) \quad (2)$$

Where $n_i(\infty)$ is the number of ions in the bulk of the solution.

The potential distribution at the interface of the double-layer is given by Poisson's equation (105).

$$\text{div} \left[\epsilon \text{grad } \psi(x) \right] = -4\pi \rho(x) \quad (3)$$

where ϵ is a constant (dielectric constant) and $\rho(x)$ is the space charge density. For one dimensional system:

$$\frac{d^2 \psi(x)}{dx^2} = - \frac{4\pi \rho(x)}{\epsilon} \quad (4)$$

but the space charge density $\rho(x)$ is made up from the individual ionic charges or:

$$\rho(x) = \sum_i z_i e n_i(x) \quad (5)$$

combining relations (2), (4), and (5) we obtain the so-called Boltzmann-Poisson equation (105) which is the basis of the double-layer theory:

$$\frac{d^2 \psi(x)}{dx^2} = - 4 \frac{\pi}{\epsilon} \sum_i z_i e n_i(\infty) \exp. \left(- \frac{z_i e \psi(x)}{kT} \right) \quad (6)$$

This may be simplified to:

$$\frac{d^2 \psi(x)}{dx^2} = \kappa^2 \psi(x) \quad (7)$$

with the use of the electroneutrality principle, namely:

$$\sum_i z_i n_i(\infty) = 0 \quad (8)$$

where κ is given by
$$\kappa^2 = \frac{4\pi e^2 \sum_i n_i(\infty) z_i^2}{\epsilon kT} \quad (9)$$

The solution of equation (7) is

$$\psi(x) = \psi(0) e^{-\kappa x} \quad (10)$$

Using boundary conditions and other relations, the surface charge can be given by:

$$\frac{d\psi(x)}{dx} = \pm \left(\frac{8\pi n kT}{\epsilon z_+ z_-} \right)^{1/2} \left(z_- e^{-\frac{z_+ e \psi(x)}{kT}} + z_+ e^{-\frac{z_- e \psi(x)}{kT}} \right)^{1/2} \quad (11)$$

where $n = z_+ n_+(\infty) = z_- n_-(\infty)$ (12)

and
$$\sigma(x) = \pm \sqrt{\frac{\epsilon n kT}{2\pi z_+ z_-}} \left(z_- e^{-\frac{z_+ e \psi(x)}{kT}} + z_+ e^{-\frac{z_- e \psi(x)}{kT}} \right) \quad (13)$$

and for a spherical interface Debye and Hückle (106) the total charge as

$$Q = 4\pi a^2 \sigma = \epsilon a \psi(a) (1 + \kappa a) \quad (14)$$

From the above treatment we can conclude that many factors affect the electrophoretic mobility.

1- Factors relative to the protein:

- a- Sign of charge,
- b- Magnitude of charge,
- c- Size, shape, and molecular weight of the protein.

2- Factors relative to the electrolyte solution:

- a- pH of the solution, (acid-base equilibria of protein)
- b- Ionic strength or effectively the thickness of the double-layer.
- c- Concentration of electrolyte (s).
- d- Temperature and viscosity of the solution.

3- Factors relative to the supporting medium :

- a- Current density, and voltage,
- b- Physical dimensions of the capillary.

A relation relating the field intensity and the voltage or current density has been found by Kunkel and Tiselius (107).

$$E = \frac{V}{l} = \frac{i}{Qk} \quad (15)$$

where V is the applied voltage, i the current density, k the conductivity of the medium, and l and Q are the length and width of the capillary. The electrophoretic mobility U , is connected with the electrophoretic potential or Zeta-potential (ζ), and this in turn is connected with the charge density.

$$U = \frac{\zeta \epsilon}{4\pi\eta} = \frac{Qd}{4\pi a^2\eta} \quad (16)$$

$$= \frac{4\pi\sigma}{\epsilon \times 0.33 \times 10^8} \mu^{\frac{1}{2}} \quad (17)$$

Combining equations (16) and (17) we obtain a relation in terms of σ only:

$$\sigma = 0.33 \times 10^8 \eta U (\mu)^{\frac{1}{2}} \quad (18)$$

Where d is the thickness of the double-layer, η is the viscosity of the medium, a is the radius of the spherical protein, ϵ is the dielectric constant of the medium, and μ is the ionic strength of the electrolyte.

Combining equations (14) and (16) we obtain a relation relating d to σ , namely:

$$d = \eta U / \sigma. \quad (19)$$

b - Technique, results and analysis

It was found, from early experiments that paper electrophoresis is not satisfactory. It was then decided to use a free-boundary electrophoretic cell, Fig. III.16.

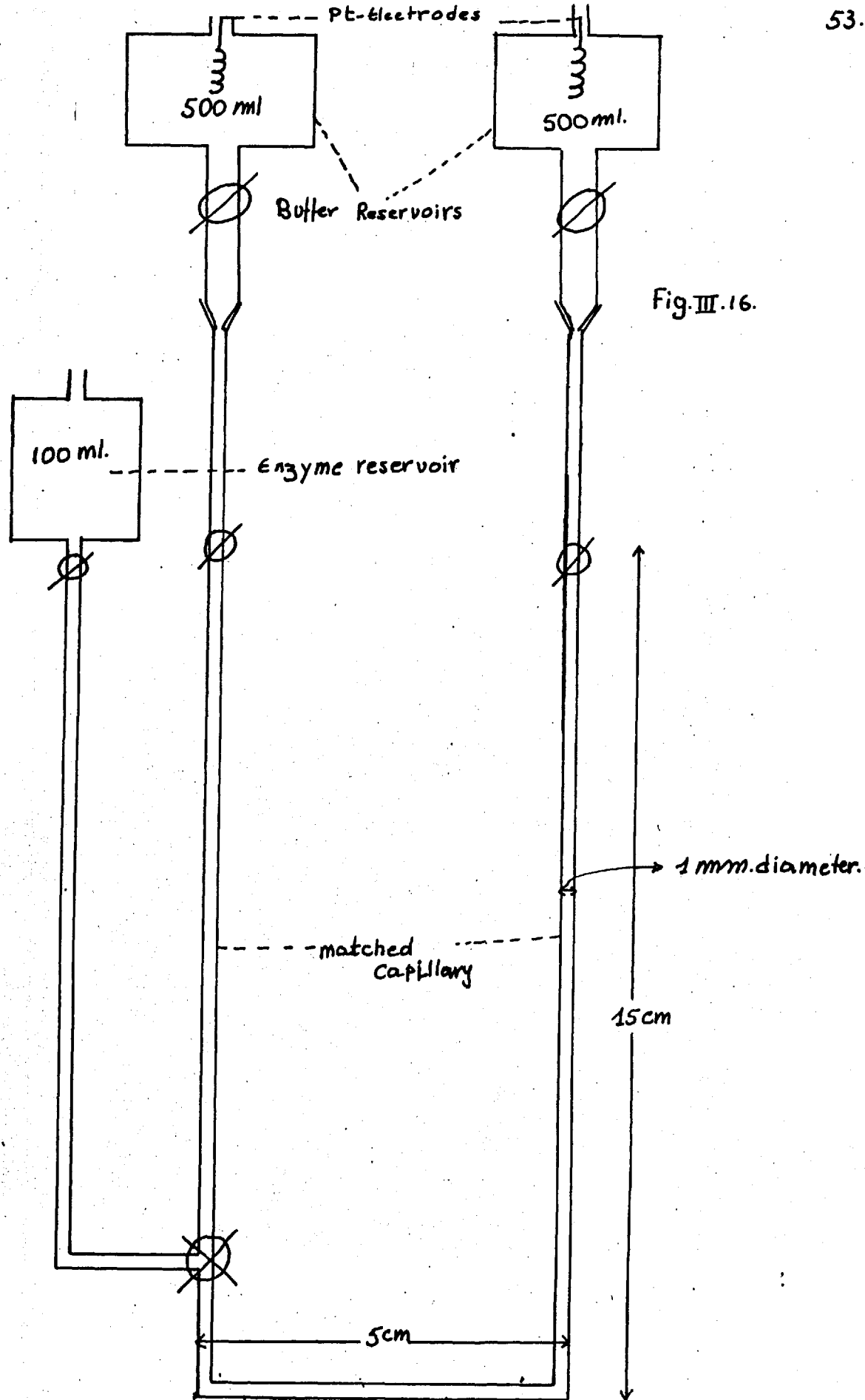
When the cell had been filled, the D.C. supply was switched on, and the movement of the boundary was plotted against the time and the mobility U calculated.

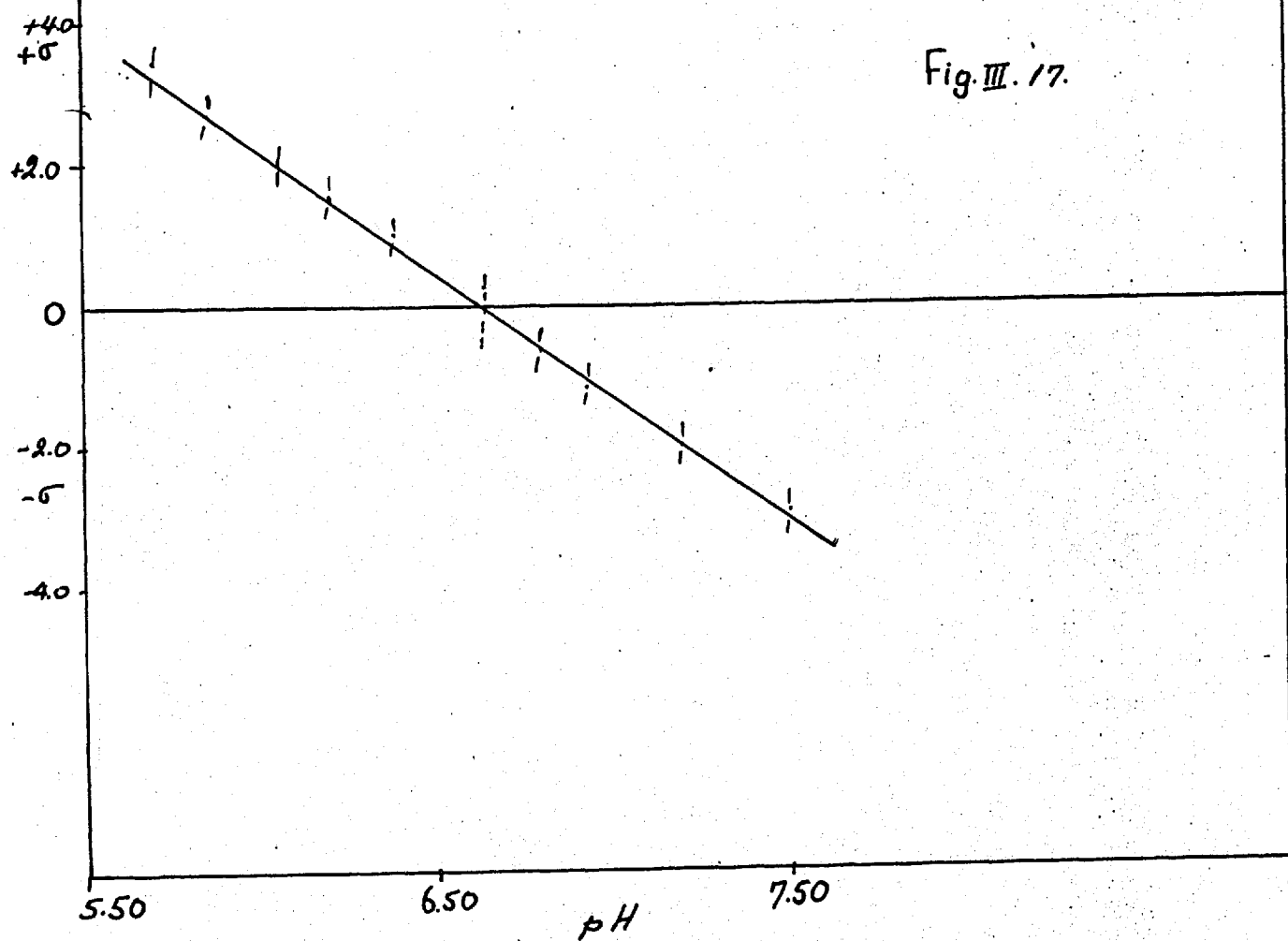
From the mobilities of the protein at different pH-values, the charge density had been estimated, Table III.3, and a plot of pH versus the charge was obtained, Fig.III.17. The isoionic point was found to be at pH = 6.64.

TABLE.III.3.

Electrophoretic mobilities of hydrogenase.

pH	U $\text{cm}^2/\text{V}/\text{sec.}$	σ
5.70	5.70	3.3
5.86	4.8	2.8
6.06	3.5	2.0
6.38	1.6	0.92
6.81	-1.0	-0.58
6.94	-1.9	-1.1
7.22	-3.5	-2.0
7.52	-5.2	-3.0





CHAPTER FOUR

Spectral properties of Hydrogenase

A - Theory of Optical Spectroscopy (109-111)

One of the great achievements of quantum mechanics is the analysis of spectra. The energy levels, hamiltonians, and finally the wave functions, that are obtained from different types of spectral measurements may be combined to give the total hamiltonian $\mathcal{H}_T$, or the wave function Ψ_T of the system.

For an ion in solution, the total wave function can be represented by the following relation:

$$\Psi_T = \Psi_S \cdot \Psi_L \cdot \Psi_V \cdot \Psi_r \cdot \Psi_e \quad (1)$$

where Ψ_S , Ψ_L , Ψ_V , Ψ_e , Ψ_r , represent the spin, orbital, vibrational, translational and rotational wave functions respectively.

Incident energy, in the form of radiation (equation(2)) may excite a molecule and a transition from $\langle \Psi_1 |$ to $\langle \Psi_2 |$ levels can be observed

$$E = h \nu \quad (2)$$

$\langle \Psi_1 |$ and $\langle \Psi_2 |$ stand for the ground and excited state wave functions respectively. These transitions are best accounted for by the electric dipole mechanism. Transitions may occur if and only if the transition moment Q , given by equation (3) is non-zero,

$$Q = \langle \Psi_1 | r | \Psi_2 \rangle \quad (3)$$

r is a vector of the same symmetry as the electric dipole.

Since Ψ_r and Ψ_e do not change in the condensed phase, the quantum mechanical selection rules are dependant on Ψ_S , Ψ_L and Ψ_V .

The spin selection rule

$$\psi_{s(1)} - \psi_{s(2)} \neq 0 \quad (4)$$

or

$$\Delta S \neq 0 \quad (5)$$

The orbital selection rule

$$\langle \psi_{L(1)} \psi_{V(1)} | r | \psi_{L(2)} \psi_{V(2)} \rangle \neq 0 \quad (6)$$

However, due to spin-orbit coupling, the spin selection rule is not fully valid, since the factorization of spin and orbital angular momenta is impossible. In other words:

$$\langle L_1, S_1 | \mathcal{H}_{SL} | L_2, S_2 \rangle \neq \langle L_1 | \mathcal{H}_L | L_2 \rangle \cdot \langle S_1 | \mathcal{H}_S | S_2 \rangle \quad (7)$$

Excitations due to spin orbit-coupling are known as spin-forbidden transitions.

The orbital selection rule is, however, based on the assumption that no vibrational interactions exist (equation (8)).

$$\langle \psi_{V(1)} | r | \psi_{V(2)} \rangle = 0 \quad (8)$$

and thus the selection rule is:

$$\langle \psi_{L(1)} | r | \psi_{L(2)} \rangle \neq 0 \quad (9)$$

If however, vibrational interactions do exist, then equation (9) can become zero and yet a transition is observed. Such forbidden excitations are known as Orbital or Laporte forbidden transitions.

The forbidden bands may be recognised if their Q factors (their transition moments) are estimated. Q, however, is related to the oscillator strength f which in turn is related to the band intensity. The intensity is given by:

$$I = I_0 e^{-k \epsilon} \quad (10)$$

where I_0 and I are the intensities of incident and transmitted light respectively. f, the oscillator strength will be

$$f = A \int_{\nu_2}^{\nu_1} \epsilon d\nu \quad (11)$$

$$= 4.6 \times 10^{-9} \epsilon_{\max} \Delta \nu$$

where ϵ is the extinction co-efficient, A is a constant, ν_1 and ν_2 are the frequencies at which the excitation starts and dies off respectively. The transition moment Q , will then be given by:

$$Q^2 = 9.124 \times 10^{-12} f / \nu_{\max} \quad (13)$$

A table of representative values for different transitions in transition metal complexes is given (112), Table IV.1.

The observed excitations of a complex can be assigned energy from a Sugano-Tanabe plot (113), and the ratio β of the G-level energy in the excited state to that of the ground state can be calculated.

$$\beta = \frac{E_{G \text{ -level in complex}}}{E_{G \text{ -level in free ion}}} \quad (14)$$

β is of great importance since it is a measure of the ionic or covalent character of the complex.

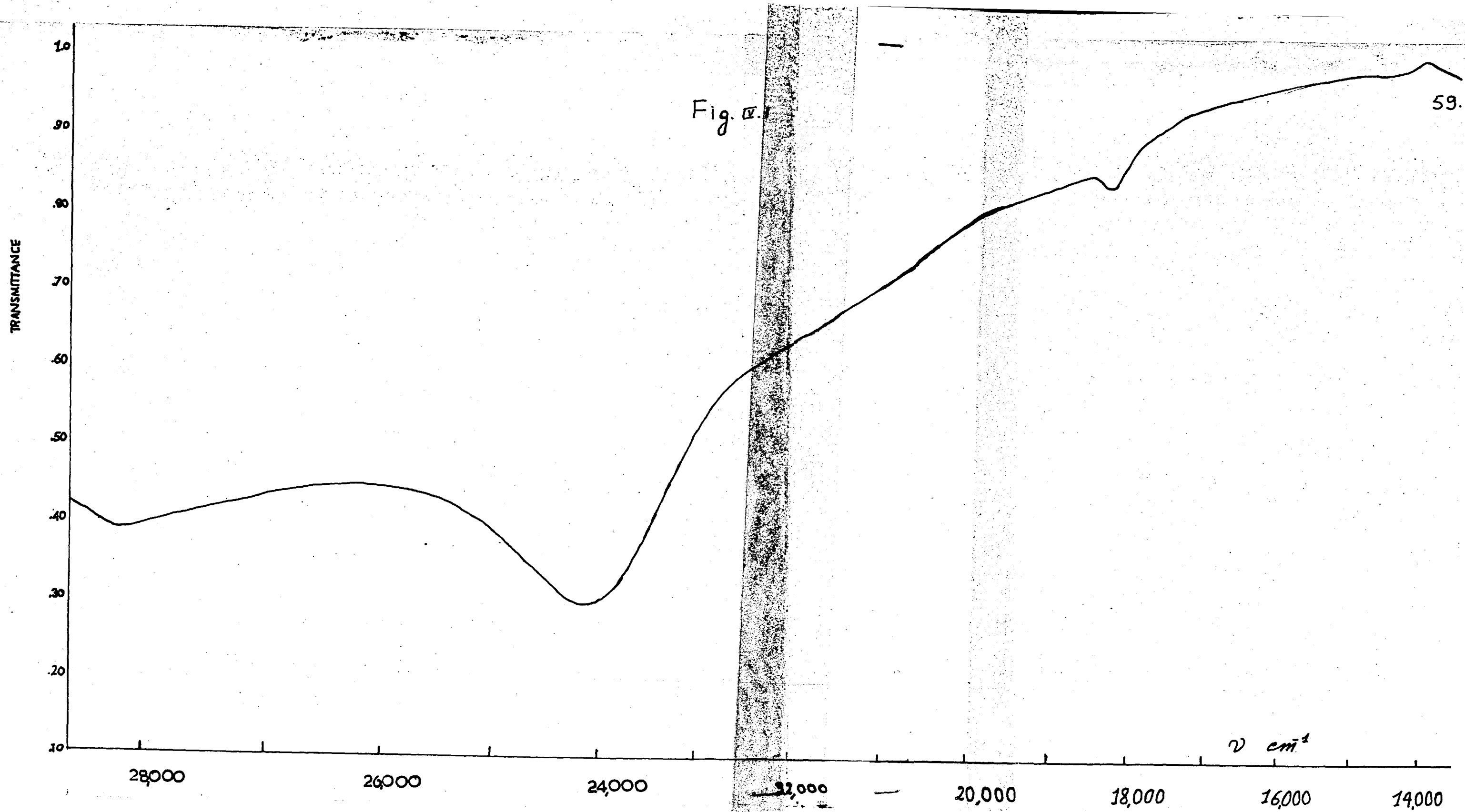
$$\beta = (1-x)^2 \quad (15)$$

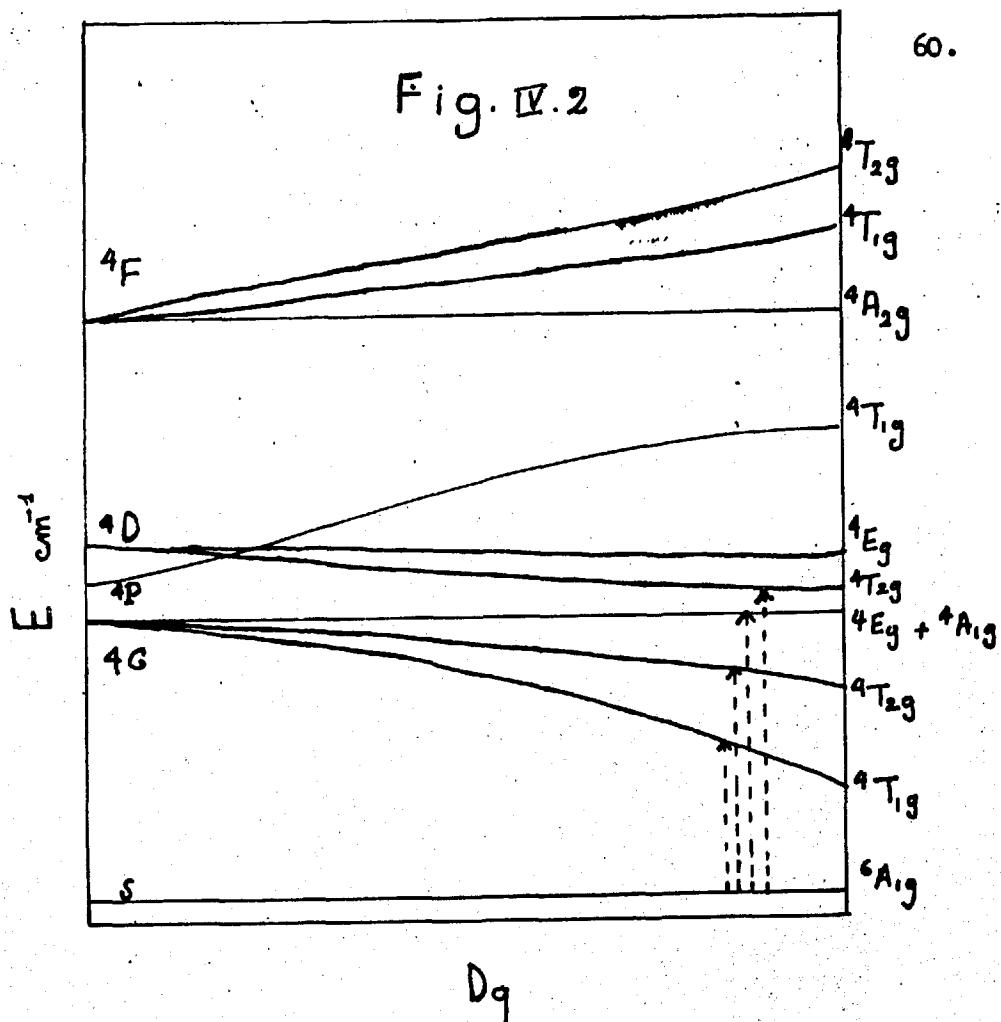
when $x = 0$ the complex is fully ionic,

while when $x = \frac{1}{2}$ the complex is fully covalent.

B - Results and Analysis

The Ultra Violet and visible spectra of hydrogenase have been obtained on the Perkin-Elmer 350 Spectrophotometer, Fig. IV.1. The protein shows two absorptions in the U.V. due to the π -electron systems of the aromatic groups. While the iron in the enzyme shows 4 transitions in the visible region of the Spectrum. The results have been analysed and tabulated, Table IV.2.





Sugano-Tanabe d^5 ion diagram. (Not drawn to scale).

TABLE IV.1

Oscillator strengths and extinction co-efficients of different transitions.

	$\sim f$	$\sim \epsilon$
spin-forbidden, Laporte-forbidden	1×10^{-7}	1×10^{-1}
spin-allowed, Laporte-forbidden	1×10^{-5}	10
spin-allowed, Laporte-forbidden + p - d mixing	1×10^{-3}	1×10^2
spin allowed, Laporte-forbidden + Intensity stealing	1×10^{-2}	1×10^3
spin allowed, Laporte-allowed	1×10^{-1}	1×10^4

TABLE IV.2

Oscillator strengths and transition moments of hydrogenase excitations

$\lambda \text{ m}\mu$	$\nu \text{ cm}^{-1}$	$\sim \epsilon$	$\Delta\nu \text{ cm}^{-1}$	$\sim f$	$\sim Q$
245	40,800	-	-	-	-
285	35,000	-	-	-	-
355	28,100	2	500	4.6×10^{-7}	1.2×10^{-6}
410-415	24,300-24,100	12	2,100	1.0×10^{-4}	1.6×10^{-5}
550	18,200	3	400	5.0×10^{-6}	2.5×10^{-6}
720	13,900	1	800	3.0×10^{-6}	2.1×10^{-6}

The d^5 Sugano-Tanabe diagram Fig. IV.2, was used and the energy levels have been assigned spectroscopic terms.

$$\begin{array}{ll}
 \langle N_1 | \text{ at } 13,900 \text{ cm}^{-1} & 4T_{1g}(G) \leftarrow {}^6A_{1g} \\
 \langle N_2 | \text{ at } 18,200 \text{ cm}^{-1} & 4T_{2g}(G) \leftarrow {}^6A_{1g} \\
 \langle N_3 | \text{ at } 24,300 \text{ cm}^{-1} & 4E_g + 4A_{1g}(G) \leftarrow {}^6A_{1g} \\
 \langle N_4 | \text{ at } 28,100 \text{ cm}^{-1} & 4T_{2g}(D) \leftarrow {}^6A_{1g}
 \end{array}$$

The β value has been estimated from the ratio of the energy of term to that of the free ion

$$\beta = \frac{24,300}{32,800} = 0.74$$

then α can be estimated from equation (15) and we obtain

$$\alpha = 0.17.$$

In other words the ferric ion in the enzyme has 34% covalent bonding character and 66% ionic bonding character.

Comparing the oscillator strengths of Table IV.2, to those of Table IV.1, we can arrive at the following conclusions:

- 1 - $\langle N_1 |$ and $\langle N_2 |$ are spin-allowed, Laporte forbidden transition
- 2 - $\langle N_3 |$ is a spin-allowed, Laporte forbidden transition with a certain amount of p - d orbitals mixing.
- and 3 - $\langle N_4 |$ is a spin-forbidden, Laporte forbidden transition.

C - Theory of Electron Spin Resonance Spectroscopy

A spinning electron gives rise to two effects: an angular momentum and a magnetic moment (equations (1) and (2) respectively).

$$M_w = \hbar S \quad (1)$$

$$M_e = \gamma \hbar S = -g \beta S \quad (2)$$

where $\hbar$ is Planck's constant, h , divided by 2π , γ is the magnetogyric ratio, g is a constant, and β is the Bohr magneton unit

$$\beta = \frac{e \hbar}{2 m c} \quad (3)$$

the g-value for a free electron is 2.0032.

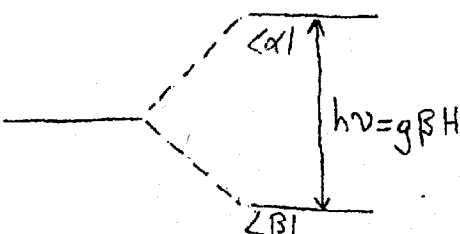
However, a spin is quantized and m_s , the spin quantum number can be:

$$m_s = \pm 1/2 \quad (4)$$

the degeneracy of the spin may be removed by applying a magnetic field, H. The hamiltonian for the system can be written then:

$$\mathcal{H} = g \beta \vec{H} \cdot \vec{S} \quad (5)$$

and a signal is observed only when the energy required to separate these levels has been supplied.

$$h\nu = g\beta H \quad (6)$$


Let these levels be called $\langle\alpha|$ and $\langle\beta|$. The intensity of these transitions can be calculated by the use of the probability theory and applying the Boltzmann distribution law. Let N_α and N_β be the number of spins in each quantum state $\langle\alpha|$ and $\langle\beta|$:

$$\text{then} \quad N_\alpha + N_\beta = N \quad (7)$$

$$\text{and} \quad \frac{N_\alpha}{N_\beta} = e^{-\frac{\Delta E}{kT}} = e^{-\frac{g\beta H}{kT}} \quad (8)$$

$$\text{or} \quad N_\alpha = \frac{1}{2} N \left(1 - \frac{g\beta H}{kT} \right) \quad (9)$$

$$N_\beta = \frac{1}{2} N \left(1 + \frac{g\beta H}{kT} \right) \quad (10)$$

The transition probability $P_{\alpha\beta}$ for the perturbing field V is

$$P_{\alpha\beta} = \frac{2\pi}{\hbar} [\langle\alpha|V|\beta\rangle]^2 \delta(E_\beta - E_\alpha - h\nu) \quad (11)$$

and equation (11) will be zero when:

$$\delta(E_\beta - E_\alpha - h\nu) = 0 \quad (12)$$

or
$$E_\beta - E_\alpha = h\nu$$

For a transition metal ion, a many-electron system, the hamiltonian may be written as:

$$\mathcal{H} = \mathcal{H}_{G.I.} + \mathcal{H}_{C.F.} + \mathcal{H}_{S.L.} \quad (13)$$

where $\mathcal{H}_{G.I.} = \sum_i \left(\frac{p_i^2}{2m} - \frac{Z_i e^2}{r_i} \right)$, the gaseous ion hamiltonian, (14)

$\mathcal{H}_{C.F.} = \sum_i \frac{e_{ij}^2}{r_{ij}^2}$, the crystal field hamiltonian, (15)

and $\mathcal{H}_{S.L.} = \sum_i \xi(r_i) \hat{l}_i \cdot \hat{s}_i$, the spin-orbit interaction hamiltonian (16)

and $\xi(r_i) = \frac{\hbar^2}{2m^2 c^2} \frac{1}{r_i} \frac{\partial U(r)}{\partial r_i}$ (17)

Although our results do not yet allow us to calculate the spin-orbit coupling constant, we will include the analysis for the sake of completeness.

Let us represent state $\langle \alpha |$ and $\langle \beta |$ by their quantum numbers,

then we can write:

$$\langle L M_L' S M_S' | \mathcal{H}_{S.L.} | L M_L S M_S \rangle = \lambda [\langle L M_L' S M_S' | \hat{L} \cdot \hat{S} | L M_L S M_S \rangle] \quad (18)$$

so $\lambda M_L M_S = \langle L M_L' S M_S' | \mathcal{H}_{S.L.} | L M_L S M_S \rangle = \sum_i \xi(r_i) m_{l_i}^i m_{s_i}^i$ (19)

and $\lambda = \frac{1}{M_L M_S} \sum_i \xi(r_i) m_{l_i}^i m_{s_i}^i$ (20)

but Hund's rule requires that $\lambda = \pm \frac{1}{2S} \xi$ (21)

so $\lambda = \frac{E(J) - E(J-1)}{J}$ where $J = L + S$

D - Technique and Results

The E.S.R. spectra of hydrogenase solutions under different conditions have been obtained on an X-band Varian V4502-15 spectrometer. Four spectra are included, Fig. IV.3 and 4. Table IV.3 shows the conditions under which these spectra have been obtained.

TABLE IV.3

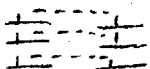
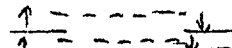
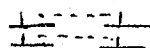
100 KC/s Channel

Solution	T°K	P.A.dB.	M.A.	S.L.	R.	Fig.Ref.
Enzyme+H ₂	77	6	8000	800	1	a
Enzyme reduced	113	10	8000	500	1	b
Enzyme+H ₂ O ₂	193	10	8000	1000	1	c
Enzyme+O ₂	193	10	4000	630	1	d

Field Sweep 1KG/5 minutes centred at 3.3 KG.

Fig. IV.3.a, shows that the enzyme does not produce free radicals, since no signal at $g=1.98$ was observed. Also the oxidised enzyme does not show any signal. The signal in Fig. B.4.c, is due to the free radical OH. While the reduced enzyme shows a signal at a rather high g -value $g=2.06$. This leads us to assume that the iron atom is highly distorted in the complex.

The reason why no signal was observed in the oxidised form of hydrogenase is not known. But to follow the idea of Hall and co-worker (114), we will assume that two or more pairs of ferric ions couple with each other in a certain fashion leading to a total spin of zero.



OR



No signals at high fields were observed on either forms of the enzyme (reduced and oxidised.)

66.

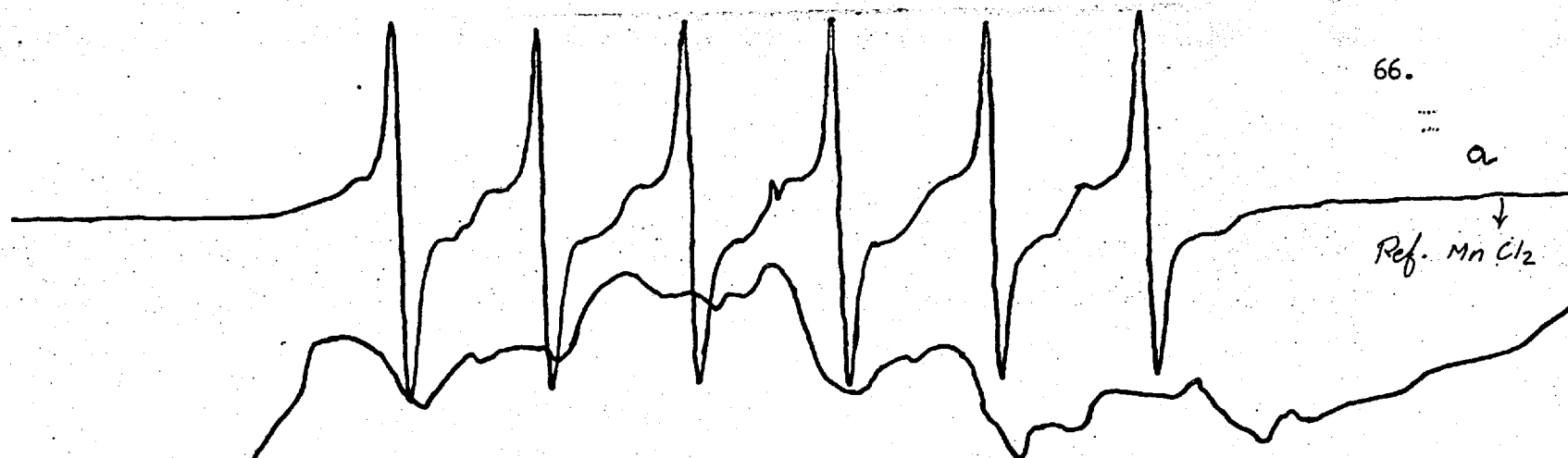
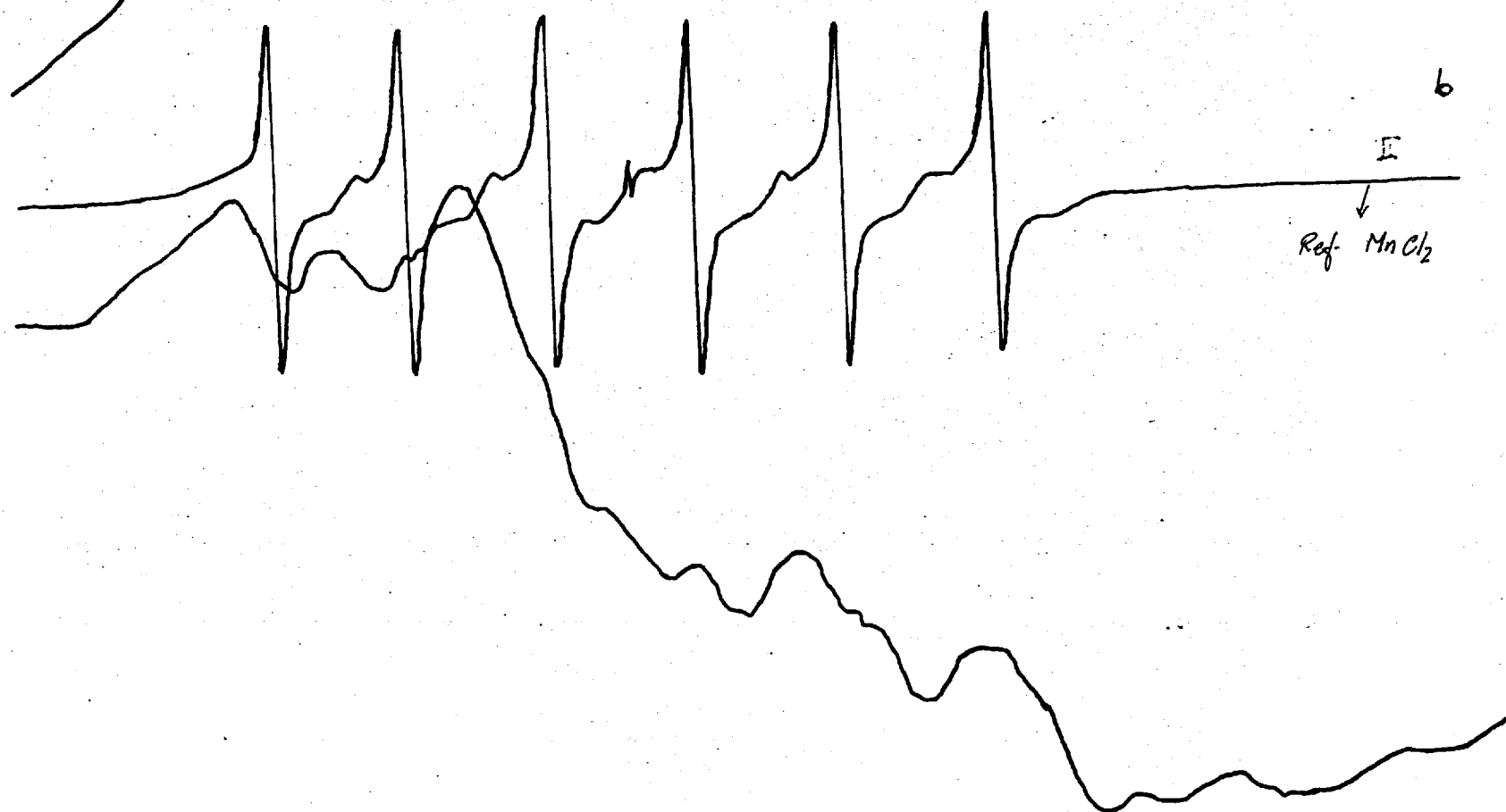


Fig. IV.3.



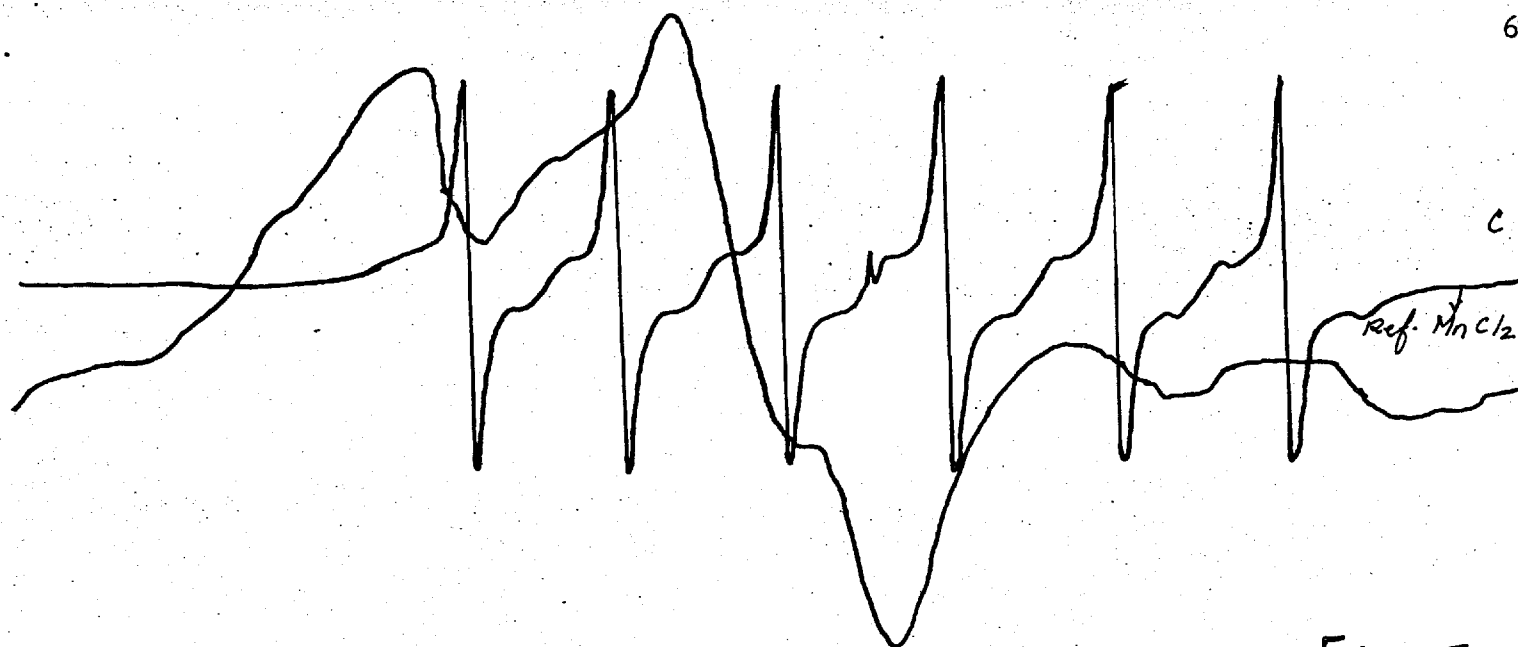
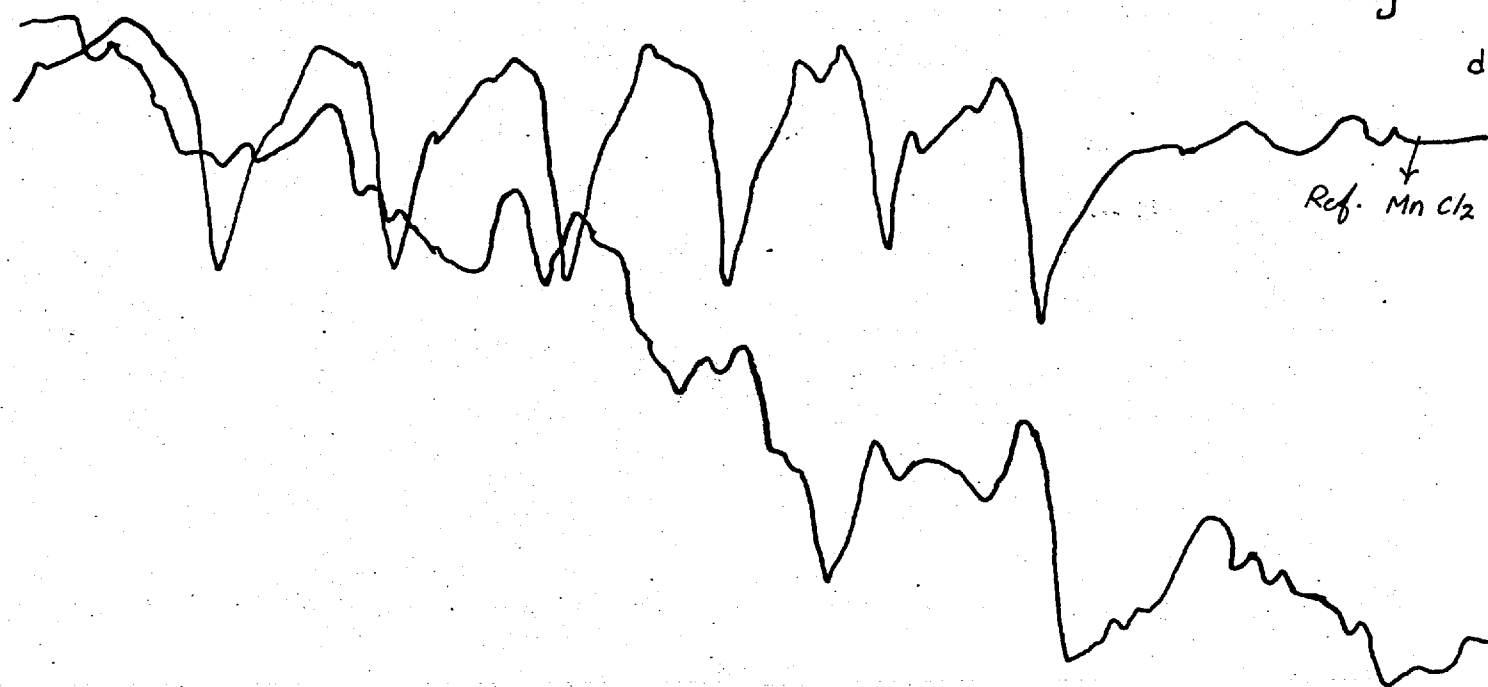


Fig. IV.4.



CHAPTER FIVE

Kinetics of Hydrogenase Catalysed Reactions

A - Introduction

Hydrogenase is the enzyme catalyzing the oxidation of hydrogen in biological systems . Like all other enzymes, hydrogenase is reversible; it is reported that hydrogenase produces hydrogen from easily oxidizable biomolecules (41)

Although hydrogenase is found in many biological systems and has been purified to a certain extent (57), its kinetic properties have not been studied extensively. The only reactions that have been studied in some detail, are the reduction of ferricyanide (40), methylene blue (39), the deuterium exchange (75), and the ortho-para conversion (64). Two methods of assaying hydrogenase activity have been used: the Warburg respirometric method based on the uptake of hydrogen gas from a closed cell and following the change of pressure in the cell (119); and the second method is based on the enrichment in heavy water when the reactions are conducted in an atmosphere of heavy hydrogen. The first of these methods is most inadequate for detailed kinetic studies because of the many sources of error, while the second is sound and elegant but requires much elaborate equipment.

Hydrogenase is of particular interest since it is present in most nitrogen-fixing systems; this is not to say however, that they require it.

The ability of hydrogenase to reduce certain ions is used in the present work to assess the activity of the enzyme. The reactions

are simple, involving the production of a basic group, the rate of production of which is measured by the consequent change in pH. Three ions have been studied: nitrate, nitrite, and sulfate ions.

B - Theory and Mechanism

Hydrogenase-catalyzed reductions can only occur through two mechanisms, one involving a hydride ion and the other a free radical namely the hydrogen atom. Both of these will be discussed in the following paragraphs.

1 - Free radical mechanism of hydrogenase catalysis

Let E, S, X, and P represent the enzyme, substrate, an intermediate, and the product respectively. Then we can write:



Mathematically the rates can be written as:

$$\frac{d[H]}{dt} = k_1[E][H_2] - k_2[E][H]^2 - k_3[S][H]^n + k_4[X] \quad (4)$$

$$\frac{d[X]}{dt} = k_3[S][H]^n - (k_4 + k_5)[X] \quad (5)$$

$$\text{and } \frac{d[P]}{dt} = k_5[X] \quad (6)$$

When the steady state is reached, the use of the steady state approximation, namely that the rate of formation of intermediates equals to zero, is justified. Equations (4) and (5) yield:

$$k_1[E][H_2] - k_2[E][H]^2 - k_3[S][H]^n + \frac{k_3 k_4 [S][H]^n}{k_4 + k_5} = 0 \quad (7)$$

$$\text{and} \quad X = \frac{k_3 [S][H]^n}{k_4 + k_5} \quad (8)$$

If we assume that the concentration of the free radical is very small, terms involving this concentration to high powers, ($n > 2$), may be neglected, and equation (7) gives:

$$[H] = \left(\frac{k_1}{k_2} \right)^{\frac{1}{2}} \cdot [H_2]^{\frac{1}{2}} \quad (9)$$

Combining equations (8) and (9) we obtain the expression:

$$[X] = \left(\frac{k_1}{k_2} \right)^{\frac{n}{2}} \cdot k_3 k_5 / (k_4 + k_5) \cdot [S][H]^{\frac{1}{2}n} \quad (10)$$

and the rate of production of products is given by:

$$\frac{d[P]}{dt} = V = \left(\frac{k_1}{k_2} \right)^{\frac{1}{2}n} \cdot k_3 k_5 / (k_4 + k_5) \cdot [S][H]^{\frac{1}{2}n} \quad (11)$$

The rate then, should be independent of the enzyme concentration, and proportional to the concentration of the substrate and the hydrogen pressure to the power $n/2$.

2 - Hydride ion mechanism for hydrogenase catalysis

Using the same notations as in paragraph B - 1, we can write the following scheme:



The rates will be given by:

$$\frac{d[E-H_2]}{dt} = k_1[E][H_2] - k_2[E-H_2] - k_3[E-H_2][S] + k_4[X] \quad (15)$$

$$-\frac{d[X]}{dt} = k_3[E-H_2][S] - (k_4 + k_5)[X] \quad (16)$$

$$\text{and } -\frac{d[P]}{dt} = k_5[X] \quad (17)$$

Using the steady state approximation, equations (15) and (16) become

$$k_1[E][H_2] - k_2[E-H_2] - k_3[E-H_2][S] + k_4[X] = 0 \quad (18)$$

$$\text{and } [X] = \frac{k_3[E-H_2][S]}{k_4 + k_5} \quad (19)$$

Combining equations (19) and (18) we obtain:

$$[X] = \frac{k_1 k_3 [E][H_2][S]}{k_2(k_4 + k_5) + k_3 k_5 [S]} \quad (20)$$

and the rate

and the rate expression becomes:

$$-\frac{d[P]}{dt} = v = \frac{k_1 k_3 k_5 [E][H_2][S]}{k_2(k_4 + k_5) + k_3 k_5 [S]} \quad (21)$$

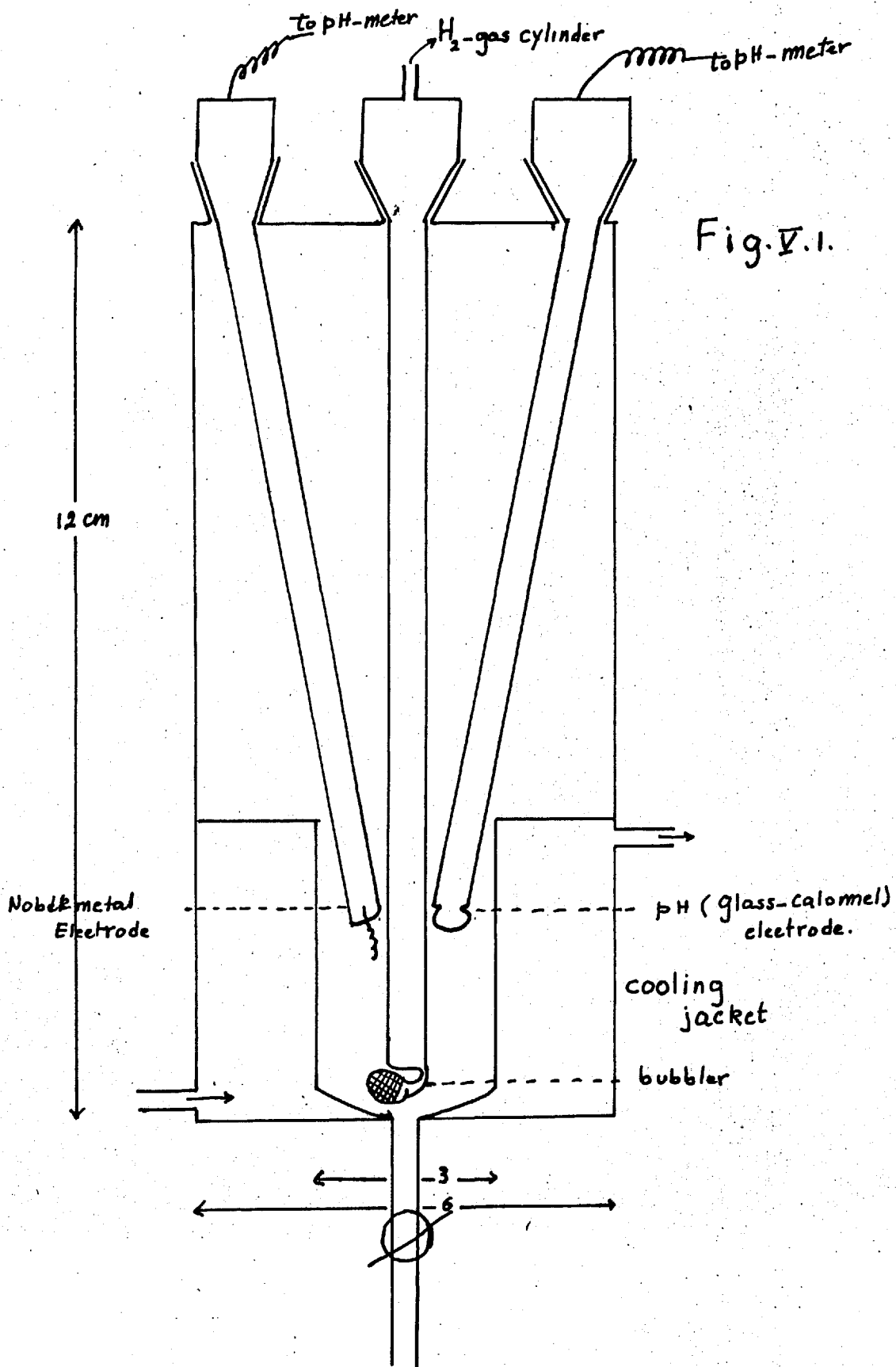
C - Technique, results and analysis

1 - Technique

A solution of known concentration of substrate and enzyme was introduced to a special cell fitted with sockets for two electrodes and a bubbler, Fig.V.1. The initial pH was read and hydrogen bubbling was started; readings were taken every 15 seconds, Fig.V.2. First order plots, $\log (pH - pH_{\infty})$ versus time, were plotted and the observed rate calculated from the slope of the line, Fig.V.3. The pH-meter, a Radiometer pH-M4, was standardized with the National Bureaus of standards buffers (115).

2 - Effect of Enzyme concentration on the observed rate

The effect of enzyme concentration on the rate of nitrite reduction has been studied. It was found that the rate is proportional



to the enzyme concentration when the latter is highly purified, Table V.4. This is in direct contradiction to equation (11), while it is in agreement with equation (22).

3 - Effect of hydrogen ion concentration on the observed rate

The pH-profile of the nitrite reduction reaction has been obtained, Table IV.2, Fig.V.5. The sharp peak is due to tertiary structure configurational changes as predicted from Chapter three.

4 - Effect of substrate concentration on the observed rate

The rates of reduction of three ions, nitrite, nitrate, and sulfate have been studied over a wide range of concentration. These show self-inhibition after reaching a maximum, Fig. V.6-8., Table V. 3-5. This optimal concentration was found, from initial studies, to be dependent on the enzyme concentration, as predicted by equation (22).

5 - Analysis of results

From plots V.4-7, it is obvious that the mechanism involving a free radical is not the true mechanism. However the mechanism involving a hydride ion predicts both the dependence on enzyme concentration, Fig.V.4, and the peculiarities of substrate self-inhibition, Fig.V.6-8. We will assume it is the actual mechanism, and try to analyze the results in the light of the Michaelis-Menten scheme (106).

The Michaelis-Menten scheme gives the following equations for V and V_I , the rates of the reaction with and without an inhibitor respectively:

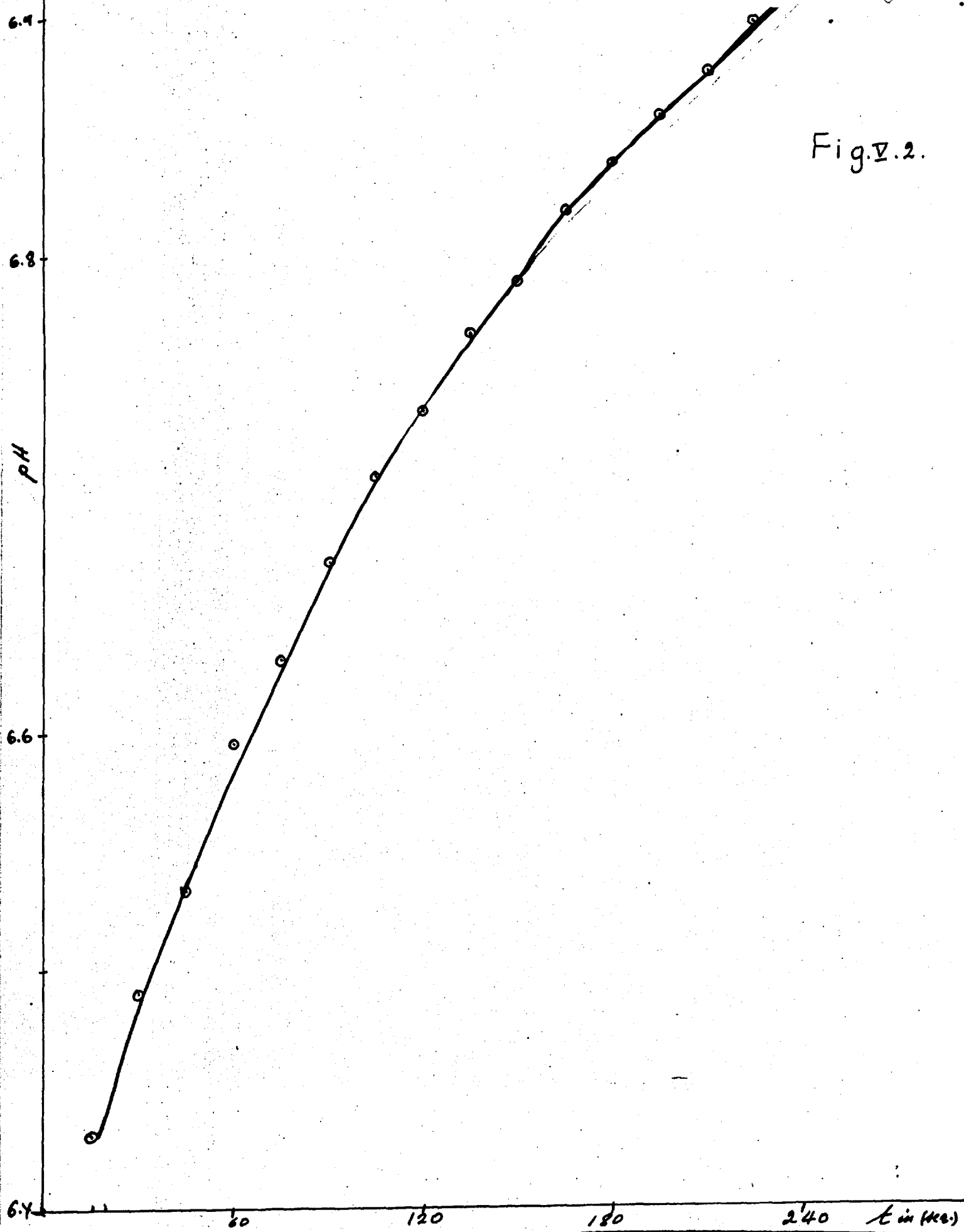


Fig. 3.

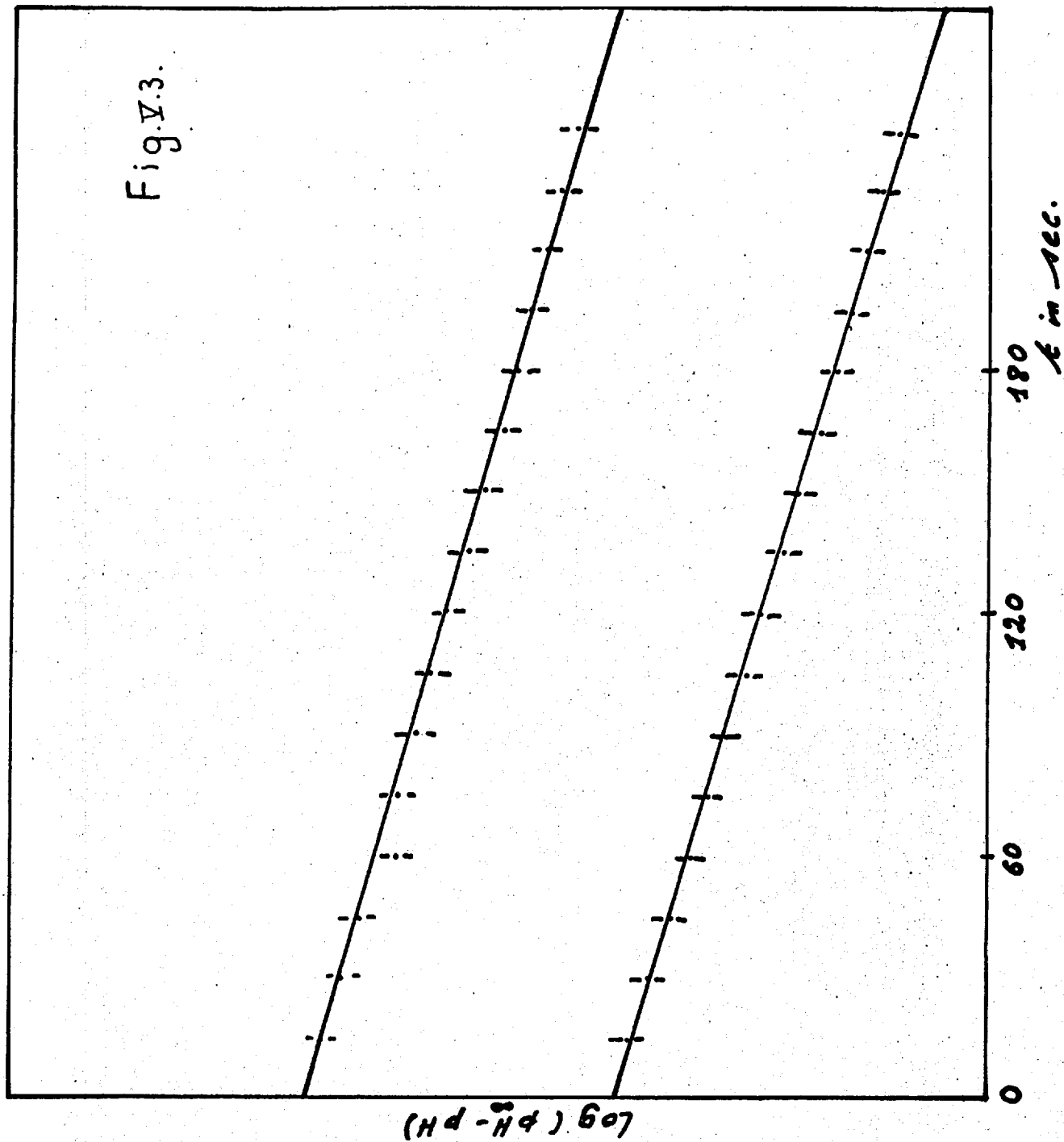
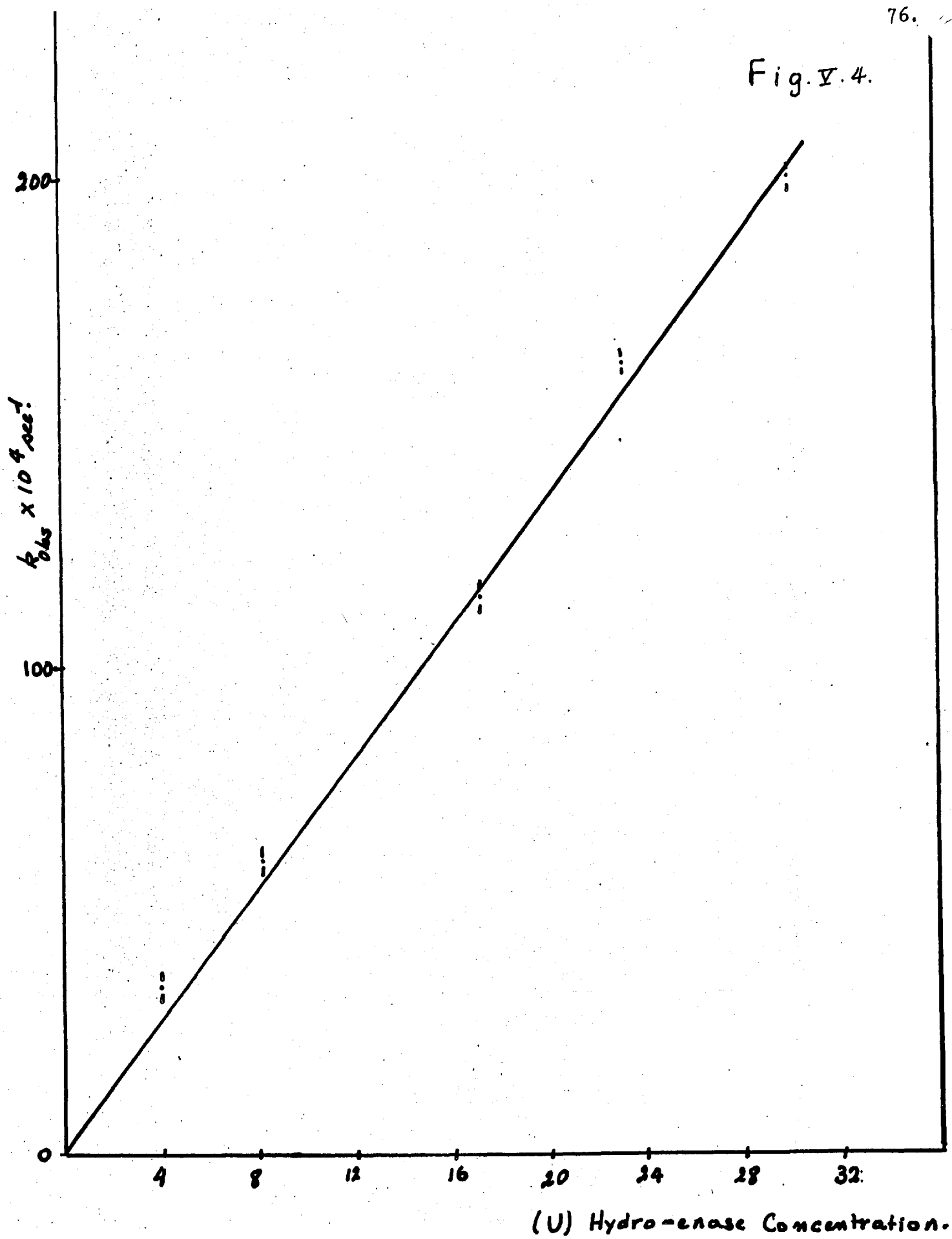
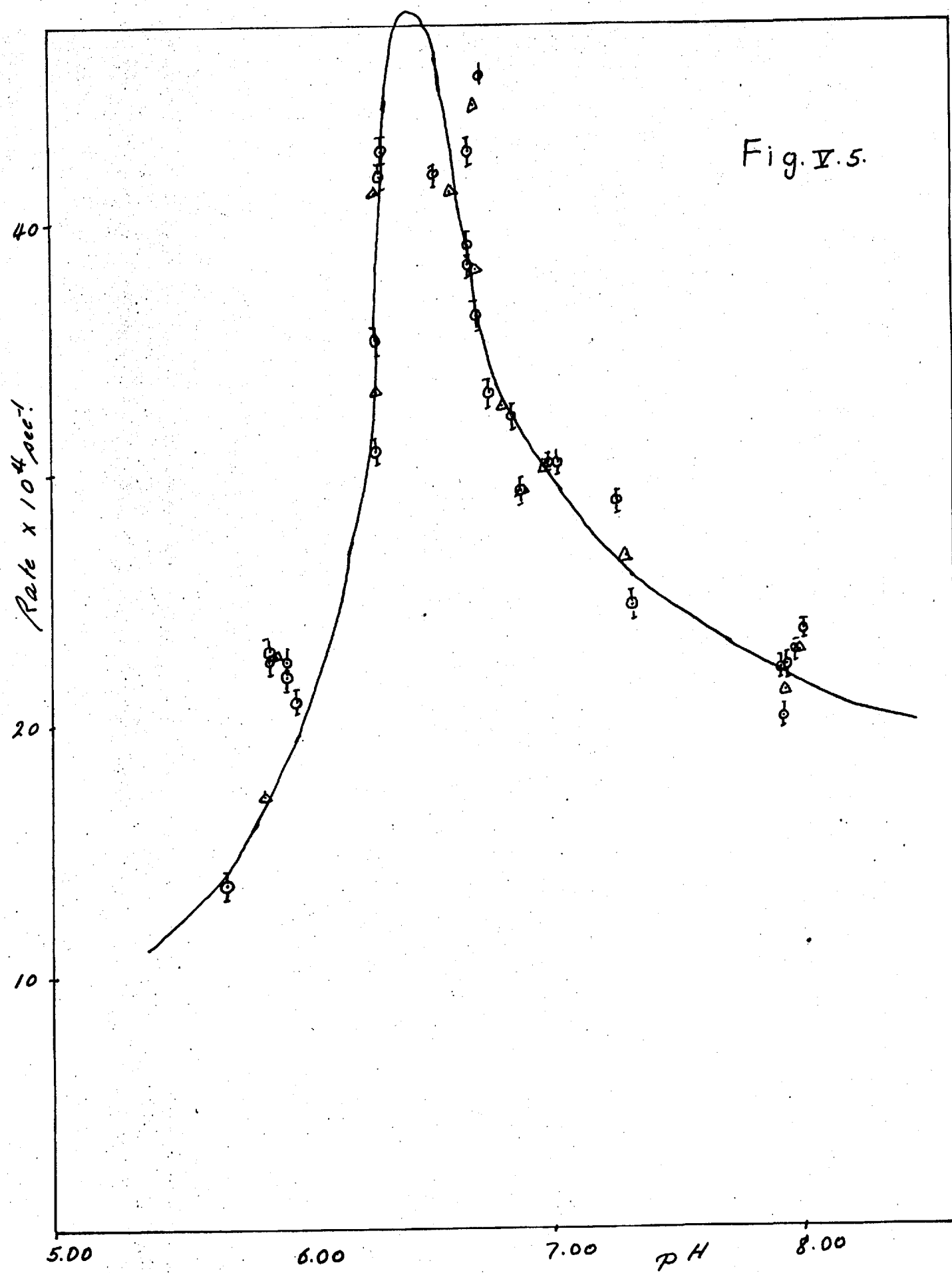
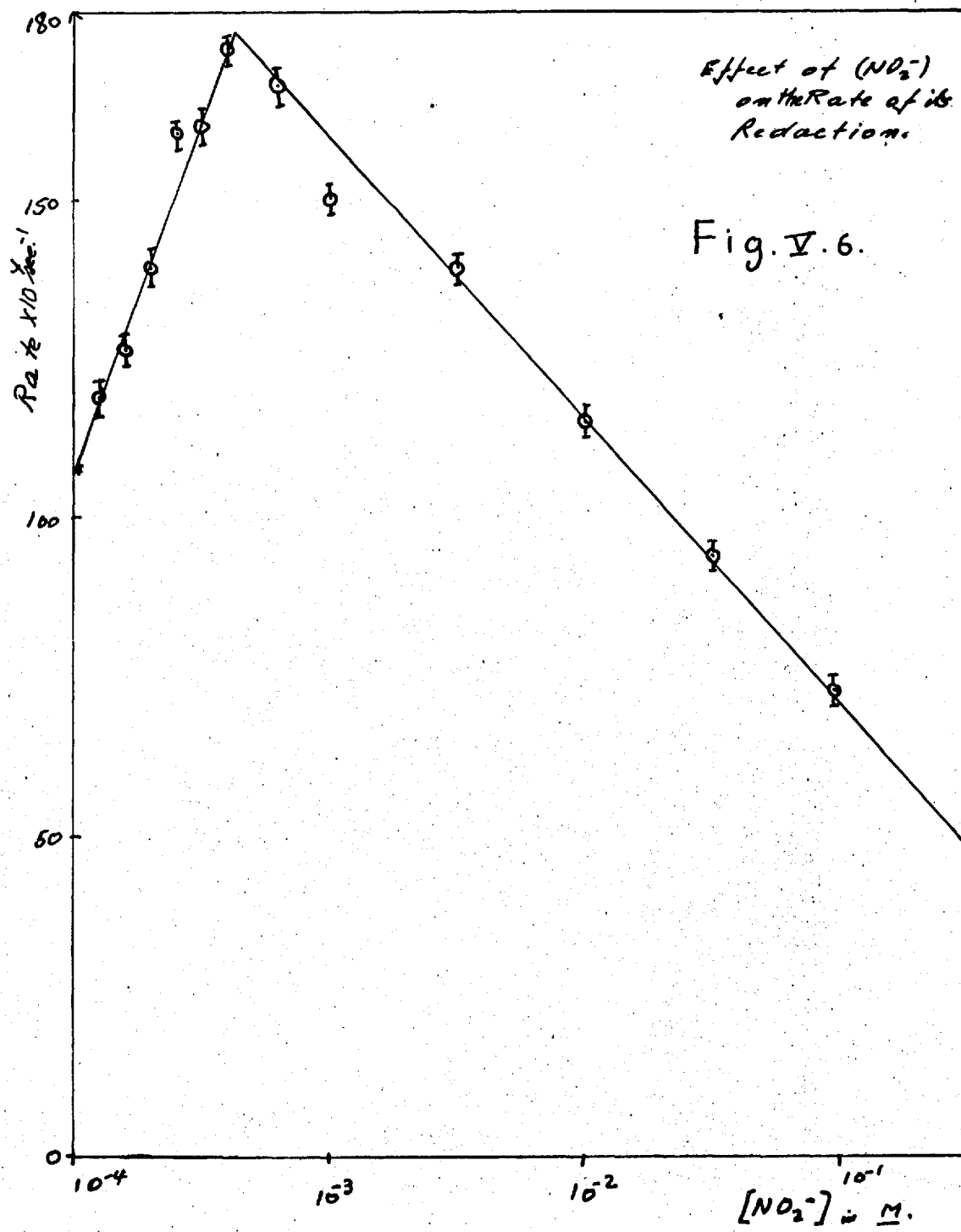


Fig. V. 4.







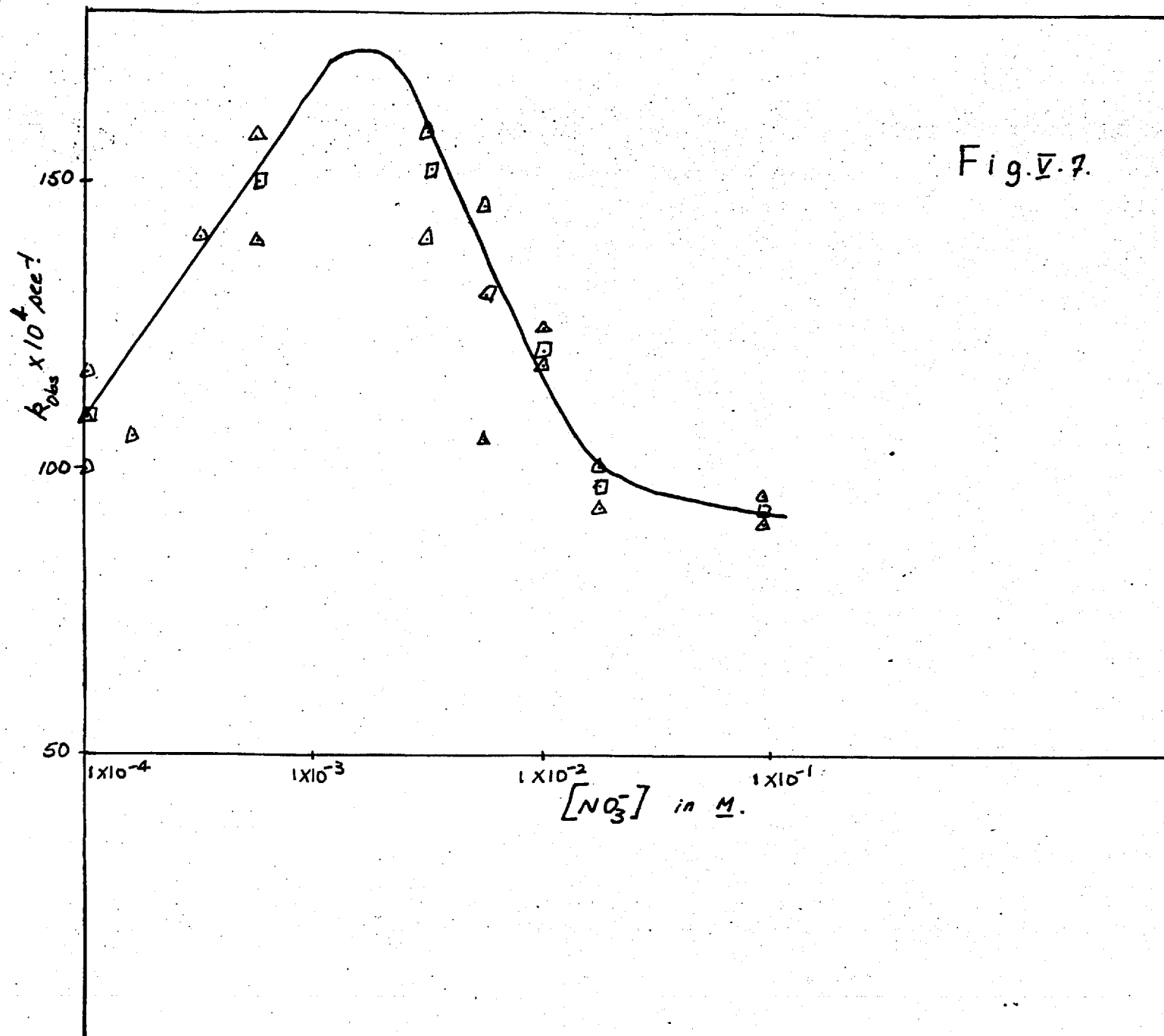
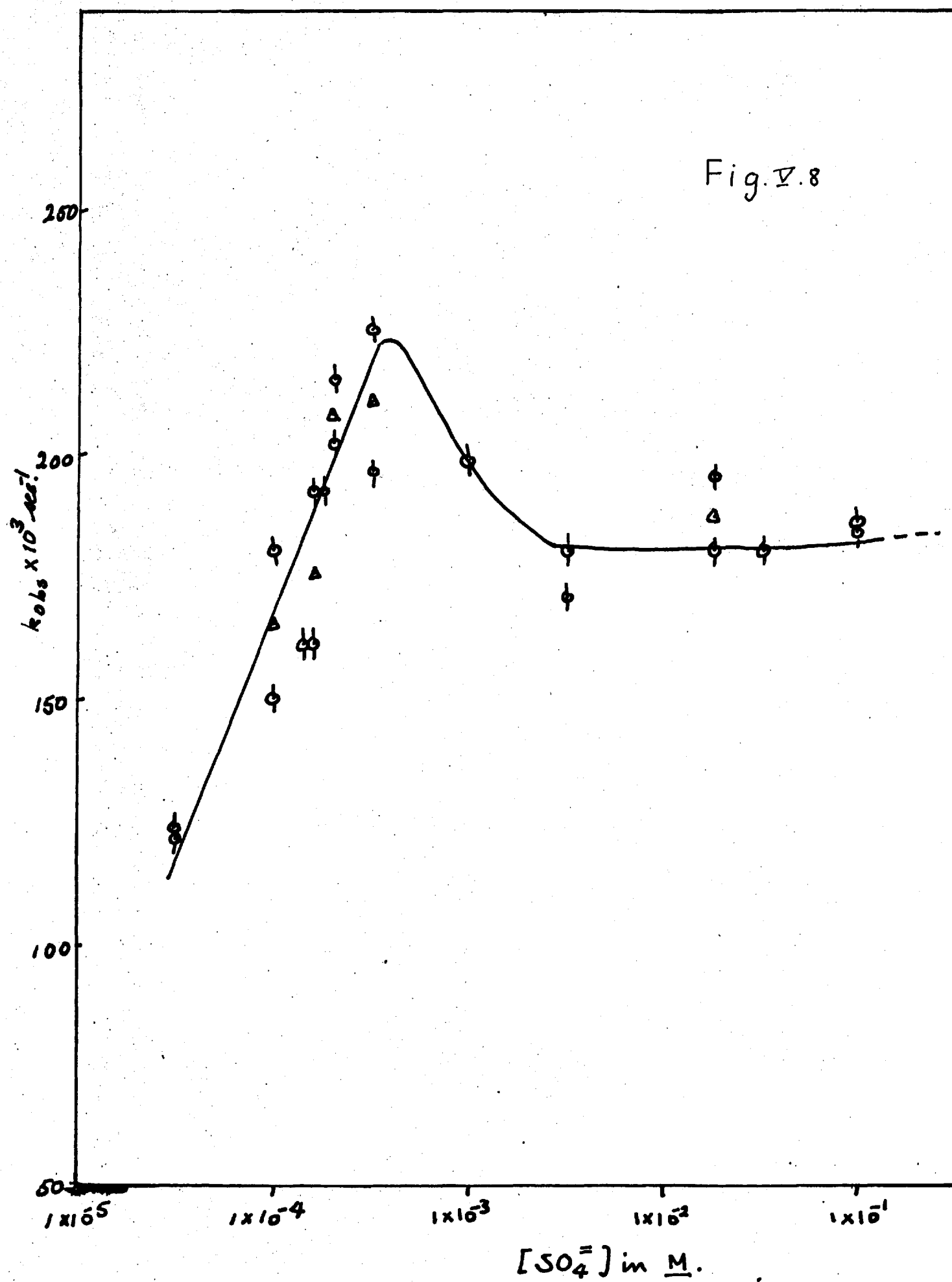


Fig. V.7.



$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_s}{V_{\max} [S]} \quad (23)$$

$$V + K_s \frac{V}{[S]} = V_{\max} \quad (24)$$

$$\text{and } \frac{1}{V_I} = \frac{1}{V_{\max}} + \frac{K_s}{V_{\max}} \left\{ 1 + \frac{[I]}{K_I} \right\} \quad (25)$$

$$\text{where } K_s = \frac{k_2 (k_4 + k_5)}{k_3 k_5} \quad (26)$$

$$\text{and } K_I = \frac{k_2 (k_4' + k_5')}{k_3 k_5} \quad (27)$$

where the prime denotes the intrinsic rate constants in the presence of aninhibitor.

We can assume that the increasing part of plot V.6 represents the rate of the reaction in the absence of any inhibitor. Then plots of equations (23) and (24) using the results of Table V.6, give Fig. V. 9 and 10.

$$K_s = 1.73 \times 10^{-4} \text{ sec.}^{-1}$$

$$V_{\max} = 220 \times 10^{-4} \text{ sec.}^{-1}$$

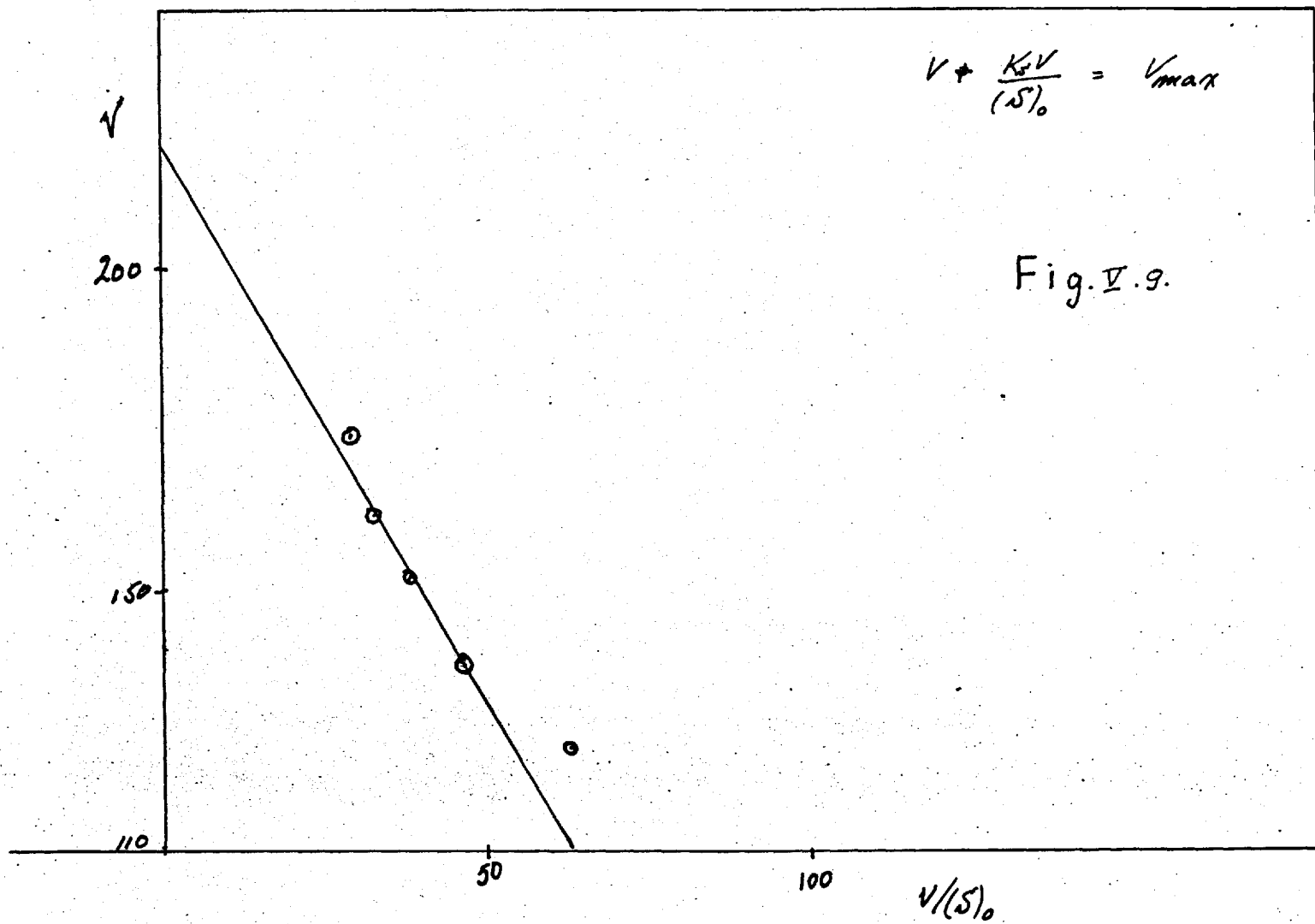
On the other hand we can assume that the decreasing part of plot V.6, represents the rate of the reaction with an inhibitor concentration given by

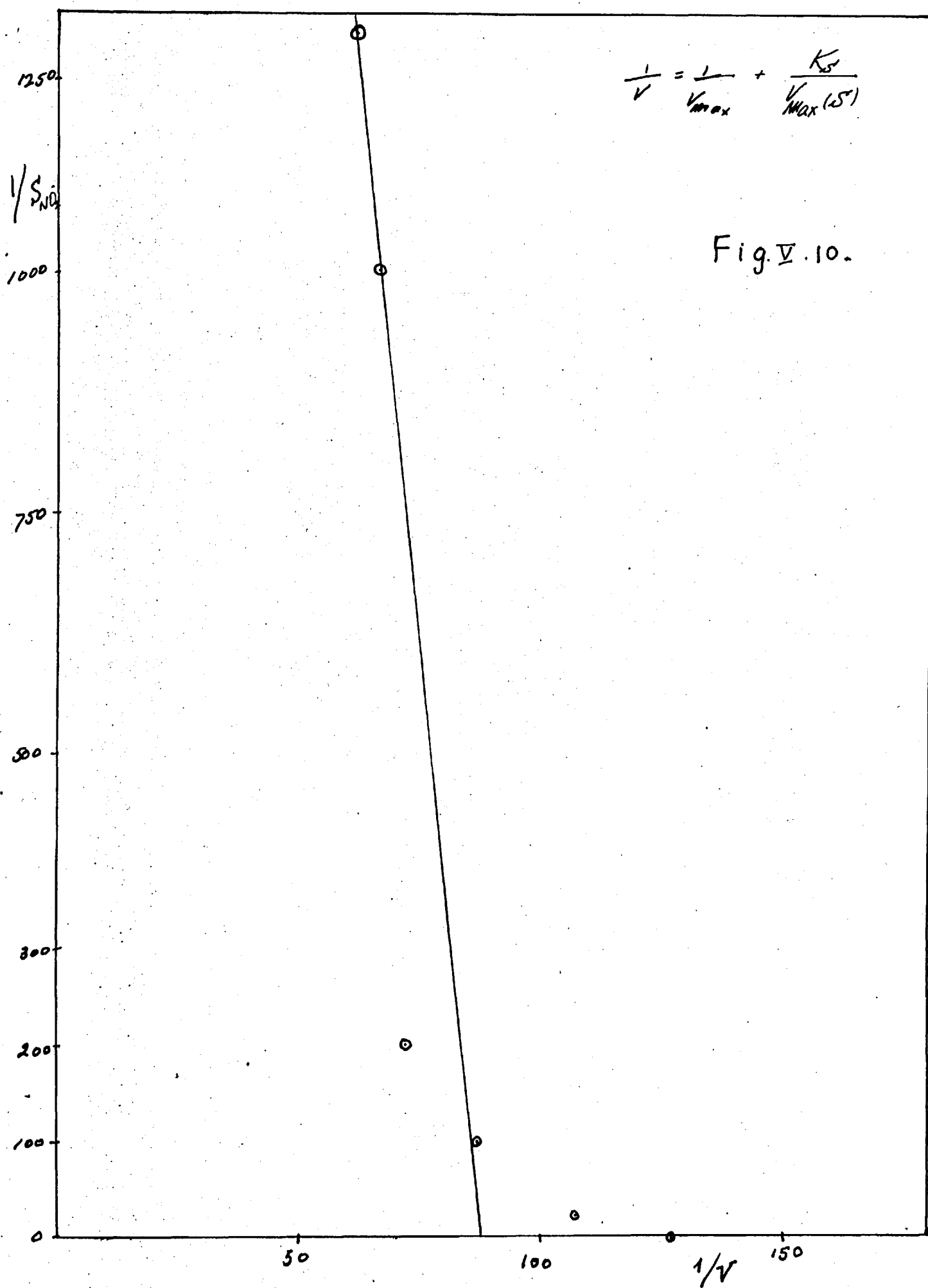
$$[I] = [S] - [S]_{\max}$$

Where $[S]$ and $[S]_{\max}$ are the actual and the optimal concentration of the substrate. A plot of equation (25) gives us Fig.V.11.

$$K_I = 1.03 \times 10^{-4} \text{ sec.}^{-1}$$

$$V_{\max} = 185 \times 10^{-4} \text{ sec.}^{-1}$$





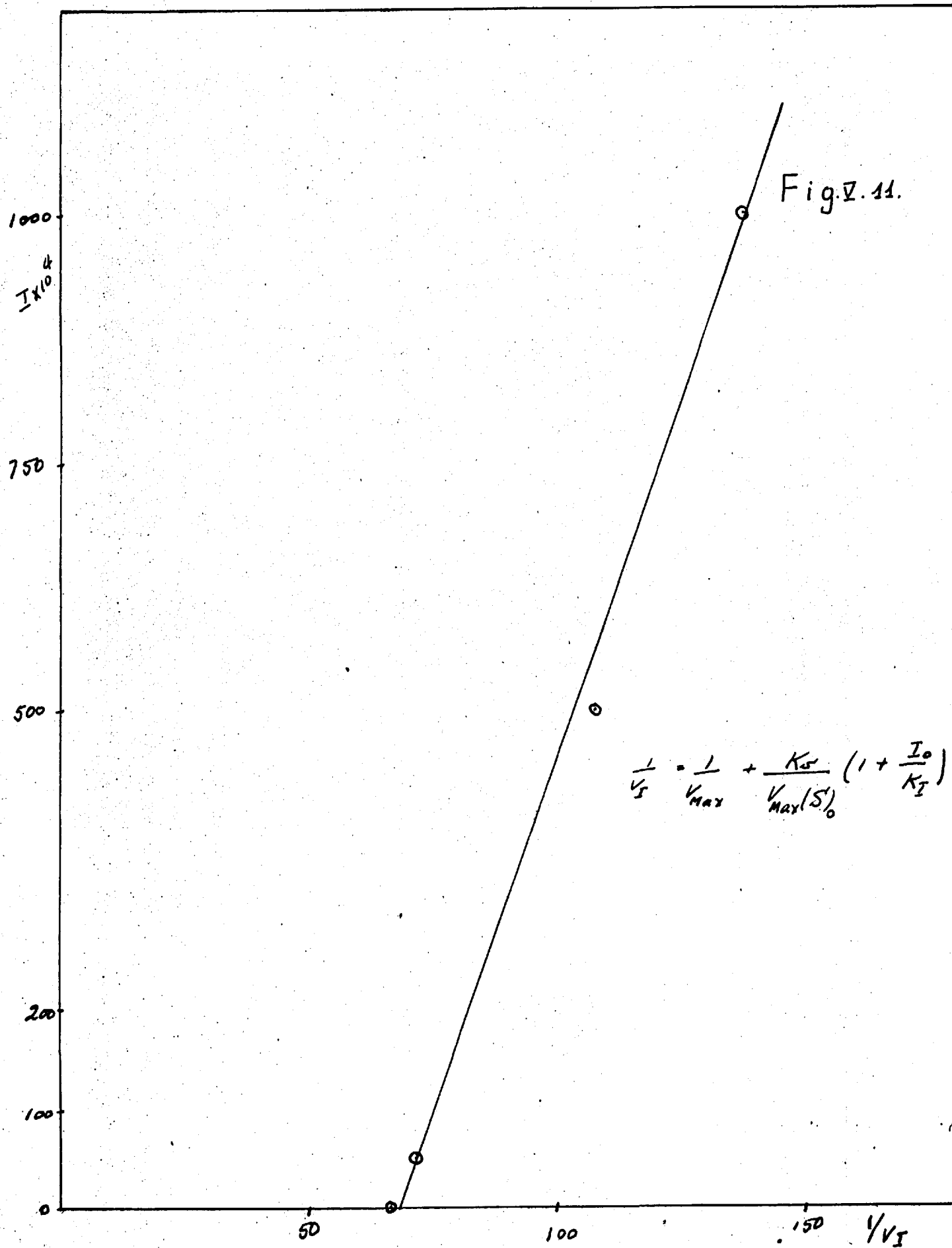


TABLE V. 1.Effect of hydrogenase concentration on the k_{obs} .
 $[E] \times 10^6 \text{ gmN}_2/\text{ml}$
 $k_{obs} \times 10^4 \text{ sec}^{-1}$

4.0	34
8.2	60
17.2	114
23.1	162
30.3	200

$$[\text{NO}_2^-] = 5 \times 10^{-4}$$

pH ~ 6 , S.T.P.TABLE V. 2.Effect of pH on k_{obs}

pH range	pH	$k_{obs} \times 10^4 \text{ sec}^{-1}$	$k_{obs} \times 10^4 \text{ sec}^{-1}$	pH
5.50-5.93	5.72	13.7		
5.79-6.17	5.98	21.0	17.4	5.85
5.75-6.12	5.84	22.6		
5.72-6.02	5.89	23.0	22.8	5.90
5.99-6.58	6.30	31.1	33.3	6.30
5.95-6.56	6.30	35.4		
6.05-6.54	6.30	43.1	41.6	6.30
6.09-6.53	6.31	40.1		
6.21-6.87	6.54	40.1	39.3	6.60
6.38-6.93	6.66	38.4		
6.39-7.10	6.75	33.3	32.9	6.80
6.38-7.10	6.84	32.4		
6.38-6.95	6.67	39.2	38.2	6.69
6.45-6.95	6.70	37.2		
6.43-6.92	6.72	43.5	44.8	6.70
6.47-6.97	6.88	46.0		
6.64-7.11	6.88	29.4	24.4	6.88
6.82-7.22	7.02	30.7	30.7	7.07
6.78-7.22	6.99	30.7		
7.12-7.43	7.26	29.0	26.9	7.29
7.19-7.46	7.32	24.8		
7.83-8.03	7.92	22.2	21.4	7.93
7.87-7.97	7.92	20.5		
7.88-8.11	8.00	23.9	23.4	7.98
7.86-8.08	7.97	23.0		

$$[E] = 92.4 \times 10^{-6} \text{ gmN}_2/\text{ml}$$

$$[\text{NO}_2^-] = 5.0 \times 10^{-2} \text{ M}$$

S.T.P.

TABLE V. 3.Effect of $[\text{NO}_2^-]$ on k_{obs}

$[\text{NO}_2^-] \text{ M}$	$k_{\text{obs}} \times 10^4 \text{ sec}^{-1}$
1.0×10^{-4}	119
2.0×10^{-4}	126
3.0×10^{-4}	139
4.0×10^{-4}	161
5.0×10^{-4}	162
6.0×10^{-4}	174
8.0×10^{-4}	168
1.0×10^{-3}	150
5.0×10^{-3}	139
1.0×10^{-2}	115
5.0×10^{-2}	94
1.0×10^{-1}	73

$[E] = 23.1 \times 10^{-6} \text{ gm N}_2/\text{ml}$
 $\text{pH} \sim 6$, S.T.P.

TABLE V. 4.Effect of $[\text{NO}_3^-]$ on k_{obs}

$[\text{NO}_3^-] \text{ M}$	$k_{\text{obs}} \times 10^4 \text{ sec}^{-1}$
1.0×10^{-4}	110; 110; 126
2.0×10^{-4}	105
5.0×10^{-4}	141
7.5×10^{-4}	140; 150; 158
5.0×10^{-3}	140; 160
7.5×10^{-3}	105; 146
1.0×10^{-2}	118; 125
2.5×10^{-2}	93; 100
1.0×10^{-1}	90; 95

$[E] = 1.16 \times 10^{-5} \text{ gm N}_2/\text{ml}$
 $\text{pH} \sim 6$,
 S.T.P.

TABLE V. 5.Effect of $[SO_4^{=}]$ on k_{obs}

$[SO_4^{=}]$ M	$k_{obs} \times 10^4 \text{sec}^{-1}$
5.0×10^{-5}	121; 123
1.0×10^{-4}	150; 180
1.5×10^{-4}	161; 161
2.0×10^{-4}	161; 192
2.5×10^{-4}	192; 192
3.0×10^{-4}	202; 216
5.0×10^{-4}	196; 225
1.0×10^{-3}	198; 196
5.0×10^{-3}	169; 180
2.5×10^{-2}	180; 195
5.0×10^{-2}	170; 180
1.0×10^{-1}	184; 186

$[E] = 1.16 \times 10^{-5} \text{ gm N}_2/\text{ml}$
 pH ~ 6,
 S.T. P.

TABLE V. 6.A Michaelis-Menten Analysis of the nitrite reduction rates

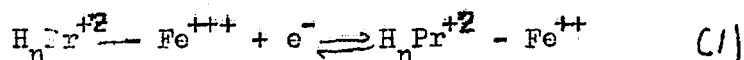
$v \times 10^4$	$[S]$	$1/v$	$1/[S]$	$v/[S]$	$I/[S] - 6.25 \times 10^{-4}$
119	1.0×10^{-4}	84	1×10^4	119	
126	2.0×10^{-4}	97.4	5.0×10^3	63	
139	3.0×10^{-4}	174.6	3.3×10^3	46.4	
161(152)	4.0×10^{-4}	62.2	2.5×10^3	38	
162	5.0×10^{-4}	61.7	2.0×10^3	32.4	
174	6.0×10^{-4}	57.6	1.67×10^3	29	
168	8.0×10^{-4}	61.3	1.25×10^3		1.25×10^{-4}
150	1.0×10^{-3}	66.7	1.0×10^3		3.75×10^{-4}
139	5.0×10^{-3}	71.9	2.0×10^2		4.38×10^{-3}
115	1.0×10^{-2}	89.0	1.0×10^2		9.38×10^{-3}
94	5.0×10^{-2}	107	20		4.94×10^{-2}
73	9.99×10^{-2}	137	10		9.93×10^{-2}

CHAPTER SIX

OXIDATION-REDUCTION POTENTIALS

A - Introduction

Whatever might be the true mechanism of hydrogenase catalyzed reactions, it is thought that at least one oxidation-reduction reaction is involved.



The estimation of the equilibrium constant and the redox potential of the first reaction seems interesting, since it may throw light *on the energetics of other* possible hydrogenase-catalyzed reactions and also elucidate some other obscure aspects of hydrogenase catalysis.

B - Theory

i - General Theory

Two species of different oxidation states in the same medium, will tend to reach equilibrium, at which point the energy of the system is minimum. Let system (1) be represented by:



Where Red., and ox. stand for the reduced and oxidised states respectively. Then the equilibrium constant may be written.

$$K = \frac{[\text{OX}]}{[\text{Red}]} \quad (4)$$

At equilibrium, the free energy of the system is given by:

$$\begin{aligned} \Delta F &= -RT \ln K \\ &= -RT \ln [\text{OX}]/[\text{Red}]. \end{aligned} \quad (5)$$

and from electrochemical considerations, ΔF may be expressed as:

$$\Delta F = -nF \cdot \Delta E = nF(E - E_0) \quad (7)$$

combining equations (6) and (7) we obtain the well known Nernst's equation:

$$E = E_0 + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]} \quad (8)$$

ii- Effect of ionic strength

It is obvious that the above derivations yield non-thermodynamic constants, since all the equations were expressed in terms of concentrations and not activities.

The thermodynamic treatment is as follows:

$$E = E_0 + \frac{RT}{nF} \ln \left(\frac{A_0}{A_R} \right) \quad (9)$$

$$= E_0 + \frac{RT}{nF} \ln \left(\frac{f_0}{f_R} \right) \frac{[O]}{[R]} \quad (10)$$

$$= E_0 + \frac{RT}{nF} \ln \frac{[O]}{[R]} + \frac{RT}{nF} \ln \left(\frac{f_0}{f_R} \right) \quad (11)$$

If the ratio of $[O]/[R]$ is unity then equation (11) becomes;

$$E = E_0 + \frac{RT}{nF} \ln \frac{f_0}{f_R}$$

If the activity coefficient is given by the Debye-Hückle theory:

$$\log f = -\frac{1}{2} z^2 (\mu)^{\frac{1}{2}} \quad (12)$$

where μ is the ionic strength; more refined derivations give f as:

$$\log f = -\frac{0.5 (\mu)^{\frac{1}{2}}}{1 + (\mu)^{\frac{1}{2}}} \quad (12)$$

rearranging equation (11) and combining it with equations (12)

and (13) we obtain respectively:

$$169.5 (E - E_0) = \frac{1}{2} (2z + 1) (\mu)^{\frac{1}{2}} \quad (13)$$

$$\text{and } 169.5 (E - E_0) = \frac{1}{2} (2z + 1) (\mu)^{\frac{1}{2}} / (1 + (\mu)^{\frac{1}{2}}). \quad (14)$$

plots of $(E-E_0)$ versus $\sqrt{\mu}$ or $\sqrt{\mu}/(1+\sqrt{\mu})$ should give an approximate measure of the charge on the iron.

iii - Effect of Temperature

Equations 5, 6, and 7 are equivalent to

$$\Delta F = \Delta H - T\Delta S \quad (16)$$

and from thermodynamic considerations:

$$\Delta S = - \left(\frac{\partial \Delta F}{\partial T} \right)_P \quad (17)$$

where subscript P is to denote that the process is at constant pressure. Combining (16) and (17) we obtain:

$$\Delta F = \Delta H + T \left(\frac{\partial \Delta F}{\partial T} \right) \quad (18)$$

while equations (20) and (7) yield:

$$- nFE = \Delta H - TnF \left(\frac{\partial E}{\partial T} \right) \quad (19)$$

$$\text{or } \Delta H = - nF \left[E - T \left(\frac{\partial E}{\partial T} \right) \right] \quad (20)$$

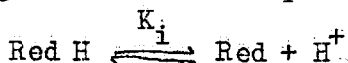
iv Effect of pH

Many redox reactions have been found to be pH-dependent. This is due to the ionizations accompanying the redox process. Let equation (3) be written:



$$\text{then } E = E_0 + \frac{RT}{nF} \ln \frac{[O]}{[R]} \quad (22)$$

Let the accompanying ionization be represented by:



$$\text{then } K_i = \frac{[R][H]}{[RH]} \quad (23)$$

and the total reduced form is

$$[R] = [RH] + [R^-] \quad (24)$$

and equations (23) and (24) yield:

$$K_i = \frac{(R^-)(H)}{(R) - (R^-)} \quad (25)$$

$$\text{or } (R^-) = \frac{(R) K_i}{(H) + K_i} \quad (26)$$

$$\text{then } E_h = E_o + \frac{RT}{nF} \log \left(1 + \frac{(H)}{K_i} \right) \cdot \frac{(O)}{(R)} \quad (27)$$

if $\frac{(O)}{(R)} = 1$ then equation (27) reduces to:

$$E_h = E_o + \frac{RT}{nF} \log \left(1 + \frac{(H)}{K_i} \right) \quad (28)$$

B - Techniques, results and analysis

a - Technique

Four electrodes, three of platinum and one of silver were prepared as described (117). The ferrocyanide-ferricyanide system (118) was used to check their reversibility. It was found that the three platinum electrodes, of which only two had a small error, were reversible, while the silver electrode was found to be completely non-reversible, Fig.VI.1., Table VI.1. The two electrodes with the small error were used throughout this work and their average E.M.F. is quoted.

Solutions of hydrogenase were prepared containing different amounts of the reduced and oxidized species, (the former prepared by addition of dithionite and dialyzing the excess)

b - Results and Analysis

i - Determination of E_o

Different amounts of reduced and oxidized species were put in

a cell (Fig. V.1.) and the E.M.F. measured. The E.M.F. obtained was plotted versus $\log (\text{Red})/(\text{OX})$ as in equation (8). The slope was found to be 0.058 and we conclude that there is only one electron transferred and that $E_0 = 520 \text{ mv}$ at $\text{pH} = 7.00$ (Table VI.2. Fig.VI.2.)

ii - Effect of Temperature

The same solutions as above were prepared and cooled to 0°C . n was still found to be unity and

$$\Delta H = - 0.32 \text{ Kcal /mole.}$$

$$\Delta F^{23} = 12 \text{ Kcal /mole.}$$

$$\Delta S = -45 \text{ cal/deg./mole}$$

(Table VI.2.)

iii - Effect of pH on E_0

Buffers of different pH values but constant ionic strengths were prepared, Table VI.3. The effect of pH on E_0 was investigated as shown in Table VI.4 and Fig VI.3.

iv - Effect of ionic strength on E_0

Solutions of the same pH, but different ionic strength were prepared and the E.M.F. of hydrogenase of these ionic strengths were obtained. Table VI.5. The results were plotted according to equations (14) and (5) Fig. VI.4 and 5.

TABLE IV. 1Redox potentials of the ferrocyanide/ferricyanide system

R = ferrocyanide/ ferricyanide	log R	E_1	E_2	E_3	E_4
1/9	-0.954	213.6	215.9	187.0	103.2
2/8	-0.602	192.9	194.6	167.1	97.4
3/7	-0.366	180.2	181.1	154.2	94.4
4/6	-0.176	170.0	170.4	144.1	90.3
5/5	0.000	160.0	160.0	134.7	86.7
6/4	0.176	151.0	151.0	125.0	80.1
7/3	0.366	140.1	139.1	113.0	72.9
8/2	0.602	127.3	126.9	101.2	61.1
9/1	0.954	110.2	108.5	81.7	48.9

Total concentration of cyanide complex = 0.0091 M.

E_0 from literature at the correctionic strength and concentration = 413.9 mv

	E°_{obs}	E°_{cal}	E corr.
Electrode (1) and (2)	160.0	402.5	11.4
Electrode (3)	134.5	376.0	37.4
Electrode (4) is irreversible.			

TABLE IV. 2 E_0 of hydrogenase

Reduced/Oxidized	E^{23}_{mv}	E°_{mv}
1/9	577	538
2/9	558	516
3/7	542	504
4/6	530	490
5/5	522	482
6/4	509	470
7/3	500	460
8/2	486	445
9/1	463	422

TABLE IV. 3

Buffers of constant ionic strength

pH Calc.	pH obs.	<u>ME. of 0.4M</u>			gm of KCl
		KH_2PO_4	K_2HPO_4	$\mu \times 10^3$	
5.9	5.86	45.0	5.0	98.0	3.7653
6.1	6.04	42.0	8.0	105.6	3.6236
6.3	6.27	39.0	11.0	115.2	3.4450
6.5	6.48	34.0	16.0	131.2	3.1464
6.7	6.64	28.0	22.0	150.4	2.7885
6.9	6.89	22.0	28.0	169.6	2.4306
7.0	6.99	20.0	30.0	176.0	2.3114
7.1	7.08	16.0	34.0	188.8	2.0728
7.3	7.27	11.0	39.0	204.8	1.7745
7.5	7.48	8.0	42.0	214.4	1.5732
7.9	7.85	3.0	47.0	230.4	1.2973

Dilute to 250 ml.

$$[\text{PO}_4] = 0.080\text{M}$$

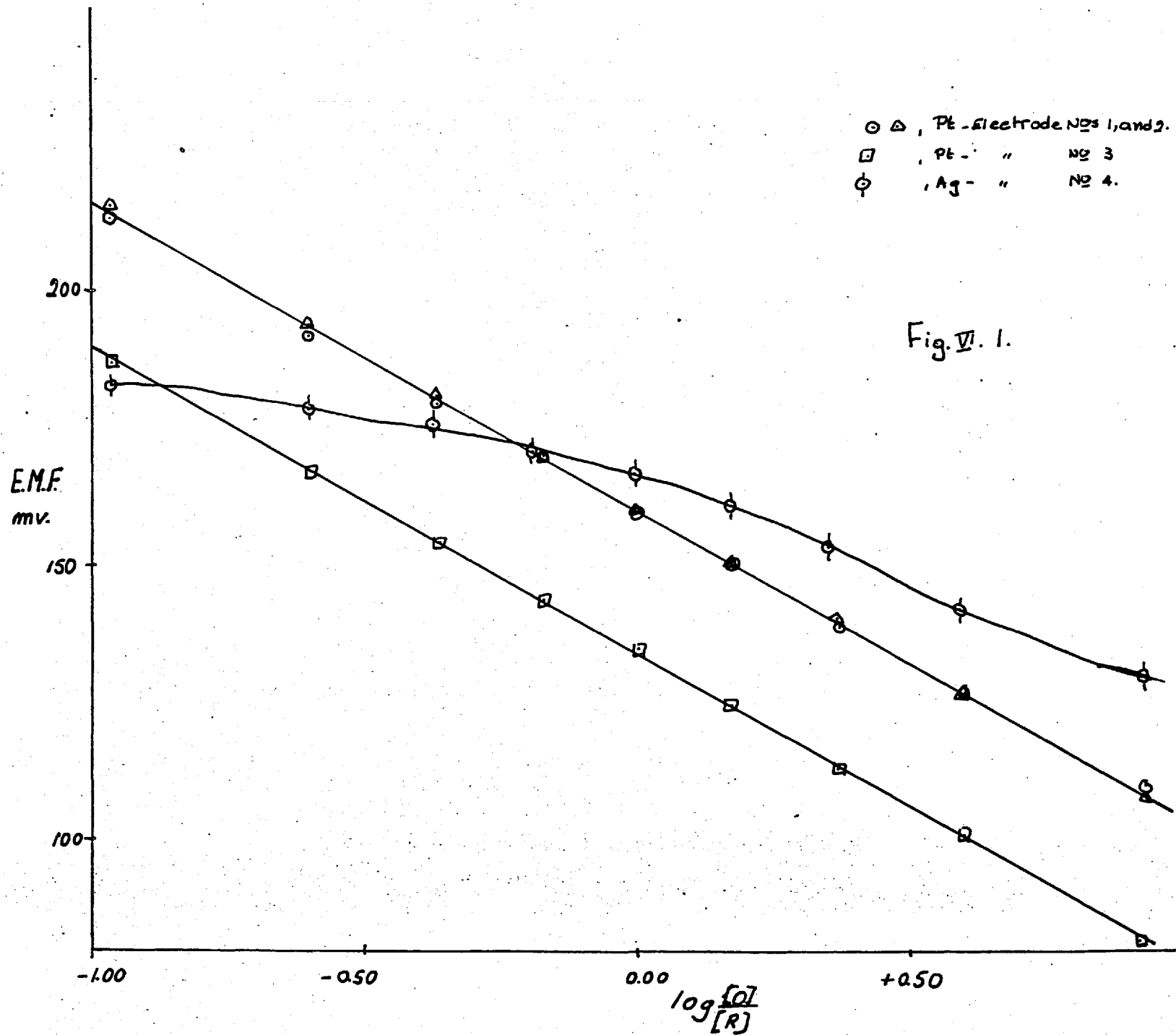
$$[\mu] = 0.300\text{M}$$

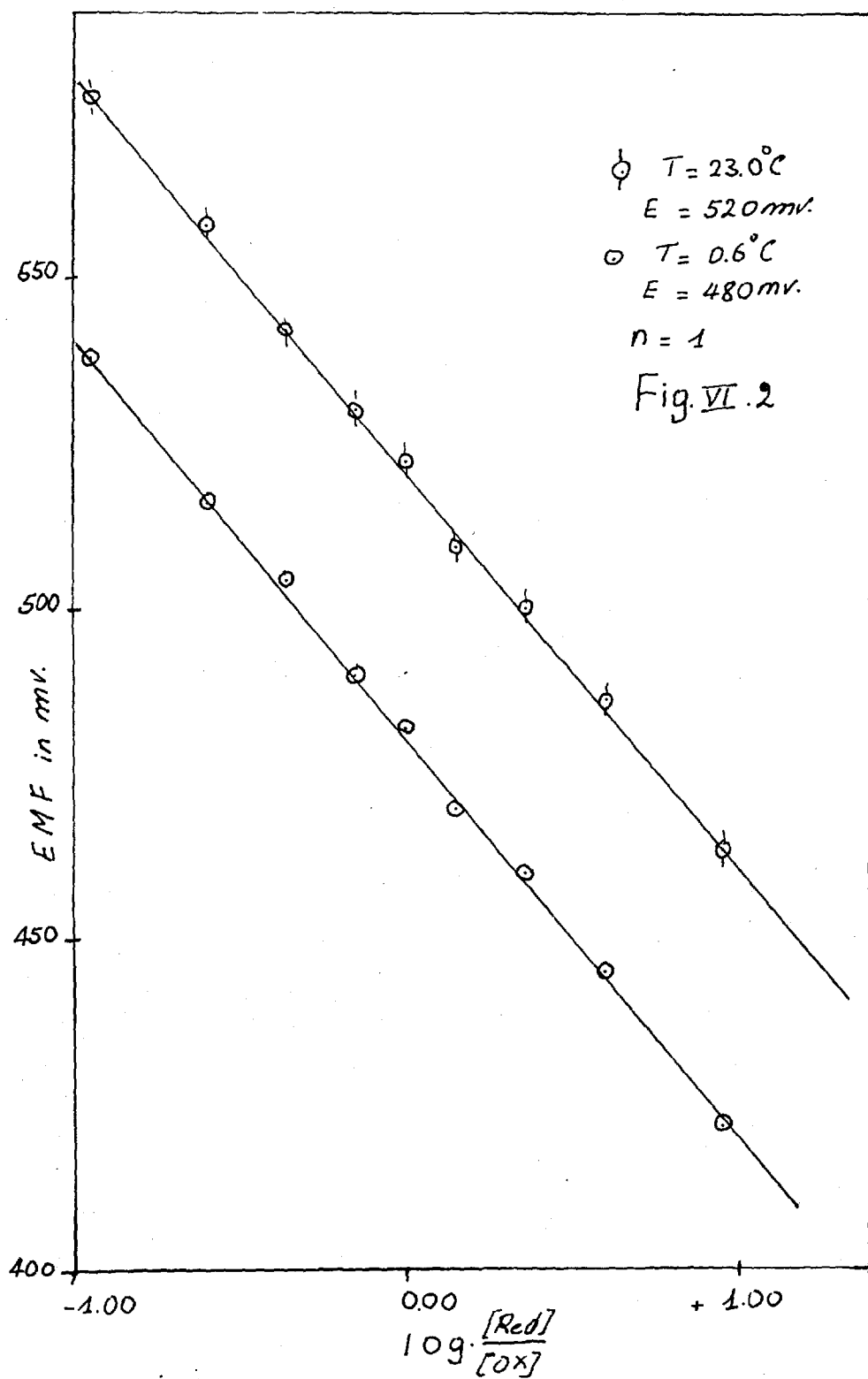
TABLE IV. 4Effect of pH on E_{obs}

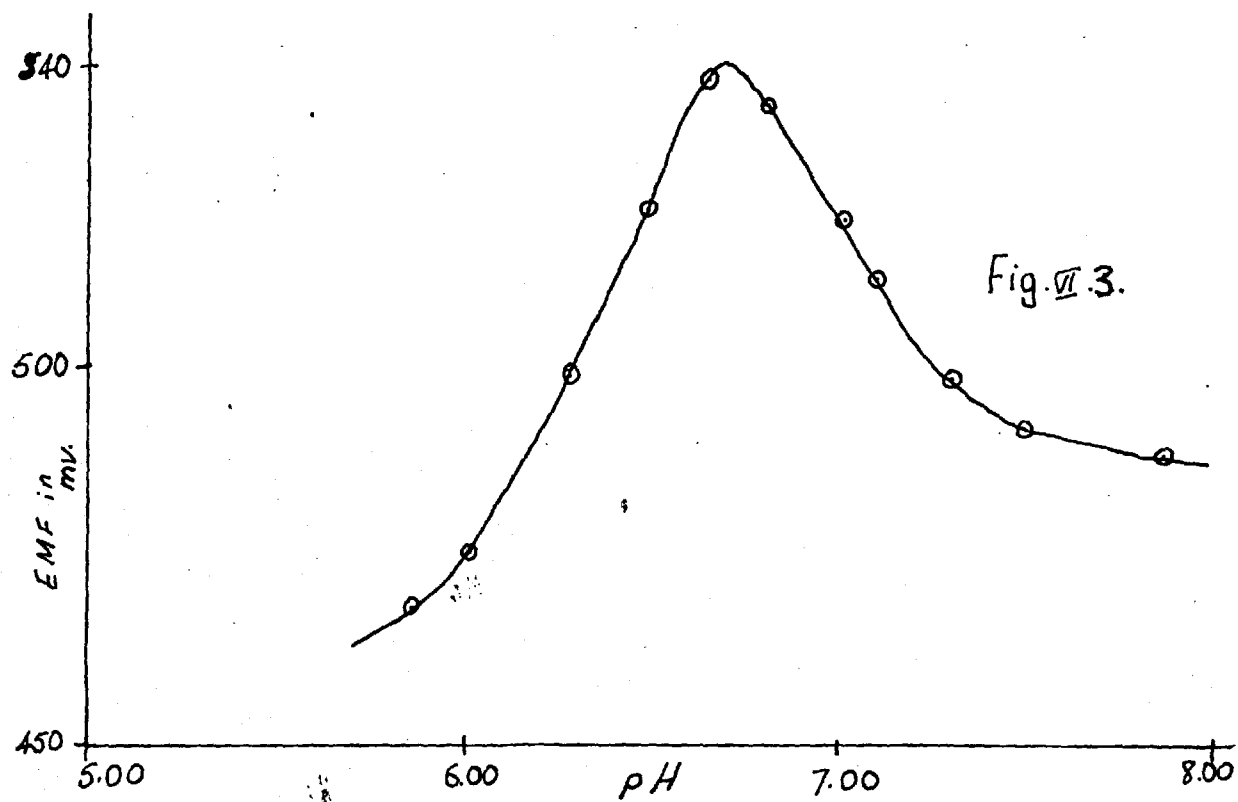
pH	E _{mu}
5.86	468
6.04	475
6.27	498
6.48	521
6.64	538
6.89	535
6.99	520
7.08	512
7.27	501
7.48	492
7.85	488

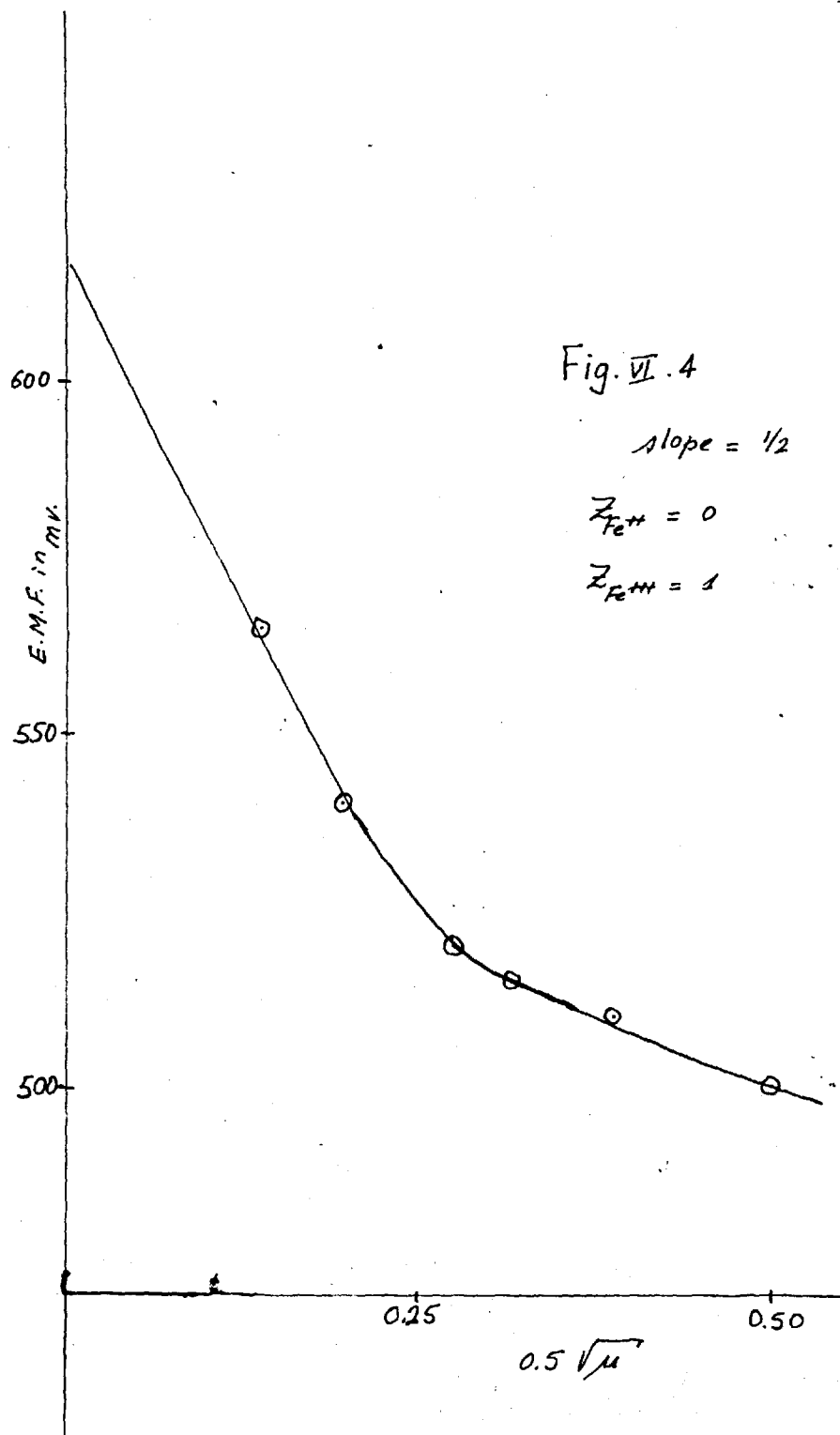
TABLE IV. 5Effect of Ionic Strength on E_{obs}

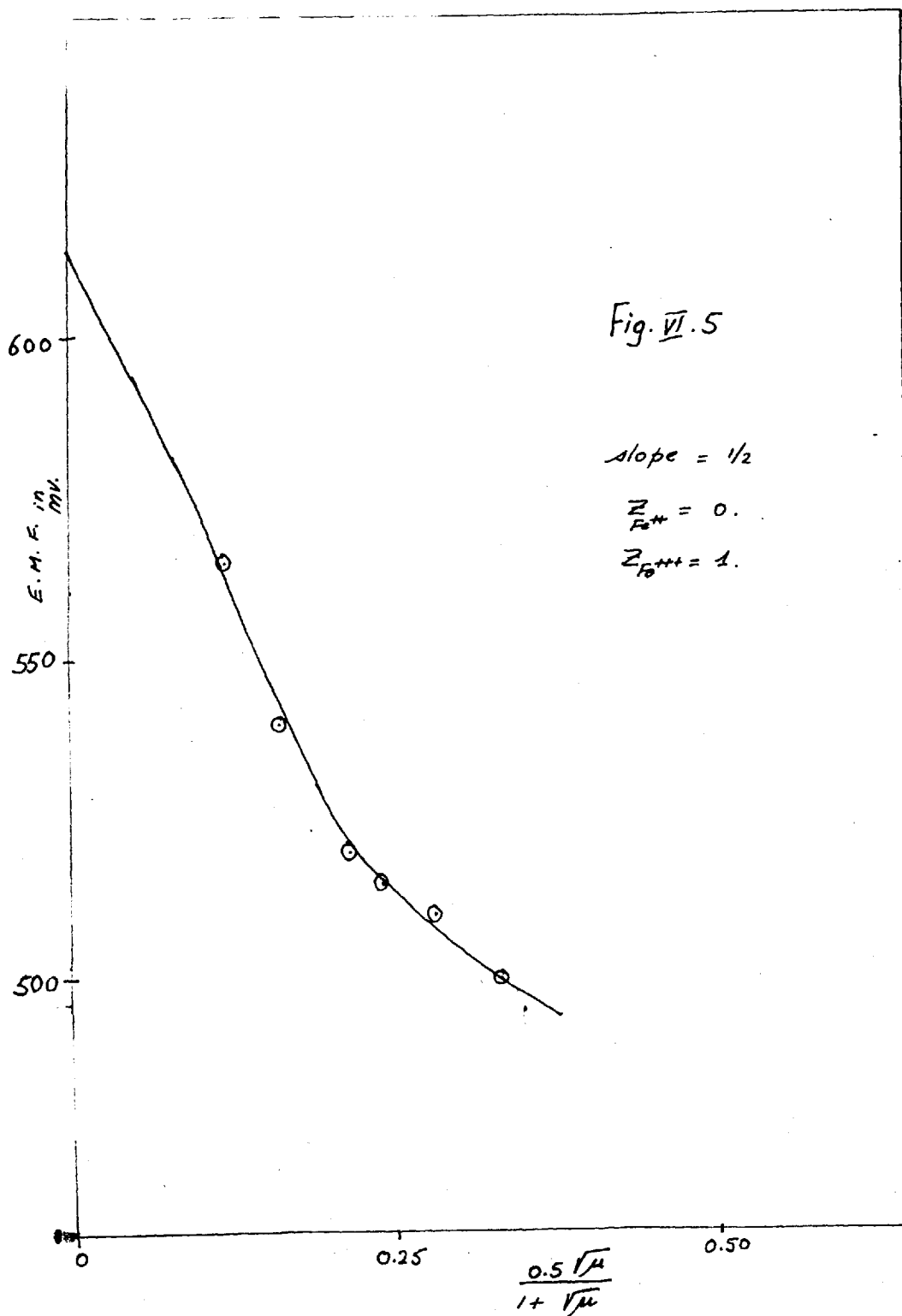
$[\mu]$	$.5(\mu)^{\frac{1}{2}}$	$\frac{.5(\mu)^{\frac{1}{2}}}{1 + (\mu)^{\frac{1}{2}}}$	E _{mv}
1.000	.500	.333	500
0.600	.387	.279	510
0.400	.316	.240	515
0.300	.273	.214	520
0.150	.194	.162	540
0.075	.137	.120	565











CHAPTER SEVEN

DISCUSSION.

It is obvious from Chapter II, that hydrogenase itself is not a highly particulate enzyme, but forms complexes with nucleic acids which may have much higher molecular weights. Also we found that this complex is held together by electrostatic forces and can be broken down by increasing the ionic strength of the solution containing it.

Other workers (57) showed that when the hydrogenase of *Desulfovibrio desulfuricans* is chromatographed on an ion-exchanger, three components are obtained, the enzymic activity of which is fairly similar.

From the K_D values obtained the molecular weights (Flodin method) the hydrated molecular weights, the Stokes radii, and the diffusion and frictional coefficients have been obtained. We can conclude with certainty, from the data that:

- 1 - The protein is highly hydrated.
- 2 - Since the Stokes radii are not in a ratio of 5:3:1 as the molecular weights are, the protein cannot be globular, but may possibly be rod-shaped.
- 3 - Three or five protein moieties may combine to form a triangular or pentagonal prism - this could be proved by ultracentrifugation, diffusion, and light scattering experiments.

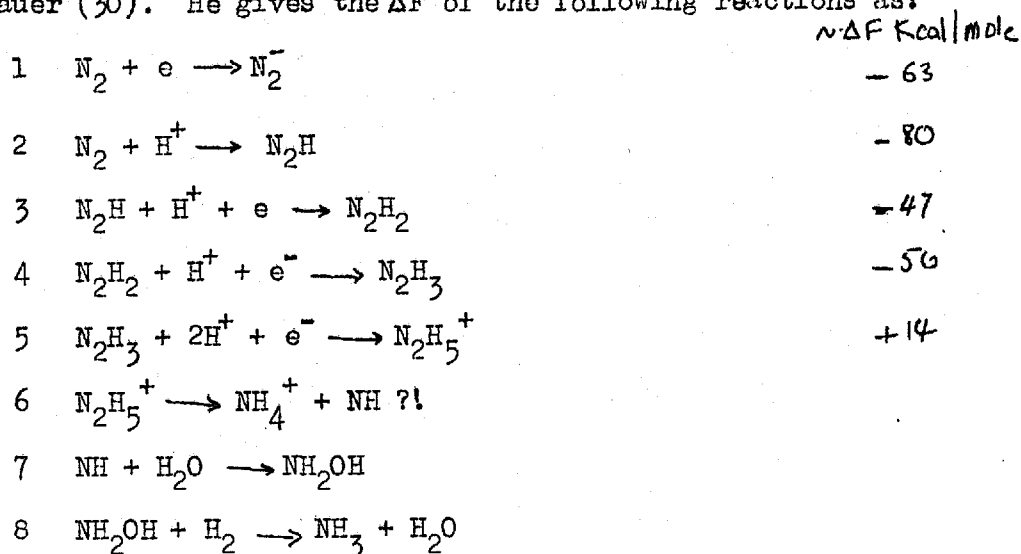
Again, we come to question the validity of these results. Is this observed Heterogeneity an artifact due to the preparation

method (namely the use of high ionic strength solutions) or is it the real state in which the protein exists? This can be established by studying the effects of ionic strength and pH on the three components of the G-75 chromatography. Changes in pH and/or ionic strength could show recombination or further dissociation of the oligomers. Haemoglobin is an ideal example ^{of} association-dissociation of polymeric groups (120-122). The results from the analysis of the titration curves show the ratio of the number of freely ionizing carboxyl group to that of the ammonium groups is 2:1. The isoionic pH, as determined from electrophoresis, is in agreement with this datum. The pK 's and pH 's of the enzyme are reasonably within the range of pK 's and pH 's of other proteins. It is worthwhile noting that the denatured proteins shows an increase of basicity and an increase in the number of titratable groups. This can be interpreted as follows: the protein unfolds by breaking the hydrogen bonds (hence increase of basicity (123)), and this change in the configuration (the increase of the number of titratable groups) is responsible for the enzyme reactivity. If the protein is subject to such changes, the sharpness of the nitrite-reduction pH-profile can be accounted for. However, the effect of pH on the helical structure should be studied in detail in order to explain the effect just described.

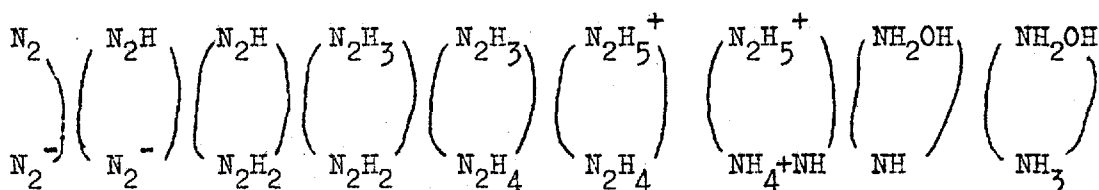
From the values of χ/β (Fig. IV14 and 15) the anomalies of the E.S.R. spectra and the high g -value, we conclude that the iron is a high spin, non haeme, highly distorted ion and coordinated to oxygen and nitrogen ligands only. The absence of signal when the enzyme is

under hydrogen pressure shows that the free radical mechanism is not operative. On the other hand, the hydride ion mechanism as suggested by Eley, is found to fit the kinetic results for the reduction of olefins catalyzed by a rhenium complex. The same features (substrate self-inhibition, absence of E.S.R., H-radical signal, and the identification of the hydride ion by N.M.R.) shows that the mechanism involving the hydride ion is the most likely one at the present time.

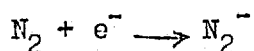
To link the present work to nitrogen fixation, we will try to see how hydrogenase can act as a reductant to the species suggested by Bauer (30). He gives the ΔF of the following reactions as:



It is obvious from our redox potentials that hydrogenase cannot act as a catalyst for these reactions, but these ΔF 's have been calculated for the species when in the gas phase. We will assume that the free energies will be lowered in the aqueous phase and that 'nitrogenase' is a surface catalyst, namely it adsorbs the nitrogen and carries out the first step. Then the nitrogen fixation cycle may be tentatively put as:



Our assumption that nitrogenase may carry out the first process namely:



is not without proof since Zacharias claimed that nitrogen fixation is higher when the nitrogen circulated has been irradiated with U.V. rays thus forming N_2^+ or N_2^- radicals (124).

We can equally recall that reduction of hydrazine (which is immediately transformed to hydrazine acetate in cultures of Azotobacter) may occur without going along the whole cycle.

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