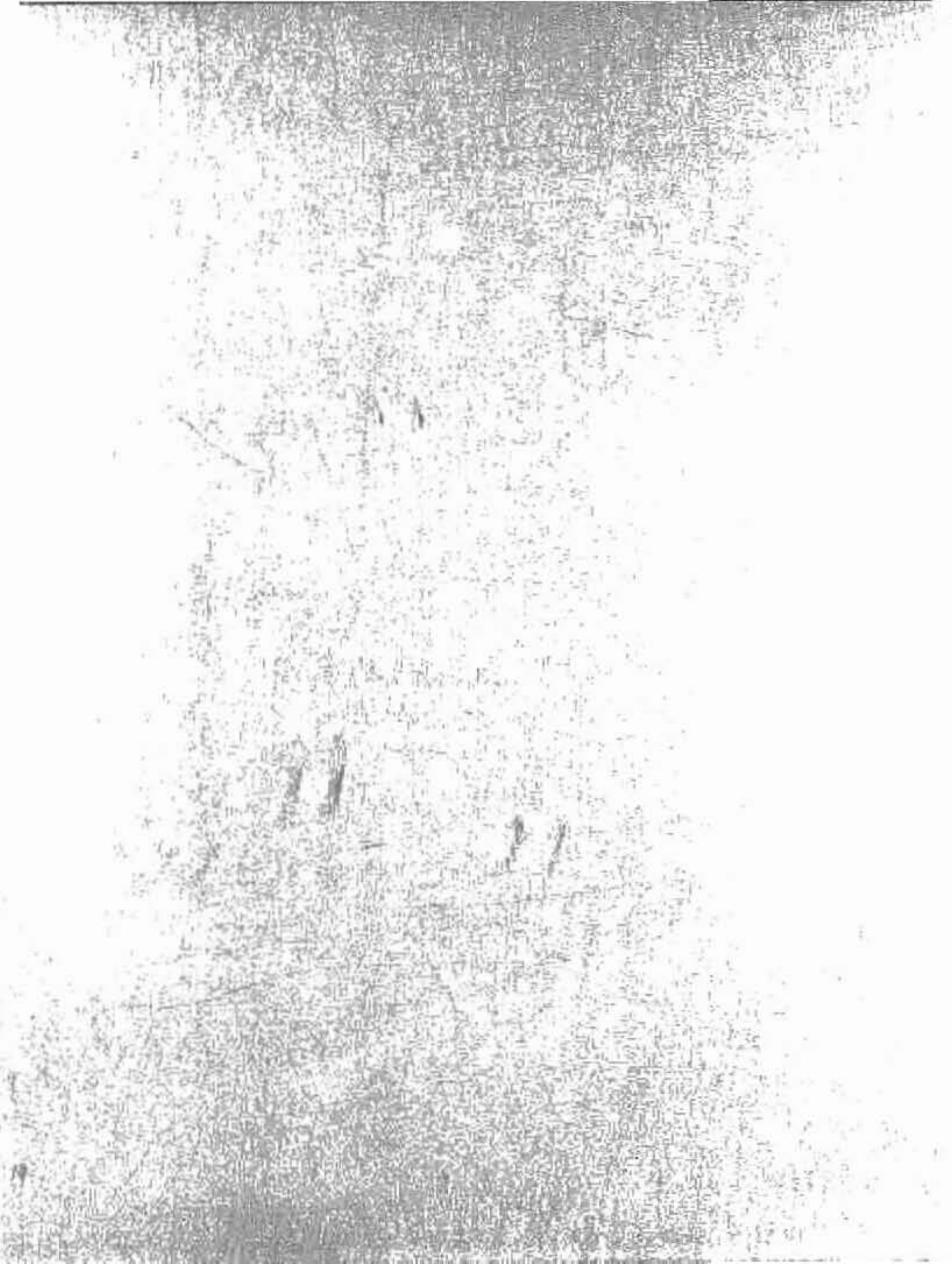

RESEARCH REPORT #1

Undergraduate Research

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INTRODUCTION

Ferredoxin, a low molecular weight electron transport protein, is found in plants and numerous anaerobic bacteria (6, 17). It was first isolated from Clostridium pasteurianum by Mortenson et al (23). Ferredoxins from various organisms all are characterized by being electron transport proteins of low molecular weight which contain equal molar amounts of non-heme iron and inorganic sulfide (34).

Bacterial Ferredoxin

Bacterial ferredoxin is found only in anaerobic organisms and is most prevalent among the Clostridia (17). Clostridium pasteurianum, C. acidiurici, C. butyricum, C. tetanomorphum, C. thermosaccharolyticum, C. kluyveri, C. cylindrosporum, C. sporogenes, and C. lactoacetophilum all contain ferredoxin, whereas C. nigrificans does not (17, 34, 48). Other organixms containing ferredoxin include Methanobacter omelianskii (44), Micrococcus lactilyticus (38, 47), Micrococcus aerogenes (44), Diplococcus glycinophilus (34), Butyribacterium rettgeri (34), Chlorobium thiosulfatoplilum (11), Chromatium (30), Rhodospirillum rubrum (30), and Desulfovibrio desulfuricans (30).

Among the bacterial ferredoxins, the clostridial ferredoxins have been the most extensively studied (34). Chromatography on DEAE cellulose, gel filtration, and fractionation with ammonium sulfate have been used in the preparation of highly purified ferredoxins (6, 17, 20, 22, 30).

Several clostridial ferredoxins (C. pasteurianum, C. tetanomorphum, C. acidiurici, C. butyricum, and C. cylindrosporum) have been crystallized (6, 17). These ferredoxins are similar in many respects, however they exhibit small differences in spectra and amino acid composition (6, 17).

The molecular weights of the clostridial ferredoxins are all approximately 6,000, with 7 atoms of non-heme iron and inorganic sulfide per molecule (17, 20). They have similar electrophoretic properties, with isoelectric points around 3.7, and an oxidation-reduction potential of - 418 mv at pH 7.11 All clostridial ferredoxins exhibit absorption maxima at 280 and 385-390 mu: however, small differences in spectra exist (6, 17).

Lovenberg et al (1963) have determined the amino acid composition of five clostridial ferredoxins. All of these ferredoxins lacked tryptophan, methionine, and histidine, and all contained a single basic amino acid except for C. butyricum, which contained no basic amino acids. High amounts of glutamate and aspartate account for the acidic nature of these proteins (17).

Tanaka et al have determined the amino acid sequence of C. pasteurianum ferredoxin. They found it contained 55 amino acid residues, of which 7 were cysteines (32, 33). Sieker and Jensen have done a preliminary X-ray analysis of Micrococcus aerogenes ferredoxin. They reported evidence that suggested Fe atoms were not arranged in a single long linear array (28).

Bacterial ferredoxins function in a number of reactions: pyruvate degradation, the oxidation and reduction of purines, pyridine nucleotide reduction, nitrogen fixation, and the reduction of inorganic compounds (34, 51).

I. The Degradation of Pyruvate

Many researchers have elucidated the various cofactors involved in the phosphoroclasic reaction (7, 8, 13, 14, 25, 26, 49, 44). However, not until Mortenson et al (1962) isolated ferredoxin was the reaction's oxidation-reduction mechanism understood (22). The proposed scheme for the overall phosphoroclastic (clastic) reaction is as follows:

$$PO_4 + \text{Pyruvate} \xrightarrow[\text{enzymes}]{\text{clastic}} \text{Acetyl-}PO_4 + CO_2 + H_2 \quad (1)$$

The clastic enzymes include phosphototransacetylase, pyruvic dehydrogenase, and hydrogenase, requiring as cofactors thiamine pyrophosphate and coenzyme A. Ferredoxin serves as the electron mediator which links pyruvic dehydrogenase with hydrogenase to yield molecular hydrogen (34). The evolution of hydrogen from α-ketoglutarate and α-ketobutyrate have also been shown to be ferredoxin-hydrogenase reactions (39, 41).

In C. pasteurianum, CO_2 - pyruvate exchange was demonstrated as being dependent upon ferredoxin (2). Recently, the synthesis of pyruvate by CO_2 fixation was demonstrated as a ferredoxin dependent reaction in cell-free extracts of several organisms (4, 11).

II. The Oxidation and Reduction of Purines

M. lactilyticus can oxidize hypoxanthine to xanthine and molecular hydrogen (38). Two components of this system were identified as xanthine oxidase and hydrogenase, and an electron carrier was postulated by Whiteley (1956) as the electron link between these enzymes (45, 46). Valentine et al (1962) later identified this component as ferredoxin (38).

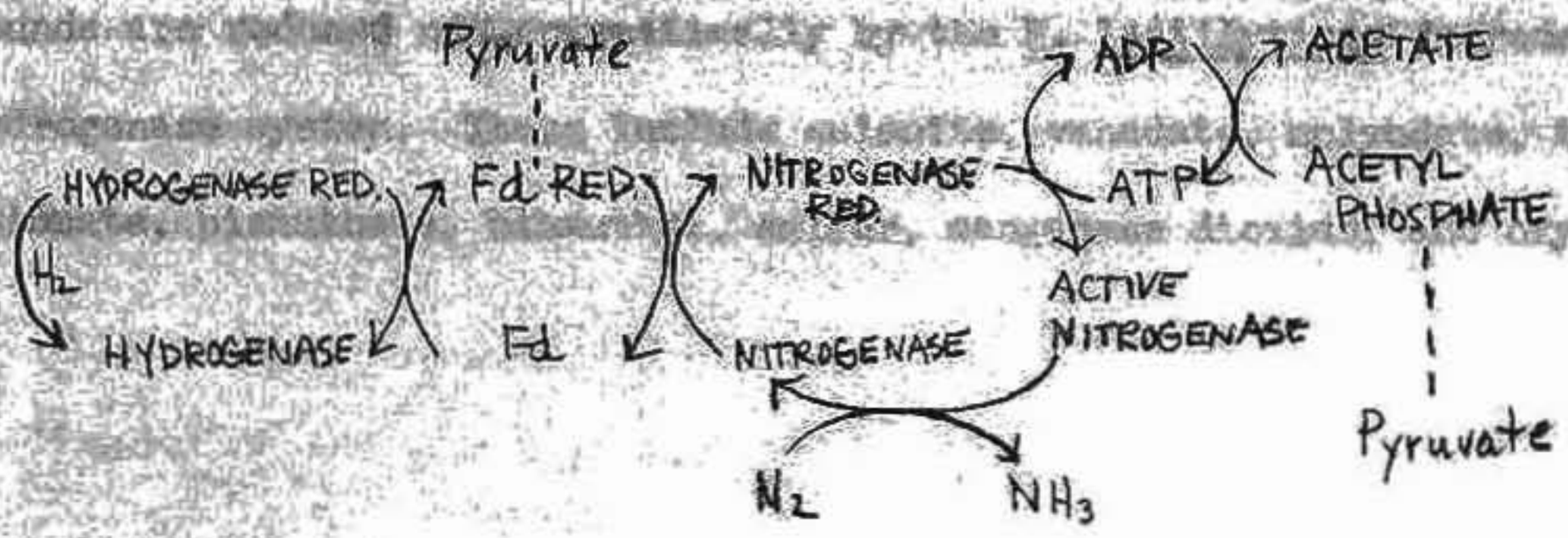
M. lactilyticus ferredoxin coupled with hydrogenase in an atmosphere of molecular hydrogen reduces uric acid and xanthine (38).

III. Pyridine Nucleotide Reduction

Bacterial ferredoxins demonstrate specificity of pyridine nucleotide reduction. C. acidiurici ferredoxin reduces NAD, while all other clostridial ferredoxins reduce NADP, but not NAD (34, 35, 36). M. lactilyticus ferredoxin reduces NADP (47), and Methanobacillus omelianskii ferredoxin utilizes NAD as a hydrogen acceptor (3).

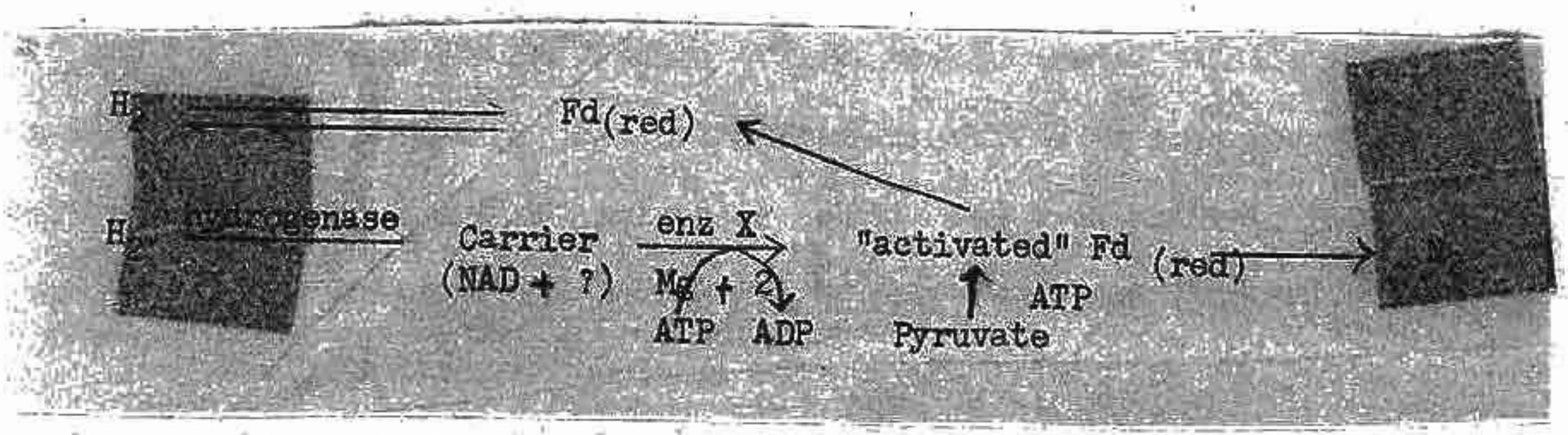
IV Nitrogen Fixation

Nitrogen fixation by C. pasteurianum has been previously reported (7, 8, 19, 24). Mortenson (1964) demonstrated ferredoxin (21) and ATP (22) dependency of nitrogen fixation in extracts of C. pasteurianum. Mortenson proposed the following model:



In this model, pyruvate serves as an electron donor as well as an ATP generator. ATP activates reduced nitrogenase which couples with N_2 to produce NH_3 (22).

Dilworth et al (1965) have proposed an alternative model to explain the ATP dependency of nitrogen fixation (10):



In this working model ferredoxin utilizes ATP supplied by pyruvate metabolism or by an enzyme X to become "activated" reduced ferredoxin. This "activated" ferredoxin may then couple with nitrogenase or become inactivated and couple with hydrogenase (10).

V. The Reduction of Inorganic Compounds

M. lactilyticus ferredoxins coupled with hydrogenase, in the presence of specific reductases, can reduce nitrate, nitrite, arsenate, hydroxylamine, metabisulfite, tellurate, and nitroprusside. However, many inorganic compounds are reduced non-enzymatically by the M. lactilyticus ferredoxin-hydrogenase system. These include selenite, vanadate, molybdate, tetrathionate, bismuthate, bismuthyl, sulfur, manganese dioxide, and oxygen (51).

Photosynthetic Ferredoxin

Photosynthetic pyridine nucleotide reductase (PPNR) and methaemoglobin reducing factor were reported separately, and are now known to be one and the same ferredoxin (9). PPNR has been isolated and crystallized by various workers (12, 27, 37). Its molecular weight is approximately 13,000 (42),and it contains 2 moles of iron and inorganic sulfide per mole (1). While PPNR and bacterial ferredoxin differ in some respects, such as spectral properties, molecular weight, and iron content, PPNR can substitute for bacterial ferredoxin in many reactions (16, 37).

PPNR and NADP play an important role in the regulation of cyclic and non-cyclic photophosphorylation in plants. Ferredoxin is reduced by chlorophyll a, and at this point the degree of cyclic or non-cyclic photophosphorylation is regulated by the availability of NADP. When NADP is abundant, then non-cyclic photophosphorylation occurs, whereas if the pool of NADP is exhausted, then ferredoxin will donate its electrons to a bound component of the grana, resulting in an increase in cyclic photophosphorylation (31).

The recent observation in this laboratory that ferredoxin can be readily eluted from M. aerogenes and C. tetanomorphum has suggested that the properties of these electron carrier proteins could be compared readily. For this purpose, preliminary studies have been made concerning the elution of ferredoxin. Two ferredoxins have been crystallized.

EXPERIMENTAL PROCEDURE

Growth and harvesting of bacteria

M. aerogenes (228) was grown in a liquid glutamate medium previously described (43). Large volumes of cells were obtained by growth in a 130 liter batch fermentor (Fermacell). Smaller volumes of cells were obtained by growing the organism in carboys or in 1 liter flasks. All cultures were incubated at 37°, and usually harvested near middle to late log phase. Large volumes of cells were harvested at 4° using Sharples centrifuges. Smaller volumes of cells were harvested at 4° in 250 ml. plastic centrifuge bottles with the aid of a Sorvall centrifuge. All cells were stored at -20° in corked centrifuge tubes.

Obtaining samples for ferredoxin purification and analysis

I. Cell free extracts:

For large volumes of cells, the Raytheon 10-kc sonic oscillator was used. Cells were suspended in 2 to 4 times their volume with distilled water, and sonicated 30 to 40 minutes. For smaller volumes of cells the MSE ultrasonic oscillator or French pressure cell were used. Cell suspensions as above were subjected to sonic oscillation for 5 minutes, or 16,000 lbs. pressure respectively. Cell debris and extraneous material were removed by centrifugation at 25,000 x g for 30 minutes at 4°. If the extracts were not used immediately, they were stored at -20° under an atmosphere of hydrogen.

II. Cell free eluates:

M. aerogenes or C. tetanomorphum which had been grown in the "Fermacell" and frozen from 3 days to 2 weeks, were thawed at 23° with approximately 3 volumes of distilled water. After approximately one half hour at room temperature the thawed cells were thoroughly suspended and allowed to stand an additional 1 to 2 hours at room temperature. The preparation was then centrifuged for 15 minutes at 25,000 x g at 4°. The resulting supernatant was brown in color and contained a relatively large percentage of the total ferredoxin. This material was used as a source of ferredoxin for purification.

Purification of ferredoxin

The methods employed here were modifications of the procedures used by Buchanan et al (6) and Sieker (29).

Step 1 Chromatography on DEAE-cellulose*:

Ferredoxin was isolated from either extracts or eluates by using short DEAE beds to adsorb the protein. All columns were washed prior to use with approximately 5 volumes of .1M Tris, pH 7.0. After samples were applied, the beds were washed with distilled water until no color was observed in the eluates.

The columns were then washed with .05M PO_4, pH 6.5, until the flavin bands were eluted. To elute ferredoxin, .15 M Tris-.67M NaCl, pH 7.3 was used.

The above operations were usually carried out at room temperature. If crude ferredoxin obtained in this manner was not used immediately, it was stored under hydrogen at -20°.

* DEAE = diethylaminoethyl cellulose

** Tris = Tris (hydroxymethyl) amino-methane

Step 2 Gel Filtration:

To desalt crude ferredoxin samples obtained from Step 1, G-25 Sephadex was used. 2 ml aliquots were subjected to filtration through a column (1.4 x 13 cm) at room temperature. Elution was performed with .1M tris, pH 7.0.

Step 3 Chromatography on DEAE-cellulose:

Desalted crude ferredoxin samples were applied to long DEAE beds (1.8 x 56 cm.) which had previously been washed with 5 volumes of .1M Tris, pH 7.0 buffer. The samples were eluted slowly at 4° with .15 Tris - .15 M NaCl buffer, pH 7.3. In M. aerogenes samples a pink fraction (probably rubredoxin) was eluted

prior to ferredoxin. To elute ferredoxins of M. aerogenes or C. tetanamorphum the eluting buffer was changed to .15M Tris-.67 M NaCl, pH 7.3.

Step 4: Crystallization of ferredoxin

The dark brown material obtained from step 3 was fractionated with ammonium sulfate at 4°. The pH was kept between 7.0 and 7.5 by the addition of crystals of Tris-HCl. Sufficient solid $(NH_4)_2 SO_4$ was added to bring the M. aerogenes preparation to 69% saturation and the C. tetanamorphum preparation to 50% saturation. Samples were allowed to stand with occasional stirring at 4°. Grey-brown precipitates which appeared after 2 hours were removed by centrifugation and additional $(NH_4)_2 SO_4$ was added to bring the supernatant fraction of both samples to 87% saturation. After 2 hours at 4°, the fractions were centrifuged and the darkly colored precipitates were dissolved in 3 ml of .1M Tris, pH 7.0. Additional $(NH_4)_2 SO_4$ was added at intervals of several days while storing the preparations at 4°. Crystals began to form at 72% saturation for the M. aerogenes preparation and at 50% for the C. tetanamorphum preparation.

Purification of rubredoxin

Step 1 Concentration:

The pink fraction obtained from Step 3 of the purification of M. aerogenes ferredoxin was diluted with an equal volume of distilled water and adsorbed to a short DEAE bed previously washed with 5 volumes of .1M Tris, pH 7.0. The dark pink band obtained by this procedure was eluted with .15 MTris-.67 M NaCl, pH 7.3.

Step 2 Removal of ferredoxin:

Solid $(NH_4)_2 SO_4$ was added to the above fraction to achieve 90% saturation. After storing for 24 hours at 4°, the sample was centrifuged yielding a faint, brown-red precipitate and a pink supernatant.

Step 3 Crystallization:

The pink supernatant from step 2 was dialyzed against 2 liters of distilled water for 2 hours at 4°. The sample was then lyophilized, and dissolved in 2 ml. of distilled water. Solid $(NH_4)_2 SO_4$ was added to bring the preparation to 50% saturation. The sample was stored at 4°C. Crystals were observed after 2 weeks.

Estimating the ferredoxin content of intact cells

To improve complete cell breakage, cell suspensions were subjected to repeated sonication and French pressure cell treatment. Crude ferredoxin was obtained by passing these extracts through DEAE beds as described above (Purification of ferredoxin, Step 1). Ferredoxin units (Fd units) in the eluted material were defined as:

$$\frac{10^2 \cdot \text{absorbance at 390 m}\mu \cdot \text{ml. crude ferredoxin}}{\text{total cell protein}}$$

Estimating the ferredoxin content of eluates

Frozen cells were eluted, eluates were adsorbed to DEAE-cellulose and eluted as described above ("Fd-eluate"). Cell free extracts were prepared from the cells remaining after elution. Ferredoxin was collected from the cell-free extracts by adsorbtion to and elution from DEAE-cellulose ("Fd-cells"). The percentage of ferredoxin eluted was defined as:

$$\frac{(10^2)\ (\text{Fd units from "Fd eluate"})}{(\text{Fd units from "Fd eluate") plus (Fd units from "Fd-cells")}}$$

It should be noted that the above methods for the estimation of the content of ferredoxin in eluates

and cells are subject to several errors. The load on DEAE-cellulose columns, the amount of buffer used for washing DEAE-cellulose columns, the length of these columns, the length of time cells were frozen before elution, and the efficiency of cell breakage are important variables. Although the methods used were directed toward the elimination of such variables, fluctuations in results still exist.*

Protoplast formation

An attempt was made to form protoplasts of M. aerogenes. Frozen cells were suspended in .03 M Tris, pH 8.0 (.1 gm/ml) and incubated at 37° in 20% sucrose with varying concentrations of lysozyme, EDTA** and NaCl. Samples were removed after one hour and 6 hours and diluted 1/10 in distilled water. The optical density of the dilutions was compared at 420 mu.

* see RESULTS (section d. and Table III).

** Ethylenediaminotetraacetic acid.

Electrophoresis

Various samples were subjected to acrylamide gel electrophoresis. The 7.5 per cent gel used was prepared according to a Canal Industrial Corp. phamphlet, (4935 Cordell Ave., Bethesda, Maryland). Samples were mixed with 2M sucrose prior to application. Electrophoresis was performed at room temperature using 4 m. amps per gel. Protein was stained with Amido Schwartz black for 1 hour, and destained electrophoretically using 7 per cent acetic acid.

Methods:

Protein was determined by the method of Lowry (14). Absorbancy was measured with either the Beckman DU spectrophotometer with Gilford attachments, or the Beckman DK-2 recording spectrophotometer.

RESULTS

I. Studies of elution conditions with M. aerogenes

a. The repeated elution of frozen cells

80.39 gm wet weight of frozen cells of M. aerogens were eluted as described in the EXPERIMENTAL PROCEDURE. They were frozen over night and eluted again, refrozen and eluted for the third time. Table I shows that 81.2% of the ferredoxin was eluted during the first thawing. Acrylamide gel electrophoresis of this eluate showed 21 distinct bands, indicating selective elution. After 3 elutions the cells remaining contained only 5% of their original ferredoxin content.

b. Volume of eluting solution

Figure I shows that ferredoxin was eluted efficiently by 15 ml. H_2O, and that the use of larger volumes yielded only a slight increase in the amount eluted. The near maximum at 15 ml. is equivalent to 1.5 volumes of H_2O per wet weight of cells.

c. Time of elution

Samples were taken periodically from a large batch of cells undergoing elution and ferredoxin was estimated by the absorbancy of the supernatant at 390 mu. Figure II shows that 1 hour of elution is required for maximum elution of ferredoxin. It is of interest that elution of total protein continues.

d. Eluting agents

Tris, EDTA, and H_2O were compared for efficiency in the elution of ferredoxin. Table II shows that EDTA and H_2O have similar elution efficiencies (approximately 70%), while Tris was less efficient (58%).

Table III shows that the total Fd units estimated by the sum of Fd units eluted and Fd units remaining vary between different experiments. However this variance should not influence comparisons of the % ferredoxin eluted by different eluting agents.

e. Protoplast formation

In order to determine if ferredoxin was located between the cell wall and cell membrane, protoplast formation was attempted. Table IV shows only a slight indication of protoplast formation.

II. Crystalline Ferredoxin and Rubredoxin

a. M. aerogenes ferredoxin

Table V shows the purification and crystallization of M. aerogenes ferredoxin. Starting with 895 mg of eluate protein only about 15 mg of crystalline ferredoxin was recovered. The 390/280 absorbancy ratio is a measure of purity. The value of .64 compares with that of .78 reported for crystalline ferredoxin from C. acidi-urici (6). As seen from Figure III, this material has a spectrum which resembles that reported for C. butyricum (17). It has absorbancy maxima at 390 mu and 280 mu. Figures V (a) and (b) are photographs of M. aerogenes ferredoxin crystals. The rod-like rosettes compare with crystalline C. acidi-urici ferredoxin crystals (17). Acrylamide gel electrophoresis of the crystalline M. aerogenes ferredoxin showed 1 dark band and 2 very faint ones.

b. C tetanomorphum ferredoxin

C. tetanomorphum ferredoxin crystals had a $\frac{390}{280}$ absorbancy ratio of .7, compared with .83 previously reported (17). Needle-like rosettes were observed which agreed with the photograph previously reported by Lovenberg et al (17).

C. M. aerogenes rubredoxin

The spectrum of rubredoxin obtained just prior to crystallization is given in Figure IV. It has absorbancy maxima at 490, 380, and 280 mu with shoulders at 570, 370, and 290 mu. A photograph of the crystals is illustrated in Figure V (C).

DISCUSSION

The percentage of cell protein that is ferredoxin in M. aerogenes can be estimated from the results shown in Table V. If one assumes a 40% recovery of crystalline ferredoxin from the initial eluate, ferredoxin comprises about 0.4% of the cell protein.

The possible mechanisms involved with ferredoxin elution are of interest. Cell lysis should be ruled out, for the per cent of cell protein eluted is negligible to the % of ferredoxin eluted. The location of ferredoxin may be an important factor. If ferredoxin is located near the cell membrane, then small punctures in cell walls by ice crystals could permit leakage.

If protoplasts could be formed, then the problem of ferredoxin localization could be considerably clarified. If ferredoxin is located between the cell wall and cell membrane, then upon protoplast formation in a osmotically stabilized environment ferredoxin would be released into the medium.

The spectrum and crystal appearance of M. aerogenes ferredoxin are only 2 properties thus far determined. In the future the amino acid content, iron content, sulfide content and molecular weight should be found for this ferredoxin, so that it may be compared with other ferredoxins.

Lovenberg and Sobel have recently crystallized rubredoxin, a pink electron transport protein from C. pasteurianum (18). An interesting similarity between rubredoxin and the pink fraction of M. aerogenes is that their spectra are identical. Thus the pink fraction is probably rubredoxin. Further research should be done with the M. aerogenes rubredoxin, to compare it with other rubredoxins, and also to compare it with M. aerogenes ferredoxin.

TABLE I

Repeated Elution of Ferredoxin by Thawing Frozen Cells

Elution #	Total Protein Eluted (mg)	Fd units	% Fd Eluted	% Total Cell Protein Eluted
1	894	0.807	81.2%	6.7%
2	154	0.139	14.0%	0.89%
3	92.1	*	*	*

* Negligible

The Fd units were estimated as outlined in EXPERIMENTAL PROCEDURE. After three elutions with H_2O the cell-free extract contained only 0.048 Fd units.

TABLE II

Elution of Ferredoxin from Frozen Cells by Different Eluting Agents

Eluting Agent	Total Protein Eluted (mg.)	Fd units Eluted	Fd units Remaining	% Efficiency
9.98×10^{-4}M EDTA*, pH 7.0	54.5	0.746	0.269	73.5%
.1M Tris-HCl, pH 7.0	39.8	0.551	0.397	58.2%
H_2O	77.6	0.753	0.329	69.5%

* Ethylenediaminotetraacetic acid

Fd units estimated as described in EXPERIMENTAL PROCEDURE.

TABLE III

Comparison of Ferredoxin Estimations

Elution Method	Fd units Eluted	Fd units Remaining	Total Fd units	% Error
.1M Tris-HCl, pH 7.0	0.551	0.397	0.948	4.4%
9.98×10^{-4}M EDTA, pH 7.0	0.746	0.269	1.015	10.3%
H_2O	0.753	0.329	1.082	19.2%
Non Eluted Cells			0.908	

The % error was based on the estimation of Fd units from non-eluted cells.

TABLE IV

The Effects of Various Agents on M. aerogenes Protoplast Formation

Tube #	Conc. lysozyme (µg/ml)	Conc. EDTA (µg/ml)	Conc. NaCl (µg/ml)	O. D. @ 1 hr.	O. D. @ 6 hrs.
C *	-	-	-	.72	.70
1	10	200	-	.69	.66
2	100	500	-	.77	.50
3	500	1,000	-	.67	.60
4	1,000	2,000	-	.79	.85
5	100	200	-	.60	.52
6	100	500	-	.60	.45
7	100	1,000	-	.62	.54
8	100	2,000	-	.64	.48
9	100	400	-	.64	.60
10	100	400	-	.70	.70
11	500	2,000	6,850	.68	.79
12	500	2,000	13,700	.68	.65

* Control (.1 mg/ml cells, 20% sucrose, in .03M Tris-pH 8.0)

The experiment was carried out as outlined in EXPERIMENTAL PROCEDURE.

TABLE V

Purification and Crystallization
of Ferredoxin from M. aerogenes

	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	A $\frac{390}{280}$	Specific* Activity
Initial Eluate	106	8.93	895	0.063	0.171
First DEAE	7.5	18.95	142	0.120	0.736
First Sephadex	17.6	7.50	132	0.131	0.531
Second DEAE	15.6	5.05	78.8	0.561	1.25
Ferredoxin Crystals	-	-	15**	0.641	1.51

* Specific activity = $\frac{(A\ 390)(ml)}{\text{total protein}}$

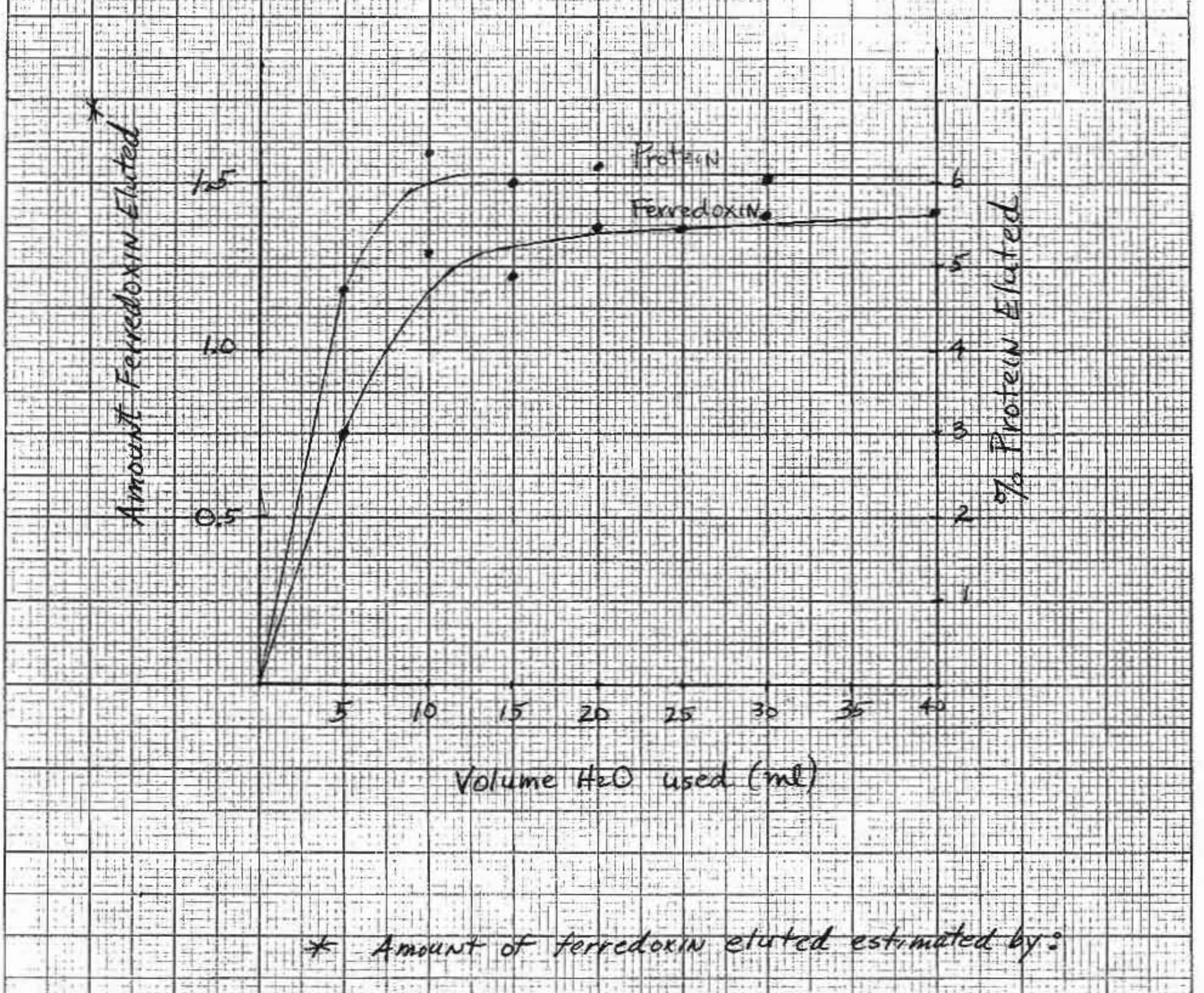
** Estimated weight

The initial eluate was obtained from 12,990 mg cell protein.

This eluate contained 70% of the ferredoxin contained by the cells.

FIGURE I

FERREDOXIN ELUTED WITH DIFFERENT VOLUMES OF H_2O

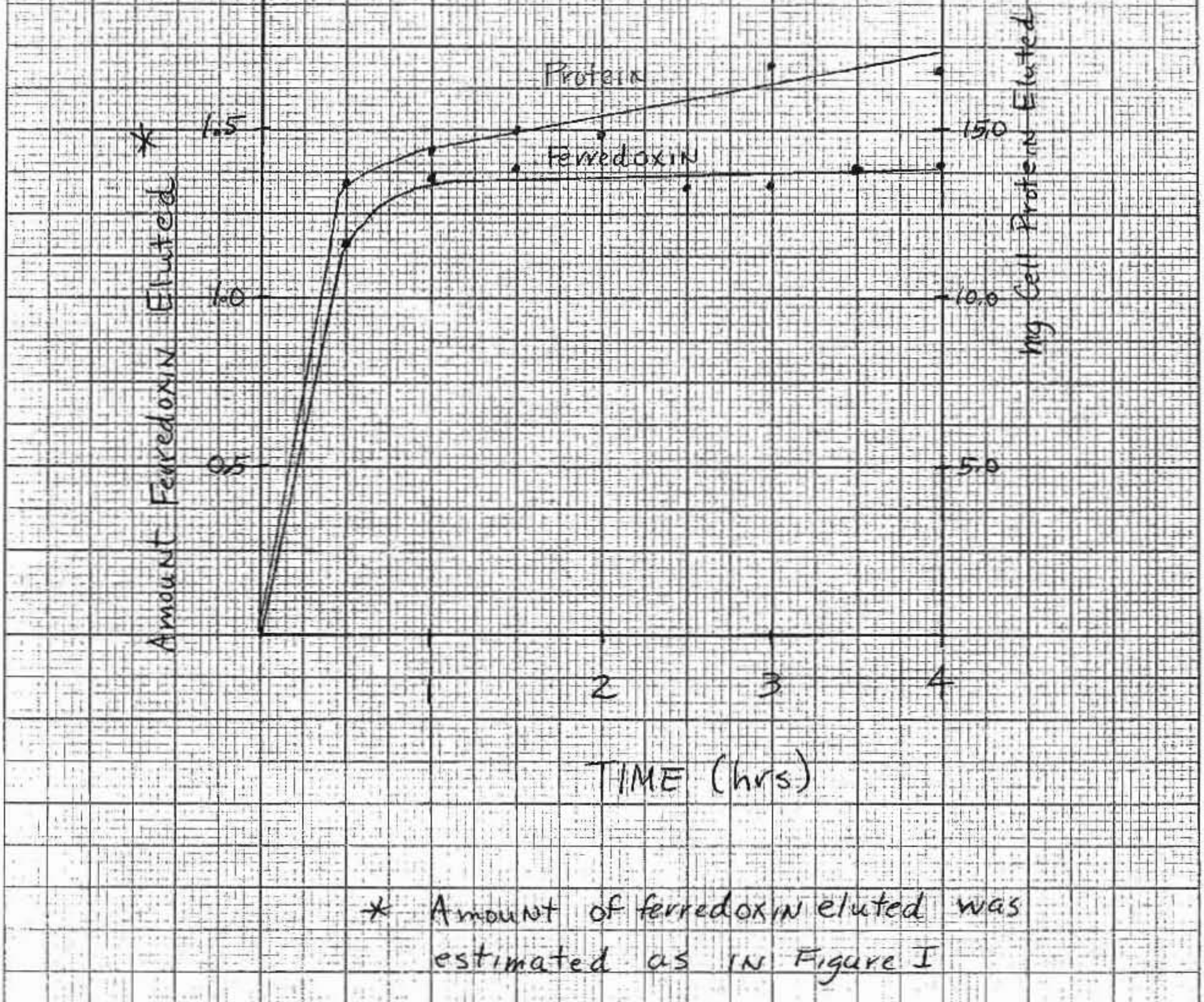


* Amount of ferredoxin eluted estimated by:

$$\frac{(A_{390}\ \text{eluate})(10^2)(\text{vol.})}{\text{total cell protein}}$$

FIGURE II

FERREDOXIN ELUTED AT DIFFERENT TIMES



* Amount of ferredoxin eluted was estimated as in Figure I

FIGURE III

SPECTRUM OF FERREDOXIN CRYSTALLIZED FROM *M. aerogenes*

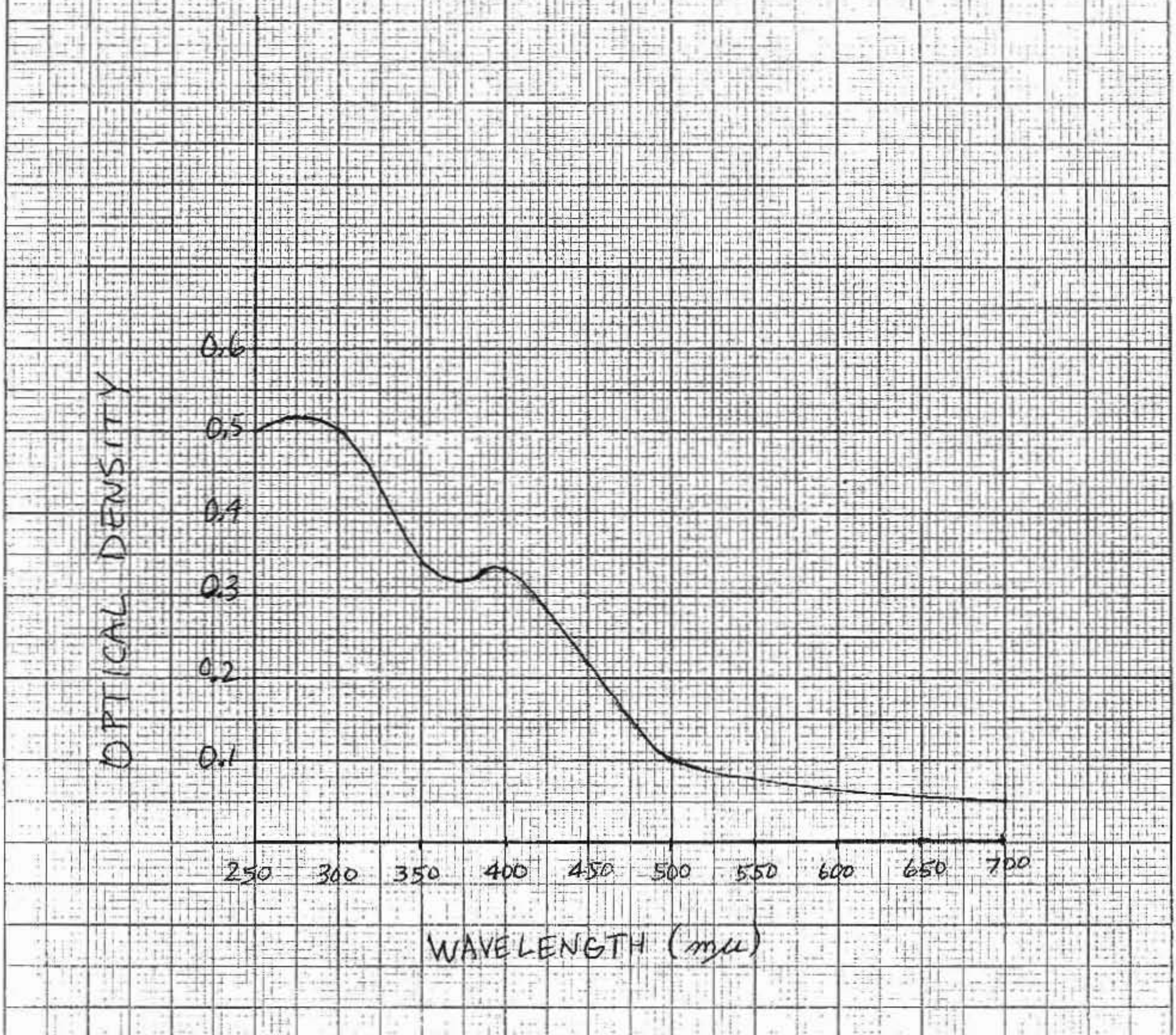


FIGURE IV

SPECTRUM OF M. aerogenes RUBREDOXIN

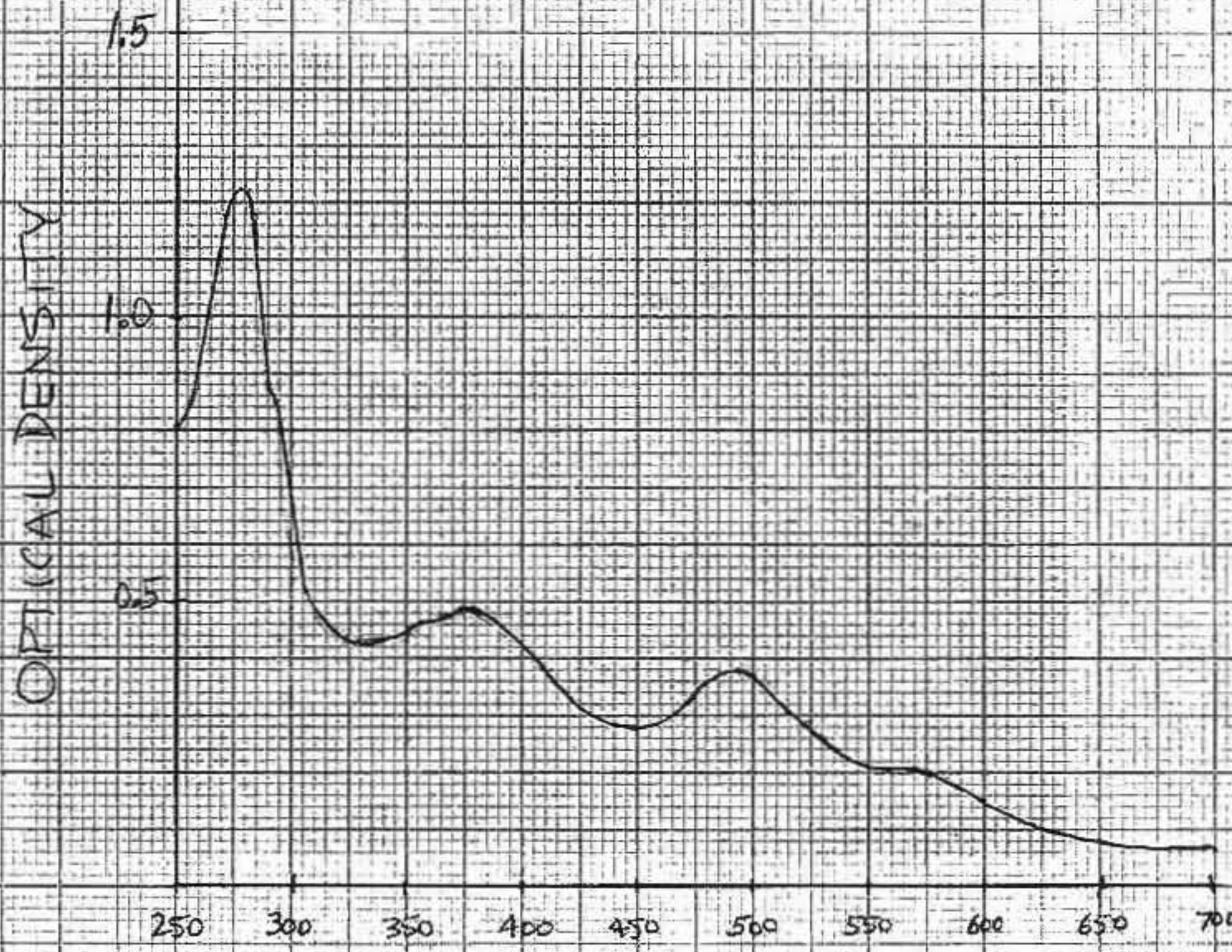
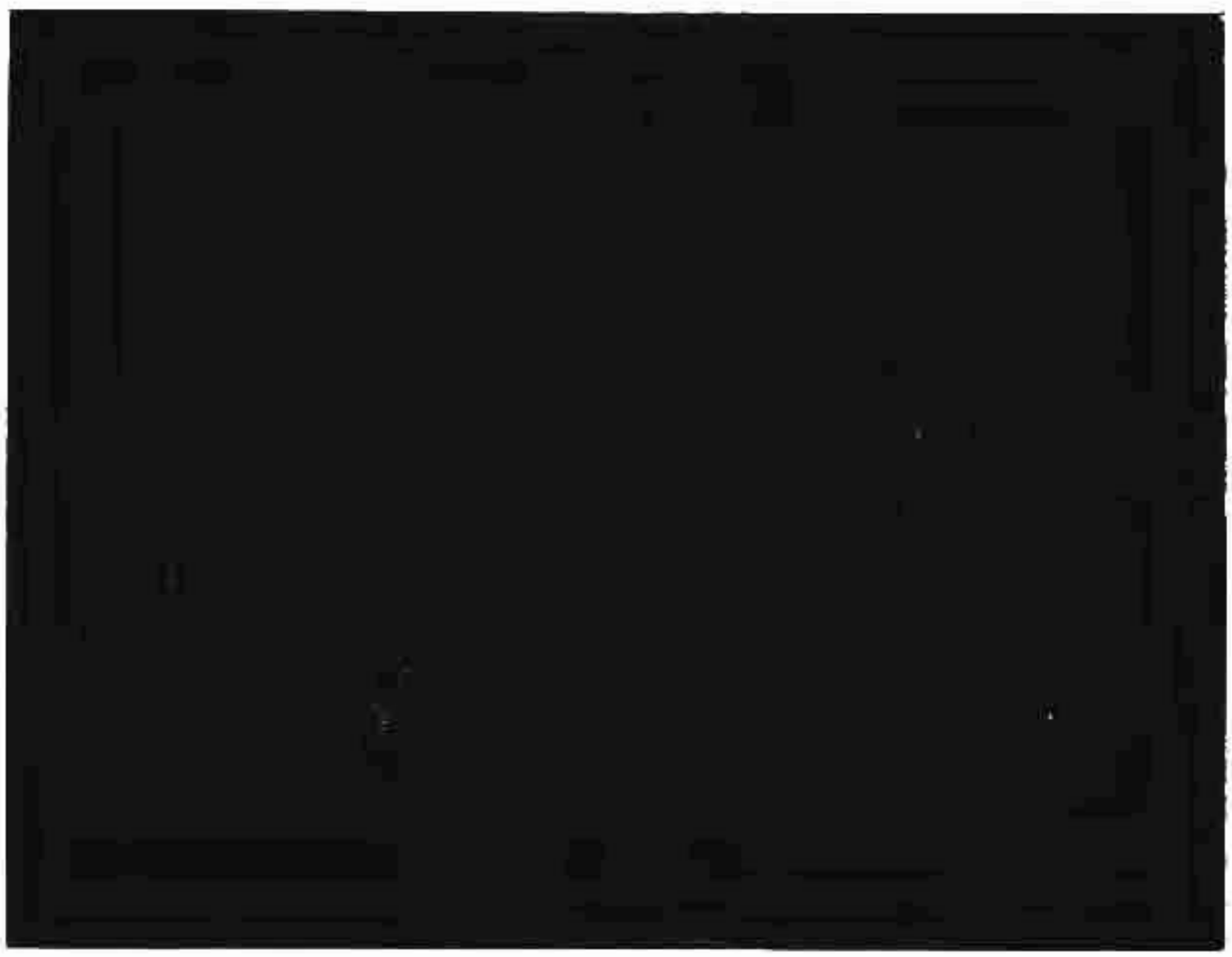


FIGURE V (a)

CRYSTALLINE M. aerogenes FERREDOXIN



10 X

The picture was taken with a Polaroid MP-3 Land Multi-purpose Industrial View Camera with a bellows attachment. (f 5.6, 0.5 second exposure, 35 mm lens).

FIGURE V (b)

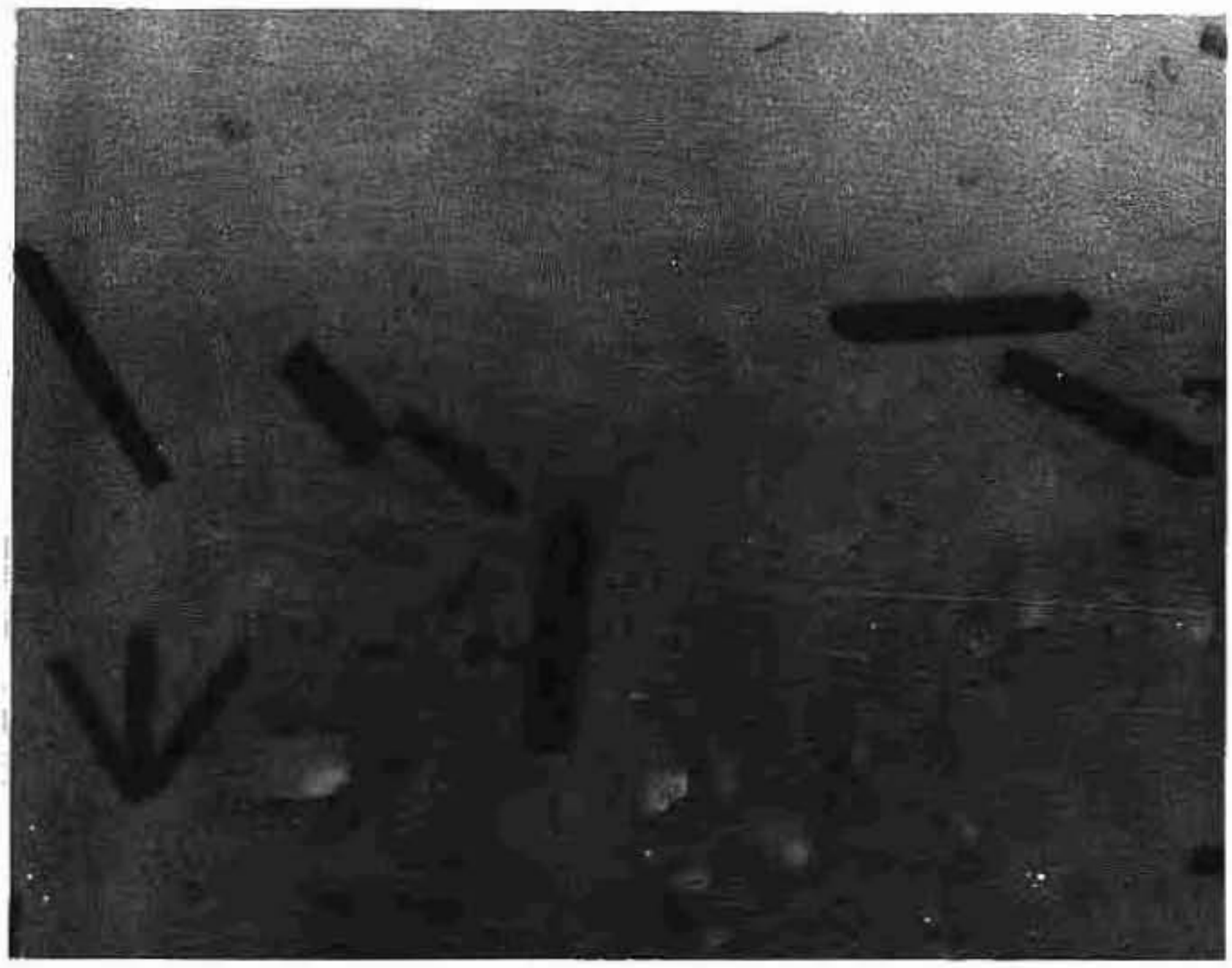
CRYSTALLINE M. aerogenes PRANDOVIN

37.5 K

This picture was taken with a Carl Zeiss Ultraphot photomicroscope.

FIGURE V (c)

CRYSTALLINE M. ASPOPCHES NITROPRUSS



100 X

This picture was taken with a Carl Zeiss Ultraphot photo-

microscope.

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Undergraduate Research

Dr. H.R. Whiteley

RESEARCH REPORT #2

Undergraduate Research

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I. METHODS

A. Growth and Harvesting of Bacteria

1. Micrococcus aerogenes growth and harvesting conditions for elution or protein purification experiments have in part been previously described (9). One hundred liter Fermacell cultures (either yeast extract or yeast hydrolysate medium) with 6% inocula were grown for 18 to 22 hrs.

Cultures used for hydrogenase studies were grown in yeast extract medium and harvested at an optical density of 0.75, which corresponded to 12 hrs incubation of a 5% inoculum. Cultures from 3 to 6 liters were harvested with a continuous flow centrifuge at 10,000 rpm at 4°, whereas 16 to 32 liter cultures were harvested with a Sharples centrifuge at 40,000 rpm at 4°. Cells used for hydrogenase studies were washed at 4° with 3 volumes of 0.1 M phosphate, pH 7.0 containing 0.005% sodium sulfide. These cells were stored under hydrogen in stoppered centrifuge tubes at -20°.

2. Micrococcus lactilyticus was grown in lactate medium as previously described (25). Sixteen liter carboys with 10% inocula were incubated at 37° for 14 to 16 hrs. Cultures were routinely harvested with a Sharples centrifuge at 4°. Cells were washed once with three times their volume of water and stored in stoppered centrifuge tubes at -20°.

B. Preparation of Cell-Free Extracts

1. The preparation of cell-free extracts of M. aerogenes for elution studies has been previously described (9). Cell-free extracts for hydrogenase studies were prepared by suspending cells in 2 to 4 volumes of 0.1 M K_2HPO_4 at pH 7.0, containing 5 um/ml dithiothreitol (DTT), and disrupting the suspension by sonic oscillation for 40 min at 4° under hydrogen using a Ratheon 10-Kc sonic oscillator (6). The extract was then centrifuged

for 30 min at 25,000 x g at 4°. The supernatant was stored under hydrogen at 4° and used within 1 to 3 hrs after preparation.

Cells were gently broken for localization or cell wall studies by shaking vials, each containing 6 ml cell suspension, 1 drop of n-tributyl citrate and 4 g of superbrite glass beads, under hydrogen for 45 min with a Mickle disintegrator at 4°. Glass beads were removed by centrifugation twice at 2,000 rpm for 3 min in a Sorvall centrifuge at 4° (11). Intact cells were removed by centrifugation at 4,000 rpm for 10 min as above. This step was repeated until intact cells were no longer sedimented, usually 2 to 3 times.

2. Cells free extracts of M. lactilyticus were prepared by suspending cells in 2 to 4 volumes of distilled water, sonicating the suspension for 45 min with the Ratheon 10-kc sonic oscillator at 4°, and centrifuging the extract at 25,000 x g for 30 min at 4°. The supernatant was stored under hydrogen at -20°, and recentrifuged as above before use.

C. Preparation of Cell Free Eluates of M. aerogenes

1. Standard Method

The standard method of freeze-thaw elution has been previously described (9). The initial samples used for ferredoxin and rubredoxin purification were prepared by two freeze-thaw elutions.

2. Varying pH

Tubes containg equal amounts of fresh cell pellets (435 mg protein per tube) were stored frozen for 46 hrs at -20°. Cell pellets were thawed by the addition of 6.0 ml of 0.1 M phosphate (pH 2-10) for 30 min at room temperature. A control tube was thawed in distilled water. Cells were then suspended, and after 2 hrs centrifuged as described above. The supernatants, pH and the Fd Eluate Units were determined as described below.

The supernatant and remaining cells from the control tube were also assayed for Fd units as described below.

The effect of pH upon crude Fd absorbancy was determined by adding 0.2 ml of salt free crude Fd to 1.8 ml of 0.1 M phosphate (pH 2-10). After 30 min at room temperature the mixtures were centrifuged. The absorbancy at 390 mu (A 390) of the supernatants were then determined.

3. Repeated Freeze-Thawing

The methods employed for this experiment have been in part previously described (9). Besides determining Fd units in the 1st, 2nd, and 3rd eluates and remaining cells, Fd units in non-treated cells were also determined as a control.

4. Osmotic Shock

The methods employed for osmotic shock of non-frozen cells were similar to those of Neu and Heppel (20). Fresh cell pellets were suspended in 10 volumes of either distilled water, 20% sucrose, or 20% sucrose-0.03 M Tris (pH 8.0) - 0.001 M EDTA. After 25 min of occasional stirring, suspensions were centrifuged, supernatants decanted and saved, and cell pellets shocked by resuspension in 9 volumes of distilled water. The preparations were stirred occasionally for 25 min, and then the suspensions were centrifuged. Supernatants were decanted and saved. All the above operations were carried out at 4°. The resulting pellets were eluted by the standard freeze-thaw method. Fd units were determined for all the above frations including the remaining cells.

In another experiment, frozen cell pellets were thawed in 8 volumes of sucrose of varying concentration between 0 and 30%. After 30 min, cell pellets were suspended, and after 2 hrs at room temperature the suspensions were centrifuged. The resulting supernatants were decanted and saved, whereas

the resulting pellets were then shocked by suspending in 5 volumes of distilled water. The samples were stirred occasionally at room temperature for 30 min and then centrifuged. Fd Eluate units were determined on all supernatants. In addition, Fd units were determined for the 1st distilled water eluate from the control tube and its corresponding remaining cells.

D. Viability Determination of M. aerogenes Cells

A 500 ml culture sample was removed aseptically from a 20 hour Fermacell culture. To determine the number of viable organisms present before and after centrifugation at 25,000 x g for 15 min at 4°, serial dilutions with sterile distilled water were made followed by plate counts in duplicate on glutamate plus 2% agar medium. Another portion of the culture sample was centrifuged as above and the pellet stored frozen for one week at -20°. The pellet was then thawed at room temperature by suspending the cells in sterile water to their original sampling volume. Plate counts were also performed on the resulting suspension as above. All plates were incubated for 84 hrs at 37° in Brewer jars under an atmosphere of 95% H_2, and 5% CO_2. All plates were prepared by the pour plate method with the agar at 45°.

E. Estimation of M. aerogenes Crude Ferredoxin

1. Present in Whole Cells

The estimation of Fd units for whole cells (eluted or non-eluted) has been previously described (9).

2. Present in Eluates

The estimation of Fd units for eluates has been in part previously described (9). A shorter method was used for determining Fd units in experiments which the effects of time, volume, pH, or sucrose concentration upon

elution were studied . First, the determination of the amount of ferredoxin present in all the eluates (Amount Eluate Fd) of a given experiment was made. Amount Eluate Fd was defined as A 390 x ml eluate. Next, the amount of crude ferredoxin (Amount Crude Fd) present in one eluate and its corresponding remaining cells was determined. Amount Crude Fd was defined as A 390 x ml Crude Fd. Knowing the total cell protein, Fd Eluate units and Fd units were calculated. These units were defined respectively as Amount Eluate or Amount Crude Fd times 100 divided by total cell protein. The ratio of Fd units over Fd Eluate units, or Amount Crude Fd over Amount Eluate Fd was used to convert Fd Eluate units to Fd units.

3. Present at Different Stages of Growth

Four 750 ml cultures (3% inoculum per flask) were grown for 12, 16, 20, and 24 hrs respectively. After harvesting, the cell pellets were stored at -20° for 72 hrs. These pellets were then eluted by thawing (standard method). Fd units were determined for the resulting eluates and the remaining cells.

For the determination of Fd units in a "step up" culture, two 750 ml flasks were grown to stationary phase. One flask was harvested and Fd units were determined. The other flask was used to inoculate fresh medium. Two hundred ml of inoculum to 600 ml of fresh medium (25% inoculum). After 3 hrs, the "step up" culture was harvested and Fd units were determined.

F. Purification of Ferredoxin and Rubredoxin

1. M. aerogenes

Purification methods employed below have in part been previously described (9).

Step a. Chromatography on DEAE-cellulose:

Cells resulting from 3-100 l Fermacell cultures (approximately

450 gm wet weight) were twice freeze-thaw eluted. The eluates were combined (1,170 ml) and adsorbed to a DEAE column (3.2 x 10 cm) which had been previously washed with 5 volumes of 0.1M Tris, pH 7.0. After eluate adsorption, the column was washed with 0.05 M KH_2PO_4, pH 6.5 until flavin bands were no longer eluted. The ferredoxin band was then eluted with 0.15 M Tris -0.67 M NaCl, pH 7.3. The above procedure was carried out at room temperature.

Step b. Gel-filtration:

To desalt the crude ferredoxin (Crude Fd) obtained by Step a, the sample (53 ml) was applied to and eluted from a G-25 Sephadex column (3.2 x 40 cm), which previously was equilibrated with and subsequently eluted by 0.1 M Tris, pH 7.0. The above operations were carried out at room temperature.

Step c. Chromatography on DEAE cellulose:

Desalted Crude Fd obtained from Step b (100 ml) was applied to a DEAE column (2.4 x 90 cm) which had been previously washed with 5 volumes of 0.1 M Tris, pH 7.0. After sample adsorption, the column was eluted with 0.15 M Tris - 0.15 M NaCl, pH 7.3 at a rate of 1 drop per 12 seconds. After 14 hrs rubredoxin had begun to be eluted. After approximately 16 hrs, rubredoxin had been completely eluted in a total volume of 178 ml. At this point the DEAE was removed by air pressure, and the ferredoxin - adsorbed DEAE was suspended in 4 volumes of distilled water. A new column (approximately 3.2 x 16 cm) was packed with this ferredoxin - adsorbed DEAE suspension. The latter column's ferredoxin was then eluted with 0.15 M Tris - 0.67 M NaCl, pH 7.3. The above procedures were performed at room temperature.

Step d. Crystallization of ferredoxin:

The ferredoxin sample (38.5 ml) obtained from Step c was brought to 85% saturation by the addition of solid $(NH_4)_2SO_4$ with constant stirring at 4°. After 30 min of constant stirring, the sample was centrifuged at 25,000 x g for 15 min at 4°. The resulting precipitate was grey-brown in color, whereas the supernatant was dark brown. Additional $(NH_4)_2SO_4$ was added as above to bring the supernatant fraction to 95% saturation. After 2 hrs at 4°, with occasional stirring, the sample was centrifuged as above. The resulting supernatant was yellow-brown in color, and the precipitate was brown-black. This precipitate was dissolved in 7.0 ml of 0.1 M Tris, pH 7.0, brought to 80% $(NH_4)_2SO_4$ saturation as above, keeping the pH between 7.0 and 7.5 by the addition of crystals of Tris, and stored at 4° until crystallization. After 4 days crystallization of ferredoxin was complete.

Step e. Crystallization of rubredoxin:

The rabredoxin from Step c (178 ml) was diluted with distilled water to 600 ml. The diluted rebredoxin sample was then adsorbed to a DEAE column (2.4 x 6 cm) which had been previously washed with 5 volumes of 0.1 M Tris, pH 7.0. The resulting dark red band of rubredoxin was eluted with 0.15 M Tris - 0.67 M NaCl, pH 7.3 in a volume of 15.5 ml. These operations were carried out at room temperature.

In order to remove any contaminating ferredoxin, the above sample was brought to 95% $(NH_4)_2SO_4$ saturation with constant stirring at 4°. After 1 hour the resulting sample was centrifuged as above. The precipitate was brown-red in color, whereas the supernatant

was red-pink. The supernatant was diluted 1: 4 and additional solid $(NH_4)_2SO_4$ was added to bring the saturation to 75%. This solution was stored at 4^{o} until crystallization. Crystallization of rubredoxin was complete after one month.

2. M. lactilyticus

The methods employed below were modifications of the procedures used by Buchanan et al. (5) and Sieker (22).

Step a. Chromatography on DEAE-cellulose:

Cells harvested from two 16 liter carboys were sonicated as described above. Sonic extract (420 ml) was applied to a DEAE column (2.4 x 15 cm) which had been previously washed with 5 volumes of 0.05 M Tris, pH 7.0. After adsorption, the column was washed with 0.05 M KH_2PO_4, pH 6.5 until flavin bands were no longer eluted. The ferredoxin was then eluted with 0.15 M Tris - 0.67 M NaCl, pH 7.3.

Step b. Gel-filtration:

To desalt the crude Fd obtained by Step a, the sample (70ml) was divided into two portions, each of which was applied to and eluted from a G-25 Sephadex column (3.2 x 40 cm) which was equilibrated with and eluted by 0.05 M tris, pH 7.0. All procedures were carried out at room temperature.

Step c. Chromatography on DEAE-cellulose:

Desalted crude Fd (148 ml) obtained from Step b was applied to and eluted from DEAE as in Step a.

Step d. Gel-filtration:

To desalt the crude Fd obtained by Step c, the sample (10 ml) was applied to and eluted from Sephadex G-25 as in Step b.

Step e. Chromatography on DEAE-cellulose:

Desalted crude Fd (61.5 ml) obtained from Step d was applied to and eluted from DEAE as in Step a.

Step f. Gel-filtration:

To desalt the crude Fd obtained by Stap e, the sample (16.5 ml) was applied to and eluted from Sephadex G-25 was in Step b.

Step g. Chromatography on DEAE-cellulose

Desalted crude Fd obtained from Step f (65.0 ml) was applied to a DEAE column (2.0 x 40 cm) which had been previously washed with 5 volumes of 0.05 M Tris, pH 7.0. After adsorption of the sample, the column was eluted with 0.075 M Tris - 0.075 M NaCl, pH 7.3 This buffer readily eluted the rubredoxin, but not ferredoxin. After the rubredoxin was collected (110 ml), the eluting buffer was changed to 0.15 M Tris - 0.15 M NaCl, pH 7.3. Ferredoxin was eluted by this buffer in a volume of 55.0 ml. All these operations were carried out at 4^{o}.

Step h. Crystallization of rubredoxin:

The rubredoxin obtained from Step g was diluted to 500 ml, adsorbed to and eluted from a DEAE bed (1.4 x 6 cm) as in Step a, yielding 5.1 ml. This sample was brought to 90% $(NH_4)_2SO_4$ saturation as previously described above. After 1 hour of occasional stirring at 4^{o} the sample was centrifuged at 25,000 x g for 15 min. The resulting precipitate was dark red, whereas the supernatant was pale yellow-pink. This precipitate was dissolved in 1 ml and brought to approximately 50% $(NH_4)_2SO_4$ saturation as previously described. After one week at 4^{o} crystallization of rubredoxin

was complete.

It should be noted that when fractions of M. aerogenes or M. lactilyticus ferredoxin or rubredoxin were not used immediately, they were stored under hydrogen at -20°.

G. Purification of Cell Walls

The methods used for preparation of M. aerogenes cell-free extracts using the Mickle disintegrator have been previously described above.

Cell walls were removed from a cell-free extract (from either fresh or frozen cells) prepared as above by centrifugation at 8,000 rpm for 10 min at 4°. The resulting supernatant was discarded, whereas the precipitate was suspended in distilled water to the original extract volume and re-centrifuged at 8,000 rpm as above. This washing procedure was repeated 5 times. Removal of any remaining whole cells was accomplished by suspending the 8,000 rpm precipitate from the above step with distilled water to the original extract volume, and centrifuging this suspension at 4,000 rpm for 10 min at 4°. The supernatant obtained by this procedure was carefully removed with a pipette. This supernatant served as a source of purified cell walls for electron microscopy.

H. Localization of Hydrogenase and Ferredoxin from M. aerogenes

Fresh, non-frozen cells of M. aerogenes, which has been grown for 12 hrs in a 16 l carboy of yeast extract-glutamate medium and harvested at an optical density of 0.75, served as a source of extract for localization studies by differential centrifugation

1. Hydrogenase

To localize hydrogenase, fresh cells were suspended in 4 volumes of 0.1 M K_2HPO_4 at pH 7.0 - 5 um/ml DTT - 0.005 M $MgCl_2$ (PDM) and broken under hydrogen using the Mickle disintegrator. Glass beads and intact cells were removed from the resulting extract by centrifugation at 4° as described above. After approximately 2.5 ml of sample was removed for an immediate hydrogenase assay, the remaining cell-free extract (8.0 ml) was diluted with PDM to a volume of 12.0 ml. This sample was then centrifuged at 25,000 rpm for 30 min at 4°. After centrifugation the precipitate was suspended in 10 ml of PDM and the above centrifugation step was repeated, whereas the resulting supernatant (10.7 ml) was subjected to centrifugation for 6 hrs at 100,000 x g. Before centrifugation, however, 2.5 ml of the sample was removed for an immediate hydrogenase assay and the remaining sample was diluted to 12.0 ml, 11.5 ml of which was centrifuged. The resulting 25,000 x g wash (10 ml) and precipitate (suspended in 10 ml of PDM) were also immediately assayed for hydrogenase activity. The resulting 100,000 x g supernatant (10.5 ml) was assayed immediately for hydrogenase, whereas the precipitate was suspended in 12 ml of PDM, 11.5 ml of which was recentrifuged at 100,000 x g for 3 hrs. The resulting wash (10.6 ml) and precipitate (suspended in 10 ml PDM) were also assayed immediately for hydrogenase. The data describing the localization experiment (Table 15) have been corrected for volume changes and dilutions. Table 15 expresses total activity as if samples had not been taken.

2. Ferredoxin

For the localization of ferredoxin, a fresh cell pellet was suspended in 4 volumes of PDM. Half of this suspension was broken by sonication, the other half by shaking with glass beads.

After centrifugation of the sonicate 3 times at 4,000 rpm for 10 min at 4°, the resulting supernatant served as a source of total ferredoxin. This supernatant was adsorbed to and eluted from DEAE, and Fd units were determined as previously described.

After the removal of glass beads and intact cells as previously described, the resulting "gentle broken" cell free extract was centrifuged for 6 hrs at 100,000 x g at 4°. The resulting precipitate was washed once by suspending with PDM and centrifuging at 100,000 x g for 3 hrs at 4°. The resulting 100,000 x g supernatant and wash served as a source of total soluble ferredoxin. These supernatants were adsorbed to and eluted from DEAE columns and Fd units were determined as previously described.

The determination of Fd ENZ units was also performed for the sonicate and glass-bead broken fractions from above (see Assays-acetyl phosphate).

I. Determination of Molecular Weights

1. Sephadex G-75 Gel-filtration

The methods used below were similar to those used by P. Andrews (3).

A G-75 Sephadex (fine grade) column (1.6 x 90 cm) was equilibrated with 0.05 M Tris - 0.1 M KCl (pH 7.0) at 4°. All column operations were subsequently carried out at 4° using the above buffer. Column calibration was carried out in 3 separate experiments by determining the elution volume required for each component of the following mixtures.

a. blue dextran and cytochrome C (V)

b. lysozyme, γ-globulin, and FMN

and, c. haemoglobin and vitamin B_{12}

One ml samples containing 3 mg each of the compounds in either, group a, b, or c, and 50 mg sucrose were subjected to gel-filtration.

Sixteen drops per tube (1.0 ml) were collected using a Packard fraction collector. Fractions were assayed spectrophotometrically at the following wavelengths: blue dextran -- 625 mu, cytochrome C (V) -- 408 mu, lysozyme -- 280 mu, γ-globulin -- 280 mu, FMN -- 445 mu, haemoglobin -- 410 mu, and vitamin B_{12} -- 360 mu. (see calibration curve, Figure 8).

For determining the molecular weights of the ultraviolet absorbing material present in the freeze-thaw eluate of M. aerogenes, 7.5 ml of eluate was lyophilized. The freeze-dryed sample was then dissolved in 1.5 ml of 0.1 M Tris, pH 7.0, and 1.0 ml (19 mg) of the resulting solution was subjected to gel-filtration as described above. Column fractions were assayed at 280 mu and 260 mu. Colored fractions were also assayed at 390 mu and 490 mu, and assayed for acetyl phosphate activity.

2. Sucrose Gradient Centrifugation

The methods employed here were similar to those of Martin and Ames (16).

Three 5-20% sucrose gradients (4.50 ml each) were prepared by a two chamber mixing device. To one gradient, 0.2 ml of eluate was applied (0.7 mg), to the other gradients, 0.2 ml of a mixture of eluate (0.7 mg) and haemoglobin (1.3 mg) was applied. Centrifugation of samples was carried out at 115,000 x g for 15 hrs at 4°. Fractionation (8 drops per tube) of each gradient yielded 45 tubes per gradient. To each fraction 0.30 ml of distilled water was added. The diluted fractions' protein concentrations were subsequently determined by the Lowry micro-method (15). Haemoglobin was localized by its absorbancy at 410 mu.

J. Assays

1. Protein

Protein was determined by the method of Lowry et al.(15), using the either a macro or micro method (7.5 or 1.5 ml final volume respectively).

2. Nucleic Acids

RNA and DNA concentrations were determined by the method of W. C. Schneider (21), as modified by Davidson and Smellie (7). It was assumed that 1 mg of RNA or DNA had an optical density of 22 or 20 repectively at 260 mu.

3. Spectrophotometry

Growth of bacteria was measured at 500 mu in a Beckman Spectronic 20. Spectral studies were performed using the Beckman DK-2 recording spectrophotometer. All other absorbancys were measured by the Beckman DU spectrophotometer with Gilford attachments.

4. RNase

RNase activity present in a freeze-thaw eluate or a cell-free extract of M. aerogenes was assayed by measuring the abilities of the above to degrade C^{14}- RNA from Bacillus subtilis. Reaction mixtures contained 0.05 ml C^{14}-RNA (1,837 counts per 10 min), 0.1 ml yeast carrier RNA (10 ug), 0.5 ml of 0.01 M Tris - 0.01 M $MgCl_2$ (pH 7.5) and either; no addition (control); 0.05 ml extract; 0.1 ml extract; 0.15 ml eluate; or 0.30 ml eluate. Final volume was 1.0 ml. Incubation time was 30 min at 37°. The reaction was terminated at 4° by the addition of 0.05 ml yeast RNA (100 ug) and 1.0 ml of cold 10% TCA. After 10 min in an ice bath, each reaction mixture's RNA was collected on separate Milipore membrane filters by suction filtration. Each filter was washed 4 times with cold 5% TCA. The filters were subsequently placed in vials and dried at 80° for 15 min. After the vials had cooled to room temperature, 10 ml per vial of toluene scintillation solution was added. Vials were counted for 10 min in an ambient temperature Nuclear Chicago scintillation counter. Background was less than 10% of the control.

5. Histidase

One percent final concentration protamine sulfate supernatants of a freeze-thaw eluate and a complete cell-free extract of M. aerogenes were

assayed for histidase activity according to the method of Wickremasinghe and Fry (26)*.

6. Acetyl Phosphate

Two methods were used for the determination of acetyl phosphate formation.

In early experiments the method of Mortenson, Valentine and Carnahan (19) was used. In latter experiments, a more sensitive assay was used. (13). One ml reaction mixtures contained 25 um KH_2PO_4, pH 6.5, 10 um sodium pyruvate, 0.05 um CoA, from 0 to 500 mg protein minus crude Fd. After 10 min incubation at room temperature the reaction was terminated by the addition of neutralized 28% hydroxylamine, and after an additional 10 min, 1.5 ml of ferric chloride reagent (12) was added. The solution was centrifuged at 25,000 x g for 10 min and the absorbance of the supernatant was measured at 540 mu. Under these conditions 1 um of acetyl hydroxamate had an absorbance of 0.045 (see standard curve, Figure 16). One unit of Fd was defined as catalysing the formation of 1 um of acetyl phosphate above the amount formed in the control reaction which had no Fd (used 4.0 mg protein minus crude Fd).

Stock protein for the above assay was prepared by centrifuging large batches of sonic cell-free extract for 3 hrs at 100,000 x g at 4°. The resulting supernatant was stored at -20° under hydrogen in vials containing 5 ml each. Vials were thawed just before use and protein was immediately depleted by passage through DEAE columns (1.2 x 4 cm per 5 ml extract) equilibrated with and eluted by 0.05 M phosphate, pH 6.5. The resulting eluted protein was used immediately and served as a source of protein minus crude Fd for the above assay.

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* The author wishes to thank Robert LeDoux for determining the histidase activity in the freeze-thaw eluates.

7. Hydrogen Evolution or Uptake

The evolution or utilization of hydrogen was measured at 30° by conventional manometric techniques (23). Vessels were flushed for 15 min, with shaking, either with nitrogen or hydrogen which had been previously passed through a hot copper columnn to remove trace amounts of oxygen. Reactions were started after an additional 15 min of shaking by the addition of substrate. All experiments were carried out at pH 7.5 in the presence of 100 um phosphate.

When methyl viologen (MV) was used, a calculated 20 um was routinely added per vessel. Of this 20 um, only 5 to 8 um was observed to be active.

Two methods were employed for assaying hydrogenase activity in crude extracts of M. aerogenes, either the evolution of hydrogen from or the uptake of hydrogen by reduced or oxidized methyl viologen respectively.

Procedures used in the evolution assay were those of McCormick (18). Each vessel contained 5 mg dithionite added as a dry powder, 6-8 active umoles of MV, 100 um phosphate, pH 7.5, 0 to 30 mg protein, 0.2 ml 20% KOH and distilled water to 2.2 ml. The gas phase was nitrogen.

For hydrogen uptake studies, each vessle contained 6-8 active umoles of MV, 100 um phosphate, pH 7.5, 0 to 30 mg protein, 0.2 ml 20% KOH, and distilled water to a final volume of 2.2 ml. The gas phase was hydrogen.

In experiments where dilute protein fractions were assayed, 10 mg of crystalline bovine serum albumin was added. Precipitates were assayed in the presence of 0.4 ml of BES (27).

8. Disc Electrophoresis

Procedures using acrylamide gel electrophoresis for the detection of protein bands have been previously reported (9).

For the detection of hydrogenase bands (2), electrophoresis was conducted at 4°. After the bromo-phenol blue marker dye had migrated 90% of the gel's length, electrophoresis was stopped and gels were immediately removed. Gels were then placed in serum stoppered test tubes (1.5 x 15 cm), each containing 1.6 mg tetrazolium violet and 7.0 ml phosphate (pH 7.0). Tubes were then evacuated with as aspirator and flushed for 1 min with either hydrogen or nitrogen using syringe needles for intake and exhaust ports. The resulting reaction mixtures were incubated at room temperature, horizontally, from 1 to 24 hrs with occasional shaking.

II. ABBREVIATIONS

$AcPO_4$	acetyl phosphate
BES	boiled extract supernatant (27)
CoA	coenzyme A
DEAE	DEAE-cellulose, diethyl aminoethyl cellulose
DNA	deoxyribonucleic acid
DPN	diphosphopyridine nucleotide
DTT	dithiothreitol
EDTA	ethylenediaminotetra-acetic acid
Fd	ferredoxin
FMN	flavin mononucleotide
FTE	freeze-thaw elution
MV	methyl viologen
Rd	rubredoxin
RNA	ribonucleic acid
RNase	ribonuclease
TCA	trichloroacetic acid
TPN	triphosphopyridine nucleotide
Tris	Tris(hydroxymethyl)amino-methane

III. DEFINITIONS

A 280/490	Absorbancy ratio used as a criterion for the purity of rubredoxin. Ratio decreases as purity increases.
A 390/280	Absorbacy ratio used as criterion for the purity of ferredoxin. Ratio increases as purity increases.
Amount Fd	A 390 x ml Fd
Amount Crude Fd	A 390 x ml crude Fd
Amount Eluate Fd	A 390 x ml Eluate
Crude Fd	A mixture of Fd, Rd, and contaminating proteins resulting from Step a of M. aerogenes Fd purification
Depleting	The process of removal of crude Fd
Fd units	Amount crude Fd x 100/mg Total cell protein
Fd Eluate units	Amount eluate Fd x 100/mg Total cell protein
Fd EWZ units	The change in absorbancy at 540 mu per 12 mg depleted protein per 10 min incubation per ml crude Fd times total ml crude Fd times 10 divided by mg total cell protein (activity per ml crude Fd times total ml crude Fd times 10 divided by mg total cell protein).
Freeze-thaw eluate	The eluate which results from thawing frozen cells of M. aerogenes
% Fd eluted	Fd units eluted x 100/total Fd units or Fd units eluted x 100/Fd units eluted plus Fd units remaining
% Protein eluted	mg protein eluted x 100/total mg protein or mg protein eluted x 100/mg protein eluted plus mg protein remaini

Specific activities

a. Ferredoxin um $AcPO_4$ formed above the control per 4 mg depleted protein per 10 min incubation at room temperature per mg ferredoxin (units per mg Fd)

b. Hydrogenase ul hydrogen evolution or uptake per min per mg protein

IV. RESULTS AND DISCUSSION

A. Elution of Ferredoxin and Other Low Molecular Weight Compounds from Micrococcus aerogenes

1. Optimum Conditions of Freeze-Thaw Elution

a. Time of Elution. Figure 1 shows that the % Fd eluted reached a maximum of 63% after 1 hour of thawing at room temperature. Additional incubation resulted in a slight increase in the % total cell protein eluted, from 3.4% to 4.0%, whereas the % Fd eluted remained constant.

b. Volume of Elution. Figure 2 shows that the % Fd eluted reached a maximum of 67% when cells were thawed by suspending in 3 volumes of distilled water. No increase in the % Fd eluted resulted from increasing the volume of water used for thawing.

The % total cell protein eluted reached a constant maximum of 3.2% after thawing in 2 volumes of water. No increase in % protein eluted resulted by using larger volumes of water for thawing.

c. Eluting Agents. Table 1 shows that approximately 70% of the cells' Fd was eluted by using distilled water, with or without the presence of 10^{-3} M EDTA. The use of either 0.1 M phosphate, pH 7.0, or 0.1 M Tris, pH 7.0, as an eluting agent resulted in the elution of approximately 60% of the cells' Fd.

Two to 4% of the total cell protein was eluted in all cases except in the presence of 10^{-3} M EDTA. When EDTA was used as aneluting agent, 7.8% of the total cell protein was eluted.

d. pH of Elution. The effect of pH upon elution was studied by using 0.1 M phosphate as the eluting agent. Figure 3 shows that the percent of non-denatured Fd eluted varied from a maximum of 83% at pH 4, to a minimum

of 61% at pH 7.

The percent of non-denatured total cell protein eluted varied from a maximum of 4.6% at pH 4 to a minimum of 3.5% at pH 6.

pHs below 4 or above 8 resulted in denaturation of protein. Figure 4 shows the effect of pH upon the absorbancy of crude Fd. Marked denaturation took place at pHs below 4.

pHs between 4 and 6 eluted Fd with high efficiency, however, Fd was unstable at these acidic pHs. Therefore, this method was not employed for the large scale purification of Fd.

2. Methods to Increase Ferredoxin Elution

a. Repeated Freeze-Thawing. Table 2 summarizes an experiment in which cells were freeze-thaw eluted 3 times. Sixty-one percent, 9 percent, and 3 percent of the cells' ferredoxin was eluted by the first, second, and third elution respectively. The remaining cells, however, contained only 3 % of their original Fd content. These results suggest that about 24% of the cells' Fd was denatured during the repeated freeze-thaw elution process.

Three and a half percent, 1.1 percent, and 0.2 percent of the total cell protein was eluted by the first, second, and third elution repectively. Thus, only about 5% of the cell protein was eluted by 3 freeze-thawings.

b. Osmotic Shock

1. Non-frozen Cells. Neu and Heppel have previously reported (20) an osmotic shocking method for the elution of various enzymes localized between the cell wall and cell membrane of E. coli. Their shocking method consisted of suspending fresh cells of E. coli in 20% sucrose - 0.03 M Tris (pH 8) - 10^{-3} M EDTA at 4° with stirring, centrifuging the resulting suspension, and suspending the cell pellet in distilled water. Their results indicated that approximately 60% of the cells' total alkaline phosphatase and 11% of the total cell protein were eluted by one shocking cycle (20).

Table 3 shows the results of an experiment employing the shocking techniques of Neu and Heppel on fresh cells of M. aerogenes. When cells were

washed twice with distilled water (control), approximately 23% Fd was eluted. When sucrose was used for the suspending solution, followed by osmotic shock with distilled water, 36% Fd was eluted. The presence of EDTA did not increase the % Fd eluted.

After shock, cells were freeze-thaw eluted. Results indicated that previous treatments of cells did not increase the total Fd eluted, which ranged from 57% to 63%.

Previous treatments of cells, however, did enhance the elution of total cell protein. Non-pretreated cells freeze-thaw eluted 3.3% total cell protein. In contrast, sucrose pretreated cells eluted 8.3% of the total cell protein.

ii. Frozen Cells. In an attempt to increase the % Fd eluted, frozen cells of M. aerogenes were thawed in the presence of sucrose of various concentrations, centrifuged, and the resulting cell pellet was osmotically shocked by suspension in distilled water.

Figure 5 (curve 1) shows that the % Fd freeze-thaw eluted was a maximum of 70% when 30% sucrose was used as the eluting agent, and a minimum of 61% when distilled water was used as the eluting agent.

Figure 5 (curve 2) shows that the % Fd eluted by osmotic shock was a maximum of 17% for the 30% sucrose pre-treated cells, and a minimum of 12% for the distilled water pre-treated cells.

Curve 3 in Figure 5 shows the sum of curves 1 and 2. The toal Fd eluted ranged from 74% to 88%.

Figure 6 (curves 1, 2, and 3) show the % total cell protein eluted by the previous experiment. The sum of the freeze-thawing and shocking steps (curve 3) resulted in a maximum of 5.8% and a minimum of 4.4% total cell protein eluted respectively.

3. Production and Elution of Ferredoxin from Cells at Different Stages of Growth

a. Freeze-Thaw Elution of Ferredoxin from Cells at Different Stages of Growth. The data from Table 4 shows that the elution of Fd from flask grown cells at different stages of growth was erratic, and below the % Fd eluted from Fermacell grown cells. Apparently cells from flask grown cultures are less susceptible to freeze-thaw elution. This could be a result of the oxygen tension. Cells grown less anaerobically may produce more rigid cell walls and/or cell membranes, or less soluble Fd.

b. Ferredoxin Production by Cells at Different Stages of Growth. Table 4 also shows that the Fd units present in a youmg culture (absorbance 0.44) were approximately twice the value of the Fd units present in cultures of latter stages of growth.

A plot of the log absorbance versus either the amount of total cell protein or total Fd produced should describe the kinetics of protein or Fd synthesis respectively (since cells are growing logarithmically, the log absorbance is directly proportional to time).

Figure 7 is such a plot. The production of protein followed an exponential curve. The production of Fd was quite different In very young cultures, Fd synthesis must have been at a rate much greater than that of total cell protein, since, from absorbancies of 0.44 to 0.68, no increase in total Fd occurred, and, in cultures with higher absorbancies Fd production followed the rate of total cell protein synthesis.

c. Ferredoxin Production by a "Step Up" Culture

The observation that Fd in young cultures was produced at a rate greater than that of total cell protein was also supported by a "step up" experiment. Table 5 shows that when stationary cells were diluted with

fresh medium and harvested after approximately one generation, the Fd units were increased 1.5 fold.

4. Properties of the Eluate

a. Physical

i. Nucleic acid composition. Table 6 shows that the eluate obtained by thawing frozen cells of M. aerogenes with distilled water contains negligible amounts of nucleic acid (cold TCA insoluble, hot TCA soluble material). Only 0.72% of the cells' RNA and 0.27% of the cells' DNA was found to be present in a freeze-thaw eluate.

ii. Molecular Size of Eluted Materials.

a) Sephadex Gel-filtration. Figure 9 shows the approximate molecular weights of the ultraviolet absorbing material present in a freeze-thaw eluate of M. aerogenes. Gel-filtration of the eluate on G-75 Sephadex resulted in 4 distinct peaks, with molecular weights: (1) greater or equal to 100,000, (2) less than or equal to 40,000, (3) less than or equal to 17,500, and (4) less than or equal to 3,500.

The protein concentration of the four resulting peaks was estimated by using Kalckar's formula (mg/ml = A 280 x 1.45 - A 260 x .74). One %, 0.25%, 20%, and 79% of the total protein was estimated to be contained in peaks 1-4 respectively.

Peak 1, greater than or equal to 100,000 molecular weight is probably a result of a small amount of cell lysis.

Peak 2, less than or equal to 40,000 molecular weight is probably mainly nucleic acid (t-RNA ?).

Peak 3, less than or equal to 17,500 molecular weight, was shown to contain all the eluates Fd and Rd by absorbancy measurements as well as acetyl phosphate synthesis activity.

The unusually large molecular weight for Fd and Rd may imply aggregation of Fd and Rd by disulfide linkage, or Fd and Rd may not migrate as spherical proteins through G-75 Sephadex.

Peak 4, less than or equal to 3,500 molecular weight, contained flavin, and probably in addition short chains of peptides and nucleotides, oligio-polysaccharides and other cellular pool substances.

b) Sucrose Gradient Centrifugation. Figure 10 shows the results of an experiment where an eluate was subjected to sucrose gradient centrifugation. Molecular weight were estimated by a control tube which had eluate plus added haemoglobin as a marker (16). The bulk of the protein, including Fd, remained at, or near the top of the gradient. Approximately 80% of the total protein had a molecular weight less than or equal to 15,000.

b. Biological

A freeze-thaw eluate and a complete cell-free extract, both derived from carboy grown M. aerogenes, were examined for the presence of 4 enzymatic activities. Hydrogenase, the complete acetyl phosphate enzymatic system, RNase, and histidase were all found to be present, and very reactive in a complete cell-free extract. However, none of the above activities were found to be present in a freeze-thaw eluate.

5. Localization of Ferredoxin

Table 7 shows the results of an experiment which employed differential centrifugation of a gently broken cell-free extract in an attempt to localize Fd. Crude Fd was assayed by both A 390 and acetyl phosphate formation.

This experiment made the assumption that a cell-free sonicate contained 100% of the cells' Fd.

Part C of Table 7 shows that 83-85% of the cells' Fd was soluble after

centrifugation at 100,000 x g for 6 hrs. After suspending the 100,000 x g precipitate in PDM buffer and repeating the 100,000 x g centrifugation for 3hrs, an additional 7-10% of the cells' Fd was rendered soluble.

These results indicate that Fd is 90-95% soluble in a gently broken cell-free extract.

6. Changes in Cells after Freeze-Thaw Elution

a. Viability

Table 8 shows that M. aerogenes retained only 0.6% of its original viability after freezing and thawing. Similar results have been reported for other bacteria.

b. Morphology

i. Whole Cells. No detectable differences could be observed between freeze-thaw eluted cells and fresh cells when examined by phase contrast microscopy.

ii. Cell Walls. Preliminary studies of the morphology of either non-frozen or frozen purified cell walls were made with the aid of the electron microscope.* The non-frozen cell walls were shown to be associated with a membrane-like material which probably was on the interior side of the cell wall. This material was not detected in the freeze-thaw eluted cell wall sample.

7. Comments

a. Eluate Contents

The 1st eluate has been shown to contain less than 1% of the cells' nucleic acid and approximately 3% of the cells' protein. Acrylamide gel electrophoresis of the 1st eluate demonstrated 23 distinct acidic protein bands. Sucrose gradient centrifugation as well as Sephadex gel-filtration of the 1st

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* The author whished to express his thanks to Dr. V. Chambers for her services in electron microscopy.

eluate showed that at least 80% of the eluates' protein had a molecular weight of less than or equal to 15,000. A preliminary examination of the 4 enzymatic activities (hydrogenase, histidase, phosphoclastic, and RNase) showed the complete absence of these enzymes in the 1st eluate.

Fd and Rd are estimated to comprise 0.84% and 0.61% respectively of the total soluble cell protein of M. aerogenes[a]. The combined 1st and 2nd eluates are estimated to contain at least 70% of the cells' Fd and Rd, whereas only 4.5% of the cell protein[b]. Fd and rubredoxin comprise approximately 22% of the combined eluates' protein (12.8% Fd and 9.2% Rd)[c]. Thus, by using an eluate instead of a cell-free extract as the starting material for Fd and Rd purification, one achieves an initial 15 fold purification advantage.

b. Mechanism of Freeze-Thaw Elution

Since the process of freeze-thaw elution (FTE) results in the leakage of a variety of the cells' low molecular weight compounds, the mechanism of FTE is of considerable interest.

Gerhardt and Judge (10) have shown that cell walls may act as molecular sieves, with permeability to low molecular weight compounds, and exclusion of high molecular weight substances (e.g. Yeast exclusion limit (EL)= 4,500; Bacillus megaterium EL = 57,000).

Since only low molecular weight substances are eluted, the types of molecules eluted are probably limited by the pore size of the cell wall of M. aerogenes.

When frozen cells were eluted with sucrose solutions of comparable osmotic pressures to that of the cell contents, no decrease in Fd elution was observed. This result is consistent with the interpretation that permeability damage

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[a] This was calculated from purification data obtained from Table 9. See APPENDIX OF CALCULATIONS

[b] See Table 2

[c] The value of 22% is supported by both Sephadex gel-filtration, and purification data. See APPENDIX OF CALCULATIONS

is a result of freeze-thawing, and not due to additional damage caused by osmotic shock by distilled water.

Marzur explains freeze-thawing damage to cells is caused by: Thermal shock, an increase in solute concentration, and injury from ice crystal formation (17). Numerous workers have reported that freezing and thawing can cause extensive damage to membranes (8).

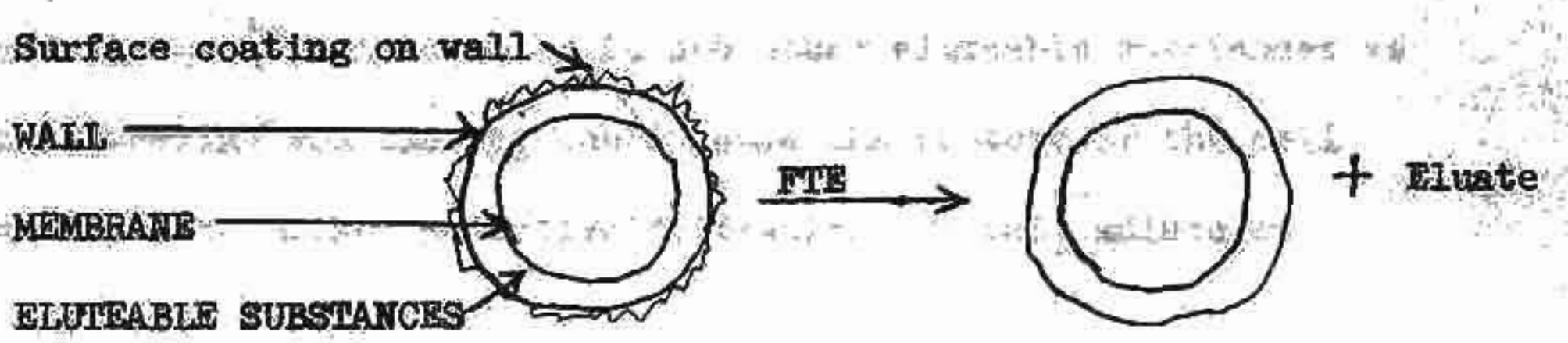
Several questions concerning the mechanism of FTE remain unanswered. Is the leakage of low molecular weight substances a result of an alteration in, cell wall permeability, cell membrane permeability, or both?

When FTE cells and fresh cells were compared by phase contrast microscopy, detectable differences between the cells were not observed.

Freeze-thaw elution may be explained by 2 possible models.

Model I

(The modification of cell wall permeability)



In this model, Fd and other eluteable substances are localized at the cell membrane or between the cell wall and cell membrane.

Freezing and thawing would effect the release of membrane bound substances, with the concomitant change in cell wall permeability (e.g. release of surface coating). The net result would be the leakage of low molecular weight substances. In this model, the membrane would remain intact.

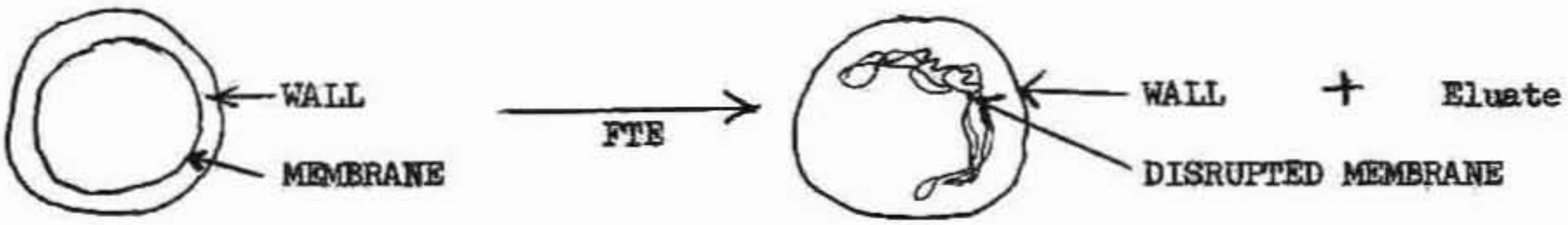
Possible support for such a model could be achieved by protoplast formation in a hypertonic solution, if Fd and Rd were released into the medium. Unfortunately, attempts to obtain protoplasts were unsuccessful(9).

Preliminary studies in electron microscopy of purified cell walls from either FTE cells of fresh cells showed a difference in morphology. Cell walls prepared from fresh bacteria had a membrane-like structure, which appeared to be present in the interior of the cell wall. This could be possible contaminating membrane..

An alternate, and much more probably explanation of FTE is Model II.

Model II

(The disruption of the cell membrane)



In this model, the localization of Fd and other eluteable substances is not important. Freezing and thawing would cause the rupture of the cell membrane, with the concomitant selective filtration of small molecules through the cell wall.

If this model were correct, then one would expect a large percentage (e.g. 70%) of all the soluble low molecular weight material of the cell to be eluted. This could be readily tested by several methods.

One method would be to treat known amounts of; an eluate, a complete extract, and an extract obtained from eluted cells with cold TCA. After centrifugation, the cold TCA soluble material would be decanted and the absorbance at 260 mu read for each of the 3 soluble fractions. By comparing

the absorbancies at 260 mu, one could readily determine just how much of the cells' pool was eluted. If model II was correct, perhaps 70% or more of the acid soluble, 260 mu absorbing material would be eluted.

An alternative experiment to support or eliminate model II would be G-25 Sephadex gel-filtration of an eluate, a complete extract, and an extract prepared from eluted cells. By comparing the amount of low molecular weight material (3,000 or less), which absorbs at 260 mu, present in each sample, one could determine the % of low molecular weight material eluted.

Presently, model II is favored, since such a large amount of low molecular weight, 260 mu absorbing material was shown to be present in an eluate in the Sephadex G-75 experiment.

The electron micrographs of cell walls could also support model II. If freeze-thaw elution disrupts cell membrane, then one would expect cell membrane only associated with fresh cell walls.

In reality, the mechanism of FTE may be a hybrid of models I and II.

B. Comparison of Ferredoxin and Rubredoxin from M. aerogenes and M. lactilyticus

1. Physical Properties

a. Methods of Purification. Tables 9 and 10 summarize the procedures and results obtained for the purification of Fd and Rd from M. aerogenes and M. lactilyticus respectively. Several differences exist between the 2 organisms' electron transport proteins. These are:

i. Since FTE cannot be used as an aid in the purification of Fd and Rd from M. lactilyticus, many more chromatography steps must be employed, in comparison with those of M. aerogenes, to achieve the same level of purity.

ii. Fd and Rd from M. lactilyticus elute much more readily from DEAE-cellulose than do the corresponding proteins from M. aerogenes. Therefore, a different buffer system must be used for their purification. For example, 0.1 M Tris, pH 7.0, elutes M. lactilyticus' Rd, but not the Rd from M. aerogenes.

iii. Rd from M. lactilyticus readily crystallizes in the presence of $(NH_4)_2SO_4$, whereas Rd from M. aerogenes does not.

iv. Fd from M. aerogenes readily crystallizes in the presence of $(NH_4)_2SO_4$, whereas M. lactilyticus' Fd does not.

v. Fd from M. aerogenes is probably more stable than Fd from M. lactilyticus.

b. Amounts Present in Cells. Using the data from Tables 9 and 10, it is possible to estimate the per cent of total soluble cell protein that is Fd or Rd in M. aerogenes and M. lactilyticus.*

Fd is estimated to comprise 0.84% and 0.47% of the total soluble cell protein of M. aerogenes and M. lactilyticus respectively.

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* See APPENDIX OF CALCULATIONS

Rubredoxin is estimated to comprise 0.62% and 0.29% of the total soluble cell protein of M. aerogenes and M. lactilyticus respectively.

Although the biological role of Rd has not as yet been established, Rd must be of fundamental importance in the metabolism of these micrococci, since Rd comprises a significant portion of the soluble protein of these bacteria.

The ratios of Fd/Rd in each organism are nearly the same, 1.35 for M. aerogenes and 1.6 for M. lactilyticus. This similarity in ratios may reflect some parallelsim in the control of the production of Fd in relationship to Rd between these micrococci.

Although the ratios of Fd/Rd are nearly the same between M. aerogenes and M. lactilyticus, the absolute per cent of Fd or Rd in M. aerogenes is is approximately twice that of M. lactilyticus. This may reflect that M. aerogenes relies more upon Fd-Rd mediated reactions as a source of energy. An alternative interpretation might be that the biological activity of the M. lactilyticus electron carriers is twice that of the M. aerogenes electron carriers.

c. Crystals

1. Ferredoxin. A photomicrograph of M. aerogenes crystalline Fd is shown in Figure 11. In addition to having a rod shaped appearance, crystalline Fd from M. aerogenes also forms rosettes under conditions where the crystallization is affected by a higher initial $(NH_4)_2SO_4$ concentration.

Unfortunately, preliminary attempts to crystallize M. lactilyticus Fd were unsuccessful. The appearance of crystalline M. aerogenes Fd, however, closely resembles that of Clostridium acidi-urici (13).

ii. Rubredoxin. Figures 12 and 13 show photomicrographs of crystalline rubredoxin from M. aerogenes and M. lactilyticus respectively. Although M. lactilyticus Rd more readily crystallizes than that of M. aerogenes, its crystals are much smaller than those of the latter organism. Both crystalline preparations

have a rod shaped appearance, and upon standing tend to form rosettes. These rubredoxins differ from that of Clostridium pasteurianum, since the latter forms cubic crystals (14).

c. Absorption Spectra

i. Ferredoxin. The absorption spectrum of crystalline M. aerogenes Fd has been previously reported (9). Figure 14 shows the spectrum of M. lactilyticus Fd. Both proteins have nearly identical absorption spectra, with maxima at 275 mu and 390 mu, and shoulders at 300 mu. Although this type of spectrum does not directly compare with any of the spectra of the clostridial ferredoxins previously reported, Clostridium acidi-urici ferredoxins absorption spectrum most closely resembles that of these micrococci (13).

The highest A 390/280 ratio thus far obtained for recrystallized M. aerogenes Fd was 0.80. In contrast, the highest A 390/280 ratio obtained for M. lactilyticus Fd was 0.69. Both ferredoxins have an A 390/mg closely resembling that of clostridial ferredoxins, which is approximately 3.4 (13).

ii. Rubredoxin. The absorption spectrum of M. aerogenes Rd has been previously reported (9). Figure 15 shows the spectrum of M. lactilyticus Rd. Both proteins have nearly identical absorption spectra, and closely compare with that of C. pasteurianum Rd (14). Absorption maxima exist at 275, 380, and 490 mu, with shoulders present at 570, 370, and 290 mu.

The lowest A 280/490 ratio thus far obtained for recrystallized M. aerogenes Rd was 2.25. In contrast, the lowest A 280/490 ratio obtained for M. lactilyticus Rd was 3.3. Both rubredoxins have an A 490/mg closely resembling the Rd of C. pasteurianum, which is approximately 1.47 (14).

e. Amino Acid Composition of M. aerogenes Ferredoxin and Rubredoxin. Table 11 shows the results of preliminary amino acid analysis studies of Fd and Rd from M. aerogenes. Since Dr. K.T. Yasunobu and associates are determining the amino acid sequence of these proteins, no discussion of these preliminary data will be made.

2. Biological

a. Synthesis of Acetyl Phosphate Catalysed by Crude Ferredoxin

i. M. aerogenes. The effect of increasing depleted protein concentration or crude Fd concentration upon acetyl phosphate synthesis from pyruvate was studied for M. aerogenes by the assay method of Lovenberg et al. (13).

Figure 17 shows the effect of increasing delpleted protein concentration upon acetyl phosphate synthesis. The reaction was dependent upon Fd and gave a sigmoidal response when the depleted protein concentration was varied from 0 to 4 mg.

Figure 18 shows that the effect of increasing crude Fd concentration gave a linear response of acetyl phosphate synthesis for a crude Fd concentration up to 75 ug.

ii. M. lactilyticus. A preliminary study of the effect of increasing depleted protein concentration upon acetyl phosphate synthesis for M. lactilyticus was undertaken by using the assay system of Mortenson et al. (19). Figure 19 shows that the reaction was dependent uponFd and gave a sigmoidal response when the depleted protein concentration was varied from 0 to 9 mg. No direct comparison of the phosphoclastic reaction can be made between M. lactilyticus and M. aerogenes, since different assay systems were used to study acetyl phosphate synthesis. The method of Lovenberg et al. (13) was found to be a much more sensitive assay.

b. Synthesis of Acetyl Phosphate Catlysed by Crystalline Ferredoxin and Rubredoxin from M. aerogenes.

The ability of recrystallized Fd or Rd from M. aerogenes to catalyse the phosphoroclastic reaction was studied. The Fd and Rd crystals used were dissolved in 0.05 M Tris, pH 7.0. Contaminating $(NH_4)_2SO_4$ was removed from the solutions by passage through G-25 Sephadex, equilibrated with and eluted

by the above buffer. The Fd sample used for this study had an A 390/280 of 0.8, whereas the Rd sample had an A 280/490 of 0.25. Fd and Rd protein concentrations used in the assay were calculated on the assumption that the A 390/mg for Fd equaled 3.4 and the A 490/mg for Rd equaled 1.473 (13, 14).

Figure 20 shows the results of an experiment where the Fd and Rd samples from above were assayed simultaneously in the same experiment by the method of Lovenberg et al. (13). Several interesting results exist. These are:

i. At low concentrations, (less than 20 ug), Rd is more efficient than Fd in acetyl phosphate synthesis.

ii. The Rd catalysed reaction is biphasic.

iii. At higher concentrations, Rd is approximately 60% as efficient as Fd for the catalysis of pyruvate.

In view of the fact that this experiment was not repeated, one cannot be sure if the results describe the actual biological phenomenon. However, this data has an interesting tentative implication.

Assuming the biphasic curve is not an artifact of the rubredoxin reaction, one might predict that an enzyme system other than the Fd mediated one exists for pyruvate degradation. This system would have to be specific for Rd (allosteric ?). When this hypothetical system becomes saturated with Rd, excess Rd added would serve to replace Fd in the Fd mediated enzyme system. This type of reasoning would explain the biphasic nature of the Rd catalysed reaction.

In the future, it might be of considerable interest to study this biphasic phenomenon. If it proves to be real then a possible biological role for Rd may be established.

C. Properties of Hydrogenase Present in Crude Extracts of M. aerogenes

1. Studies of Hydrogen Uptake or Evolution using Methyl Viologen

a. Effect of Cell Disruption by Different Methods. Preliminary experiments were performed to study the effects of various methods of cell disruption upon the hydrogen uptake activity present in crude extracts of M. aerogenes. These experiments established that sonication under hydrogen was the best method to prepare active extracts. No hydrogen uptake activity was observed when young cells were broken by sonication in the absence of a hydrogen atmosphere or by the french press. It was later found that pre-incubation under hydrogen in the presence of 5 um/ml dithiothreitol (DTT) restored the hydrogen uptake activity of extracts prepared in the absence of hydrogen.

b. Effect of Storage Conditions. Table 12 describes the results of an experiment where an extract prepared by sonication under hydrogen in the absence of DTT was subjected to various storage conditions. Samples were stored for 48 hrs at either 4° or -20° in the presence of either, no additions, 5 um/ml dithionite, or 40% glycerol. After storage, samples were assayed for both hydrogen uptake and hydrogen evolution activities.

The results of the experiment indicated that:

i. The hydrogen evolution activity was stable to storage either at 4° or -20° in all cases. In addition, storage in the presence of dithionite activated the reaction. This is probably due to the increase in substrate concentration of the assay.

ii. The hydrogen uptake activity was unstable to storage at 4°, except in the presence of 40% glycerol.

iii. The hydrogen uptake activity was stable to storage at -20°.

Since glycerol prevented the loss of hydrogen uptake activity at 4°, and storage at -20° also protected this activity, hydrogenase may be composed of subunits. The previous observation that a reducing atmosphere was necessary to maintain uptake activity suggests the presence of free -SH groups on the hydrogenase molecule.

c. Effect of Reducing Agents and Storage. The previous observation that hydrogen uptake activity was dependent upon reducing conditions suggested the following experiment.

Since disrupting cells in the absence of a hydrogen atmosphere resulted in the loss of the hydrogen uptake activity, it was of interest to prepare an extract by that method, and then test the abilities of various reducing agents to restore the hydrogen uptake activity.

Table 13 shows the results of such an experiment. This experiment established that DTT was the only reducing agent tested that replentished the hydrogen uptake activity. Even after freezing and thawing the pretreated DTT extract twice, the hydrogen uptake activity had only lost 30% of its original activity.

In contrast, although the hydrogen evolution activity was initially activated by DTT, it appeared to undergo a more drastic loss in activity upon storage.

The above experiments established the proper preparative conditions of cell-free extracts for hydrogen uptake experiments. In all the following experiments, extracts were prepared by sonication under hydrogen in the presence of 5 um/ml DTT.

d. Kinetics of Hydrogen Uptake. McCormick previously observed that under his conditions, the hydrogen uptake activity of M. aerogenes was inconsistent and non-linear with respect to enzyme concentration (18).

In constrast, Figure 21 shows that under the preparative conditions described above, hydrogen uptake activity is consistent and linear with increasing protein concentration.

2. Reduction of Inorganic or Organic Substrates

a. Inorganic. Figure 22 shows the results of an experiment where various inorganic anions were tested for their ability to act as electron acceptors from the hydrogenase activity present in crude extracts of M. aerogenes. The specific activity of these reactions is reported in Table 14. Of the 8 anions tested, only tellurite served as an active electron acceptor. However, the reaction stopped abruptly before 50% of the tellurite was reduced. This is probably a result of poisoning by the production of elemental tellurium.

b. Organic. Figure 23 shows the results of an experiment where various organic substrates were tested for their ability to act as electron acceptors from the M. aerogenes hydrogenase activity present in crude extracts. The specific activity of these reaction is also reported in Table 14. Of the 7 substrates tested, 5 served as significant electron acceptors.

The observation that the specific activity for DPN reduction is over 3 fold greater than that of TPN is quite significant. Only two other anaerobic bacteria have been reported which utilize DPN as the primary pyridine nucleotide acceptor, Clostridium acidi-urici (24) and Methanobacillus omelianskii (4).

3. Presence of DPN-TPN Transhydrogenase

Since DPN was preferentially reduced instead of TPN, it was of interest to determine whether TPN reduction was a result of DPN-TPN transhydrogenase activity.

Figure 24 shows the results of an experiment which studied the effects of various concentrations of DPN upon hydrogen uptake. DPN was probably being regenerated, since after the initial added amount of DPN was utilized, there was a reduction in rate of hydrogen uptake, but not a plateau.

Furthermore, when 2 um of TPN was added to a DPN reaction mixture which

had gone to completion, a synergistic enhancement of hydrogen uptake was observed.

In an effort to study the presence of DPN-TPN transhydrogenase without the problems of interpretation created by endogenous DPN and TPN, attempts to deplete extracts of these nucleotides using G-25 Sephadex were performed. Sephadex G-25 was equilibrated with and eluted by 0.1 M phosphate, pH 7.0, containing 5 um/ml DTT. It was found that extracts passed through Sephadex G-25 as above were entirely absent in DPN reductase activity, even in the presence of BES. In contrast, hydrogenase activity still remained. Thus, either DPN reductase is very labile, or BES did not completely replenish absent cofactors from the system.

An alternative experiment was performed in an effort to establish the presence of DPN-TPN transhydrogenase activity in crude extracts of M. aerogenes.

If M. aerogenes has this enzyme, then a catalytic amount of DPN should enhance the rate of TPN reduction. Figure 25 shows that when 0.5 um of DPN was initially added to 10 um of TPN, the rate of TPN reduction was increased more than that expected if transhydrogenase was not present.

4. Hydrogenase Linked, Ferredoxin Dependent Reactions

An attempt was made to elucidate the dependency of Fd and/or Rd in the xanthine, hydroxylamine, and DPN reductive reactions mediated by hydrogenase in M. aerogenes.

Extracts were depleted of crude Fd and tested for their abilities to catalyse the above 3 reductions in the absence or presence of added crude Fd (1 mg). In only 2 out of 5 experiments was any reductive activity restored.

In contrast, in all 5 experiments hydrogenase retained approximately 80% of its original activity. Furthermore, when the 3 reductive activities were

restored, they occurred after a lag period and were less than 10% of their original, non-treated extract activity.

These observations indicated that the specific reductases were either depleted or inactivated by DEAE treatment.

Additional experiments were performed which tested the ability of 5, 10, or 15 um DTT - o.1 M phosphate buffer to protect these reductase activities during depletion. It was found that the reductase activities were not protected by DTT.

Another experiment was performed where the DEAE column previously used to deplete an extract was eluted, and the eluate added to reaction mixtures. This procedure did not restore reductase activities.

In another experiment, BES was added to reaction mixtures in addition to crude Fd. This addition also did not restore reductase activites.

In all cases it was found that the crude Fd added was active in the acetyl phosphate assay, that hydrogenase activity was present in the depleted extracts used (as assayed by reduction of methyl viologen), and that the 3 reductase activities were present prior to depletion.

These observations are consistent with the interpretation that these 3 reductase activities are all labile to DEAE treatment.

Future experiments which might protect or restore reductase activity should include the use of 40% glycerol during crude Fd depletion. Another feasible experiment might be the preincubation of the depleted extract with the added electron transport protein and DTT, under hydrogen for 60 min at 4° before assaying.

5. Localization of Hydrogenase

Table 15 describes the results of an experiment where differential

centrifugation was employed in an attempt to localize M. aerogenes hydrogenase. After centrifugation, 3 different protein concentrations of each fraction were immediately assayed, and specific activities were calculated for the linear portion of the reaction.

The recovery of hydrogenase activity was 87%. It is of interest that only 9% of the original activity was sedimented at 25,000 x g. Furthermore, all the activity that sedimented at 100,000 x g was released by a repeated wash, which accounted for 27% of the total activity of the 25,000 x g supernatant.

This data may indicate that hydrogenase, in the native state, is loosely bound to the membrane. However, during cell disruption, it may readily be released.

6. Acrylamide Gel electrophoresis as a Method to Detect M. lactilyticus Hydrogenase

Four preliminary experiments using crude extracts or 100,000 x g supernatants of M. lactilyticus for electrophoresis according to the method of Ackrell and Mower (2) were performed. The results indicated that M. lactilyticus had one soluble species of hydrogenase with an average Rf of 0.77 under the conditions employed.

Ackrell, Asato, and Mower have recently indicated that M. lactilyticus has two species of soluble hydrogenases, with Rfs of 0.92 and 0.96 (1). Since they reported that the species with an Rf of 0.92 comprises only a small percentage of the total hydrogenase activity, this may explain the failure to detect this band by this paper's author.

In the future, M. aerogenes hydrogenase(s) may possibly be detected by this procedure. Employing DTT in the gel and buffer system may aid to stabilize the activity.

Also, specific reductases could be studies by the above method. For example, pyruvate dehydrogenase or xanthine oxidase could be localized by the addition of pyruvate or hypoxanthine repectively to the incubation mixtures. These

assays should be done in an atmosphere of nitrogen.

V. APPENDIX OF CALCULATIONS

A. M. aerogenes

1. Ferredoxin

From Table 9, 67 mg of crystalline Fd with a 33% recovery was derived from 34,400 mg cell protein by 2 FTE steps. The combined first and second eluates contained a total of 1,580 mg protein. From Table 2 at least 70% of the cells' Fd is eluted by 2 FTE.

% of total soluble cell protein that is Fd is equal to (100)(67/0.33)(1/0.7)(1/ 34,400). This equals 0.84 %.

% of total combined eluate protein that is Fd equals (100)(67/0.33)(1/1,580). This equals 12.8%.

2. Rubredoxin

From Table 9, 48.3 mg of Rd was derived from 34,400 mg cell protein by 2 FTE steps. Assume 33% recovery of Rd. Assume 70% of the cells' Rd is eluted by 2 FTE. The combined first and second eluates contained a total of 1,580 mg protein.

% of the total soluble cell protein that is Rd equals (100)(48.3/0.33)(1/34,400) which equals 0.62%.

% of total combined eluate protein that if Rd is equal to (100) (48.3/0.33) (1/1,580) which equals 9.2%.

B. M. lactilyticus

1. Ferredoxin

From Table 10, 10.8 mg of Fd with a 22.4% recovery was derived from 10,300 mg of cell protein.

% of total soluble cell protein that is Fd equals (100)(10.8/0.244)(1/10,300). This is equal to 0.47%.

2. Rubredoxin

From Table 10, 3.0 mg of Rd with an assumed recovery of 10% was derived from 10,300 mg of cell protein.

% of total soluble cell protein that is Rd is equal to (100) (3.0/0.10)(1/10,300). This is equal to 0.29%.

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Table 1. Freeze thaw elution of ferredoxin and protein from M. aerogenes by different eluting agents[a]

Eluting Agent	Cell Protein (mg)	Eluate Protein (mg)	% Protein eluted	Total Fd Units[b]	Fd Units Eluted[c]	% Fd Eluted
0.1 M Tris pH 7.0	2,043	77.6	3.8	0.95	0.55	58.2
0.1 M K_2HPO_4 pH 7.0	445	13.8	3.1	1.04	0.64	61.5
1 x 10^{-3} M EDTA pH 7.0	697	54.5	7.8	1.02	0.75	73.5
H_2O pH 4.0	1,895	39.8	2.1	1.08	0.75	69.5

[a] Part of this data was reported incorrectly in Research Report #1.

[b,c] Fd units were determined as outlined in Research Report #1. Fd units in this table and in following tables are defined in the Methods section.

Table 2. The repeated elution of ferredoxin from Micrococcus aerogenes by thawing frozen cells

Sample	Protein (mg)	% Protein	Fd units	% Fd
Intact Cells	2,320.0	100	2.16	100
1st Eluate	80.5	3.5	1.32	61.0
2nd Eluate	25.0	1.1	0.20	9.1
3rd Eluate	5.1	0.2	0.06	2.7
Remaining Cells	2,245.0	97.0	0.07	3.0
Recovery	2,356.0	101.8	1.65	75.8

Table 3. Elution of ferredoxin by osmotic shock and freeze-thawing from M. aerogenes

pending lution	Steps [a]	Protein (mg)	% Protein	Fd Units	% Fd
ONE	A	--	--	--	--
	B	--	--	--	--
	C	9.1	3.3	1.59	57.0
	D	266.0	96.7	0.99	43.0
	Sum	275.1	100.0	2.58	100.0
$_2O$	AA	2.7	1.3	0.24	8.2
	B	1.4	0.7	0.45	15.2
	C	5.7	2.8	1.07	37.4
	D	195.8	95.2	1.10	39.2
	Sum	204.8	100.0	2.85	100.0
20% Sucrose	A	2.4	1.7	0.38	11.3
	B	3.8	2.7	0.83	24.8
	C	11.9	8.3	0.87	26.0
	D	126.0	87.2	1.27	37.9
	Sum	144.1	100.0	3.36	100.0
20% Sucrose - . x 10^{-3} M DTA - 0.03 M ris (pH 8)	AA	4.7	2.4	0.16	5.5
	B	5.3	2.8	0.76	25.2
	C	7.6	3.9	0.97	32.2
	D	176.0	90.9	1.13	37.1
	Sum	193.6	100.0	3.02	100.0

[a] Steps: (A) suspending, (B) shocking with distilled water, (C) freeze-thaw elution, (D) sonication.

Table 4. Production and freeze-thaw elution of ferredoxin from M. aerogenes at different stages of growth

		Culture[a] I	II	III	IV
Absorbancy at harvest (500 mu)		0.44	0.68	0.83	1.30
Protein (mg)		41.9	77.5	95.4	151.3
Amount crude Fd[b]		1.96	2.01	2.83	4.06
Fd units	eluted	2.48	0.74	1.39	0.59
	remaining	2.20	1.95	1.58	2.08
	total	4.68	2.59	2.97	2.68
% Fd eluted		53.0	28.6	46.7	22.2
Protein (mg)	eluted	2.0	2.5	3.8	3.8
	remaining	40.0	75.0	91.6	147.5
% Protein eluted		4.7	3.2	4.0	2.5

[a] When 0.025% wt/vol ferrous ammonium sulfate was initially added to a culture which was harvested at an absorbancy of 0.66, no increase in ferredoxin production or elution was observed.

[b] Amouht Fd used in this table and in following tables is defined in the Methods section.

Table 5. Ferredoxin production by *M. aerogenes* after step up [a]

Culture	Absorbancy (500 mu)	Protein (mg)	Amount crude Fd	Fd units
Stationary	1.50	272.0	5.43	2.0
Step up (initial)	0.35	72.5	1.45	2.0
Step up (final)	0.75	108.7	3.47	3.5

[a] Step up is defined in the Methods section

Table 6. RNA, DNA, and protein analysis of the eluate obtained by thawing frozen cells of M. aerogenes

Sample	Protein		RNA		DNA	
	mg	%	mg	%	mg	%
Intact Cells	5150	100.0	680.0	100.0	184.0	100.0
Eluate	107	2.1	4.2	0.72	0.49	0.27
Remaining Cells	5140	99.8	664.0	97.5	182.0	99.0

Table 7. Localization of ferredoxin from *M. aerogenes*

A. DIFFERENTIAL CENTRIFUGATION [a]

100,000xg Fraction	Vol.(ml)	Protein (mg/ml)	Total Protein (mg)
Supernatant	9.2	6.7	61.6
Wash	10.2	3.6	36.7
Precipitate	9.2	16.6	152.8
		SUM	251.1

B. ISOLATION OF CRUDE FERREDOXIN

DEAE Fraction	Vol.(ml)	Protein (mg/ml)	Total Protein (mg)
Sonicate [b]	3.8	2.85	10.8
100,000xg Sup	9.2	2.0	18.4
100,000xg Wash	2.7	1.8	4.9

C. ASSAY OF CRUDE FERREDOXIN FRACTIONS BY:

1. Optical Density

DEAE Fraction	Vol.(ml)	A390	Amt. Crude Fd	Fd Units	% Fd
Sonicate	3.8	0.966	3.67	2.60	100.0
100,000xg Sup	9.2	0.604	5.55	2.21	85.0
100,000xg Wash	2.7	0.232	0.63	0.25	9.6
			% TOTAL SOLUBLE FD		94.6

2. Acetyl Phosphate Formation

DEAE Fraction	Vol.(ml)	Activity/ml [c]	Total Activity	Fd ENZ units [d]	% Fd
Sonicate	3.8	4.90	18.60	1.32	100.0
100,000xg Sup	9.2	2.97	27.30	1.09	82.6
100,000xg Wash	2.7	0.83	2.24	0.09	6.8
100,000xg ppt	9.2	0.21	1.90	0.08	6.1
			% TOTAL SOLUBLE FD		89.4

[a] Starting material was a glass bead broken, cell free extract.

[b] Sonicate used assumed to represent 100% of the cells' Fd, and contained 141.0 mg protein.

[c] Activity/ml expressed as the change in O.D. 540 per 10 min per ml Fd, using 12.0 mg protein minus ferredoxin.

[d] Fd ENZ units expressed as total activity divided by total protein times 10.

Table 8. Viability of M. aerogenes after freezing and thawing

Sample	Colony counts x 10^{-6} Exp. #1	#2	Average	% Viability
Before centrifugation [a]	338	302	320	--
After centrifugation	323	300	312	100.0
After freezing	2.0	1.7	1.85	0.6

[a] Cells were harvested by centrifugation at 15,000 rpm for 15 minutes at 4°.

TABLE 9

Purification and crystallization of ferredoxin from Micrococcus aerogenes

Fraction	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	A 390	A 390/280	A 390/mg	Specific[a] Activity (units/mg)	Total Activity (units)	Yield %
Combined eluates[b]	1,170.0	1.35	1,580.0	1.09	0.078	0.81	7.55	11,910	100
1st DEAE	53.0	13.58	719.8	14.09	0.118	1.04	13.15	9,450	79
1st Sephadex	100.0	6.85	685.0	6.55	0.150	0.96	15.50	10,600	89
2nd DEAE[c]	38.5	3.82	147.1	11.54	0.460	3.02	25.50	3,742	31
Crystals	8.0	8.40[d]	67.0	28.45	0.792	3.40	58.50	3,920	33

[a] Units expressed as um acetyl phosphate synthesized per 10 min incubation period at toom remperature per 4.0 mg 39,000 rpm supernatant-depleted extract.

[b] 1st and 2nd eluates from 450 gm wet weight cells (approximately 34,400 mg protein).

[c] The Rd obtained by this step had a volume of 178 ml, an absorbance of 0.40 at 490 mu, an A 280/490 of 11.05, and contained 151 mg protein. Assuming the extinction coefficient (490) of 8.85×10^3 as reported by Lovenberg and Sobel (14), then A 490/mg equals 1.473. Thus (178)(0.4)/1.473 equals 48.3 mg pure Rd is present in this fraction.

[d] The protein content was based on the assumption that A 390/mg is approximately 3.4 as reported for five clostridial ferredoxins (13).

TABLE 10

Purification of ferredoxin from Micrococcus lactilyticus

Fraction	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	A 390	A 390/280	A 390/mg	Amount Fd	Yield %
Sonic extract	420.0	24.5	10,300	2.7	0.025	-	-	-
1st DEAE	70.0	10.0	699	2.34	0.058	0.234	164.0	100.0 [a]
1st Sephadex	148.0	3.05	451	0.74	0.048	0.242	109.7	66.9
2nd DEAE	10.0	20.2	202	8.05	0.083	0.399	80.5	49.1
2nd Sephadex	61.5	1.76	108	1.22	0.089	0.693	75.2	45.9
3rd DEAE	16.5	5.82	96	4.18	0.098	0.719	69.0	42.0
3rd Sephadex	65.0	1.36	88.5	0.82	0.092	0.603	53.3	32.5
4th DEAE [b]	55.0	0.28	15.4 [c]	0.67	0.693	2.39	36.8	22.4

[a] Since M. lactilyticus contains large quantities of other brown protein(s), the 1st DEAE fraction was taken as 100% ferredoxin based on A 390. Even this value is probably too high.

[b] Rd obtained from this step had a volume of 110.0 ml. After diluting 1:4 and concentrating the sample (by adsorption to and elution from DEAE), the samples volume was 5.1 ml and had an absorbance of 0.874 at 490 mu, with an A 280/490 of 3.3. Assuming an A 490/mg of 1.473, this fraction contains (5.1)(0.874)/1.473 equals 3.0 mg protein.

[c] Assuming an A 390/mg equals 3.4, this fraction probably contains (55)(0.67)/3.4 equals 10.8 mg of pure Fd.

Table 11. Comparison of amino acid compositions of ferredoxin and rubredoxin of *M. aerogenes* [a]

(Proposed residues per mole)

Amino acid	Ferredoxin	Rubredoxin
Lysine	1	2
Aspartic	8	8
Threonine	-	2
Serine	4	1
Glutamic	4	12
Proline	5	4
Glycine	4	5
Alanine	7	3
Half-cystine	7-8	3
Valine	4	3-4
Isoleucine	6	1
Tyrosine	2	3
Phenylalanine	?	3
Methionine	-	1
Arginine	-	-
Tryptophan	?	?
Histidine	?	?
Leucine	?	3
TOTAL	53 +	54 +

[a] These are preliminary results kindly supplied by University of Washington Biochemistry Department for ferredoxin and Yasunobu's group for rubredoxin.

Table 12. Effects of storage conditions on hydrogenase activity in crude extracts of M. aerogenes

		Assay [a]			
		Hydrogen evolution		Hydrogen uptake	
Initial specific activity [b]		3.73		2.56	
Storage temperature [c]		4°	-20°	4°	-20°
Specific activity after storage in the presence of:	None	3.34	1.96	0	2.02
	5 um /ml Dithionite	5.43	4.40	0.11	1.98
	40% glycerol	1.93	3.05	1.43	1.54

[a] Each Warburg cup contained 100 um K_2HPO_4, pH 7.5, 5 mg dithionite for hydrogen evolution, an observed 8 um of methyl viologen, 8.58 mg protein with additions as indicated above, 0.2 ml 20% KOH, and water to a final volume of 2.2 ml.

[b] Specific activity expressed as ul hydrogen uptake or evolution per min per mg protin.

[c] Samples were stored for 48 hrs.

Table 13. Effects of reducing agents and storage on hydrogenase activity in crude extracts of M. aerogenes

		Assay [a]					
		Hydrogen evolution			Hydrogen uptake		
Hrs stored at - 20°		0	18	90	0	18	90
Specific activity[b] (after the following additions)[c]	None	1.31	1.02	0.88	-[d]	-	-
	2-Mercaptoethanol	1.07	0.92	0.74	-	-	-
	Glutathione	1.39	1.27	0.85	-	-	-
	Dithionite	1.76	0.94	0.95	-	-	-
	Dithiothreitol	2.52	1.99	0.66	1.52	1.31	1.10

[a] Each Warburg cup contained 100 um K_2HPO_4, pH 7.5, 5 mg dithionite for hydrogen evolution, an observed 8 um of methyl viologen, 0.5 ml of extract (14.1 mg protein) with additions as indicated above, 0.2 ml 20% KOH, and H_2O to a final volume of 2.2 ml.

[b] Specific activity expressed as ul H_2 uptake or evolution per min per mg protein.

[c] Each reducing agents' final concentration was 5 um/ml and samples were preincubated for 90 min under H_2 at 4° before the initial assay.

[d] The lines indicate less than 4% of the original activity.

Table 14 Investigation of the reduction of inorganic and organic substrates catalyzed by crude extracts of M. aerogenes [a]

Inorganic substrate	Specific activity [b]
Arsenate	0.051
Dithionite	--
Metabisulfite	0.26
Molybdate	0.051
Nitrate	0.11
Nitrite	--
Selenite	--
Tellurite	0.49
Organic substrate	
2, 4 Dinitrophenol	--
DPN	0.74
FMN	--
Hydroxylamine	0.58
Methyl Viologen	2.40
TPN	0.20
Xanthine	0.81

[a] Each warburg cup contained 100 um K_2HPO_4, pH 7.5, 6.9 mg protein, 0.2 ml 20% KOH, and are substrate of the following concentration: 10 um for inorganic anions, and DPN, TPN, FMN, and 2, 4 dinitrophenol, 15 um hydroxylamine, 7 um xanthine, an observed 5 um methyl viologen, and H_2O to a final volume of 2.2 ml.

[b] Specific activity expressed as ul H_2 uptake per min per mg protein.

Table 15. Localization of hydrogenase from M. aerogenes

Fraction		Units/ml[a]	mg/ml	Specific Activity[b]	Total[c] Activity	% Recovery
Cell free extract		75.0	37.3	2.0	600.0	100
15,000 RPM	Supernatant	48.0	17.2	2.8	515.0	86
	Wash	3.0	1.7	1.7	42.3	7
	Precipitate	3.3	8.5	0.4	52.6	9
39,000 RPM	Supernatant	18.0	5.8	3.1	278.0	54[d]
	Wash	8.5	3.0	2.8	138.5	27
	Precipitate	0.3	6.6	0.5	5.0	1

[a] Units expressed as ul H_2 uptake per min.

[b] Specific activity expressed as units per mg.

[c] Total activity expressed as $\frac{\text{units}}{\text{ml}}$ x total volume (ml), (corrected to compensate for sampling), see Methods.

[d] % Recovery for 39,000 Supernatant is eg. $(\frac{278.0}{515.0} \times 100 = 54\%)$

Figure 1. Effect of time on the elution of ferredoxin and protein by thawing frozen cells of M. aerogenes. This data was reported in Research Report #1 and has been replotted. Percentage of ferredoxin is based on a known percentage of 61% for 2 hrs thawing.

——— ferredoxin, ----- protein

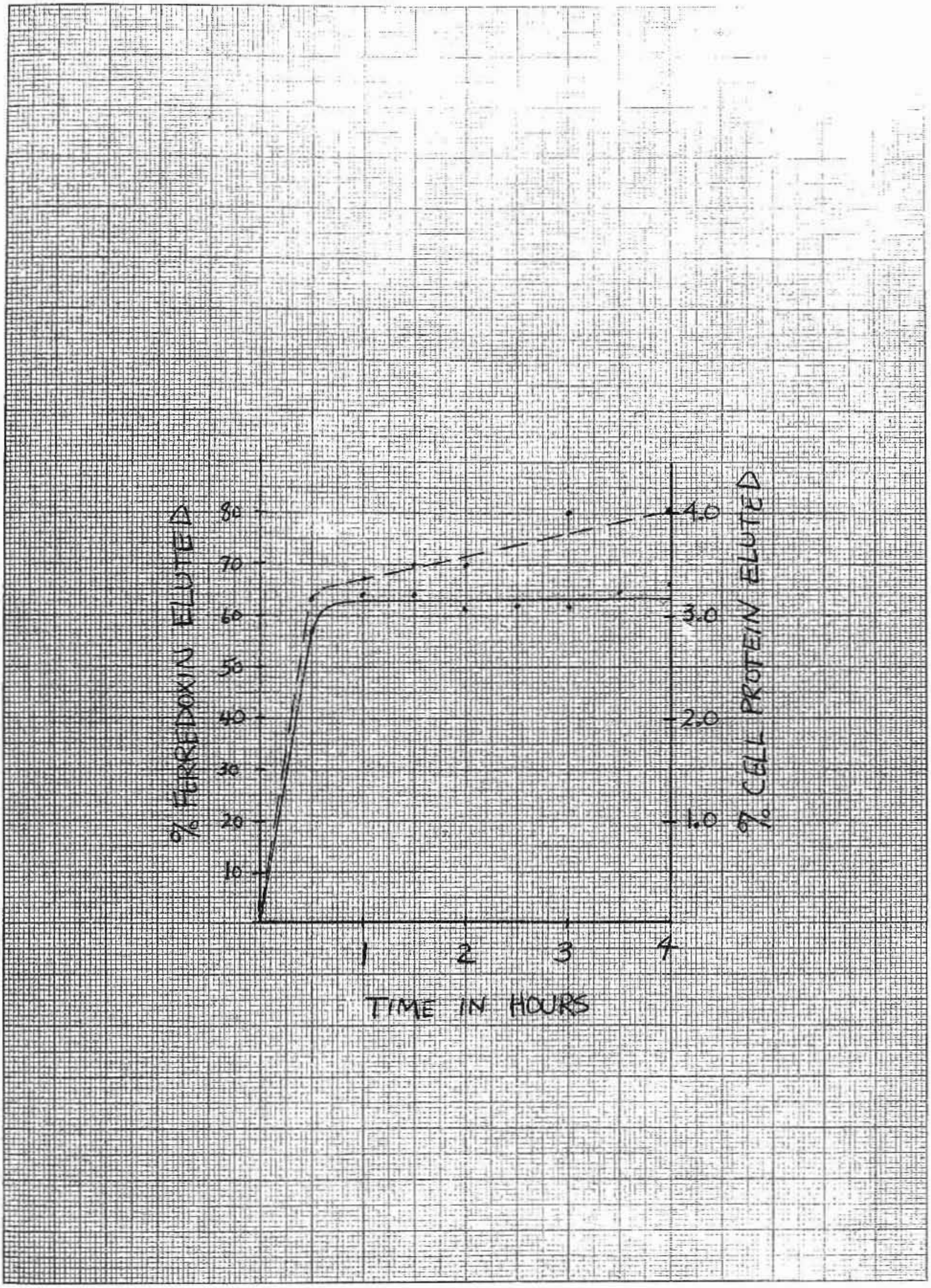

Δ HEAD
80
70
60
50
40
30
20
10
% HEKRED OXID
4.0
3.0
2.0
1.0
Δ CELL PROTEIN
1
2
3
4
TIME IN HOURS

Figure 2. Effect of volume on the elution of ferredoxin and protein by thawing frozen cells of M. aerogenes. This data in part was reported in Research Report #1 and has been replotted.

——— ferredoxin, ----- protein

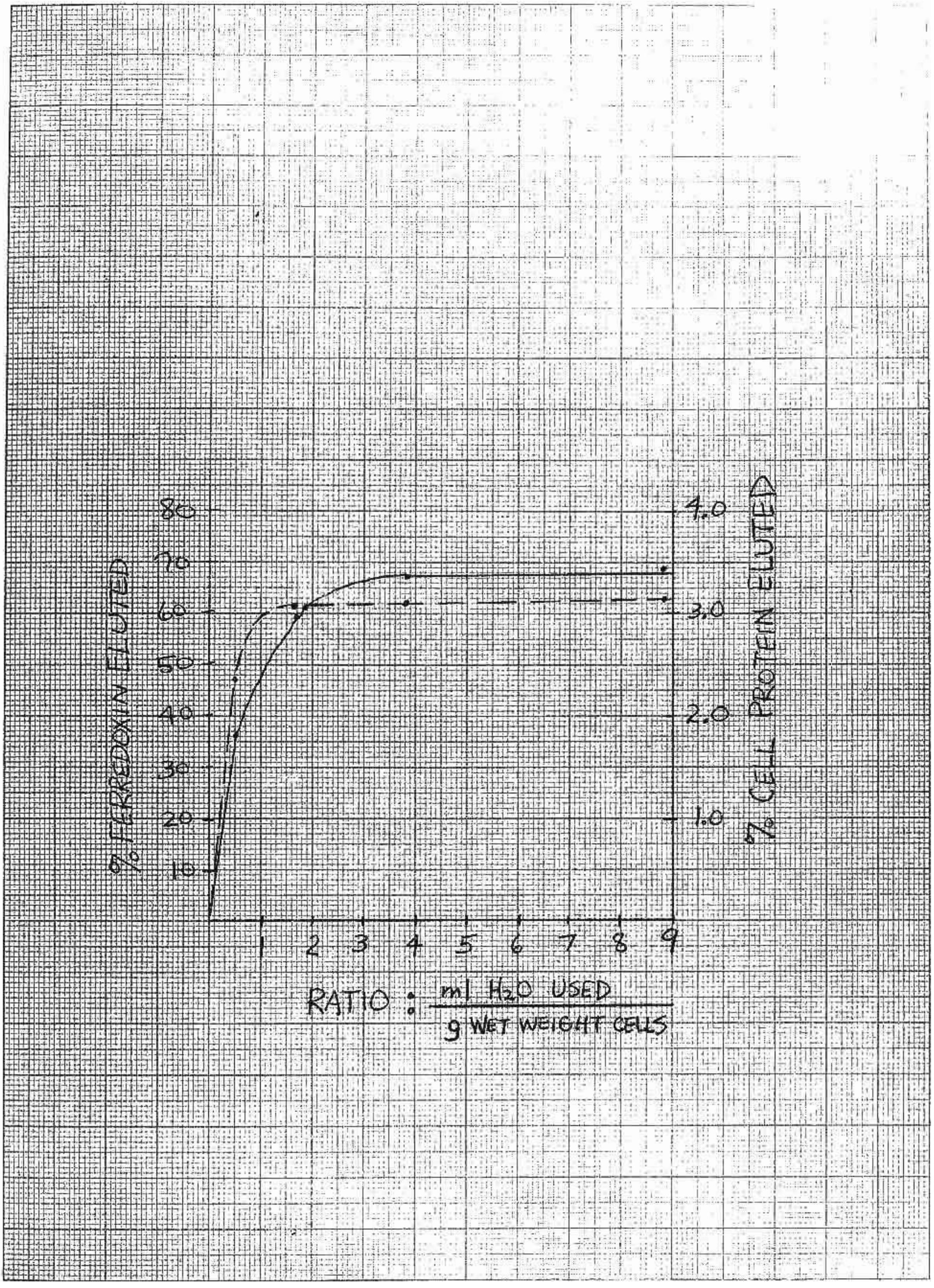

% FERREDOXIN
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
8
9
RATIO : ml H2O USED / 9 WET WEIGHT CELLS
4.0
3.0
2.0
1.0
% CELL PROTEIN

Figure 3. Effect of pH on the elution of ferredoxin and protein from frozen cells of M. aerogenes. ——— ferredoxin, ------ protein

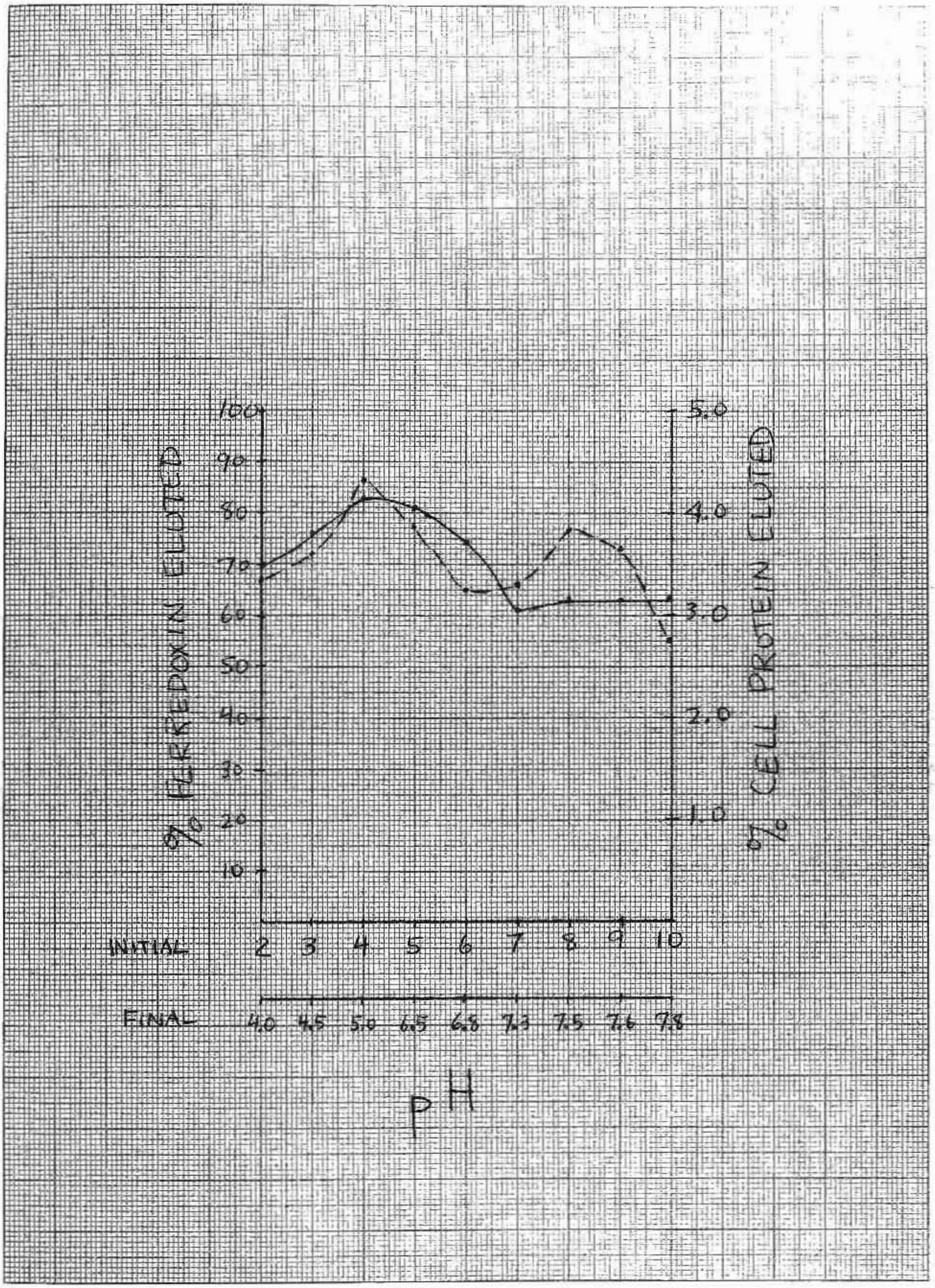

100
90
80
70
60
50
40
30
20
10
0
% REDUCED ZINC
5.0
4.0
3.0
2.0
1.0
0
CELL DROP, VOLTS
INITIAL
2 3 4 5 6 7 8 9 10
FINAL
4.0 4.5 5.0 6.5 6.8 7.3 7.5 7.6 7.8
pH

Figure 4. Effect of pH on the absorbancy of crude ferredoxin from *M. aerogenes*.

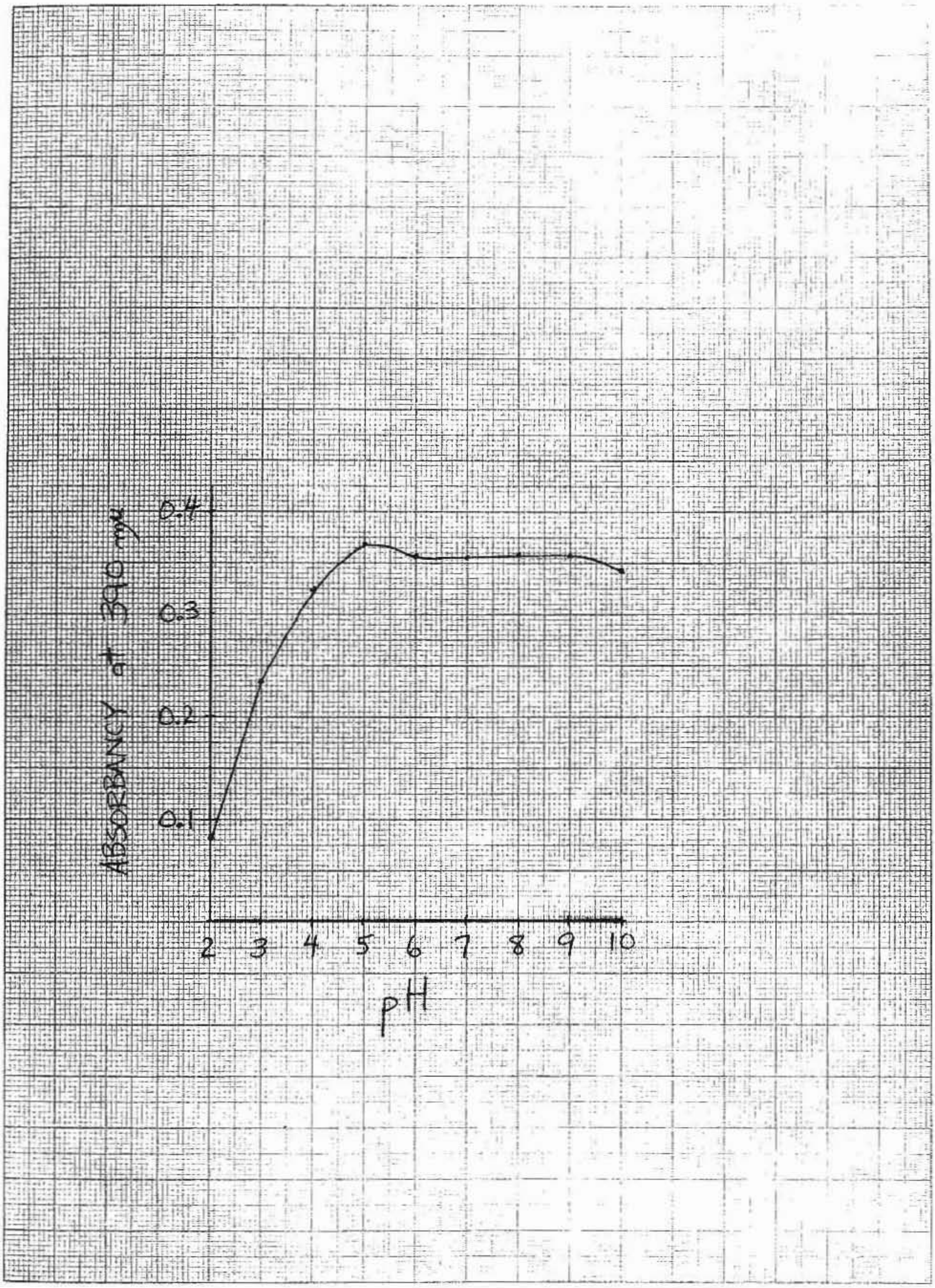

0.4
0.3
0.2
0.1
ABSORBANCY
2
3
4
5
6
7
8
9
10
pH

Figure 5. Effects of sucrose and osmotic shock on the elution of ferredoxin from frozen cells of M. aerogenes. Curves: (1) freeze-thaw elution with corresponding sucrose concentration, (2) shock with water, (3) sum of curves 1 and 2.

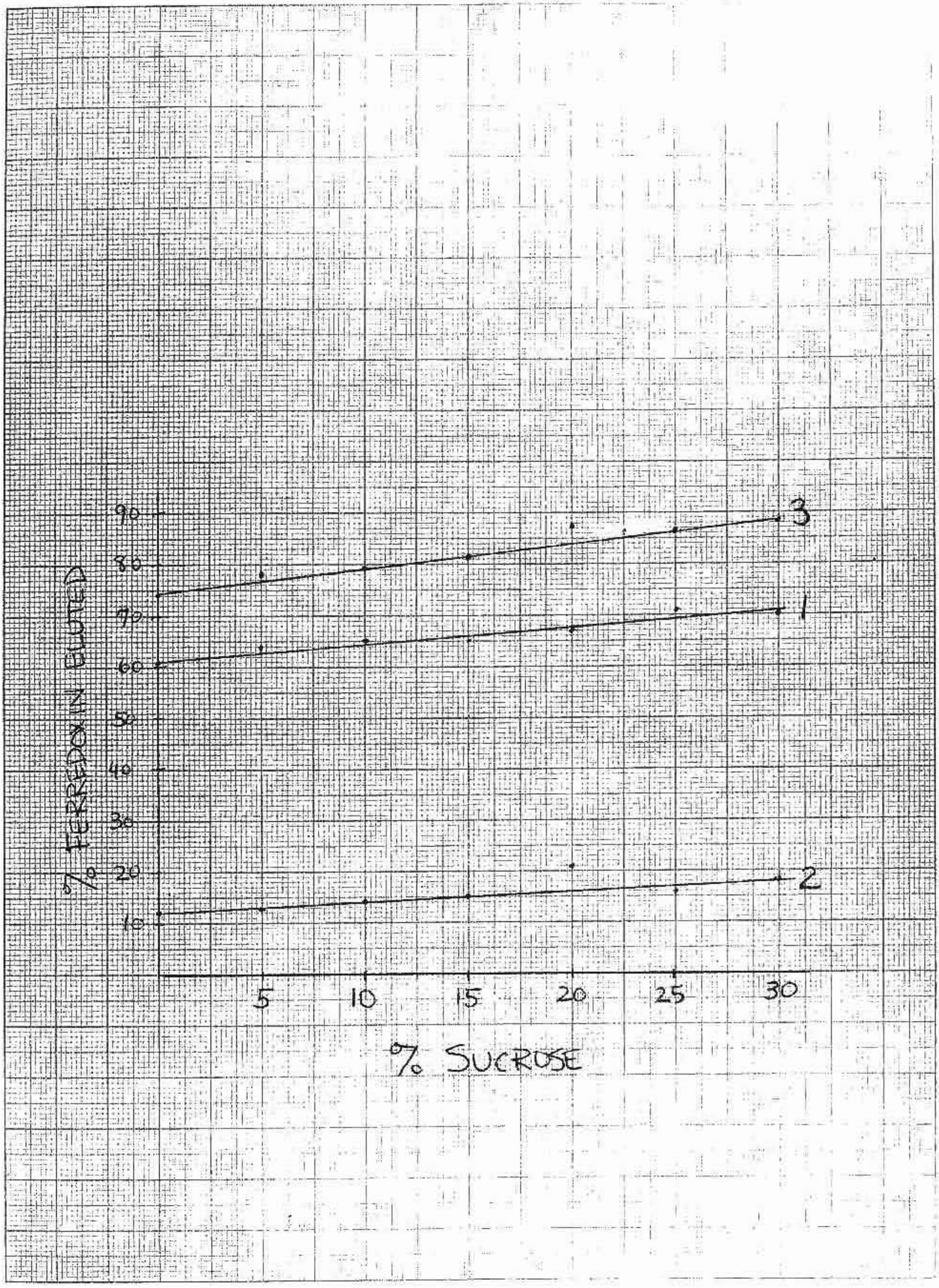

90
80
70
60
50
40
30
20
10
3
1
2
5
10
15
20
25
30
% SUCROSE

Figure 6. Effects of sucrose and osmotic shock on the elution of protein from frozen cells of M. aerogenes. Curves: (1) freeze-thaw elution with corresponding sucrose concentration, (2) shock with water, (3) sum of curves 1 and 2.

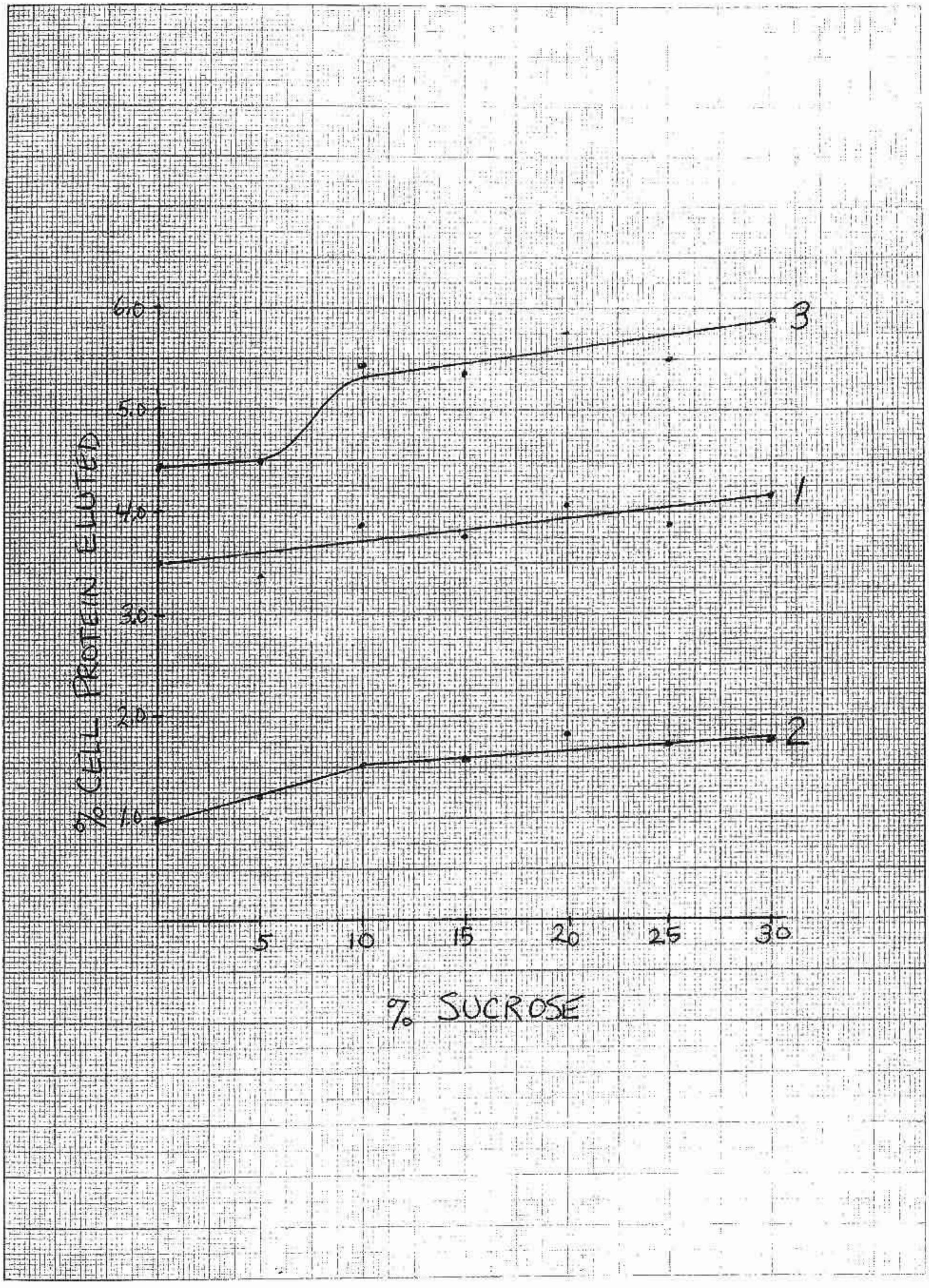

6.0
5.0
4.0
3.0
2.0
1.0
CUT-1 (RELATIVE)
3
1
2
5
10
15
20
25
30
% SUCROSE

Figure 7. Production of ferredoxin and protein by M. aerogenes at different stages of growth. ——— ferredoxin, ------ protein

AMOUNT PROTEIN (mg)

50 70 90 110 130 150

30

.3 .4 .5 .6 .7 .8 .9 1.0 1.25

ABSORBANCE (560 mμ)

1.0 3.0 5.0 7.0 9.0 11.0

AMOUNT CRUDE FERREDOXIN

KEUFFEL & ESSER CO. MADE IN U.S.A.

Figure 8. Sephadex G-75 calibration curve.

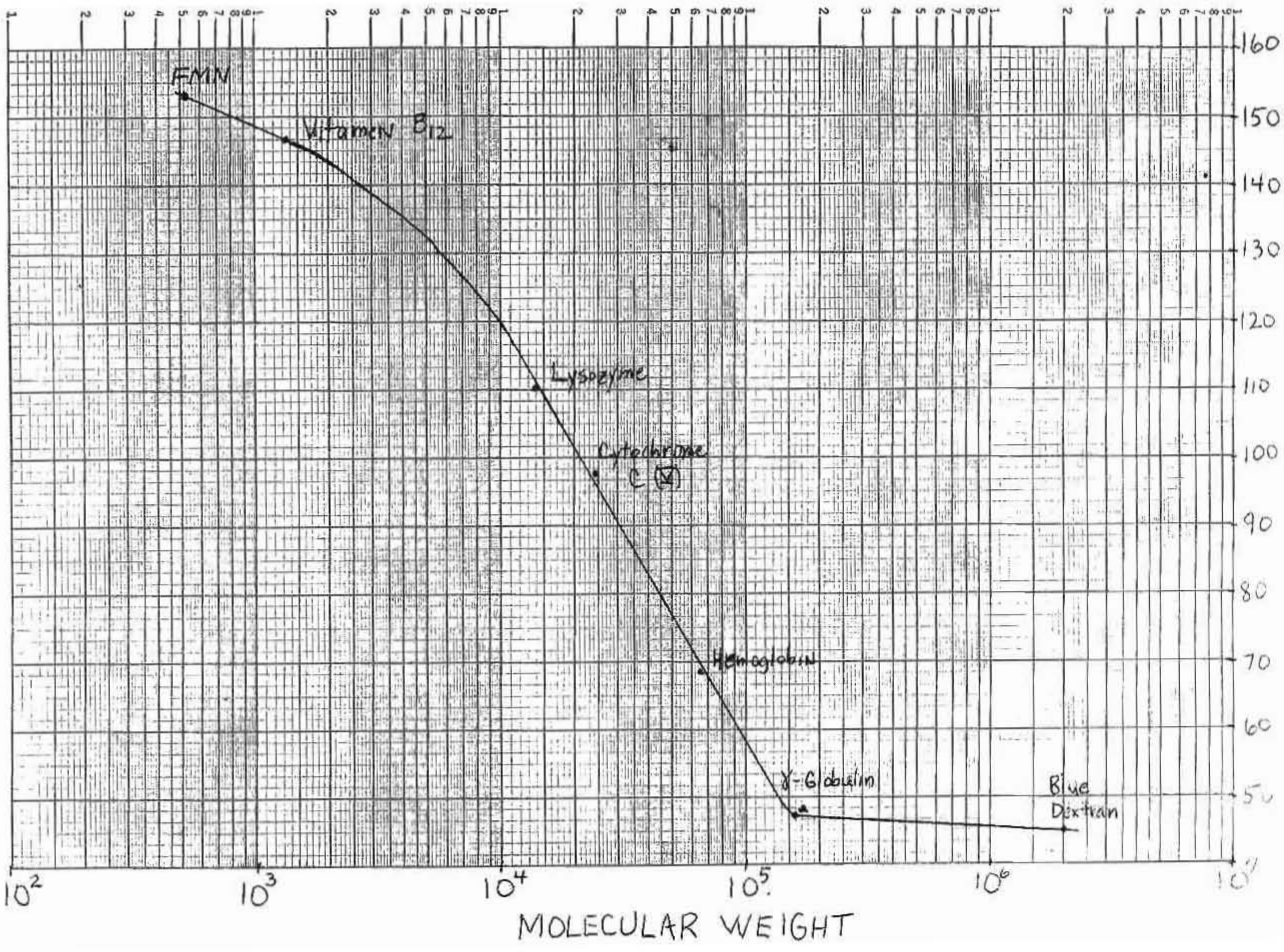

FMN
Vitamen B12
Lysozyme
Cytochrome C
Hemoglobin
γ-Globulin
Blue Dextran
160
150
140
130
120
110
100
90
80
70
60
50
10^2
10^3
10^4
10^5
10^6
MOLECULAR WEIGHT
VOLUME COLLECTED

Figure 9. Estimation of molecular weights of ultraviolet absorbing material present in the freeze-thaw eluate of M. aerogenes by G-75 Sephadex gel-filtration. —— A 280, ---- A 260. Molecular weights are given above the peaks.

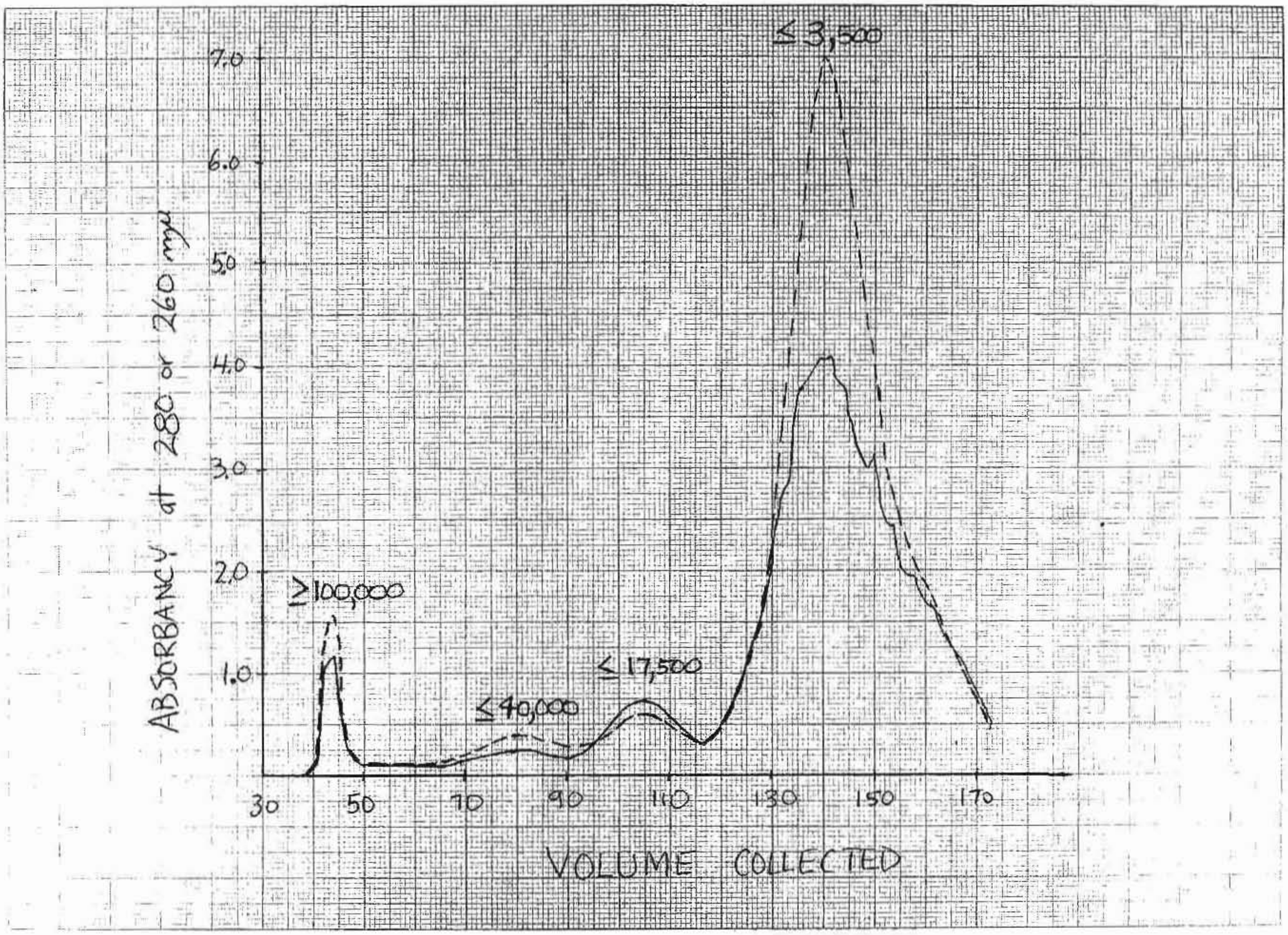

≤ 3,500
≥ 100,000
≤ 40,000
≤ 17,500
7.0
6.0
5.0
4.0
3.0
2.0
1.0
ABSORBANCY at 280 or 260 mμ
30
50
70
90
110
130
150
170
VOLUME COLLECTED

Figure 10. Estimation of the molecular weights of eluted protein using sucrose density gradient centrifugation. Molecular weights above peaks were estimated from a control tube with added haemoglobin as a reference marker (16).

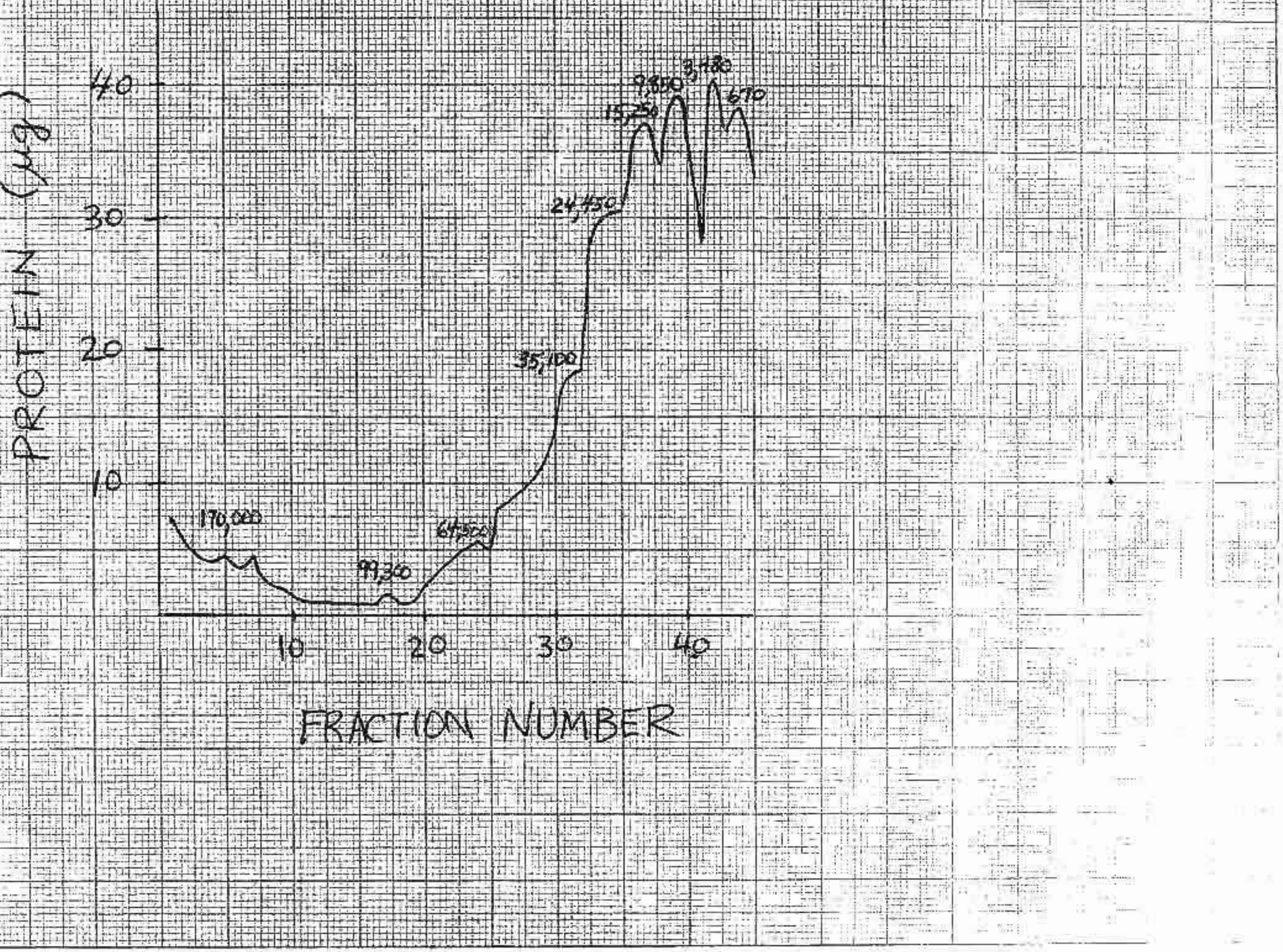

PROTEIN (mg)
40
30
20
10
10
20
30
40
FRACTION NUMBER
170,000
99,300
64,500
35,100
24,450
15,250
9,850
3,180
670

Figure 11. Crystalline ferredoxin from M. aerogenes.*

* 25 X

This picture and the following pictures were taken with a Carl Zeiss Ultraphot photomicroscope equipped with polaroid attachments.

Figure 12. Crystalline rubredoxin from M. aerogenes.*

* 25 X

Figure 13. Crystalline rubredoxin from M. lactilyticus.*

* 304 X

Figure 14. Absorption spectrum of ferredoxin from M. lactilyticus.

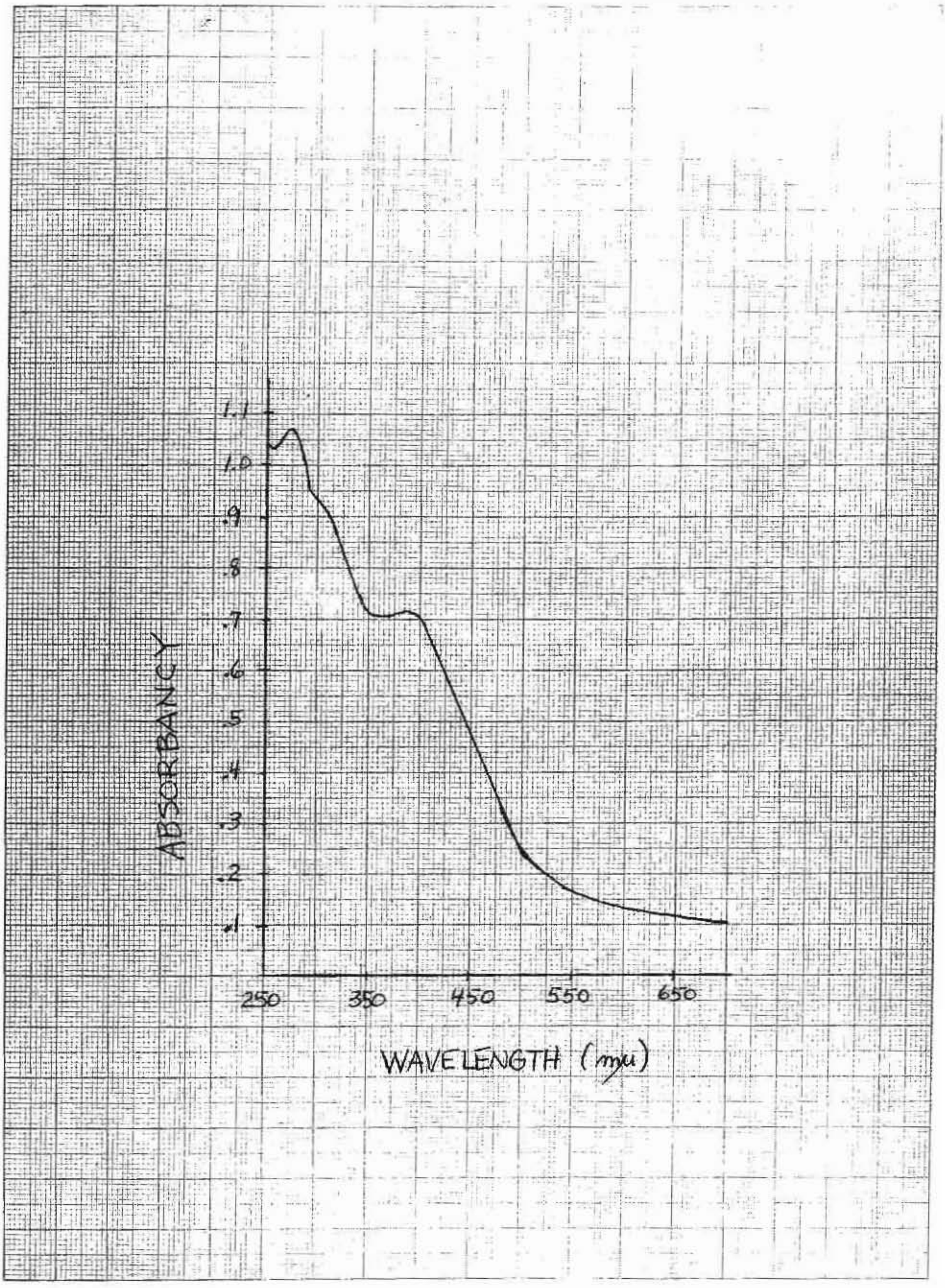

1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
ABSORBANCY
250
350
450
550
650
WAVELENGTH (mμ)

Figure 12: Specific Heat Capacity of Unreacted Epoxy Resin

Figure 15. Absorption spectrum of rubredoxin from M. lactilyticus.

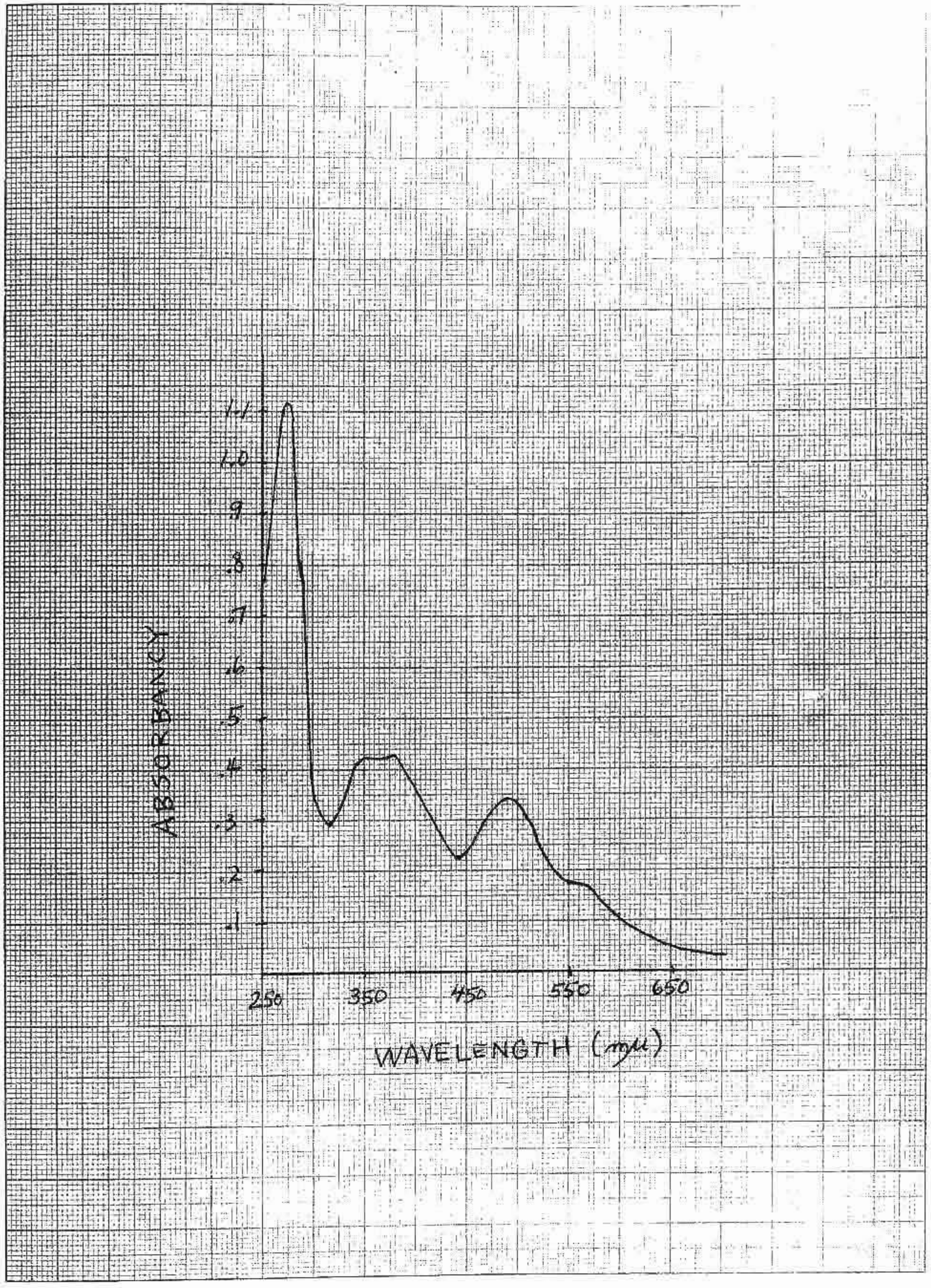

1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
ABSORBANCY
250
350
450
550
650
WAVELENGTH (mμ)

Figure 16. Acetyl phosphate standard curve.

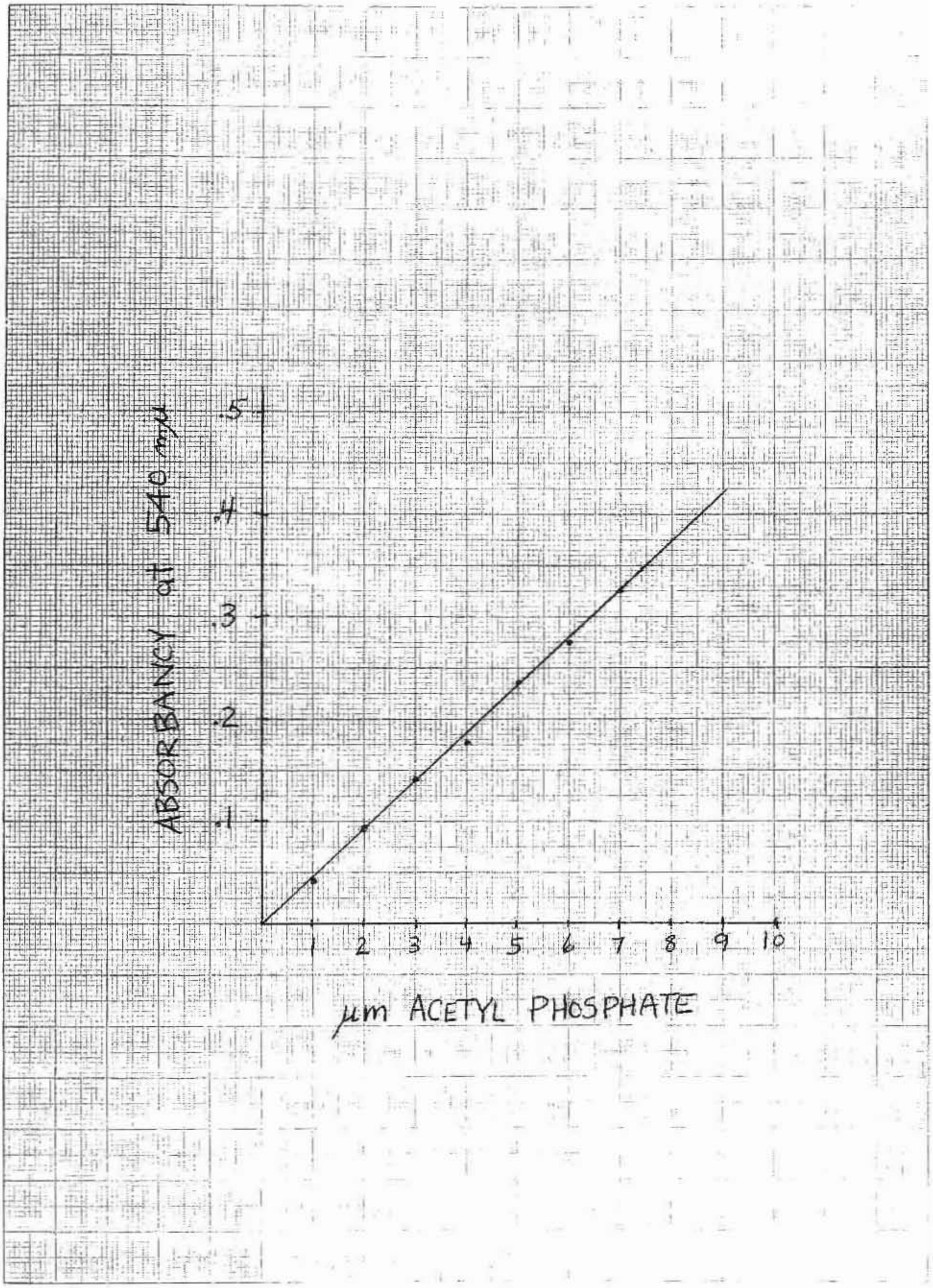

ABSORBANCY at 540 mμ
.5
.4
.3
.2
.1
1
2
3
4
5
6
7
8
9
10
μm ACETYL PHOSPHATE

Figure 17. Effect of increasing protein concentration on acetyl phosphate formation by M. aerogenes. Reaction mixture contained 324 ug of M. aerogenes "crude Fd", 25 um KH_2PO_4, pH 6.5, 10 um sodium pyruvate, 0.05 um CoA, and M. aerogenes protein minus "crude Fd" from 0 to 40 mg. Final volume was 1.0 ml. Incubation time was 10 min at room temperature. ——— plus crude ferredoxin, ------ minus crude ferredoxin

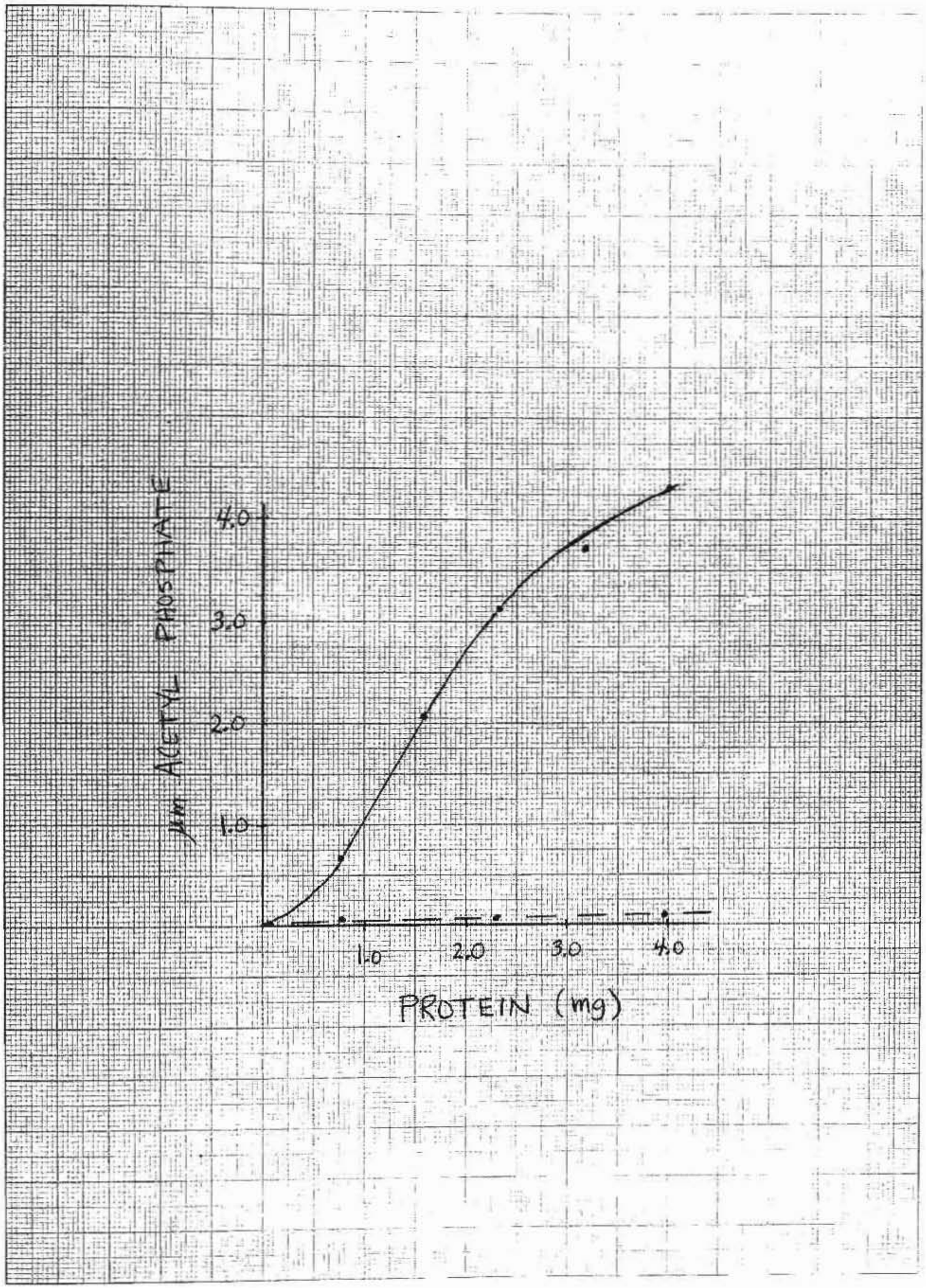

μM ACETYL PHOSPHATE
4.0
3.0
2.0
1.0
1.0
2.0
3.0
4.0
PROTEIN (mg)

Figure 18. Ability of "crude Fd" from M. aerogenes to stimulate acetyl phosphate formation. The reaction mixture contained 25 um KH_2PO_4, pH 6.5, 10 um sodium pyruvate, 0.05 um CoA, 4.15 mg M. aerogenes protein minus "crude Fd", and from 0 to 325 ug M. aerogenes "crude Fd". Final volume was 1.0 ml. Incubation time was 10 min at room temperature.

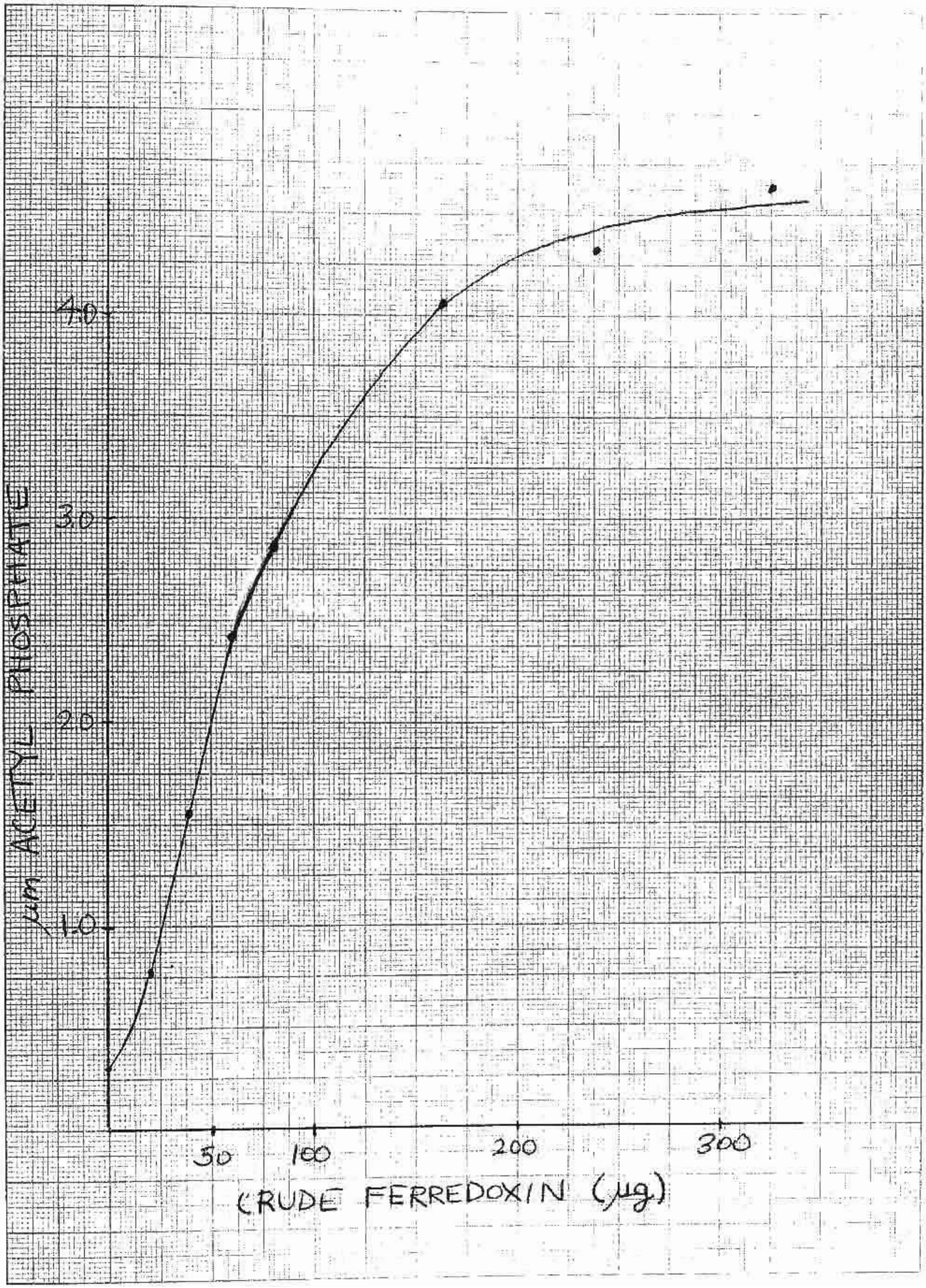

40
30
20
10
μm ACETYL PHOS
50
100
200
300
CRUDE FERREDOXIN (μg)

Figure 19. Effect of increasing protein concentration on acetyl phosphate formation by M. lactilyticus. Reaction mixture contained 250 ug of "crude Fd" from M. lactilyticus, 110 um sodium pyruvate, 50 um KH_2PO_4, pH 6.5, 0.13 um CoA, and M. lactilyticus protein minus "crude Fd" from 0 to 9.0 mg. Final volume was 1.0 ml. Incubation time was 15 min at room temperature. ——— plus ferredoxin, ------ minus ferredoxin

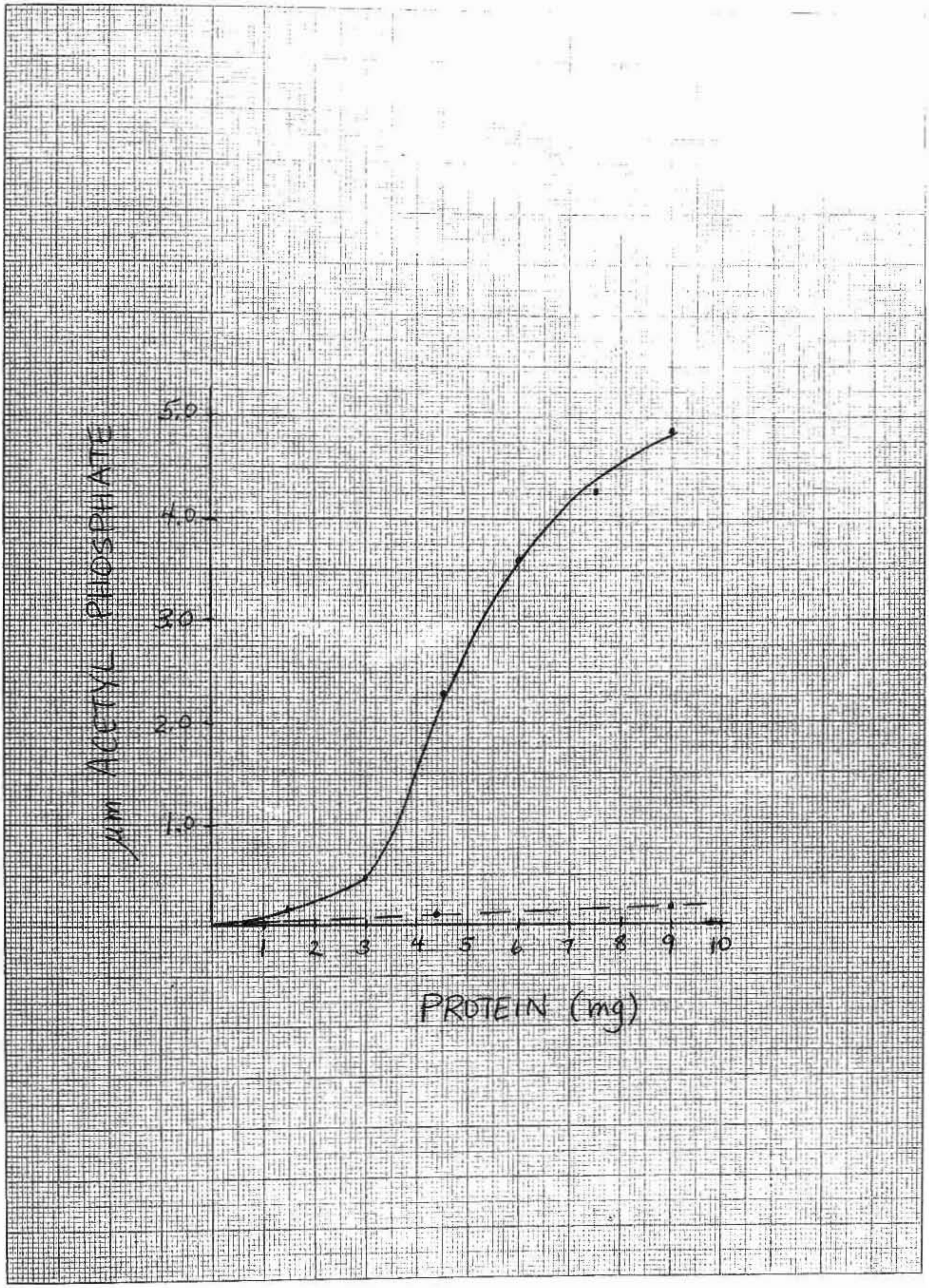

5.0
4.0
3.0
2.0
1.0
1 2 3 4 5 6 7 8 9 10
PROTEIN (mg)

Figure 20. Synthesis of acetyl phosphate catalysed by crystalline ferredoxin or rubredoxin from M. aerogenes. Reaction mixture contained 25 um KH_2PO_4, pH 6.5, 10 um sodium pyruvate, 0.05 um CoA, 12.0 mg M. aerogenes protein minus "crude Fd", and M. aerogenes crystalline ferredoxin or rubredoxin as indicated. Final volume was 1.0 ml. Reaction was incubated at room temperature for 10 min.

_____ ferredoxin, ------ rubredoxin

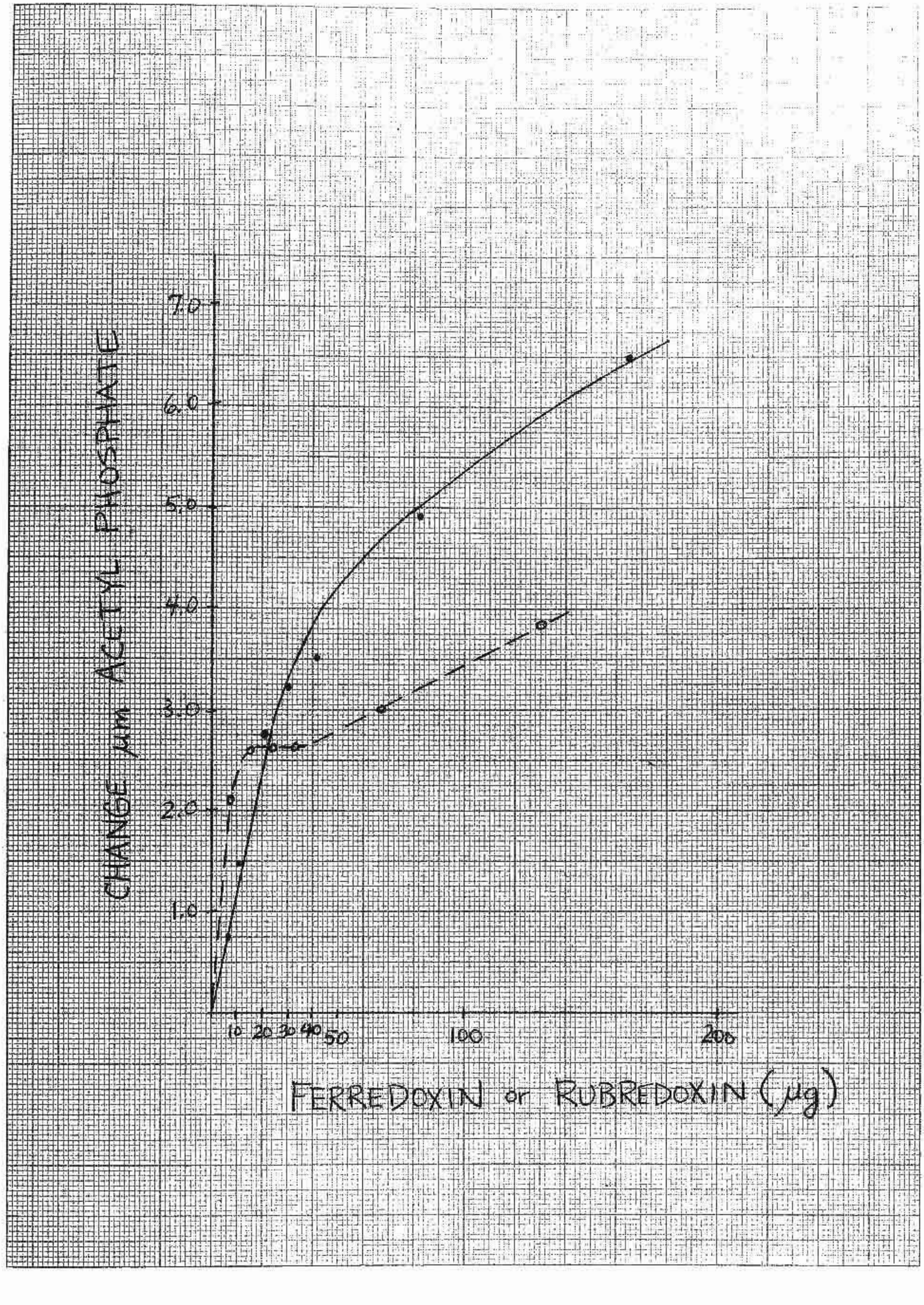

7.0
6.0
5.0
4.0
3.0
2.0
1.0
CHANGE IN ΔE 410 mμ
10
20
30
40
50
100
200
FERREDOXIN or RUBREDOXIN (μg)

Figure 21. Kinetics of hydrogen uptake by crude extracts of M. aerogenes using methyl viologen as an acceptor.

A. Hydrogen uptake at three different extract concentrations. Each Warburg cup contained 100 um K_2HPO_4, pH 7.5, an observed 5 um of methyl viologen, either 4.8, 9.6, or 14.4 mg protein, 0.2 ml 20% KOH, and water to a final volume of 2.2 ml.

B. The effect of increasing crude extract concentration upon hydrogen uptake (slope of curves from A. plotted).

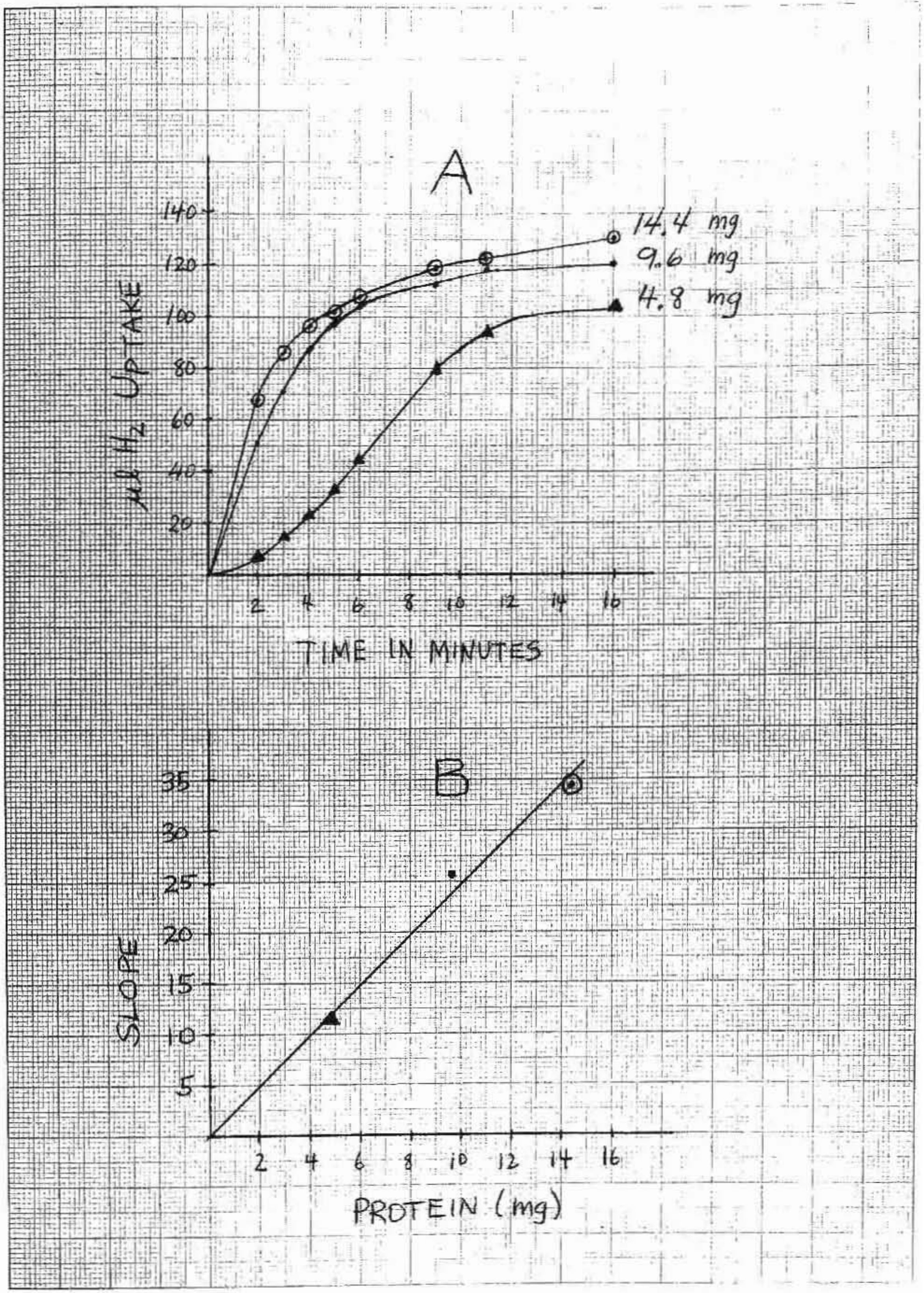

A
140
120
100
80
60
40
20
μL H_2 UPTAKE
14.4 mg
9.6 mg
4.8 mg
2
4
6
8
10
12
14
16
TIME IN MINUTES
B
35
30
25
20
15
10
5
μL H_2 UPTAKE
2
4
6
8
10
12
14
16
PROTEIN (mg)

Figure 22. Reduction of inorganic anions catalysed by crude extracts of M. aerogenes. Reaction mixtures and results are summarized in Table 14.

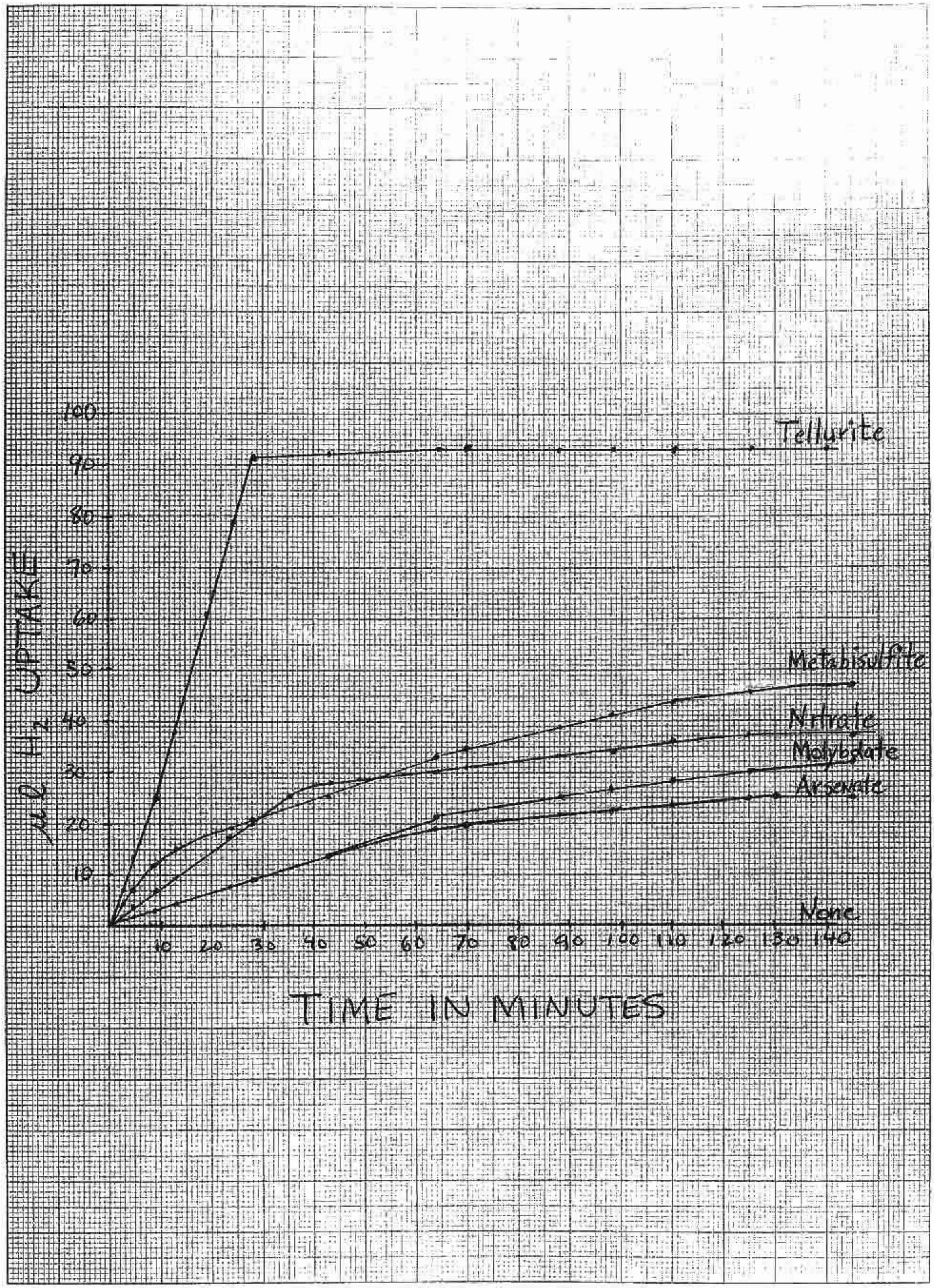

100
90
80
70
60
50
40
30
20
10
Tellurite
Metabisulfite
Nitrate
Molybdate
Arsenate
None
10
20
30
40
50
60
70
80
90
100
110
120
130
140
TIME IN MINUTES

Figure 23. Reduction of organic substrates catalysed by crude extracts of *M. aerogenes*. Reaction mixtures and results are summarized in Table 14.

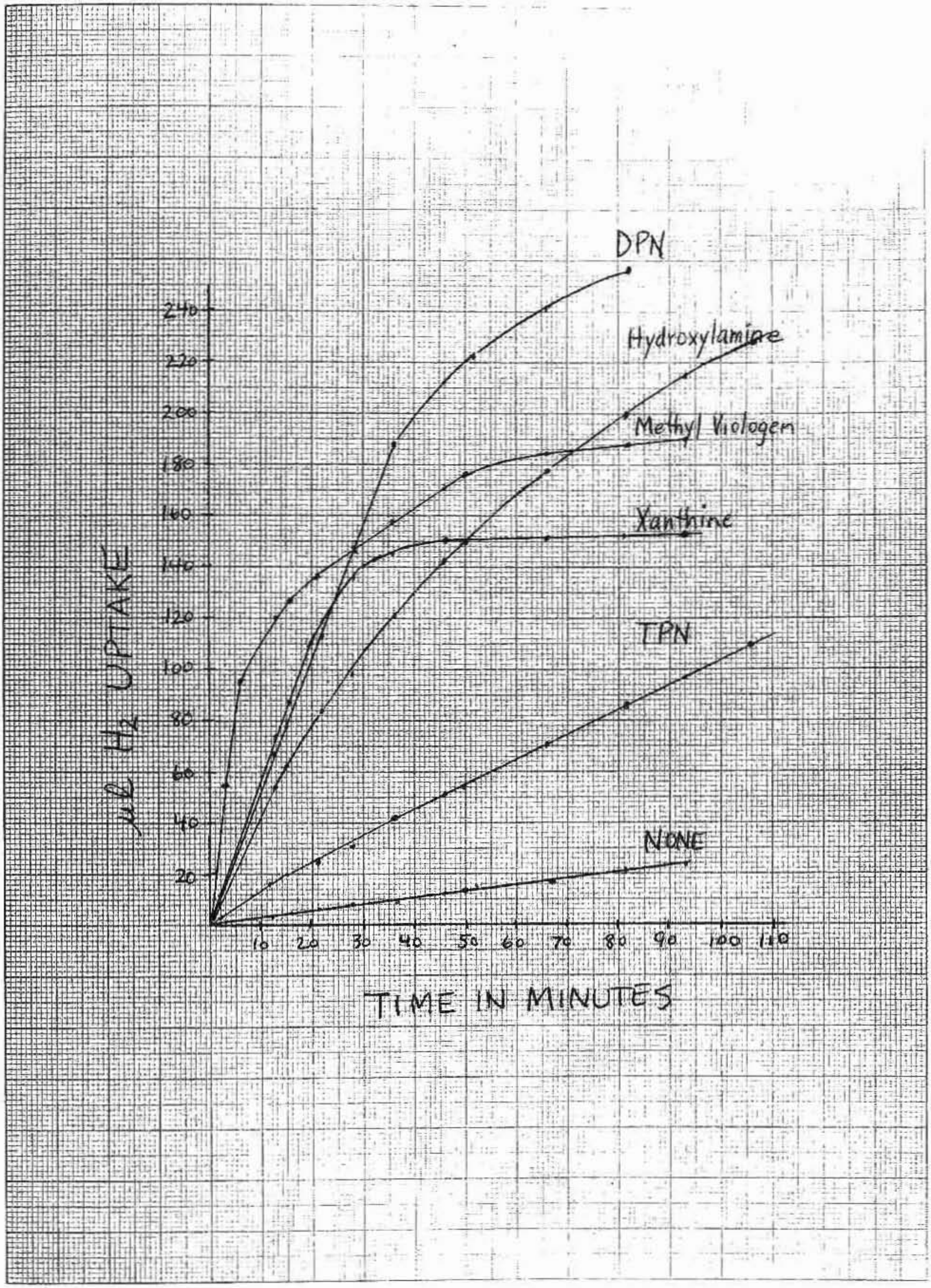

DPN
Hydroxylamine
Methyl Viologen
Xanthine
TPN
NONE
240
220
200
180
160
140
120
100
80
60
40
20
10
20
30
40
50
60
70
80
90
100
110
TIME IN MINUTES

Figure 24. Possible presence of DPN - TPN transhydrogenase in crude extracts of M. aerogenes. Each Warburg cup contained 100 um K_2HPO_4, pH 7.5 6.4 mg protein, 0.2 ml 20% KOH, and either 5, 7.5, or 10 um DPN, or 10 um TPN, and water to a final volume of 2.2 ml. After 44 min 2 um of TPN was added to the cup which initially had 5 um DPN present.

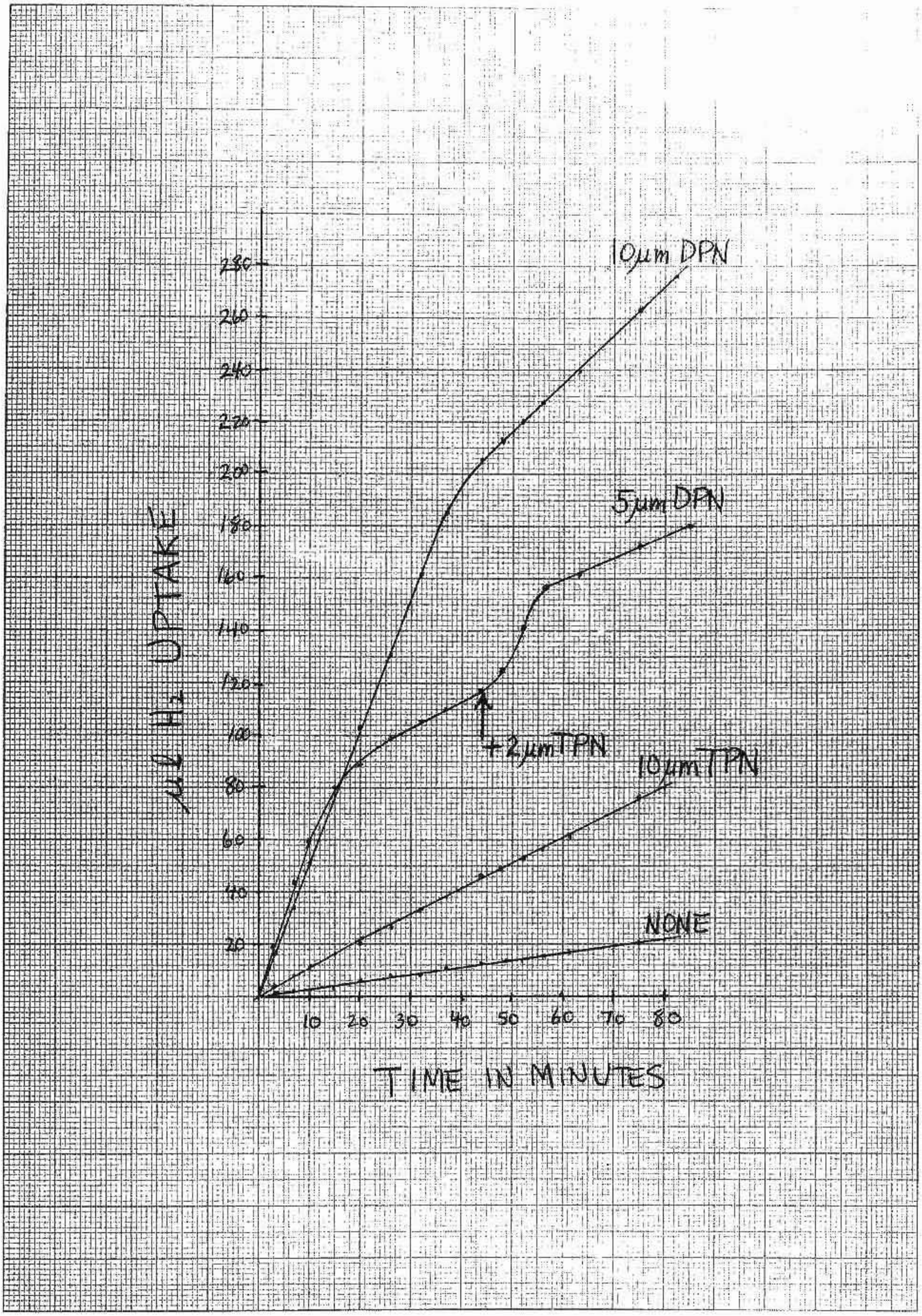
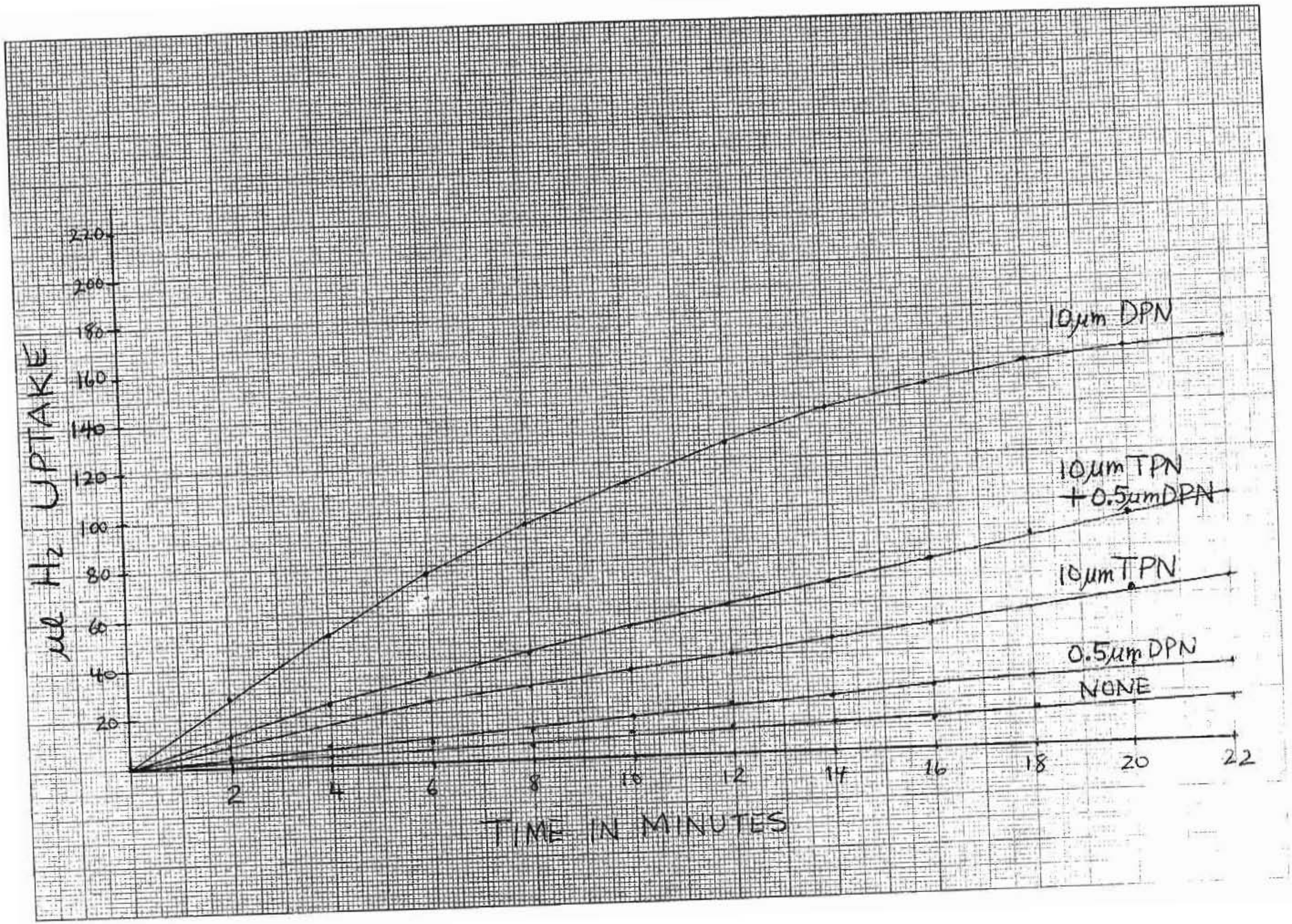

280
260
240
220
200
180
160
140
120
100
80
60
40
20
VELOCITY IN FPS
10 20 30 40 50 60 70 80
TIME IN MINUTES
0µm DPN
5µm DPN
+2µm TPN
10µm TPN
NONE

Figure 25. Presence of DPN - TPN transhydrogenase in crude extracts of M. aerogenes. Each Warburg cup contained 100 um K_2HPO_4, pH 7.5, 15.2 mg protein, 0.2 ml 20% KOH, either 10 um DPN, 10 um TPN plus 0.5 um DPN, 10 um TPN, or 0.5 um DPN, and water to a final volume of 2.2 ml.


UPTAKE H_2 µl
220
200
180
160
140
120
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
22
TIME IN MINUTES
10µm DPN
10µm TPN
+0.5µm DPN
10µm TPN
0.5µm DPN
NONE

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